UNIVERSITAT DE BARCELONA

Effects of alternative dietary lipid sources on lipid metabolism and regulation of food intake in larvae and juvenile senegalese sole *(Solea senegalensis)*

Kruno Bonacic

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Effects of Alternative Dietary Lipid Sources on Lipid Metabolism and Regulation of Food Intake in Larvae and Juvenile Senegalese Sole (*Solea senegalensis*)

DOCTORAL THESIS

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Barcelona, 2015

The thesis was performed under the direction of Dr. Sofia Morais at Institut de Recerca i Tecnologia Agroalimentàries (IRTA)—Sant Carles de la Rápita as part of the Aquaculture doctoral program at the Department of physiology and immunology, Faculty of Biology, University of Barcelona (2012–2015).

"Effects of Alternative Dietary Lipid Sources on Lipid Metabolism and Regulation of food Intake in Larvae and Juvenile Senegalese sole (*Solea senegalensis*)"

This dissertation is submitted by Kruno Bonacic, doctoral degree candidate at the University of Barcelona.

Tesis realizada bajo la dirección de la Dra. Sofia Morais de l'Institut de Recerca i Tecnologia Agroalimentàries (IRTA)—Sant Carles de la Rápita, Programa de doctorado de Acuicultura, Departamento de Fisiología i Inmunología de la Facultad de Biología de la Universidad de Barcelona (2012–2015).

"Efectos de fuentes de lípidos alternativos de la dieta sobre el metabolismo lipídico y regulación de la ingestión en larvas y juveniles del lenguado Senegalés (*Solea senegalensis*)"

Memoria presentada por Kruno Bonacic para optar al grado de Doctor por la Universidad de Barcelona.

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Report on paper impact factors

Dr. Sofia Morais, as the supervisor of the Doctoral Thesis titled **"Effects of alternative dietary lipid sources on lipid metabolism and regulation of food intake in larvae and juvenile Senegalese sole (*Solea senegalensis*)"** performed by Kruno Bonacic, hereby certifies the veracity of the Impact Factors and the implication of the doctoral student in each of the scientific papers that are part of this Thesis, as manifested below. Furthermore, it is declared that none of the papers have been included in any other Doctoral Thesis apart from the present.

1. AUTHORS: Bonacic, K., Estevez, A., Bellot, O., Conde–Sieira, M., Gisbert, E., Morais, S.

TITLE: ***Dietary fatty acid metabolism is affected more by lipid level than source in Senegalese sole juveniles: Interactions for optimal dietary formulation***

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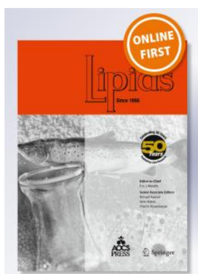

IF: 1.854 (Q3–NUTRITION & DIETETICS)

Kruno Bonacic participated in setting up the experimental design, organizing the trial and sampling, performed most of the analytical work (molecular biology and analysis of enzyme activity), carried out all data analysis and interpretation, and had the primary responsibility of writing up the manuscript.

2. AUTHORS: Bonacic, K., Campoverde, C., Sastre, M., Hachero–Cruzado, I., Ponce, M., Manchado, M., Estevez, A., Gisbert, E., Morais, S.

TITLE: ***Mechanisms of lipid metabolism and transport underlying superior performance of Senegalese sole (Solea senegalensis, Kaup 1858) larvae fed diets containing n–3 polyunsaturated fatty acids.***

JOURNAL: Aquaculture, 450: 383–396 (2016),

DOI:10.1016/j.aquaculture.2015.07.009

IF: 1.878 (Q1–FISHERIES)

Kruno Bonacic participated in setting up the experimental design, was in charge of running the larval experiment, including the enrichment of live preys, organized and participated in the samplings, performed most of the molecular work (RNA extraction, cDNA synthesis and RT–qPCR), carried out all data analysis (except for the OpenArray data) and interpretation, and had the primary responsibility of writing up the manuscript.

3. AUTHORS: Bonacic, K., Martínez, A., Martín–Robles, A.J., Muñoz–Cueto, J.A., Morais, S.

TITLE: ***Characterization of seven cocaine– and amphetamine–regulated transcripts (CART) differentially expressed in the brain and peripheral tissues of Solea senegalensis (Kaup)***

JOURNAL: General and Comparative Endocrinology DOI:10.1016/j.ygcen.2015.08.017

IF: 2.470 (Q3–ENDOCRINOLOGY & METABOLISM)

Kruno Bonacic performed all the "*in silico*" and part of the molecular work (RNA extraction, cDNA synthesis and PCR) necessary to find and characterize the 7 different *cart* transcripts, was in charge of running the feeding experiment, carried out all phylogenetic analysis and participated in writing up the manuscript.

4. AUTHORS: Conde–Sieira, M., Bonacic, K., Velasco, C., Valente, L.M.P., Morais, S., Soengas, J.L.

TITLE: ***Hypothalamic fatty acid sensing in Senegalese sole (Solea senegalensis): response to long–chain saturated, monounsaturated, and polyunsaturated (n–3) fatty acids***

JOURNAL: American Journal of Physiology, Regulatory Integrative and Comparative Physiology, DOI: 10.1152/ajpregu.00386.2015

IF: 3.106 (Q2– PHYSIOLOGY)

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Kruno Bonacic participated in setting up the experimental design, had primary responsibility in the day–to–day running of the experiment, participated in the samplings and performed part of the analytical work (enzyme analysis).

5. AUTHORS: Bonacic, K., Campoverde, C., Gómez–Arbonés, J., Gisbert, E., Estevez, A., Morais, S..

TITLE: ***Dietary fatty acid composition affects food intake and gut–brain satiety signaling in Senegalese sole (Solea senegalensis, Kaup 1858) larvae and post–larvae***

JOURNAL: General and Comparative Endocrinology, in revision

IF: 2.470

Kruno Bonacic participated in setting up the experimental design, optimized the methodology for fluorescently labeling live preys and quantify larval food intake, was in charge of running the larval experiment, including the enrichment of live preys, organized and participated in the samplings, performed all of the molecular work, carried out most data analysis and interpretation, and had the primary responsibility of writing up the manuscript.

6. AUTHORS: Bonacic, K., Martínez, A., Gisbert, E., Estevez, A., Morais, S.

TITLE: ***Food intake and appetite regulation in juvenile Solea senegalensis (Kaup, 1858) in response to dietary lipid level and oil source***

JOURNAL: Aquaculture, preparing for submission

IF: 1.878 (Q1–FISHERIES)

Kruno Bonacic participated in setting up the experimental design, organizing the trial and sampling, performed most of the molecular work, carried out all data analysis and interpretation, and had the primary responsibility of writing up the manuscript.

Abbreviations

AA – amino acid
ACAA2 – 3–ketoacyl–CoA thiolase
ACAD – acyl–CoA dehydrogenase
ACC – acetyl–CoA carboxylase
ACLY – ATP–citrate lyase
ACOX1 – acyl–CoA oxidase 1
ACTH – adrenocorticotropic hormone
AGPAT – 1–acylglycerol–3–phosphate O–acyltransferase
AGRP – agouti related peptide
ALA – α–linolenic acid
AMP – adenosine monophosphate
AP – alkaline phosphatase
APO – apolipoprotein
APOER – apolipoprotein E receptor
ARA – arachidonic acid
ARC – arcuate nucleus
ATGL – adipose triglyceride lipase
ATP – adenosine triphosphate
BP – base pair
BSS – bombesin
BW – body weight
CART – cocaine– and amphetamine–related transcript
CAT – carnitine–acylcarnitine translocase
CCK – cholecystokinin
CD36 – cluster of differentiation 36 family
CLO – cod liver oil
CNRQ – calibrated normalized relative quantity
CNS – central nervous system
CoA – coenzyme A
CPT1 – carnitine palmitoyl transferase type 1
CRF (CRH) – corticotropin–releasing factor (hormone)
CSTF1 – cleavage stimulation factor subunit 1
DAG – diacylglycerol
DHA – docosahexaenoic acid
DM – dry mass
DPH – days post hatching
DW – dry weight
ECH – enoyl–CoA hydratase
eEF1A1 – eukaryotic elongation factor 1 alpha 1
EFA – essential fatty acid
EL – endothelial lipase
ELOVL – fatty acid elongase
EPA – eicosapentaenoic acid
FA – fatty acid

FABP – fatty acid binding protein
FAS – fatty acid synthase
FAT – fatty acid translocase
FATP – fatty acid transport protein
FBW – final body weight
FCR – food conversion ratio
FM – fish meal
FO – fish oil
FXR – farnesoid X receptor
GAL – galanin
GLP–1 – glucagon–like peptide 1
GnRH – gonadotropin–releasing hormone
GRP – G protein–coupled receptor
HBP – hydroxypropyl–β–cyclodextrin
HDL – high density lipoprotein
HOAD – 3–hydroxy acyl–CoA dehydrogenase
HPA – hypothalamo–pituitary–adrenal
HSI – hepatosomatic index
HTGL – hepatic triacylglycerol lipase precursor
IDL – intermediate density lipoprotein
IBW – initial body weight
KIR6.X – inward rectifier K+ channel pore type 6.x
LCLAT – lysocardiolipin acyltransferase
LC–PUFA – long–chain polyunsaturated fatty acid
LDL – low density lipoprotein
Leu–Ala – leucine–alanine peptidase
LH – lateral hypothalamus
LH – luteinizing hormone
LMP2 – fatty acid translocase lysosome membrane protein 2–like
LNA – linoleic acid
LPCAT – lysophosphatidylcholine acyltransferase
LPIN1 – lipin 1
LPL – lipoprotein lipase
LPP – lipid phosphate phosphohydrolase
LPPR – lipid phosphate phosphatase–related protein
LRA – lauric acid
LSO – linseed oil
LXR – liver X receptor
MA – myristic acid
MAG – monoacylglycerol
MCH – melanocyte–concentrating hormone
MOGAT – monoacylglycerol O–acyltransferase
MS222 – tricaine methanesulphonate
MSH – melanocyte–stimulating hormone
MTP – microsomal trygliceride transfer protein
MUFA – monounsaturated fatty acid
NAc – nucleus accumbens

NFκB – nuclear factor κB
nMLF – nucleus of the medial longitudinal fascicle
NPY – neuropeptide Y
NRL – nucleus of the lateral recess
NRQ – normalized relative quantity
NTS – nucleus of the solitary tract
OA – oleic acid
OEA – oleoylethanolamide
OO – olive oil
ORF – open reading frame
PA – palmitic acid
PC – phosphatydilcholine
PCR – polymerase chain reaction
PCYT – ethanolamine–phosphate cytidylyltransferase
PE – phosphatydilethanolamine
PG12 – secretory phospholipase A2–like protein
PG4L – fatty acid translocase platelet glycoprotein 4–like
PISD – phosphatidylserine decarboxylase
PL – phospholipid
PLA2G1B – phospholipase A2 group 1B
PLA2G3 – group 3 secretory phospholipase A2 precursor
PLD1 – phospholipase D2
PNPLA2 – adipose triglyceride lipase
POMC – proopiomelanocortin
PPAR – peroxisome proliferator–activated receptor type
PS – phosphatydilserine
PSS – CDP–diacylglycerol–serine O–phosphatidyl transferase
PTDSS1 – phosphatidylserine synthase 1
PUFA – polyunsaturated fatty acid
PVN – paraventricular nucleus
PYY – peptide YY
qPCR – quantitative polymerase chain reaction
RACE – rapid amplification of cDNA ends
RPS4 – ribosomal protein S4
RT–qPCR – real–time quantitative polymerase chain reaction
RXR – retinoid X receptor
SA – stearic acid
SBO – soybean oil
SCD1 – stearoyl–CoA desaturase–1
SFA – saturated fatty acid
SGR – specific growth rate
SREBP1C – sterol regulatory element–binding protein type 1c
SUR – sulfonylurea receptor
TAG – triacylglycerol (triglyceride)
TFA – total fatty acids
TRH – thyrotropin–releasing hormone
UBQ – ubiquitin

UTR – untranslated region
VFI – voluntary food intake
VLDL – very low density lipoprotein
VM – vegetable meal
VMN – ventromedial hypothalamic nucleus
VO – vegetable oil
VSI – viscerosomatic index
WW – wet weight
Δ4FAD – Δ4 fatty acid desaturase

CHAPTER 1

General introduction

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1. *Status of global aquaculture and fish meal and oil production*

Aquaculture is a rapidly growing industry that has been responsible for a 3.2% annual growth rate of global fisheries since the 1990s (Fig. 1) and today already accounts for around 40% of total world fishery production (66.6 million tons in 2012). This trend is outpacing even world population growth (1.6%) and is reflected in the global fish consumption per capita, which has almost doubled since 1960 (FAO, 2014).

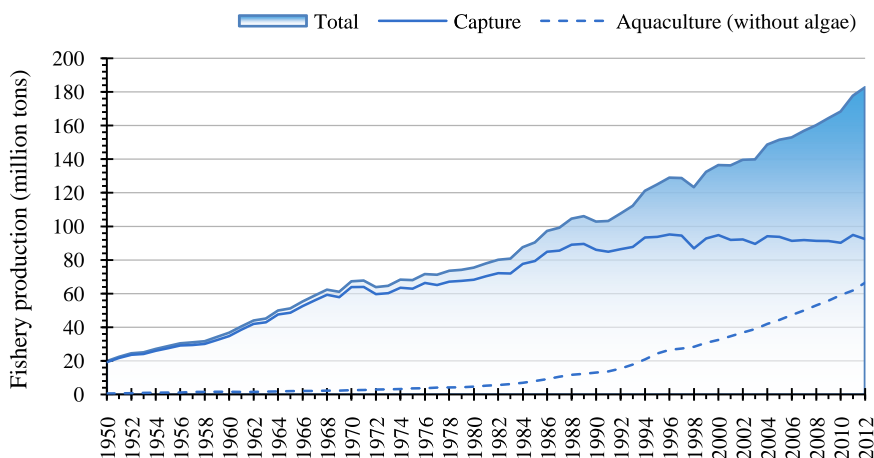

Figure 1. World capture, aquaculture and total fishery production from 1950 to 2012 (FAO, 2014).

The proportion of total world fisheries production used for direct human consumption has increased from about 71% in the 1980s to more than 86% (136 million tons) in 2012, with the remainder, destined for non–food uses, being stagnant (21.7 million tons in 2012; Table 1). A large part of these non–food uses includes the production of fish meal (FM) and fish oil (FO) used in compound feed formulation for aquaculture and terrestrial animal farming. These ingredients are mainly obtained from small pelagic fish stocks, which are currently being fully exploited. However, due to the increased demand of the growing aquaculture industry, combined with the disruption of capture yields due to natural disasters such as the El Niño phenomenon and stricter regulation of the anchoveta (*Engraulis ringens*) fisheries, prices of FM and FO are increasing (FAO, 2014).

Recent decades have seen significant efforts to establish other alternative ingredients, such as plant proteins and vegetable oils (VO) for aquafeed production. Today we are already seeing a considerable decline of FM and FO use in compound feeds (Tacon and Metian, 2008). In fact, fish–based products are increasingly being used more as strategic than conventional ingredients and are added only in small amounts to the diet. However, not all cultured fish species accept alternative oil sources equally well, and dedicated research must be performed on a case by case basis to identify the optimal quantities and ratios of different types of VO required to prepare nutritionally adequate feed formulations for individual species (Turchini et al., 2010). Therefore, we must first

familiarize ourselves with the nature of these oils and the general nutritional lipid requirements of fish species in aquaculture.

Table 1. Fate of global fishery products in relation to world population and consumption per capita (FAO, 2014).

	2007	2008	2009	2010	2011	2012
Human consumption	117.3	120.9	123.7	128.2	131.2	136.2
Non–food uses	23.4	22.2	22.1	19.9	24.5	21.7
Population (billions)	6.7	6.8	6.8	6.9	7.0	7.1
Per capita food fish supply (kg)	17.6	17.9	18.1	18.5	18.7	19.2

2. *Lipids in fish nutrition*

Structure and function of lipids

Lipids are diverse in structure and biological function, but are generally classified into neutral and polar lipids, according to their solubility characteristics. Collectively, they provide energy and cellular building blocks, and act as mediators for numerous physiological processes in all organisms, including fish (Tocher, 2003; Turchini et al., 2010). Some major classes of neutral lipids include: triacylglycerols (TAG; triglycerides), wax esters, sterols, steryl esters and free fatty acids (FA), while polar lipids encompass phospholipids (phosphoglycerides), sphingolipids, sulpholipids or glycolipids (Gurr and Harwood, 1991; Sargent et al., 2002). The most abundant of these classes are TAG (a glycerol backbone esterified with three FA molecules) and phospholipids (glycerol esterified with two FA molecules and a phosphate group). Triglycerides are the major form of energy storage in animals and plants; in fish appearing invariably as oils (Tocher, 2003). Phospholipids are polar molecules and, as such, the key components of the lipid bilayers surrounding cells, but are also involved in metabolism and cell signaling. The hydrophilic head group consists of a phosphodiester link of phosphoric acid to glycerol and either choline, ethanolamine, serine, glycerol or inositol, thus creating the 5 main families of phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylinositol (PI), respectively (Lehninger et al., 2008).

Fatty acids are the basic constituents of complex lipids, but also appear in the free form. They are carboxylic acids with a long aliphatic chain that can vary in length and degree of saturation (number of double bonds between C atoms). In this regard, they can be divided into short–chain (<6 C atoms), medium–chain (6–12 C atoms), long–chain (13–21 C atoms) and very long–chain FA (>21 C atoms), and in relation to degree of saturation into: saturated (SFA; no double bonds), monounsaturated (MUFA; one double bond) and polyunsaturated FA (PUFA; two or more double bonds). Unsaturated FA are further differentiated by the start position of the first double bond from the methyl end of the molecule, which in fish are most commonly located after the 9th (n–9), 6th (n–6) or 3rd (n–3) C atom from the terminal methyl end. Each subsequent double bond, if present, is separated by a single methylene (CH_2) group from the previous one. The accepted FA nomenclature, based on these characteristics, has the form "18:3n–6",

where 18 is the number of C atoms in the aliphatic chain, 3 is the number of double bonds and 6 is the ordinal number of starting point of the double bond series (Fig. 2). Finally, the double bonds can have different spatial orientation, depending on the position of the adjacent hydrogen atoms. In a *cis* configuration the two hydrogen atoms protrude on the same side of the chain, causing it to bend (Fig. 2a). This limits the ability of these FA to be closely packed together when forming parts of larger lipid formations, changing the fluidity of the membrane or fat, which affects its melting and freezing temperatures. In contrast, *trans* configurations are characterized by hydrogen atoms being located on opposite sides of the chain, resulting in a straight formation resembling that of SFA (Fig. 2b). However, in naturally occurring unsaturated FA, bonds mainly have a *cis* orientation, while *trans* configurations are generally a result of human processing (trans fats).

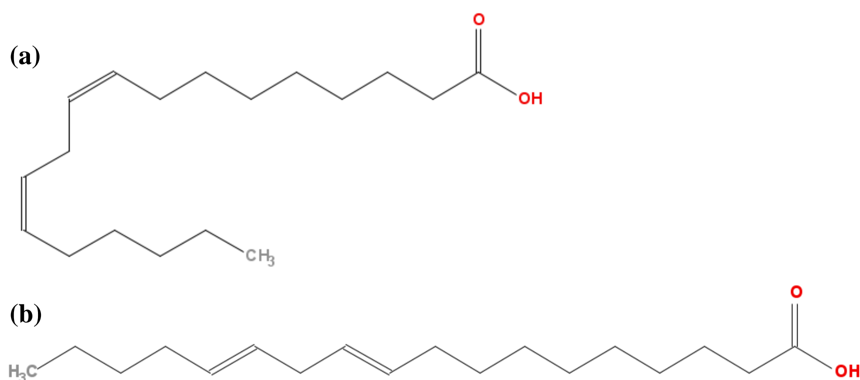

Figure 2. Molecular structure and orientation of *cis* (a) and *trans* (b) linoleic acid, 18:2n–6.

Characteristics of fish and vegetable oils

Fatty acids found in FO generally have an even number of C atoms (12–24) and the most abundant are 14:0, 16:0, 16:1n–7, 18:1n–9, 20:5n–3 and 22:6n–3 (Table 2) (Turchini et al., 2010). However, undoubtedly the most important and sought–after are the long–chain polyunsaturated fatty acids (LC–PUFA): eicosapentaenoic acid (20:5n–3), docosahexaenoic acid (DHA, 22:6n–3) and arachidonic acid (20:4n–6, ARA).

Conversely, VO are void of LC–PUFA, but can be abundant in SFA, MUFA such as oleic acid (OA, 18:1n–9) and C_{18} PUFA such as linoleic acid (LNA, 18:2n–6) and/or α–linolenic acid (ALA, 18:3n–3). Fatty acid profiles vary greatly between different types of VO, but for easier referencing they can be grouped into several classes and sub–classes based on their predominant FA types (Table 3) (Dubois et al., 2007). The most abundant VO produced are palm, soybean, rapeseed and sunflower oil (Table 3), although not all of these are interesting as ingredients for aquafeed production (USDA, 2014). The highest levels of the sought–after ALA are found in linseed oil (9.9% total FA) (Dubois et al., 2007). Nevertheless, besides having a higher market price, global production of linseed oil was estimated to be less than 0.23 million tons in 2013; calculated from total crops processed for linseed oil production (0.56 million tons; FAOSTAT, 2015) and the upper margin of oil content in flax seeds (40%; El–Beltagi et al., 2007). Thus, when

formulating commercial fish diets, a compromise has to be made between the desired FA profile, raw material availability and market price of the oil.

Table 2. Fatty acid composition of the main finfish oils produced worldwide, expressed in % total FA weight (FAO, 2014).

	Anchovy	Herring	Capelin	Menhaden
14:00	6.5–9.0	4.6–8.4	6.2–7.0	7.2–12.1
16:00	17.0–19.4	10.1–18.6	10.0	15.3–25.6
18:00	4.2	1.4	1.2	4.2
16:1n–9	9.0–13.0	6.2–12.0	10.0–14.3	9.3–15.8
18:1n–9	10.0–22.0	9.7–25.2	14.0–15.0	8.3–13.8
20:1n–9	0.9–10.	7.3–19.9	17.0	n/a–1.0
22:1n–9	1.0–2.1	6.9–30.6	15.4	n/a–1.4
18:2n–6	2.8	0.1–0.6	0.7	0.7–2.8
20:4n–6	0.1	<1.0	0.2	0.2
18:3n–3	1.8	n/a–2.0	0.2	0.8–2.3
20:5n–3	7.6–22.0	3.9–15.2	6.1–8.0	11.1–16.3
22:5n–3	1.6–2.0	0.8	0.6	2.0
22:6n–3	9.0–12.7	2.0–7.8	3.7–6.0	4.6–13.8

It has generally been shown that a 100% replacement of dietary FO with VO blends has a negative effect on the growth of most marine finfish species (Sales and Glencross, 2011). However, a large fraction (60–75%) of FO can generally be substituted without affecting growth and feed efficiency, if essential fatty acid (EFA) requirements are met (Turchini et al., 2009).

Table 3. World production of major VO in 2014/2015 (USDA, 2014) and their classification in relation to dominant FA constituents according to Dubois et al. (2007).

Oil	Production (million tons)	Class	Subclass
Palm	61.46	SFA	PA
Soybean	48.57	PUFA	LNA + MUFA
Rapeseed	27.13	MUFA	MUFA
Sunflower	15.22	PUFA	LNA + MUFA
Palm kernel	7.21	SFA	LRA + MA
Peanut	5.58	MUFA	MUFA + SFA + LNA
Cotton seed	5.14	PUFA	LNA + SFA
Coconut	3.35	SFA	LRA + MA
Olive	2.37	MUFA	MUFA

FA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LRA, lauric acid (12:0); MA, myristic acid (14:0); PA, palmitic acid (16:0); LNA, linoleic acid (18:2n–6)

Essential fatty acids

In fish, as in most vertebrates, LNA and ALA are the main EFA, as they cannot be synthesized endogenously and can only be obtained from dietary sources. However, LC–

PUFA such as EPA, DHA and ARA are also widely considered as EFA in fish, as numerous species have either completely lost the ability to biosynthesize them endogenously, or cannot produce them in enough quantities to meet physiological demands (Sargent et al., 1995). This dependence on dietary LC–PUFA, as opposed to C_{18} PUFA has been shown to increase with environmental salinity—likely as an adaptation to the high availability of LC–PUFA in marine food webs—and trophic level (Turchini et al., 2009). The structural and physiological importance of dietary LC–PUFA in fish has become so recognized that they are commonly regarded as the main EFA, especially in marine species, with C_{18} PUFA receiving much less attention. Numerous efforts have been made over the years to identify the EFA requirements of several fish species with aquaculture interest, which differ greatly from one life stage to the other (Izquierdo, 1996). An overview of some established dietary n–3 LC–PUFA recommendations for optimal fish performance are presented in Table 4. However, despite substantial accumulated knowledge, there is still a need to complete or re–define the EFA requirements of most species in aquaculture, as new feed production technologies arise and new analytical methods become available (Glencross, 2009).

Table 4. Optimal dietary n–3 LC–PUFA levels for juveniles of different marine fish species.

Fish species	Fish weight (g)	Dietary lipid level (%)	Optimal n–3 LC–PUFA (%)	Source
Sparus aurata	1.0	12.0	0.9	Kalogeropoulos et al. (1992)
	11.5	10.0	1.0	Ibeas et al. (1996)
Pagrus major	7.5	10.0	1.5	Takeuchi et al. (1992)
	–	20.0	3.7	Teshima et al. (1992)
Sparidentex hasta	13.3	15.0	0.6–0.8	Mozanzadeh et al. (2015)
Dicentrarchus labrax	–	12.5	1.0	Coutteau et al. (1996)
Lates calcalifer	–	13.0	1.0–1.7	Boonyaratpalin (1997)
Scopthalamus maximus	–	15.0	3.5	Castell et al. (1994)
Paralichthys olivaceus	8.5	6.5	0.8–1.0	Kim and Lee (2004)
Platyicthys stellatus	1.9	10.0	0.9	Lee et al. (2003)
Pleuronectes ferrugineus	6.8	10.0	2.5	Whalen et al. (1999)
Rachycentron canadum	62.0	11.0	0.8–1.5	Trushenski et al. (2010)
Sebastes schlegeli	43.7	14.0	0.9	Lee (2001)
Pseudocaranx dentex	–	–	1.7	Takeuchi et al. (1992)
Epinephelus malabaricus	10.3	4.0	0.4	Lin and Shiau (2007)
Larmichthys crocea	9.8	11.0	1.0	Zuo et al. (2012)
Sciaenops ocellatus	60	7.0	0.3–0.6	Lochmann and Gatlin (1993)

These FA play crucial roles as bioactive molecules in numerous physiological processes (mainly through conversion into eicosanoids, derivatives of C_{20} LC–PUFA), but are also structurally important as integral components of membrane phospholipids, where they contribute to membrane fluidity (of special significance in neural tissue). High concentrations of DHA can, therefore, be found in the olfactory nerve, eyes and central nervous system (CNS) of fish larvae, enhancing the detection and capture of prey (Furuita et al., 1998; Sargent et al., 1999b; Sargent et al., 1993). In fact, EFA are vital to developing fish and necessary in high dietary amounts, to meet the demands of rapid growth and intense organogenesis of fish larvae (Hamre et al., 2013; Tocher, 2010). On

the other hand, a lack of EFA in broodstock diets was observed to reduce fecundity and fertilization rates, and can also cause embryo deformities and lower larval quality, as the FA composition and total lipid content of the eggs are a direct reflection of the maternal diet (Fernández–Palacios et al., 1995; Izquierdo et al., 2001; Mourente and Odriozola, 1990). Although EFA are particularly critical dietary components for broodstock and larvae, it is also very important to consider them in juvenile and adult fish diets, outside the context of spawning. In fact, in cases of low dietary EFA levels, fish were observed to have poor growth rate, lower FCR, increased sensitivity to stress and increased mortality (reviewed by Glencross, 2009; Turchini et al., 2009). Several pathologies caused by lack of EFA were observed in rainbow trout, including erosion of the caudal fin, myocarditis and shock syndrome (Castell et al., 1972) and Atlantic salmon had fatty livers (Ruyter et al., 2006). Finally, ingested PUFA are incorporated into cellular membranes and fat stores of farmed fish and therefore determine the fillet FA composition at harvest (Francis et al., 2014). This defines the quality of produced fish for the end consumer, as DHA has significant health and developmental benefits for human beings (Horrocks and Yeo, 1999).

Importance of the n–3/n–6 ratio

Besides quantifying absolute requirements of EFA, it is very important to define optimal dietary n–3 to n–6 ratios (Sargent et al., 1999a). Various PUFA and eicosanoids from the n–3 class compete with those of the n–6 FA class not only to be incorporated into cellular membranes, but also for binding sites as natural ligands for nuclear receptors that regulate numerous physiological processes (Fig. 3) (Farooqui, 2009). In addition, ARA and EPA also compete for the same enzymes in order to produce their respective class of eicosanoids (Tocher, 2003). Generally, n–6 based compounds promote, while n–3 suppress, inflammatory, atherogenic and prothrombotic effects of cellular functions, with n–3 FA generally being more potent ligands for nuclear receptors than n–6 FA (Schmitz and Ecker, 2008). Various inflammatory signaling pathways are regulated through the nuclear factor κB (NFκB), which is a transcription factor that is inhibited directly by n–3 LC–PUFA (Weldon et al.). Furthermore, n–3 LC–PUFA have also been shown to have a hypotriglyceridemic effect both in fish (Bell et al., 2001; Jordal et al., 2005; Morais et al., 2012b) and mammals (Davidson, 2006). Catabolic pathways are regulated by peroxisome proliferator–activated receptors (PPAR), of which PPARα is the one most important in activating genes encoding enzymes involved in β–oxidation (reviewed by Nielsen et al., 2006). Eicosanoids (prostaglandins and leukotrienes) are stronger activators of this ligand–activated nuclear transcription factor than n–3 LC–PUFA. Upon activation, PPAR form heterodimers with the retinoid X receptor (RXR), which then bind to PPAR responsive elements in the regulatory region of target genes and thus influence their expression (Schmitz and Ecker, 2008). Sterol regulatory element binding protein 1c (SREBP–1c) is a major mediator of lipogenesis. Polyunsaturated FA (both n–3 and n–6) inhibit transcription of lipogenic genes by suppressing SREBP–1c gene expression or inhibiting the proteolytic release of nuclear SREBP–1c. Additionally, liver X receptor (LXR) is a major activator of SREBP–1c and is also regulated by binding of n–3 and n–6 PUFA (Schmitz and Ecker, 2008).

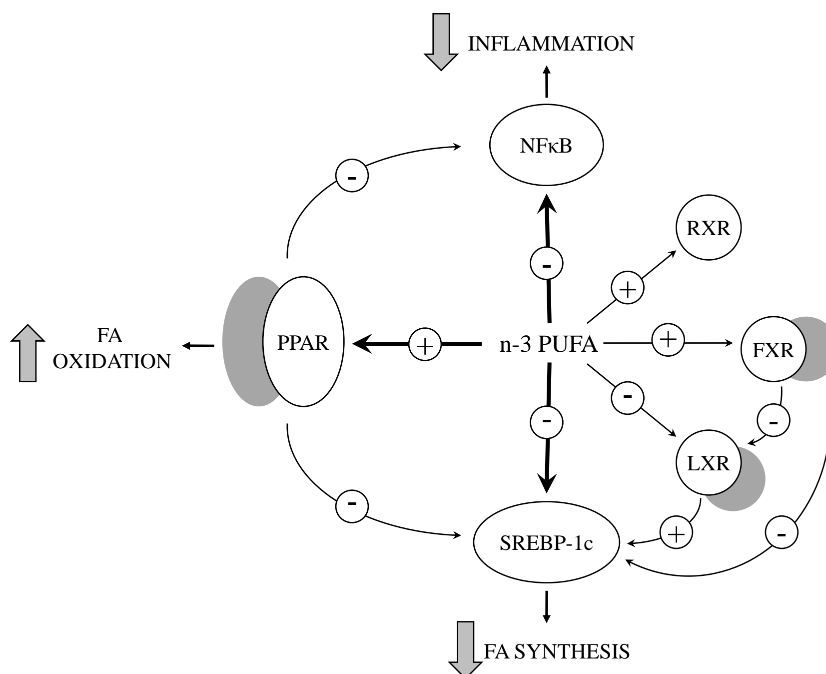

Figure 3. Nuclear receptors influenced by PUFA; n–3 being more potent ligands than n–6. FXR, farnesoid X receptor; LXR, liver X receptor; NFκB; nuclear factor κB; PPAR, peroxisome proliferator–activated receptor; RXR, retinoid X receptor; SREBP–1c, sterol regulatory element binding protein 1c (adapted from Davidson, 2006; Schmitz and Ecker, 2008).

It is especially important to consider the effects of the n–3/n–6 ratio when substituting dietary FO with VO. One of the most dominant FA in numerous plant oils is LNA, which has been shown to negatively affect fish growth performance (Montero et al., 2008; Montero et al., 2010), non–specific immunity (Wu and Chen, 2012; Zuo et al., 2015), spawning, larval quality (Liang et al., 2014) and FA and lipid metabolism (Berge et al., 2009; Robaina et al., 1998; Rollin et al., 2003), when representing a high fraction of total dietary FA. However, these negative effects of LNA could be eliminated by increasing the n–3/n–6 PUFA ratio. Dietary inclusions of both n–3 LC–PUFA and ALA were able to efficiently eliminate the negative effects of LNA in salmon and freshwater fish (Berge et al., 2009; Blanchard et al., 2008; Chen et al., 2013; Menoyo et al., 2007; Rollin et al., 2003; Senadheera et al., 2010; Tan et al., 2009). The beneficial properties of ALA as a standalone bioactive molecule (rather than as a precursor of LC–PUFA) have still not been determined, especially in marine fish that generally lack the capacity to biosynthesis C_{20} from C_{18} (Zuo et al., 2015). However, recent research has shown that the efficacy of ALA in the modulation of inflammation and oxidative stress in a marine fish was similar to that of LC–PUFA, and that the ALA/LNA ratio modulated the expression of genes involved in inflammation, FA oxidation and FA synthesis (Zuo et al., 2015). These results are of great interest as they suggest that C_{18}, and not just LC–PUFA have the potential to affect lipid metabolism, and thus the internal energy homeostasis of the organism. As will be discussed in further sections, these mechanisms

can determine the expenditure of energy directed for somatic growth and physiological processes, but also regulate energy intake by modulating appetite. Thus, it is of vital importance to understand and control these regulatory pathways in farmed fish in order develop nutritionally favorable, sustainable and economically viable feed formulations which would optimize the culture process and produce a higher quality food product.

Dietary lipid level

Fish species differ in their ability to efficiently digest, absorb and metabolize lipids (Tocher, 2003). For example, capelin (*Mallotus villosus*) feed on lipid–rich zooplankton, and as a result have developed the capability to efficiently assimilate, utilize and store large quantities of lipids (up to 20% wet body weight), which has also caused similar adaptations in their predators (e.g. Atlantic salmon, *Salmo salar*) (Tocher, 2003). On the other hand, demersal fish exposed to benthic food webs, which include polychaetes, bivalves and crustaceans, have developed a different digestive, absorptive and metabolic lipid capacity adapted to their ecology (Kapoor et al., 1976; Yúfera and Darías, 2007). Species like turbot (*Scophthalmus maximus*) (Koven et al., 1997; Regost et al., 2001) and Senegalese sole (*Solea senegalensis*) (Borges et al., 2013a) were shown to have high apparent digestibility coefficients for dietary lipids, but still did not respond well to high–lipid diets, suggesting potential issues in metabolic, rather than digestive, processes.

High dietary lipid inclusions (when using FO) have been observed to promote growth in several cultured species due to a protein sparing effect, which is characterized by almost exclusive catabolism of lipids for energy, maximizing the deposition of proteins for growth (Boujard et al., 2004a; Caballero et al., 1999; Company et al., 1999; Kaushik and Médale, 1994; Vergara et al., 1996; Watanabe, 1982). However, this was not the case in species like common sole (*Solea solea)* (Bonvini et al., 2015)*,* Senegalese sole (Borges et al., 2013b), turbot (Regost et al., 2001), red drum (*Sciaenops ocellatus*) (McGoogan and Gatlin III, 1999) or meagre (*Argyrosomus regius*) (Chatzifotis et al., 2010), where increased dietary lipid levels had no beneficial effects on fish performance.

3. *The lipid metabolism of fish*

Intestinal lipid digestion and absorption

The different capacities of fish to digest and absorb dietary nutrients depend greatly on the anatomical features of their gastrointestinal tract and the accompanying digestive physiology (Kapoor et al., 1976). The main part of lipolytic activity and lipid absorption seems to take place in the anterior part of the intestine and pyloric caeca (if present), but can extend to posterior parts of the intestine with a gradual decrease in activity (Koven et al., 1994; Tocher, 2003). Lipids are mainly hydrolyzed in the intestinal lumen by lipases, a group of pancreatic and hepatopancreatic enzymes (Kapoor et al., 1976). They are released in conjunction with bile salts that aid emulsification of luminal lipids to increase lipolytic efficiency, but are also needed to activate certain enzymes and/or enhance their activity (Olsen and Ringø, 1997). Some fish have also been shown to actively secrete a gastric (non–pancreatic) lipase in the stomach, which may partially digest lipids before

they enter into the intestine, but the physiological significance of these enzymes in fish is still unclear (Gisbert et al., 1999; Olsen and Ringø, 1997). Nevertheless, ingested lipids reach the intestine mostly in the form of TAG, which are hydrolyzed mainly into free FA and 2–monoacylglycerols, and are absorbed by enterocytes of the intestinal epithelia in this form (Tocher, 2003).

The mechanisms of long–chain FA uptake from the intestinal lumen in mammals are still quite poorly understood, with even less knowledge being available for fish. While the basic physiological processes are assumed to be generally similar (Ho et al., 2006; Tocher, 2003), it has been observed that the absorption rate of lipids tends to be much slower in fish, compared to mammals, as a result of low body temperatures influencing nutrient digestibility (Kapoor et al., 1976). Luminal uptake was initially thought to operate only by diffusion, but over the past couple of decades an additional protein mediated transfer has been suggested (Iqbal and Hussain, 2009; Pohl et al., 2004). While evidence in mammals has been obtained for both of these pathways, the relative importance of each appears to be highly dependent on the microenvironment and phenotype of the analyzed tissue (Niot et al., 2009). A standing hypothesis is that when luminal FA concentrations are low, the main component of uptake is active transport (involving protein transporters), while in conditions of high FA levels the majority of the FA are taken up passively (Tso et al., 2004). A couple of proteins have been proposed to take part in this active uptake: cluster of differentiation 36 (CD36), also known as fatty acid translocase (FAT), fatty acid transport protein 4 (FATP4) and caveolin–1 (Abumrad et al., 1999; Iqbal and Hussain, 2009; McArthur et al., 1999; Pohl et al., 2004; Su and Abumrad, 2009). However, the involvement of these peptides has not been unequivocally confirmed, and some studies have even shown that intestinal uptake was not affected in CD36–knockout or FATP4–knockout mice (Goudriaan et al., 2002; Shim et al., 2009). Additionally, these peptides have been attributed roles in intestinal lipid sensing, although the two processes are likely closely linked (Berthoud, 2008; Miguel–Aliaga, 2012; Schwartz, 2011).

Once FA enter the enterocytes, they are directed to the endoplasmatic reticulum by fatty acid binding proteins (FABP) to be assembled into complex lipids (Her et al., 2004). Pathways homologous to those in mammals have recently been described in fish (Oxley et al., 2005). They involve the synthesis of diacylglycerol (DAG), which is then directed to form TAG or phospholipids. However, there are two main pathways of diacylglycerol (DAG) synthesis: i) the monoacylglycerol (MAG) pathway, which involves the addition of a fatty acyl–CoA molecule to *sn*–2–monoacylglycerol with the aid of acyltransferase enzymes, and ii) the glycerol–3–phosphate pathway, which involves *de novo* synthesis of phosphatidic acid from glycerol–3–phosphate (originating from glucose metabolism), and its subsequent transformation into DAG through the activity of lipid phosphate phosphohydrolases (Brindley and Waggoner, 1998; Johnston, 1977). Both pathways converge at this point, and are now directed either to synthesize TAG through DAG acyltransferase activity, PE through the activity of DAG ethanolaminephosphotransferase, or PC through DAG cholinephosphotransferase (Coleman, 1992; Coleman and Lee, 2004). The preferred route of TAG synthesis under normal feeding conditions in Atlantic salmon was shown to be via the MAG pathway (Oxley et al., 2007).

Transport and storage of lipids

As dietary TAG become re–esterified in enterocytes they are packaged into lipoprotein particles in the lumen of the endoplasmatic reticulum and thus prepared for transport to other bodily tissues (Sheridan, 1988). Lipoproteins produced in the intestine of fish are mainly chylomicron–like particles and a small percentage of very low density lipoprotein–like (VLDL) particles, although these are mainly produced in the liver (Tocher, 2003; Turchini et al., 2009). Chylomicrons are the largest lipoprotein particles and consist of TAG and cholesteryl esters, encased in a PL matrix containing a series of embedded apolipoproteins (Apo). Given its important structural role, PC has been recognized as crucial for chylomicron assembly and TAG clearance from enterocytes (Mansbach, 2001). In fish, it is not yet clearly understood which route lipoproteins take upon leaving the enterocytes (Turchini et al., 2009), but in mammals they are secreted from the basolateral membrane (as nascent chylomicrons) into the lymphatic system, from which they enter circulation, bypassing the hepatic portal vein. In fish, nascent chylomicron–like particles contain Apoa1, Apoa4 and Apob (Kamalam et al., 2013), and remain in circulation for several hours following a meal before being cleared by the liver (Arnold–Reed et al., 1997). It is considered that during this period chylomicron–like particles interact with high–density lipoproteins (HDL), from which they receive Apoc2 (Shen et al., 2000), but seemingly not Apoe (Arnold–Reed et al., 1997), as is the case in mammals, and become mature chylomicrons. The Apoc2 lipoprotein is an activator of lipoprotein lipase (Lpl) in peripheral, non–hepatic, tissues, which hydrolyze core TAG molecules and surface phospholipids of the lipoprotein particle. As a result of Lpl activity, these particles, in mammals now called chylomicron remnants, become depleted in lipids and have a significantly different composition. They are finally directed to the liver to be further hydrolyzed by hepatic lipase and absorbed for processing (Tocher, 2003).

The liver stores useful lipids or repackages them into VLDL–like particles, which have a different set of apolipoproteins to chylomicrons, and are the most common circulating lipoprotein in fish (Babin and Vernier, 1989). Earlier studies observed the presence of an Apob–like protein in fish VLDL (Rogie and Roy Skinner, 1985; Smith et al., 1988) and suggested that Apoc2 (Babin and Vernier, 1989; Sheridan, 1988) and Apoe (Poupard et al., 2000) were also associated with these lipoproteins. When assembled, VLDL–like particles are released into the bloodstream to distribute nutrients to other tissues in a process using Lpl–dependent or independent transporting, with the former involving incorporation of whole lipoproteins into target tissue cells, likely through lipoprotein–receptor–mediated endocytosis, and the previous through Lpl–induced hydrolysis that releases FA, which are then bound to albumin and transported to target tissues (Alam et al., 2004; Tocher, 2003). As VLDL release their lipid content, they also pass Apoc and Apoe proteins onto HDL particles and become known as intermediate density lipoproteins (IDL), which are then either reabsorbed by the liver or further hydrolyzed into low–density lipoproteins (LDL) (Gjøen and Berg, 1993; Tocher, 2003).

Based on dietary input, energy demands and homeostatic state of the animal, nutrients are directed to be hydrolyzed close to adipocytes or, alternatively, near muscle and other high energy–producing tissues, in favor of anabolic (lipogenesis) or catabolic (β–oxidation) pathways, respectively. In fact, it has been observed that dietary FA can

affect Lpl expression in this manner, although the exact mechanisms are not known (Liang et al. 2002).

Lipogenesis

In fish, neutral lipids are mainly deposited in the visceral cavity, subcutaneous tissue, muscle and/or liver (Fernandes et al., 2012b; Jobling and Johansen, 2003; Santinha et al., 1999; Sheridan, 1988; Valente et al., 2011; Weil et al., 2013), but the preferential site of lipogenesis is the hepatic tissue (Lin et al., 1977; Sargent et al., 2002). This mainly refers to *de novo* synthesis of FA from acetyl–CoA, but also to the esterification of FA into TAG—processes which have been characterized in fish and are very similar to those observed in mammals (Henderson, 1996; Sargent, 1989). As summarized by Sul and Smith (2008), the major site for FA synthesis is the cytosol, but acetyl–CoA is produced from pyruvate in the mitochondria (Fig. 4). It enters the citric acid cycle and is subsequently translocated to the cytoplasm as citrate. Citrate is cleaved into acetyl–CoA and oxaloacetate—a reaction catalyzed by ATP–citrate lyase (ACLY). However, the first committed reaction of FA synthesis is catalyzed by acetyl–CoA carboxylase (ACC), which produces malonyl–CoA from acetyl–CoA (Henderson, 1996). This is a key regulatory point in FA synthesis, as malonyl–CoA is a potent inhibitor of CPT1 (Fig. 4). However, ACC is highly regulated at the transcriptional level by SREBP–1c, PPAR, LXRα and other transcriptional factors, but also by allosteric control mechanisms (inhibited by long–chain fatty acyl–CoA and stimulated by citrate). Furthermore, it can be inactivated and activated by phosphorylation (e.g. via AMP–activated protein kinase, glucagon or catecholamines) and de–phosphorylation (e.g. via insulin), respectively (Brownsey et al., 2006; Munday, 2002). The final phase in FA synthesis is catalyzed by fatty acid synthase (FAS), in order to combine acetyl–CoA and malonyl–CoA molecules into longer chain fatty acyl–CoA, of which the end product is palmitoyl–CoA, the precursor of palmitic acid (16:0) (Henderson, 1996; Sargent, 1989). High dietary lipid levels (Shimeno et al., 1996), as well as LC–PUFA (Alvarez et al., 2000; Morais et al., 2012b; Zuo et al., 2015), especially DHA (Kjær et al., 2008), have been shown to inhibit lipogenesis in fish. Moreover, in mammals, n–3 LC–PUFA (especially EPA) were observed to suppressed hepatic lipogenesis through inhibition of SREBP–1c and PPAR (Davidson, 2006; Jump et al., 1996).

β–oxidation

Fish generally have a low ability to efficiently utilize carbohydrates as an energy source, although this seems to greatly depend on the species' ecology and its adaptation to the composition of natural feed and environmental characteristics. On the other hand, energy contained in neutral fat stores represent a very important metabolic fuel for fish (Sargent et al., 2002). In order to be oxidized for energy, TAG are primarily lipolyzed into free FA locally (e.g. intramuscular energy reserves) or in fat storing tissues and then transported through circulation to the site of use, by forming complexes with serum albumin (Bernard et al., 1999; Bilinski and Lau, 1969; van der Vusse, 2009). Similar cellular uptake mechanisms to those earlier described for the intestine take place, and once FA enter the cell, they travel to the mitochondria or peroxisomes by diffusion or are transported by specific FABP homologues (Chmurzyńska, 2006; Schulz, 2008). To

undergo β–oxidation (Fig. 4), long–chain fatty acyl–CoA molecules need to enter these organelles with the aid of carnitine acyltransferase enzymes, which are specific for mitochondria and peroxisomes (Derrick and Ramsay, 1989; Tocher, 2003; Tocher et al., 2003). The acyl group is transferred to a carnitine molecule on the outer membrane of mitochondria or peroxisomes by carnitine palmitoyltransferase 1 (CPT1) and subsequently shuttled into the organelle by carnitine–acylcarnitine translocase (CAT). Once inside, the fatty acylcarnitine complex is processed by the enzyme CPT2 on the inner membrane of the organelle in order to replace carnitine with the CoA thiol (Schulz, 2008). Fatty acid β–oxidation involves a series of cycles in which long–chain fatty acyl–CoA are broken down to numerous acetyl–CoA molecules (1 per cycle). This process involves a variety of enzymes shown in Fig. 4 and results in the production of acetyl–CoA molecules (1 for every two C atoms in the fatty–acyl chain), which are then used

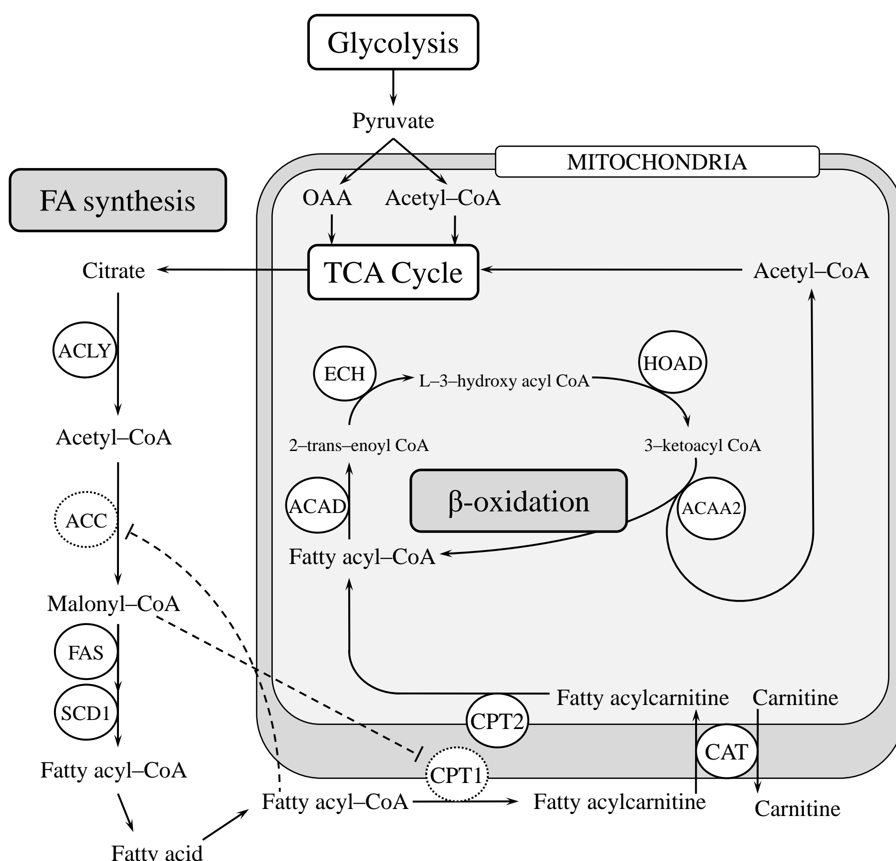

Figure 4. A simplified overview of the main enzymes involved in FA synthesis and β–oxidation (in mitochondria), with proposed regulatory mechanism in liver (modified from Schulz, 2008). ACLY, ATP–citrate lyase; ACC, acetyl–CoA carboxylase; FAS, fatty acid synthase; SCD1, stearoyl–CoA desaturase–1; CPT carnitine–palmitoyltransferase; CAT, carnitine–acylcarnitine translocase; ACAD, acyl–CoA dehydrogenase; ECH, enoyl–CoA hydratase; HOAD, 3–hydroxy acyl–CoA dehydrogenase; ACAA2, 3–ketoacyl–CoA thiolase.

for energy production through the citric acid cycle and subsequent electron transport chain (Fig. 4) (Tocher, 2003).

In hepatic cells, where both fatty acid synthesis and oxidation occur, the rate of β–oxidation is determined by CPT1 activity, which is controlled by malonyl–CoA—an intermediate of FA synthesis. When animals switch from a fed to a fasted state, hepatic metabolism shifts from glycolysis to gluconeogenesis and citrate is not supplied for FA synthesis, thus malonyl–CoA is not produced and CPT1 activity is relieved (Fig. 4). Furthermore, mitochondrial and peroxisomal FA oxidation is regulated through nuclear transcription factors opposite to lipogenesis, with LC–PUFA promoting catabolic reactions. In this respect, Turchini et al. (2003) observed that n–3 LC–PUFA stimulated FA uptake into mitochondria through both Cpt1 and Cpt2 in brown trout (*Salmo trutta*). Furthermore, dietary FO, as opposed to VO, promoted β–oxidation by affecting several key enzymes and transcription factors in Atlantic salmon (Jordal et al., 2005; Torstensen et al., 2004; Torstensen et al., 2009).

Fish readily use MUFA and C18 PUFA as substrates for oxidation (Crockett and Sidell, 1993; Kiessling and Kiessling, 1993), but also LC–PUFA if present in dietary surplus during periods of fast growth (Stubhaug et al., 2007). Thus, by altering dietary FA compositions, it is possible to promote specific FA to be the dominant energy substrate (Turchini et al., 2009). In this respect, Atlantic salmon fed VO retained more than 70% dietary EPA and DHA, while those fed an FO–based diet retained only 30% (Stubhaug et al., 2007).

Biosynthesis of long–chain PUFA

All vertebrates, including fish, have a strict dietary requirement for C_{18} PUFA (LNA and ALA), since they cannot be synthesized *de novo*. Subsequent synthesis of LC–PUFA (ARA, EPA and DHA) using C_{18} PUFA as precursors is conditionally possible and involves sequential desaturation and elongation processes (Cook and McMaster, 2002). The degree to which animals can perform these conversions varies greatly and depends on the presence and activity of several fatty acid elongases (ELOVL) and desaturases (FAD) (Fig. 5). Freshwater fish seem to have the necessary Δ5 and Δ6 desaturases and elongases to perform these reactions, but with varying levels of potency, and often LC–PUFA are still considered EFA, as biosynthetic pathways do not meet the high demands for these FA (Sargent et al., 1995). Furthermore, the activity of these enzymes seems to be dictated by feeding habits and dietary EFA availability (Castro et al., 2012). Conversely, marine fish have very limited or no capability of LC–PUFA biosynthesis, which is generally attributed to the LC–PUFA–rich lower trophic levels of the marine food web (Sargent et al., 1999a). However, genes encoding a desaturase enzyme with a dual Δ6/Δ5 activity has been revealed in rabbitfish (*Siganus canaliculatus*) (Li et al., 2010), a marine herbivorous species that feeds exclusively on benthic algae and seagrasses, suggesting that trophic level and diet, above other environmental parameters, are the main determinants of LC–PUFA biosynthesis capability (Li et al., 2010). Similar dual activities had previously been observed in a freshwater fish (Hastings et al., 2001), but this was the first time a Δ5Fad activity was reported in any marine fish, although a couple of studies had previously observed that turbot was able to synthesize small amounts of DHA from injected ^{14}C–ALA (Linares and Henderson, 1991; Tocher et al., 1992). Furthermore, a Δ*4fad* gene, that converts EPA into DHA, was also identified for

the first time in rabbitfish (Li et al., 2010; Zhang et al., 2014), but since then also in Senegalese sole (Morais et al., 2012a; Morais et al., 2015b).

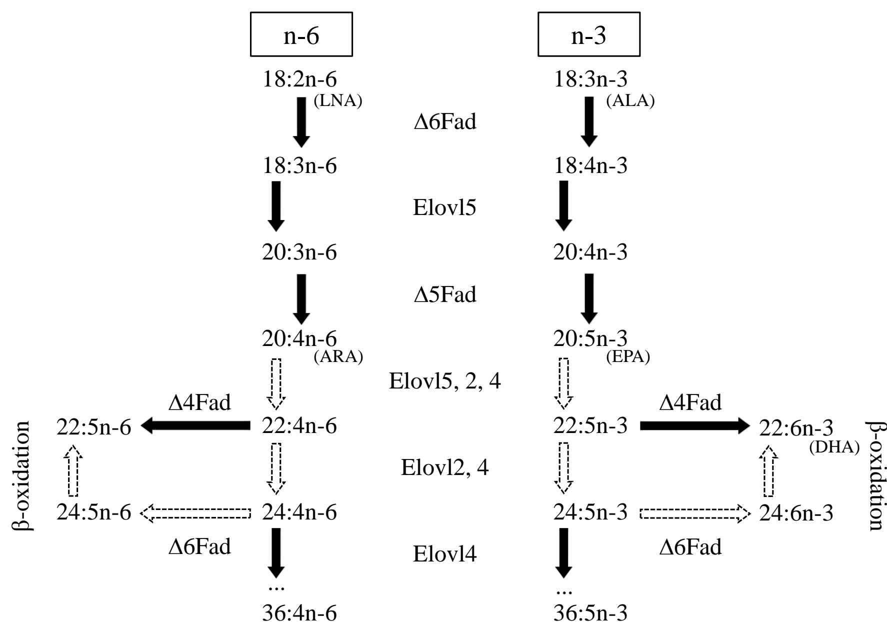

Figure 5. A step by step representation of LC–PUFA biosynthesis pathway and the accompanying enzymes (not all are necessarily present in the same species). Dashed arrows correspond to the Sprecher pathway (Voss et al., 1991). Image is adapted from Navarro–Guillén et al. (2014).

4. *Appetite and regulation of food intake*

Appetite directly governs food intake, which has a central role in determining the growth and body composition of cultured animals (Forbes, 2007). Under farming conditions, fish are generally fed a fixed ration and therefore cannot adjust their food intake to meet specific requirements for a particular nutrient or energy content (Saravanan et al., 2012). This may lead to under– or, alternatively, over–feeding, which can have negative effects on fish performance and health or cause feed wastage and degradation of water quality. With this in mind, it is very important to determine the voluntary food intake (VFI) of the cultured species on specific dietary formulations and to understand the effects dietary nutrients and energy levels have on appetite–regulatory mechanisms, in order to tailor feeds according to individual species and/or life stage requirements (Saravanan et al., 2012).

Energy–dense, lipid–rich diets have commonly been shown to reduce food intake in both adult and juvenile fish (Boujard et al., 2004b; Gélineau et al., 2002; Sæther and Jobling, 2001), but an increasing number of studies show that this is often not the case. For example, European seabass (Peres and Oliva–Teles, 1999a, b), Senegalese sole (Guerreiro et al., 2012) and rainbow trout (Saravanan et al., 2012) seemed to adjust

better food intake in relation to protein than to lipid level, although a case of hyperphagia towards very high lipid diets in the first two species was observed (Borges et al., 2009; Peres and Oliva–Teles, 1999a). Similarly, turbot ingested more of a high–, compared to low–lipid diet, when dietary protein levels were low (Cho et al., 2005). On the other hand, no adjustment of food intake towards dietary lipid–level was observed in Atlantic salmon (Helland and Grisdale–Helland, 1998) and in another study with Senegalese sole (Dias et al., 2004). These results indicate that food intake in fish is far from just energy–dependent and is likely governed by a complex set of appetite regulating mechanisms that are also sensitive to individual nutrients and macronutrients, as has been suggested in mammals (Karhunen et al., 2008; Raben et al., 2003). However, this has barely been studied in fish, especially in the scope of nutritional studies, where diets are ingested orally and can affect FA sensors throughout the gastrointestinal tract, putatively affecting appetite before and during absorption.

Lipid sensing in the intestine

Lipids are a major stimuli for the secretion of intestinal satiety peptides. A model of gastrointestinal peptide release through intestinal FA sensing mechanisms has been established in mammals (Steinert and Beglinger, 2011), but the exact regulatory mechanisms are still not known, and practically no researched has been performed in fish. Enteroendocrine chemosensory cells, scattered along the epithelial lining of the intestine, that have direct access to the luminal contents and afferent nerve terminals in their close vicinity, are thought to contain receptors relevant for lipid sensing (Steinert and Beglinger, 2011). The main FA sensors in these cells are considered to be fatty acid translocase (FAT/CD36), FATP4 and several G protein–coupled receptors (GRP40, GRP43, GPR119, GRP120) (Berthoud, 2008; Guijarro et al., 2010; Khan and Besnard, 2009; Laugerette et al., 2005; Piomelli, 2013; Schwartz et al., 2008; Yonezawa et al., 2013), which differ in their specificity for types of FA, FA derivatives such as oleoylethanolamide (OEA) and for oxidized FA (Little and Feinle–Bisset, 2010; Miyauchi et al., 2010; Vangaveti et al., 2010; Yonezawa et al., 2013). These have been associated to the release of specific satiety factors, including peptide YY (PYY), cholecystokinin (CCK) and glucagon–like peptide 1 (GLP–1), which will be described in the following section (Diep et al., 2011; Fu et al., 2003; Gaetani et al., 2003; Lauffer et al., 2009; Oveisi et al., 2004; Yonezawa et al., 2013). Finally, post–absorptive processing of long–chain FA, including their packaging into chylomicrons and the generation of Apoa4 also seem to stimulate the release of CCK (Little and Feinle–Bisset, 2010).

Intestinal satiety signals

In fish, a number of appetite–regulating peptides, homologous to those found in mammals, have been isolated predominantly from the intestine: Cck, Pyy, Glp–1, galanin (Gal) and bombesin (Bss) (reviewed by Volkoff et al., 2005). They are synthesized in chemosensory cells of the intestinal parenchyma, likely as a reaction to lipid sensing (Abumrad et al., 1999; Little and Feinle–Bisset, 2010). Once secreted, they can excite vagal afferents (paracrine activity) or enter systemic circulation (endocrine activity) and transmit the relevant signals to the CNS (Steinert and Beglinger, 2011).

Sensory information passing through the vagus nerve first reaches the hindbrain and is mostly relayed to specific neuroendocrine areas of the hypothalamus. On the other hand, peptides released as humoral factors travel through circulation to the CNS over a longer period of time. Upon arrival, they cross the blood–brain barrier and activate specific receptors on neuronal cells (Steiner and Beglinger, 2011). There is also a third pathway by which ingested food can trigger responses in the brain, bypassing the gut–brain axis. This occurs via free circulating macronutrients that activate central sensing mechanisms (discussed further below) (López et al., 2007; Polakof et al., 2011b).

There is a significant lack of information in fish on the effects of dietary FA composition on the expression of these peptides, although a couple of studies with Atlantic salmon (*Salmo salar*) and yellowtail (*Seriola quinqueradiata*), linked dietary macronutrient composition to the activity of Cck and Pyy (Hevrøy et al., 2008; Murashita et al., 2008). On the other hand, numerous studies have been performed on the response to feeding and/or starvation in a variety of species, which generally confirmed an anorexigenic function for *pyy* (Gonzalez and Unniappan, 2010), *glp1* (Silverstein et al., 2001 {Polakof, 2011 #1334; Sundby et al., 1991) and *cck* (Aldman and Holmgren, 1995; Murashita et al., 2007; Murashita et al., 2008; Peyon et al., 1999), and orexigenic function of *gal* (Nelson and Sheridan, 2006) in fish.

Central FA sensing

This term refers to the capacity of specific hypothalamic neurons to detect changes in levels of free circulating FA, which can be a result of lipid metabolic activity (fat–store mobilization) and/or post–absorptive changes following a meal. Fatty acid sensing mechanisms, established both in mammals (Blouet and Schwartz, 2010; López et al., 2007) and fish (Soengas, 2014), that detect increased concentration of plasma long–chain FA, include: i) FA metabolism through inhibition of CPT–1; ii) binding to FAT/CD36 and modulation of transcription factors PPARα and SREBP1c; iii) activation of protein kinase C–θ; and iv) mitochondrial production of reactive oxygen species (ROS) by electron leakage, resulting in an inhibition of ATP–dependent inward rectifier potassium channel (KATP) activity. Previous studies have identified the presence of these mechanisms in rainbow trout (*Oncorhynchus mykiss*) and showed that food intake was modified by circulating levels of FA (Librán–Pérez et al., 2013a; Librán–Pérez et al., 2012). These systems responded to changes in the levels of not only long–chain FA such as OA but, unlike mammals, also to medium–chain FA like octanoic acid (Librán–Pérez et al., 2013a; Librán–Pérez et al., 2012). However, to date, the presence of FA sensing systems in other fish species has not been investigated.

Central appetite–regulating peptides

The focal point of appetite control is generally considered to be the hypothalamus; mainly the arcuate nucleus (ARC) (Dhillo et al., 2002), which in fish corresponds to the lateral tuberal nucleus (Agulleiro et al., 2014). However, several studies also suggest that the hindbrain (specifically the nucleus of the solitary tract) additionally plays an important role in appetite regulation as a nexus for incoming vagal signals, in both mammals and fish (Alhadeff and Grill, 2014; Polakof et al., 2011a). In order to maintain whole–body energy homeostasis, an impressive array of peripheral signals

pertaining to energy balance are received centrally. These include, but are not limited to: i) endocrine (and possibly vagal) signals reporting on the status of long–term fat stores (via adipokines such as leptin, from adipose tissue and liver), reported in fish (Chisada et al., 2014; Volkoff et al., 2003) similarly to mammals (Berthoud, 2002); ii) vagal signals from liver reporting on the status of the hepatic oxidative metabolism, although this pathway has only been suggested in fish (Figueiredo–Silva et al., 2012b) and the exact mechanisms in mammals remain largely unknown (Allen et al., 2009; Berthoud, 2008); and iii) circulating levels of certain metabolites and compounds (e.g. free FA, endocannabinoids, eicosanoids) that activate specific receptors in the hypothalamus, continuously reporting on the metabolic state of the organism (Naughton et al., 2013; Soengas, 2014). This information is integrated with nonfood cues like sensory input from the environment, circadian control systems and food expectancy (Berthoud, 2002; Schwartz et al., 2000), and interpreted through complex, redundant, and distributed neural systems that utilize a large number of peptides as endocrine effectors (Dhillo et al., 2002; Lau and Herzog, 2014; Vicentic and Jones, 2007). Thus, "arousing" signals may include e.g. the visual detection of food and/or low fuel availability sensed peripherally or centrally, or the lack of a particular nutrient, which would generate a central orexigenic effect, leading to initiation of ingestion and/or simply adjustments in steady state energy balance (Berthoud, 2002). If food is ingested, a cascade of endocrine, paracrine and vagal signals are produced in the gastroenteropancreatic system (Nelson and Sheridan, 2006; Volkoff et al., 2005) and, with some delay, levels of circulating metabolites are augmented, affecting other peripheral organs and the brain directly, causing the generation of central anorexigenic signals in order to lower appetite and cease ingestion (Berthoud, 2002; Naughton et al., 2013; Soengas, 2014). In the case of neural and endocrine activation of metabolic processes, the resulting changes in metabolism and fuel availability are detected by internal sensors, and in both cases the initial arousal is satisfied or neutralized (Berthoud, 2002).

Information on the regulatory mechanisms of these neuropeptides in fish is growing, but is still limited, and studies often rely on mammalian literature as a starting point (Volkoff, 2006; Volkoff et al., 2005). Thus, the most common central peptides established in mammals were the first to be studied in fish. These include the putatively anorexigenic: cocaine– and amphetamine–regulated transcript (CART), melanocyte–stimulating hormones (MSH; originating from proopiomelanocortin—POMC), corticotropin–releasing factor (CRF) and CCK (expressed in brain as well as in the gastrointestinal tract); and the orexigenic: neuropeptide Y (NPY), agouti–related protein (AgRP), melanin–concentrating hormone (MCH), GAL and orexins (Volkoff et al., 2005). Numerous studies have been performed to characterize the action of these peptides in fish, mainly by the use of peptide injections followed by measurements of food intake or by gene expression studies in relation to fasting and feeding conditions. In this regard, an anorexigenic–like regulation of *cart* was confirmed in channel catfish (*Ictalurus punctatus*) (Kobayashi et al., 2008; Peterson et al., 2012), catfish (*Clarias gariepinus*) (Subhedar et al., 2011), zebrafish (*Danio rerio*) (Nishio et al., 2012), Atlantic salmon (Murashita et al., 2009a; Valen et al., 2011) and Atlantic cod (*Gadus morhua*) (Kehoe and Volkoff, 2007); of *pomc* in goldfish (Cerdá–Reverter et al., 2003b), zebrafish (Song et al., 2003) and Atlantic salmon (Murashita et al., 2011; Valen et al., 2011); of *cck* in goldfish (Volkoff et al., 2003) and rainbow trout (Gélineau and Boujard, 2001; Jensen et al., 2001); and for *crf* in rainbow trout (Doyon et al., 2003). On the other

hand, an orexigenic activity of *npy* has been observed in channel catfish (Peterson et al., 2012) and goldfish (de Pedro et al., 2000; López–Patiño et al., 1999; Narnaware and Peter, 2001; Narnaware et al., 2000); of *agrp* in channel catfish (Peterson et al., 2012), goldfish (Cerdá–Reverter et al., 2003a), zebrafish (Song et al., 2003) and Atlantic salmon (Valen et al., 2011); of *gal* in goldfish (de Pedro et al., 1995; Unniappan et al., 2004; Volkoff and Peter, 2001b); and of orexins in goldfish (Volkoff et al., 1999; Volkoff et al., 2003; Volkoff and Peter, 2000; Volkoff and Peter, 2001b). However, very few studies have focused on the nutritional regulation of these peptides, especially with regard to FA composition. This is surprising and needs to be urgently addressed, given that food intake is perhaps one of the most influential parameters for the success of any animal production operation, given its role in determining growth and body composition.

5. *Senegalese sole*

Senegalese sole was chosen as the model organism for all the experiments performed in the scope of this thesis. It is a predominantly littoral marine flatfish (order *Pleuronectiformes*, family *Soleidae*), with a main distribution along the eastern Atlantic coast, from France to Senegal and slightly less in the western Mediterranean. As a benthic species, it lives on sandy or muddy bottoms, up to 100 m depth, where it feeds on invertebrates, such as small polychaetes, bivalves and crustaceans (Fishbase, 2015).

Some of its most interesting characteristics are its particular nutritional requirements and metabolism. Contrary to many cultured marine finfish, Senegalese sole adults and juveniles do not perform well with high dietary lipid inclusions (≥12%; Borges et al., 2009; Valente et al., 2011), but are tolerant to high levels of FO substitution (100% replacement at 9% dietary lipids, although with presence of FM; Borges et al., 2014). Conversely, it seems capable of metabolizing carbohydrates with no detrimental effects on growth performance, while this is not the case for most carnivorous fish (Borges et al., 2013b). In this respect, it fits perfectly within the sustainability–oriented aquaculture production trends of today. Even larvae have proven to have relatively low LC–PUFA requirements, compared to other marine finfish, which appear to decrease even further after metamorphosis (Conceição et al., 2007; Dâmaso–Rodrigues et al., 2010; Morais and Conceição, 2009; Morais et al., 2004; Villalta et al., 2005b). Moreover, Senegalese sole is one of the rare carnivorous fish that has been shown to synthesize DHA from EPA via a Δ4 desaturation pathway (Morais et al., 2015b).

Moreover, Senegalese sole is also an economically important species, commercially cultured in southern Europe (France, Spain, Portugal and Italy), and is important for the wider European market in which it shares a market niche with the common sole (*Solea solea*) (Morais et al., 2015a). For the past two decades, it has been a candidate for diversifying European aquaculture production due to its high market value and demand (Dinis et al., 1999). Consequently, in the last ten years it has already seen a growth in popularity among fish producers of Spain and Portugal, and production is rapidly increasing—from only 8 t in 2002 to 640 t in 2013 (FAO, 2015b).

In the years since early culture attempts, significant work has been done in order to resolve some issues that have prevented the aquaculture development of this species. As a result of these efforts, mass production of juveniles was achieved more than a decade

ago (Dinis et al., 1999). Initial problems with bacterial diseases (tenacibaculosis, formerly flexibacteriosis; photobacteriosis, formerly pasteurellosis; and vibriosis) have been greatly minimized by changing from outdoor ponds to dedicated recirculating aquaculture systems (Cañavate, 2011), and it was established that by maintaining culture temperatures below 20–22°C it was possible to greatly prevent disease outbreaks (Zorrilla et al., 1999). Furthermore, although suitable vaccines have still not been developed, research is ongoing (Arijo et al., 2005). The issues that remain problematic include weaning to compound diets, pigmentation abnormalities and malformations, variable growth of juvenile fish and reproduction difficulties of G1 stocks (reviewed in Morais et al., 2015a). Natural spawning of wild–caught broodstock has been possible for several decades, although problems persist with reproduction of individuals from the first (F1) and second (F2) rearing generations, resulting in a strong dependency of wild–caught broodstock (Cañavate, 2005). It was recently confirmed by Carazo (2013) that this was due to lack of egg fertilization and thus connected to male reproductive dysfunction. While the synthesis and release of all relevant reproductive hormones seemed to be correct and in correlation to gonad development, plasma levels of reproductive hormones were consistently lower in cultured G1 (and G2) stocks compared with wild fish (Agulleiro, 2007; Guzmán et al., 2009). Furthermore, G1 males did not exhibit the typical reproductive behavioral cues (e.g. courtship) necessary for successful spawning (Carazo, 2013).

In current aquaculture setups (Fig. 6), females reach sexual maturation after 3 years of life (later than males) and spawn during spring to early summer, although a second spawning period in autumn is often achieved through manipulation of ambient parameters. Individual females of wild–caught broodstock were observed to spawn in intervals of 1–56 days, generally producing around 100,000 eggs kg^{-1} day^{-1} (Martín et al., 2014). Hatching time depends on water temperature, and ranges from 34 h at 21°C to 62 h at 15°C (Campos et al., 2013). Newly hatched larvae are small, altricial, planktonic predators with bilateral symmetry and measure around 2.4 ± 0.1 mm (Dinis et al., 1999; Morais et al., 2015a). The established practice is to feed rotifers to newly–hatched larvae from 2–6 dph, which are gradually substituted by *Artemia* (completely by 8 dph). Enriched *Artemia* metanauplii are administered to the larvae until around 19 dph and then gradually changed to frozen *Artemia* metanauplii over a period of 6 days (Dinis et al., 1999; Villalta et al., 2008).

Around 15 days post hatching (depending on the temperature), larvae begin metamorphosis, gradually losing their bilateral symmetry. This process is characterized by the flattening of the body, development of asymmetrical pigmentation and migration of the left eye to the occular side (Fig. 7b–d). During this period, larvae migrate to the bottom, orient their body with the left side facing downwards and commence a benthic lifestyle (Fernández–Díaz et al., 2001). They continue to ingest prey during metamorphosis, but not as effectively (Cañavate et al., 2006), and therefore need to increase their hepatic energy stores beforehand, to utilize during this period of extreme morphological and physiological change (Yúfera et al., 1999). Additionally, maturation of the digestive tract is only fully achieved at the end of metamorphosis (around 25 dph), although the main pancreatic and intestinal enzymes are efficient from the early stages (Ribeiro et al., 1999b). The larvae are generally fast growing and resistant, and readily complete metamorphosis with high survival rates (Dinis et al., 1999).

The weaning period, characterized by the transition from live prey to inert feed is another critical step in the lifecycle of cultured Senegalese sole (Dinis et al., 1999). Different feeding strategies have been tested during this sensitive step in sole culture (Engrola et al., 2007; Engrola et al., 2010; Engrola et al., 2009a; Engrola et al., 2009b) and co–feeding larvae with inert diets from mouth opening produced larger and better quality post–larvae at the end of weaning (Engrola et al., 2009a). In spite of these and other advances in rearing practices, considerable research is still centered on the early life stages, given that suboptimal larval rearing or nutritional conditions could have profound effects on later juvenile quality (Conceição et al., 2007; Dinis et al., 1999; Imsland et al., 2003). However, studies aimed at determining the specific nutritional requirements of Senegalese sole at different life stages will enable the formulation and commercialization of improved weaning and on–growing species–specific diets and, through the development of optimal dietary regimes, it will hopefully be possible to optimize the production cycle and reduce phenotypic abnormalities and growth dispersion of sole juveniles (Morais et al., 2015a).

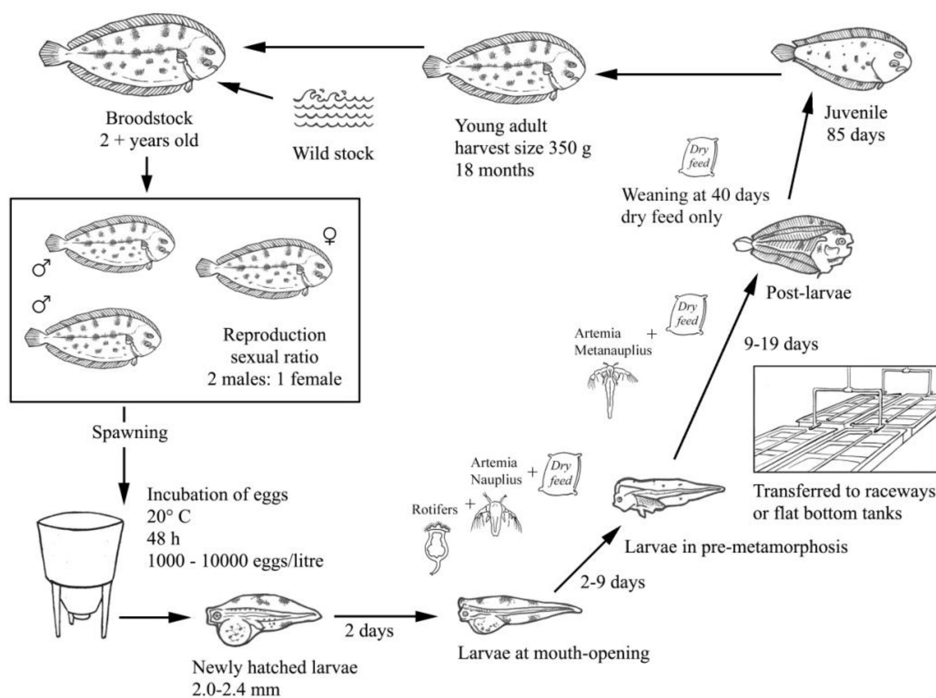

Figure 6. Production cycle of *Solea spp*. (FAO, 2015a).

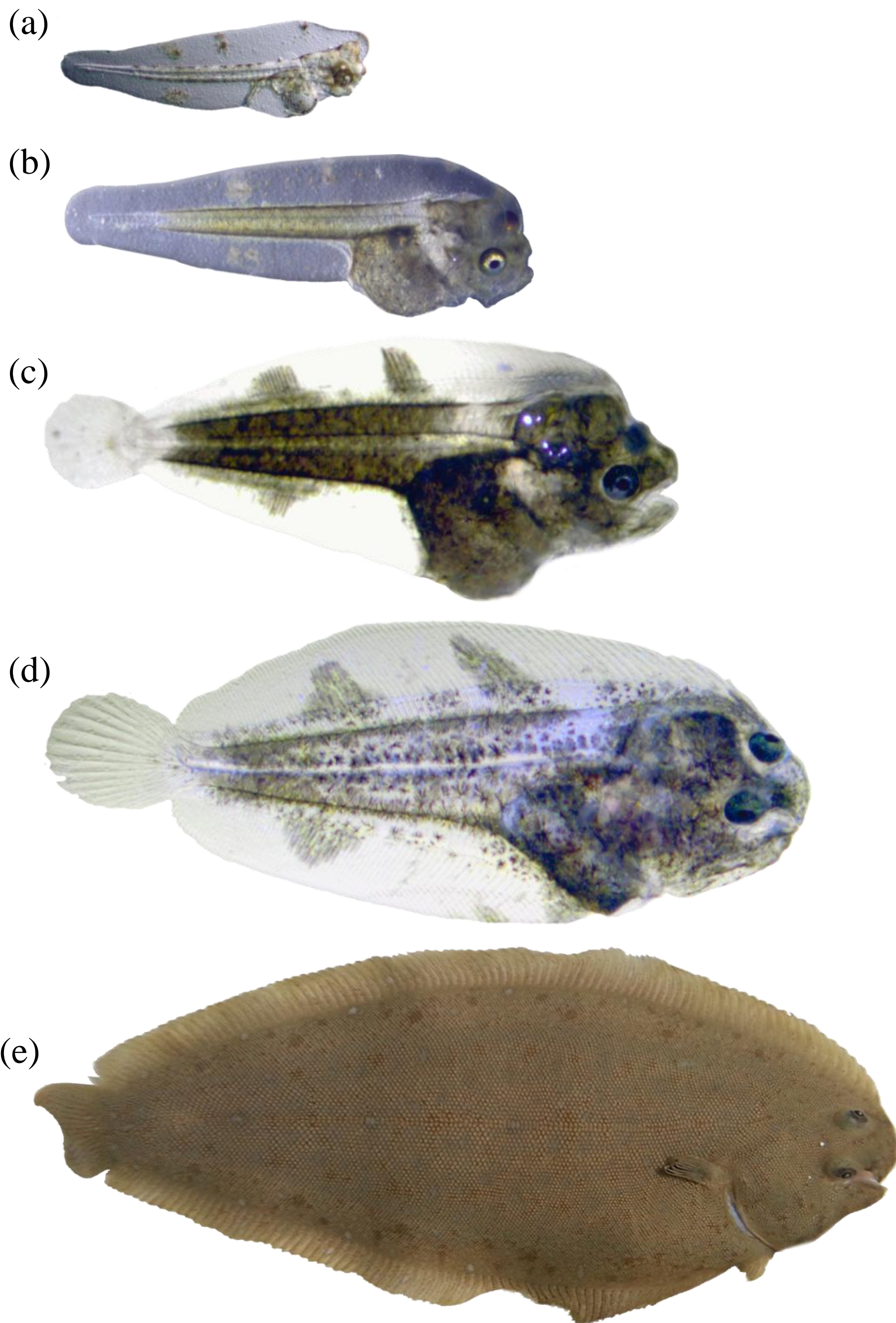

Figure 7. Senegalese sole larvae (a) before mouth opening (2 dph), (b) before (9 dph), (c) during (19 dph) and (d) after metamorphosis (22 dph), and (e) a juvenile specimen. Images are not to scale.

6. *Objectives*

Many studies have been performed looking at the partial or total replacement of FM and FO in aquafeeds by vegetable alternatives. These generally focus on several aspects of growth performance, fish health, changes in biochemical composition/product quality and metabolism. Although the effects have not always been consistent, it has been established relatively well that these dietary replacements can affect lipid metabolism and energy homeostasis. However, even though these changes are likely associated with potential effects on the regulation of appetite and food intake, this question has hardly been investigated in fish. Given the importance of food intake to the successful culture of any animal, the main objective of this thesis was to evaluate the potential impacts of alternative VO–based feed formulations on the regulation of food intake and attempt to uncover some of the possible physiological regulatory mechanisms behind it.

In order to address these questions, experiments combining a nutritional, physiological and molecular approach were performed with both larval and juvenile stages of the marine flatfish Senegalese sole (*Solea senegalensis*). These have been prepared as scientific manuscripts and are presented in this format in the following chapters:

CHAPTER 2: ***Dietary fatty acid metabolism is affected more by lipid level than source in Senegalese sole juveniles: Interactions for optimal dietary formulation***

The objective of this study was to observe the effects of FO replacement with VO, at different lipid levels (8% and 18%), on growth performance and key aspects of lipid absorption and metabolism in Senegalese sole juveniles.

CHAPTER 3: ***Mechanisms of lipid metabolism and transport underlying superior performance of Senegalese sole (Solea senegalensis, Kaup 1858) larvae fed diets containing n–3 polyunsaturated fatty acids***

This study was designed to analyze the effects of dietary FA composition (prevalent LC–PUFA, C_{18} PUFA or MUFA) on growth, development, lipid absorption and metabolism of Senegalese sole larvae and post–larvae.

CHAPTER 4: ***Characterization of seven cocaine– and amphetamine–regulated transcripts (CART) differentially expressed in the brain and peripheral tissues of Solea senegalensis (Kaup)***

Although most studies required the development of specific molecular tools (obtaining information on complete mRNA sequences for the design of RT–qPCR primers) in order to analyze the expression of key genes in Senegalese sole, and these often had several homologues, CARTs were especially interesting due to the unusually large amount of genes found in this species. Thus, the objective of this study was to describe their structure, brain and tissue distribution, basic regulation (response to feeding) and phylogenic relations to homologues in other species.

CHAPTER 5: ***Hypothalamic fatty acid sensing in Senegalese sole (Solea senegalensis): Response to long–chain saturated, monounsaturated, and polyunsaturated (n–3) fatty acids***

This experiment was performed to determine the presence of FA–sensing mechanisms in the hypothalamus of Senegalese sole juveniles and to additionally examine whether they respond differently to circulating FA, differing in chain length and degree of saturation.

CHAPTER 6: ***Food intake and appetite regulation in juvenile Solea senegalensis (Kaup, 1858) in response to dietary lipid level and oil source***

This study was performed in order to observe the effects of diets containing varying lipid levels (8% or 18%) and oil sources (100% FO or 75% VO) on food intake, and to attempt relating it with the expression of several gastrointestinal and neuropeptides in Senegalese sole juveniles, to gain further knowledge on their potential implication on appetite regulation mechanisms.

CHAPTER 7: ***Dietary fatty acid composition affects food intake and gut–brain satiety signaling in Senegalese sole (Solea senegalensis, Kaup 1858) larvae and post–larvae***

The objective of this study was to develop a method for the quantification of live prey intake and use it with Senegalese sole larvae and post–larvae to evaluate the effects of diets differing in FA composition on food intake. Furthermore, as in chapter 6, the transcriptional regulation of several gastrointestinal and neuropeptides was assessed, in an attempt to uncover possible underlying appetite–regulating mechanisms mediated by such peptides.

CHAPTER 2

Dietary fatty acid metabolism is affected more by lipid level than source in Senegalese sole juveniles: Interactions for optimal dietary formulation

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Abstract

This study analyses the effects of dietary lipid level and source on lipid absorption and metabolism in Senegalese sole (*Solea senegalensis*). Juvenile fish were fed 4 experimental diets containing either 100% fish oil (FO) or 25% FO and 75% vegetable oil (VO; rapeseed, linseed and soybean oils) at two lipid levels (~8% or ~18%). Effects were assessed on fish performance, body proximate composition and lipid accumulation, activity of hepatic lipogenic and fatty acid oxidative enzymes and, finally, on the expression of genes related to lipid metabolism in liver and intestine, and to intestinal absorption, both pre– and post–prandially. Increased dietary lipid level had no major effects on growth and feeding performance (FCR), although fish fed FO had marginally better growth. Nevertheless, diets induced significant changes in lipid accumulation and metabolism. Hepatic lipid deposits were higher in fish fed VO, associated to increased hepatic ATP citrate lyase activity and up–regulated carnitine palmitoyltransferase 1 (*cpt1*) mRNA levels post–prandially. However, lipid level had a larger effect on gene expression of metabolic (lipogenesis and β–oxidation) genes than lipid source, mostly at fasting. High dietary lipid level down–regulated fatty acid synthase expression in liver and intestine, and increased *cpt1* mRNA in liver. Large lipid accumulations were observed in the enterocytes of fish fed high lipid diets. This was possibly a result of a poor capacity to adapt to high dietary lipid level, as most genes involved in intestinal absorption were not regulated in response to the diet.

Keywords: fish oil, lipogenesis, beta–oxidation, fish nutrition, lipid absorption, fatty acid binding proteins, lipid trafficking, molecular biology

1. *Introduction*

Traditionally, fish oil (FO) has been used as the main dietary lipid source for cultured marine finfish (Turchini et al., 2009). However, the steady growth of aquaculture production worldwide, paired with reduced availability of raw materials from wild fisheries, has led to a rising need to replace FO, at least partly, with alternative and more sustainable ingredients, such as vegetable oils (VO) (Turchini et al., 2010). Therefore, in the past years numerous studies have focused on identifying suitable candidate VO for use in aquaculture by investigating their effect on growth and feed efficiency, health, reproduction and physiology of cultured organisms (Montero et al., 2003; Nasopoulou and Zabetakis, 2012; Sargent et al., 1999a; Turchini et al., 2009; Zuo et al., 2015). It has generally been shown that a 100% replacement of dietary FO with VO has a negative effect on the growth of most marine finfish species (Sales and Glencross, 2011). However, a large fraction (60–75%) of FO can be substituted without affecting growth and feed efficiency, if essential fatty acid requirements are met (Turchini et al., 2009). Nevertheless, not all species necessarily respond equally to dietary FO replacements and although these generalizations may be used as a benchmark, effects of dietary VO should be evaluated on a case by case basis (Turchini et al., 2009). Even then, results obtained for a species may vary depending on numerous factors such as trial duration, type of VO, amount of fish meal (source of FO) in the diet, fish size, age or developmental stage (Sales and Glencross, 2011), but also dietary lipid level (Figueiredo–Silva et al., 2012a; Jobling et al., 2002; Kenari et al., 2011; Kim et al., 2012; Tocher et al., 2003).

Most studies that focus on alternative dietary lipid sources use isolipidic diets and test various degrees of FO substitution with VO. However, studies that take into account the interrelation between lipid sources and lipid levels are fewer in number and address only a few aspects of lipid metabolism and growth performance. Furthermore, such types of studies are lacking in Senegalese sole (*Solea senegalensis*) – an interesting species for lipid–related research, due to some particularities in its nutritional physiology, and one of high commercial aquaculture importance in southern Europe (Morais et al., 2015a). Senegalese sole is a lean flatfish (1–4 g fat / 100 g of flesh) with a limited ability to efficiently use high dietary lipid levels (≥12%), at both juvenile (Borges et al., 2009) and adult stages (Valente et al., 2011). Other demersal lean fish, including different species of sole, flounder and catfish (Borges et al., 2013a; Borges et al., 2013b; Borges et al., 2009; Guerreiro et al., 2012; Lee et al., 2000) generally do not cope well with high fat diets, contrary to salmonids (Einen and Roem, 1997; Hemre and Sandnes, 1999) and more active marine species like sparids or European seabass (*Dicentrarchus labrax*) (Peres and Oliva–Teles, 1999a; Skalli et al., 2004; Vergara et al., 1996) which utilize dietary lipids more efficiently. On the other hand, even complete replacement of dietary FO in Senegalese sole with different VO (at 9% dietary lipids) did not seem to affect fish performance negatively (Borges et al., 2014a). Nevertheless, other criteria should also be considered to assess the physiologic and metabolic effects of different lipid sources. For instance, Montero et al. (2008) and Caballero et al. (2006) observed no detrimental effect of FO substitution on growth of gilthead seabream (*Sparus aurata*), but several types of intestinal morphological alterations, like lipid droplet accumulation, were observed in fish fed high contents of VO–based diets. Considering that any adverse effects of new dietary formulations on the cultured organism can potentially have significant economic

repercussions when applied in large–scale aquaculture production, this type of research is not only interesting from a scientific point of view, but also of great importance to the development of the commercial sector.

This study was designed to further study the mechanisms of lipid absorption and metabolism in Senegalese sole and contribute to the growing knowledge of the nutritional physiology of this interesting species. Since both the type and quantity of ingested fats are known to regulate hepatic gene expression (Jump, 2004), we investigated if the effects of alternative dietary lipid sources were modulated by their levels of inclusion in the diet and whether their long–term administration had different effects pre– and post–prandially. To address this, we tested 4 experimental diets containing either 100% FO or 25% FO and 75% VO at two lipid levels (~8% or ~18%). The effects of these dietary treatments were assessed by analyzing growth performance, hepatosomatic index (HSI), viscerosomatic index (VSI), whole–body proximate composition, lipid accumulation in specific tissues, activity of lipogenic and fatty acid (FA) oxidative enzymes in the liver and, finally, expression of genes related to lipid metabolism in both liver and intestine, and also of genes involved in lipid uptake, intracellular transport and basolateral secretion in the intestine. The fatty acyl elongation and desaturation activities, as well as the FA profiles of key tissues, were previously reported by Morais et al. (2015).

2. *Materials and methods*

Experimental diets

Four isoproteic extruded diets were formulated and manufactured by Sparos Lda. (Olhão, Portugal). They differed in total lipid level (~8% or ~18%) and fatty acid composition, using FO or VO as the main lipid source. The 8FO and 18FO diets had 100% of the lipid supplied by FO, while 75% of the FO in diets 8VO and 18VO was replaced by a VO blend (rapeseed, soybean, and linseed oil in a ratio of 1:1:1; Table 1). Proximate and FA composition of the experimental diets were analyzed in triplicate, as described below, and are presented in Tables 1 and 2, respectively.

Growth trial and sampling

Senegalese sole juveniles with an average body weight (BW) of 5.0 ± 0.1 g were distributed into twelve rectangular flat bottom 20 l tanks (50 fish per tank) connected to a recirculation system at CCMAR, University of Faro, Portugal, and maintained at a temperature of 19.3 ± 1.2 ºC, a salinity of 32, and a 12 h light/12 h dark photoperiod for 13 weeks. They were fed 2 mm extruded diets using automatic feeders 22 h per day. Feed doses were adjusted daily to ensure the fish were fed to satiety. In the case of excess uneaten feed, rations were reduced by 10% and in the absence of uneaten feed increased by 10%. All fish were sampled after fasting for 24 hours. Prior to sampling fish were euthanized with a lethal dose of tricaine methanesulfonate (MS222; Sigma, Sintra, Portugal). Fish were weighed at 28, 53, 77 and 91 days after the start of the experiment (n = 3 pools of 30 individuals), with the additional weighing of whole fish, liver and viscera of 8 fish per tank in the final sampling to calculate HSI and VSI (n = 24

Table 1. Formulation and proximate composition of the experimental diets.

	8FO	8VO	18FO	18VO
Ingredients (%)				
Fishmeal 70 LT[1]	22.00	22.00	22.00	22.00
Fishmeal 60[2]	15.00	15.00	15.00	15.00
Fish protein hydrolisate[3]	5.00	5.00	5.00	5.00
Squid meal[4]	5.00	5.00	5.00	5.00
Pea protein concentrate[5]	4.00	4.00	4.00	4.00
Soy protein concentrate[6]	2.00	2.00	2.00	2.00
Soybean meal 48[7]	9.80	9.80	10.00	10.00
Wheat gluten[8]	7.00	7.00	10.10	10.10
Corn gluten meal[9]	5.00	5.00	4.50	4.50
Pea grits[10]	11.10	11.10	2.50	2.50
Wheat meal	9.00	9.00	4.80	4.80
Fish oil[11]	2.60	0.65	12.60	3.15
Rapeseed oil[12]	–	0.65	–	3.15
Soybean oil[12]	–	0.65	–	3.15
Linseed oil[12]	–	0.65	–	3.15
Vitamin & Mineral Premix[13]	1.00	1.00	1.00	1.00
Binder (guar gum)[14]	1.00	1.00	1.00	1.00
Proximate composition				
Moisture (%)	5.5	4.6	4.3	4.4
Crude Protein (% DM)	56.0	56.9	58.0	57.2
Crude Fat (% DM)	7.9	7.4	17.6	17.4
Ash (% DM)	10.5	10.7	10.4	10.3

[1] Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru.
[2] Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF, COFACO, Portugal.
[3] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[4] Super prime squid meal: 80% CP, 3.5% CF, Sopropêche, France.
[5] Lysamine GP: 78% CP, 8% CF, ROQUETTE, France.
[6] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[7] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal.
[8] VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.
[9] Corn gluten feed: 61% CP, 6% CF, COPAM, Portugal.
[10] Aquatex G2000: 24% CP, 0.4% CF, SOTEXPRO, France.
[11] COPPENS International, The Netherlands.
[12] Henry Lamotte Oils GmbH, Germany.
[13] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL–alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL–cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.
[14] Guar gum 101 HV– E412, Seah International, France.

fish per treatment). Specific growth rate (SGR) was calculated based on values at the end of the experimental period (n = 3 pools of 30 fish). To analyze whole–body proximate composition, 2 fish were taken from each tank, frozen and later homogenized (n = 3 homogenates per treatment). Total lipids of intestine, liver and muscle tissues were analyzed in pools from 3 fish per tank (n = 3 pools per treatment). The intestine and liver were sampled from 3 fish per tank (n = 9 per treatment) for optical histology, stored in 10% buffered formalin for 24 h, and subsequently transferred to 70% ethanol until analysis. A section of liver from 5 fish per tank (n = 15 per treatment) was taken for measurement of lipogenic and FA oxidative enzyme activities. Tissue samples for enzyme, proximate and total lipid analysis were instantly frozen on dry ice and stored at –80 ºC until analysis.

The study was conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE).

Table 2. Fatty acid composition of the experimental diets, expressed as mean %mol ± SD (n = 3).

	8FO	8VO	18FO	18VO
14:0	2.6 ± 0.4	1.8 ± 0.7	4.8 ± 0.8	1.7 ± 0.1
15:0	0.4 ± 0.0	0.3 ± 0.1	0.5 ± 0.0	0.2 ± 0.0
16:0	18.4 ± 0.6	16.2 ± 0.7	19.1 ± 0.2	13 ± 0.8
18:0	4.3 ± 0.1	4.1 ± 0.1	4.7 ± 0.1	3.9 ± 0.4
Total saturated	25.8 ± 1.1	22.5 ± 1.3	29 ± 0.6	18.8 ± 1.2
16:1	5.6 ± 0.6	4.5 ± 0.6	6.2 ± 1.0	3.8 ± 0.0
18:1n–9	14.2 ± 0.2	18.5 ± 0.2	12.6 ± 0	23.5 ± 0.7
18:1n–7	3.0 ± 0.8	2.7 ± 0.6	3 ± 0.6	3.1 ± 1.4
20:1	4.8 ± 0.1	4.4 ± 0.1	3.6 ± 0.2	2.4 ± 0.2
Total monounsaturated	27.6 ± 0.3	30.2 ± 0.1	25.3 ± 1.8	32.8 ± 0.5
18:2n–6	12.4 ± 0.4	17.7 ± 0.5	6.8 ± 0.1	20.5 ± 0.2
18:3n–6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:3n–6	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:4n–6	1.0 ± 0.2	0.6 ± 0.0	1.2 ± 0.0	0.4 ± 0.0
22:4n–6	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
22:5n–6	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.3	0.2 ± 0.0
Total n–6 PUFA	14.2 ± 0.4	18.9 ± 0.5	8.8 ± 0.2	21.4 ± 0.1
18:3n–3	1.8 ± 0.1	5.6 ± 0.1	1.3 ± 0.0	12.1 ± 0.6
18:4n–3	2.0 ± 0.0	1.5 ± 0.1	2.4 ± 0.2	1.2 ± 0.1
20:4n–3	0.6 ± 0.2	0.4 ± 0.0	0.8 ± 0.0	0.3 ± 0.0
20:5n–3	14.7 ± 0.3	10.4 ± 0.5	17.2 ± 0.7	7.0 ± 0.1
21:5n–3	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0
22:5n–3	1.0 ± 0.0	0.7 ± 0.0	1.3 ± 0.2	0.5 ± 0.0
22:6n–3	11.2 ± 0.4	8.6 ± 0.3	11.3 ± 0.9	5.2 ± 0.1
Total n–3 PUFA	31.5 ± 0.5	27.4 ± 0.7	34.8 ± 1.9	26.3 ± 0.7
Total PUFA	45.6 ± 0.9	46.3 ± 1.3	43.6 ± 2.1	47.7 ± 0.8
n–3/n–6 PUFA	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.1
DHA/EPA	0.2 ± 0.1	0.6 ± 0.1	0.8 ± 0.3	0.3 ± 0.2
ARA/EPA	0.4 ± 0.0	0.2 ± 0.0	0.8 ± 00	0.2 ± 0.1

Feeding trial and sampling for molecular analysis

A feeding trial was performed during week 13 in order to measure pre– and post–prandial expression levels of genes involved in lipid metabolism and absorption. Two fish per tank were sampled after 24 hours of fasting (t0, pre–prandial; n = 6 per treatment) and another two were then fed their respective diets and sampled 6 h after feeding (t6, post–prandial; n = 6 per treatment). In order to ensure that all fish ate an equal amount of feed at the appointed time, and considering the highly variable voluntary feed intake of sole (Dias et al., 2010), a force–feeding method was used. Plastic tubes were filled with 10 pellets each (~0.15% BW) and inserted up to the back of the oral cavity of previously anesthetized (MS222, 50 ppm) fish. A piston was then used to push the contents of the tube into the esophagus and the fish were monitored for a few minutes before being returned to the tanks, in order to ensure that the pellets were not expelled. At both time points 100–150 mg samples of intestine and liver were collected and transferred into eppendorf tubes with 1.5 ml of RNAlater stabilization buffer (Ambion, Life Technologies, Madrid, Spain). Samples were kept in agitation at 4 °C for 24 h, and then stored at –80 ºC.

Whole body proximate composition and total lipid levels of tissues

Protein content in extruded diets was carried out by the Dumas method using a protein standard (LECO FP–528) (AOAC, 2005b). Water and ash content were calculated in subsamples of each diet (n = 6) and whole body homogenates by drying them at 105 °C until a constant weight was obtained, or after ashing in a muffle furnace for 5 h at 550 °C, respectively (AOAC, 2005a). For the analysis of protein content (according to Lowry et al., 1951; following overnight hydrolysis in sodium–hydroxide solution), carbohydrates (Dubois et al., 1956), and lipids (extracted as in Folch et al., 1957) and quantified gravimetrically), 5 subsamples were taken for each analysis from each homogenate (n = 15 per treatment). Total lipids from intestine, liver and muscle pooled from 3 fish per tank (3 pools per treatment) were also extracted following the method of Folch et al., 1957) and quantified gravimetrically. The tissue total lipid results are presented as both percentage (%) of dry weight (DW) to reduce variability associated with water content of tissue, but also as wet weight (WW) for discussion purposes, as numerous studies use these values. Water content was calculated as the difference between WW and DW and presented as a percentage of WW. Finally, total lipid content of liver and intestine was also expressed as mg 100 g^{-1} fish.

Histological analyses

Samples were serially sectioned at 6–7 μm and stained with Harris haematoxylin and eosin. Sections of the intestine and liver were photographed at x40 magnification (300 dpi; 3 photos per tissue, n = 27 photos per treatment) with an Olympus DP70 digital camera connected to a Leica DM 2000™ microscope. All digital image analyses were performed using ImageJ (U.S. National Institutes of Health, Bethesda, USA; http://rsbweb.nih.gov/ij/index.html). A semi–automatic quantitative analysis was employed in order to measure the percentage of intestinal epithelium (longitudinal transects) and hepatocytes occupied by fat deposits. By adjusting the color threshold settings to brightness values between 195 and 255, the program was configured to select

all surfaces covered with a white to light pink color, which corresponded to the area of fat deposits (Gisbert et al., 2008). These values were used to calculate the percentage of area occupied by lipids as in Boglino et al. (2012). For liver samples, the total area of analyzed tissue was a rectangular selection, while transects of the intestinal epithelium were selected manually with the “polygon selection” tool and the diameters of intestinal lipid droplets were measured using the "straight" tool of ImageJ.

Lipogenic and FA oxidative enzyme activities

Activities of ATP citrate lyase (Acly; lipogenic) and 3–hydroxyacyl–CoA dehydrogenase (Hoad; FA oxidative) and carnitine palmitoyltransferase 1 (Cpt1; FA oxidative) were assessed in 100 mg liver samples homogenized by ultrasonic disruption with 7 volumes of ice–cold buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4–dithiothreitol, and a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA; P–2714). The homogenate was centrifuged and the supernatant taken and immediately frozen on dry ice and stored at –80 ºC until analysis. Enzyme activities were determined in a microplate reader Elx–tec (Biotek). Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (10 µl, 7.5 µl (+7.5 µl of H_2O) and 5 µl for Acly, Hoad and Cpt1, respectively) to buffer solutions containing the substrates (0.10 mM of CoA, 0.05 mM of Acetoacetil–CoA and 1.00 mM of L–carnitine hydrochloride for Acly, Hoad and Cpt1, respectively), which were omitted in control wells (final volume 275–280 µl). The reactions were allowed to proceed at 37 °C for pre–established periods of time (10–30 min). Enzymatic analyses were carried out at maximum rates, with the reaction mixtures optimized in preliminary tests to render optimal activities by adapting methods previously described for Acly (Alvarez et al., 2000), Hoad (Kolditz et al., 2008) and Cpt1 (Ditlecadet and Driedzic, 2013). Total protein content in homogenates was assayed in duplicate, according to the bicinchoninic acid method (Smith et al., 1985), using bovine serum albumin (Sigma) as a standard. Enzyme activities were normalized by mg of protein.

Primer design

Primers for real time quantitative PCR (RT–qPCR) for most genes analyzed in this study (Table 3) are from previous work (our own unpublished data), but primers for *cpt1* and for a transcript belonging to the cluster of differentiation 36 family, the platelet glycoprotein 4–like (*cd36*) were designed specifically for this study. Sequences of target genes were searched by gene annotation in the SoleaDB database (http://www.scbi.uma.es/soleadb) *Solea senegalensis* v4.1 global assembly and the retrieved fragments were assembled *in silico* into contigs using the BioEdit Sequence Alignment Editor (Hall, 1999). A blastx search was performed in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) to compare with orthologs in other fish and vertebrate species and identify the open reading frames (ORF) and 3’ and 5’ untranslated regions (UTR) of the sequences. When 5’ and 3' UTR's were missing, rapid amplification of cDNA ends (RACE) PCR was performed using the FirstChoice® RLM–RACE kit (Ambion). The obtained fragments were separated by

Table 3. Primers used for real–time quantitative PCR (qPCR). Shown are sequences and annealing temperature (Ta) of the primer pairs, size of the fragment produced, average reaction efficiency (E) and accession number of the target and reference genes.

Gene	Primer sequence (5'–3')	Amplicon size	Ta	E*	Accession number
Lipid absorption					
cd36	TGAATGAGACGGCTGAGTTG	168 bp	64 ºC	100,3%	KR872889
	TGTTGTTTCTGCTCCTCACG				
fabp1	GCTCATCCAGAAAGGCAAAG	199 bp	62 °C	102,1%	KP842779
	GGAGACCTTCAGCTTGTTGC				
fabp2a	ACACACATGACCTTAGCACACTG	70 bp	60 °C	102,3%	KP842780
	TGCGAGTATCAAAATCCGGTA				
apoa4	AGGAACTCCAGCAGAACCTG	122 bp	60 °C	100,2%	KP842775
	CTGGGTCATCTTGGAGAAGG				
mtp	CAGGCGTACACCACATGTAAA	150 bp	60 °C	102,3%	KP842778
	GTGATCAGGCTTCTGCAGTG				
Lipid metabolism					
acox1	GGTCCATGAATCTTTCCACAA	168 bp	60 °C	104,1%	KP842776
	ACAAGCCTGACGTCTCCATT				
fas	CACAAGAACATCAGCCGAGA	197 bp	60 °C	103,1%	KP842777
	GAAACATTGCCGTCACACAC				
cpt1	TAACAGCCACCGTCGACATA	156 bp	63 ºC	101,1%	KR872890
	AGCGATTCCCTTGTGTCACT				
Reference genes					
ubq	AGCTGGCCCAGAAATATAACTGCGACA	93 bp	70 °C	101,3%	AB291588
	ACTTCTTCTTGCGGCAGTTGACAGCAC				
rps4	GTGAAGAAGCTCCTTGTCGGCACCA	83 bp	70 °C	100,9%	AB291557
	AGGGGGTCGGGGTAGCGGATG				
eef1a1	GATTGACCGTCGTTCTGGCAAGAAGC	142 bp	70 °C	100,5%	AB326302
	GGCAAAGCGACCAAGGGGAGCAT				

*Efficiency corresponds to an average of 2 runs (intestine tissue at t0 and t6) for lipid absorption and 4 runs (intestine and liver tissues at t0 and t6) for lipid metabolism genes.

cd36, cluster of differentiation 36 family (platelet glycoprotein 4–like); *fabp*, fatty acid binding protein; *apoa4*, apolipoprotein A–IV; *mtp*, microsomal trygliceride transfer protein; *acox1*, acyl–CoA oxidase 1; *fas*, fatty acid synthase; *cpt1*, carnitine palmitoyltransferase 1; *ubq*, ubiquitin; *rps4*, 40S ribosomal protein S4; *eef1a1*, elongation factor 1 alpha.

gel electrophoresis and resulting bands of the expected length were cut, purified (Illustra GFX™ PCR DNA and gel band purification kit, GE Healthcare, Barcelona, Spain) and sequenced (SCSIE, University of Valencia, Spain). The transcript sequences thus obtained were used as templates to design primers for qPCR, using Primer3 v. 0.4.0 (Koressaar and Remm, 2007). The qPCR conditions were then optimized and the obtained amplicons were sequenced to confirm their identity and the specificity of the qPCR assay. Several homolog genes were obtained for Senegalese sole *cd36* and *cpt1*, but only one transcript of each was selected for use in this study primarily based on the pattern of tissue distribution (unpublished data) and highest sequence homology to the specific gene of interest, chosen based on previous studies in other species. In this

respect, the *cpt1* gene was equally similar to both *cpt1a* and *cpt1b* which are the genes most commonly assayed in rainbow trout (*Onchorhynchus mykiss*) liver to study effects of FA composition on lipid metabolism (Librán–Pérez et al., 2012).

RNA extraction and real time qPCR

For RNA extraction, samples were homogenized in 1 ml of TRIzol (Ambion) with 50 mg of 1mm diameter zirconium glass beads (Mini–Beadbeater, Biospec Products Inc., U.S.A.). Solvent extraction was performed following manufacturer's instructions and RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain). Two micrograms of total RNA per sample were reverse transcribed into cDNA using the High–Capacity cDNA RT kit (Applied Biosystems, Life Technologies, U.S.A.), following manufacturer's instructions, but using a mixture of random primers (1.5 µl as supplied) and anchored oligo–dT (0.5 µl at 400 ng/µl, Eurogentec, Cultek, S.L., Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. A pool was created from 2 µl of cDNA from all samples of a single tissue to use for dilution series and samples were then diluted 60–fold with water.

Amplifications were carried out in duplicate on a CFX96 Touch Real–Time PCR Detection System (Bio–Rad, Alcobendas, Spain) in a final volume of 20 µl containing 5 µl of diluted (1/60) cDNA, 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio–Rad) and 500 nM primers (except for *cpt1*, *cd36* and fatty acid–binding protein 1 (*fabp1*), where 150 nM, 200 nM and 150 nM were used, respectively). A systematic negative control (NTC–non template control) was also included. The qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 35 cycles: 15 s at 95 °C, 1 min at the corresponding annealing temperature (Ta; Table 2). After the amplification phase, a melt curve was performed enabling confirmation of the amplification of a single product in each reaction. Non–occurrence of primer–dimer formation in the NTC was also confirmed. The amplification efficiency of each primer pair was assessed from serial dilutions of the cDNA pool.
The qPCR results were imported into the software qBase+ (Biogazelle, Zwijnaarde, Belgium), where normalized relative quantities (NRQ) were calculated employing target and run–specific amplification efficiencies and using the geometric mean of 3 reference genes (elongation factor 1 alpha, ubiquitin and 40S ribosomal protein; *eef1a1, ubq* and *rps4,* respectively; Infante *et al*., 2008). The stability of the reference genes was checked (Vandesompele et al., 2002) and results for the combination of the 3 genes at t0/t6 were: M = 0.276/0.260, coefficient of variance – CV = 0.110/0.102 for intestine and M = 0.149/0.134, CV = 0.061/0.053 for liver.

Statistical analysis

All statistical analyses were performed with SPSS v.20 (SPSS Inc., Chicago, IL, USA). A two–way ANOVA, with factors lipid source and lipid level, at a significance level of 0.05, was used to analyze the results.

3. *Results*

Diet fatty acid composition

As shown in Table 2, the 18FO diet provided the highest relative and absolute levels of the long–chain polyunsaturated fatty acids (LC–PUFA) eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) and saturated fatty acids (SFA), but lowest levels of C_{18} polyunsaturated fatty acids (PUFA). On the other hand, the 18VO diet provided the lowest relative levels (but not in absolute content) of LC–PUFA, and had the highest relative and absolute linoleic acid (LNA), α–linolenic acid (ALA) and monounsaturated fatty acid (MUFA) content.

Fish performance and body composition

At the end of the 3–month experimental period, a four–fold size increase was recorded in juvenile Senegalese sole from all treatments (Table 4). Fish cultured on FO diets grew slightly more, albeit non–significantly ($p = 0.06$ for final BW and SGR), compared to those fed diets with 75% VO, while no differences were observed with regard to lipid level. However, the food conversion ratio (FCR) did not differ between treatments. The HSI was significantly higher in fish fed high lipid diets, with fish fed 18VO having the largest livers, and interaction between the dietary factors was only marginally non–significant ($p = 0.06$). The results for VSI showed a significant interaction between the two dietary factors, and fish from the 18VO group had the highest values.

In juvenile sole fed high lipid diets, crude lipids constituted a significantly higher proportion of the whole body compared to those fed a low lipid diet (Table 4). However, no noticeable effect of lipid source was observed. Conversely, the moisture content and protein fraction were significantly lower in fish fed high lipid diets. Carbohydrate levels were generally low, but a significant interaction between the dietary factors was observed, with values being higher in the 8FO and 18VO treatments.

Total lipid content of different tissues was also measured (Table 4). In the liver and muscle, no significant differences were observed between treatments, but in the intestine fish fed 18% lipid diets showed significantly higher lipid accumulation than those fed lower levels of dietary lipids.

Histological assessment of fat accumulation in the intestine and liver

Fish fed the VO diets had a significantly higher percentage of fat within the hepatic parenchyma compared to fish fed FO, showing a more compact hepatic parenchyma, a reduction in size of hepatic sinusoids and higher size of lipid deposits within hepatocytes (Table 4, Fig. 1). In the intestine, clear differences were observed between treatments with regard to lipid level (Fig. 2). Large lipid accumulations (numerous lipid droplets with up to 16 μm in diameter) were observed in fish fed the 18% lipid diets, while the 8% lipid groups had no or very little lipid inclusions of a very small diameter (~1.5 μm). This was also confirmed by image analysis (Table 4), although in this case there was also a significant interaction between the two dietary factors.

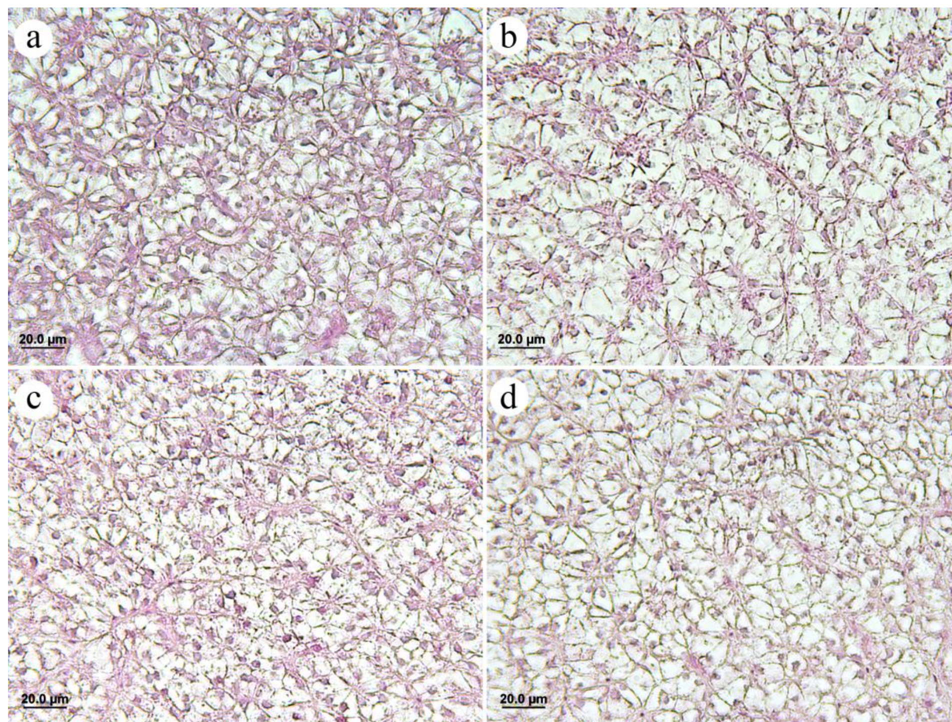
Table 4. Growth and feeding performance, HSI, VSI, proximate composition, lipid content of selected tissues and tissue area occupied by lipid droplets (surface occupied, as percentage of total tissue surface) in liver and intestine of juvenile Senegalese sole cultured on 4 different diets during a period of 13 weeks. Values are expressed as mean ± SD. Results of 2–way ANOVA analysis in relation to dietary factors lipid level (LL), lipid source (LS) and their interaction (LL×LS) are also presented.

		Dietary treatment				P–value (2–way ANOVA)		
	Day	8FO	8VO	18FO	18VO	LL	LS	LL×LS
BW (g)	0	5.0 ± 0.07	5.0 ± 0.05	5.0 ± 0.70	4.9 ± 0.08	0.141	1.000	0.438
	28	10.7 ± 0.14	10.4 ± 0.30	10.7 ± 0.50	10.0 ± 0.02	0.401	0.012	0.178
	53	16.6 ± 1.68	16.0 ± 0.75	16.7 ± 1.70	15.0 ± 0.50	0.571	0.140	0.461
	77	20.8 ± 1.82	18.6 ± 0.92	17.6 ± 1.70	17.9 ± 0.74	0.040	0.278	0.162
	91	23.4 ± 2.80	21.7 ± 1.16	23.7 ± 2.00	20.7 ± 0.95	0.721	0.058	0.555
SGR (%/day)		1.69 ± 0.14	1.60 ± 0.06	1.71 ± 0.09	1.57 ± 0.04	0.925	0.060	0.639
FCR		1.31 ± 0.12	1.30 ± 0.06	1.16 ± 0.10	1.30 ± 0.09	0.195	0.303	0.208
HSI (% BW)		1.04 ± 0.28	1.00 ± 0.25	1.10 ± 0.21	1.27 ± 0.28	0.002	0.228	0.057
VSI (% BW)		3.68 ± 0.73	3.56 ± 0.56	3.57 ± 0.38	4.03 ± 0.41	0.111	0.134	0.009
Whole–body proximate composition (% DW)								
Crude Fat		16.4 ± 1.13	17.1 ± 1.02	23.2 ± 0.80	21.5 ± 0.28	<0.001	0.251	0.006
Crude Protein		50.3 ± 3.01	50.9 ± 3.53	45.0 ± 2.92	45.5 ± 2.65	<0.001	0.989	0.909
Carbohydrates		1.4 ± 0.46	1.3 ± 0.30	1.3 ± 0.21	1.4 ± 0.37	0.948	0.772	0.020
Ash		2.4 ± 0.05	2.4 ± 0.05	2.3 ± 0.38	2.4 ± 0.16	0.484	0.834	0.830
Moisture†		74.4 ± 0.50	75.2 ± 0.43	72.4 ± 1.14	73.1 ± 1.13	<0.001	0.010	0,747
Total lipids in tissues								
Liver	% WW	4.2 ± 0.8	4.1 ± 1.0	5.2 ± 0.4	5.8 ± 2.1	0.130	0.749	0.665
	% DW	13.1 ± 2.6	12.5 ± 3.5	15.2 ± 1.8	15.6 ± 5.5	0.279	0.967	0.818
	mg 100 g^{-1} fish*	44.0	41.4	57.3	74.0			
Intestine	% WW	2.1 ± 0.1	1.7 ± 0.5	2.8 ± 0.6	3.4 ± 0.2	0.001	0.768	0.070
	% DW	8.8 ± 1.5	8.7 ± 3.7	12.7 ± 2.7	12.8 ± 1.2	0.026	0.991	0.953
	mg 100 g^{-1} fish*	77.1	60.9	101.1	135.1			
Muscle	% WW	0.4 ± 0.0	0.6 ± 0.3	0.5 ± 0.1	0.5 ± 0.1	0.703	0.400	0.400
	% DW	1.7 ± 0.3	2.3 ± 1.1	2.2 ± 0.4	2.3 ± 0.2	0.461	0.333	0.415
Tissue area occupied by lipid droplets (%)								
Liver		54.8 ± 4.72	58.5 ± 4.43	56.0 ± 4.37	58.0 ± 2.37	0.610	<0.001	0.294
Intestine		8.4 ± 3.25	3.8 ± 3.86	25.4 ± 4.59	26.8 ± 8.37	<0.001	0.238	0.032

*Calculated as (lipid % of tissue WW /100) × HSI (or VSI) × 1000

†Moisture content is the difference of WW and DW, expressed as percentage of WW

Figure 1. Lipid droplet accumulation in hepatocytes of Senegalese sole juveniles fed dietary treatments differing in lipid source and level of lipid inclusion: 8FO (a), 8VO (b), 18FO (c), 18VO (d).



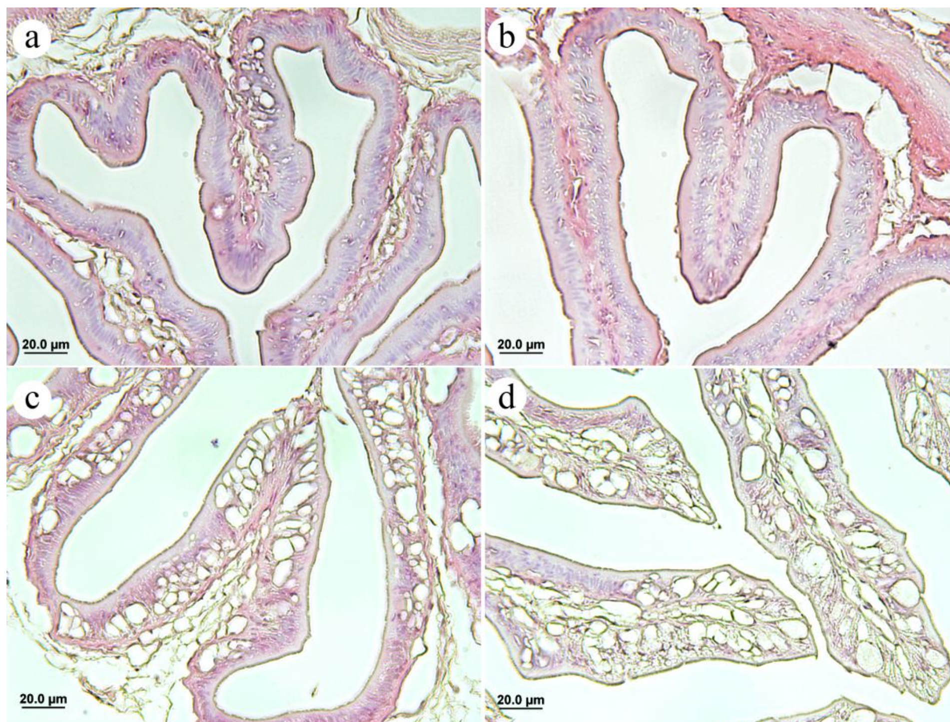
Activity of lipogenic and FA oxidative enzymes

Significant differences were observed only in the relative hepatic activity of Acly, where fish fed the VO diets had higher activity than those fed FO (Table 5). There was a high variability in the results and no significant differences were observed in the relative activity of Hoad or Cpt1.

Table 5. Relative activity of lipogenic enzymes (mUI/mg protein) measured in the liver of Senegalese sole juveniles fed 4 different dietary treatments. Values are shown as means ± SD (n = 15 per treatment). Results of 2–way ANOVA analysis in relation to dietary factors lipid level (LL), lipid source (LS) and their interaction (LL×LS) are also presented.

	Dietary treatment				P–value (2–way ANOVA)		
	8FO	8VO	18FO	18VO	LL	LS	LL×LS
Hoad	31.9 ± 15.83	34.0 ± 12.76	28.8 ± 13.98	35.7 ± 10.00	0.855	0.240	0.525
Acly	17.9 ± 9.21	23.3 ± 15.80	18.8 ± 10.88	30.1 ± 10.89	0.238	0.014	0.371
Cpt1	21.4 ± 12.30	21.4 ± 10.06	25.3 ± 8.64	26.3 ± 15.15	0.169	0.874	0.882

Figure 2. Lipid droplet accumulation in enterocytes of the posterior intestine in Senegalese sole juveniles fed dietary treatments differing in lipid source and level of lipid inclusion: 8FO (a), 8VO (b), 18FO (c), 18VO (d).



Gene expression assessed by RT–qPCR

The expression levels of key genes involved in lipid absorption and metabolism were measured by RT–qPCR in the intestine and liver of Senegalese sole juveniles before (t0; Table 6) and 6 h after feeding (t6; Table 7). In the liver, fatty acid synthase (*fas*) was down–regulated and *cpt1* up–regulated in fish fed high lipid diets at t0. However, at t6, *cpt1* expression was marginally higher ($p = 0.06$) in fish fed the FO diets, while no changes were observed with regard to LL.

In the intestine, high lipid diets also caused a down–regulation of *fas* at t0, while at t6, *cpt1* was the only gene with significant differences in transcript levels, being elevated only in the 8VO treatment (showing a significant effect of lipid level and lipid source, but also a significant interaction between the two factors).

Regarding genes related to lipid absorption, *cd36* was significantly more expressed in fish fed low lipid diets at t0, while microsomal trygliceride transfer protein (*mtp*) was significantly affected by dietary lipid level, whereas highest mRNA levels were observed in the 18VO treatment. At t6, *cd36* was significantly affected by lipid level and lipid source, being more highly expressed in fish fed lower lipid levels and FO diets, hence resulting in highest mRNA levels in the 8FO treatment.

No significant differences were observed in transcript levels of *acox1*, *fabp1*, *fabp2a* or *apoa4* in relation to any of the dietary factors.

Table 6. Pre–prandial expression of lipid metabolism and absorption genes in Senegalese sole juveniles assessed by RT–qPCR at t0 (after 24 h of fasting) regulated by dietary lipid level (LL; 8% or 18% lipids) and/or source (LS; VO or FO). Values are mean NRQs ± SD (n = 6 per treatment).

	8FO	8VO	18FO	18VO	p–value (2–way ANOVA)		
					LL	LS	LL×LS
Liver							
Lipid metabolism							
acox1	1.20 ± 0.40	1.11 ± 0.27	0.94 ± 0.28	1.09 ± 0.61	0.402	0.863	0.495
fas	3.40 ± 4.64	2.22 ± 1.35	0.74 ± 0.22	0.59 ± 0.33	0.042	0.809	0.609
cpt1	0.88 ± 0.38	0.73 ± 0.21	1.47 ± 0.54	1.51 ± 0.95	0.010	0.832	0.704
Intestine							
Lipid absorption							
cd36	1.26 ± 0.38	1.17 ± 0.21	0.99 ± 0.25	0.77 ± 0.21	0.006	0.164	0.567
fabp1	0.87 ± 0.38	1.04 ± 0.18	1.36 ± 0.54	1.04 ± 0.45	0.164	0.639	0.158
fabp2a	1.03 ± 0.36	1.13 ± 0.17	0.93 ± 0.28	1.04 ± 0.15	0.371	0.335	0.955
mtp	0.93 ± 0.21	0.94 ± 0.1	0.99 ± 0.13	1.22 ± 0.21	0.026	0.112	0.122
apoa4	0.94 ± 0.18	0.98 ± 0.17	1.10 ± 0.30	1.1 ± 0.32	0.187	0.846	0.800
Lipid metabolism							
acox1	1.10 ± 0.38	1.01 ± 0.31	1.08 ± 0.22	0.95 ± 0.27	0.745	0.385	0.871
fas	1.16 ± 0.33	1.18 ± 0.22	0.90 ± 0.11	0.85 ± 0.11	0.003	0.849	0.679
cpt1	1.07 ± 0.35	1.09 ± 0.27	0.95 ± 0.16	1.02 ± 0.33	0.440	0.730	0.812

4. *Discussion*

Effect of lipid level and source on growth performance

In this study, no significant dietary effects were observed on growth (SGR), although a strong trend (p = 0.06) suggested that fish fed FO diets grew slightly better than those fed diets containing 75% VO, irrespective of lipid level. Our results showing no significant effects of lipid level on growth of sole juveniles were surprising, considering how an increase in dietary lipid level has been shown to be detrimental to growth performance in this species (Borges et al., 2009; Mandrioli et al., 2012). It could be suggested that 8% dietary lipid levels might have already been excessive for sole but this hypothesis is not supported by a previous study which did not indicate differences in growth performance between fish fed 4% and 8% lipid diets, while superior growth was observed in those fed 8% compared to 12% dietary lipids and higher (Borges et al., 2009). On the other hand, in a study by Borges et al. (2014), growth of juvenile Senegalese sole was not compromised when substituting dietary FO up to 100% with a blend of VO (rapeseed, soybean, and linseed oil in a ratio of 3:2:5) at 9% lipid levels. However, Benítez–Dorta et al. (2013) observed a lower growth performance when sole

were fed 12% lipid diets formulated exclusively with soybean oil when compared to FO or linseed oil–based diets. These results suggest that both dietary lipid level and lipid source are important and the interaction between both factors can determine how much lipid from a vegetable origin can be included in sole diets without affecting growth. Furthermore, LNA has been observed to negatively affect fish performance compared to ALA (Zuo et al., 2015). Hence, it may be possible that soybean oil had a negative effect on Senegalese sole performance, most likely due to its high LNA content (Benítez–Dorta et al., 2013). If this was indeed the case, results from Borges et al. (2014) and the present study (using the same VO blend, but at a 1:1:1 ratio) indicate that if the soybean oil component is diluted by dietary inclusion of ALA–rich linseed and (to a lesser extent) rapeseed oil, similar growth performance can be achieved to that of fish fed FO.

Table 7. Post–prandial expression of lipid metabolism and absorption genes in Senegalese sole juveniles assessed by RT–qPCR at t6 (6 h after feeding) regulated by dietary lipid level (LL; 8% or 18% lipids) and/or source (LS; VO or FO). Values are mean NRQs ± SD (n = 6 per treatment).

	8FO	8VO	18FO	18VO	p–value (2–way ANOVA)		
					LL	LS	LL×LS
Liver							
Lipid metabolism							
acox1	1.02 ± 0.38	1.06 ± 0.24	1.14 ± 0.21	0.94 ± 0.29	0.982	0.507	0.305
fas	1.19 ± 0.69	2.27 ± 1.93	0.8 ± 0.63	1.24 ± 1.11	0.167	0.140	0.523
cpt1	1.5 ± 0.74	0.81 ± 0.13	1.25 ± 0.77	0.96 ± 0.49	0.849	0.057	0.414
Intestine							
Lipid absorption							
cd36	1.22 ± 0.18	1.10 ± 0.22	1.03 ± 0.26	0.77 ± 0.10	0.004	0.030	0.367
fabp1	0.78 ± 0.32	1.15 ± 0.35	1.11 ± 0.30	1.21 ± 0.23	0.135	0.073	0.303
fabp2a	0.94 ± 0.32	1.06 ± 0.23	1.11 ± 0.16	1.04 ± 0.38	0.521	0.809	0.421
mtp	0.94 ± 0.08	1.00 ± 0.24	1.12 ± 0.10	0.98 ± 0.12	0.213	0.477	0.115
apoa4	0.96 ± 0.16	1.09 ± 0.24	0.99 ± 0.40	1.08 ± 0.13	0.938	0.297	0.868
Lipid metabolism							
acox1	0.99 ± 0.18	1.13 ± 0.56	1.13 ± 0.25	0.94 ± 0.27	0.827	0.860	0.264
fas	1.18 ± 0.45	1.44 ± 1.03	0.81 ± 0.15	0.94 ± 0.14	0.076	0.400	0.776
cpt1	0.91 ± 0.38	1.47 ± 0.23	0.94 ± 0.25	0.91 ± 0.22	0.032	0.031	0.017

Lipid deposition in whole body and specific tissues

In teleosts, lipid stores can generally be found in the muscle, subcutaneous tissue, liver, head, viscera and other organs (Sheridan, 1988), but the overall proximate lipid content of the body and preferential sites of lipid deposition vary greatly among species (as reviewed by Tocher, 2003). In this study, whole–body lipids ranged from 4.1 to 6.4% WW, similar to what has previously been reported (Borges et al., 2009; Cabral et al.,

2011; Fernandes et al., 2012a), and were significantly higher in fish fed high lipid diets, mainly at the expense of protein. According to Fernandes et al. (2012), most of the whole body lipids in Senegalese sole were located in muscle (31%) and head (8%), while liver and viscera accounted for only 2% and 1%, respectively. However, even though muscle adds to total body lipids more than other tissues, it is not considered a preferential site for fat deposition in this species. Accordingly, no changes in total muscle lipid levels in response to dietary input were observed in this or previous studies (Borges et al., 2009; Fernandes et al., 2012b; Valente et al., 2011). On the other hand, liver is considered the primary tissue for fat deposition in this species (Fernandes et al., 2012b; Valente et al., 2011) and indeed had the highest total lipid level (4.1–5.8% WW) of all tissues analyzed in this study, but no significant differences were observed in total lipid content between treatments, as previously reported by Borges et al. (2009) in response to varying dietary lipid level. However, other studies with juvenile Senegalese sole observed significant differences when the dietary lipid/carbohydrate ratio was increased (Dias et al., 2004), but also when fish were fed higher levels of plant protein (Fernandes et al., 2012a), where total lipid levels in the liver were around 17.5–30% WW and 5.5–11% WW, respectively. These high variations in hepatic fat content indicate that other dietary factors, such as carbohydrate content and protein amino acid profile, can influence fat accumulation in the liver as much as, or even more, than lipid levels and fatty acid composition. On the other hand, histological observations of hepatocytes revealed that, although dietary lipid levels did not affect the fat content in the liver, fish fed VO had a more compact hepatic parenchyma and significantly larger surface of tissue occupied by fat deposits compared to those fed FO, similarly to what was described by Caballero et al. (2002) in the liver of rainbow trout. In our study the histological method was probably more precise than lipid analysis, which may not have been able to detect subtle changes in lipid content among different experimental groups.
In spite of a lack of an effect of lipid level on hepatic lipid accumulation, sole fed higher dietary lipid level had a significantly higher HSI. Although other authors have reported similar results in response to increases in lipid level (Cabral et al., 2013; Dias et al., 2004; Rueda–Jasso et al., 2004), it is noteworthy that in our study these differences were mainly caused by the 18VO diet (which also induced the highest VSI). However, an elevated HSI may also be an indicator of glycogen accumulation and not just increased lipid reserves (Gaylord and Gatlin, 2000). Moreover, high dietary lipid level was shown to have a hyperglycaemic effect in Senegalese sole juveniles and has been associated with significantly higher glycogen content in the liver of this species (Borges et al., 2014b). Therefore, although carbohydrate composition was not measured specifically in liver, we cannot exclude the possibility of a greater glycogen accumulation in liver of sole fed the 18VO diet having at least partly contributed towards the HSI results in our study, in addition to FA biosynthesis and lipid storage.

More lipids were found in the intestine (60.9 to 135.1 mg 100 g^{-1} fish) than in the liver (41.4 to 74.0 mg 100 g^{-1} fish), similarly to what was reported by Borges et al. (2009), although these are generally considered transient accumulations rather than lipid deposition (energetic stores) as in liver. These accumulations can be caused by high dietary lipid content and are suggested to be a temporary storage of re–esterified FA, rather than products of lipogenesis, in cases when the rate of lipid absorption exceeds the rate of lipoprotein synthesis (Sheridan, 1988) or because of an inability to metabolize lipids (Caballero et al., 2006; Kjørsvik et al., 1991). Thus, total lipid levels in the

intestine, as well as accumulation of lipids in the enterocytes were higher in fish fed the 18% lipid diets, irrespective of the lipid source. These accumulations consisted of numerous large intracellular lipid droplets of up to 16 µm in diameter, while fish fed low lipid diets had no or very few small droplets (~1.5 µm in diameter), similar to what was observed by Bonvini et al. (2015) in common sole (*S. solea*).

Effect of dietary lipid level and source on liver metabolism

Lipogenesis in mammals is known to be regulated by dietary lipid level, as well as by lipid source (Clarke and Jump, 1994; Davidson, 2006; Kim et al., 2004), and good evidence is also starting to accumulate in fish. Two key lipogenic enzymes were analyzed in this study, at the transcriptional level (*fas*) or by determining its activity (Acly). High lipid diets caused a down–regulation of hepatic *fas* mRNA levels in fish that were fasting for 24 h. Similarly, previous studies with fasted Senegalese sole (Borges et al., 2013b; Dias et al., 2004), channel catfish *Ictalurus punctatus* (Likimani and Wilson, 1982), Atlantic salmon *Salmo salar* (Arnesen et al., 1993), common carp *Cyprinus carpio* (Shimeno et al., 1995) and European seabass (Boujard et al., 2004b; Dias et al., 1998) also showed an inhibition of hepatic lipogenesis by high dietary lipid levels. However, no differences in *fas* expression were observed 6 h after a meal, contrary to previous studies in mammals (Ferramosca et al., 2014; Kelley et al., 1987), and also some salmonid species (Ducasse–Cabanot et al., 2007; Lin et al., 1977) that reported an inhibitory effect of high dietary lipid level on lipogenesis during the post–prandial phase. However, conclusions in these studies were based on the activity of different lipogenic enzymes, which may be affected differently depending on their place/role in the pathway and may have temporal differences in activity. In rainbow trout, for example, Fas activity did not vary between treatments 8 h after feeding, while acetyl–CoA carboxylase (Acc) did (Ducasse–Cabanot et al., 2007).

When looking at the effects of lipid source, no differences were observed in *fas* expression between treatments either pre– or post–prandially. Nevertheless, the hepatic activity of Acly was lower in fish fed FO diets. These results are partly in agreement with the general consensus across species that dietary PUFA act on multiple nuclear receptors and transcription factors to decrease anabolic pathways and increase catabolism (Davidson, 2006; Jump, 2008). In mammals, the effects of PUFA in inhibiting the transcription of *fas* and decreasing lipogenesis in liver have been well documented (Clarke et al., 1977; Kim et al., 2004). However, studies in fish have been inconsistent in this respect and this might be explained by the fact that poikilotherms appear to have poor regulatory mechanisms of lipogenesis (Iritani et al., 1984). Hence, while no effect of dietary FO substitution by VO has been reported on lipogenesis in studies with Atlantic salmon (Torstensen et al., 2004), rainbow trout (Richard et al., 2006a) or European seabass (Richard et al., 2006b), another study reported an inhibitory effect of dietary FO on hepatic *fas* mRNA levels compared to VO–based diets in fasting Atlantic salmon (Morais et al., 2011). Furthermore, hepatocytes of rainbow trout *in vitro* had lower Fas and Acc relative activity when EPA and DHA were added, compared to LNA, and also reduced Acly activity in response to EPA, but not DHA (Alvarez et al., 2000). Considering these results, and the reduced amounts of lipid deposits within hepatocytes from fish fed FO diets, we cannot ignore the potential effect that dietary LC–PUFA may have on reducing hepatic lipogenesis in this species.

In order to undergo β–oxidation, long–chain fatty acyl–CoA enter the mitochondria and peroxisomes with the aid of carnitine acyltransferase enzymes, which are specific for each organelle (Derrick and Ramsay, 1989). The mitochondrial *cpt1* gene analyzed in this study showed significantly higher transcript levels in fasted fish (t0) fed high lipid levels compared to those fed a low lipid diet – opposite to what was observed for *fas*. This is not surprising considering how catabolic pathways are usually regulated in opposite directions to anabolic pathways to maintain energy homeostasis. For example, malonyl–CoA, the substrate of Fas in lipogenesis, acts as an inhibitor of Cpt1 (Kim, 1997). Inside the peroxisome or mitochondria, β–oxidation is catalyzed by enzymes such as acyl–coA oxidase (Acox1; peroxisomal) and Hoad. In this study no significant differences in the activity of Cpt1 or Hoad enzymes were observed and no differences in *acox1* expression were observed either, at none of the analyzed time points. This, as well as the lack of significant effects in some of the other parameters measured in this study, is possibly due to the high variability of the results. While a force feeding method was used to ensure fish were fed equally (in terms of amount of food and timing of feeding), it is possible that individual differences in basal metabolism established over the whole experimental period might have caused these variations. High individual variability in feeding and metabolism can be typically found in several fish species but might be exacerbated in Senegalese sole, as a strong hierarchical population structure is characteristic for this species (Morais et al. 2015). In the liver of fasted juvenile haddock *Melanogrammus aeglefinus*, similarly to what was observed here in sole, no difference in β–oxidation was observed when fish were fed diets varying in lipid content from 12 to 24% (Nanton et al., 2003). However, in rainbow trout, both *cpt1* and *acox1* were up–regulated in the liver of fish fed high lipid diets, but also in diets containing LC–PUFA compared to medium–chain FA (Figueiredo–Silva et al., 2012a). In sum, similarly to effects of lipid level or lipid source in lipogenesis, effects on β–oxidation appear to be more variable in fish species than in mammals, but present results indicate that a high lipid diet can stimulate import of fatty acyl–CoA's for mitochondrial oxidation in Senegalese sole juveniles.

As previously noted, an important metabolism modulating effect has been reported for dietary LC–PUFA in mammals, acting on multiple nuclear receptors and transcription factors to coordinately depress anabolism and increase catabolic pathways, such as β–oxidation (Davidson, 2006; Jump, 2008). Furthermore, in fish, saturated and monounsaturated fatty acids have been shown to be a preferred substrate over PUFA for β–oxidation (Henderson, 1996), although, when given in surplus, PUFA are also readily oxidized (Stubhaug et al., 2007). In this study a strong trend ($p = 0.06$) for higher *cpt1* mRNA levels was observed in fish fed FO diets, although only during the post–prandial phase, possibly because its pre–prandial expression was more strongly influenced by dietary lipid level. This correlated well with the lower lipid accumulation observed in the liver of fish fed FO diets. Similarly, in Atlantic salmon, EPA (although not DHA) was shown to stimulate hepatic β–oxidation (Kjær et al., 2008; Vegusdal et al., 2005), while in rainbow trout, SFA, MUFA, ALA, ARA and DHA up–regulated, while LNA and EPA down–regulated *cpt1* expression *in vitro*(Coccia et al., 2014).

Effect of dietary lipid level and source on lipid metabolism in the intestine

The liver is generally considered a more relevant organ for lipid metabolism compared to the intestine (Henderson and Tocher, 1987). Thus, it is not surprising that in this study *fas* and *cpt1* mRNA levels were substantially lower in the intestine compared to the liver, although *acox1* expression was comparable in both tissues (based on C_t values; data not shown). On the other hand, the existing data collectively suggests that some aspects of lipid metabolism in the intestine might be expressed similarly or even more than in the liver (Fonseca–Madrigal et al., 2006; Tocher et al., 2002), and that this organ likely has numerous other roles beyond the simple reacylation and packaging of lipids for transfer into body tissues (Bell et al., 2003; Morais et al., 2012b; Teitelbaum and Walker, 2001). Still, studies specifically looking at lipid metabolism in fish intestine are still insufficient. In response to dietary lipid level, pre–prandial expression of *fas* was down–regulated in fish fed high lipid diets, similarly to what was observed in liver. Not much work has been done in similar conditions in fish, but an increase in gene expression and enzyme activity of the β–oxidation pathway was observed in the intestine of mice fed high fat diets (Kondo et al., 2006).

On the other hand, the hypotriglyceridemic effect of FO (through increased β–oxidation and/or reduced lipogenesis) observed in the liver of Senegalese sole in this study was not noticeable in the intestine. Namely, no changes in fas, cpt1 or acox1 expression were observed in fasted fish, and cpt1 was unexpectedly up–regulated post–prandially, only in the 8VO treatment. Such effects have been reported in the intestine of fish and mammals, such as in Atlantic salmon (Morais et al., 2012b), where intestinal fas expression was down–regulated by dietary LC–PUFA, and in mice fed LC–PUFA rich diets, which exhibited an increase in β–oxidation in the small intestine (van Schothorst et al., 2009). Considering the variability of the responses measured in fish liver so far, and the scarcity of studies focusing on intestinal lipid metabolism, more work should be performed to address the effects of lipid level and lipid source on metabolism in this important organ.

Effect of dietary lipid level on intestinal lipid absorption

Sole fed high lipid diets clearly showed a higher lipid accumulation in the intestinal mucosa, while the source of the dietary lipids did not affect these results. These differences did not seem to have major long term effects in the animal as the FCR did not differ significantly between treatments, and no significant changes were observed in lipid content in muscle or liver in relation to dietary lipid level. However, they indicate that sole were not able to deal with a high dietary supply of lipids by adjusting their intestinal lipid absorption mechanisms and, in order to investigate this in further detail, we looked at the expression of several genes that are key for these processes.

The *CD36* peptide, also known as fatty acid translocase (*FAT*), is a multiligand scavenger receptor that has been implicated in numerous biological processes (Silverstein and Febbraio, 2000). In mammals it is quite abundant in numerous tissues, where it may bind to native or modified lipoproteins, thronspondin–1, collagen and other compounds (Endemann et al., 1993; Febbraio et al., 2001). In addition, it has also been shown to play an important role in intestinal absorption, where it facilitates long–chain FA uptake by cells (Chen et al., 2001; Lobo et al., 2001; Nassir and Abumrad, 2009) and

has been associated with intestinal (Schwartz et al., 2008) and orosensory (Gaillard et al., 2008; Khan and Besnard, 2009) perception of dietary fat and satiety signaling. The platelet glycoprotein 4–like transcript, exclusively expressed in the intestine and brain (>20 times lower than in intestine) of Senegalese sole (although its expression in the oral epithelium was not evaluated; unpublished data), showed high sequence homology to the mammalian *FAT/CD36* and was used for the purpose of this study. Our results showed that intestinal *cd36* expression was significantly affected by dietary lipid level, but the results were opposite to what might be expected. Namely, transcript levels were lower in fish fed high lipid diets pre– and post–prandially, while, higher levels of CD36/FAT were recorded in mouse fed high lipid diets (Petit et al., 2007). On the other hand, this gene was also significantly affected by lipid source post–prandially (and at t0 a similar trend was observed), being up–regulated in fish fed FO diets. There was no interaction between lipid level and lipid source and, in fact, the effect appeared cumulative, resulting in the highest and lowest expression being measured in the 8FO and 18VO treatments, respectively. Presently, it is difficult to determine the exact mechanisms explaining the regulation of this *cd36* homolog in Senegalese sole, but considering one of its roles being in the detection of FA, we hypothesize that the responses measured here could be more related to gastrointestinal lipid sensing than lipid absorption.

Once FA enter the enterocytes, they are re–esterified and packaged into lipoproteins before being released into circulation. FABPs are proteins which facilitate intracellular transport of free FA from the microvillus membrane to the endoplasmatic reticulum for re–esterification (Ockner and Manning, 1976). They have a complementary function to CD36/FAT and were equally up–regulated in the intestine of mouse fed high–lipid diets (Poirier et al., 1996). In zebrafish, *fabp* mRNA levels have been correlated with intracellular storage of lipid droplets and synthesis of VLDL particles in enterocytes (Andre et al., 2000). However, in this study neither of the two analyzed *fabp* transcripts in the intestine were affected by dietary lipid level.

The MTP is a protein that is essential for correct formation of primordial apoB apolipoproteins in the endoplasmatic reticulum of enterocytes (Hussain et al., 2003). In hamster, a liver–type *FABP* homolog (corresponding to *fabp1* in our study) and *MTP* were up–regulated after one day of feeding a high lipid diet, but after prolonged exposure (9 days), only *MTP* levels remained elevated (Lin et al., 1994). In our study, long–term feeding of Senegalese sole juveniles with high lipid diets increased fasting levels of *mtp* in the intestine. However, this response was only noticeable in fish fed the 18VO diet, indicating that an interaction between lipid level and its FA composition (lipid source) was necessary for it to occur. Therefore, present results suggest that rather than being affected by generally high lipid levels, it may be that absolute values of individual dietary FA components that affected *mtp* expression. This hypothesis is likely, considering results from an *in vitro* study with HepG2 cells that showed that administration of oleic acid (which was by far higher in the 18VO diet) up–regulated *MTP*, while no effect was observed when the same cells were exposed to palmitate, ARA or LNA (Qiu et al., 2005). Nevertheless, *mtp* is regulated at the transcriptional, translational and/or post–translational level by different macronutrients, and also by hormones (insulin, leptin) and other factors (Hussain et al., 2011), and therefore other mechanisms could explain our results.

Finally, the expression of apolipoprotein A4 (*apoa4*) was also analyzed. This protein has an essential role in enhancing triacylglycerol (TAG) packaging into chylomicrons by

increasing their size and, which can lead to an increased basolateral TAG secretion in the intestine (Lu et al., 2006). In sole larvae, a coordinated up–regulation of several apolipoprotein–related genes (including *apoa4*) in response to a higher dietary lipid intake has been described (Hachero–Cruzado et al., 2014). However, in this study no pre– or post–prandial changes were observed in *apoa4* in response to lipid level.

In summary, results from this and other studies suggest that sole can cope well with a high inclusion of VO (up to 100%) in their ongrowing diets, although this might vary somewhat depending on the oil type or blend which is used. Furthermore, although best growth performance is usually obtained at low (8–10%) dietary lipid levels, results from this experiment suggest that lipid level can probably be increased in the diets with no major effects on growth and feeding performance, using diets containing fish meal and up to 75% of FO replaced by an equal blend of linseed, rapeseed and soybean oil. However, the diets had different effects on HSI, VSI, lipid deposition and metabolism and, in a few cases, results suggest an interaction between dietary lipid level and lipid source. In particular, the increase in HSI and VSI in fish fed high lipid diets appeared to be mainly (HSI) or exclusively (VSI) associated with the high content of VO in the diet. In general, dietary lipid level seemed to have a larger effect on the expression of genes involved in lipogenesis or β–oxidation compared to lipid source, with most changes observed at fasting. The results show that Senegalese sole juveniles responded to diets containing high lipid levels and conversely, reduced carbohydrate contents, by down–regulating FA synthesis in the liver and intestine, and enhancing β–oxidation in liver. Furthermore, although less clearly, results suggest that LC–PUFA can also affect these pathways in sole liver, as indicated by a reduced Acly enzymatic activity at fasting and up–regulation of *cpt1* 6h after feeding, as well as by a significantly lower accumulation of lipids in hepatocytes (observed histologically), when fish were fed FO diets. Finally, analysis of the transcriptional changes of several genes that are collectively responsible for FA uptake from the intestinal lumen, intracellular transport and packaging into lipoproteins, did not indicate a strong response to dietary lipid level or lipid source (except in the case of *cd36*, possibly unrelated to FA absorption, and in *mtp*, but specifically associated with the 18VO diet). This might suggest a poor capacity of Senegalese sole to regulate their intestinal FA absorption in order to adapt to high dietary lipid levels and could explain the important accumulation of lipid droplets in the intestinal epithelia of sole fed high lipid diets.

Acknowledgements

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CHAPTER 3

Mechanisms of lipid metabolism and transport underlying superior performance of Senegalese sole (*Solea senegalensis*, Kaup 1858) larvae fed diets containing n–3 polyunsaturated fatty acids

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Abstract

To date, most research on larval lipid nutrition has been centered on essential fatty acid requirements. However, less is known about effects of dietary fatty acid (FA) composition on lipid absorption and metabolism, as addressed in this study. Senegalese sole were fed live preys enriched with different oils: cod liver oil (CLO), linseed oil (LSO), soybean oil (SBO), and olive oil (OO), that are rich in long–chain polyunsaturated FAs (LC–PUFA), n–3 PUFA, n–6 PUFA or monounsaturated FAs (MUFA), respectively. Larvae reared on CLO showed significantly improved growth and survival, faster onset of metamorphosis and maturation of the intestine, lower lipid accumulation in liver after metamorphosis and an up–regulation of genes involved in lipid transport and phospholipid metabolism, while key lipogenesis genes were down–regulated. From the remaining treatments, the LSO diet induced the closest performance to CLO, with larvae completing metamorphosis at a similar time and having the second best growth and survival by the end of the experiment. They were also grouped closer to the CLO treatment than the remaining vegetable oil treatments, based on the patterns of gene expression. These results showed that oil sources rich in LC–PUFA and n–3 PUFA were superior to those having high n–6 PUFA or MUFA levels in the larval nutrition of Senegalese sole and indicate that this effect might be at least partly explained by an up–regulation of phospholipid metabolism and apolipoprotein synthesis, likely leading to enhanced lipid transport and mobilization, as well as tissue growth and remodeling.

Keywords: Vegetable oil, Flatfish, PUFA, Phospholipid metabolism, Apolipoprotein, Openarray

1. *Introduction*

Dietary lipids are essential for fish larvae to meet the high structural and energy demands of rapid growth and intense organogenesis associated to development and metamorphosis (Hamre et al., 2013; Tocher, 2010). Long–chain polyunsaturated fatty acids (LC–PUFA), namely eicosapentaenoic acid (20:5 n–3, EPA), docosahexaenoic acid (22:6 n–3, DHA) and arachidonic acid (20:4 n–6, ARA), are considered essential fatty acids (EFA) in teleosts, as they either cannot be synthesized endogenously, or can but at insufficient rates to meet physiological demands. These EFAs are of great importance as they are integral components of phospholipids (PLs), which are in turn fundamental building blocks of membrane bilayers. Furthermore, they also act as precursors to bioactive eicosanoids and regulate gene expression related to inflammatory responses and lipid metabolism (Schmitz and Ecker, 2008; Tocher, 2010). Thus, it is not surprising that a deficiency of LC–PUFA in larval diets has been shown to result in reduced growth and survival rates, pigmentation abnormalities, altered visual acuity (affecting the capacity to capture live prey), abnormal behavior and susceptibility to disease and stress (Bell and Dick, 1993; Bransden et al., 2005; Furuita et al., 1998; Lund et al., 2007; Sargent et al., 1999b; Shields et al., 1999; Tocher, 2010; Villalta et al., 2005a; Watanabe, 1993).

However, aside from EFAs, numerous other dietary FAs also have important roles in larval development. Lipids are the main energy source for developing fish and it has been well–established that saturated (SFA) and monounsaturated fatty acids (MUFA), like oleic acid (OA), are preferred to polyunsaturated fatty acids (PUFA) as substrates for β–oxidation (Crockett and Sidell, 1993; Kiessling and Kiessling, 1993). Lipid requirements of larvae should, therefore, be considered not only in terms of absolute and relative levels of EFAs, but rather the optimal balance between LC–PUFA and other dietary FAs, which are the main source of metabolic energy.

Furthermore, a standing question refers to the nutritional and physiological roles that dietary C_{18} PUFA, such as α–linolenic (18:3 n–3, ALA) and linoleic (18:2 n–6, LNA) acids play in numerous fish species. Their effects in the absence of LC–PUFA have rarely been investigated in fish, particularly in larvae, as it is unfeasible to culture most marine species during these critical stages of development in such extreme and adverse dietary conditions. However, Senegalese sole (*Solea senegalensis*), a marine species of commercial aquaculture importance in southern Europe (Morais et al., 2015a), has been shown to have relatively low LC–PUFA requirements during the larval stage compared to other marine finfish, which appeared to decrease even further after metamorphosis (Conceição et al., 2007; Dâmaso–Rodrigues et al., 2010; Morais and Conceição, 2009; Morais et al., 2004; Villalta et al., 2005b), making it a good model species to investigate this subject.

In order to address these questions and help understand the effects that different FAs might have on lipid metabolism and absorption, an experiment was designed with larvae and post–larvae of Senegalese sole fed live prey enriched with one of four oil emulsions differing in FA composition (rich in either LC–PUFA, ALA, LNA or MUFA). A holistic approach was implemented to determine the effects these FAs might have on fish performance and metabolism, with analyses including the assessment of growth, development (completion of metamorphosis), intestinal maturation (evaluated by

digestive enzyme activity), lipid and FA composition, hepatic and intestinal lipid accumulation (assessed by histology) and expression of genes involved in lipid metabolism, absorption and transport (qPCR).

2. *Materials and methods*

Larval rearing, dietary treatments and feeding trial

Newly hatched Senegalese sole larvae were obtained from Stolt Sea Farm S.A. (Carnota, A Coruña, Spain) and distributed into sixteen (4 replicates per treatment) 100 L cylindroconical tanks at a density of 90 larvae L^{-1}. The tanks were connected to a recirculation system (IRTAMAR®) with 50% daily water renewal and regulated temperature (18–20 ºC), salinity (35 ppt) and dissolved oxygen (7.5 mg L^{-1}). The photoperiod was 16 h light: 8 h dark with a light intensity of <500 lux at the water surface. Larvae were fed enriched rotifers, at a density of 10 rotifers mL^{-1}, from 2 days post–hatching (dph) to 8 dph. Enriched *Artemia* metanauplii were introduced at 6 dph and fed as detailed in Boglino et al. (2012a) until 37 dph. In short, enriched and hydrogen peroxide–disinfected (5 min at 8000 ppm) *Artemia* metanauplii were fed from 6 to 14 dph in quantities gradually increasing from 0.5 to 4 metanauplii mL^{-1} and from 15 to 25 dph, as larvae became benthonic, live *Artemia* were gradually substituted with frozen enriched metanauplii, and fed at 4–6 metanauplii mL^{-1} in total. From 25 dph onwards, the post–larvae were fed exclusively frozen enriched *Artemia,* at 6–12 metanauplii mL^{-1}. Feeding was performed twice a day.

Four emulsions (Table 1) were prepared following the methodology described in Villalta et al. (2005b) and were used to enrich rotifers and *Artemia* metanauplii (EG type, Sep–Art, GSL; INVE, Belgium). Each emulsion was prepared with one of the following oils: cod (*Gadus morhua*) liver oil – CLO, linseed oil – LSO, soybean oil – SBO, and olive oil – OO. Live prey culture and enrichment were performed as described in Boglino et al. (2012a). Briefly, rotifers were fed daily with microalgae (*Tetraselmis chuii)* at $4x10^5$ cells mL^{-1} and yeast (Mauripan, Categgio Lieviti, Italy) at 0.7 g per million rotifers. Each day, rotifers were enriched, at a density of 500 rotifers mL^{-1}, with 0.1 g L^{-1} of each experimental emulsions for 2 h (first meal of the day) or 6 h (second meal). *Artemia* nauplii were hatched daily during the larval pelagic stage to be enriched and fed live. Additionally, two large batches of enriched metanauplii were produced and frozen to later feed the benthic post–larvae. *Artemia* enrichments were performed for 16 h in standard conditions, at a concentration of 150 metanauplii mL^{-1}, with 0.6 g L^{-1} of emulsion.

A feeding trial was performed at 16 dph with pre–metamorphic pelagic larvae and again at 34 dph with benthic post–larvae. One group of fish per tank (200 larvae and 50 post–larvae) was transferred into a 2 L container placed inside the tank, with mesh allowing for water circulation but preventing *Artemia* in the tanks from entering. A second group of fish (200 larvae and 50 post–larvae) was transferred to a separate 12 L container with clean aerated seawater, for easier manipulation. Both groups of fish were kept unfed and after 16 h fasting (t0) the first group was sampled. The fish in the 12 L containers were then re–fed with enriched *Artemia* (live at 16 dph and frozen at 34 dph)

Table 1. Formulation of the experimental emulsions and the resulting total lipid content, total FA content and FA composition of enriched *Artemia* (means ± SD; n = 4 pools).

	CLO	LSO	SBO	OO
Emulsion formulation (mg g^{-1})				
Cod–liver oil[1]	528	0	0	0
Linseed oil[2]	0	528	0	0
Soybean oil[3]	0	0	528	0
Olive oil[4]	0	0	0	528
Soy lecithin[5]	40	40	40	40
α–Tocopherol[6]	12	12	12	12
Distilled water	420	420	420	420
Total lipid and FA content of enriched Artemia (mg per g of DW)				
Total lipids	107.1 ± 19.3	100.6 ± 6.3	106.1 ± 20.4	112.6 ± 6.8
Total FA	61.6 ± 10.8	61.2 ± 11.0	69.3 ± 22.3	71.7 ± 7.5
FA composition of enriched Artemia (% of TFA)*				
16:0	10.0± 0.3	7.4 ± 0.5	8.3 ± 1.0	8.1 ± 0.7
18:0	5.4 ± 0.4	6.5 ± 1.0	6.0 ± 0.9	5.0 ± 0.5
Total SFA	16 ± 0.7	14 ± 1.4	14.4 ± 1.2	13.3 ± 1.3
16:1	2.5 ± 0.5	0.4 ± 0.2	0.5 ± 0.1	0.6 ± 0.2
18:1n–9	24.4 ± 0.5	25.1 ± 1.1	24.7 ± 0.5	49 ± 0.1
18:1n–7	7.6 ± 0.5	5.7 ± 1.0	4.6 ± 1.3	7.7 ± 1.0
20:1	2.0 ± 2.3	0.2 ± 0.3	0.1 ± 0.2	0.0 ± 0.0
Total MUFA	40.4 ± 2.7	31.3 ± 1.6	30.0 ± 0.8	57.3 ± 1.0
18:2n–6	6.0 ± 0.4	11.7 ± 1.9	28.8 ± 5.0	7.9 ± 2.2
20:4n–6	0.5 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
Total n–6 PUFA	9.1 ± 3.3	12.4 ± 1.6	29.9 ± 4.5	8.7 ± 2.2
18:3n–3	19.8 ± 2.2	38.2 ± 2.1	21.5 ± 2.8	17.1 ± 0.7
20:5n–3	6.3 ± 0.8	0.9 ± 0.3	0.8 ± 0.2	0.7 ± 0.1
22:5n–3	0.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:6n–3	3.9 ± 0.8	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0
Total n–3 PUFA	34.4 ± 0.7	42.2 ± 1.7	25.6 ± 3.2	20.6 ± 0.9
Total PUFA	43.5 ± 2.9	54.6 ± 2.6	55.5 ± 1.6	29.3 ± 1.4
DHA/EPA	0.6 ± 0.1	0.0 ± 0.0	0.2 ± 0.4	0.0 ± 0.0
ARA/EPA	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0

*Not all FAs are shown.

[1]Sigma–Aldrich Co., St Louis, MO, Germany; [2]Biolasi Products Naturals, S.L., Guipùzcoa, Spain; [3]Huilerie Emile Noël, S.A.S., Pont Saint Esprit, France; [4]Borges Pont, Lleida, Spain; [5]Laboratorios Korott, Alicante, Spain; [6]Sigma Aldrich Co., Germany.

from their respective treatments and were left to feed undisturbed for 1 h, after which any uneaten live feed was removed by filtering the fish through a submerged 500 µm mesh. Larvae/post–larvae were then transferred to a new container (12 L) with clean aerated seawater and sampled 3 h later (t3), which is estimated to correspond to the post–prandial period when the meal had been digested. Sampled fish were quickly euthanized with MS–222 and preserved in 3 mL of RNAlater stabilization buffer (Ambion, Life Technologies, Alcobendas, Madrid, Spain) following manufacturer's instructions.

All experimentation on live fish was performed according to the European and National legislation with protocols approved by the ethics committee of IRTA.

Larval performance

Standard length (SL) and dry weight (DW) of 30 larvae per tank were measured at 2, 9, 16, 21, 30 and 37 dph. Larvae were euthanized using a lethal dose of MS–222 (Sigma–Aldrich Co., St Louis, MO, Germany; 1000 mg L^{-1}). Standard length was measured using a Nikon SMZ800 dissecting microscope (Nikon, Spain) connected to an Olympus DP25 digital camera (Olympus Corporation, Germany) and image analysis software (AnalySIS Gmbh, Olympus, Germany). The same larvae were then pooled, washed with distilled water, oven–dried at 60 °C for 24 h and weighed (one pool per tank) to determine DW to the nearest µg on a Mettler A–20 microbalance (Mettler Toledo, Columbus, OH, USA). To assess developmental progress of the larvae, eye migration was observed during metamorphosis. Samples of 20 specimens were taken from each tank prior to, during and post metamorphosis (at 12, 14, 16, 18, 22, 23, 25, 28, 30 dph) and examined under a dissecting microscope (as for SL measurements). Developmental stages were classified into five categories according to Fernández–Díaz et al. (2001): from "0" representing bilaterally symmetric larvae with a vertical swimming plane to "4" larvae with eye translocation completed and orbital arch clearly visible. The eye migration index (I_{EM}), was calculated according to Solbakken et al. (1999).

At the end of the experiment (37 dph), larvae remaining in the tanks were counted to estimate the survival rates. These values were then corrected for all larvae sampled during the experiment using a formula from Laurence (1974), modified to account for irregular sampling on a daily, instead of weekly, basis.

Lipid and fatty acid analyses

To evaluate the lipid content and fatty acid (FA) composition of the experimental diets, samples of enriched *Artemia* metanauplii from each treatment were taken on four consecutive days, carefully washed and immediately frozen at –20 ºC. For biochemical analysis of the larvae, pools of 100 larvae and 50 post–larvae were taken per tank at 22 dph and 37 dph, respectively. Sampled larvae were euthanized with MS–222, washed with distilled water and immediately frozen at –20 ºC. Total lipids were extracted using the method of Folch et al. (1957) and quantified gravimetrically, as detailed in Boglino et al. (2012a). Acid catalyzed transmethylation was carried out using the method of (Christie, 1982b) and methyl esters were analyzed by gas–liquid chromatography as described in Morais et al. (2015). Results of fatty acid contents were expressed as a percentage of total fatty acids.

Digestive enzyme activity

Activity of the intestinal brush border membrane enzyme alkaline phosphatase (AP) and of the intracellular leucine–alanine peptidase (Leu–ala) was measured in pools of 30 whole larvae per tank sampled at 37 dph, quickly frozen and stored at –80 ºC. Samples were homogenized in cold 50 mM manniol, 2mM Tris–HCl buffer, pH 7.0 and processed as described in Gisbert et al. (2009) for brush border purification. The enzymatic activities of AP and Leu–ala were quantified according to Bessey et al. (1946) and Nicholson and Kim (1975), respectively, and expressed as specific units (mU mg^{-1} protein). Soluble protein of crude enzyme extracts was quantified by the Bradford method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate.

Histological analyses

Five larvae from each tank were processed at 22 and 37 dph. They were euthanized as previously described, fixed in 4% formalin buffered to pH 7.0 with 0.1 M phosphate buffer for 24 h and then preserved in 70% ethanol. Samples were serially sectioned at 6–7 µm and stained with Harris haematoxylin and eosin. Sections of the posterior intestine and liver were photographed at x40 magnification (300 dpi; 1 or 3 photos per tissue, respectively; n = 20 and n = 60 photos per treatment, respectively) with an Olympus DP70 digital camera connected to a Leica DM 2000™ microscope. All digital image analyses were performed using ImageJ (U.S. National Institutes of Health, Bethesda, USA; http://rsbweb.nih.gov/ij/index.html). A semi–automatic quantitative analysis was employed in order to measure the percentage of intestinal epithelium (longitudinal transects) and hepatocytes occupied by fat deposits. By adjusting the color threshold settings to brightness values between 180 and 255, the program was configured to select all surfaces covered with a white to light pink color, which corresponded to the area of fat deposits (Gisbert et al., 2008). These values were used to calculate the percentage of area occupied by lipids as in Boglino et al. (2012b). For liver samples, the total area of analyzed tissue was a rectangular selection, while longitudinal transects of the intestinal epithelium were selected manually with the “polygon selection” tool.

Gene expression analysis

For RNA extraction, pelagic larvae aged 16 dph were homogenized whole, while the benthic post–larvae (34 dph) were dissected on ice, under a microscope, in order to separate the head from the body, and only the latter compartment was used in this study. Samples were transferred to 2 mL screw–cap tubes containing 1 mL of TRIzol (Ambion, Life Technologies, Madrid, Spain) and approximately 50 mg of 1 mm diameter zirconium glass beads and homogenized (Mini–Beadbeater, Biospec Products Inc., USA). Solvent extraction was performed following manufacturer’s instructions and final RNA concentrations were determined by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Madrid, Spain). RNA quality and integrity was assessed from the ratio of absorbances at 260 and 280nm and by gel electrophoresis, respectively.

In order to study the expression of a few selected genes involved in lipid transport and metabolism by RT–qPCR, a search for candidate transcripts was performed in the SoleaDB online database (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/; Benzekri et al.; 2014) using the *Solea senegalensis* v4.1 global assembly. The retrieved transcripts were aligned and assembled *in silico* using the BioEdit Sequence Alignment Editor (Hall, 1999), and completed with rapid amplification of cDNA ends (RACE) PCR, if needed, using the FirstChoice® RLM–RACE kit (Ambion, Life Technologies, Alcobendas, Madrid, Spain). The obtained fragments were confirmed by sequencing (SCSIE, University of Valencia, Spain) and used as templates to design primers for RT–qPCR with Primer3 v. 0.4.0 (Koressaar and Remm, 2007; Untergasser et al., 2012) (Table 2). The resulting amplicons were, once again, sequenced to confirm their identity and the specificity of the assay.

Two micrograms of total RNA were reverse transcribed into cDNA using the High–Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life technologies, USA), following manufacturer's instructions, but using a mixture of random primers (1.5 µL as supplied) and anchored oligo–dT (0.5 µL at 400 ng μL^{-1}, Eurogentec, Cultek, S.L., Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. A pool of 2 µL from each cDNA sample was created to be used for standard dilutions and the remaining cDNA was diluted 60–fold with water. Amplifications were carried out in duplicate on a CFX96 Touch Real–Time PCR Detection System (Bio–Rad, Alcobendas, Spain) in a final volume of 20 µL containing 5 µL of diluted (1/60) cDNA, 0.5 µM of each primer (except for *fabp1*, where 150 µM was used) and 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio–Rad) and included a systematic negative control (NTC–non template control). The RT–qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 35 cycles: 15 s at 95 °C, 1 min at the corresponding annealing temperature (Ta; Table 2). After the amplification phase, a melt curve was performed, enabling confirmation of the amplification of a single product in each reaction. Non–occurrence of primer–dimer formation in the NTC was also checked. The amplification efficiency of each primer pair was assessed from serial dilutions of the cDNA pool (Table 2). For data analysis, the RT–qPCR results were imported into the software qBase+ (Biogazelle, Zwijnaarde, Belgium), where normalized relative quantities (NRQ) were calculated employing target and run–specific amplification efficiencies and using the geometric mean of 2–3 reference genes (*eef1a1, ubq* and *rps4*) established by Infante *et al.* (2008). For larval samples, all 3 reference genes were used for t0 (M = 0.222; coefficient of variance – CV = 0.087), while for t3 only *rps4* and *ef1a1* were selected (M = 0.222; CV = 0.077). For the post–larval samples, all 3 reference genes were used for both t0 (M = 0.130; CV = 0.052) and t3 (M = 0.126; CV = 0.050).

To complete the gene expression analysis, we performed a qPCR assay using a 112x24 high–performance openarray chip, as detailed in Hachero–Cruzado et al. (2014). Samples were loaded in triplicate into OpenArray plates with the OpenArray® AccuFill™ System following manufacturer's protocols. Each subarray was loaded with 5.0 µL of master mix containing a specific cDNA and TaqMan® Gene Expression Master Mix. For gene expression analysis, raw data was imported into the Datassist v3.01 software and Ct values were exported and analyzed using the $2^{-(\Delta\Delta Ct)}$ method

(Livak and Schmittgen, 2001) in which values were normalized with the geometric mean of 3 reference genes (*ubq*, *eef1a1* and *gadph2*), and calibrated to the CLO treatment.

Table 2. Primers used for real–time quantitative PCR (RT–qPCR). Shown are sequences and annealing temperature (Ta) of the primer pairs, size of the fragment produced, average reaction efficiency (E) and accession number of the target and reference genes (Infante, 2008).

Transcript	Primer sequence (5'–3')	Amplicon size	Ta	E*	Accession number
Lipid metabolism					
aco	GGTCCATGAATCTTTCCACAA ACAAGCCTGACGTCTCCATT	168 bp	60°C	103.8%	KP842776
fas	CACAAGAACATCAGCCGAGA GAAACATTGCCGTCACACAC	197 bp	60°C	104.9%	KP842777
Δ4fad	AAGCCTCTGCTGATTGGAGA GGCTGAGCTTGAAACAGACC	131 bp	60°C	101.8%	JN673546
Lipid absorption and transport					
apoa4	AGGAACTCCAGCAGAACCTG CTGGGTCATCTTGGAGAAGG	122 bp	60°C	102.6%	KP842775
fabp1	GCTCATCCAGAAAGGCAAAG GGAGACCTTCAGCTTGTTGC	199 bp	62°C	102.6%	KP842779
fabp2a	ACACACATGACCTTAGCACACTG TGCGAGTATCAAAATCCGGTA	70 bp	60°C	103.6%	KP842780
fabp2b	ATTCTCATGGGCTTCCACTG CCTCTCAGCTTCCCTGCTTT	154 bp	60°C	101.8%	KP842781
fabp3	GTCAGGGAAGTCAACGGAGA ATAAAGAGATGGCGGGAGGT	225 bp	60°C	103.4%	KP842782
mtp	CAGGCGTACACCACATGTAAA GTGATCAGGCTTCTGCAGTG	150 bp	60°C	103.5%	KP842778
Reference genes					
ubq	AGCTGGCCCAGAAATATAACTGCGACA ACTTCTTCTTGCGGCAGTTGACAGCAC	93 bp	70°C	101,3%	AB291588
rps4	GTGAAGAAGCTCCTTGTCGGCACCA AGGGGGTCGGGGTAGCGGATG	83 bp	70°C	100,9%	AB291557
eef1a1	GATTGACCGTCGTTCTGGCAAGAAGC GGCAAAGCGACCAAGGGGAGCAT	142 bp	70°C	100,5%	AB326302

aco, acyl–CoA oxidase; *fas*, fatty acid synthase; *Δ4fad,* delta 4 fatty acid desaturase*; apoa4*, apolipoprotein A–IV; *fabp*, fatty acid binding protein; *mtp*, microsomal trygliceride transfer protein; *ubq*, ubiquitin; *rps4*, 40S ribosomal protein S4; *ef1a1*, elongation factor 1 alpha.

*Efficiency corresponds to an average of 2 runs (larval and post–larval samples; each run contained both t0 and t3).

Statistical analyses

All statistical analyses were performed with SPSS v.20 (SPSS Inc., Chicago, IL, USA). Results of DW, FA composition, survival, I_{EM}, enzymatic activity and RT–qPCR (NRQ values exported from qBase+) analysis, performed on pools of specimens (n = 4 per treatment) and those of SL and fat accumulation (n = 120 or n = 12 individual fish per treatment, respectively) were analyzed by one–way ANOVA. The $2^{-(\Delta\Delta Ct)}$ data from the openarray analysis, also performed on pools (n = 3), was log transformed and

processed using a One–way multivariate analysis of variance (MANOVA) approach ($p<0.05$). When data passed the Levene's test of equal variances ($p>0.05$), the Tukey's post–hoc test was performed to assess significant differences between dietary treatments at each age or time point, at a significance level of 0.05. When variances were not equally distributed, the Games–Howell test was used instead. For clustering analysis, the permutMatrix software (Caraux and Pinloche, 2005) was used to compare log2 transformed fold changes, with parameters set as following: dissimilarity – Euclidean distance; hierarchical – McQuitty's criteria Method; and seriation – multiple–fragment heuristic (MF).

3. *Results*

Larval performance and maturation of the digestive function

The enrichment emulsions caused significant differences in fish DW at 22 and 30 dph (Fig. 1a), but not in SL (Fig. 1b). Larvae fed the CLO diet were significantly heavier than those fed OO at 22 dph and 30 dph, but no significant differences in DW or SL were found at the end of the experiment due to large size dispersion. At this point, larvae fed CLO still tended to be larger and heavier, but those fed LSO appeared to be catching up in terms of growth.

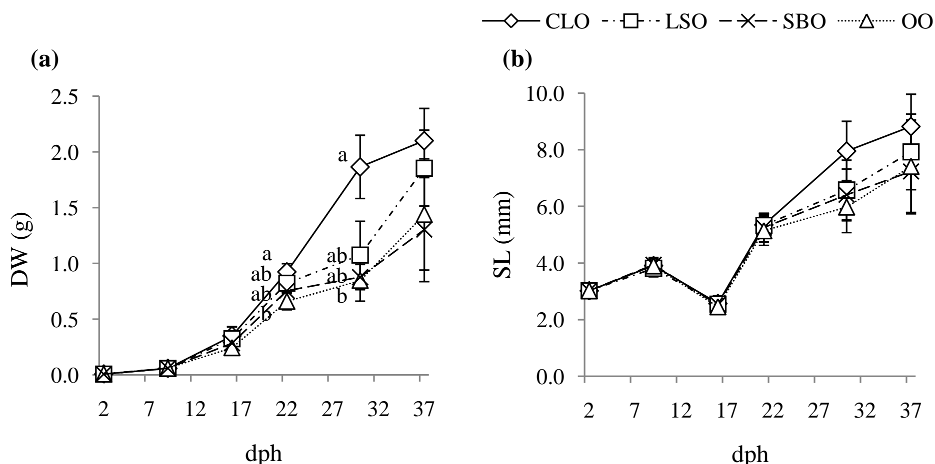

Figure 1. Dry weight (a) and standard length (b) of Senegalese sole larvae and post–larvae fed live preys enriched with different oil emulsions. Values are means ± SD (n = 4 pools for DW or n = 120 individuals for SL, per treatment and time point). Letters show significant differences ($p<0.05$) between treatments for individual days.

Metamorphosis started earlier in larvae fed the CLO diet (at 14 dph) than those from the other 3 treatments. Larvae fed CLO and LSO, progressed through metamorphosis faster than those fed the SBO and OO (more evident at 23 dph). At 30 dph, most treatments had completed metamorphosis, except the OO group, which was significantly delayed with respect to the CLO treatment (Table 5).

Table 3. Eye migration index (I_{EM}) of Senegalese sole larvae and post larvae fed live prey enriched with CLO, LSO, SBO or OO at 12, 14, 16, 18, 22, 23, 25, 28 and 30 dph. Values are means ± SD (n = 4 pools) and different letters indicate significant differences (ANOVA, p<0.05) between the treatments within one column.

I_{EM}	12dph	14dph	16dph	18dph	22dph	23dph	25dph	28dph	30dph
CLO	0,10 ± 0,10	$0,16 \pm 0,06^{a}$	0,93 ± 0,54	1,71 ± 0,36	3,34 ± 0,12	$3,58 \pm 0,26^{a}$	$3,88 \pm 0,18^{a}$	3,95 ± 0,04	$3,98 \pm 0,03^{a}$
LSO	0,01 ± 0,03	$0,04 \pm 0,03^{b}$	0,96 ± 0,53	1,65 ± 0,36	3,17 ± 0,09	$3,49 \pm 0,25^{ab}$	$3,78 \pm 0,15^{ab}$	3,89 ± 0,17	$3,93 \pm 0,03^{ab}$
SBO	0,00 ± 0,00	$0,01 \pm 0,02^{b}$	0,43 ± 0,31	1,47 ± 0,30	2,94 ± 0,44	$3,01 \pm 0,21^{c}$	$3,45 \pm 0,22^{b}$	3,60 ± 0,25	$3,93 \pm 0,05^{ab}$
OO	0,00 ± 0,00	$0,01 \pm 0,02^{b}$	0,40 ± 0,12	1,45 ± 0,12	3,00 ± 0,06	$3,11 \pm 0,14^{bc}$	$3,5 \pm 0,02^{b}$	3,72 ± 0,23	$3,62 \pm 0,09^{b}$

Larval survival was significantly higher in larvae fed CLO (25.2 ± 5.0%) than those fed OO (7.6 ± 6.0%). Survival of larvae fed LSO and SBO was 19.6 ± 8.4% and 13.0 ± 4.9%, respectively, and not significantly different from the other treatments.

Looking at enzymatic activity, post–larvae from the CLO treatment showed a significantly higher AP/Leu–ala ratio than the other treatments at 37 dph (Fig. 2), mainly due to the significantly higher specific activity of AP.

Lipid and fatty acid composition of enriched Artemia, larvae and post–larvae

Live preys were enriched with emulsions prepared with either a marine oil, CLO, or one of three vegetable oils (VO): LSO, SBO or OO. As expected, the enriched *Artemia* did not show significant differences in total lipid and total FA content between treatments, but had significantly different FA profiles that reflected their oil source (Table 1). *Artemia* enriched with CLO supplied the larvae with the highest levels of LC–PUFA (ARA 0.5%, EPA 6.3%, DHA 3.9% of TFA), while the VO treatments had negligible amounts of these EFAs (0–0.9% of TFA). The VO treatments also differed substantially: the LSO treatment had the highest levels of n–3 PUFA, as a result of high ALA content (2–fold higher than other treatments); the SBO enrichment supplied the larvae with the highest n–6 PUFA levels, due to its LNA concentration (3–fold more than other treatments); and *Artemia* enriched with the OO emulsion presented the highest levels of MUFA, given its high content in OA, and had the lowest PUFA levels.

Total lipid content was higher in larvae, but there were no significant differences between treatments at either age (Tables 4 and 5). On the other hand, the FA profile of larvae and post–larvae closely reflected the composition of their diets (Table 1) and did not change greatly from 22 to 37 dph (Tables 4 and 5). At both ages, fish fed the CLO diet had a significantly higher

percentage of n–3 PUFA, mostly EPA and DHA, closely followed by the LSO treatment due to high levels of ALA. Although fish fed the LSO diet had significantly higher ARA levels than the remaining treatments, those fed SBO showed the highest n–6 PUFA levels due to a significantly higher percentage of LNA. The OO treatment had significantly higher levels of MUFA than the other treatments, reflecting the oleic acid (OA) content. No significant differences in SFA levels were found between treatments at 22 dph, but at 37 dph they were significantly lower in post–larvae from the OO treatment.

Fat accumulation in the intestine and liver

The percentage of epithelium occupied by fat deposits in the posterior intestine and liver, at 22 dph and 37 dph was assessed by histological procedures (Table 6). The posterior intestine was chosen as the area to assess intestinal lipid accumulation since there was a marked longitudinal gradient, increasing in an anterior–posterior direction (Appendix A.1). No significant differences were observed in lipid accumulation in the intestinal mucosa between treatments at either age (Table 6). Large intracellular lipid inclusions were scattered throughout the entire cytoplasm from the apical to the basal end of enterocytes, with some having a diameter of over 20 µm. In extreme cases, these inclusions created very large aggregations resulting in the deformation of enterocytes and villi (Fig. 3a, c, e, g and 4a, c, e, g).

The percentage of liver tissue covered by lipids was not significantly different between the 4 dietary treatments at 22 dph (Table 6). At this time hepatocyte nuclei, where visible, were always displaced to the periphery of the cells. Hepatocytes were enlarged, homogeneous in size and evenly distributed across the whole hepatic parenchyma along sinusoids, compressing the vascular lumen (Fig. 3b, d, f, h). By 37 dph, the hepatocytes of post–larvae from the CLO treatment had no to very little visible lipid droplets (Fig. 4b). This was also apparent in the area occupied by fat deposits, which was significantly lower in the CLO treatment compared to the VO treatments (Table 6). Contrary to the CLO treatment, the degree of fat accumulation in the VO treatments was similar to that observed at 22 dph; however, although the entire cytoplasm of hepatocytes was completely filled with fat, the cells did not seem as enlarged as at 22 dph; the nuclei were clearly visible and, in numerous cases, centrally located (Fig. 4b, d, f, h).

Gene expression analysis

In order to evaluate the transcriptional responses of fasted and fed larvae (at 16 dph) and post–larvae (at 34 dph), the transcript levels of key lipid metabolism, absorption and transport genes were measured by RT–qPCR after a 16h fasting period (t0; Table 7) and in the post–pandrial stage (3 h after providing a meal; t3; Table 8).

At t0, *fas*, *apoa4* and *Δ4fad* levels were significantly different across diets both in pelagic larvae and benthonic post–larvae. At 16 dph, *fas* was significantly down–regulated and *apoa4* up–regulated in the CLO compared to OO treatment. Similar to *fas*, *Δ4fad* was significantly down–regulated in the CLO treatment, but only in comparison to the LSO treatment. At 34dph, *fas* expression was significantly lower in the CLO treatment, compared to the SBO and OO treatments, while *apoa4* was inversely

regulated, with significantly higher values in post–larvae fed the CLO and LSO diets than in the SBO and OO treatments. The expression of *Δ4fad* at 34 dph was similar to that at 16 dph, but it was also down–regulated in the OO treatment as well as in CLO compared to LSO.

Table 4. Area of fat deposits in the intestinal epithelia and liver of Senegalese sole larvae (22 dph) and post–larvae (37 dph) fed CLO, LSO, SBO or OO. Values are expressed as percentage of total tissue surface and presented as means ± SD (n = 12 larvae per treatment, 1 photo per larvae for intestine and 3 for liver). Letters show significant differences (ANOVA, $p<0.05$) between dietary treatments for each tissue at each age.

	Tissue area occupied by lipid droplets (%)	
Diet	Posterior intestine	Liver
22dph		
CLO	56.5 ± 4.7	64.3 ± 5.9
LSO	63.1 ± 7.8	57.0 ± 8.9
SBO	57.9 ± 6.5	63.4 ± 6.6
OO	62.4 ± 6.8	62.5 ± 8.1
37dph		
CLO	62.5 ± 6.9	33.5 ± 8.9[a]
LSO	62.5 ± 3.2	64.5 ± 6.8[b]
SBO	57.9 ± 8.1	65.4 ± 3.9[b]
OO	65.6 ± 4.8	65.4 ± 3.8[b]

Expression results at t3 only showed significant differences in mRNA amounts of *Δ4fad* and *fabp3* at 16 dph, when both genes had significantly lower expression levels in the CLO, compared to the OO treatment, while SBO and LSO had intermediate values.
To characterize the transcriptional responses of larvae fed the different diets, a set of 109 transcripts was analyzed (complete list presented in Hachero–Cruzado et al.; 2014). Statistical analysis identified 19 and 26 transcripts to be differentially expressed between different dietary treatments in larvae (16 dph) and post–larvae (34 dph), respectively.

Generally, larvae from the LSO treatment exhibited similar expression values to those from the CLO group at 16 dph, while the SBO and OO treatments had opposite values (Appendix A.2). Twelve transcripts had significantly higher levels in larvae fed CLO compared to SBO:1–acylglycerol–3–phosphate O–acyltransferases 3 and 9 (*agpat3*, *agpat9*), lysophosphatidylcholine acyltransferase 1(*lpcat1*), ethanolamine–phosphate cytidylyltransferase 2 (*pcyt2*), lysocardiolipin acyltransferase 1 (*lclat1*), lipid phosphate phosphatase–related protein type 1(*lppr1*), phospholipase D2 (*pld1*), secretory phospholipase A2–like proteins A and B (*pg12a*, *pg12b*), lipid phosphate phosphohydrolase 1 (*lpp1*) and apolipoproteins b and c2 (*apob*, *apoc2*). Transcripts *pg12a*, *pg12b* and *apoc2* were also significantly up–regulated in the CLO, compared to OO treatment, as were another 2 transcripts (not significantly different to SBO): monoacylglycerol O–acyltransferase 1 (*mogat1*), and apolipoprotein E (*apoe*). Adipose triglyceride lipase (*pnpla2*) had higher expression levels in larvae from the LSO

compared to the SBO treatment. The 2 remaining differentially expressed transcripts had an inverse expression pattern, with larvae from the SBO and OO treatments having higher levels of lipin 1 (*lpin1*) and endothelial lipase (*el*) with relation to CLO, respectively.

Table 5. Total lipid content, total FA content and FA composition of Senegalese sole larvae at 22 dph fed Artemia enriched with CLO, LSO, SBO or OO. Values are means ± SD (n = 4 pools). Different letters indicate significant differences between treatments (ANOVA, p<0.05).

	CLO	LSO	SBO	OO
Total lipid and total FA content of larvae (mg g^{-1} DW)				
Total lipids	93.2 ± 31.9	111.3 ± 11.4	101.8 ± 2.4	103.7 ± 13.7
Total FA	48.9 ± 15.9	65.2 ± 9.8	59.1 ± 5.8	57.8 ± 8.8
FA composition of larvae (% TFA)*				
16:0	10.6 ± 0.8	8.8 ± 0.8	9.5 ± 0.8	9.6 ± 0.6
18:0	7.1 ± 0.2	8.3 ± 0.3	8.3 ± 0.6	6.9 ± 0.4
Total SFA	18.0 ± 0.9[a]	17.3 ± 1.1[a]	17.9 ± 1.1[a]	16.6 ± 0.5[a]
16:1	3.0 ± 0.3	1.0 ± 0.3	1.3 ± 0.2	2.2 ± 0.9
18:1n–9	22.8 ± 0.2	23.9 ± 0.4	22.5 ± 0.6	38.7 ± 1.4
18:1n–7	7.2 ± 0.4	5.7 ± 0.4	5.6 ± 0.7	6.0 ± 1.2
20:1	3.3 ± 0.2	1.7 ± 0.2	1.2 ± 0.0	1.5 ± 0.1
Total MUFA	36.4 ± 0.5[c]	32.4 ± 0.3[b]	30.5 ± 0.7[a]	48.4 ± 1.4[d]
18:2n–6	7.4 ± 0.5[a]	14.9 ± 0.8[c]	22.7 ± 0.5[d]	9.4 ± 0.5[b]
20:4n–6	2.9 ± 0.2[ab]	3.4 ± 0.0[c]	3.0 ± 0.2[b]	2.5 ± 0.2[a]
Total n–6 PUFA	11.3 ± 0.5[a]	19.4 ± 0.7[c]	26.6 ± 0.7[d]	12.9 ± 0.7[b]
18:3n–3	15.2 ± 0.5[b]	20.9 ± 0.7[d]	16.8 ± 0.8[c]	13.7 ± 0.2[a]
20:5n–3	5.4 ± 0.3[c]	1.7 ± 0.3[b]	0.8 ± 0.1[a]	1.2 ± 0.2[a]
22:5n–3	3.0 ± 0.2	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
22:6n–3	5.6 ± 0.3[d]	2.0 ± 0.2[c]	1.4 ± 0.1[a]	1.6 ± 0.3[b]
Total n–3 PUFA	32.6 ± 1.0[d]	28.7 ± 0.8[c]	22.5 ± 0.8[b]	20.0 ± 0.8[a]
Total PUFA	43.9 ± 1.0[b]	48.1 ± 0.9[c]	49.2 ± 0.5[c]	32.8 ± 1.1[a]
DHA/EPA	1.04 ± 0.02[a]	1.18 ± 0.15[ab]	1.80 ±0.07[b]	1.35 ± 0.04[c]
ARA/EPA	0.53 ± 0.05[a]	2.01 ± 0.36[b]	3.74 ± 0.55[c]	2.17 ± 0.33[b]

*Not all FAs are shown. Only FAs and FA groups with letters were statistically analyzed.

At 34 dph, similarly to 16 dph, expression profiles of post–larvae fed CLO tended to be significantly different to SBO and OO (Appendix A.3). Post–larvae from the OO treatment had similar expression levels to those fed SBO, with several transcripts having reduced levels compared to CLO and/or LSO: 1–acyl–sn–glycerol–3–phosphate acyltransferase beta (*agpat2*), lysophosphatidylcholine acyltransferase 5 (*lpcat5*), *pcyt2*, phosphatidylserine decarboxylase (*pisd*), group 3 secretory phospholipase A2 precursor

(*pla2g3*), *pg12a*, *pg12b*, hepatic triacylglycerol lipase precursor (*htgl*), lipoprotein lipase (*lpl*), fatty acid binding protein 1 (*fabp1*), apolipoprotein A1 (*apoa1*), *apoa4* and 3 *apoe* transcripts. Conversely, post–larvae from the SBO treatment showed higher expression, compared to the CLO treatment, for several transcripts: phosphatidylserine synthase 1 (*ptdss1*), CDP–diacylglycerol–serine O–phosphatidyl transferase (*pss*), lipid phosphate phosphatase–related protein type 1 (*lppr1*), apolipoprotein E receptor 2 (*apoer2*) and cleavage stimulation factor subunit 1 (*cstf1*). Furthermore, adipose triglyceride lipase (*atgl*) had higher expression levels in the LSO and OO treatments compared to CLO. Finally, phospholipase A2 group 1B (*pla2g1b*), was expressed at higher levels in post–larvae from the OO compared to the SBO treatment.

Table 6. Total lipid content, total FA content and FA composition of Senegalese sole post–larvae at 37 dph fed *Artemia* enriched with CLO, LSO, SBO or OO. Values are means ± SD (n = 4 pools). Different letters indicate significant differences between treatments (ANOVA, $p<0.05$).

	CLO	LSO	SBO	OO
Total lipid and total FA content of post–larvae (mg g^{-1} DW)				
Total lipids	73.1 ± 19.8	60.6 ± 17.9	69.1 ± 14.5	74.7 ± 7.1
Total FA	43.5 ± 13.1	37.8 ± 8.4	39.3 ± 8.0	48.4 ± 6.9
FA composition of post–larvae (% TFA)*				
16:0	12.5 ± 0.8	9.6 ± 1.3	10.6 ± 0.4	9.5 ± 0.3
18:0	6.7 ± 0.5	6.7 ± 0.9	7.0 ± 0.4	5.9 ± 0.5
Total SFA	19.7 ± 1.2^{b}	16.5 ± 2.2^{ab}	17.9 ± 0.7^{b}	15.6 ± 0.7^{a}
16:1	3.5 ± 0.2	2.1 ± 0.1	1.8 ± 0.3	2.6 ± 0.8
18:1n–9	22.6 ± 0.4	22.3 ± 0.7	21.2 ± 0.5	35.8 ± 0.8
18:1n–7	6.5 ± 0.4	4.5 ± 0.3	5.0 ± 0.5	5.7 ± 0.9
20:1	2.8 ± 0.2	1.1 ± 0.1	1.0 ± 0.0	1.5 ± 0.1
Total MUFA	35.4 ± 0.6^{b}	30.0 ± 1.0^{a}	29 ± 0.7^{a}	45.6 ± 1.3^{c}
18:2n–6	7.8 ± 0.7^{a}	14.9 ± 0.2^{c}	22.9 ± 0.5^{d}	10.1 ± 0.3^{b}
20:4n–6	3.0 ± 0.1^{a}	3.9 ± 0.3^{c}	3.2 ± 0.0^{b}	2.7 ± 0.4^{ab}
Total n–6 PUFA	11.7 ± 1.0^{a}	19.8 ± 0.7^{c}	27.4 ± 0.3^{d}	14.2 ± 0.7^{b}
18:3n–3	13.1 ± 1.1^{a}	25.2 ± 3.6^{b}	15.1 ± 1.5^{a}	14.3 ± 0.8^{a}
20:5n–3	4.9 ± 0.2^{b}	0.9 ± 0.2^{a}	1.0 ± 0.2^{a}	1.2 ± 0.2^{a}
22:5n–3	2.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.2
22:6n–3	7.2 ± 0.2^{b}	2.3 ± 0.6^{a}	2.7 ± 0.5^{a}	3.1 ± 1.2^{a}
Total n–3 PUFA	30.7 ± 1.2^{a}	31.6 ± 3.5^{a}	22.4 ± 0.7^{b}	22.4 ± 0.9^{b}
Total PUFA	42.4 ± 1.9^{b}	51.4 ± 3.5^{c}	49.8 ± 1^{c}	36.6 ± 1.2^{a}
DHA/EPA	1.48 ± 0.05^{a}	2.37 ± 0.20^{b}	2.87 ± 0.11^{c}	2.45 ± 0.64^{abc}
ARA/EPA	0.62 ± 0.03^{a}	4.25 ± 0.76^{c}	3.51 ± 0.67^{c}	2.26 ± 0.51^{b}

*Not all FAs are shown. Only FAs and FA groups with letters were statistically analyzed.

Clustering analysis of significant qPCR results generally led to a good separation of the treatments. Overall, larvae fed CLO and LSO were grouped separately from the SBO and OO treatments at both stages of larval development (Fig. 5a and b).

4. *Discussion*

Fish performance and digestive function maturity

In the present study Senegalese sole larvae fed CLO exhibited the highest growth and survival rates followed by those fed LSO and SBO, with main differences observed in the period between 22 and 30 dph. During the pre–metamorphic stage, up to 21 dph, larvae showed very similar growth performance, independent of their diet, whereas the absence of significant differences at the end of the experiment was due to a high variability in growth leading to a lack of statistical power to detect differences. Nevertheless, larvae from the LSO treatment appeared to show a compensatory growth from 30 to 37 dph and almost “caught up” to the CLO treatment. These results seem to corroborate the standing hypothesis that dietary levels of LC–PUFA, particularly EPA and DHA, might be crucial for this species during a developmentally critical window just before metamorphosis, but are less important once the larvae metamorphose and acquire the benthic life stage (Dâmaso–Rodrigues et al., 2010; Morais et al., 2012a; Morais et al., 2004). In fact, metamorphosis was initiated earlier in larvae fed CLO and only the LSO treatment reached similar values towards the end of the metamorphosis window (23–25 dph at 18–20ºC). It is generally accepted that larval size at metamorphosis is a major factor determining the energy reserves and amount of time that an individual requires to fulfill the transition to demersal post–larvae (Geffen et al., 2007). Under our experimental conditions the onset of metamorphosis in Senegalese sole seemed to be diet–dependent, as significant differences were observed in I_{EM} values between treatments without significant variation in weight or length during this period suggesting that dietary EFAs could be influencing metamorphosis, as previously described in flatfish larvae (Boglino et al., 2012a; Lund et al., 2008; Shields et al., 1999; Villalta et al., 2005a).

The activity of intestinal enzymes provides a reliable marker for assessing the development of the digestive function in fish larvae (Cahu and Zambonino–Infante, 1994). The AP/Leu–ala ratio was, therefore, used as an indicator of the intestinal maturation of larvae at 37 dph (Cahu and Zambonino–Infante, 1994; Martínez et al., 1999; Ribeiro et al., 1999b). Post–metamorphic specimens from the CLO treatment had the highest values for the AP/Leu–ala ratio, mostly as a result of the higher AP activity in this treatment compared to the VO diets. This reflected the development of the brush border membrane of the enterocytes and indicated an increasing relevance of the absorptive processes mediated by these enzymes (Boglino et al., 2012a; Martínez et al., 1999). Furthermore, these results correlated well with the fastest growth and development, assessed by the I_{EM} in the CLO treatment.

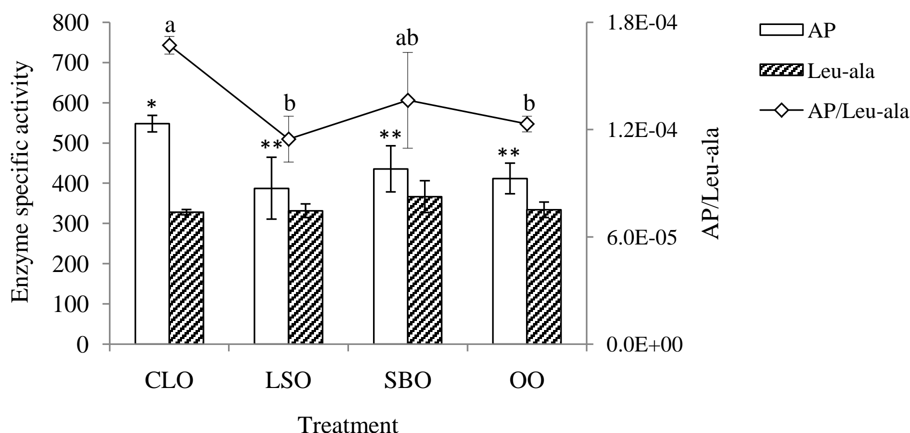

Figure 2. Specific activities of alkaline phosphatase (AP) and leucine–alanine (Leu–ala) and their mutual ratio for each dietary treatment. Values were measured at 37 dph in *Solea senegalensis* post–larvae and are presented as means ± SD (n = 4 pools per treatment) in U mg^{-1} protein × 10,000 and U mg^{-1} protein for AP and Leu–ala, respectively. Letters and asterisk indicate significant differences between treatments for each individual data series.

Effect of diets on FA composition of larvae and post–larvae

In accordance with other studies, the FA profile of Senegalese sole larvae and post–larvae closely reflected the diet composition (Boglino et al., 2012a; Dâmaso–Rodrigues et al., 2010; Morais et al., 2012a; Villalta et al., 2005a). Nonetheless, some deviations from the expected profile were observed in the content of both n–3 and n–6 LC–PUFA. At 22 dph, larvae fed the LSO diet had significantly higher levels of EPA and DHA than the SBO treatment, although both diets were equally deficient in these LC–PUFA. However, these differences were not evident at 37 dph. On the other hand, larvae fed LSO (and not CLO, which was the richest dietary source of ARA) showed the highest ARA levels (3.4%) at 22 dph, which were even higher at 37 dph (3.9%). In addition, larvae fed the SBO and OO diets had ARA levels that were not significantly different from the CLO treatment. These results raise the question of whether the LC–PUFA might have been synthesized from lower chain precursors, possibly from C_{18} PUFA, as previously hypothesized by Navarro–Guillén et al. (2014), and as further discussed below.

Effects of diets on fat accumulation in target tissues

The intestine and liver are considered target organs when evaluating the nutritional and physiological status of fish (Gisbert et al., 2008). As observed by optical microscopy in this study, the largest aggregations of lipids were visible in enterocytes of the posterior intestine (Appendix A.1) as in Morais et al. (2006), instead of anterior and/or mid intestine, which have been described as the main areas of luminal lipid absorption in the digestive tract of Senegalese sole (Boglino et al., 2012b; Ribeiro et al., 1999a) and other teleost species (Boulhic and Gabaudan, 1992; Deplano et al., 1989; Gisbert et al., 2005; Iwai, 1969; Kjørsvik et al., 1991).

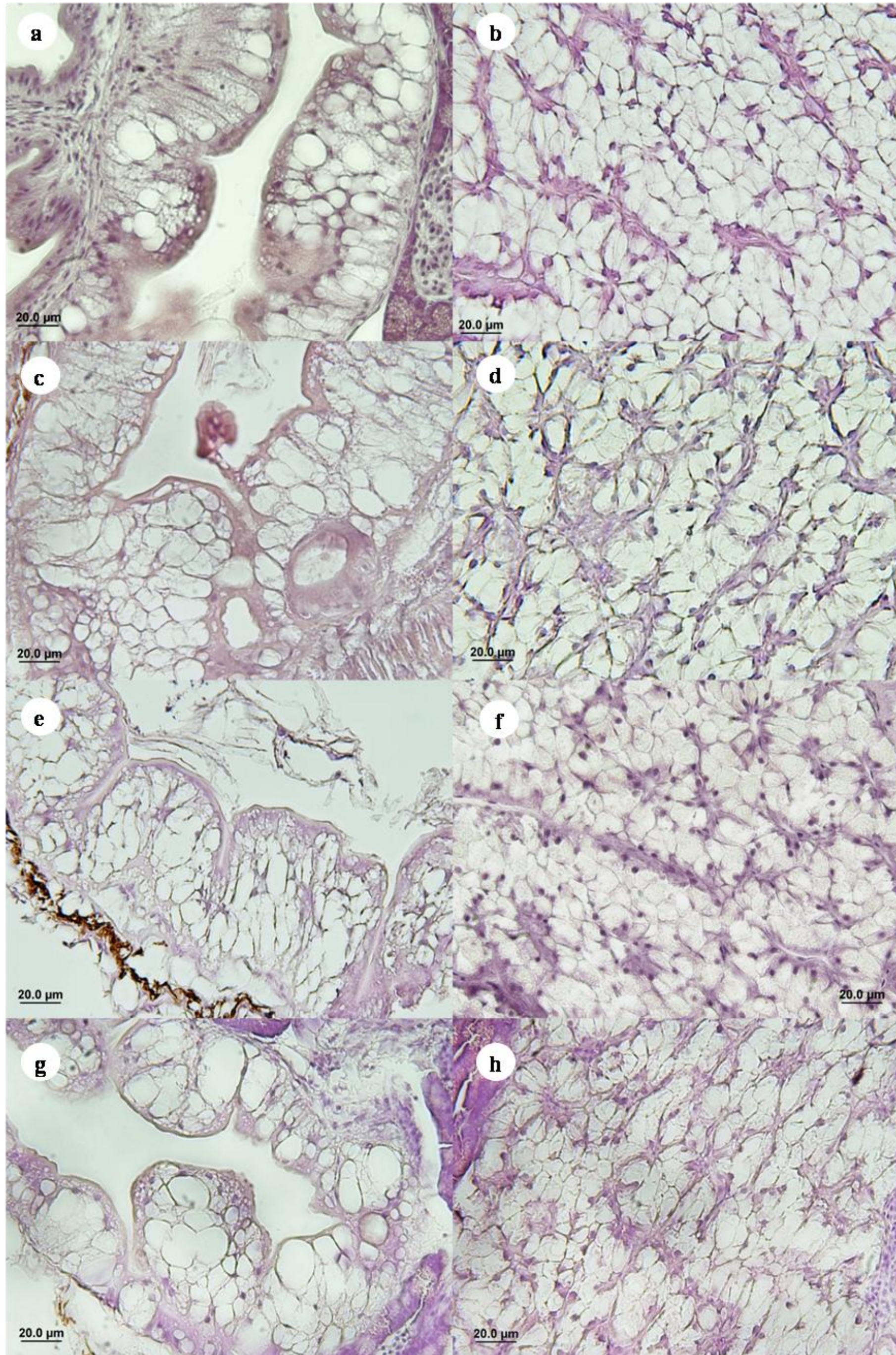

Figure 3. Lipid droplet accumulation in target tissues of Senegalese sole larvae at 22 dph. Images are x40 magnifications of the posterior intestine (a, c, e, g) and liver (b, d, f, h) of Senegalese sole larvae fed the CLO (a, b), LSO (c, d), SBO (e, f) and OO (g, h) diets. The scale bars represent 20 µm.

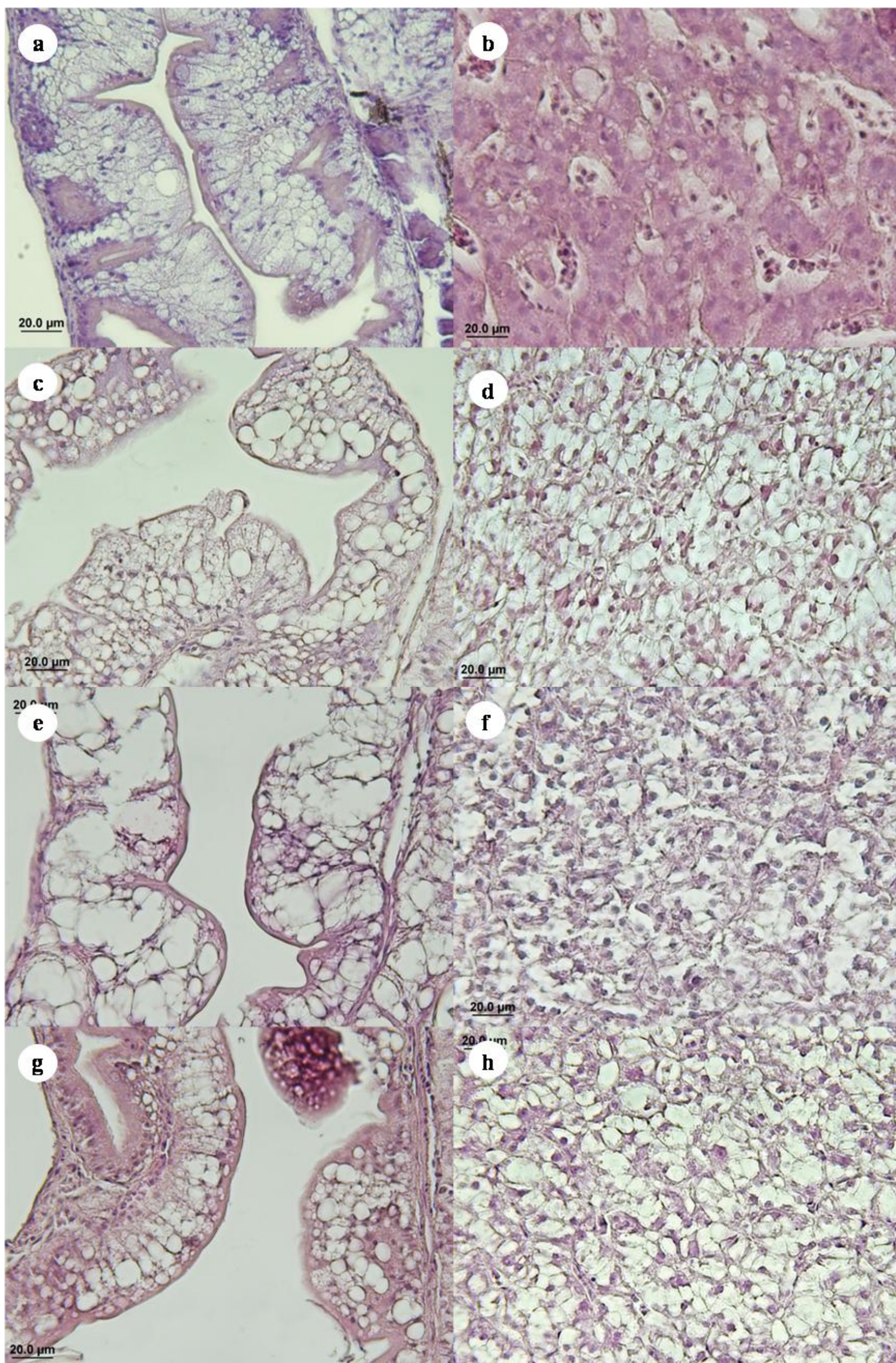

Figure 4. Lipid droplet accumulation in target tissues of Senegalese sole post–larvae at 37 dph. Images are x40 magnifications of the posterior intestine (a, c, e, g) and liver (b, d, f, h) of Senegalese sole post–larvae fed the CLO (a, b), LSO (c, d), SBO (e, f) and OO (g, h) diets. Scale bars represent 20 µm.

Both larvae and post–larvae, at 22 dph and 37 dph, respectively, had high levels of fat accumulation in the posterior intestine that did not vary significantly between treatments. Very large lipid droplets with diameters of up to ~20 µm were observed, which were much larger than the 5–8 µm droplet sizes previously reported in larvae of other teleosts (Boglino et al., 2012b; Deplano et al., 1991; Diaz et al., 1997). Lipid droplets are believed to be caused by high dietary FA content and are suggested to be temporary storage of re–esterified FAs in cases when the rate of lipid absorption exceeds the rate of lipoprotein synthesis (Sheridan, 1988), or because of an inability to metabolize lipids (Kjørsvik et al., 1991). Extensive lipid droplet formation is often denominated as intestinal steatosis, but is rarely considered a pathogenic state, although in extreme cases it may cause pathological damage to the intestinal epithelium (Gisbert et al., 2008). In this study, large fat deposits in the intestinal mucosa did not seem to affect their integrity, neither result in focal lesions; thus, differences in mortalities among groups cannot be attributed to intestinal damage caused by different diets.

The hepatic tissue of larvae from all treatments had similarly large accumulations of lipids at 22 dph. However, at 37 dph, post–larvae from the CLO treatment exhibited large intracellular spaces and very little to no cytoplasmatic lipid inclusions in the hepatic parenchyma. Larvae from the VO treatments retained a high degree of fat within the tissue, although the hepatocytes didn't seem as enlarged. A similar liver appearance to that of CLO post–larvae in this study was observed by Boglino et al. (2012b) in Senegalese sole post–larvae at 38 dph, fed *Artemia* enriched with a commercial product – Aquagrow Gold©. It is interesting to note that this experimental group had the best growth (DW at 38 dph was similar to that of CLO at 37 dph in our study under similar rearing conditions) and maturation of the digestive system of all the tested treatments and was also the only treatment without any hepatic lipid inclusion at 38 dph. However, the FA profile of the *Artemia* enriched with Aquagrow Gold© was somewhat different to that of the CLO, and the worst performing treatment from Boglino et al. (2012b), Easy Selco©, had an almost identical FA profile to our CLO treatment. This observation may seem contradictory, but it is important to note that total lipids in all experimental diets from Boglino et al. (2012b) were 2–fold higher than in our study. Therefore, it may be that total dietary lipid levels modulated the effect that specific FA profiles had on larval performance.

Effects of diets on the expression of genes involved in lipid absorption and metabolism

In order to examine the effect of the highly contrasting dietary FA profiles in larval and post–larval lipid absorption and metabolism, we analyzed the expression of a series of genes with RT–qPCR. Of these, 4 showed significant diet–dependent differences: *Δ4fad*, *fas*, *apoa4* and *fabp3*.

The *Δ4fad* is a key gene for LC–PUFA biosynthesis, namely of DHA, in Senegalese sole (Morais et al., 2012a; Morais et al., 2015b). It has been suggested that the enzyme it encodes might also have a residual Δ6/Δ5 Fad activity under particular nutritional conditions and/or life stages (Morais et al., 2012a; Navarro–Guillén et al., 2014), but so far DHA synthesis has only been demonstrated to occur from EPA and not from C_{18} PUFA in sole juveniles (Morais et al., 2015b). In this study, *Δ4fad* was down–regulated in fish fed CLO, particularly under fasting conditions. As previously reported, this was

probably due to a negative feedback effect of higher dietary end product (DHA) levels (Clarke and Jump, 1994; Morais et al., 2012a; Morais et al., 2015b). On the other hand, fish fed LSO had highest basal (t0) levels of Δ*4fad* transcripts at both 16 dph (although significantly different only from CLO) and 34 dph (significantly different from CLO and OO). It would be tempting to suggest that these results could explain some of the unexpected differences in larvae LC–PUFA profiles, as discussed above, but in face of the present lack of evidence of Δ6/Δ5 Fad activity in sole juveniles this should be interpreted with caution.

The expression of *fas*, an enzyme that catalyzes the *de novo* biosynthesis of FAs, was lowest in sole from the CLO treatment at both 16 and 34 dph, although only when fasting (t0). This could probably be explained by the well established hypotriglycemic effect of dietary fish oils (FO) in mammals. Dietary n–6 and n–3 PUFA, but not MUFA or SFA are known to suppress the transcription of FAS and other lipogenic enzymes in rats (Teran–Garcia et al., 2007; Wilson et al., 1990), with LC–PUFA being even more efficient in this respect (Davidson, 2006; Willumsen et al., 1993). Similar expression patterns have been found in teleost species fed FO compared to VO–based diets, which supports the existence of a similar effect in fish (Alvarez et al., 2000; Morais et al., 2012b). Interestingly, we observed that *fas* levels in the LSO group were comparable to that of CLO–fed fish, which only presented significant differences with OO–fed fish at 16 dph, and both OO and SBO treatments at 34 dph. Zuo *et al.* (2015) observed a similar down–regulation of *fas* in yellow croaker (*Larimichthys crocea*) fed a ALA–rich VO diet compared to those fed a LNA–based diet.

Differences in *apoa4* expression were also noted between treatments, which likely resulted in a different efficiency of lipid absorption from the gut lumen. In mammals, APOA4 aids triacylglycerol (TAG) packaging into chylomicrons by increasing their size, which greatly enhances basolateral TAG secretion in the intestine, subsequently increasing luminal FA absorption and flux of lipids through the intestine (Lu et al., 2006). In turn, an increase in intestinal fat absorption has been shown to stimulate APOA4 synthesis (Apfelbaum et al., 1987). At 16 dph, *apoa4* expression was significantly higher in the CLO compared to OO treatment, while at 34 dph it was higher in fish fed CLO and LSO compared to the SBO and OO treatments, but significant effects were only observed after fasting (t0) in both ages. Again, post–larvae fed LSO had values similar to CLO at 34 dph which coincided with a period of elevated growth for this group. Seeing as post–prandial effects on expression were not observed, this suggests that the different dietary FAs did not affect *apoa4* transcription acutely, but had a more chronic effect on mechanisms of lipid absorption instead. Furthermore, it may also be possible that a more mature and efficient intestine, as observed in larvae fed the CLO diet, was able to absorb lipids at a higher rate, leading to *apoa4* up–regulation.

A more acute effect of the different diets was visible in the post–prandial changes in expression of *fabp3* at 16 dph, when an up–regulation of this gene was observed in larvae fed CLO compared to OO. Unlike the remaining *fabp* transcripts (*fabp1*, *fabp2a* and *fabp2b*) and *mtp*, which are involved in lipid transport through the gastrointestinal mucosa, *fapb3* is mainly expressed in the ovary, liver and heart of Senegalese sole (our own unpublished results) and other fish species (Ando et al., 1998). Although we are not able to explain these results at present, we suggest that this expression could be associated with lipid metabolism in the liver. The encoded Fabp3 facilitates the transfer

Table 7. Expression of lipid metabolism, absorption and transport genes in Senegalese sole larvae (16 dph) and post–larvae (34 dph) from the CLO, LSO, SBO and OO treatments at t0 (after 16 h fasting). Values are mean NRQs ± SD (n = 4 per treatment). Letters represent significant differences in mRNA levels between treatments ($p<0.05$), for each gene and age separately.

	Larvae (16 dph)				Post–larvae (34 dph)			
	CLO	LSO	SBO	OO	CLO	LSO	SBO	OO
Lipid metabolism								
aco	1.03 ± 0.09	1.07 ± 0.18	1.00 ± 0.28	0.98 ± 0.24	0.97 ± 0.16	1.03 ± 0.38	1.06 ± 0.15	1.06 ± 0.29
fas	0.75 ± 0.06^{a}	1.12 ± 0.12^{ab}	0.99 ± 0.24^{ab}	1.18 ± 0.21^{b}	0.56 ± 0.07^{a}	1.08 ± 0.24^{ab}	1.53 ± 0.4^{b}	1.15 ± 0.24^{b}
Δ4fad	0.58 ± 0.14^{a}	1.31 ± 0.42^{b}	1.05 ± 0.27^{ab}	1.19 ± 0.23^{ab}	0.61 ± 0.09^{a}	1.76 ± 0.44^{b}	1.18 ± 0.46^{ab}	0.94 ± 0.36^{a}
Lipid absorption and transport								
apoa4	1.31 ± 0.12^{a}	1.03 ± 0.07^{ab}	1.18 ± 0.21^{ab}	0.76 ± 0.34^{b}	1.18 ± 0.08^{a}	1.11 ± 0.08^{a}	0.88 ± 0.14^{b}	0.89 ± 0.10^{b}
fabp1	1.17 ± 0.47	1.04 ± 0.45	1.04 ± 0.31	0.99 ± 0.29	1.25 ± 0.24	1.09 ± 0.39	0.92 ± 0.29	0.89 ± 0.04
fabp2a	1.07 ± 0.17	1.14 ± 0.21	1.13 ± 0.22	0.87 ± 0.4	1.09 ± 0.12	1.13 ± 0.37	0.99 ± 0.31	0.91 ± 0.07
fabp2b	1.49 ± 0.65	1.16 ± 0.49	0.97 ± 0.3	0.80 ± 0.23	0.99 ± 0.17	0.95 ± 0.33	1.08 ± 0.35	1.15 ± 0.34
fabp3	0.98 ± 0.13	1.12 ± 0.19	0.9 ± 0.33	1.09 ± 0.12	1.04 ± 0.15	1.08 ± 0.11	0.87 ± 0.1	1.06 ± 0.18
mtp	1.27 ± 0.01	1.24 ± 0.27	1.07 ± 0.3	0.88 ± 0.51	1.01 ± 0.08	1.03 ± 0.28	1.01 ± 0.21	1.01 ± 0.15

Table 8. Post–prandial expression (t3; 3 h after a meal) of lipid metabolism, absorption and transport genes in Senegalese sole larvae (at 16dph) and post–larvae (at 34dph) fed CLO, LSO, SBO or OO. Values are mean NRQs ± SD (n = 4 per treatment). Letters represent significant differences in mRNA levels between treatments ($p<0.05$), for each gene and age separately.

	Larvae (16 dph)				Post–larvae (34 dph)			
	CLO	LSO	SBO	OO	CLO	LSO	SBO	OO
Lipid metabolism								
aco	0.92 ± 0.18	1.09 ± 0.24	1.03 ± 0.16	1.02 ± 0.08	1.06 ± 0.21	1.04 ± 0.60	1.00 ± 0.19	1.35 ± 0.64
fas	0.90 ± 0.10	1.01 ± 0.15	0.96 ± 0.07	1.17 ± 0.18	1.21 ± 1.02	1.06 ± 0.60	1.22 ± 0.25	1.07 ± 0.18
Δ4fad	0.65 ± 0.18[a]	1.20 ± 0.37[ab]	1.09 ± 0.32[ab]	1.30 ± 0.17[b]	0.75 ± 0.21	1.56 ± 0.83	1.24 ± 0.38	1.03 ± 0.56
Lipid absorption and transport								
apoa4	1.01 ± 0.10	1.03 ± 0.09	1.01 ± 0.10	0.97 ± 0.14	1.21 ± 0.81	1.34 ± 0.48	1.40 ± 0.16	1.26 ± 0.10
fabp1	0.77 ± 0.26	1.26 ± 0.31	1.07 ± 0.39	1.19 ± 0.46	1.22 ± 0.88	1.43 ± 0.28	1.44 ± 0.28	1.31 ± 0.10
fabp2a	0.95 ± 0.30	1.05 ± 0.04	0.99 ± 0.21	1.09 ± 0.27	1.09 ± 0.75	1.33 ± 0.49	1.38 ± 0.22	1.13 ± 0.11
fabp2b	0.89 ± 0.48	1.32 ± 0.22	0.95 ± 0.26	1.05 ± 0.30	1.09 ± 0.29	0.96 ± 0.45	1.09 ± 0.18	1.03 ± 0.09
fabp3	0.87 ± 0.11[a]	1.08 ± 0.16[ab]	0.95 ± 0.1[ab]	1.13 ± 0.08[b]	1.18 ± 0.20	1.01 ± 0.33	0.91 ± 0.13	1.02 ± 0.25
mtp	0.85 ± 0.18	1.08 ± 0.06	1.12 ± 0.22	1.01 ± 0.12	1.03 ± 0.66	1.28 ± 0.75	1.36 ± 0.20	1.33 ± 0.08

of FAs across cellular membranes (Spener et al., 1989; Veerkamp et al., 1991) and in zebrafish (*Danio rerio*) liver it has been found to mediate FA transport for lipogenesis (Liu et al., 2003). Although we did not find significant differences in post–prandial expression of *fas*, this gene showed a similar trend in expression to *fabp3*, and was similarly and significantly regulated at t0 in 16 dph larvae. Therefore, both results could be potentially related to diet–induced differences in lipogenic pathways in the liver, and might at least partly explain the lower lipid accumulation in the liver observed histologically at 37 dph. Another possible, but not mutually exclusive explanation, could be an enhanced lipid mobilization from the liver in the CLO treatment through increased lipoprotein synthesis, as further discussed below.

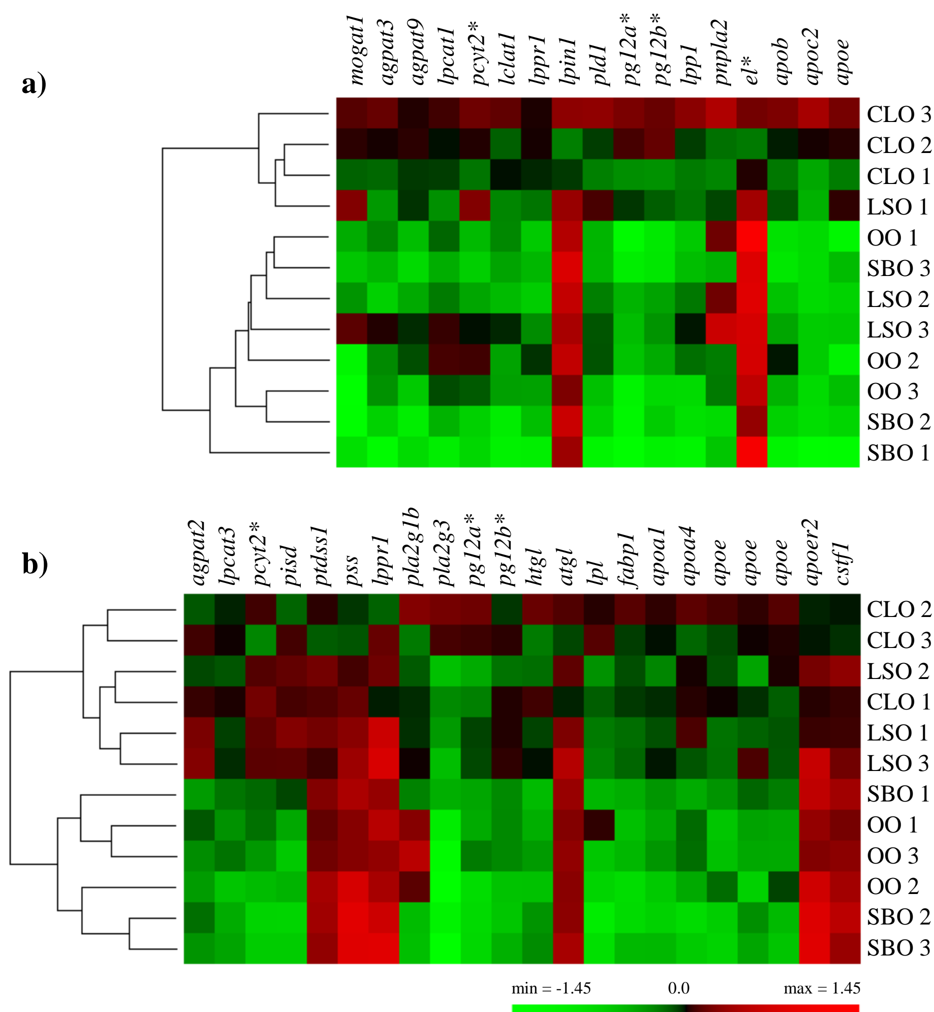

Figure 5. Hierarchical clustering analysis of differentially regulated transcripts related to lipid metabolism and absorption in Senegalese sole larvae. Expression levels were measured with an openarray chip in *Senegalese sole* larvae at 16 dph (a) and post–larvae at 34 dph (b) fed CLO, LSO, SBO or OO. Data is expressed as log2 fold change of each sample in relation to the average

Expression analysis of 109 transcripts related to lipid metabolism and transport (Hachero–Cruzado et al., 2014) revealed a set of differentially expressed genes between treatments mostly involved in lipid synthesis (acyltransferases and phosphatases), lipid hydrolysis (lipases and hydrolases) and apolipoproteins. Cluster analysis at 16 and 34 dph clearly separated the CLO treatment. However, the LSO treatment grouped more closely to CLO at both ages, especially at 34 dph, whereas the SBO and OO treatments behaved more similarly to each other and tended to form an opposite group. This data suggests that the identified set of differentially expressed genes could explain the beneficial effects CLO had on larval performance, followed by the LSO treatment. The CLO/LSO group showed higher mRNA levels for transcripts involved in the synthesis of PLs at 16 and 34 dph, namely of phosphatidylcholine (PC; *lpcat1, lpcat5*), phosphatidylethanolamine (PE; *pcyt2*, *pisd*), PL precursors such as monoacylglycerol phosphate (*agpat9*) and diacylglycerol phosphate (*agpat2*, *agpat3*) and other membrane components such as cardiolipin (*lclat1*) and dihydroxyoctadecanoate (*lppr1*, involved in sphingolipid metabolism). Furthermore, genes involved in PL hydrolysis (*pld1*, *pg12a*, *pg12b*, *lpp1*, *pla2g3*, *htgl*) and encoding apolipoproteins (*apoa1*, *apoa4*, *apob*, *apoc2*, *apoe*) were also up–regulated. Within this group, CLO generally had the highest levels of expression, except at 34 dph when, interestingly, LSO exhibited higher mRNA levels for almost all genes involved in PC and PE synthesis, matching a period in which larvae fed LSO had the fastest growth rate and appeared to be catching up to CLO–fed larvae in terms of size. Phospholipids are key

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of the CLO treatment. Asterisks represent differentially expressed transcripts observed both at 16 dph and 34 dph. The column labels are abbreviations of the following genes and their assay IDs (Hachero–Cruzado et al., 2014): *mogat1* – monoacylglycerol O–acyltransferase (1AICSWAJ), *agpat3* – 1–acyl–sn–glycerol–3–phosphate acyltransferase gamma–like (AIS08JC), *agpat9* – glycerol–3–phosphate O–acyltransferase (AII1M5Ml), *lpcat1* – lysophosphatidylcholine acyltransferase 1 (AI0IXKQ), *pcyt2* – ethanolamine–phosphate cytidylyltransferase 2 (AII1M5O), *lclat1* – lysocardiolipin acyltransferase 1 (AIQJB6Y), *pnpla2* – adipose triglyceride lipase (AI5IP9K), *lppr1* – lipid phosphate phosphatase–related protein type 1 (AIKALBV), *lpin1* – lipin 1 isoform 2 (AIT96PM), *el* – endothelial lipase (AIWR212), *pld1* – phospholipase D2 (AI6ROFV), *lpp1* – lipid phosphate phosphohydrolase 1 (AIHSOZF), *apob* – apolipoprotein B (AICSWAH), *apoc2* – apolipoprotein C–II (AIHSOZD), *apoe* – apolipoprotein E (AIPAD0P), *gpat1* – glycerol–3–phosphate acyltransferase 1 (AIMSHOA), *dgat1* – diacylglycerol O–acyltransferasehomolog 1b (AI5IP9L), *agpat2* – 1–acyl–sn–glycerol–3–phosphate acyltransferase beta (AIX008A), *lpcat2* – lysophospholipid acyltransferase 2 (AI70ML2), *lpcat5* – lysophospholipid acyltransferase 5 (AI5IP9M), *pcyt2* – ethanolamine–phosphate cytidylyltransferase 2 (AII1M5O), *pisd* – phosphatidylserine decarboxylase (AI89KSB), *ptdss1* – phosphatidylserine synthase 1 (AICSWAK), *pss* – CDP–diacylglycerol–serine O–phosphatidyltransferase (AIAAZX4), *lppr1* – lipid phosphate phosphatase–related protein type 1 (AIKALBV), *pla2g1b* – phospholipase A2; phosphatidylcholine 2–acylhydrolase (AIVI4VV), *pla2g3*– phospholipase A2 (AI1RVQZ), *pg12a* – secretory phospholipase A2–like protein (AIY9ZEJ), *pg12b* – secretory phospholipase A2–like protein (AIWR213), *htgl* – hepatic lipase (AIN1FUI), *atgl* – adipose triglyceride lipase (AI39R3C), *el* – endothelial lipase (AIWR212), *lpl* – lipoprotein lipase (AIVI4VU), *fabp1* – fatty acid binding protein 1 (AID1UGQ), *apoa1*– apolipoprotein A–I (AIAAZX1), *apoa4* – apolipoprotein A–IV (AI70ML0), *apoe* – apolipoprotein E (AIN1FUH, AIQJB6X, AIS08JD), *apoer2* – apolipoprotein E receptor 2 (AIX0079), *cstf1* – cleavage stimulation factor subunit 1 (AIFASM0).

to larval development, as they are building blocks for biomembrane synthesis and essential components of lipoproteins (Tocher et al., 2008), which in turn promote neutral lipid absorption from the gut and transport between tissues. Furthermore, lipid mobilization is essential in faster growing fish for the energy allocation to tissue differentiation and somatic growth, especially during metamorphosis when feeding is arrested (Boglino et al., 2012b; Brewster, 1987; Geffen et al., 2007). In this respect, PLs are considered essential nutrients for fish larvae, as these early stages seem to have an insufficient PL biosynthesis capacity to maintain their high rates of growth and morpho–anatomical development (Cahu et al., 2009). Therefore, not surprisingly, diets leading to a coordinated up–regulation of PL and apolipoprotein synthesis were also associated with a higher growth and faster development in this study. Previous work had similarly reported the ability of young Senegalese sole larvae to deal with higher dietary lipid concentrations by increasing the expression of apolipoproteins, leading to better absorption throughout the enterocytes and lower lipid accumulation in liver and gut (Hachero–Cruzado et al., 2014). In our study we did not observe differences between treatments in lipid droplet accumulation in the intestinal mucosa but the coordinated increase in PL and apolipoprotein synthesis in the CLO treatment most likely resulted in higher rates of lipoprotein transport leading to a lower accumulation of lipid in the liver in post–metamorphosed larvae.

On the other hand, the few genes that were up–regulated in the SBO and OO treatments were involved mostly in phosphatidylserine (PS), sphingolipid and TAG metabolism. At 16 dph only 2 genes were up–regulated (in relation to the CLO treatment): *lpin1*, involved in diacylglycerol synthesis and *el*, a gene with a high density lipoprotein (HDL) hydrolyzing function, acting on PLs as well as TAG. In mouse, up–regulation of *Lpin1* has been associated with a higher expression of *Fas* and other genes involved in FA synthesis, as well as with low dietary PUFA content (Martin et al., 2007), which is in agreement with the results from this study. At 34 dph, a total of 6 up–regulated genes were observed, of which some were involved in TAG hydrolysis (*atgl*), PL hydrolysis (*pla2g1b*; only in the OO treatment), lipoprotein metabolism (*apoer2*), PS synthesis (*ptdss1*, *pss*) and sphingolipid metabolism (*lppr1*). The up–regulation of some genes related to lipogenesis, particularly in the OO treatment, was surprising considering that these diets supply high levels of FAs (especially OA) that are considered preferential substrates for β–oxidation (Crockett and Sidell, 1993; Kiessling and Kiessling, 1993). We could therefore speculate that, in spite of the high dietary contents of energy–yielding FAs in the OO diet, the deficiency in EFA might have led to an energy deprived state, which could be caused by insufficient rates of PL synthesis for lipoprotein production, hence leading to low rates of intestinal absorption and endogenous lipid mobilization.

5. *Conclusions*

Results from this study clearly indicated that larval diets with higher levels of LC–PUFA, and possibly also C_{18} n–3 PUFA, promote growth, development and survival in Senegalese sole larvae and post–larvae. Furthermore, the data suggested that this effect was at least partly mediated by an up–regulation of PL metabolism and apolipoprotein

synthesis, potentially leading to higher rates of cellular membrane synthesis and enhancing intestinal lipid transport, most likely driving higher luminal lipid absorption, as well as transport and mobilization of lipid reserves from hepatic cells to other tissues throughout the body. However, it remains to be unequivocally established whether the moderately more beneficial effects of the LSO diet, compared to the SBO treatment, were due to a higher nutritional value of ALA compared to LNA and its involvement in different metabolic pathways, or whether this effect could be explained by some degree of bioconversion of ALA into biologically more active n–3 LC–PUFA by Senegalese sole larvae and post–larvae**.**

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Appendix A

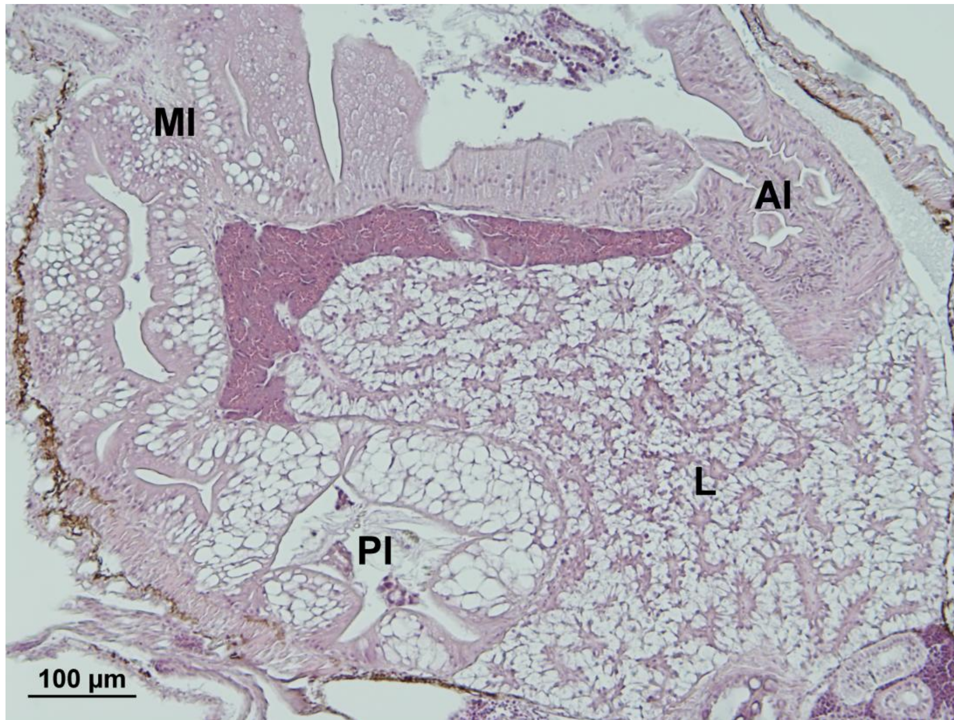

Figure A.1. Longitudinal transect of the abdominal cavity of a Senegalese sole larvae at 22 dph fed the OO diet. A gradual increase of lipid droplet inclusion in the intestinal epithelium from the anterior (AI), through mid (MI) to posterior intestine (PI) is clearly visible. The liver is marked as "L".

Appendix B

Table B.1. Expression of the 17 transcripts found to be significantly differentially regulated in the openarray chip between 16 dph–old Senegalese sole larvae raised on different dietary treatments. The activity of listed transcripts corresponds directly or indirectly to the function they have been categorized under. Data are expressed as means ± SD (n = 3) of fold change with respect to the CLO treatment. Expression results were normalized by gapdh2, ubq and eef1a1 mRNA levels. Letters indicate significant differences ($p < 0.05$) between dietary treatments.

		Dietary treatment			
Transcript	Assay ID†	CLO	LSO	SBO	OO
Lipid synthesis					
mogat1	AICSWAJ	1.00 ± 0.09[a]	1.02 ± 0.20[a]	0.53 ± 0.14[ab]	0.52 ± 0.20[b]
agpat3	AIS08JC	1.00 ± 0.11[a]	0.81 ± 0.20[ab]	0.58 ± 0.15[b]	0.82 ± 0.02[ab]
agpat9	AII1M5M	1.00 ± 0.03[a]	0.90 ± 0.13[a]	0.55 ± 0.05[b]	0.75 ± 0.16[ab]
lpcat1	AI0IXKQ	1.00 ± 0.04[a]	0.90 ± 0.12[ab]	0.65 ± 0.13[b]	0.96 ± 0.08[a]
pcyt2	AII1M5O	1.00 ± 0.13[a]	0.98 ± 0.22[a]	0.61 ± 0.02[b]	0.89 ± 0.18[ab]
lclat1	AIQJB6Y	1.00 ± 0.10[a]	0.83 ± 0.14[ab]	0.58 ± 0.15[b]	0.79 ± 0.04[ab]
lppr1	AIKALBV	1.00 ± 0.01[a]	0.77 ± 0.12[ab]	0.62 ± 0.12[b]	0.79 ± 0.17[ab]
lpin1	AIT96PM	1.01 ± 0.19[b]	1.37 ± 0.12[ab]	1.51 ± 0.21[a]	1.35 ± 0.16[ab]
Lipid hydrolysis					
pld1	AI6ROFV	1.01 ± 0.20[a]	0.94 ± 0.11[a]	0.59 ± 0.13[b]	0.77 ± 0.14[ab]
pg12a	AIY9ZEJ	1.01 ± 0.18[a]	0.79 ± 0.15[ab]	0.44 ± 0.06[c]	0.52 ± 0.13[bc]
pg12b	AIWR213	1.01 ± 0.18[a]	0.82 ± 0.08[ab]	0.54 ± 0.08[c]	0.61 ± 0.11[bc]
lpp1	AIHSOZF	1.01 ± 0.19[a]	0.91 ± 0.07[a]	0.56 ± 0.11[b]	0.70 ± 0.16[ab]
pnpla2	AI5IP9K	1.03 ± 0.30[ab]	1.21 ± 0.31[a]	0.65 ± 0.07[b]	0.95 ± 0.16[ab]
el	AIWR212	1.01 ± 0.14[b]	1.59 ± 0.25[ab]	1.74 ± 0.48[ab]	1.82 ± 0.47[a]
Lipid transport					
apob	AICSWAH	1.01 ± 0.16[a]	0.78 ± 0.13[ab]	0.53 ± 0.08[b]	0.75 ± 0.22[ab]
apoc2	AIHSOZD	1.03 ± 0.29[a]	0.64 ± 0.07[ab]	0.51 ± 0.10[b]	0.60 ± 0.03[b]
apoe	AIPAD0P	1.01 ± 0.15[a]	0.76 ± 0.23[ab]	0.56 ± 0.15[ab]	0.53 ± 0.13[b]

†Assay ID from Hachero–Cruzado et al. (2014);

mogat1, monoacylglycerol O–acyltransferase; *agpat3*, 1–acyl–sn–glycerol–3–phosphate acyltransferase gamma–like; *agpat9*, glycerol–3–phosphate O–acyltransferase 9; *lpcat1*, lysophosphatidylcholine acyltransferase 1; *pcyt2*, ethanolamine–phosphate cytidylyltransferase 2; *lclat1*, lysocardiolipin acyltransferase 1; *lppr1*, lipid phosphate phosphatase–related protein type 1; *lpin1*, lipin 1 isoform 2; *pld1*, phospholipase D2; *pg12a*, secretory phospholipase A2–like protein; *pg12b*, secretory phospholipase A2–like protein; *lpp1*, lipid phosphate phosphohydrolase 1; *pnpla2*, adipose triglyceride lipase; *apob*, apolipoprotein B; *apoc2*, apolipoprotein C–II; *apoe*, apolipoprotein E

Table B.2. Expression of the 22 transcripts found to be significantly differentially regulated in the openarray chip between 34 dph–old Senegalese sole post–larvae raised on different dietary treatments. The activity of listed transcripts corresponds directly or indirectly to the function they have been categorized under. Data are expressed as means ± SD (n = 3) of fold change with respect to the CLO treatment. Expression results were normalized by *gapdh2*, *ubq* and *eef1a1* mRNA levels. Letters indicate significant differences ($p < 0.05$) between dietary treatments.

		Dietary treatment			
Transcript	Assay ID†	CLO	LSO	SBO	OO
Lipid synthesis					
agpat2	AIX008A	1.00 ± 0.10^{ab}	1.16 ± 0.21^{a}	0.75 ± 0.07^{b}	0.77 ± 0.10^{b}
lpcat5	AI5IP9M	1.00 ± 0.02^{a}	0.93 ± 0.04^{ab}	0.70 ± 0.10^{b}	0.69 ± 0.14^{b}
pcyt2	AII1M5O	1.02 ± 0.24^{ab}	1.14 ± 0.02^{a}	0.61 ± 0.20^{b}	0.70 ± 0.12^{ab}
pisd	AI89KSB	1.01 ± 0.13^{ab}	1.20 ± 0.09^{a}	0.63 ± 0.25^{b}	0.59 ± 0.07^{b}
ptdss1	AICSWAK	1.00 ± 0.12^{b}	1.17 ± 0.09^{ab}	1.39 ± 0.10^{a}	1.30 ± 0.19^{ab}
pss	AIAAZX4	1.01 ± 0.15^{b}	1.29 ± 0.19^{ab}	2.13 ± 0.49^{a}	1.60 ± 0.48^{ab}
lppr1	AIKALBV	1.01 ± 0.16^{b}	1.79 ± 0.52^{ab}	1.94 ± 0.51^{a}	1.53 ± 0.15^{ab}
Lipid hydrolysis					
pla2g1b	AIVI4VV	1.02 ± 0.26^{ab}	0.95 ± 0.06^{ab}	0.63 ± 0.12^{b}	1.39 ± 0.30^{a}
pla2g3	AI1RVQZ	1.02 ± 0.25^{a}	0.6 ± 0.08^{ab}	0.39 ± 0.20^{b}	0.28 ± 0.07^{b}
pg12a	AIY9ZEJ	1.02 ± 0.22^{a}	0.83 ± 0.17^{ab}	0.52 ± 0.12^{b}	0.62 ± 0.18^{ab}
pg12b	AIWR213	1.00 ± 0.05^{a}	0.96 ± 0.12^{ab}	0.63 ± 0.11^{c}	0.68 ± 0.12^{bc}
htgl	AIN1FUI	1.01 ± 0.13^{a}	0.76 ± 0.04^{ab}	0.45 ± 0.12^{b}	0.68 ± 0.31^{ab}
atgl	AI39R3C	1.00 ± 0.10^{b}	1.36 ± 0.27^{a}	1.34 ± 0.04^{ab}	1.43 ± 0.09^{a}
lpl	AIVI4VU	1.01 ± 0.20^{a}	0.92 ± 0.09^{ab}	0.68 ± 0.09^{bc}	0.62 ± 0.07^{c}
Lipid transport					
fabp1	AID1UGQ	1.00 ± 0.11^{a}	0.86 ± 0.04^{a}	0.55 ± 0.09^{b}	0.53 ± 0.08^{b}
apoa1	AIAAZX1	1.00 ± 0.04^{a}	0.88 ± 0.12^{ab}	0.59 ± 0.11^{c}	0.62 ± 0.10^{bc}
apoa4	AI70ML0	1.01 ± 0.15^{a}	1.00 ± 0.10^{a}	0.53 ± 0.10^{b}	0.77 ± 0.11^{ab}
apoe	AIN1FUH	1.00 ± 0.09^{a}	0.84 ± 0.05^{ab}	0.56 ± 0.13^{b}	0.64 ± 0.17^{b}
apoe	AIPAD0P	1.00 ± 0.04^{a}	0.87 ± 0.22^{ab}	0.74 ± 0.10^{ab}	0.60 ± 0.10^{b}
apoe	AIS08JD	1.01 ± 0.13^{a}	0.93 ± 0.07^{ab}	0.63 ± 0.14^{b}	0.74 ± 0.16^{ab}
apoer2	AIX0079	1.00 ± 0.02^{b}	1.39 ± 0.43^{ab}	2.17 ± 0.33^{a}	1.57 ± 0.39^{ab}
Other					
cstf1	AIFASM0	1.00 ± 0.05^{b}	1.21 ± 0.15^{ab}	1.55 ± 0.17^{a}	1.37 ± 0.14^{a}

†Assay ID from Hachero–Cruzado et al. (2014);
agpat2, 1–acyl–sn–glycerol–3–phosphate acyltransferase beta; *lpcat5*, lysophospholipid acyltransferase 5; *pcyt2*, ethanolamine–phosphate cytidylyltransferase 2; *pisd*, phosphatidylserine decarboxylase; *ptdss1*, phosphatidylserine synthase 1; *pss*, CDP–diacylglycerol–serine O–phosphatidyltransferase; *lppr1*, lipid phosphate phosphatase–related protein type 1; *pla2g1b*, phospholipase A2, phosphatidylcholine 2–acylhydrolase; *pla2g3*, phospholipase A2; *pg12a*, secretory phospholipase A2–like protein; *pg12b*, secretory phospholipase A2–like protein; *htgl*, hepatic lipase; *atgl*, adipose triglyceride lipase; *lpl*, lipoprotein lipase; *fabp1*, fatty acid binding protein 1; *apoa1*, apolipoprotein A–I; *apoa4*, apolipoprotein A–IV; *apoe*, apolipoprotein E; *apoer2*,apolipoprotein E receptor 2; *cstf1*, cleavage stimulation factor subunit 1.

CHAPTER 4

Characterization of seven cocaine– and amphetamine–regulated transcripts (CART) differentially expressed in the brain and peripheral tissues of *Solea senegalensis* (Kaup)

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Abstract

CART (cocaine– and amphetamine–regulated transcript) is a peptide with neurotransmitter and neuroendocrine functions with several key roles, both centrally and peripherally. In mammals there is a single gene that produces two alternatively spliced variants in rat and a single transcript in human but in teleosts multiple genes have been found. In the present study we report the existence of seven transcripts in Senegalese sole and characterize their sequences and phylogenetic relationships, as well as their expression patterns in the brain and peripheral tissues, and in response to feeding. Both *cart2a* and *cart4* showed a ubiquitous expression in the brain, while *cart1a*, *cart1b* and *cart3a* were similarly expressed and had higher transcript levels in the mesencephalon, followed by the diencephalon. On the other hand, *cart2b* showed a main expression in the olfactory bulbs, and *cart3b* was predominantly expressed in the spinal cord. The expression profile in peripheral tissues differed substantially between *cart*'s, even between more recently duplicated genes. Collectively, all the tissues examined, except the muscle, express at least one of the different *cart*'s, although the highest transcript levels were found in the brain, gonads (ovary and testis) and, in some cases, eye and kidney. Concerning the feeding response, only brain *cart1a*, *cart2a* and *cart4* showed a significant post–prandial regulation, although future studies are necessary to assess potential confounding effects of stress imposed by the force feeding technique employed. Senegalese sole exhibits the highest number of *cart* genes reported to date in a vertebrate species. Their differential expression patterns and feeding regulation suggest that multiple *cart* genes, resulting from at least 3 rounds of whole genome duplication, have been retained in fish genomes through subfunctionalization, or possibly even through neofunctionalization.

Keywords: Neuropeptide; Fish; Tissue distribution; Appetite; Regulation of food intake; Gene evolution

1. *Introduction*

The cocaine– and amphetamine–regulated transcript (CART) was discovered and named by Douglass et al. (1995) as a mRNA that was highly up–regulated in the rat striatum following acute administration of cocaine or amphetamine. In the last two decades it has emerged as a key neurotransmitter and hormone involved in the regulation of a variety of important physiological processes. Considering its initial description, CART was associated early on with the effects of psychostimulants, and subsequent studies found CART mRNA and peptides to be present in important regions associated with drug–mediated reward, reinforcement and behaviour, particularly in areas of the mesolimbic dopaminergic system (reviewed by Vicentic and Jones, 2007). Furthermore, CART–containing cells were observed to form synapses with neurons producing neurotransmitters associated with addiction, such as dopamine and GABA, and to colocalize with neurons that synthesize neurochemicals implicated in the pharmacological actions of phychostimulants (Vicentic and Jones, 2007).

However, the best documented action of CART has been with regard to its involvement in the regulation of feeding and energy balance, promoting energy expenditure. CART is expressed in key areas of the brain that regulate these actions, such as the ventromedial hypothalamic nucleus (VMN), lateral hypothalamus (LH), arcuate nucleus (Arc), paraventricular nucleus (PVN), nucleus of the solitary tract (NTS) and nucleus accumbens (NAc). Moreover, CART is co–expressed with peptides that are involved in the regulation of feeding, pituitary hormone secretion and energy metabolism, such as pro–opiomelanocortin (POMC), melanin–concentrating hormone (MCH), thyrotropin–releasing hormone (TRH), corticotrophin–releasing factor (CRF) and neuropeptide Y (Vicentic and Jones, 2007; Lau and Herzog, 2014). In addition, effects of intracerebroventricular injections of CART on feeding, gastric emptying, body weight gain, plasma insulin and leptin levels, lipid oxidation and thermogenesis support the anorexic role of CART as a mediator of feeding and metabolic regulation (reviewed in Murphy, 2005; Vicentic and Jones, 2007; Rogge et al., 2008; Lau and Herzog, 2014). Other emerging roles of CART are its implication in sensory processing, endocrine regulation, stress and anxiety, cardiovascular function and bone remodelling (Rogge et al., 2008). In this respect, CART mRNA and peptides are abundant along the hypothalamo–pituitary–adrenal (HPA) axis and sympatho–adrenal system, where peptide expression can be altered by stress (Balkan et al., 2012).

Since its discovery, CART has been intensively studied in several mammalian species but has also been identified and characterized in amphibians, birds and fish (e.g., Volkoff and Peter, 2001; Tachibana et al., 2003; Lázár et al., 2004). Its important physiological role is supported by the high level of conservation of its mRNA and protein sequences from fish to humans. Interestingly, genomic studies in teleosts revealed the existence of higher number of *cart* genes than in tetrapods, which might have arisen as a result of a specific whole genome duplication event in teleost evolution (Volff, 2005). So far, different studies on CART mRNA and/or peptide have been performed in goldfish (*Carassius auratus*), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*) and two species of catfish (*Ictalurus punctatus* and *Clarias batrachus*). These studies focused on the expression patterns in different brain regions

and peripheral tissues, as well as in the transcriptional response of *cart* genes to starvation and food intake, both during early ontogeny and in adult animals (Volkoff and Peter, 2001; Kehoe and Volkoff, 2007; Singru et al., 2007; Kobayashi et al., 2008; Murashita et al., 2009; Barsagade et al., 2010; Murashita and Kurokawa, 2011; Valen et al., 2011; Mukherjee et al., 2012; Nishio et al., 2012; Wan et al., 2012; Akash et al., 2014). However, in most cases only 1–2 gene products have been studied, with the exception of medaka and zebrafish, where the differential expression of 6 and 4 transcripts, respectively, was reported (Murashita and Kurokawa, 2011; Nishio et al., 2012; Akash et al., 2014). Given the involvement of CART peptides in multiple and varied physiological processes, the enlargement of the CART family in teleost species might have led to redundancy or, alternatively, conferred subfunctionalization of roles or even development of new functions, which facilitate the retention of duplicated genes in the genome. In order to better understand this, and possibly uncover specific functions of different *cart* gene products, a comprehensive characterization of the differential expression patterns and transcriptional regulation is of paramount importance. With this objective in mind, we have thoroughly mined a Senegalese sole (*Solea senegalensis*) transcriptomic database for *cart*–like transcripts. We then characterized the different gene products and present original data on their differential expression in different brain areas and in an extensive collection of peripheral tissues, as well as on transcriptional responses to a 24 h fasting and post–prandial changes up to 12 h after feeding.

2. *Materials and methods*

cDNA sequence mining

Sequences of cocaine– and amphetamine–regulated transcripts were searched by gene annotation in the SoleaDB transcriptomic database (http://www.scbi.uma.es/soleadb) *Solea senegalensis* v4.1 global assembly and then grouped and assembled *in silico* into contigs using the BioEdit Sequence Alignment Editor (Hall, 1999). When open reading frames (ORF) were incomplete, 5’ and 3’ rapid amplification of cDNA ends (RACE) PCR was performed using the FirstChoice® RLM–RACE kit (Ambion, Life Technologies, Alcobendas, Madrid, Spain) on 5’ and 3’RACE cDNA synthesized from a 1:1 mixture of Senegalese sole brain and eye total RNAs. The obtained fragments were separated by gel electrophoresis and resulting bands were cut, purified using the Illustra GFX™ PCR DNA and gel band purification kit (GE Healthcare, Barcelona, Spain) and sequenced (SCSIE, University of Valencia, Spain) to confirm their identity. Finally, PCR specific primers were designed on the extremities of each transcript to amplify and confirm, by sequencing, the whole length of the seven *cart* cDNA’s.

Sequence and phylogenetic analysis

The ORF region of the Senegalese sole *cart* cDNA sequences were compared with each other, with the zebrafish (GeneBank accession numbers: GU057833.2 for *cart1*, GU057834.2 for *cart2a*, GU057835.2 for *cart2b*, and GU057836.1 for *cart3*; Nishio et al., 2012) human (NM_004291.3) and rat short variant (XM_006231843.2) sequences,

and an identity matrix was obtained in ClustalW2 (not shown). Similarly, the prepropeptide CART sequences deduced, using the online EMBL–EBI translation tool, from the *S. senegalensis cart*'s were compared with zebrafish (ADB12484.2, ADB12485.2, ADB12486.2 and ADB12487.1), rat (XP_006231905.1) and human (NP_004282.1) CART prepropeptides and aligned with the human and rat (short variant) counterparts in ClustalW2. The putative cleavage site of the signal peptide was estimated for each Senegalese sole deduced protein using the SignalP 4.1 software (http://www.cbs.dtu.dk/services/SignalP/).

Protein blast or tblastn searches were performed in the GenBank database in order to identify complete CART prepropeptides in several teleost species, together with a selection of tetrapod sequences. A phylogenetic tree was built based on the alignment of all the amino acid sequences (accession numbers shown in Fig. 4) and the evolutionary history was inferred using the Neighbor–Joining method (Saitou and Nei, 1987) in MEGA6 (Tamura et al., 2013). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was calculated according to Felsenstein (1985) and the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965), being presented as the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated.

Samples for characterization of cart expression in different brain regions

In order to analyze central *cart* expression in Senegalese sole, juvenile specimens (n=4) from 101 to 172 g in body weight and 20 to 25 cm in length were sampled from the "Laboratorio de Cultivos Marinos" (University of Cádiz, Puerto Real, Spain). They were maintained in 250 L tanks under natural photoperiod, with continuous seawater renovation, at a constant temperature and salinity of 19±1ºC and 39 ppt, respectively. Fish were fed with commercial dry pellets (LE–3 Skretting, Burgos, Spain) once a day. Animals were anaesthetized with tricaine methanesulfonate MS–222 (Sigma–Aldrich, Madrid, Spain; 100–200 mg/L water) and sacrificed by decapitation after 14 h fasting. The following brain regions were dissected, as depicted in Fig. 1: olfactory bulbs, telencephalic cerebral hemispheres, diencephalon (preoptic area, thalamus, hypothalamus and posterior tubercle), mesencephalon (optic tectum and tegmentum), rhombencephalon (cerebellum, vestibulolateral lobe and medulla oblongata), spinal cord and pituitary. All samples were rapidly frozen in liquid nitrogen and stored at –80°C until used. This study was approved by the Animal Experimentation and Ethics Committee of the University of Cádiz (Spain) and was conducted according to international ethical standards.

Samples for characterization of cart expression in peripheral tissues

Tissue samples were collected from 3 different Senegalese sole juveniles (average weight: 251 g) held in the experimental culture facilities of IRTA, Center of Sant Carles de la Rápita (Spain) in 16 m^3 tanks, with natural thermo–photoperiod, a salinity of 36 ppt and fed once a day a standard commercial feed (LE–3, Skretting, Burgos, Spain), supplemented twice a week with natural feeds (mussels and polychaetes). Fish were fasted for 24 h prior to sampling and sacrificed with a lethal dose of MS–222. A

homogeneous sample of about 100 mg of tissue, from the same relative position in all animals, was collected from: stomach (Sto), anterior intestine (AI), posterior intestine (PI), liver (L), spleen (Spl), anterior kidney–interrenal tissue (K), heart (H), muscle (M), dorsal skin (DS), ventral skin (VS), and ovaries (O). Other tissues including eye (E, closest to mouth), brain (B), olfactory rosettes (OR) and one testis (T), were sampled whole, and for gills (G) one gill arch was taken from the middle region. Samples were immediately frozen in dry ice and kept at –80ºC pending analysis.

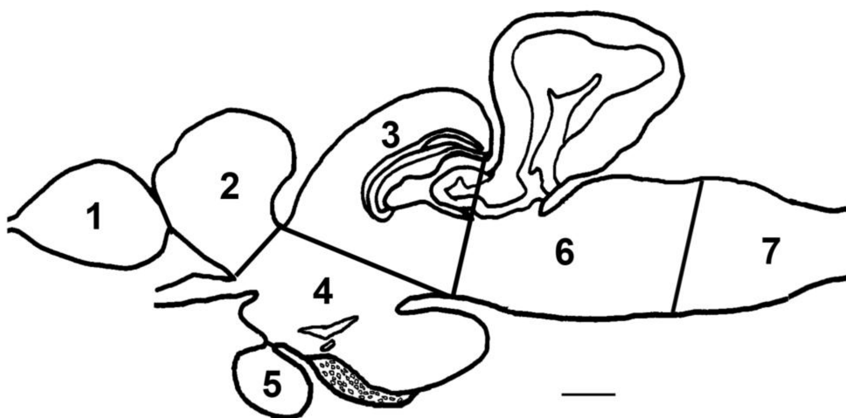


Figure 1. Schematic representation that depicts the dissections used in the present study to carry out the *cart* expression studies in the sole brain (sagittal view). 1: Olfactory bulbs; 2: Telencephalon; 3: Mesencephalon (optic tectum–tegmentum); 4: Diencephalon (preoptic area–thalamus–hypothalamus–posterior tubercle); 5: Pituitary; 6: Rhombencephalon (cerebellum–vestibulolateral lobe–isthmus–medulla oblongata); and 7: Spinal cord. Bar scale: 1 mm.

Feeding trial

Senegalese sole juveniles were cultured during 4 months, from an average body weight (BW) of 27.6 g until 67.7 g, and fed an extruded diet meeting Senegalese sole's nutritional requirements, containing 56% and 8% crude protein and lipids, respectively, formulated with 100% fish oil (diet formulation and composition can be found in Morais et al., 2015). Fish were grown in 4 replicate 500 L tanks (with 55 fish per tank) in a recirculation system at the IRTA experimental facility in Sant Carles de la Rápita. At the end of the experiment a feeding trial was performed. In order to ensure that all fish ate an equal amount of feed at the appointed time, and considering the highly variable voluntary feed intake of sole, a force–feeding method was used. Animals were fasted for 24 h after the last meal and then one fish per tank (n=4) was sacrificed with a lethal dose of MS222 and whole brains were removed. Afterwards, fish were force–fed with a silicone tube, to deposit a single meal composed of 6–10 feed pellets (depending on the size of the fish; ~0.15% BW) into the esophagus. Fish were monitored to ensure that the pellets were not expelled and were returned to the tanks were they remained for 1h, 3h, 6h, 9h and 12h before being sacrificed (n=4 per time point). Whole brain samples were immediately frozen in dry ice and kept at –80ºC pending analysis. Experimentation on

live fish (feeding trial and sampling for peripheral tissues) was performed according to the European and National legislation with protocols approved by the ethics committee of IRTA.

Real time quantitative PCR (qPCR)

Total RNA was isolated from samples by homogenizing in 1ml of TRIzol (Ambion, Life Technologies, Madrid, Spain) and performing solvent extraction according to manufacturer's instructions. Total RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain), respectively. Two micrograms of total RNA per sample were reverse transcribed into cDNA using the High–Capacity cDNA RT kit (Applied Biosystems, Life Technologies, USA), following manufacturer's instructions, but using a mixture of random primers (1.5 µl as supplied) and anchored oligo–dT (0.5 µl at 400 ng/µl, Eurogentec, Cultek, SL, Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. For studying *cart* expression in different brain regions, and due to the small amount of tissue, 100 ng of total RNA were reverse transcribed using the Quantitec Reverse Transcription kit (Qiagen, Hiden, Germany), which includes a genomic DNA elimination step. A similar amount of cDNA was pooled from all samples from the feeding trial (to prepare a dilution series and determine the efficiency of the PCR amplification) and the remaining cDNA was diluted 60–fold with water. The cDNA used for the brain and tissue expression profile studies were diluted 10– and 20–fold, respectively.

Primers for qPCR were designed using Primer3 v. 0.4.0 (http://bioinfo.ut.ee/primer3–0.4.0/) (Table 1). The resulting qPCR amplicons were sequenced to confirm their identity and the specificity of the qPCR assay. To normalize the results, previously validated reference genes were used (Infante et al., 2008): ubiquitin (*ubq*), 40S ribosomal protein S4 (*rps4*) and elongation factor 1 alpha (*ef1a1*) for the feeding trial (normalization factor generated with the 3 genes by geNorm, giving an M value of 0.103–0.129; Vandesompele et al., 2002), *rps4* for the brain expression study, and 18S rRNA (*18s*) for the tissue distribution profile. Expression of the *cart* genes was quantified in duplicate on a CFX96 Touch Real–Time PCR Detection System (Bio–Rad, Alcobendas, Spain) in a final volume of 20 µl containing 5 µl of diluted cDNA (except for *18s*: 1 µl; and all genes in the brain expression study: 4 µl), 0.5 µM (or 0.2 µM for *cart2a* and *cart3a*) of each primer and 10 µl of SsoAdvanced Universal SYBR Green Supermix (Bio–Rad) and included a systematic negative control (non template control or NTC). The qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 35–40 cycles: 15 s at 95 °C and 30 s at the Ta (60 ºC for target genes; 70 °C for reference genes). After the amplification phase, a melting curve was performed enabling confirmation of the amplification of a single product in each reaction and non–occurrence of primer–dimer formation in the NTC.

The expression levels of the different *cart* transcripts in different brain regions and tissues were determined using the delta–delta C_T method ($2^{-\Delta\Delta CT}$) describing the normalized (by *rps4* or *18s*, respectively) relative expression of the target genes in each region or tissue in relation to the average across all regions or tissues (Livak and Schmittgen, 2001). In the case of the feeding trial, the amplification efficiency of the primer pairs and arbitrary copy numbers in each sample were assessed by serial dilutions

of the cDNA pool, and the average normalized (by geNorm normalization factor calculated using *rps4, ef1a1, ubq* expression) values were compared by one–way ANOVA in SPSS v20 (SPSS Inc., Chicago, IL, USA), followed by the Tukey's post hoc test to perform multiple comparisons of the values between time points.

Table 1. Primers used for real–time quantitative PCR (qPCR). Shown are sequence and annealing temperature (Ta) of the primer pairs, size of the fragment produced, reaction efficiency (in the feeding trial) and accession number of the target and reference genes.

Transcript	Primer sequence	Fragment	Ta	E	Accession No.
cart1a	CGTCCACCACTGTCATTCTG CTTTCTCCTCCTGCGTCTTG	147 bp	60ºC	101.0%	KT189188
cart1b	TCGCTGAAAAGTCAACAAGAAA GCCAAGCTTTTTCTCCAGTG	99 bp	60ºC	99.2%	KT189189
cart2a	TCGCGTATCATCGAACACAT GTGACACTGAGCCACAGCAC	86 bp	60ºC	101.1%	KT189190
cart2b	AGGACCATGCAGAGTTCCAG GGACTCGGTGTCCATCACTT	99 bp	60ºC	104.2%	KT189191
cart3a	CAACAATCAGCTGGACGAGA AGCGCAGGAAGAAGGTGTTA	230 bp	60ºC	101.2%	KT189192
cart3b	TGTGGAAGAGCAGCAAACTG CACAGACAGGGTCAGGGTTT	153 bp	60ºC	99.4%	KT189193
cart4	GTGAGCGAGAGCAGGAAACT TCGTGGTGAAATAAGGCAAA	144 bp	60ºC	100.6%	KT189194
ubq[a]	AGCTGGCCCAGAAATATAACTGCGACA ACTTCTTCTTGCGGCAGTTGACAGCAC	93 bp	70ºC	101.2%	AB291588
rps4[a, b]	GTGAAGAAGCTCCTTGTCGGCACCA AGGGGGTCGGGGTAGCGGATG	83 bp	70ºC	100.8%	AB291557
ef1a1[a]	GATTGACCGTCGTTCTGGCAAGAAGC GGCAAAGCGACCAAGGGGAGCAT	142 bp	70ºC	100.9%	AB326302
18s[c]	GAATTGACGGAAGGGCACCACCAG ACTAAGAACGGCCATGCACCACCAC	148 bp	70ºC	–	AM882675

[a]Feeding trial; [b]Brain expression study; [c]Tissue distribution.

3. *Results*

CART sequence and phylogenetic analysis

Database mining followed by 3' and 5' extension of cDNA ends led us to the identification of seven *cart*–like transcripts containing a coding region of 291, 324, 309, 381, 333, 324 and 321 bp, which translate into a prepropeptide of 96, 107, 102, 126, 110, 107 and 106 amino acids (aa), respectively. These transcripts were named following the zebrafish nomenclature suggested by Nishio et al. (2012) and taking into account their position in the phylogenic tree as *cart1a*, *cart1b*, *cart2a*, *cart2b*, *cart3a*, *cart3b* and

cart4, and were deposited in GenBank with accession numbers KT189188, KT189189, KT189190, KT189191, KT189192, KT189193 and KT189194, respectively.

Comparison of the ORF region of the cDNA sequences of Senegalese sole with those of human, rat and 4 zebrafish genes (not shown) revealed that the percentage of identity between the different sole transcripts and that of mammalian (human and rat) genes varied from 67.6% to 51.6%, and from 73.9% to 47.8% between the different *cart* genes of sole and zebrafish. Between the seven Senegalese sole *cart* transcripts, homology varies between 71.8% and 45.7%, being highest for the pair *cart2a–cart2b*, followed by *cart1a–cart1b* (69.7% identity), and lowest for *cart3a–cart4* pair. Similar comparisons were performed with the prepropeptide sequences and the obtained identity matrix is shown in Fig. 2. In amino acid sequence, conservation between the sole CART's and the mammalian CART varied between 54.3% and 34.0%, and the different sole and zebrafish CART prepropeptide sequences have 70.6%–29.4% identity between each other. When compared to each other, sole CART's vary in identity from 62.1% to 30.1%, with highest homology being found between SsCART1a and SsCART1b, followed by SsCART2a and SsCART2b (59.4%), and lowest between SsCART3a and SsCART4 (30.1%), followed by SsCART3b and SsCART4 (31.1%). The zebrafish prepropeptides showed a similar lower range of identities between DrCART1 and DrCART3 (30.4%), although a higher homology exists between DrCART2a and DrCART2b (69.2%).

The translated prepropeptides of the sole *cart* sequences were all predicted to contain a N–terminal signal peptide of variable size (from 19–aa to 30–aa). When compared to rat and human CART prepropeptides, a high degree of conservation was found in the carboxy–termini of the sequence, where 6 cysteine residues are strictly conserved. Between the highly divergent signal peptide and the conserved carboxy–termini end, the mammalian CARTs typically present two sites of processing by prohormone convertases composed by pairs of basic aa's (KR and KK). In Senegalese sole, the second pair is also strictly conserved in all CART prepropeptides, except in SsCART3b, where KR replaces KK, while the first (KR) processing site found in mammals is only conserved in SsCART2b (Fig. 3).

The reconstruction of the evolutionary history of this gene in vertebrates is shown in the phylogenetic tree presented in Fig. 4. Although a single gene exists in several mammalian species, up to 4 genes could be found in other tetrapods, such as in *Xenopus (Silurana) tropicalis*. Furthermore, 3 CART–like peptides were found for the lobe–finned fish *Latimeria chalumnae*, one of the two only living representatives of the class Sarcopterygii, which is closer to reptiles and mammals than to ray–finned fish. In the case of teleosts, multiple CART–like peptides were identified in several species. For instance, for *Maylandia zebra*, *Oreochromis niloticus*, *Pundamilia nyererei*, *Haplochromis burtoni* and *Xiphophorus maculates*, 6 different CART–like peptides were found grouping closely together in different clades of the phylogenetic tree. In the case of zebrafish, *Danio rerio*, only 4 genes have been found. Interestingly, a cyprinid–specific gene duplication was identified in zebrafish (CART2a and CART2b), goldfish, *Carassius auratus* (CART type I and type II) and common carp, *Cyprinus carpio* (CART type I and type II).

		1 HsCART	2 RnCART	3 SsCART1a	4 SsCART1b	5 DrCART1	6 DrCART2b	7 SsCART2b	8 DrCART2a	9 SsCART2a	10 SsCART4	11 DrCART3	12 SsCART3a	13 SsCART3b
HsCART	1	100.0												
RnCART	2	94.8	100.0											
SsCART1a	3	54.2	54.2	100.0										
SsCART1b	4	53.3	54.2	62.1	100.0									
DrCART1	5	54.3	55.2	64.6	64.4	100.0								
DrCART2b	6	46.9	48.7	47.9	42.3	43.1	100.0							
SsCART2b	7	53.9	53.9	50.5	50.9	50.0	71.8	100.0						
DrCART2a	8	50.9	54.3	49.0	45.8	46.7	69.2	70.6	100.0					
SsCART2a	9	49.0	51.9	43.8	44.8	45.3	63.6	59.4	61.8	100.0				
SsCART4	10	42.5	40.6	43.2	40.0	36.9	41.9	40.0	41.5	41.1	100.0			
DrCART3	11	37.1	38.1	29.8	34.0	30.4	38.8	35.6	34.3	36.8	30.1	100.0		
SsCART3a	12	34.0	34.0	33.7	33.7	31.1	33.0	33.0	32.1	31.3	30.1	61.0	100.0	
SsCART3b	13	40.2	41.1	34.4	31.7	29.4	39.4	36.8	37.4	36.8	31.1	44.7	39.4	100.0

Figure 2. Percent identities between *Solea senegalensis* (Ss) and other vertebrate CART prepropeptides (computed by ClustalW2). GenBank accession numbers are as follows: human HsCART, NP_004282.1; rat short variant RnCART, XP_006231905.1; zebrafish DrCART1, ADB12484.2; zebrafish DrCART2a, ADB12485.2; zebrafish DrCART2b, ADB12486.2; zebrafish DrCART3, ADB12487.1.

cart mRNA expression in areas of Senegalese sole brain

Relative levels of *cart* mRNA in different brain areas were determined by qPCR (Fig. 5). Both *cart2a* and *cart4* were found in all brain areas and fairly equally distributed, except for slightly higher transcript levels of *cart2a* in diencephalon and a slightly lower expression of both genes in the pituitary. The expression profile of *cart1a*, *cart1b* and *cart3a* in the brain was quite similar, with a main expression in the mesencephalon, followed by the diencephalon, although the third region showing conspicuous expression differed between the genes: olfactory bulbs in the case of *cart1a*, telencephalic cerebral hemispheres for *cart1b* and spinal cord for *cart3a*. On the other hand, *cart2b* showed a main expression in the olfactory bulbs, and *cart3b* was predominantly expressed in the spinal cord.

Peripheral tissue distribution of cart mRNA

The relative expression of the different *cart* genes was also assessed by qPCR in whole brain and different peripheral tissues, and their pattern of expression differed considerably (Fig. 6). For *cart1b*, *cart3a* and *cart3b*, the predominant expression was in brain. For *cart1b* and *cart3a* a similar pattern was also found in the high expression in the eye and gonads (ovary, followed by testis, for *cart1b* and only testis in the case of *cart3a*). However, *cart1b* was not expressed in any other tissues, while *cart3a* also showed a relevant expression in liver, kidney and stomach. For *cart3b*, besides the main expression in the brain, a lower expression was also found in kidney and posterior intestine, followed by dorsal and ventral skin, anterior intestine and stomach. In the case of *cart2b* and *cart4*, similar mRNA levels were found in brain as in gonads (ovary for *cart2b* and testis for *cart4*). These two genes were also characterized by having an important expression in kidney, followed by a more minor expression in dorsal and ventral skin, stomach and in the olfactory rosettes. They differed mostly in the eye, where only *cart2b* was expressed, and in the gill where low levels of *cart4* mRNA were detected. Finally, *cart1a* and *cart2a* were both characterized by having a higher expression in gonads (testis in *cart1a* and ovary in *cart2a*) than in the brain. Furthermore, *cart1a* was also expressed at an important level in the ovary, followed by a more minor expression in posterior intestine, heart and stomach. On the other hand, *cart2a* mRNA was also found at low levels in testis, kidney, spleen and olfactory rosettes.

Post–prandial variations of cart mRNA expression in Senegalese sole brain

The levels of *cart* mRNA in sole brain were measured at different times after a single meal and compared to basal levels (24 h fasting; sampling at scheduled feeding time, just before feeding) (Fig. 7). Of the seven cart genes, only *cart1a*, *cart2a* and *cart4* showed a significant post–prandial regulation, being significantly up–regulated at 1 h post–feeding in the case of *cart1a* and 3 h after feeding, in the case of *cart4*. As for *cart2a*, differences were only significant at 12 h post–feeding with respect to unfed animals, but this is likely due to the variability of the data, which shows a strong trend for up–regulation already at 3 h after feeding. Overall, in spite of being a non–significant trend in the case of *cart1a* and *cart4*, mRNA levels remain still high 12 h after the meal.

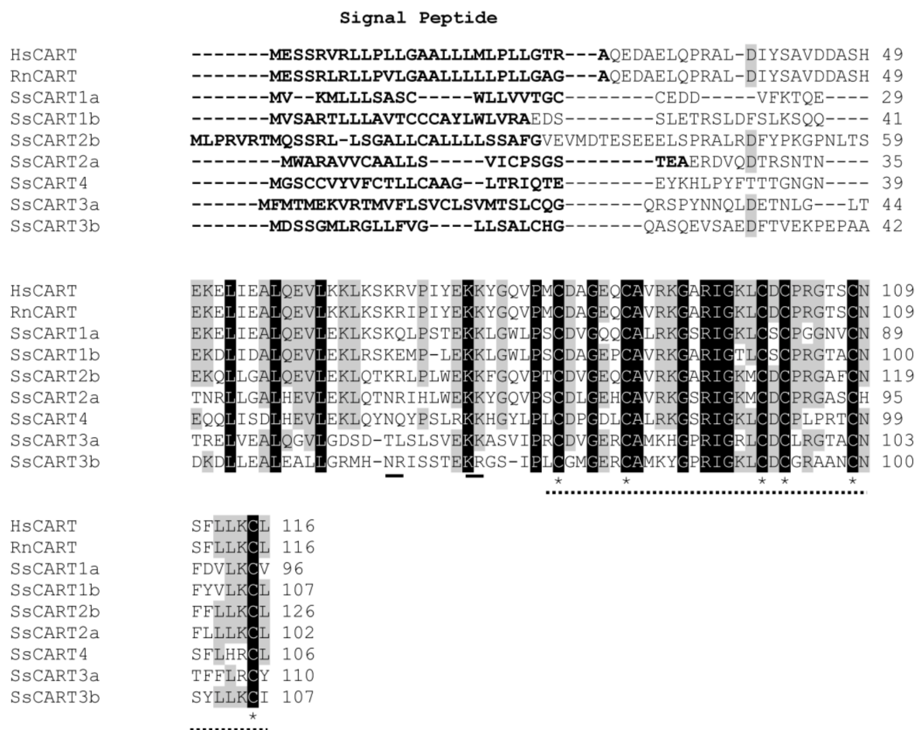


Figure 3. Alignment of the deduced amino acid sequences of CART prepropeptides in ClustalW2. Black areas indicate residues shared by all sequences and shaded areas indicate residues identical in >66% of the sequences. The putative signal peptide is indicated in bold, potential proteolytic cleavage sites (KR and KK) are underlined, and sequence corresponding to exon 3 of the mammalian gene (showing highest conservation amongst vertebrates) is dashed underlined. Indicated (*) are also the 6 cysteine residues that form disulphide bridges responsible for the protein's tertiary structure. GenBank accession numbers are as follows: *Homo sapiens*, HsCART, NP_004282.1; *Rattus norvegicus* short variant, RnCART, XP_006231905.1.

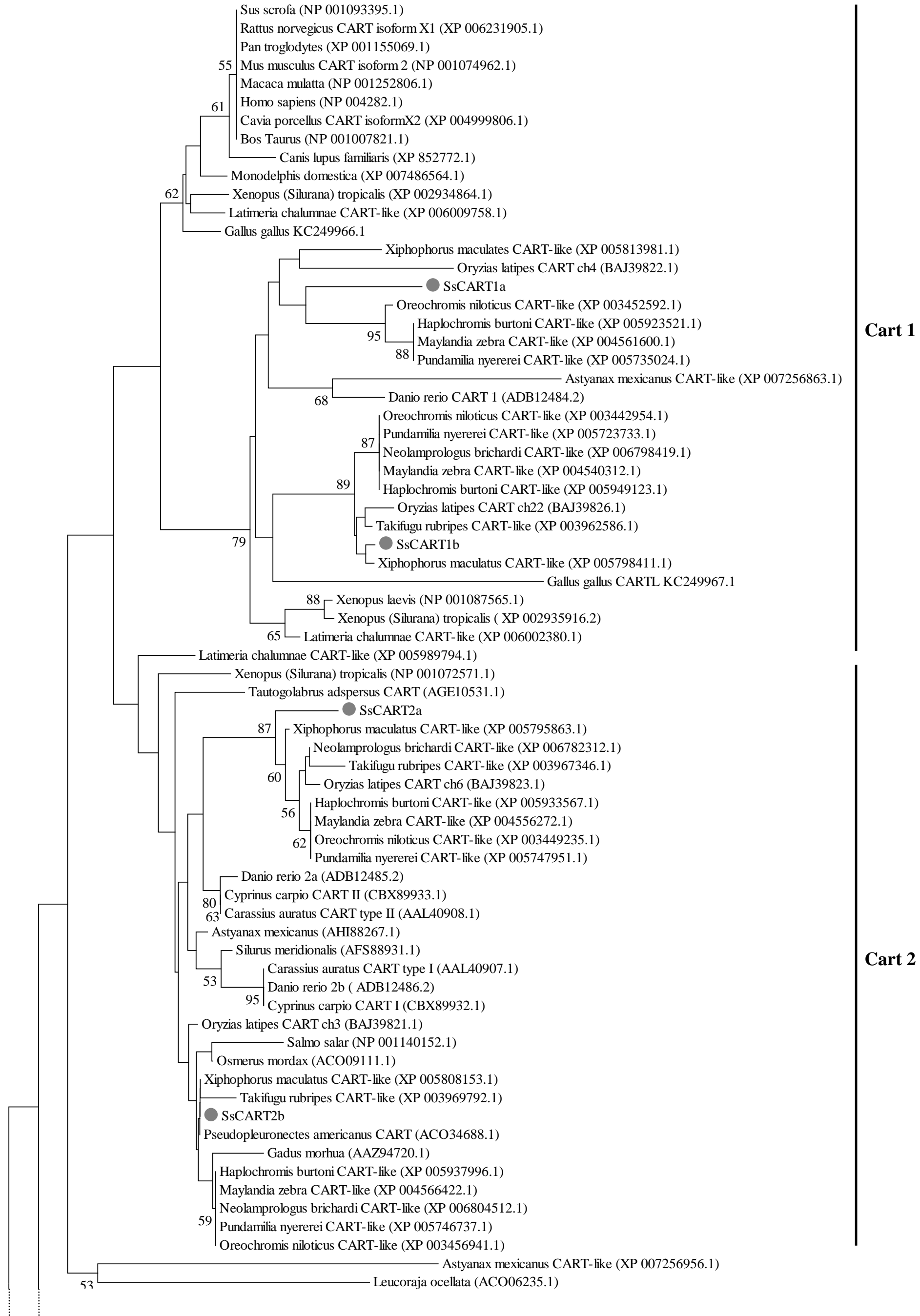
4. *Discussion*

Many of the established or suggested actions and functional roles of CART have been inferred by studies of its anatomical central and peripheral location and co–expression with other neurotransmitters (e.g., dopamine and GABA), and some of its putative physiological effects were then later corroborated by studies examining effects of intracerebroventricular injection, overexpression or injection of other peptides (e.g., leptin, glucocorticoids), KO studies and analysis of gene polymorphisms associated to obesity (Murphy, 2005; Vicentic and Jones, 2007; Rogge et al., 2008; Lau and Herzog, 2014; Subhedar et al., 2014). Therefore, in order to gain further knowledge on the physiological roles of CART in teleosts, and how these might have diversified in species presenting many *cart* genes, we present data on the expression of seven Senegalese sole *cart* gene products in different brain areas and peripheral tissues. Furthermore, a potential involvement in the regulation of food intake was also investigated by studying transcriptional changes in response to feeding.

Structure and phylogenetic analysis

In rats, transcription of a single *Cart* gene results in two alternatively spliced mRNAs, while in humans a single transcript is produced, corresponding to the short variant of rat mRNA (Douglass et al., 1995; Douglass and Daoud, 1996). In both cases, the mRNA is translated into CART propeptides, which are then processed into smaller active forms, in a tissue–specific manner. At least six CART active peptides, which result from this post–translational processing of the CART protein, have been identified so far in mammals, and seem to have different potencies (Kuhar and Yoho, 1999; Dylag et al., 2006). However, only two of these have been more extensively investigated (Murphy, 2005; Rogge et al., 2008). Human and rat CART are very highly conserved, with only 6–aa differences, of which 5 are within the signal peptide that targets the protein for entry into the secretory pathway (Douglass et al., 1995; Douglass and Daoud, 1996). Other conserved features are 6 cysteine residues that form disulphide bridges leading to the tertiary structure that is essential for the protein to maintain its activity, and two pairs of basic aa's (KR and KK) that are the sites of cleavage by prohormone convertases (Rogge et al., 2008). The translated prepropeptides of the Senegalese sole *cart*'s were all predicted to contain a N–terminal signal peptide of variable size (from 19–aa to 30–aa). Furthermore, they all presented a strict conservation of the 6 cysteine residues within the carboxy–termini, which indicates that the resulting peptides should conserve biological activity. This was also the area showing the highest conservation among the sole CART sequences, as well as between sole CARTs and mammalian proteins. This is not surprising given that this sequence is found in all biologically active CART peptides described so far (Rogge et al., 2008). Of the seven deduced proteins characterized here, only SsCART2b contains two similar convertase cleavage sites, which indicates that this protein undergoes similar post–translational processing to become biologically active as in mammals. The remaining propeptides conserve the second (KK) putative cleavage site that, in the case of the sequence translated from *cart3b*, has been replaced by KR. This could mean that a different number of active peptides are produced from these transcripts but needs to be experimentally verified.

The reconstruction of the evolutionary history of the *cart* gene in vertebrates supports the hypothesis of the two–round (2R) whole genome duplication, also known as the 1:4 rule, occurring at the base of the vertebrate lineage, with a variable number of genes being retained during tetrapod evolution (Dehal and Boore, 2005). In this case, we have found 3 CART–like peptides in the lobe–finned fish *Latimeria chalumnae* and 4 in *Xenopus (Silurana) tropicalis*, but a single CART peptide is present in several mammals, indicating that 3 genes have been lost in the mammalian lineage. The number of CART peptides found in teleost species, including the 7 Senegalese sole CARTs, 6 CART–like peptides described previously in medaka (Murashita and Kurokawa, 2011) and in *Maylandia zebra*, *Oreochromis niloticus*, *Pundamilia nyererei*, *Haplochromis burtoni* and *Xiphophorus maculatus*, and 4 CARTs in zebrafish and *Takifugu rubripes*, for instance, evidence a third–round (3R) of whole genome duplication after the appearance of the teleost lineage (Volff, 2005). The phylogenetic tree presented in this study also suggests a later duplication of a *cart* gene in the cyprinid line and, based on this, we would propose that the nomenclature suggested by Nishio et al. (2012) for *Danio rerio* and by Volkoff and Peter (2001) for *Carassius auratus* should be revised, and that


Sus scrofa (NP 001093395.1)
Rattus norvegicus CART isoform X1 (XP 006231905.1)
Pan troglodytes (XP 001155069.1)
Mus musculus CART isoform 2 (NP 001074962.1)
Macaca mulatta (NP 001252806.1)
Homo sapiens (NP 004282.1)
Cavia porcellus CART isoformX2 (XP 004999806.1)
Bos Taurus (NP 001007821.1)
Canis lupus familiaris (XP 852772.1)
Monodelphis domestica (XP 007486564.1)
Xenopus (Silurana) tropicalis (XP 002934864.1)
Latimeria chalumnae CART-like (XP 006009758.1)
Gallus gallus KC249966.1
Xiphophorus maculates CART-like (XP 005813981.1)
Oryzias latipes CART ch4 (BAJ39822.1)
SsCART1a
Oreochromis niloticus CART-like (XP 003452592.1)
Haplochromis burtoni CART-like (XP 005923521.1)
Maylandia zebra CART-like (XP 004561600.1)
Pundamilia nyererei CART-like (XP 005735024.1)
Astyanax mexicanus CART-like (XP 007256863.1)
Danio rerio CART 1 (ADB12484.2)
Oreochromis niloticus CART-like (XP 003442954.1)
Pundamilia nyererei CART-like (XP 005723733.1)
Neolamprologus brichardi CART-like (XP 006798419.1)
Maylandia zebra CART-like (XP 004540312.1)
Haplochromis burtoni CART-like (XP 005949123.1)
Oryzias latipes CART ch22 (BAJ39826.1)
Takifugu rubripes CART-like (XP 003962586.1)
SsCART1b
Xiphophorus maculatus CART-like (XP 005798411.1)
Gallus gallus CARTL KC249967.1
Xenopus laevis (NP 001087565.1)
Xenopus (Silurana) tropicalis (XP 002935916.2)
Latimeria chalumnae CART-like (XP 006002380.1)
Latimeria chalumnae CART-like (XP 005989794.1)
Xenopus (Silurana) tropicalis (NP 001072571.1)
Tautogolabrus adspersus CART (AGE10531.1)
SsCART2a
Xiphophorus maculatus CART-like (XP 005795863.1)
Neolamprologus brichardi CART-like (XP 006782312.1)
Takifugu rubripes CART-like (XP 003967346.1)
Oryzias latipes CART ch6 (BAJ39823.1)
Haplochromis burtoni CART-like (XP 005933567.1)
Maylandia zebra CART-like (XP 004556272.1)
Oreochromis niloticus CART-like (XP 003449235.1)
Pundamilia nyererei CART-like (XP 005747951.1)
Danio rerio 2a (ADB12485.2)
Cyprinus carpio CART II (CBX89933.1)
Carassius auratus CART type II (AAL40908.1)
Astyanax mexicanus (AHI88267.1)
Silurus meridionalis (AFS88931.1)
Carassius auratus CART type I (AAL40907.1)
Danio rerio 2b (ADB12486.2)
Cyprinus carpio CART I (CBX89932.1)
Oryzias latipes CART ch3 (BAJ39821.1)
Salmo salar (NP 001140152.1)
Osmerus mordax (ACO09111.1)
Xiphophorus maculatus CART-like (XP 005808153.1)
Takifugu rubripes CART-like (XP 003969792.1)
SsCART2b
Pseudopleuronectes americanus CART (ACO34688.1)
Gadus morhua (AAZ94720.1)
Haplochromis burtoni CART-like (XP 005937996.1)
Maylandia zebra CART-like (XP 004566422.1)
Neolamprologus brichardi CART-like (XP 006804512.1)
Pundamilia nyererei CART-like (XP 005746737.1)
Oreochromis niloticus CART-like (XP 003456941.1)
Astyanax mexicanus CART-like (XP 007256956.1)
Leucoraja ocellata (ACO06235.1)
55
61
62
95
88
68
87
89
79
88
65
87
60
56
62
80
63
53
95
59
53
Cart 1
Cart 2

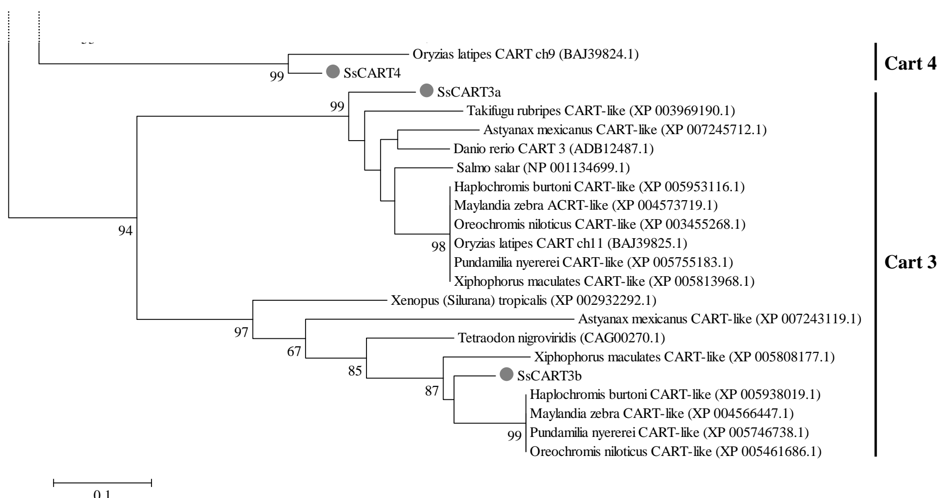


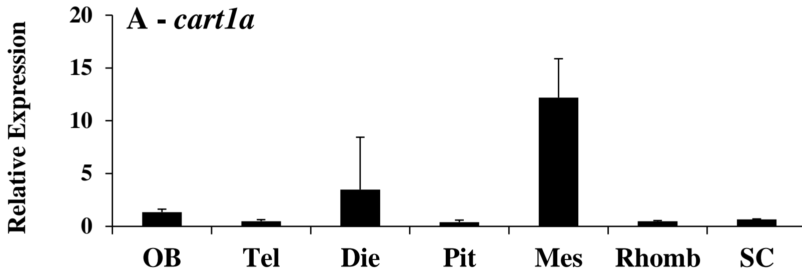
Figure 4. Phylogenetic analysis of CART amino acid sequences in vertebrates. Protein IDs (GeneBank accession numbers) are indicated in the tree, after the species name. The tree is drawn to scale and the scale bar represents the substitution rate per residue. Numbers at nodes indicate the bootstrap value, as percentages, obtained for 1000 replicates, in MEGA6.

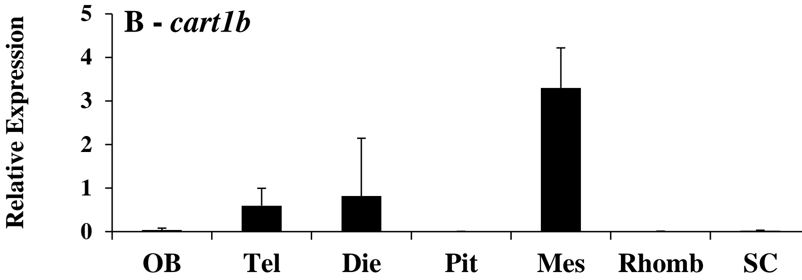
DrCART2a and DrCART2b, as well as CaCART I and CaCART II, should be renamed as CART2a1 and CART2a2. The retention of 6–7 *cart* genes in highly evolved perciform and pleuronectiform teleost species suggests that different gene products might have adopted a new function (‘‘neofunctionalization’’) or partitioned old functions (‘‘subfunctionalization’’), which in mammals are performed by a single gene. This supports the theory that, in teleosts, which make up roughly half of all existing vertebrate species, differential loss or subfunction partitioning of gene duplicates might have been involved in the generation of such fish biodiversity (Volff, 2005).

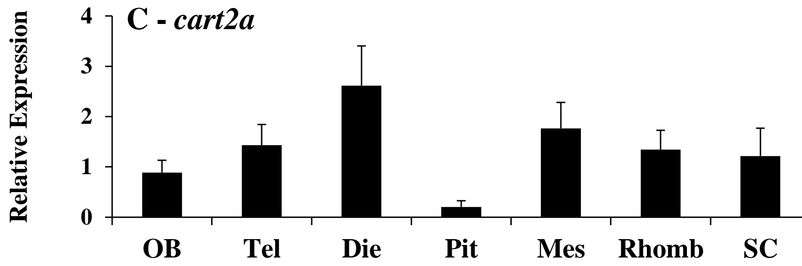
cart mRNA expression in areas of Senegalese sole brain

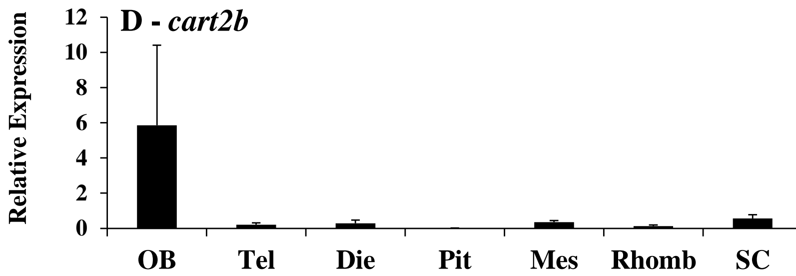
When it was first described in the rat, CART mRNA’s were found predominantly in the hypothalamus and pituitary, followed by the thalamus/midbrain and significantly less in the cortex, striatum, hippocampus, and hindbrain (Douglass et al., 1995). These authors also described the pattern of expression in human brain, which generally does not differ much from that of rat. Many detailed studies have been performed since to further localize CART mRNA and peptides in specific neuroanatomical regions (reviewed by Subhedar et al., 2014).

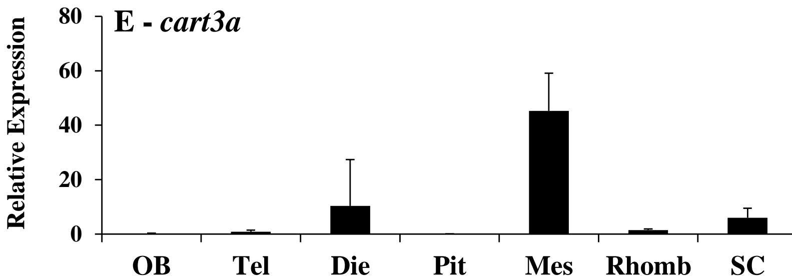
In our study we have generally characterized the expression of seven putative *cart* genes in different brain regions of Senegalese sole by quantifying the relative levels of mRNA’s by qPCR. Our study revealed that *cart2a* and *cart4* genes exhibited the most widespread distribution in the brain of sole. In the goldfish brain a differential expression was found between two *cart* transcripts, with form I being more abundant in the olfactory bulbs and hypothalamus, whereas form II presented higher mRNA levels in the


A - cart1a
Relative Expression
20
15
10
5
0
OB
Tel
Die
Pit
Mes
Rhomb
SC


B - cart1b
Relative Expression
5
4
3
2
1
0
OB
Tel
Die
Pit
Mes
Rhomb
SC


C - cart2a
Relative Expression
4
3
2
1
0
OB
Tel
Die
Pit
Mes
Rhomb
SC


D - cart2b
Relative Expression
12
10
8
6
4
2
0
OB
Tel
Die
Pit
Mes
Rhomb
SC


E - cart3a
Relative Expression
80
60
40
20
0
OB
Tel
Die
Pit
Mes
Rhomb
SC

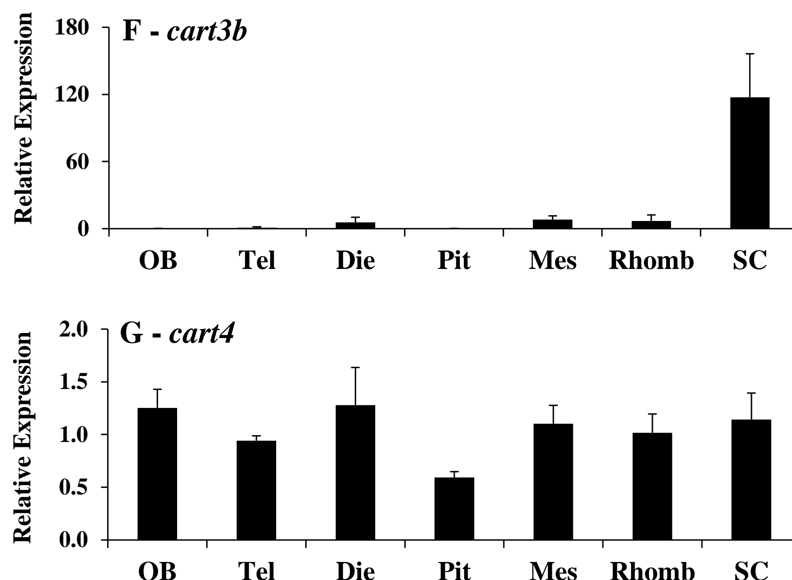
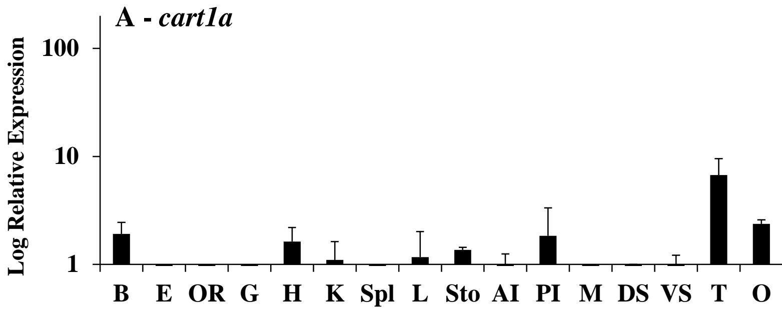
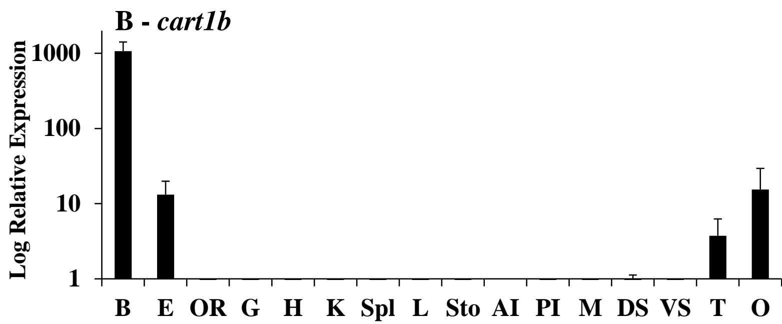
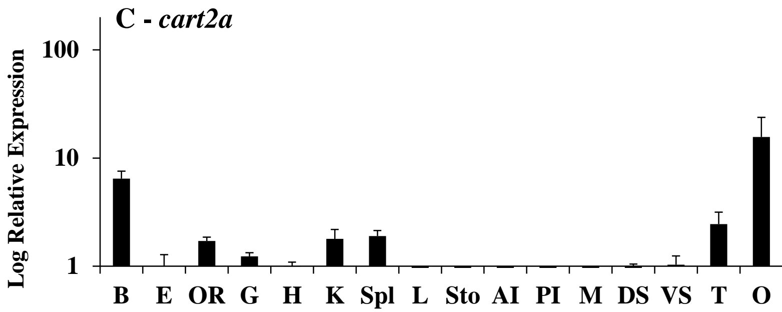


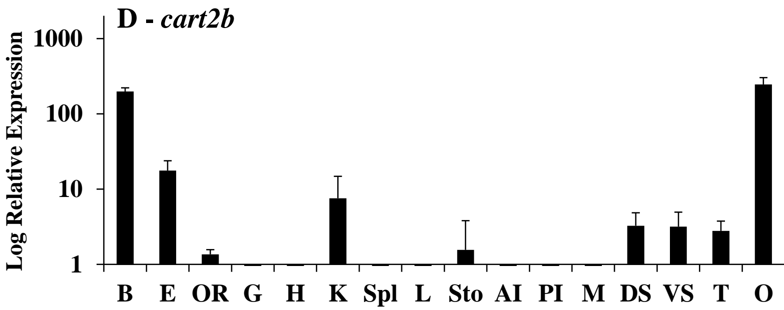
Figure 5. *cart* mRNA expression in different Senegalese sole brain areas, determined by qPCR. Graphs show the normalized (by *rps4*) relative expression of *cart1a* (A), *cart1b* (B), *cart2a* (C), *cart2b* (D), *cart3a* (E), *cart3b* (F), and *cart4* (G) in each brain region (n = 4 individuals ± standard deviation, SD) in relation to the average across all regions. OB – olfactory bulbs, Tel – telencephalic cerebral hemispheres, Die – diencephalon, Pit – pituitary, Mes – mesencephalon, Rhomb – rhombencephalon, and SC – spinal cord.

optic tectum–thalamus (Volkoff and Peter, 2001). In Senegalese sole, the transcript presenting higher homology to both goldfish transcript forms is *cart2a*, which was found in the diencephalon, mesencephalon, cerebral hemispheres, rhombencephalon, spinal cord and olfactory bulbs. However, similarly to the goldfish genes, a low expression was found in the pituitary. A detailed study on the differential distribution of four *cart* genes has been recently performed in zebrafish (Akash et al., 2014). As in sole, *cart2* showed a widespread distribution in the zebrafish brain. The diencephalon of zebrafish also contained the highest expression of *cart2*, with cells being evident in many cell masses from the preoptic area, ventral and dorsal thalamus, tuberal hypothalamus and posterior tubercle. Similarly to sole, the optic tectum and mesencephalic tegmentum of zebrafish also contained abundant *cart2*–expressing cells (Akash et al., 2014). In the present study we have also found an important *cart2a* expression in the hindbrain of sole. This rhombencephalic expression could be restricted to the medulla oblongata because this part of the brain but not the cerebellum contained *cart2* cells in zebrafish (Akash et al., 2014), as well as immunoreactive CART cells in catfish (Singru et al., 2007). The *cart4* gene also exhibited a profuse expression in the brain of sole. It is interesting to note that *cart4*–expressing cells (=*cart3* following the nomenclature from Nishio et al., 2012) also showed a broad distribution from the olfactory bulbs to the rhombencephalon in


A - *cart1a*
Log Relative Expression
100
10
1
B E OR G H K Spl L Sto AI PI M DS VS T O


B - *cart1b*
Log Relative Expression
1000
100
10
1
B E OR G H K Spl L Sto AI PI M DS VS T O


C - *cart2a*
Log Relative Expression
100
10
1
B E OR G H K Spl L Sto AI PI M DS VS T O


D - *cart2b*
Log Relative Expression
1000
100
10
1
B E OR G H K Spl L Sto AI PI M DS VS T O

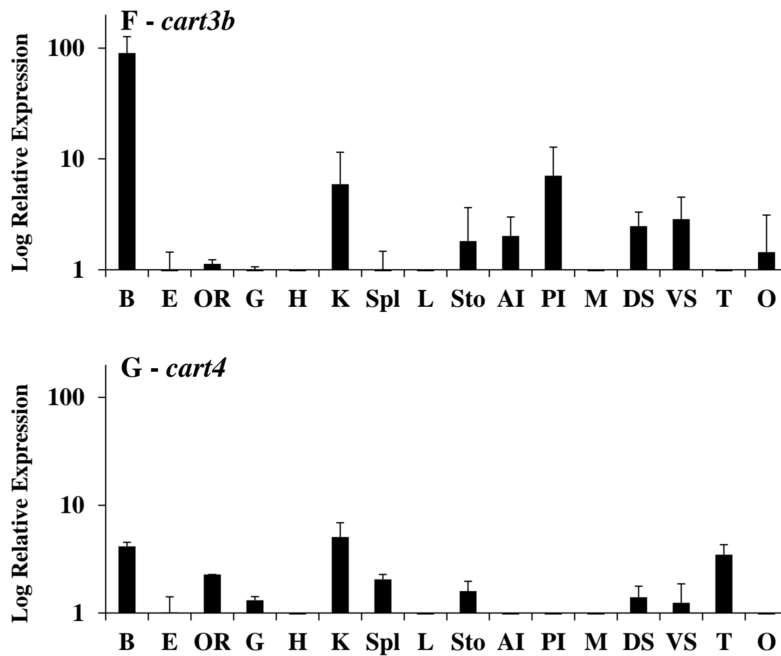


Figure 6. *cart* mRNA expression in different Senegalese sole peripheral tissues, determined by qPCR. Graphs show the normalized (by *18s*) relative expression, in logarithmic scale, of *cart1a* (A), *cart1b* (B), *cart2a* (C), *cart2b* (D), *cart3a* (E), *cart3b* (F), and *cart4* (G) in each tissue (n = 4 individuals ± standard deviation, SD) in relation to the average across all tissues. B – brain, E – eye, OR – olfactory rosettes, G – gills, H – heart, K – anterior kidney–interrenal tissue, Spl – spleen, L – liver, Sto – stomach, AI – anterior intestine, PI – posterior intestine, M – muscle, DS – dorsal skin, VS – ventral skin, T – testis, and O – ovaries.

zebrafish (Akash et al., 2014) and that this gene represents the most divergent gene from this family in both species. On the contrary, the zebrafish *cart1* gene is exclusively expressed in cells from the nucleus of the medial longitudinal fascicle (nMLF), in the mesencephalic tegmentum. In agreement with these results, both sole *cart1a* and *cart1b* genes exhibit the highest mRNA expression levels in samples from the mesencephalon, where nMLF is contained. In addition, *cart1a* and *cart1b* expression was evident in the diencephalon and rostral forebrain (olfactory bulbs and cerebral hemispheres) from sole but no *cart1*–expressing cells were detected in these brain areas of zebrafish by in situ hybridization (Akash et al., 2014). If these dissimilarities represent real differences between species or differences in the sensitivities of the techniques used remain to be elucidated. The expression of sole *cart2b* was restricted to the rostral forebrain and, distinctly, to the olfactory bulbs. It should be noted that *cart3*–expressing cells (=*cart2b* following the nomenclature from Nishio et al., 2012) appear also restricted to the anterior pole of the brain, but in this case within the telencephalic entopeduncular nucleus (Akash et al., 2014). In sole, *cart3a* and *cart3b* brain expression patterns were clearly dissimilar between each other and in relation to its counterpart zebrafish gene (Akash et al., 2014). The expression pattern of sole *cart3a* was quite similar to that of *cart1a and cart1b* (mainly in mesencephalon and diencephalon), whereas *cart3b*

expression pattern was unique and restricted to the spinal cord. Further studies using in situ hybridization appears necessary to elucidate if *cart1a, cart1b* and *cart3b* are expressed in the same cell masses from these regions.

The general pattern of distribution of the different sole *cart* transcripts in the brain suggests that, although subfunctionalization probably occurs, overall, CART peptides are likely to have similar roles in fish as in mammals, in regulating feeding and energy homeostasis, but also in neuroendocrine/hypophysiotropic functions and in processing peripheral sensory information.

Peripheral tissue distribution of cart mRNA

In contrast to the distribution of CART mRNA and peptides in the central nervous system, much less information exists on its peripheral distribution and possible modes of action. When it was first described in rat, CART mRNA was only found in the eye and adrenal glands (Douglass et al., 1995) but many studies conducted since then suggest a widespread distribution of CART, regulating many functions of the body, acting either locally or synergistically with central CART (Lau and Herzog, 2014). For instance, an important expression has been found in the enteric nervous system of mammals, particularly in the submucosal and myenteric plexuses of the stomach, small and large intestines, as well as in cell bodies and nerve fibers in the extrahepatic biliary tract (Couceyro et al., 1998; Ellis and Mawe, 2003; Ekblad, 2006). The precise role of this local enteric CART release within the gastrointestinal tract is still not clear, as Ekblad et al. (2003) failed to demonstrate an effect in intestinal motility. A hormonal role has also been suggested since CART is expressed in several pancreatic islet cell types in rat and was found up–regulated in β–cells of diabetic animals (Wierup et al., 2006) and, in humans, it is found in pancreatic ganglia and in islet endocrine cells (Kasacka et al., 2012). It is also believed to play a role in renovascular hypertension in neuroendocrine cells of adrenal glands of rat (Kasacka et al., 2014). Finally, CART has been found in subcutaneous and visceral white adipose tissues in humans and rat, where it has a role in lipid and glucose utilization (Banke et al., 2013).

Similar to what was initially described in rats, the eye also seems to be a major site of *cart* expression in teleosts. In Atlantic salmon, common carp (CART I), goldfish (particularly CART I) and medaka (only for *ch3* and *ch6*) the eye was one of the tissues showing highest *cart* expression (Volkoff and Peter, 2001; Murashita et al., 2009; Murashita and Kurokawa, 2011; Wan et al., 2012). Furthermore, CART immunoreactivity has been found in different elements of the zebrafish retina and optic nerve system from 4 days of age until adulthood (Mukherjee et al., 2012). In Senegalese sole, *cart1b*, *cart2b* and *cart3a* were highly expressed in the eye. It is noteworthy that for *cart1b* and *cart3a* there was also a predominant central expression in the optic tectum–tegmentum (mesencephalon). However, for *cart2b*, the main central expression was in the olfactory bulbs, which could be related to expression found in the olfactory rosettes, albeit at low level. Two other sole genes, *cart2a* and *cart4*, that showed an important expression in the olfactory bulbs were also expressed in the olfactory rosettes. These results are consistent with a suggested important role of CART in processing sensory information, both visual and olfactory, as indicated previously in zebrafish (Mukherjee et al., 2012; Akash et al., 2014; Subhedar et al., 2014).

Evidence is starting to accumulate suggesting a specific role of CART in fish reproduction, although the exact mechanism in which it is implicated is still not clear. High levels of expression have been found in ovary in goldfish (Volkoff and Peter, 2001), common carp (Wan et al., 2012) and Atlantic cod (Kehoe and Volkoff, 2007), in testis of channel catfish (Kobayashi et al., 2008), in gonads of winter flounder and of the elasmobranch winter skate (MacDonald and Volkoff, 2009a,b), and at low levels in testis of Atlantic salmon (Murashita et al., 2009). In sole, all of the *cart* transcripts were found either in testis (*cart3a* and *cart4*) or ovary (*cart3b*), or both in testis and ovary (*cart1a*, *cart1b*, *cart2a*, *cart2b*), in some cases at a similar level or even higher than in the brain (*cart1a*, *cart2a*, *cart2b*, *cart4*). Variations in CART immunoreactivity in the brain of female catfish were studied over the annual sexual cycle and the pattern of change was correlated to that of luteinizing hormone (LH) cells in the pituitary and gonadosomatic index. Important changes correlated with the reproductive cycle were observed in brain areas associated with the central control of reproductive processes as well as in the olfactory bulbs and other neural areas associated with olfaction (Barsagade et al., 2010). It was therefore suggested that CART reproductive–related roles might be to process energetic and reproductive status–related information, trigger the brain–pituitary–ovary axis, and signal seasonal–dependent changes in the quality or availability of food and/or the presence of sex steroids. In mammals there is also indication that CART peptides might affect the reproductive hypothalamic–pituitary–gonadal axis, being involved in the release of gonadotropin–releasing hormone (GnRH) (Lebrethon et al., 2000; Parent et al., 2000), the reproductive behaviour (Douglass et al., 1995) and the brain steroid feedback (Sohn et al., 2002). In addition, *CART* mRNA was found in the bovine ovary, where it is thought to be involved in regulating follicular atresia (Kobayashi et al., 2004).

In mammals, although an important CART immunoreactivity has been found in stomach and intestine, *cart* mRNA has not been detected in these tissues, suggesting that CART peptides are synthesized elsewhere and transported to the gut, possibly via sensory fibers or the vagus nerve (Murphy et al., 2000). Previous studies in fish found moderate levels of *cart* mRNA in the stomach, gut and liver of winter flounder (MacDonald and Volkoff, 2009a) and a low expression of *cart* in Atlantic salmon midgut (Murashita et al., 2009). In Senegalese sole, moderate levels of mRNA were found for *cart3b* in posterior and anterior intestine, as well as in the stomach. Furthermore, low levels of expression were found in stomach for *cart1a*, *cart2b*, *cart3a* and *cart4*. However, considering how these organs were sampled in fish for our qPCR analysis, we cannot rule out that the mRNA is located in fibers of the enteric nervous system embedded in the gastrointestinal mucosa, as described in mammals (Couceyro et al., 1998; Ellis and Mawe, 2003; Ekblad, 2006). On the other hand, from the seven analyzed transcripts, only *cart3a* was expressed in liver but, similarly to the gastrointestinal tract, we cannot exclude that this expression might be specific of the hepatic nerves.

Other relevant sites of *cart* expression in sole are the kidney (*cart2a*, *cart2b*, *cart3a*, *cart3b* and *cart4*), skin (*cart2b*, *cart3b* and *cart4*), spleen and gills (in both tissues *cart2a* and *cart4* were found at relatively low levels), and heart (*cart1a*). The fairly high levels of expression in the kidney of some of these transcripts matches the high expression found in the mammalian adrenal glands (Douglass et al., 1995; Kasacka et al., 2014), which in fish are replaced by interrenal cells that appear interspersed within the kidney, and suggests a conserved role of CART. Expression in the kidney and gill

(mostly of CART I) was also previously described in goldfish, and *cart* mRNA was also found at low levels in salmon and winter flounder kidney, gill and heart, as well as in spleen in winter flounder and gill in medaka (*ch9*) (Volkoff and Peter, 2001; MacDonald

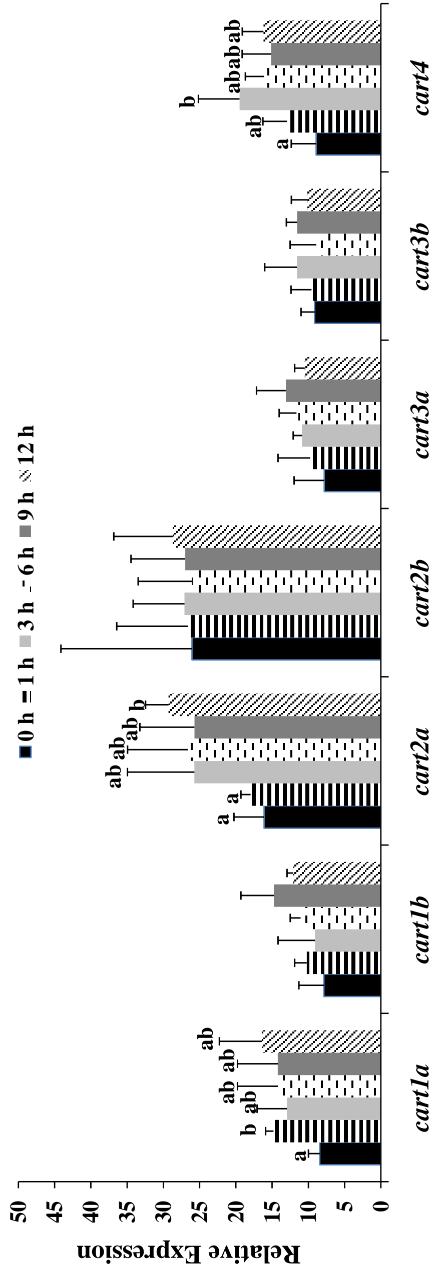


Figure 7. Post–prandial variations of *cart* mRNA expression in Senegalese sole whole brain, determined by qPCR. Graphs show the normalized (by a normalization factor computed from the

averaged expression of *rps4, ef1a1, ubq*) relative expression of *cart1a*, *cart1b*, *cart2a*, *cart2b*, *cart3a*, *cart3b*, and *cart4* in the brain of 4 individuals (± SD) at 0 h (24 h fasting; sampling at scheduled feeding time) and again at 1 h, 3 h, 6 h, 9 h and 12 h after feeding.

and Volkoff, 2009a; Murashita et al., 2009; Murashita and Kurokawa, 2011). CART immunoreactivity has also been found in the rat's cardiac nervous system (Richardson et al., 2006) and CART peptide injections into the heart of *Rana ridibunda* have been associated with the regulation of cardiac function in frog (Ivanova et al., 2007). Therefore, the, expression in the heart could be associated to the cardiac nervous system, as suggested for gastrointestinal tract and liver. Skin was the main site of expression of medaka *ch9* (Murashita and Kurokawa, 2011). In Senegalese sole, a low expression of the medaka's *ch9* closest homologue (*cart4*), but also of *cart2b* and *cart3b*, was detected in dorsal (pigmented) and ventral (unpigmented) skin. The only examined tissue for which there was no expression of any of the sole *cart* genes was in muscle. A lack of expression in muscle was also previously reported in goldfish and Atlantic salmon (Volkoff and Peter, 2001; Murashita et al., 2009), but a residual expression was described in medaka (for *ch3* and *ch9*) and winter flounder (MacDonald and Volkoff, 2009a; Murashita and Kurokawa, 2011).

Post–prandial variations of cart mRNA expression in Senegalese sole brain

The effect of CART on feeding and behaviour of fish has been fairly well studied and its anorexigenic role appears to be well conserved across the vertebrate lineage. Intracerebroventricular injections of CART peptides inhibited food intake in goldfish (Volkoff and Peter, 2000). In addition, prolonged fasting and feeding were associated to a significant reduction or up–regulation, respectively, of *cart* expression in goldfish (CART I), Atlantic salmon, Atlantic cod, channel catfish, African catfish, medaka (*ch3*), common carp and zebrafish brain (Volkoff and Peter, 2001; Kehoe and Volkoff, 2007; Kobayashi et al., 2008; Murashita et al., 2009; Subhedar et al., 2011; Valen et al., 2011; Nishio et al., 2012; Peterson et al., 2012). However, not all *cart* genes in fish are affected by fasting or feeding, as shown in winter skate, goldfish (CART II), zebrafish (*cart1* and *cart3*) and medaka (five out of six *cart* genes) (Volkoff and Peter, 2001; MacDonald and Volkoff, 2009b; Murashita and Kurokawa, 2011; Akash et al., 2014), which indicates other non–feeding regulation roles for these genes, probably explained by their divergence to acquire or retain non–redundant functions.

Compared to the effects of prolonged fasting, fewer studies have examined post–prandial changes in *cart* expression in the fish brain (Volkoff and Peter, 2001; Kehoe and Volkoff, 2007; Valen et al., 2011; Peterson et al., 2012). In this study we examined the effect of feeding a single meal on *cart* brain expression at 1 h, 3 h, 6 h, 9 h and 12 h after feeding, and compared it with 24 h fasted fish (sampled at scheduled feeding time – 0 h). As previously seen in goldfish (Volkoff and Peter, 2001), not all Senegalese sole *cart* genes responded similarly to feeding and only *cart1a*, *cart2a* and *cart4* showed significant post–prandial changes in expression. An increase in *cart* mRNA levels was observed at 1 h (*cart1a*) or 3 h (for *cart4* and tendentially for *cart2a*) after feeding and by 12 h the values still appeared fairly elevated (significantly for *cart2a*). However, the usage of the force feeding method, although enabling a good control of the amount of food ingested and time of feeding, is likely to have introduced some variability in the results, associated with the stress imposed by the technique. From what is known of the

response of Senegalese sole to acute stress, plasma cortisol (primary stress response) and glucose (secondary stress response) levels increase rapidly and peak at 1 h following stress, dropping close to basal levels by 4 h (Costas et al., 2011). Therefore, the increase in expression of at least *cart2a* and *cart4* is likely to have been unrelated to stress. Nevertheless, it remains to be assessed how the different *cart* genes respond to stress and whether a differential expression in different areas of the brain might have masked the response to feeding in the whole brain for some of the genes. This question was outside of the scope of the present study but deserves careful consideration and further studies. Still, the timings of the response observed are within what has been observed in other studies in teleosts, although the response appears to be variable depending on the species. In goldfish hypothalamus and olfactory bulbs the expression of CART I was increased 2 h after a meal and returned to basal levels after 6 h (Volkoff and Peter, 2001). Similarly, in Atlantic salmon there was a significant up–regulation of *cart* expression in the brain 1.5 h after a meal but the high variability in the data make it difficult to assess when *cart* mRNA returned to basal levels (Valen et al., 2011). A fast post–prandial up–regulation, only 30 min after feeding, was also measured in channel catfish, and *cart* mRNA levels remained elevated during at least 4 h (Peterson et al., 2012). In Atlantic cod, on the other hand, although a 7–day fasting led to the expected decline in *cart* mRNA levels, its expression was surprisingly down–regulated 2 h after a meal (Kehoe and Volkoff, 2007). Valen et al. (2011) suggested that satiety signals are associated to stomach fullness and gastric emptying, so that anorexigenic signals are associated with the initial phase after feeding, when the stomach is full, while orexigenic signals arise once most of the stomach content is evacuated. Considering the large diversity of teleost species, with important differences in diets and feeding habits affecting the dynamics of feeding, it is likely that the pattern of post–prandial change and response to fasting of *cart* mRNA levels in fish brain can be highly species–specific and probably also diet–dependent. Nevertheless, results gathered so far support a conserved role of CART in regulating both short term and long term (fasting studies) appetite in fish.

The localization of *cart1a*, *cart2a* and *cart4* in the brain and peripheral tissues does not present significant clues to explain why only these genes were transcriptionally affected by feeding. While *cart2a* and *cart4* presented a more ubiquitous expression, including the telencephalon and diencephalon that contains the putative centers of regulation of food intake, *cart1a* was predominantly expressed in the mesencephalon, although a significant expression was also found in diencephalon. Nevertheless, *cart1b* and *cart3a* shared a very similar profile of expression in the brain as that of *cart1a*, and the latter but not the formers were significantly affected by feeding. In zebrafish, for instance, different *cart* genes have been shown to respond differently to fasting in specific, and often non–overlapping, brain areas or even cell populations within a brain region (Nishio et al., 2012; Akash et al., 2014). Thus, starvation resulted in a significant decrease in CART2–positive cells in the telencephalic entopeduncular nucleus and the hypothalamic nucleus of the lateral recess (NRL), as well as in a reduction of CART4–expressing neurons from the lateral tuberal nucleus of the hypothalamus, suggesting a function in energy homeostasis for these neurons (Akash et al., 2014). More anatomically detailed studies are necessary in the future in order to establish the mechanisms through which different *cart* genes and specific brain regions might be involved in the regulation of food intake in teleosts. Until then, the results presented here

on the response of the different *cart* genes to feeding should be only taken as preliminary, although it is very likely that the response differs between genes, which again points to a probable subfunctionalization of the multiple *cart*'s retained in fish genomes across evolution.

5. *Conclusion*

Our study has shown that Senegalese sole, a pleuronectiform teleost, exhibits seven *cart* genes with distinct patterns of expression and feeding responses. Comparison of results presented here with previous studies performed in other teleost species indicates the existence of common patterns of expression, which suggests conserved evolutionary trends among vertebrate species and functions analogous to those found in mammals. However, there is also considerable variability in the expression of homologous genes in different teleost species, or even in closely related genes within the same species, possibly indicating divergence in *cart* gene subfunctionalization or development of new roles across evolution. In fact, some of the *cart* genes of sole, but not others, appear affected by food intake. This evidence, together with the differential pattern of expression in central and peripheral tissues suggest that, in addition to feeding and energy homeostasis, *cart* genes from sole could also be involved in other relevant physiological functions that remain to be elucidated in future studies.

Authors' contributions

S.M. conceived the study. K.B., A.M., and S.M. performed the *in silico* and molecular laboratory work, including obtaining samples for analyzing the tissue expression and post–prandial profile. A.J.M.R. and J.A.M.C. obtained the samples for the brain expression study. All authors analyzed the data, wrote, read and approved the final manuscript.

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CHAPTER 5

Hypothalamic fatty acid sensing in Senegalese sole (*Solea senegalensis*): response to long–chain saturated, monounsaturated, and polyunsaturated (n−3) fatty acids

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Abstract

We assessed the presence of fatty acid (FA) sensing mechanisms in hypothalamus of Senegalese sole (*Solea senegalensis*) and investigated their sensitivity to FA chain length and/or level of unsaturation. Stearate (SA, saturated FA), oleate (OA, monounsaturated FA of the same chain length), α–linolenate (ALA, a n–3 PUFA of the same chain length), and eicosapentanoate (EPA, a n–3 PUFA of a larger chain length) were injected intraperitoneally. Parameters related to FA sensing and neuropeptide expression in the hypothalamus were assessed after 3h and changes in accumulated food intake after 4, 24, and 48 h. Three FA sensing systems characterized in rainbow trout were also found in Senegalese sole, and were activated by OA in a way similar to that previously characterized in rainbow trout and mammals. These hypothalamic FA sensing systems were also activated by ALA, differing from mammals, where n–3 PUFA do not seem to activate FA sensors. This might suggest additional roles and highlights the importance of n–3 PUFA in fish diets, especially in marine species. The activation of FA sensing seems to be partially dependent on acyl chain length and degree of saturation, as no major changes were observed after treating fish with SA or EPA. The activation of FA sensing systems by OA and ALA, but not SA or EPA, is further reflected in the expression of hypothalamic neuropeptides involved in the control of food intake. Both OA and ALA enhanced anorexigenic capacity compatible with the activation of FA sensing systems.

Key words: Senegalese sole, hypothalamus, PUFA, fatty acid sensing, food intake

1. *Introduction*

This study focuses on the metabolic control of food intake in hypothalamus. This term refers to the capacity of specific neurons located in the hypothalamus of detecting changes in the levels of metabolites such as glucose or fatty acids through nutrient–sensing mechanisms (Blouet and Schwartz, 2010; Christie, 1982a; López et al., 2007). When the levels of nutrients rise, this increase is detected through nutrient sensing mechanisms in these neurons and as a result one of the populations (the so–called glucose–excited, GE neurons) is stimulated resulting in increased release of the neuropeptides POMC and CART synthesized by these neurons. In contrast, the other population (the so–called glucose–inhibited, GI neurons) is inhibited resulting in decreased release of the neuropeptides AgRP and NPY synthesized by these neurons (Blouet and Schwartz, 2010; Christie, 1982a; López et al., 2007). The global balance of the response is an increased anorectic potential and subsequent decrease in food intake. Conversely, when the levels of nutrients decline the nutrient sensing systems are inhibited resulting in increased production of AgRP/NPY and decreased production of POMC/CART resulting in increased food intake (Blouet and Schwartz, 2010; Christie, 1982a; López et al., 2007). The nutrient sensing mechanisms characterized so far in mammals (Blouet and Schwartz, 2010; Christie, 1982a; López et al., 2007) and fish (Soengas, 2014) include both different types of glucosensors and fatty acid sensing mechanisms.

Fatty acid (FA) sensing mechanisms detect increases in plasma concentration of long–chain fatty acids (LCFA) through several processes (Blouet and Schwartz, 2010; Christie, 1982a; López et al., 2007), including i) FA metabolism through inhibition of carnitine palmitoyltransferase 1 (CPT–1) that imports FA–CoA into the mitochondria for oxidation; ii) binding to FA translocase (FAT/CD36) and modulation of transcription factors like peroxisome proliferator–activated receptor type α (PPARα) and sterol regulatory element–binding protein type 1c (SREBP1c); iii) activation of protein kinase C–θ; and iv) mitochondrial production of reactive oxygen species (ROS) by electron leakage, resulting in an inhibition of ATP–dependent inward rectifier potassium channel (K_{ATP}) activity (Blouet and Schwartz, 2010; Christie, 1982a; López et al., 2007). The 18 carbon monounsaturated FA (MUFA) oleate (OA, C18:1 n–9) is the most studied LCFA in mammals that typically activates all these systems (Blouet and Schwartz, 2010; Christie, 1982a; López et al., 2007). However, the ability of different types of LCFA, differing in acyl chain length or degree of unsaturation, to elicit the activation of these systems has been scarcely assessed to date. Fatty acid unsaturation appears to be important since the saturated FA palmitate (C16:0) does not seem to activate hypothalamic FA sensing systems (Greco et al., 2014; López et al., 2007; Ross et al., 2010). Moreover, the presence of more than one double bond does not seem to induce comparable activations of FA sensing systems in mammals, as demonstrated by studies carried out with linoleate (C18:2 n–6) (41) or docosahexanoate (DHA, C22:6 n–3) (Folch et al., 1957; Gaillard et al., 2008; Ross et al., 2010) although no studies that we are aware of have examined both FAs in parallel.

Appetite regulation in fish is a complex process in which the hypothalamus integrates metabolic and endocrine information to elicit changes in food intake through modulation of neuropeptide synthesis and release (Soengas, 2014; Volkoff et al., 2010) (Volkoff et

al., 2009). As for the regulation of food intake elicited by the sensing of changes in circulating concentrations of FA, we have characterized the presence and function of FA–sensing systems in the hypothalamus of the freshwater species rainbow trout (*Oncorhynchus mykiss*) (Librán–Pérez et al., 2013a; Librán–Pérez et al., 2015a; Librán–Pérez et al., 2013b; Librán–Pérez et al., 2013c; Librán–Pérez et al., 2014a; Librán–Pérez et al., 2015b; Librán–Pérez et al., 2014b; Librán–Pérez et al., 2015c). These systems responded to changes in the levels of not only LCFA such as OA but, unlike mammals, also to medium–chain FA (MCFA) like octanoate (Librán–Pérez et al., 2013a; Librán–Pérez et al., 2015a; Librán–Pérez et al., 2013b; Librán–Pérez et al., 2013c; Librán–Pérez et al., 2014a; Librán–Pérez et al., 2015b; Librán–Pérez et al., 2014b; Librán–Pérez et al., 2015c). The activation of these systems was associated with an upregulation of the expression of anorexigenic neuropeptides and food intake as in mammalian species (Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012). To date, the presence of FA sensing systems in other fish species has never been investigated.

All vertebrate species have absolute dietary requirements for certain polyunsaturated FA (PUFA), which are termed essential fatty acids since they cannot be synthesized *de novo* and are required for normal body functioning (Sargent et al., 2002). On the other hand, lipids in aquatic food webs are rich in PUFA, and in marine environments fish diets are particularly rich in long chain PUFA(Sargent et al., 2002). Furthermore, PUFA of the n–6 and particularly n–3 series are predominant in tissues of most fish, but especially in marine species (Mourente and Tocher, 1992; Tocher, 2003). The brain of marine fish is particularly rich in n–3 PUFA, mainly in α–linolenate (C18:3 n–3), eicosapentanoate (C20:5 n–3), and DHA (C22:6 n–3) (Betancor et al., 2014; Tocher et al., 1992). Therefore, it is interesting to assess whether fish hypothalamic FA sensing systems, particularly in marine species, could differ from those of mammals in the ability to sense changes in levels of these PUFA.

With this in mind, we wanted to investigate the presence of FA sensing systems in the hypothalamus of a marine fish species. Moreover, we also aimed to know if the ability of hypothalamic FA sensing might vary as a function of LCFA chain length and/or degree of unsaturation. As a model marine fish species we used a lean flatfish, the Senegalese sole (*Solea senegalensis* Kaup). The choice of Senegalese sole as a model species for this study had both academic and commercial reasons. More specifically, we selected this marine species because 1) it has high commercial interest and therefore studying the physiology of its feeding is important for aquaculture, and 2) its lipid nutrition has been quite well–studied, due to its particular nutritional physiology and metabolism, in what concerns lipid and fatty acid requirements: it does not perform well with high lipid levels in the diet and has unique capacities to biosynthesize PUFAs (Borges et al., 2013a; Morais et al., 2015a). To achieve these objectives, we injected intraperitoneally (IP) 1) stearate (SA, C18:0) – a saturated FA ; 2) oleate (OA, C18:1 n9) – a MUFA of the same chain length, which has been demonstrated to activate FA sensing systems and to modulate food intake in rainbow trout (Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012); 3) α–linolenate (ALA, C18:3 n–3) – an n–3 PUFA of the same chain length; and 4) eicosapentanoate (EPA, C20:5 n–3) – an n–3 PUFA of a longer chain length and higher degree of unsaturation. We then evaluated food intake and the hypothalamic mRNA abundance of neuropeptides related to the metabolic control of food intake, such as *agrp, npy, pomc* and *cart.* Furthermore, variables related to putative FA–sensing systems were also evaluated based on 1) FA metabolism, such as

activities of ATP–citrate lyase (ACLY) and CPT–1, and mRNA abundance of acetyl–CoA carboxylase (*acc*), *acly, cpt–1c*, and fatty acid synthase (*fas*); 2) binding to FAT/CD36 and subsequent modulation of transcription factors, such as mRNA abundance of *fat/cd36*, liver X receptor α (*lxrα*), *pparα*, and *srebp1c*; and, 3) mitochondrial activity, such as 3–hydroxyacyl–CoA dehydrogenase (HOAD) activity, and mRNA abundance of inward rectifier K^+ channel pore type 6.x (*kir6.x*) and sulfonylurea receptor (*sur*). We have evaluated mRNA abundance of neuropeptides and not protein levels because appropriate antibodies for Western Blot were not available in Senegalese sole at the time of carrying out the study.

2. *Materials and methods*

Fish

Senegalese sole obtained from a commercial fish farm (Aquacria Piscícolas, S.A., Aveiro, Portugal) were kept under quarantine conditions for a 3–week period. The fish were then individually weighed (88.3 ± 1.5 g), measured and distributed (17 fish per tank in the first phase of the experiment, and 8 fish per tank in the second phase of the experiment) among fiberglass rectangular tanks (0.5 m x 0.4 m) in a closed recirculation system. The system was supplied with filtered and heated (20.0 ± 1.0 ºC) seawater (24 $^{0}/_{00}$) at a flow rate of 1.5 $L.min^{-1}$. An artificial photoperiod of 12 h light:12 h dark was established. Fish were fed by hand twice daily (09.00 h and 17.00 h) to satiety with a diet manufactured by Sparos, Portugal (proximate food analysis was 57.9% crude protein, 0.8% fiber, 15.5% starch, 8.6% crude fat, and 8.5% ash; 20.3 MJ/kg of feed). The experiment was directed by trained scientists (following category C FELASA recommendations) and conducted according to the European guidelines on protection of animals used for scientific purposes (directive 2010/63/UE of European Parliament and of the Council of European Union).

Experimental design

Following a 2–week acclimation period, the first phase of the experiment was carried out. We used a total number of 170 fish. These fish were randomly divided into 5 groups (control, SA, OA, ALA, or EPA) of 34 fish each. Each treatment was carried out in duplicate tanks (2 replicate tanks per treatment), and each tank contained 17 fish. Fish were fasted for 24h before the experiment to ensure that basal hormone levels were achieved and then fish were anaesthetized with MS–222 (75 $mg \cdot l^{-1}$, Sigma, St Louis, Mo, USA), weighed and IP injected (10 ml/kg) with saline solution alone (control) or containing SA, OA, ALA, or EPA (all from Sigma Chemical Co.) at a dose of 300 $\mu g.kg^{-1}$. The FA dose was selected based on previous studies carried out in rainbow trout (Le Foll et al., 2009) and in preliminary experiments carried out in Senegalese sole (data not shown). To safely deliver the FAs, they were solubilized in 45% hydroxypropyl–β–cyclodextrin (HBP, Sigma) to a final concentration of 17 mM (Morgan et al., 2004). The HPB–FA solution was diluted in saline to the appropriate concentration used for each injection. HPB was also added in the control group at a similar concentration to the remaining treatments. Three hours after IP injection (established based on previous

unpublished results), fish were lightly anaesthetized with MS–222 (75 mg·l^{-1}) and sampled. Blood was collected by caudal puncture with ammonium–heparinized syringes, and plasma samples were obtained after blood centrifugation, then deproteinized (using 0.6 M perchloric acid) and neutralized (using 1 M potassium bicarbonate) before storage at –80ºC until analysis. Fish were sacrificed by decapitation and dissected to remove the hypothalamus, which was snap–frozen in dry ice, and stored at –80 °C. At each time point, in each tank, 6 fish were used to assess enzyme activities and metabolite levels, 8 fish were used to evaluate the hypothalamic FA profile and the remaining 3 fish were used for assessment of mRNA levels by RT–qPCR. Since we used replicate tanks, for each experimental group we had 12 fish for the assessment of metabolite levels and enzyme activities, 16 fish for the assessment of FA profile, and 6 fish for the assessment of mRNA abundance. For molecular analysis, samples of hypothalamus were collected in tubes containing 0.5 ml of RNAlater stabilization buffer (Sigma) and were kept in agitation at 4 °C for 24 h, before storing at –80ºC.

In the second phase of the experiment we used 160 fish. The fish were divided into 5 treatment groups as described above of 32 fish each. Each treatment was carried out in fish of 4 tanks (4 replicate tanks per treatment) and each tank contained 8 fish. Fish were fed by hand twice per day (09.00 h and 17.00 h) to satiety during 3 weeks. Food intake was quantified for 3 days before IP injection (to collect basal line data) and then 4, 24 and 48h after an IP treatment with pure saline–HPB or containing SA, OA, ALA or EPA as described above. After each meal, any remaining uneaten pellets were removed from the tanks. The amount of feed consumed in each tank was calculated as the difference between the number of offered and uneaten pellets. Results are shown as the mean ± SEM of the data obtained in 4 tanks per treatment.

Assessment of metabolite levels and enzyme activities

Levels of total FA and triglycerides in plasma, were determined enzymatically using commercial kits (Wako Chemicals, Neuss, Germany and Spinreact, Barcelona, Spain; respectively), following manufacturer's instructions, adapted to a microplate format.
Samples used to assess hypothalamic metabolite levels were homogenized immediately by ultrasonic disruption in 5.5 vols of ice–cooled 0.6 M perchloric acid, and neutralized (using 1 M potassium bicarbonate). The homogenate was centrifuged (10,000 g), and the supernatant used to assay tissue metabolites. Hypothalamic FA and triglyceride levels were determined enzymatically using commercial kits as described above for plasma samples.

Samples for evaluation of enzyme activity were homogenized by ultrasonic disruption with 7 vols of ice–cold–buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4–dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (900 g) and the supernatant used immediately for enzyme assays. Enzyme activities were determined using the INFINITE 200 Pro microplate reader (Tecan, Männedorf, Switzerland). Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or, in the case of CPT–1 activity, of 5,5′–Dithiobis(2–nitrobenzoic acid)–CoA complex at 412 nm. The reactions were started by the addition of sample supernatant (10–15 µl) at a pre–established protein concentration, omitting the substrate in control wells (final volume 275–295 µl), and allowing the reactions to proceed at 20°C for pre–established times (10–25 min).

Enzyme activities are expressed in terms of mg protein. Protein was assayed in triplicate in homogenates using microplates according to the bicinchoninic acid method with bovine serum albumin (Sigma) as standard. Enzyme activities were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. ACLY (*EC* 4.1.3.8), HOAD (*EC* 1.1.1.35), and CPT–1 (*EC* 2.3.1.21) activities were determined by adapting previously described methods (Librán–Pérez et al., 2012).

Determination of total lipids and FA profile in the hypothalamus

In order to have enough tissue mass for the analysis of FA profile in the hypothalamus, the 16 fish sampled for this purpose from each treatment group were grouped into 4 pools of 4 fish each. In each pool, total lipids in the hypothalamus were extracted by chloroform/methanol (2:1, v/v) according to Folch et al. (1997) and quantified gravimetrically after evaporation of the solvent under nitrogen flow followed by vacuum desiccation overnight. Total lipids were resuspended at 20 mg/ml in chloroform/methanol (2:1) containing 0.01 % BHT and 100 µl subjected to acid–catalyzed transesterification with 21:0 internal standard (Duca and Yue, 2014). FAME were extracted using isohexane/diethyl ether (1:1, v/v), purified by TLC (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas–liquid chromatography on a Thermo Electron–TraceGC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 m × 0.25 mm id; SGE, UK), using a two–stage thermal gradient initially at 40 °C/min from 50 °C (injection temperature) to 150 °C and then to 250 °C at 2 ºC/min. Helium (1.2 ml/min constant flow rate) was used as the carrier gas and on–column injection and flame ionization detection was performed at 250 °C. Fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well–characterized fish oil (Marinol, Stepan Specialty Products, LLC, USA) and quantified using Chrom–card for Windows (TraceGC, Thermo Finnigan, Italy).

Primer design

Search for selected candidate genes was performed in the SoleaDB data base (Benzekri et al., 2014) (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/) using the *Solea senegalensis* v4.1 global assembly. The retrieved transcripts were aligned and assembled *in silico* using the BioEdit Sequence Alignment Editor and, in some cases, completed with rapid amplification of cDNA ends (RACE) PCR using the FirstChoice RLM–RACE kit (Ambion, Life Technologies, Alcobendas, Madrid, Spain), and the resulting fragments were sequenced (SCSIE, University of Valencia, Spain). Primers for RT–qPCR were designed with Primer3 v. 0.4.0 (Koressaar and Remm, 2007; Untergasser et al., 2012) (Table 1) and the resulting amplicons were sequenced to confirm their identity and the specificity of the assay.

Table 1. Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by RT–PCR (qPCR).

Gene	Primer sequence	Ta	Data Base	Reference
acc	CAGCTGGGTGGAATTCAGAT ATGGGATCTTTGGCACTGAG	60°C	SoleaDB[1]	solea_v4.1_unigene15555
acly	CCACAGATTCACACCATTGC GCCAGGATGTTATCCAGCAT	60°C	SoleaDB[1]	solea_v4.1_unigene11536
agrp2	CAGGTCAGACTCCGTGAGCCC GTCGACACCGACAGGAGGCAC	64°C	SoleaDB[1]	solea_v4.1_unigene32957
cart2b	AGGACCATGCAGAGTTCCAG GGACTCGGTGTCCATCACTT	60°C	GenBank	KT189191
cart4	GTGAGCGAGAGCAGGAAACT CGTGGTGAAATAAGGCAAA	60°C	GenBank	KT189194
cpt1.1	TAACAGCCACCGTCGACATA AGCGATTCCCTTGTGTCACT	63°C	GenBank	KR872890
cpt1.2	TCGCCAAGAACTAACCGAAC AGACCTGGCGTAGAGCTTCA	64°C	GenBank	KR872891
cpt1.3	CCTGACTGTTGACCCCAAGT TCACTCACAGTTACGCAGCA	60°C	GenBank	KR872892
ef1α	GATTGACCGTCGTTCTGGCAAGAAGC GGCAAAGCGACCAAGGGGAGCAT	70°C	GenBank	CAB326302
fas	CACAAGAACATCAGCCGAGA GAAACATTGCCGTCACACAC	60°C	GenBank	KP842777
fat/cd36 (lmp2)	TATGTGGCGGTAATGGATCA GCCGGTGTGGAATACAAACT	60°C	GenBank	KR872888
fat/cd36 (pg4l)	TGAATGAGACGGCTGAGTTG TGTTGTTTCTGCTCCTCACG	64°C	GenBank	KR872889
kir6.x	AGATGTTGGCGAGAAAGAGC GCTCGCGGATGTTCTTGT	60°C	SoleaDB[1]	solea_v4.1_unigene120423
lxrα	AAAGCAGGGCTTCAGTTTGA CAGCCTCTCCACCAGATCAT	60°C	SoleaDB[1]	solea_v4.1_unigene47872
npy	GAGGGATACCCGATGAAACC GCTGGACCTCTTCCCATACC	60°C	SoleaDB[1]	solea_v4.1_unigene466117
pomca	AAGGCAAAGAGGCGTTGTAT TTCTTGAACAGCGTGAGCAG	60°C	GenBank	FR851915
pomcb	GTCGAGCAACACAAGTTCCA GTCAGCTCGTCGTAGCGTTT	60°C	GenBank	FR851916
pparα	AAACCGCCTCTCATCATCC CACACCTGGAAACACATCTCC	60°C	GenBank	JX4240810
srebp1c	TCCAAGGCTTTCAGCAAGAT CTCCTCTGTCTTGGCTCCAG	60°C	SoleaDB[1]	solea_v4.1_unigene4060
sur	GCAGCACCTTCCGTTACCTA GCAGCAGCTTAGAGGACGAC	60°C	SoleaDB[1]	solea_v4.1_unigene446925
ubq	AGCTGGCCCAGAAATATAACTGCGACA ACTTCTTCTTGCGGCAGTTGACAGCAC	70°C	GenBank	CAB291588

[1]SoleaDB: http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/

acc, Acetyl–CoA carboxylase; *acly*, ATP–citrate lyase; *agrp2*, Agouti related peptide 2; *cart*, cocaine– and amphetamine–related transcript; *cpt1.1*, carnitine palmitoyl transferase type 1, isoform 1; *cpt–1.2*, carnitine palmitoyl transferase type 1, isoform 2; *cpt1.3*, carnitine palmitoyl transferase type 1, isoform 3;

mRNA abundance analysis by real–time quantitative RT–PCR

Total RNA was extracted from tissues (approx. 20 mg) using Trizol reagent (Life Technologies, Grand Island, NY, USA), following manufacturer's instructions and treated with RQ1–DNAse (Promega), and RNA quantity and quality was evaluated by spectrophotometry (NanoDrop 2000). Two µg of total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Promega, Madison, Wi, USA) and random hexaprimers (Promega). Gene expression levels were determined by real–time quantitative RT–PCR (q–PCR) using the iCycler iQ (BIO–RAD, Hercules, CA, USA). Analyses were performed on 5 µl of diluted (1/50) cDNA using the MAXIMA SYBR Green qPCR Mastermix (Life Technologies), in a total PCR reaction volume of 15 µl, containing 120–500 nM of each primer. Sequences of the forward and reverse primers used for each gene expression assay are shown in Table 1. Relative quantification of the target genes was done using elongation factor 1 alpha (*ef1a*) or ubiquitin (*ubq*) as reference genes (Infante et al., 2008). Thermal cycling was initiated with incubation at 95°C for 2 min using hot–start iTaq DNA polymerase activation; 35 steps of qPCR were performed, each one consisting of heating at 95°C for 15s, 30s at each specific annealing temperature (Table 1) and 30s at 72 ºC. Following the final PCR cycle, melting curves were systematically monitored (temperature gradient at 0.5°C/s from 55 to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without cDNA were run as negative controls, which were indeed negative, and confirmed no amplificacion of genomic DNA. Relative expression of the target genes was calculated using the delta–delta CT method ($2^{-\Delta\Delta CT}$), following Pffafl (2001).

Statistics

Comparisons among groups were carried out using the statistical package SigmaStat with one–way ANOVA with treatment (control, SA, OA, ALA and EPA) as the main factor. In cases where a significant effect was noted, post–hoc comparisons were carried out by a Student–Newman–Keuls test. Differences were considered statistically significant at $P<0.05$.

ef1α, elongation factor 1α; *fas*, fatty acid synthase; *fat/cd36* (*lmp2*), fatty acid translocase lysosome membrane protein 2–like; fat/cd36 (*pg4l*), fatty acid translocase platelet glycoprotein 4–like; *kir6.x*, inward rectifier K+ channel pore type 6.x; *lxrα*, liver X receptor α; *npy*, neuropeptide Y; *pomc*, pro–opio melanocortin A1; *pparα*, peroxisome proliferator–activated receptor type α; *srebp1c*, sterol regulatory element–binding protein type 1c; *sur*, sulfonylurea receptor; *ubq*, ubiquitin.

3. *Results*

Fatty acid composition of hypothalamus from fish treated with IP injections is shown in table 2.

All fish treated with FAs tended to have a lower food intake compared to the control group, although no significant differences were observed among treatments (Fig. 1).

Levels of total FA in plasma increased in the SA and EPA groups (Fig. 2A) compared to the control. In the hypothalamus, total FA levels (Fig. 2B) increased in the OA treatment compared to the control and SA treatments, and were also higher in fish injected with ALA compared with the control, SA, and EPA treatments. Triglyceride levels (Fig. 2C) were higher in the OA, ALA, and EPA treatments compared with the control and SA groups. Total lipid levels (Fig. 2D) were higher in the ALA group compared to control and SA groups. ACLY activity (Fig. 2E) decreased in the OA and ALA groups compared with the control, and the activity in the ALA group was also lower than in the SA group. CPT–1 activity (Fig. 2F) decreased in OA and ALA groups compared with the control.

The mRNA abundance of genes related to FA metabolism are shown in Fig. 3. The expression of *acc* (Fig. 3A) decreased in the OA and ALA groups compared with the control. *acly* expression in the ALA group was lower than that of the control group (Fig. 3B). *fas* mRNA abundance was lower in the OA group compared to the control (Fig. 3C). The expression of *cpt1.3* was higher in the SA and EPA groups compared with the control (Fig.3F). Finally, no significant changes were observed in mRNA abundance of *cpt1.1* (Fig. 3D) and *cpt1.2* (Fig. 3E).

Transcript levels of genes related to FA transport and regulation of gene expression (transcription factors) is shown in Fig. 4. Transcription of *fat/cd36*–like genes, thrombospondin receptor or platelet glycoprotein 4–like (*pg4l*) (Fig. 4A) and lysosome membrane protein 2–like (*lmp2*) (Fig. 4B), was not affected by any FA treatment. *lxrα* mRNA abundance (Fig. 4C) was decreased in the OA and EPA groups compared with the control group. Expression of *pparα* (Fig. 4D) and *srebp1c* (Fig. 4E) was lower in OA and ALA groups compared to the control.

The parameters related to mitochondrial activity and K_{ATP} channel are shown in Fig. 5. HOAD activity (Fig. 5A) in the OA and EPA groups was lower than that of the control and SA groups. On the other hand, the OA and ALA groups displayed a lower expression of *kir6.x* (Fig. 5B) and *sur* (Fig. 5C) than the control.

The mRNA abundance of neuropeptides involved in the regulation of food intake is shown in Fig. 6. *Agrp2* expression in the OA group was lower than in the control, SA, and EPA groups, while in the ALA group it was only lower than the control (Fig. 6A). No significant changes were noted for mRNA abundance of *npy* (Fig. 6B), *pomca* (Fig. 6C), *pomcb* (Fig. 6D), and *cart4* (Fig. 6F). *cart2b* mRNA levels (Fig. 6E) were higher in the OA, ALA, and EPA groups, whereas the expression in the EPA group was also higher than in the SA group.

Table 2. Fatty acid composition (µg.mg^{-1} dry weight) of Senegalese sole hypothalamus 3 h after intraperitoneal administration of saline alone (control, CTR) or containing 300 µg·kg^{-1} of stearate (SA) oleate (OA), α–linolenate (ALA) or eicosapentanoate (EPA). Each value is the mean ± SEM of n = 4 pools of 4 hypothalamus per treatment. Different letters indicate significant differences (P<0.05) between groups.

Fatty acid	CTR	SA	OA	ALA	EPA
C14:0	0.56±0.13^{b}	0.89±0.41^{b}	0.58±0.07^{b}	1.13±0.13^{a}	0.46±0.08^{b}
C16:0	16.48±1	19.76±3.46	17.35±0.44	25.43±4.67	18.22±0.99
C18:0	14.52±0.98	14.66±1.42	14.95±0.4	19.3±1.95	16.5±1.36
C24:0	1.31±0.16	1.47±0.16	2.24±0.83	1.87±0.28	1.27±0.18
Σ SFA[1]	32.97±2.09^{b}	37.05±5.15^{ab}	35.53±1.72^{b}	52.75±6.47^{a}	36.63±2.27^{b}
C16:1	0.6±0.14	1.17±0.43	0.82±0.33	1.13±0.5	0.65±0.21
C18:1n–9	15.6±1.23	18.46±3.13	18.27±2.8	24.28±4.73	16.87±1.03
C20:1	0.1±0.06^{ab}	0.3±0.08^{ab}	0.2±0.12^{ab}	0.45±0.17^{a}	0.16±0.01^{b}
Σ MUFA[2]	16.3±1.35^{b}	19.93±3.64^{ab}	19.29±3.22^{ab}	25.86±5.32^{a}	17.68±0.99^{ab}
C18:2n–6	1.44±0.1	1.54±0.21	1.55±0.23	2.03±0.53	1.5±0.14
C20:4n–6	1.93±0.08	1.85±0.24	1.93±0.2	2.6±0.75	2.19±0.16
Σ n–6 PUFA[3]	3.37±0.16	3.43±1	3.48±0.44	4.56±1.05	3.69±0.29
C18:3n–3	0±0	0.02±0.02	0±0	0.14±0.08	0±0
C18:4n–3	0.18±0.1	0.33±0.02	0.4±0.07	0.77±0.39	0.27±0.04
C20:5n–3	2.07±0.12	2.3±0.34	2.19±0.28	4.07±1.73	2.33±0.06
C22:5n–3	0.95±0.08	1±0.07	1.27±0.36	1.35±0.19	1.03±0.08
C22:6n–3	34.14±1.04	33.34±3.78	33.52±0.84	43.33±5.73	36.95±1.39
Σ n–3 PUFA[4]	37.33±1.19	36.91±4.08	37.39±1.52	49.47±7.59	40.58±1.35
Σ PUFA[5]	40.71±1.35	40.35±4.7	40.87±1.95	54.04±8.57	44.27±1.31

Different letters indicate significant differences (p<0.05) between FA treatments
[1]C15:0, C22:0 are also included
[2]C18:1n7, C22:1, C24:1n9 are also included
[3]C18:3n6, C20:2n6, C20:3n6, C22:4n6, C22:5n6 are also included
[4]C20:3n3, C20:4n3, C21:5n3, C22:4n3 are also included
SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids

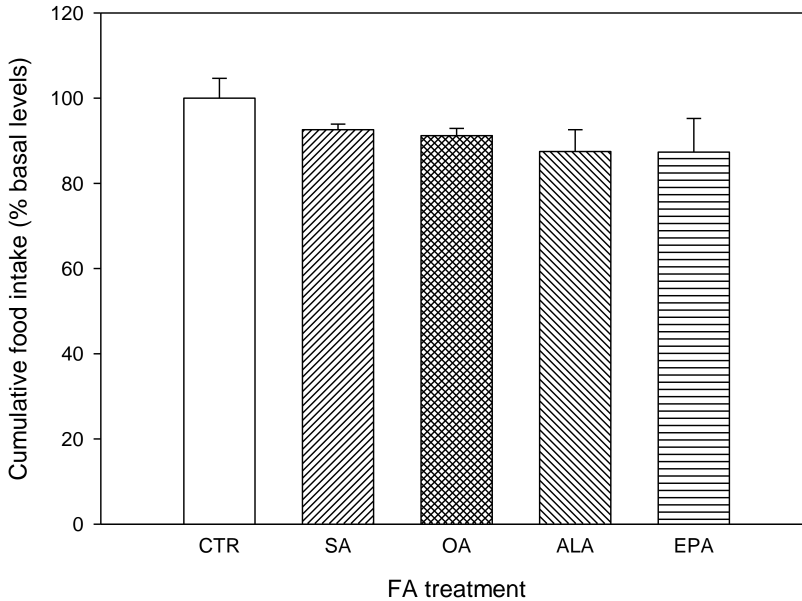

Figure 1. Effect of intraperitoneal treatment with saline solution alone (control, CTR) or containing 300 $\mu g \cdot kg^{-1}$ of stearate (SA) oleate (OA), α–linolenate (ALA) or eicosapentanoate (EPA) on cumulative food intake of Senegalese sole. All fish (n=8) in a tank were fed and food intake was monitored in the whole tank at 4, 24, and 48 h after feeding. Food intake is displayed as percentage of food ingested with respect to basal levels (calculated as the average food intake in the same tank the three days preceding the experiment). The results are shown as mean ± SEM of the results obtained in four different tanks per treatment.

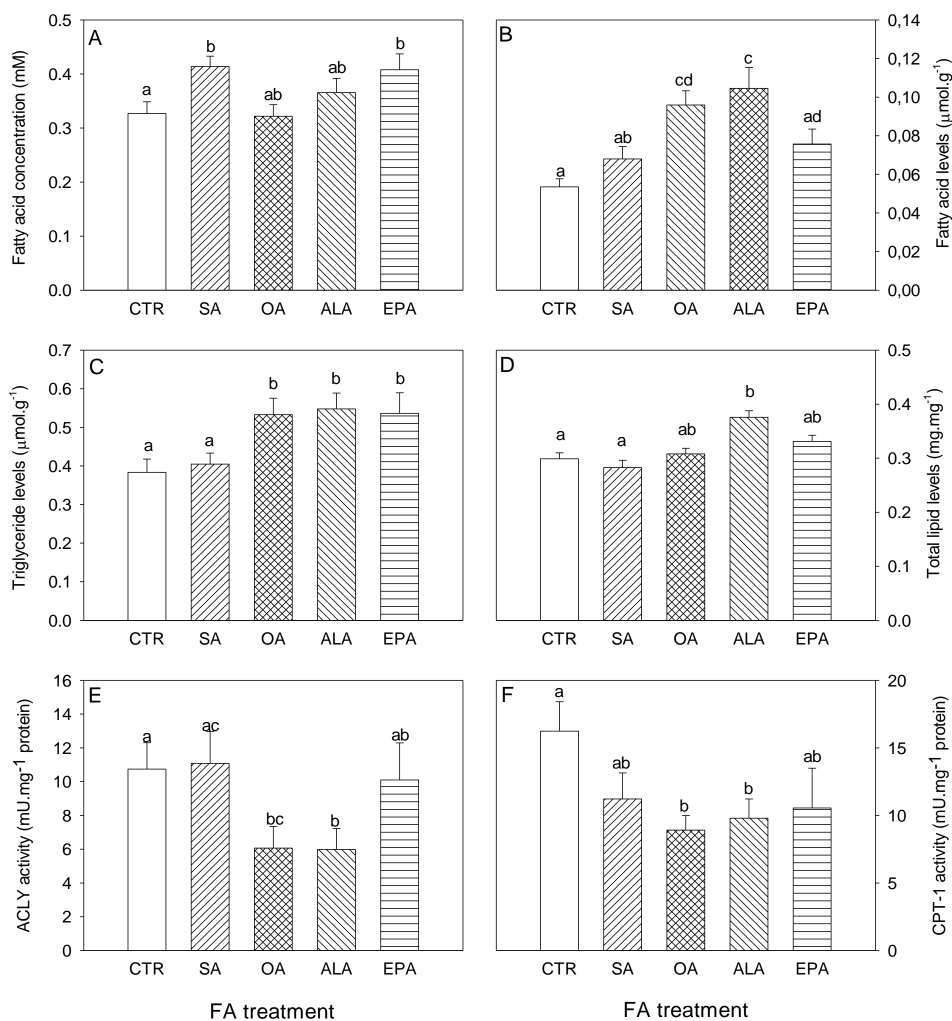

Figure 2. Concentration of total FA in plasma (A), and levels of total FA (B), triglyceride (C) and total lipid (D), and activities of ACLY (E) and CPT–1 (F), in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (control, CTR) or containing 300 $\mu g \cdot kg^{-1}$ of stearate (SA) oleate (OA), α–linolenate (ALA) or eicosapentanoate (EPA). Each value is the mean ± SEM of n = 12 fish per treatment. Different letters indicate significant differences (P<0.05) between groups.

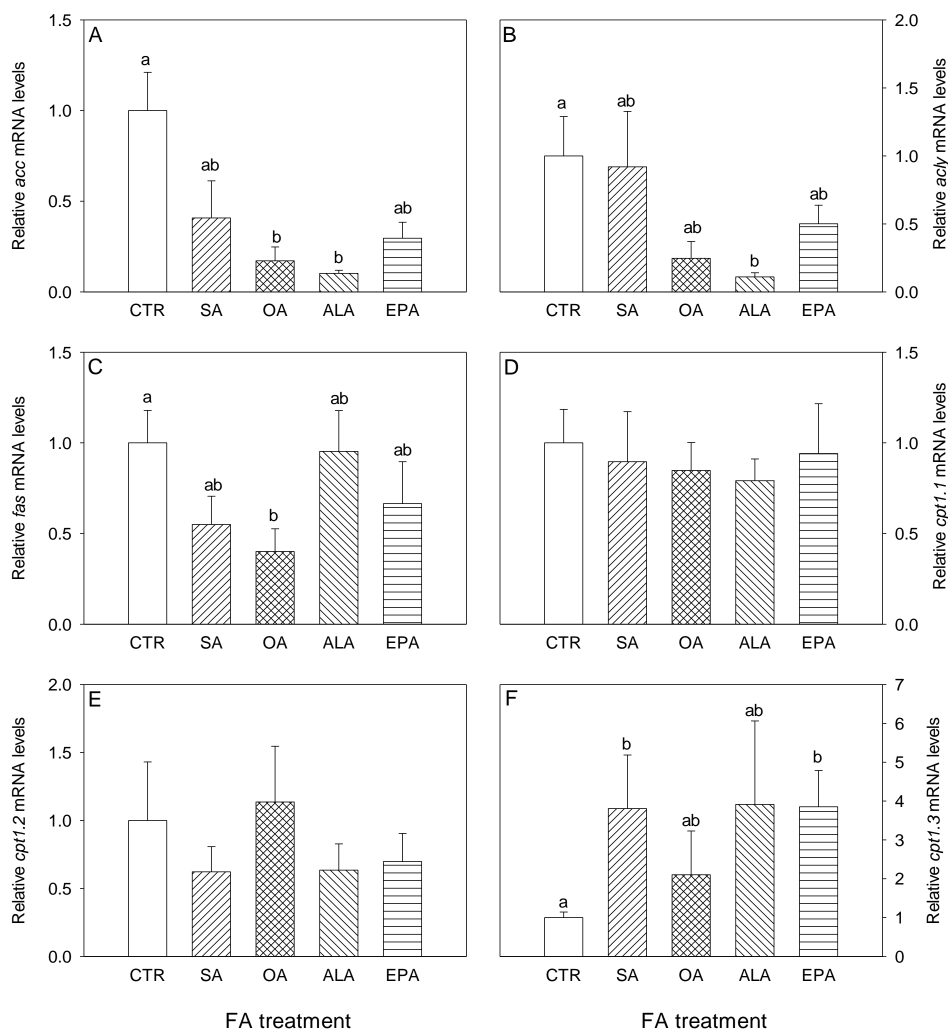

Figure 3. mRNA abundance of *acc* (A), *acly* (B), *fas* (C), *cpt1.1* (D), *cpt1.2* (E), and *cpt1.3* (F) in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (control, CTR) or containing 300 $\mu g \cdot kg^{-1}$ of stearate (SA) oleate (OA), α–linolenate (ALA) or eicosapentanoate (EPA). Gene expression results are expressed relative to the control group and are normalized by *ef1α* or *ubq* expression. Each value is the mean ± SEM of n = 6 fish per treatment. Different letters indicate significant differences (P<0.05) between groups.

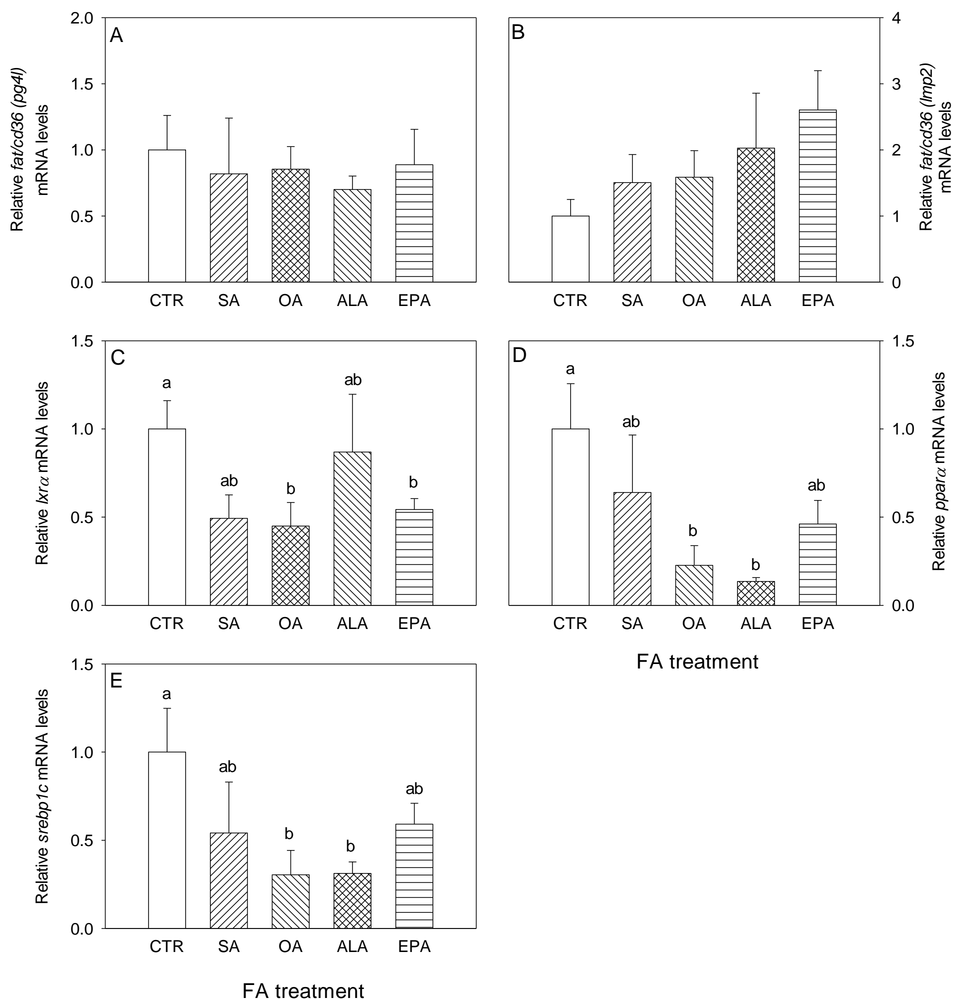

Figure 4. mRNA abundance of *fat/cd36 (pg4l)* (A), *fat/cd36 (lmp2)* (B), *lxrα* (C), *pparα* (D), and *srebp1c* (E) in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (control, CTR) or containing 300 $\mu g \cdot kg^{-1}$ of stearate (SA) oleate (OA), α–linolenate (ALA) or eicosapentanoate (EPA). Gene expression results are expressed relative to the control group and are normalized by *ef1α* or *ubq* expression. Each value is the mean ± SEM of n = 6 fish per treatment. Different letters indicate significant differences (P<0.05) between groups.

4. *Discussion*

We have previously demonstrated in rainbow trout, a freshwater fish species, the presence of FA sensing systems in the hypothalamus and their response to increased levels of OA or octanoate (Librán–Pérez et al., 2013a; Librán–Pérez et al., 2015a; Librán–Pérez et al., 2013b; Librán–Pérez et al., 2013c; Librán–Pérez et al., 2014a; Librán–Pérez et al., 2015b; Librán–Pérez et al., 2012; Librán–Pérez et al., 2014b; Librán–Pérez et al., 2015c). However, there was no evidence to date of the existence of such mechanisms in other fish species. PUFA are a major constituent of marine fish diets, particularly important essential nutrients, and major constituents of fish brains and other body tissues. Therefore, we wanted to further investigate whether (if present) FA sensing systems in the hypothalamus of a marine fish species could respond to changes in PUFA levels, as well as to different types of LCFA differing in chain length and degree of saturation.

First of all, it was necessary to setup the techniques required for the evaluation of parameters related to FA sensing systems, including enzyme activities and gene expression in Senegalese sole. In some cases, multiple transcripts were found for the genes of interest and preliminary work was performed to select those that would be more relevant for the objectives of this study. Thus, in the case of *cart*, 7 different transcripts were found (Bonacic et al., 2015b), of which we selected *cart2b* and *cart4* to examine in this study. The first gene is the closest homologue to the trout *cart* that has been studied so far (Librán–Pérez et al., 2012) and also to medaka ch3, which was the only *cart* transcript (out of 6) that responded to fasting and re–feeding in this species, and was suggested to have an anorexic role (Murashita and Kurokawa, 2011). On the other hand, *cart4* was selected given that it is expressed relatively well in Senegalese sole hypothalamus and appears to respond to feeding (Bonacic et al., 2015b). On the other hand, we found 5 different transcripts for *cpt1* (2 of them being splice variants of the same gene – isoform 4), of which we decided to assess the 3 most similar to the trout *cpt1* isoforms involved in FA sensing (Librán–Pérez et al., 2012). Finally, we also obtained evidence for the presence of multiple *fat/cd36* genes, of which we focused on LMP2 and PG4L. The first gene resembled the transcript most commonly studied in relation to FA sensing in trout (Librán–Pérez et al., 2012), while the second is closest to a mammalian *fat/cd36*–like gene with roles in the orosensory detection of FA and mediating effects of FA on hypothalamic food intake regulation in rats (Gaillard et al., 2008; Laugerette et al., 2005; Moullé et al., 2013).

The parameters related to the FA sensing system based on FA metabolism displayed changes depending on the FA assessed, and we clearly distinguished two patterns of response. The first pattern included the actions of OA and ALA that activated this system, as demonstrated by the decrease observed in ACLY activity and expression, CPT–1 activity, *fas* mRNA abundance (only after OA treatment), and *acc* expression. The response of these parameters is generally comparable to that previously observed in rainbow trout (Librán–Pérez et al., 2013c; Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012) and in mammals (Christie, 1982a; López et al., 2007) in response to OA, thus supporting the presence of this system in a marine fish species like Senegalese sole. The lack of changes in mRNA abundance of the different isoforms tested for CPT1 might indicate a failure in selecting the isoform in this species functionally similar to the

CPT1c in rainbow trout (Librán–Pérez et al., 2012) or mammals (Borges et al., 2013a; López et al., 2007), in responding to changes in FA levels.

The response observed after ALA IP injection, basically comparable to that of OA, suggest for the first time in any vertebrate species that this hypothalamic FA sensing system responds to increased levels of a n–3 PUFA differently to that observed to date in mammals (Gomez–Pinilla and Ying, 2010; Greco et al., 2014; Ross et al., 2010). There are no other studies that we are aware of in fish hypothalamus. The effect of n–3 PUFA like ALA, EPA and DHA have only been elucidated in peripheral tissues such as liver, where an inhibition of lipogenic enzymes (similar to that herein observed) has been recorded in rainbow trout (Alvarez et al., 2000; Morash et al., 2009).

The second pattern of response included the actions of SA and EPA which in general did not induce any change in FA metabolism parameters, or the changes induced (e.g., increased expression of *cpt1.3*, similarly to the OA and ALA groups) were unexpected. The lack of response to SA is not surprising considering that in mammals another saturated LCFA like palmitate did not induce changes in parameters related to this FA sensing system (Ross et al., 2010; Schwinkendorf et al., 2011). This could mean that the response requires the presence of at least one double bond. On the other hand, the lack of changes observed in response to IP injection of EPA is very interesting considering that this FA is also an n–3 PUFA (like ALA) but of a longer acyl chain and higher degree of saturation. We have no explanation for these differences. They could be associated with the fact that LC–PUFA (such as EPA and DHA) and C18 PUFA (such as ALA) may differentially affect key regulatory pathways of lipid metabolism, binding to nuclear receptors or transcription factors and modulating the expression of genes with different potencies, as has been observed in mammals (Jump, 2008; Yoshikawa et al., 2002). In this respect, a strong hypotriglyceridemic effect has been attributed to EPA in rats, through simultaneous effects on FA oxidation and lipogenic pathways (Willumsen et al., 1993). In fish this possibility has not been assessed yet. However, evidence supporting the existence of similar effects (at least in lipogenesis) is accumulating in Senegalese sole (Bonacic et al., 2016) and salmonid species (Alvarez et al., 2000; Morais et al., 2011).

The FA sensing mechanisms based on binding of FA to FAT/CD36 and subsequent modulation of transcription factors was also differentially affected by FA treatments. Two *fat*/*cd36*–like genes were investigated in Senegalese sole. The first one is the putative homologue of the gene that has been studied so far in trout (Librán–Pérez et al., 2013c; Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012). The second one is closest to a mammalian *fat/cd36* implicated in the orosensory detection of FA and affecting food intake through LCFA sensing in the hypothalamus (Gaillard et al., 2008; Laugerette et al., 2005; Moullé et al., 2013). However, no changes were observed in the mRNA abundance of any of the genes after treatment with the tested FA. Since other *fat*/*cd36*–like genes were identified in the transcriptomic SoleaDB (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/), these should also be investigated in the future. If we focus on the remaining parameters related to this FA sensing system (mRNA abundance of *pparα*, *srebp1c*, and *lxrα*), we can observe a similar response to that outlined above. Both OA and ALA altered the mRNA abundance of the transcription factors *lxrα* (although not ALA in this case), *srebp1c*, and *pparα* in a

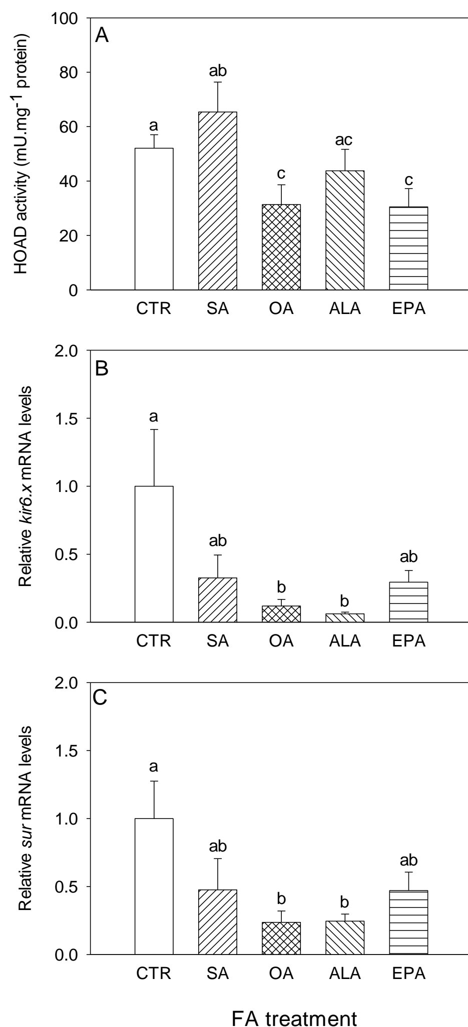

Figure 5. Activity of HOAD (A), and mRNA abundance of kir6.x (A) and sur (B) in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (control, CTR) or containing 300 $\mu g \cdot kg^{-1}$ of stearate (SA) oleate (OA), α–linolenate (ALA) or eicosapentanoate (EPA). Gene expression results are expressed relative to the control group and are normalized by ef1α or ubq expression. Each value is the mean ± SEM of n = 12 (enzyme activity) or n = 6 (mRNA levels) fish per treatment. Different letters indicate significant differences (P<0.05) between groups.

way comparable to that previously observed after OA treatment in mammals (Le Foll et al., 2015; Le Foll et al., 2009; Migrenne et al., 2011) and rainbow trout(Librán–Pérez et al., 2013c; Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012). These results support once again the presence of these mechanisms in the hypothalamus of Senegalese sole, as well as its response not only to an unsaturated C18 FA such as OA, but also to a C18 PUFA such as ALA. As far as we are aware, the response to an n–3 PUFA is absolutely novel in any vertebrate species to date. Very few studies exist in fish, although when Atlantic salmon were fed a DHA–enriched diet, no changes were observed in *lxrα* expression, while *srebp1c* was up–regulated in whole brain tissue (Betancor et al., 2014). In spite of a trend for lower expression of these transcription factors in the SA and EPA treatments in relation to the control group, these changes were not significant. A notable exception was *lxrα*, for which IP injection with EPA, and not ALA, induced a significant down–regulation.

The assessed parameters of FA sensing based on mitochondrial activity also displayed a generally similar pattern to the other FA sensing systems. The IP injection of OA and ALA induced changes (decreased expression of the components of the K_{ATP} channel and HOAD activity, although only significantly for OA in the latter case) comparable to those observed in rainbow trout (Librán–Pérez et al., 2013c; Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012) and in mammals (Benani et al., 2007; Christie, 1982a) after treatment with OA. In contrast, neither SA nor EPA induced any change in the mRNA levels of *kir6.x* or *sur*, but the EPA treatment led to a significant reduction of HOAD activity, compared to the control.

The activation of FA–sensing systems is typically associated with the inhibition of the orexigenic factors AgRP and NPY, and the enhancement of the anorexigenic factors POMC and CART, ultimately leading to decreased food intake in mammals (Christie, 1982a; Migrenne et al., 2011) as well as in rainbow trout (Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012). In Senegalese sole, IP injections of both OA and ALA induced a decrease in the mRNA abundance of *agrp2* and an increase in the mRNA abundance of *cart2b* (which was also significantly increased by the EPA treatment), without significantly affecting *npy* or *pomc* mRNA abundance. These changes in gene expression are consistent with a general anorexigenic balance that is usually associated with decreases in food intake, as observed in mammals (Blouet and Schwartz, 2010; Christie, 1982a) or rainbow trout (Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012) after treatment with OA. Furthermore, similar changes in anorexigenic potential have also been recorded in rat, after treatment with DHA (Schwinkendorf et al., 2011), or after feeding an n–3 PUFA enriched–diet (Dziedzic et al., 2007), while palmitate failed to induce effects in neuropeptide expression (Schwinkendorf et al., 2011), as was the case with SA in the current study. However, despite a slight decrease observed in food intake, non–significant differences in food intake were observed after FA treatment.

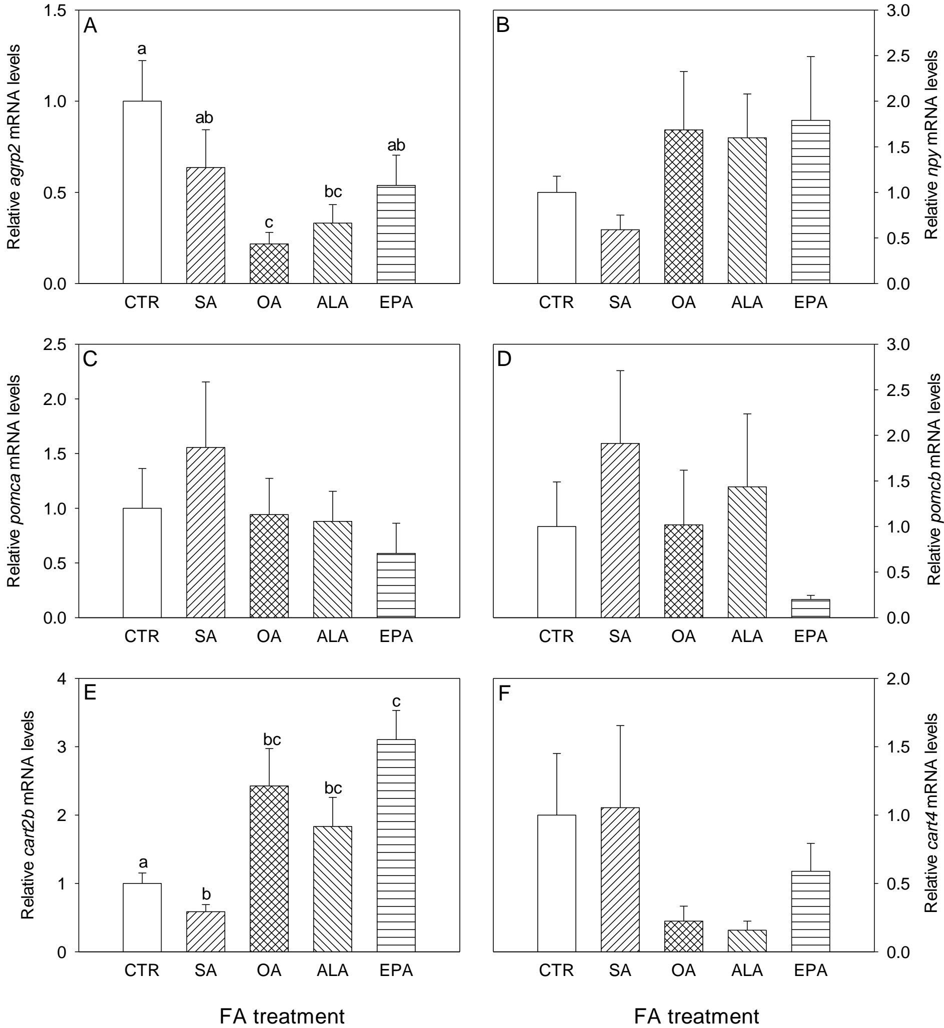

Figure 6. mRNA abundance of *agrp2* (A), *npy* (B), *pomca* (C), *pomcb* (D), *cart2b* (E), and *cart4* (F) in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (control, CTR) or containing 300 µg·kg^{-1} of stearate (SA) oleate (OA), α–linolenate (ALA) or eicosapentanoate (EPA). Gene expression results are expressed relative to the control group and are normalized by *ef1α* or *ubq* expression. Each value is the mean ± SEM of n = 6 fish per treatment. Different letters indicate significant differences (P<0.05) between groups.

5. *Perspectives and significance*

In summary, in the present study we demonstrated that the three FA sensing systems characterized in rainbow trout are also present in Senegalese sole hypothalamus, indicating that this capacity is not specific to rainbow trout and can also be found in marine fish species like Senegalese sole. These systems were activated by OA in a way similar to that previously described not only in rainbow trout but also in mammals, highlighting their importance in the phylogeny of vertebrates. On the other hand, we provide for the first time in any vertebrate species (as far as we are aware) evidence of the activation of these hypothalamic FA sensing systems by an n–3 PUFA such as ALA. This is different to that reported in mammals (Folch et al., 1957; Gaillard et al., 2008; Ross et al., 2010; Schwinkendorf et al., 2011). This different response may relate to the importance of n–3 PUFA in fish, especially in marine species. Thus, PUFA may be sensed in the hypothalamus of marine fish and possibly promote changes in food intake. However, in spite of the particularly high levels of LC–PUFA (including EPA) found in the brain tissues of teleosts, the overall lack of effects of EPA on fatty acid sensing systems in the hypothalamus of Senegalese sole indicates that the response might be specific to certain PUFA. This differential response may be related to different (yet undetermined) roles of LC–PUFA in fish. Still, IP injections of EPA led to a few changes in some of the assessed parameters, such as increased plasma triglyceride levels and hypothalamic *cpt1.3* and *cart2b* expression, and decreased *lxrα* mRNA levels and HOAD activity. These effects are probably unrelated to a role in FA sensing and might be explained by other mechanisms, such as the activation of transcription factors and modulation of the expression of lipid metabolism genes with effects on energy homeostasis. Finally, we also obtained evidence on the lack of response of FA sensing systems to a saturated FA such as SA. This response is comparable to the lack of effect in mammals of other saturated LCFA, such as palmitate (Greco et al., 2014; Ross et al., 2010). However, we previously observed that a MCFA like octanoate was able to activate these systems in rainbow trout. Therefore, both level of unsaturation and chain length of FA seem to be important factors for the hypothalamic sensing capacity in sole but are probably not the only factors involved.

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Author contributions

M.C–S., L.M.P.V., S.M., and J.L.S. conception and design of research; S.M. designed primers, M.C–S. and K.B. performed experiments; M.C–S., K.B., S.M. and C.V. analyzed data; M.C–S., K.B., C.V., L.M.P.V., S.M., and J.L.S. interpreted results of experiments; M.C–S., K.B., C.V. and J.L.S. prepared figures; M.C–S., K.B., C.V., L.M.P.V., S.M., and J.L.S. edited and revised manuscript; M.C–S., K.B., C.V., L.M.P.V., S.M., and J.L.S. drafted manuscript. M.C–S., L.M.P.V., S.M., and J.L.S. approved final version of manuscript.

CHAPTER 6

Effect of dietary lipid level and oil source on food intake and appetite regulation in juvenile *Solea senegalensis* (Kaup, 1858)

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Abstract

Vegetable oils (VO) are increasingly being used to substitute fish oil (FO) in aquafeeds, in order to meet demands of the rapidly growing aquaculture industry. However, effects of dietary VO on appetite and food intake have hardly been investigated, despite the importance of these factors for determining growth and body composition of cultured fish. This study analyzes the effects of dietary lipid level (8% or 18%) and source (100% FO or 75% FO replaced by VO) on voluntary food intake (VFI) and on the expression of putative appetite–regulating genes in the intestine and brain of Senegalese sole juveniles before and after feeding. Decreased VFI was observed only in fish fed the 18FO diet, indicating a higher satiating effect of FO, compared to VO at equally high lipid levels. However, no obvious relationship was found between the VFI results and mRNA levels of the peripheral or central genes. Several putatively anorexigenic peripheral genes (*pyya*, *pyyb*, *glp1*) had higher basal expression in fish fed lower lipid diets while, 6 h after feeding, only *pyyb* and the orexigenic *gal* showed higher expression in the 8% lipid treatments. Conversely, basal mRNA levels of central neuropeptides were not regulated by diet, but most showed temporal changes post–prandially, with some slight differences in relation to lipid level. Of all the studied peptides, only the anorexigenic *cart1a* and *cart1b* were affected by dietary lipid source, with higher post–prandial mRNA levels in fish fed FO, 1 h and 3 h after feeding the 18FO and 8FO diets, respectively, possibly relating to the decreased VFI of the 18FO treatment. This study generally provides new information on several key genes believed to be involved in the regulation of appetite and how they are affected by dietary lipid properties in Senegalese sole.

Keywords: fatty acid, satiety, gastrointestinal, neuropeptide, *glp*, *cck*, *pyy*, *cart*, *npy*

1. *Introduction*

Wild capture of small pelagic fish used for the production of fish meal (FM) and fish oil (FO) has declined in recent decades, due to dwindling natural stocks and natural phenomenon such as the “El Niño”, which has led to stricter management of fisheries and increased FM and FO prices (FAO, 2014). In order to meet increased demand of the growing aquaculture industry, efforts to establish alternative ingredients are ongoing, and in recent years we have already seen a substantial decline of FM and FO use in compound feeds (Tacon and Metian, 2008). Today, but especially in the near future, FM and FO will be used as strategic ingredients at low dietary inclusion levels and differently for specific stages of production (FAO, 2014). Most studies aiming at identifying suitable candidate vegetable oils (VO) for the complete or partial replacement of FO in aquaculture feeds have focused on their effects on growth, feed efficiency, fish health, reproduction and physiology, but results vary (Montero et al., 2003; Nasopoulou and Zabetakis, 2012; Sargent et al., 1999a; Turchini et al., 2010; Turchini et al., 2009; Zuo et al., 2015). Not all species perform well on these alternative oil sources, due to their lack of essential n–3 long–chain polyunsaturated fatty acids (LC–PUFA) and high levels of n–6 PUFA, which leads to imbalances in the n–3/n–6 ratio (Turchini et al., 2010). While differences in performance mainly occur between species, numerous other factors such as trial duration, type of VO used, amount of fish meal (source of FO) in the diet, fish size, age or developmental stage, but also dietary lipid level, can lead to intra–species variation, and thus effects of dietary VO inclusions should be evaluated on a case by case basis (Figueiredo–Silva et al., 2012a; Jobling et al., 2002; Kenari et al., 2011; Kim et al., 2012; Sales and Glencross, 2011; Tocher et al., 2003; Turchini et al., 2009).

An important factor that has received little attention in the context of dietary FO substitution is appetite. This is quite surprising, since appetite directly governs food intake, which has a central role in determining growth and body composition of cultured animals (Forbes, 2007). It has been stated that fish regulate their food intake based on dietary energy density (Boujard et al., 2004b; Gélineau et al., 2002; Sæther and Jobling, 2001). However, specific fatty acids (FA) and other macronutrients have also been shown to generate different responses in appetite–regulating mechanisms (Francis et al., 2014; Narnaware and Peter, 2002; Varricchio et al., 2015; Varricchio et al., 2012). Furthermore, because fish under farming conditions are generally fed pre–set amounts of feed, they cannot compensate their food intake for the lack of a particular nutrient or for energy content, which may cause lower performance (Saravanan et al., 2012). Therefore, in order to avoid wasting feed and degrading water quality, while keeping fish healthy and within an optimal window of growth performance, it is very important to determine the voluntary food intake (VFI) of the cultured species on specific diets, but also the effects that dietary nutrients have on appetite–regulatory mechanisms (Saravanan et al., 2012). This regulation occurs in the central nervous system (CNS), mainly in the hypothalamus, by neuroendocrine secretion of several anorexigenic and orexigenic peptides that mutually interact to inhibit or stimulate appetite, respectively (Volkoff et al., 2005). It results from a combination of signals originating from sensory cues from the environment, the gastrointestinal tract (quality and quantity of ingested food), homeostatic regulatory mechanisms (lipostatic and glycostatic signals), and circadian

control systems (Arora and Anubhuti, 2006; Rønnestad et al., 2013). However, the core of appetite regulation lies in the gut–brain axis and gastrointestinal endocrine signals will be most affected by changes in diet composition and in relation to feed entering the gut (Cummings and Overduin, 2007). These consist of anorexigenic and orexigenic peptides that transmit signals to the CNS by activating vagal afferents or entering the circulatory system, both of which elicit responses in the hypothalamus to release relevant appetite–regulating neuropeptides (Breen et al., 2011).

There are few studies in fish analyzing the effects of dietary FO substitution at different lipid levels and these are mostly related to growth performance, general indicators of health and condition and a few aspects of lipid metabolism (Jobling et al., 2002; Kenari et al., 2011; Kim et al., 2012; Tocher et al., 2003), with few reporting on the effects on VFI and the underlying mechanisms of peripheral and central appetite regulation (Figueiredo–Silva et al., 2012a; Morais et al., 2006). To approach this subject, we have chosen the marine flatfish Senegalese sole (*Solea senegalensis*) as a model organism. It is a commercially important aquaculture species in southern Europe, which in itself brings substantial value to any research towards the development of a sustainable feed (Morais et al., 2015a). Furthermore, contrary to many cultured marine finfish species, Senegalese sole is very sensitive to high dietary lipid inclusions (≥12%; Borges et al., 2009; Valente et al., 2011), but tolerant to even total FO substitution (at 9% dietary lipids)(Borges et al., 2014a), with unique capabilities of synthesizing docosahexaenoic (DHA) from eicosapentaenoic acid (EPA) via the Δ4 desaturation pathway (Morais et al., 2015b). With this in mind, Senegalese sole is a very interesting candidate to investigate how appetite–regulating mechanisms potentially sensitive to lipid level might function in varying dietary conditions. This study was set up to analyze the effects of diets differing in lipid level and oil source on the voluntary food intake of Senegalese sole juveniles during a 13 week culture period. At the end of the experiment, fish were fasted for 24 h and basal mRNA levels of key gastrointestinal and central appetite–regulating peptides, responding to the long–term feeding of the different diets, were evaluated. In addition, we assessed how subsequent re–feeding of the fish with their respective diets would affect transcript levels of the analyzed peptides.

2. *Materials and methods*

Experimental diets and fish culture

Four isoproteic extruded diets were formulated and manufactured by Sparos Lda. (Olhão, Portugal). They differed in total lipid level (~8% or ~18%) and FA composition, using FO or VO as the main lipid source. The 8FO and 18FO diets had 100% of the lipid supplied by FO, while 75% of the FO in diets 8VO and 18VO was replaced by a VO blend (rapeseed, soybean, and linseed oil in a ratio of 1:1:1; Table 1). Formulation and proximate analysis (n = 3) of the experimental diets are presented in Table 1 and FA composition (n = 3) in Table 2. The analyses were performed using standard methods, as described in Bonacic et al. (2015a) .

Table 1. Formulation and proximate composition of the experimental diets.

	8FO	8VO	18FO	18VO
Ingredients (%)				
Fishmeal 70 LT[1]	22.00	22.00	22.00	22.00
Fishmeal 60[2]	15.00	15.00	15.00	15.00
Fish protein hydrolisate[3]	5.00	5.00	5.00	5.00
Squid meal[4]	5.00	5.00	5.00	5.00
Pea protein concentrate[5]	4.00	4.00	4.00	4.00
Soy protein concentrate[6]	2.00	2.00	2.00	2.00
Soybean meal 48[7]	9.80	9.80	10.00	10.00
Wheat gluten[8]	7.00	7.00	10.10	10.10
Corn gluten meal[9]	5.00	5.00	4.50	4.50
Pea grits[10]	11.10	11.10	2.50	2.50
Wheat meal	9.00	9.00	4.80	4.80
Fish oil[11]	2.60	0.65	12.60	3.15
Rapeseed oil[12]	–	0.65	–	3.15
Soybean oil[12]	–	0.65	–	3.15
Linseed oil[12]	–	0.65	–	3.15
Vitamin & Mineral Premix[13]	1.00	1.00	1.00	1.00
Binder (guar gum)[14]	1.00	1.00	1.00	1.00
Proximate composition				
Moisture (%)	5.5	4.6	4.3	4.4
Crude Protein (% DM)	56.0	56.9	58.0	57.2
Crude Fat (% DM)	7.9	7.4	17.6	17.4
Ash (% DM)	10.5	10.7	10.4	10.3

[1] Peruvian fishmeal LT: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru.
[2] Fair Average Quality (FAQ) fishmeal: 62% CP, 12%CF, COFACO, Portugal.
[3] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[4] Super prime squid meal: 80% CP, 3.5% CF, Sopropêche, France.
[5] Lysamine GP: 78% CP, 8% CF, ROQUETTE, France.
[6] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[7] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal.
[8] VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.
[9] Corn gluten feed: 61% CP, 6% CF, COPAM, Portugal.
[10] Aquatex G2000: 24% CP, 0.4% CF, SOTEXPRO, France.
[11] COPPENS International, The Netherlands.
[12] Henry Lamotte Oils GmbH, Germany.
[13] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL–alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL–cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.
[14] Guar gum 101 HV– E412, Seah International, France.

Table 2. Fatty acid composition of the 4 experimental diets, expressed as % of total FA and µg/mg DW (in parenthesis); n = 3.

	8FO	8VO	18FO	18VO
14:0	2.1 (1.0)	1.5 (0.8)	3.8 (3.8)	1.4 (1.6)
15:0	0.3 (0.2)	0.3 (0.2)	0.4 (0.4)	0.2 (0.2)
16:0	16.5 (7.9)	14.5 (8.0)	17.2 (17.2)	11.8 (13.5)
18:0	4.2 (2.0)	4.1 (2.2)	4.6 (4.6)	3.9 (4.5)
Total saturated	23.1 (11.1)	20.4 (11.2)	26.1 (26.1)	17.3 (19.9)
16:1	5.0 (2.4)	4.0 (2.2)	5.5 (5.5)	3.4 (3.9)
18:1n–9	14 (6.7)	18.3 (10.0)	12.4 (12.4)	23.5 (27)
18:1n–7	2.9 (1.4)	2.7 (1.5)	3.0 (2.9)	3.1 (3.5)
20:1	5.2 (2.5)	4.8 (2.6)	3.9 (3.9)	2.7 (3.1)
Total monounsaturated	27.1 (13.0)	29.8 (16.3)	24.8 (24.8)	32.7 (37.5)
18:2n–6	12.1 (5.8)	17.4 (9.5)	6.7 (6.7)	20.4 (23.4)
18:3n–6	0.2 (0.1)	0.2 (0.1)	0.2 (0.2)	0.2 (0.2)
20:3n–6	0.1 (0.1)	0.1 (0.0)	0.1 (0.1)	0.1 (0.2)
20:4n–6	1.1 (0.5)	0.7 (0.4)	1.2 (1.3)	0.4 (0.5)
22:4n–6	0.1 (0.0)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)
22:5n–6	0.4 (0.2)	0.3 (0.2)	0.6 (0.6)	0.2 (0.2)
Total n–6 PUFA	14.0 (6.7)	18.7 (10.2)	8.9 (8.9)	21.3 (24.5)
18:3n–3	1.7 (0.8)	5.5 (3.0)	1.3 (1.3)	11.9 (13.7)
18:4n–3	1.9 (0.9)	1.5 (0.8)	2.3 (2.4)	1.1 (1.3)
20:4n–3	0.6 (0.3)	0.4 (0.2)	0.8 (0.8)	0.3 (0.3)
20:5n–3	15.5 (7.5)	11.1 (6.1)	18.3 (18.3)	7.5 (8.6)
21:5n–3	0.3 (0.1)	0.2 (0.1)	0.5 (0.5)	0.1 (0.2)
22:5n–3	1.1 (0.5)	0.8 (0.5)	1.5 (1.5)	0.6 (0.7.0)
22:6n–3	12.8 (6.2)	9.8 (5.4)	13.0 (13.1)	6.1 (7.0)
Total n–3 PUFA	34.0 (16.3)	29.3 (16)	37.8 (37.8)	27.6 (31.6)
Total PUFA	48.0 (23.1)	48.0 (26.3)	46.7 (46.7)	48.9 (56.1)
n–3/n–6 PUFA	2.4	1.6	4.2	1.3
DHA/EPA	0.8	0.9	0.7	0.8
EPA/ARA	14.2	16.3	14.6	17.5

Senegalese sole juveniles with an average initial body weight (IBW) of 5.0 ± 0.1 g were distributed into twelve rectangular flat bottom 20 l tanks (50 fish per tank) connected to a recirculation system at CCMAR, University of Faro, Portugal, and maintained at a temperature of 19.3 ± 1.2 ºC, a salinity of 32, and a 12 h light/12 h dark photoperiod for 13 weeks. They were fed 2 mm extruded diets using automatic feeders in several meals spread throughout 22 h of the day, in order to avoid possible interference of feed expectation, as has been observed in some central neuropeptides (Kehoe and Volkoff, 2007). Administered feed was recorded and adjusted daily to ensure the fish were fed to satiety. In the case of excess uneaten feed, rations were reduced by 10% and in the absence of uneaten feed increased by 10%. Fish were weighed (FBW) at the end of the experiment (n = 3 pools of 30 individuals) and specific growth rate, SGR = [(ln FBW (g) − ln IBW(g)) / t] × 100, where t is experimental period

(91 days); food conversion ratio, FCR = feed intake (g) / [FBW (g) – IBW (g)]; and voluntary feed intake, VFI = {(feed intake (g) × 100) / [(IBW (g) + FBW(g)) / 2]} / t (days), were calculated.

The study was conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

Sampling for molecular analysis

During week 13, fish were sampled for molecular analysis of putative gastrointestinal satiety–related peptides and central appetite–regulating neuropeptides. The expression of peripheral peptides was analyzed in pre– and post–prandial conditions. For this, samples of intestine (100–150 mg) were taken from two fish per tank (n = 6 per treatment) after a fasting period of 24 h, after which the fish were fed and similarly sampled 6 h after feeding. In the case of central neuropeptides, the experiment was setup to analyze the effects of feeding the diets in more detail. Samples of whole brain were collected from two fish per tank (n = 6 per treatment) at the end of a 24 h fasting period, and then again 1, 3 and 6 h after feeding (n = 6 per treatment at each time). In order to ensure that all fish ate an equal amount of feed at the exact appointed time, and considering the highly variable voluntary feed intake of sole (Dias et al., 2010), a tube–feeding method was used. Plastic capillaries were filled with 10 pellets each (~0.15% BW) and inserted into the back of the oral cavity of fish that were previously anesthetized with 50 ppm of tricaine methanesulfonate (MS222; Sigma, Sintra, Portugal). A piston was then used to push the contents of the tube into the esophagus and the fish were monitored for a few minutes before being returned to the tanks, in order to ensure that the pellets were not expelled. Tissue samples were collected into 1.5 ml of RNAlater stabilization buffer (Ambion, Life Technologies, Madrid, Spain) and kept in agitation at 4 °C for 24 h, and then stored at –80 ºC.

RNA extraction and RT–qPCR

For RNA extraction, samples were homogenized in 1 ml of TRIzol (Ambion) with 50 mg of 1 mm diameter zirconium glass beads in a Mini–Beadbeater (Biospec Products Inc., U.S.A.). Solvent extraction was performed following manufacturer’s instructions and RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain), respectively. Two micrograms of total RNA per sample were reverse transcribed into cDNA using the High–Capacity cDNA RT kit (Applied Biosystems, Life Technologies, U.S.A.), following manufacturer’s instructions, but using a mixture of random primers (1.5 µl as supplied) and anchored oligo–dT (0.5 µl at 400 ng/µl, Eurogentec, Cultek, S.L., Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. A pool was created with 2 µl of cDNA from all samples of each tissue, to later prepare standard curves with serially diluted cDNA, and samples were then diluted 60–fold with water.

Table 3. Primers used for real–time quantitative PCR (RT–qPCR). Shown are sequences and annealing temperature (Ta) of the primer pairs, size of the fragment produced, average reaction efficiency (E) and accession number of the target and reference genes.

Transcript	Primer sequence (5'–3')	Amplicon	Ta	E*	Reference
Peripheral					
pyya	AGCAGCACAGCAAGATCTCA	174 bp	60ºC	99.8%	KT626438[a]
	CCTTCCCACAGATTCTGCAT				
pyyb	CAACTTGTGCTGCATGGAAT	237 bp	60ºC	101.3%	KT626439[a]
	AGCAGCAAACATGAGACGTG				
glp1	CCTCACGGACCAGGCTATTA	209 bp	60ºC	104.4%	KT626440[a]
	TACTGTCCCCTGAGGGTTTG				
*cckl***	TTCTCCTGGTCCAAACCAAC	100 bp	60ºC	101.9%	KT626441[a]
	AGGGCAGACTCAGAGAACCA				
gal	CTTAACGGTTTCCCCATGC	101 bp	60ºC	100.5%	FF290428[a]
	CCAGAGTCGGAGTGTGAGTG				
Central					
cart1a	CGTCCACCACTGTCATTCTG	147 bp	60ºC	101.7%	KT189188[b]
	CTTTCTCCTCCTGCGTCTTG				
cart1b	TCGCTGAAAAGTCAACAAGAAA	99 bp	60ºC	100.3%	KT189189[b]
	GCCAAGCTTTTTCTCCAGTG				
cart2a	TCGCGTATCATCGAACACAT	86 bp	60ºC	100.3%	KT189190[b]
	GTGACACTGAGCCACAGCAC				
cart4	GTGAGCGAGAGCAGGAAACT	144 bp	60ºC	100.5%	KT189194[b]
	TCGTGGTGAAATAAGGCAAA				
pomca	AAGGCAAAGAGGCGTTGTAT	127 bp	60ºC	100.0%	FR851915[c]
	TTCTTGAACAGCGTGAGCAG				
npy	GAGGGATACCCGATGAAACC	129 bp	60ºC	106.4%	solea_v4.1_ unigene466117[d]
	GCTGGACCTCTTCCCATACC				
agrp2	CAGGTCAGACTCCGTGAGCCC	104 bp	64ºC	99.2%	solea_v4.1_ unigene32957[d]
	GTCGACACCGACAGGAGGCAC				
Reference genes					
ubq	AGCTGGCCCAGAAATATAACTGCGACA	93 bp	70ºC	100.3%	AB291588[e]
	ACTTCTTCTTGCGGCAGTTGACAGCAC				
rps4	GTGAAGAAGCTCCTTGTCGGCACCA	83 bp	70ºC	102,5%	AB291557[e]
	AGGGGGTCGGGGTAGCGGATG				
ef1a1	GATTGACCGTCGTTCTGGCAAGAAGC	142 bp	70ºC	100.9%	AB326302[e]
	GGCAAAGCGACCAAGGGGAGCAT				

pyy, peptide YY; *glp1*, glucagon–like peptide 1; *cckl*, cholecystokinin (Leu) precursor; *gal*, galanin; *cart*, cocaine– and amphetamine–related transcript; *pomc*, proopiomelanocortin; *npy*, neuropeptide Y; *agrp2*, agouti–related protein 2; *ubq*, ubiquitin; *rps4*, 40S ribosomal protein S4; *ef1a1*, elongation factor 1 alpha.
*Efficiency corresponds to the average of 4 runs for each tissue (for the reference genes and cckl it is 8 in total).
**Also analyzed in the brain
[a]Bonacic et al., unpublished results
[b]Bonacic et al. (2015b)

Primers for the anorexigenic and orexigenic peptides used in this study are presented in Table 3. These include two homologues of peptide yy – *pyya* and *pyyb* (formerly called *py*; Sundström et al., 2013), glucagon–like peptide 1 – *glp1,* cholecystokinin (Leu) precursor (similar to that described in rainbow trout *Oncorhynchus mykiss* by Jensen et al., 2001)) – *cckl,* 4 cocaine– and amphetamine–related transcript homologues (Bonacic et al., 2015b) – *cart1a*, *cart1b, cart2a* and *cart4*, proopiomelanocortin homologue a (Wunderink et al., 2012) – *pomca* , galanin – *gal*, neuropeptide y – *npy* and agouti–related protein 2 – *agrp2*. Amplifications were carried out in duplicate on a CFX96 Touch Real–Time PCR Detection System (Bio–Rad, Alcobendas, Spain) in a final volume of 20 µl containing 5 µl of diluted (1/60) cDNA, 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio–Rad) and 500 nM primers (except for *pomca*, and *cart2a* where 200 nM, and *agrp2* and *npy* where 120 nM were used). A systematic negative control (NTC – non–template control) was also included. The qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 35 cycles: 15 s at 95 °C, 1 min at the corresponding annealing temperature (Ta; Table 3). After the amplification phase, a melt curve was performed enabling confirmation of the amplification of a single product in each reaction. Non–occurrence of primer–dimer formation in the NTC was also confirmed. The amplification efficiency of each primer pair was assessed from serial dilutions of the cDNA pool.

The RT–qPCR results were imported into the software qBase+ (Biogazelle, Zwijnaarde, Belgium), where normalized relative quantities (NRQ) were calculated employing target and run–specific amplification efficiencies and using the geometric mean of 3 reference genes (elongation factor 1 alpha, ubiquitin and 40S ribosomal protein; *eef1a1, ubq* and *rps4,* respectively; Infante *et al.*). Interrun calibration was performed for brain samples, which were analyzed in 4 plates (one for each time point), by including three samples in triplicate in each plate for the purpose of calibration. The stability of the reference genes was evaluated using the geNorm algorithm incorporated in qBase+ (Vandesompele et al., 2002). The combination of the 3 reference genes in samples of intestine pre–/post–prandially gave M = 0.276/0.260, coefficient of variance – CV = 0.110/0–102, while only 2 of the genes (*ubq* and *rps4*) were used for normalization of brain results across all four intercalibrated plates, with M = 0.279 and CV = 0.097.

Statistical analysis

All statistical analyses were performed with SPSS v.20 (SPSS Inc., Chicago, IL, USA). A two–way ANOVA, with factors lipid source and lipid level, at a significance level of 0.05, was used to analyze fish performance and the RT–qPCR results of intestine, separately for the pre– and post–prandial times. A three–way ANOVA, with factors lipid source, lipid level and time, was used to analyze samples of brain, at a significance level of 0.05.

[c]Wunderink et al. (2012)
[d]Martín–Robles & Muñoz–Cueto, unpublished
[e]Infante et al. (2008)

3. *Results*

Fish performance and body composition

At the end of the 13–week experimental period, a four–fold size increase was recorded in juvenile Senegalese sole from all treatments (Table 4). Fish cultured on FO diets grew slightly more, although marginally non–significantly ($p = 0.06$ for final body weight and SGR), compared to those fed the VO treatments, while no differences were observed in relation to lipid level. The FCR did not differ between treatments, while VFI was significantly lower ($p = 0.04$) in fish fed high lipid diets. However, this was only due to differences observed between the 8FO and 18FO treatments, as fish fed the VO diets had equal VFI, independent of lipid level (Table 4).

Table 4. Growth and feeding performance of juvenile Senegalese sole cultured on 4 different diets during a period of 13 weeks. Values are expressed as mean ± SD. Results of 2–way ANOVA analysis in relation to dietary factors lipid level (LL), lipid source (LS) and their interaction (LL×LS) are also presented.

	Dietary treatment				P–value (2–way ANOVA)		
	8FO	8VO	18FO	18VO	LL	LS	LL×LS
IBW (g)	5.0 ± 0.07	5.0 ± 0.05	5.0 ± 0.70	4.9 ± 0.08	0.141	1.000	0.438
FBW (g)	23.4 ± 2.80	21.7 ± 1.16	23.7 ± 2.00	20.7 ± 0.95	0.721	0.058	0.555
SGR (%/day)	1.69 ± 0.14	1.60 ± 0.06	1.71 ± 0.09	1.57 ± 0.04	0.925	0.060	0.639
FCR	1.31 ± 0.12	1.30 ± 0.06	1.16 ± 0.10	1.30 ± 0.09	0.195	0.303	0.208
VFI (% BW/day)	1.88 ± 0.05	1.78 ± 0.01	1.65 ± 0.10	1.77 ± 0.12	0.037	0.851	0.068

IBW, initial body weight; FBW, final body weight; SGR, specific growth rate; FCR, food conversion ratio; VFI, voluntary feed intake

Gene expression in the intestine

Expression levels of putative enteroendocrine satiety peptides were measured by RT–qPCR in the intestine of Senegalese sole juveniles pre–prandially (24 h fasting; Fig. 1) and post–prandially (6 h after feeding; Fig. 2). Based on results of two–way ANOVA, pre–prandial mRNA levels of the anorexigenic peptides *pyya*, *pyyb* and *glp1* showed significant differences in response to lipid level, with fish fed 18% lipid diets showing a lower expression of these genes than those fed the 8% lipid diets. In the case of *cckl*, a similar trend (marginally non–significant; $p = 0.06$) was observed. Post–prandially, levels of *pyyb* were also significantly lower in fish fed the 18%, compared to 8% diets, while the remaining anorexigenic genes did not show any significant differences, in spite of a similar trend being noticeable. The orexigenic peptide *gal* was also significantly lower in fish fed higher lipid diets post–prandially. None of the analyzed genes showed a transcriptional response to the oil source, neither pre– nor post–prandially.

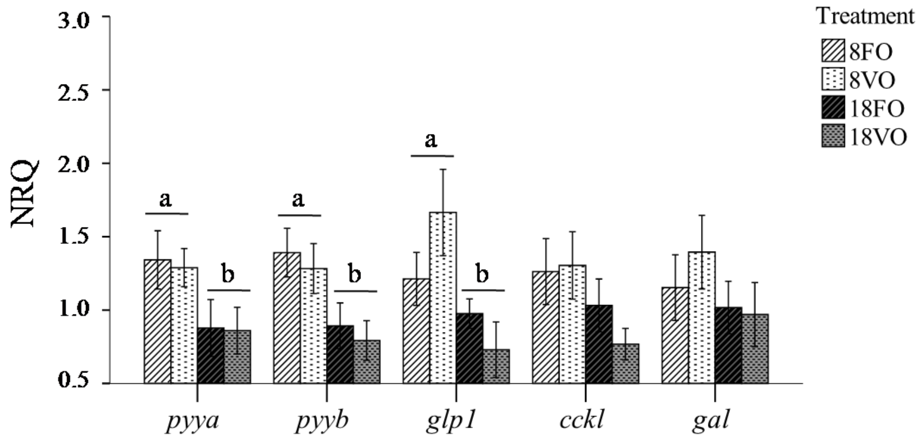


Figure 1. Pre–prandial mRNA levels of putative anorexigenic (*pyya*, *pyyb*, *glp1* and *cckl*) and orexigenic (*gal*) peptides in the intestine of Senegalese sole juveniles after a 24 h fasting period. Fish were fed one of 4 dietary treatments (8FO, 8VO, 18FO and 18VO) over a period of 13 weeks. Significant results of two–way ANOVA analysis in relation to lipid level are marked with letters. No significant differences were observed in lipid source. Values are calibrated normalized relative quantities (NRQ) ± SE (n = 6).

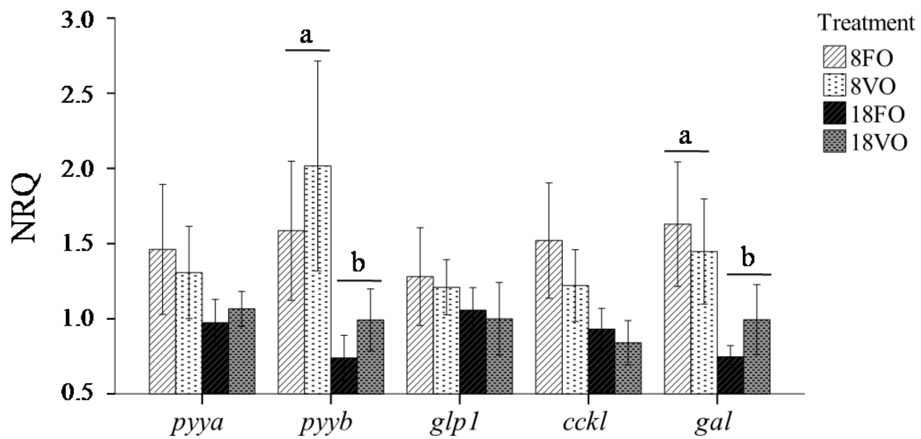


Figure 2. Post–prandial mRNA levels of putative anorexigenic (*pyya*, *pyyb*, *glp1* and *cckl*) and orexigenic (*gal*) peptides in the intestine of Senegalese sole juveniles 6 h following a single meal. Fish were fed one of 4 experimental diets (8FO, 8VO, 18FO or 18VO) over a period of 13 weeks, fasted for 24 h and then re–fed with their respective diet. Significant results of two–way ANOVA analysis in relation to lipid level are marked with letters. No significant differences were observed in lipid source. Values are calibrated normalized relative quantities (NRQ) ± SE (n = 6).

Gene expression in the brain

Transcript levels of neuropeptides putatively involved in appetite regulation were measured by RT–qPCR in the brain of Senegalese sole juveniles before and 1, 3 and 6 h after feeding (Fig. 3). Most showed significant differences in relation to the factor time (*cart1b*, *cart4*, *pomca*, *cckl*, *npy* and *agrp2*) and only a few (*pomca*, *cckl* and *agrp2*) were significantly affected by lipid level or by lipid source (*cart1a* and *cart1b*). Expression patterns of *cart1a* (Fig. 3a) and *cart1b* (Fig. 3b) were quite similar, and overall, both genes had significantly higher expression in fish fed the FO diets, with mRNA levels in the 18FO treatment peaking 1 h after feeding and in the 8FO treatment 3 h after feeding. Transcript levels of *cart1b* were also significantly lower at 1 h, compared to 6 h after feeding (with the exception of the 18FO treatment) and a significant interaction was observed between the factors time and lipid level. On the other hand, *cart4* expression (Fig. 3c) increased after feeding, reaching significantly higher values 3 h after the meal, and then dropped back to the pre–prandial level 6 h after feeding. Transcript levels of both *pomca* and *cckl* (Fig. 3e and Fig. 3f) were generally significantly higher in fish fed 8%, compared to 18%, lipid diets, mainly due to the 8FO treatment in the later post–prandial phase. Furthermore, the expression of *pomca* was significantly higher at 6 h, compared to 1 h after feeding. Transcript levels of *cckl* dropped significantly 1 h after feeding, and generally remained similar up to 3 h after the meal, after which (at 6 h after feeding) they increased back to pre–prandial values. Transcript levels of *npy* (Fig. 3g) were significantly lower 1 h, compared to 3 and 6 h after feeding, while in the case of *agrp2* (Fig. 3h) values were significantly lower at 1 h compared to 6 h post–prandially. However, in both cases the low values observed 1 h after feeding were mostly influenced by a drop in the 18VO treatment. Finally, diets containing lower lipid level seemed to significantly increase the expression of *agrp2,* although mainly due to values observed before and 6 h after feeding.

4. *Discussion*

Effect of lipid level and source on fish performance and voluntary food intake

In this study, a higher inclusion of dietary lipids (18%) did not seem to have a negative effect on juvenile performance, although lower dietary lipid levels (≤12%) have been established as optimal for Senegalese sole (Borges et al., 2013b; Borges et al., 2009; Campos et al., 2010; Dias et al., 2004; Mandrioli et al., 2012; Valente et al., 2011). On the other hand, a marginally non–significant trend in SGR suggested that in the current study fish fed FO diets grew slightly better than those fed diets containing 75% VO. We have previously hypothesized that eventual negative effects of FO substitution may be caused by the properties of the VO used, especially taking into account the ratio of linoleic acid (LNA; 18:2n–6) to α–linolenic acid (ALA; 18:3n–3) (Bonacic et al., 2015a). Accordingly, other studies showed that a lower growth performance was observed in Senegalese sole fed 12% lipid diets formulated exclusively with soybean oil (high in LNA), compared to FO or linseed oil–based (high in ALA) diets (Benítez–Dorta et al., 2013), or that no negative effects of 100% FO substitution with a VO blend

containing rapeseed, soybean, and linseed oil at a ratio of 3:2:5 (at 9% dietary lipid level) was observed (Borges et al., 2014a).

In the current study, a significant effect of lipid level was observed on VFI, which was reduced in sole fed higher lipid diets, but this result was exclusively caused by the 18FO treatment. Although energy–dense, lipid–rich diets have commonly been shown to reduce food intake in both adult and juvenile fish (Boujard et al., 2004b; Gélineau et al., 2002; Hevrøy et al., 2008; Murashita et al., 2008; Sæther and Jobling, 2001; Tocher, 2010), in the current study, the effects of dietary lipid level were apparently influenced by lipid source. Namely, equally high levels of VO in the diet did not seem to satiate the animals as effectively as FO. As a result of the interaction between these two dietary factors, the diets had distinct profiles in terms of absolute FA quantities (µg/mg DW). This led to 18FO having around 2–3 times more EPA and DHA than the remaining treatments, which could relate to the low VFI of sole on this diet. Several studies have shown that fish do not always regulate their food intake based on dietary digestible energy content as is widely accepted (Figueiredo–Silva et al., 2012a; Geurden et al., 2006; Helland and Grisdale–Helland, 1998; Peres and Oliva–Teles, 1999a; Saravanan et al., 2012). In mammals, it has been suggested that these processes are governed by complex appetite regulating mechanisms based, at least partly, on specific secretory responses generated by ingested macronutrients (Karhunen et al., 2008; Raben et al., 2003), with some findings in fish supporting this hypothesis (Cho et al., 2005; Narnaware and Peter, 2002; Peres and Oliva–Teles, 1999a, b). In a study by Guerreiro et al. (2012), Senegalese sole seemed to adjust its intake to dietary protein more than to energy content, when fed diets of varying total protein and lipid levels. However, in other studies, no changes in food intake of Senegalese sole juveniles were observed when dietary lipids were increased (from 11% to 21%) at the expense of carbohydrates (Dias et al., 2004), while food intake even increased proportionately with dietary lipid level (4% to 20%, in 4% increments) at the expense of carbohydrates (Borges et al., 2009). On the other hand, common sole (*Solea solea*) showed a reduction in food intake in proportion to increasing lipid level (8% to 20%, in 4% increments) at the expense of carbohydrates (Bonvini et al., 2015). Although variable, these results indicate that Senegalese sole do not simply adjust their VFI to dietary energy density. However, these studies also do not enable verifying a possible regulation by specific dietary nutrients, e.g. by FA composition, as suggested in the current study.

In a study by Morais et al. (2006), one of the few looking at the combined effects of dietary lipid level and source on appetite, gilthead seabream (*Sparus aurata*) larvae conversely ingested more low– than high–lipid diets when the dietary lipid fraction was from soybean oil, but this was reversed when FO was used (Morais et al., 2006). However, care should be taken when considering these results, as early ontogenic stages generally have different FA requirements than juvenile fish and may also have less developed appetite control mechanisms (Gomes et al., 2015). On the other hand, in a study with rainbow trout juveniles, Figueiredo–Silva et al. (2012) did not observe any variation in food intake between fish fed diets differing in lipid level (5% and 15%) and source (FO and coconut oil—high in SFA). However, in another study, rainbow trout showed a preference towards FO, rapeseed oil or a blend of FO and olive oil (OO), compared to linseed oil (LSO), in a demand feeding system (Geurden et al., 2007). Although it is uncertain whether the lower demand for LSO was a result of satiating feedback mechanisms of the ingested feed or of its sensory properties, this study showed

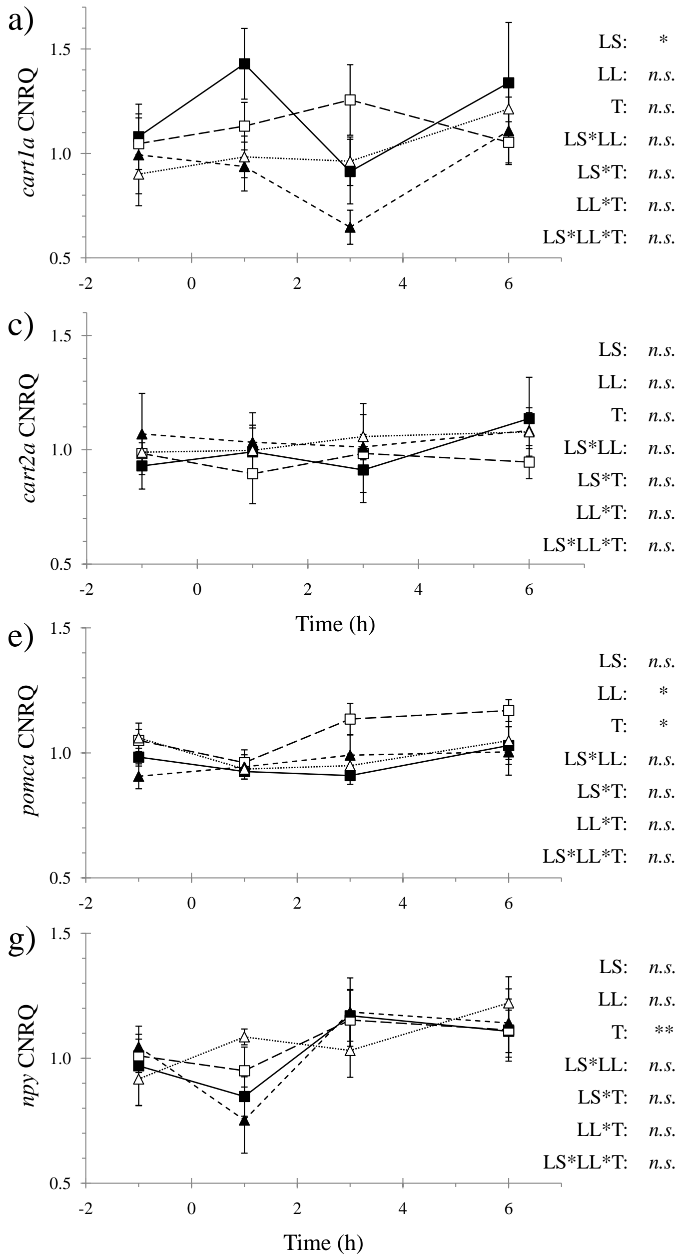
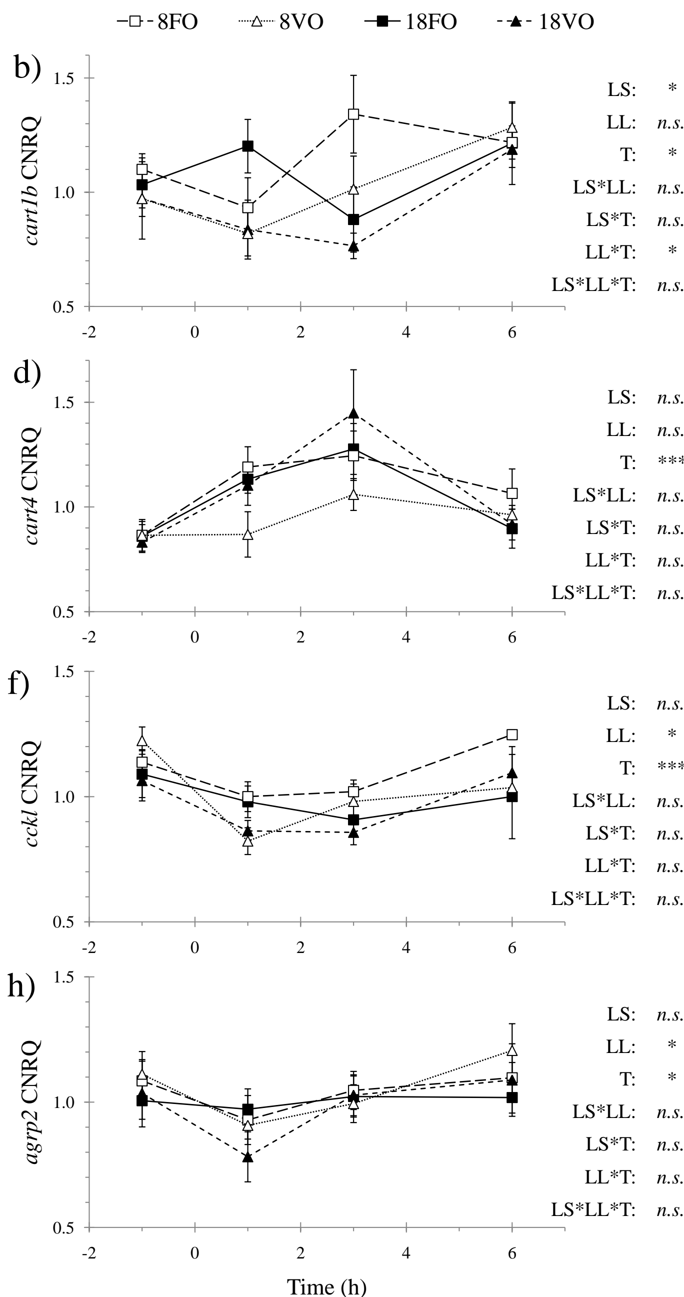


Figure 3. Expression of the putative anorexigenic *cart1a* (a), *cart1b* (b), *cart2a* (c), *cart4* (d), *pomca* (e), *cckl* (f) and of the orexigenic *npy* (g) and *agrp2* (h) genes in the brain of Senegalese sole juveniles, in response to time of feeding and diet. Samples were taken just before the usual feeding time (after 24 h fasting), then fed for one hour (from –1 to 0 h) on their respective dietary treatment (8FO, 8VO, 18FO or 18VO) and sampled 1, 3 and 6 h after the end of the meal.



Results of 3–way ANOVA analysis are presented beside each chart, where *n.s.* is not significant, * is p<0.05, ** is p<0.01 and *** p<0.001. Values are calibrated normalized relative quantities (CNRQ) ± SE (n = 6).

that appetitive behaviour in trout can be mediated by dietary oil source. In the context of the current study, Senegalese sole juveniles were tube–fed when assessing the expression of appetite–related genes, thus bypassing all sensory stimuli prior to feed entering the esophagus. Nevertheless, we cannot rule out possible effects of the diet's olfactory or gustatory properties on the VFI results, which were calculated from measurements performed in normal feeding conditions over the 3–month experimental period. Finally, studies on FA sensing mechanisms in rainbow trout reported different effects of circulating medium–chain (octanoic acid) and long–chain (oleic acid; OA) FA on the expression of hypothalamic appetite–regulating peptides and appetite (Soengas, 2014), while variations in dietary lipid level did not seem to have an effect (Librán–Pérez et al., 2015a).

In order to further investigate the effects and interaction of dietary lipid level and source on food intake in Senegalese sole juveniles, the following sections discuss aspects related to the gut–brain satiety signaling axis, which is considered crucial for appetite regulation (Cummings and Overduin, 2007). For this purpose, the mRNA expression of key gastrointestinal genes and central neuropeptides was quantified and analyzed in relation to the dietary factors and time of feeding.

Effects of dietary lipid level and source on satiety signals in the intestine

Studies in fish investigating effects of dietary FA composition on peripheral satiety factors have mainly focused on the peptides ghrelin and leptin (Francis et al., 2014; Ganga et al., 2005; Varricchio et al., 2012). Results showed that post–prandial plasma levels of these peptides in rainbow trout (Varricchio et al., 2012) and gilthead seabream (Ganga et al., 2005) changed when fish were fed diets differing in FA composition. Other studies, with Atlantic salmon (*Salmo salar*) and yellowtail (*Seriola quinqueradiata*), linked dietary macronutrient composition to the activity of the enteroendocrine peptides Cck and Pyy (Hevrøy et al., 2008; Murashita et al., 2008). However, to our knowledge, expression of the five intestinal satiety peptides *pyya*, *pyyb*, *glp1*, *cckl* and *gal*, analyzed in this study, has not been previously investigated in fish in relation to dietary lipid level and source. Results showed that *pyya*, *pyyb* and *glp1* mRNA levels were elevated in fish fed low–lipid diets (8%) in basal conditions (after a period of fasting). At 6 h after feeding this trend was possibly continued, but with more variability in the values and significant differences only visible in *pyyb* and *gal* (both higher in the 8% diets). On the other hand, no differences were observed in relation to lipid source either before or after feeding.

The expression of *cckl* was not significantly affected by lipid level, either pre– or post–prandially, which was somewhat unexpected, as in other fish species it has been observed to increase in response to nutrients entering the intestine, especially in the case of high lipid diets (Aldman and Holmgren, 1995; Murashita et al., 2008; Peyon et al., 1999). In yellowtail, *cck* mRNA levels increased immediately following a meal but returned to basal levels within 6 h after feeding (Murashita et al., 2007). There is a realistic possibility that this may have also happened in the current study, signifying that an eventual increase in *cckl* expression after feeding could have been missed. The other analyzed peptides, however, have generally been characterized as having a slower reaction time (Murashita et al., 2007; Sundby et al., 1991) and were more likely to have been affected by food that was very probably still present in the intestine. This is

supported by the fact that more than 75% of the total feed digesta was still located in the anterior and mid intestine 6 h after Senegalese sole juveniles were fed a diet containing 55% protein and 13% lipids (Dias et al., 2010).

Galanin (*gal*) was the only gastrointestinal gene that responded as expected in the current study (high mRNA levels in low–lipid diets), which correlated well with a previously described orexigenic function of this peptide (Nelson and Sheridan, 2006; Volkoff and Peter, 2001b). Conversely, the levels of p*yya*, *pyyb* and *glp*–1 and post–prandial levels of *pyyb* were significantly higher in fish fed low lipid diets, which was quite puzzling, considering how these peptides are believed to have an anorexigenic effect in several fish species (Gonzalez and Unniappan, 2010; Polakof et al., 2011a; Rubio et al., 2008; Silverstein et al., 2001; Sundby et al., 1991). In the case of Pyy, however, contradicting results have been obtained in different species. Similarly to mammals, an anorexigenic function has been implied for this peptide in goldfish (*Carassius auratus*), in which intracerebroventricular and intraperitoneal injections caused reductions in food intake (Gonzalez and Unniappan, 2010). On the other hand, Atlantic salmon showed no differences in *pyy* expression after 6 days of starvation or re–feeding (Murashita et al., 2009b). Furthermore, Murashita et al. (2007) reported that *pyyb* mRNA levels decreased in yellowtail after being fed a commercial diet, and remained low for more than 12 h, until returning to basal levels by 24 h after feeding. These authors also observed a post–prandial decrease in *pyyb* levels in yellowtail after oral administration of casein, oleic acid and triolein, but not starch, which further suggests different nutrient–related regulating mechanisms in this species (Murashita et al., 2008). These results could indicate an alternative orexigenic function for this peptide in yellowtail and highlight the complexity and lack of knowledge on the possible roles of Pyy in fish, as well as on other appetite–related peptides, and how they might be affected by dietary composition.

One of the possible mechanisms regulating gastrointestinal peptide secretion is through intestinal FA sensing, which has been relatively well established in mammals (Steinert and Beglinger, 2011), but hardly investigated in fish. The main FA sensors involved in these processes include fatty acid translocase/cluster of differentiation 36 (FAT/CD36) and several G protein–coupled receptors (GRP40, GRP43, GPR119, GRP120) (Guijarro et al., 2010; Khan and Besnard, 2009; Laugerette et al., 2005; Piomelli, 2013; Schwartz et al., 2008; Yonezawa et al., 2013), which differ in their specificity for types of FA, FA derivatives such as oleoylethanolamide (OEA) and for oxidized FA (Little and Feinle–Bisset, 2010; Miyauchi et al., 2010; Vangaveti et al., 2010; Yonezawa et al., 2013), and are associated to the release of specific satiety factors, including PYY, CCK and GLP–1 (Yonezawa et al., 2013). Expression of *cd36* was previously reported in Senegalese sole juveniles from this experiment (Bonacic et al., 2015a), where it was down–regulated in fish fed high lipid diets (pre– and post–prandially) and additionally up–regulated in the FO treatments post–prandially. Interestingly, this expression pattern coincided with most satiety peptides analyzed in the current study, in response to dietary lipid level, although this was not the case in relation to oil source. In mammals, the action of CD36 has been associated with the inhibition of food intake (most notably via GLP–1) through stimulation by OEA (Diep et al., 2011; Fu et al., 2003; Gaetani et al., 2003; Guijarro et al., 2010; Oveisi et al., 2004; Piomelli, 2013; Schwartz et al., 2008), but there are hardly any studies on CD36 in the context of intestinal FA sensing in fish. However, Tinoco et al. (2014) recently indicated the

involvement of OEA in the regulation of feeding, swimming and lipid metabolism of goldfish and suggested a conservation of the role of OEA in vertebrates. Thus, the expression of genes coding for gastrointestinal satiety peptides (especially *glp1*) may have been related to that of *cd36* reported in Bonacic et al. (2015a). Furthermore, secretion of GLP–1 in mice was also stimulated by LNA, ALA, DHA, EPA and palmitoleic acid (16:1n–7), but not by OA, stearic or octanoic acid (Adachi et al., 2006; Morishita et al., 2008), via activation of intestinal GRP120, leading to reduced appetite (Adachi et al., 2006; Hirasawa et al., 2005). Although these results support a sensitivity of the GLP–1 production mechanism to dietary PUFA composition in mammals, it seems that both C_{18} PUFA and LC–PUFA elicited similar responses, which could similarly explain the lack of differences between fish fed different lipid sources in the current study.

In sum, the reason for the higher expression of some of the assessed gut peptides when sole were fed lower lipid diets remains elusive, although a possible involvement of gastrointestinal FA sensing mechanisms could be suggested. However, this remains very speculative at present and future studies should be performed to elucidate these mechanisms in fish and how they might respond to diet composition. On the other hand, there was also a lack of correlation between the expression of the gastrointestinal peptides assessed in this study and VFI, which showed a particularly marked decrease in the 18FO treatment. This may suggests the activity of other regulatory mechanisms affecting food intake that do not involve the direct action of these gastrointestinal peptides.

Effects of dietary lipid level and source on the expression of appetite–regulating peptides in the brain

Besides receiving neural and humoral signals originating from the gastrointestinal tract, the brain, more specifically the hypothalamus, has the capacity to sense free circulating FA. This enables it to continuously assess the energy status of the body, along with post–prandial variations caused by ingested food (Soengas, 2014). There are several processes through which circulating FAs can signal the brain (reviewed in detail in Soengas et al., 2014) and the activation of these systems can cause the inhibition of the orexigenic factors AgRP and NPY, and the enhancement of the anorexigenic factors POMC and CART, ultimately leading to a decreased food intake. In the present study we have not looked into the different hypothalamic FA sensing systems, since these have been recently investigated in detail in Senegalese sole (Conde–Sieira et al., 2015), and shown to operate similarly as in trout and mammals (Blouet and Schwartz, 2010; López et al., 2007; Soengas, 2014), and also to have different sensitivities to FA differing in chain length and degree of saturation (Conde–Sieira et al., 2015). In the current study, we report on the expression of the main putatively anorexigenic (*cart1a*, *cart1b*, *cart2a*, *cart4*, *pomca* and *cckl*) and orexigenic (*npy* and *agrp2*) neuropeptides, whose expression could reflect central FA sensing processes. These peptides interact in elaborate positive or negative feedback loops (Dhillo et al., 2002; Lau and Herzog, 2014; Vicentic and Jones, 2007), which often complicates the interpretation of results in nutritional experiments, such as the one in the current study. The analyzed neuropeptides were affected differently by diet composition (lipid level and source), as well as in response to feeding (time series including several post–prandial time points). However, no

differences were observed pre–prandially between treatments, which suggests that dietary lipid level and oil source did not have a long–term effect on basal neuropeptide mRNA levels, contrary to what was observed with gastrointestinal peptides. Thus, changes in expression during the post–prandial phase were likely a direct result of ingested feed (short–term effects).

Of all the analyzed peripheral and central genes, dietary lipid source only affected the expression of the anorexigenic neuropeptides *cart1a* and *cart1b*, with both having increased expression in sole juveniles fed the FO diets. In addition, both genes had a similar expression pattern, with mRNA levels in the 18FO and 8FO treatments peaking 1 h and 3 h after feeding, respectively. This indicates that activation of anorexigenic pathways may have occurred at different times during the post–prandial period in the FO treatments; earlier when FO was present at higher level in the diet. This result was unexpected given previous results on the hypothalamic FA sensing responsiveness of Senegalese sole, where OA and ALA where found to enhance the sole's anorexigenic capacity, while stearic acid and EPA did not appear to have an effect (Conde–Sieira et al., 2015). Therefore, considering how the 18VO diets had by far the highest OA and ALA contents, we would expect this diet to have the highest satiating effect. However, the results might not be directly comparable due to important differences in the experimental designs; while in the previous study results were assessed following intraperitoneal injections of free FA, in the present experiment there is much higher complexity resulting from the fact that we tested the ingestion of a complete diet, containing macronutrients at different levels, that can interact and affect lipid metabolism and energy balance, including the metabolites that eventually get into circulation and are sensed by the brain. On the other hand, mammalian studies have clearly implicated CART as a mediator of metabolic regulation, responding to changes in the metabolic state of the animals, through mechanisms involving, for instance, interaction with leptin (Vicentic and Jones, 2007). In this respect, hypothalamic CART mRNA levels have been found greatly increased in rats fed a high energy diet, which was positively correlated with a reduction in lipid storage, enhanced lipid mobilization (stimulation of catabolic pathways) and increased circulating leptin levels (Wortley et al., 2004). However, a similar association was not found in the present study, since no significant differences were observed in the expression of the two *cart1* genes with respect to dietary lipid level, although basal levels of hepatic β–oxidation (estimated by *cpt1* expression) in Senegalese sole juveniles was increased in fish fed high lipid diets (Bonacic et al., 2015a). While a marginally non-significant post–prandial increase in *cpt1* expression was also measured in the FO treatments in animals from this experiment (Bonacic et al., 2015a), these results more likely reflected short term effects associated to ingestion of the feed rather than chronic effects of diet on energy balance mediated by lipostatic signals. Therefore, although the early (i.e., before new feed becoming available and the onset of the following meal) potential activation of anorexigenic pathways through cart1a and cart1b up-regulation could at least partly explain the lower VFI of the 18FO diet, we cannot presently speculate on the nature of these mechanisms, and further studies should be performed in the future to confirm this hypothesis.

In the case of *cart4*, a fairly consistent increase in expression in all treatments except 8VO was observed in the post–prandial phase, peaking 3 h after feeding, after which it returned to basal levels, which fits well with the post–prandial expression pattern previously described in sole juveniles fed a diet equivalent to the 8FO diet in this

experiment (Bonacic et al., 2015b). In light of these and previous results, we can hypothesize that *cart4* may be regulated by feeding through processes that are not affected by dietary composition. In addition, the expression of *cart* homologues observed in this study generally seemed to agree with the putative anorexigenic effect of these peptides established in other fish species (Kehoe and Volkoff, 2007; Kobayashi et al., 2008; Murashita et al., 2009a; Nishio et al., 2012; Peterson et al., 2012; Subhedar et al., 2011; Valen et al., 2011; Volkoff et al., 2005; Volkoff and Peter, 2001a). However, individual homologues in this study behaved differently, and not all (e.g., *cart2a*) were affected by fasting or feeding, similar to what has previously been reported for Senegalese sole (Bonacic et al., 2015b) and other teleost species (MacDonald and Volkoff, 2009; Murashita and Kurokawa, 2011; Volkoff and Peter, 2001b) where more than one *cart* homologue was identified. It is important to note that besides its role in the regulation of feeding, CART is a key neurotransmitter and hormone involved in the regulation of many physiological processes in mammals, and one of its best documented actions is in the regulation of energy expenditure (Vicentic and Jones, 2007). However, much less is known regarding the physiological roles of CART in teleosts, although they are generally believed to be conserved across vertebrates (Bonacic et al., 2015b). Moreover, the results reported here further advance knowledge on *cart* genes in fish by demonstrating that some *cart* homologues (such as cart1) can also be differently regulated in response to dietary lipid composition (particularly oil source, and hence FA composition).

The co–expression of *Cart* and *Pomc* has been well established in hypothalamic neurons of mammals, where they act together as potent anorexigenic factors (Dhillo et al., 2002; Elias et al., 1998). Both genes were assessed in the current study but the expression pattern of *pomca* was not similar to any of the *cart* homologues that were analyzed. Two *pomc* homologues have previously been identified in Senegalese sole, with *pomca* giving rise to adrenocorticotropic hormone (Acth) and the other, *pomcb*, to α– or β–melanocyte–stimulating hormone (Msh) (Wunderink et al., 2012). Although both transcripts were analyzed and Msh peptides are considered more interesting in the context of appetite regulation, *pomcb* showed extremely high individual variations (up to 1000–fold differences in some samples) in the current study, unrelated to dietary treatment or feeding (hence not shown here). This was somewhat puzzling as *pomcb* was presumably the homologue more likely to respond to changes in diet, while *pomca* is possibly more involved in stress–response reactions through Acth (Wunderink et al., 2012). A significant effect of lipid level was observed in the expression of *pomca*, although this was exclusively due to the higher mRNA levels observed in the 8FO treatment at 3 h and 6 h after feeding, compared to the remaining treatments. Furthermore, a significant effect of time was also found, attributable to the overall lowest expression levels at 1 h, compared to 6 h after feeding. These changes were very subtle and do not agree with the putative anorexigenic activity of *pomc* peptides, established in numerous fish species (Cerdá–Reverter et al., 2003b; Murashita et al., 2011; Song et al., 2003; Valen et al., 2011), which could mean that their expression in the present study might have been affected by other, undetermined, physiological processes.

Apart from the gastrointestinal tract, *cck* is also expressed in the brain of teleosts, where it has been demonstrated to have an anorexigenic role in appetite regulation (Gélineau and Boujard, 2001; Jensen et al., 2001; Volkoff et al., 2003). In the current

study, central expression of *cckl* generally showed a decline 1 hour after feeding and a slow increase back to basal levels 6 h after the meal, independently of the dietary treatment. This pattern of expression was not consistent with previous results in goldfish (Peyon et al., 1999), Atlantic salmon (Valen et al., 2011) and channel catfish (Peterson et al., 2012), where *cck* expression increased post–prandially within 2 h (in the hypothalamus), 0.5 h (in the whole brain) and 1 h (in the whole brain), respectively, and was lower in fasted, compared to fed goldfish (Volkoff et al., 2003), which is more in line with its role as an anorexigenic factor. Although we cannot offer an explanation for these results at present, significant differences were found in *cckl* expression in the brain with relation to dietary lipid level, mostly caused by higher transcript levels in the diets containing 8% lipids before and 3 h after feeding, which was consistent with trends observed in the expression of this peptide in the intestine.

The neuropeptides *npy* and *agrp* have been established in numerous fish species as potent orexigenic factors (Cerdá–Reverter et al., 2003a; Doyon et al., 2003; Kehoe and Volkoff, 2007; Narnaware and Peter, 2002; Peterson et al., 2012; Song et al., 2003; Valen et al., 2011; Volkoff, 2006; Volkoff et al., 2005). In mammals and birds, both transcripts are co–localized in the same neurons within the ARC (Boswell et al., 2002; Broberger et al., 1998; Hahn et al., 1998). However, in fish, localization has been somewhat variable, with data on central expression of *npy* being inconsistent (Cerdá–Reverter et al., 2000; Narnaware and Peter, 2002; Peng et al., 1994; Pickavance et al., 1992; Vecino et al., 1994). In the current study, in accordance with their orexigenic role, transcript levels of both genes generally decreased (with a few exceptions, such as 8VO in the case of *npy* and 18FO in the case of *agrp2*) at the beginning of the post–prandial phase, but quickly recovered 3 h after feeding. The observed changes were fairly subtle and might suggest a relatively short–term lowered appetite after ingestion of a single meal. In other species like goldfish (Narnaware and Peter, 2001) and catfish (Peterson et al., 2012) the decline in *npy* mRNA levels in re–fed compared to fasted fish was more pronounced and lasted for over 2 h and 4 h, respectively. On the other hand, a study with Atlantic salmon showed a completely opposite pattern, with a slight stagnation in *npy* expression just after feeding, which then increased rapidly at 3 h and returned to initial levels by 6 h in the post–prandial phase, with higher levels being found at all post–prandial times compared to unfed fish (Valen et al., 2011). An interesting observation in the current study was that the decrease in *npy* expression, shortly after feeding, appeared slightly (non–significantly) more pronounced in the diets containing 18% lipids, particularly 18VO, which could suggest higher satiation properties of high lipid diets. However, the differences were only very subtle and did not correlate with the highest reduction in VFI observed in the 18FO treatment, and therefore future studies should be performed to confirm this hypothesis. Additionally, *npy* seems to be strongly regulated by food expectancy, and exhibited periprandial expression peaks in studies with channel catfish (Peterson et al., 2012), Atlantic cod (*Gadus morhua*) (Kehoe and Volkoff, 2007) and Atlantic salmon (Murashita et al., 2009a). However, this should not have interfered with the results in the present study, as the feed was distributed during 22 h throughout the day.

Two *agrp* homologues have recently been described in teleosts (Murashita et al., 2009a), while in tetrapods one of them seems to have been lost in evolution (Braasch and Postlethwait, 2011). These homologues, along with agouti–signaling proteins, are endogenous antagonists of melanocortin receptors and compete with Msh and Acth for

receptor binding sites (Agulleiro et al., 2014; Sánchez et al., 2009). However, they differ in tissue distribution and bind to specific receptors; *agrp1* is expressed in the tuberal hypothalamus (homologous to the mammalian ARC, where most appetite–related activity is centered), but also in several peripheral tissues, while *agrp2* has been observed in the telencephalon (pineal gland), tectum mesencephali, pituary and also in several peripheral organs, with some differences in individual species (Agulleiro et al., 2014; Cerdá–Reverter and Peter, 2003; Kurokawa et al., 2006; Murashita et al., 2009a; Song et al., 2003; Wan et al., 2012; Zhang et al., 2010). Thus, it is generally considered that hypothalamic expression of *agrp1* regulates energy homeostasis (Cerdá–Reverter and Peter, 2003; Song et al., 2003; Zhang et al., 2010), whereas the pineal expression of *agrp2* is involved in pigment back– ground adaptation (Zhang et al., 2010). Unfortunately, a full characterization of *agrp* has not yet been performed in Senegalese sole and the necessary molecular tools to analyze *agrp1* expression were not available for this study, so we could not evaluate potential differences between the homologues. In other fish species, such as European seabass (*Dicentrarchus labrax*), progressive fasting caused an increase in *agrp1* (after 8 days) and decrease in *agrp2* (after 15 days) mRNA levels in the brain (Agulleiro et al., 2014). Furthermore, in common carp, a decrease in *agrp2* levels was observed after 1 day of fasting, followed by an increase after re–feeding (Wan et al., 2012), while in Atlantic salmon a decrease was observed in *agrp1* expression, but *agrp2* did not change even after 6 days of fasting (Murashita et al., 2009a). Although somewhat varied, results of these studies generally show that fasting causes a decline in *agrp2* levels in some teleost species, contrary to what was observed in the current study, where a slight decrease in *agrp2* mRNA levels was observed only after re–feeding. This may signify a possible orexigenic effect of this homologue in Senegalese sole, instead of a function in pigment back–ground adaptation as suggested by Zhang et al. (2010). On the other hand, a significant effect of lipid level was also observed in the present study, mostly due to lower levels of *agrp2* in fish fed high–lipid diets in basal (fasting) conditions and 6 h after feeding. While this might indicate a regulatory mechanism to control dietary energy intake, these hypotheses remain very speculative, and physiological studies on both homologues are necessary to ascertain their biological roles in Senegalese sole.

5. *Conclusion*

In the present study, Senegalese sole juveniles decreased their food intake at higher lipid levels, but only when fish were fed the 18FO diet, which may indicate that FO had a higher satiating effect than corresponding amounts of VO. Therefore, high dietary contents of LC–PUFA, characteristic of the 18FO diet, may have had specific effects on the regulation of appetite in this species. These actions were likely exerted through mechanisms that do not involve the secretion of the peripheral gastrointestinal peptides or central neuropeptides examined in the present study, since no obvious relationship was found between the expression of these genes and food intake in sole. The only exception was in the expression of *cart1a* and *cart1b*, which was significantly affected by dietary lipid source. In both cases the transcripts showed an increase in expression in the FO treatments after feeding, in line with their putative anorexigenic role.

Furthermore, mRNA levels for *cart1a* and *cart1b* showed an earlier peak (at 1 h post–feeding) in the 18FO treatment, compared to 8FO (at 3 h after feeding), which might suggest that the activation of anorexigenic pathways could occur earlier when FO is present at higher level in the diet. On the other hand, the long term feeding of high lipid diets tended to reduce the basal (pre–prandial) expression of putatively anorexigenic enteroendocrine peptides (significantly for *pyya*, *pyyb*, *glp1*), and the expression of *pyyb* and of the orexigenic *gal* were similarly affected 6 h after feeding. Conversely, the basal expression of neuropeptides was not modulated by any of the tested dietary factors, suggesting no long–term adaptation to lipid level or source. Finally, most of the analyzed neuropeptides (*cart1b*, *cart4*, *pomca*, *cckl*, *npy*, *agrp2*) appeared to transcriptionally respond to feeding and showed temporal changes in expression during the post–prandial phase. This study provides new information on several key players of appetite–regulating pathways and how they are affected by the quantity and quality of dietary lipids in Senegalese sole. However, more work clearly needs to be done to fully understand these complicated regulatory mechanisms in fish.

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CHAPTER 7

Dietary fatty acid composition affects food intake and gut–brain satiety signaling in Senegalese sole (*Solea senegalensis*, Kaup 1858) larvae and post–larvae

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Abstract

Little is known how dietary lipids affect food intake during larval development of fish, especially with regard to fatty acid (FA) composition. In fact, very little work has been done on appetite regulation and food intake in fish larvae in general, due to biological and technical difficulties associated with this type of studies. A new method using fluorescent microspheres as markers was developed in this study to evaluate food intake and prey selectivity of Senegalese sole larvae and post–larvae. Food intake was quantified in fish fed *Artemia* metanauplii enriched with oils differing in FA profile: cod liver oil (CLO), linseed oil (LSO), soybean oil (SBO) or olive oil (OO). The fish did not preferentially ingest a specific diet when presented with a choice. However, pre–metamorphic larvae from the CLO treatment ingested more metanauplii per g body weight, while differences in post–larvae were not significant. These findings were developed further by analyzing mRNA levels of a range of putative anorexigenic (*pyya, pyyb, glp1, cckl, cart1a, cart1b, cart2a, cart4, pomca, pomcb, crf*) and orexigenic (*gal*, *npy*, *agrp2*) genes, to identify those which are significantly affected by feeding and/or dietary FA composition. The variety of expression patterns observed highlighted the complexity of appetite regulatory mechanisms. In general, fish fed the CLO diet tended to show gene expression patterns most dissimilar to the remaining treatments. Expression in pre–metamorphic larvae was generally less in accordance with the putative function of the peptides than in post–larvae, which could suggest a yet underdeveloped regulatory system.

Keywords: appetite, selectivity, neuropeptide, gastrointestinal peptide, live feed, fluorescent microspheres, flow cytometry

1. *Introduction*

Appetite and food intake are factors that greatly impact larval growth and development, as they determine the amount of nutrients available to larvae for the high structural and energy demands of rapid growth and organogenesis (Hamre et al., 2013). However, due to biological and technical constraints, particularly in terms of accurately determining food intake, studies in larvae are difficult to perform. This has resulted in a lack of knowledge concerning the mechanisms governing appetite and food intake in these early life stages (Rønnestad et al., 2013). Regulation of appetite and food intake involves a complex set of mechanisms that integrate external (sensory cues from the environment) and internal signals (endocrine and neuronal signals from peripheral organs) with the circadian and homeostatic control systems in the central nervous system (CNS), mainly the hypothalamus (Arora and Anubhuti, 2006; Rønnestad et al., 2013). The CNS transmits neuroendocrine factors that either inhibit or stimulate food intake (Kulczykowska and Sánchez Vázquez, 2010). Satiety signals can therefore be divided by place of synthesis into peripheral and central, and by function into anorexigenic (appetite–suppressing) and orexigenic (appetite–stimulating; Volkoff et al., 2005).

Dietary lipids have been recognized as one of the most important nutritional factors affecting larval growth and survival (Tocher, 2010), but their effects on food intake have hardly been studied in larvae. A previous study by Morais *et al*. (2006) suggested that dietary fatty acid (FA) composition might affect food intake in gilthead sea bream (*Sparus aurata*) larvae, by modulating the effects of dietary total lipid levels. On the other hand, energy–dense, lipid–rich diets have commonly been shown to reduce food intake in both adult and juvenile fish (Boujard et al., 2004b; Gélineau et al., 2002; Sæther and Jobling, 2001; Tocher, 2010), but less is known regarding the effects of dietary nutrients beyond their energy value (Hevrøy et al., 2008; Murashita et al., 2008). Mammalian studies have established that macronutrients can also affect the secretion of gut peptides in different ways, independent of energy densities, which suggests the existence of complex mechanisms that generate specific secretory responses potentially affecting appetite (Karhunen et al., 2008; Raben et al., 2003). Only a few studies investigating the effects of dietary FA composition on peripheral satiety factors have been performed (Francis et al., 2014; Varricchio et al., 2015; Varricchio et al., 2012). On the other hand, hypothalamic mechanisms of lipid sensing have been shown to detect changes in plasma levels of long–chain FA in mammals as well as in fish, and in turn regulate anorexigenic and orexigenic neuropeptide activity (Gao et al., 2013; Librán–Pérez et al., 2013a; Migrenne et al., 2007). In fish larvae, the expression of genes coding for appetite–regulating peptides has been shown to change with development (Gomes et al., 2015; Kortner et al., 2011), but few studies have examined the mechanisms of their regulation with regard to feeding (Gomes et al., 2015).

Altricial marine fish larvae are very small planktonic visual predators in their early stages of development and, in commercial aquaculture conditions, are still fed on live prey before being weaned into inert artificial diets (Hamre et al., 2013; Rønnestad et al., 2013). To date, several direct and indirect methods of quantifying food consumption have been tested with live prey, including measuring clearance rate from the water column or the fish digestive tract (Fushimi, 1983; Keckeis and Schiemer, 1992; Pedersen, 1984), gravimetric methods (Kamler et al., 1986), staining (Lessard et al.,

1996; Planas and Cunha, 1999), using inert markers (Cook et al., 2008; Garcia et al., 1998) and radioactive or stable isotopes (Boehlert and Yoklavich, 1984; Conceição et al., 1998; Govoni et al., 1982; Morais et al., 2006; Sorokin and Panov, 1966; Tandler and Mason, 1984). However, these have proven difficult to standardize and implement on a regular basis as they are either imprecise, laborious, expensive or require special equipment and licenses (in the case of radioactive isotopes), which are not accessible to many research groups. Therefore, the first goal of this study was to develop a suitable method for live prey labeling using fluorescent microspheres as markers and to implement it to quantify live food intake in Senegalese sole (*Solea senegalensis*) larvae. Additionally, using different colours of microspheres also allowed the evaluation of prey selectivity. The second objective of the study was to examine the effects of changes in dietary FA composition on food intake in pre– and post–metamorphic sole larvae, and analyze a wide range of genes coding for central and peripheral peptides to investigate potential differences in putative appetite–regulating mechanisms with regard to time of feeding and FA composition of the diet.

2. *Materials and methods*

Larval rearing and dietary treatments

Four groups of larvae, each distributed into 4 replicate 100 L tanks at a density of 90 larvae.L^{-1}, were cultured from 2 to 37 days post hatching (dph) on different dietary treatments. The tanks were connected to a recirculation system (IRTAmar®) with 50% daily water renewal and regulated temperature (18–20 ºC), salinity (35), and dissolved oxygen (7.5 mg L^{-1}), and a photoperiod of 16 h light: 8 h dark. The experimental dietary treatments consisted of rotifers (from 2 to 8 dph) and *Artemia* (fresh metanauplii up to 25 dph and frozen from 25 dph onwards, with a transition period of decreasing and increasing quantities of fresh and frozen metanauplii, respectively, from 15 to 25 dph), as suggested by (Dinis et al., 1999). Live prey were enriched in standard conditions with one of 4 lipid emulsions that contained either cod liver oil (CLO), linseed oil (LSO), soybean oil (SBO) or olive oil (OO) as the main lipid source. Previous results using larvae from this experiment have been presented in Bonacic et al. (2016), where further details on the rearing system and diets can be found. Table 1 shows the formulation of the experimental emulsions used to enrich both rotifers and *Artemia*, as well as the total lipid and FA profile of the enriched *Artemia* metanauplii. Rotifers were not analyzed, as they were fed only for a short duration post hatching and not used in any of the additional experiments.

All experiments with live fish were performed according to the European and National legislation with protocols approved by the IRTA ethics committee.

Quantifying live prey intake

Four small batches of *Artemia* metanauplii were enriched with experimental emulsions for 16 h, after which they were harvested, washed and concentrated (200 metanauplii mL^{-1}) in well aerated 300 mL cylindroconical containers immersed in a water bath at 28 ± 1 ºC. Fluorescent microspheres (FluoSpheres® Polystyrene

Microspheres, 10 µm, 3.6×10^6 beads mL^{-1}, Life Technologies, Alcobendas, Spain) were sonicated (Vibra–Cell©, Sonics; 30 sec; pulse 5 sec on/ 1 sec off) and vigorously vortexed before adding them to the labelling containers, at a concentration of 5.4×10^3 spheres μl^{-1}. Fifteen minutes after adding the microspheres (duration of labelling was optimized in preliminary studies), the *Artemia* was thoroughly washed and filtered. The labeled metanauplii were then quickly fed to the pelagic sole larvae alive, or flash frozen in dry ice, with as little water as possible, and stored at – 80ºC for later feeding to benthic post–larvae. Aliquots of labeled *Artemia* metanauplii were taken and fixed in 70% ethanol to count the number of spheres per *Artemia* using a fluorescent microscope (Leica DMLB, Leica, Spain) and quantify the number of spheres or total fluorescence per *Artemia* by flow cytometry and fluorescent spectrophotometry, respectively, as described below.

Sixteen hours prior to each experiment, around 180 larvae (at 19 dph) and 120 post–larvae (at 35 dph) from each tank were moved into 2 L containers placed inside the tank, with an adjustable mesh window that, when open, allowed *Artemia* to pass through and, when closed, retained them. Ten thousand live labeled metanauplii (for pelagic larvae) or 24,000 frozen labeled metanauplii (for benthic larvae), previously enriched with the different emulsions, were added to containers of the respective treatment. Larvae were allowed to feed during a period of 1 h after which 50 pelagic or 35 benthic larvae per container were sampled (t1). Sampled fish were quickly euthanized with MS–222 (1,000 mg L^{-1}) and rinsed with distilled water to eliminate any residual labelled *Artemia* on the outside, and then flash frozen with dry ice and stored at –20°C. After the t1 sampling, labeled metanauplii remaining in the containers were washed out by opening the mesh window and directing a flow of water through the container for a duration of 10 min. Following this, the windows were closed in order to perform a "cold chase", which is a method used to determine the evacuation rate of labelled *Artemia*, that is assumed to be affected by the ingestion rate of non–labelled *Artemia* (Werner and Blaxter, 1980). Therefore, unlabelled enriched metanauplii were added to the closed containers in the same amount as before and larvae were allowed to feed for 1 h (t2) and 2 h (t3), after which 50 pelagic or 35 benthic larvae were sampled in order to determine the amount of labelled prey still remaining in their gut.

Diet selectivity experiment

To differently label food items from each dietary treatment, *Artemia* were enriched and fluorescently labeled as described above, but using microspheres of four colors (blue–green, red, orange and yellow–green), one per treatment. This method permitted feeding a mixture of the 4 dietary treatments in equal proportions and to evaluate possible diet preferences when fish are presented with a choice of enriched *Artemia* with different lipid compositions. The selectivity trial was performed with benthic post–larvae in two consecutive days, at 29 and 30 dph, in which the fluorescent dyes used to label each treatment were swapped, to avoid the possibility of a bias associated to microsphere colour. Sixteen hours prior to the trial, 50 post–larvae were transferred from each tank into the same closed container used for evaluating food intake and fasted. On the morning of the experiment, an equal amount of frozen labelled *Artemia* from each treatment were thawed and added to each container to obtain a total concentration of 6 metanauplii mL^{-1}. The post–larvae were allowed to feed for one hour, after which they

were collected and euthanized with a lethal dose of MS222, gently rinsed with distilled water to eliminate any residual prey sticking to the outside body, flash frozen with dry ice and stored at –20°C until analysis.

Table 1. Formulation of the experimental emulsions and the resulting total lipid content, total FA content and FA composition of enriched *Artemia* (means ± SD; n = 4 pools).

	CLO	LSO	SBO	OO
Emulsion formulation (mg/g)				
Cod–liver oil[1]	528	0	0	0
Linseed oil[2]	0	528	0	0
Soybean oil[3]	0	0	528	0
Olive oil[4]	0	0	0	528
Soy lecithin[5]	40	40	40	40
α–Tocopherol[6]	12	12	12	12
Distilled water	420	420	420	420
Total lipid and FA content of enriched Artemia (mg per g of DW)				
Total lipids	107.1 ± 19.3	100.6 ± 6.3	106.1 ± 20.4	112.6 ± 6.8
Total FA	61.6 ± 10.8	61.2 ± 11.0	69.3 ± 22.3	71.7 ± 7.5
FA composition of enriched Artemia (% of TFA)*				
16:0	10.0± 0.3	7.4 ± 0.5	8.3 ± 1.0	8.1 ± 0.7
18:0	5.4 ± 0.4	6.5 ± 1.0	6.0 ± 0.9	5.0 ± 0.5
Total SFA	16 ± 0.7	14 ± 1.4	14.4 ± 1.2	13.3 ± 1.3
16:1	2.5 ± 0.5	0.4 ± 0.2	0.5 ± 0.1	0.6 ± 0.2
18:1n–9	24.4 ± 0.5	25.1 ± 1.1	24.7 ± 0.5	49 ± 0.1
18:1n–7	7.6 ± 0.5	5.7 ± 1.0	4.6 ± 1.3	7.7 ± 1.0
20:1	2.0 ± 2.3	0.2 ± 0.3	0.1 ± 0.2	0.0 ± 0.0
Total MUFA	40.4 ± 2.7	31.3 ± 1.6	30.0 ± 0.8	57.3 ± 1.0
18:2n–6	6.0 ± 0.4	11.7 ± 1.9	28.8 ± 5.0	7.9 ± 2.2
20:4n–6	0.5 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
Total n–6 PUFA	9.1 ± 3.3	12.4 ± 1.6	29.9 ± 4.5	8.7 ± 2.2
18:3n–3	19.8 ± 2.2	38.2 ± 2.1	21.5 ± 2.8	17.1 ± 0.7
20:5n–3	6.3 ± 0.8	0.9 ± 0.3	0.8 ± 0.2	0.7 ± 0.1
22:5n–3	0.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:6n–3	3.9 ± 0.8	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0
Total n–3 PUFA	34.4 ± 0.7	42.2 ± 1.7	25.6 ± 3.2	20.6 ± 0.9
Total PUFA	43.5 ± 2.9	54.6 ± 2.6	55.5 ± 1.6	29.3 ± 1.4
DHA/EPA	0.6 ± 0.1	0.0 ± 0.0	0.2 ± 0.4	0.0 ± 0.0
ARA/EPA	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0

*Not all FAs are shown.1Sigma–Aldrich Co., Germany; 2Biolasi Products Naturals, S.L., Guipùzcoa, Spain; 3Huilerie Emile Noël, S.A.S., Pont Saint Esprit, France; 4Borges Pont, Lleida, Spain; 5Laboratorios Korott, Alicante, Spain; 6Sigma Aldrich Co., Germany.

Processing of samples for analysis by spectrophotometry and flow cytometry

The tissue of larvae collected for the determination of food intake and diet selectivity was processed using the method described by (Van Oosterhout et al., 1995), in order to quantify food intake by spectrophotometry and selectivity by flow cytometry. Spectrophotometry was used for quantifying total absorbance, but was judged imprecise for simultaneous quantification of several colours of microspheres due to overlap of

emmission wavelengths. Cytometry, on the other hand, resulted to be a more precise method, but was limited to relative quantification given that only a fraction of the larval sample could be measured. In addition, 4 subsamples of labelled *Artemia* (from aliquotes taken immediately after labelling and fixed in 70% ethanol) with at least 150 exactly counted metanauplii of each treatment were also collected and processed for both fluorometry and cytometry analysis.

Firstly, to digest the tissues, 4 mL of 4M KOH were added to the samples that were then incubated for 48 h at 37 ºC in the dark with agitation. The tubes were vortexed vigorously after the first 24 h of incubation and at the end of the digestion process samples were sonicated (20 sec) to break down any remaining tissue. Microspheres were sedimented by centrifugation at 3,000 g for 20 min at room temperature. The supernatant was removed and samples were then washed with 9 mL 0.25% Tween 80 (Sigma–Aldrich Co., St Louis, MO, France) in distilled water (pre–heated at 60 ºC) and then vortexed until completely ressuspending the pellet. The sample was centrifuged again as described above, the supernatant removed, the pellet rinsed with 9 mL of distilled water and vortexed. After centrifuging again at 3,000 g for 20 min the supernatant was removed leaving 500 µl for cytometry or spectrophotometry analyses.

For flow cytometry, digested *Artemia* and larvae samples were resuspended and filtered through a 50 µm screen. The number of fluorospheres was quantified in aliquots of larvae samples and in the whole *Artemia* samples using a Navios® flow cytometer (Beckman–Coulter®, Miami, FL, USA) with 3 solid–state lasers, forward–scatter and side–scatter detectors and 10 fluorescence detectors. Readings were carried out in fluorescence channels where the detection and identification was optimal for each fluorosphere colour.

In the case of the spectrophotometric analysis, the fluorescent dye first had to be extracted by dissolving the microsphere polystyrene wall. This was performed by adding 3 mL of 2–ethoxyethyl acetate (98%, Sigma Aldrich) to the samples, which were then vortexed and left to stand for 24 h, protected from light. After incubation, vortexing and centrifugation at 3,000 g for 10 min, triplicate aliquots of 250 µl were taken from the supernatant of each sample and read in a fluorescent spectrophotometer (Synergy HT, BioTek Instruments, Winooski, VT, USA) at excitation and emission wavelengths of 565 nm and 600 nm (corresponding to the red dye of microspheres used for quantifying food intake), respectively. Values were calculated as fluorescence intensity per *Artemia* and per g wet weight (WW) of larvae.

Effects of diet on basal and post–prandial gene expression

A feeding trial was performed at 16 dph with pre–metamorphic pelagic larvae, and again at 34 dph with benthic post–larvae, and samples of fish were collected for real time quantitative PCR (qPCR) analysis. This trial has been described in detail in Bonacic et al. (2016). Briefly, two groups of fish were transferred into separate containers in the evening before the trial and were kept fasted for 16 h. The first group (t0), containing 200 larvae (at 16 dph) or 50 post–larvae (at 34 dph) per tank, was kept in a 2 L container placed inside the tank, with a mesh preventing *Artemia* in the tanks from entering. Fish were sampled after 16 h of fasting, at the usual feeding time (9 am), shortly after lights were turned on. The second group (600 larvae or 150 post–larvae per tank) was transferred into a separate 12 L container with clean aerated seawater and, after 16 h

fasting fed with enriched *Artemia* metanauplii from their respective dietary treatment (live at 16 dph and frozen at 34 dph). Sole specimens were allowed to feed undisturbed for 1 h and then filtered through a submerged 500 μm mesh in order to remove uneaten metanauplii, after which they were transferred to a new container with clean aerated seawater. A sample of 200 larvae or 50 post–larvae was then taken from the container 30 min (t1), 1 h 30 min (t2) and 3 h (t3) after the transfer (end of feeding). Sampled fish were quickly euthanized with MS–222 and preserved in 3 mL of RNAlater stabilization buffer (Ambion, Life Technologies, Alcobendas, Madrid, Spain) following manufacturer's instructions.

Real time quantitative PCR (qPCR)

A series of genes coding for anorexigenic (peptide yy – *pyy*, glucagon–like peptide 1 – *glp1,* cholecystokinin – *cckl,* cocaine– and amphetamine–related transcript *cart,* proopiomelanocortin – *pomc,* corticotropin–releasing factor – *crf*) and orexigenic (galanin – *gal*, neuropeptide y – *npy*, agouti–related protein 2 – *agrp2*) peptides involved in gut–brain satiety signaling in mammals, and putatively also in fish, were selected for analysis by qPCR (Table 2). In a few cases primers had to be specifically developed for this study. This was done by searching for candidate transcripts in the SoleaDB database, *Solea senegalensis* v4.1 global assembly (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/) (Benzekri et al., 2014), followed by *in silico* assemblage into contigs using the BioEdit Sequence Alignment Editor (Hall, 1999). For some transcripts rapid amplification of cDNA ends (RACE) PCR was performed using the FirstChoice® RLM–RACE kit (Ambion, Life Technologies, Alcobendas, Madrid, Spain), in order to obtain and sequence (SCSIE, University of Valencia, Spain) the 3' untranslated regions (UTR's) which, whenever possible, were used as templates to design primers for qPCR, using Primer3 v. 0.4.0 (Koressaar and Remm, 2007; Untergasser et al., 2012). Amplicons of newly designed qPCR assays were sequenced to confirm their identity and the specificity of the assay.

Total RNA was extracted from whole pelagic larvae (16 dph), while the head was separated from the body of benthic post–larvae (34 dph) and RNA was extracted from the two dissected compartments. Pools of larvae/tissues were homogenized (Mini–Beadbeater, Biospec Products Inc., USA) in 1 mL of TRIzol (Ambion, Life Technologies, Madrid, Spain) and solvent extraction was performed following manufacturer's instructions. Final RNA concentrations were determined by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Madrid, Spain) and RNA quality and integrity was assessed from the ratio of absorbances at 260 and 280 nm and by gel electrophoresis, respectively. Two micrograms of total RNA were then reverse transcribed into cDNA using the High–Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life technologies, USA), following manufacturer's instructions, but using a mixture of random primers (1.5 μL as supplied) and anchored oligo–dT (0.5 μL at 400 ng μL^{-1}, Eurogentec, Cultek, S.L., Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. A pool of 2 μL from each cDNA sample was created to be used for standard curves of serial dilutions and the remaining cDNA was diluted 60–fold with water. Amplifications were carried out in duplicate on a CFX96 Touch Real–Time PCR Detection System (Bio–Rad, Alcobendas, Spain) in a final volume of 20 μL containing 5 μL of diluted

Table 2. Primers used for real–time quantitative PCR (RT–qPCR). Shown are sequences and annealing temperature (Ta) of the primer pairs, size of the fragment produced, average reaction efficiency (E) and accession number of the target and reference genes.

Transcript	Primer sequence (5'–3')	Amplicon	Ta	E*	Reference
Peripheral anorexigenic					
pyya	AGCAGCACAGCAAGATCTCA CCTTCCCACAGATTCTGCAT	174 bp	60ºC	100.6%	KT626438
pyyb	CAACTTGTGCTGCATGGAAT AGCAGCAAACATGAGACGTG	237 bp	60ºC	100.5%	KT626439
glp1	CCTCACGGACCAGGCTATTA TACTGTCCCCTGAGGGTTTG	209 bp	60ºC	99.7%	KT626440
*cckl***	TTCTCCTGGTCCAAACCAAC AGGGCAGACTCAGAGAACCA	100 bp	60ºC	104.1%	KT626441
Peripheral orexigenic					
*gal***	CTTAACGGTTTCCCCATGC CCAGAGTCGGAGTGTGAGTG	101 bp	60ºC	103.7%	FF290428
Central anorexigenic					
cart1a	CGTCCACCACTGTCATTCTG CTTTCTCCTCCTGCGTCTTG	147 bp	60ºC	101.0%	KT189188[a]
cart1b	TCGCTGAAAAGTCAACAAGAAA GCCAAGCTTTTTCTCCAGTG	99 bp	60ºC	101.3%	KT189189[a]
cart2a	TCGCGTATCATCGAACACAT GTGACACTGAGCCACAGCAC	86 bp	60ºC	101.1%	KT189190[a]
cart4	GTGAGCGAGAGCAGGAAACT TCGTGGTGAAATAAGGCAAA	144 bp	60ºC	103.0%	KT189194[a]
pomca	AAGGCAAAGAGGCGTTGTAT TTCTTGAACAGCGTGAGCAG	127 bp	60ºC	100.2%	FR851915[b]
pomcb	GTCGAGCAACACAAGTTCCA GTCAGCTCGTCGTAGCGTTT	110 bp	60ºC	103.6%	FR851916[b]
crf	CCTGACCTTCCACCTGCTAC GAGATCTTTGGCGGAGTGAA	135 bp	60ºC	103.3%	FR745427[c]
Central orexigenic					
npy	GAGGGATACCCGATGAAACC GCTGGACCTCTTCCCATACC	129 bp	60ºC	99.9%	solea_v4.1_ unigene466117[d]
agrp2	CAGGTCAGACTCCGTGAGCCC GTCGACACCGACAGGAGGCAC	104 bp	64ºC	102.9%	solea_v4.1_ unigene32957[d]
Reference genes					
ubq	AGCTGGCCCAGAAATATAACTGCGACA ACTTCTTCTTGCGGCAGTTGACAGCAC	93 bp	70ºC	100.9%	AB291588[e]
rps4	GTGAAGAAGCTCCTTGTCGGCACCA AGGGGGTCGGGGTAGCGGATG	83 bp	70ºC	101.7%	AB291557[e]
ef1a1	GATTGACCGTCGTTCTGGCAAGAAGC GGCAAAGCGACCAAGGGGAGCAT	142 bp	70ºC	101.9%	AB326302[e]

pyy, peptide YY; *glp1*, glucagon–like peptide 1; *cckl*, cholecystokinin (Leu) precursor; *gal*, galanin; *cart*, cocaine– and amphetamine–related transcript; *pomc*, proopiomelanocortin; *crf*, corticotropin–releasing factor; *npy*, neuropeptide Y; *agrp2*, agouti–related protein 2; *ubq*, ubiquitin; *rps4*, 40S ribosomal protein S4; *ef1a1*, elongation factor 1 alpha.

(1/60) cDNA, 500 nM of each primer (except for *pomca*, *pomcb*, *crf*, and *cart2a* where 200 nM, and *agrp2* and *npy* where 120 nM were used) and 10 µL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio–Rad), and included a systematic negative control (NTC–non template control). The RT–qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 35 cycles: 15 s at 95 °C, 1 min at the corresponding annealing temperature (Ta; Table 2). After the amplification phase, a melt curve was performed, enabling confirmation of the amplification of a single product in each reaction. Non–occurrence of primer–dimer formation in the NTC was also checked. The amplification efficiency of each primer pair was assessed from serial dilutions of the cDNA pool (Table 2).

For data analysis, qPCR results were imported into the software qBase+ (Biogazelle, Zwijnaarde, Belgium), where normalized relative quantities (NRQ) were calculated employing target and run–specific amplification efficiencies and using the geometric mean of 2–3 reference genes (elongation factor 1 alpha, *eef1a1*; ubiquitin, *ubq*; and 40S ribosomal protein S4, *rps4*; Infante *et al*., 2008). Two reference genes (*rps4* and *eef1a1*) were used to normalize the results at collected at 16 dph from whole larvae (M = 0.331; coefficient of variance – CV = 0.115) and at 34 dph from the body compartment of post–larvae (M = 0.160, CV = 0.055). For samples of the head compartment collected at 34 dph, all 3 reference genes were employed (M = 0.158, CV = 0.063). All genes and tissues were analyzed in two plates so that the samples were grouped by time point (t0 and t1 in one plate and t2 and t3 in the other). An inter–run calibration was performed between these two plates using the standard curves as calibration factors, producing calibrated normalized relative quantities (CNRQ).

Statistical analysis

All analyses were performed with SPSS v.20 (SPSS Inc., Chicago, IL, USA). Gene expression results (CNRQ values; n = 4) were analyzed by two–way ANOVA examining effects of the factors “diet” and “time”. Data for food intake obtained with spectrophotometry (n = 4) was analyzed by ANOVA at a significance level of 0.05 for each timepoint separately. Results of food selectivity obtained by flow cytometry (n = 4) were also analyzed by one–way ANOVA. When data passed the Levene’s test of equal variances ($p>0.05$), the Tukey’s post–hoc test was performed to assess significant differences between dietary treatments at each age or time point. When variances were not equally distributed, the Games–Howell test was used instead.

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*Efficiency corresponds to an average of 4 runs (for larval and post–larval samples, each with two runs).
**Although primarily peripheral, these genes were also analyzed in the head compartments of post–larvae
a(Bonacic et al., 2015b)
b(Wunderink et al., 2012)
c(Wunderink et al., 2011)
dMartín–Robles & Muñoz–Cueto, unpublished
e(Infante et al., 2008)

3. *Results*

Food intake

The results obtained from the ingestion trials performed at 19 dph and 35 dph are shown in Fig. 1. At 19 dph, larvae from the CLO treatment ingested significantly (around 3 times) more enriched labeled *Artemia* during 1 h of feeding than those fed vegetable oil–enriched diets (t1). During the "cold chase" period (t2 and t3), i.e., 1 h and 2 h after replacing the labeled diet by non–labeled enriched *Artemia*, there was a similarly low and variable evacuation of all the dietary treatments. Nevertheless, a similar trend of higher amounts of labeled *Artemia* being present in the gut of larvae fed the CLO treatment (significantly compared to OO at t3) was observed. At 35 dph, post–larvae fed CLO–enriched *Artemia* metanauplii also showed a trend for higher intake at t0 and the evacuation of this diet appeared to be more accelerated than the remaining treatments. However, results were not significant, at any of the time points, due to the large variability of the data.

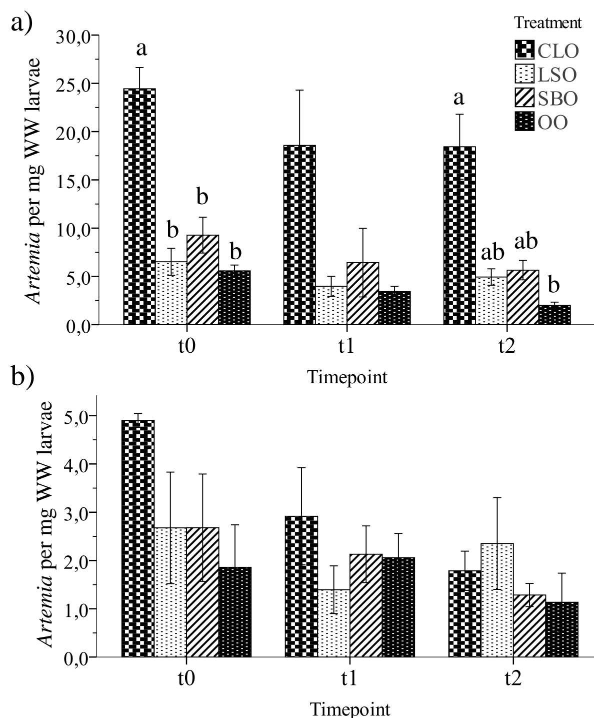


Figure 1. Feed intake (number of metanauplii) of enriched and fluorescently labeled *Artemia* after 1 h of feeding (t0) and subsequent retention in the gut after replacing by non–labeled enriched *Artemia* and feeding a further 1 h and 2 h (t1 and t2, respectively) of Senegalese sole larvae at 19 dph (a) and post larvae at 35 dph (b). One–way ANOVA was performed to compare dietary treatments at individual timepoints. Values are means of pools collected from each replicate tank (n=4) ± SE.

Diet selectivity

The relative proportion of each diet in the larval gut after 1 h of feeding on *Artemia* metanauplii enriched with different lipid sources did not differ significantly between treatments at 29 dph (Fig. 2a) or 30 dph (Fig. 2b). However, *Artemia* enriched with OO generally tended to be less ingested than the other diets, independently of the dietary treatment the larvae came from.

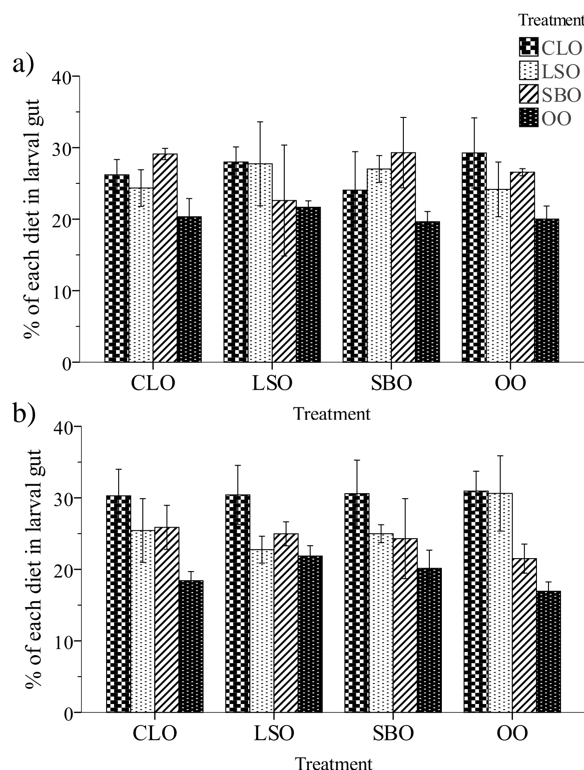


Figure 2. Diet selectivity (% of each diet present in the gut, relative to the total) in Senegalese sole post–larvae at 29 dph (a) and 30 dph (b). Enriched *Artemia* diets were labeled with fluorescent microspheres of different colours which were swapped between 29 dph (a; CLO – blue–green, LSO – orange, SBO – red, OO – yellow–green) and 30 dph (b; CLO – orange, LSO – blue–green, SBO – yellow–green; OO –red). No significant differences were observed between treatments, analyzed by one–way ANOVA, at either day. Values are means of pools collected from each replicate tank (n=4) ± SE.

Gene expression

At 16 dph, mRNA levels of *glp1*, *cart1a*, *cart1b*, *cart2a*, *pomca*, *pomcb*, *crf*, *npy* and *agrp2* in whole pelagic larvae were significantly affected by the factor "time", while *pyyb*, *gal*, *cart1b*, *cart2a* and *cart4* were expressed differently with regard to the factor "diet", and transcript levels of *pyya* and *cckl* did not change in response to any of the analyzed factors (Fig. 3, 4 and 5). Transcript levels of *pyyb* (Fig. 3b) and *gal* (Fig. 5a) were significantly higher in the CLO, compared to OO treatment, primarily due to

differences observed at 30 min after feeding in the case of *pyyb*. The anorexigenic genes *glp1* (Fig. 3c) and *cart1a* (Fig. 4a) had very similar expression patterns, with values decreasing 30 min after feeding in all treatments except for the OO group (CLO being the most pronounced) and then increasing until 3 h post–feeding. Transcript levels of *cart1b* (Fig. 4b) generally increased at 1 h 30 min post–feeding, but differences were more pronounced with regard to the factor "diet", with lowest values observed in larvae fed the CLO diet and highest in those fed *Artemia* metanauplii enriched with OO. Expression of *cart2a* (Fig. 4c) and *pomca* (Fig. 4e) showed a similar pattern, with a peak in mRNA levels at 30 min after feeding in fish fed the CLO treatment. This led to a significantly higher overall expression of *cart2a* in the CLO compared to the SBO treatment. Expression of *cart4* (Fig. 4d) was also significantly higher in larvae fed *Artemia* metanauplii enriched with CLO compared to the SBO group, with differences being visible in all post–prandial time points. Transcript levels of *pomcb* (Fig. 4f) in all treatments except for the CLO group slowly decreased throughout the post–prandial phase, while *crf* (Fig. 4g) levels increased in all treatments. In the case of the orexigenic genes, mRNA levels of *npy* (Fig. 5b) in the CLO and LSO treatments tended to slightly increase, and remain elevated, just after feeding (with a tendency for higher expression in the CLO–fed larvae), while in the SBO and OO treatments *npy* expression appeared to somewhat increase only at 1h 30 min after feeding. Transcript levels of *agrp2* (Fig. 5c) slowly decreased throughout the post–prandial phase in all treatments.

Among the genes analyzed in the body compartment of post–larvae at 34 dph, only *pyya* was significantly affected by the factor "time", with highest mRNA values observed 30 min after the end of the meal in fish from all treatments, except in the LSO group (Fig. 6). Expression of *pyya*, *glp1* and *cckl* (Fig. 6a, c and d) was significantly affected by the factor "diet", with lower *pyya* transcript levels being observed in the CLO and LSO treatments (compared to SBO and OO), highest *glp1* mRNA levels in fish fed the CLO–based diet (significantly higher than SBO and OO) and higher values of *cckl* expression in fish fed the CLO compared to the LSO and SBO diets. Transcript levels of *gal* and *pyyb* did not change in relation to either analyzed factor.

In the head compartment of 34 dph post–larvae, the expression of the neuropeptides *cart2a, cart4* and *agrp2* was significantly affected by the factor "time", and *cart1a*, *cart1b*, *cart2a*, *pomca*, *pomcb*, *cckl*, *npy* and *agrp2* were significantly affected by "diet", whereas no significant differences in relation to either factor were observed in *gal* or *crf* (Fig. 7 and 8). Transcript levels of the anorexigenic genes *cart2a* (Fig. 7c) and *cart4* (Fig. 7d) tended to increase post–prandially, most noticeably at 30 min or 1h 30min after feeding depending on the treatment. Other anorexigenic genes, *cart1a*, *cart1b*, *pomca*, *pomcb* and *cckl* (Fig. 7a, b, e, f and h), had generally lower expression, unaffected by feeding, when fish were fed CLO–enriched *Artemia*. It may be important to note that fish fed the SBO treatment showed a prominent peak in expression of *cart1a*, *cart4* and *pomca* at 30 min after feeding, although values were only significantly higher than in the remaining treatments for the *pomca* gene. Transcript levels of the orexigenic gene *npy* (Fig. 8b) were higher in fish fed the LSO treatment than in those fed the OO–based diet. Finally, the expression of *agrp2* (Fig. 8c) was significantly lower at 3 h compared to 30 min after a meal in all treatments except CLO. Furthermore, lower *agrp2* levels were generally found in post–larvae fed the CLO–based diet, compared to the OO and SBO diets.

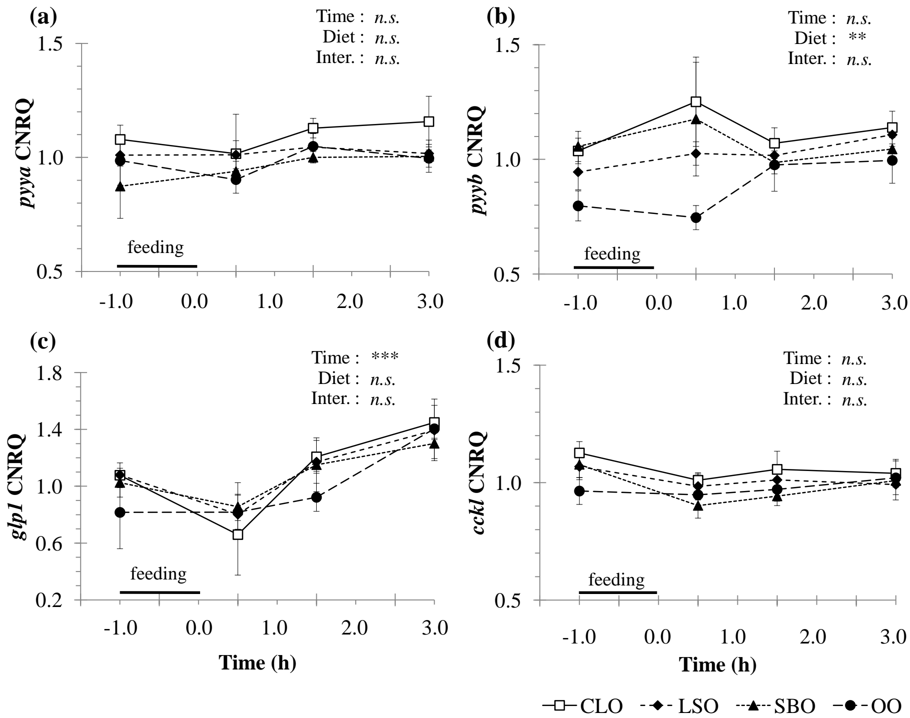


Figure 3. Expression of putative anorexigenic genes *pyya* (a), *pyyb* (b), *glp1* (c) *and cckl* (d) in Senegalese sole whole larvae at 16 dph, in relation to time of feeding and diet. Larvae were sampled just before the usual feeding time (16 h fasted), then fed for one hour (horizontal bar) on their respective dietary treatment (*Artemia* enriched with either CLO, LSO, SBO or OO) and sampled 0.5, 1.5 and 3 h after end of the meal. Results of 2–way ANOVA analysis are presented beside each chart, where *n.s.* is not significant, * is p<0.05, ** is p<0.01 and *** p<0.001. Values are calibrated normalized relative quantities (CNRQ) ± SE.

4. *Discussion*

Effect of dietary fatty acid composition on food intake

Altricial marine fish larvae, such as those of Senegalese sole, are planktonic visual predators and seem to feed almost constantly if prey is available, which differs from the feeding behaviour in juvenile and adult stages (Rønnestad et al., 2013). Larvae may select live prey based on factors such as size, shape, mobility, color and odor (Checkley, 1982; Mayer and Wahl, 1997; Pavlov and Kasumyan, 1990). Furthermore, there are internal feedback mechanisms that regulate appetite and food intake, which are believed to operate in fish similarly as in higher vertebrate species, although there is still a great paucity of information in teleosts, and on larval stages in particular (Gomes et al., 2015; Kortner et al., 2011). Such mechanisms are based on signals generated by ingested food and energy balance of the organism, and travel from peripheral tissues to relevant centers of homeostatic control in the CNS (Volkoff, 2006). The larvae and post–larvae in the

food intake experiment reported here were fed exclusively on *Artemia* metanauplii, and thus no differences were associated to visual stimulus. However, since each prey group was enriched with a different oil emulsion, they may have given off specific sensorial cues (olfactory and/or taste signals) to the fish, which could have interfered with internal homeostatic mechanisms in determining food intake. To address this possibility, a selectivity trial was performed, which showed that Senegalese sole larvae did not preferentially ingest any of the diets when offered with a choice, regardless of the FA profile of the enriched *Artemia* metanauplii, although the OO–based diet might have been slightly less preferred. Therefore, the food intake results most likely resulted from a combination of post–ingestion appetite regulating factors (gut–brain signaling) and lipid metabolism.

Dietary lipid level, by determining dietary energy content, has commonly been shown to affect food intake in adult and juvenile fish and it is believed that fish regulate their food intake to meet their energy requirements (Boujard et al., 2004b; Gélineau et al., 2002; Sæther and Jobling, 2001; Tocher, 2010). However, results from Morais *et al.* (2006) suggest that dietary FA composition may also have played a role in the control of ingestion in larvae of gilthead sea bream, since the effects of lipid level depended on the lipid source (fish oil or soybean oil) that was used. The current study similarly suggests an effect of lipid source on food intake, as 19 dph larvae fed CLO–enriched *Artemia* ingested significantly more prey per mg of their body weight (i.e., disregarding differences between treatments in larval body weight) than those fed vegetable oil–based diets. A similar trend was observed in 34 dph post–larvae, although differences were not significant at this point, possibly due to high variability in the data. The CLO treatment provided the larvae with higher levels of long–chain polyunsaturated fatty acids (LC–PUFA), particularly eicosapentaenoic acid (EPA; 20:5 n–3) and docosahexaenoic acid (DHA; 22:6 n–3), while the LSO, SBO and OO treatments were especially rich in α–linolenic acid (ALA; 18:3 n–3), linoleic acid (LNA; 18:2 n–6) and oleic acid (OA; 18:1 n–9), respectively. However, total lipid or FA content did not differ between diets, which implies that the increased feed intake in larval (19 dph) Senegalese sole fed CLO–enriched *Artemia* was a response to just the qualitative (FA profile) rather than the quantitative (total lipid) properties of the diet. Nevertheless, we cannot rule out that the two factors might be interrelated since not all FAs are utilized as energy substrates with the same efficiency. In fact, it has been fairly well established that saturated and monounsaturated FA (including OA) are preferential substrates for mitochondrial and peroxisomal β–oxidation in marine and freshwater fish (Crockett and Sidell, 1993; Kiessling and Kiessling, 1993). In rainbow trout (*Onchorhynchus mykiss*), PUFA such as LNA and ALA were also readily oxidized (Qiu et al., 2005), but had a slower oxidation rate (Kiessling and Kiessling, 1993). Furthermore, a metabolic study performed with Senegalese sole post–larvae showed that catabolic oxidation of a tube fed DHA radiotracer was minimal, and it tended to be mostly deposited and retained in body tissues, while OA was mostly oxidized (Morais et al., 2005). Therefore, we could hypothesize that, in spite of similar lipid (hence gross energy) levels, the CLO prey might have had a lower energetic value to the larvae, which could also have been a factor governing food intake. On the other hand, free circulating medium– and long–chain FAs have also been shown to affect appetite of juvenile rainbow trout through hypothalamic lipid sensing mechanisms (Librán–Pérez et al., 2012), and our own unpublished results confirmed the presence of these mechanisms in Senegalese sole

juveniles and their sensitivity to OA and ALA, but not stearic acid and EPA, which further highlights the importance of the dietary FA profile on appetite regulation.

Bonacic et al. (2016) reported that significant differences in the size of larvae used in the current experiment were observed between 22 and 30 dph (larvae fed the CLO diet were significantly heavier than those fed OO), which became insignificant at 37 dph, mainly due to a large dispersion in size, typical of the early juvenile stage (Morais et al., 2015a). While this suggests a correlation between food intake (appetite), lipid metabolism and growth, it has still not been clearly established when internal appetite–regulating mechanisms become functional in altricial fish larvae (Rønnestad et al., 2013). In this respect, it is difficult to ascertain if larval growth was affected by differences in appetite (caused by dietary FA composition in the early life stages), or if appetite was modulated by enhanced growth (caused by nutritional value of the dietary treatment and effects on lipid metabolism, as discussed in Bonacic et al., 2016). The answer to this question is probably very complex, but may partly lie in the satiety regulatory mechanisms discussed below.

Transcriptional regulation of genes coding putative satiety peptides in larvae and post–larvae fed diets containing different fatty acid compositions

Although information on food intake regulation via neuro– and gastrointestinal peptides in fish is growing, it is still very limited, and studies on this issue often rely on mammalian literature as a starting point (Volkoff, 2006; Volkoff et al., 2005). However, whole genome duplication events in teleosts have resulted in the appearance of numerous homologues of a wide variety of appetite regulating genes, leading to sub–specialization of duplicated genes and likely more complex regulatory mechanisms than in mammals (de Souza et al., 2005; Gonzalez et al., 2010; Murashita and Kurokawa, 2011; Sundström et al., 2013). The complexity of these mechanisms in fish was also apparent in the present study, where numerous homologues of some of the analyzed peripheral and central genes were identified. Therefore, it is essential to perform more dedicated work in fish and not rely on information obtained from higher vertebrates, which in some cases may differ considerably from what is observed in teleosts (Mommsen, 2000).

Peripheral anorexigenic peptides

Initially characterized as a gastrointestinal peptide, Pyy has also been identified in certain brain areas of mammals and teleosts (Cerdá–Reverter et al., 2000; Söderberg et al., 2000). While at 16 dph no significant differences were observed in response to feeding, at 34 dph transcript levels of *pyya* were higher at 30 min after having ingested a meal (significantly when compared to 3 h), in all treatments except LSO (due to high variability). Furthermore, the SBO and OO treatments had higher overall levels of *pyya* compared to the CLO and LSO groups. Similar post–prandial effects were observed in goldfish *Carassius auratus* (Gonzalez and Unniappan, 2010) and in the brain of Atlantic halibut *Hippoglossus hipppoglossus* larvae (Gomes et al., 2015), which is generally in accordance with the putative anorexigenic properties of Pyy (Volkoff, 2006). On the other hand, transcript levels of *pyyb* were higher in 16 dph larvae fed a CLO–based diet

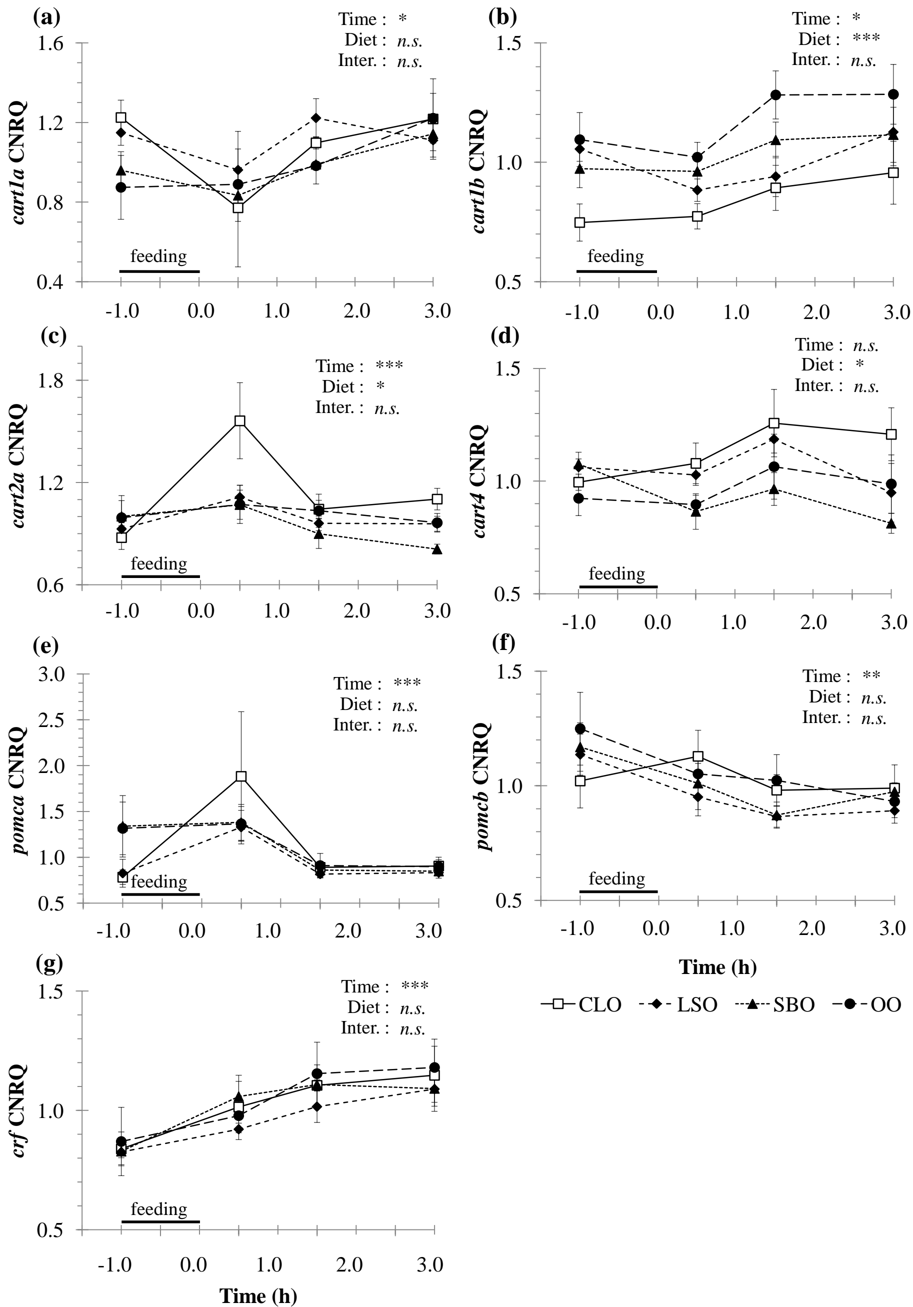


Figure 4. Expression of putative anorexigenic genes *cart1a* (a), *cart1b* (b), *cart2a* (c), *cart4* (d), *pomca* (e), *pomcb* (f) and *crf* (g) in Senegalese sole whole larvae at 16 dph, in relation to time of feeding and diet. Larvae were sampled just before the usual feeding time (16 h fasted), then fed for one hour (horizontal bar) on their respective dietary treatment (Artemia enriched with either CLO, LSO, SBO or OO) and sampled 0.5, 1.5 and 3 h after end of the meal. Results of 2–way ANOVA analysis are presented beside each chart, where n.s. is not significant, * is p<0.05, ** is p<0.01 and *** p<0.001. Values are calibrated normalized relative quantities (CNRQ) ± SE.

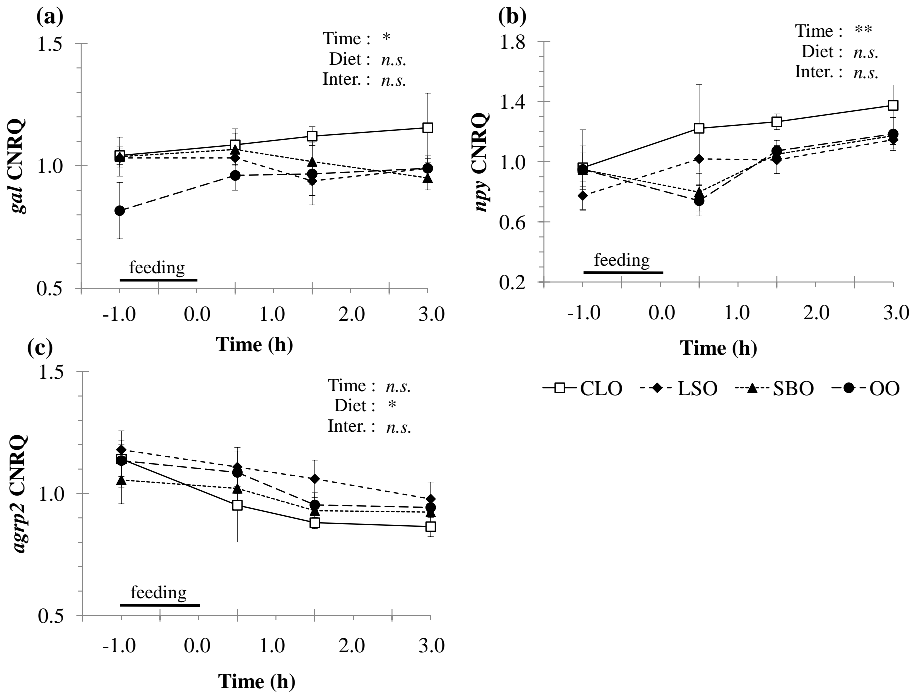


Figure 5. Expression of putative orexigenic *gal* (a), *npy* (b) and *agrp2* (c) in Senegalese sole whole larvae at 16 dph, in relation to time of feeding and diet. Larvae were sampled just before the usual feeding time (16 h fasted), then fed for one hour (horizontal bar) on their respective dietary treatment (*Artemia* enriched with either CLO, LSO, SBO or OO) and sampled 0.5, 1.5 and 3 h after end of the meal. Results of 2–way ANOVA analysis are presented beside each chart, where *n.s.* is not significant, * is p<0.05, ** is p<0.01 and *** p<0.001. Values are calibrated normalized relative quantities (CNRQ) ± SE.

compared to OO, but no changes were visible in response to dietary FA composition at 34 dph. In larvae, the CLO and, to an extent, the SBO treatments had a subtle increase in post–prandial *pyyb* expression, which was in accordance with an anorexigenic function, but it would be very speculative to conclude anything due to the ambiguous expression patterns of the remaining dietary treatments. Nevertheless, these results indicate that *pyyb* was regulated differently under different dietary conditions.

At 16 dph, transcript levels of *glp1* were generally lowest shortly (30 min) after feeding and highest at 3 h. At 34 dph, post–larvae fed *Artemia* metanauplii enriched with SBO and OO had a similar expression pattern as 16 dph larvae, while those fed the CLO and LSO treatments did not exhibit any post–prandial changes. However, post–larvae fed CLO tended to have the highest transcript levels of all treatments, both pre– and post–prandially (especially 30 mins post feeding). These results are somewhat puzzling, as the expression pattern of *glp1* appeared more in line with an orexigenic, rather than anorexigenic activity observed in other teleost species (Silverstein et al., 2001; Sundby et al., 1991). Nevertheless, changes in expression observed at 30 mins post–prandially were subtle and variable, and hence further studies are required to ascertain this.

In the case of *cckl*, no transcriptional changes were observed at 16 dph, but in older post–larvae its expression was significantly affected by dietary composition, in both peripheral and central tissues. However, in the body compartment, highest levels were observed in fish fed CLO (significantly higher than LSO and SBO), while in the head compartment, fish fed CLO had lowest expression (significantly lower than SBO and OO). Nonetheless, these differences were quite subtle and since this peptide usually produces a strong anorexigenic feedback signal when food is present in the intestine (Aldman and Holmgren, 1995; Peyon et al., 1999), it may be that *cckl* regulation was not fully developed at either of the developmental stages.

Peripheral orexigenic peptides

Galanin expression has been observed in both peripheral tissues and brain of teleosts (Johnsson et al., 2001; Unniappan et al., 2004). However, intracerebroventricular administration of this peptide stimulated food intake in goldfish and tench (*Tinca tinca*), while intraperitoneal administration had no effect in either species (de Pedro et al., 1995; Guijarro et al., 1999; Volkoff and Peter, 2001b). Although this may suggest a higher *gal* activity in the CNS, in the current study, no regulation was observed at 34 dph in either body or head compartments of post–larvae. Nevertheless, in whole larvae at 16 dph *gal* was up–regulated in fish fed CLO compared to OO, which is in accordance with results of food intake.

Central anorexigenic peptides

This group of peptides contains several genes with multiple homologues in Senegalese sole, most of which seemed to have been regulated differently in the present study. On the other hand, some homologues had very similar expression patterns to those from other genes, potentially suggesting a coordinated action in regulatory pathways.

Intracerebroventricular injections of CART have generally been shown to have an anorexigenic effect on feeding (including gastric emptying) and metabolic regulation (body weight gain, plasma insulin and leptin levels, lipid oxidation and thermogenesis; reviewed in Murphy, 2005; Vicentic and Jones, 2007; Rogge et al., 2008; Lau and Herzog, 2014). However, in several instances where more than one *cart* homologue was identified in teleosts, not all were affected by fasting or feeding (MacDonald and Volkoff, 2009; Murashita and Kurokawa, 2011; Volkoff and Peter, 2001b) and, when injected into discrete hypothalamus nuclei of rats, even an opposite (orexigenic) effect has been observed (Dhillo et al., 2002). In a previous study with Senegalese sole juveniles, 7 *cart* homologues were identified and characterized, but only 3 (*cart1a*, *cart2a* and *cart4*) showed a significant transcriptional response to feeding in the brain (Bonacic et al., 2015b). The former authors also established that *cart1a* and *cart4* are abundant in numerous peripheral tissues in juvenile sole, in levels comparable to the brain, while *cart2a* is mostly expressed in the brain and gonads, but also in the kidney and spleen. An additional transcript, *cart1b* was analyzed in this study due to a more restricted and predominant expression in the brain, with some expression also in the eye and gonads. In the present study, these 4 homologues (*cart1a*, *cart1b*, *cart2a* and *cart4*) showed very different expression patterns. However, some coincided with other

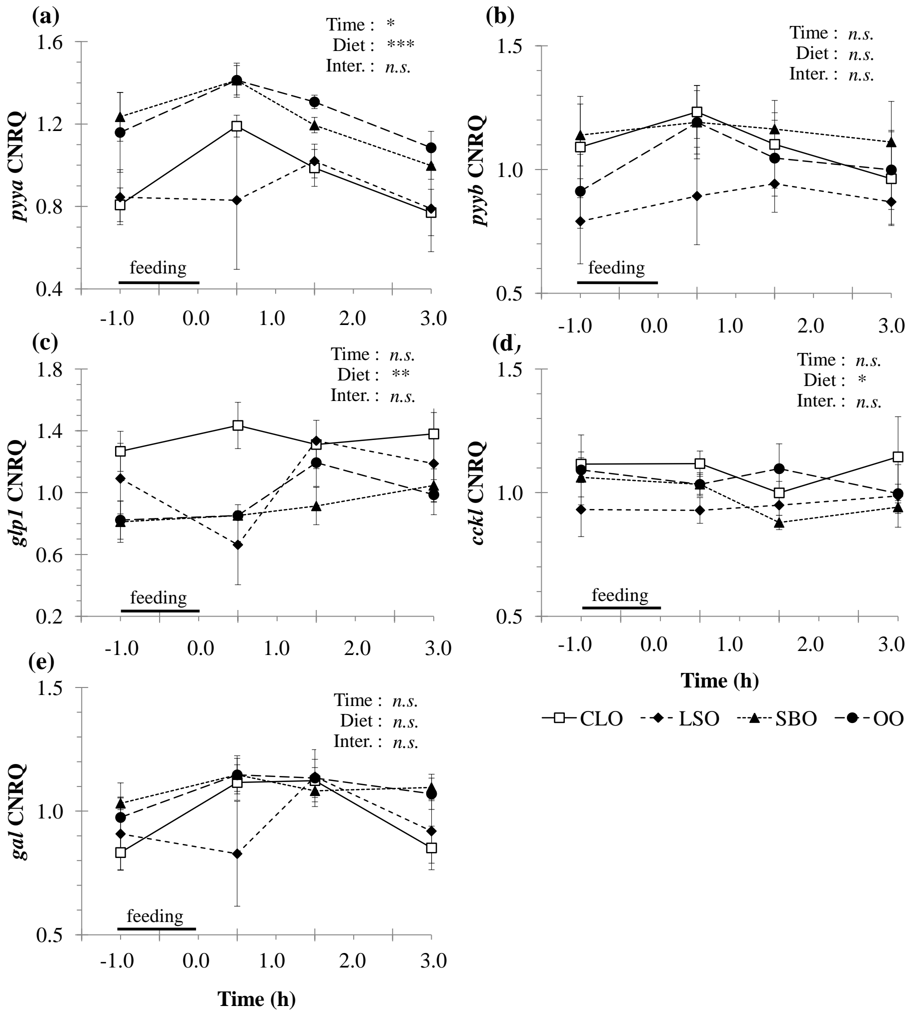


Figure 6. Expression of putative anorexigenic genes *pyya* (a), *pyyb* (b), *glp1* (c) and *cckl* (d) and the orexigenic *gal* (e) genes in the body compartment of Senegalese sole post–larvae at 34 dph, in relation to time of feeding and diet. Larvae were sampled just before the usual feeding time (16 h fasted), then fed for one hour (horizontal bar) on their respective dietary treatment (*Artemia* enriched with either CLO, LSO, SBO or OO) and sampled 0.5, 1.5 and 3 h after end of the meal. Results of 2–way ANOVA analysis are presented beside each chart, where *n.s.* is not significant, * is p<0.05, ** is p<0.01 and *** p<0.001. Values are calibrated normalized relative quantities (CNRQ) ± SE.

analyzed genes and will therefore be discussed in conjunction, since they could reflect responses of different interconnected regulatory mechanisms (Volkoff et al., 2005). Interestingly, *cart1b* expression was unrelated to any of the other genes and, although it did not appear to be regulated by feeding, it was markedly affected by "diet" at both ages, with fish fed *Artemia* metanauplii enriched with CLO having significantly lower transcript levels (at 16 dph compared to OO and at 34 dph to both LSO and SBO treatments). Considering how the CLO diet resulted in the highest food intake, growth and most dissimilar metabolism of the 4 dietary treatments (Bonacic et al., 2016), the results suggest that this *cart* homologue responds to changes in the FA composition of the diet in sole larval and post–larval stages, although the exact mechanism through which it exerts its effect (e.g., if related to an anorexigenic function, roles in energy homeostasis, or some other mechanisms) remains unknown.

Two *pomc* homologues previously identified in the pituitary of Senegalese sole were predicted to give rise to either ACTH (pomca) or α– and β–MSH (*pomcb*; Wunderink et al., 2012). It is well established that POMC and CART are co–expressed in the hypothalamus (Dhillo et al., 2002) and hindbrain (Polakof et al., 2011a) and act together as some of the most potent anorexigenic factors (Volkoff et al., 2006). Since α–MSH is involved in energy balance in fish (Cerdá–Reverter et al., 2003a; Yada et al., 2002), *pomcb* (its putative precursor) is arguably the more relevant *pomc* homolog to consider in the scope of this study. However, this gene did not show any expression similarities to the *cart* genes. On the other hand, *pomca* and *cart2a* had very similar expression patterns in larvae (at 16 dph), with transcript levels in fish fed the CLO diet peaking 30 min after feeding, while levels in the other treatments remained lower. The CLO treatment was, therefore, the only one in which these genes had a response consistent with an anorexigenic function. Post–larvae, on the other hand, had similar expression patterns of *pomca*, *cart1a* and *cart4*. Even though *cart4* was the only transcript significantly affected by the factor "time" (expression increased 1 h 30 min post–prandially compared to t0), all 3 transcripts showed a similar peak in expression 30 min after feeding, specifically in the SBO treatment. However, presently available data does not enable us to speculate on the significance of this result.

Another analyzed gene was *crf*, which codes a peptide that has primarily been associated with stress–related appetite loss (Bale and Vale, 2004)(Bale and Vale, 2004; Matsuda, 2013) and was shown to mediate α–MSH–induced anorexigenic action in the hypothalamus (Matsuda, 2013). In this study, *crf* significantly responded to feeding in 16 dph larvae, but not at 34 dph. However, transcript levels slowly increased with time post–prandially, which differed from the expression patterns of both *pomc* homologues.

Central orexigenic peptides

The neuropeptides NPY and AgRP2 have been established in numerous fish species as potent orexigenic factors, similarly to mammals (Cerdá–Reverter et al., 2000; Doyon et al., 2003; Kehoe and Volkoff, 2007; Peterson et al., 2012; Valen et al., 2011; Volkoff, 2006; Volkoff et al., 2005). However, in this study, the observed changes in gene expression did not always mirror the putative appetite–stimulating role of the peptides.

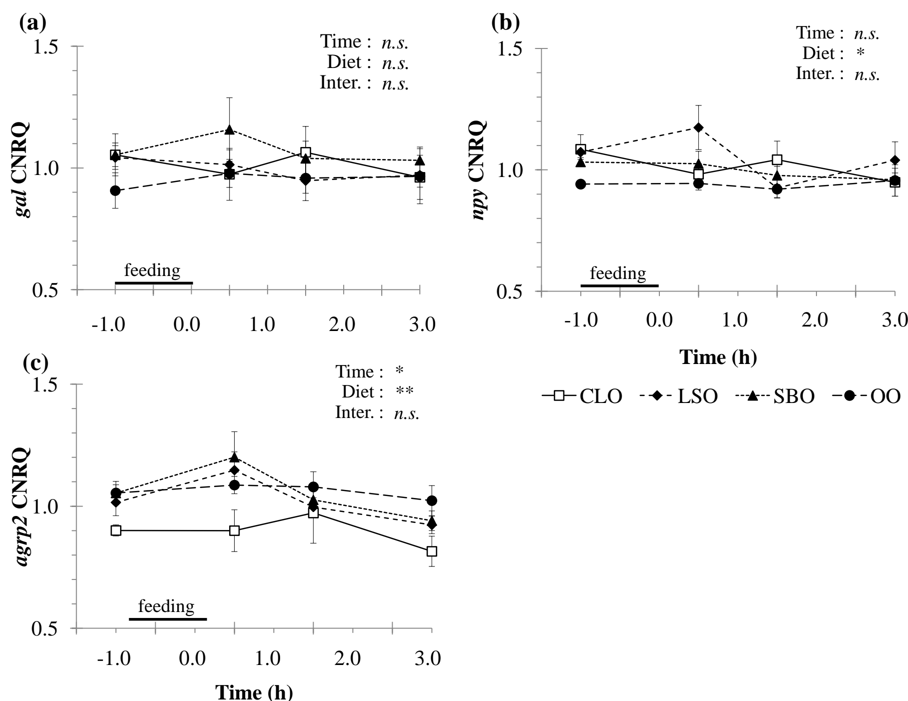


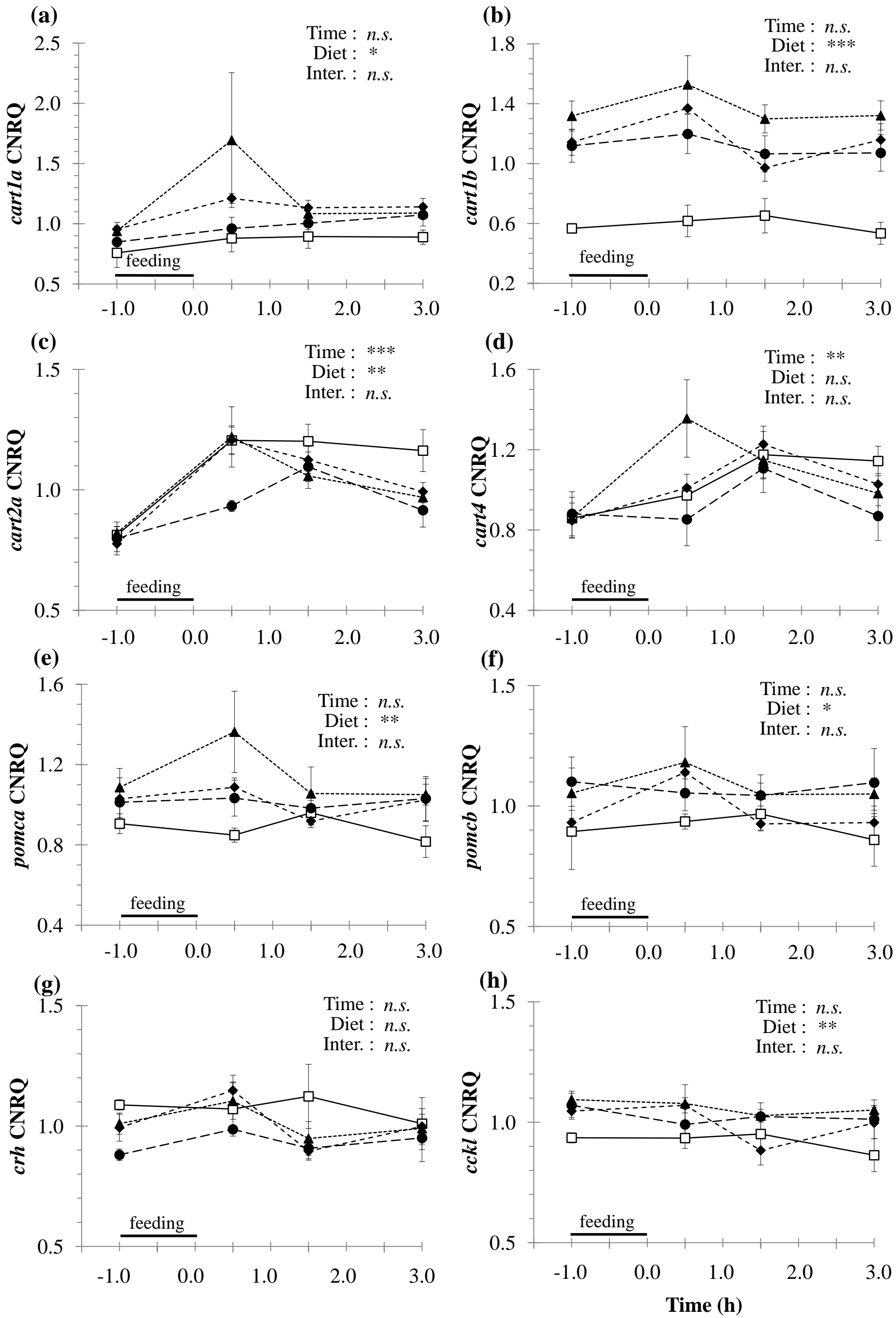
Figure 8. Expression of putative orexigenic genes *gal* (a), *npy* (b) and *agrp2* (c) in the head compartment of Senegalese sole post–larvae at 34 dph in relation to time of feeding and diet. Larvae were sampled just before the usual feeding time (16 h fasted), then fed for one hour (horizontal bar) on their respective dietary treatment (*Artemia* enriched with either CLO, LSO, SBO or OO) and sampled 0.5, 1.5 and 3 h after end of the meal. Results of 2–way ANOVA analysis are presented beside each chart, where *n.s.* is not significant, * is $p<0.05$, ** is $p<0.01$ and *** $p<0.001$. Values are calibrated normalized relative quantities (CNRQ) ± SE.

At 16 dph, the expression of *npy* was significantly affected by the factor "time", and was characterized by a slow but steady increase from just before feeding (16 h fasting) to 3 h after feeding, although this was mainly observed in the LSO and CLO treatments. Furthermore, larvae fed *Artemia* enriched with CLO had the highest post–prandial expression of *npy*. However, in the post–larval stages *npy* was not regulated by feeding, while fish fed the LSO diet had higher expression than those fed the OO diet, mainly due to an increase in the LSO treatment at 30 min post–prandially. Overall, transcriptional changes in *npy* were quite subtle in both stages of development, and apparently affected more by dietary FA profile than by feeding. However, effects were not consistent at the two analyzed ages. Similarly, in Atlantic cod larvae, diet specific modulations of *npy* expression were observed in larvae at 16 dph, while no changes were evident at 29 dph (Kortner et al., 2011). In addition, *npy* levels increased post–prandially in fish fed rotifers enriched with *Rhodomonas baltica*, while no changes were observed in larvae fed unenriched rotifers (Kortner et al., 2011). Furthermore, in Atlantic halibut larvae, food deprivation increased *npy* levels that stayed elevated until 5 h after refeeding (Gomes et al., 2015). These authors suggest that the role of *npy* on appetite regulation is still not fully developed in larvae, which is consistent with the results in this study.

On the other hand, *agrp2* slowly declined post–prandially in larvae at 16 dph, while at 34 dph transcript levels started to drop slightly from 30 mins after feeding onwards (but only in the SBO and LSO treatments). This slow post–prandial decline could be related with its putative orexigenic function. However, *agrp2* levels were generally lowest in sole fed *Artemia* metanauplii enriched with CLO, and significantly so at 34 dph, when expression was unaffected by feeding, which could suggest other possible effects beyond its orexigenic role. In goldfish and zebrafish, *agrp* in the hypothalamus was up–regulated by fasting (Cerdá–Reverter et al., 2003a; Song et al., 2003). However, two *agrp* homologues have recently been described in teleosts (Murashita et al., 2009a). While the *agrp2* homolog seems to have been lost in tetrapod evolution (Braasch and Postlethwait, 2011), in teleosts it has been implicated in the regulation of pigmentation and background adaptation (Zhang et al., 2010). These authors found *agrp2* in zebrafish (*Danio rerio*) to be specifically expressed in the pineal gland and not regulated by metabolic state of the organism. On the other hand, in salmon, *agrp2* was ubiquitously expressed in numerous central and peripheral tissues (Murashita et al., 2009a) and in pufferfish (*Takifugu rubripes*) *agrp2* levels were higher in the tectum mesencephali and hypothalamus, but was also expressed in heart and kidney (Kurokawa et al., 2006). Therefore, differences in the tissue distribution of *agrp2* between fish species might suggest some variation in function, and potentially multiple roles. A characterization of *agrp* homologues has not been performed in Senegalese sole and the necessary molecular tools to analyze the *agrp1* homolog were not available for this study. However, it is noteworthy that the expression pattern of *agrp2* in the current study was very similar to that of *pomcb* (the putative propetide of α–Msh), particularly at 34 dph (at 16 dph the response to feeding, but not to diet, also closely resembled), which could suggest a coordinated activity between the two peptides these genes encode. Although speculative, this hypothesis is worth bearing in mind in the future especially considering how the melanocortin 4 receptor (mc4r) displayed a constitutive activity regulated by agrp binding that may work as a competitive antagonist or inverse agonist to α–Msh in European sea bass *Dicentrarchus labrax* (Sánchez et al., 2009).

Appetite–regulating mechanisms during ontogenetic development

In the natural environment larvae may not require strict appetite regulatory mechanisms as the prey encounter rate may be so low that satiety signals would not be required to prevent overfeeding (Rønnestad et al., 2013). However, in a commercial environment, under constant lighting and food availability, a lack of functional feedback mechanisms could be detrimental. Therefore, it is of great importance to clearly establish when appetite regulation becomes functional in altricial fish larvae (Rønnestad et al., 2013). In a pleuronectiform fish such as Senegalese sole, the drastic ontogenic changes during metamorphosis are accompanied by important behavioral changes with regard to feeding (Fernández–Díaz et al., 2001). These changes are likely to affect appetite–regulating mechanisms, as was observed in the brain of Atlantic halibut larvae, where *cart* levels decreased from before to after metamorphosis, while *npy*, *pyy* and *pomc–c* levels did not change (Gomes et al., 2015), and in whole larvae of cod (*Gadus morhu*a), where *cart* levels increased during this period (Kortner et al., 2011). Similarly, differences were observed here in the patterns of gene expression between the two developmental stages, especially in *cart* homologues (with the exception of *cart1b*).


(a)
Time : n.s.
Diet : *
Inter. : n.s.
cart1a CNRQ
feeding
(b)
Time : n.s.
Diet : ***
Inter. : n.s.
cart1b CNRQ
feeding
(c)
Time : ***
Diet : **
Inter. : n.s.
cart2a CNRQ
feeding
(d)
Time : **
Diet : n.s.
Inter. : n.s.
cart4 CNRQ
feeding
(e)
Time : n.s.
Diet : **
Inter. : n.s.
pomca CNRQ
feeding
(f)
Time : n.s.
Diet : *
Inter. : n.s.
pomcb CNRQ
feeding
(g)
Time : n.s.
Diet : n.s.
Inter. : n.s.
crh CNRQ
feeding
(h)
Time : n.s.
Diet : **
Inter. : n.s.
cckl CNRQ
feeding
Time (h)

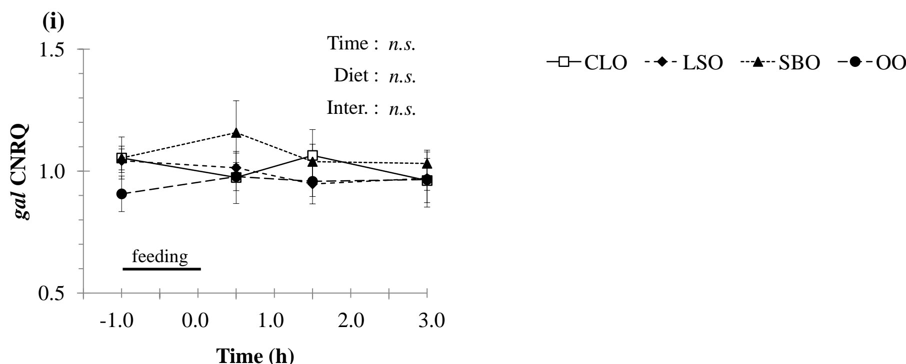


Figure 7. Expression of putative anorexigenic genes *cart1a* (a), *cart1b* (b), *cart2a* (c), *cart4* (d), *pomca* (e), *pomcb* (f), *crf* (g) and *cckl* (h) and the orexigenic gal (i) in the head compartment of Senegalese sole post–larvae at 34 dph, in relation to time of feeding and diet. Larvae were sampled just before the usual feeding time (16 h fasted), then fed for one hour (horizontal bar) on their respective dietary treatment (Artemia enriched with either CLO, LSO, SBO or OO) and sampled 0.5, 1.5 and 3 h after end of the meal. Results of 2–way ANOVA analysis are presented beside each chart, where n.s. is not significant, * is $p<0.05$, ** is $p<0.01$ and *** $p<0.001$. Values are calibrated normalized relative quantities (CNRQ) ± SE.

Furthermore, genes in the pre–metamorphosis larval stage were generally not regulated as would be anticipated from their putative functions, while in post–larvae gene expression was more in line with expected results. However, results from the present study cannot be used to assess potential changes in the mechanisms controlling appetite and energy balance in fish larvae during ontogeny, given the fact that the small size of pre–metamorphic larvae meant that they had to be analyzed whole, while in post–larvae head and body compartments were analyzed separately. Hence, while these differences may be explained by more developed appetite control mechanisms in post–larvae (Rønnestad et al., 2013), we cannot rule out the possibility that sampling of the whole body might have diluted or modified the expression of genes with a wide tissue distribution (Sæle et al., 2009).

5. *Conclusions*

It was previously reported that Senegalese sole pre– and post–metamorphic larvae fed a CLO–enriched diet, richer in FA that are essential for the development of marine fish larvae, such as EPA and DHA, showed superior performance and altered lipid metabolism and transport compared to sole fed prey enriched with vegetable oils (LSO, SBO and OO) (Bonacic et al., 2016). In this study, we further demonstrated that this LC–PUFA–rich diet was associated with a higher food intake, particularly in the larval stage prior to bottom settlement. Additionally, the lack of selectivity or preference towards one type of enriched prey indicated that these results were probably unrelated to specific sensorial cues (olfactory and/or taste signals) associated to the lipid source. Therefore, dietary FA composition likely affected the different mechanisms in the gut–

brain axis involved in food intake regulation and energy homeostasis. Analysis of the transcriptional changes of peripheral and neuropeptides showed a variety of expression patterns in response to both feeding (at fasting and different time points post–prandially) and diet. However, these were often not in accordance with the putative function of the peptides encoded by the analyzed genes (most notably *glp1*), especially during the pre–metamorphic stage. This could suggest a yet underdeveloped appetite regulating system in the early larval stages but it needs to be further addressed in future studies, given the complexity of appetite regulatory mechanisms, in general, and how the interpretation of the results is made difficult by the lack of specific information on the roles played by some of these genes in fish, especially during the larval stages. Nevertheless, it was interesting to note that both larvae and post–larvae fed the CLO diet tended to show the most dissimilar patterns of gene expression, compared to the other dietary treatments, which ties well with the remaining results from this experiment, even if causative links cannot be established at present. Therefore, besides establishing that a diet containing higher levels of LC–PUFA was associated with an increased food intake in Senegalese sole larvae, this study has identified genes that are significantly affected by feeding and/or dietary FA composition and provided a stepping stone for further research to advance the sparse knowledge on appetite regulation in early life stages of fish.

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CHAPTER 8

General discussion

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One of the biggest challenges of modern aquaculture is the necessity to replace fish meal (FM) and fish oil (FO) in aquafeed formulations with more sustainable alternatives. This need has been accentuated by escalating requirements of the rapidly growing aquaculture industry, combined with limited supply and increasing prices of ingredients deriving from capture fisheries (Turchini et al., 2009). Therefore, significant efforts have been directed towards the development of more sustainable aquafeed formulations, and in recent years we have seen an increasing use of vegetable oil (VO) and vegetable meal (VM) ingredients. This has been enabled by intense research initiatives dedicated to understanding and mitigating potential negative effects of these dietary changes, in particular those concerning impacts on fish health, metabolism and product quality (Bell et al., 2001; Jordal et al., 2005; Montero et al., 2003; Nasopoulou and Zabetakis, 2012; Sargent et al., 1999a; Turchini et al., 2010; Turchini et al., 2009; Zuo et al., 2015). However, even though there is some evidence suggesting that vegetable–based diets might have poorer acceptability and reduce food intake, little research has been performed in this area (Benedito–Palos et al., 2007; Ganga et al., 2005; Geurden et al., 2007).

Moreover, information pertaining to mechanisms of appetite regulation in fish is scarce, especially in connection to the nutritional profile of the diet. This is paradoxical considering that appetite directly governs food intake, which has a central role in determining the growth and body composition of cultured fish and that feed is the single highest running cost in semi–intensive and intensive farming operations (Tacon, 1995). Since fish under farming conditions are generally fed pre–set amounts of feed, they cannot compensate for lack of a particular nutrient or energy content by increasing intake, which may lead to lower culture performance (Saravanan et al., 2012). Thus, a primary concern among aquaculturists is to deliver feeds that meet the nutritional requirements of the fish at ration sizes that optimize both growth and FCR, while reducing feed wastage, keeping water quality optimal and generally reducing economic losses (Hasan and New, 2013; Saravanan et al., 2012). In this context, determining the voluntary food intake (VFI) of a cultured species on specific diets is of great importance when developing new feed formulations and establishing optimal feeding practices (Saravanan et al., 2012).

It has been established relatively well that dietary replacements can affect lipid metabolism and energy homeostasis (Turchini et al., 2009), which are interrelated with pathways of appetite regulation (Kaushik and Médale, 1994; Saravanan et al., 2012). Lipogenesis was shown to be inhibited (Alvarez et al., 2000; Kjær et al., 2008; Morais et al., 2012b; Zuo et al., 2015), and β–oxidation promoted (Jordal et al., 2005; Torstensen et al., 2004; Torstensen et al., 2009; Turchini et al., 2003) by diets rich in n–3 long–chain polyunsaturated fatty acids (LC–PUFA), compared to VO–based diets. Furthermore, previous studies have identified the presence of fatty acid (FA) sensing mechanisms in trout and showed that food intake was modified by circulating levels of a long–chain and medium–chain FA (Librán–Pérez et al., 2013a; Librán–Pérez et al., 2012).

Considering the tight interrelations of these processes, several studies involving analyses of food intake, lipid absorption, peripheral satiety signaling (via gastrointestinal peptides), lipid metabolism, hypothalamic lipid sensing and central appetite regulation were performed in the scope of this thesis, in relation to the qualitative and/or

quantitative properties of dietary lipids fed to Senegalese sole (*Solea senegalensis*) at different life stages—larvae, post–larvae and juveniles.

1. *Development of molecular tools and characterization of genes containing multiple transcripts*

The effects associated to the level and FA composition of dietary lipids on genes coding for gastrointestinal and central appetite–related peptides, as well as on genes involved in processes of lipid absorption, metabolism and sensing, were assessed in this thesis by measuring their mRNA levels. For this purpose, molecular tools needed to be developed for Senegalese sole, as most of these genes had not been previously identified in this species. Specific, often fragmented, mRNA sequences of target genes were found using the publicly available transcriptomic database of Senegalese sole (SoleaDB, http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/), which were then assembled *in silico* and completed by rapid amplification of cDNA ends (RACE) PCR. In several cases, more than one transcript was identified, and further characterization work needed to be done to assess which one(s) could be more relevant to focus on, in the context of the objectives of the thesis.

Evidence for the presence of several fatty acid translocase/cluster of differentiation 36 (*fat/cd36*)–like genes was found, but the complete sequence was obtained for only two that seemed more relevant for the objectives of this thesis: lysosome membrane protein 2–like (*lmp2*) and platelet glycoprotein 4–like (*pg4l*). The first homologue resembled a transcript most commonly studied in relation to FA sensing in trout (Librán–Pérez et al., 2012), while the second was closest to the mammalian *Fat/Cd36* gene with roles in orosensory detection of FA and mediating effects of FA on hypothalamic food intake regulation in rats (Gaillard et al., 2008).

Furthermore, 5 different transcripts were found for carnitine palmitoyltransferase 1 (*cpt1*), two of them being splice variants of the same gene. Thus, the 4 genes were annotated as *cpt1.1*, *cpt1.2*, *cpt1.3* and *cpt1.4*, although in 2 of the studies (**Chapter 1 and 2**) where a single *cpt1* gene was analyzed, it was referred to just as "*cpt1*", but corresponded to *cpt1.1*. This transcript was chosen for analysis in the liver and intestine as it showed most similarity to both *cpt1a* and *cpt1b*, described in rainbow trout (*Oncorhynchus mykiss*), where it has been studied in association with liver (Librán–Pérez et al., 2012) and Brockmann bodies (Librán–Pérez et al., 2013c). On the other hand, the *cpt1.2* and *cpt1.3* transcripts had highest similarity with trout *cpt1c* and *cpt1d*, respectively, used in hypothalamic analyses in the context of FA sensing (Librán–Pérez et al., 2013c). The splice variants of *cpt1.4* were the least similar to the transcripts analyzed in trout and had higher expression in the brain of Senegalese sole compared to liver and intestine (our own unpublished results), but were not analyzed in any of the studies.

A total of 7 genes from the fatty acid binding protein (*fabp*) family were identified in Senegalese sole: *fabp1*, *fabp2a*, *fabp2b*, *fabp3*, *fabp6*, *fabp7* and *fabp11b* (our own unpublished results), generally comparable to what has been observed in vertebrates (Storch and Thumser, 2010). Based on transcript distributions examined in adult Senegalese sole tissues, we opted for the analysis of *fabp1*, *fabp2a*, *fabp2b* and/or *fabp3*

in the scope of this thesis (**Chapter 1 and 2**), due their high expression in intestine and liver (our own unpublished results).

A particularly interesting case was that of cocaine– and amphetamine–regulated transcript (*cart*) genes. A total of 7 different homologues were identified for this peptide in Senegalese sole, which presented the highest number of *cart* homologues observed in a vertebrate species, to date. While only one gene has been described in humans (Douglass and Daoud, 1996) and other mammals (Douglass et al., 1995) (excluding an alternatively spliced variant in some rodents), fish seem to show a much greater diversity. Using phylogenetic analyses to observe the evolutionary history of the *cart* gene in vertebrates, supporting evidence for a two–round (2R) whole genome duplication event, occurring at the base of the vertebrate lineage, can be found (**Chapter 4**), with a variable number of genes being lost during tetrapod evolution (Dehal and Boore, 2005). However, the 7 *cart*'s found in sole, 6 *cart*–like transcripts previously identified in medaka (*Oryzias latipes*) (Murashita and Kurokawa, 2011), *Maylandia zebra*, *Oreochromis niloticus*, *Pundamilia nyererei*, *Haplochromis burtoni* and *Xiphophorus maculatus*, and 4 *cart*'s in zebrafish (*Danio rerio*) and *Takifugu rubripes*, suggest a third–round (3R) of whole genome duplication after the appearance of the teleost lineage (Volff, 2005). The retention of 6–7 *cart* genes in highly evolved perciform and pleuronectiform teleost species suggests that different gene products might have adopted a new function ("neofunctionalization") or partitioned old functions ("subfunctionalization") which in mammals are performed by a single gene. While the anorexigenic role of CART on feeding and behavior appears to have been well conserved across the teleost lineage (Kehoe and Volkoff, 2007; Kobayashi et al., 2008; Murashita et al., 2009a; Nishio et al., 2012; Peterson et al., 2012; Subhedar et al., 2011; Valen et al., 2011; Volkoff and Peter, 2001a), not all *cart* homologues seem to be affected by fasting or feeding, as has been observed in several species, including goldfish (CART II), zebrafish (*cart1* and *cart3*) and medaka (five out of six *cart* genes) (Akash et al., 2014; Murashita and Kurokawa, 2011; Volkoff and Peter, 2001a). Furthermore, a different pattern of expression was observed in the brain compartments and peripheral tissues between the 7 characterized homologues in Senegalese sole (**Chapter 4**), which suggests that in addition to feeding and energy homeostasis, *cart* genes from sole could also be involved in other physiological functions that are still unknown. In order to identify the homologues involved in central appetite regulation, expression of all 7 *cart* genes was analyzed in the brain of juvenile sole before and at several times after feeding. Results revealed that only *cart1a*, *cart2a* and *cart4* appeared to be affected by feeding and hence were chosen for later analysis in subsequent studies. A fourth homologue, *cart1b*, was additionally analyzed due to its predominant expression in the brain.

A few other important neuropeptides, for which dedicated characterization was not performed in the scope of this study, have also been described by other authors as having multiple genes. For instance, two proopiomelanocortin (*pomc*) homologues were previously identified in the pituitary of Senegalese sole by Wunderink et al. (2012), which putatively give rise to either ACTH (*pomca*) or α– and β–MSH (*pomcb*). Seeing as α–MSH is involved in energy balance in fish (Cerdá–Reverter et al., 2003a; Yada et al., 2002), *pomcb* (its putative precursor) was arguably the more relevant *pomc* homolog to consider in the scope of this thesis. Nevertheless, both genes were analyzed, but in the nutritional experiment with juvenile sole (**Chapter 6**) the expression of *pomcb* was

highly variable and showed huge fold differences between individuals, unrelated to dietary treatment, which rendered the data unusable. On the other hand, two agouti–related protein (*agrp*) homologues have recently been described in teleosts (Murashita et al., 2009a), one of which seems to have been lost in tetrapod evolution (Braasch and Postlethwait, 2011). It is believed that expression of *agrp1*, characterized in the hypothalamus, regulates energy homeostasis (Cerdá–Reverter and Peter, 2003; Song et al., 2003; Zhang et al., 2010), whereas expression of *agrp2*, located in the pineal gland, is involved in pigment background adaptation (Zhang et al., 2010). Unfortunately, it was not possible to perform a full characterization of both homologues in Senegalese sole in this thesis and RT–qPCR primers were only available for *agrp2*.

2. *Effects of diet composition of food intake in juvenile sole*

In numerous cases, adult and juvenile fish have been shown to adjust their food intake towards dietary energy density (Boujard et al., 2004b; Gélineau et al., 2002; Sæther and Jobling, 2001), but an increasing number of studies have revealed that this is often not the case. Senegalese sole (Guerreiro et al., 2012), European seabass (*Dicentrarchus labrax*) (Peres and Oliva–Teles, 1999a, b) and rainbow trout (Saravanan et al., 2012) have been shown to adjust their food intake more towards dietary protein, than lipid level. On the other hand, a case of hyperphagia towards very high dietary lipids was also observed in another study with Senegalese sole (22% compared to 17%, 14%, 8% and 4% dietary lipids) (Borges et al., 2009) and in gilthead seabream (*Sparus aurata*) (30% compared to 24%, 18% and 12% dietary lipids) (Peres and Oliva–Teles, 1999a). Similarly, turbot (*Scophthalmus maximus*) ingested more of a 17%, compared to 7% lipid diet, although only when dietary protein levels were low (55%, compared to 60% and 65%) (Cho et al., 2005). However, in yet another study with Senegalese sole, food intake was not regulated in response to dietary lipid–to–carbohydrate levels (Dias et al., 2004), similar to what was observed in both Atlantic salmon (*Salmo salar*) (Helland and Grisdale–Helland, 1998) and in a study with rainbow trout (Figueiredo–Silva et al., 2012a). These results, although variable, indicate that food intake in fish is more than just energy–dependent and likely governed by complex appetite regulating mechanisms that are also sensitive to specific nutrients, as has been suggested in mammals (Karhunen et al., 2008; Raben et al., 2003). Nevertheless, the effects of dietary energy and macronutrient content are likely closely interrelated.

In the present thesis, food intake in Senegalese sole juveniles (**Chapter 6**) was likely influenced by both lipid level and lipid source, since equally high levels of VO in the diet did not seem to satiate the animals as effectively as FO. This suggests that absolute dietary FA quantities (µg/mg dry weight; DW) may have been the determinant for lower VFI values observed in the high lipid (18%) FO diet. However, it is important to note that both VO diets had only 75% of their FO replaced by VO, and a certain amount of LC–PUFA originating from the lipid component of the FM was also present. Figueiredo–Silva et al. (2012) performed a similar study with rainbow trout juveniles fed diets differing in lipid level (5% and 15%) and source (FO and coconut oil—high in saturated fatty acids; SFA), but did not observe any variation in food intake between treatments. Conversely, results from another similar study, although with larvae of

gilthead seabream, showed that fish ingested more of a low– than high–fat diet when the lipid fraction was based on soybean oil (SBO), but more of the high– than low–fat diet when FO was used (Morais et al., 2006). Both studies were contradictory to what was observed in sole juveniles in this thesis, although the high importance of dietary LC–PUFA to fish during early developmental stages may have influenced the results reported by Morais et al. (2006). On the other hand, in another study with rainbow trout, a preference towards FO, rapeseed oil or a blend of FO and olive oil (OO) was observed, compared to linseed oil (LSO), in a demand feeding system (Geurden et al., 2007). Although it is uncertain whether the lower demand for LSO was a result of satiating feedback mechanisms of the ingested feed, or its sensory properties, this study showed that appetitive behaviour in trout was mediated by the dietary oil source. Finally, studies with FA sensing mechanisms in rainbow trout reported different effects between circulating medium–chain (octanoic acid) and long–chain (oleic acid; OA) FA on the expression of hypothalamic appetite–regulating peptides and appetite (Soengas, 2014), while variations in dietary lipid level did not seem to have an effect (Librán–Pérez et al., 2015a).

3. *Absorption of lipids and gastrointestinal peptide expression in juvenile Senegalese sole*

In juvenile Senegalese sole, transcriptional changes of several genes collectively responsible for FA uptake from the intestinal lumen, intracellular transport and packaging into lipoproteins, did not indicate a response to either dietary lipid level or lipid source. This lack of response may have been indicative of an overall poor capacity to transcriptionally regulate intestinal FA absorption in order to adapt to high dietary lipid levels and might explain the accumulation of lipid droplets in the intestinal epithelia of sole fed high lipid diets (**Chapter 2**). However, Senegalese sole juveniles were previously characterized as having high apparent digestibility coefficients for dietary lipids and an increased flux of triglycerides through the intestinal epithelia when fed high lipid diets (Borges et al., 2013a). Nevertheless, the increase in secretion observed by Borges et al. (2013a) may have not been sufficient to transport all absorbed lipids from the enterocytes, leading to significant lipid accumulations when fish were exposed to high–fat diets over a long period of time.

One of the few regulated exceptions was the *pg4l* (*fat/cd36*–like) gene, which was characterized by having lower mRNA levels in fish fed high lipid diets (pre– and post–prandially), and by being up–regulated in fish fed the FO treatments post–prandially. This was opposite to an expected up–regulation stimulated by high dietary lipid levels, as seen in rats (Petit et al., 2007; Poirier et al., 1996; Sukhotnik et al., 2001), suggesting that it may have been unrelated to FA absorption. In mammals, this gene has very commonly been related to orosensory and intestinal perception of fats (Gaillard et al., 2008; Khan and Besnard, 2009; Sundaresan and Abumrad, 2015) and associated with secretion of GLP–1 through stimulation by oleoylethanolamide (Diep et al., 2011; Fu et al., 2003; Gaetani et al., 2003; Guijarro et al., 2010; Lauffer et al., 2009; Oveisi et al., 2004; Piomelli, 2013; Schwartz et al., 2008). Interestingly, in this thesis (**Chapter 1**), *pg4l* expression in juvenile sole was similar to that of most of the analyzed

gastrointestinal peptides (in response to dietary lipid level; **Chapter 6**), which may suggest a regulation via luminal FA sensing mechanisms that activate the enteroendocrine release of gastrointestinal satiety peptides. Although we cannot conclude anything from this circumstantial evidence, these results may help the design of future studies in fish, which should focus on the implication of *fat*/*cd36* genes (particularly *pg4l*) in these systems, but also that of G protein–coupled receptors (Little and Feinle–Bisset, 2010; Miyauchi et al., 2010; Vangaveti et al., 2010; Yonezawa et al., 2013).

Table 1. Summary of gene expression and enzymatic activity related to lipid absorption, metabolism and enteroendocrine satiety signaling in juvenile Senegalese sole, that presented significant (or only marginally non–significant) differences pre–prandially (0 h) and at 6 h post–prandially, based on a two–way ANOVA (lipid level × source).

Tissue	Analysis type	Gene/ Enzyme	0 h				6 h			
			8%	18%	FO	VO	8%	18%	FO	VO
Gut sensing and/or absorption										
Int	gene	*cd36 (pg4l)*	↑	↓	–	–	↑	↓	↑	↓
Extracellular transport										
Int	gene	*mtp*	–	↑18VO	–	–	–	–	–	–
Lipogenesis										
Liver	enzyme	Acly	–	–	↓	↑	n/a	n/a	n/a	n/a
Liver	gene	*fas*	↑	↓	–	–	–	–	–	–
Int	gene	*fas*	↑	↓	–	–	–	–	–	–
β–oxidation										
Liver	gene	*cpt1*	↓	↑	–	–	–	–	↑*	↓*
Int	gene	*cpt1*	–	–	–	–	↑8VO	–	–	↑8VO
Satiety signaling										
Int	gene	*pyya*	↑	↓	–	–	–	–	–	–
Int	gene	*pyyb*	↑	↓	–	–	↑	↓	–	–
Int	gene	*glp–1*	↑	↓	–	–	–	–	–	–
Int	gene	*cckl*	↑*	↓*	–	–	–	–	–	–
Int	gene	*gal*	–	–	–	–	↑	↓	–	–

*marginally non–significant (p=0.06); *cd36* (*pg4l*), cluster of differentiation 36 family (platelet glycoprotein 4–like); *mtp*, microsomal trygliceride transfer protein; Acly, ATP citrate lyase; *fas*, fatty acid synthase; *cpt1*, carnitine palmitoyltransferase 1; *pyy*, peptide YY; *glp1*, glucagon–like peptide 1; *cckl*, cholecystokinin (Leu) precursor; *gal*, galanin.

Diets containing high levels of lipids also tended to reduce the basal expression of putatively anorexigenic enteroendocrine peptides (significantly for two peptide yy transcripts *pyya* and *pyyb*; and for glucagon–like peptide 1, *glp1*) in Senegalese sole juveniles, while expression of *pyyb* and the orexigenic galanin (*gal*) were similarly affected 6 h after feeding (Table 1). However, it is possible that the post–prandial window of activity, induced by food entering the gut, was missed for these genes (further discussed below in the context of larvae). Furthermore, this was not in accordance with

VFI values observed in this experiment (**Chapter 6**), at both basal and post–prandial timepoints, where fish fed 18FO had lower food intake than the remaining treatments, which may suggest the involvement of other satiety signaling mechanisms, including: increases in post–prandial circulating metabolites (activating hypothalamic FA sensors), hepatic oxidative lipid metabolism and/or lipostatic signals associated with long–term lipid stores.

Although most differences were observed pre–prandially, which may imply a long–term adaptation to the properties of these diets, the regulatory effects of dietary lipid level (no regulation by lipid source was observed) on these genes was generally not in accordance with their putative functions (further discussed below in the context of larvae). Overall, there is not enough information to conclude on the cause(s) of the seemingly inverse regulation and effects of the peripheral peptides in juvenile sole, although we can speculate on a few possibilities: potential alternative roles and/or regulatory mechanisms of the analyzed peptides; existence of additional post–transcriptional regulatory mechanisms (although a lack of regulation at the transcriptional level is usually characteristic in this case) or desensitization of intestinal FA receptors, as observed in mice fed high–fat diets (Richards et al., 2015). However, these are just speculations and the regulatory mechanisms behind gastrointestinal peptides in Senegalese sole juveniles still remain elusive, but our results draw attention to an interesting area for future research, on which information in teleosts is scarce.

4. *Juvenile performance and lipid metabolism*

Other studies have generally shown that both adult and juvenile Senegalese sole do not perform well with high dietary lipid levels (≥12%) (Borges et al., 2009; Valente et al., 2011). Borges et al. (2013a) suggested that potential issues concerning growth performance of juveniles fed high–lipid diets may lie in metabolic, rather than digestive, processes. However, in the experimental conditions of the current thesis, a high dietary lipid level (18%) did not appear to affect growth performance of Senegalese sole juveniles. On the other hand, fish grew slightly (marginally non–significantly) better when fed FO–, compared to VO–based diets. In a previous study, growth of juvenile Senegalese sole was not compromised when substituting dietary FO up to 100% with a blend of VO (rapeseed, soybean, and linseed oil in a ratio of 3:2:5) at 9% lipid levels (Borges et al., 2014a). However, Benítez–Dorta et al. (2013) observed a lower growth performance when sole were fed 12% lipid diets formulated exclusively with SBO, compared to FO or linseed oil–based diets. Taking into account that the dietary VO used in the current thesis was a blend of rapeseed, SBO and LSO in equal proportions (1:1:1), this could suggest that juvenile sole performance can vary somewhat depending on the oil type or blend used, as has often been observed in other fish species (Turchini et al., 2009), with high inclusions of the n–6 polyunsaturated FA (PUFA)–rich SBO possibly having negative effects (further discussed below in the context of larvae). However, while growth was only slightly affected in this study, the diets had different effects on hepatosomatic index (HSI), viscerosomatic index (VSI), lipid deposition and

metabolism. Values of HSI and VSI were higher in fish fed high–lipid diets, although this seemed mainly (HSI) or exclusively (VSI) caused by the VO content.

The effects of lipostatic signals (i.e. via leptin) on food intake and energy homeostasis were not investigated in these studies. However, the additional histological, biochemical and biometric data obtained from the fish may allow for an approximation of the animals' fat reserves, which have been positively correlated with leptin production and the accompanying anorexigenic stimulus in mammals (Brennan and Mantzoros, 2006). Similar evidence has also been reported in fish (Johansen et al., 2002; Johnson et al., 2000), although leptin in teleosts seems to be mainly expressed in the hepatic and not adipose tissue (Chisada et al., 2014; Kurokawa and Murashita, 2009).

Lipid metabolism, as assessed by gene expression and activity of a few key enzymes, seemed to be affected more by dietary lipid level than source, especially at basal levels, where fish fed the higher lipid diets had increased catabolic (β–oxidation) and suppressed anabolic (lipogenesis) pathways (Table 1). This was likely a result of endogenous homeostatic regulation, affected by feedback mechanisms from significantly higher total body lipid stores in fish fed high lipid diets. Although less clearly, results also suggest that LC–PUFA affected these pathways in the liver, as indicated by a reduced ATP–citrate lyase (Acly) enzymatic activity at fasting. This could have been related to a significantly lower accumulation of lipids in hepatocytes, observed histologically in fish fed FO diets, and would be in accordance with the hypotriglyceridemic effect of n–3 LC–PUFA that has been well established in both fish (Bell et al., 2001; Jordal et al., 2005; Morais et al., 2012b) and mammals (Davidson, 2006). Post–prandially (6 h after a meal), only an effect of lipid source was visible on gene expression of *cpt1* in liver (marginally non–significantly higher in FO diets) and intestine (higher only in the 8VO treatment), probably as a short–term effect of the ingested feed.

A possibility that the anabolic and catabolic pathways in the liver influence food intake through hepatic vagal afferents in trout was suggested by Figueiredo–Silva et al. (2012), based on mammalian models (Allen et al., 2009; Berthoud, 2008). Furthermore, results from mammalian studies suggest that fat that is oxidized has a satiating effect, whereas fat that is stored does not, which may link lipid fuel partitioning in the liver and the control of eating behavior (Friedman, 1998). However, no interaction of lipid level and source was observed that may have been correlated to the low VFI exclusively in juvenile sole fed the 18FO treatment (**Chapter 6**). On the other hand, hepatic β–oxidation (*cpt1* expression) was generally higher and lipogenesis (*fas* expression) lower in the 18% lipid diets, while lipogenesis (Acly activity) was lower in fish fed FO.

Nevertheless, this trend in intermediate metabolism was only visible in fish pre–prandially (during fasting), while fish exhibited almost no post–prandial (6 h after feeding) effects. Thus, these values were likely the result of steady–state homeostatic regulation and not the other way around. In any case, given the present experimental setup and the tight integration of lipid metabolism in the complex and dynamic mechanisms constantly regulating energy homeostasis, it is very difficult to isolate this pathway as a source of appetite regulation.

5. *Hypothalamic FA sensing mechanisms in juveniles*

Nutrient sensing is the capability of specific neurons in the hypothalamus to detect changes in the levels of circulating metabolites (Blouet and Schwartz, 2010; López et al., 2007). In the context of this thesis, a hypothalamic mechanism of controlling appetite through processes that sense the presence of long–chain free FA, is discussed. It is a very novel area of research in fish that, to date, has only been examined in one species—rainbow trout (Librán–Pérez et al., 2013a; Librán–Pérez et al., 2015a; Librán–Pérez et al., 2012). Oleic acid is the most studied long–chain FA in this context and has been shown to activate several lipid sensing systems in both mammals (Blouet and Schwartz, 2010; López et al., 2007) and rainbow trout (Librán–Pérez et al., 2012). However, these systems in rainbow trout responded not only to OA but, unlike mammals, also to octanoic acid (a medium–chain FA) (Soengas, 2014). On the other hand, the ability of other types of long–chain FA to elicit the activation of these systems has scarcely been studied, especially in fish.

In trout, activation of FA sensing systems was associated with an up–regulation of anorexigenic neuropeptide expression and decreased food intake (Librán–Pérez et al., 2013a; Librán–Pérez et al., 2013b; Librán–Pérez et al., 2013c; Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012). In the scope of this thesis, an experiment was performed to investigate the presence and regulation of these mechanisms in Senegalese sole juveniles, as well as their effects on food intake and central appetite regulation (**Chapter 5**). The array of tested long–chain FA was expanded to include stearic acid (SA), OA, ALA and EPA, in order to additionally test the sensitivity of these systems to changes in FA chain length and degree of saturation. These FA were administered through intraperitoneal injections, after which food intake and the hypothalamic mRNA abundance of the neuropeptides *agrp, npy, pomc* and *cart*, was measured, in addition to evaluating other components of the putative FA–sensing systems: i) FA metabolism through inhibition of Cpt1; ii) FA binding to Fat/Cd36 and modulation of transcription factors like peroxisome proliferator–activated receptor type α (Pparα) and sterol regulatory element–binding protein type 1c (Srebp1c); iii) activation of protein kinase C–θ; and iv) mitochondrial production of reactive oxygen species by electron leakage, resulting in an inhibition of ATP–dependent inward rectifier potassium channel activity (Blouet and Schwartz, 2010; López et al., 2007).

Results showed that the FA sensing systems characterized in rainbow trout were also active in the hypothalamus of Senegalese sole, indicating that this capacity can also be found in a marine fish species (**Chapter 5**). The FA–sensing systems were activated by OA in a way similar to that previously described in both trout (Librán–Pérez et al., 2013c; Librán–Pérez et al., 2014a; Librán–Pérez et al., 2012) and mammals (Blouet and Schwartz, 2010; López et al., 2007), highlighting their important role in vertebrates. However, for the first time in any vertebrate species, an n–3 PUFA (in this case ALA) was shown to activate transcription factor components (*srebp1c* and *ppar*α) of the hypothalamic FA sensing system. Furthermore, OA and ALA had similar effects on hypothalamic intermediate metabolism to what was previously observed in trout after IP injections of OA (Librán–Pérez et al., 2012). These FA mainly promoted hypolipidemic pathways (down–regulation of acetyl–CoA carboxylase, *acc*; *acly* and lower activity of Acly in comparison to the control; down–regulation of *fas* in only in OA), although

lower activity of Cpt1 was also observed. Lipid sensing signals mediated through the intermediate lipid metabolism of the hypothalamus are likely integrated with the malonyl–CoA mechanism of regulating appetite (Wolf, 2006; Wolfgang and Lane, 2006). This is in accordance with the results of this experiment, as malonyl–CoA is an intermediate of lipogenesis, produced by the activity of Acly and Acc, that inhibits Cpt1 (Henderson, 1996). High levels of hypothalamic malonyl–CoA are indicative of an energy surplus in the body and stimulate the release of anorexigenic peptides, putatively through the inhibition of a brain–specific Cpt1 (Obici et al., 2003; Price et al., 2002; Wolf, 2006; Wolfgang and Lane, 2006). Furthermore, it is important to note that this is also connected to increased peripheral energy expenditure (Wolfgang and Lane, 2006), and that Cpt1 inhibition in the liver has an opposite effect on appetite, contrary to the hypothalamic Cpt1 (Obici et al., 2003). Activation of these mechanisms may have resulted in the down–regulation of the orexigenic *agrp2* in the hypothalamus, observed after IP injections of both OA and ALA, which suggested a decrease in appetite.

On the other hand, IP injections of EPA also caused several changes, but in fewer and different parameters than OA and ALA, with less clear effects. On one hand, they induced a reduction in β–oxidation through reduced Hoad activity but, on the other, increased *cpt1.3* expression. However, EPA significantly increased mRNA levels of the anorexigenic *cart2b*, although OA and ALA exhibited a similar, but not significant, trend.

Finally, the study also obtained evidence on the lack of response of FA sensing systems to a saturated FA such as SA. This was comparable to the lack of effects of palmitic (Greco et al., 2014) and SA in mammals (Schwinkendorf et al., 2011). Thus, while OA and ALA activation of FA sensing systems was observed in Senegalese sole juveniles, possibly causing subsequent changes in appetite regulation, eventual effects of circulating EPA were not very evident, and SA didn't seem to elicit any response. However, despite the different effects on FA sensing mechanisms caused by the different FA in this study, there were no significant reductions in food intake, although a slight decrease was observed in all treatments compared to the control. Thus, while results of this study demonstrate the existence of FA sensing systems in Senegalese sole that respond differently to FA differing in chain length and degree of saturation, additional work is necessary to further characterize the possible involvement of these mechanisms in the regulation of food intake and how they are integrated with other established pathways.

6. *Central appetite regulation in juvenile Senegalese sole*

As discussed above, Senegalese sole juveniles exhibited lower food intake when fed a high–lipid FO diet. The expression of central neuropeptides putatively involved in appetite control was expected to closely relate to VFI values, but instead showed mixed results in response to the nutritional properties of the diets (**Chapter 6**). Interestingly, the basal expression of neuropeptides was not modulated by any of the tested dietary factors, suggesting no long–term adaptation to lipid level or source. In this context, we can assume that transcriptional responses were the result of the ingested diets, rather than being driven by steady–state feedback mechanisms from bodily fat reserves. In fact,

while whole–body lipid reserves were higher in fish fed high–lipid diets, hepatic levels were lower in the FO treatments, which goes against a lipostatic control of appetite (**Chapter 2**). However, there was also no obvious relation between the expression of genes coding for gastrointestinal appetite–related peptides and VFI (**Chapter 2**) and the effect of circulating FA on mechanisms likely to suppress appetite was more pronounced when OA and ALA, rather than EPA, were injected intraperitoneally (**Chapter 5**). Furthermore, it is important to note that the fish were tube fed when collecting samples for RT–qPCR analysis of appetite–related genes, thus bypassing all sensory stimuli prior to feed entering the esophagus. Nevertheless, we cannot rule out possible effects of the diet's olfactory or gustatory properties on the VFI results, which were calculated from measurements performed in normal feeding conditions over the 3–month culture period.

In Senegalese sole juveniles, only the expression of *cart1a* and *cart1b* was significantly affected by dietary lipid source (**Chapter 6**). Both genes showed an increase in mRNA levels in the FO treatments after feeding, which was in line with their putative anorexigenic role. Furthermore, transcript levels of *cart1a* and *cart1b* peaked earlier (1 h after eating a single meal) in fish fed 18FO, compared to 8FO (3 h after feeding), which might suggest that the activation of anorexigenic pathways occurred earlier when FO was present at higher level in the diet. These results might offer a mechanistic explanation to the lower VFI measured in the 18FO treatment throughout the experiment. Regarding the remaining central appetite–related genes, most (*cart1b*, *cart4*, *pomca*, *cckl*, *npy*, *agrp2*) appeared to respond transcriptionally to feeding and showed temporal changes in expression during the post–prandial phase, with only slight differences being noticed in relation to lipid level.

7. *Food intake and selectivity in larvae and post–larvae*

Numerous methods for quantifying ingestion of live prey in altricial marine larvae have been used over the years, including measuring clearance rate from the water column or the fish digestive tract (Fushimi, 1983; Keckeis and Schiemer, 1992; Pedersen, 1984), gravimetric methods (Kamler et al., 1986), staining (Lessard et al., 1996; Planas and Cunha, 1999), using inert markers (Cook et al., 2008; Garcia et al., 1998) and radioactive or stable isotopes (Boehlert and Yoklavich, 1984; Conceição et al., 1998; Govoni et al., 1982; Morais et al., 2006; Sorokin and Panov, 1966; Tandler and Mason, 1984). However, these have proven difficult to standardize and implement on a regular basis as they were either imprecise, laborious, expensive or required special equipment and licenses (in the case of radioactive isotopes), which are not accessible to many research groups. Thus, for the purpose of this thesis, a new method of labeling live prey (in this case *Artemia* metanauplii) using fluorescent microspheres was developed and implemented to quantify intake of larvae and post–larvae of Senegalese sole (**Chapter 7**) fed diets differing in FA composition.

Unlike the experiment with juvenile fish, Senegalese sole larvae and post–larvae were fed isolipidic diets (around 100 mg g^{-1} DW of enriched *Artemia*), but with a wider range of FA profiles (**Chapter 3** and **7**). The low LC–PUFA requirements of Senegalese sole larvae (Conceição et al., 2007; Dâmaso–Rodrigues et al., 2010; Morais and Conceição, 2009; Morais et al., 2004; Villalta et al., 2005b), allowed for the rare

opportunity of testing diets practically void of these FA in marine fish during early stages of development. These were prepared by enriching *Artemia* metanauplii with an emulsion of one of four different oils: cod liver oil (CLO), LSO, SBO, and olive oil (OO), which are predominantly rich in LC–PUFA, n–3 C_{18} PUFA, n–6 C_{18} PUFA or monounsaturated FA (MUFA), respectively. Based on these findings, and also those of juvenile Senegalese sole, it seems that dietary FA composition (possibly in absolute, rather than just relative, terms) plays an important role in food intake regulation in this species.

Furthermore, it is important to note that the larvae and post–larvae in the food intake experiment (**Chapter 7**) were fed exclusively on *Artemia* metanauplii, and thus no differences in intake between treatments could be associated to visual stimulus. However, since each prey group was enriched with a different oil emulsion, they may have given off specific sensorial cues (olfactory and/or taste signals) to the fish, which could have interfered with internal homeostatic mechanisms in determining food intake. To account for this possibility, an experiment to determine if Senegalese sole larvae would preferentially ingest one dietary treatment over another, had to be performed. This was also tested using fluorescent microspheres which, given their availability in different colours, allowed for the differential labeling of prey items enriched with different emulsions. An equal mixture of the different preys was then administered to the larvae, and later individually quantified in larvae samples using fluorescent spectrophotometry or flow cytometry. The results from this experiment did not show differences in ingestion of any of the diets, regardless of the FA profile of the enriched *Artemia* metanauplii, although the OO–based diet might have been slightly less preferred. Therefore, we could rule out interference from exogenous sensory cues that might have affected some of the regulatory mechanisms of appetite analyzed in larvae and post–larvae in the scope of this thesis.

8. *Expression of peripheral satiety peptides*

In order to tackle the question of whether dietary FA composition could affect feeding via putative peripheral mechanisms of appetite regulation during early ontogenic development, the expression of peripheral satiety peptides was analyzed in both larvae (whole body; Table 2) and post–larvae (body without head; Table 3), before and 0.5 h, 1.5 h and 3 h after a meal (**Chapter 7**). Interestingly, the most pronounced differences between treatments were generally observed 30 min after feeding and mainly in post–larvae (in larvae only *pyyb* was significantly affected). This would be in accordance with a fast reaction of these genes to food entering the gut, as has been observed in later life stages of other fish species (Aldman and Holmgren, 1995; Murashita et al., 2007; Murashita et al., 2008; Peyon et al., 1999), and may signify the presence of gastrointestinal satiety feedback mechanisms sensitive to dietary FA composition in younger life stages of Senegalese sole. Furthermore, it further suggests that analyses performed in juvenile sole (**Chapter 6**) may have been measured after this short–term post–prandial window of activity.

Table 2. Summary of gene expression related to lipid absorption, metabolism and enteroendocrine satiety signaling in Senegalese sole larvae (16 dph). Only significant results from one–way (in relation to diet; pre–prandially—0 h, or at 6 h post–prandially) or 2–way ANOVA (diet × time; 0 h – before feeding; 0.5 h, 1.5 h and 3 h – after feeding) are presented.

Gene	Time	Diet				Time			
		CLO	LSO	SBO	OO	0 h	0.5 h	1.5 h	3 h
Intercellular transport									
fabp3	3 h	↓	–	–	↑	n/a	n/a	n/a	n/a
Extracellular transport									
apoa4	0 h	↑	–	–	↓	n/a	n/a	n/a	n/a
Lipogenesis									
fas	0 h	↓	–	–	↑	n/a	n/a	n/a	n/a
Biosynthesis									
Δ4fad	0 h	↓	↑	–	–	n/a	n/a	n/a	n/a
Δ4fad	3 h	↓	–	–	↑	n/a	n/a	n/a	n/a
Satiety signaling									
pyyb	n/a	↑	–	–	↓	–	–	–	–
glp–1	n/a	–	–	–	–	–	↓	–	↑
gal	n/a	↑	–	–	↓	–	–	–	–

fabp, fatty acid binding protein; *apoa4*, apolipoprotein A4; *fas*, fatty acid synthase; *Δ4fad*, Δ4 fatty acid desaturase; *pyy*, peptide YY; *glp1*, glucagon–like peptide 1; *gal*, galanin.

Table 3. Summary of gene expression related to lipid absorption, metabolism and enteroendocrine satiety signaling in Senegalese sole post–larvae (34 dph). Only significant results from one–way (in relation to diet; only in the pre–prandial phase; 0h) or 2–way ANOVA (diet × time; 0 h – before feeding; 0.5 h, 1.5 h and 3 h – after feeding) are presented.

Gene	Time	Diet				Time			
		CLO	LSO	SBO	OO	0 h	0.5 h	1.5 h	3 h
Extracellular transport									
apoa4	t0	↑	–	–	↓	n/a	n/a	n/a	n/a
Lipogenesis									
fas	t0	↓	–	↑	↑	n/a	n/a	n/a	n/a
Biosynthesis									
Δ4fad	t0	↓	↑	–	↓	n/a	n/a	n/a	n/a
Satiety signaling									
pyyb	n/a	↓	↓	↑	↑	–	↑	–	↓
glp–1	n/a	↑	–	↓	↓	–	–	–	–

apoa4, apolipoprotein A4; *fas*, fatty acid synthase; *Δ4fad*, Δ4 fatty acid desaturase; *pyy*, peptide YY; *glp1*, – glucagon–like peptide 1.

Given the putative anorexigenic function of *glp1* (Polakof et al., 2011a; Silverstein et al., 2001; Sundby et al., 1991) and *cck* (Rubio et al., 2008) in other fish species, the high expression levels in post–larvae fed the CLO treatment were not in accordance with results of food intake (although the higher trend of CLO ingestion was not significantly different from the other treatments after metamorphosis). On the other hand, mRNA levels of *pyya* in post–larvae generally peaked slightly after feeding and were also higher in the SBO and OO treatments compared to CLO and LSO, which, on both accounts seemed in accordance with an anorexigenic function. However, the regulation of this peptide has shown contradicting results in different fish species (Gonzalez and Unniappan, 2010; Murashita et al., 2008; Murashita et al., 2009b).

The results of this study may suggest that Senegalese sole might have underdeveloped peripheral appetite regulating systems in the early larval stages, as has been considered by Rønnestad et al. (2013) but, given the complexity of appetite regulatory mechanisms in general, and how the interpretation of the results is made difficult by the lack of specific information on the roles played by some of these genes in fish, this needs to be further addressed in future studies.

9. *Performance and lipid metabolism of larvae and post–larvae*

As might have been expected, larvae reared on the CLO treatment showed significantly improved growth and survival, faster onset of metamorphosis and maturation of the intestine, as observed in the relative activities of the intestinal brush border membrane enzyme alkaline phosphatase (AP) in relation to the intracellular leucine–alanine peptidase (Leu–ala) (**Chapter 3**). However, they were closely followed in performance by those fed the LSO treatment, which completed metamorphosis at a similar time and had the second best growth and survival by the end of the experiment. Interestingly, gene expression profiles of larvae, and especially post–larvae, from the LSO treatment were similar to those in larvae fed CLO. This included decreased transcript levels of genes involved in anabolic pathways such as lipogenesis (fas) and LC–PUFA biosynthesis (Δ4 fatty acid desaturase; Δ4fad; Table 2 and 3) and increased of those related to phospholipid metabolism, lipoprotein secretion, lipid transport and hydrolysis (Chapter 3). These results suggested that apart from oil sources rich in LC–PUFA, those rich in n–3 C18 PUFA (ALA) may be beneficial to Senegalese sole larvae, compared to diets with prevailing n–6 C18 PUFA (LNA) or MUFA. Similarly, LNA has previously been shown to have a negative effect on juvenile and adult fish growth performance (Montero et al., 2008; Montero et al., 2010), non–specific immunity (Wu and Chen, 2012; Zuo et al., 2015), spawning, larval quality (Liang et al., 2014) and FA and lipid metabolism (Berge et al., 2009; Robaina et al., 1998; Rollin et al., 2003), when representing a high fraction of total dietary FA. On the other hand, both n–3 LC–PUFA and ALA were able to mitigate the negative effects of LNA in salmon and freshwater fish (Berge et al., 2009; Blanchard et al., 2008; Chen et al., 2013; Menoyo et al., 2007; Rollin et al., 2003; Senadheera et al., 2010; Tan et al., 2009). In the context of this study, we suggest that an enhanced phospholipid metabolism and apolipoprotein synthesis in the CLO and LSO treatments likely represents the main underlying mechanisms explaining the superior performance of Senegalese sole larvae and post–larvae fed LC–

PUFA–rich, and to an extent, ALA–rich diets. In this respect, it has been well established that dietary phospholipids enhance growth and development of larval fish, as well as preventing accumulation of lipid droplets in the intestine and enhancing transport of lipids from enterocytes to hepatocytes (Izquierdo et al., 2000; Salhi et al., 1999), and it has been reasoned that these effects were due to a limited ability of larval fish to biosynthesize phospholipids *de novo* (Coutteau et al., 1997; Geurden et al., 1995; Tocher et al., 2008). On the other hand, it remains to be unequivocally established whether the moderately beneficial effects of the LSO diet, compared to the SBO treatment, were due to a higher nutritional value of ALA compared to LNA and its involvement in different metabolic pathways, or whether this effect could be explained by some degree of bioconversion of ALA into biologically more active n–3 LC–PUFA by Senegalese sole larvae and post–larvae, especially since *Δ4fad* activity was highest in fish fed the LSO treatments.

10. *Central appetite regulation in Senegalese sole larvae and post–larvae*

It has been argued that fish larvae in the natural environment may not require strict appetite regulatory mechanisms as the prey encounter rate may be so low that satiety signals would not be required to prevent overfeeding (Rønnestad et al., 2013). However, given the constant lighting and food availability in a commercial environment, a lack of functional feedback mechanisms could be detrimental. Therefore, it is of great importance to clearly establish when appetite regulation becomes functional in altricial fish larvae (Rønnestad et al., 2013). In a pleuronectiform fish such as Senegalese sole, the drastic ontogenic changes during metamorphosis are accompanied by important behavioral changes with regard to feeding (Fernández–Díaz et al., 2001). These changes are likely to affect appetite–regulating mechanisms, which may explain the results observed in larvae of Senegalese sole (**Chapter 7**). Beside results of peripheral peptides being regulated more by dietary FA properties after metamorphosis, the examined central appetite–regulating neuropeptides (*cart1a*, *cart1b*, *cart2a*, *cart4*, *pomca*, *pomcb*, *crf*, *cckl*, *agrp2*, *npy*, *gal*) showed a variety of expression patterns in response to the diet, fasting and re–feeding, which were often not in accordance with their putative functions, especially during the pre–metamorphic stage. Even though significant variability has also been observed in juvenile fish, the differences in expression patterns between larvae and post–larvae, similar to what has been observed in the brain of Atlantic halibut (*Hippoglossus hippoglossus*) larvae (Gomes et al., 2015) and in whole larvae of cod (*Gadus morhu*a) (Kortner et al., 2011), may suggest a yet underdeveloped appetite regulating system in the pre–metamorphic stage. Nevertheless, we should not exclude the possibility that sampling of the whole body might have diluted or modified the expression of genes with a wide tissue distribution (Sæle et al., 2009).

Furthermore, no causative links were found between the differential effects of dietary FA composition on larval food intake and the expression of central peptides. Therefore, there must have been some other mechanisms regulating this process. The fact that LC–PUFA decreased anabolic lipid pathways and potentially enhanced intestinal lipid absorption and transport, as well as mobilization of endogenous lipid reserves may have

affected appetite through pathways reflecting changes in energy homeostasis and circulating metabolites. This is in accordance with an established connection between Apoa4 activity (chylomicron production) and food intake in rodent models (Fujimoto et al., 1992; Fujimoto et al., 1993), but also observed in zebrafish (Otis et al., 2015).

11. *Concluding remarks*

Finally, it should be recognized that the results obtained in this thesis, in particular the expression of genes putatively involved in the peripheral and central regulation of food intake, were often difficult to interpret and relate to the observed phenotypic effects, including VFI in juveniles and number of ingested prey per mg of BW in larvae and post larvae. This is justified by the extreme complexity of the multiple interrelated pathways, as it is well known in mammals, and by the shortage of specific information in fish species. Additionally, we have to take into account the possibility of post–transcriptional regulation mechanisms affecting the function of these peptides. Furthermore, it is important to highlight that the genes analyzed in this thesis have only been putatively connected to appetite control and energy metabolism based on previous research in mammals (Dhillo et al., 2002; Kulczykowska and Sánchez Vázquez, 2010) and a few other fish species (Volkoff, 2006; Volkoff et al., 2005). However, numerous other unrelated functions have also been attributed to these genes. These can be, although are not limited to, regulation of pigmentation and background adaptation (*agrp2*, *pomcb*) (Wunderink et al., 2012; Zhang et al., 2010), endocrine stress response (*pomca*, *crf*) (Wunderink et al., 2012; Wunderink et al., 2011), release of pancreatic secretions, gallbladder contraction, gastric emptying, gut peristalsis (*cck*) (Olsson et al., 1999; Volkoff, 2006), sensory processing, endocrine regulation, stress and anxiety, cardiovascular function, bone remodelling (*cart* in mammals) (Rogge et al., 2008) and reproduction (*cart* in fish) (Kehoe and Volkoff, 2007; MacDonald and Volkoff, 2009; Murashita et al., 2009a; Volkoff and Peter, 2001a; Wan et al., 2012). Therefore, it is obvious that present knowledge on these mechanisms in fish only enables us to “scratch the surface” and infer on whether some of these genes and pathways might be affected by dietary lipid composition. Having this in mind, the present thesis was meant to provide a more descriptive, rather than mechanistic, view of these pathways and lay the foundations for future research. In this context, the data collected in this thesis contributes to knowledge in lipid metabolism, homeostatic and appetite regulation in Senegalese sole larvae, post–larvae and juveniles, which can serve as a basis for future studies.

Conclusions

Molecular tools

1. mRNA sequences were described and RT–qPCR primers were developed in Senegalese sole for a wide range of genes related to: lipid absorption, lipid metabolism, peripheral satiety signaling and central appetite–regulation.
2. A total of 7 homologues of the *cart* gene were identified in this species—the highest number encountered in any vertebrate to date. Their expression in peripheral tissues and brain regions, and regulation related to fasting and re–feeding (several post–prandial timepoints) suggests multiple and highly diverse physiological roles.

Juvenile fish

3. Juvenile sole decreased their food intake at higher lipid levels, but only when the lipid source was FO (i.e., the effect was only clear in the 18FO treatment, compared to 8FO), which may indicate a higher satiating effect of FO than similar amounts of VO.
4. In juvenile sole, transcriptional changes of several genes collectively responsible for FA uptake from the intestinal lumen, intracellular transport and packaging into lipoproteins, did not indicate a strong response to dietary lipid level or lipid source.
5. Long term feeding of high lipid diets tended to reduce the basal (pre–prandial) expression of putatively anorexigenic enteroendocrine peptides (significantly for *pyya*, *pyyb*, *glp1*) in Senegalese sole juveniles, and the expression of *pyyb* and the orexigenic *gal* were similarly affected 6 h after feeding. These results were not in accordance with the food intake results or the regulatory mechanisms based on dietary lipids that have been described in mammals.
6. The lipid metabolism in the liver of juvenile sole seemed to be affected more by dietary lipid level than source, especially at basal levels (pre–prandial), where high dietary lipid inclusions promoted catabolic (β–oxidation) and suppressed anabolic (lipogenesis) pathways.
7. LC–PUFA also affected these pathways in sole liver, as indicated by a reduced Acly enzymatic activity at fasting and a lower accumulation of lipids in hepatocytes.
8. The presence of hypothalamic FA–sensing systems was confirmed in Senegalese sole, and shown to be activated by OA similarly as in trout.
9. For the first time in a vertebrate species, evidence of hypothalamic FA sensing system activation by an n–3 PUFA (ALA) is reported. However, EPA injections affected related systems only slightly and did not result in activation.
10. No differences in the basal (pre–prandial) expression of central appetite–regulating neuropeptides were observed between treatments.
11. Most of the central neuropeptides (*cart1b*, *cart4*, *pomca*, *cckl*, *npy*, *agrp2*) responded to feeding and showed temporal changes in expression during the post–prandial phase, with only slight differences in relation to lipid level.

12. mRNA levels of *cart1a* and *cart1b* were higher in fish fed the FO, compared to VO diets. Furthermore, expression of both genes peaked earlier (at 1 h post–feeding) in fish fed 18FO, compared to 8FO (at 3 h after feeding), which might suggest that the activation of anorexigenic pathways could occur earlier when FO is present at higher level in the diet.

Larvae and post–larvae

13. A method for quantifying live prey intake in larval fish using fluorescent microspheres as markers has been developed. Moreover, the method also allows using different coloured spheres to simultaneously label prey with different properties to perform selectivity experiments.
14. Larvae fed live prey enriched with CLO had higher food intake values than those fed on live prey enriched with ALA, LNA or OA.
15. Larvae fed the CLO diet showed improved growth and survival, faster onset of metamorphosis and maturation of the intestine.
16. Gene expression profile of both larvae and post–larvae fed the CLO diet stood out most from the other treatments, with decreased transcript levels of genes involved in anabolic pathways such as lipogenesis (*fas*) and LC–PUFA biosynthesis (*Δ4fad*), and increased of those related to phospholipid metabolism, lipoprotein secretion, lipid transport and hydrolysis.
17. The increased expression of genes involved in phospholipid metabolism and apolipoprotein synthesis in the CLO and LSO treatments likely represents the main underlying mechanisms related to the superior performance of fish fed LC–PUFA and, to an extent, ALA–rich diets.
18. The most pronounced differences between treatments in the expression of genes coding for peripheral peptides was generally observed just 30 min after feeding, and mainly in post–larvae (in larvae only *pyyb* was significantly affected).
19. Peripheral peptides generally did not mirror their putative function or explain lower food intake in fish fed the VO diets.
20. Larvae and post–larvae fed the CLO diet tended to show the most dissimilar pattern of central appetite–regulating gene expression, compared to the other dietary treatments.

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ORIGINAL ARTICLE

Dietary Fatty Acid Metabolism is Affected More by Lipid Level than Source in Senegalese Sole Juveniles: Interactions for Optimal Dietary Formulation

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Abstract This study analyses the effects of dietary lipid level and source on lipid absorption and metabolism in Senegalese sole (*Solea senegalensis*). Juvenile fish were fed 4 experimental diets containing either 100 % fish oil (FO) or 25 % FO and 75 % vegetable oil (VO; rapeseed, linseed and soybean oils) at two lipid levels (~8 or ~18 %). Effects were assessed on fish performance, body proximate composition and lipid accumulation, activity of hepatic lipogenic and fatty acid oxidative enzymes and, finally, on the expression of genes related to lipid metabolism in liver and intestine, and to intestinal absorption, both pre- and postprandially. Increased dietary lipid level had no major effects on growth and feeding performance (FCR), although fish fed FO had marginally better growth. Nevertheless, diets induced significant changes in lipid accumulation and metabolism. Hepatic lipid deposits were higher in fish fed VO, associated to increased hepatic ATP citrate lyase activity and up-regulated carnitine palmitoyltransferase 1 (*cpt1*) mRNA levels post-prandially. However, lipid level had a larger effect on gene expression of metabolic (lipogenesis and β-oxidation) genes than lipid source, mostly at fasting. High dietary lipid level down-regulated fatty acid synthase expression in liver and intestine, and increased *cpt1* mRNA in liver. Large lipid accumulations were observed in the enterocytes of fish fed high lipid diets. This was possibly a result of a poor capacity to adapt to high dietary lipid level, as most genes involved in intestinal absorption were not regulated in response to the diet.

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Keywords Fish oil · Lipogenesis · Beta-oxidation · Fish nutrition · Lipid absorption · Fatty acid binding proteins · Lipid trafficking · Molecular biology

Abbreviations

ACOX1	Acyl-CoA oxidase 1
ALA	α-Linolenic acid
APOA4	Apolipoprotein A4
ARA	Arachidonic acid
CD36	Cluster of differentiation 36 family
CPT1	Carnitine palmitoyltransferase 1
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acid
FABP	Fatty acid binding protein
FAS	Fatty acid synthase
FCR	Food conversion ratio
HSI	Hepatosomatic index
MTP	Microsomal triglyceride transfer protein

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MUFA	Monounsaturated fatty acid
LC-PUFA	Long-chain polyunsaturated fatty acid
LNA	Linoleic acid
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
VSI	Viscerosomatic index

Introduction

Traditionally, fish oil (FO) has been used as the main dietary lipid source for cultured marine finfish [1]. However, the steady growth of aquaculture production worldwide, paired with reduced availability of raw materials from wild fisheries, has led to a rising need to replace FO, at least partly, with alternative and more sustainable ingredients, such as vegetable oils (VO) [2]. Therefore, in the last few years, numerous studies have focused on identifying suitable candidate VO for use in aquaculture by investigating their effect on growth and feed efficiency, health, reproduction and physiology of cultured organisms [1, 3–6]. It has generally been shown that a 100 % replacement of dietary FO with VO has a negative effect on the growth of most marine finfish species [7]. However, a large fraction (60–75 %) of FO can be substituted without affecting growth and feed efficiency, if essential fatty acid requirements are met [1]. Nevertheless, not all species necessarily respond equally to dietary FO replacements and although these generalizations may be used as a benchmark, effects of dietary VO should be evaluated on a case by case basis [1]. Even then, results obtained for a species may vary depending on numerous factors such as trial duration, type of VO, amount of fish meal (source of FO) in the diet, fish size, age or developmental stage [7], but also dietary lipid level [8–12].

Most studies that focus on alternative dietary lipid sources use isolipidic diets and test various degrees of FO substitution with VO. However, studies that take into account the interrelation between lipid sources and lipid levels are fewer in number and address only a few aspects of lipid metabolism and growth performance. Furthermore, such types of studies are lacking in Senegalese sole (*Solea senegalensis*)—an interesting species for lipid-related research, due to some particularities in its nutritional physiology, and one of high commercial aquaculture importance in southern Europe [13]. Senegalese sole is a lean flatfish (1–4 g fat/100 g of flesh) with a limited ability to efficiently use high dietary lipid levels (≥12 %), at both juvenile [14] and adult stages [15]. Other demersal lean fish, including different species of sole, flounder and catfish [14, 16–19] generally do not cope well with high fat diets, contrary to salmonids [20, 21] and more active marine species like sparids or European seabass (*Dicentrarchus labrax*) [22–24] which utilize dietary lipids more efficiently. On the other hand, even complete replacement of dietary FO in Senegalese sole with different VO (at 9 % dietary lipids) did not seem to affect fish performance negatively [25]. Nevertheless, other criteria should also be considered to assess the physiologic and metabolic effects of different lipid sources. For instance, Montero *et al*. [4] and Caballero *et al*. [26] observed no detrimental effect of FO substitution on growth of gilthead seabream (*Sparus aurata*), but several types of intestinal morphological alterations, like lipid droplet accumulation, were observed in fish fed high contents of VO-based diets. Considering that any adverse effects of new dietary formulations on the cultured organism can potentially have significant economic repercussions when applied in large-scale aquaculture production, this type of research is not only interesting from a scientific point of view, but also of great importance to the development of the commercial sector.

This study was designed to further study the mechanisms of lipid absorption and metabolism in Senegalese sole and contribute to the growing knowledge of the nutritional physiology of this interesting species. Since both the type and quantity of ingested fats are known to regulate hepatic gene expression [27], we investigated if the effects of alternative dietary lipid sources were modulated by their levels of inclusion in the diet and whether their long-term administration had different effects pre- and post-prandially. To address this, we tested 4 experimental diets containing either 100 % FO or 25 FO and 75 % VO at two lipid levels (~8 or ~18 %). The effects of these dietary treatments were assessed by analyzing growth performance, hepatosomatic index (HSI), viscerosomatic index (VSI), whole-body proximate composition, lipid accumulation in specific tissues, activity of lipogenic and fatty acid (FA) oxidative enzymes in the liver and, finally, expression of genes related to lipid metabolism in both liver and intestine, and also of genes involved in lipid uptake, intracellular transport and basolateral secretion in the intestine. The fatty acyl elongation and desaturation activities, as well as the FA profiles of key tissues, were previously reported by Morais *et al*. [28].

Materials and Methods

Experimental Diets

Four isoproteic extruded diets were formulated and manufactured by Sparos Lda. (Olhão, Portugal). They differed in total lipid level (~8 or ~18 %) and fatty acid composition, using FO or VO as the main lipid source. The 8FO and 18FO diets had 100 % of the lipid supplied by FO, while 75 % of the FO in diets 8VO and 18VO was replaced by a VO blend (rapeseed, soybean, and linseed oil in a ratio

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of 1:1:1; Table 1). Proximate and FA composition of the experimental diets were analyzed in triplicate, as described below, and are presented in Tables 1 and 2, respectively.

Growth Trial and Sampling

Senegalese sole juveniles with an average body weight (BW) of 5.0 ± 0.1 g were distributed into twelve rectangular flat bottom 20 l tanks (50 fish per tank) connected to a recirculation system at CCMAR, University of Faro, Portugal, and maintained at a temperature of 19.3 ± 1.2 °C, a salinity of 32, and a 12 h light/12 h dark photoperiod for 13 weeks. They were fed 2 mm extruded diets using automatic feeders 22 h per day. Feed doses were adjusted daily to ensure the fish were fed to satiety. In the case of excess uneaten feed, rations were reduced by 10 % and in the absence of uneaten feed increased by 10 %.

All fish were sampled after fasting for 24 h. Prior to sampling fish were euthanized with a lethal dose of tricaine methanesulfonate (MS222; Sigma, Sintra, Portugal). Fish were weighed at 28, 53, 77 and 91 days after the start of the experiment ($n = 3$ pools of 30 individuals), with the additional weighing of whole fish, liver and viscera of eight fish per tank in the final sampling to calculate HSI and VSI ($n = 24$ fish per treatment). Specific growth rate (SGR) was calculated based on values at the end of the experimental period ($n = 3$ pools of 30 fish). To analyze whole-body proximate composition, two fish were taken from each tank, frozen and later homogenized ($n = 3$ homogenates per treatment). Total lipids of intestine, liver and muscle tissues were analyzed in pools from three fish per tank ($n = 3$ pools per treatment). The intestine and liver were sampled from three fish per tank ($n = 9$ per treatment) for optical histology, stored in 10 % buffered formalin for 24 h, and subsequently transferred to 70 % ethanol until analysis. A section of liver from five fish per tank ($n = 15$ per treatment) was taken for measurement of lipogenic and FA oxidative enzyme activities. Tissue samples for enzyme, proximate and total lipid analysis were instantly frozen on dry ice and stored at −80 °C until analysis.

The study was conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE).

Feeding Trial and Sampling for Molecular Analysis

A feeding trial was performed during week 13 in order to measure pre- and post-prandial expression levels of genes involved in lipid metabolism and absorption. Two fish per tank were sampled after 24 h of fasting (t0, pre-prandial; $n = 6$ per treatment) and another two were then fed their respective diets and sampled 6 h after feeding (t6, post-prandial; $n = 6$ per treatment). In order to ensure

Table 1 Formulation and proximate composition of the experimental diets

	8FO	8VO	18FO	18VO
Ingredients (%)				
Fishmeal 70 LT[a]	22.00	22.00	22.00	22.00
Fishmeal 60[b]	15.00	15.00	15.00	15.00
Fish protein hydrolysate[c]	5.00	5.00	5.00	5.00
Squid meal[d]	5.00	5.00	5.00	5.00
Pea protein concentrate[e]	4.00	4.00	4.00	4.00
Soy protein concentrate[f]	2.00	2.00	2.00	2.00
Soybean meal 48[g]	9.80	9.80	10.00	10.00
Wheat gluten[h]	7.00	7.00	10.10	10.10
Corn gluten meal[i]	5.00	5.00	4.50	4.50
Pea grits[j]	11.10	11.10	2.50	2.50
Wheat meal	9.00	9.00	4.80	4.80
Fish oil[k]	2.60	0.65	12.60	3.15
Rapeseed oil[l]	–	0.65	–	3.15
Soybean oil[l]	–	0.65	–	3.15
Linseed oil[l]	–	0.65	–	3.15
Vitamin and mineral premix[m]	1.00	1.00	1.00	1.00
Binder (guar gum)[n]	1.00	1.00	1.00	1.00
Proximate composition				
Moisture (%)	5.5	4.6	4.3	4.4
Crude protein (% DM)	56.0	56.9	58.0	57.2
Crude fat (% DM)	7.9	7.4	17.6	17.4
Ash (% DM)	10.5	10.7	10.4	10.3

[a] Peruvian fishmeal LT: 71 % crude protein (CP), 11 % crude fat (CF), EXALMAR, Peru

[b] Fair average quality (FAQ) fishmeal: 62 % CP, 12 % CF, COFACO, Portugal

[c] CPSP 90: 84 % CP, 12 % CF, Sopropêche, France

[d] Super prime squid meal: 80 % CP, 3.5 % CF, Sopropêche, France

[e] Lysamine GP: 78 % CP, 8 % CF, ROQUETTE, France

[f] Soycomil P: 65 % CP, 0.8 % CF, ADM, The Netherlands

[g] Solvent extracted dehulled soybean meal: 47 % CP, 2.6 % CF, SORGAL SA, Portugal

[h] VITEN: 85.7 % CP, 1.3 % CF, ROQUETTE, France

[i] Corn gluten feed: 61 % CP, 6 % CF, COPAM, Portugal

[j] Aquatex G2000: 24 % CP, 0.4 % CF, SOTEXPRO, France

[k] COPPENS International, The Netherlands

[l] Henry Lamotte Oils GmbH, Germany

[m] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings

[n] Guar gum 101 HV- E412, Seah International, France

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Table 2 Fatty acid composition of the 4 experimental diets, expressed as mean %mol ± SD ($n = 3$)

	8FO	8VO	18FO	18VO
14:0	2.6 ± 0.4	1.8 ± 0.7	4.8 ± 0.8	1.7 ± 0.1
15:0	0.4 ± 0.0	0.3 ± 0.1	0.5 ± 0.0	0.2 ± 0.0
16:0	18.4 ± 0.6	16.2 ± 0.7	19.1 ± 0.2	13 ± 0.8
18:0	4.3 ± 0.1	4.1 ± 0.1	4.7 ± 0.1	3.9 ± 0.4
Total saturated	25.8 ± 1.1	22.5 ± 1.3	29 ± 0.6	18.8 ± 1.2
16:1	5.6 ± 0.6	4.5 ± 0.6	6.2 ± 1.0	3.8 ± 0.0
18:1n-9	14.2 ± 0.2	18.5 ± 0.2	12.6 ± 0	23.5 ± 0.7
18:1n-7	3.0 ± 0.8	2.7 ± 0.6	3 ± 0.6	3.1 ± 1.4
20:1	4.8 ± 0.1	4.4 ± 0.1	3.6 ± 0.2	2.4 ± 0.2
Total monounsaturated	27.6 ± 0.3	30.2 ± 0.1	25.3 ± 1.8	32.8 ± 0.5
18:2n-6	12.4 ± 0.4	17.7 ± 0.5	6.8 ± 0.1	20.5 ± 0.2
18:3n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:3n-6	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20:4n-6	1.0 ± 0.2	0.6 ± 0.0	1.2 ± 0.0	0.4 ± 0.0
22:4n-6	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
22:5n-6	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.3	0.2 ± 0.0
Total n-6 PUFA	14.2 ± 0.4	18.9 ± 0.5	8.8 ± 0.2	21.4 ± 0.1
18:3n-3	1.8 ± 0.1	5.6 ± 0.1	1.3 ± 0.0	12.1 ± 0.6
18:4n-3	2.0 ± 0.0	1.5 ± 0.1	2.4 ± 0.2	1.2 ± 0.1
20:4n-3	0.6 ± 0.2	0.4 ± 0.0	0.8 ± 0.0	0.3 ± 0.0
20:5n-3	14.7 ± 0.3	10.4 ± 0.5	17.2 ± 0.7	7.0 ± 0.1
21:5n-3	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0
22:5n-3	1.0 ± 0.0	0.7 ± 0.0	1.3 ± 0.2	0.5 ± 0.0
22:6n-3	11.2 ± 0.4	8.6 ± 0.3	11.3 ± 0.9	5.2 ± 0.1
Total n-3 PUFA	31.5 ± 0.5	27.4 ± 0.7	34.8 ± 1.9	26.3 ± 0.7
Total PUFA	45.6 ± 0.9	46.3 ± 1.3	43.6 ± 2.1	47.7 ± 0.8
n-3/n-6 PUFA	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.1
DHA/EPA	0.2 ± 0.1	0.6 ± 0.1	0.8 ± 0.3	0.3 ± 0.2
ARA/EPA	0.4 ± 0.0	0.2 ± 0.0	0.8 ± 00	0.2 ± 0.1

that all fish ate an equal amount of feed at the appointed time, and considering the highly variable voluntary feed intake of sole [29], a force-feeding method was used. Plastic tubes were filled with 10 pellets each (~0.15 % BW) and inserted up to the back of the oral cavity of previously anesthetized (MS222, 50 ppm) fish. A piston was then used to push the contents of the tube into the esophagus and the fish were monitored for a few minutes before being returned to the tanks, in order to ensure that the pellets were not expelled. At both time points 100–150 mg samples of intestine and liver were collected and transferred into eppendorf tubes with 1.5 ml of RNA later stabilization buffer (Ambion, Life Technologies, Madrid, Spain). Samples were kept in agitation at 4 °C for 24 h, and then stored at −80 °C.

Whole Body Proximate Composition and Total Lipid Levels of Tissues

Protein content in extruded diets was carried out by the Dumas method using a protein standard (LECO FP-528) [30]. Water and ash content were calculated in subsamples of each diet ($n = 6$) and whole body homogenates by drying them at 105 °C until a constant weight was obtained, or after ashing in a muffle furnace for 5 h at 550 °C, respectively [31]. For the analysis of protein content (according to Lowry *et al.* [32], following overnight hydrolysis in sodium-hydroxide solution), carbohydrates [33], and lipids (extracted as in Folch *et al.* [34]) and quantified gravimetrically), five subsamples were taken for each analysis from each homogenate ($n = 15$ per treatment). Total lipids from intestine, liver and muscle pooled from three fish per tank (three pools per treatment) were also extracted following the method of Folch *et al.* [34]) and quantified gravimetrically. The tissue total lipid results are presented as both percentage (%) of dry weight (DW) to reduce variability associated with water content of tissue, but also as wet weight (WW) for discussion purposes, as numerous studies use these values. Water content was calculated as the difference between WW and DW and presented as a percentage of WW. Finally, total lipid content of liver and intestine was also expressed as mg 100 g^{-1} fish.

Histological Analyses

Samples were serially sectioned at 6–7 µm and stained with Harris hematoxylin and eosin. Sections of the intestine and liver were photographed at 40× magnification (300 dpi; 3 photos per tissue, $n = 27$ photos per treatment) with an Olympus DP70 digital camera connected to a Leica DM 2000™ microscope. All digital image analyses were performed using ImageJ (U.S. National Institutes of Health, Bethesda, USA; http://rsbweb.nih.gov/ij/index.html). A semi-automatic quantitative analysis was employed in order to measure the percentage of intestinal epithelium (longitudinal transects) and hepatocytes occupied by fat deposits. By adjusting the color threshold settings to brightness values between 195 and 255, the program was configured to select all surfaces covered with a white to light pink color, which corresponded to the area of fat deposits [35]. These values were used to calculate the percentage of area occupied by lipids as in Boglino *et al.* [36]. For liver samples, the total area of analyzed tissue was a rectangular selection, while transects of the intestinal epithelium were selected manually with the "polygon selection" tool and the diameters of intestinal lipid droplets were measured using the "straight" tool of ImageJ.

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Lipogenic and FA Oxidative Enzyme Activities

Activities of ATP citrate lyase (Acly; lipogenic) and 3-hydroxyacyl-CoA dehydrogenase (Hoad; FA oxidative) and carnitine palmitoyltransferase 1 (Cpt1; FA oxidative) were assessed in 100 mg liver samples homogenized by ultrasonic disruption with 7 volumes of ice-cold buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA; P-2714). The homogenate was centrifuged and the supernatant taken and immediately frozen on dry ice and stored at −80 °C until analysis. Enzyme activities were determined in a microplate reader Elx-tec (Biotek). Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (10 µl, 7.5 µl (+7.5 µl of H_2O) and 5 µl for Acly, Hoad and Cpt1, respectively) to buffer solutions containing the substrates (0.10 mM of CoA, 0.05 mM of Acetoacetil-CoA and 1.00 mM of L-carnitine hydrochloride for Acly, Hoad and Cpt1, respectively), which were omitted in control wells (final volume 275–280 µl). The reactions were allowed to proceed at 37 °C for pre-established periods of time (10–30 min). Enzymatic analyses were carried out at maximum rates, with the reaction mixtures optimized in preliminary tests to render optimal activities by adapting methods previously described for Acly [37], Hoad [38] and Cpt1 [39]. Total protein content in homogenates was assayed in duplicate, according to the bicinchoninic acid method [40], using bovine serum albumin (sigma) as a standard. Enzyme activities were normalized by mg of protein.

Primer Design

Primers for real time quantitative PCR (RT-qPCR) for most genes analyzed in this study (Table 3) are from previous work (our own unpublished data), but primers for *cpt1* and for a transcript belonging to the cluster of differentiation 36 family, the platelet glycoprotein 4-like (*cd36*) were designed specifically for this study. Sequences of target genes were searched by gene annotation in the SoleaDB database (http://www.scbi.uma.es/soleadb) *Solea senegalensis* v4.1 global assembly and the retrieved fragments were assembled in silico into contigs using the BioEdit Sequence Alignment Editor [41]. A blastx search was performed in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) to compare with orthologs in other fish and vertebrate species and identify the open reading frames (ORF) and 3′ and 5′ untranslated regions (UTR) of the sequences. When 5′ and 3′ UTR's were missing, rapid amplification of cDNA ends (RACE) PCR was performed using the FirstChoice® RLM-RACE kit (Ambion). The obtained fragments were separated by gel electrophoresis and resulting bands of the expected length were cut, purified (Illustra GFX™ PCR DNA and gel band purification kit, GE Healthcare, Barcelona, Spain) and sequenced (SCSIE, University of Valencia, Spain). The transcript sequences thus obtained were used as templates to design primers for qPCR, using Primer3 v. 0.4.0 [42]. The qPCR conditions were then optimized and the obtained amplicons were sequenced to confirm their identity and the specificity of the qPCR assay. Several homolog genes were obtained for Senegalese sole *cd36* and *cpt1*, but only one transcript of each was selected for use in this study primarily based on the pattern of tissue distribution (unpublished data) and highest sequence homology to the specific gene of interest, chosen based on previous studies in other species. In this respect, the *cpt1* gene was equally similar to both *cpt1a* and *cpt1b* which are the genes most commonly assayed in rainbow trout (*Oncorhynchus mykiss*) liver to study effects of FA composition on lipid metabolism [43].

RNA Extraction and Real Time qPCR

For RNA extraction, samples were homogenized in 1 ml of TRIzol (Ambion) with 50 mg of 1 mm diameter zirconium glass beads (Mini-Beadbeater, Biospec Products Inc., USA). Solvent extraction was performed following manufacturer's instructions and RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain). Two micrograms of total RNA per sample were reverse transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Life Technologies, USA), following manufacturer's instructions, but using a mixture of random primers (1.5 µl as supplied) and anchored oligo-dT (0.5 µl at 400 ng/µl, Eurogentec, Cultek, S.L., Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. A pool was created from 2 µl of cDNA from all samples of a single tissue to use for dilution series and samples were then diluted 60-fold with water.

Amplifications were carried out in duplicate on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Alcobendas, Spain) in a final volume of 20 µl containing 5 µl of diluted (1/60) cDNA, 10 µl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and 500 nM primers (except for *cpt1*, *cd36* and fatty acid-binding protein 1 (*fabp1*), where 150, 200 and 150 nM were used, respectively). A systematic negative control (NTC-non template control) was also included. The qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 35 cycles: 15 s at 95 °C, 1 min at the corresponding annealing temperature (*T*a; Table 2). After the amplification phase,

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Table 3 Primers used for real-time quantitative PCR (qPCR)

Transcript	Primer sequence (5′–3′)	Amplicon size (bp)	*Ta* (°C)	E^{a} (%)	Accession number
Lipid absorption					
cd36	TGAATGAGACGGCTGAGTTG	168	64	100.3	KR872889
	TGTTGTTTCTGCTCCTCACG				
fabp1	GCTCATCCAGAAAGGCAAAG	199	62	102.1	KP842779
	GGAGACCTTCAGCTTGTTGC				
fabp2a	ACACACATGACCTTAGCACACTG	70	60	102.3	KP842780
	TGCGAGTATCAAAATCCGGTA				
apoa4	AGGAACTCCAGCAGAACCTG	122	60	100.2	KP842775
	CTGGGTCATCTTGGAGAAGG				
mtp	CAGGCGTACACCACATGTAAA	150	60	102.3	KP842778
	GTGATCAGGCTTCTGCAGTG				
Lipid metabolism					
acox1	GGTCCATGAATCTTTCCACAA	168	60	104.1	KP842776
	ACAAGCCTGACGTCTCCATT				
fas	CACAAGAACATCAGCCGAGA	197	60	103.1	KP842777
	GAAACATTGCCGTCACACAC				
cpt1	TAACAGCCACCGTCGACATA	156	63	101.1	KR872890
	AGCGATTCCCTTGTGTCACT				
Reference genes					
ubq	AGCTGGCCCAGAAATATAACTGCGACA	93	70	101.3	AB291588
	ACTTCTTCTTGCGGCAGTTGACAGCAC				
rps4	GTGAAGAAGCTCCTTGTCGGCACCA	83	70	100.9	AB291557
	AGGGGGTCGGGGTAGCGGATG				
eef1a1	GATTGACCGTCGTTCTGGCAAGAAGC	142	70	100.5	AB326302
	GGCAAAGCGACCAAGGGGAGCAT				

Shown are sequences and annealing temperature (*T*a) of the primer pairs, size of the fragment produced, average reaction efficiency (*E*) and accession number of the target and reference genes

cd36 cluster of differentiation 36 family (platelet glycoprotein 4-like), *fabp* fatty acid binding protein, *apoa4* apolipoprotein A-IV, *mtp* microsomal triglyceride transfer protein, acox1 acyl-CoA oxidase 1 palmitoyl, *fas* fatty acid synthase, *cpt1* carnitine palmitoyltransferase 1, *ubq* ubiquitin, *rps4* 40S ribosomal protein S4, *eef1a1* elongation factor 1 alpha

[a] Efficiency corresponds to an average of 2 runs (intestine tissue at t0 and t6) for lipid absorption and 4 runs (intestine and liver tissues at t0 and t6) for lipid metabolism genes

a melt curve was performed enabling confirmation of the amplification of a single product in each reaction. Non-occurrence of primer-dimer formation in the NTC was also confirmed. The amplification efficiency of each primer pair was assessed from serial dilutions of the cDNA pool.

The qPCR results were imported into the software qBase + (Biogazelle, Zwijnaarde, Belgium), where normalized relative quantities (NRQ) were calculated employing target and run-specific amplification efficiencies and using the geometric mean of 3 reference genes (elongation factor 1 alpha, ubiquitin and 40S ribosomal protein; *eef1a1, ubq* and *rps4,* respectively; Infante et al. [44]). The stability of the reference genes was checked [45] and results for the combination of the 3 genes at t0/t6 were: $M = 0.276/0.260$, coefficient of variance-CV = 0.110/0.102 for intestine and $M = 0.149/0.134$, CV = 0.061/0.053 for liver.

Statistical Analysis

All statistical analyses were performed with SPSS v.20 (SPSS Inc., Chicago, IL, USA). A two-way ANOVA, with factors lipid source and lipid level, at a significance level of 0.05, was used to analyze the results.

Results

Diet Fatty Acid Composition

As shown in Table 2, the 18FO diet provided the highest relative and absolute levels of the long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic

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acid (ARA) and saturated fatty acids (SFA), but lowest levels of C_{18} polyunsaturated fatty acids (PUFA). On the other hand, the 18VO diet provided the lowest relative levels (but not in absolute content) of LC-PUFA, and had the highest relative and absolute linoleic acid (LNA), α-linolenic acid (ALA) and monounsaturated fatty acid (MUFA) content.

Fish Performance and Body Composition

At the end of the 3-month experimental period, a fourfold size increase was recorded in juvenile Senegalese sole from all treatments (Table 4). Fish cultured on FO diets grew slightly more, albeit non-significantly ($p = 0.06$ for final BW and SGR), compared to those fed diets with 75 % VO,

Table 4 Growth and feeding performance, HSI, VSI, proximate composition, lipid content of selected tissues and tissue area occupied by lipid droplets (surface occupied, as percentage of total tissue surface) in liver and intestine of juvenile Senegalese sole cultured on 4 different diets during a period of 13 weeks

Day	Dietary treatment				*P* value (2-way ANOVA)		
	8FO	8VO	18FO	18VO	LL	LS	LL × LS
BW (g)							
0	5.0 ± 0.07	5.0 ± 0.05	5.0 ± 0.70	4.9 ± 0.08	0.141	1.000	0.438
28	10.7 ± 0.14	10.4 ± 0.30	10.7 ± 0.50	10.0 ± 0.02	0.401	0.012	0.178
53	16.6 ± 1.68	16.0 ± 0.75	16.7 ± 1.70	15.0 ± 0.50	0.571	0.140	0.461
77	20.8 ± 1.82	18.6 ± 0.92	17.6 ± 1.70	17.9 ± 0.74	0.040	0.278	0.162
91	23.4 ± 2.80	21.7 ± 1.16	23.7 ± 2.00	20.7 ± 0.95	0.721	0.058	0.555
SGR (%/day)	1.69 ± 0.14	1.60 ± 0.06	1.71 ± 0.09	1.57 ± 0.04	0.925	0.060	0.639
FCR	1.31 ± 0.12	1.30 ± 0.06	1.16 ± 0.10	1.30 ± 0.09	0.195	0.303	0.208
HSI (% BW)	1.04 ± 0.28	1.00 ± 0.25	1.10 ± 0.21	1.27 ± 0.28	0.002	0.228	0.057
VSI (% BW)	3.68 ± 0.73	3.56 ± 0.56	3.57 ± 0.38	4.03 ± 0.41	0.111	0.134	0.009
Whole-body proximate composition (% DW)							
Crude fat	16.4 ± 1.13	17.1 ± 1.02	23.2 ± 0.80	21.5 ± 0.28	<0.001	0.251	0.006
Crude protein	50.3 ± 3.01	50.9 ± 3.53	45.0 ± 2.92	45.5 ± 2.65	<0.001	0.989	0.909
Carbohydrates	1.4 ± 0.46	1.3 ± 0.30	1.3 ± 0.21	1.4 ± 0.37	0.948	0.772	0.020
Ash	2.4 ± 0.05	2.4 ± 0.05	2.3 ± 0.38	2.4 ± 0.16	0.484	0.834	0.830
Moisture[a]	74.4 ± 0.50	75.2 ± 0.43	72.4 ± 1.14	73.1 ± 1.13	<0.001	0.010	0.747
Total lipids in tissues							
Liver							
% WW	4.2 ± 0.8	4.1 ± 1.0	5.2 ± 0.4	5.8 ± 2.1	0.130	0.749	0.665
% DW	13.1 ± 2.6	12.5 ± 3.5	15.2 ± 1.8	15.6 ± 5.5	0.279	0.967	0.818
mg 100 g^{-1} fish[b]	44.0	41.4	57.3	74.0			
Intestine							
% WW	2.1 ± 0.1	1.7 ± 0.5	2.8 ± 0.6	3.4 ± 0.2	0.001	0.768	0.070
% DW	8.8 ± 1.5	8.7 ± 3.7	12.7 ± 2.7	12.8 ± 1.2	0.026	0.991	0.953
mg 100 g^{-1} fish[b]	77.1	60.9	101.1	135.1			
Muscle							
% WW	0.4 ± 0.0	0.6 ± 0.3	0.5 ± 0.1	0.5 ± 0.1	0.703	0.400	0.400
% DW	1.7 ± 0.3	2.3 ± 1.1	2.2 ± 0.4	2.3 ± 0.2	0.461	0.333	0.415
Tissue area occupied by lipid droplets (%)							
Liver	54.8 ± 4.72	58.5 ± 4.43	56.0 ± 4.37	58.0 ± 2.37	0.610	<0.001	0.294
Intestine	8.4 ± 3.25	3.8 ± 3.86	25.4 ± 4.59	26.8 ± 8.37	<0.001	0.238	0.032

Values are expressed as mean ± SD. Results of 2-way ANOVA analysis in relation to dietary factors lipid level (LL), lipid source (LS) and their interaction (LL × LS) are also presented

[a] Moisture content is the difference of WW and DW, expressed as percentage of WW

[b] Calculated as (lipid % of tissue WW/100) × HSI (or VSI) × 1000

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while no differences were observed with regard to lipid level. However, the food conversion ratio (FCR) did not differ between treatments. The HSI was significantly higher in fish fed high lipid diets, with fish fed 18VO having the largest livers, and interaction between the dietary factors was only marginally non-significant ($p = 0.06$). The results for VSI showed a significant interaction between the two dietary factors, and fish from the 18VO group had the highest values.

In juvenile sole fed high lipid diets, crude lipids constituted a significantly higher proportion of the whole body compared to those fed a low lipid diet (Table 4). However, no noticeable effect of lipid source was observed. Conversely, the moisture content and protein fraction were significantly lower in fish fed high lipid diets. Carbohydrate levels were generally low, but a significant interaction between the dietary factors was observed, with values being higher in the 8FO and 18VO treatments.

Total lipid content of different tissues was also measured (Table 4). In the liver and muscle, no significant differences were observed between treatments, but in the intestine fish fed 18 % lipid diets showed significantly higher lipid accumulation than those fed lower levels of dietary lipids.

Histological Assessment of Fat Accumulation in the Intestine and Liver

Fish fed the VO diets had a significantly higher percentage of fat within the hepatic parenchyma compared to fish fed FO, showing a more compact hepatic parenchyma, a reduction in size of hepatic sinusoids and higher size of lipid deposits within hepatocytes (Table 4; Fig. 1). In the intestine, clear differences were observed between treatments with regard to lipid level (Fig. 2). Large lipid accumulations (numerous lipid droplets with up to 16 µm in diameter) were observed in fish fed the 18 % lipid diets, while the 8 % lipid groups had no or very little lipid inclusions of a very small diameter (~1.5 µm). This was also confirmed by image analysis (Table 4), although in this case there was also a significant interaction between the two dietary factors.

Activity of Lipogenic and FA Oxidative Enzymes

Significant differences were observed only in the relative hepatic activity of Acly, where fish fed the VO diets had higher activity than those fed FO (Table 5). There was a

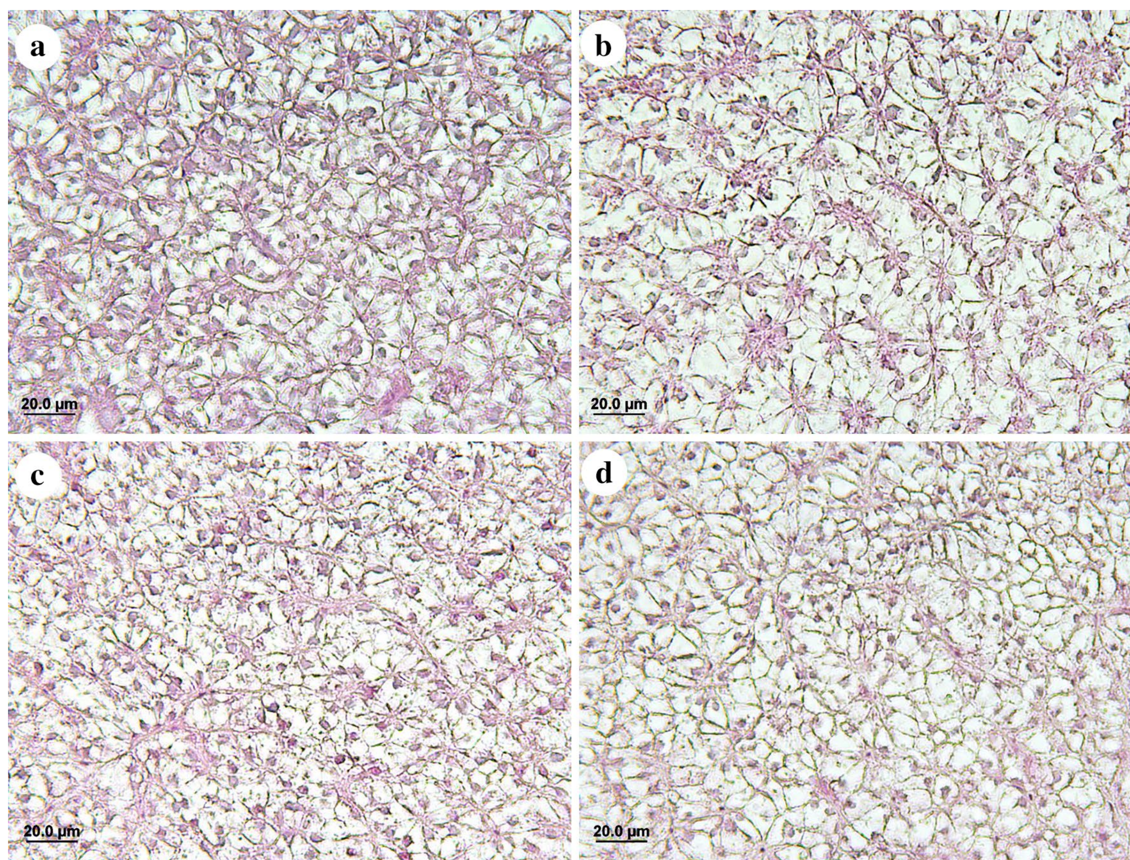

Fig. 1 Lipid droplet accumulation in hepatocytes of Senegalese sole juveniles fed dietary treatments differing in lipid source and level of lipid inclusion: 8FO (**a**), 8VO (**b**), 18FO (**c**), 18VO (**d**)

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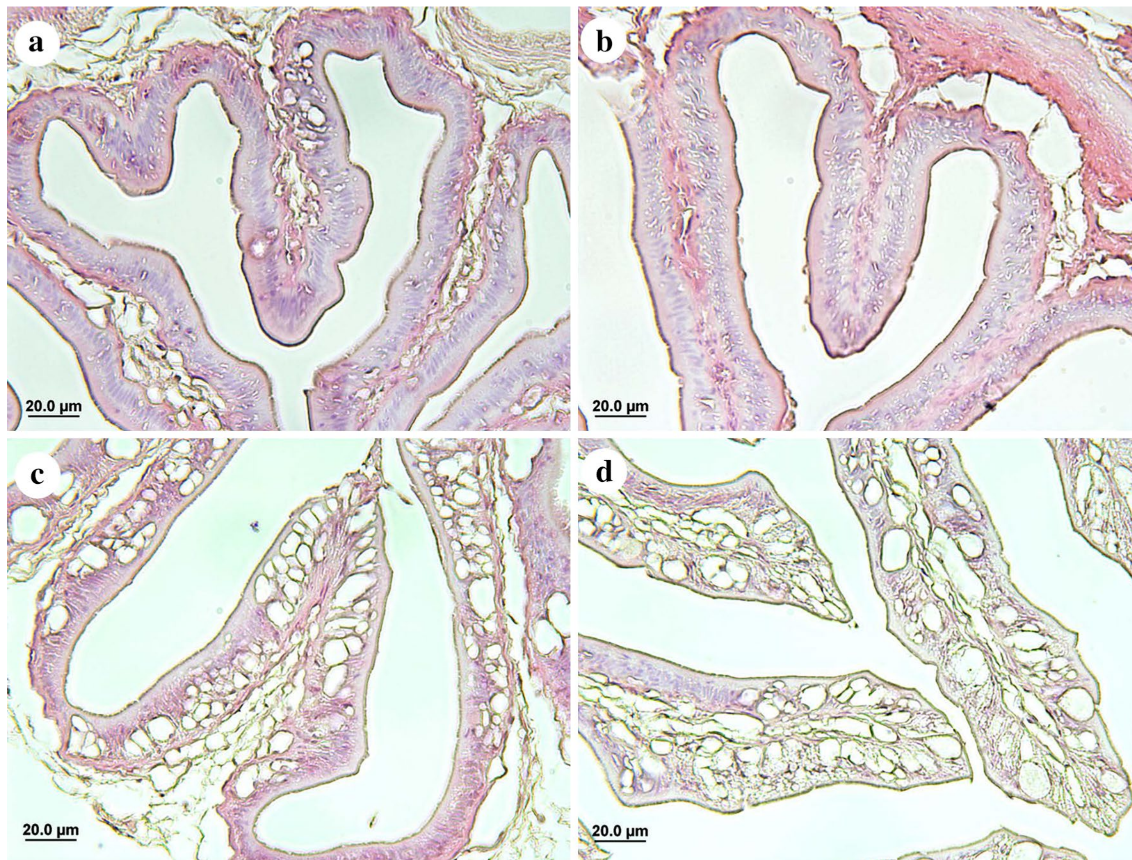

Fig. 2 Lipid droplet accumulation in enterocytes of the posterior intestine in Senegalese sole juveniles fed dietary treatments differing in lipid source and level of lipid inclusion: 8FO (**a**), 8VO (**b**), 18FO (**c**), 18VO (**d**)

Table 5 Relative activity of lipogenic enzymes (mUI/mg protein) measured in the liver of Senegalese sole juveniles fed four different dietary treatments

	Dietary treatment				*P* value (2-way ANOVA)		
	8FO	8VO	18FO	18VO	LL	LS	LL × LS
Hoad	31.9 ± 15.83	34.0 ± 12.76	28.8 ± 13.98	35.7 ± 10.00	0.855	0.240	0.525
Acly	17.9 ± 9.21	23.3 ± 15.80	18.8 ± 10.88	30.1 ± 10.89	0.238	0.014	0.371
Cpt1	21.4 ± 12.30	21.4 ± 10.06	25.3 ± 8.64	26.3 ± 15.15	0.169	0.874	0.882

Values are shown as mean ± SD ($n = 15$ per treatment). Results of 2-way ANOVA analysis in relation to dietary factors lipid level (LL), lipid source (LS) and their interaction (LL × LS) are also presented

high variability in the results and no significant differences were observed in the relative activity of Hoad or Cpt1.

Gene Expression Assessed by RT-qPCR

The expression levels of key genes involved in lipid absorption and metabolism were measured by RT-qPCR in the intestine and liver of Senegalese sole juveniles before (t0; Table 6) and 6 h after feeding (t6; Table 7). In the liver, fatty acid synthase (*fas*) was down-regulated and *cpt1* up-regulated in fish fed high lipid diets at t0. However, at t6, *cpt1* expression was marginally higher ($p = 0.06$) in fish fed the FO diets, while no changes were observed with regard to LL.

In the intestine, high lipid diets also caused a down-regulation of *fas* at t0, while at t6, *cpt1* was the only gene with significant differences in transcript levels, being elevated only in the 8VO treatment (showing a significant effect of lipid level and lipid source, but also a significant interaction between the two factors).

Regarding genes related to lipid absorption, *cd36* was significantly more expressed in fish fed low lipid diets at t0, while microsomal triglyceride transfer protein (*mtp*) was significantly affected by dietary lipid level, whereas highest mRNA levels

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Table 6 Pre-prandial expression of lipid metabolism and absorption genes in Senegalese sole juveniles assessed by RT-qPCR at t0 (after 24 h of fasting) regulated by dietary lipid level (LL; 8 or 18 % lipids) and/or source (LS; VO or FO)

	8FO	8VO	18FO	18VO	*P* value (2-way ANOVA)		
					LL	LS	LL × LS
Liver							
Lipid metabolism							
acox1	1.20 ± 0.40	1.11 ± 0.27	0.94 ± 0.28	1.09 ± 0.61	0.402	0.863	0.495
fas	3.40 ± 4.64	2.22 ± 1.35	0.74 ± 0.22	0.59 ± 0.33	0.042	0.809	0.609
cpt1	0.88 ± 0.38	0.73 ± 0.21	1.47 ± 0.54	1.51 ± 0.95	0.010	0.832	0.704
Intestine							
Lipid absorption							
cd36	1.26 ± 0.38	1.17 ± 0.21	0.99 ± 0.25	0.77 ± 0.21	0.006	0.164	0.567
fabp1	0.87 ± 0.38	1.04 ± 0.18	1.36 ± 0.54	1.04 ± 0.45	0.164	0.639	0.158
fabp2a	1.03 ± 0.36	1.13 ± 0.17	0.93 ± 0.28	1.04 ± 0.15	0.371	0.335	0.955
mtp	0.93 ± 0.21	0.94 ± 0.1	0.99 ± 0.13	1.22 ± 0.21	0.026	0.112	0.122
apoa4	0.94 ± 0.18	0.98 ± 0.17	1.10 ± 0.30	1.1 ± 0.32	0.187	0.846	0.800
Lipid metabolism							
acox1	1.10 ± 0.38	1.01 ± 0.31	1.08 ± 0.22	0.95 ± 0.27	0.745	0.385	0.871
fas	1.16 ± 0.33	1.18 ± 0.22	0.90 ± 0.11	0.85 ± 0.11	0.003	0.849	0.679
cpt1	1.07 ± 0.35	1.09 ± 0.27	0.95 ± 0.16	1.02 ± 0.33	0.440	0.730	0.812

Values are mean NRQ ± SD ($n = 6$ per treatment)

Table 7 Post-prandial expression of lipid metabolism and absorption genes in Senegalese sole juveniles assessed by RT-qPCR at t6 (6 h after feeding) regulated by dietary lipid level (LL; 8 or 18 % lipids) and/or source (LS; VO or FO)

	8FO	8VO	18FO	18VO	*P* value (2-way ANOVA)		
					LL	LS	LL × LS
Liver							
Lipid metabolism							
acox1	1.02 ± 0.38	1.06 ± 0.24	1.14 ± 0.21	0.94 ± 0.29	0.982	0.507	0.305
fas	1.19 ± 0.69	2.27 ± 1.93	0.8 ± 0.63	1.24 ± 1.11	0.167	0.140	0.523
cpt1	1.5 ± 0.74	0.81 ± 0.13	1.25 ± 0.77	0.96 ± 0.49	0.849	0.057	0.414
Intestine							
Lipid absorption							
cd36	1.22 ± 0.18	1.10 ± 0.22	1.03 ± 0.26	0.77 ± 0.10	0.004	0.030	0.367
fabp1	0.78 ± 0.32	1.15 ± 0.35	1.11 ± 0.30	1.21 ± 0.23	0.135	0.073	0.303
fabp2a	0.94 ± 0.32	1.06 ± 0.23	1.11 ± 0.16	1.04 ± 0.38	0.521	0.809	0.421
mtp	0.94 ± 0.08	1.00 ± 0.24	1.12 ± 0.10	0.98 ± 0.12	0.213	0.477	0.115
apoa4	0.96 ± 0.16	1.09 ± 0.24	0.99 ± 0.40	1.08 ± 0.13	0.938	0.297	0.868
Lipid metabolism							
acox1	0.99 ± 0.18	1.13 ± 0.56	1.13 ± 0.25	0.94 ± 0.27	0.827	0.860	0.264
fas	1.18 ± 0.45	1.44 ± 1.03	0.81 ± 0.15	0.94 ± 0.14	0.076	0.400	0.776
cpt1	0.91 ± 0.38	1.47 ± 0.23	0.94 ± 0.25	0.91 ± 0.22	0.032	0.031	0.017

Values are mean NRQ ± SD ($n = 6$ per treatment)

were observed in the 18VO treatment. At t6, *cd36* was significantly affected by lipid level and lipid source, being more highly expressed in fish fed lower lipid levels and FO diets, hence resulting in highest mRNA levels in the 8FO treatment.

No significant differences were observed in transcript levels of *acox1*, *fabp1*, *fabp2a* or *apoa4* in relation to any of the dietary factors.

Discussion

Effect of Lipid Level and Source on Growth Performance

In this study, no significant dietary effects were observed on growth (SGR), although a strong trend ($p = 0.06$)

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suggested that fish fed FO diets grew slightly better than those fed diets containing 75 % VO, irrespective of lipid level. Our results showing no significant effects of lipid level on growth of sole juveniles were surprising, considering how an increase in dietary lipid level has been shown to be detrimental to growth performance in this species [14, 46]. It could be suggested that 8 % dietary lipid levels might have already been excessive for sole but this hypothesis is not supported by a previous study which did not indicate differences in growth performance between fish fed 4 and 8 % lipid diets, while superior growth was observed in those fed 8 % compared to 12 % dietary lipids and higher [14]. On the other hand, in a study by Borges *et al*. [25], growth of juvenile Senegalese sole was not compromised when substituting dietary FO up to 100 % with a blend of VO (rapeseed, soybean, and linseed oil in a ratio of 3:2:5) at 9 % lipid levels. However, Benítez-Dorta *et al*. [47] observed a lower growth performance when sole were fed 12 % lipid diets formulated exclusively with soybean oil when compared to FO or linseed oil-based diets. These results suggest that both dietary lipid level and lipid source are important and the interaction between both factors can determine how much lipid from a vegetable origin can be included in sole diets without affecting growth. Furthermore, LNA has been observed to negatively affect fish performance compared to ALA [6]. Hence, it may be possible that soybean oil had a negative effect on Senegalese sole performance, most likely due to its high LNA content [47]. If this was indeed the case, results from Borges *et al.* [25] and the present study (using the same VO blend, but at a 1:1:1 ratio) indicate that if the soybean oil component is diluted by dietary inclusion of ALA-rich linseed and (to a lesser extent) rapeseed oil, similar growth performance can be achieved to that of fish fed FO.

Lipid Deposition in Whole Body and Specific Tissues

In teleosts, lipid stores can generally be found in the muscle, subcutaneous tissue, liver, head, viscera and other organs [48], but the overall proximate lipid content of the body and preferential sites of lipid deposition vary greatly among species (as reviewed by Tocher [49]). In this study, whole-body lipids ranged from 4.1 to 6.4 % WW, similar to what has previously been reported [14, 50, 51], and were significantly higher in fish fed high lipid diets, mainly at the expense of protein. According to Fernandes *et al*. [51], most of the whole body lipids in Senegalese sole were located in muscle (31 %) and head (8 %), while liver and viscera accounted for only 2 and 1 %, respectively. However, even though muscle adds to total body lipids more than other tissues, it is not considered a preferential site for fat deposition in this species. Accordingly, no changes in total muscle lipid levels in response to dietary input were observed in this or previous studies [14, 15, 52]. On the other hand, liver is considered the primary tissue for fat deposition in this species [15, 52] and indeed had the highest total lipid level (4.1–5.8 % WW) of all tissues analyzed in this study, but no significant differences were observed in total lipid content between treatments, as previously reported by Borges *et al.* [14] in response to varying dietary lipid level. However, other studies with juvenile Senegalese sole observed significant differences when the dietary lipid/carbohydrate ratio was increased [53], but also when fish were fed higher levels of plant protein [51], where total lipid levels in the liver were around 17.5–30 % WW and 5.5–11 % WW, respectively. These high variations in hepatic fat content indicate that other dietary factors, such as carbohydrate content and protein amino acid profile, can influence fat accumulation in the liver as much as, or even more, than lipid levels and fatty acid composition. On the other hand, histological observations of hepatocytes revealed that, although dietary lipid levels did not affect the fat content in the liver, fish fed VO had a more compact hepatic parenchyma and significantly larger surface of tissue occupied by fat deposits compared to those fed FO, similarly to what was described by Caballero *et al.* [54] in the liver of rainbow trout. In our study the histological method was probably more precise than lipid analysis, which may not have been able to detect subtle changes in lipid content among different experimental groups.

In spite of a lack of an effect of lipid level on hepatic lipid accumulation, sole fed higher dietary lipid level had a significantly higher HSI. Although other authors have reported similar results in response to increases in lipid level [53, 55, 56], it is noteworthy that in our study these differences were mainly caused by the 18VO diet (which also induced the highest VSI). However, an elevated HSI may also be an indicator of glycogen accumulation and not just increased lipid reserves [57]. Moreover, high dietary lipid level was shown to have a Hyperglycemic effect in Senegalese sole juveniles and has been associated with significantly higher glycogen content in the liver of this species [58]. Therefore, although carbohydrate composition was not measured specifically in liver, we cannot exclude the possibility of a greater glycogen accumulation in liver of sole fed the 18VO diet having at least partly contributed towards the HSI results in our study, in addition to FA biosynthesis and lipid storage.

More lipids were found in the intestine (60.9 to 135.1 mg 100 g^{-1} fish) than in the liver (41.4 to 74.0 mg 100 g^{-1} fish), similarly to what was reported by Borges *et al.* [14], although these are generally considered transient accumulations rather than lipid deposition (energetic stores) as in liver. These accumulations can be caused by high dietary lipid content and are suggested to be a temporary storage of re-esterified FA, rather than products

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of lipogenesis, in cases when the rate of lipid absorption exceeds the rate of lipoprotein synthesis [48] or because of an inability to metabolize lipids [26, 59]. Thus, total lipid levels in the intestine, as well as accumulation of lipids in the enterocytes were higher in fish fed the 18 % lipid diets, irrespective of the lipid source. These accumulations consisted of numerous large intracellular lipid droplets of up to 16 µm in diameter, while fish fed low lipid diets had no or very few small droplets (~1.5 µm in diameter), similar to what was observed by Bonvini *et al*. [60] in common sole (*S. solea*).

Effect of Dietary Lipid Level and Source on Liver Metabolism

Lipogenesis in mammals is known to be regulated by dietary lipid level, as well as by lipid source [61–63], and good evidence is also starting to accumulate in fish. Two key lipogenic enzymes were analyzed in this study, at the transcriptional level (*fas*) or by determining its activity (Acly). High lipid diets caused a down-regulation of hepatic *fas* mRNA levels in fish that were fasting for 24 h. Similarly, previous studies with fasted Senegalese sole [17, 53], channel catfish *Ictalurus punctatus* [64], Atlantic salmon *Salmo salar* [65], common carp *Cyprinus carpio* [66] and European seabass [67, 68] also showed an inhibition of hepatic lipogenesis by high dietary lipid levels. However, no differences in *fas* expression were observed 6 h after a meal, contrary to previous studies in mammals [69, 70], and also some salmonid species [71, 72] that reported an inhibitory effect of high dietary lipid level on lipogenesis during the post-prandial phase. However, conclusions in these studies were based on the activity of different lipogenic enzymes, which may be affected differently depending on their place/role in the pathway and may have temporal differences in activity. In rainbow trout, for example, Fas activity did not vary between treatments 8 h after feeding, while acetyl-CoA carboxylase (Acc) did [72].

When looking at the effects of lipid source, no differences were observed in *fas* expression between treatments either pre- or post-prandially. Nevertheless, the hepatic activity of Acly was lower in fish fed FO diets. These results are partly in agreement with the general consensus across species that dietary PUFA act on multiple nuclear receptors and transcription factors to decrease anabolic pathways and increase catabolism [63, 73]. In mammals, the effects of PUFA in inhibiting the transcription of *fas* and decreasing lipogenesis in liver have been well documented [62, 74]. However, studies in fish have been inconsistent in this respect and this might be explained by the fact that poikilotherms appear to have poor regulatory mechanisms of lipogenesis [75]. Hence, while no effect of dietary FO substitution by VO has been reported on lipogenesis in studies with Atlantic salmon [76], rainbow trout [77] or European seabass [78], another study reported an inhibitory effect of dietary FO on hepatic *fas* mRNA levels compared to VO-based diets in fasting Atlantic salmon [79]. Furthermore, hepatocytes of rainbow trout in vitro had lower Fas and Acc relative activity when EPA and DHA were added, compared to LNA, and also reduced Acly activity in response to EPA, but not DHA [37]. Considering these results, and the reduced amounts of lipid deposits within hepatocytes from fish fed FO diets, we cannot ignore the potential effect that dietary LC-PUFA may have on reducing hepatic lipogenesis in this species.

In order to undergo β-oxidation, long-chain fatty acyl-CoA enter the mitochondria and peroxisomes with the aid of carnitine acyltransferase enzymes, which are specific for each organelle [80]. The mitochondrial *cpt1* gene analyzed in this study showed significantly higher transcript levels in fasted fish (t0) fed high lipid levels compared to those fed a low lipid diet-opposite to what was observed for *fas*. This is not surprising considering how catabolic pathways are usually regulated in opposite directions to anabolic pathways to maintain energy homeostasis. For example, malonyl-CoA, the substrate of Fas in lipogenesis, acts as an inhibitor of Cpt1 [81]. Inside the peroxisome or mitochondria, β-oxidation is catalyzed by enzymes such as acyl-coA oxidase (Acox1; peroxisomal) and Hoad. In this study no significant differences in the activity of Cpt1 or Hoad enzymes were observed and no differences in *acox1* expression were observed either, at none of the analyzed time points. This, as well as the lack of significant effects in some of the other parameters measured in this study, is possibly due to the high variability of the results. While a force feeding method was used to ensure fish were fed equally (in terms of amount of food and timing of feeding), it is possible that individual differences in basal metabolism established over the whole experimental period might have caused these variations. High individual variability in feeding and metabolism can be typically found in several fish species but might be exacerbated in Senegalese sole, as a strong hierarchical population structure is characteristic for this species [13]. In the liver of fasted juvenile haddock *Melanogrammus aeglefinus*, similarly to what was observed here in sole, no difference in β-oxidation was observed when fish were fed diets varying in lipid content from 12 to 24 % [82]. However, in rainbow trout, both *cpt1* and *acox1* were up-regulated in the liver of fish fed high lipid diets, but also in diets containing LC-PUFA compared to medium-chain FA [11]. In sum, similarly to effects of lipid level or lipid source in lipogenesis, effects on β-oxidation appear to be more variable in fish species than in mammals, but present results indicate that a high lipid diet can stimulate import of fatty acyl-CoA's for mitochondrial oxidation in Senegalese sole juveniles.

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As previously noted, an important metabolism modulating effect has been reported for dietary LC-PUFA in mammals, acting on multiple nuclear receptors and transcription factors to coordinately depress anabolism and increase catabolic pathways, such as β-oxidation [63, 73]. Furthermore, in fish, saturated and monounsaturated fatty acids have been shown to be a preferred substrate over PUFA for β-oxidation [83], although, when given in surplus, PUFA are also readily oxidized [84]. In this study a strong trend ($p = 0.06$) for higher *cpt1* mRNA levels was observed in fish fed FO diets, although only during the post-prandial phase, possibly because its pre-prandial expression was more strongly influenced by dietary lipid level. This correlated well with the lower lipid accumulation observed in the liver of fish fed FO diets. Similarly, in Atlantic salmon, EPA (although not DHA) was shown to stimulate hepatic β-oxidation [85, 86], while in rainbow trout, SFA, MUFA, ALA, ARA and DHA up-regulated, while LNA and EPA down-regulated *cpt1* expression in vitro [87].

Effect of Dietary Lipid Level and Source on Lipid Metabolism in the Intestine

The liver is generally considered a more relevant organ for lipid metabolism compared to the intestine [88]. Thus, it is not surprising that in this study *fas* and *cpt1* mRNA levels were substantially lower in the intestine compared to the liver, although *acox1* expression was comparable in both tissues (based on C_t values; data not shown). On the other hand, the existing data collectively suggests that some aspects of lipid metabolism in the intestine might be expressed similarly or even more than in the liver [89, 90], and that this organ likely has numerous other roles beyond the simple reacylation and packaging of lipids for transfer into body tissues [91–93]. Still, studies specifically looking at lipid metabolism in fish intestine are still insufficient. In response to dietary lipid level, pre-prandial expression of *fas* was down-regulated in fish fed high lipid diets, similarly to what was observed in liver. Not much work has been done in similar conditions in fish, but an increase in gene expression and enzyme activity of the β-oxidation pathway was observed in the intestine of mice fed high fat diets [94].

On the other hand, the hypotriglyceridemic effect of FO (through increased β-oxidation and/or reduced lipogenesis) observed in the liver of Senegalese sole in this study was not noticeable in the intestine. Namely, no changes in *fas*, *cpt1* or *acox1* expression were observed in fasted fish, and *cpt1* was unexpectedly up-regulated post-prandially, only in the 8VO treatment. Such effects have been reported in the intestine of fish and mammals, such as in Atlantic salmon [93], where intestinal *fas* expression was down-regulated by dietary LC-PUFA, and in mice fed LC-PUFA rich diets, which exhibited an increase in β-oxidation in the small intestine [95]. Considering the variability of the responses measured in fish liver so far, and the scarcity of studies focusing on intestinal lipid metabolism, more work should be performed to address the effects of lipid level and lipid source on metabolism in this important organ.

Effect of Dietary Lipid Level on Intestinal Lipid Absorption

Sole fed high lipid diets clearly showed a higher lipid accumulation in the intestinal mucosa, while the source of the dietary lipids did not affect these results. These differences did not seem to have major long term effects in the animal as the FCR did not differ significantly between treatments, and no significant changes were observed in lipid content in muscle or liver in relation to dietary lipid level. However, they indicate that sole were not able to deal with a high dietary supply of lipids by adjusting their intestinal lipid absorption mechanisms and, in order to investigate this in further detail, we looked at the expression of several genes that are key for these processes.

The CD36 peptide, also known as fatty acid translocase (FAT), is a multiligand scavenger receptor that has been implicated in numerous biological processes [96]. In mammals it is quite abundant in numerous tissues, where it may bind to native or modified lipoproteins, thrombospondin-1, collagen and other compounds [97, 98]. In addition, it has also been shown to play an important role in intestinal absorption, where it facilitates long-chain FA uptake by cells [99–101] and has been associated with intestinal [102] and orosensory [103, 104] perception of dietary fat and satiety signaling. The platelet glycoprotein 4-like transcript, exclusively expressed in the intestine and brain (>20 times lower than in intestine) of Senegalese sole (although its expression in the oral epithelium was not evaluated; unpublished data), showed high sequence homology to the mammalian *Cd36/Fat* and was used for the purpose of this study. Our results showed that intestinal *cd36* expression was significantly affected by dietary lipid level, but the results were opposite to what might be expected. Namely, transcript levels were lower in fish fed high lipid diets pre- and post-prandially, while, higher levels of *Cd36/Fat* were recorded in mouse fed high lipid diets [105]. On the other hand, this gene was also significantly affected by lipid source post-prandially (and at t0 a similar trend was observed), being up-regulated in fish fed FO diets. There was no interaction between lipid level and lipid source and, in fact, the effect appeared cumulative, resulting in the highest and lowest expression being measured in the 8FO and 18VO treatments, respectively. Presently, it is difficult to determine the exact mechanisms explaining the regulation of this *cd36* homolog in Senegalese sole, but considering one of its roles being in the detection of FA, we

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hypothesize that the responses measured here could be more related to gastrointestinal lipid sensing than lipid absorption.

Once FA enter the enterocytes, they are re-esterified and packaged into lipoproteins before being released into circulation. FABPs are proteins which facilitate intracellular transport of free FA from the microvillus membrane to the endoplasmatic reticulum for re-esterification [106]. They have a complementary function to CD36/FAT and were equally up-regulated in the intestine of mouse fed high-lipid diets [107]. In zebrafish, *fabp* mRNA levels have been correlated with intracellular storage of lipid droplets and synthesis of VLDL particles in enterocytes [108]. However, in this study neither of the two analyzed *fabp* transcripts in the intestine were affected by the dietary lipid level.

The *Mtp* is a protein that is essential for correct formation of primordial apoB apolipoproteins in the endoplasmatic reticulum of enterocytes [109]. In hamster, a liver-type *Fabp* homolog (corresponding to *fabp1* in our study) and *Mtp* were up-regulated after one day of feeding a high lipid diet, but after prolonged exposure (9 days), only *Mtp* levels remained elevated [110]. In our study, long-term feeding of Senegalese sole juveniles with high lipid diets increased fasting levels of *mtp* in the intestine. However, this response was only noticeable in fish fed the 18VO diet, indicating that an interaction between lipid level and its FA composition (lipid source) was necessary for it to occur. Therefore, present results suggest that rather than being affected by generally high lipid levels, it may be that absolute values of individual dietary FA components that affected *mtp* expression. This hypothesis is likely, considering results from an *in vitro* study with HepG2 cells that showed that administration of oleic acid (which was by far higher in the 18VO diet) up-regulated *Mtp*, while no effect was observed when the same cells were exposed to palmitate, ARA or LNA [111]. Nevertheless, *mtp* is regulated at the transcriptional, translational and/or post-translational level by different macronutrients, and also by hormones (insulin, leptin) and other factors [112], and therefore other mechanisms could explain our results.

Finally, the expression of apolipoprotein A4 (*apoa4*) was also analyzed. This protein has an essential role in enhancing triacylglycerol (TAG) packaging into chylomicrons by increasing their size and, which can lead to an increased basolateral TAG secretion in the intestine [113]. In sole larvae, a coordinated up-regulation of several apolipoprotein-related genes (including *apoa4*) in response to a higher dietary lipid intake has been described [114]. However, in this study no pre- or post-prandial changes were observed in *apoa4* in response to lipid level.

In summary, results from this and other studies suggest that sole can cope well with a high inclusion of VO (up to 100 %) in their ongrowing diets, although this might vary somewhat depending on the oil type or blend which is used. Furthermore, although best growth performance is usually obtained at low (8–10 %) dietary lipid levels, results from this experiment suggest that lipid level can probably be increased in the diets with no major effects on growth and feeding performance, using diets containing fish meal and up to 75 % of FO replaced by an equal blend of linseed, rapeseed and soybean oil. However, the diets had different effects on HSI, VSI, lipid deposition and metabolism and, in a few cases, results suggest an interaction between dietary lipid level and lipid source. In particular, the increase in HSI and VSI in fish fed high lipid diets appeared to be mainly (HSI) or exclusively (VSI) associated with the high content of VO in the diet. In general, dietary lipid level seemed to have a larger effect on the expression of genes involved in lipogenesis or β-oxidation compared to lipid source, with most changes observed at fasting. The results show that Senegalese sole juveniles responded to diets containing high lipid levels and conversely, reduced carbohydrate contents, by down-regulating FA synthesis in the liver and intestine, and enhancing β-oxidation in liver. Furthermore, although less clearly, results suggest that LC-PUFA can also affect these pathways in sole liver, as indicated by a reduced Acly enzymatic activity at fasting and up-regulation of *cpt1* 6 h after feeding, as well as by a significantly lower accumulation of lipids in hepatocytes (observed histologically), when fish were fed FO diets. Finally, analysis of the transcriptional changes of several genes that are collectively responsible for FA uptake from the intestinal lumen, intracellular transport and packaging into lipoproteins, did not indicate a strong response to dietary lipid level or lipid source (except in the case of *cd36*, possibly unrelated to FA absorption, and in *mtp*, but specifically associated with the 18VO diet). This might suggest a poor capacity of Senegalese sole to regulate their intestinal FA absorption in order to adapt to high dietary lipid levels and could explain the important accumulation of lipid droplets in the intestinal epithelia of sole fed high lipid diets.

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Mechanisms of lipid metabolism and transport underlying superior performance of Senegalese sole (*Solea senegalensis*, Kaup 1858) larvae fed diets containing n-3 polyunsaturated fatty acids

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To date, most research on larval lipid nutrition has been centered on essential fatty acid requirements. However, less is known about effects of dietary fatty acid (FA) composition on lipid absorption and metabolism, as addressed in this study. Senegalese sole were fed live preys enriched with different oils: cod liver oil (CLO), linseed oil (LSO), soybean oil (SBO), and olive oil (OO), that are rich in long-chain polyunsaturated FAs (LC-PUFA), n-3 PUFA, n-6 PUFA or monounsaturated FAs (MUFA), respectively. Larvae reared on CLO showed significantly improved growth and survival, faster onset of metamorphosis and maturation of the intestine, lower lipid accumulation in liver after metamorphosis and an up-regulation of genes involved in lipid transport and phospholipid metabolism, while key lipogenesis genes were down-regulated. From the remaining treatments, the LSO diet induced the closest performance to CLO, with larvae completing metamorphosis at a similar time and having the second best growth and survival by the end of the experiment. They were also grouped closer to the CLO treatment than the remaining vegetable oil treatments, based on the patterns of gene expression. These results showed that oil sources rich in LC-PUFA and n-3 PUFA were superior to those having high n-6 PUFA or MUFA levels in the larval nutrition of Senegalese sole and indicate that this effect might be at least partly explained by an up-regulation of phospholipid metabolism and apolipoprotein synthesis, likely leading to enhanced lipid transport and mobilization, as well as tissue growth and remodeling.

Statement of relevance: The nutritional and physiological roles of dietary C18 PUFA in the absence of LC-PUFA have rarely been investigated in fish, particularly in larvae, as it is unfeasible to culture most marine species in such extreme dietary conditions. Senegalese sole (Solea senegalensis), a marine species of high commercial aquaculture importance in southern Europe, has relatively low LC-PUFA requirements during the larval stage compared to other marine finfish, making it a good model species to investigate this subject. A holistic approach was implemented to determine the effects these FAs may have on fish performance and metabolism, by analyzing growth, development, intestinal maturation, lipid and FA composition, hepatic and intestinal lipid accumulation, and expression of genes involved in lipid metabolism, absorption and transport (qPCR).

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1. Introduction

Dietary lipids are essential for fish larvae to meet the high structural and energy demands of rapid growth and intense organogenesis associated to development and metamorphosis (Hamre et al., 2013; Tocher, 2010). Long-chain polyunsaturated fatty acids (LC-PUFA), namely eicosapentaenoic acid (20:5 n-3, EPA), docosahexaenoic acid (22:6 n-3, DHA) and arachidonic acid (20:4 n-6, ARA), are considered essential fatty acids (EFAs) in teleosts, as they either cannot be synthesized endogenously, or can but at insufficient rates to meet physiological demands. These EFAs are of great importance as they are integral components of phospholipids (PLs), which are in turn fundamental building blocks of membrane bilayers. Furthermore, they also act as

Abbreviations: CLO, cod liver oil; LSO, linseed oil; SBO, soybean oil; OO, olive oil; FO, fish oil; VO, vegetable oil; PUFA, polyunsaturated fatty acid; LC-PUFA, long-chain polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid; EFA, essential fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; ALA, α-linolenic acid; LNA, linoleic acid; OA, oleic acid; TFA, total fatty acids; UTR, untranslated region; ORF, open reading frame; TAG, triacylglicerol (triglyceride); PL, phospholipid; PC, phosphatydilcholine; PE, phosphatydilethanolamine; PS, phosphatydilserine; HDL, high density lipoprotein; FAS, fatty acid synthase; APOA4, apolipoprotein A4; Δ4FAD, Δ4 fatty acid desaturase; FABP3, fatty acid binding protein 3.

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precursors to bioactive eicosanoids and regulate gene expression related to inflammatory responses and lipid metabolism (Schmitz and Ecker, 2008; Tocher, 2010). Thus, it is not surprising that a deficiency of LC-PUFA in larval diets has been shown to result in reduced growth and survival rates, pigmentation abnormalities, altered visual acuity (affecting the capacity to capture live prey), abnormal behavior and susceptibility to disease and stress (Bell and Dick, 1993; Bransden et al., 2005; Furuita et al., 1998; Lund et al., 2007; Sargent et al., 1999; Shields et al., 1999; Tocher, 2010; Villalta et al., 2005a; Watanabe, 1993).

However, aside from EFAs, numerous other dietary FAs also have important roles in larval development. Lipids are the main energy source for developing fish and it has been well-established that saturated (SFA) and monounsaturated fatty acids (MUFA), like oleic acid (OA), are preferred to polyunsaturated fatty acids (PUFA) as substrates for β-oxidation (Crockett and Sidell, 1993; Kiessling and Kiessling, 1993). Lipid requirements of larvae should, therefore, be considered not only in terms of absolute and relative levels of EFAs, but rather the optimal balance between LC-PUFA and other dietary FAs, which are the main source of metabolic energy.

Furthermore, a standing question refers to the nutritional and physiological roles that dietary C_{18} PUFA, such as α-linolenic (18:3 n-3, ALA) and linoleic (18:2 n-6, LNA) acids play in numerous fish species. Their effects in the absence of LC-PUFA have rarely been investigated in fish, particularly in larvae, as it is unfeasible to culture most marine species during these critical stages of development in such extreme and adverse dietary conditions. However, Senegalese sole (*Solea senegalensis*), a marine species of commercial aquaculture importance in southern Europe (Morais et al., 2015a), has been shown to have relatively low LC-PUFA requirements during the larval stage compared to other marine finfish, which appeared to decrease even further after metamorphosis (Conceição et al., 2007; Dâmaso-Rodrigues et al., 2010; Morais and Conceição, 2009; Morais et al., 2004; Villalta et al., 2005b), making it a good model species to investigate this subject.

In order to address these questions and help understand the effects that different FAs might have on lipid metabolism and absorption, an experiment was designed with larvae and post-larvae of Senegalese sole fed live prey enriched with one of four oil emulsions differing in FA composition (rich in either LC-PUFA, ALA, LNA or MUFA). A holistic approach was implemented to determine the effects these FAs might have on fish performance and metabolism, with analyses including the assessment of growth, development (completion of metamorphosis), intestinal maturation (evaluated by digestive enzyme activity), lipid and FA composition, hepatic and intestinal lipid accumulation (assessed by histology) and expression of genes involved in lipid metabolism, absorption and transport (qPCR).

2. Materials and methods

2.1. Larval rearing, dietary treatments and feeding trial

Newly hatched Senegalese sole larvae were obtained from Stolt Sea Farm S.A. (Carnota, A Coruña, Spain) and distributed into sixteen (4 replicates per treatment) 100 L cylindroconical tanks at a density of 90 larvae L^{-1}. The tanks were connected to a recirculation system (IRTAMAR®) with 50% daily water renewal and regulated temperature (18–20 °C), salinity (35 ppt) and dissolved oxygen (7.5 mg L^{-1}). The photoperiod was 16 h light:8 h dark with a light intensity of <500 lx at the water surface. Larvae were fed enriched rotifers, at a density of 10 rotifers mL^{-1}, from 2 days post-hatching (dph) to 8 dph. Enriched *Artemia* metanauplii were introduced at 6 dph and fed as detailed in Boglino et al. (2012a) until 37 dph. In short, enriched and hydrogen peroxide-disinfected (5 min at 8000 ppm) *Artemia* metanauplii were fed from 6 to 14 dph in quantities gradually increasing from 0.5 to 4 metanauplii mL^{-1} and from 15 to 25 dph, as larvae became benthonic, live *Artemia* were gradually substituted with frozen enriched metanauplii, and fed at 4–6 metanauplii mL^{-1} in total. From 25 dph onwards, the post-larvae were fed exclusively frozen enriched *Artemia*, at 6–12 metanauplii mL^{-1}. Feeding was performed twice a day.

Four emulsions (Table 1) were prepared following the methodology described in Villalta et al. (2005b) and were used to enrich rotifers and *Artemia* metanauplii (EG type, Sep-Art, GSL; INVE, Belgium). Each emulsion was prepared with one of the following oils: cod (*Gadus morhua*) liver oil — CLO, linseed oil — LSO, soybean oil — SBO, and olive oil — OO. Live prey culture and enrichment were performed as described in Boglino et al. (2012a). Briefly, rotifers were fed daily with microalgae (*Tetraselmis chuii*) at 4×10^5 cells mL^{-1} and yeast (Mauripan, Categgio Lieviti, Italy) at 0.7 g per million rotifers. Each day, rotifers were enriched, at a density of 500 rotifers mL^{-1}, with 0.1 g L^{-1} of each experimental emulsions for 2 h (first meal of the day) or 6 h (second meal). *Artemia* nauplii were hatched daily during the larval pelagic stage to be enriched and fed live. Additionally, two large batches of enriched metanauplii were produced and frozen to later feed the benthic post-larvae. *Artemia* enrichments were performed for 16 h in standard conditions, at a concentration of 150 metanauplii mL^{-1}, with 0.6 g L^{-1} of emulsion.

A separate short-term feeding trial was performed at two different ages: 16 dph with pre-metamorphic pelagic larvae and 34 dph with benthic post-larvae from the 4 dietary treatments. On the evening before the experiment, two groups of fish per tank were transferred into separate containers, each group containing 200 larvae and 50

Table 1
Formulation of the experimental emulsions and the resulting total lipid content, total FA content and FA composition of enriched *Artemia* (means ± SD; n = 4 pools).

	CLO	LSO	SBO	OO
Emulsion formulation (mg g^{-1})				
Cod-liver oil[1]	528	0	0	0
Linseed oil[2]	0	528	0	0
Soybean oil[3]	0	0	528	0
Olive oil[4]	0	0	0	528
Soy lecithin[5]	40	40	40	40
α-Tocopherol[6]	12	12	12	12
Distilled water	420	420	420	420
Total lipid and FA content of enriched Artemia (mg per g of DW)				
Total lipids	107.1 ± 19.3	100.6 ± 6.3	106.1 ± 20.4	112.6 ± 6.8
Total FA	61.6 ± 10.8	61.2 ± 11.0	69.3 ± 22.3	71.7 ± 7.5
FA composition of enriched Artemia[a] (% of TFA)				
16:0	10.0 ± 0.3	7.4 ± 0.5	8.3 ± 1.0	8.1 ± 0.7
18:0	5.4 ± 0.4	6.5 ± 1.0	6.0 ± 0.9	5.0 ± 0.5
Total SFA	16 ± 0.7	14 ± 1.4	14.4 ± 1.2	13.3 ± 1.3
16:1	2.5 ± 0.5	0.4 ± 0.2	0.5 ± 0.1	0.6 ± 0.2
18:1n-9	24.4 ± 0.5	25.1 ± 1.1	24.7 ± 0.5	49 ± 0.1
18:1n-7	7.6 ± 0.5	5.7 ± 1.0	4.6 ± 1.3	7.7 ± 1.0
20:1	2.0 ± 2.3	0.2 ± 0.3	0.1 ± 0.2	0.0 ± 0.0
Total MUFA	40.4 ± 2.7	31.3 ± 1.6	30.0 ± 0.8	57.3 ± 1.0
18:2n-6	6.0 ± 0.4	11.7 ± 1.9	28.8 ± 5.0	7.9 ± 2.2
20:4n-6	0.5 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
Total n-6 PUFA	9.1 ± 3.3	12.4 ± 1.6	29.9 ± 4.5	8.7 ± 2.2
18:3n-3	19.8 ± 2.2	38.2 ± 2.1	21.5 ± 2.8	17.1 ± 0.7
20:5n-3	6.3 ± 0.8	0.9 ± 0.3	0.8 ± 0.2	0.7 ± 0.1
22:5n-3	0.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:6n-3	3.9 ± 0.8	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0
Total n-3 PUFA	34.4 ± 0.7	42.2 ± 1.7	25.6 ± 3.2	20.6 ± 0.9
Total PUFA	43.5 ± 2.9	54.6 ± 2.6	55.5 ± 1.6	29.3 ± 1.4
DHA/EPA	0.6 ± 0.1	0.0 ± 0.0	0.2 ± 0.4	0.0 ± 0.0
ARA/EPA	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0

[a] Not all FAs are shown.
[1] Sigma-Aldrich Co., St Louis, MO, Germany.
[2] Biolasi Products Naturals, S.L., Guipùzcoa, Spain.
[3] Huilerie Emile Noël, S.A.S., Pont Saint Esprit, France.
[4] Borges Pont, Lleida, Spain.
[5] ADD.
[6] ADD.

post-larvae, and were left with no food overnight. The first group was kept in a 2 L container placed inside the tank, with mesh allowing for water circulation but preventing *Artemia* in the tanks from entering, and all larvae were sampled at the end of a 16 h fasting period (t0). The second group of fish was kept in a separate 12 L container with clean aerated seawater, for easier manipulation. In the second group, after the 16 h fasting period, the fish were fed with enriched *Artemia* (live at 16 dph and frozen at 34 dph) from their respective dietary treatments and were left to feed undisturbed for 1 h, after which any uneated live feed was removed by filtering the fish through a submerged 500 μm mesh. Larvae and post-larvae were then transferred to a new 12 L container with clean aerated seawater and sampled postprandially, at 3 h after the end of feeding (t3). Sampled fish were quickly euthanized with MS-222 and preserved in 3 mL of RNAlater stabilization buffer (Ambion, Life Technologies, Alcobendas, Madrid, Spain) following manufacturer's instructions.

All experimentations on live fish were performed according to the European and National legislation with protocols approved by the ethics committee of IRTA.

2.2. Larval performance

Standard length (SL) and dry weight (DW) of 30 larvae per tank were measured at 2, 9, 16, 21, 30 and 37 dph. Larvae were euthanized using a lethal dose of MS-222 (Sigma-Aldrich Co., St Louis, MO, Germany; 1000 mg L^{-1}). Standard length was measured using a Nikon SMZ800 dissecting microscope (Nikon, Spain) connected to an Olympus DP25 digital camera (Olympus Corporation, Germany) and image analysis software (AnalySIS Gmbh, Olympus, Germany). The same larvae were then pooled, washed with distilled water, oven-dried at 60 °C for 24 h and weighed (one pool per tank) to determine DW to the nearest μg on a Mettler A-20 microbalance (Mettler Toledo, Columbus, OH, USA). To assess developmental progress of the larvae, eye migration was observed during metamorphosis. Samples of 20 specimens were taken from each tank prior to, during and post metamorphosis (at 12, 14, 16, 18, 22, 23, 25, 28, 30 dph) and examined under a dissecting microscope (as for SL measurements). Developmental stages were classified into five categories according to Fernández-Díaz et al. (2001): from "0" representing bilaterally symmetric larvae with a vertical swimming plane to "4" larvae with eye translocation completed and orbital arch clearly visible. The eye migration index (I_{EM}), was calculated according to Solbakken et al. (1999).

At the end of the experiment (37 dph), larvae remaining in the tanks were counted to estimate the survival rates. These values were then corrected for all larvae sampled during the experiment using a formula from Laurence (1974), modified to account for irregular sampling on a daily, instead of weekly, basis.

2.3. Lipid and fatty acid analyses

To evaluate the lipid content and fatty acid (FA) composition of the experimental diets, samples of enriched *Artemia* metanauplii from each treatment were taken on four consecutive days, carefully washed and immediately frozen at −20 °C. For biochemical analysis of the larvae, pools of 100 larvae and 50 post-larvae were taken per tank at 22 dph and 37 dph, respectively. Sampled larvae were euthanized with MS-222, washed with distilled water and immediately frozen at −20 °C. Total lipids were extracted using the method of Folch et al. (1957) and quantified gravimetrically, as detailed in Boglino et al. (2012a). Acid catalyzed transmethylation was carried out using the method of (Christie, 1982) and methyl esters were analyzed by gas–liquid chromatography as described in Morais et al. (2015). Results of fatty acid contents were expressed as a percentage of total fatty acids.

2.4. Digestive enzyme activity

Activity of the intestinal brush border membrane enzyme alkaline phosphatase (AP) and of the intracellular leucine–alanine peptidase (Leu–ala) was measured in pools of 30 whole larvae per tank sampled at 37 dph, quickly frozen and stored at −80 °C. Samples were homogenized in cold 50 mM mannitol, 2 mM Tris–HCl buffer, pH 7.0 and processed as described in Gisbert et al. (2009) for brush border purification. The enzymatic activities of AP and Leu–ala were quantified according to Bessey et al. (1946) and Nicholson and Kim (1975), respectively, and expressed as specific units (mU mg^{-1} protein). Soluble protein of crude enzyme extracts was quantified by the Bradford method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate.

2.5. Histological analyses

Five larvae from each tank were processed at 22 and 37 dph. They were euthanized as previously described, fixed in 4% formalin buffered to pH 7.0 with 0.1 M phosphate buffer for 24 h and then preserved in 70% ethanol. Samples were serially sectioned at 6–7 μm and stained with Harris hematoxylin and eosin. Sections of the posterior intestine and liver were photographed at × 40 magnification (300 dpi; 1 or 3 photos per tissue, respectively; n = 20 and n = 60 photos per treatment, respectively) with an Olympus DP70 digital camera connected to a Leica DM 2000™ microscope. All digital image analyses were performed using ImageJ (U.S. National Institutes of Health, Bethesda, USA; http://rsbweb.nih.gov/ij/index.html). A semi-automatic quantitative analysis was employed in order to measure the percentage of intestinal epithelium (longitudinal transects) and hepatocytes occupied by fat deposits. By adjusting the color threshold settings to brightness values between 180 and 255, the program was configured to select all surfaces covered with a white to light pink color, which corresponded to the area of fat deposits (Gisbert et al., 2008). These values were used to calculate the percentage of area occupied by lipids as in Boglino et al. (2012b). For liver samples, the total area of analyzed tissue was a rectangular selection, while longitudinal transects of the intestinal epithelium were selected manually with the "polygon selection" tool.

2.6. Gene expression analysis

For RNA extraction, pelagic larvae aged 16 dph were homogenized whole, while the benthic post-larvae (34 dph) were dissected on ice, under a microscope, in order to separate the head from the body, and only the latter compartment was used in this study. Samples were transferred to 2 mL screw-cap tubes containing 1 mL of TRIzol (Ambion, Life Technologies, Madrid, Spain) and approximately 50 mg of 1 mm diameter zirconium glass beads and homogenized (Mini-Beadbeater, Biospec Products Inc., USA). Solvent extraction was performed following manufacturer's instructions and final RNA concentrations were determined by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Madrid, Spain). RNA quality and integrity was assessed from the ratio of absorbances at 260 and 280 nm and by gel electrophoresis, respectively.

In order to study the expression of a few selected genes involved in lipid transport and metabolism by RT-qPCR, a search for candidate transcripts was performed in the SoleaDB online database (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/; Benzekri et al., 2014) using the *S. senegalensis* v4.1 global assembly. The retrieved transcripts were aligned and assembled *in silico* using the BioEdit Sequence Alignment Editor (Hall, 1999), and completed with rapid amplification of cDNA ends (RACE) PCR, if needed, using the FirstChoice® RLM-RACE kit (Ambion, Life Technologies, Alcobendas, Madrid, Spain). The obtained fragments were confirmed by sequencing (SCSIE, University of Valencia, Spain) and used as templates to design primers for RT-qPCR with Primer3 v. 0.4.0 (Koressaar and Remm, 2007; Untergasser et al.,

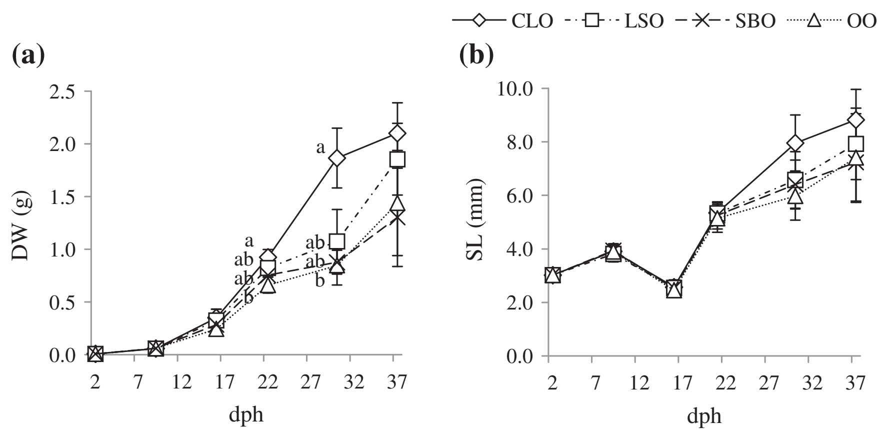

Fig. 1. Dry weight (a) and standard length (b) of Senegalese sole larvae and post-larvae fed live preys enriched with different oil emulsions. Values are means ± SD (n = 4 pools for DW or n = 120 individuals for SL, per treatment and time point). Letters show significant differences ($p < 0.05$) between treatments for individual days.

2012) (Table 2). The resulting amplicons were, once again, sequenced to confirm their identity and the specificity of the assay.

Two micrograms of total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life technologies, USA), following manufacturer's instructions, but using a mixture of random primers (1.5 μL as supplied) and anchored oligo-dT (0.5 μL at 400 ng μL^{-1}, Eurogentec, Cultek, S.L., Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. A pool of 2 μL from each cDNA sample was created to be used for standard dilutions and the remaining cDNA was diluted 60-fold with water. Amplifications were carried out in duplicate on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Alcobendas, Spain) in a final volume of 20 μL containing 5 μL of diluted (1/60) cDNA, 0.5 μM of each primer (except for *fabp1*, where 150 μM was used) and 10 μL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and included a systematic negative control (NTC-non template control). The RT-qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 35 cycles: 15 s at 95 °C, 1 min at the corresponding annealing temperature (Ta; Table 2). After the amplification phase, a melt curve was performed, enabling confirmation of the amplification of a single product in each reaction. Non-occurrence of primer-dimer formation in the NTC was also checked. The amplification efficiency of each primer pair was assessed from serial dilutions of the cDNA pool (Table 2). For data analysis, the RT-qPCR results were imported into the software qBase + (Biogazelle, Zwijnaarde, Belgium), where normalized relative quantities (NRQ) were calculated employing target and run-specific amplification efficiencies and using the geometric mean of 2–3 reference genes (*eef1a1*, *ubq* and *rps4*) established by Infante et al. (2008). For larval samples, all 3 reference genes were used for t0 (M = 0.222; coefficient of variance — CV = 0.087), while for t3 only *rps4* and *ef1a1* were selected (M = 0.222; CV = 0.077). For the post-larval samples,

Table 2
Primers used for real-time quantitative PCR (RT-qPCR). Shown are sequences and annealing temperature (Ta) of the primer pairs, size of the fragment produced, average reaction efficiency (E) and accession number of the target and reference genes (Infante et al., 2008).

Transcript	Primer sequence (5′–3′)	Amplicon size	Ta	E[a]	Accession number
Lipid metabolism					
aco	GGTCCATGAATCTTTCCACAA	168 bp	60°C	103.8%	**KP842776**
	ACAAGCCTGACGTCTCCATT				
fas	CACAAGAACATCAGCCGAGA	197 bp	60°C	104.9%	**KP842777**
	GAAACATTGCCGTCACACAC				
Δ4fad	AAGCCTCTGCTGATTGGAGA	131 bp	60°C	101.8%	**JN673546**
	GGCTGAGCTTGAAACAGACC				
Lipid absorption and transport					
apoa4	AGGAACTCCAGCAGAACCTG	122 bp	60°C	102.6%	**KP842775**
	CTGGGTCATCTTGGAGAAGG				
fabp1	GCTCATCCAGAAAGGCAAAG	199 bp	62°C	102.6%	**KP842779**
	GGAGACCTTCAGCTTGTTGC				
fabp2a	ACACACATGACCTTAGCACACTG	70 bp	60°C	103.6%	**KP842780**
	TGCGAGTATCAAAATCCGGTA				
fabp2b	ATTCTCATGGGCTTCCACTG	154 bp	60°C	101.8%	**KP842781**
	CCTCTCAGCTTCCCTGCTTT				
fabp3	GTCAGGGAAGTCAACGGAGA	225 bp	60°C	103.4%	**KP842782**
	ATAAAGAGATGGCGGGAGGT				
mtp	CAGGCGTACACCACATGTAAA	150 bp	60°C	103.5%	**KP842778**
	GTGATCAGGCTTCTGCAGTG				
Reference genes					
ubq	AGCTGGCCCAGAAATATAACTGCGACA	93 bp	70°C	101.1%	**AB291588**
	ACTTCTTCTTGCGGCAGTTGACAGCAC				
rps4	GTGAAGAAGCTCCTTGTCGGCACCA	83 bp	70°C	101.7%	**AB291557**
	AGGGGGTCGGGGTAGCGGATG				
ef1a1	GATTGACCGTCGTTCTGGCAAGAAGC	142 bp	70°C	101.9%	**AB326302**
	GGCAAAGCGACCAAGGGGAGCAT				

aco, acyl-CoA oxidase; *fas*, fatty acid synthase; *Δ4fad*, delta 4 fatty acid desaturase; *apoa4*, apolipoprotein A-IV; *fabp*, fatty acid binding protein; *mtp*, microsomal trygliceride transfer protein; *ubq*, ubiquitin; *rps4*, 40S ribosomal protein S4; *ef1a1*, elongation factor 1 alpha.
[a] Efficiency corresponds to an average of 2 runs (larval and post-larval samples; each run contained both t0 and t3).

all 3 reference genes were used for both t0 (M = 0.130; CV = 0.052) and t3 (M = 0.126; CV = 0.050).

To complete the gene expression analysis, we performed a qPCR assay using a 112 × 24 high-performance OpenArray chip, as detailed in Hachero-Cruzado et al. (2014). Samples were loaded in triplicate into OpenArray plates with the OpenArray® AccuFill™ System following manufacturer's protocols. Each subarray was loaded with 5.0 μL of master mix containing a specific cDNA and TaqMan® Gene Expression Master Mix. For gene expression analysis, raw data was imported into the Datassist v3.01 software and Ct values were exported and analyzed using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) in which values were normalized with the geometric mean of 3 reference genes (*ubq*, *eef1a1* and *gadph2*), and calibrated to the CLO treatment.

2.7. Statistical analyses

All statistical analyses were performed with SPSS v.20 (SPSS Inc., Chicago, IL, USA). Results of DW, FA composition, survival, I_{EM}, enzymatic activity and RT-qPCR (NRQ values exported from qBase+) analysis, performed on pools of specimens (n = 4 per treatment) and those of SL and fat accumulation (n = 120 or n = 12 individual fish per treatment, respectively) were analyzed by one-way ANOVA. The $2^{-(\Delta\Delta Ct)}$ data from the openarray analysis, also performed on pools (n = 3), was log transformed and processed using a One-way multivariate analysis of variance (MANOVA) approach ($p < 0.05$). When data passed the Levene's test of equal variances ($p > 0.05$), the Tukey's post-hoc test was performed to assess significant differences between dietary treatments at each age or time point, at a significance level of 0.05. When variances were not equally distributed, the Games–Howell test was used instead. For clustering analysis, the permutMatrix software (Caraux and Pinloche, 2005) was used to compare log2 transformed fold changes, with parameters set as following: dissimilarity — Euclidean distance; hierarchical — McQuitty's criteria Method; and seriation — multiple-fragment heuristic (MF).

3. Results

3.1. Larval performance and maturation of the digestive function

The enrichment emulsions caused significant differences in fish DW at 22 and 30 dph (Fig. 1a), but not in SL (Fig. 1b). Larvae fed the CLO diet were significantly heavier than those fed OO at 22 dph and 30 dph, but no significant differences in DW or SL were found at the end of the experiment due to large size dispersion. At this point, larvae fed CLO still tended to be larger and heavier, but those fed LSO appeared to be catching up in terms of growth.

Metamorphosis started earlier in larvae fed the CLO diet (at 14 dph) than those from the other 3 treatments. Larvae fed CLO and LSO, progressed through metamorphosis faster than those fed the SBO and OO (more evident at 23 dph). At 30 dph, most treatments had completed metamorphosis, except the OO group, which was significantly delayed with respect to the CLO treatment (Table 3).

Larval survival was significantly higher in larvae fed CLO (25.2 ± 5.0%) than those fed OO (7.6 ± 6.0%). Survival of larvae fed LSO and SBO was 19.6 ± 8.4% and 13.0 ± 4.9%, respectively, and not significantly different from the other treatments.

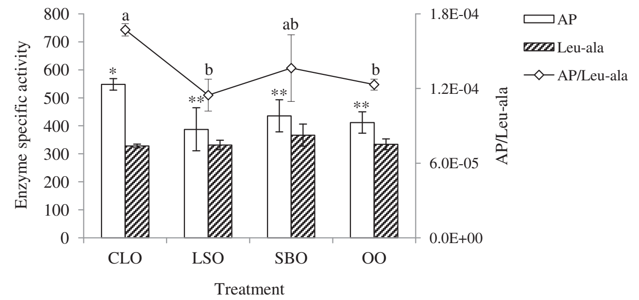

Fig. 2. Specific activities of alkaline phosphatase (AP) and leucine-alanine (Leu-ala) and their mutual ratio for each dietary treatment. Values were measured at 37 dph in *Solea senegalensis* post-larvae and are presented as means ± SD (n = 4 pools per treatment) in U mg^{-1} protein ∗ 10,000 and U mg^{-1} protein for AP and Leu–ala, respectively. Letters and asterisk indicate significant differences between treatments for each individual data series.

Looking at enzymatic activity, post-larvae from the CLO treatment showed a significantly higher AP/Leu–ala ratio than the other treatments at 37 dph (Fig. 2), mainly due to the significantly higher specific activity of AP.

3.2. Lipid and fatty acid composition of enriched Artemia, larvae and post-larvae

Live preys were enriched with emulsions prepared with either a marine oil, CLO, or one of three vegetable oils (VO): LSO, SBO or OO. As expected, the enriched *Artemia* did not show significant differences in total lipid and total FA content between treatments, but had significantly different FA profiles that reflected their oil source (Table 1). *Artemia* enriched with CLO supplied the larvae with the highest levels of LC-PUFA (ARA 0.5%, EPA 6.3%, DHA 3.9% of TFA), while the VO treatments had negligible amounts of these EFAs (0–0.9% of TFA). The VO treatments also differed substantially: the LSO treatment had the highest levels of n-3 PUFA, as a result of high ALA content (2-fold higher than other treatments); the SBO enrichment supplied the larvae with the highest n-6 PUFA levels, due to its LNA concentration (3-fold more than other treatments); and *Artemia* enriched with the OO emulsion presented the highest levels of MUFA, given its high content in OA, and had the lowest PUFA levels.

Total lipid content was higher in larvae, but there were no significant differences between treatments at either age (Tables 4 and 5). On the other hand, the FA profile of larvae and post-larvae closely reflected the composition of their diets (Table 1) and did not change greatly from 22 to 37 dph (Tables 4 and 5). At both ages, fish fed the CLO diet had a significantly higher percentage of n-3 PUFA, mostly EPA and DHA, closely followed by the LSO treatment due to high levels of ALA. Although fish fed the LSO diet had significantly higher ARA levels than the remaining treatments, those fed SBO showed the highest n-6 PUFA levels due to a significantly higher percentage of LNA. The OO treatment had significantly higher levels of MUFA than the other treatments, reflecting the oleic acid (OA) content. No significant differences in SFA levels were found between treatments at 22 dph, but at 37 dph they were significantly lower in post-larvae from the OO treatment.

Table 3
Eye migration index (I_{EM}) of Senegalese sole larvae and post larvae fed live prey enriched with CLO, LSO, SBO or OO at 12, 14, 16, 18, 22, 23, 25, 28 and 30 dph. Values are means ± SD (n = 4 pools) and different letters indicate significant differences (ANOVA, $p < 0.05$) between the treatments within one column.

I_{EM}	12 dph	14 dph	16 dph	18 dph	22 dph	23 dph	25 dph	28 dph	30 dph
CLO	0.10 ± 0.10	0.16 ± 0.06[a]	0.93 ± 0.54	1.71 ± 0.36	3.34 ± 0.12	3.58 ± 0.26[a]	3.88 ± 0.18[a]	3.95 ± 0.04	3.98 ± 0.03[a]
LSO	0.01 ± 0.03	0.04 ± 0.03[b]	0.96 ± 0.53	1.65 ± 0.36	3.17 ± 0.09	3.49 ± 0.25[ab]	3.78 ± 0.15[ab]	3.89 ± 0.17	3.93 ± 0.03[ab]
SBO	0.00 ± 0.00	0.01 ± 0.02[b]	0.43 ± 0.31	1.47 ± 0.30	2.94 ± 0.44	3.01 ± 0.21[c]	3.45 ± 0.22[b]	3.60 ± 0.25	3.93 ± 0.05[ab]
OO	0.00 ± 0.00	0.01 ± 0.02[b]	0.40 ± 0.12	1.45 ± 0.12	3.00 ± 0.06	3.11 ± 0.14[bc]	3.5 ± 0.02[b]	3.72 ± 0.23	3.62 ± 0.09[b]

3.3. Fat accumulation in the intestine and liver

The percentage of epithelium occupied by fat deposits in the posterior intestine and liver, at 22 dph and 37 dph was assessed by histological procedures (Table 6). The posterior intestine was chosen as the area to assess intestinal lipid accumulation since there was a marked longitudinal gradient, increasing in an anterior–posterior direction (Appendix A.1). No significant differences were observed in lipid accumulation in the intestinal mucosa between treatments at either age (Table 6). Large intracellular lipid inclusions were scattered throughout the entire cytoplasm from the apical to the basal end of enterocytes, with some having a diameter of over 20 μm. In extreme cases, these inclusions created very large aggregations resulting in the deformation of enterocytes and villi (Fig. 3a, c, e, g and 4a, c, e, g).

The percentage of liver tissue covered by lipids was not significantly different between the 4 dietary treatments at 22 dph (Table 6). At this time hepatocyte nuclei, where visible, were always displaced to the periphery of the cells. Hepatocytes were enlarged, homogeneous in size and evenly distributed across the whole hepatic parenchyma along sinusoids, compressing the vascular lumen (Fig. 3b, d, f, h). By 37 dph, the hepatocytes of post-larvae from the CLO treatment had no to very little visible lipid droplets (Fig. 4b). This was also apparent in the area occupied by fat deposits, which was significantly lower in the CLO treatment compared to the VO treatments (Table 6). Contrary to the CLO treatment, the degree of fat accumulation in the VO treatments was similar to that observed at 22 dph; however, although the entire cytoplasm of hepatocytes was completely filled with fat, the cells did not seem as enlarged as at 22 dph; the nuclei were clearly visible and, in numerous cases, centrally located (Fig. 4b, d, f, h).

3.4. Gene expression analysis

In order to evaluate the transcriptional responses of fasted and fed larvae (at 16 dph) and post-larvae (at 34 dph), the transcript levels of key lipid metabolism, absorption and transport genes were measured by RT-qPCR after a 16 h fasting period (t0; Table 7) and in the postpandrial stage (3 h after providing a meal; t3; Table 8).

At t0, *fas*, *apoa4* and *Δ4fad* levels were significantly different across diets both in pelagic larvae and benthonic post-larvae. At 16 dph, *fas* was significantly down-regulated and *apoa4* up-regulated in the CLO compared to OO treatment. Similar to *fas*, *Δ4fad* was significantly down-regulated in the CLO treatment, but only in comparison to the LSO treatment. At 34 dph, *fas* expression was significantly lower in the CLO treatment, compared to the SBO and OO treatments, while *apoa4* was inversely regulated, with significantly higher values in post-larvae fed the CLO and LSO diets than in the SBO and OO treatments. The expression of *Δ4fad* at 34 dph was similar to that at 16 dph, but it was also down-regulated in the OO treatment as well as in CLO compared to LSO.

Expression results at t3 only showed significant differences in mRNA amounts of *Δ4fad* and *fabp3* at 16 dph, when both genes had significantly lower expression levels in the CLO, compared to the OO treatment, while SBO and LSO had intermediate values.

To characterize the transcriptional responses of larvae fed the different diets, a set of 109 transcripts was analyzed (complete list presented in Hachero-Cruzado et al., 2014). Statistical analysis identified 19 and 26 transcripts to be differentially expressed between different dietary treatments in larvae (16 dph) and post-larvae (34 dph), respectively.

Table 4
Total lipid content, total FA content and FA composition of Senegalese sole larvae at 22 dph fed *Artemia* enriched with CLO, LSO, SBO or OO. Values are means ± SD (n = 4 pools). Different letters indicate significant differences between treatments (ANOVA, p < 0.05).

	CLO	LSO	SBO	OO
Total lipid and total FA content of larvae (mg g^{-1} DW)				
Total lipids	93.2 ± 31.9	111.3 ± 11.4	101.8 ± 2.4	103.7 ± 13.7
Total FA	48.9 ± 15.9	65.2 ± 9.8	59.1 ± 5.8	57.8 ± 8.8
FA composition of larvae (% TFA)*				
16:0	10.6 ± 0.8	8.8 ± 0.8	9.5 ± 0.8	9.6 ± 0.6
18:0	7.1 ± 0.2	8.3 ± 0.3	8.3 ± 0.6	6.9 ± 0.4
Total SFA	18.0 ± 0.9^a	17.3 ± 1.1^a	17.9 ± 1.1^a	16.6 ± 0.5^a
16:1	3.0 ± 0.3	1.0 ± 0.3	1.3 ± 0.2	2.2 ± 0.9
18:1n-9	22.8 ± 0.2	23.9 ± 0.4	22.5 ± 0.6	38.7 ± 1.4
18:1n-7	7.2 ± 0.4	5.7 ± 0.4	5.6 ± 0.7	6.0 ± 1.2
20:1	3.3 ± 0.2	1.7 ± 0.2	1.2 ± 0.0	1.5 ± 0.1
Total MUFA	36.4 ± 0.5^c	32.4 ± 0.3^b	30.5 ± 0.7^a	48.4 ± 1.4^d
18:2n-6	7.4 ± 0.5^a	14.9 ± 0.8^c	22.7 ± 0.5^d	9.4 ± 0.5^b
20:4n-6	2.9 ± 0.2^{ab}	3.4 ± 0.0^c	3.0 ± 0.2^b	2.5 ± 0.2^a
Total n-6 PUFA	11.3 ± 0.5^a	19.4 ± 0.7^c	26.6 ± 0.7^d	12.9 ± 0.7^b
18:3n-3	15.2 ± 0.5^b	20.9 ± 0.7^d	16.8 ± 0.8^c	13.7 ± 0.2^a
20:5n-3	5.4 ± 0.3^c	1.7 ± 0.3^b	0.8 ± 0.1^a	1.2 ± 0.2^a
22:5n-3	3.0 ± 0.2	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
22:6n-3	5.6 ± 0.3^d	2.0 ± 0.2^c	1.4 ± 0.1^a	1.6 ± 0.3^b
Total n-3 PUFA	32.6 ± 1.0^d	28.7 ± 0.8^c	22.5 ± 0.8^b	20.0 ± 0.8^a
Total PUFA	43.9 ± 1.0^b	48.1 ± 0.9^c	49.2 ± 0.5^c	32.8 ± 1.1^a
DHA/EPA	1.04 ± 0.02^a	1.18 ± 0.15^{ab}	1.80 ± 0.07^b	1.35 ± 0.04^c
ARA/EPA	0.53 ± 0.05^a	2.01 ± 0.36^b	3.74 ± 0.55^c	2.17 ± 0.33^b

* Not all FAs are shown. Only FAs and FA groups with letters were statistically analyzed.

Table 5
Total lipid content, total FA content and FA composition of Senegalese sole post-larvae at 37 dph fed *Artemia* enriched with CLO, LSO, SBO or OO. Values are means ± SD (n = 4 pools). Different letters indicate significant differences between treatments (ANOVA, p < 0.05).

	CLO	LSO	SBO	OO
Total lipid and total FA content of post-larvae (mg g^{-1} DW)				
Total lipids	73.1 ± 19.8	60.6 ± 17.9	69.1 ± 14.5	74.7 ± 7.1
Total FA	43.5 ± 13.1	37.8 ± 8.4	39.3 ± 8.0	48.4 ± 6.9
FA composition of post-larvae (% TFA)*				
16:0	12.5 ± 0.8	9.6 ± 1.3	10.6 ± 0.4	9.5 ± 0.3
18:0	6.7 ± 0.5	6.7 ± 0.9	7.0 ± 0.4	5.9 ± 0.5
Total SFA	19.7 ± 1.2^b	16.5 ± 2.2^{ab}	17.9 ± 0.7^b	15.6 ± 0.7^a
16:1	3.5 ± 0.2	2.1 ± 0.1	1.8 ± 0.3	2.6 ± 0.8
18:1n-9	22.6 ± 0.4	22.3 ± 0.7	21.2 ± 0.5	35.8 ± 0.8
18:1n-7	6.5 ± 0.4	4.5 ± 0.3	5.0 ± 0.5	5.7 ± 0.9
20:1	2.8 ± 0.2	1.1 ± 0.1	1.0 ± 0.0	1.5 ± 0.1
Total MUFA	35.4 ± 0.6^b	30.0 ± 1.0^a	29 ± 0.7^a	45.6 ± 1.3^c
18:2n-6	7.8 ± 0.7^a	14.9 ± 0.2^c	22.9 ± 0.5^d	10.1 ± 0.3^b
20:4n-6	3.0 ± 0.1^a	3.9 ± 0.3^c	3.2 ± 0.0^b	2.7 ± 0.4^{ab}
Total n-6 PUFA	11.7 ± 1.0^a	19.8 ± 0.7^c	27.4 ± 0.3^d	14.2 ± 0.7^b
18:3n-3	13.1 ± 1.1^a	25.2 ± 3.6^b	15.1 ± 1.5^a	14.3 ± 0.8^a
20:5n-3	4.9 ± 0.2^b	0.9 ± 0.2^a	1.0 ± 0.2^a	1.2 ± 0.2^a
22:5n-3	2.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.2
22:6n-3	7.2 ± 0.2^b	2.3 ± 0.6^a	2.7 ± 0.5^a	3.1 ± 1.2^a
Total n-3 PUFA	30.7 ± 1.2^a	31.6 ± 3.5^a	22.4 ± 0.7^b	22.4 ± 0.9^b
Total PUFA	42.4 ± 1.9^b	51.4 ± 3.5^c	49.8 ± 1^c	36.6 ± 1.2^a
DHA/EPA	1.48 ± 0.05^a	2.37 ± 0.20^b	2.87 ± 0.11^c	2.45 ± 0.64^{abc}
ARA/EPA	0.62 ± 0.03^a	4.25 ± 0.76^c	3.51 ± 0.67^c	2.26 ± 0.51^b

* Not all FAs are shown. Only FAs and FA groups with letters were statistically analyzed.

Table 6
Area of fat deposits in the intestinal epithelia and liver of Senegalese sole larvae (22 dph) and post-larvae (37 dph) fed CLO, LSO, SBO or OO. Values are expressed as percentage of total tissue surface and presented as means ± SD (n = 12 larvae per treatment, 1 photo per larvae for intestine and 3 for liver). Letters show significant differences (ANOVA, p < 0.05) between dietary treatments for each tissue at each age.

Diet	Tissue area occupied by lipid droplets (%)	
	Posterior intestine	Liver
22 dph		
CLO	56.5 ± 4.7	64.3 ± 5.9
LSO	63.1 ± 7.8	57.0 ± 8.9
SBO	57.9 ± 6.5	63.4 ± 6.6
OO	62.4 ± 6.8	62.5 ± 8.1
37 dph		
CLO	62.5 ± 6.9	33.5 ± 8.9^a
LSO	62.5 ± 3.2	64.5 ± 6.8^b
SBO	57.9 ± 8.1	65.4 ± 3.9^b
OO	65.6 ± 4.8	65.4 ± 3.8^b

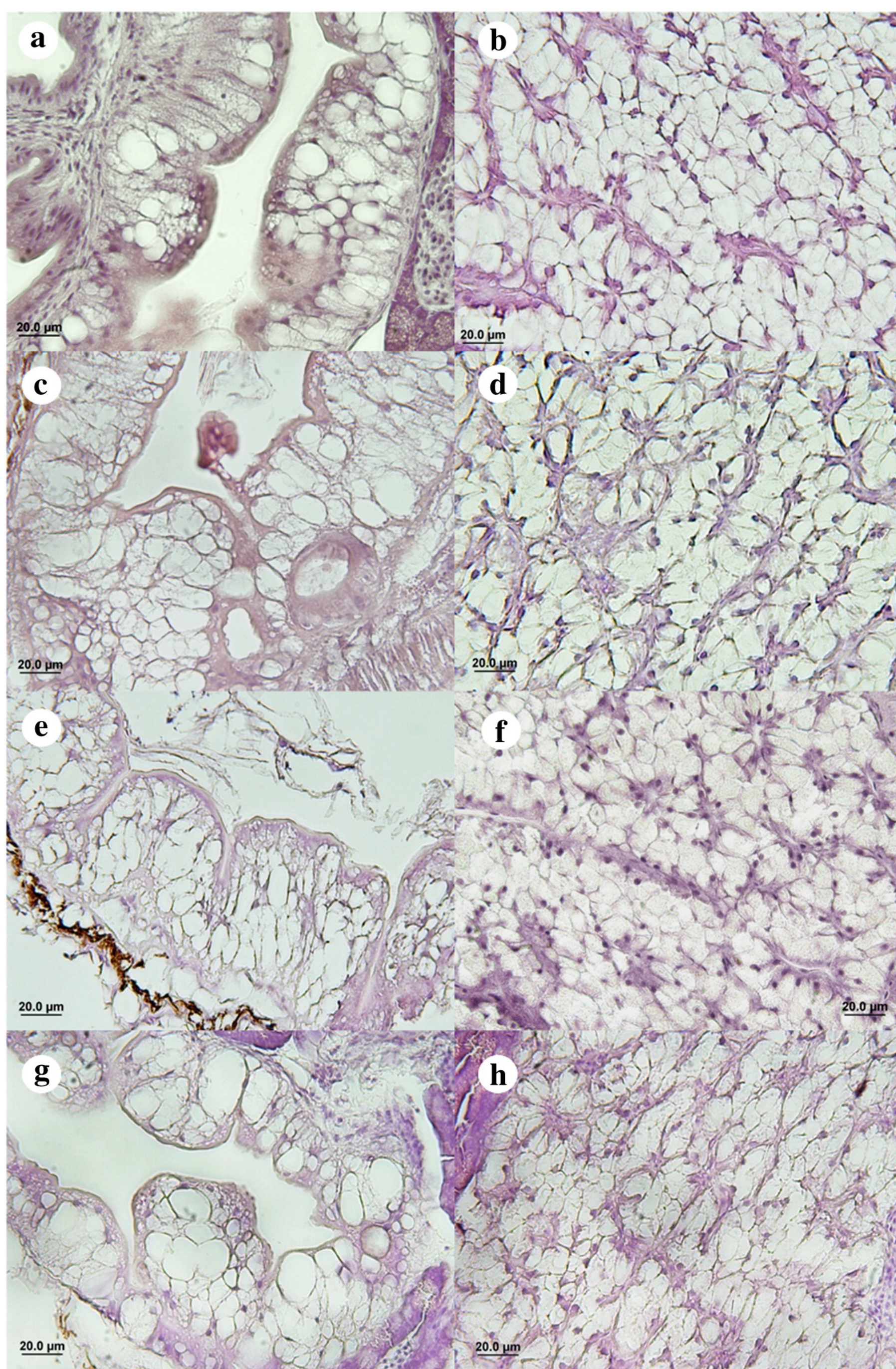

Fig. 3. Lipid droplet accumulation in target tissues of Senegalese sole larvae at 22 dph. Images are × 40 magnifications of the posterior intestine (a, c, e, g) and liver (b, d, f, h) of Senegalese sole larvae fed the CLO (a, b), LSO (c, d), SBO (e, f) and OO (g, h) diets. The scale bars represent 20 μm.

Generally, larvae from the LSO treatment exhibited similar expression values to those from the CLO group at 16 dph, while the SBO and OO treatments had opposite values (Appendix B.1). Twelve transcripts had significantly higher levels in larvae fed CLO compared to SBO:1-acylglycerol-3-phosphate O-acyltransferases 3 and 9 (*agpat3*, *agpat9*), lysophosphatidylcholine acyltransferase 1(*lpcat1*), ethanolamine-phosphate cytidylyltransferase 2 (*pcyt2*), lysocardiolipin acyltransferase 1 (*lclat1*), lipid phosphate phosphatase-related protein type 1(*lppr1*), phospholipase D2 (*pld1*), secretory phospholipase A2-like proteins A and B (*pg12a*, *pg12b*), lipid phosphate phosphohydrolase 1(*lpp1*) and apolipoproteins b and c2 (*apob*, *apoc2*). Transcripts *pg12a*, *pg12b* and *apoc2* were also significantly upregulated in the CLO, compared to OO treatment, as were another 2 transcripts (not significantly different to SBO): monoacylglycerol O-acyltransferase 1 (*mogat1*), and apolipoprotein E (*apoe*). Adipose triglyceride lipase (*pnpla2*) had higher expression levels in larvae from the LSO compared to the SBO treatment. The 2 remaining differentially expressed transcripts had an inverse expression pattern, with larvae from the SBO and OO treatments having higher levels of lipin 1 (*lpin1*) and endothelial lipase (*el*) with relation to CLO, respectively.

At 34 dph, similarly to 16 dph, expression profiles of post-larvae fed CLO tended to be significantly different to SBO and OO (Appendix B.2). Post-larvae from the OO treatment had similar expression levels to those fed SBO, with several transcripts having reduced levels compared to CLO and/or LSO: 1-acyl-sn-glycerol-3-phosphate acyltransferase beta (*agpat2*), lysophosphatidylcholine acyltransferase 5 (*lpcat5*), *pcyt2*, phosphatidylserine decarboxylase (*pisd*), group 3 secretory

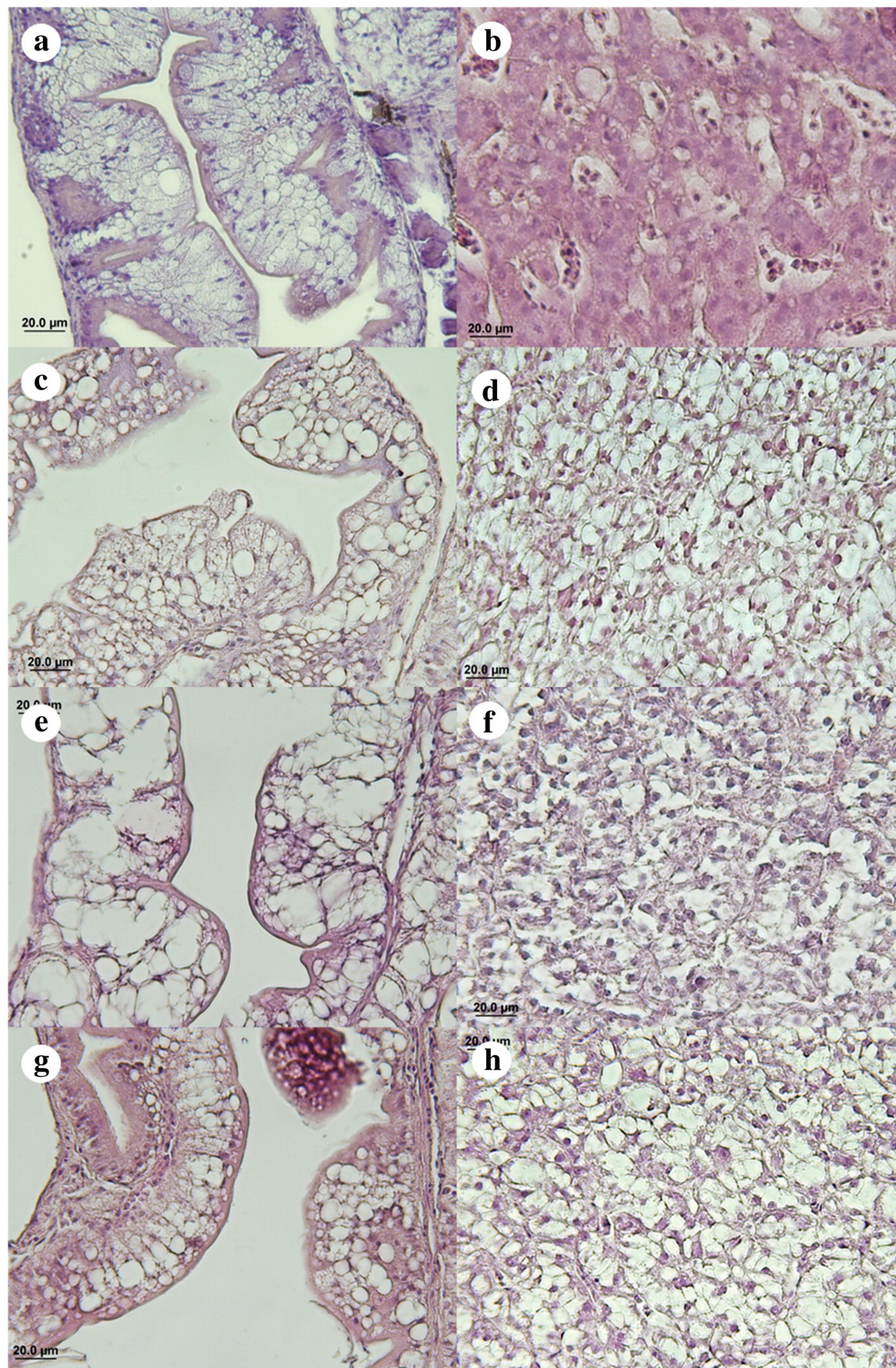

Fig. 4. Lipid droplet accumulation in target tissues of Senegalese sole post-larvae at 37 dph. Images are × 40 magnifications of the posterior intestine (a, c, e, g) and liver (b, d, f, h) of Senegalese sole post-larvae fed the CLO (a, b), LSO (c, d), SBO (e, f) and OO (g, h) diets. Scale bars represent 20 μm.

phospholipase A2 precursor (*pla2g3*), *pg12a*, *pg12b*, hepatic triacylglycerol lipase precursor (*htgl*), lipoprotein lipase (*lpl*), fatty acid binding protein 1 (*fabp1*), apolipoprotein A1 (*apoa1*), *apoa4* and 3 *apoe* transcripts. Conversely, post-larvae from the SBO treatment showed higher expression, compared to the CLO treatment, for several transcripts: phosphatidylserine synthase 1 (*ptdss1*), CDP-diacylglycerol-serine O-phosphatidyl transferase (*pss*), lipid phosphate phosphatase-related protein type 1 (*lppr1*), apolipoprotein E receptor 2 (*apoer2*) and cleavage stimulation factor subunit 1 (*cstf1*). Furthermore, adipose triglyceride lipase (*atgl*) had higher expression levels in the LSO and OO treatments compared to CLO. Finally, phospholipase A2 group 1B (*pla2g1b*), was expressed at higher levels in post-larvae from the OO compared to the SBO treatment.

Clustering analysis of significant qPCR results generally led to a good separation of the treatments. Overall, larvae fed CLO and LSO were grouped separately from the SBO and OO treatments at both stages of larval development (Fig. 5a and b).

4. Discussion

4.1. Fish performance and digestive function maturity

In the present study Senegalese sole larvae fed CLO exhibited the highest growth and survival rates followed by those fed LSO and SBO, with main differences observed in the period between 22 and 30 dph. During the pre-metamorphic stage, up to 21 dph, larvae showed very

Table 7
Expression of lipid metabolism, absorption and transport genes in Senegalese sole larvae (16 dph) and post-larvae (34 dph) from the CLO, LSO, SBO and OO treatments at t0 (after 16 h fasting). Values are mean NRQs ± SD (n = 4 per treatment). Letters represent significant differences in mRNA levels between treatments (p < 0.05), for each gene and age separately.

	Larvae (16 dph)				Post-larvae (34 dph)			
	CLO	LSO	SBO	OO	CLO	LSO	SBO	OO
Lipid metabolism								
aco	1.03 ± 0.09	1.07 ± 0.18	1.00 ± 0.28	0.98 ± 0.24	0.97 ± 0.16	1.03 ± 0.38	1.06 ± 0.15	1.06 ± 0.29
fas	0.75 ± 0.06[a]	1.12 ± 0.12[ab]	0.99 ± 0.24[ab]	1.18 ± 0.21[b]	0.56 ± 0.07[a]	1.08 ± 0.24[ab]	1.53 ± 0.4[b]	1.15 ± 0.24[b]
Δ4fad	0.58 ± 0.14[a]	1.31 ± 0.42[b]	1.05 ± 0.27[ab]	1.19 ± 0.23[ab]	0.61 ± 0.09[a]	1.76 ± 0.44[b]	1.18 ± 0.46[ab]	0.94 ± 0.36[a]
Lipid absorption and transport								
apoa4	1.31 ± 0.12[a]	1.03 ± 0.07[ab]	1.18 ± 0.21[ab]	0.76 ± 0.34[b]	1.18 ± 0.08[a]	1.11 ± 0.08[a]	0.88 ± 0.14[b]	0.89 ± 0.10[b]
fabp1	1.17 ± 0.47	1.04 ± 0.45	1.04 ± 0.31	0.99 ± 0.29	1.25 ± 0.24	1.09 ± 0.39	0.92 ± 0.29	0.89 ± 0.04
fabp2a	1.07 ± 0.17	1.14 ± 0.21	1.13 ± 0.22	0.87 ± 0.4	1.09 ± 0.12	1.13 ± 0.37	0.99 ± 0.31	0.91 ± 0.07
fabp2b	1.49 ± 0.65	1.16 ± 0.49	0.97 ± 0.3	0.80 ± 0.23	0.99 ± 0.17	0.95 ± 0.33	1.08 ± 0.35	1.15 ± 0.34
fabp3	0.98 ± 0.13	1.12 ± 0.19	0.9 ± 0.33	1.09 ± 0.12	1.04 ± 0.15	1.08 ± 0.11	0.87 ± 0.1	1.06 ± 0.18
mtp	1.27 ± 0.01	1.24 ± 0.27	1.07 ± 0.3	0.88 ± 0.51	1.01 ± 0.08	1.03 ± 0.28	1.01 ± 0.21	1.01 ± 0.15

similar growth performance, independent of their diet, whereas the absence of significant differences at the end of the experiment was due to a high variability in growth leading to a lack of statistical power to detect differences. Nevertheless, larvae from the LSO treatment appeared to show a compensatory growth from 30 to 37 dph and almost "caught up" to the CLO treatment. These results seem to corroborate the standing hypothesis that dietary levels of LC-PUFA, particularly EPA and DHA, might be crucial for this species during a developmentally critical window just before metamorphosis, but are less important once the larvae metamorphose and acquire the benthic life stage (Dâmaso-Rodrigues et al., 2010; Morais et al., 2004, 2012a). In fact, metamorphosis was initiated earlier in larvae fed CLO and only the LSO treatment reached similar values towards the end of the metamorphosis window (23–25 dph at 18–20 °C). It is generally accepted that larval size at metamorphosis is a major factor determining the energy reserves and amount of time that an individual requires to fulfill the transition to demersal post-larvae (Geffen et al., 2007). Under our experimental conditions the onset of metamorphosis in Senegalese sole seemed to be diet-dependent, as significant differences were observed in I_{EM} values between treatments without significant variation in weight or length during this period suggesting that dietary EFAs could be influencing metamorphosis, as previously described in flatfish larvae (Boglino et al., 2012a; Lund et al., 2008; Shields et al., 1999; Villalta et al., 2005a).

The activity of intestinal enzymes provides a reliable marker for assessing the development of the digestive function in fish larvae (Cahu and Zambonino-Infante, 1994). The AP/Leu–ala ratio was, therefore, used as an indicator of the intestinal maturation of larvae at 37 dph (Cahu and Zambonino-Infante, 1994; Martínez et al., 1999; Ribeiro et al., 1999b). Post-metamorphic specimens from the CLO treatment had the highest values for the AP/Leu–ala ratio, mostly as a result of the higher AP activity in this treatment compared to the VO diets. This reflected the development of the brush border membrane of the enterocytes and indicated an increasing relevance of the absorptive processes mediated by these enzymes (Boglino et al., 2012a; Martínez et al., 1999). Furthermore, these results correlated well with the fastest growth and development, assessed by the I_{EM} in the CLO treatment.

4.2. Effect of diets on FA composition of larvae and post-larvae

In accordance with other studies, the FA profile of Senegalese sole larvae and post-larvae closely reflected the diet composition (Boglino et al., 2012a; Dâmaso-Rodrigues et al., 2010; Morais et al., 2012a; Villalta et al., 2005a). Nonetheless, some deviations from the expected profile were observed in the content of both n-3 and n-6 LC-PUFA. At 22 dph, larvae fed the LSO diet had significantly higher levels of EPA and DHA than the SBO treatment, although both diets were equally deficient in these LC-PUFA. However, these differences were not evident at 37 dph. On the other hand, larvae fed LSO (and not CLO, which was the richest dietary source of ARA) showed the highest ARA levels (3.4%) at 22 dph, which were even higher at 37 dph (3.9%). In addition, larvae fed the SBO and OO diets had ARA levels that were not significantly different from the CLO treatment. These results raise the question of whether the LC-PUFA might have been synthesized from lower chain precursors, possibly from C_{18} PUFA, as previously hypothesized by Navarro-Guillén et al. (2014), and as further discussed below.

4.3. Effects of diets on fat accumulation in target tissues

The intestine and liver are considered target organs when evaluating the nutritional and physiological status of fish (Gisbert et al., 2008). As observed by optical microscopy in this study, the largest aggregations of lipids were visible in enterocytes of the posterior intestine (Appendix A.1) as in Morais et al. (2006), instead of anterior and/or

Table 8
Postprandial expression (t3; 3 h after a meal) of lipid metabolism, absorption and transport genes in Senegalese sole larvae (at 16 dph) and post-larvae (at 34 dph) fed CLO, LSO, SBO or OO. Values are mean NRQs ± SD (n = 4 per treatment). Letters represent significant differences in mRNA levels between treatments (p < 0.05), for each gene and age separately.

	Larvae (16 dph)				Post-larvae (34 dph)			
	CLO	LSO	SBO	OO	CLO	LSO	SBO	OO
Lipid metabolism								
aco	0.92 ± 0.18	1.09 ± 0.24	1.03 ± 0.16	1.02 ± 0.08	1.06 ± 0.21	1.04 ± 0.60	1.00 ± 0.19	1.35 ± 0.64
fas	0.90 ± 0.10	1.01 ± 0.15	0.96 ± 0.07	1.17 ± 0.18	1.21 ± 1.02	1.06 ± 0.60	1.22 ± 0.25	1.07 ± 0.18
Δ4fad	0.65 ± 0.18[a]	1.20 ± 0.37[ab]	1.09 ± 0.32[ab]	1.30 ± 0.17[b]	0.75 ± 0.21	1.56 ± 0.83	1.24 ± 0.38	1.03 ± 0.56
Lipid absorption and transport								
apoa4	1.01 ± 0.10	1.03 ± 0.09	1.01 ± 0.10	0.97 ± 0.14	1.21 ± 0.81	1.34 ± 0.48	1.40 ± 0.16	1.26 ± 0.10
fabp1	0.77 ± 0.26	1.26 ± 0.31	1.07 ± 0.39	1.19 ± 0.46	1.22 ± 0.88	1.43 ± 0.28	1.44 ± 0.28	1.31 ± 0.10
fabp2a	0.95 ± 0.30	1.05 ± 0.04	0.99 ± 0.21	1.09 ± 0.27	1.09 ± 0.75	1.33 ± 0.49	1.38 ± 0.22	1.13 ± 0.11
fabp2b	0.89 ± 0.48	1.32 ± 0.22	0.95 ± 0.26	1.05 ± 0.30	1.09 ± 0.29	0.96 ± 0.45	1.09 ± 0.18	1.03 ± 0.09
fabp3	0.87 ± 0.11[a]	1.08 ± 0.16[ab]	0.95 ± 0.1[ab]	1.13 ± 0.08[b]	1.18 ± 0.20	1.01 ± 0.33	0.91 ± 0.13	1.02 ± 0.25
mtp	0.85 ± 0.18	1.08 ± 0.06	1.12 ± 0.22	1.01 ± 0.12	1.03 ± 0.66	1.28 ± 0.75	1.36 ± 0.20	1.33 ± 0.08

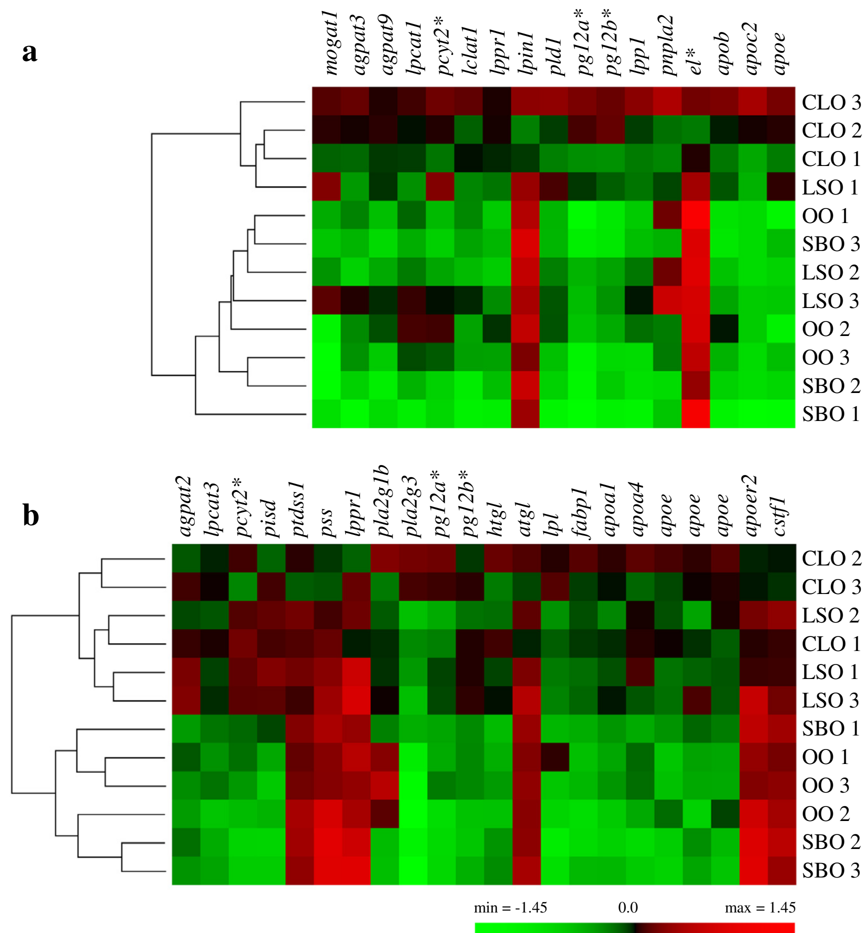

Fig. 5. Hierarchical clustering analysis of differentially regulated transcripts related to lipid metabolism and absorption in Senegalese sole larvae. Expression levels were measured with an OpenArray chip in *Senegalese sole* larvae at 16 dph (a) and post-larvae at 34 dph (b) fed CLO, LSO, SBO or OO. Data is expressed as log2 fold change of each sample in relation to the average of the CLO treatment. Asterisks represent differentially expressed transcripts observed both at 16 dph and 34 dph. The column labels are abbreviations of the following genes and their assay IDs (Hachero-Cruzado et al., 2014): *mogat1* — monoacylglycerol O-acyltransferase (1AICSWAJ), *agpat3* — 1-acyl-sn-glycerol-3-phosphate acyltransferase gamma-like (AIS08JC), *agpat9* — glycerol-3-phosphate O-acyltransferase (AII1M5MI), *lpcat1* — lysophosphatidylcholine acyltransferase 1 (AI0IXKQ), *pcyt2* — ethanolamine-phosphate cytidylyltransferase 2 (AII1M5O), *lclat1* — lysocardiolipin acyltransferase 1 (AIQJB6Y), *pnpla2* — adipose triglyceride lipase (AI5IP9K), *lppr1* — lipid phosphate phosphatase-related protein type 1 (AIKALBV), *lpin1* — lipin 1 isoform 2 (AIT96PM), *el* — endothelial lipase (AIWR212), *pld1* — phospholipase D2 (AI6ROFV), *lpp1* — lipid phosphate phosphohydrolase 1 (AIHSOZF), *apob* — apolipoprotein B (AICSWAH), *apoc2* — apolipoprotein C-II (AIHSOZD), *apoe* — apolipoprotein E (AIPAD0P), *gpat1* — glycerol-3-phosphate acyltransferase 1 (AIMSHOA), *dgat1* — diacylglycerol O-acyltransferasehomolog 1b (AI5IP9L), *agpat2* — 1-acyl-sn-glycerol-3-phosphate acyltransferase beta (AIX008A), *lpcat2*-lysophospholipidacyltransferase 2 (AI70ML2), *lpcat5* — lysophospholipidacyltransferase 5 (AI5IP9M), *pcyt2* — ethanolamine-phosphate cytidylyltransferase 2 (AII1M5O), *pisd* — phosphatidylserinedecarboxylase (AI89KSB), *ptdss1* — phosphatidylserinesynthase 1 (AICSWAK), *pss* — CDP-diacylglycerol-serine O-phosphatidyltransferase (AIAAZX4), *lppr1* — Lipidphosphatephosphatase-relatedproteintype 1 (AIKALBV), *pla2g1b* — phospholipase A2; phosphatidylcholine 2-acylhydrolase (AIVI4VV), *pla2g3* — phospholipase A2 (AI1RVQZ), *pg12a* — secretory phospholipase A2-like protein (AIY9ZEJ), *pg12b* — secretory phospholipase A2-like protein (AIWR213), *htgl* — hepaticlipase (AIN1FUI), *atgl* — adiposetriglyceridelipase (AI39R3C), *el* — endothelial lipase (AIWR212), *lpl* — lipoproteinlipase (AIVI4VU), *fabp1* — fatty acid binding protein 1(AID1UGQ), *apoa1* — apolipoprotein A-I (AIAAZX1), *apoa4* — apolipoprotein A-IV (AI70ML0), *apoe* — apolipoprotein E (AIN1FUH, AIQJB6X, AIS08JD), *apoer2*-apolipoprotein E receptor 2 (AIX0079), and *cstf1* — cleavagestimulation factor subunit 1 (AIFASM0).

mid intestine, which have been described as the main areas of luminal lipid absorption in the digestive tract of Senegalese sole (Boglino et al., 2012b; Ribeiro et al., 1999a) and other teleost species (Boulhic and Gabaudan, 1992; Deplano et al., 1989; Gisbert et al., 2005; Iwai, 1969; Kjørsvik et al., 1991).

Both larvae and post-larvae, at 22 dph and 37 dph, respectively, had high levels of fat accumulation in the posterior intestine that did not vary significantly between treatments. Very large lipid droplets with diameters of up to ~20 µm were observed, which were much larger than the 5–8 µm droplet sizes previously reported in larvae of other teleosts (Boglino et al., 2012b; Deplano et al., 1991; Diaz et al., 1997). Lipid droplets are believed to be caused by high dietary FA content and are suggested to be temporary storage of re-esterified FAs in cases when the rate of lipid absorption exceeds the rate of lipoprotein synthesis (Sheridan, 1988), or because of an inability to metabolize lipids (Kjørsvik et al., 1991). Extensive lipid droplet formation is often denominated as intestinal steatosis, but is rarely considered a pathogenic state, although in extreme cases it may cause pathological damage to the intestinal epithelium (Gisbert et al., 2008). In this study, large fat deposits in the intestinal mucosa did not seem to affect their integrity, neither result in focal lesions; thus, differences in mortalities among groups cannot be attributed to intestinal damage caused by different diets.

The hepatic tissue of larvae from all treatments had similarly large accumulations of lipids at 22 dph. However, at 37 dph, post-larvae from the CLO treatment exhibited large intracellular spaces and very little to no cytoplasmatic lipid inclusions in the hepatic parenchyma. Larvae from the VO treatments retained a high degree of fat within the tissue, although the hepatocytes didn't seem as enlarged. A similar liver appearance to that of CLO post-larvae in this study was observed by Boglino et al. (2012b) in Senegalese sole post-larvae at 38 dph, fed *Artemia* enriched with a commercial product — Aquagrow Gold©. It is interesting to note that this experimental group had the best growth (DW at 38 dph was similar to that of CLO at 37 dph in our study under similar rearing conditions) and maturation of the digestive system of all the tested treatments and was also the only treatment

without any hepatic lipid inclusion at 38 dph. However, the FA profile of the *Artemia* enriched with Aquagrow Gold© was somewhat different to that of the CLO, and the worst performing treatment from Boglino et al. (2012b), Easy Selco©, had an almost identical FA profile to our CLO treatment. This observation may seem contradictory, but it is important to note that total lipids in all experimental diets from Boglino et al. (2012b) were 2-fold higher than in our study. Therefore, it may be that total dietary lipid levels modulated the effect that specific FA profiles had on larval performance.

4.4. Effects of diets on the expression of genes involved in lipid absorption and metabolism

In order to examine the effect of the highly contrasting dietary FA profiles in larval and post-larval lipid absorption and metabolism, we analyzed the expression of a series of genes with RT-qPCR. Of these, 4 showed significant diet-dependent differences: Δ*4fad*, *fas*, *apoa4* and *fabp3*.

The Δ*4fad* is a key gene for LC-PUFA biosynthesis, namely of DHA, in Senegalese sole (Morais et al., 2012a; Morais et al., 2015b). It has been suggested that the enzyme it encodes might also have a residual Δ6/Δ5 Fad activity under particular nutritional conditions and/or life stages (Morais et al., 2012a; Navarro-Guillén et al., 2014), but so far DHA synthesis has only been demonstrated to occur from EPA and not from C_{18} PUFA in sole juveniles (Morais et al., 2015b). In this study, Δ*4fad* was down-regulated in fish fed CLO, particularly under fasting conditions. As previously reported, this was probably due to a negative feedback effect of higher dietary end product (DHA) levels (Clarke and Jump, 1994; Morais et al., 2012a, 2015b). On the other hand, fish fed LSO had highest basal (t0) levels of Δ*4fad* transcripts at both 16 dph (although significantly different only from CLO) and 34 dph (significantly different from CLO and OO). It would be tempting to suggest that these results could explain some of the unexpected differences in larvae LC-PUFA profiles, as discussed above, but in face of the present lack of evidence of Δ6/Δ5 Fad activity in sole juveniles this should be interpreted with caution.

The expression of *fas*, an enzyme that catalyzes the *de novo* biosynthesis of FAs, was lowest in sole from the CLO treatment at both 16 and 34 dph, although only when fasting (t0). This could probably be explained by the well established hypotriglycemic effect of dietary fish oils (FO) in mammals. Dietary n-6 and n-3 PUFA, but not MUFA or SFA are known to suppress the transcription of FAS and other lipogenic enzymes in rats (Teran-Garcia et al., 2007; Wilson et al., 1990), with LC-PUFA being even more efficient in this respect (Davidson, 2006; Willumsen et al., 1993). Similar expression patterns have been found in teleost species fed FO compared to VO-based diets, which supports the existence of a similar effect in fish (Alvarez et al., 2000; Morais et al., 2012b; Zuo et al., 2015). Interestingly, we observed that *fas* levels in the LSO group were comparable to that of CLO-fed fish, which only presented significant differences with OO-fed fish at 16 dph, and both OO and SBO treatments at 34 dph. Zuo et al. (2015) observed a similar down-regulation of *fas* in yellow croaker (*Larimichthys crocea*) fed ALA-rich VO diet compared to those fed a LNA-based diet.

Differences in *apoa4* expression were also noted between treatments, which likely resulted in a different efficiency of lipid absorption from the gut lumen. In mammals, APOA4 aids triacylglycerol (TAG) packaging into chylomicrons by increasing their size, which greatly enhances basolateral TAG secretion in the intestine, subsequently increasing luminal FA absorption and flux of lipids through the intestine (Lu et al., 2006). In turn, an increase in intestinal fat absorption has been shown to stimulate APOA4 synthesis (Apfelbaum et al., 1987). At 16 dph, *apoa4* expression was significantly higher in the CLO compared to OO treatment, while at 34 dph it was higher in fish fed CLO and LSO compared to the SBO and OO treatments, but significant effects were only observed after fasting (t0) in both ages. Again, post-larvae fed LSO had values similar to CLO at 34 dph which coincided with a period of elevated growth for this group. Seeing as post-prandial effects on expression were not observed, this suggests that the different dietary FAs did not affect *apoa4* transcription acutely, but had a more chronic effect on mechanisms of lipid absorption instead. Furthermore, it may also be possible that a more mature and efficient intestine, as observed in larvae fed the CLO diet, was able to absorb lipids at a higher rate, leading to *apoa4* up-regulation.

A more acute effect of the different diets was visible in the postprandial changes in expression of *fabp3* at 16 dph, when an up-regulation of this gene was observed in larvae fed CLO compared to OO. Unlike the remaining *fabp* transcripts (*fabp1*, *fabp2a* and *fabp2b*) and *mtp*, which are involved in lipid transport through the gastrointestinal mucosa, *fapb3* is mainly expressed in the ovary, liver and heart of Senegalese sole (our own unpublished results) and other fish species (Ando et al., 1998). Although we are not able to explain these results at present, we suggest that this expression could be associated with lipid metabolism in the liver. The encoded Fabp3 facilitates the transfer of FAs across cellular membranes (Spener et al., 1989; Veerkamp et al., 1991) and in zebrafish (*Danio rerio*) liver it has been found to mediate FA transport for lipogenesis (Liu et al., 2003). Although we did not find significant differences in postpandrial expression of *fas*, this gene showed a similar trend in expression to *fabp3*, and was similarly and significantly regulated at t0 in 16 dph larvae. Therefore, both results could be potentially related to diet-induced differences in lipogenic pathways in the liver, and might at least partly explain the lower lipid accumulation in the liver observed histologically at 37 dph. Another possible, but not mutually exclusive explanation, could be an enhanced lipid mobilization from the liver in the CLO treatment through increased lipoprotein synthesis, as further discussed below.

Expression analysis of 109 transcripts related to lipid metabolism and transport (Hachero-Cruzado et al., 2014) revealed a set of differentially expressed genes between treatments mostly involved in lipid synthesis (acyltransferases and phosphatases), lipid hydrolysis (lipases and hydrolases) and apolipoproteins. Cluster analysis at 16 and 34 dph clearly separated the CLO treatment. However, the LSO treatment grouped more closely to CLO at both ages, especially at 34 dph, whereas the SBO and OO treatments behaved more similarly to each other and tended to form an opposite group. This data suggests that the identified set of differentially expressed genes could explain the beneficial effects CLO had on larval performance, followed by the LSO treatment. The CLO/LSO group showed higher mRNA levels for transcripts involved in the synthesis of PLs at 16 and 34 dph, namely of phosphatidylcholine (PC; *lpcat1*, *lpcat5*), phosphatidylethanolamine (PE; *pcyt2*, *pisd*), PL precursors such as monoacylglycerol phosphate (*agpat9*) and diacylglycerol phosphate (*agpat2*, *agpat3*) and other membrane components such as cardiolipin (*lclat1*) and dihydroxyoctadecanoate (*lppr1*, involved in sphingolipid metabolism). Furthermore, genes involved in PL hydrolysis (*pld1*, *pg12a*, *pg12b*, *lpp1*, *pla2g3*, *htgl*) and encoding apolipoproteins (*apoa1*, *apoa4*, *apob*, *apoc2*, *apoe*) were also up-regulated. Within this group, CLO generally had the highest levels of expression, except at 34 dph when, interestingly, LSO exhibited higher mRNA levels for almost all genes involved in PC and PE synthesis, matching a period in which larvae fed LSO had the fastest growth rate and appeared to be catching up to CLO-fed larvae in terms of size. Phospholipids are key to larval development, as they are building blocks for biomembrane synthesis and essential components of lipoproteins (Tocher et al., 2008), which in turn promote neutral lipid absorption from the gut and transport between tissues. Furthermore, lipid mobilization is essential in faster growing fish for the energy allocation to tissue differentiation and somatic growth, especially during metamorphosis when feeding is arrested (Boglino et al., 2012b; Brewster, 1987; Geffen et al., 2007). In this respect, PLs are considered essential nutrients for fish larvae, as these early stages seem to have an insufficient PL biosynthesis capacity to maintain their high rates of growth and morpho-anatomical development (Cahu et al., 2009). Therefore, not surprisingly, diets leading to a coordinated up-regulation of PL and apoliprotein synthesis were also associated with a higher growth and faster development in this study. Previous work had similarly reported the ability of

young Senegalese sole larvae to deal with higher dietary lipid concentrations by increasing the expression of apolipoproteins, leading to better absorption throughout the enterocytes and lower lipid accumulation in liver and gut (Hachero-Cruzado et al., 2014). In our study we did not observe differences between treatments in lipid droplet accumulation in the intestinal mucosa but the coordinated increase in PL and apolipoprotein synthesis in the CLO treatment most likely resulted in higher rates of lipoprotein transport leading to a lower accumulation of lipid in the liver in post-metamorphosed larvae.

On the other hand, the few genes that were up-regulated in the SBO and OO treatments were involved mostly in phosphatidylserine (PS), sphingolipid and TAG metabolism. At 16 dph only 2 genes were up-regulated (in relation to the CLO treatment): *lpin1*, involved in diacylglycerol synthesis and *el*, a gene with a high density lipoprotein (HDL) hydrolysing function, acting on PLs as well as TAG. In mouse, up-regulation of *Lpin1* has been associated with a higher expression of *Fas* and other genes involved in FA synthesis, as well as with low dietary PUFA content (Martin et al., 2007), which is in agreement with the results from this study. At 34 dph, a total of 6 up-regulated genes were observed, of which some were involved in TAG hydrolisis (*atgl*), PL hydrolysis (*pla2g1b*; only in the OO treatment), lipoprotein metabolism (*apoer2*), PS synthesis (*ptdss1*, *pss*) and sphingolipid metabolism (*lppr1*). The up-regulation of some genes related to lipogenesis, particularly in the OO treatment, was surprising considering that these diets supply high levels of FAs (especially OA) that are considered preferential substrates for β-oxidation (Crockett and Sidell, 1993; Kiessling and Kiessling, 1993). We could therefore speculate that, in spite of the high dietary contents of energy-yielding FAs in the OO diet, the deficiency in EFA might have led to an energy deprived state, which could be caused by insufficient rates of PL synthesis for lipoprotein production, hence leading to low rates of intestinal absorption and endogenous lipid mobilization.

5. Conclusions

Results from this study clearly indicated that larval diets with higher levels of LC-PUFA, and possibly also C_{18} n-3 PUFA, promote growth, development and survival in Senegalese sole larvae and post-larvae. Furthermore, the data suggested that this effect was at least partly mediated by an up-regulation of PL metabolism and apolipoprotein synthesis, potentially leading to higher rates of cellular membrane synthesis and enhancing intestinal lipid transport, most likely driving higher luminal lipid absorption, as well as transport and mobilization of lipid reserves from hepatic cells to other tissues throughout the body. However, it remains to be unequivocally established whether the moderately more beneficial effects of the LSO diet, compared to the SBO treatment, were due to a higher nutritional value of ALA compared to LNA and its involvement in different metabolic pathways, or whether this effect could be explained by some degree of bioconversion of ALA into biologically more active n-3 LC-PUFA by Senegalese sole larvae and post-larvae.

Acknowledgments

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Appendix A

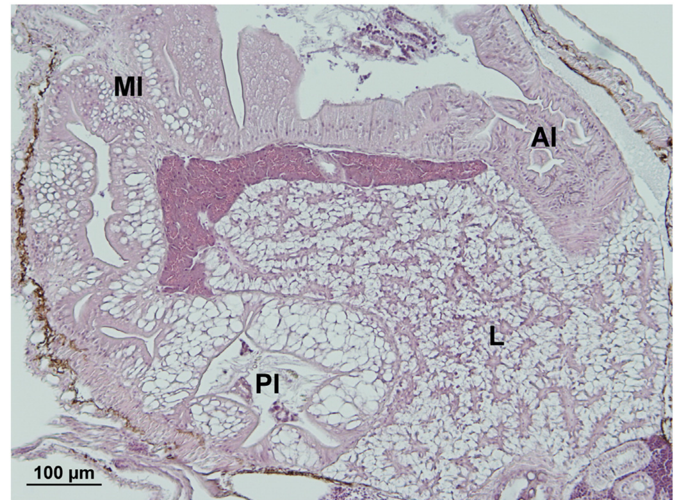

Fig. A.1. Longitudinal transect of the abdominal cavity of a Senegalese sole larvae at dph fed the OO diet. A gradual increase of lipid droplet inclusion in the intestinal epithelium from the anterior (AI), through mid (MI) to posterior intestine (PI) is clearly visible. The liver is marked as “L”.

Appendix B

Table B1
Expression of the 17 transcripts found to be significantly differentially regulated in the OpenArray chip between 16 dph-old *Senegalese sole* larvae raised on different dietary treatments. The activity of listed transcripts corresponds directly or indirectly to the function they have been categorized under. Data are expressed as means ± SD (n = 3) of fold change with respect to the CLO treatment. Expression results were normalized by *gapdh2*, *ubq* and *eef1a1* mRNA levels. Letters indicate significant differences (p < 0.05) between dietary treatments.

		Dietary treatment			
Transcript	Assay ID[†]	CLO	LSO	SBO	OO
Lipid synthesis					
mogat1	AICSWAJ	1.00 ± 0.09^{a}	1.02 ± 0.20^{a}	0.53 ± 0.14^{ab}	0.52 ± 0.20^{b}
agpat3	AIS08JC	1.00 ± 0.11^{a}	0.81 ± 0.20^{ab}	0.58 ± 0.15^{b}	0.82 ± 0.02^{ab}
agpat9	AII1M5M	1.00 ± 0.03^{a}	0.90 ± 0.13^{a}	0.55 ± 0.05^{b}	0.75 ± 0.16^{ab}
lpcat1	AI0IXKQ	1.00 ± 0.04^{a}	0.90 ± 0.12^{ab}	0.65 ± 0.13^{b}	0.96 ± 0.08^{a}
pcyt2	AII1M5O	1.00 ± 0.13^{a}	0.98 ± 0.22^{a}	0.61 ± 0.02^{b}	0.89 ± 0.18^{ab}
lclat1	AIQJB6Y	1.00 ± 0.10^{a}	0.83 ± 0.14^{ab}	0.58 ± 0.15^{b}	0.79 ± 0.04^{ab}
lppr1	AIKALBV	1.00 ± 0.01^{a}	0.77 ± 0.12^{ab}	0.62 ± 0.12^{b}	0.79 ± 0.17^{ab}
lpin1	AIT96PM	1.01 ± 0.19^{b}	1.37 ± 0.12^{ab}	1.51 ± 0.21^{a}	1.35 ± 0.16^{ab}
Lipid hydrolisis					
pld1	AI6ROFV	1.01 ± 0.20^{a}	0.94 ± 0.11^{a}	0.59 ± 0.13^{b}	0.77 ± 0.14^{ab}
pg12a	AIY9ZEJ	1.01 ± 0.18^{a}	0.79 ± 0.15^{ab}	0.44 ± 0.06^{c}	0.52 ± 0.13^{bc}
pg12b	AIWR213	1.01 ± 0.18^{a}	0.82 ± 0.08^{ab}	0.54 ± 0.08^{c}	0.61 ± 0.11^{bc}
Lipid hydrolisis					
lpp1	AIHSOZF	1.01 ± 0.19^{a}	0.91 ± 0.07^{a}	0.56 ± 0.11^{b}	0.70 ± 0.16^{ab}
pnpla2	AI5IP9K	1.03 ± 0.30^{ab}	1.21 ± 0.31^{a}	0.65 ± 0.07^{b}	0.95 ± 0.16^{ab}
el	AIWR212	1.01 ± 0.14^{b}	1.59 ± 0.25^{ab}	1.74 ± 0.48^{ab}	1.82 ± 0.47^{a}
Lipid transport					
apob	AICSWAH	1.01 ± 0.16^{a}	0.78 ± 0.13^{ab}	0.53 ± 0.08^{b}	0.75 ± 0.22^{ab}
apoc2	AIHSOZD	1.03 ± 0.29^{a}	0.64 ± 0.07^{ab}	0.51 ± 0.10^{b}	0.60 ± 0.03^{b}
apoe	AIPAD0P	1.01 ± 0.15^{a}	0.76 ± 0.23^{ab}	0.56 ± 0.15^{ab}	0.53 ± 0.13^{b}

[†] Assay ID from Hachero-Cruzado et al. (2014); *mogat1*, monoacylglycerol O-acyltransferase; *agpat3*, 1-acyl-sn-glycerol-3-phosphate acyltransferase gamma-like; *agpat9*, glycerol-3-phosphate O-acyltransferase 9; *lpcat1*, lysophosphatidylcholine acyltransferase 1; *pcyt2*, ethanolamine-phosphate cytidylyltransferase 2; *lclat1*, lysocardiolipin acyltransferase 1; *lppr1*, lipid phosphate phosphatase-related protein type 1; *lpin1*, lipin 1 isoform 2; *pld1*, phospholipase D2; *pg12a*, secretory phospholipase A2-like protein; *pg12b*, secretory phospholipase A2-like protein; *lpp1*,lipid phosphate phosphohydrolase 1; *pnpla2*, adipose triglyceride lipase; *apob*, apolipoprotein B; *apoc2*, apolipoprotein C-II; *apoe*, apolipoprotein E.

Table B2
Expression of the 22 transcripts found to be significantly differentially regulated in the openarray chip between 34 dph-old *Senegalese sole post*-larvae raised on different dietary treatments. The activity of listed transcripts corresponds directly or indirectly to the function they have been categorized under. Data are expressed as means ± SD (n = 3) of fold change with respect to the CLO treatment. Expression results were normalized by *gapdh2*, *ubq* and *eef1a1* mRNA levels. Letters indicate significant differences ($p < 0.05$) between dietary treatments.

Transcript	Assay ID[†]	CLO	Dietary treatment LSO	SBO	OO
Lipid synthesis					
agpat2	AIX008A	1.00 ± 0.10^{ab}	1.16 ± 0.21^{a}	0.75 ± 0.07^{b}	0.77 ± 0.10^{b}
lpcat5	AI5IP9M	1.00 ± 0.02^{a}	0.93 ± 0.04^{ab}	0.70 ± 0.10^{b}	0.69 ± 0.14^{b}
pcyt2	AII1M5O	1.02 ± 0.24^{ab}	1.14 ± 0.02^{a}	0.61 ± 0.20^{b}	0.70 ± 0.12^{ab}
pisd	AI89KSB	1.01 ± 0.13^{ab}	1.20 ± 0.09^{a}	0.63 ± 0.25^{b}	0.59 ± 0.07^{b}
ptdss1	AICSWAK	1.00 ± 0.12^{b}	1.17 ± 0.09^{ab}	1.39 ± 0.10^{a}	1.30 ± 0.19^{ab}
pss	AIAAZX4	1.01 ± 0.15^{b}	1.29 ± 0.19^{ab}	2.13 ± 0.49^{a}	1.60 ± 0.48^{ab}
lppr1	AIKALBV	1.01 ± 0.16^{b}	1.79 ± 0.52^{ab}	1.94 ± 0.51^{a}	1.53 ± 0.15^{ab}
Lipid hydrolysis					
pla2g1b	AIVI4VV	1.02 ± 0.26^{ab}	0.95 ± 0.06^{ab}	0.63 ± 0.12^{b}	1.39 ± 0.30^{a}
pla2g3	AI1RVQZ	1.02 ± 0.25^{a}	0.6 ± 0.08^{ab}	0.39 ± 0.20^{b}	0.28 ± 0.07^{b}
pg12a	AIY9ZEJ	1.02 ± 0.22^{a}	0.83 ± 0.17^{ab}	0.52 ± 0.12^{b}	0.62 ± 0.18^{ab}
pg12b	AIWR213	1.00 ± 0.05^{a}	0.96 ± 0.12^{ab}	0.63 ± 0.11^{c}	0.68 ± 0.12^{bc}
htgl	AIN1FUI	1.01 ± 0.13^{a}	0.76 ± 0.04^{ab}	0.45 ± 0.12^{b}	0.68 ± 0.31^{ab}
atgl	AI39R3C	1.00 ± 0.10^{b}	1.36 ± 0.27^{a}	1.34 ± 0.04^{ab}	1.43 ± 0.09^{a}
lpl	AIVI4VU	1.01 ± 0.20^{a}	0.92 ± 0.09^{ab}	0.68 ± 0.09^{bc}	0.62 ± 0.07^{c}
Lipid transport					
fabp1	AID1UGQ	1.00 ± 0.11^{a}	0.86 ± 0.04^{a}	0.55 ± 0.09^{b}	0.53 ± 0.08^{b}
apoa1	AIAAZX1	1.00 ± 0.04^{a}	0.88 ± 0.12^{ab}	0.59 ± 0.11^{c}	0.62 ± 0.10^{bc}
apoa4	AI70ML0	1.01 ± 0.15^{a}	1.00 ± 0.10^{a}	0.53 ± 0.10^{b}	0.77 ± 0.11^{ab}
apoe	AIN1FUH	1.00 ± 0.09^{a}	0.84 ± 0.05^{ab}	0.56 ± 0.13^{b}	0.64 ± 0.17^{b}
apoe	AIPAD0P	1.00 ± 0.04^{a}	0.87 ± 0.22^{ab}	0.74 ± 0.10^{ab}	0.60 ± 0.10^{b}
apoe	AIS08JD	1.01 ± 0.13^{a}	0.93 ± 0.07^{ab}	0.63 ± 0.14^{b}	0.74 ± 0.16^{ab}
apoer2	AIX0079	1.00 ± 0.02^{b}	1.39 ± 0.43^{ab}	2.17 ± 0.33^{a}	1.57 ± 0.39^{ab}
Other					
cstf1	AIFASM0	1.00 ± 0.05^{b}	1.21 ± 0.15^{ab}	1.55 ± 0.17^{a}	1.37 ± 0.14^{a}

[†] Assay ID from Hachero-Cruzado et al. (2014); *agpat2*, 1-acyl-sn-glycerol-3-phosphate acyltransferase beta; *lpcat5*, lysophospholipidacyltransferase 5; *pcyt2*, ethanolamine-phosphate cytidylyltransferase 2; *pisd*, phosphatidylserine decarboxylase; *ptdss1*, phosphatidylserinesynthase 1; *pss*, CDP-diacylglycerol-serine O-phosphatidyltransferase; *lppr1*, Lipidphosphatephosphatase-relatedproteintype 1; *pla2g1b*, phospholipase A2; phosphatidylcholine 2-acylhydrolase; *pla2g3*, phospholipase A2; *pg12a*, secretory phospholipase A2-like protein; *pg12b*, secretory phospholipase A2-like protein; *htgl*, hepaticlipase; *atgl*, adiposetriglyceridelipase; *lpl*, lipoproteinlipase; *fabp1*, fatty acid binding protein 1; *apoa1*, apolipoprotein A-I; *apoa4*, apolipoprotein A-IV; *apoe*, apolipoprotein E; *apoer2*,apolipoprotein E receptor 2; *cstf1*, cleavagestimulation factor subunit 1.

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Characterization of seven cocaine- and amphetamine-regulated transcripts (CARTs) differentially expressed in the brain and peripheral tissues of *Solea senegalensis* (Kaup)

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ABSTRACT

CART (cocaine- and amphetamine-regulated transcript) is a peptide with neurotransmitter and neuroendocrine functions with several key roles, both centrally and peripherally. In mammals there is a single gene that produces two alternatively spliced variants in rat and a single transcript in human but in teleosts multiple genes have been found. In the present study we report the existence of seven transcripts in Senegalese sole and characterize their sequences and phylogenetic relationships, as well as their expression patterns in the brain and peripheral tissues, and in response to feeding. Both *cart2a* and *cart4* showed a ubiquitous expression in the brain, while *cart1a*, *cart1b* and *cart3a* were similarly expressed and had higher transcript levels in the mesencephalon, followed by the diencephalon. On the other hand, *cart2b* showed a main expression in the olfactory bulbs, and *cart3b* was predominantly expressed in the spinal cord. The expression profile in peripheral tissues differed substantially between *cart*'s, even between more recently duplicated genes. Collectively, all the tissues examined, except the muscle, express at least one of the different *cart*'s, although the highest transcript levels were found in the brain, gonads (ovary and testis) and, in some cases, eye and kidney. Concerning the feeding response, only brain *cart1a*, *cart2a* and *cart4* showed a significant postprandial regulation, although future studies are necessary to assess potential confounding effects of stress imposed by the force feeding technique employed. Senegalese sole exhibits the highest number of *cart* genes reported to date in a vertebrate species. Their differential expression patterns and feeding regulation suggest that multiple *cart* genes, resulting from at least 3 rounds of whole genome duplication, have been retained in fish genomes through subfunctionalization, or possibly even through neofunctionalization.

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1. Introduction

The cocaine- and amphetamine-regulated transcript (CART) was discovered and named by Douglass et al. (1995) as a mRNA that was highly up-regulated in the rat striatum following acute administration of cocaine or amphetamine. In the last two decades it has emerged as a key neurotransmitter and hormone involved in the regulation of a variety of important physiological processes. Considering its initial description, CART was associated early on with the effects of psychostimulants, and subsequent studies found CART mRNA and peptides to be present in important regions associated with drug-mediated reward, reinforcement and behavior, particularly in areas of the mesolimbic dopaminergic system (reviewed by Vicentic and Jones, 2007). Furthermore, CART-containing cells were observed to form synapses with neurons producing neurotransmitters associated with addiction, such as dopamine and GABA, and to colocalize with neurons that synthesize neurochemicals implicated in the pharmacological actions of psychostimulants (Vicentic and Jones, 2007).

However, the best documented action of CART has been with regard to its involvement in the regulation of feeding and energy balance, promoting energy expenditure. CART is expressed in key areas of the brain that regulate these actions, such as the ventromedial hypothalamic nucleus (VMN), lateral hypothalamus (LH), arcuate nucleus (Arc), paraventricular nucleus (PVN), nucleus of the solitary tract (NTS) and nucleus accumbens (NAc). Moreover, CART is co-expressed with peptides that are involved in the regulation of feeding, pituitary hormone secretion and energy

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metabolism, such as pro-opiomelanocortin (POMC), melanin-concentrating hormone (MCH), thyrotropin-releasing hormone (TRH), corticotrophin-releasing factor (CRF) and neuropeptide Y (Vicentic and Jones, 2007; Lau and Herzog, 2014). In addition, effects of intracerebroventricular injections of CART on feeding, gastric emptying, body weight gain, plasma insulin and leptin levels, lipid oxidation and thermogenesis support the anorexic role of CART as a mediator of feeding and metabolic regulation (reviewed in Murphy, 2005; Vicentic and Jones, 2007; Rogge et al., 2008; Lau and Herzog, 2014). Other emerging roles of CART are its implication in sensory processing, endocrine regulation, stress and anxiety, cardiovascular function and bone remodeling (Rogge et al., 2008). In this respect, CART mRNA and peptides are abundant along the hypothalamic–pituitary–adrenal (HPA) axis and sympatho-adrenal system, where peptide expression can be altered by stress (Balkan et al., 2012).

Since its discovery, CART has been intensively studied in several mammalian species but has also been identified and characterized in amphibians, birds and fish (e.g., Volkoff and Peter, 2001; Tachibana et al., 2003; Lázár et al., 2004). Its important physiological role is supported by the high level of conservation of its mRNA and protein sequences from fish to humans. Interestingly, genomic studies in teleosts revealed the existence of higher number of *cart* genes than in tetrapods, which might have arisen as a result of a specific whole genome duplication event in teleost evolution (Volff, 2005). So far, different studies on CART mRNA and/or peptide have been performed in goldfish (*Carassius auratus*), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*), medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*) and two species of catfish (*Ictalurus punctatus* and *Clarias batrachus*). These studies focused on the expression patterns in different brain regions and peripheral tissues, as well as in the transcriptional response of *cart* genes to starvation and food intake, both during early ontogeny and in adult animals (Volkoff and Peter, 2001; Kehoe and Volkoff, 2007; Singru et al., 2007; Kobayashi et al., 2008; Murashita et al., 2009; Barsagade et al., 2010; Murashita and Kurokawa, 2011; Valen et al., 2011; Mukherjee et al., 2012; Nishio et al., 2012; Wan et al., 2012; Akash et al., 2014). However, in most cases only 1–2 gene products have been studied, with the exception of medaka and zebrafish, where the differential expression of 6 and 4 transcripts, respectively, was reported (Murashita and Kurokawa, 2011; Nishio et al., 2012; Akash et al., 2014). Given the involvement of CART peptides in multiple and varied physiological processes, the enlargement of the CART family in teleost species might have led to redundancy or, alternatively, conferred subfunctionalization of roles or even development of new functions, which facilitate the retention of duplicated genes in the genome. In order to better understand this, and possibly uncover specific functions of different *cart* gene products, a comprehensive characterization of the differential expression patterns and transcriptional regulation is of paramount importance. With this objective in mind, we have thoroughly mined a Senegalese sole (*Solea senegalensis*) transcriptomic database for *cart*-like transcripts. We then characterized the different gene products and present original data on their differential expression in different brain areas and in an extensive collection of peripheral tissues, as well as on transcriptional responses to a 24 h fasting and postprandial changes up to 12 h after feeding.

2. Materials and methods

2.1. cDNA sequence mining

Sequences of cocaine- and amphetamine-regulated transcripts were searched by gene annotation in the SoleaDB transcriptomic database (http://www.scbi.uma.es/soleadb) *S. senegalensis* v4.1 global assembly and then grouped and assembled *in silico* into contigs using the BioEdit Sequence Alignment Editor. When open reading frames (ORF) were incomplete, 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR was performed using the FirstChoice® RLM-RACE kit (Ambion, Life Technologies, Alcobendas, Madrid, Spain) on 5′ and 3′ RACE cDNA synthesized from a 1:1 mixture of Senegalese sole brain and eye total RNAs. The obtained fragments were separated by gel electrophoresis and resulting bands were cut, purified using the Illustra GFX™ PCR DNA and gel band purification kit (GE Healthcare, Barcelona, Spain) and sequenced (SCSIE, University of Valencia, Spain) to confirm their identity. Finally, PCR specific primers were designed on the extremities of each transcript to amplify and confirm, by sequencing, the whole length of the seven *cart* cDNA's.

2.2. Sequence and phylogenetic analysis

The ORF region of the Senegalese sole *cart* cDNA sequences were compared with each other, with the zebrafish (GenBank accession numbers: GU057833.2 for *cart1*, GU057834.2 for *cart2a*, GU057835.2 for *cart2b*, and GU057836.1 for *cart3*; Nishio et al., 2012) human (NM_004291.3) and rat short variant (XM_006231843.2) sequences, and an identity matrix was obtained in ClustalW2 (not shown). Similarly, the prepropeptide CART sequences deduced, using the online EMBL-EBI translation tool, from the *S. senegalensis cart*'s were compared with zebrafish (ADB12484.2, ADB12485.2, ADB12486.2 and ADB12487.1), rat (XP_006231905.1) and human (NP_004282.1) CART prepropeptides and aligned with the human and rat (short variant) counterparts in ClustalW2. The putative cleavage site of the signal peptide was estimated for each Senegalese sole deduced protein using the SignalP 4.1 software (http://www.cbs.dtu.dk/services/SignalP/).

Protein blast or tblastn searches were performed in the GenBank database in order to identify complete CART prepropeptides in several teleost species, together with a selection of tetrapod sequences. A phylogenetic tree was built based on the alignment of all the amino acid sequences (accession numbers shown in Fig. 4) and the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) in MEGA6 (Tamura et al., 2013). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was calculated according to Felsenstein (1985) and the evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965), being presented as the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated.

2.3. Samples for characterization of cart expression in different brain regions

In order to analyze central *cart* expression in Senegalese sole, juvenile specimens ($n = 4$) from 101 to 172 g in body weight and 20 to 25 cm in length were sampled from the "Laboratorio de Cultivos Marinos" (University of Cádiz, Puerto Real, Spain). They were maintained in 250 L tanks under natural photoperiod, with continuous seawater renovation, at a constant temperature and salinity of 19 ± 1 °C and 39 ppt, respectively. Fish were fed with commercial dry pellets (LE-3 Skretting, Burgos, Spain) once a day. Animals were anaesthetized with tricaine methanesulfonate MS-222 (Sigma–Aldrich, Madrid, Spain; 100–200 mg/L water) and sacrificed by decapitation after 14 h fasting. The following brain regions were dissected, as depicted in Fig. 1: olfactory bulbs, telencephalic cerebral hemispheres, diencephalon (preoptic area, thalamus, hypothalamus and posterior tubercle), mesencephalon (optic tectum and tegmentum), rhombencephalon (cerebellum,

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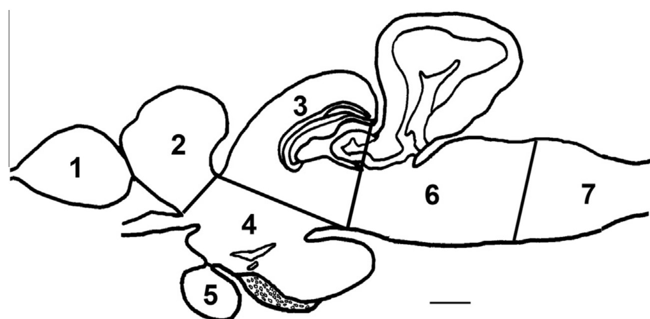

Fig. 1. Schematic representation that depicts the dissections used in the present study to carry out the *cart* expression studies in the sole brain (sagittal view). (1) Olfactory bulbs; (2) telencephalon; (3) mesencephalon (optic tectum-tegmentum); (4) diencephalon (preoptic area-thalamus-hypothalamus-posterior tubercle); (5) pituitary; (6) rhombencephalon (cerebellum-vestibulolateral lobe-isthmus-medulla oblongata); and (7) spinal cord. Bar scale: 1 mm.

vestibulolateral lobe and medulla oblongata), spinal cord and pituitary. All samples were rapidly frozen in liquid nitrogen and stored at −80 °C until used. This study was approved by the Animal Experimentation and Ethics Committee of the University of Cádiz (Spain) and was conducted according to international ethical standards.

2.4. Samples for characterization of cart expression in peripheral tissues

Tissue samples were collected from 3 different Senegalese sole juveniles (average weight: 251 g) held in the experimental culture facilities of IRTA, Center of Sant Carles de la Ràpita (Spain) in 16 m^3 tanks, with natural thermo-photoperiod, a salinity of 36 ppt and fed once a day a standard commercial feed (LE-3, Skretting, Burgos, Spain), supplemented twice a week with natural feeds (mussels and polychaetes). Fish were fasted for 24 h prior to sampling and sacrificed with a lethal dose of MS-222. A homogeneous sample of about 100 mg of tissue, from the same relative position in all animals, was collected from: stomach (Sto), anterior intestine (AI), posterior intestine (PI), liver (L), spleen (Spl), anterior kidney-interrenal tissue (K), heart (H), muscle (M), dorsal skin (DS), ventral skin (VS), and ovaries (O). Other tissues including eye (E, closest to mouth), brain (B), olfactory rosettes (OR) and one testis (T), were sampled whole, and for gills (G) one gill arch was taken from the middle region. Samples were immediately frozen in dry ice and kept at −80 °C pending analysis.

2.5. Feeding trial

Senegalese sole juveniles were cultured during 4 months, from an average body weight (BW) of 27.6 g until 67.7 g, and fed an extruded diet meeting Senegalese sole's nutritional requirements, containing 56% and 8% crude protein and lipids, respectively, formulated with 100% fish oil (diet formulation and composition can be found in Morais et al., 2015). Fish were grown in 4 replicate 500 L tanks (with 55 fish per tank) in a recirculation system at the IRTA experimental facility in Sant Carles de la Ràpita. At the end of the experiment a feeding trial was performed. In order to ensure that all fish ate an equal amount of feed at the appointed time, and considering the highly variable voluntary feed intake of sole, a force-feeding method was used. Animals were fasted for 24 h after the last meal and then one fish per tank ($n = 4$) was sacrificed with a lethal dose of MS222 and whole brains were removed. Afterwards, fish were force-fed with a silicone tube, to deposit a single meal composed of 6–10 feed pellets (depending on the size of the fish; ~0.15% BW) into the esophagus. Fish were monitored to ensure that the pellets were not expelled and were returned to the tanks were they remained for 1 h, 3 h, 6 h, 9 h and 12 h before being sacrificed ($n = 4$ per time point). Whole brain samples were immediately frozen in dry ice and kept at −80 °C pending analysis. Experimentation on live fish (feeding trial and sampling for peripheral tissues) was performed according to the European and National legislation with protocols approved by the ethics committee of IRTA.

2.6. Real time quantitative PCR (qPCR)

Total RNA was isolated from samples by homogenizing in 1 ml of TRIzol (Ambion, Life Technologies, Madrid, Spain) and performing solvent extraction according to manufacturer's instructions. Total RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain), respectively. Two micrograms of total RNA per sample were reverse transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Life Technologies, USA), following manufacturer's instructions, but using a mixture of random primers (1.5 µl as supplied) and anchored oligo-dT (0.5 µl at 400 ng/µl, Eurogentec, Cultek, SL, Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. For studying *cart* expression in different brain regions, and due to the small amount of tissue, 100 ng of total RNA were reverse transcribed using the Quantitec Reverse Transcription kit (Qiagen, Hiden, Germany), which includes a genomic DNA elimination step. A similar amount of cDNA was pooled from all samples from the feeding trial (to prepare a dilution series and determine the efficiency of the PCR amplification) and the remaining cDNA was diluted 60-fold with water. The cDNA used for the brain and tissue expression profile studies were diluted 10- and 20-fold, respectively.

Primers for qPCR were designed using Primer3 v. 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/) (Table 1). The resulting qPCR amplicons were sequenced to confirm their identity and the specificity of the qPCR assay. To normalize the results, previously validated reference genes were used (Infante et al., 2008): ubiquitin (*ubq*), 40S ribosomal protein S4 (*rps4*) and elongation factor 1 alpha (*ef1a1*) for the feeding trial (normalization factor generated with the 3 genes by geNorm, giving an *M* value of 0.103–0.129; Vandesompele et al., 2002), *rps4* for the brain expression study, and 18S rRNA (*18s*) for the tissue distribution profile. Expression of the *cart* genes was quantified in duplicate on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Alcobendas, Spain) in a final volume of 20 µl containing 5 µl of diluted cDNA (except for *18s*: 1 µl; and all genes in the brain expression study: 4 µl), 0.5 µM (or 0.2 µM for *cart2a* and *cart3a*) of each primer and 10 µl of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and included a systematic negative control (non template control or NTC). The qPCR profiles contained an initial activation step at 95 °C for 2 min, followed by 35–40 cycles: 15 s at 95 °C and 30 s at the Ta (60 °C for target genes; 70 °C for reference genes). After the amplification phase, a melting curve was performed enabling confirmation of the amplification of a single product in each reaction and non-occurrence of primer-dimer formation in the NTC.

The expression levels of the different *cart* transcripts in different brain regions and tissues were determined using the delta-delta C_T method ($2^{-\Delta\Delta C_T}$) describing the normalized (by *rps4* or *18s*, respectively) relative expression of the target genes in each region or tissue in relation to the average across all regions or tissues (Livak and Schmittgen, 2001). In the case of the feeding trial, the amplification efficiency of the primer pairs and arbitrary copy numbers in each sample were assessed by serial dilutions of the cDNA pool, and the average normalized (by geNorm normalization factor

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Table 1
Primers used for real-time quantitative PCR (qPCR). Shown are sequence and annealing temperature (Ta) of the primer pairs, size of the fragment produced, reaction efficiency (in the feeding trial) and accession number of the target and reference genes.

Transcript	Primer sequence	Fragment (bp)	Ta (°C)	Efficiency (%)	Accession No.
cart1a	CGTCCACCACTGTCATTCTG CTTTCTCCTCCTGCGTCTTG	147	60	101.0	KT189188
cart2a	TCGCGTATCATCGAACACAT GTGACACTGAGCCACAGCAC	86	60	101.1	KT189190
cart2b	AGGACCATGCAGAGTTCCAG GGACTCGGTGTCCATCACTT	99	60	104.2	KT189191
cart3a	CAACAATCAGCTGGACGAGA AGCGCAGGAAGAAGGTGTTA	230	60	101.2	KT189192
cart3b	TGTGGAAGAGCAGCAAACTG CACAGACAGGGTCAGGGTTT	153	60	99.4	KT189193
cart4	GTGAGCGAGAGCAGGAAACT TCGTGGTGAAATAAGGCAAA	144	60	100.6	KT189194
ubq[a]	AGCTGGCCCAGAAATATAACTGCGACA ACTTCTTCTTGCGGCAGTTGACAGCAC	93	70	101.2	AB291588
rps4[a,b]	GTGAAGAAGCTCCTTGTCGGCACCA AGGGGGTCGGGGTAGCGGATG	83	70	100.8	AB291557
ef1a1[a]	GATTGACCGTCGTTCTGGCAAGAAGC GGCAAAGCGACCAAGGGGAGCAT	142	70	100.9	AB326302
18s[c]	GAATTGACGGAAGGGCACCACCAG ACTAAGAACGGCCATGCACCACCAC	148	70	–	AM882675

[a] Feeding trial.
[b] Brain expression study.
[c] Tissue distribution.

calculated using *rps4*, *ef1a1*, *ubq* expression) values were compared by one-way ANOVA in SPSS v20 (SPSS Inc., Chicago, IL, USA), followed by the Tukey's post hoc test to perform multiple comparisons of the values between time points.

3. Results

3.1. CART sequence and phylogenetic analysis

Database mining followed by 3′ and 5′ extension of cDNA ends led us to the identification of seven *cart*-like transcripts containing a coding region of 291, 324, 309, 381, 333, 324 and 321 bp, which translate into a prepropeptide of 96, 107, 102, 126, 110, 107 and 106 amino acids (aa), respectively. These transcripts were named following the zebrafish nomenclature suggested by Nishio et al. (2012) and taking into account their position in the phylogenic tree as *cart1a*, *cart1b*, *cart2a*, *cart2b*, *cart3a*, *cart3b* and *cart4*, and were deposited in GenBank with accession numbers KT189188, KT189189, KT189190, KT189191, KT189192, KT189193 and KT189194, respectively.

Comparison of the ORF region of the cDNA sequences of Senegalese sole with those of human, rat and 4 zebrafish genes (not shown) revealed that the percentage of identity between the different sole transcripts and that of mammalian (human and rat) genes varied from 67.6% to 51.6%, and from 73.9% to 47.8% between the different *cart* genes of sole and zebrafish. Between the seven Senegalese sole *cart* transcripts, homology varies between 71.8% and 45.7%, being highest for the pair *cart2a-cart2b*, followed by *cart1a-cart1b* (69.7% identity), and lowest for *cart3a-cart4* pair. Similar comparisons were performed with the prepropeptide sequences and the obtained identity matrix is shown in Fig. 2. In amino acid sequence, conservation between the sole CART's and the mammalian CART varied between 54.3% and 34.0%, and the different sole and zebrafish CART prepropeptide sequences have 70.6–29.4% identity between each other. When compared to each other, sole CART's vary in identity from 62.1% to 30.1%, with highest homology being found between SsCART1a and SsCART1b, followed by SsCART2a and SsCART2b (59.4%), and lowest between SsCART3a and SsCART4 (30.1%), followed by SsCART3b and SsCART4 (31.1%). The zebrafish prepropeptides showed a similar lower range of identities between DrCART1 and DrCART3 (30.4%), although a higher homology exists between DrCART2a and DrCART2b (69.2%).

The translated prepropeptides of the sole *cart* sequences were all predicted to contain a N-terminal signal peptide of variable size (from 19-aa to 30-aa). When compared to rat and human CART prepropeptides, a high degree of conservation was found in the carboxy-termini of the sequence, where 6 cysteine residues are strictly conserved. Between the highly divergent signal peptide and the conserved carboxy-termini end, the mammalian CARTs typically present two sites of processing by prohormone convertases composed by pairs of basic aa's (KR and KK). In Senegalese sole, the second pair is also strictly conserved in all CART prepropeptides, except in SsCART3b, where KR replaces KK, while the first (KR) processing site found in mammals is only conserved in SsCART2b (Fig. 3).

The reconstruction of the evolutionary history of this gene in vertebrates is shown in the phylogenetic tree presented in Fig. 4. Although a single gene exists in several mammalian species, up to 4 genes could be found in other tetrapods, such as in *Xenopus* (*Silurana*) *tropicalis*. Furthermore, 3 CART-like peptides were found for the lobe-finned fish *Latimeria chalumnae*, one of the two only living representatives of the class Sarcopterygii, which is closer to reptiles and mammals than to ray-finned fish. In the case of teleosts, multiple CART-like peptides were identified in several species. For instance, for *Maylandia zebra*, *Oreochromis niloticus*, *Pundamilia nyererei*, *Haplochromis burtoni* and *Xiphophorus maculatus*, 6 different CART-like peptides were found grouping closely together in different clades of the phylogenetic tree. In the case of zebrafish, *D. rerio*, only 4 genes have been found. Interestingly, a cyprinid-specific gene duplication was identified in zebrafish (CART2a and CART2b), goldfish, *C. auratus* (CART type I and type II) and common carp, *C. carpio* (CART type I and type II).

3.2. cart mRNA expression in areas of Senegalese sole brain

Relative levels of *cart* mRNA in different brain areas were determined by qPCR (Fig. 5). Both *cart2a* and *cart4* were found in all brain areas and fairly equally distributed, except for slightly higher transcript levels of *cart2a* in diencephalon and a slightly lower

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		1	2	3	4	5	6	7	8	9	10	11	12	13
		HsCART	RnCART	SsCART1a	SsCART1b	DrCART1	DrCART2b	SsCART2b	DrCART2a	SsCART2a	SsCART4	DrCART3	SsCART3a	SsCART3b
HsCART	**1**	100.0												
RnCART	**2**	94.8	100.0											
SsCART1a	**3**	54.2	54.2	100.0										
SsCART1b	**4**	53.3	54.2	62.1	100.0									
DrCART1	**5**	54.3	55.2	64.6	64.4	100.0								
DrCART2b	**6**	46.9	48.7	47.9	42.3	43.1	100.0							
SsCART2b	**7**	53.9	53.9	50.5	50.9	50.0	71.8	100.0						
DrCART2a	**8**	50.9	54.3	49.0	45.8	46.7	69.2	70.6	100.0					
SsCART2a	**9**	49.0	51.9	43.8	44.8	45.3	63.6	59.4	61.8	100.0				
SsCART4	**10**	42.5	40.6	43.2	40.0	36.9	41.9	40.0	41.5	41.1	100.0			
DrCART3	**11**	37.1	38.1	29.8	34.0	30.4	38.8	35.6	34.3	36.8	30.1	100.0		
SsCART3a	**12**	34.0	34.0	33.7	33.7	31.1	33.0	33.0	32.1	31.3	30.1	61.0	100.0	
SsCART3b	**13**	40.2	41.1	34.4	31.7	29.4	39.4	36.8	37.4	36.8	31.1	44.7	39.4	100.0

Fig. 2. Percent identities between *Solea senegalensis* (Ss) and other vertebrate CART prepropeptides (computed by ClustalW2). GenBank accession numbers are as follows: human HsCART, NP_004282.1; rat short variant RnCART, XP_006231905.1; zebrafish DrCART1, ADB12484.2; zebrafish DrCART2a, ADB12485.2; zebrafish DrCART2b, ADB12486.2; zebrafish DrCART3, ADB12487.1.

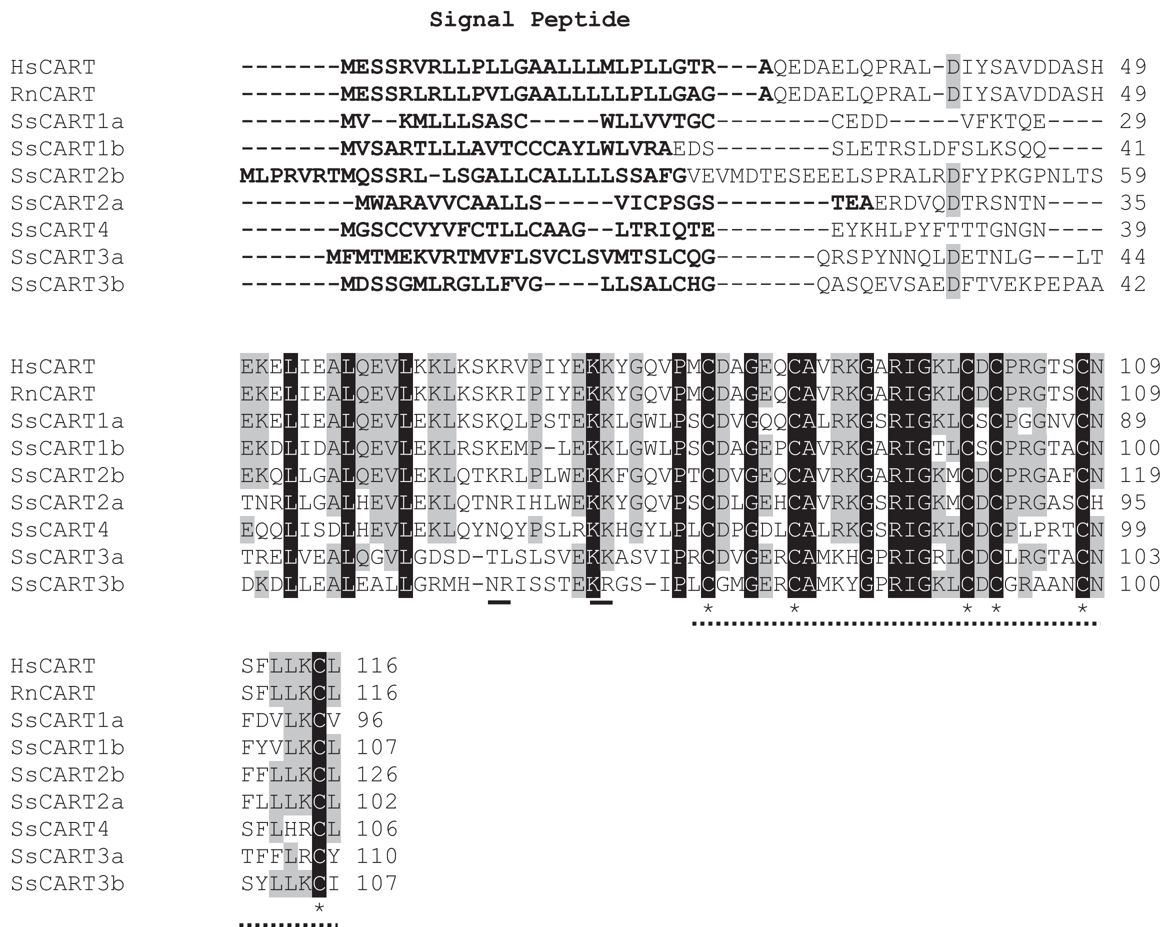

Fig. 3. Alignment of the deduced amino acid sequences of CART prepropeptides in ClustalW2. Black areas indicate residues shared by all sequences and shaded areas indicate residues identical in >66% of the sequences. The putative signal peptide is indicated in bold, potential proteolytic cleavage sites (KR and KK) are underlined, and sequence corresponding to exon 3 of the mammalian gene (showing highest conservation amongst vertebrates) is dashed underlined. Indicated (*) are also the 6 cysteine residues that form disulfide bridges responsible for the protein's tertiary structure. GenBank accession numbers are as follows: *Homo sapiens*, HsCART, NP_004282.1; *Rattus norvegicus* short variant, RnCART, XP_006231905.1.

expression of both genes in the pituitary. The expression profile of *cart1a*, *cart1b* and *cart3a* in the brain was quite similar, with a main expression in the mesencephalon, followed by the diencephalon, although the third region showing conspicuous expression differed between the genes: olfactory bulbs in the case of *cart1a*, telencephalic cerebral hemispheres for *cart1b* and spinal cord for *cart3a*. On the other hand, *cart2b* showed a main expression in the olfactory bulbs, and *cart3b* was predominantly expressed in the spinal cord.

3.3. Peripheral tissue distribution of cart mRNA

The relative expression of the different *cart* genes was also assessed by qPCR in whole brain and different peripheral tissues,

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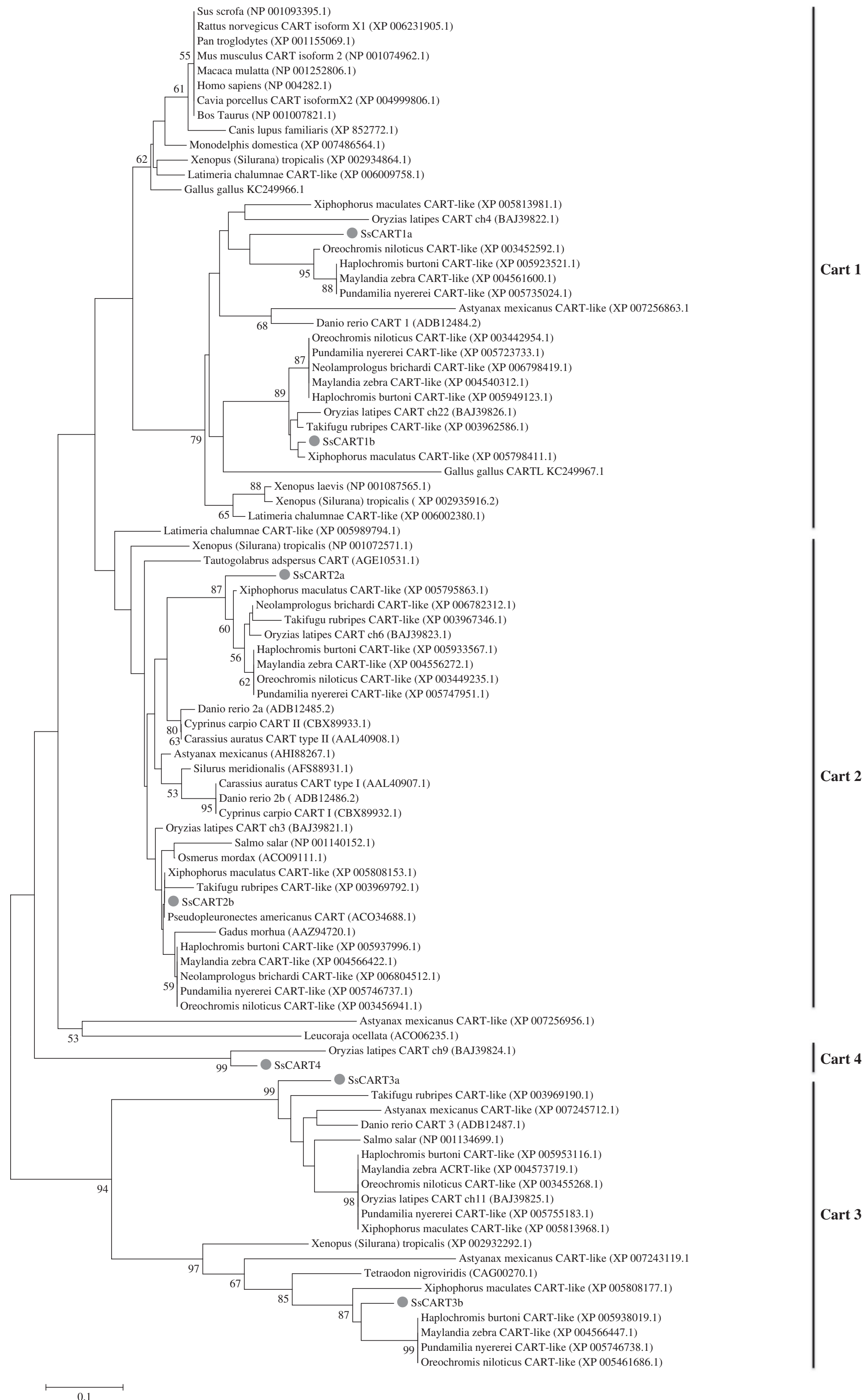

Fig. 4. Phylogenetic analysis of CART amino acid sequences in vertebrates. Protein IDs (GenBank accession numbers) are indicated in the tree, after the species name. The tree is drawn to scale and the scale bar represents the substitution rate per residue. Numbers at nodes indicate the bootstrap value, as percentages, obtained for 1000 replicates, in MEGA6.

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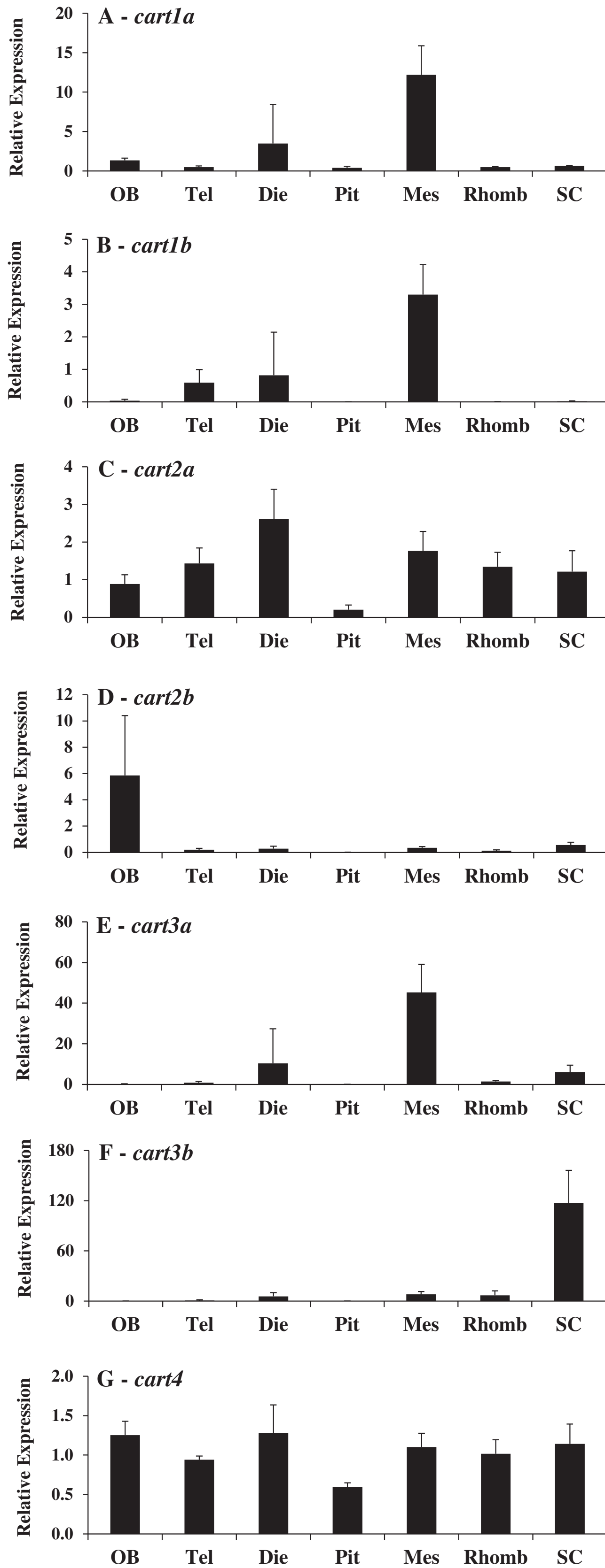

Fig. 5. *cart* mRNA expression in different Senegalese sole brain areas, determined by qPCR. Graphs show the normalized (by *rps4*) relative expression of *cart1a* (A), *cart1b* (B), *cart2a* (C), *cart2b* (D), *cart3a* (E), *cart3b* (F), and *cart4* (G) in each brain region ($n = 4$ individuals ± standard deviation, SD) in relation to the average across all regions. OB – olfactory bulbs, Tel – telencephalic cerebral hemispheres, Die – diencephalon, Pit – pituitary, Mes – mesencephalon, Rhomb – rhombencephalon, and SC – spinal cord.

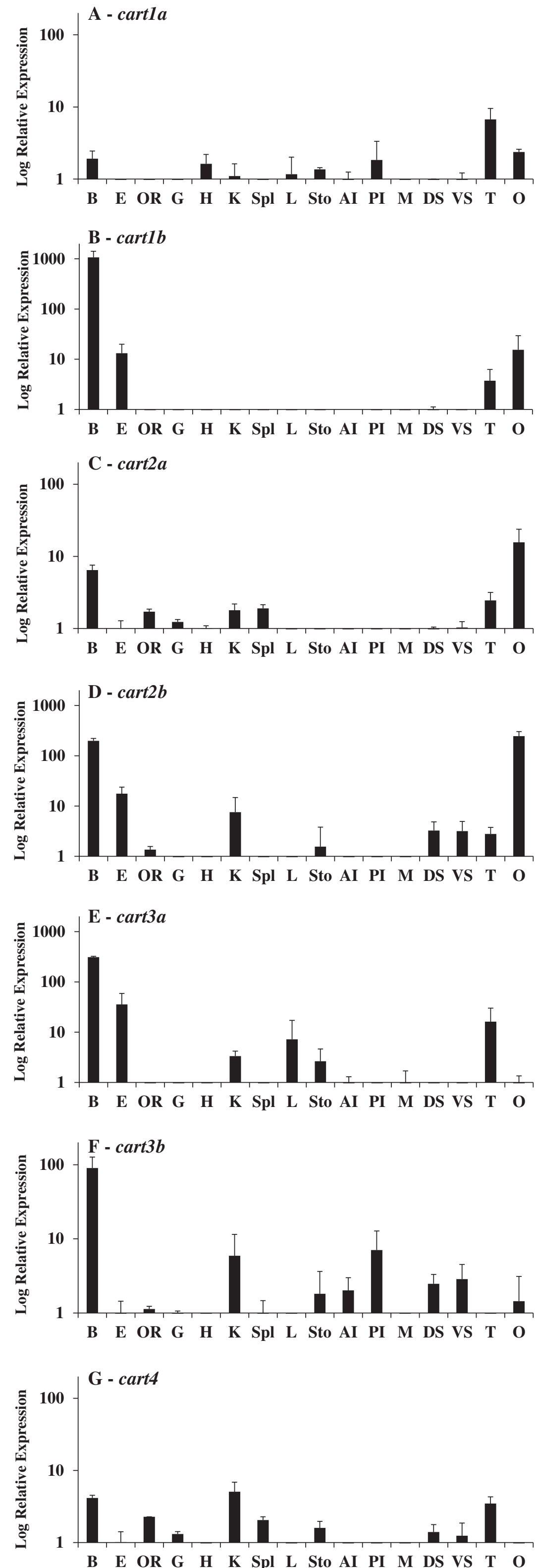

Fig. 6. *cart* mRNA expression in different Senegalese sole peripheral tissues, determined by qPCR. Graphs show the normalized (by *18s*) relative expression, in logarithmic scale, of *cart1a* (A), *cart1b* (B), *cart2a* (C), *cart2b* (D), *cart3a* (E), *cart3b* (F), and *cart4* (G) in each tissue ($n = 4$ individuals ± standard deviation, SD) in relation to the average across all tissues. B – brain, E – eye, OR – olfactory rosettes, G – gills, H – heart, K – anterior kidney-interrenal tissue, Spl – spleen, L – liver, Sto – stomach, AI – anterior intestine, PI – posterior intestine, M – muscle, DS – dorsal skin, VS – ventral skin, T – testis, and O – ovaries.

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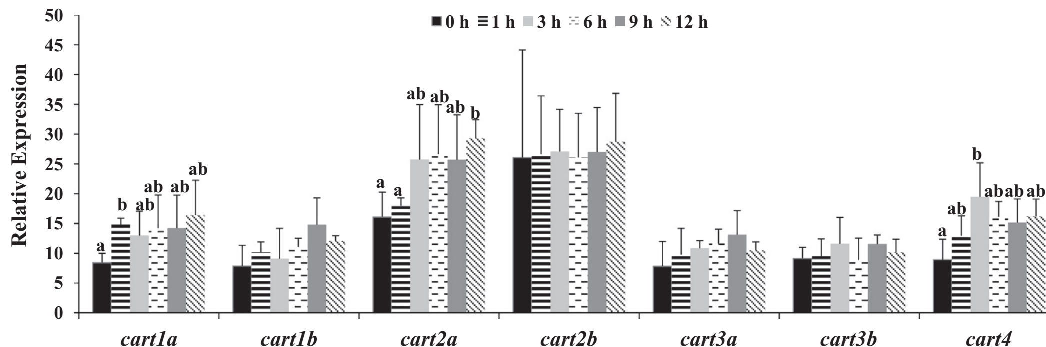

Fig. 7. Postprandial variations of *cart* mRNA expression in Senegalese sole whole brain, determined by qPCR. Graphs show the normalized (by a normalization factor computed from the averaged expression of *rps4*, *ef1a1*, *ubq*) relative expression of *cart1a*, *cart1b*, *cart2a*, *cart2b*, *cart3a*, *cart3b*, and *cart4* in the brain of 4 individuals (±SD) at 0 h (24 h fasting; sampling at scheduled feeding time) and again at 1 h, 3 h, 6 h, 9 h and 12 h after feeding.

and their pattern of expression differed considerably (Fig. 6). For *cart1b*, *cart3a* and *cart3b*, the predominant expression was in brain. For *cart1b* and *cart3a* a similar pattern was also found in the high expression in the eye and gonads (ovary, followed by testis, for *cart1b* and only testis in the case of *cart3a*). However, *cart1b* was not expressed in any other tissues, while *cart3a* also showed a relevant expression in liver, kidney and stomach. For *cart3b*, besides the main expression in the brain, a lower expression was also found in kidney and posterior intestine, followed by dorsal and ventral skin, anterior intestine and stomach. In the case of *cart2b* and *cart4*, similar mRNA levels were found in brain as in gonads (ovary for *cart2b* and testis for *cart4*). These two genes were also characterized by having an important expression in kidney, followed by a more minor expression in dorsal and ventral skin, stomach and in the olfactory rosettes. They differed mostly in the eye, where only *cart2b* was expressed, and in the gill where low levels of *cart4* mRNA were detected. Finally, *cart1a* and *cart2a* were both characterized by having a higher expression in gonads (testis in *cart1a* and ovary in *cart2a*) than in the brain. Furthermore, *cart1a* was also expressed at an important level in the ovary, followed by a more minor expression in posterior intestine, heart and stomach. On the other hand, *cart2a* mRNA was also found at low levels in testis, kidney, spleen and olfactory rosettes.

3.4. Postprandial variations of cart mRNA expression in Senegalese sole brain

The levels of *cart* mRNA in sole brain were measured at different times after a single meal and compared to basal levels (24 h fasting; sampling at scheduled feeding time, just before feeding) (Fig. 7). Of the seven cart genes, only *cart1a*, *cart2a* and *cart4* showed a significant postprandial regulation, being significantly up-regulated at 1 h post-feeding in the case of *cart1a* and 3 h after feeding, in the case of *cart4*. As for *cart2a*, differences were only significant at 12 h post-feeding with respect to unfed animals, but this is likely due to the variability of the data, which shows a strong trend for up-regulation already at 3 h after feeding. Overall, in spite of being a non-significant trend in the case of *cart1a* and *cart4*, mRNA levels remain still high 12 h after the meal.

4. Discussion

Many of the established or suggested actions and functional roles of CART have been inferred by studies of its anatomical central and peripheral location and co-expression with other neurotransmitters (e.g., dopamine and GABA), and some of its putative physiological effects were then later corroborated by studies examining effects of intracerebroventricular injection, overexpression or injection of other peptides (e.g., leptin, glucocorticoids), KO studies and analysis of gene polymorphisms associated to obesity (Murphy, 2005; Vicentic and Jones, 2007; Rogge et al., 2008; Lau and Herzog, 2014; Subhedar et al., 2014). Therefore, in order to gain further knowledge on the physiological roles of CART in teleosts, and how these might have diversified in species presenting many *cart* genes, we present data on the expression of seven Senegalese sole *cart* gene products in different brain areas and peripheral tissues. Furthermore, a potential involvement in the regulation of food intake was also investigated by studying transcriptional changes in response to feeding.

4.1. Structure and phylogenetic analysis

In rats, transcription of a single *Cart* gene results in two alternatively spliced mRNAs, while in humans a single transcript is produced, corresponding to the short variant of rat mRNA (Douglass et al., 1995; Douglass and Daoud, 1996). In both cases, the mRNA is translated into CART propeptides, which are then processed into smaller active forms, in a tissue-specific manner. At least six CART active peptides, which result from this post-translational processing of the CART protein, have been identified so far in mammals, and seem to have different potencies (Kuhar and Yoho, 1999; Dylag et al., 2006). However, only two of these have been more extensively investigated (Murphy, 2005; Rogge et al., 2008). Human and rat CART are very highly conserved, with only 6-aa differences, of which 5 are within the signal peptide that targets the protein for entry into the secretory pathway (Douglass et al., 1995; Douglass and Daoud, 1996). Other conserved features are 6 cysteine residues that form disulfide bridges leading to the tertiary structure that is essential for the protein to maintain its activity, and two pairs of basic aa's (KR and KK) that are the sites of cleavage by prohormone convertases (Rogge et al., 2008). The translated prepropeptides of the Senegalese sole *cart*'s were all predicted to contain a N-terminal signal peptide of variable size (from 19-aa to 30-aa). Furthermore, they all presented a strict conservation of the 6 cysteine residues within the carboxy-termini, which indicates that the resulting peptides should conserve biological activity. This was also the area showing the highest conservation among the sole CART sequences, as well as between sole CARTs and mammalian proteins. This is not surprising given that this sequence is found in all biologically active CART peptides described so far (Rogge et al., 2008). Of the seven deduced proteins characterized here, only SsCART2b contains two similar convertase cleavage sites, which indicates that this protein undergoes similar post-translational processing to become biologically active as in mammals. The remaining propeptides conserve the second (KK) putative cleavage site that, in the case of the sequence translated

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from *cart3b*, has been replaced by KR. This could mean that a different number of active peptides are produced from these transcripts but needs to be experimentally verified.

The reconstruction of the evolutionary history of the *cart* gene in vertebrates supports the hypothesis of the two-round (2R) whole genome duplication, also known as the 1:4 rule, occurring at the base of the vertebrate lineage, with a variable number of genes being retained during tetrapod evolution (Dehal and Boore, 2005). In this case, we have found 3 CART-like peptides in the lobe-finned fish *Latimeria chalumnae* and 4 in *Xenopus* (*Silurana*) *tropicalis*, but a single CART peptide is present in several mammals, indicating that 3 genes have been lost in the mammalian lineage. The number of CART peptides found in teleost species, including the 7 Senegalese sole CARTs, 6 CART-like peptides described previously in medaka (Murashita and Kurokawa, 2011) and in *M. zebra*, *O. niloticus*, *P. nyererei*, *H. burtoni* and *X. maculatus*, and 4 CARTs in zebrafish and *Takifugu rubripes*, for instance, evidence a third-round (3R) of whole genome duplication after the appearance of the teleost lineage (Volff, 2005). The phylogenetic tree presented in this study also suggests a later duplication of a *cart* gene in the cyprinid line and, based on this, we would propose that the nomenclature suggested by Nishio et al. (2012) for *D. rerio* and by Volkoff and Peter (2001) for *C. auratus* should be revised, and that DrCART2a and DrCART2b, as well as CaCART I and CaCART II, should be renamed as CART2a1 and CART2a2. The retention of 6–7 *cart* genes in highly evolved perciform and pleuronectiform teleost species suggests that different gene products might have adopted a new function (“neofunctionalization”) or partitioned old functions (“subfunctionalization”), which in mammals are performed by a single gene. This supports the theory that, in teleosts, which make up roughly half of all existing vertebrate species, differential loss or subfunction partitioning of gene duplicates might have been involved in the generation of such fish biodiversity (Volff, 2005).

4.2. cart mRNA expression in areas of Senegalese sole brain

When it was first described in the rat, CART mRNA’s were found predominantly in the hypothalamus and pituitary, followed by the thalamus/midbrain and significantly less in the cortex, striatum, hippocampus, and hindbrain (Douglass et al., 1995). These authors also described the pattern of expression in human brain, which generally does not differ much from that of rat. Many detailed studies have been performed since to further localize CART mRNA and peptides in specific neuroanatomical regions (reviewed by Subhedar et al., 2014).

In our study we have generally characterized the expression of seven putative *cart* genes in different brain regions of Senegalese sole by quantifying the relative levels of mRNA’s by qPCR. Our study revealed that *cart2a* and *cart4* genes exhibited the most widespread distribution in the brain of sole. In the goldfish brain a differential expression was found between two *cart* transcripts, with form I being more abundant in the olfactory bulbs and hypothalamus, whereas form II presented higher mRNA levels in the optic tectum-thalamus (Volkoff and Peter, 2001). In Senegalese sole, the transcript presenting higher homology to both goldfish transcript forms is *cart2a*, which was found in the diencephalon, mesencephalon, cerebral hemispheres, rhombencephalon, spinal cord and olfactory bulbs. However, similarly to the goldfish genes, a low expression was found in the pituitary. A detailed study on the differential distribution of four *cart* genes has been recently performed in zebrafish (Akash et al., 2014). As in sole, *cart2* showed a widespread distribution in the zebrafish brain. The diencephalon of zebrafish also contained the highest expression of *cart2*, with cells being evident in many cell masses from the preoptic area, ventral and dorsal thalamus, tuberal hypothalamus and posterior tubercle. Similarly to sole, the optic tectum and mesencephalic tegmentum of zebrafish also contained abundant *cart2*-expressing cells (Akash et al., 2014). In the present study we have also found an important *cart2a* expression in the hindbrain of sole. This rhombencephalic expression could be restricted to the medulla oblongata because this part of the brain, but not the cerebellum, contained *cart2* cells in zebrafish (Akash et al., 2014), as well as immunoreactive CART cells in catfish (Singru et al., 2007). The *cart4* gene also exhibited a profuse expression in the brain of sole. It is interesting to note that *cart4*-expressing cells (=*cart3* following the nomenclature from Nishio et al., 2012) also showed a broad distribution from the olfactory bulbs to the rhombencephalon in zebrafish (Akash et al., 2014) and that this gene represents the most divergent gene from this family in both species. On the contrary, the zebrafish *cart1* gene is exclusively expressed in cells from the nucleus of the medial longitudinal fascicle (nMLF), in the mesencephalic tegmentum. In agreement with these results, both sole *cart1a* and *cart1b* genes exhibit the highest mRNA expression levels in samples from the mesencephalon, where nMLF is contained. In addition, *cart1a* and *cart1b* expression was evident in the diencephalon and rostral forebrain (olfactory bulbs and cerebral hemispheres) from sole but no *cart1*-expressing cells were detected in these brain areas of zebrafish by in situ hybridization (Akash et al., 2014). If these dissimilarities represent real differences between species or differences in the sensitivities of the techniques used remain to be elucidated. The expression of sole *cart2b* was restricted to the rostral forebrain and, distinctly, to the olfactory bulbs. It should be noted that *cart3*-expressing cells (=*cart2b* following the nomenclature from Nishio et al., 2012) appear also restricted to the anterior pole of the brain, but in this case within the telencephalic entopeduncular nucleus (Akash et al., 2014). In sole, *cart3a* and *cart3b* brain expression patterns were clearly dissimilar between each other and in relation to its counterpart zebrafish gene (Akash et al., 2014). The expression pattern of sole *cart3a* was quite similar to that of *cart1a* and *cart1b* (mainly in mesencephalon and diencephalon), whereas *cart3b* expression pattern was unique and restricted to the spinal cord. Further studies using in situ hybridization appears necessary to elucidate if *cart1a*, *cart1b* and *cart3b* are expressed in the same cell masses from these regions.

The general pattern of distribution of the different sole *cart* transcripts in the brain suggests that, although subfunctionalization probably occurs, overall, CART peptides are likely to have similar roles in fish as in mammals, in regulating feeding and energy homeostasis, but also in neuroendocrine/hypophysiotropic functions and in processing peripheral sensory information.

4.3. Peripheral tissue distribution of cart mRNA

In contrast to the distribution of CART mRNA and peptides in the central nervous system, much less information exists on its peripheral distribution and possible modes of action. When it was first described in rat, CART mRNA was only found in the eye and adrenal glands (Douglass et al., 1995) but many studies conducted since then suggest a widespread distribution of CART, regulating many functions of the body, acting either locally or synergistically with central CART (Lau and Herzog, 2014). For instance, an important expression has been found in the enteric nervous system of mammals, particularly in the submucosal and myenteric plexuses of the stomach, small and large intestines, as well as in cell bodies and nerve fibers in the extrahepatic biliary tract (Couceyro et al., 1998; Ellis and Mawe, 2003; Ekblad, 2006). The precise role of this local enteric CART release within the gastrointestinal tract is still not clear, as Ekblad et al. (2003) failed to demonstrate an effect in intestinal motility. A hormonal role has also been suggested since CART is expressed in several pancreatic

islet cell types in rat and was found up-regulated in β-cells of diabetic animals (Wierup et al., 2006) and, in humans, it is found in pancreatic ganglia and in islet endocrine cells (Kasacka et al., 2012). It is also believed to play a role in renovascular hypertension in neuroendocrine cells of adrenal glands of rat (Kasacka et al., 2014). Finally, CART has been found in subcutaneous and visceral white adipose tissues in humans and rat, where it has a role in lipid and glucose utilization (Banke et al., 2013).

Similar to what was initially described in rats, the eye also seems to be a major site of *cart* expression in teleosts. In Atlantic salmon, common carp (CART I), goldfish (particularly CART I) and medaka (only for *ch3* and *ch6*) the eye was one of the tissues showing highest *cart* expression (Volkoff and Peter, 2001; Murashita et al., 2009; Murashita and Kurokawa, 2011; Wan et al., 2012). Furthermore, CART immunoreactivity has been found in different elements of the zebrafish retina and optic nerve system from 4 days of age until adulthood (Mukherjee et al., 2012). In Senegalese sole, *cart1b*, *cart2b* and *cart3a* were highly expressed in the eye. It is noteworthy that for *cart1b* and *cart3a* there was also a predominant central expression in the optic tectum-tegmentum (mesencephalon). However, for *cart2b*, the main central expression was in the olfactory bulbs, which could be related to expression found in the olfactory rosettes, albeit at low level. Two other sole genes, *cart2a* and *cart4*, that showed an important expression in the olfactory bulbs were also expressed in the olfactory rosettes. These results are consistent with a suggested important role of CART in processing sensory information, both visual and olfactory, as indicated previously in zebrafish (Mukherjee et al., 2012; Akash et al., 2014; Subhedar et al., 2014).

Evidence is starting to accumulate suggesting a specific role of CART in fish reproduction, although the exact mechanism in which it is implicated is still not clear. High levels of expression have been found in ovary in goldfish (Volkoff and Peter, 2001), common carp (Wan et al., 2012) and Atlantic cod (Kehoe and Volkoff, 2007), in testis of channel catfish (Kobayashi et al., 2008), in gonads of winter flounder and of the elasmobranch winter skate (MacDonald and Volkoff, 2009a,b), and at low levels in testis of Atlantic salmon (Murashita et al., 2009). In sole, all of the *cart* transcripts were found either in testis (*cart3a* and *cart4*) or ovary (*cart3b*), or both in testis and ovary (*cart1a*, *cart1b*, *cart2a*, *cart2b*), in some cases at a similar level or even higher than in the brain (*cart1a*, *cart2a*, *cart2b*, *cart4*). Variations in CART immunoreactivity in the brain of female catfish were studied over the annual sexual cycle and the pattern of change was correlated to that of luteinizing hormone (LH) cells in the pituitary and gonadosomatic index. Important changes correlated with the reproductive cycle were observed in brain areas associated with the central control of reproductive processes as well as in the olfactory bulbs and other neural areas associated with olfaction (Barsagade et al., 2010). It was therefore suggested that CART reproductive-related roles might be to process energetic and reproductive status-related information, trigger the brain-pituitary-ovary axis, and signal seasonal-dependent changes in the quality or availability of food and/or the presence of sex steroids. In mammals there is also indication that CART peptides might affect the reproductive hypothalamic–pituitary–gona dal axis, being involved in the release of gonadotropin-releasing hormone (GnRH) (Lebrethon et al., 2000; Parent et al., 2000), reproductive behavior (Douglass et al., 1995) and brain steroid feedback (Sohn et al., 2002). In addition, *CART* mRNA was found in the bovine ovary, where it is thought to be involved in regulating follicular atresia (Kobayashi et al., 2004).

In mammals, although an important CART immunoreactivity has been found in stomach and intestine, *cart* mRNA has not been detected in these tissues, suggesting that CART peptides are synthesized elsewhere and transported to the gut, possibly via sensory fibers or the vagus nerve (Murphy et al., 2000). Previous studies in fish found moderate levels of *cart* mRNA in the stomach, gut and liver of winter flounder (MacDonald and Volkoff, 2009a) and a low expression of *cart* in Atlantic salmon midgut (Murashita et al., 2009). In Senegalese sole, moderate levels of mRNA were found for *cart3b* in posterior and anterior intestine, as well as in the stomach. Furthermore, low levels of expression were found in stomach for *cart1a*, *cart2b*, *cart3a* and *cart4*. However, considering how these organs were sampled in fish for our qPCR analysis, we cannot rule out that the mRNA is located in fibers of the enteric nervous system embedded in the gastrointestinal mucosa, as described in mammals (Couceyro et al., 1998; Ellis and Mawe, 2003; Ekblad, 2006). On the other hand, from the seven analyzed transcripts, only *cart3a* was expressed in liver but, similarly to the gastrointestinal tract, we cannot exclude that this expression might be specific of the hepatic nerves.

Other relevant sites of *cart* expression in sole are the kidney (*cart2a*, *cart2b*, *cart3a*, *cart3b* and *cart4*), skin (*cart2b*, *cart3b* and *cart4*), spleen and gills (in both tissues *cart2a* and *cart4* were found at relatively low levels), and heart (*cart1a*). The fairly high levels of expression in the kidney of some of these transcripts matches the high expression found in the mammalian adrenal glands (Douglass et al., 1995; Kasacka et al., 2014), which in fish are replaced by interrenal cells that appear interspersed within the kidney, and suggests a conserved role of CART. Expression in the kidney and gill (mostly of CART I) was also previously described in goldfish, and *cart* mRNA was also found at low levels in salmon and winter flounder kidney, gill and heart, as well as in spleen in winter flounder and gill in medaka (*ch9*) (Volkoff and Peter, 2001; MacDonald and Volkoff, 2009a; Murashita et al., 2009; Murashita and Kurokawa, 2011). CART immunoreactivity has also been found in the cardiac nervous system of rats (Richardson et al., 2006) and CART peptide injections into the heart of *Rana ridibunda* have been associated with the regulation of cardiac function in frog (Ivanova et al., 2007). Therefore, the expression in the heart could be associated to the cardiac nervous system, as suggested for gastrointestinal tract and liver. Skin was the main site of expression of medaka *ch9* (Murashita and Kurokawa, 2011). In Senegalese sole, a low expression of the medaka's *ch9* closest homologue (*cart4*), but also of *cart2b* and *cart3b*, was detected in dorsal (pigmented) and ventral (unpigmented) skin. The only examined tissue for which there was no expression of any of the sole *cart* genes was in muscle. A lack of expression in muscle was also previously reported in goldfish and Atlantic salmon (Volkoff and Peter, 2001; Murashita et al., 2009), but a residual expression was described in medaka (for *ch3* and *ch9*) and winter flounder (MacDonald and Volkoff, 2009a; Murashita and Kurokawa, 2011).

4.4. Postprandial variations of cart mRNA expression in Senegalese sole brain

The effect of CART on feeding and behavior of fish has been fairly well studied and its anorexigenic role appears to be well conserved across the vertebrate lineage. Intracerebroventricular injections of CART peptides inhibited food intake in goldfish (Volkoff and Peter, 2000). In addition, prolonged fasting and feeding were associated to a significant reduction or up-regulation, respectively, of *cart* expression in goldfish (CART I), Atlantic salmon, Atlantic cod, channel catfish, African catfish, medaka (*ch3*), common carp and zebrafish brain (Volkoff and Peter, 2001; Kehoe and Volkoff, 2007; Kobayashi et al., 2008; Murashita et al., 2009; Subhedar et al., 2011; Valen et al., 2011; Nishio et al., 2012; Peterson et al., 2012). However, not all *cart* genes in fish are affected by fasting or feeding, as shown in winter skate, goldfish (CART II), zebrafish (*cart1* and *cart3*) and medaka (five out of six *cart* genes) (Volkoff and Peter, 2001; MacDonald and Volkoff, 2009b; Murashita and Kurokawa, 2011; Akash et al., 2014), which indicates other

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non-feeding regulation roles for these genes, probably explained by their divergence to acquire or retain non-redundant functions.

Compared to the effects of prolonged fasting, fewer studies have examined postprandial changes in *cart* expression in the fish brain (Volkoff and Peter, 2001; Kehoe and Volkoff, 2007; Valen et al., 2011; Peterson et al., 2012). In this study we examined the effect of feeding a single meal on *cart* brain expression at 1 h, 3 h, 6 h, 9 h and 12 h after feeding, and compared it with 24 h fasted fish (sampled at scheduled feeding time – 0 h). As previously seen in goldfish (Volkoff and Peter, 2001), not all Senegalese sole *cart* genes responded similarly to feeding and only *cart1a*, *cart2a* and *cart4* showed significant postprandial changes in expression. An increase in *cart* mRNA levels was observed at 1 h (*cart1a*) or 3 h (for *cart4* and tendentially for *cart2a*) after feeding and by 12 h the values still appeared fairly elevated (significantly for *cart2a*). However, the usage of the force feeding method, although enabling a good control of the amount of food ingested and time of feeding, is likely to have introduced some variability in the results, associated with the stress imposed by the technique. From what is known of the response of Senegalese sole to acute stress, plasma cortisol (primary stress response) and glucose (secondary stress response) levels increase rapidly and peak at 1 h following stress, dropping close to basal levels by 4 h (Costas et al., 2011). Therefore, the increase in expression of at least *cart2a* and *cart4* is likely to have been unrelated to stress. Nevertheless, it remains to be assessed how the different *cart* genes respond to stress and whether a differential expression in different areas of the brain might have masked the response to feeding in the whole brain for some of the genes. This question was outside of the scope of the present work but deserves careful consideration and further studies. Still, the timings of the response observed are within what has been observed in other studies in teleosts, although the response appears to be variable depending on the species. In goldfish hypothalamus and olfactory bulbs the expression of CART I was increased 2 h after a meal and returned to basal levels after 6 h (Volkoff and Peter, 2001). Similarly, in Atlantic salmon there was a significant up-regulation of *cart* expression in the brain 1.5 h after a meal but the high variability in the data make it difficult to assess when *cart* mRNA returned to basal levels (Valen et al., 2011). A fast postprandial up-regulation, only 30 min after feeding, was also measured in channel catfish, and *cart* mRNA levels remained elevated during at least 4 h (Peterson et al., 2012). In Atlantic cod, on the other hand, although a 7-day fasting led to the expected decline in *cart* mRNA levels, its expression was surprisingly down-regulated 2 h after a meal (Kehoe and Volkoff, 2007). Valen et al. (2011) suggested that satiety signals are associated to stomach fullness and gastric emptying, so that anorexigenic signals are associated with the initial phase after feeding, when the stomach is full, while orexigenic signals arise once most of the stomach content is evacuated. Considering the large diversity of teleost species, with important differences in diets and feeding habits affecting the dynamics of feeding, it is likely that the pattern of postprandial change and response to fasting of *cart* mRNA levels in fish brain can be highly species-specific and probably also diet-dependent. Nevertheless, results gathered so far support a conserved role of CART in regulating both short term and long term (fasting studies) appetite in fish.

The localization of *cart1a*, *cart2a* and *cart4* in the brain and peripheral tissues does not present significant clues to explain why only these genes were transcriptionally affected by feeding. While *cart2a* and *cart4* presented a more ubiquitous expression, including the telencephalon and diencephalon that contains the putative centers of regulation of food intake, *cart1a* was predominantly expressed in the mesencephalon, although a significant expression was also found in diencephalon. Nevertheless, *cart1b* and *cart3a* shared a very similar profile of expression in the brain as that of *cart1a*, and the latter but not the formers were significantly affected by feeding. In zebrafish, for instance, different *cart* genes have been shown to respond differently to fasting in specific, and often non-overlapping, brain areas or even cell populations within a brain region (Nishio et al., 2012; Akash et al., 2014). Thus, starvation resulted in a significant decrease in CART2-positive cells in the telencephalic entopeduncular nucleus and the hypothalamic nucleus of the lateral recess (NRL), as well as in a reduction of CART4-expressing neurons from the lateral tuberal nucleus of the hypothalamus, suggesting a function in energy homeostasis for these neurons (Akash et al., 2014). More anatomically detailed studies are necessary in the future in order to establish the mechanisms through which different *cart* genes and specific brain regions might be involved in the regulation of food intake in teleosts. Until then, the results presented here on the response of the different *cart* genes to feeding should be only taken as preliminary, although it is very likely that the response differs between genes, which again points to a probable subfunctionalization of the multiple *cart*'s retained in fish genomes across evolution.

5. Conclusion

Our study has shown that Senegalese sole, a pleuronectiform teleost, exhibits seven *cart* genes with distinct patterns of expression and feeding responses. Comparison of results presented here with previous studies performed in other teleost species indicates the existence of common patterns of expression, which suggests conserved evolutionary trends among vertebrate species and functions analogous to those found in mammals. However, there is also considerable variability in the expression of homologous genes in different teleost species, or even in closely related genes within the same species, possibly indicating divergence in *cart* gene subfunctionalization or development of new roles across evolution. In fact, some of the *cart* genes of sole, but not others, appear affected by food intake. This evidence, together with the differential pattern of expression in central and peripheral tissues suggest that, in addition to feeding and energy homeostasis, *cart* genes from sole could also be involved in other relevant physiological functions that remain to be elucidated in future studies.

Authors' contributions

S.M. conceived the study. K.B., A.M., and S.M. performed the *in silico* and molecular laboratory work, including obtaining samples for analyzing the tissue expression and postprandial profile. A.J. M.R. and J.A.M.C. obtained the samples for the brain expression study. All authors analyzed the data, wrote, read and approved the final manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

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Hypothalamic fatty acid sensing in Senegalese sole (*Solea senegalensis*): response to long-chain saturated, monounsaturated, and polyunsaturated (n-3) fatty acids

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AQ: au

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AQ:4

Conde-Sieira M, Bonacic K, Velasco C, Valente LM, Morais S, Soengas JL. Hypothalamic fatty acid sensing in Senegalese sole (*Solea senegalensis*): response to long-chain saturated, monounsaturated, and polyunsaturated (n-3) fatty acids. *Am J Physiol Regul Integr Comp Physiol* 309: R000–R000, 2015. First published October 14, 2015; doi:10.1152/ajpregu.00386.2015.—We assessed the presence of fatty acid (FA)-sensing mechanisms in hypothalamus of Senegalese sole (*Solea senegalensis*) and investigated their sensitivity to FA chain length and/or level of unsaturation. Stearate (SA, saturated FA), oleate (OA, monounsaturated FA of the same chain length), α-linolenate [ALA, a n-3 polyunsaturated fatty acid (PUFA) of the same chain length], and eicosapentanoate (EPA, a n-3 PUFA of a larger chain length) were injected intraperitoneally. Parameters related to FA sensing and neuropeptide expression in the hypothalamus were assessed after 3 h and changes in accumulated food intake after 4, 24, and 48 h. Three FA sensing systems characterized in rainbow trout were also found in Senegalese sole and were activated by OA in a way similar to that previously characterized in rainbow trout and mammals. These hypothalamic FA sensing systems were also activated by ALA, differing from mammals, where n-3 PUFAs do not seem to activate FA sensors. This might suggest additional roles and highlights the importance of n-3 PUFA in fish diets, especially in marine species. The activation of FA sensing seems to be partially dependent on acyl chain length and degree of saturation, as no major changes were observed after treating fish with SA or EPA. The activation of FA sensing systems by OA and ALA, but not SA or EPA, is further reflected in the expression of hypothalamic neuropeptides involved in the control of food intake. Both OA and ALA enhanced anorexigenic capacity compatible with the activation of FA sensing systems.

Senegalese sole; hypothalamus; polyunsaturated fatty acid; fatty acid sensing; food intake

THIS STUDY FOCUSES ON THE metabolic control of food intake in the hypothalamus. This term refers to the capacity of specific neurons located in the hypothalamus to detect changes in the levels of metabolites, such as glucose or fatty acids through nutrient-sensing mechanisms (8, 10, 31). When the levels of nutrients rise, this increase is detected through nutrient sensing mechanisms in these neurons, and as a result, one of the populations (the so-called glucose-excited, GE neurons) is stimulated, resulting in increased release of the neuropeptides proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) synthesized by these neurons. In contrast, the other population (the so-called glucose-inhibited, GI neurons) is inhibited, resulting in decreased release of the neuropeptides Agouti-related peptide (AgRP) and neuropeptide Y (NPY) synthesized by these neurons (8, 10, 31). The global balance of the response is an increased anorectic potential and subsequent decrease in food intake. Conversely, when the levels of nutrients decline, the nutrient-sensing systems are inhibited, resulting in increased production of AgRP/NPY and decreased production of POMC/CART, resulting in increased food intake (8, 10, 31). The nutrient sensing mechanisms characterized so far in mammals (8, 10, 31) and fish (44) include both different types of glucosensors and fatty acid sensing mechanisms.

Fatty acid (FA) sensing mechanisms detect increases in plasma concentration of long-chain fatty acids (LCFA) through several processes (8, 10, 31), including *1*) FA metabolism through inhibition of carnitine palmitoyltransferase 1 (CPT-1) that imports FA-CoA into the mitochondria for oxidation; *2*) binding to FA translocase (FAT/CD36) and modulation of transcription factors like peroxisome proliferator-activated receptor type α and sterol regulatory element-binding protein type 1c (SREBP1c); *3*) activation of protein kinase C-θ; and *4*) mitochondrial production of reactive oxygen species by electron leakage, resulting in an inhibition of ATP-dependent inward rectifier potassium channel (K_{ATP}) activity (8, 10, 31). The 18 carbon monounsaturated FA (MUFA) oleate (OA; C18:1 n-9) is the most studied LCFA in mammals that typically activates all of these systems (8, 10, 31). However, the ability of different types of LCFA, differing in acyl chain length or degree of unsaturation, to elicit the activation of these systems has been scarcely assessed to date. Fatty acid unsaturation appears to be important since the saturated FA palmitate (C16:0) does not seem to activate hypothalamic FA sensing systems (15, 41, 43). Moreover, the presence of more than one double bond does not seem to induce comparable activations of FA sensing systems in mammals, as demonstrated by studies carried out with linoleate (C18:2 n-6) (39) or docosahexanoate (DHA; C22:6 n-3) (12, 13, 41), although no studies that we are aware of have examined both FAs in parallel.

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Appetite regulation in fish is a complex process in which the hypothalamus integrates metabolic and endocrine information to elicit changes in food intake through modulation of neuropeptide synthesis and release (44, 48, 49). As for the regulation

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of food intake elicited by the sensing of changes in circulating concentrations of FA, we have characterized the presence and function of FA sensing systems in the hypothalamus of the freshwater species rainbow trout (*Oncorhynchus mykiss*) (23–30). These systems responded to changes in the levels of not only LCFA such as OA but, unlike mammals, also to medium-chain FA (MCFA) like octanoate (23–30). The activation of these systems was associated with an upregulation of the expression of anorexigenic neuropeptides and food intake as in mammalian species (22, 26). To date, the presence of FA sensing systems in other fish species has never been investigated.

All vertebrate species have absolute dietary requirements for certain polyunsaturated FAs (PUFAs), which are termed essential fatty acids since they cannot be synthesized de novo and are required for normal body functioning (42). On the other hand, lipids in aquatic food webs are rich in PUFA, and in marine environments, fish diets are particularly rich in long-chain PUFA (42). Furthermore, PUFA of the n-6 and particularly n-3 series are predominant in tissues of most fish, but especially in marine species (38, 45). The brain of marine fish is particularly rich in n-3 PUFA, mainly in α-linolenate (C18:3 n-3), eicosapentanoate (C20:5 n-3), and DHA (C22:6 n-3) (4, 46). Therefore, it is interesting to assess whether fish hypothalamic FA sensing systems, particularly in marine species, could differ from those of mammals in the ability to sense changes in levels of these PUFA.

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With this in mind, we wanted to investigate the presence of FA sensing systems in the hypothalamus of a marine fish species. Moreover, we also aimed to know whether the ability of hypothalamic FA sensing might vary as a function of LCFA chain length and/or degree of unsaturation. As a model marine fish species, we used a lean flatfish, the Senegalese sole (*Solea senegalensis* Kaup). The choice of Senegalese sole as a model species for this study had both academic and commercial reasons. More specifically, we selected this marine species because *1*) it has high commercial interest, and therefore, studying the physiology of its feeding is important for aquaculture, and *2*) its lipid nutrition has been quite well studied, due to its particular nutritional physiology and metabolism, as it concerns lipid and fatty acid requirements: it does not perform well with high lipid levels in the diet and has unique capacities to biosynthesize PUFAs (7, 34). To achieve these objectives, we injected intraperitoneally *1*) stearate (SA; C18:0)—a saturated FA; *2*) oleate (OA, C18:1 n9)—a MUFA of the same chain length, which has been demonstrated to activate FA sensing systems and to modulate food intake in rainbow trout (22, 26); *3*) α-linolenate (ALA; C18:3 n-3), an n-3 PUFA of the same chain length; and *4*) eicosapentanoate (EPA; C20:5 n-3)—an n-3 PUFA of a longer chain length and a higher degree of unsaturation. We then evaluated food intake and the hypothalamic mRNA abundance of neuropeptides related to the metabolic control of food intake, such as *agrp, npy, pomc*, and *cart*. Furthermore, variables related to putative FA-sensing systems were also evaluated on the basis of *1*) FA metabolism, such as activities of ATP-citrate lyase (ACLY) and CPT-1, and mRNA abundance of acetyl-CoA carboxylase (*acc*), *acly, cpt-1c*, and fatty acid synthase (*fas*); *2*) binding to FAT/CD36 and subsequent modulation of transcription factors, such as mRNA abundance of *fat/cd36*, liver X receptor α (*lxrα*), *pparα*, and *srebp1c*; and, *3*) mitochondrial activity, such as 3-hydroxyacyl-CoA dehydrogenase (HOAD) activity, and mRNA abundance of inward rectifier K^+ channel pore type 6.x (*kir6.x*) and sulfonylurea receptor (*sur*). We have evaluated mRNA abundance of neuropeptides and not protein levels because appropriate antibodies for Western blot analysis were not available in Senegalese sole at the time this study was carried out.

MATERIALS AND METHODS

Fish

Senegalese sole obtained from a commercial fish farm (Aquacria Piscícolas, Aveiro, Portugal) were kept under quarantine conditions for a 3-wk period. The fish were then individually weighed (88.3 ± 1.5 g), measured, and distributed (17 fish per tank in the first phase of the experiment, and 8 fish per tank in the second phase of the experiment) among fiberglass rectangular tanks (0.5 m × 0.4 m) in a closed recirculation system. The system was supplied with filtered and heated (20.0 ± 1.0°C) seawater (24‰) at a flow rate of 1.5 l/min. An artificial photoperiod of 12:12 h light-dark was established. Fish were fed by hand twice daily (0900 and 1700) to satiety with a diet manufactured by Sparos, Portugal (proximate food analysis was 57.9% crude protein, 0.8% fiber, 15.5% starch, 8.6% crude fat, and 8.5% ash; 20.3 MJ/kg of feed). The experiment was directed by trained scientists (following category C FELASA recommendations) and conducted according to the European guidelines on protection of animals used for scientific purposes (directive 2010/63/UE of European Parliament and of the Council of European Union).

AQ: 7

Experimental design. Following a 2-wk acclimation period, the first phase of the experiment was carried out. We used a total number of 170 fish. These fish were randomly divided into five groups (control, SA, OA, ALA, or EPA) of 34 fish each. Each treatment was carried out in duplicate tanks (two replicate tanks per treatment), and each tank contained 17 fish. Fish were fasted for 24 h before the experiment to ensure that basal hormone levels were achieved, and then fish were anesthetized with MS-222 (75 mg/l; Sigma, St. Louis, MO), weighed and injected (10 ml/kg ip) with saline solution alone (control) or containing SA, OA, ALA, or EPA (all from Sigma Chemical) at a dose of 300 μg/kg. The FA dose was selected on the basis of previous studies carried out in rainbow trout (20) and in preliminary experiments carried out in Senegalese sole (data not shown). To safely deliver the FAs, they were solubilized in 45% hydroxypropyl-β-cyclodextrin (HBP; Sigma) to a final concentration of 17 mM (36). The HPB-FA solution was diluted in saline to the appropriate concentration used for each injection. HPB was also added in the control group at a similar concentration to the remaining treatments. Three hours after intraperitoneal injection (established on the basis of previous unpublished results), fish were lightly anesthetized with MS-222 (75 mg/l) and sampled. Blood was collected by caudal puncture with ammonium-heparinized syringes, and plasma samples were obtained after blood centrifugation, then deproteinized (using 0.6 M perchloric acid) and neutralized (using 1 M potassium bicarbonate) before storage at −80°C until analysis. Fish were killed by decapitation and dissected to remove the hypothalamus, which was snap-frozen in dry ice and stored at −80°C. At each time point, in each tank, six fish were used to assess enzyme activities and metabolite levels, eight fish were used to evaluate the hypothalamic FA profile, and the remaining three fish were used for assessment of mRNA levels by RT-quantitative PCR. Because we used replicate tanks for each experimental group, we had 12 fish for the assessment of metabolite levels and enzyme activities, 16 fish for the assessment of FA profile, and 6 fish for the assessment of mRNA abundance. For molecular analysis, samples of hypothalamus were collected in tubes containing 0.5 ml of RNAlater stabilization buffer (Sigma) and were kept in agitation at 4°C for 24 h, before storing at −80°C.

In the second phase of the experiment, we used 160 fish. The fish were divided into five treatment groups of 32 fish each (as described

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above). Each treatment was carried out in fish of four tanks (four replicate tanks per treatment), and each tank contained eight fish. Fish were fed by hand twice per day (0900 and 1700) to satiety during 3 wk. Food intake was quantified for 3 days before intraperitoneal injection (to collect baseline data) and then 4, 24 and 48 h after an intraperitoneal treatment with pure saline-HPB or containing SA, OA, ALA, or EPA, as described above. After each meal, any remaining uneaten pellets were removed from the tanks. The amount of feed consumed in each tank was calculated as the difference between the number of offered and uneaten pellets. Results are shown as means ± SE of the data obtained in four tanks per treatment.

Assessment of metabolite levels and enzyme activities. Levels of total FA and triglycerides in plasma, were determined enzymatically using commercial kits (Wako Chemicals, Neuss, Germany and Spinreact, Barcelona, Spain; respectively), following manufacturer's instructions, adapted to a microplate format.

Samples used to assess hypothalamic metabolite levels were homogenized immediately by ultrasonic disruption in 5.5 vols of ice-cooled 0.6 M perchloric acid and were neutralized (using 1 M potassium bicarbonate). The homogenate was centrifuged (10,000 *g*), and the supernatant was used to assay tissue metabolites. Hypothalamic FA and triglyceride levels were determined enzymatically using commercial kits, as described above for plasma samples.

Samples for evaluation of enzyme activity were homogenized by ultrasonic disruption with 7 vols of ice-cold-buffer consisting of 50 mM Tris (pH 7.6), 5 mM EDTA, 2 mM 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged (900 *g*), and the supernatant was used immediately for enzyme assays. Enzyme activities were determined using the INFINITE 200 Pro microplate reader (Tecan, Männedorf, Switzerland). Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm or, in the case of CPT-1 activity, of 5,5′-dithiobis(2-nitrobenzoic acid)-CoA complex at 412 nm. The reactions were started by the addition of sample supernatant (10–15 μl) at a preestablished protein concentration, omitting the substrate in control wells (final volume 275–295 μl) and allowing the reactions to proceed at 20°C for preestablished times (10–25 min). Enzyme activities are expressed in terms of milligrams of protein. Protein was assayed in triplicate in homogenates using microplates, according to the bicinchoninic acid method with BSA (Sigma) as a standard. Enzyme activities were assessed at maximum rates by preliminary tests to determine optimal substrate concentrations. ACLY (*EC* 4.1.3.8), HOAD (*EC* 1.1.1.35), and CPT-1 (*EC* 2.3.1.21) activities were determined by adapting previously described methods (22).

Determination of total lipids and FA profile in the hypothalamus. To have enough tissue mass for the analysis of FA profile in the hypothalamus, the 16 fish sampled for this purpose from each treatment group were grouped into 4 pools of 4 fish each. In each pool, total lipids in the hypothalamus were extracted by chloroform/methanol (2:1, vol/vol) according to Folch et al. (12) and quantified gravimetrically after evaporation of the solvent under nitrogen flow followed by vacuum desiccation overnight. Total lipids were resuspended at 20 mg/ml in chloroform/methanol (2:1) containing 0.01% BHT and 100 μl subjected to acid-catalyzed transesterification with 21:0 internal standard (9). FAME was extracted using isohexane/diethyl ether (1:1, vol/vol), purified by TLC (Silica gel 60; VWR, Lutterworth, UK) and analyzed by gas-liquid chromatography on a Thermo Electron-Trace GC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 m × 0.25 mm ID; SGE Analytical Science, Chester, UK), using a two-stage thermal gradient initially at 40°C/min from 50°C (injection temperature) to 150°C and then to 250°C at 2°C/min. Helium (1.2 ml/min constant flow rate) was used as the carrier gas and on-column injection, and flame ionization detection was performed at 250°C. FAs were identified by comparison with known standards (Supelco, Spain) and a well-characterized fish oil (MARINOL, Stepan Specialty Products, Anaheim, CA) and quantified using Chrom-card for Windows (TraceGC, Thermo Finnigan, Milan, Italy).

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Primer design. Search for selected candidate genes was performed in the SoleaDB database (3) (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/) using the *Solea senegalensis* v4.1

EQ:1

Table 1. *Nucleotide sequences of the PCR primers used to evaluate mRNA abundance by quantitative RT-PCR*

Gene	Forward Primer	Reverse Primer	Annealing Temperature, °C	Database	Reference
acc	CAGCTGGGTGGAATTCAGAT	ATGGGATCTTTGGCACTGAG	60	SoleaDB[1]	solea_v4.1_unigene15555
acly	CCACAGATTCACACCATTGC	GCCAGGATGTTATCCAGCAT	60	SoleaDB[1]	solea_v4.1_unigene11536
agrp2	CAGGTCAGACTCCGTGAGCCC	GTCGACACCGACAGGAGGCAC	64	SoleaDB[1]	solea_v4.1_unigene32957
cart2b	AGGACCATGCAGAGTTCCAG	GGACTCGGTGTCCATCACTT	60	GenBank	KT189191
cart4	GTGAGCGAGAGCAGGAAACT	CGTGGTGAAATAAGGCAAA	60	GenBank	KT189194
cpt1.1	TAACAGCCACCGTCGACATA	AGCGATTCCCTTGTGTCACT	63	GenBank	KR872890
cpt1.2	TCGCCAAGAACTAACCGAAC	AGACCTGGCGTAGAGCTTCA	64	GenBank	KR872891
cpt1.3	CCTGACTGTTGACCCCAAGT	TCACTCACAGTTACGCAGCA	60	GenBank	KR872892
ef1α	GATTGACCGTCGTTCTGGCAAGAAGC	GGCAAAGCGACCAAGGGGAGCAT	70	GenBank	CAB326302
fas	CACAAGAACATCAGCCGAGA	GAAACATTGCCGTCACACAC	60	GenBank	KP842777
fat/cd36 (lmp2)	TATGTGGCGGTAATGGATCA	GCCGGTGTGGAATACAAACT	60	GenBank	KR872888
fat/cd36 (pg4l)	TGAATGAGACGGCTGAGTTG	TGTTGTTTCTGCTCCTCACG	64	GenBank	KR872889
kir6.x	AGATGTTGGCGAGAAAGAGC	GCTCGCGGATGTTCTTGT	60	SoleaDB[1]	solea_v4.1_unigene120423
lxrα	AAAGCAGGGCTTCAGTTTGA	CAGCCTCTCCACCAGATCAT	60	SoleaDB[1]	solea_v4.1_unigene47872
npy	GAGGGATACCCGATGAAACC	GCTGGACCTCTTCCCATACC	60	SoleaDB[1]	solea_v4.1_unigene466117
pomca	AAGGCAAAGAGGCGTTGTAT	TTCTTGAACAGCGTGAGCAG	60	GenBank	FR851915
pomcb	GTCGAGCAACACAAGTTCCA	GTCAGCTCGTCGTAGCGTTT	60	GenBank	FR851916
pparα	AAACCGCCTCTCATCATCC	CACACCTGGAAACACATCTCC	60	GenBank	JX4240810
srebp1c	TCCAAGGCTTTCAGCAAGAT	CTCCTCTGTCTTGGCTCCAG	60	SoleaDB[1]	solea_v4.1_unigene4060
sur	GCAGCACCTTCCGTTACCTA	GCAGCAGCTTAGAGGACGAC	60	SoleaDB[1]	solea_v4.1_unigene446925
ubq	AGCTGGCCCAGAAATATAACTGCGACA	ACTTCTTCTTGCGGCAGTTGACAGCAC	70	GenBank	CAB291588

[1]SoleaDB: http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/. *acc, acetyl-CoA carboxylase; acly*, ATP-citrate lyase; *agrp*, Agouti-related peptide 2; *cart*, cocaine- and amphetamine-related transcript; *cpt1.1*, carnitine palmitoyl transferase type 1, isoform 1; *cpt-1.2*, carnitine palmitoyl transferase type 1, isoform 2; *cpt1.3*, carnitine palmitoyl transferase type 1, isoform 3; *ef1α*, elongation factor 1α; *fas*, fatty acid synthetase; *fat/cd36 (lmp2)*, fatty acid translocase lysosome membrane protein 2-like; *fat/cd36 (pg4l)*, fatty acid translocase platelet glycoprotein 4-like; *kir6.x*, inward rectifier K^+ channel pore type 6.x; *lxrα*, liver X receptor α; *npy*, neuropeptide Y; *pomc*, proopiomelanocortin A1; *pparα*, peroxisome proliferator-activated receptor type α; *srebp1c*, sterol regulatory element-binding protein type 1c; sur, sulfonylurea receptor; *ubq*, ubiquitin.

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Table 2. *Fatty acid composition (μg/mg dry weight) of Senegalese sole hypothalamus 3 h after intraperitoneal administration of saline alone, or saline + 300 μg/kg of stearate, oleate, α-linolenate, or eicosapentanoate*

Fatty Acid	Treatment				
	CTR	SA	OA	ALA	EPA
C14:0	0.56 ± 0.13 b	0.89 ± 0.41 b	0.58 ± 0.07 b	1.13 ± 0.13 a	0.46 ± 0.08 b
C16:0	16.48 ± 1.00	19.76 ± 3.46	17.35 ± 0.44	25.43 ± 4.67	18.22 ± 0.99
C18:0	14.52 ± 0.98	14.66 ± 1.42	14.95 ± 0.40	19.30 ± 1.95	16.50 ± 1.36
C24:0	1.31 ± 0.16	1.47 ± 0.16	2.24 ± 0.83	1.87 ± 0.28	1.27 ± 0.18
Σ SAFA[1]	32.97 ± 2.09 b	37.05 ± 5.15 ab	35.53 ± 1.72 b	52.75 ± 6.47 a	36.63 ± 2.27 b
C16:1	0.60 ± 0.14	1.17 ± 0.43	0.82 ± 0.33	1.13 ± 0.50	0.65 ± 0.21
C18:1n-9	15.60 ± 1.23	18.46 ± 3.13	18.27 ± 2.80	24.28 ± 4.73	16.87 ± 1.03
C20:1	0.10 ± 0.06 ab	0.30 ± 0.08 ab	0.20 ± 0.12 ab	0.45 ± 0.17 a	0.16 ± 0.01 b
Σ MUFA[2]	16.30 ± 1.35 b	19.93 ± 3.64 ab	19.29 ± 3.22 ab	25.86 ± 5.32 a	17.68 ± 0.99 ab
C18:2n-6	1.44 ± 0.10	1.54 ± 0.21	1.55 ± 0.23	2.03 ± 0.53	1.50 ± 0.14
C20:4n-6	1.93 ± 0.08	1.85 ± 0.24	1.93 ± 0.20	2.60 ± 0.75	2.19 ± 0.16
Σ n-6 PUFA[3]	3.37 ± 0.16	3.43 ± 1.00	3.48 ± 0.44	4.56 ± 1.05	3.69 ± 2.09
C18:3n-3	0.00 ± 0.00	0.02 ± 0.02	0.00 ± 0.00	0.14 ± 0.08	0.00 ± 0.00
C18:4n-3	0.18 ± 0.10	0.33 ± 0.02	0.40 ± 0.07	0.77 ± 0.39	0.27 ± 0.04
C20:5n-3	2.07 ± 0.12	2.30 ± 0.34	2.19 ± 0.28	4.07 ± 1.73	2.33 ± 0.06
C22:5n-3	0.95 ± 0.08	1.00 ± 0.07	1.27 ± 0.36	1.35 ± 0.19	1.03 ± 0.08
C22:6n-3	34.14 ± 1.04	33.34 ± 3.78	33.52 ± 0.84	43.33 ± 5.73	36.95 ± 1.39
Σ n-3 PUFA[4]	37.33 ± 1.19	36.91 ± 4.08	37.39 ± 1.52	49.47 ± 7.59	40.58 ± 1.35
Σ PUFA[5]	40.71 ± 1.35	40.35 ± 4.70	40.87 ± 1.95	54.04 ± 8.57	44.27 ± 1.31

Each value is the mean ± SE of n = 4 pools of four hypothalami per treatment. CTR, control; SA, stearate; OA, oleate; ALA, α-linolenate; EPA, eicosapentanoate; SAFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Different letters indicate significant differences ($P < 0.05$) between groups. Different lowercase letters indicate significant differences ($P < 0.05$) between FA treatments. [1]C15:0, C22:0 are also included. [2]C18:1n7, C22:1, C24:1n9 are also included. [3]C18:3n6, C20:2n6, C20:3n6, C22:4n6, C22:5n6 are also included. [4]C20:3n3, C20:4n3, C21:5n3, C22:4n3 are also included.

global assembly. The retrieved transcripts were aligned and assembled in silico using the BioEdit Sequence Alignment Editor and, in some cases, completed with rapid amplification of cDNA ends (RACE) PCR using the FirstChoice RLM-RACE kit (Ambion, Life Technologies, Alcobendas, Madrid, Spain), and the resulting fragments were sequenced (SCSIE, University of Valencia, Valencia, Spain). Primers for quantitative RT-PCR were designed with Primer3 v. 0.4.0 (18, 47) (Table 1) and the resulting amplicons were sequenced to confirm their identity and the specificity of the assay.

AQ: 10 T1

mRNA abundance analysis by real-time quantitative RT-PCR. Total RNA was extracted from tissues (~20 mg) using TRIzol reagent (Life Technologies, Grand Island, NY), following manufacturer's instructions and treated with RQ1-DNase (Promega), and RNA quantity and quality were evaluated by spectrophotometry (NanoDrop 2000). Two micrograms of total RNA were reverse transcribed into cDNA using Superscript II reverse transcriptase (Promega, Madison, WI) and random hexaprimers (Promega). Gene expression levels were determined by real-time quantitative RT-PCR (q-PCR) using the iCycler iQ (Bio-Rad, Hercules, CA). Analyses were performed on 5 μl of diluted (1/50) cDNA using the MAXIMA SYBR Green qPCR Mastermix (Life Technologies), in a total PCR reaction volume of 15 μl, containing 120–500 nM of each primer. Sequences of the forward and reverse primers used for each gene expression assay are shown in Table 1. Relative quantification of the target genes was done using elongation factor 1 alpha (*ef1a*) or ubiquitin (*ubq*) as reference genes (16). Thermal cycling was initiated with incubation at 95°C for 2 min using hot-start iTaq DNA polymerase activation; 35 steps of qPCR were performed, each one consisting of heating at 95°C for 15 s, 30 s at each specific annealing temperature (Table 1), and 30 s at 72°C. Following the final PCR cycle, melting curves were systematically monitored (temperature gradient at 0.5°C/s from 55 to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without cDNA were run as negative controls, which were indeed negative and confirmed no amplification of genomic DNA. Relative expression of the target genes was calculated using the delta-delta CT method ($2^{-\Delta\Delta CT}$), following Pfaffl (40).

Statistics. Comparisons among groups were carried out using the statistical package SigmaStat with one-way ANOVA with treatment (control, SA, OA, ALA, and EPA) as the main factor. In cases where a significant effect was noted, post hoc comparisons were carried out by a Student-Newman-Keuls test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Fatty acid composition of hypothalamus from fish treated with intraperitoneal injections is shown in Table 2.

All fish treated with FAs tended to have a lower food intake compared with the control group, although no significant differences were observed among treatments (Fig. 1).

T2 F1

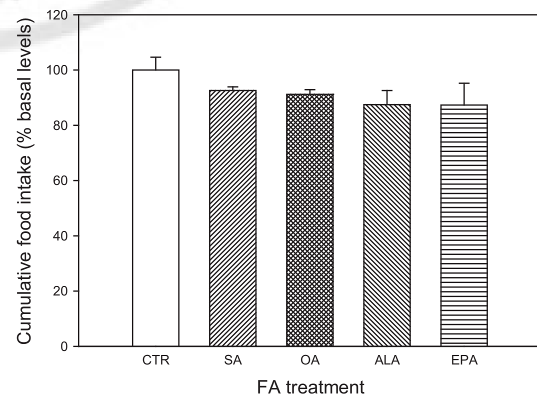

Fig. 1. Effect of intraperitoneal treatment with saline solution alone (control, CTR) or containing 300 μg/kg of stearate (SA) oleate (OA), α-linolenate (ALA), or eicosapentanoate (EPA) on cumulative food intake of Senegalese sole. All fish (n = 8) in a tank were fed, and food intake was monitored in the whole tank at 4, 24, and 48 h after feeding. Food intake is displayed as a percentage of food ingested with respect to basal levels (calculated as the average food intake in the same tank the 3 days preceding the experiment). The results are shown as mean ± SE of the results obtained in four different tanks per treatment.

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Concentrations of total FA in plasma increased in the SA and EPA groups (Fig. 2*A*) compared with the control. In the hypothalamus, total FA levels (Fig. 2*B*) increased in the OA treatment compared with the control and SA treatments and were also higher in fish injected with ALA compared with the control, SA, and EPA treatments. Triglyceride levels (Fig. 2*C*) were higher in the OA, ALA, and EPA treatments compared with the control and SA groups. Total lipid levels (Fig. 2*D*) were higher in the ALA group compared with control and SA groups. ACLY activity (Fig. 2*E*) decreased in the OA and ALA groups compared with the control, and the activity in the ALA group was also lower than in the SA group. CPT-1 activity (Fig. 2*F*) decreased in OA and ALA groups compared with the control.

The mRNA abundance of genes related to FA metabolism is shown in Fig. 3. The expression of *acc* (Fig. 3*A*) decreased in the OA and ALA groups compared with the control. *acly* expression in the ALA group was lower than that of the control group (Fig. 3*B*). *fas* mRNA abundance was lower in the OA group compared with the control (Fig. 3*C*). The expression of *cpt1.3* was higher in the SA and EPA groups compared with the control (Fig. 3*F*). Finally, no significant changes were observed in mRNA abundance of *cpt1.1* (Fig. 3*D*) and *cpt1.2* (Fig. 3*E*).

Transcript levels of genes related to FA transport and regulation of gene expression (transcription factors) are shown in Fig. 4. Transcription of *fat/cd36*-like genes, thrombospondin receptor or platelet glycoprotein 4-like (*pg4l*) (Fig. 4*A*) and lysosome membrane protein 2-like (*lmp2*) (Fig. 4*B*), was not affected by any FA treatment. *lxrα* mRNA abundance (Fig. 4*C*) was decreased in the OA and EPA groups compared with the control group. Expression of *pparα* (Fig. 4*D*) and *srebp1c* (Fig. 4*E*) was lower in OA and ALA groups compared with the control.

The parameters related to mitochondrial activity and K_{ATP} channel are shown in Fig. 5. HOAD activity (Fig. 5*A*) in the OA and EPA groups was lower than that of the control and SA groups. On the other hand, the OA and ALA groups displayed a lower expression of *kir6.x* (Fig. 5*B*) and *sur* (Fig. 5*C*) than the control.

The mRNA abundance of neuropeptides involved in the regulation of food intake is shown in Fig. 6. *Agrp2* expression in the OA group was lower than in the control, SA, and EPA groups, while in the ALA group, it was only lower than the control (Fig. 6*A*). No significant changes were noted for mRNA abundance of *npy* (Fig. 6*B*), *pomca* (Fig. 6*C*), *pomcb* (Fig. 6*D*), and *cart4* (Fig. 6*F*). *cart2b* mRNA levels (Fig. 6*E*) were higher in the OA, ALA, and EPA groups, whereas the

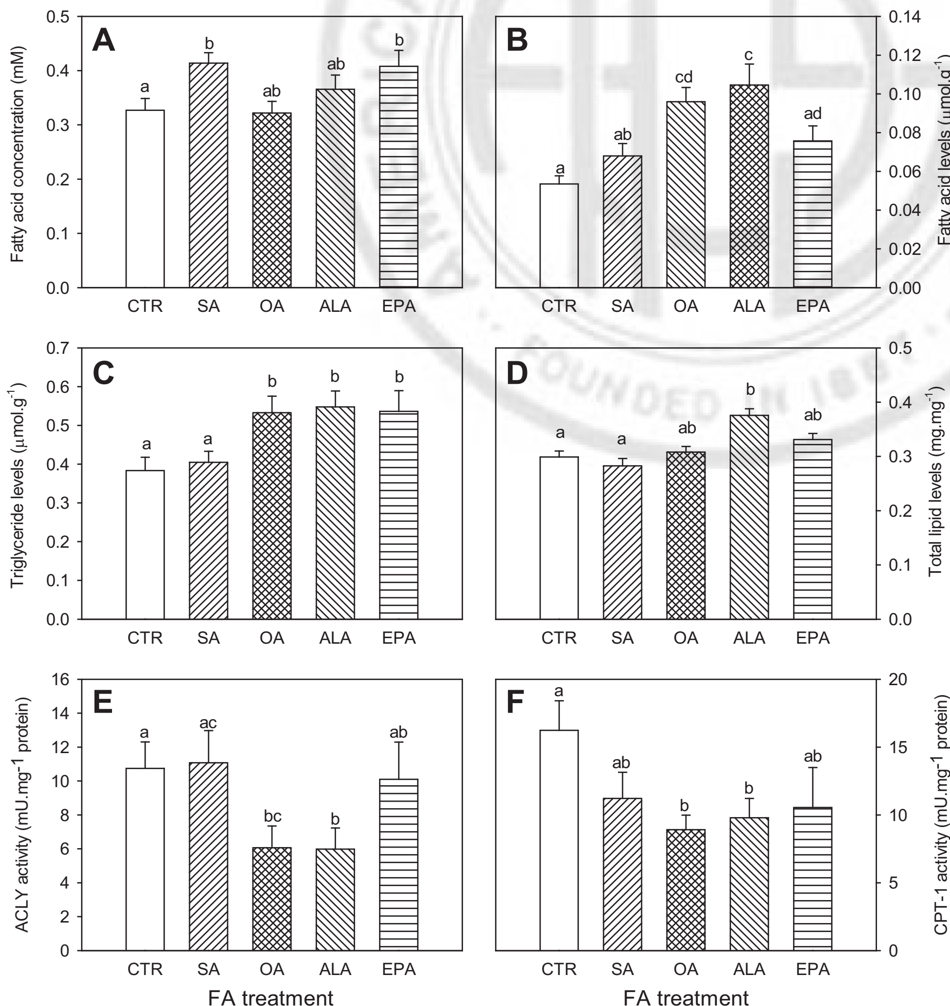

Fig. 2. Concentration of total FA in plasma (*A*), and levels of total FA (*B*), triglyceride (*C*) and total lipid (*D*), and activities of ATP-citrate lyase (ACLY; *E*) and carnitine palmitoyltransferase 1 (CPT-1; *F*), in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (CTR) or containing 300 µg/kg of SA, OA, ALA, or EPA. Each value is the mean ± SE of $n = 12$ fish per treatment. Different lowercase letters above bars indicate significant differences ($P < 0.05$) between groups.

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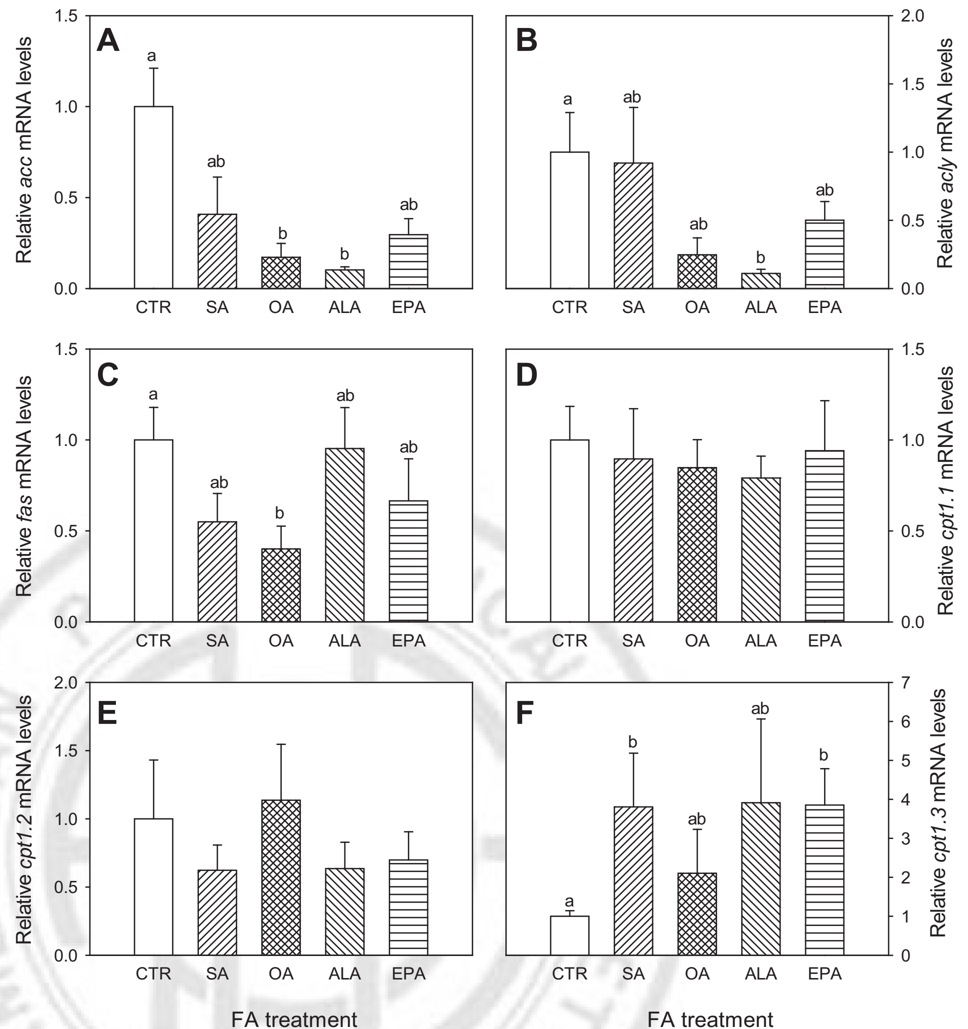

Fig. 3. mRNA abundance of *acc* (*A*), *acly* (*B*), *fas* (*C*), *cpt1.1* (*D*), *cpt1.2* (*E*), and *cpt1.3* (*F*) in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (CTR) or containing 300 μg/kg of SA, OA, ALA, or EPA. Gene expression results are expressed relative to the control group and are normalized by *ef1α* or *ubq* expression. Each value is the mean ± SE of $n = 6$ fish per treatment. Different letters indicate significant differences ($P < 0.05$) between groups.

expression in the EPA group was also higher than in the SA group.

DISCUSSION

We have previously demonstrated in rainbow trout, a freshwater fish species, the presence of FA sensing systems in the hypothalamus and their response to increased levels of OA or octanoate (22–30). However, there was no evidence to date of the existence of such mechanisms in other fish species. PUFAs are a major constituent of marine fish diets, particularly important essential nutrients, and major constituents of fish brains and other body tissues. Therefore, we wanted to further investigate whether (if present) FA sensing systems in the hypothalamus of a marine fish species could respond to changes in PUFA levels, as well as to different types of LCFA differing in chain length and degree of saturation.

First of all, it was necessary to set up the techniques required for the evaluation of parameters related to FA sensing systems, including enzyme activities and gene expression in Senegalese sole. In some cases, multiple transcripts were found for the genes of interest, and preliminary work was performed to select those that would be more relevant for the objectives of this study. Thus, in the case of *cart*, seven different transcripts were found (5), of which we selected *cart2b* and *cart4* to examine in this study. The first gene is the closest homolog to the trout *cart* that has been studied so far (22) and also to medaka ch3, which was the only *cart* transcript (out of six) that responded to fasting and refeeding in this species, and was suggested to have an anorexic role (39). On the other hand, *cart4* was selected given that it is expressed relatively well in Senegalese sole hypothalamus and appears to respond to feeding (5). On the other hand, we found five different transcripts for *cpt1* (two of them being splice variants of the same gene: isoform 4), of which we decided to assess the three most similar to the trout *cpt1* isoforms involved in FA sensing (22). Finally, we also obtained evidence for the presence of multiple *fat/cd36* genes, of which we focused on LMP2 and PG4L. The first gene resembled the transcript most commonly studied in relation to FA sensing in trout (22), while the second is closest to a mammalian *fat/cd36*-like gene with roles in the orosensory detection of FA and mediating effects of FA on hypothalamic food intake regulation in rats (13, 19, 37).

AQ: 12

The parameters related to the FA sensing system based on FA metabolism displayed changes depending on the FA assessed, and we clearly distinguished two patterns of response. The first pattern included the actions of OA and ALA that activated this system, as demonstrated by the decrease observed in ACLY activity and expression, CPT-1 activity, *fas*

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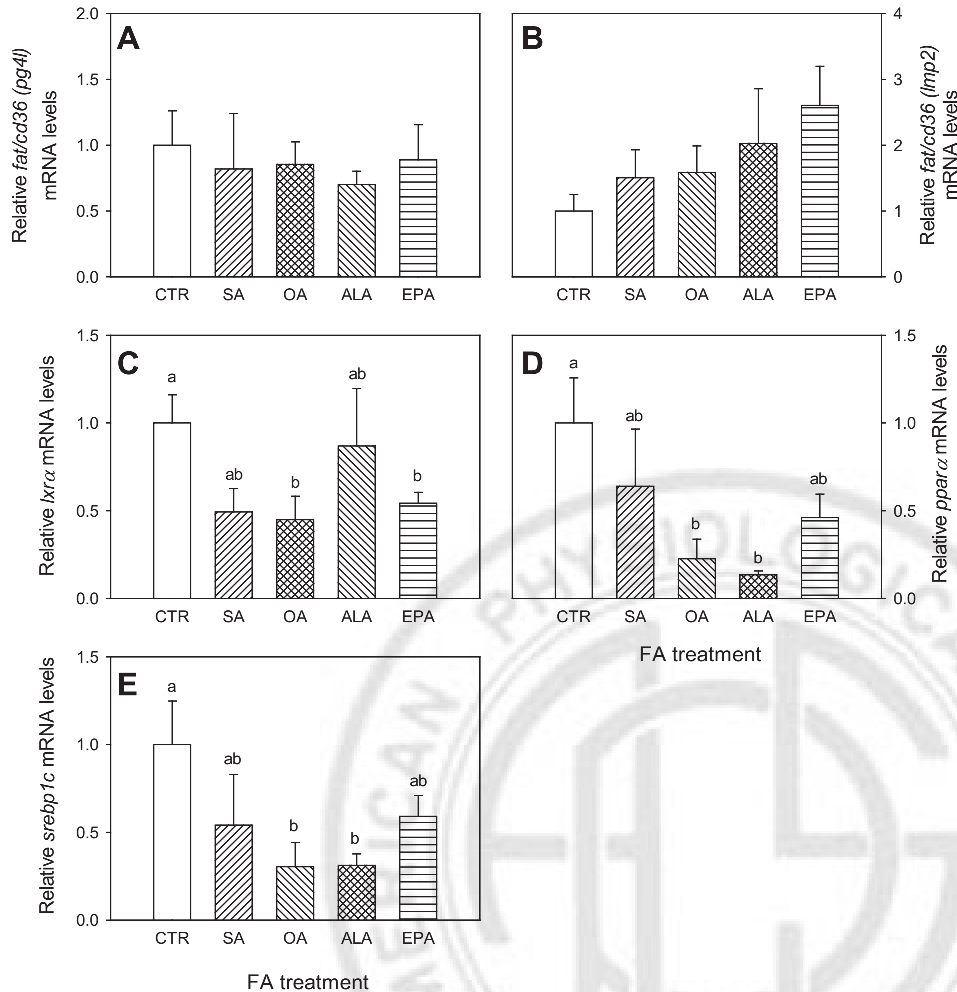

Fig. 4. mRNA abundance of *fat/cd36 (pg4l)* (*A*), *fat/cd36 (lmp2)* (*B*), *lxrα* (*C*), *pparα* (*D*), and *srebp1c* (*E*) in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (CTR) or containing 300 μg/kg of stearate (SA) oleate (OA), α-linolenate (ALA) or eicosapentanoate (EPA). Gene expression results are expressed relative to the control group and are normalized by *ef1α* or *ubq* expression. Each value is the mean ± SE of $n = 6$ fish per treatment. Different lowercase letters above bars indicate significant differences ($P < 0.05$) between groups.

mRNA abundance (only after OA treatment), and *acc* expression. The response of these parameters is generally comparable to that previously observed in rainbow trout (22, 24, 26) and in mammals (8, 31) in response to OA, thus supporting the presence of this system in a marine fish species like Senegalese sole. The lack of changes in mRNA abundance of the different isoforms tested for CPT1 might indicate a failure in selecting the isoform in this species functionally similar to the CPT1c in rainbow trout (22) or mammals (7, 31), in responding to changes in FA levels.

The response observed after ALA IP injection, basically comparable to that of OA, suggest for the first time in any vertebrate species that this hypothalamic FA sensing system responds to increased levels of a n-3 PUFA differently to that observed to date in mammals (14, 15, 41). There are no other studies that we are aware of in fish hypothalamus. The effects of n-3 PUFA like ALA, EPA, and DHA have only been elucidated in peripheral tissues such as the liver, where an inhibition of lipogenic enzymes (similar to that herein observed) has been recorded in rainbow trout (1, 35).

The second pattern of response included the actions of SA and EPA, which, in general, did not induce any change in FA metabolism parameters, or the changes induced (e.g., increased expression of *cpt1.3*, similarly to the OA and ALA groups) were unexpected. The lack of response to SA is not surprising considering that in mammals, another saturated LCFA like palmitate did not induce changes in parameters related to this FA sensing system (41, 43). This could mean that the response requires the presence of at least one double bond. On the other hand, the lack of changes observed in response to intraperitoneal injection of EPA is very interesting considering that this FA is also an n-3 PUFA (like ALA) but of a longer acyl chain and higher degree of saturation. We have no explanation for these differences. They could be associated with the fact that LC-PUFA (such as EPA and DHA) and C18 PUFA (such as ALA) may differentially affect key regulatory pathways of lipid metabolism, binding to nuclear receptors or transcription factors and modulating the expression of genes with different potencies, as has been observed in mammals (17, 51). In this respect, a strong hypotriglyceridemic effect has been attributed to EPA in rats, through simultaneous effects on FA oxidation and lipogenic pathways (50). In fish, this possibility has not been assessed yet. However, evidence supporting the existence of similar effects (at least in lipogenesis) is accumulating in Senegalese sole (6) and salmonid species (1, 33).

The FA sensing mechanisms based on binding of FA to FAT/CD36 and subsequent modulation of transcription factors was also differentially affected by FA treatments. Two *fat/cd36*-like genes were investigated in Senegalese sole. The first one is the putative homologue of the gene that has been studied

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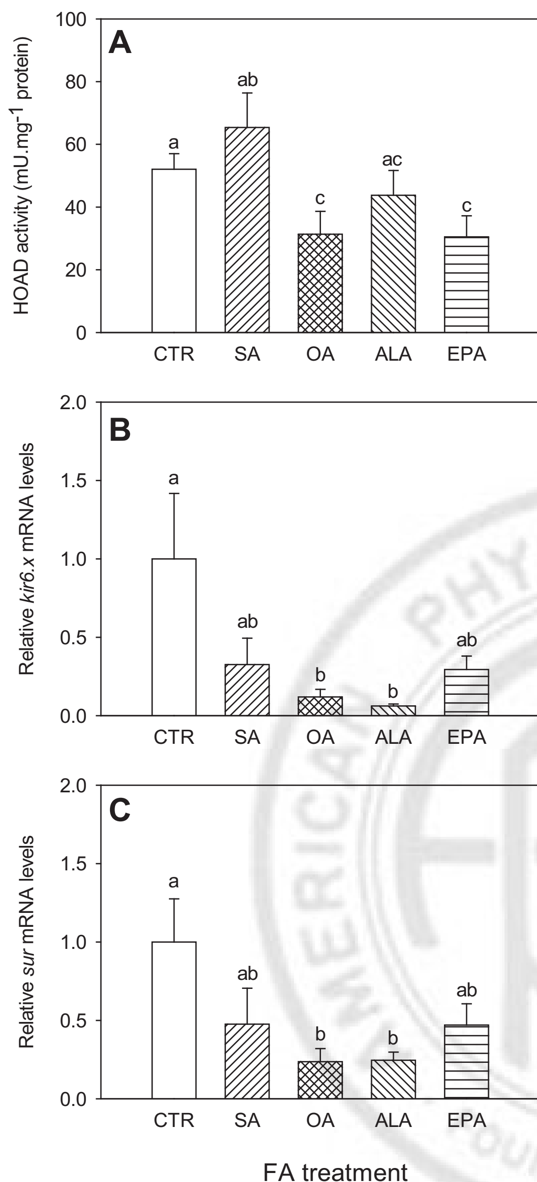

Fig. 5. Activity of 3-hydroxyacyl-CoA dehydrogenase (HOAD; *A*), and mRNA abundance of *kir6.x* (*B*) and *sur* (*C*) in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (CTR) or containing 300 μg/kg of SA, OA, ALA, or EPA. Gene expression results are expressed relative to the control group and are normalized by *ef1α* or *ubq* expression. Each value is the mean ± SE of $n = 12$ (enzyme activity) or $n = 6$ (mRNA levels) fish per treatment. Different lowercase letters above bars indicate significant differences ($P < 0.05$) between groups.

so far in trout (22, 24, 26). The second one is closest to a mammalian *fat/cd36* implicated in the orosensory detection of FA and affecting food intake through LCFA sensing in the hypothalamus (13, 19, 37). However, no changes were observed in the mRNA abundance of any of the genes after treatment with the tested FA. Because other *fat/cd36*-like genes were identified in the transcriptomic SoleaDB (http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/), these should also be investigated in the future. If we focus on the remaining parameters related to this FA sensing system (mRNA abundance of *pparα*, *srebp1c*, and *lxrα*), we can observe a similar response to that outlined above. Both OA and ALA altered the mRNA abundance of the transcription factors *lxrα* (although not ALA in this case), *srebp1c*, and *pparα* in a way comparable to that previously observed after OA treatment in mammals (20, 21, 32) and rainbow trout (22, 24, 26). These results support once again the presence of these mechanisms in the hypothalamus of Senegalese sole, as well as its response not only to an unsaturated C18 FA such as OA, but also to a C18 PUFA such as ALA. As far as we are aware, the response to an n-3 PUFA is absolutely novel in any vertebrate species to date. Very few studies exist in fish, although when Atlantic salmon were fed a DHA-enriched diet, no changes were observed in *lxrα* expression, while *srebp1c* was upregulated in whole brain tissue (4). In spite of a trend for lower expression of these transcription factors in the SA and EPA treatments in relation to the control group, these changes were not significant. A notable exception was *lxrα*, for which intraperitoneal injection with EPA, and not ALA, induced a significant downregulation.

The assessed parameters of FA sensing based on mitochondrial activity also displayed a generally similar pattern to the other FA sensing systems. The intraperitoneal injection of OA and ALA induced changes (decreased expression of the components of the K_{ATP} channel and HOAD activity, although only significantly for OA in the latter case) comparable to those observed in rainbow trout (22, 24, 26) and in mammals (2, 8) after treatment with OA. In contrast, neither SA nor EPA induced any change in the mRNA levels of *kir6.x* or *sur*, but the EPA treatment led to a significant reduction of HOAD activity, compared with the control.

The activation of FA sensing systems is typically associated with the inhibition of the orexigenic factors AgRP and NPY, and the enhancement of the anorexigenic factors POMC and CART, ultimately leading to decreased food intake in mammals (8, 32), as well as in rainbow trout (22, 26). In Senegalese sole, intraperitoneal injections of both OA and ALA induced a decrease in the mRNA abundance of *agrp2* and an increase in the mRNA abundance of *cart2b* (which was also significantly increased by the EPA treatment), without significantly affecting *npy* or *pomc* mRNA abundance. These changes in gene expression are consistent with a general anorexigenic balance that is usually associated with decreases in food intake, as observed in mammals (8, 10) or rainbow trout (22, 26) after treatment with OA. Furthermore, similar changes in anorexigenic potential have also been recorded in rat, after treatment with DHA (43), or after feeding an n-3 PUFA enriched-diet (11), while palmitate failed to induce effects in neuropeptide expression (43), as was the case with SA in the current study. However, despite a slight decrease observed in food intake, nonsignificant differences in food intake were observed after FA treatment.

Perspectives and Significance

In summary, in the present study, we demonstrated that the three FA sensing systems characterized in rainbow trout are also present in Senegalese sole hypothalamus, indicating that this capacity is not specific to rainbow trout and can also be found in marine fish species like Senegalese sole. These systems were activated by OA in a way similar to that previously described not only in rainbow trout but also in mammals, highlighting their importance in the phylogeny of vertebrates.

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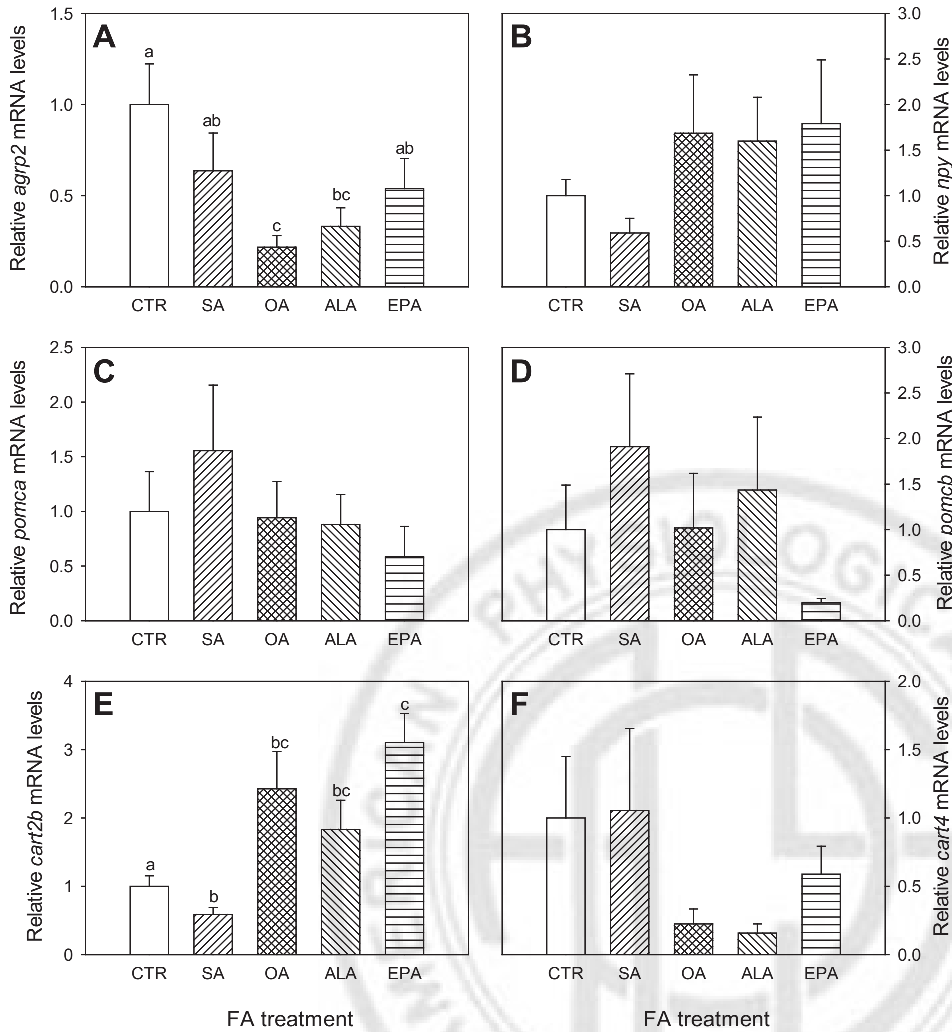

Fig. 6. mRNA abundance of *agrp2* (*A*), *npy* (*B*), *pomca* (*C*), *pomcb* (*D*), *cart2b* (*E*), and *cart4* (*F*) in the hypothalamus of Senegalese sole 3 h after intraperitoneal administration of saline alone (CTR) or containing 300 μg/kg of SA, OA, ALA, or EPA. Gene expression results are expressed relative to the control group and are normalized by *ef1α* or *ubq* expression. Each value is the mean ± SE of $n = 6$ fish per treatment. Different lowercase letters above bars indicate significant differences ($P < 0.05$) between groups.

On the other hand, we provide for the first time in any vertebrate species (as far as we are aware) evidence of the activation of these hypothalamic FA sensing systems by an n-3 PUFA such as ALA. This is different to that reported in mammals (12, 13, 39, 41). This different response may relate to the importance of n-3 PUFA in fish, especially in marine species. Thus, PUFA may be sensed in the hypothalamus of marine fish and possibly promote changes in food intake. However, in spite of the particularly high levels of LC-PUFA (including EPA) found in the brain tissues of teleosts, the overall lack of effects of EPA on fatty acid sensing systems in the hypothalamus of Senegalese sole indicates that the response might be specific to certain PUFA. This differential response may be related to different (yet undetermined) roles of LC-PUFA in fish. Still, intraperitoneal injections of EPA led to a few changes in some of the assessed parameters, such as increased plasma triglyceride levels and hypothalamic *cpt1.3* and *cart2b* expression, and decreased *lxrα* mRNA levels and HOAD activity. These effects are probably unrelated to a role in FA sensing and might be explained by other mechanisms, such as the activation of transcription factors and modulation of the expression of lipid metabolism genes with effects on energy homeostasis. Finally, we also obtained evidence on the lack of a response of FA sensing systems to a saturated FA such as SA. This response is comparable to the lack of effect in mammals of other saturated LCFA, such as palmitate (15, 41). However, we previously observed that a MCFA-like octanoate was able to activate these systems in rainbow trout. Therefore, both the level of unsaturation and chain length of FA seem to be important factors for the hypothalamic sensing capacity in sole but are probably not the only factors involved.

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DISCLOSURES

AQ: 14 No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.C.-S., L.M.V., S.M., and J.L.S. conception and design of research; M.C.-S. and K.B. performed experiments; M.C.-S., K.B., C.V., and S.M. analyzed data; M.C.-S., K.B., C.V., L.M.V., S.M., and J.L.S. interpreted results of experiments; M.C.-S., K.B., C.V., and J.L.S. prepared figures; M.C.-S., K.B., C.V., L.M.V., S.M., and J.L.S. drafted manuscript; M.C.-S., K.B., C.V., L.M.V., S.M., and J.L.S. edited and revised manuscript; M.C.-S., L.M.V., S.M., and J.L.S. approved final version of manuscript.

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