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Tesis Doctoral

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Dra. Josefina Blasco Mínguez Dr. Jaume Fernández Borràs Miguel Martín Pérez

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“Aquel que no sabe lo que está buscando
no comprenderá lo que encuentra”

agradecimientos

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Han sido muchos los esfuerzos para llegar hasta aquí. Durante este camino ha habido muchas personas que han contribuido de una u otra manera a llegar a la meta que representa esta tesis.

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prologue

PROLOGUE

The work this thesis is based on was carried out within the Group of Energy and Intermediary Metabolism of Fish, Department of Physiology and Immunology, Faculty of Biology, University of Barcelona (UB), Barcelona, under the Physiology Program during the period 2007-2012. The main objective of this thesis is to study the physiological processes occurring in the muscle of sea bream under different culture conditions by means of emerging tools such as stable isotopes analysis and proteomic techniques. To that end, the candidate took part in studies of the effects of distinct dietary composition, different hatchery conditions, and continuous swimming, on the natural abundance of stable isotopes of nitrogen (^{15}N) and carbon (^{13}C) and the proteome of muscle tissues in gilthead sea bream (*Sparus aurata*).

The thesis presented here is structured in three chapters/papers:

Chapter I: M. Martin-Perez, J. Fernandez-Borras, A. Ibarz, O. Felip, R. Fontanillas, J. Gutierrez and J. Blasco. **Naturally occurring stable isotopes are a valuable tool for determining the optimum dietary requirements for gilthead sea bream (*Sparus aurata*) juveniles**. *Food Chemistry*, submitted.

Chapter II: M. Martin-Perez, J. Fernandez-Borras, A. Ibarz, O. Felip, J. Gutierrez and J. Blasco. **Stable Isotope Analysis Combined with Metabolic Indices Discriminates between Gilthead Sea Bream (*Sparus aurata*) Fingerlings Produced in Various Hatcheries**. *Journal of Agricultural and Food Chemistry* **2011,** *59* (18), 10261-10270

Chapter III: M. Martin-Perez, J. Fernandez-Borras, A. Ibarz, A. Millan-Cubillo, O. Felip, E. Oliveira and J. Blasco. **New insights into fish swimming: a proteomic and isotopic approach in gilthead sea bream**. *Journal of Proteome Research* **2012**, *11*(7), 3533-3547

During the course of the candidate work on this thesis, a review of the physiological consequences of swimming performance on the proteome of muscle in fish was published as a book chapter:

J.V. Planas, M. Martín-Pérez, L.J. Magnoni, J. Blasco, A. Ibarz, J. Fernandez-Borras, A.P. Palstra. **Transcriptomic and proteomic response of skeletal muscle to swimming-induced exercise in fish**. In Palstra, Arjan P.; & Planas, Josep V. (Eds.), *Swimming Physiology of Fish* **2012**, Chapter 11. ISBN 978-3-642

The results contained in this thesis have also been presented at different scientific meetings and conferences as posters or oral presentations: I Simposi d´Aqüicultura de Catalunya, Barcelona, Spain (February 2008); II Simposi d´Aqüicultura de Catalunya, Sant Carles de la Ràpita, Spain (October 2009); XII National Spanish Conference on Aquaculture, Madrid, Spain (November 2009); FitFish - Workshop on the Swimming Physiology of Fish, Barcelona, Spain (July 2010); 9th International Congress on the Biology of Fish, Barcelona, Spain (July 2010); and, 5th ESS – "Proteomic Basics", Brixen, Italy (August 2011).

In addition, a six-month stay in a foreign research institution was carried out during the preparation of this thesis at the Proteomics and Functional Genomics Research Group (PFG) in the Faculty of Veterinary Science at the University of Liverpool, Liverpool, United Kingdom, under the supervision of Dr. Phillip Whitfield, Dr. Mary Doherty and Dr. Iain Young. During that period, the candidate developed expertise in the design and execution of SILAC (Stable Isotopes Labelling by Aminoacids in Cell culture) experiments aimed at the analysis of protein turnover in human cell lines and fish muscle cells. The candidate also gained considerable competency in cell culture handling, LC-MS/MS analysis (nanoUPLC-LTQ Orbitrap system) and the bioinformatic processing of large quantitative proteomic datasets This stay was supported by a travel grant from the Generalitat de Catalunya (BE Program 2010)

Miguel Martín was funded by a pre-doctoral fellowship from the 2007 FI Program (Generalitat de Catalunya). The author of this thesis also acknowledges the financial support of XRAq (Generalitat de Catalunya) and the consolidated research group of the Generalitat de Catalunya (SGR-2009).

abbreviations

ABBREVIATIONS

2D-PAGE: two-dimensional polyacrylamide gel electrophoresis

AC: Atlantic coast

ADN: ácido desoxirribonucleico

ANOVA: analysis of variance

ARN: ácido ribonucleico

CC: Cantabric coast

CF: condition factor

COX: cytochrome-C-oxidase

CS: citrate synthase

DAA: dispensable amino acid

DIGE: difference gel electrophoresis

DM: dry matter

DNA: deoxyribonucleic acid

EA: elemental analyzer

ESI-QTOF: electrospray ionization – quadrupole time-of-flight

ESTs: expressed sequence tags

FAO: Food and Agriculture Organization

FE: feed efficiency

GO: gene ontology

HIS: hepatosomatic index

IAA: indispensable amino acid

IGF: insulin-like growth factor

IRMS: isotopic ratio mass spectometry

LC: liquid chromatography

LP: lipid

MALDI: matrix-assisted laser desorption/ionization

MB: músculo blanco

MC: Mediterranean coast

MCPs: myogenic precursor cells

MFI: mesenteric fat index

MR: músculo rojo

MS: mass spectrometry

MSI: musculosomatic index

NCBI: National Center for Biotechnology Information

PBS: phosphate buffered saline

PCA: principal components analysis

PDB: pee dee belemnite

PER: protein efficiency ratio

PR: protein

PTMs: post-translational modifications

RM: red muscle

RNA: ribonucleic acid

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

SGR: specific growth rate

SIA: stable isotope analysis

TOF: time-of-flight

w.w.: wet weight

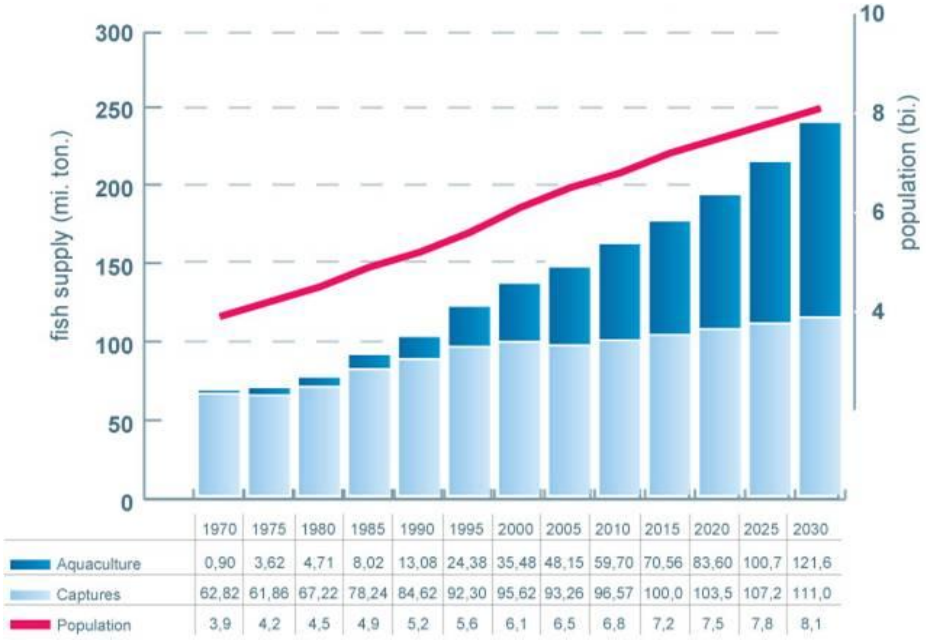
WM: white muscle

introduction

INTRODUCTION

1. Current status of Aquaculture

According to the Food and Agriculture Organization of the United Nations (FAO), aquaculture: "is understood to mean the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated". With the decline in wild fish stocks caused by over fishing, habitat loss and the increased demand for fish as a food source, the aquaculture industry has become an important component in the global economy, not only with regard to consumable goods but also as a means of stock replenishment through release programs.



	1970	1975	1980	1985	1990	1995	2000	2005	2010	2015	2020	2025	2030
Aquaculture	0,90	3,62	4,71	8,02	13,08	24,38	35,48	48,15	59,70	70,56	83,60	100,7	121,6
Captures	62,82	61,86	67,22	78,24	84,62	92,30	95,62	93,26	96,57	100,0	103,5	107,2	111,0
Population	3,9	4,2	4,5	4,9	5,2	5,6	6,1	6,5	6,8	7,2	7,5	7,8	8,1

Figure 1. FAO projection for world aquaculture and fisheries captures (FAO, 2010).

During recent decades global aquaculture production has grown rapidly, from 0.6 million metric tonnes (MMT) in 1950 to about 60 MMT in 2010 (**Figure 1**), being the food industry with the fastest growth worldwide. In addition to the use of aquaculture for livestock feed manufacture and other purposes, almost a half of all aquatic products currently consumed by the world's population come from this industry. Capture fisheries reached their maximum yield of about 90 MMT more than ten years ago, and the demand for seafood has shown no signs of abating (FAO, 2010). It is therefore clear that traditional fishery would be unsustainable to cover population requirements and that these should be fulfilled by the

aquaculture sector. The FAO anticipates that in 2030 about 65% of aquatic food consumed in the world will come from aquaculture. However, a gradual reduction in the growth rate of aquaculture production is noticeable, from annual increments of 9% in the 1980s and 1990s, to 6% in the first decade of the XXI century. Current projections for aquaculture indicate that previous growth is unlikely to be sustained in the future as a result of limits to the availability of wild marine fish for aquaculture feed (FAO, 2010). Small pelagic fish make up 37% of the total landings of marine capture fisheries. Of this, 90% (or 27% of total landings) are processed into fishmeal and fish oil, with the remaining 10% used directly for animal feed (Alder et al. 2008). Thus, it is necessary to find new ways to maintain the continuous development of aquaculture in a sustainable way, mitigating its environmental impact, and ensuring both the safety and quality of the products obtained.

1.1. Gilthead sea bream farming

Gilthead sea bream is a protandrous hermaphrodite species. It is common in the Mediterranean Sea, present along the Eastern Atlantic coasts from Great Britain to Senegal, and rare in the Black Sea. Until intensive rearing systems were developed during the 1980s, gilthead sea bream, like other sparids, was traditionally farmed extensively in coastal lagoons and saltwater ponds, because of the euryhaline and eurythermal habits of this species. Artificial breeding was successfully achieved from 1981-82 and large-scale production of gilthead sea bream juveniles was definitively achieved from 1988-89. The hatchery production and farming of this fish is one of the success stories of aquaculture. This species very quickly demonstrated high adaptability to intensive rearing conditions, both in ponds and cages, becoming a highly suitable species for aquaculture in the Mediterranean as a result of its good market price and high survival rate, reaching a marketable size (approx. 400 g) 18-24 months after hatching.

In production terms, the sea bream farming industry has been a spectacular success in less than two decades, equal to that of salmon farming. With a global aquaculture production of 139,925 tonnes in 2010 (**Figure 2**), gilthead sea bream (15.3%) is the third main fish-farmed species in the EU, after rainbow trout (*Onchorynchus mykiss*, 32.1%) and Atlantic salmon (*Salmo salar*, 23.3%) (FAO, 2010). Most production occurs in Mediterranean countries, with Greece (51.5%) being by far the largest producer in the region, followed by Turkey (15.0%) and Spain (14.6%). Although capture fisheries landings still occur and their volume remains relatively constant, fluctuating between 5000 and 8000 tonnes per year, farmed sea bream accounts for 95% of total production.

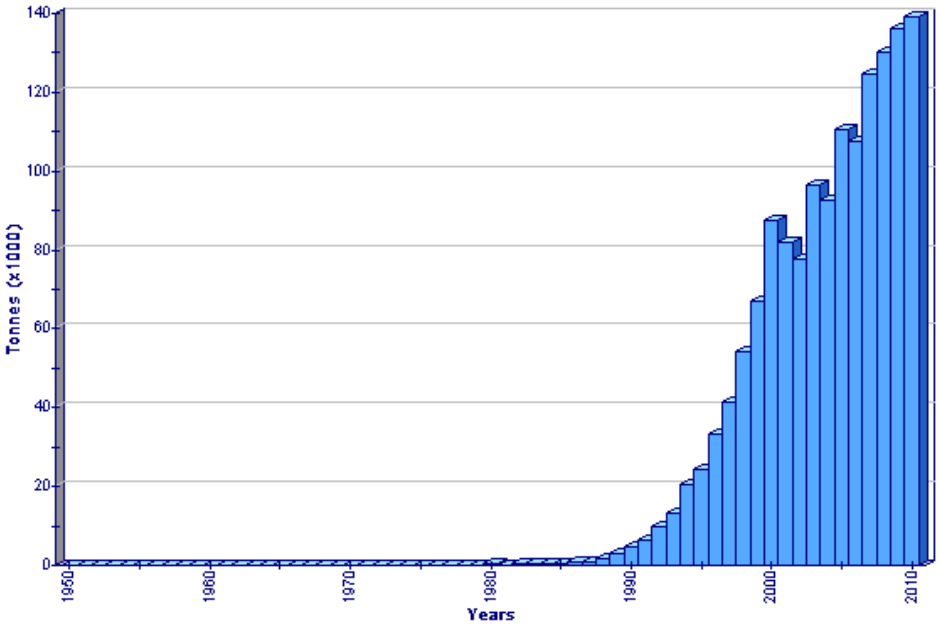


Figure 2. Global aquaculture production of *Sparus aurata* (FAO Fishery Statistic, 2010)

However, the farming of gilthead sea bream in the Mediterranean region is undergoing a transformation, moving from being an industry of high margins and low volumes to one of low margins and high volumes. The rapid development of production in sea cages has led to declining prices; the price of this species decreased by approximately 60% between 1990 and 2000 and continues to fall. In recent years, the price of farmed gilthead sea bream has collapsed; the European market price is currently fluctuating around 5.50 €/kg for the 350-g size category. Thus, the conventional market for sea bream has become saturated. For future growth, the Mediterranean aquaculture industry needs to adopt more sophisticated marketing methods. The development of alternative markets and value-added products is likely to lead to greater requirements for controlled and predictable quality and for larger fish. The sea bream industry could therefore be described as a sector that is entering its mature phase but that still requires more efficient production systems to increase fish growth or to reduce production costs.

2. Fish muscle

Skeletal muscle is the largest organ system in fish and corresponds to the edible part of the animal. The major function of this contractile tissue is swimming activity. Gram for gram, fish has more muscle than any other vertebrate, comprising about 40-60% of the total body weight

(Bone 1978). In gilthead sea bream of commercial sizes, muscle accounts for 35-48% and it is composed mainly of water (69-80%), protein (18-23%), and lipid (1-11%), although these values can vary according to lifecycle and lifestyle (reviewed in Grigorakis 2007). Excluding water, protein is the largest component in fish muscle, with a ratio between indispensable to dispensable amino acids close to one, and it is distributed into sarcoplasmic (10-30%), myofibrillar (70-90%) and stroma (3-10%) proteins (reviewed in Espe 2009).

2.1. Fish muscle anatomy and function

Compared to those of terrestrial animals, the muscles of fish are layered rather than bundled. Fish muscle is very simply constructed and lacks the tendinous system that connects muscle bundles to the skeleton present in other vertebrates. Instead, fish have muscle fibers running in parallel along the length axis and firmly attached to sheaths of connective tissue, which are anchored to the skeleton and the skin (Love 1970). Each segment or individual block of muscle is called a myomere or myotome, and it is separated from its neighbor by a thin sheet of connective tissue called myocommata or myoseptum (**Figure 3a**). Other sheets of connective tissue, called septum, occur along the vertical midline of the body, separating the muscles of the left and right sides of the body and horizontally separating the muscles of the upper (dorsal or epaxial) and lower (ventral or hypaxial) halves of the body (Bone 1978). The connective tissue in fish, unlike mammals, accounts for only a small percentage of the total muscle weight.

Along the body muscle, the myotomes run in an oblique pattern perpendicular to the long axis of the fish, from the skin to the spine, with the innermost edge nearer the front of the body (anterior or rostral) and the outermost edge nearer the tail (posterior or caudal), giving rise to the characteristic W-shape appearance in the longitudinal section (Van Leeuwen, 1999) (**Figure 3a and 3b**). Each myotome contains a superficial wedge-shaped region lying directly beneath the lateral line, where the muscle fibers run parallel to the body axis, and a deeper part where the muscle fibers are arranged in a helical fashion, forming angles of up to 40º to the longitudinal axis of the fish (Alexander, 1969). This anatomy enables all fibers across the body section to undergo similar strain during body bending, and it is ideally suited for the flexing muscle movements required to propel fish through water (Videler, 1993).

These muscle fibers are made up of many myofibrils. A cylindrical myofibril consists of thousands of microscopic units called sarcomeres, which are connected end-to-end (**Figure 3d**). The components of the sarcomere include thin and thick myofilaments, made of proteins called actin and myosin, respectively. These proteins or filaments are arranged in a characteristic alternating system bordered by Z-lines, which make the muscle appear striated upon

microscopic examination. When a nerve impulse reaches a skeletal muscle fiber, it causes a change in electric potential across the plasma membrane. Skeletal muscle fibers quickly convert this signal into depolarization through a rise in cytosolic Ca^{2+} from the sarcoplasmic reticulum and then initiate contraction. Myosin is a motor protein that interacts with actin filaments and couples ATP hydrolysis. When the Ca^{2+} concentration increases at the active enzyme site on the myosin filament, the enzyme ATP-ase is activated. This ATP-ase splits the ATP found between the actin and myosin filaments, thereby causing a release of energy. Most of this energy is used as contractile energy, making the actin filaments slide in between the myosin filaments in a telescopic fashion, contracting the muscle fiber. When the reaction is reversed (i.e., when the Ca^{2+} is pumped back, the contractile ATP-ase activity stops and the filaments are allowed to slip passively past each other), the muscle is relaxed. In skeletal muscle, contraction is regulated by four proteins associated with the actin filament: tropomyosin and troponins C, I and T. The cytolosic Ca^{2+} concentration influences the position of these proteins on the thin filaments, which in turn controls myosin-actin interaction (Purves et al. 2001).

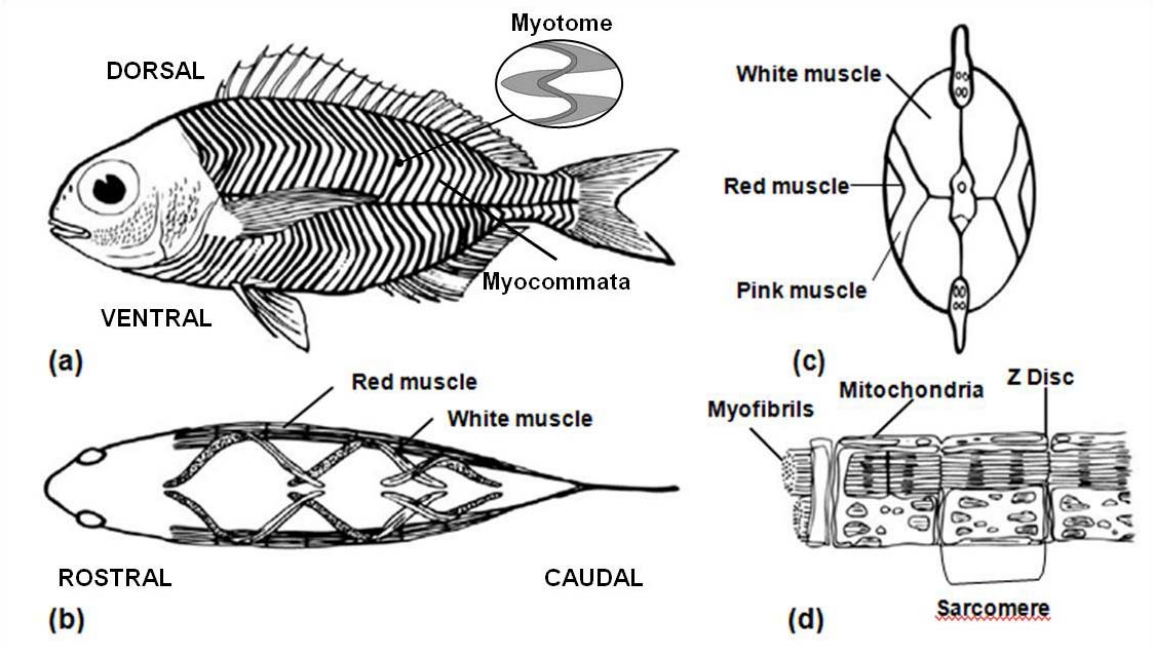


Figure 3: Myotomal muscle in fish. Myotomes are shown as they appear in the lateral **(a)** and dorsal view **(b)** of fish. A longitudinal band of slow-red muscle runs down the midline of each side of the fish (b). The fast-white muscle is visible above (epaxial) and below (hypaxial) the slow-red muscle band. When viewed transversely **(c)**, the lateral bands of slow-red muscle are "V" shaped, while the bulk of the myotome is fast-white muscle. In some fish species, an intermediate-pink muscle is observed between these layers. The muscle fibers are composed of smaller cylindrical units called myofibrils **(d),** which are made of many small structures called sarcomeres. Figures based on: (a) Bone 1978; (b) Rome et al. 1988; (c) Johnston et al. 1975; (d) Johnston 2001.

It is worth noting that despite the extreme organization of muscle, this tissue is not static, but its components are situated on a dynamic equilibrium, with constant coordinated alterations of protein synthesis and degradation, as well as assembly and maintenance (Clark et al. 2002).

2.2. Muscle types in fish

In contrast to mammalian muscle, which is characterized by a mosaic pattern of fiber types, the axial muscle of teleosts shows anatomically distinct zones of fiber types. The muscle fibers are separated in two discrete layers: subdermal slow-red muscle, with oxidative metabolism and slow contraction; and deep fast-white muscle, with glycolytic metabolism and fast contraction (**Figure 3c**). In several fish, intermediate-pink muscle located in the transitional zone between slow-red and fast-white muscle can be distinguished, with fibers characterized as rapidly contracting with intermediate resistance to fatigue and intermediate speed of shortening (Johnston et al. 1975; Bone 1978). The relative amount of intermediate-pink muscle differs between fish species and between their development stage (Sänger & Stoiber 2001) and shows features in-between those of slow-red and fast-white fibers, although their precise function is not clear (Johnston et al. 1977). Like the slow-red and fast-white muscle fibers, the intermediate-pink muscle fibers of most teleosts are multiply innervated (Bone 1978).

Fast-white fibers comprise the major part of fish skeletal muscle. These fibers are relatively large (50 -100 μm or even higher) compared to slow-red fibers (25-45 μm), which usually comprise around 10-30% of the myotomal muscle, depending on the locomotion mode (Sänger & Stoiber 2001). Active pelagic families have a higher proportion of slow fibers compared to demersal species, which are almost entirely composed of fast muscle (Johnston 1981).The ratio between slow-red and white-fast fibers increases from rostral to caudal regions to aid bending of the caudal fin (Bone 1978; Sänger & Stoiber 2001), although it depends upon lifestyle. Typically, compared with fast-white fibers, slow-red fibers have more capillaries and mitochondria and higher concentrations of lipid droplets and myoglobins, which endow its red color (Bone 1978). These features are reflected by the relatively high concentrations of respiratory and citric acid cycle enzymes in red muscle, required for energy generation during slow sustained aerobic swimming, which is fueled mostly by lipids and/or carbohydrates (Sänger & Stoiber 2001). Thus, glycogen and lipid content is also higher in this muscle type. In addition, red muscle is reported to exert functions similar to those found in the liver (Johnston 1981). In contrast, white muscle is generally used at high swimming speeds. However, in many teleost fish, electromyographic evidence indicates that both slow-red and fast-white muscle, specially the more superficial fast-white fibers, contribute to sustained intermediate speed swimming (Bone 1978), although this may not be the case for all species (Johnston et al. 1977;

Bone 1978; Johnston & Moon 1980a). Fast-white muscle is powered mainly by phosphagen hydrolysis, followed by the activation of anaerobic glycogenolysis, resulting in the accumulation of lactic acid, which is then transported to the liver for further metabolism (Sänger & Stoiber 2001).

3. Fish muscle growth

Fish growth is commonly determined by changes in body weight, body length or condition factor, which give an indirect but fast estimation of muscle growth. Muscle cellularity is frequently used as an indicator of changes in growth (Johnston 1999). Moreover, myosin isoforms are useful markers of muscle development in fish because they show development transitions characteristic of the distinct muscle fiber types (Veggetti et al. 1993; Johnston & Horne 1994; Mascarello et al. 1995). Biochemical indices are also valuable to understand muscle growth in fish, and given their capacity to predict fish growth potential, they are useful in studies relating to aquaculture production (Arndt et al. 1994). The relative contents or rates of synthesis of protein and nucleic acids in muscle are commonly used to estimate muscle growth. In general, the rate of (protein) growth is well correlated with the RNA concentration and with the RNA:DNA ratio (Grant 1996; Rowlerson & Veggetti 2001; Alami-Durante et al. 2007; Tanaka et al. 2007). Hypertrophic growth can be indirectly measured using protein:DNA ratios (Tanaka et al. 2007) whereas an increase in DNA concentration indicates a rise in hyperplastic growth (Valente et al. 1998).

Fish growth in many species is indeterminate, which means that fish, as opposed to higher vertebrates, never cease to grow. Most fish growth is invested in accretion of muscle tissue, particularly white muscle. This observation, together with the highest contribution to total body mass, low fractional rate of protein synthesis and highest growth rate efficiency of muscle, compared to other tissues, make increases in muscle mass an excellent overall measure of fish growth. It is known that post-larval muscle growth in fish involves both the enlargement of existing muscle fibers (hypertrophy) and the recruitment of new fibers (hyperplasia) (reviewed in Johnston 2001). This situation differs from that found in mammals and most terrestrial vertebrates, where the definitive number of muscle fibers is fixed at birth, leaving hypertrophy the main mechanism during the postnatal life (Goldspink 1972). Myogenic progenitor cells (MPCs), analogous to satellite cells in the adult mammalian muscle, are responsible for both mechanisms of muscle growth in teleost species (Fauconneau and Paboeuf 2001; Johnston 2001). The massive increase in size between hatching and maturity is achieved through the

hypertrophy of muscle fibers since hyperplasic growth declines gradually until the fish reaches a characteristic fraction of body size, after which further growth occurs only by hypertrophy (Johnston 1999). This observation was also made when studying gilthead sea bream (Rowlerson et al. 1995). However, rapid somatic growth is commonly associated with a higher rate of hyperplasia (Alami-Durante et al. 1997; Valente et al. 1999; Johnston et al. 2011). Thus, the plasticity of muscle growth under different production conditions is not only an interesting question in terms of biological mechanisms, but also of great practical relevance in aquaculture.

3.1. Intrinsic factors affecting muscle growth

Growth in fish and most vertebrates is largely controlled by growth hormones. A number of endocrine and paracrine factors affect muscle growth in fish, including growth hormone (GH), insulin-like growth factor I (IGF-I), thyroid hormone, and sex steroids (reviewed in Mommsen & Moon 2001). Previous studies found that supplementation of GH promotes hyperplasic growth in rainbow trout (Weatherley & Gill 1982), Atlantic salmon (Fauconneau et al. 1996), coho salmon (Hill et al. 2000) and Arctic charr (Pitkänen et al. 2000). Other studies addressing IGF expression under distinct physiological conditions support the notion that this growth factor is involved in fish muscle growth (Chauvigne et al. 2003; Peterson et al. 2004; Benedito-Palos et al. 2007; Montserrat et al. 2007a,b).

Individual genetic characteristics influence hormonal regulation, thereby potentially resulting in intra-specific variations in growth rate and improved "fitness" for aquaculture conditions. Strain differences within species have been noted in several fish families, thus evidencing genetic influences on muscle growth patterns. Valente et al. (1998) determined the RNA, DNA and protein concentrations in skeletal muscle of fast and slow growing strains of farmed juvenile rainbow trout. The higher DNA:protein ratio in the muscle of the fast growing strain suggested a smaller cell size. A subsequent morphometric study validated this biochemical result since smaller fast-white fiber diameters and a greater number of small fibers were observed in the fast-white muscle of this strain (Valente et al. 1999).

3.2. Extrinsic factors affecting muscle growth

Gilthead sea bream is found in marine habits that often show complex temporal-spatial variations in temperature, salinity, oxygen content, pH, light availability and water flow. Environmental inputs affect skeletal muscle physiology, resulting in phenotypic changes associated with locomotion, metabolism and growth. This muscle plasticity, which often

involves structural changes in cellular organelles or supporting structures such as capillaries, typically requires a period of time for a new steady state to be reached (Johnston 2006). During embryonic and larval stages, phenotypic changes in muscle are usually irreversible due to rapid ontogenetic development. In contrast, these changes are completely reversible following the establishment of the adult body plan. Factors controlling other energy-demanding processes will have a bearing on energy potentially allocated to growth, for example growth is slowed during breeding or even arrested when fish stop feeding. In addition, water quality parameters like pH, salinity, and oxygen availability may affect fish growth. For example, a decreased osmoregulatory demand has been shown to increase growth (Mommsen and Moon 2001). However, in this part we are concerned mainly with the factors affecting muscle growth and these will be further discussed in the following chapters of this Thesis.

3.2.1. Diet

As might be expected, diet is the major factor affecting fish somatic growth. The development and profitability of intensive fish farming largely depends on sufficient dietary inputs of all essential nutrients for optimum growth. Moreover, dietary factors such as feeding regime and diet composition not only influence muscle growth but also flesh quality (Johnston 1999). Muscle growth depends on a positive balance between protein synthesis and degradation since muscle proteins are continuously being replaced. In this regard, biochemical studies have identified increased net protein synthesis in muscle linked to a larger dietary intake (Houlihan et al. 1993; 1995), and low protein turnover as a strategy for efficient growth (Carter et al. 1998; Carter and Houlihan 2001). Atrophy of white muscle fibers occurs under conditions of severe nutritional restriction (Johnston 1981), and reduced ration size reduces muscle growth (reviewed in Johnston 1981; Rowlerson and Veggetti 2001).

Protein fraction is the main dietary component affecting fish growth. Traditionally, fish protein requirements have been considered to be higher than in other vertebrates. The greater protein fraction needed in fish diets seems to originate from fish preference to use amino acids as an energy source rather than carbohydrates or lipids (Bowen 1987). It is commonly accepted that high protein diets improve fish growth, especially in carnivorous species (Watanabe 2002). Optimal diet composition has been established for several aquaculture species at various life stages. In gilthead sea bream, optimum dietary protein requirement is estimated to be around 55% for fry (Vergara et al. 1996a) and 45-46% in larger fish (Santinha et al. 1996; Vergara et al. 1996b), denoting, as expected, a reduction of requirement with lifecycle. The source of protein, and especially its amino acid composition, has a particularly important effect on fish growth and the replacement of "*high quality*" fish components by cheaper alternative plant sources is

currently a hot topic in aquaculture research (Watanabe 2002; Tacon and Meitan 2008).

Other dietary components, such as lipids and carbohydrates, do not have as much impact as protein on fish growth; however, they must be included in the diet in proper proportions to cover the nutritional requirements of each species in order to achieve optimal growth. Nevertheless, lipids and carbohydrates can be used to reduce protein content in feeds while maintaining rapid growth rates. The so called "*protein sparing"* effect is achieved by increasing the energetic non-protein fraction, thereby reducing protein waste as an energy source and promoting dietary protein for muscle growth. Although both components can be used, most studies have opted to use lipids because of the difficulties encountered by many fish species to use high amounts of dietary carbohydrates (reviewed in Moon 2001), and because carnivorous fish, as the major farmed species, are generally able to use higher lipid levels than omnivorous or herbivorous species (Seiliez et al., 2006). In gilthead sea bream an optimum dietary lipid level of 15-16% was established (Vergara and Jauncey 1993; Vergara et al. 1996b). Further research reported that weight gain improved with an increase in dietary lipid content from 15 to 22%, although higher lipid levels are not advisable as they may lead to liver abnormalities (Vergara et al. 1999). Regarding carbohydrates, in general, a 20% content of dietary digestible carbohydrate appears optimal for growth in marine and coldwater fish, whereas higher levels could be used for freshwater or warm water species (Wilson 1994). This can be applied for carnivorous fish like gilthead sea bream, although it has been found to perform satisfactory with diets including up to 40% of wheat starch (Venou et al. 2003), probably because their natural diet mainly consists of mollusks, particularly mussels, which have high carbohydrate levels, and these fish can be accessorily herbivorous as well (Basurco et al. 2011).

In addition, the effects of diet on growth may be influenced by several culture conditions, such as temperature and photoperiod, which affect feeding behavior. Social dominance has also been found to interact with feeding behavior since during stress conditions, like overcrowding, fish usually stop eating with the consequent effects on muscle growth. For example, social dominance in Atlantic salmon is thought to separate fast- and slow-growing fish (Metcalfe et al. 1989). In contrast, gilthead sea bream kept in low numbers display social hierarchies, thus resulting in inefficient growth (Tort et al. 2011).

3.2.2. Temperature

Fish are poikilothermic animals and therefore temperature is one of the most important extrinsic factors that modulate growth. Increased temperature favors growth up to an optimal value, above which net growth falls (because of the cost of the increasing metabolic rate) before

reaching the survival limit (reviewed by Jobling 1997). The effects of increasing temperature on muscle growth at various life stages have also been described for several species. These effects include acclimation responses such as phenotype transformations of existing muscle fibers, effects on protein gain and retention efficiency, as well as alterations in muscle growth dynamics (reviewed by Johnston 1994; 2006). In temperate species, several weeks of cold-acclimation results in a shift to a more aerobic phenotype in all fiber types and is associated with an increase in the density of mitochondria and muscle capillary supply (Johnston 1982; Egginton and Sidell 1989). In juveniles and adults of some fish species such as common carp, which have a wide thermal range, changes in the temperature regime result in alterations in myofibrillar ATP-ase activity (Heap et al. 1985), expression of contractile protein isoforms (Crockford & Johnston 1990) and myosin heavy chain gene expression (Gerlach et al. 1990; Goldspink et al. 1992). Thermal imprinting during early stages of development affects somatic growth of muscle fibers in juvenile and adult fish, although the underlying mechanisms remain unknown (reviewed by Johnston et al. 2011).

3.2.3. Exercise

Fish can generally be easily forced to swim against a current, which makes them ideal subjects for exercise training. However, comparisons between studies are challenging due to differences in the duration and intensity of swimming training protocols. Additionally, the results are species-specific; relating to the life styles and swim styles of each species (Davison 1997; Johnston 2001). Swimming in fish is typically characterized as either aerobic or anaerobic (Beamish 1978). During sustained exercise, muscle metabolism is generally aerobic and is supported by the red musculature (Davison 1997). During events such as predator-prey interactions and spawning migrations, the capacity of red muscle is exceeded and burst-type exercise occurs. This burst-type action is supported largely by anaerobic glycolysis within white muscle (Beamish 1978; Kieffer 2000). This section deals only with sustained exercise, which is the main cause of exercise-related growth in fish muscle, and it will be further discussed in the following chapters of this thesis.

Numerous studies have shown that endurance training modifies muscle morphology, enzyme activity, and aerobic capacity in fish (Davison 1997; Kieffer 2000). It is generally assumed that at favorable swimming speeds ($\leq$ 1.5 body length per second), exercise training leads to increased growth, whereas training at substantially higher speeds has distinct negative effects on fish growth (Davison 1997). Although speeds vary among fish species and reflect differences in training methods, exercise has been shown to improve growth in many species (reviewed in Davison 1997; Palstra and Planas 2011). In the few studies where growth seemed unaffected or

retarded (Kiessling et al. 1994; Davison 1997; Bjornevik et al. 2003), the confined conditions of the experimental raceway may have stressed the fish, thus inhibiting growth. In general, endurance exercise commonly leads to an increase in cell diameter and number of aerobic fibers (Davison, 1997). The increase in fiber size and number leads to a greater proportion of total red muscle along with increased capillarization (Young and Cech Jr. 1993a; Sanger and Stoiber 2001). There is also some evidence of hypertrophy in white muscle (Davison 1997; Ibarz et al. 2011), whose oxidation potential may also be affected in some species, even at low speeds (Mommsen and Moon, 2001). Interestingly, in salmonids, these changes are not reported (Davison 1997; Sanger and Stoiber 2001). Particularly important for aquaculture fish destined for human consumption, exercise was found to increase flesh firmness in Atlantic salmon and sea bream and long-term exercise (9 months) did not affect flesh quality of farmed cod (Bjornevik et al., 2003). Changes in non-growth parameters, such as aerobic capacity, behavior, and internal body chemistry, also occur as a result of exercise training. In many salmonids and a few non-salmonid species, exercise increases feed conversion efficiency. On the whole, during exercise training, appetite is stimulated and the fish eat more. Although exercise may increase energy demand, a unit mass of growth is generally achieved at a faster rate while requiring less food as compared to unexercised fish (Davison 1997). This phenomenon was manifested in Arctic charr, where exercised fish experienced a decreased food ration with no negative effects on growth (Hammer 1995). Mommsen and Moon (2001) suggested that at least a part of the increased growth could be due to decreased aggressiveness, which reduces the dominance structure and with it feeding dominance, consequently increasing appetite. Particularly in subordinate individuals, swimming may cause a reduction of energy expenditure as a result of relief from chronic stress and a subsequent increase in growth rate (Yogata and Oku 2000). Reduced aggression during exercise has been observed in several studies, including salmonids (reviewed in Davison 1997), Arctic charr (Adams et al. 1995) and yellowtail (Yogata and Oku 2000). Moreover, schooling behavior has been shown to reduce chronic stress caused by farming conditions, thus increasing growth rates (Palstra and Planas 2011). This decreased stress could be related to lower cortisol levels reported in steelhead trout, Atlantic salmon, Arctic charr, and striped bass as a result of sustained exercise training (Young and Cech Jr. 1994b; Davison 1997; Herbert et al. 2011). In support of this notion, spontaneous swimming costs in gilthead sea bream are similar or even higher than those of moderate swimming (Steinhausen et al. 2010).

Much of the biochemical work done on swimming muscles in fish is somewhat contradictory but general trends are noted (Davison 1997; Kieffer 2000; Sanger and Stoiber 2001). Exercise training increases protein turnover in most tissues in rainbow trout (Houlihan and Laurent, 1987). Exercise also raises hematocrit, and consequently increases hemoglobin concentration,

thus increasing blood oxygen carrying capacity. When coupled with increased myoglobin in muscles, this enhanced capacity allows for better transport of oxygen in muscle tissue (Davison 1997; Sanger and Stoiber 2001). There is strong evidence supporting that endurance exercise training increases aerobic potential in red – and sometimes white – muscle as a result of improved tissue capillary supply, increased mitochondrial and lipid content, and enhanced enzyme activity (Sanger and Stoiber 2001). The enzymes predominantly affected are those involved in lipid metabolism, thereby suggesting that lipids provide the primary fuel for swimming (Davison 1997; Sanger and Stoiber 2001), although recent estimates of fuel utilization indicate that sustained swimming is maintained by the oxidation of approximately 45% carbohydrate, 35% lipid and 20% protein in the red muscle of rainbow trout (Richards et al. 2002). An increase in the glycolytic potential of white muscle suggests increased lactate production; along with the corresponding capillary supply, there is an enhanced ability to clear lactate (Sanger and Stoiber 2001). After exercise, rainbow trout show higher levels of enzymes associated with aerobic metabolism in red, white, and cardiac muscle, with high levels of 3-hydroxyacyl-coenzyme A dehydrogenase (HOAD) in white muscle, indicating a shift toward lipid metabolism (Sanger and Stoiber 2001). In striped bass, trained fish reached peak lactate levels later and lactate returned to resting values faster, with the improvement of the muscle buffering capacity resulting in a smaller decrease in pH (Davison 1997).

It is important to note that all these results present a general consensus for most species; however, there are contradictory results, particularly in regard to muscle morphology and biochemistry. Discrepancies between studies may be a result of the differences in training procedures (condition and duration of exercise), the use of species unaccustomed to aerobic swimming in the wild (benthic fish), and differences in a variety of environmental factors (Davison 1997; Kieffer 2000; Sanger and Stoiber 2001).

4. Application of stable isotope analysis in fish physiology studies.

Isotopes are atoms with the same number of protons and electrons but differing in the number of neutrons. Stable isotopes are defined as those that are not radioactive and are usually found in a very low abundance. The isotopes in biological research are dominated by the lighter elements, such as H, C, O, N, and S, because they are the most abundant in biological compounds. Among these, ^{13}C and ^{15}N are the most commonly used since they appear to be the most sensitive to changes in metabolic state (reviewed in Hatch 2012), accounting for 1.11 and 0.36% of the total C and N, respectively (**Table 1**).

Stable isotope composition is calculated as the ratio between the heavier and the lighter isotopes (i.e. $^{13}C/^{12}C$, $^{15}N/^{14}N$). Given the large difference in the proportion of distinct isotopes at natural abundance levels, these ratios are quite small. Therefore, stable isotope ratios are given in parts per thousand and are expressed in normalized delta (δ) notation. These delta values (e.g. $\delta^{13}C$ and $\delta^{15}N$) are the difference between the value of the sample and that of international standards, which are set at 0‰ by convention. A positive δ value indicates that the sample has more of the heavy isotope than the standard whereas a negative δ value denotes that the sample has less of the heavy isotope than the standard. The international standards used are air and Pee Dee Belemnite (PDB) for nitrogen and carbon, respectively. In the case of carbon, PDB is a mineral very rich in ^{13}C, therefore negative delta values result for most biological materials.

Table 1. Natural abundance of the most commonly used stable isotopes in biology

^{2}H	0.016%				
^{13}C	1.111%				
^{15}N	0.366%				
^{17}O	0.037%	**^{18}O**	0.204%		
^{33}S	0.750%	**^{34}S**	4.210%	**^{36}S**	0.014%

Heavy isotopes form more stable and stronger bonds (Hoefs 1980), leading to fractionations, or isotopic differences between the source and product compounds of a chemical transformation. Therefore, the abundance of stable isotopes varies among biological materials because of the faster enzymatic reaction rates on substrates containing lighter isotopic forms. Among biological compounds, lipids are depleted in ^{13}C because of decarboxylation processes during lipogenesis (De Niro and Epstein 1977). Moreover, deamination and transamination processes favor the mobilization of light isotopes (Gaebler et al. 1966; Macko et al. 1986; Hare et al. 1991), ^{14}N being preferentially excreted and ^{15}N retained in tissue (Steele and Daniel 1978). Overall, the characteristic isotopic composition of an organism results from the summation of feed ingested plus the fractionation occurring in metabolism. For these reasons, the higher the position of the organism in the trophic chain, the higher the proportion of the heavier isotope.

Since the late 1980s, the field of stable isotopes has expanded tremendously because of the development of isotope ratio mass spectrometry (IRMS). The natural abundance of stable isotopes can be examined to find patterns and mechanisms at the single organism level as well as to trace food webs, understand paleodiets, and follow nutrient cycling in both terrestrial and marine systems. Consequently, isotopic analysis has become a standard tool for physiologists

and ecologists. The application of stable isotope analysis (SIA) relies on the fact that the tissue of an organism strongly reflects the isotopic composition of its diet, as well as that of its environment, plus a discrimination factor (isotopic fractionation: $\Delta = \delta_{tissue} - \delta_{diet}$) (DeNiro and Epstein 1978; 1981). The isotopic fractionation varies depending on the tissue or element being studied, and also on differences in tissue composition and physiology between species and individuals (Post 2002; McCutchan et al. 2003; Vanderklift and Ponsard 2003). In aquatic systems, ^{13}C fractionation ($\Delta\delta^{13}C$) is assumed to be around 1‰ and may be caused by loss of $^{12}CO_2$ during respiration and by the uptake of ^{13}C compounds during digestion or biosynthesis (DeNiro and Epstein 1981; Tieszen 1983). Fractionation of ^{15}N ($\Delta\delta^{15}N$), in turn, results from the selective excretion of ^{15}N-depleted compounds, and it is estimated to be larger (around 3.2‰), representing the trophic level (DeNiro and Epstein 1981; Minagawa and Wada 1984).

Most of the previous assumptions are based on field studies and should be adopted with care since isotopic fractionation values in fish can vary widely depending on the species and tissue studied, and there are very few laboratory studies to support these assumptions (reviewed in Martinez del Rio et al. 2009). Thus, the effects of nutritional and environmental factors on isotopic fractionation remain poorly understood in fish. The main findings on this issue in fish studies are presented below. Trueman et al. (2005) demonstrated an inverse relationship between $\Delta\delta^{15}N$ and growth rate in salmon reared under controlled conditions and fed to satiety. The effects of feeding level have been studied in tilapia (Gaye-Siesseger et al. 2003) and carp (Gaye Siesseger et al. 2004a), showing in both cases an increase in $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$, along with an enrichment in ^{13}C of the lipid fraction as feeding level increases. Starvation also caused an increase in body $\Delta\delta^{15}N$ and ^{13}C content of lipids in tilapia (Gaye Siessger et al. 2007). However, Focken (2001) observed higher fractionation values for N and C isotopes at higher feeding rates in tilapia. The effects of dietary protein levels have also received mixed support. In tilapia fed isoenergetic and isolipidic diets varying in protein content, Gaye Siesseger et al. (2004b) observed that $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$ decreased with increasing protein accretion. Kelly and Martinez del Rio (2010), in turn, observed in the same species a positive relationship between dietary protein content and $\Delta\delta^{15}N$ when protein quality remained unchanged. Protein quality also affects isotopic fractionation by increasing $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$ in those fish fed diets with higher plant protein content. In this regard, SIA has been successfully used to differentiate between fish fed on wheat- (C3) or corn- (C4) based diets (Focken 2004), fish fed diets based on different proportions of plant protein (Beltran et al. 2009) and between farmed and wild specimens (Dempson and Power 2004; Bell et al. 2007). On the basis of differences in dietary protein sources, Molkentin et al. (2007), measuring $\delta^{15}N$ and $\delta^{13}C$ in raw fillets, were able to differentiate organic and commercially farmed salmon and their geographic origin. Furthermore, Sweeting et al. (2007a,b) assessed the effects of non-dietary parameters, such as

body mass, age and temperature, on the isotopic fractionation in several tissues of sea bass, showing specific tissue responses to these factors. The only study on the natural abundance of stable isotopes in gilthead sea bream was carried out by Beltran et al. (2009), showing greater values for ^{15}N and ^{13}C isotopes in tissues with higher protein and lower lipid content like muscle. These observations are in accordance with the enrichment in heavier isotopes of tissues that are less metabolically active (Pinnegar and Polounin 1999; Focken 2001).

As a summary of expected variations in the natural abundance of stable isotopes in animal tissues, Martinez del Rio et al. (2009) stated that: (i) differences in tissue isotopic incorporation rates (and hence in isotopic composition) seem to be explained by variations in protein turnover and growth rate; (ii) isotopic routing, explained as the different allocation of isotopically distinct dietary components to different tissues (Schwarcz 1991), profoundly influences the C isotopic composition of tissues; (iii) according to the following premises, $\Delta\delta^{15}N$ should: 1) decrease with increased dietary protein quality; 2) increase with dietary protein content; 3) decrease with the efficiency of nitrogen deposition, measured as the ratio between protein assimilation and protein loss; and 4) increase with fasting time; and (iv) inter-tissue differences in protein isotopic composition depend on the tissue amino acid profile and on the differences in isotopic content among amino acids.

Given the variability of results (inter- and intra-species, and even inter-tissue in the same individual), SIA might not serve as an index or diagnostic tool for detecting metabolic states under distinct physiological conditions; however, this technique might be indicative of these changes (Hatch 2012). Therefore, SIA is proving to be considerably more promising as a tool to study how physiological conditions affect the metabolic state and use of nutrients in fish. However, further laboratory studies are needed to test current assumptions, validate new ones, and further explore emerging applications of SIA, as claimed by several authors (Gannes et al. 1997; Martinez del Rio et al. 2009; Hatch, 2012).

5. Application of proteomics in fish physiology studies

Traditionally, most approaches used to study fish at the molecular level include biochemistry, and molecular biology, and more lately genomics techniques, such as microarray and gene expression analysis. In recent years, dramatic improvements in mass spectrometry (MS) have led to the emergence of proteomics as a powerful comparative tool for studying biological systems and their dynamics in a range of conditions. Thus this technology has been increasingly

used in fish biology research (reviewed in Parrington and Coward 2002; Piñeiro et al. 2003; Martyniuk and Denslow 2009; Martin 2009; Forné et al. 2010; Zhou et al. 2012; Rodrigues et al. 2012).

The concept of proteome, as the protein complement expressed by a genome, was established recently (Wasingern et al. 1995). Compared with the genome, the proteome varies with time, so, in general, proteomics is the study of the entire complement of proteins expressed spatially and temporally in an organism, including protein variants and post-translational modifications, and the characterization of protein-protein interactions (Martyniuk and Denslow 2009). Proteomic techniques expand the experimental focus from targeted proteins to a whole range of proteins at the same time and allow the assessment of changes in their relative abundance in response to a given environmental modification. Therefore, proteomics offers the opportunity to gain a far deeper understanding of the cellular and molecular mechanisms involved in fish physiological processes, since it allows global analysis of the molecular pathways underlying physiological responses and identification of novel proteins and fish homologs of known proteins shown to play key roles in other animal groups.

The molecular basis of physiological states can now be revealed at the protein level, thus complementing transcript and genomics data. Compared to genomics, proteomics can capture changes in protein activity, measured as post-translational modifications (PTMs), thus providing information about the regulation level of gene expression that is missed by the transcriptome. In this regard, there is some concern over the correlation between protein expression and mRNA (Pandey and Mann 2000). Proteomics, nevertheless, has its own limitations. The proteome magnitude is larger than that of the genome (ranging from 10000 to 150000 gene products depending on the organism); however, the proteome coverage compared to the genome remains comparatively small because of the extreme dynamic range of protein expression levels (up to 10000-fold), together with the low analytical sensitivity and the lack of an amplification method for proteins with a low abundance. Concerning fish proteomics, a major limiting factor is the lack of complete and annotated genome sequences, since the identification of proteins depends on database searches. At present, full genomes are available only for some model species, such as zebrafish, fugu, tetraodon, medaka, coelacanth and stickleback, although recently more research effort is being devoted to the genomes of commercial species (like tilapia, cod and salmon) (Zhou et al. 2012). For gilthead sea bream, the number of nucleotides (1473), expressed sequence tags or ESTs (74877) and protein (614) sequences available in public databases (NCBI, 2012) is relatively small compared to zebra fish or human (**Table 2**). However, increasing efforts are being made to boost these numbers (Perez-Sanchez et al. 2011; Garcia de la Serrana et al. 2012)

Proteomic strategies use a combination of efficient separation techniques, high resolution MS and powerful bioinformatic tools to characterize and quantify proteins from tissues. Quantitative proteomic approaches include gel-based (2D polyacrylamide gel electrophoresis: 2D-PAGE; and difference gel electrophoresis: DIGE) and non-gel based methods, which can be further separated into labeling and label-free approaches. Due to the complexity of the proteome, the separation of protein mixtures is important for identifying and quantifying proteins. 2D-PAGE, which combines the separation of proteins on the basis of their isoelectric point (by isoelectric focusing) and molecular weight (by SDS-PAGE), followed by identification of the proteins of interest by MS (Görg et al. 2000; 2004), is the most common strategy used nowadays for proteome analysis in fish (Martin, 2009) (**Figure 4**). The main advantage of gel-based approaches is that information on PTMs can be readily extracted. In contrast, gel-based methods can reveal hundreds or even thousands of proteins, which is low proteomic coverage compared with non-gel based methods. Gel-based methods have limited capacity to quantify proteins that are highly acidic/basic, have low/high molecular weight or are membrane-bound proteins and not easily solubilized (Martyniuk and Denslow 2009). A second obstacle to overcome is gel to gel variation during separation, which increases the difficulty in correlating spots across gels. DIGE improves this variation by providing a protein standard across all gels (Unlu et al. 1997). In addition, the co-migration of multiple proteins in overlapping spots and detection of low abundance proteins are also difficulties that remain to be tackled.

Table 2. Number of nucleotides, ESTs, and protein sequences available in NCBI (2012)

	Nucleotide	**ESTs**	**Protein**
Gilthead sea bream (*Sparus aurata*)	1473	74877	614
Zebra fish (*Danio rerio*)	126047	1488275	71657
Human (*Homo sapiens*)	9926198	8692723	674969

The application of proteomics in fish research studies is in its infancy, despite the large number of potential applications. Since the main focus of aquaculture is to produce fish with an optimal growth rate and health status, proteomics can shed light, from a physiological point of view, on the fish growth processes, which, in addition to genetic background, depend on other mutually interdependent processes, such as development, nutrition, metabolism and stress. Most proteomics research in farmed fish over the last decade has addressed various questions regarding welfare, nutrition, health, quality, and safety (reviewed in Parrington and Coward 2002; Martyniuk and Denslow 2009; Forné et al. 2010; Zhou et al. 2012; Rodrigues et al. 2012).

Relatively few proteomics studies conducted in sea bream have been reported. These have addressed very different issues, such oocyte maturation (Ziv et al. 2008), postmortem changes in muscle (Schiavone et al. 2008), salinity effects in heart (Varó et al. 2009), muscle characterization of wild and farmed specimens (Addis et al. 2010), effects of bacterial infection in kidney (Addis et al. 2010a), hormonal regulation in pituitary (Ibarz et al. 2010), liver response to chronic handling and crowding stress (Alves et al. 2010b), cold exposure (Ibarz et al. 2010), therapeutic treatments (Varó et al. 2010) and maslinic acid intake (Rufino-Palomares et al. 2011), and the effect of Cu exposure on serum (Isani et al. 2011).

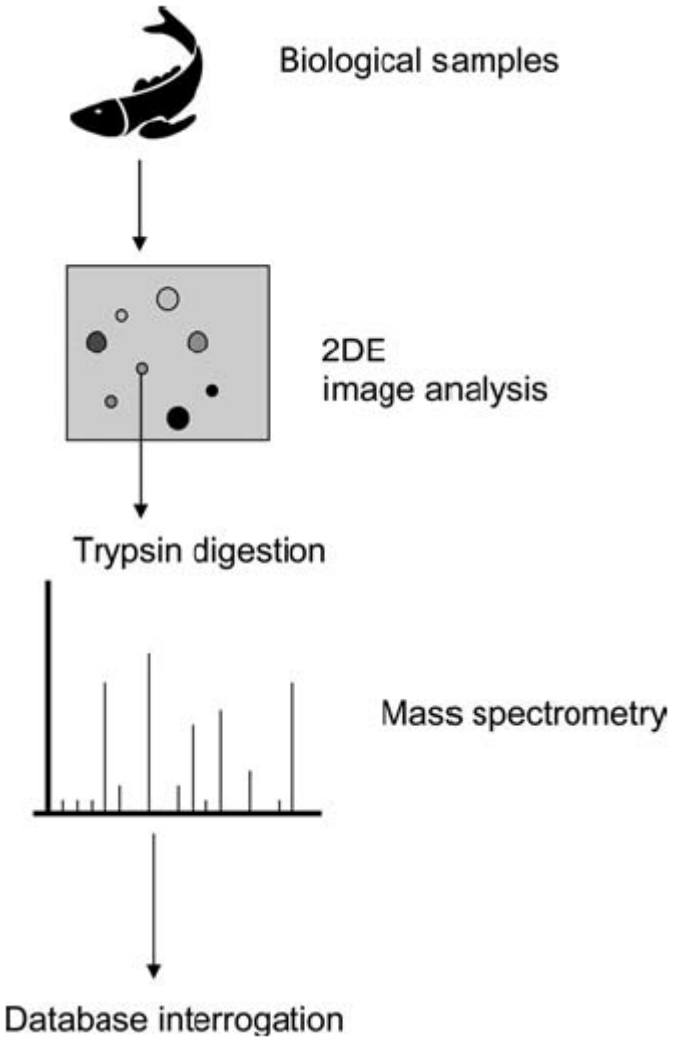


Figure 6.1. Flow diagram of the general approach taken for proteome analysis. Proteins are extracted from the biological samples followed by 2DE and gel image analysis. Protein spots of interest are excised from the gel and subjected to trypsin digestion and separation of peptides on mass spectrometer. Finally, the fragment sizes are used to search protein and nucleic acid databases. Figure from Martin (2009).

6. Future research trends in farmed fish species

In aquaculture, there is a pressing need for information relating growth mechanisms in muscle to other factors (e.g. culture conditions such as nutrition temperature and exercise) that influence overall growth performance. To date, the use of emerging tools, such as SIA and proteomics techniques, is still limited in fish biology. However, the studies performed until now have demonstrated the potential of these two approaches to reveal physiologically relevant mechanisms in response to environmental conditions. The capacity to combine SIA with other metabolic or proteomic techniques will provide a wider vision and greater understanding of fish physiology.

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objectives

OBJECTIVES

The general aim of this thesis is to increase knowledge about the physiological changes that occur in the muscle of gilthead sea bream (*Sparus aurata*), the most farmed fish in the Mediterranean area, when it is subjected to different rearing conditions. There is a need in the aquaculture sector for information relating the growth mechanism to other factors (e.g., conditions such as nutrition and exercise) that influence overall growth performance. This thesis combines emerging methods of stable isotope analysis and proteomic techniques with the study of several physiological parameters, such as details of the amino acid profile, the nucleic acid contents and metabolic enzyme activities, thus shedding light on the relationship between changes in natural occurring stable isotopes, protein expression and physiological status. Moreover, the potential use of stable isotopes as indicators or biomarkers of different physiological conditions is evaluated.

Considering the principal objective as given above, the following more specific objectives were established:

1. To evaluate the use of stable isotopes (^{15}N, ^{13}C) in determining optimal growing conditions for gilthead sea bream juveniles when fed diets with different protein-to-lipid ratio (**Chapter I**).

2. To analyze the potential of stable isotopes, combined with metabolic and growth parameters (DNA/RNA, COX/CS activities), as a discriminatory tool for determining the rearing conditions of gilthead sea bream fingerlings from different hatcheries (**Chapter II**).

3. To explore the molecular mechanism underlying muscle function of gilthead sea bream during moderate and sustained swimming by means of an integrative strategy combining metabolic parameters, isotopic analysis and proteomic techniques (2D-PAGE) (**Chapter III**).

objetivos

OBJETIVOS

El objetivo principal de esta tesis es aumentar el conocimiento acerca de los cambios fisiológicos que se producen en el músculo de dorada (*Sparus aurata*), la especie más cultivada en el entorno mediterráneo, cuando ésta es sometida a diferentes condiciones de cultivo. En el sector de la acuicultura, existe una necesidad de información que relacione los mecanismos de crecimiento con otros factores (por ejemplo, la nutrición y el ejercicio) que afectan al rendimiento general del crecimiento. Esta tesis combina métodos emergentes de análisis de isotopos estables y técnicas proteómicas con el estudio de distintos parámetros fisiológicos, como el perfil de aminoácidos, el contenido de ácidos nucleicos y la actividad de enzimas metabólicos, esclareciendo de este modo las relaciones entre los cambios en la abundancia natural de isótopos estables, la expresión de proteínas y el estado fisiológico. Además, se evalúa el uso potencial de los isótopos estables como indicadores o biomarcadores de diferentes condiciones fisiológicas.

Teniendo en cuenta el objetivo principal indicado anteriormente, se establecieron los siguientes objetivos específicos:

1. Evaluar el uso de isótopos estables (^{15}N, ^{13}C) para la determinación de condiciones óptimas de crecimiento en juveniles de dorada cuando son alimentados con diferentes proporciones de proteína y lípido en la dieta (**Capítulo I**).

2. Analizar el potencial de los isótopos estables, junto con otros parámetros metabólicos y de crecimiento (ADN / ARN y actividades COX / CS), como una herramienta discriminatoria para determinar las condiciones de cría de alevines de dorada de criaderos diferentes (**Capítulo II**).

3. Explorar el mecanismo molecular que subyace a la función muscular en la dorada durante la natación moderada y sostenida, a través de una estrategia integrativa que combina parámetros metabólicos, análisis isotópico, y técnicas proteómicas (2D-PAGE) (**Capítulo III**).

índice de impacto

ÍNDICE DE IMPACTO

La Dra. **Josefina Blasco Mínguez** y el Dr. **Jaume Fernàndez-Borràs**, como directores de la Tesis Doctoral titulada "Physiological changes in the muscle of gilthead sea bream induced by culture and feeding conditions: a stable isotopes (^{15}N and ^{13}C) and proteomic study" y realizada por **Miguel Martin Pérez,** manifiestan la veracidad del factor de impacto y la implicación del doctorando en cada uno de los artículos científicos que se presentan en esta tesis doctoral.

Artículo 1 (Chapter I)

Título: Natural-occurring stable isotopes as a valuable tool for determining the optimum dietary requirements in gilthead sea bream (*Sparus aurata*) juveniles.

Autores (p.o. de firma): M. Martin-Perez, J. Fernandez-Borras, A. Ibarz, O. Felip, R. Fontanillas, J. Gutierrez and J. Blasco.

Revista: *Food Chemistry* (en proceso de revisión editorial)

I.F. (2011): 3.655 (Food Science Technology 6/128: **Q1**) **5 Years I.F.** (2011): 4.268

Participación del doctorando: Miguel Martín ha obtenido las muestras y las ha analizado en su totalidad, colaborando en la interpretación de los resultados. Ha redactado la primera versión del manuscrito y colaborado activamente en el proceso de revisión por iguales.

Artículo 2 (Chapter II)

Título: Stable Isotope Analysis Combined with Metabolic Indices Discriminates between Gilthead Sea Bream (*Sparus aurata*) Fingerlings Produced in Various Hatcheries.

Autores (p.o. de firma): M. Martin-Perez, J. Fernandez-Borras, A. Ibarz, O. Felip, J. Gutierrez and J. Blasco.

Revista: *Journal of Agricultural and Food Chemistry* **2011,** 59 (18), 10261-10270

I.F. (2011): 2.823 (Food Science Technology 14/128: **Q1**) **5 Years I.F.** (2011): 3.239

Participación del doctorando: Miguel Martín ha participado en el diseño experimental, y ha realizado el trabajo experimental en cuanto obtención de muestras, análisis posterior e interpretación de los resultados. Ha redactado la primera versión del manuscrito y colaborado activamente en el proceso de revisión por iguales.

Artículo 3 (Chapter III)

Título: New insights into fish swimming: a proteomic and isotopic approach in gilthead sea bream.

Autores (p.o. de firma): M. Martin-Perez, J. Fernandez-Borras, A. Ibarz, A. Millan-Cubillo, O. Felip, E. de Oliveira and J. Blasco.

Revista: *Journal of Proteome Research* **2012**, 11(7), 3533-3547

I.F. (2011): 5.113 (Biochemical Research Methods 10/72: **Q1**) **5 Years I.F.** (2011): 5.413

Participación del doctorando: Miguel Martín ha participado en el diseño experimental, en la obtención de las muestras y en la realización de las técnicas descritas, generando e interpretando los resultados con especial énfasis en las técnicas de proteómica. Ha redactado la primera versión del manuscrito y colaborado activamente en el proceso de revisión por iguales.

Otras publicaciones adicionales que no forman parte de la tesis:

D. García de la Serrana, R. Fontanillas, W. Koppe, J. Fernández-Borràs, J. Blasco, M. Martín-Pérez, I. Navarro and J. Gutiérrez. **Effects of variable protein and lipid proportion in gilthead sea bream (*Sparus aurata*) diets on fillet structure and quality**. *Aquaculture Nutrition* **2012**, in press, DOI: 10.1111/j.1365-2095.2012.00966.x.

O. Felip, A. Ibarz, J. Fernández-Borràs, M. Beltrán, M. Martín-Pérez, JV. Planas and J. Blasco. **Tracing metabolic routes of dietary carbohydrate and protein in rainbow trout (*Oncorhynchus mykiss*) using stable isotopes ([^{13}C]starch and [^{15}N]protein): effects of gelatinisation of starches and sustained swimming**. *British Journal of Nutrition* **2012**, 107, 834-844.

A. Ibarz, O. Felip, J. Fernández-Borràs, M. Martín-Pérez, J. Blasco and J. Torrella. **Sustained swimming improves muscle growth and cellularity in gilthead sea bream**. *Journal of Comparative Physiology Part B* **2011**, *181*, 209-217.

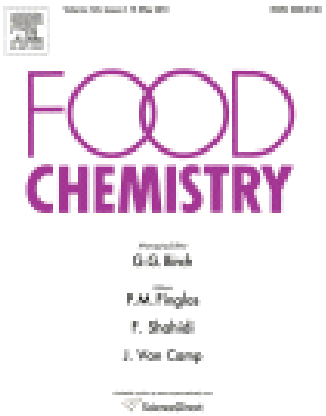
A. Ibarz, M. Martin-Perez , J. Blasco , D. Bellido , E. Oliveira, J. Fernández-Borràs. **Gilthead sea bream liver proteome altered at low temperatures by oxidative stress**. *Proteomics* **2010**, *10*, 1-13.

Josefina Blasco Mínguez Jaume Fernández-Borràs

publications

CHAPTER I

Naturally-occurring stable isotopes are a valuable tool for determining the optimum dietary protein-to-lipid ratio for gilthead sea bream (*Sparus aurata*) juveniles



Food Chemistry (submitted)

Naturally-occurring stable isotopes are a valuable tool for determining the optimum dietary protein-to-lipid ratio for gilthead sea bream (*Sparus aurata*) juveniles

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Running title

Optimal dietary requirements by stable isotopes analysis

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Abstract

There is currently much pressure to lower the protein content of farmed fish diets due to economic and environmental issues; however, apart from growth studies, there are no reliable methods available for determining optimum nutritional conditions for growing fish. Here we examine the potential use of stable isotopes (^{15}N and ^{13}C) to define the optimum dietary protein-to-lipid ratio for farmed gilthead sea bream juveniles. Muscle $\delta^{15}N$ increased as dietary protein-to-lipid ratio decreased. This increase was mainly a result of a higher protein turnover, but also partly due to changes in the muscle amino acid profile. Muscle $\delta^{13}C$, in turn, showed no differences. The inverse relationship observed between muscle ^{15}N-fractionation and growth rate indicates a higher protein gain in muscle as the dietary protein content increases, although no differences were found in either parameter over a dietary ratio of 44% protein / 20% lipid. PCA analysis of isotopic parameters also established discrimination at this point. These findings show that muscle isotopic composition, especially ^{15}N, which responds to changes in protein metabolism, allows optimum nutritional conditions for fish growth to be determined.

Keywords: stable isotopes, isotopic fractionation, white muscle amino acid composition, *Sparus aurata*, protein turnover, dietary components, optimal growth

1. INTRODUCTION

Fish growth is affected mainly by the dietary protein fraction, which represents the largest proportion of feed production costs (Tacon & Metian 2008). It is commonly accepted that high-protein diets improve fish growth, especially in carnivorous species (Watanabe 2002). However, such diets have several drawbacks with respect to the aquaculture industry. Protein is the most expensive component of fish feed and the by-products of protein catabolism are the most important source of nitrogen loading to waters. Moreover, there is an additional problem regarding the unreliability of the material used for fish meal production. One approach to minimizing feed costs and achieving a reduction in feed and metabolic wastes is to improve protein sparing by dietary inclusion of energy sources like lipids or carbohydrates.

Gilthead sea bream (*Sparus aurata*) is a major finfish species farmed in the Mediterranean area, and its production increases annually (FAO 2010). While the production of this species is a well-controlled process, knowledge of its nutritional requirements is still incomplete compared to other species like salmonids. Commercial diets for sparids like gilthead sea bream commonly include 20-23% lipids combined with 48-53% protein (Grigorakis 2007), although these proportions can vary during the lifecycle. However, many studies have focused on determining the minimum percentage of dietary protein required to obtain satisfactory growth rates and thus reduce feed production costs for this species. The optimum level of dietary protein was first determined as being around 40% for sea bream juveniles (Sabaut & Luquet 1973). Later, it was estimated to be 55% for fry (Vergara et al. 1996a) and 45-46% in larger fish (Vergara et al. 1993, Santinha et al. 1996; Vergara et al. 1996b), denoting, as expected, a reduction of requirement with increased body size. The efficiency of protein utilization for growth can be improved in sea bream by replacing dietary protein with non-protein energy sources, such as lipids (Vergara et al. 1996b; Santinha et al. 1999; Vergara et al. 1999; Company et al. 1999; Lupatsch et al. 2001; Morais et al. 2006) and carbohydrates (Marais and Kissil 1979; Meton et al. 1999; Venou et al. 2003; Fernández et al. 2007), thus producing a protein-sparing effect. This approach allows the protein content of diets to be reduced without seriously affecting growth rates.

Stable isotopes have been widely used in fishery sciences to authenticate and determine the geographical origin of food (Martínez et al. 2009; Sant'Ana et al. 2010; Martin-Perez et al. 2011), and to study the feeding ecology and movement of wild stocks (Moore and Semmens 2008; Carlisle et al. 2012). There is also growing use of stable isotopes in fish nutrition studies since the isotopic composition of animals reflects that of their diets plus a discrimination factor caused by the fractionation that occurs during chemical, physical, and biological processes (reviewed Gannes et al. 1998). Experimental studies have been performed mainly in larval

nutrition using stable isotopes as direct measures of feeding efficiency, nutrient incorporation and turnover (reviewed in Le Vay and Gamboa-Delgado 2011). However, few studies have been performed on larger fish, and these have addressed ecological issues. Several studies analyzed the effect of feeding level on tilapia (Focken 2001; Gaye-Siesseger et al. 2003; Gaye-Siesseger et al. 2007), carp (Gaye-Siesseger et al. 2004a) and sea bass (Barnes et al. 2007) and generally reported an inverse relationship between the $\delta^{15}N$ and $\delta^{13}C$ content of the fish and the amount of food ingested. Others, based on the quality of dietary sources, allowed isotopic discrimination between fish fed on animal or plant proteins (Gaye-Siesseger et al. 2003; Beltrán et al. 2009) and on C3 or C4 plant-derived ingredients (Focken 2004). Only two studies, both of tilapia, compared the effect of distinct dietary protein content on the stable isotope composition of fish. Gaye-Siesseger et al. (2004b) showed a decrease in N and C isotope fractionation in fish with a higher protein retention, whereas a recent study by Kelly and Martinez del Rio (2010) proposed a positive correlation between ^{15}N fractionation in tissues and protein intake. The generality of these results, which are conflicting, awaits further experimental work. Therefore, given that changes in the proportion of dietary components affect fish isotopic composition, we propose that isotope determination be considered a useful tool to ensure adequate levels of dietary components for fish growth. To date, little attention has been devoted to this issue. Optimum fish production via an optimal dietary balance is especially important for the aquaculture sector because of the high economic and environmental costs of the high-protein diets used.

Here, we evaluate the use of stable isotopes as a tool to determine the optimum dietary requirements for growing fish. In order to test their usefulness, we examined the variations in the natural abundance of stable isotopes in gilthead sea bream juveniles in response to changes in the protein-to-lipid ratio (i.e. the ratio protein/energy) of their diet. White muscle was selected as the target tissue for isotopic analyses ($\delta^{15}N$ and $\delta^{13}C$) since it reflects physiological changes in isotopic composition and is less variable than whole body (Martin-Perez et al. 2011) or other tissues that are metabolically more active, such as liver (Pinnegar and Polounin 1999; Focken 2001). In addition, we studied the muscle amino acid (AA) profile with the aim of ascertaining its potential implication in isotopic changes. Thus relationships between diet and muscle indispensible AA (IAA) profiles were established in order to detect AA imbalances between the experimental groups.

2. MATERIAL AND METHODS

2.1. Animal conditions and sampling

Gilthead sea breams (*Sparus aurata*), with a weight of around 70 g and a length of 14 cm, were kept in IRTA installations (Sant Carles de la Ràpita, Tarragona, Spain) under natural conditions of temperature, oxygen level, salinity and photoperiod throughout the study (May to July). The fish were initially adapted to the installation conditions over 2 weeks using a standard commercial fish feed. Following this period, they were randomly distributed into three 400-L fiberglass tanks (30 fish per tank) for each experimental diet (6 diets × 3 tanks = 18 tanks in total). The experimental diets were formulated by Skretting Aquaculture to be isoenergetic (22.8 kJ/g, calculated from gross composition: protein 24 kJ/g, lipid 39 kJ/g, carbohydrate 17 kJ/g) and to contain a gradual increase in the protein/lipid ratio from diet A (38/24) to diet G (53/19), as summarized in **Table 1**. The proportions of distinct protein sources were kept constant in the diets. Dietary pellets were extruded and the size (4 mm) was adequate for the corresponding fish weight. Fish were fed to satiation twice a day, 7 days a week for 12 weeks. Feed was automatically delivered for 1 h in each feeding session. Satiation was ensured by calculating estimated daily feed intake and allowing a ration 20% above this value. Feed delivery was recorded daily and uneaten feed was collected daily and then dried and weighed to calculate true feed intake. Total biomass from triplicate tanks was recorded in order to evaluate weight gain during the experimental period and used to calculate feed efficiency (FE = weight gain/feed intake as dry matter). To monitor growth, the fish from each tank were individually weighed and length was measured at the beginning, in the middle and at the end of the trial. Specific growth rates (SGRs) were calculated as follows: SGR (% day^{-1}) = 100 × (lnW_2 – lnW_1)/ t; where W_1 and W_2 are the initial and final weight of animals respectively, after t days.

After the experimental period, the fish were fasted for 24 h before sampling. Nine fish (3 per tank) were captured at random from each condition and anesthetized with 2-phenoxyethanol (100 ppm) diluted in seawater. Fish weight and size were measured. The animals were then killed by cutting the spinal column and eviscerated to measure mesenteric fat and liver weight. Epaxial white skeletal muscle (under the dorsal fin and above the lateral line) was dissected and frozen immediately in liquid nitrogen before storing at -80ºC until analysis. The experiments complied with the Guidelines of the European Union Council (86/609/EU), the Spanish Government (RD 1201/2005), and the University of Barcelona (Spain) for the use of laboratory animals.

Table 1. Formulation and chemical composition of the experimental diets fed to gilthead sea bream.

Diets (%protein/%lipid)	A (38/24)	B (41/23)	C (44/22)	D (47/21)	E (50/20)	F (53/19)
			Raw Material (g / kg)			
Wheat[1]	325	292	259	225	192	158
Wheat gluten[2]	107	117	128	139	149	160
Soybean concentrate[3]	107	117	128	139	149	160
Fish meal[4]	267	294	320	347	373	400
Fishoil Nordic[5]	190	176	162	147	133	119
Vitamin premix[6]	2.6	2.6	2.6	2.6	2.6	2.6
			Nutrient Analysis (g / kg w.w)			
Moisture	79	78	77	77	76	75
Crude protein	380	410	440	470	500	530
Crude fat	242	231	219	208	197	186
NFE[7]	252	234	217	199	181	163
Ash	47	51	55	58	62	65
Crude energy (MJ / kg DM)[8]	23.3	23.3	22.9	23	22.7	22.9
			Amino Acid analysis (g / 100 g AA pool)			
Arginine	5.3	5.4	5.4	5.4	5.5	5.6
Cysteine	0.7	0.8	0.8	0.8	0.8	0.8
Histidine	2.1	2.1	2.2	2	2	2.1
Isoleucine	2.9	3	3	3	3	3
Leucine	7.3	7.5	7.5	7.4	7.4	7.5
Lysine	6.8	6.7	6.7	6.7	6.7	6.8
Methionine	2.9	2.9	3	3	3.1	3.2
Phenylalanine	4.5	4.8	4.7	4.7	4.6	4.6
Threonine	3.5	3.6	3.6	3.6	3.8	3.8
Tyrosine	2.6	2.6	2.7	2.7	2.7	2.7
Valine	3.3	3.3	3.3	3.7	3.3	3.3
Alanine	5.3	5.3	5.3	5.6	5.4	5.5
Asparagine(Asn+Asp)	8.7	8.8	8.7	9	9.1	9.2
Glutamine (Gln + Glu)	25.3	25.1	24.8	24.5	24.6	24.1
Glycine	5.4	5.3	5.3	5.3	5.3	5.4
Proline	7.6	7.6	7.5	7.3	7.3	7.1
Serine	5.1	5.3	5.2	5.2	5.3	5.4
Total IAA[9]	42.2	42.7	42.9	43	42.9	43.2
Total DAA[10]	57.5	57.4	56.8	56.9	57	56.8
ratio *IAA* : DAA	0.74	0.74	0.76	0.76	0.75	0.76
			Isotopic Analysis (‰)[11]			
Raw $\delta^{13}C$	-25.86 ± 0.13 a	-25.50 ± 0.19 ab	-25.09 ± 0.18 bc	-24.95 ± 0.13 bc	-24.87 ± 0.27 c	-24.58 ± 0.29 c
Raw $\delta^{15}N$	6.42 ± 0.25	6.39 ± 0.48	6.69 ± 0.05	6.48 ± 0.39	6.48 ± 0.22	6.66 ± 0.05
Protein $\delta^{13}C$	-24.96 ± 0.01	-25.15 ± 0.07	-24.79 ± 0.21	-24.88 ± 0.30	-24.68 ± 0.09	-24.34 ± 0.04
Protein $\delta^{15}N$	6.43 ± 0.21	6.33 ± 0.08	6.71 ± 0.21	6.38 ± 0.12	6.30 ± 0.11	6.79 ± 0.19
Lipid $\delta^{13}C$	-26.95 ± 0.09	-27.00 ± 0.09	-26.99 ± 0.01	-27.18 ± 0.06	-27.22 ± 0.12	-27.31 ± 0.14

Skretting designed the experimental diets and also performed nutrient analysis (for captions see below)

Captions for **Table 1**:
Skretting Aquaculture designed the experimental diets and also performed nutrient analysis
[1]Statkorn, Norway (comp. in g/kg: moisture, 132; protein, 149; fat, 30; NFE, 650; ash, 17)
[2]Cerestar Scandinavia AS, Denmark (comp. in g/kg: moisture, 71; protein, 783; fat, 50; NFE, 80; ash, 8)
[3]Imcopa, Brazil (comp. in g/kg: moisture, 72; protein, 615; fat, 18; NFE, 299; ash, 59)
[4]Consortio, Peru (comp. in g/kg: moisture, 82; protein, 668; fat, 131; ash, 130)
[5] Nordsildmel, Norway (comp. in g/kg: fat, 1000)
[6]Proprietary formula of Skretting (Norway). Vitamin and mineral supplementation is estimated to cover requirements according to NRC, 1993
[7]NFE, Nitrogen Free Extract
[8]Analyzed values using a bomb calorimeter
[9]*IAA*, Indispensable Amino Acids
[10]DAA, Dispensable Amino Acids
[11]Values are expressed as Mean ± S.E.M (n=3). Letters indicate significant differences ($p<0.05$)

2.2. Muscle composition

Prior to analysis, muscle samples were ground in a mortar with liquid nitrogen. These samples were then separated in several fractions for composition (lipid, protein, glycogen and water), isotopic and AA measurements. Tissue water content was determined gravimetrically after drying the samples at 100ºC for 24 h. Glycogen was evaluated by a spectrophotometer using the anthrone method. Total lipid content was purified from two methanol–chloroform (2:1) extractions. The washed lipid extracts were dried under N_2 and the lipid content was determined gravimetrically. Protein was purified using defatted samples via precipitation with trifluoroacetic acid (100 ml L^{-1}) and after centrifugation at 1060 *g* for 30 min. The protein content was calculated from the nitrogen obtained by elemental analysis (Elemental Analyzer Flesh 1112, ThermoFinnigan, Bremen, Germany) using the coefficient 6.25.

2.3. $\delta^{15}N$ and $\delta^{13}C$ determination

Samples of diets and muscle tissue were lyophilized and ground into a homogenous powder for isotopic analysis. Aliquots (from 0.3 to 0.6 mg) from these samples, as well as their purified lipid and protein, were accurately weighed in small tin capsules (3·3 x 5 mm, Cromlab, Barcelona, Spain). Samples were analyzed to determine the carbon and nitrogen isotope composition using a Mat Delta C IRMS (Finnigan MAT, Bremen, Germany) coupled to Flash 1112 Elemental Analyzer at the *Serveis Científico-Tècnics* at the University of Barcelona. Isotope ratios (^{15}N /^{14}N, ^{13}C /^{12}C) are given in ‰, parts *per* thousand, on a δ-scale and refer to the deviation in measured ratio from the international accepted standards VPDB (Vienna Pee Dee Belemnite) for carbon and AIR for nitrogen. Delta values were determined as follows:

$$\delta = [(R_{sa}/R_{st}) - 1] \times 1000$$

where R_{sa} = ^{15}N /^{14}N or ^{13}C /^{12}C of samples and R_{st} = ^{15}N /^{14}N or ^{13}C /^{12}C of the international standards. The following standards certified by the International Atomic Energy Agency (IAEA, Vienna, Austria) were used: IAEA-N1 ($\delta^{15}N_{air}$=0.4‰), IAEA-NO-3 ($\delta^{15}N_{air}$=4.7‰) and IAEA-N2 ($\delta^{15}N_{air}$=20.3‰) for nitrogen; and LSVEC ($\delta^{13}C_{VPDB}$= -46.6 ‰), IAEA-CH-7 ($\delta^{13}C_{VPDB}$= -32.15 ‰), USGS40 ($\delta^{13}C_{VPDB}$= -26.4‰), IAEA-CH-6 ($\delta^{13}C_{VPDB}$= -10.45‰) for carbon. Every ten measurements, three standard samples were analyzed to compensate for machine drift and as a quality control measure. The same reference material examined over the analysis period was measured with ±0.2‰ precision. Nitrogen and carbon isotopic fractionation ($\Delta\delta^{15}N$ and $\Delta\delta^{13}C$) values for muscle and its reserves (protein and lipid) were calculated as the difference between δ tissue and δ diet for each component.

2.4. Amino acid analysis

Three fish for each dietary condition were randomly selected and muscle samples were taken from the epaxial area (under dorsal fin) for AA measurement. These samples were frozen in liquid nitrogen and then freeze-dried at -80ºC and weighed. Protein-bound AA samples were hydrolyzed in 6 M HCl at 108ºC over 24 h in nitrogen-flushed glass vials. We applied reversed-phase high pressure liquid chromatography (HPLC) in a Waters Pico-Tag AA analysis system, using norleucine as an internal standard. The resulting chromatograms were analyzed with Breeze software (Waters, USA). Results for tryptophan are not reported here since this AA is destroyed by acid hydrolysis. Glutamine is converted to glutamate during acid hydrolysis so these AAs are reported as their sum. The same occurs for asparagine and aspartate. IAA and dispensable AA (DAA) data are expressed in weight percentage of the total AA pool, i.e. (weight of one AA) × 100 / (weight of all AA), to allow comparisons between food and muscle AA profiles. Relationships between the two profiles were established for IAA.

2.5. Statistical analysis

Statistical differences between treatments were analyzed by nested one-way analysis of variance (ANOVA) with tank as random factor to test possible tank effect. When tank effect was not found, an ANOVA followed by Tukey's or Dunnett's posthoc test was used when variances were uniform or not, respectively. Statistical differences were considered significant when *p*-values were less than 0.05. The Shapiro-Wilk test was previously used to ensure the normal distribution of data, and the uniformity of variances was determined by the statistical Levene's

test. The relationships between muscle isotopic parameters and dietary protein content or SGR were tested using linear regression. Linear correlations between parameters were accepted as significant when the *p*-value was <0.05. The Pearson correlation index was calculated for all significant correlations found; significant correlations are indicated in all cases when found. Principal component analysis (PCA) was performed to study the structure of the isotopic data. Score plots from the PCA explore the main trends in the data, and their respective loading reveals variables with a significant loading. All statistical analyses were done using commercial software (PASW 17.0, SPSS Inc., Chicago, IL).

3. RESULTS

3.1. Animal growth and body parameters

Final body weight and SGR of gilthead sea bream juveniles rose as the dietary protein-to-lipid ratio increased, although no differences were found between groups fed above 44 % dietary protein (**Table 2**). Changes in body length followed the same pattern as body weight, so condition factor did not differ between groups. Feed efficiency was also very similar between groups, with the only difference being between groups B and F, although groups fed low protein/high lipid diets showed a slight decrease in feed intake. The amount of protein ingested increased from group A to F. No differences were observed for the mesenteric fat index (MFI) between groups, although group A, fed the highest lipid diet, presented the highest hepatosomatic index (HSI).

3.2. Proximal composition and amino acid profile of muscle

The proximal composition of white muscle did not show any changes between groups (**Table 3**). Regarding the AA composition of muscle, only the percentage of valine showed lower values in group B with respect to E and F, whereas higher glycine values were found in groups fed low protein diets (A, B and C) compared to those on high protein ones (D, E and F). To show dietary imbalances for a given AA, a comparison of the IAA profiles between muscle and diet for each experimental condition is given in **Figure 1**. These plots show no differences in the AA balance between experimental groups. This observation is attributed to the small variation in the AA profiles of the experimental diets. The most limiting IAAs in all diets were lysine and valine because they presented the highest difference between the AA content in muscle and that of diet.

Table 2. Body parameters and index in gilthead sea bream fed experimental diets for 12 weeks

Diets (%protein/%lipid)	A (38/24)	B (41/23)	C (44/22)	D (47/21)	E (50/20)	F (53/19)
initial body weight (g)	71.7 ± 2.28	72.0 ± 1.03	72.3 ± 0.85	72.5 ± 0.99	72.5 ± 1.19	71.4 ± 0.66
final body weight (g)	189.0 ± 1.4 ab	181.1 ± 6.2 a	200.3 ± 2.5 abc	204.4 ± 5.7 bc	216.8 ± 3.3 c	208.6 ± 3.2 c
final body length (cm)	21.9 ± 0.22 a	21.8 ± 0.17 a	22.5 ± 0.04 ab	22.7 ± 0.19 b	22.9 ± 0.13 b	22.9 ± 0.17 b
CF (%)[1]	1.00 ± 0.03	1.02 ± 0.01	0.98 ± 0.04	0.93 ± 0.02	0.97 ± 0.02	0.95 ± 0.01
weight gain (kg)[2]	3.45 ± 0.29 ab	3.2 ± 0.39 a	3.84 ± 0.13 abc	3.83 ± 0.25 abc	4.23 ± 0.16 c	4.07 ± 0.23 bc
SGR (%)[3]	1.14 ± 0.03 ab	1.08 ± 0.03 a	1.20 ± 0.02 bc	1.22 ± 0.03 bc	1.29 ± 0.02 c	1.26 ± 0.02 c
feed intake (kg)[4]	4.29 ± 0.10 ab	4.03 ± 0.17 a	4.45 ± 0.03 abc	4.42 ± 0.08 abc	4.84 ± 0.08 c	4.56 ± 0.13 bc
protein intake (kg)[5]	1.77 ±0.39 a	1.79 ± 0.76 a	2.12 ± 0.15 b	2.25 ± 0.40 b	2.62 ± 0.45 c	2.62 ± 0.76 c
FE[6]	0.80 ± 0.03 ab	0.80 ± 0.03 a	0.86 ± 0.01 ab	0.87 ± 0.02 ab	0.87 ± 0.01 ab	0.89 ± 0.01 b
PER[7]	1.95 ± 0.06 a	1.79 ± 0.07 ab	1.81 ± 0.02 ab	1.70 ± 0.04 bc	1.62 ± 0.01 bc	1.56 ± 0.02 c
HSI (%)[8]	1.79 ± 0.07 a	1.54 ± 0.07 ab	1.52 ± 0.09 ab	1.36 ± 0.07 b	1.41 ± 0.08 b	1.29 ± 0.03 b
MFI (%)[9]	1.43 ± 0.17	1.34 ± 0.17	1.46 ± 0.12	1.44 ± 0.14	1.62 ± 0.04	1.20 ± 0.14

Values are expressed as Mean ± S.E.M (n=3 tanks, except for HIS and MFI n=9 fish). Letters indicate significant differences (p<0.05).

[1]CF (Condition Factor) = (weight / size^3) × 100.

[2]In biomass per tank.

[3]SGR (Specific Growth Rate) = (ln initial weight - ln final weight) × 100 / days.

[4]Total feed intake = kg of feed consumed as DM / tank.

[5]Total protein intake = kg of protein consumed / tank.

[6]FE (Feed Efficiency) = weight gain / feed intake as DM.

[7]PER (Protein Efficiency Ratio) = weight gain / crude protein intake.

[8]HSI (Hepatosomatic Index) = (liver weight / animal weight) × 100.

[9]MFI (Mesenteric Fat Index) = (fat weight / animal weight) × 100

Table 3. Proximal and amino acid composition of white muscle of gilthead sea bream after receiving the expiremental diets.

Diets (%protein/%lipid)	A (38/24)	B (41/23)	C (44/22)	D (47/21)	E (50/20)	F (53/19)
			Proximal composition (g / 100g muscle)			
Moisture	76.31 ± 0.29	76.44 ± 0.31	76.59 ± 0.27	76.40 ± 0.13	76.03 ± 0.16	76.75 ± 0.28
Protein	20.80 ± 0.28	20.84 ± 0.25	20.85 ± 0.18	21.01 ± 0.20	21.16 ± 0.20	20.87 ± 0.30
Lipid	1.93 ± 0.15	1.84 ± 0.06	1.87 ± 0.13	1.82 ± 0.12	1.76 ± 0.17	1.67 ± 0.07
Glycogen	0.35 ± 0.04	0.42 ± 0.03	0.41 ± 0.04	0.39 ± 0.03	0.48 ± 0.03	0.46 ± 0.02
			Amino acid composition (g / 100 g AA pool)			
Arginine	6.87 ± 0.03	6.84 ± 0.07	6.79 ± 0.10	6.92 ± 0.05	6.81 ± 0.03	6.74 ± 0.01
Cysteine	0.43 ± 0.01	0.41 ± 0.01	0.56 ± 0.14	0.55 ± 0.06	0.50 ± 0.06	0.58 ± 0.06
Histidine	2.98 ± 0.02	2.96 ± 0.01	2.90 ± 0.06	2.98 ± 0.04	2.98 ± 0.02	2.98 ± 0.03
Isoleucine	4.60 ± 0.03	4.59 ± 0.05	4.48 ± 0.09	4.59 ± 0.01	4.66 ± 0.04	4.60 ± 0.00
Leucine	8.21 ± 0.05	8.22 ± 0.07	8.25 ± 0.04	8.37 ± 0.02	8.29 ± 0.03	8.32 ± 0.04
Lysine	10.28 ± 0.04	10.26 ± 0.09	10.28 ± 0.11	10.59 ± 0.13	10.57 ± 0.02	10.50 ± 0.04
Methionine	3.40 ± 0.05	3.36 ± 0.06	3.35 ± 0.08	3.40 ± 0.02	3.34 ± 0.03	3.30 ± 0.09
Phenylalanine	4.49 ± 0.02	4.47 ± 0.03	4.45 ± 0.05	4.49 ± 0.01	4.54 ± 0.02	4.50 ± 0.02
Threonine	4.99 ± 0.03	4.94 ± 0.06	4.91 ± 0.07	4.98 ± 0.06	4.93 ± 0.05	4.95 ± 0.02
Tyrosine	3.10 ± 0.01	3.11 ± 0.01	3.13 ± 0.06	3.19 ± 0.05	3.14 ± 0.02	3.12 ± 0.04
Valine	5.30 ± 0.03 ab	5.24 ± 0.03 a	5.27 ± 0.02 ab	5.27 ± 0.04 ab	5.40 ± 0.03 b	5.38 ± 0.02 b
Alanine	5.95 ± 0.09	5.96 ± 0.02	5.99 ± 0.04	5.94 ± 0.00	5.94 ± 0.03	6.03 ± 0.04
Asparagine (Asn + Asp)	10.42 ± 0.26	10.55 ± 0.27	10.47 ± 0.22	10.73 ± 0.29	10.62 ± 0.19	10.69 ± 0.16
Glutamine (Gln + Glu)	15.62 ± 0.04	15.79 ± 0.08	15.81 ± 0.05	15.81 ± 0.03	15.59 ± 0.06	15.60 ± 0.04
Glycine	5.16 ± 0.13 a	5.20 ± 0.02 a	5.21 ± 0.06 a	4.78 ± 0.11 b	4.61 ± 0.03 b	4.66 ± 0.05 b
Proline	3.46 ± 0.02	3.44 ± 0.08	3.50 ± 0.04	3.51 ± 0.07	3.51 ± 0.09	3.47 ± 0.07
Serine	4.74 ± 0.03	4.65 ± 0.05	4.64 ± 0.07	4.70 ± 0.08	4.58 ± 0.01	4.68 ± 0.01
Total IAA[1]	54.65 ± 0.07	54.40 ± 0.12	54.38 ± 0.32	55.05 ± 0.14	55.16 ± 0.17	54.88 ± 0.15
Total DAA[2]	45.35 ± 0.07	45.60 ± 0.12	45.62 ± 0.32	44.95 ± 0.14	44.84 ± 0.17	45.12 ± 0.15
ratio *IAA* : DAA	1.21 ± 0.01	1.19 ± 0.01	1.19 ± 0.02	1.22 ± 0.01	1.23 ± 0.01	1.22 ± 0.01

Values expressed as Mean ± S.E.M (n= 9 fish for proximal composition; n=3 fish for AA composition). Letters indicate significant differences ($p<0.05$). [1]*IAA* = Indispensable Amino Acid. [2]DAA = Dispensable Amino Acid.

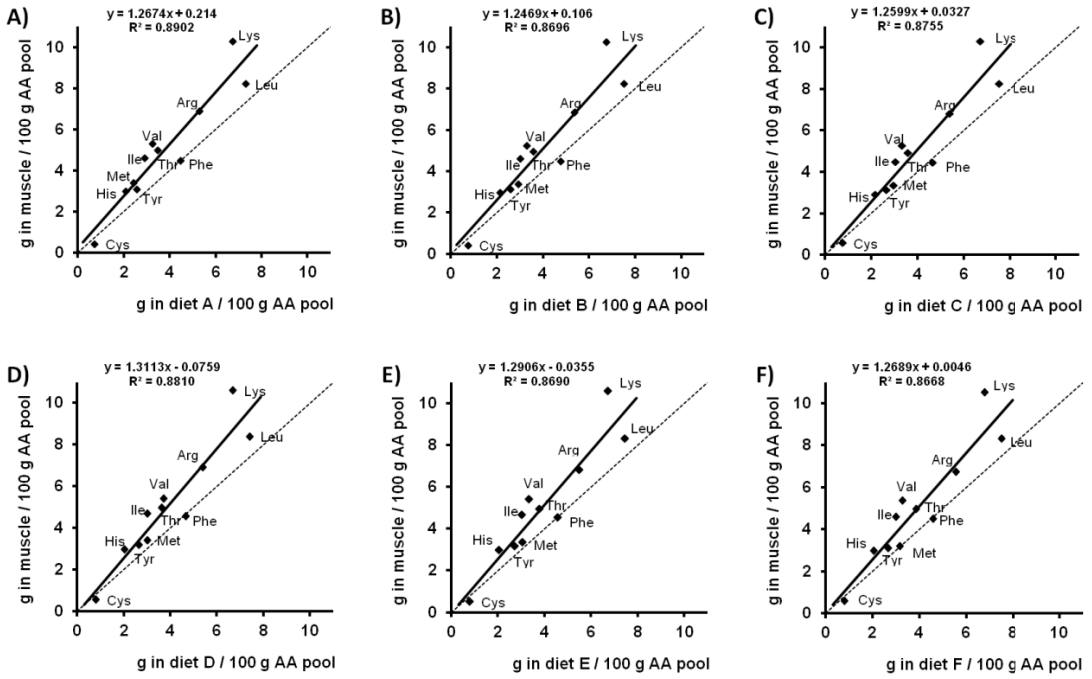

Figure 1. Comparison between IAA profiles of muscle from gilthead sea bream juveniles and the experimental diets (A, diet A; B, diet B; C, diet C; D, diet D; E, diet E; F, diet F). Linear regression (bold line) corresponds to the observed relationship which, can be compared with the line of equality (dotted line). Points above line of equality suggest restrictions for that AA in the food. Each point represents a mean of three fish.

3.3. Muscle isotopic composition

The relationships between the dietary protein content and the isotopic composition of muscle tissue and muscle fractions (protein, lipid and glycogen), and the isotopic fractionation, i.e. the isotopic difference between delta values of diet and tissue ($\Delta\delta = \delta_{muscle} - \delta_{diet}$), are shown in **Figure 2**. Fish fed high protein diets presented lower $\delta^{15}N$ in muscle tissue and in the muscle protein fraction, both parameters showing an inverse relationship with the dietary protein content. In contrast, $\delta^{13}C$ values showed no differences in muscle tissue or in the protein, lipid or glycogen fractions of muscle. Since the $\delta^{15}N$ values of raw diets and the dietary protein fraction did not change, as indicated by the observation that the calculated proportion of plant over animal protein was practically equal among diets (ranging from 0.92 to 1.10), the ^{15}N fractionation ($\Delta\delta^{15}N$) of muscle tissue and muscle protein fraction followed the same pattern as their counterparts for N isotopic composition, even though there were no differences between the groups fed over 44% protein. A similar correlation was found for protein-$\Delta\delta^{13}C$ of muscle, showing a slope almost identical to that of protein-$\Delta\delta^{15}N$ of muscle. The $\Delta\delta^{13}C$ of muscle tissue also presented an inverse relationship with the dietary protein content but with a higher slope because of the decline in raw $\delta^{13}C$ of diets with increasing lipid content (**Table 1**). The lipid-

$\Delta\delta^{13}C$ of muscle, on his part, showed no trend. The muscle isotopic parameter with the best correlation factor ($\Delta\delta^{15}N$ of muscle tissue) was selected to relate growth (SGR), and showed an inverse relationship (**Figure 3**).

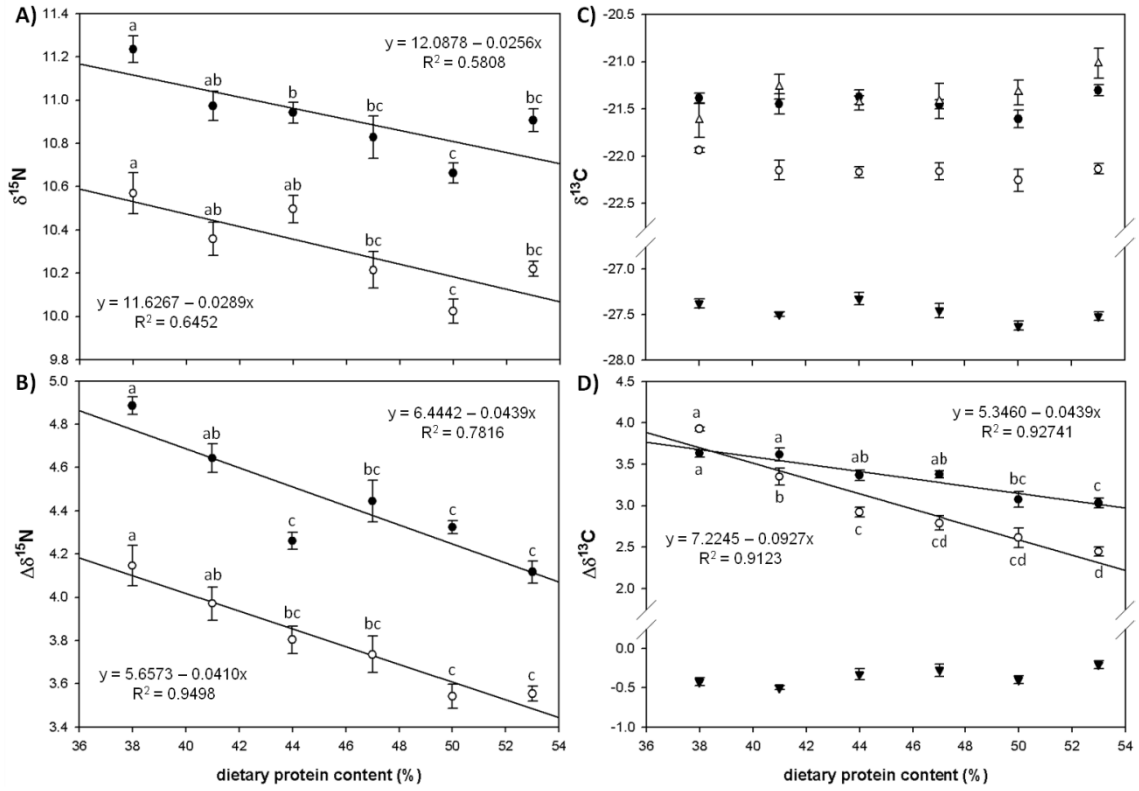

Figure 2. Relationship between dietary protein content and muscle isotopic δ values (A, $\delta^{15}N$; C, $\delta^{13}C$) or muscle isotopic fractionation (B, $\Delta\delta^{15}N$; D, $\Delta\delta^{13}C$). Values are represented as the mean and SEM (error bars) of nine fish (letters indicate significant differences, $p<0.05$). Open circles correspond to tissue values, filled circles to protein values, open triangles to glycogen values, and filled triangles to lipid values. Significant ($p<0.05$) linear regression equations are indicated.

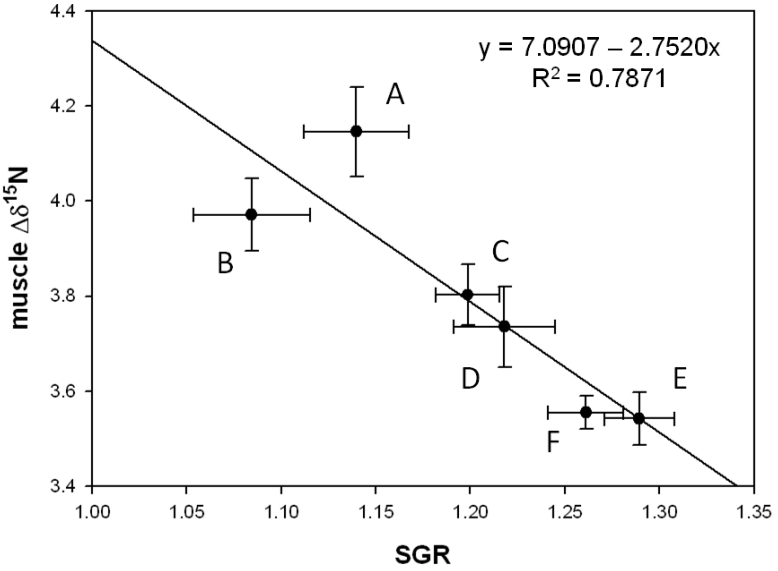

Figure 3. Relationship between SGR (n=3) and muscle $\Delta\delta^{15}N$ (n=9) of the six experimental groups ($p<0.01$). Each point represents the mean and SEM (error bars).

PCA analysis of muscle isotopic variables provided good discrimination between experimental groups (**Figure 4**), those fed low protein/high lipid diets (A and B groups) being clearly separated from the other groups, with factor 1 providing the greatest discrimination. The first three factors accounted for over 70 % of the variability in the data (**Table S1**). The variables with the highest load on the first factor (**Table S2**) were related mainly to the protein fraction (protein-$\delta^{15}N$, muscle-$\Delta\delta^{15}N$, protein-$\Delta\delta^{15}N$, muscle-$\Delta\delta^{13}C$, protein-$\Delta\delta^{13}C$ and muscle-$\delta^{15}N$).

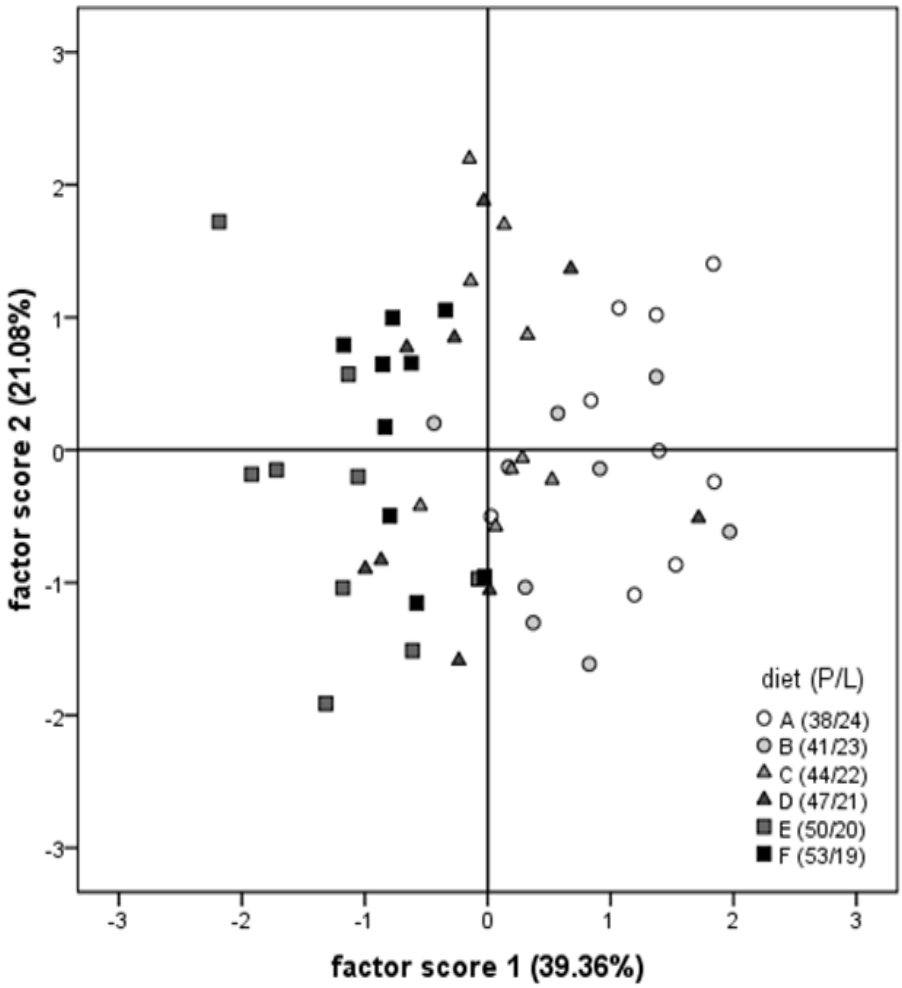

Figure 4. Principal Component Analysis (PCA) plot of muscle isotopic data from gilthead sea bream juveniles fed the experimental diets (diet A to diet F; P/L = protein / lipid content in %). Factors 1 and 2 represent the first and second principal components, with the percentage of explained variance indicated in parentheses.

4. DISCUSSION

All experimental groups were fed *ad libitum* to avoid dietary restrictions. However, fish fed on low protein/high lipid diets (groups A and B) showed lower growth. The negative effect of high lipid diets on growth has been previously reported in gilthead sea bream (Company et al. 1999; Lupatsch et al. 2001) and has been related to the ingestion of excessive energy, which reduces food consumption (Lupatsch et al. 2001). The total lipid content of the diet negatively affects food intake in rainbow trout juveniles (Yamamoto et al. 2002), possibly because of the negative feedback on appetite by the accumulation of lipids in depots (lipostatic control). Since the diets used in the present study were designed to be isoenergetic, the excess of energy would not cause the reduction in food intake observed in the groups on high lipid diets (especially in group B, which showed less hunger) but rather induce lipostatic control. Although no differences were found in MFI and liver composition was not analyzed in the present study, the increase in the HSI observed in fish on high lipid diets indicates fat deposition in liver. In this regard, steatosis in liver has been described in gilthead sea bream fed diets containing 22% lipid or higher (Caballero et al. 1999; Santinha et al. 1999), with the negative physiological consequences involved (Ibarz et al. 2010). Feed efficiency suggests that all our experimental diets had a similar degree of digestibility, although practical difficulties precluded the collection of feces and digestibility analysis. The formulation of the diets also supports the idea of similar digestibility because they all comprised the same raw materials and had a constant proportion of animal-to-plant protein. Previous studies in gilthead sea bream have demonstrated that high dietary wheat content, like in the present study, does not reduce the high digestibility of this cereal or affect the growth of this species (Venou et al. 2003).

Differences in the dietary protein-to-lipid ratio between experimental groups modified the muscle isotopic composition, especially $\delta^{15}N$, which is linked mainly to the protein fraction. Changes in muscle-$\delta^{15}N$ mirrored those in $\delta^{15}N$ of the protein fraction, the latter values being higher since lipid extraction during protein purification led to <1% isotope shift in $\delta^{15}N$ (Sotiropoulos et al. 2004). The inverse relationship found between muscle-$\delta^{15}N$ and the dietary protein content evidenced differences in nitrogen balance between experimental groups. Tissue enrichment in $\delta^{15}N$ has been observed in fish subjected to high protein turnover conditions, such as fasting or restricted protein intake (Gaye Siesseger et al. 2003, 2004a, 2007; Barnes et al. 2007), since ^{14}N is preferentially excreted during deamination and transamination while ^{15}N is retained in newly synthesized AAs and proteins (Gaebler et al. 1966; Macko et al. 1986, 1987; Hare et al. 1991). Therefore, we propose that the higher muscle-$\delta^{15}N$ values observed in fish fed low-protein diets in our study were caused by a higher protein turnover in muscle induced by a restriction in the balanced input of AAs in muscle. This restriction is reflected in the lower growth of these fish. Furthermore, differences in $\delta^{15}N$ between fish tissues can be explained by

variations in AA composition and by the isotopic content of individual AAs (Pinnegar and Polounin, 1999). Popp et al. (2007) classified the muscle AAs of yellowfin tuna in two groups: the relatively ^{15}N-enriched or 'trophic' AAs and the relatively ^{15}N-depleted or 'source' AAs. DAAs and IAAs can be found in both groups, although it appears that the dispensable and N-promiscuous AAs involved in the transport of nitrogen tend to be ^{15}N-enriched, while those that are essential and not easily transaminated tend to be relatively ^{15}N-depleted (reviewed in Martinez del Rio et al. 2009). Unfortunately, we were unable to measure the isotopic composition for each individual AA and therefore cannot provide major conclusions in this regard. However, despite the similar muscle AA profile observed between experimental groups, the higher content of dispensable glycine and the lower content of indispensable valine in muscle of fish fed low protein diets could partly explain the higher muscle-$\delta^{15}N$ values found in these animals. These results also indicate that fish on low protein diets have a higher AA turnover as a result of the lower dietary availability of AAs compared to fish on high protein diets. The experimental diets were formulated to present a similar AA balance, the AA profile being comparable to the optimum requirements calculated by Peres and Oliva-Teles (2009) for gilthead sea bream juveniles. Moreover, the muscle AA concentrations reported here are similar to those reported by Kaushik (1998) for whole body of immature gilthead sea bream.

The isotopic fractionation ($\Delta\delta = \delta_{tissue} - \delta_{diet}$), as the effect of all physiological processes that lead to differences between an animal and its diet, can vary in function of differences in tissue composition and physiology between individuals (Post 2002, McCutchan et al. 2003). The N and C isotope fractionation values found in our experiment were in accordance with previously reported values in gilthead sea bream (Beltran et al 2009). In the case of ^{15}N, fractionation values ($\Delta\delta^{15}N_{muscle\text{-}diet}$) reflected the differences observed in muscle-$\delta^{15}N$ between experimental groups, because diet-$\delta^{15}N$ values were equal among them. Martinez del Rio and Wolf (2005) predicted that $\Delta\delta^{15}N$ should increase with diet protein content and decrease with the efficiency of N deposition (measured as the ratio between protein assimilation and protein loss). The inverse relationship between $\Delta\delta^{15}N$ and dietary protein content observed in the present study seems to contradict this first prediction. In fact, this prediction has not been consistently supported empirically (reviewed in Martinez del Rio et al. 2009), because the variation in the magnitude of $\Delta^{15}N$ depends not only on the protein intake but also on protein quality and thus protein use efficiency. Recently, in tilapia fingerlings, Kelly and Martinez del Rio (2010) found a positive decelerating relationship between $\Delta\delta^{15}N$ of muscle and dietary protein content when protein quality remained constant. In that experiment, the dietary protein content of the groups (3.75, 7.5, 15 and 30 %) was below the optimum protein level (35%) for tilapia fry (Santiago et al. 1983). In contrast, the protein content of our experimental diets was around the optimum values for gilthead sea bream juveniles (Sabaut and Luquet 1973; Santinha et al. 1996; Vergara

et al. 1996). Therefore we postulate that the inverse relationship between muscle-$\Delta^{15}N$ and dietary protein content observed in our experiment was determined mainly by the efficiency of N deposition. This notion implies that protein accretion increases as ^{15}N fractionation decreases, since more dietary AAs are used directly for protein synthesis and hence the $\delta^{15}N$ values of the fish approach the isotopic values of the diet, as observed in carp (Gaye Siesseger et al. 2003) and tilapia (Gaye Siesseger et al. 2004a, 2004b). In support of this assumption, we found that the fish growth rate (as an indirect estimation of muscle growth and hence protein gain) was inversely related to muscle-$\Delta\delta^{15}N$. This observation has also been made in blue crabs (Fantle et al. 1999) and salmon (Trueman et al. 2005). Moreover, ^{15}N and ^{13}C fractionation of the protein fraction showed a similar behavior to muscle-$\Delta\delta^{15}N$, which strengthens this conclusion.

Regarding ^{13}C fractionation, the $\Delta\delta^{13}C$ values of muscle tissue also showed an inverse relationship with dietary protein content. However, unlike $\Delta\delta^{15}N$, this finding was essentially due to the increase in $\delta^{13}C$ of raw diets as dietary fat content decreased, since lipids are ^{13}C-depleted compared to other components (DeNiro and Epstein 1977). The $\delta^{13}C$ of muscle, in turn, showed no differences despite the dietary isotopic differences observed. This finding could be explained by the differential allocation of isotopically distinct dietary components (i.e. 'isotopic routing', Schwarcz 1991) and the similar lipid content observed in the muscle among the experimental groups. Moreover, unchanged values in $\delta^{13}C$ of muscle energy reserves (i.e. glycogen and lipid) would point to a similar use of them since their enhanced use would lead to isotopic enrichment of tissue reserves as observed in the white muscle of gilthead sea bream under exercise training (Martin-Perez et al. 2012).

The PCA plot shows that groups with lower growth (A and B) are clearly separated from the rest, protein-related isotopic variables (especially ^{15}N) being those with the highest load in the main discrimination factor. The border line seems to be in group C, fed a 44% protein / 22% lipid diet, proportions that are consistent with established optimum values of these components in gilthead sea bream (Vergara et al. 1996; Santinha et al. 1996, 1999; Caballero et al. 1999). From this proportion on, no differences were observed in either fish growth or ^{15}N fractionation of muscle. Therefore, $\Delta\delta^{15}N$ may indicate the state where catabolism and recycling of protein significantly increases in muscle due to the decrease in the amount of dietary protein. Beyond this threshold the dietary protein-to-lipid ratio is not enough to maintain maximal growth, although the protein efficiency ratio (expressed per unit of protein intake) increased as dietary protein content decreased. This is explained because the highest protein retention efficiency is generally achieved under restricted protein feeding, as previously shown in gilthead sea bream (Company et al. 1999). Therefore our results indicate that the protein sparing effect of dietary lipids could be primed by a slight decrease in the dietary protein-to-lipid ratio. In summary, the present data show that stable isotope analysis (especially $\Delta\delta^{15}N$) is a reliable indicator of

nutritional status in fish and therefore may be a valuable tool for the rapid and efficient determination of the optimal protein-to-lipid ratio in diets for farmed fish.

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Appendix

View supplementary Tables S1 and S2 for eigenvalues and factor loadings of PCA analysis, respectively (see Annex section).

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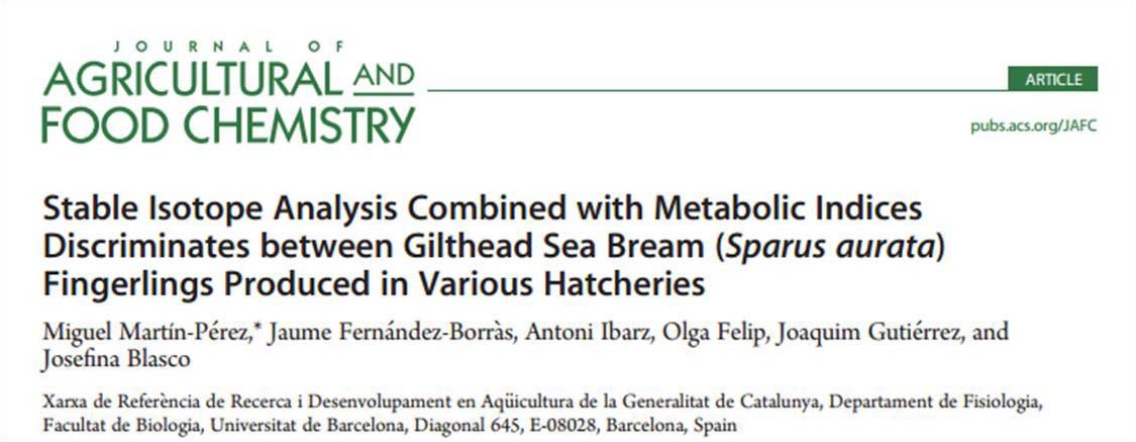
CHAPTER II

Stable isotope analysis combined with metabolic indices discriminates between gilthead sea bream (*Sparus aurata*) fingerlings produced in various hatcheries



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Stable isotope analysis combined with metabolic indices discriminates between gilthead sea bream (*Sparus aurata*) fingerlings produced in various hatcheries

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Running title:

Sea bream discrimination between hatcheries

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Abstract

There are few traceability systems other than genetic markers capable of distinguishing between sea products of different origin and quality. Here we address the potential of stable isotopes combined with metabolic and growth parameters as a discriminatory tool for the selection of fish seeds with high growth capacity. For this purpose, sea bream fingerlings produced in three hatcheries (Spanish Mediterranean coast, MC; Cantabrian coast, CC; and South-Iberian Atlantic coast, AC) were subjected to isotopic analysis ($\delta^{15}N$ and $\delta^{13}C$) and indices of growth (RNA, DNA) and energy metabolism (cytochrome-*c*-oxidase, COX, and citrate synthase, CS, activities) were calculated. These analyses were performed prior to and after a "homogenization" period of 35 days under identical rearing conditions. After this period, fingerlings were discriminated between hatcheries, with isotopic measures (especially $\delta^{15}N$), metabolic parameters (COX and CS) and proximal composition (fat content) in muscle providing the highest discriminatory capacity. Therefore particular rearing conditions and/or genetic divergence between hatcheries, affecting the growth capacity of fingerlings, are defined mainly by the isotopic imprint. Moreover, muscle isotopic signature is a more suitable indicator than whole fish for discrimination purposes.

Key words: Sea bream (*Sparus aurata*), $\delta^{13}C$/ $\delta^{15}N$, isotopic fractionation, RNA:DNA, citrate synthase, cytochrome-c-oxidase, growth capacity

1. INTRODUCTION

Gilthead sea bream (*Sparus aurata*) aquaculture was first introduced in the 1970s and it is now the Mediterranean species most widely farmed in this way. Given the market relevance of this relatively new field, the traceability of fishery and aquaculture products is becoming increasingly important. Recently, intense efforts have been devoted to developing analytical tools to distinguish between farmed fish and those caught in the wild. In particular, stable isotope analysis (SIA), often combined with fatty acid composition, has been used successfully to discriminate between cultured and wild gilthead sea bream (*1–3*) and in studies on marine fish species like salmon (*4*), sea bass (*5*) and turbot (*6*). The differences between farmed and wild fish presumably reflect variations in diet, which have a major influence on isotopic profiles because natural food and commercial diets differ in their isotopic ratios. In particular, the ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes, derived directly from the diet (*7, 8*), are extremely valuable when attempting to associate a specific animal with a particular food source, being often characteristics for production systems and feeding intensity. Furthermore, given their dependence on environmental water conditions, $\delta^{18}O$ and $\delta^{2}H$ are very useful for determining the geographical origin of fish.

Little information is available about the differentiation between fish from different farms on the basis of rearing systems or region. Muscle $\delta^{15}N$ is correlated with the geographical origin of farmed sea bream (*2*), but this relationship seems to be more related to the feed ingredients available in the farming areas than to the geographical origin itself. Stable isotope ^{15}N analysis also allows discrimination between fish fed an artificial diet formulated with plant protein and those receiving fish protein (*9*). It was recently reported that stable isotopes are the most informative variables in discriminating between fish from distinct farms (*10*). In particular, measures of $\delta^{13}C$ or $\delta^{15}N$ in flesh can be linked with those of a specific commercial diet, while $\delta^{18}O$ can be used to associate fish with a specific water source. Thus, the combined information from these isotopes allows discrimination between fish origins. In addition, tissue fatty acid and proximate compositions, and several morphological parameters are useful to distinguish between farmed and wild fish (*1*); however, these parameters are less informative for discriminating fish origin (*10*).

The quality of fish produced by aquaculture varies greatly between farms and is influenced mainly by the quality of the rearing environment, the quantity, quality and management of feed, and the culture methods used (*11, 12*). Similarly, variability in fish isotopic parameters is related to environmental factors and culture conditions, such as temperature, meal composition and ration (*13, 14*). Therefore, while it is now possible to have a high degree of confidence in defining whether fish are wild or farmed, it is not as straightforward to determine their

geographical origin or growth potential. There is an increasing need to develop analytical methods to discriminate between fish reared in distinct systems in different locations. Furthermore, the capacity to discriminate between fish 'seeds' on the basis of growth performance is highly relevant for the aquaculture industry. Recent studies show that isotopic fractionation of ^{15}N varies inversely with growth in salmon (*15*) and that increases in ^{15}N are related to stress conditions, such as fasting (*16*) and low protein diets (*13*). Therefore, stable isotope analysis combined with other complementary physiological variables that are well established as metabolic indicators, such as nucleic acid content and enzyme activities may be a useful approach to determine the quality of fish seeds. The RNA:DNA ratio (the amount of RNA per cell unit) is indicative of the status of the cell transcription rate, whereas the RNA:protein ratio is often used as an indicator of protein synthesis capacity (*17*). The aerobic capacity of fish muscle is estimated by the activities of mitochondrial enzymes, such as cytochrome-*c*-oxidase (COX) and citrate synthase (CS), and both activities have been positively correlated with growth rate in many fish species (*18, 19*).

Most of the studies dealing with fish 'origin' discrimination have focused on the isotopic analysis of fish and environmental factors (mainly food and water). However, no study has addressed the relationship between the isotopic composition of fish and metabolic and growth variables, an approach that goes beyond the discrimination of geographical origin.

Here we sought to discriminate gilthead sea bream fingerlings from distinct hatcheries on the basis of stable isotope analysis ($\delta\ ^{15}N$ and $\delta\ ^{13}C$) combined with metabolic (CS and COX activities) and growth parameter (RNA and DNA concentration) analyses in order to select the fish seed with the highest growth potential. To ensure that the rearing conditions (food composition and ration, temperature, etc.) did not interfere in the discrimination of the seeds with the highest growth potential, we analyzed these parameters before and after submitting fingerlings of the three farms to equal rearing conditions (homogenization period) for 35 days, a period long enough to circumvent the influence of these effects. To the best of our knowledge, this is the first study to address the use of stable isotope analysis for this purpose. Muscle is one of the organs that reflect the largest isotopic variations (20), and therefore it is one of the most suitable tissues in which to study differences between fish fed in a range of 'isotopic-labeling' conditions. This tissue tends to integrate isotopic signatures of food assimilated over a longer period than faster metabolic tissues like liver (21). However, we also analyzed whole fish to determine the contribution of muscle to the body isotopic composition.

2. MATERIAL AND METHODS

2.1 Animal, experimental conditions and sampling protocol

In May 2007, gilthead sea bream (*Sparus aurata*) fingerlings (3.5- 4.5 g) were obtained from three farms in Spain specialized in sea bream fingerling production. Hatcheries were located far from each other (Mediterranean coast, MC; Cantabrian coast, CC; and South-Iberian Atlantic coast, AC) (**Figure 1**) and differed in rearing conditions (**Table 1**). Thirty fish from each farm were frozen *in situ*, transported in dry ice and stored at -80 ºC until required. Frozen fish were dissected on a tray set on ice and muscle tissue samples consisting of the entire dorsal fillet above the lateral line were obtained. Due to the small size of the fish, fifteen animals were pooled into three groups for further analyses in muscle and whole body samples to determine the conditions in the hatcheries.

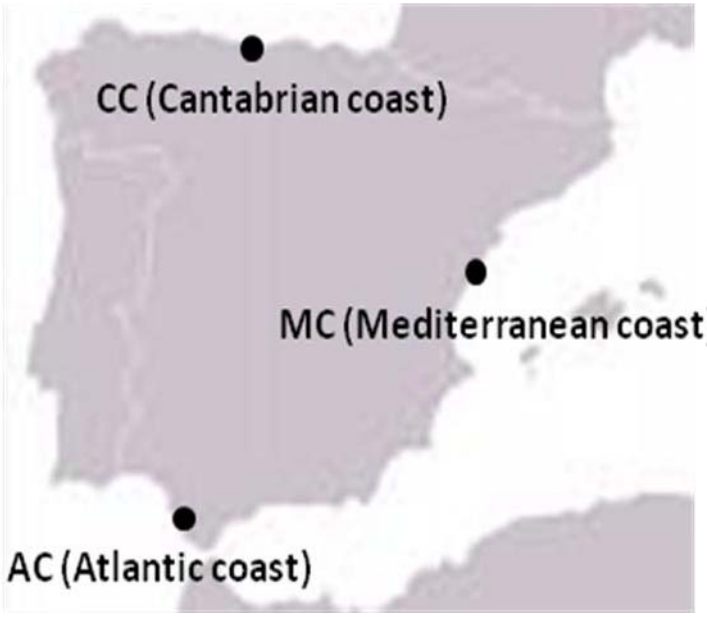

Figure 1. Schematic map of Iberian Peninsula with the location of the three sea bream hatcheries.

Three hundred fingerlings from each hatchery were transported to our facilities (Faculty of Biology, University of Barcelona, Spain) and randomly distributed with a similar density (~1 kg/m^3) into three 200-L fiberglass tanks per condition. Fish were reared with filtered seawater in a closed system with physical and biological filters, ozone skimmers and continuous aeration under controlled temperature (22± 0.2ºC), salinity (31-38 ‰), oxygenation (<90% saturation) and photoperiod (12L/12D). Before starting the experiment, fish were acclimated to the experimental facilities for two weeks, during which they were fed hatchery diets. During the 35-

day homogenization period, they were fed to satiation (nearly to 3 % of body weight) three times, and food intake was measured daily. Although the compositions of the farm diets were isoproteic, isolipidic and isoenergetic, the diet from the MC hatchery was selected for the experiment because of its higher quality (the highest values of $\delta^{15}N$, Table 1). After the experimental period, 18 animals from each hatchery (six fish per tank) were fasted for 24 hours and then killed by severing the spinal cord. Entire dorsal fillet samples were taken from 9 fish, while the other 9 fish were kept intact. Muscle and whole fish samples were immediately frozen in liquid nitrogen and stored at -80ºC until analysis. The experiments complied with the Guidelines of the European Union Council (86/609/EU), the Spanish Government (RD 1201/2005) and the University of Barcelona (Spain) for the use of laboratory animals.

Table 1. General characteristics of the three sea bream hatcheries and proximate (mg g^{-1}) and isotopic (‰) composition of the commercial diets used.

		Hatchery		
		MC	**CC**	**AC**
Water temperature (º C)		20.5	24	19
Ration size[1]		4.5	6	3
Diet digestible energy (MJ/kg)		21	20	19.6
Diet proximate composition[2]				
	protein	54	56	55
	lipid	18	18	18
	ash	11	10	11
	NFE[3]	17	16	16
Diet isotopic composition (‰)[4]				
$\delta^{15}N$	bulk diet	12.4 ± 0.2 a	9.4 ± 0.1 c	10.8 ± 0.3 b
	protein	12.8 ± 0.1 a	10.0 ± 0.1 c	10.8 ± 0.1 b
$\delta^{13}C$	bulk diet	-20.9 ± 0.1 a	-21.0 ± 0.1 a	-21.8 ± 0.2 b
	protein	-20.7 ± 0.1 a	-20.6 ± 0.1 a	-22.1 ± 0.2 b
	lipid	-23.8 ± 0.1 a	-24.6 ± 0.1 c	-24.1 ± 0.1 b

[1] g food × 100g body $weight^{-1}$ × day^{-1}

[2] g × $100g^{-1}$ dry matter

[3] NFE, Nitrogen Free Extract calculated by difference

[4] Values are reported as means ± SEM (N = 3). Values with different letter in each row are significantly different (Tukey post-hoc test, $p < 0.05$)

2.2 Growth performance

Fish from each tank were bulk weighed weekly to readjust feed ration and to control growth. Food intake was recorded daily and specific growth rates (SGR) were calculated:

$$SGR\ (\%\ day^{-1}) = 100 \times (\ln W_2 - \ln W_1)/\ t$$

where W_1 and W_2 are the initial and final weight of animals respectively, after t days.

2.3 Principal components of tissue samples and diets

Diets and muscle and whole fish samples were homogenized in liquid nitrogen using a pestle and mortar to obtain a fine powder. Tissue water content was determined gravimetrically after drying the samples at 100ºC for 24h. Total lipid content was purified from two methanol–chloroform (2:1) extractions. The washed lipid extracts were dried under N_2 and the lipid content was determined gravimetrically. Protein purification was carried out using defatted samples via precipitation with trifluoroacetic acid (100 ml L^{-1}) and after centrifugation at 1060 *g* for 30 min. The protein content was calculated from the nitrogen obtained by elemental analysis (Elemental Analyzer Flesh 1112, ThermoFinnigan, Bremen, Germany) as follows:

$$\text{g protein/100g dry matter} = (\text{g N/100g dry matter}) \times 6.25.$$

2.4 $\delta^{15}N$ and $\delta^{13}C$ determination

Samples of diets and whole tissues were lyophilized and ground into a homogenous powder for isotopic analysis. Aliquots (from 0.3 to 0.6 mg) from these samples, as well as their purified lipid and protein, were accurately weighed in small tin capsules (3·3 x 5 mm, Cromlab, Barcelona, Spain). Samples were analyzed to determine the carbon and nitrogen isotope composition using a Mat Delta C IRMS (Finnigan MAT, Bremen, Germany) coupled to Flash 1112 Elemental Analyzer at the *Serveis Científico-Tècnics* at the University of Barcelona. Isotope ratios (^{15}N /^{14}N, ^{13}C /^{12}C) are given in ‰, parts *per* thousand, on a δ-scale and refer to the deviation in measured ratio from the international accepted standards VPDB (Vienna Pee Dee Belemnite) for carbon and AIR for nitrogen. Delta values were determined as follows:

$$\delta = [(R_{sa}/R_{st}) - 1] \times 1000$$

where R_{sa} = ^{15}N /^{14}N or ^{13}C /^{12}C of samples and R_{st} = ^{15}N /^{14}N or ^{13}C /^{12}C of the international standards. The following standards certified by the International Atomic Energy Agency (IAEA, Vienna, Austria) were used: IAEA-N1 ($\delta^{15}N_{air}$=0.4 ‰), IAEA-NO-3 ($\delta^{15}N_{air}$=4.7 ‰) and IAEA-N2 ($\delta^{15}N_{air}$=20.3 ‰) for nitrogen; and LSVEC ($\delta^{13}C_{VPDB}$= -46.6 ‰), IAEA-CH-7 ($\delta^{13}C_{VPDB}$= -32.15 ‰), USGS40 ($\delta^{13}C_{VPDB}$= -26.4 ‰), IAEA-CH-6 ($\delta^{13}C_{VPDB}$= -10.45 ‰) for carbon. Every ten measurements, three standard samples were analyzed to compensate for machine drift and as a quality control measure. The same reference material examined over the analysis period was measured with ±0.2‰ precision. Nitrogen and Carbon Isotopic Fractionation ($\Delta\delta^{15}N$ and $\Delta\delta^{13}C$) values were calculated as the difference between δ tissue and δ diet.

2.5 Measurements of RNA/DNA ratios

Bulk RNA and DNA content were determined in muscle using a modification of the Schimdt-Thannhauser UV-based method (22), following the procedures for fish samples described Buckley and Bulow (23). Muscle samples were removed from liquid nitrogen and homogenized with a polytron (Kinematica, Luzern, Switzerland) in cold $HClO_4$ 0.2N to precipitate nucleic acids. Centrifugation was done at 12000 *g* for 15 min at 4 ºC and the supernatant was discarded. The process was repeated to effectively remove free nucleotides, amino acids and other acid-soluble compounds. The resulting sediment was resuspended in dilute alkali (0.3N NaOH), which also achieved hydrolysis of RNA and its chemical partitioning from protein and DNA. The hydrolysate was acidized with cold 1.32N $HClO_4$ to remove RNA from DNA and protein. DNA was then hydrolyzed and separated from the remaining protein by addition of hot 0.6N $HClO_4$. RNA and DNA were estimated from the absorbance of the appropriate hydrolysate at 260 nm using the following extinction coefficient: A260 of a 1 μg ml^{-1} solution of hydrolyzed RNA or DNA is 0·3. Absorbance was measured using a using a spectrophotometer (Spectronic Genesys 2/Milton Roy Company, Rochester, New York). Nucleic acid contents were recorded as μg RNA or DNA per mg wet tissue.

2.6 Muscle enzyme assays

Enzyme activities were assayed from crude extracts of muscle obtained by homogenizing frozen tissue (50 mg/mL) in detergent solution (1.24 mM TRITON X-100, 1 mM EDTA and 1mM $NaHCO_3$) and stabilizing solution (0.27 M EDTA and 5 mM 2-β-mercaptoethanol), 1:1 v/v. Homogenates for measuring citrate synthase (CS, EC 2.3.3.1) and cytochrome-*c*-oxidase (COX, EC 1.9.3.1) activity were centrifuged at 700 *g* at 4 °C for 10 min. CS activity was determined from absorbance increases at 412 nm of DTNB reagent, using oxalacetic acid as substrate, following the method described by Srere (*24*). COX activity was obtained by adapting a commercial kit (CYTOC-OX1, Sigma-Aldrich Inc., Sant Louis, Missouri). This colorimetric assay measures the decrease in ferrocytochrome *c* absorbance caused by oxidation of the latter by cytochrome-*c*-oxidase.

2.7 Statistical analysis

One-way analysis of variance (ANOVA) was used to test significant differences among fish from the three origins and the Tukey post-hoc test was used when significant differences were found. Initial and final data for each group were compared by an independent two-sample *t*-test.

The Shapiro-Wilk test was previously used to ensure the normal distribution of data, and the equality of variances was determined by statistical Levene's test. The relationships between lipid content and $\delta^{13}C$ of whole body and muscle tissue were tested using linear regression. Principal component analysis (PCA) was performed to study the structure of the data. Score plots from the PCA explore the main trends in the data, and their respective loading reveals variables with a significant loading. All statistical analyses were done using commercial software (SPSS 16.0, SPSS Inc., Chicago, Illinois).

3. RESULTS

3.1 Growth parameters and changes in the proximate composition

Although no significant differences in SGR or feed conversion efficiency were observed between groups, AC fingerlings showed the lowest values of these two variables (**Table 2**). Consistent with the greater body mass at the beginning and end of the experiment, the MC group showed the highest lipid content in whole fish and muscle throughout the homogenization period. In contrast, the smallest AC fingerlings also showed the lowest lipid content (**Table 3**). During the homogenization period the lipid content of whole fish and muscle samples from the AC and CC groups increased significantly. At the end of this period, the CC group registered the highest muscle protein content while the AC group showed the lowest.

Table 2. Weight, feed intake, feed efficiency and growth rate of fingerlings from the three hatcheries during the 35 days of the experiment.

	Hatchery		
	MC	**CC**	**AC**
Initial weight (g)	4.63 ± 0.03 a	3.40 ± 0.05 b	3.45 ± 0.2 b
Final weight (g)	9.10 ± 0.51 a	6.50 ± 0.33 b	6.03 ± 0.29 b
Feed intake[1]	2.54 ± 0.02 b	2.68 ± 0.04 a	2.69 ± 0.02 a
Feed efficiency[2]	0.87 ± 0.05	0.73 ± 0.06	0.58 ± 0.01
SGR*3*	1.96 ± 0.10	1.80 ± 0.10	1.37 ± 0.04

Values are reported as means ± SEM (N = 3 tanks). Values with a different letter in each row are significantly different (Tukey post-hoc test, $p < 0.05$). Results without any letter indicate no significant differences.

[1]Feed intake: g 100× g body weight^{-1} × day^{-1}

[2]Feed efficiency: g fish weight gain × g feed offered^{-1}

[3]SGR: Specific Growth Rate = 100 × [(Ln final weight – Ln initial weight) × days^{-1} of experiment]

Table 3 – Initial (hatchery) and final (after the homogenization period) whole fish and muscle composition (g $100g^{-1}$) of sea bream fingerlings from the three hatcheries.

		WHOLE FISH		MUSCLE	
		Initial	**Final**	**Initial**	**Final**
Protein	MC	14.1 ± 0.5	15.4 ± 0.3	16.0 ± 0.6	17.3 ± 0.4 ab
	CC	13.7 ± 1.2	15.1 ± 0.4	17.3 ± 0.1	18.3 ± 0.3 a
	AC	14.9 ± 0.6	16.2 ± 0.4	15.2 ± 0.8	16.5 ± 0.5 b
Lipid	MC	8.1 ± 0.3 a	9.2 ± 0.6 a	4.9 ± 0.3 a	5.2 ± 0.4 a
	CC	5.5 ± 0.5 b	8.3 ± 0.5 ab,*	3.4 ± 0.1 b	4.2 ± 0.2 a,*
	AC	3.8 ± 0.3 c	6.9 ± 0.4 b,*	2.3 ± 0.2 c	3.1 ± 0.1 b,*
Moisture	MC	73.6 ± 0.1 b	71.3 ± 0.3 b,*	76.0 ± 0.6 b	74.8 ± 0.4 b
	CC	76.6 ± 0.1 a	72.3 ± 0.6 ab,*	77.4 ± 0.3 ab	75.1 ± 0.3 b,*
	AC	77.4 ± 0.5 a	73.3 ± 0.4 a,*	78.4 ± 0.6 a	77.0 ± 0.2 a,*

Values are reported as means ± SEM (initial N = 3 pools of 5 fish; final N = 9 fish). Values with a different letter in each row are significantly different (Tukey post-hoc test; $p < 0.05$) and asterisk denotes significant differences (*t*-test; $p < 0.05$) after (initial) and before (final) the homogenization period. Results without any letter or symbol indicate no significant differences.

3.2. Isotopic composition and fractionation

The stable isotope composition of the commercial diets used by the three hatcheries varied because of the dietary ingredients used in the formulation (**Table 1**). Differences in $\delta^{15}N$ of bulk diet reflected those of the dietary protein. These differed as much as 3‰ between the MC (12.4‰) and the CC (9.4‰) diets. $\delta^{13}C$ varied between diets, with the AC diet showing the lowest values (-21.8‰). These changes also paralleled the variation found in protein $\delta^{13}C$ because protein was the major dietary component. However, variations in lipid $\delta^{13}C$ differed between the three diets. Initial $\delta^{15}N$ of whole fish samples did not differ significantly among the three groups (**Figure 2**). However, the MC group showed the highest muscle $\delta^{15}N$ while the AC group showed the lowest. After submitting all three groups to identical rearing conditions for 35 days, the $\delta^{15}N$ of whole fish and muscle samples increased in a similar manner (~2‰), thus the initial differences between the groups were maintained. However, no differences in $\delta^{13}C$ of whole fish or muscle samples were found at the beginning of the experiment. After the homogenization period, the $\delta^{13}C$ of whole fish and muscle also increased in all groups but was significant only in the latter (~0.7‰). Nevertheless, whole MC fish continued to show lower $\delta^{13}C$ of bulk tissue than AC fish ($p<0.05$) because of the lower lipid content of the latter.

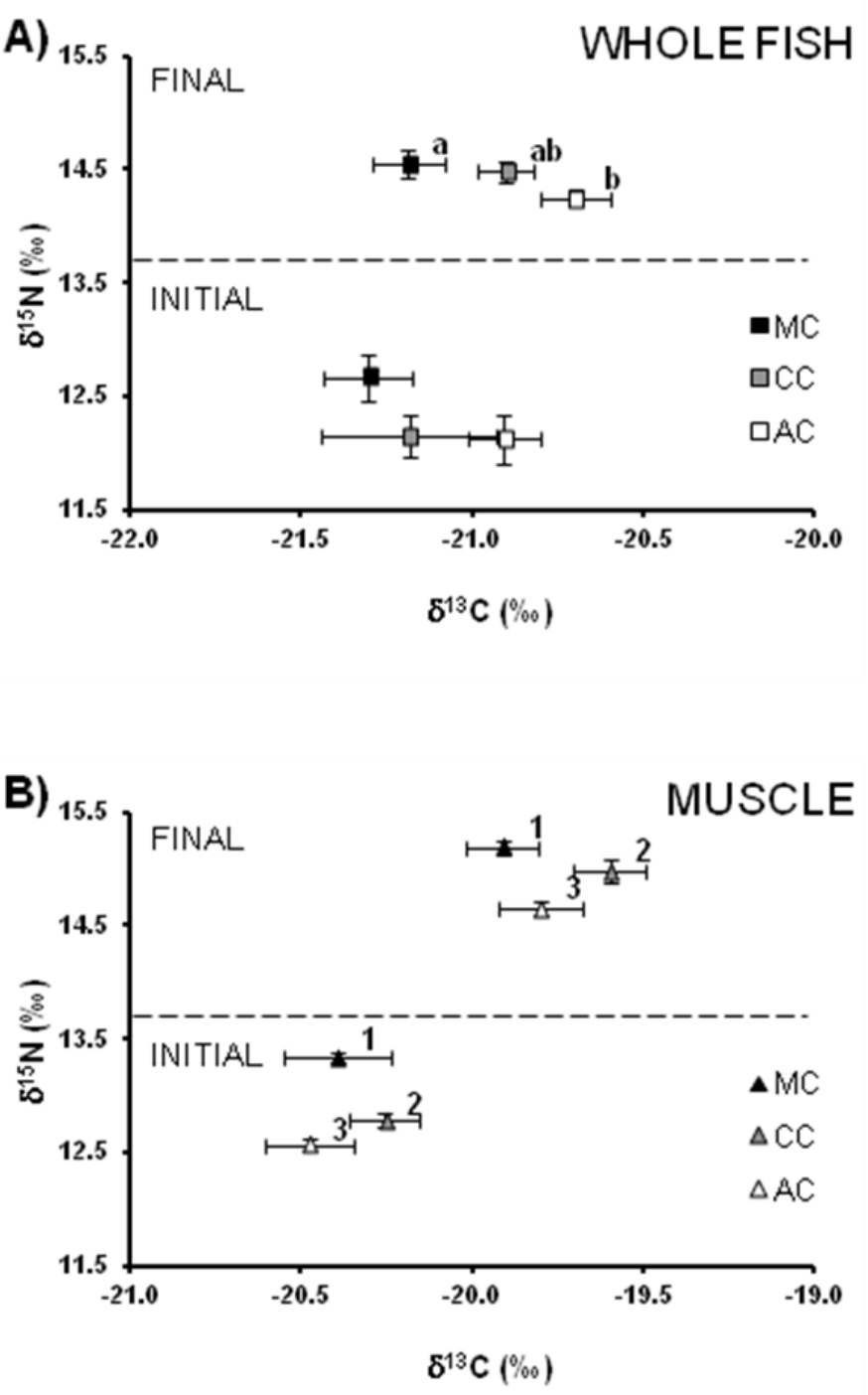

Figure 2. Evolution of the isotopic signature of bulk samples of whole fish (A) and muscle (B) of sea bream fingerlings produced in three hatcheries during the homogenization period. Symbols are reported as means and bars as SEM (initial N = 3 pools of 5 fish; final N = 9 fish per group), a different letter indicates significant differences in $\delta^{13}C$ ($p < 0.05$) while different numbers indicate significant differences in $\delta^{15}N$ ($P < 0.05$).

Protein $\delta^{15}N$ also increased significantly and reflected the same differences observed in bulk $\delta^{15}N$, showing the AC group with the lowest levels ($p<0.05$) in whole fish and muscle samples throughout the homogenization period (**Table 4**). Similarly, protein $\delta^{13}C$ showed the same pattern as protein $\delta^{15}N$, but only in whole fish was the increase in protein $\delta^{13}C$ significant. In contrast, lipid $\delta^{13}C$ increased significantly in whole fish and muscle samples throughout the homogenization period, and initial differences disappeared. The changes in lipid content modified bulk $\delta^{13}C$, showing an inverse relationship between these variables in whole fish (r=0.589, n=27, $p<0.001$) and in muscle (r=0.532, n=27, $p<0.005$) samples after homogenization (**Figure 3**).

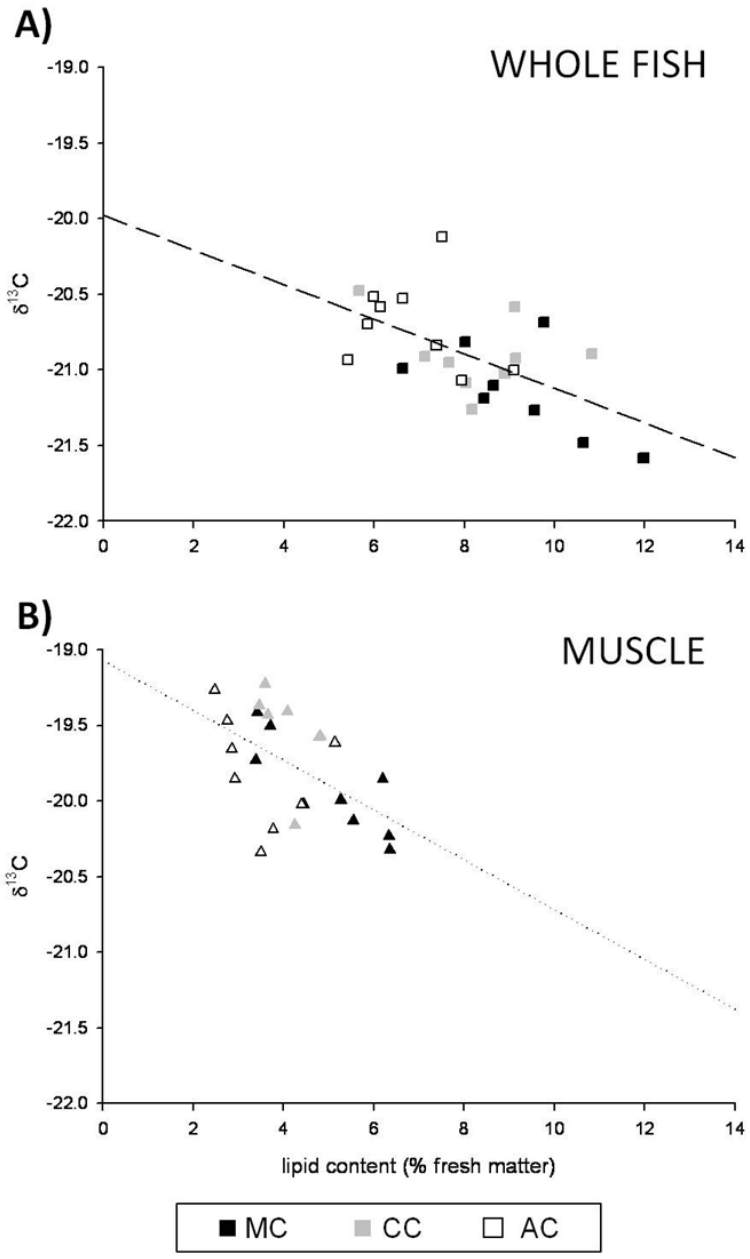

Figure 3. Relationship between lipid concentration and $\delta^{13}C$ in whole fish (A, dashed line: r = 0.589, $P < 0.001$) and muscle (B, dotted line: r = 0.532, $p < 0.005$) of sea bream fingerlings after the homogenization period.

Table 4 – Initial (hatchery) and final (after homogenization period) stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) of reserves and isotopic fractionation ($\Delta\delta^{13}C$ and $\Delta\delta^{15}N$) of bulk samples in whole fish and muscle of sea bream fingerlings from the three hatcheries.

		WHOLE FISH				MUSCLE			
		Initial		Final		Initial		Final	
		Isotopic composition of reserves							
Protein $\delta^{15}N$	MC	14.12 ± 0.12 a		15.44 ± 0.06 a,*		14.19 ± 0.10 a		16.36 ± 0.04 a,*	
	CC	12.86 ± 0.05 b		15.49 ± 0.08 a,*		13.75 ± 0.03 b		16.21 ± 0.06 a,*	
	AC	13.29 ± 0.18 b		15.19 ± 0.06 b,*		13.55 ± 0.07 b		15.96 ± 0.08 b,*	
Protein $\delta^{13}C$	MC	-19.52 ± 0.01 a		-19.33 ± 0.05 a,*		-19.69 ± 0.04 a		-19.85 ± 0.04 a	
	CC	-19.82 ± 0.04 b		-19.35 ± 0.09 a,*		-19.96 ± 0.06 b		-19.88 ± 0.04 a	
	AC	-20.13 ± 0.05 c		-19.71 ± 0.05 b,*		-20.29 ± 0.09 b		-20.23 ± 0.04 b	
Lipid $\delta^{13}C$	MC	-24.45 ± 0.06		-24.16 ± 0.05 *		-24.47 ± 0.07 a		-24.13 ± 0.02 *	
	CC	-24.53 ± 0.09		-24.11 ± 0.03 *		-24.54 ± 0.03 ab		-24.14 ± 0.06 *	
	AC	-24.70 ± 0.04		-24.20 ± 0.03 *		-24.76 ± 0.06 b		-24.21 ± 0.03 *	
		Bulk isotopic fractionation							
$\Delta\delta^{15}N$	MC	0.25 ± 0.21 c		2.13 ± 0.12 *		0.93 ± 0.04 c		2.78 ± 0.06 a,*	
	CC	2.71 ± 0.19 a		2.07 ± 0.09 *		3.34 ± 0.06 a		2.57 ± 0.05 b,*	
	AC	1.34 ± 0.21 b		1.83 ± 0.07 *		1.79 ± 0.05 b		2.24 ± 0.06 c,*	
$\Delta\delta^{13}C$	MC	-0.41 ± 0.13 b		-0.29 ± 0.11 b		0.50 ± 0.16 b		0.98 ± 0.10 *	
	CC	-0.18 ± 0.25 b		0.01 ± 0.08 ab		0.75 ± 0.10 ab		1.29 ± 0.11 *	
	AC	0.88 ± 0.11 a		0.20 ± 0.10 a,*		1.31 ± 0.13 a		1.09 ± 0.12	

Values are reported as means ± SEM (initial N = 3 pools of 5 fish; final N = 9 fish). Values with different letter in each row are significantly different (Tukey post-hoc test; $p < 0.05$) and asterisk indicates significant differences (*t*-test, $p < 0.05$) after (initial) and before (final) the homogenization period. Results without any letter or symbol indicate no significant differences.

The differences in $\delta^{15}N$ between the diets were greater than those observed in fish samples. Thus, the isotopic fractionation of nitrogen ($\Delta\delta^{15}N$: $\delta^{15}N_{tissue}$- $\delta^{15}N_{diet}$) in initial hatchery conditions was highest ($p<0.05$) for the CC group (2.71 and 3.34 ‰) and lowest for the MC group (0.25 and 0.93 ‰) in whole fish and muscle respectively (**Table 4**). After the homogenization period with the same diet and ration, $\Delta\delta^{15}N$ converged to ~2‰ and ~2.5‰ for these tissues, respectively, decreasing in the CC group while increasing in the other two. In spite of this convergence, significant differences were observed in the $\Delta\delta^{15}N$ of muscle samples. $\Delta\delta^{13}C$ in initial conditions was highest for the AC group (1.34 and 1.79 ‰) and lowest for the MC group (-0.41 and 0.50 ‰) in whole fish and muscle samples respectively ($p<0.05$). After homogenization, $\Delta\delta^{13}C$ also converged (~0‰ and ~1.1‰ for whole fish and muscle tissue respectively), but only the AC group showed a decrease in $\Delta\delta^{13}C$ in both kinds of tissue.

3.3. Changes in metabolic parameters of muscle

The lowest RNA/protein and RNA/DNA ratios under initial conditions were also found in the AC group, indicating lower protein synthesis and transcriptional capacity (**Figure 4**). COX and CS activity did not differ significantly between groups in initial conditions. This observation indicates that energy production capacity of the three hatcheries was similar, although the CC group tended to show higher activity for these two enzymes.

After the homogenization period, the RNA/protein ratio decreased in all groups as a consequence of growth. The transcriptional capacity of the AC group increased significantly, but the protein synthesis capacity of this group was still the lowest. The COX:CS ratio decreased in all groups ($p<0.05$), without significant differences among them (**Figure 4**). Nevertheless, the enzyme activities of the AC group showed a different pattern to that of the other two groups. While CS and COX activities decreased significantly in the MC and CC groups, the CS activity of AC fingerlings increased significantly and COX activity remained unchanged. These observations suggest that the AC group presented higher energy waste.

3.4. Principal components analysis (PCA)

PCA was used to determine the capacity of the variables (chemical, metabolic and isotopic measurements in muscle) to discriminate between fingerlings from the three hatcheries after the homogenization period. The first three factors accounted for over 70% of the variability within the data (**Table 5**) and bulk $\delta^{15}N$ was the variable with the highest load on the first factor (**Table 6**). Plots of factor 1 versus factors 2, 3 and 4 all provided good discrimination among fingerlings

from the three hatcheries (**Figure 5**); in particular AC fingerlings were clearly separated from the other two groups, with factor 1 providing the greatest discrimination.

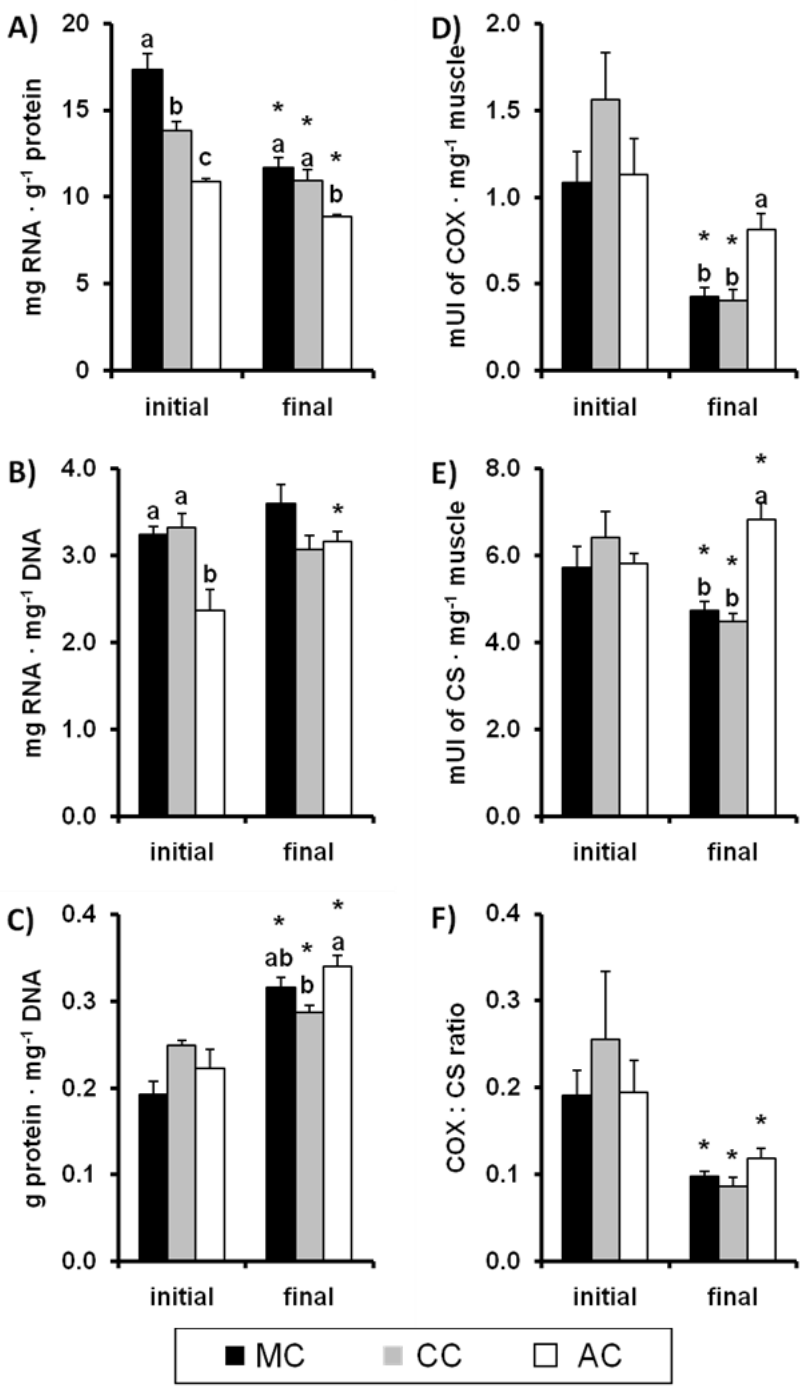

Figure 4. Initial (hatchery) and final (after the homogenization period) muscle growth (RNA:protein, A; RNA:DNA, B; and protein:DNA, C, ratios) and metabolic (COX activity, D; CS activity, E; and COX:CS ratio, F) indices of muscle samples of sea bream fingerlings produced in the three farms. Columns assigned a different letter are significantly different between groups ($p < 0.05$) and an asterisk indicates significant differences after (initial) and before (final) the homogenization period ($p < 0.05$).

Table 5. Table of eigenvalues for PCA of chemical, metabolic and isotopic data from muscle of sea bream fingerlings after the homogenization period.

	Eigenvalue	Variability (%)	Cumulative eigenvalue	Cumulative %
F1	6.88	43.01	6.88	43.01
F2	2.75	17.18	9.63	60.18
F3	1.74	10.88	11.37	71.06
F4	1.26	7.90	12.63	78.96
F5	0.93	5.80	13.56	84.75
F6	0.82	5.11	14.38	89.87
F7	0.51	3.22	14.89	93.09

Table 6. Factor loadings for muscle of seabream fingerlings.

Variable	F1	F2
Bulk $\delta^{15}N$	**0.868**	-0.007
$\Delta\delta^{15}N$	**0.866**	-0.008
Protein $\delta^{13}C$	**0.863**	0.025
CS	**-0.850**	-0.097
Protein $\delta^{15}N$	**0.845**	-0.144
% Water	**-0.826**	0.240
% Lipid	**0.817**	-0.456
COX	**-0.816**	-0.121
COX·CS $^{-1}$	-0.680	-0.276
RNA·protein^{-1}	0.581	0.190
Bulk $\delta^{13}C$	0.020	**0.962**
$\Delta\delta^{13}C$	0.000	**0.958**
% Protein	0.350	0.570
Protein·DNA^{-1}	-0.352	-0.175
RNA·DNA^{-1}	0.219	-0.357
Lipid $\delta^{13}C$	0.295	0.011

Bold type indicates loadings > 0.7 and <-0.7

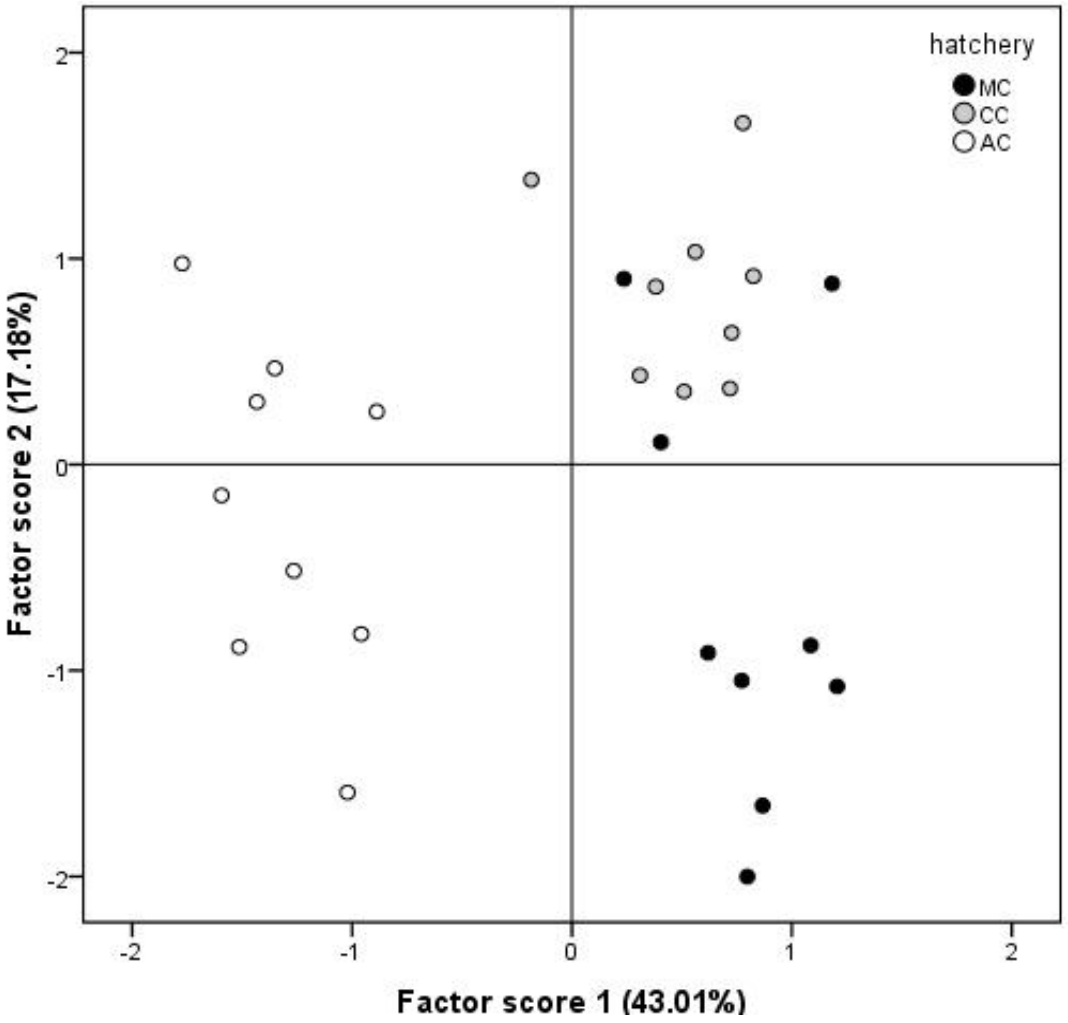

Figure 5. Principal component analysis (PCA) plot of combined chemical, metabolic and isotopic data of muscle from sea bream fingerlings from the three hatcheries after the homogenization period. Factors 1 and 2 represent the first and second principal components, with the percentage of explained variance indicated in parentheses.

4. DISCUSSION

4.1 Isotopic characterization of fingerlings in hatchery conditions

The initial ^{15}N and ^{13}C composition of juveniles from the three fish farms differed. Given the differences in diets and rearing conditions, especially food ration and water temperature, these isotopic differences at origin must be supported by other metabolic markers. The three diets in the initial condition were isoproteic and isolipidic, but the significant differences in isotopic composition should reflect differences in the proportion and type of the raw materials used. When the plant component in a diet increases, total $\delta^{15}N$ falls and the $\delta^{13}C$ values of the lipid fraction also decreases (*9, 13*). Plant meal based on C3-plants (i.e.: wheat) gives lower $\delta^{13}C$ values than C4-plants (i.e.: corn) (*21*). Therefore, the diet given to the CC group, which had the

lowest $\delta^{15}N$, had the highest proportion of plant ingredients, whereas that given to the AC group, which had the lowest $\delta^{13}C$ values, had a higher amount of C3-plant-based ingredients. Despite the wide range of variation in $\delta^{15}N$ among these fish-meal based diets, the differences in whole fish and muscle $\delta^{15}N$ between the three groups were small. Consequently, variations in isotopic fractionation of nitrogen ($\Delta\delta^{15}N_{tissue\text{-}diet}$), within the range reported in sea bream (*9*) or in other species (*25, 26*), would indicate differences in fish metabolism (*15, 16*), these possibly related to rearing conditions. The isotopic composition of animals has been reported to depend mainly on food composition (*7, 8*); however, temperature and ration also influence $\delta^{15}N$ and $\delta^{13}C$ of sea bass tissues (*14*). Protein intake is proportional to ^{15}N enrichment in fish muscle (*27*), and tilapias fed diets with a high plant meal content show increased $\delta^{15}N$ (*28*), thereby suggesting greater transformation of plant-derived dietary amino acids into animal protein. This observation could explain the finding that the CC group with the highest $\Delta\delta^{15}N$ (2.71 ‰ and 3.34 ‰ for whole fish and muscle, respectively) was fed the highest ration (6 % b.w·day^{-1}) of the diet most depleted in $\delta^{15}N$, i.e. with the highest plant protein content. In fact, this group also presented greater energy demands, an observation consistent with the direct relationship between ration and COX and CS activities, as previously found in saithe (*18*). The MC group showed the lowest $\Delta\delta^{15}N$, presenting values of 0.25 ‰ in whole fish and 0.93 ‰ in muscle, much lower values than the general 3‰ assumed for one trophic shift in the food web (*8*). This finding would suggest that this group had the lowest transformation of dietary protein, which is consistent with the diet having the highest fish meal content.

In contrast to ^{15}N, the $\delta^{13}C$ values of consumer tissues depends not only on protein but also on carbohydrates, and especially on lipids, which are depleted in ^{13}C (*29*). Furthermore, the carbon skeletons of several dietary constituents (proteins, lipids and carbohydrates) can be routed to diverse tissue constituents, i.e. “isotopic routing” (*30*). Indeed, the differences in lipid $\delta^{13}C$ between the three groups reflected those of dietary protein more closely than dietary lipid. This finding is consistent with results reported by Kelly and Martinez del Rio (2010), who observed that more than 50% of the carbon in lipids extracted from muscle was derived from dietary protein. All these findings explain the lack of differences in initial bulk $\delta^{13}C$ in muscle and whole fish between the three groups, despite variations in dietary isotopic composition and lipid content of tissues. Concerning ^{13}C fractionation, our results were in agreement with reviewed literature (*25, 26*). Gaye-Siesseger et al. (*13*) proposed changes in the anabolism-catabolism ratio, which could be referred to the inverse relationship between $\Delta\delta^{13}C$ and ration. Hence, the observation that the AC group with the lowest ration in hatchery conditions (3 % b.w·day^{-1}) showed the highest $\Delta\delta^{13}C$ in the two tissues sampled can be explained by the observation that it showed the lowest protein synthesis capacity of the three groups. The AC group was subjected to the lowest rearing temperature, which would explain this lower capacity, as also observed in

Atlantic wolffish (*31*).

4.2 Evolution of isotopic composition after the homogenization period

After submitting the fingerlings to the same diet and identical rearing conditions for 35 days, the isotopic signature changed as a result of growth, but the initial differences observed in isotopic signature remained. These differences were maintained, especially in muscle $\delta^{15}N$, which was the variable with the highest load on the first principal component of the PCA analysis. The time lag required to reflect diet isotopic composition in fish depends on the speed at which new material is added to tissue (growth rate) and the replacement of materials exported from tissues as a result of catabolism (metabolic turnover) (*32*). Half-lives for ^{13}C and ^{15}N in juvenile fish are under one month, although the half-life of the former is shorter than that of the latter (*33 – 36*). Thus, for the fingerlings in this study, which were in a fast growth phase, a trial lasting 35 days was sufficient to reflect the new conditions of isotopic composition in tissues. At this point, the isotopic composition of these fish, which follows an asymptotic evolution after a diet switch (*33 – 36*), should be in the plateau phase (i.e. in isotopic equilibrium with diet). Moreover, all fish were fed the same ration to prevent an influence of this variable on isotopic composition (*13, 16*). Therefore, we propose that the final isotopic differences between the three fish groups are the result of the 'isotopic imprint' that remains from the initial hatchery conditions (which is consequence of the previous physiological or metabolic conditions). In this regard, systematic differences in isotope composition have been found in individual animals over long periods of time compared to other individuals with identical rearing (food and water) conditions (*37*). These observations may reflect differences in physiology. However, the use of ^{15}N and ^{13}C isotopes as a discrimination tool, while useful at early growth stages, may be complicated by maturity and growth rate. Long-term studies in laboratory controlled conditions have to be carried out in order to clarify how long this isotopic imprint lasts.

Direct relationships between $\delta^{15}N$ and body weight, irrespective of dietary switches, have been interpreted as metabolic or physiological changes associated with age in walleye (*38*) and sea bass (*39*). However, growth enhances *de novo* biosynthesis of dispensable amino acids, which become ^{13}C-enriched as a result of isotopic discrimination steps, and could significantly alter consumer tissue $\delta^{13}C$, as reported in mummichog (40). Accordingly, the observation that all the fish in our study almost doubled their weight after the homogenization period, thereby leading to an increase in bulk $\delta^{15}N$ and $\delta^{13}C$ of whole body and muscle samples, mirrored the delta values of deposited protein. These isotopic enrichments are linked to the decrease in the RNA:protein ratio observed in all groups at the end of the homogenization period. As body weight increases, the RNA content decreases and a larger proportion of the RNA pool is

involved in protein turnover (*17*). Furthermore, deamination and transamination, together with decarboxylation, lead to isotopic enrichment of newly synthesized protein (*41, 42*). Therefore, the increase in protein turnover that occurs with growth could explain the increase in $\delta^{15}N$ and $\delta^{13}C$ in the three groups. Nonetheless, the group previously eating the diet used in the homogenization period showed smaller changes in $\delta^{15}N$ and $\delta^{13}C$ in whole fish and muscle samples, thus increases in its isotopic fractionation of ^{15}N and ^{13}C can be explained solely by growth. In this regard, the dietary switch to an isotopically heavier diet in the other two groups caused greater increases in tissue isotopic values. However, of the groups that switched diet, the changes were slightly lower in the one with the lowest $\Delta\delta^{15}N$ values. As a result, this group maintained the lowest ^{15}N and ^{13}C protein values. In contrast, the isotopic values of the other group (CC) approached those of the one previously eating the diet used in the homogenization period. It is well accepted that growth and protein turnover are determinant factors of the rate of isotopic incorporation after a dietary switch in fish (*43*). Therefore, the group with lowest protein turnover, indicated by its lower $\Delta\delta^{15}N$, together with a lower growth rate, could explain this discordance. Furthermore, the dietary switch of these two groups affected the evolution of their isotopic fractionation differentially. Martinez del Rio et al. (*43*) hypothesized that $\Delta\delta^{15}N$ decreases with increasing protein quality in diet and/or with the efficiency of nitrogen deposition. Thus, the observed decline of $\Delta\delta^{15}N$ in fingerlings previously fed the diet most depleted in ^{15}N can be related to the improved quality of the new diet during the homogenization period, as indicated by the higher dietary $\delta^{15}N$ (*9*). However, the increase in $\Delta\delta^{15}N$ in the group could be consequence of high metabolic waste, reflected in the highest COX and CS activities, which were maintained after homogenization. In contrast, these activities decreased in the other two groups (MC and CC) in an inverse relationship with growth, as observed in saithe (*18*). Therefore, the group with highest metabolic cost for growth would show lower protein deposition efficiency, also evidenced by a trend to lower feed conversion efficiency and the lowest content of muscle protein. In agreement with this, this group showed the lowest RNA:protein ratio, which reflected lower protein synthesis capacity. Moreover, this group would show a higher proportion of growth by processes of hypertrophy instead of hyperplasia, as a result of its higher increase in protein:DNA and RNA:DNA ratios. In contrast, lipid-$\delta^{13}C$ increased in whole fish and muscle samples of the three groups, although unlike protein, differences in lipid $\delta^{13}C$ in muscle were not maintained after the homogenization period. This change would be produced by the 'isotopic routing' of carbon skeletons described above.

Here we demonstrate that after 35-day homogenization period, fingerlings can be discriminated between hatcheries mainly on the basis of isotopic measures (especially $\delta^{15}N$) combined with metabolic parameters (COX and CS activities) and proximal composition (fat content) in

muscle, as shown in PCA analysis. The group with the lowest isotopic values in hatchery conditions reached a distinct isotopic equilibrium to that of the other two groups. Lower protein synthesis capacity, lower hyperplasic processes and higher energy metabolic costs of this group could explain these differences. Muscle isotopic signature is a more suitable indicator than whole fish for the discrimination between fingerlings produced in distinct hatcheries, although additional physiological information is required to determine fish seeds with the highest growth potential. Therefore particular rearing conditions and/or genetic divergence between hatcheries, affecting the growth capacity of fingerlings, are defined mainly by the isotopic imprint.

In summary, we conclude that the set of parameters analyzed here could be useful for potential buyers of aquaculture products.

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CHAPTER III

New insights into fish swimming: a proteomic and isotopic approach in gilthead sea bream

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New Insights into Fish Swimming: A Proteomic and Isotopic Approach in Gilthead Sea Bream

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New insights into fish swimming: a proteomic and isotopic approach in gilthead sea bream

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Proteomics in fish swimming

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Abstract

Moderate exercise enhances fish growth, although underlying physiological mechanisms are not fully known. Here we performed a proteomic and metabolic study in white (WM) and red (RM) muscle of gilthead sea bream juveniles swimming at 1.5 body lengths per second. Continuous swimming for four weeks enhanced fish growth without increasing food intake. Exercise affected muscle energy stores by decreasing lipid and glycogen contents in WM and RM respectively. Protein synthesis capacity (RNA/protein), energy use (estimated by lipid-$\delta^{13}C$ and glycogen-$\delta^{13}C$) and enzymatic aerobic capacity increased in WM, while protein turnover (expressed by $\delta^{15}N$-fractionation) did not change. RM showed no changes in any of these parameters. 2D-PAGE analysis showed that almost 15% of sarcoplasmic protein spots from WM and RM differed in response to exercise, most being over-expressed in WM and under-expressed in RM. Protein identification by MALDI-TOF/TOF-MS and LC-MS/MS revealed exercise-induced enhancement of several pathways in WM (carbohydrate catabolism, protein synthesis, muscle contraction and detoxification) and under-expression of others in RM (energy production, muscle contraction and homeostatic processes). The mechanism underpinning the phenotypic response to exercise sheds light on the adaptive processes of fish muscles, being the sustained-moderate swimming induced in gilthead sea bream achieved mainly by WM, thus reducing the work load of RM and improving swimming performance and food conversion efficiency.

Key words: exercise, sarcoplasmic proteins, 2D-PAGE, $\delta^{13}C/\delta^{15}N$, RNA/DNA, COX/CS, *Sparus aurata*

1. INTRODUCTION

Fish swim against a water stream as a reflex action, and they are thus ideal models for studying the effects of exercise during long periods. Researchers have categorized swimming in these animals into three broad groups: sustained, prolonged, and burst-type[1]. The effects induced by exercise are specific to the activity performed since the metabolic pathways activated by exercise depend on the intensity and duration of the training. It is generally assumed that exercise training at favorable swimming speeds improves the growth rate and food conversion efficiency in various fish species without any harmful consequence on animal welfare or stress[2]. In contrast, sprint training is highly stressful[2]. Furthermore, fish exposed to moderate water currents exhibit less aggressive behavior and show lower levels of circulating stress hormones, the latter suggesting an energy saving effect through the action of these hormones on metabolic rate[3].

Fish muscle, the main promoter of swimming, undergoes extensive molecular and structural changes in response to sustained exercise. It is widely accepted that only slow oxidative fibers (red muscle, RM) are recruited during sustained swimming while fast glycolytic fibers (white muscle, WM) are recruited for sprint swimming. However, white fibers may also be used to a lesser extent during sustained swimming[4,5]. Growth changes induced by moderate sustained exercise are associated with WM and RM hypertrophy, which is caused by continuous physical stimulation of the muscle[2]. Metabolic changes induced by exercise in fish muscle have traditionally been assessed by bromatological and histological analyses and enzyme activity measurements. Continuous swimming increases the oxidative capacity of both muscles[4], although few studies signaled that effect in WM[5-6], the increase of lipid oxidation in RM is well documented[7-10]. Although lipids are considered to be the primary fuel used during prolonged exercise, energy obtained from carbohydrates gains relevance at higher swimming speeds while that obtained from protein may decrease with increased exercise intensity[8,11–13]. Moreover, sustained swimming increases glycolytic potential in RM and WM in fish[4,14–16], although the responses of these muscles differ with the training regime and species. Fish subjected to continuous exercise also show enhanced muscle protein turnover[17] and buffering capacity[18]. More recent genetic studies report that sustained exercise enhances muscle expression of genes involved in aerobic metabolism[19–21], growth[22] and immune and oxidative protections[23]. Despite these findings, the molecular mechanisms underlying the changes induced by exercise in fish are not fully understood, partly because the metabolic changes recorded in swimming muscles are not consistent between studies.

Emerging tools such as proteome and stable isotope analyses can provide new insights into the metabolic changes that occur in fish muscles during prolonged training. The application of proteomics to exercise is in its infancy and only a few studies have been published, all of which

were performed in mammals. These studies show a shift from glycolysis toward greater fatty acid oxidation and exercise-induced oxidative stress[24] in response to endurance training. In contrast, the study of the natural abundance of stable isotopes (^{13}C and ^{15}N) in tissue stores allows the evaluation of reserve turnover, because enzymes involved in catabolic processes, such as decarboxylation and deamination, show a preference for light isotopes[25]. Thus, tissue constituents become enriched in heavier isotopes (^{13}C and ^{15}N). In particular, the fractionation of ^{15}N ($\Delta\delta^{15}N$), that is to say the difference in ^{15}N content between fish and diet, decreases in parallel with the efficiency of nitrogen deposition and increases with fasting time[26]. Thus $\Delta\delta^{15}N$ is a suitable biomarker of protein turnover[27]. In this regard, lipids tend to be depleted in ^{13}C because of ^{13}C-discrimination during lipogenesis[28].

Gilthead sea bream (*Sparus aurata*) is intensely farmed in Europe. It is studied as a model in marine fish physiology because of the vast knowledge accumulated, being thus a suitable species for studies addressing muscle metabolism, in particular the effects of exercise. Here we explored the molecular mechanism underlying muscle function during moderate and continuous exercise by means of an integrative strategy combining metabolic (DNA/RNA quantification, and cytochrome c oxidase (COX) and citrate synthase (CS) activities), isotopic ($\delta^{15}N$ and $\delta^{13}C$), and proteomic (2D-PAGE) approaches. To our knowledge, this is the first study to report proteome analyses of fish muscle in response to exercise. Here we identify the mechanism underpinning the phenotypic response to moderate and continuous exercise in fish and provide new insight into the adaptive processes of fish muscles.

2. MATERIAL AND METHODS

2.1 Animal Maintenance and Exercise Training

One hundred juveniles of gilthead sea bream (*Sparus aurata* L.) from a local fish farm (Cripesa, Tarragona, Spain) were adapted to the indoor facilities of the Faculty of Biology (University of Barcelona, Barcelona, Spain). Fish were kept in six 200-L seawater tanks equipped with a semi-closed recirculation system with physical and biological filters, ozone skimmers and continuous aeration at 20°C and 12L:12D, with a 35% renewal of seawater each week. Fish were first adapted to the experimental diet rich in carbohydrate (37% protein; 13% lipid; 40% carbohydrate; 1.8% fiber; and 9% ash) by feeding to apparent satiety twice a day for 2 weeks. After the acclimation period, fish were slightly anaesthetized, weighed and randomly distributed in 6 trial tanks. Data on initial body weight and length are shown in **Table 1**. Control groups (C: 12 fish per tank, in triplicate) were kept under normal rearing conditions in 200-L circular tanks with a water flow of 350 L/h (vertical water entrance), a condition that produces voluntary swimming. Exercise groups (E: 12 fish per tank, in triplicate) were kept in 400-L circular tanks

in the same semi-closed circuit with a cylindrical tube in the central area. This approach resulted in a living area corresponding to an effective space of 200 L with the same fish density as in the control tanks. To achieve sustained activity, water flow was 700 L/h with a circular and uniformly distributed flow induced by a perpendicular water entrance and one additional submerged water pump (at the bottom of the tank and isolated from the living area). This design resulted in a swimming velocity of 1.5 body lengths per second (BL/s) measured and adjusted at three tank depths (at the surface, mid-tank and near the bottom) using a low speed mechanical flow meter (General Oceanics Inc., Miami, FL, USA). This velocity is approximately 35 % of the absolute critical swimming speed (U_{crit}), i.e. maximum sustained speed, for gilthead sea bream[29]. During the four weeks of experimental period all groups were fed twice a day (9:30 a.m. and 5:30 p.m.) until apparent satiety and food intake was recorded daily for each tank.

Table 1. Growth, body indices and proximate composition of white (WM) and red (RM) muscle of gilthead sea bream voluntarily swimming (control) or subjected to sustained exercise (1.5 BL/s, for 4 weeks).

	Control			Exercise			
Growth[a]							
Initial weight (g)	88.39	±	0.98	90.46	±	1.17	
final weight (g)	98.22	±	2.41	107.15	±	2.19	*
SGR[1]	0.56	±	0.10	0.78	±	0.07	*
Food intake[2]	2.75	±	0.05	2.61	±	0.12	
Body indices[b]							
CF[3]	1.50	±	0.03	1.41	±	0.06	
HSI[4]	2.06	±	0.16	2.09	±	0.15	
PFI[5]	2.15	±	0.13	2.01	±	0.13	
MSI[6]	40.9	±	0.78	40.6	±	0.65	
WM composition (%)[b]							
moisture	75.72	±	0.14	76.65	±	0.37	*
lipid	2.17	±	0.14	1.68	±	0.15	*
glycogen	0.53	±	0.02	0.46	±	0.04	
protein	20.52	±	0.17	20.58	±	0.18	
RM composition (%)[b]							
moisture	59.53	±	0.62	59.96	±	0.86	
lipid	21.50	±	0.65	21.11	±	1.24	
glycogen	0.91	±	0.05	0.64	±	0.09	*
protein	14.32	±	0.33	13.96	±	0.29	

[a] Values are mean ± SEM, n = 3 tanks. [b] Values are mean ± SEM, n = 12 fish. (*) Significant divergence by *t*-test ($p < 0.05$). [1]*SGR,* specific growth rate in % per day = 100 × [ln (final weight) – ln (initial weight)]/24 days; [2]Ration size = g food/100g fish; [3]*CF,* condition factor = body weight × 100 × total length^{-3}; [4]*HSI,* hepatosomatic index = liver weight × 100 × body weight^{-1}; [5]*PFI,* perivisceral fat index = fat weight × 100 × body weight^{-1}; [6]*MSI,* musculosomatic index = total muscle weight × 100 × body weight^{-1}

2.2 Sample Preparation

After the experimental period, 12 fish from the C group and another 12 from the E group were randomly selected and killed by severing their spinal cord. Immediately after, we recorded morphometric parameters (hepatosomatic and muscle-somatic indexes and perivisceral fat content) and swiftly dissected samples from WM (epaxial region) and RM. These samples were then immediately frozen and stored at −80°C until analysis of tissue components (water, protein, lipid and glycogen percentages), nucleic acid analysis (RNA and DNA content), enzyme activities (COX and CS) and stable isotopic composition ($\delta^{13}C$ and $\delta^{15}N$). To minimize protein modification or degradation, all dissecting and sampling procedures were performed on ice and completed within 5 min.

The soluble protein fraction was extracted from samples of RM and WM and prepared for the further proteomic analysis. Approximately 0.3 g of frozen muscle tissue was mechanically powdered in a mortar cooled with nitrogen liquid and homogenized in 3 mL ice-cold phosphate buffer (50 mM, pH 7.0) containing Protease Inhibitor Cocktail (SIGMA-ALDRICH, St. Louis, USA). An aliquot of 0.3 mL of each sample was stored for bulk protein determination. Homogenates were centrifuged at 15 000×*g* for 45 min at 4ºC and supernatants (soluble fraction) were collected. This soluble fraction consists mainly of sarcoplasmic proteins that are easily solubilized at low ionic strength[30]. The protein content of supernatants was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Madrid, Spain) and samples were aliquoted and stored at −80°C until required. Further protein purification was performed by precipitating the samples in 4 volumes of ice-cold acetone.

2.3 Proximal Composition and Metabolic Analyses

2.3.1 Proximal Composition Analysis

Briefly, the protein content was estimated on the basis of elemental nitrogen by burning the samples in an elemental analyzer (Elemental Analyzer Flesh 1112, ThermoFinnigan, Bremen, Germany) and using a correction factor of 6.25 g of protein per g of nitrogen. The total lipid content was measured gravimetrically after purification of methanol-chloroform extracts (2:1). Glycogen was obtained from tissue samples by alkaline-hydrolysis extraction following ethanol precipitation and was analyzed by a colorimetric reaction of anthrone-sulfuric acid using mussel glycogen as standard.

2.3.2 Nucleic Acid Quantification and Metabolic Enzyme Activity

Muscle nucleic acid content (RNA and DNA) was determined using a UV-based method following the procedures for fish samples described by Buckley and Bulow[31]. RNA and DNA from muscle samples were hydrolyzed to nucleotides and their concentration was calculated from their absorbance at 260 nm. Nucleic acid concentrations were expressed as μg RNA or DNA per mg of wet tissue.

Enzyme activities were assayed from crude muscle extracts obtained by homogenizing frozen tissue (50 mg/mL) in detergent solution (1.24 mM TRITON X-100, 1 mM EDTA, and 1 mM NaHCO3) and stabilizing solution (0.27 M EDTA and 5 mM 2-β-mercaptoethanol), 1:1 v/v. Homogenates for measuring CS (EC 2.3.3.1) and COX (EC 1.9.3.1) activity were centrifuged at 700×*g* at 4 °C for 10 min. CS activity was determined from absorbance increases at 412 nm of DTNB reagent, using oxalacetic acid as the substrate, following the method described by Srere[32]. We determined COX activity by adapting a commercial kit (CYTOC-OX1, Sigma-Aldrich Inc., St. Louis, MO). This colorimetric assay measures the decrease in ferrocytochrome c absorbance caused by oxidation of the latter by COX. Enzymatic activity measurements were performed in duplicate and expressed in milliunits (mUI) per mg wet tissue. One unit corresponds to the amount of the enzyme that converts 1 μmol of substrate into product per minute.

2.3.3 Isotopic Composition Analysis ($\delta^{15}N$ and $\delta^{13}C$)

Samples of diet and bulk tissues, together with the purified tissue fractions (glycogen, lipid and protein) of RM and WM, were lyophilized and ground into a homogenous powder for isotopic analysis. Aliquots ranging from 0.3 to 0.6 mg were weighed in small tin capsules. Samples were analyzed to determine the carbon and nitrogen isotope composition using a Mat Delta C Isotope Ratio mass spectrometer (Finnigan MAT, Bremen, Germany) coupled to a Flash 1112 Elemental Analyzer. Isotope ratios (^{15}N /^{14}N, ^{13}C /^{12}C) determined by isotope-ratio mass spectrometry are expressed in delta (δ) units (parts per thousand, ‰), as follows:

$$\delta = [(R_{sa}/R_{st}) - 1] \times 1000$$

where R_{sa} is the ^{15}N /^{14}N or ^{13}C /^{12}C ratio of samples and R_{st} is the ^{15}N /^{14}N or ^{13}C /^{12}C ratio of the international standards (Viena Pee Dee Belemnite for carbon and air for nitrogen). The same reference material analyzed over the experimental period was measured with ±0.2‰ precision. Nitrogen and carbon isotopic fractionation values ($\Delta\delta^{15}N$ and $\Delta\delta^{13}C$) were calculated as the difference between the δ value in tissue and the δ value in diet.

2.3.4 Statistics

The results from proximal composition, nucleic acid quantification, enzymatic activities and isotopic analyses are presented as mean ± standard error of the mean (SEM). Statistical unpaired sample t-test was used to compare samples from the E and C groups after checking normal distribution of data and homogeneity of variances by the Shapiro-Wilk and Levene's test respectively. Principal component analysis (PCA) was performed with all the variables analyzed to study the structure of the data and select individual candidates for proteomic analysis (**Figure 1**, and **Tables S1** and **S2** of Supporting Information). All statistical analyses were performed using SPSS v.16 (SPSS Inc., Chicago, IL).

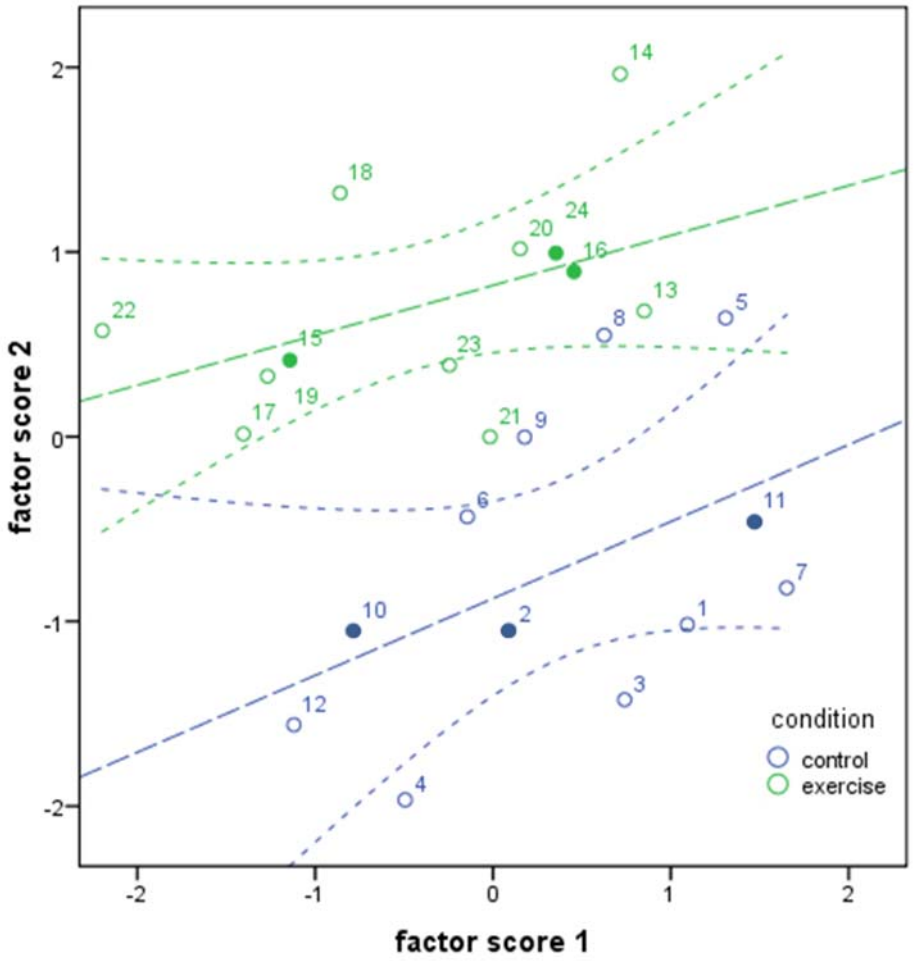

Figure 1. Principal Components Analysis of non-proteomic data set from all the fish of the study. Linear regression curves (dashed lines) with corresponding 95% confidence intervals (dotted lines) fitted to control ($r=0.500$, $p<0.05$) and exercise ($r=0.497$, $p<0.05$) groups. Filled circles indicate those fish selected for 2D-PAGE analysis.

2.4 Proteome Analysis of Muscle Soluble Fraction

The three most representative individuals from each condition according to PCA (**Figure 1**) were selected for proteomic analysis. Samples of the protein soluble fraction from each condition (C and E) and muscle type were used in the 2D-PAGE analyses. An amount of 300 µg of purified protein was dissolved into 450 µl of rehydration solution containing 7 M urea, 2 M thiourea, 2% w/v CHAPS and 0.5% v/v IPG buffer pH 3–10NL (Amersham Biosciences Europe, now GE Healthcare, Madrid, Spain), 80 mM DTT and 0.002% of bromophenol blue. The solution was then loaded onto 24-cm, pH 3–10NL IPG strips. Isoelectric focusing was performed using an IPGphor instrument (Amersham Biosciences), following the manufacturer's instructions (active rehydration at 50 V for 12 h followed by a linear gradient from 500 to 8000 V until 48000 V/h). Focused strips were equilibrated in two steps as follows: 15 min with equilibration buffer I (65 mM DTT, 50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue) and then 15 min with equilibration buffer II (135 mM iodoacetamide, 50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue). The equilibrated strips were applied directly onto 12.5% polyacrylamide gels, sealed with 0.5% w/v agarose and separated at a constant voltage of 50 V for 30 min followed by 200 V for about 6 h, until the blue dye reached the bottom on an Ettan DALT II system (Amersham Biosciences, Stockholm, Sweden). The resolved proteins were fixed for 1 h in 40% v/v methanol containing 10% v/v acetic acid and stained overnight using colloidal Coomassie Blue G-250. Gel staining was removed by washing steps with distilled water until the best visualization was achieved.

2.5 Gel Image Analysis and Statistics

Coomassie blue gels were scanned in a calibrated ImageScanner^{TM} III densitometer (GE Healthcare, Barcelona, Spain) and digital images captured at a resolution of 300 dpi in gray scale mode by Labscan 6.0 software (GE Healthcare, Barcelona, Spain) were saved as uncompressed TIFF files. Gels from three independent biological replicates were analyzed using the software package ImageMaster 2D version 6.01 (GE Healthcare, Barcelona, Spain), which can be used to detect and obtain normalized volume values of the protein spots present in the gels. This detection was performed by the automated routines in the software, combined with manual editing to remove artifacts. The protein spots were quantified using the percentage volume criterion (% Vol), which is automatically calculated by the ImageMaster software. After spot detecting and editing, a scatter plot of normalized spot volumes was performed to verify reproducibility of 2D-gels. Relative quantification of each matched spot represented as % Vol. in each gel was plotted against the rest of gels in a mode all vs. all (**Figure S1** of Supporting Information).

Protein spots that varied in abundance between C and E samples were analyzed for significance by unpaired sample *t*-test (SPSS v.16; Chicago, IL, USA). The Shapiro-Wilk test was previously used to ensure the normal distribution of data, and the equality of variances was determined by statistical Levene's test. The candidate spots selected for MS analysis presented ≥ 2-fold change in normalized volume. Exceptionally, spots with 1.9-fold changes and *p*-values ≤0.01 were also considered as well as spot 80 (1.9-fold and $p \leq 0.05$) which is the neighbor to spot 81 and was identified as the same protein. We used the Perseus program in the MaxQuant environment software (http://www.maxquant.org) to generate and view hierarchical clustering and protein abundance heat maps.

2.6 Protein Identification

2.6.1 In-gel Digestion

In-gel digestion was performed automatically (Progest, Genomic Solutions, Cambridgeshire, UK). Protein spots were excised with a razor blade and then washed sequentially with ammonium bicarbonate buffer (25 mM) and acetonitrile (ACN). The proteins were reduced (10 mM DTT, 30 min, 56ºC) and alkylated (55 mM Iodoacetamide, 15 min, 21ºC, in dark). After sequential washings with buffer and ACN, proteins were digested with trypsin (Trypsin Gold, Promega; 80 ng/sample, 37°C, overnight). The resulting peptides were collected from the gel matrix with 10% formic acid (FA) and ACN. The extracts were then pooled and dried in a vacuum centrifuge. Trypsin-digested peptide samples were analyzed by MALDI-TOF/TOF MS (4700 Proteomics Analyzer, Applied Biosystems, CA, USA) or LC-MS/MS. Proteins that were not successfully identified by MALDI were further analyzed by LC-MS/MS using CapLC-ESI-Q-TOF (Micromass-Waters, Manchester, UK) for WM or nanoHPLC (nanoAcquity; Waters, Milford, MA, USA) coupled to mass spectrometer (Orbitrap-Velos; Thermo-Scientific, Bremen, Germany) for RM.

2.6.2 MALDI-TOF/TOF-MS Analysis

The trypsin-digested samples were dissolved in 5 µL 0.1% trifluoroacetic acid (TFA) in 50% ACN. Typically, a 0.5-µL aliquot was mixed with the 0.8 µL of a matrix solution (5 mg/mL α-CHCA [Waters, Barcelona, Spain] in 0.1% TFA in 50% ACN) and directly spotted onto a MALDI-plate. MS spectra were acquired in a mass range of 900 to 4000 *m/z* in positive reflector mode (a voltage of 20 kV in Source 1 and a laser intensity range from 3000 to 4500). Typically, 500 shots per spectrum were taken and up to seven major peaks were selected to be

further characterized by MS/MS analysis. MS/MS spectra were acquired using CID with atmospheric air as the collision gas. An MS/MS 1 kV positive mode was used. MS and MS/MS spectra from the same spot were merged in a single *mgf* file prior to being submitted for a database search.

2.6.3 LC-MS/MS Analysis

Cap-LC-ESI-Q-TOF. Samples of the trypsin-digested peptide were suspended in 25 µL 1% FA solution, and 4 µL were analyzed by on-line LC-MS/MS chromatographic separation into a reverse phase capillary C_{18} column (75 µm of internal diameter, 15 cm length, PepMap column, LC Packings, Amsterdam, The Nederlands). The elution gradient was 5-65%B in 30 min (A: 2%MeCN / 98% water, 0.1% FA; B: 90% MeCN, 0.1% FA). The eluted peptides were ionized via coated nano-ES needles (PicoTip™, New Objective, Celta Ingenieros, A Coruña, Spain). Applied voltage was: 2200V to the capillary and 80V to the cone voltage. Collision energy in the CID (collision-induced dissociation) was 20-35 eV and argon was the collision gas. Data were generated as *pkl* format.

NanoHPLC Orbitrap-Velos. The resulting peptides from the digestion were dissolved in 50-100 µl of 0.1% FA and 4 µl was injected for the chromatographic separation into a reverse phase capillary C_{18} column (75 µm of internal diameter, 10 cm length, nano Acquity, 1.7 µm BEH column, Waters, Madrid, Spain). The elution gradient was from 1% to 40% of mobile phase B (0.1% FA in ACN) for 20 min followed by a gradient from 40% to 60% B for 5 min, with a flow of 250 nL/min (complementary mobile phase A: 0.1% FA). Eluted peptides were ionized via electrospray using coated nano-ES needles. A capillary voltage ranging from 1900 to 2100 V was applied. Mass range acquisition in MS mode was from 350 to 1700 m/z and was measured in a full scan MS mode of the Orbitrap with a resolution of 60,000 FWHM at 400 m/z. Five additional abundant peptides (minimum intensity of 500 counts) were selected in each MS analysis to be fragmented in the CID with helium as collision gas (38% of collision energy). Data were acquired with Thermo Xcalibur (v.2.1.0.1140; Thermo Electron, San Jose, CA, USA) software in *raw* data format and converted into *mgf* files by Proteome Discoverer v.1.3.0.339 (Thermo Fisher Scientific, Waltham, MA, USA) for further identification analysis.

2.6.4 Database Search

MALDI and Q-TOF data were analyzed using MASCOT *on-line* (http://www.matrixscience.com) server, while MASCOT *in-house* search engine (Version 2.3,

Matrixscience, London, UK) was used for Orbitrap data because of high number of spectra. All files were searched against the NCBI Actinopterygii database (*on line* search: 214,185 sequences at January 2012; *in house*: 186,963 sequences, September 9, 2011) along with "contaminants" subset (*on line*: 262 sequences at January 2012; *in house*: 248 sequences, May 13, 2010) in order to discard common contaminants. In case of no significant protein match hits, further search against the EST_vertebrates Actynopterygii nucleic acid database (31,127,172 sequences, at January 2012) was performed. We used the following parameters for the searches: 2 missed cleavages, carbamidomethyl of cysteine as fixed modifications and oxidation of methionine and pyro-Glu (N-term glutamine) as variable modifications. Peptide tolerance was 100 ppm and 0.25 Da for MS and MS/MS spectra respectively, from MALDI and Q-TOF data whereas for Orbitrap was 10 ppm and 0.6 Da for MS and MS/MS spectra respectively. An automatic decoy database was included in the search of the latter data. Protein identifications were accepted when they were established at > 95% probability. All possible protein identifications from analyses that met the above criteria were reported for each gel spot (**Table S3** of Supporting Information). However, the protein identification with the highest score, discarding contaminants, was selected in the case of redundant protein identifications. Enrichment analyses of Gene Ontology (GO) annotation terms for biological processes were performed by the Batch-Genes tool produced by GOEAST (http://omicslab.genetics.ac.cn/GOEAST/).

3. RESULTS

3.1 Body Indices and Muscle Metabolic and Isotopic Analyses

Fish subjected to a swimming speed of 1.5 BL/s for 1 month presented a significantly higher growth rate (SGR) and showed no differences in food intake (**Table 1**). Other body indices, such as the condition factor, hepatosomatic index, body percentage of perivisceral fat and muscle-somatic index (MSI), did not alter significantly. Exercising fish showed a 20% reduction in lipid content of WM while glycogen content decreased by 30% in RM compared to the C group values; however, protein content in RM and WM samples did not differ despite the differences observed in the protein soluble fraction of these two muscle types (WM: 28.5±0.8 vs. RM: 35.8±1.2 g of soluble protein/100g of total protein, $p < 0.05$). The increment in total body weight with similar MSI and protein percentage implies increased muscle mass with a net gain of total protein content in muscle. Accordingly, protein synthesis (RNA:protein ratio) and transduction (RNA:DNA ratio) capacities were enhanced in WM of fish under sustained activity

(**Figure 2**). Furthermore, exercise increased the COX activity of WM by about 60% while CS activity decreased by nearly 25%. These observations thus explain the two-fold increase in the COX:CS ratio. In contrast to WM, RM showed no changes in nucleic acid ratios or oxidative/aerobic enzyme activities between the C and E groups.

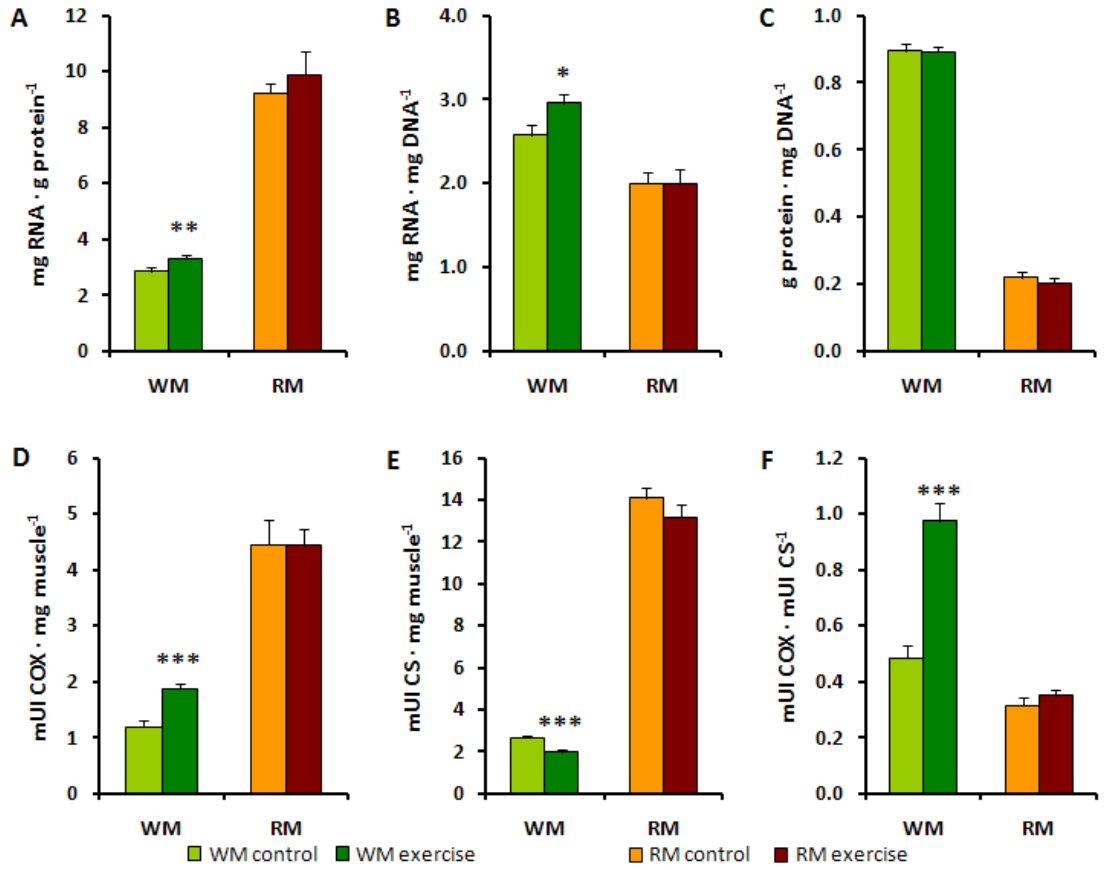

Figure 2. Changes in nucleic acid ratios (**A**, RNA:protein; **B**, RNA:DNA and **C**, DNA:protein ratios) and enzyme activities (**D**, cytochrome *c* oxidase (COX); **E**, cytrate synthase (CS) and **F**, COX:CS ratio) in white (WM) and red (RM) muscles of gilthead sea bream voluntarily swimming (control) or subjected to sustained exercise (1.5 BL/s, for 4 weeks). Values are mean ± SEM, n = 12 fish. Asterisks indicate significant differences between conditions (t-test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Exercise increased the $\delta^{13}C$ of WM (**Figure 3**) as a result of the use of lipid depots. Specifically, the lipid and glycogen contents of WM were enriched in $\delta^{13}C$ in response to exercise (**Table 2**). In contrast, no differences in ^{15}N fractionation ($\Delta\delta^{15}N$, calculated as the difference in $\delta^{15}N$ between tissue and diet) of WM (C: 0.96±0.04 vs. E: 0.92±0.06 ‰) or RM (C: 0.54±0.10 vs. E: 0.55±0.09 ‰) were observed between groups, thereby indicating a similar protein turnover for the two groups.

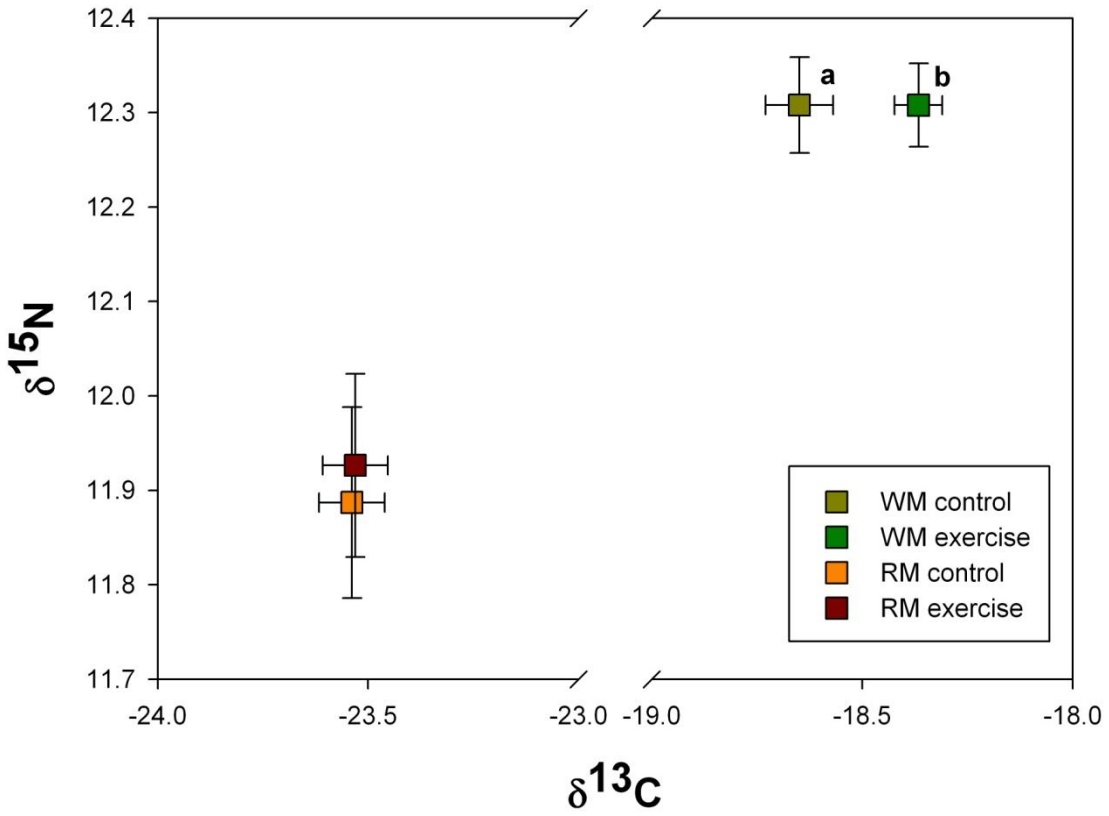

Figure 3. Isotopic signature of white (WM) and red (RM) muscles in gilthead seabream voluntarily swimming (control) or subjected to sustained exercise (1.5 BL/s, for 4 weeks). Values are mean ± SEM, n = 12 fish. Letters indicate significant differences in $\delta^{13}C$ between conditions (t-test; $p<0.01$). Food isotopic signature: $\delta^{15}N$, 11.3±0.4‰; $\delta^{13}C$, -20.9±0.4‰.

The plot of factor 1 versus factor 2 of the PCA analysis (**Figure 1**) provided a good discrimination between fish from C and E groups (see **Table S1** and **S2** of Supporting Information for further data about factor variability and factor loadings respectively). Both groups can be fitted to a linear regression, being those individuals closest to the fitted line the most representative of each population.

Table 2. Isotopic composition of reserves in white and red muscles of gilthead sea bream voluntarily swimming (control) or subjected to sustained exercise (1.5 BL/s, for 4 weeks).

	White muscle			Red muscle		
	Control	Exercise		Control	Exercise	
protein $\delta^{15}N$	13.10 ± 0.04	13.09 ± 0.04		12.76 ± 0.10	12.56 ± 0.06	
protein $\delta^{13}C$	-18.16 ± 0.06	-18.04 ± 0.06		-21.04 ± 0.64	-21.29 ± 0.59	
lipid $\delta^{13}C$	-25.80 ± 0.05	-25.65 ± 0.04	*	-25.79 ± 0.03	-25.78 ± 0.02	
glycogen $\delta^{13}C$	-22.31 ± 0.17	-21.51 ± 0.25	*	-22.35 ± 0.25	-22.16 ± 0.17	

Values are mean ± SEM, n = 12 fish. (*) Significant divergence by t-test, $p<0.05$

3.2 Detection and Identification of Differentially Expressed Proteins and Grouping by Putative Functionality

The sarcoplasmic protein profiles in an extended range of p*I* (3-10) from a representative gel of WM and RM are shown in **Figures 4** and **5** respectively. These gels were chosen as a reference gel for the study, and the profiles of the other gels were matched against them. The number of spots detected on each gel varied between WM (716 spots) and RM (432 spots). Around 12 – 13% of total spots in the WM and RM proteome of exercised fish (91 and 50 spots, respectively) presented significant differences (*t*-test, $p<0.05$). Of these, 68 protein spots were over-expressed in response to continuous swimming and 23 were under-expressed in the WM proteome. In contrast, most of the altered spots were under-expressed in the RM proteome and only 8 spots were over-expressed. The candidate spots selected for MS analysis are depicted in **Figures 4** (WM) and **5** (RM) along with the hierarchical agglomerative cluster of Z-score-transformed intensities (%Vol.). The variability of proteins differentially expressed in individual samples can be clearly appreciated from the PCA analysis (**Figure 6**). Identities of spots analyzed along with further identification information and fold-change (E vs. C) are summarized in **Table 3** (WM) and in **Table 4** (RM). All digested spots were identified as protein sequences that have been described in teleost species. Top score identifications (excluding contaminants) were selected for those spots where more than one protein identification were possible (**Table S4** of Supporting Information), except for spot 53 of RM because of most of the peptides matched were shared with a contaminant protein hit. In this case the next protein hit with higher score was selected.

Putative actions of the identified proteins, on the basis of their proximity in function or their involvement in the same metabolic pathway, are shown in **Figure 7** for both muscle types. Proteins were clustered to elucidate changes in the muscular function of gilthead sea bream under an exercise challenge. The results of GO enrichment analysis are available in Supporting Information (**Tables S5** and **S6**).

3.2.1 Protein Expression Profiling of WM

Five of the seven altered spots grouped in the GO term called “muscle contraction” (GO:0006963; $p=6.94\cdot10^{-9}$) increased under exercise and were identified as myomesin (spots 3, 5 and 7) and phosphoglycerate mutase (371 and 373), while myosin light chains 1 and 2 (466 and 477 respectively) were under-expressed. A spot identified as parvalbumin (457), a Ca binding protein involved in muscle relaxation, was also under-expressed whereas two spots identified as creatine kinase (244 and 410) were over-expressed.

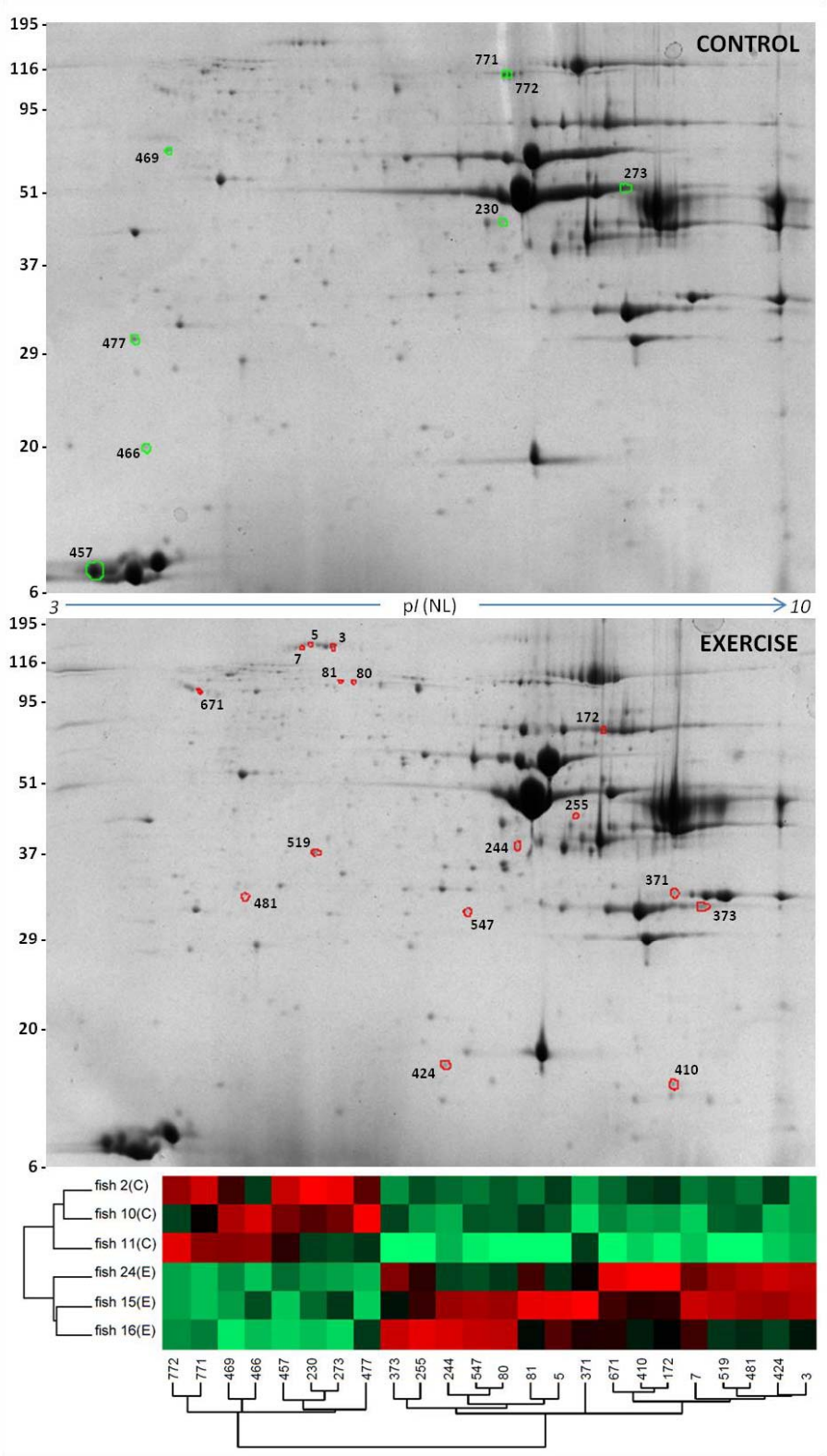

Figure 4. Representative 2D gels showing the protein expression profiles obtained from white muscle soluble protein fractions of control and exercise groups. Proteins (300 µg) were separated in the first dimension on pH 3-10 non-linear IPG strips, followed by SDS-PAGE on 12.5% w/v gels. Gels were stained with colloidal CBB. Candidate spots listed in the gels were identified by MS. Spots outlined in red and green indicate the proteins that were over-expressed and under-expressed by exercise, respectively. Below each gel, the expression data of selected spots is shown in agglomerative hierarchical cluster of Z-score transformed intensity values. Cluster coloration indicates protein abundance in the sample (red indicates higher abundance, green lower abundance and black unchanged abundance).

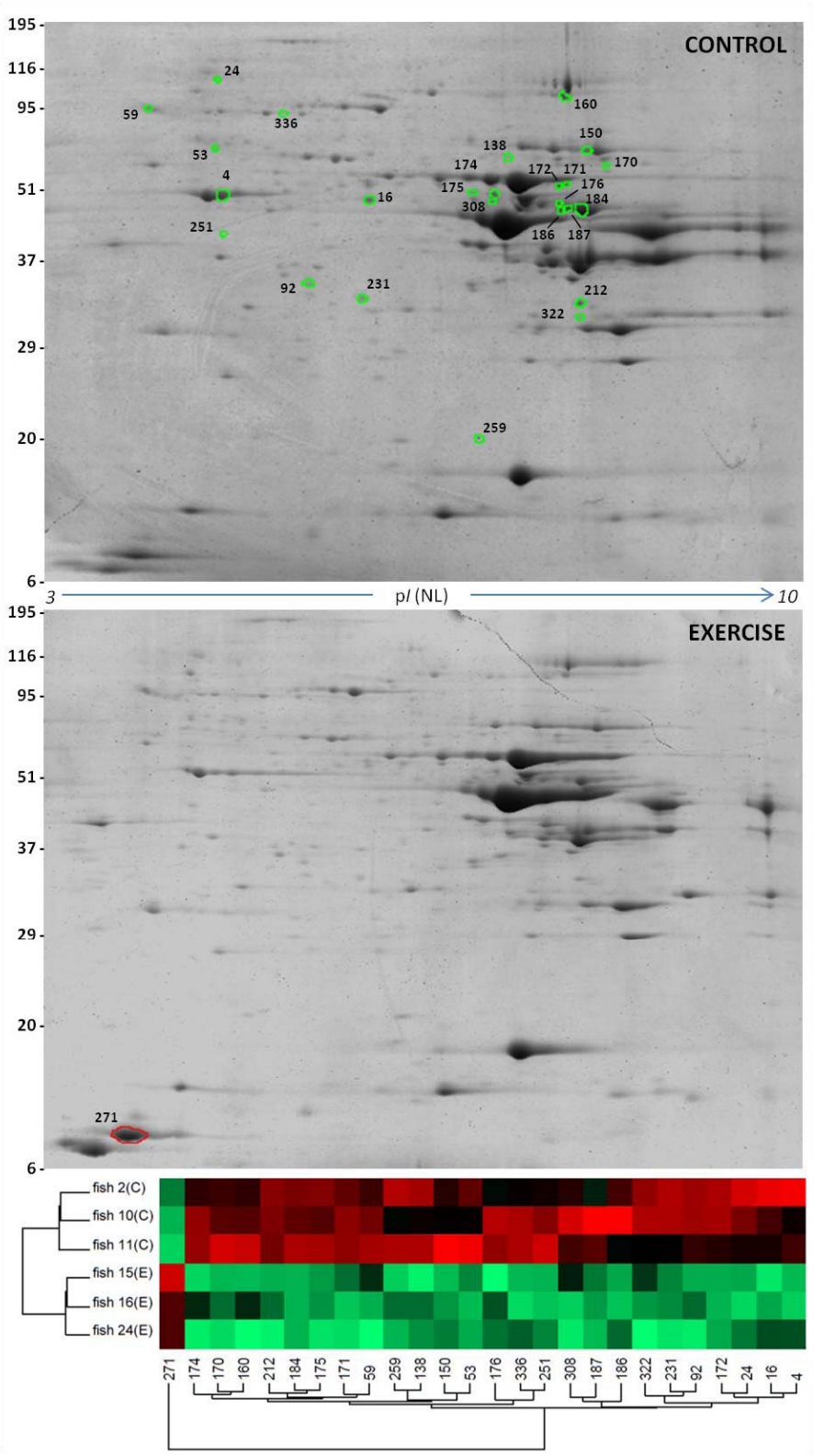

Figure 5. Representative 2D gels showing the protein expression profiles obtained from red muscle soluble protein fractions of control and exercise groups. Proteins (300 µg) were separated in the first dimension on pH 3-10 non-linear IPG strips, followed by SDS-PAGE on 12.5% w/v gels. Gels were stained with colloidal CBB. Candidate spots listed in the gels were identified by MS. Spots outlined in red and green indicate the proteins that were over-expressed and under-expressed by exercise, respectively. Below each gel, the expression data of selected spots is shown in agglomerative hierarchical cluster of Z-score transformed intensity values. Cluster coloration indicates protein abundance in the sample (red indicates higher abundance, green lower abundance and black unchanged abundance).

Table 3. Identification of differentially expressed protein spots in white muscle of sea bream in response to sustained exercise (1.5 BL/s, for 4 weeks)

Spot[a]	Accession no.	Protein name[b]	Species	Symbol[c]	GO term[d]	theorical kDa / pI	Score[e]	Peptides[f]	SC[g] (%)	Fold[h] change	*p*-val.[i]
					Over-expressed						
3	gi|348503713	PREDICTED: myomesin-1-like	*O. niloticus*	MYOM1	GO:0006936 muscle contraction	166.3 / 6.0	426 / 37	11(8) / 9(6)	6	11.3	≤ 0.01
7	gi|348503713	PREDICTED: myomesin-1-like	*O. niloticus*	MYOM1	GO:0006936 muscle contraction	166.3 / 6.0	241 / 39	4(4) / 4(4)	3	4.4	≤ 0.01
373	gi|348513753	PREDICTED: phosphoglycerate mutase 2-like	*O. niloticus*	PGAM2	GO:0006096 glycolysis	28.9 / 8.8	440 / 37	18(11) / 8(6)	27	3.2	≤ 0.05
481*	gi|207898058	EM_EST:FM153890 (glutathione S-transferase)	*Sparus aurata*	GSTM2	GO:0006749 glutathione metabolic process	26.5 / 5.4	96 / 87	15(10) / 7(2)	28	3.2	≤ 0.05
424	gi|31335193	lactate dehydrogenase-A	*C. punctipinnis*	LDHA	GO:0006096 glycolysis	36.6 / 6.9	104 / 36	3(2) / 2(2)	6	3.1	≤ 0.05
255	gi|348518580	PREDICTED: glycogen phosphorylase, muscle form-like	*O. niloticus*	PYGM	GO:0005977 glycogen metabolic process	97.7 / 6.6	193 / 37	5(1) / 4(1)	3	3.0	≤ 0.05
172	gi|45501385	Pkm2a protein	*Danio rerio*	PKM2	GO:0006096 glycolysis	58.6 / 6.4	184 / 36	5(3) / 4(2)	7	2.9	≤ 0.05
671	gi|224551742	warm temperature acclimation-related 65 kDa protein	*Sparus aurata*	wap65	GO:0006879 cellular iron ion homeostasis	49.8 / 5.4	273 / 36	7(3) / 6(3)	13	2.9	≤ 0.05
244*	gi|268315573	muscle-type creatine kinase CKM1	*P. stellatus*	CKM	GO:0006600 creatine metabolic process	43.2 / 6.2	234 / 66	23(11) / 7(4)	24	2.7	≤ 0.05
410	gi|126211553	creatine kinase 1	*P. reticulata*	CKM	GO:0006600 creatine metabolic process	43.2 / 6.4	171 / 36	6(2) / 4(1)	6	2.6	≤ 0.05
371*	gi|226441997	phosphoglycerate mutase 2	*G.mirabilis*	PGAM2	GO:0006096 glycolysis	25.2 / 6.7	94 / 66	18(8) / 7(2)	28	2.5	≤ 0.05
5	gi|348503713	PREDICTED: myomesin-1-like	*O. niloticus*	MYOM1	GO:0006936 muscle contraction	166.3 / 6.0	156 / 38	3(3) / 3(3)	2	2.3	≤ 0.05
519*	gi|51011067	pyruvate kinase, muscle, b	*Danio rerio*	PKM2	GO:0006096 glycolysis	27.0 / 6.4	67 / 66	15(6) / 5(2)	25	2.2	≤ 0.05
81	gi|317419952	Glycyl-tRNA synthetase	*D. labrax*	GARS	GO:0006412 translation	86.3 / 6.2	393 / 36	12(8) / 9(6)	11	2.0	≤ 0.05
547*	gi|298361180	peroxiredoxin 6	*Sparus aurata*	PRDX6	GO:0006979 response to oxidative stress	24.7 / 6.3	132 / 66	21(9) / 7(2)	24	2.0	≤ 0.05
80*	gi|317419952	Glycyl-tRNA synthetase	*D. labrax*	GARS	GO:0006412 translation	86.3 / 6.2	89 / 66	19(12) / 7(4)	12	1.9	≤ 0.05
					Under-expressed						
771	gi|57547484	adenosine monophosphate deaminase	*P. flesus*	AMPD	GO:0006144 purine base metabolic process	72.5 / 7.1	314 / 38	9(4) / 9(4)	15	-7.1	≤ 0.01
772	gi|57547484	adenosine monophosphate deaminase	*P. flesus*	AMPD	GO:0006144 purine base metabolic process	72.5 / 7.1	222 / 39	7(2) / 6(2)	8	-5.9	≤ 0.05
230	gi|47221527	unnamed protein product (fructose-1,6-bisphosphatase)	*T. nigroviridis*	FBP1	GO:0006094 gluconeogenesis	39.0 / 8.4	160 / 38	5(3) / 3(2)	9	-3.1	≤ 0.05
477	gi|7678732	myosin light chain 1	*P. argentata*	MYL1	GO:0006936 muscle contraction	20.8 / 4.6	277 / 36	7(5) / 6(4)	37	-2.6	≤ 0.05
466	gi|5852838	myosin light chain 2	*Sparus aurata*	MYL2	GO:0006942 reg. of striated muscle contraction	19.2 / 4.6	63 / 38	1(1) / 1(1)	5	-2.2	≤ 0.05
457	gi|48476449	parvalbumin-like protein	*Sparus aurata*	PVALB	GO:0005509 calcium ion binding	11.6 / 4.6	129 / 37	3(2) / 3(2)	35	-2.1	≤ 0.01
273	gi|197631857	phosphoglycerate kinase 1	*Salmo salar*	PGK1	GO:0006096 glycolysis	44.9 / 8.3	248 / 38	4(4) / 4(4)	7	-2.0	≤ 0.05
469	gi|348506725	PREDICTED: ATP synthase subunit beta, mitochondrial-like	*O. niloticus*	ATP5B	GO:0006091 gen.of prec. metabolites & energy	55.2 / 5.1	380 / 38	8(5) / 7(4)	18	-1.9	≤ 0.01

[a]Asterisks indicate those spots analyzed by MALDI, the rest of spots were analyzed by Q-TOF. [b]Proteins in parentheses derived from BLASTp sequence analysis. [c]Symbol of gene product from GeneCards v3.07. [d]Associated Gene Ontology term. [e]MASCOT protein score / protein score cutoff or ion score identity threshold ($p<0.05$) for MALDI or Q-TOF identifications respectively. [f]For MALDI identifications: no. ions submitted for PMF (no. ions matched) / no. ions fragmented (no. MS ions matched); for Q-TOF identifications: no. ions matched (significant) / no. sequences matched (significant). [g]Sequence Coverage. [h]C vs E (>0, up-reg.;<0, down-reg.). [i]*t*-test (n=3).

Table 4. Identification of differentially expressed protein spots in red muscle of sea bream in response to sustained exercise (1.5 BL/s, for 4 weeks)

Spot[a]	Accession no.	Protein name[b]	Species	Symbol[c]	GO term[d]	theorical kDa / pI	Score[e]	Peptides[f]	SC[g] (%)	Fold[h] change	*p*-val.[i]
					Over-expressed						
271*	gi|261825915	parvalbumin	*Sparus aurata*	PVALB	GO:0005509 calcium ion binding	11.6 / 4.8	86 / 66	10(6) / 4(3)	31	2.8	≤ 0.01
					Under-expressed						
175	gi|93115142	mitochondrial isocitrate dehydrogenase 2-like	*O. mossambicus*	IDH2	GO:0006099 tricarboxylic acid cycle	50.9 / 7.6	5109 / 13	86(58) / 16(13)	31	-4.8	≤ 0.001
187	gi|41388972	Pgk1 protein	*Danio rerio*	PGK1	GO:0006096 glycolysis	45.1 / 6.5	3610 / 13	103(82) / 12(9)	32	-3.8	≤ 0.05
16*	gi|50539866	pyruvate dehydrogenase E1 alpha 1	*Danio rerio*	PDHA1	GO:0006096 glycolysis	44.1 / 7.6	76 / 66	21(9) / 7(3)	10	-2.9	≤ 0.05
172	gi|41055718	fumarate hydratase, mitochondrial precursor	*Danio rerio*	FH	GO:0006099 tricarboxylic acid cycle	55.0 / 9.0	10259 / 13	162(117) / 12(8)	20	-2.8	≤ 0.01
24	gi|146448775	cell division cycle 48	*P. olivaceus*	VCP	GO:0006511 ubiquitin-dep. protein catabolic proc	89.9 / 5.2	7125 / 13	64(51) / 34(28)	47	-2.7	≤ 0.01
160*	gi|225706838	Short chain 3-hydroxyacyl-CoA dehydrogenase, mitoc. prec.	*O. mordax*	HADH	GO:0006635 fatty acid beta-oxidation	33.3 / 8.4	73 / 66	12(5) / 5(2)	9	-2.7	≤ 0.05
170	gi|14009437	mitochondrial ATP synthase alpha-subunit	*Cyprinus carpio*	ATP5A1	GO:0006091 gen.of prec. metabolites and energy	59.7 / 9.3	2657 / 13	41(27) / 24(17)	35	-2.7	≤ 0.01
176	gi|213514668	medium-chain specific acyl-CoA dehydrogenase, mitoc.	*Salmo salar*	ACADM	GO:0006635 fatty acid beta-oxidation	46.4 / 7.5	430 / 13	22(11) / 5(3)	9	-2.7	≤ 0.05
53	gi|209153384	disulfide-isomerase A3 precursor	*Salmo salar*	PDIA3	GO:0045454 cell redox homeostasis	55.4 / 5.5	328 / 13	4(3) / 4(3)	8	-2.6	≤ 0.01
251	gi|41055718	fumarate hydratase, mitochondrial precursor	*Danio rerio*	FH	GO:0006099 tricarboxylic acid cycle	55.0 / 9.0	461 / 13	4(4) / 1(1)	3	-2.6	≤ 0.01
308	gi|93115142	mitochondrial isocitrate dehydrogenase 2-like	*O. mossambicus*	IDH2	GO:0006099 tricarboxylic acid cycle	50.9 / 7.6	2884 / 13	44(34) / 15(12)	26	-2.6	≤ 0.05
212	gi|209730966	Hydroxyacyl-coenzyme A dehydrogenase, mitoc. prec.	*Salmo salar*	HADH	GO:0006635 fatty acid beta-oxidation	33.1 / 8.4	991 / 13	60(26) / 7(4)	18	-2.4	≤ 0.001
174*	gi|41054651	isocitrate dehydrogenase [NADP], mitochondrial	*Danio rerio*	IDH2	GO:0006099 tricarboxylic acid cycle	50.9 / 8.4	158 / 66	19(9) / 7(4)	11	-2.3	≤ 0.01
259	gi|328677135	hypothetical protein (cofilin-2)	*E. bruneus*	CFL2	GO:0003779 actin binding	19.0 / 6.8	578 / 13	12(4) / 8(3)	34	-2.3	≤ 0.01
92	gi|47225516	unnamed prot. prod. (3-hydroxyisobutyrate dehydrogenase)	*T. nigroviridis*	HIBADH	GO:0006098 pentose-phosphate shunt	32.5 / 6.2	5242 / 13	56(50) / 11(11)	43	-2.1	≤ 0.01
138	gi|47215116	unnamed protein product (adenylosuccinate lyase)	*T. nigroviridis*	ADSL	GO:0006163 purine nucleotide metabolic process	55.2 / 6.3	316 / 13	12(6) / 8(5)	14	-2.1	≤ 0.01
171	gi|47225614	unnamed protein product (fumarate hydratase)	*T. nigroviridis*	FH	GO:0006099 tricarboxylic acid cycle	48.9 / 9.3	3816 / 13	45(28) / 14(13)	19	-2.1	≤ 0.001
184*	gi|31322103	creatine kinase mitochondrial isoform	*C. aceratus*	CKMT2	GO:0006936 muscle contraction	47.1 / 8.5	192 / 66	49(13) / 7(3)	28	-2.1	≤ 0.01
336	gi|119692141	glucose regulated protein 75	*Sparus aurata*	HSPA9	GO:0006950 response to stress	69.1 / 5.6	1511 / 13	29(25) / 17(13)	31	-2.1	≤ 0.01
4*	gi|6653228	skeletal alpha-actin	*Sparus aurata*	ACTA1	GO:0006936 muscle contraction	42.2 / 5.3	340 / 66	22(14) / 7(6)	29	-2.0	≤ 0.05
150	gi|74096033	pyruvate kinase	*T. rubripes*	PKM2	GO:0006096 glycolysis	58.6 / 8.0	1757 / 13	59(35) / 16(12)	28	-2.0	≤ 0.05
186	gi|31322103	creatine kinase mitochondrial isoform	*C. aceratus*	CKMT2	GO:0006936 muscle contraction	47.1 / 8.5	8844 / 13	228(126) / 17(11)	38	-2.0	≤ 0.05
322	gi|47221217	unnamed protein product (nipsnap-like 2)	*T. nigroviridis*	GBAS	GO:0005515 protein binding	32.0 / 9.3	1692 / 13	24(15) / 7(6)	19	-2.0	≤ 0.05
59	gi|224551742	warm temperature acclimation-related 65 kDa protein	*Sparus aurata*	wap65	GO:0006879 cellular iron ion homeostasis	49.8 / 5.4	2516 / 13	89(40) / 26(13)	53	-1.9	≤ 0.01
231	gi|225706936	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitoc. prec.	*O. mordax*	ECH1	GO:0006635 fatty acid beta-oxidation	33.5 / 6.5	1286 / 13	19(15) / 10(9)	25	-1.9	≤ 0.01

[a]Asterisks indicate those spots analyzed by MALDI, the rest of spots were analyzed by Orbitrap. [b]Proteins in parentheses derived from BLASTp sequence analysis. [c]Symbol of gene product from GeneCards v3.07. [d]Associated Gene Ontology term. [e]MASCOT protein score / protein score cutoff or ion score identity threshold (p<0.05) for MALDI or Orbitrap identifications respectively. For Percolator score for Orbitrap identifications) [f]For MALDI identifications: no. ions submitted for PMF (no. ions matched) / no. ions fragmented (no. MS ions matched); for Orbitrap identifications: no. ions matched (significant) / no. sequences matched (significant). [g]Sequence Coverage. [h]C vs E (>0, up-reg.;<0, down-reg.). [i]*t*-test (n=3).

Most of the spots associated with "carbohydrate metabolic process" (GO:0005975; p=4.15·10^{-5}) were over-expressed by exercise: namely glycogen phosphorylase (255) and three enzymes of glycolysis (GO:0006091; p=1.31·10^{-8}): lactate dehydrogenase (424), phosphoglycerate mutase (371 and 373) and pyruvate kinase (172 and 519). In contrast, phosphoglycerate kinase (273), a glycolytically reversible enzyme, was under-expressed together with fructose bis-phosphatase (230), both involved in gluconeogenesis (GO:0006094, p=6.54·10^{-6}). All the spots related to carbohydrate metabolism, except fructose bis-phosphatase, were also grouped into the "generation of precursor of metabolites and energy" term (GO:0006091, p=9.70·10^{-8}), together with the beta-subunit of ATP synthase (469).

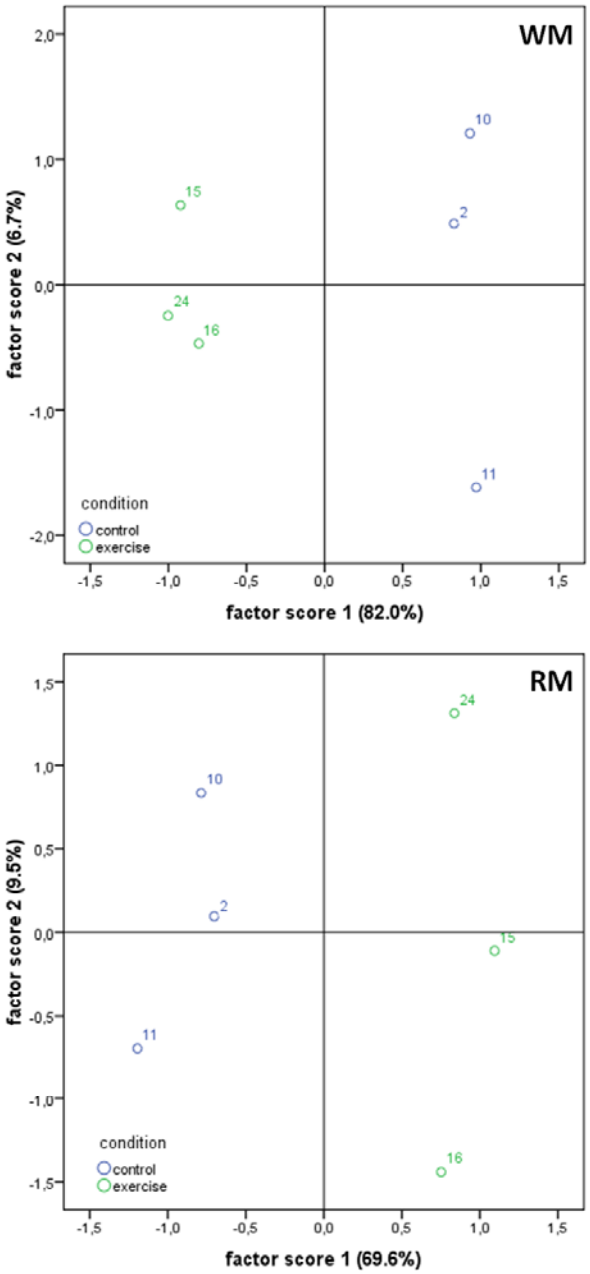

Figure 6. Principal Components Analysis of intensity values (%Vol) of proteins differentially expressed by exercise in individual samples from white (WM) and red (RM) muscle. Factors 1 and 2 represent the first and second principal components, with the percentage of explained variance indicated in parentheses.

Finally, a heterogeneous group includes several proteins related to detoxification pathways (glutathione-S-transferase, 481 and peroxirredoxin-6, 547) and stress response (warm temperature acclimation-related protein-65, 671) together with protein synthesis (glycyl-tRNA synthetase, 80 and 81) which were all over-expressed in the E group. In contrast, AMP deaminase (772 and 771), which is involved in nucleotide metabolism, was under-expressed under moderate and sustained swimming.

3.2.2 Protein Expression Profiling of RM

All altered RM spots grouped into the "muscle contraction" term (p=0.013) were under-expressed in response to sustained swimming: one spot identified as alpha-actin (4) and two as mitochondrial creatine kinase (184 and 186). Moreover, one spot identified as cofilin-2 (259), which is involved in actin filament dynamics, were under-expressed while a form of parvalbumin (271), unlike the form found in WM, was over-expressed.

In contrast to WM, in RM all the protein spots related to the "carbohydrate metabolic process" (GO:0005975; $p=7.41\cdot10^{-5}$) were under-expressed. Among these, all the spots involved in "glycolysis" (GO:0006096; p=0.003), were under-expressed by exercise: namely pyruvate dehydrogenase-alpha1 (16), pyruvate kinase (150), and phosphoglycerate kinase (187). Other proteins included in carbohydrate metabolism, such as disulfide isomerase (53), were also under-expressed. Similarly, proteins related to other energy generation pathways were under-expressed in RM under sustained swimming, such as 3-hydroxybutyrate dehydrogenase (92), which is involved in pentose-shunt pathway; isocitrate dehydrogenase (174, 175 and 308), and fumarate hydratase (171, 172 and 251), both belonging to the 'TCA cycle' (GO:0006099; $p=1.38\cdot10^{-9}$); and 3-hydroxyacyl-CoA dehydrogenase short chain (160 and 212), acyl-CoA dehydrogenase medium chain (176) and Δ3,5-Δ2,4-dienoyl-CoA isomerase (231), which are all involved in 'fatty acid β-oxidation' (GO:0006635; $p=3.56\cdot10^{-4}$). Most of these enzymes were grouped in the "generation of precursor metabolites and energy" term (GO:0006091; $p=5.36\cdot10^{-12}$), together with adenylosuccinate lyase (138) and ATP-synthase α-subunit (170), which were also under-expressed.

Including the latter two proteins, an arbitrary group of heterogeneous proteins was formed on the basis of their potential relation to homeostatic processes. All these proteins were also under-expressed by exercise, such as one isoform of WAP65 (59), a couple of chaperons so-called cell division cycle 48 (24) and glucose regulated protein 75 (336), and nipsnap-2 (322) which is involved in vesicular trafficking.

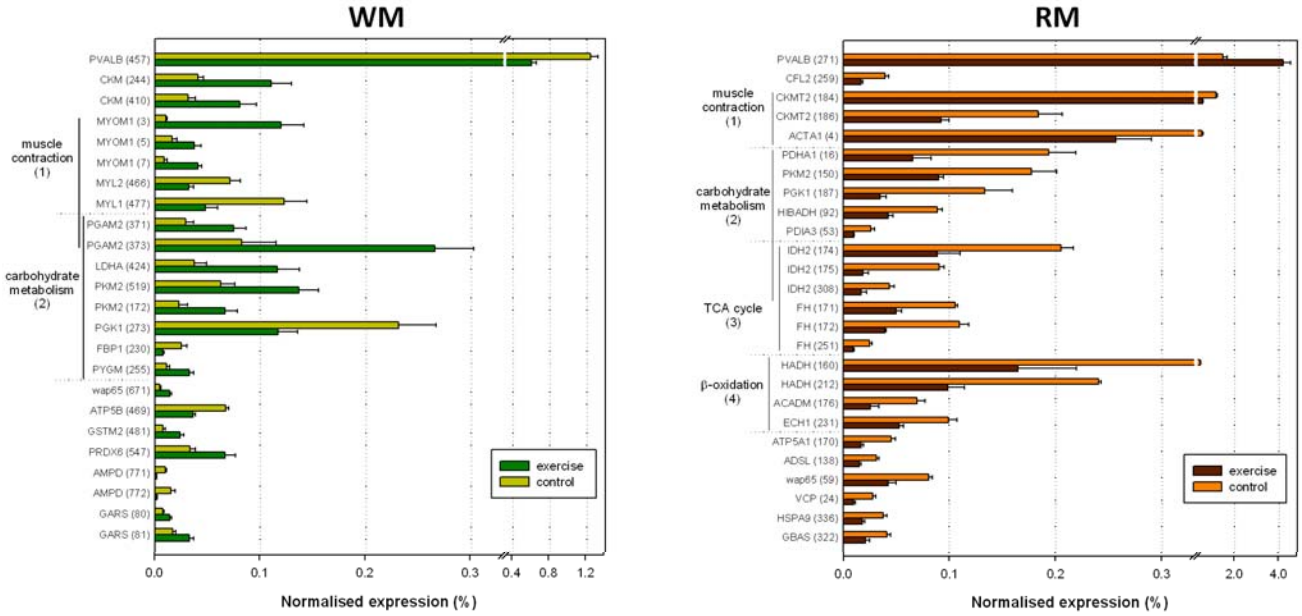

Figure 7. Physiological grouping of white (WM) and red (RM) muscle protein spots differentially expressed (*t*-test; $p<0.05$) in response to sustained exercise (1.5 BL/s, for 4 weeks). Following identification, spots were grouped on the basis of their main cellular function. Data are presented as the mean ± SEM of % Vol. Vertical lines indicate functionality related proteins ($p<0.05$) from Gene Ontology enrichment analysis by GOEAST (NODES, WM: 1-GO:0006936, ''muscle contraction''; 2-GO:0005975, ''carbohydrate metabolic process''; RM: 1-GO:0006936, ''muscle contraction''; 2-GO:0005975, ''carbohydrate metabolic process''; 3-GO:0006099, ''TCA cycle''; 4-GO:0006635, ''fatty acid beta-oxidation'').

4. DISCUSSION

The growth of gilthead sea bream increased as a result of moderate swimming but no changes in food intake were detected. This observation is consistent with improved food conversion efficiency reported in other fish species subjected to moderate sustained exercise[2]. Given that neither muscle protein turnover (estimated by $\delta^{15}N$ fractionation), body indices nor food intake differed between the C and E groups, we propose that a positive protein balance occurred in muscle. Since the protein turnover rate in a balanced nutritional status is equal to the rate of protein degradation[33], an increase in protein retention efficiency is achieved by a higher rate of protein synthesis. This observation was made in the WM and RM of rainbow trout trained at 1 BL/s for 6 weeks[17]. The enhancement of synthesis processes was consistent with the increased ribosomal (RNA:protein) and transcriptional (RNA:DNA) capacities in WM found in exercised fish. This observation is also consistent with the increased expression in the WM proteome of glycyl tRNA synthetase, associated with translation processes as other tRNA synthethases[34]. In blackspot sea bream, a lower expression of myosin light chains (MLCs) has been described when cell diameter increases[35]. The hypertrophy of epaxial WM in gilthead sea bream under sustained swimming[36] is consistent with the under-expression of MLCs found in the present study. RM showed no signs of anabolic enhancement by exercise.

RM is metabolically more active than WM in fish, thus correlating with the higher protein synthesis capacity (indicated by nucleic acid ratios), higher proportion of the soluble protein fraction (mostly including enzymes) and higher COX and CS activity levels (3 and 7 fold respectively) detected. The lower $\delta^{15}N$ fractionation of RM than WM, as previously reported in rainbow trout[37], may indicate a greater turnover rate of the former, as proposed by the inter tissue isotopic differences observed in summer flounder[38]. The metabolic differences between these two muscle types have led researchers to consider that WM is involved in burst swimming and RM in continuous swimming. Unexpectedly, our study provides evidence that gilthead sea bream submitted to moderate sustained exercise showed greater enhancement of contractile machinery in WM than in RM. Several forms of myomesin, a protein involved in sarcomer stabilization of vertebrates during continuous expansion[39], were over-expressed in WM. In addition, parvalbumin isoforms, which are involved in muscle relaxation, were over-expressed in RM and under-expressed in WM. An enhanced expression of parvalbumin isoforms and a high relaxation rate as a result of the Ca^{2+} sequestering activity of this molecule has been described in carp muscle fibers[40]. Moreover, MLCs were under-expressed in exercised WM. In agreement with our results, parvalbumin and fast isoforms of myosin regulatory and essential light chains are less abundant in exercise-trained muscle of rats, which is consistent with a shift towards a more fatigue-resistant aerobic phenotype[41]. Finally, we found that actin as well as cofilin, a regulator of actin dynamics during the contraction and relaxation cycle in mammalian

muscle[42], showed a reduced expression in exercised RM. Actin as well as other contractile proteins have also been observed in the soluble fraction of carp muscle despite they are normally associated with the myofibrillar structure[43]. It is known that soluble cytosolic actin monomers, which are in constant exchange with actin monomers within actin filaments of the sarcomere, play a fundamental role during muscle contraction[42]. In addition, exercise led to an over-expression of two creatine kinase (CK) fragments in WM and the under-expression of several CK forms in RM. The increase in CK activity maintains the immediate energy supply for contraction in muscle of coalfish[5], when fish were exercised continuously at 2.1 BL/s, while conditions of increased energy demand causes the accumulation of partially degraded products of CK in carp muscle[43].

An increase in contractile effort during continuous swimming in WM and a decrease in RM imply greater energy cost in the former, which should be reflected in the use of energy stores. We observed an increased use of lipids in WM, as indicated by the ^{13}C enrichment of lipids and the decline in lipid depots. The increased glycogen $\delta^{13}C$ values of WM are also indicative of a higher use of this store, thereby agreeing with results in rainbow trout under sustained and moderate exercise[11–13]. Increased use of carbohydrate stores was reflected by the over-expression in WM of many enzymes of carbohydrate catabolism, such as glycogen phosphorylase, pyruvate kinase (a key glycolytic enzyme) and lactate dehydrogenase. The latter indicates an increase of anaerobic glycolysis in this muscle type, which has been reported in other fish species subjected to sustained swimming[14–16,20]. In addition, fructose bisphosphatase, a key enzyme of the gluconeogenic pathway, was under-expressed by exercise in WM, which is consistent with the reduction of gluconeogenic pathway, as reported in horse mackerel subjected to long-term cruiser swimming[44]. In spite of decreasing gluconeogenesis, the high carbohydrate experimental diet enhanced glucose disposal without reducing the glycogen store of WM. To switch to carbohydrate energy stores in order to cover the energy requirements of exercise, the higher rate of glycolytic versus gluconeogenic fluxes in WM preserves gluconeogenic amino acids for protein synthesis. Enhanced use of energy stores is consistent with the increase of COX/CS ratio, which indicates relative oxidative capacity[45]. Increased aerobic capacity also occurs in WM of distinct fish species under moderate and continuous swimming[4–7,10,19–21]. Interestingly, CS and COX activities in WM showed distinct responses to exercise, in contrast to what findings in zebrafish submitted to moderate swimming[20]. The concomitant decrease in CS activity associated with a large increase in COX activity points to changes in mitochondrial compartments and proportions between respiratory chain (inner membrane) and TCA cycle enzymes (matrix compartment). This observation may indicate a functional adaptation of mitochondria to adjust to tissue-specific demands in response to exercise and to the availability of energy sources. In this regard, rainbow trout fed a low protein/high carbohydrate diet, like the

experimental diet used in the present study, present lower activities of enzymes that introduce amino acids into the TCA cycle[46]. In spite of the increase in COX activity, the ATP-synthase-β subunit was under-expressed by exercise in WM, as well as two forms of AMP-deaminase. Changes in the expression of adenylate pools (ATP/ADP+AMP) may explain these results. For example, increased amounts of ADP caused by an increased breakdown of ATP during high workload will lead to greater substrate saturation of ATP-synthase[47]. However, the amount of ATP required for continuous swimming activity in WM must be met by an increase in the above-mentioned metabolic activities.

The isotopic composition of reserves and also the enzymes activities measured were not modified in RM in response to sustained swimming. Therefore, in contrast to WM, the energy metabolism of RM was not enhanced. Indeed, in RM we detected lower expression of proteins involved in several pathways related to energy production, such as glycolysis (piruvate dehydrogenase, phosphoglycerate kinase and pyruvate kinase), the TCA cycle (fumarate hydratase and isocitrate dehydrogenase), beta oxidation (hydroxyacyl-CoA dehydrogenase, acyl-CoA dehydrogenase and dienoyl-CoA isomerase), the pentose shunt (hydroxyisobutyrate dehydrogenase), the purine nucleotide cycle (adenylosuccinate lyase) and ATP synthesis (ATP synthase-alpha subunit). The under-expression of GBAS, which is found in mitochondria and participates in oxidative phosphorylation[48], also evidenced the decline of RM metabolism with exercise.

The enhanced energy metabolism in WM in response to exercise may increase oxidative stress and lactate production, with their associated harmful effects. Accordingly, exercise over-expresses several anti-oxidant enzymes, such as peroxiredoxin-6 and glutathione-S-transferase. Peroxiredoxin-6 has an important role in the basal defense metabolism against oxidative insults in WM of gilthead sea bream[49], while glutathione-S-transferase catalyzes the detoxification of peroxidation products[50]. Moreover, WAP65 which is the homolog to mammalian hemopexin in poikilotherm[51], was over expressed. This iron binding protein, as well as other proteins that bind to pro-oxidant metal ions, may protect against free radical-induced oxidative damage[52]. As a result of the decline in the energy metabolism in RM, the expression of several proteins involved in detoxification pathways reduced. These proteins were as follows: glucose regulated protein-75, a heat-shock protein expressed in oxidative stress[53]; cdc48, another chaperon acting during ubiquitin-dependent protein degradation[54]; disulfide isomerase, also involved in the defense of oxidative protein modifications[55]; and one form of WAP-65 with the same function as in WM.

In conclusion, our proteomic study, combined with metabolic parameters (isotopic composition and enzyme activities) and growth indices, sheds light on the activity of fish muscles during

swimming. Contrary to what is commonly believed, the greatest effort of locomotion during sustained and moderate swimming in gilthead sea bream was made by WM, while RM showed a decreased work load. Neither muscle type acted as closed systems during continuous swimming but instead formed a complex relationship, complementing the work done by the other. Thus we propose that workload shifts by the two muscle types allows more efficient swimming performance. In support of this notion, spontaneous swimming costs in sea bream are similar or even higher than those of moderate swimming[56]. Since the effects induced by exercise are specific to the activity performed, the results of the present study are not applicable to burst swimming conditions but should be true for wild fish during sustained swimming. However, the nutritional record of the fish determines the level and composition of reserves, which will affect the energy fueling during moderate exercise. The composition of the experimental diet in our study contributed to enhancing food conversion efficiency during sustained and moderate swimming in gilthead sea bream.

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Supporting Information Available (view ANNEX section): Tables S1 and S2 for PCA analysis of non-proteomic data; Figure S1 for reproducibility of 2D-gels; Tables S3, S4 and Figure S2 for further protein identification information; and Tables S5 and S6 for GO enrichment analysis. This material is available free of charge via the Internet at http://pubs.acs.org/doi/abs/10.1021/pr3002832.

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resúmenes

CAPÍTULO I

La abundancia natural de los isótopos estables como herramienta para para determinar la proporción dietética óptima de proteína y lípido en juveniles de dorada (*Sparus aurata*)

Actualmente existe una fuerte presión para disminuir el contenido proteico en las dietas de peces cultivados por razones económicas y medioambientales, pero no hay métodos alternativos a los estudios de crecimiento, para determinar las condiciones alimenticias óptimas en peces en crecimiento. El presente trabajo examina el potencial de los isótopos estables (^{15}N y ^{13}C) para definir la proporción óptima de proteína y lípido (ratio PR/LP) en la dieta de juveniles de dorada. El aumento del $\delta^{15}N$ del músculo a medida que la ratio PR/LP en dieta disminuye estaba relacionado principalmente con un mayor *turnover* proteico, pero también, parcialmente, con los cambios en el perfil aminoacídico del músculo. El $\delta^{13}C$ del músculo, por el contrario, no mostró ninguna diferencia. La relación inversa observada entre el fraccionamiento de ^{15}N del músculo y la tasa de crecimiento indica una mayor ganancia de proteina en músculo a medida que el nivel de proteína de la dieta aumenta, aunque no se encontró ninguna diferencia entre los grupos en ambos parámetros por encima de la ratio 44% PR / 20% LP en dieta. El análisis de componentes principales (PCA) de los parámetros isotópicos también establece la discriminación en este punto. Estos resultados demuestran que la composición isotópica del músculo, especialmente el ^{15}N (el cual responde directamente a los cambios en el metabolismo proteico), permite determinar la condición nutricional óptima para el crecimiento de juveniles de dorada.

CAPÍTULO II

El análisis de isótopos estables combinado con índices metabólicos discrimina entre los alevines de dorada (*Sparus aurata*) producidos en varios criaderos

Existen pocos sistemas de trazabilidad, a excepción de los marcadores genéticos, capaces de distinguir entre productos marinos de diverso origen y calidad. Este estudio trata el potencial de los isótopos estables, combinados con parámetros metabólicos y del crecimiento, como herramienta discriminatoria para la selección de alevines de peces con mejor capacidad de crecimiento. Para este propósito, alevines de dorada producidos en tres criaderos de la península Ibérica con distinto origen geográfico (costa mediterránea, MC; costa cantábrica, CC; y costa atlántica sur, AC) fueron sometidos a un análisis isotópico ($\delta^{15}N$ y $\delta^{13}C$) y se calcularon además índices de crecimiento (ARN, ADN) y del metabolismo energético (actividades citocromo-c-oxidasa, COX, y citrato sintasa, CS). Estos análisis fueron realizados antes y después de un período de homogeneización de 35 días bajo idénticas condiciones de cultivo. Después de este período, los alevines pudieron ser discriminados en función del criadero de origen, siendo la composición isotópica (especialmente $\delta^{15}N$), los parámetros metabólicos (actividad COX y CS) y el contenido graso del músculo los que proporcionaban la capacidad discriminatoria más alta. Por tanto, las condiciones particulares de cultivo y/o las divergencias genéticas entre los distintos criaderos, que afectan a la capacidad de crecimiento de los alevines, quedan mayormente definidas por la imprenta o huella isotópica. Por otra parte, la signatura isotópica del músculo es un indicador más conveniente que la del pez entero a efectos discriminatorios.

CAPÍTULO III

La natación de peces desde un nuevo enfoque: estudio proteómico e isotópico en dorada

El ejercicio moderado mejora el crecimiento de los peces, aunque los mecanismos fisiológicos subyacentes no son completamente conocidos. En este trabajo, se realizó un estudio proteómico y metabólico de los músculos blanco (MB) y rojo (MR) de juveniles de dorada que nadaban a 1.5 longitudes corporales por segundo. La natación continua durante cuatro semanas mejoró el crecimiento de las doradas sin aumentar su ingesta. El ejercicio afectó a las reservas energéticas del músculo disminuyendo el contenido de lípido y de glucógeno en MB y MR respectivamente. La capacidad de síntesis proteica (ARN/proteína), el uso energético de reservas (estimado mediante el $\delta^{13}C$ del lípido y del glucógeno) y la capacidad aeróbica del MB aumentaron, mientras que el *turnover* proteico (expresado por el fraccionamiento del $\delta^{15}N$) no cambió. El MR no mostró ningún cambio en ninguno de estos parámetros. El análisis 2D-PAGE demostró que la expresión de casi el 15% de las proteinas sarcoplasmáticas de MB y de MR cambiaba en respuesta a ejercicio, la mayoría de ellas incrementado en MB y disminuyendo en MR. La identificación de las proteínas por MALDI-TOF/TOF-MS y LC-MS/MS reveló el aumento inducido por el ejercicio de varias vías metabólicas en MB (catabolismo de carbohidratos, síntesis de la proteína, contracción del músculo y desintoxicación) y la caída de otras en MR (producción energética, contracción del músculo y procesos homeostáticos). El mecanismo que subyace a la respuesta fenotípica al ejercicio esclarece los procesos adaptativos que tienen lugar en los músculos de peces, siendo la natación moderada y sostenida en dorada lograda principalmente mediante el MB, reduciendo así la cantidad de trabajo del MR y mejorando la natación y la eficiencia de conversión del alimento.

discusión general

DISCUSIÓN GENERAL

La acuicultura necesita nuevos métodos para incrementar su productividad debido a la creciente demanda de productos marinos principalmente para consumo humano, ya que la probabilidad de que las pesquerías aumenten en el futuro es prácticamente nula (FAO 2010). En el caso del cultivo de la dorada, la bajada de los precios y la saturación del mercado hacen necesario un aumento de la eficiencia productiva, ya sea mejorando el crecimiento de los peces o reduciendo los costos de producción (FAO 2010). El manejo de las granjas y la optimización de las condiciones de cultivo es esencial para incrementar la productividad acuícola. El conocimiento de los procesos fisiológicos que tienen lugar en el pez y de cómo les afectan las distintas condiciones de cultivo, puede ayudar en gran medida a dicho fin. En la presente Tesis se aborda el efecto que pueden tener distintas condiciones de cultivo sobre el crecimiento y la fisiología de doradas desde dos perspectivas emergentes en el estudio de los peces: el análisis de isótopos estables y la proteómica. Para ello se llevaron a cabo tres experimentos que abarcan distintos aspectos del proceso productivo como son: la cría y selección de alevines, la alimentación durante la fase de engorde, y las condiciones hidrodinámicas de cultivo (ejercicio). En dichos experimentos se evaluó la potencialidad de los isótopos estables como indicadores de condiciones fisiológicas óptimas para el crecimiento del pez, relacionándolos con otros parámetros metabólicos y con la expresión del proteoma del músculo. A continuación se discute la relación entre los resultados obtenidos en los tres experimentos. Más allá de hacer una discusión específica de cada capítulo, lo que aquí se pretende es analizar todos los resultados en su conjunto, cuando sea posible, y extraer así conclusiones generales.

1. El ^{15}N como indicador de cambios fisiológicos en peces

Cualquier material biológico presenta una composición isotópica característica, que viene determinada por la composición de los materiales de los que están formadas y la proporción de los mismos. En el caso del N, los valores de $\delta^{15}N$ de un tejido reflejan directamente aquellos de la fracción proteica ($\delta^{15}N$-proteína), aunque éstos últimos suelen presentar un incremento debido al proceso de delipidación durante la purificación de la proteína, el cual es inferior en músculo (<1‰) que en el pez entero (<2.8‰) (Sotiropoulos et al. 2004). Este fenómeno se observó tanto en las dietas como en los diferentes tejidos analizados en los tres experimentos presentados (**Capítulos I**, **II** y **III**). En cuanto a las dietas para nutrición en peces, se sabe que el $\delta^{15}N$ desciende a medida que aumenta la cantidad de ingredientes vegetales (Beltrán et al. 2009). Esto

es debido a la diferencia de nivel trófico entre las fuentes proteicas de origen animal y vegetal (DeNiro and Epstein 1981). Por lo tanto, las dietas del primer experimento (**Capitulo I**), que mantenían la proporción proteína animal/vegetal constante (1:1), presentaban un $\delta^{15}N$ muy similar (alrededor de 6.5‰), mientras que en los experimentos del los **Capítulos II y III**, el elevado $\delta^{15}N$ (alrededor de 10-12‰) indica un alto contenido de harina de pescado. Además, las diferencias observadas en el $\delta^{15}N$ de las dietas de doradas de distinta procendecia u origen (**Capitulo II**), indican que la dieta del Cantábrico (CC) presentaba el mayor contenido vegetal y la del Mediterráneo el menor (MC).

La composición isotópica de un animal viene determinada principalmente por la dieta más un factor discriminante causado por los procesos químicos, físicos y biológicos que experimenta el alimento hasta formar parte del cuerpo del consumidor (también llamado salto trófico o fraccionamiento: $\Delta = \delta_{tejido} - \delta_{dieta}$) (DeNiro y Epstein 1978, 1981). Este fraccionamiento produce generalmente un enriquecimiento isotópico en el consumidor que en sistemas marinos está en torno al 3.2‰ para el ^{15}N, el cual se usa para estimar niveles tróficos (DeNiro y Epstein 1981). En nuestro caso los valores de $\Delta\delta^{15}N$ del músculo blanco de las doradas de los tres experimentos: 3.5 – 4.2% (**Capítulo I**), 0.9 – 3.3‰ (**Capítulo II**), 0.9 – 1.0‰ (**Capítulo III**), se encuentran dentro del rango relatado para dorada (Beltrán et al. 2009) y otras especies de peces (Post 2002, McCutchan et al. 2003). El mayor $\Delta\delta^{15}N$ observado en el músculo del primer experimento (**Capítulo I**), vendría marcado por el mayor contenido vegetal de las dietas, como se ha observado indicado anteriormente. Por otro lado, tanto el $\Delta\delta^{15}N$ del pez entero (**Capítulo II**) como el del músculo rojo (**Capítulo III**) presentaban valores más bajos que en el músculo blanco, los cuales responden al menor $\delta^{15}N$ observado en ambos tejidos. Pinnegar y Polounin (1999) en trucha, y Beltrán et al. (2009) en dorada, también observaron diferencias similares en la composición isotópica entre los distintos tejidos del pez, y lo atribuyeron a la abundancia relativa de los distintos aminoácidos (AA) en los tejidos y a la composición isotópica de cada aminoácido. Popp et al. (2007), clasificaron los AA del músculo del atún de aleta amarilla en dos grupos: los enriquecidos en ^{15}N (AA 'tróficos') y los empobrecidos en ^{15}N (AA 'fuente'). Tanto aminoácidos dispensables (DAA) como indispensables (IAA) pueden encontrase en cualquiera de estos dos grupos, aunque los AA dispensables relacionados con el transporte de N tienden a estar enriquecidos en ^{15}N, mientras que aquellos que son esenciales y no fácilmente transaminados tienden a estar empobrecidos en ^{15}N (revisado en Martínez del Río et al. 2009). Lamentablemente, no pudimos medir la composición isotópica individual de cada AA en ningún experimento, aunque si se obtuvo el perfil aminoacídico del musculo blanco de las doradas del primer experimento (**Capítulo I**). Los perfiles apenas mostraban diferencias entre los distintos grupos experimentales, pero el mayor contenido de glicina (DAA) y el menor de valina (IAA) del músculo de doradas alimentadas con dietas bajas en proteína podrían explicar en parte el

mayor $\delta^{15}N$ observado en el músculo de estos peces. Estos resultados sugieren además un mayor *turnover* aminoacídico en las doradas alimentados con dietas bajas en proteína (como se verá más adelante), debido a la menor disponibilidad de AA en la dieta en comparación a las doradas alimentadas con dietas con alto contenido proteico.

Existen distintos factores, aparte de la dieta, como el nivel de ingesta (Gaye-Siesseger et al. 2003, 2004a), la tasa de crecimiento (Trueman et al. 2005) y las condiciones ambientales (Barnes et al. 2007) que afectan al metabolismo del pez y por tanto al fraccionamiento isotópico que ocurre en el organismo y que queda reflejado en la composición final de los tejidos. En el experimento del **Capítulo I**, a pesar de que las dietas presentaban el mismo $\delta^{15}N$, el $\delta^{15}N$ del músculo descendía a medida que aumentaba la ratio proteína/lípido de las dietas, lo que estaría relacionado con diferencias en el balance nitrogenado entre grupos. En peces sometidos a condiciones de elevado *turnover* proteico como el ayuno o la restricción de ingesta proteica se ha observado un enriquecimiento de ^{15}N en los tejidos (Gaye-Siesseger et al. 2003, 2004, 2007; Barnes et al. 2007), ya que el ^{14}N es excretado preferencialmente durante procesos de desaminación y transaminación y el ^{15}N es retenido en los nuevos aminoácidos y proteínas sintetizados (Gaebler et al. 1966; Macko et al. 1986; Hare et al. 1991). Por lo tanto, el elevado $\delta^{15}N$ observado en el músculo de las doradas del estudio alimentadas con dietas bajas en proteína son debidos a un mayor *turnover* proteico en el músculo inducido por una restricción de la entrada de aminoácidos en músculo. Este hecho se refleja además en el mayor fraccionamiento ($\Delta\delta^{15}N$) de estos peces que estaría indicando una menor deposición proteica y por tanto una mayor diferencia entre la composición isotópica de la dieta y la del músculo, como observaron Gaye et al. en carpa (2004a) y tilapia (2003,2004b). La relación inversa encontrada entre el $\Delta\delta^{15}N$ y la tasa de crecimiento, también observada en salmón (Trueman et al. 2005), confirma esta hipótesis. En el **Capítulo II**, se observó un aumento general del $\delta^{15}N$ en los alevines tras el periodo de homogenización de las condiciones de cultivo y alimentación, ligeramente superior en los grupos CC y AC que cambiaron a la dieta del grupo MC (con mayor $\delta^{15}N$). Dicho aumento respondería a cambios alométricos del *turnover* proteico y de la tasa de crecimiento durante el periodo experimental en el que los alevines doblaron su peso. El aumento del $\delta^{15}N$ con el peso corporal también se ha observado en perca (Overman y Parrish 2001) y lubina (Barnes et al. 2008), lo cual es debido a la bioacumulación de ^{15}N en los tejidos a lo largo de la vida del animal por la excreción preferencial de ^{14}N (Martínez del Río et al. 2009). Además se sabe que a medida que incrementa la masa corporal en peces, la concentración de RNA disminuye y la proporción de RNA empleada en *turnover* proteico aumenta (Houlihan et al. 1993). En los alevines de nuestro estudio, la caída de la capacidad de síntesis proteica (RNA:proteína) del músculo tras el periodo experimental indica por tanto un aumento del *turnover* proteico y estaría directamente relacionada con el aumento del $\delta^{15}N$. Por otro lado, en

el mismo experimento, el cambio de dieta afectó de manera diferente al fraccionamiento ($\Delta\delta^{15}N$) en los grupos CC y AC, que partían con un peso inicial similar. El grupo CC redujo el $\Delta\delta^{15}N$, de acuerdo con el cambio a una dieta de mejor calidad (Martínez del Rio et al. 2009), por el contrario el $\Delta\delta^{15}N$ del grupo AC incrementó sugiriendo una menor tasa de deposición proteica. Esto estaría relacionado con la diferencia de capacidad de síntesis proteica (RNA:proteína), mantenida desde la situación inicial y reflejada en el menor crecimiento del grupo AC. Por tanto, el grupo AC probablemente presenta un mayor coste metabólico como indican las elevadas actividades COX y CS. Finalmente, no se observaron diferencias en el $\delta^{15}N$ de ambos músculos (blanco y rojo), ni en su fraccionamiento, entre las doradas sometidas a natación sostenida y las de natación voluntaria (**Capítulo III**). Estos resultados indicarían que el *turnover* proteico sería similar en los dos grupos experimentales, puesto que ambos grupos fueron alimentados con la misma dieta y no se observaron diferencias en el nivel de ingesta. Sin embargo, las doradas sometidas a una natación moderada y sostenida mostraban un mayor crecimiento, reflejado en la mayor capacidad de síntesis proteica (RNA:proteína) del músculo blanco y la sobreexpresión de proteínas relacionadas con ella (tRNA-sintetasas), sugiriendo una mayor deposición proteica. Por tanto, aunque el $\delta^{15}N$ no pueda ser utilizado como un índice directo de crecimiento, ya que no se comporta igual en todas las situaciones, el análisis de los cambios que experimenta bajo distintas condiciones de cultivo puede ayudarnos interpretar fisiológicamente los cambios metabólicos que se producen en el músculo.

2. El ^{13}C como indicador de cambios fisiológicos en peces

Los valores de $\delta^{13}C$ de cualquier material biológico, a diferencia del $\delta^{15}N$, no sólo dependen de la proteína ($\delta^{13}C$-proteína) sino también de los carbohidratos ($\delta^{13}C$-glucógeno) y, principalmente, de los lípidos ($\delta^{13}C$-lípido). Éstos últimos presentan unos valores de $\delta^{13}C$ más bajos que en proteínas o carbohidratos, debido a la discriminación negativa hacia el ^{13}C que realiza la piruvato deshidrogenasa durante la lipogénesis (DeNiro and Epstein 1977). Por tanto, aparte de la dieta, la composición isotópica ($\delta^{13}C$) de un tejido está condicionada por su composición proximal, especialmente por sus niveles de lípidos. Así, en el **Capítulo I**, la composición isotópica de las dietas ($\delta^{13}C$) disminuía a medida que incrementaba su contenido graso, mientras que en el **Capítulo II** se observó una correlación inversa entre el $\delta^{13}C$ del músculo y del pez entero, y su contenido lipídico. Además, como en el caso del N, el $\delta^{13}C$-proteína suele presentar valores superiores a los del tejido debido al proceso de delipidación necesario para su purificación. Estas diferencias son menores en músculo (<0.5‰) que en el pez

entero (<3.4‰) (Sotiropoulos et al. 2004). Por otro lado, las harinas vegetales de plantas C3 (como el trigo) presentan un $\delta^{13}C$ más bajo que las de plantas C4 (como el maíz) (Focken 2004), debido de nuevo a la discriminación en detrimento del ^{13}C que realiza la enzima rubisco de las plantas C3 (Farquhar et al. 1989). Puesto que las dietas de las doradas en origen (**Capítulo II)** eran isoproteicas e isolipídicas, el menor $\delta^{13}C$ de la dieta de los alevines del Atlántico, derivado de la composición isotópica de la fracción proteica que era la mayoritaria, respondería a un mayor contenido de ingredientes de plantas C3. Por otro lado, la dieta empleada en el experimento de ejercicio (**Capítulo III**), que contenía un elevado contenido de harina de trigo, mostraba un elevado $\delta^{13}C$ similar a las dietas del experimento del **Capítulo II**. Esto es consecuencia del bajo contenido en lípidos de la dieta del experimento de ejercicio, que hace que el $\delta^{13}C$ de la delta aumente. Por otro lado, el $\delta^{13}C$-lípido de los piensos para peces desciende a medida que aumenta la cantidad de ingredientes vegetales (Gaye-Siesseger et al. 2003), ya que las fuentes de C de origen terrestre presentan valores de $\delta^{13}C$ más bajos que las de origen marino (Chisholm et al 1982). Los menores valores de $\delta^{13}C$-lípido de la dieta en origen de los alevines del Cantábrico (**Capítulo II)** indicarían por tanto una mayor proporción de ingredientes vegetales, de acuerdo con lo visto para el $\delta^{15}N$, mientras que la uniformidad en los deltas de las fracciones lipídica y proteica de las dietas del **Capítulo I** evidencia que todas las dietas fueron confeccionadas con los mismos materiales.

Los factores que afectan el fraccionamiento del ^{15}N también son aplicables para el ^{13}C pero, a diferencia del N, otros factores como el contenido lipídico y el *'isotopic routing'* complican aún más la predictibilidad de la composición isotópica ($\delta^{13}C$) de un tejido a partir de los valores de la dieta (revisado en Gannes et al.1998). Schwarcz (1991) bautizó como '*isotopic routing'* a la distribución diferencial de los esqueletos carbonatados de los diferentes componentes dietéticos (proteínas, lípidos, y carbohidratos) hacia los distintos tejidos. Por ejemplo, si los átomos de C de todos los componentes dietéticos se mezclaran durante la síntesis proteica, manteniendo la misma proporción, el $\delta^{13}C$-proteína corporal debería parecerse al valor de $\delta^{13}C$ global de la dieta. El grado de mezcla depende de la ingesta proteica y del balance nitrogenado (Gannes et al. 1998). La distribución diferencial de los distintos componentes dietéticos ('*isotopic routing*') junto con el similar contenido lipidico observado en el músculo de los distintos grupos experimentales del **Capitulo I**, podrían explicar porqué el $\delta^{13}C$ del tejido muscular no presenta diferencias entre los grupos a pesar de las diferencias observadas en el $\delta^{13}C$ de las dietas experimentales, mencionado anteriormente. Los valores de $\delta^{13}C$ de las distintas fracciones del músculo (proteína, lípido y glucógeno) tampoco mostraban diferencias entre las doradas del mismo experimento. En el segundo experimento (**Capitulo II**) los valores de $\delta^{13}C$ del pez entero y del músculo no mostraban inicialmente diferencias entre las doradas de distinta procedencia, a pesar de las diferencias observadas en la composición isotópica entre las dietas.

Esto puede explicarse por las diferencias en el contenido graso. Así, el mayor contenido de lípidos de los peces del Mediterráneo compensaría el elevado δ^{13}C-proteína que presentaban respecto a los otros dos grupos. Además, tras el periodo de homogenización de las condiciones de cultivo y alimentación, estas diferencias del contenido graso de los peces se hacen evidentes en el δ^{13}C del pez entero. El '*isotopic routing*' del ^{13}C, por su parte, podría explicar porque los valores iniciales de δ^{13}C-lípido en el músculo muestran un patrón de diferencias distinto al encontrado en el δ^{13}C-lípido de las dietas originales. De hecho las diferencias en el δ^{13}C-lípido del músculo entre los grupos de distinto origen se acercan más a las observadas en el δ^{13}C-proteína de la dieta, sugiriendo el uso de los esqueletos carbonatados de la proteína dietética para la síntesis lipídica. Esto es consistente con los hallazgos realizados por Kelly y Martinez del Río (2010), los cuáles observaron que hasta el 50% del C de los lípidos del músculo de tilapia procedía de la proteína de la dieta. Además, tras la unificación a una misma dieta de los grupos de alevines de distinto orígen, las diferencias iniciales en el δ^{13}C-lípido desaparecieron. Finalmente en el tercer experimento (**Capitulo III**), el menor δ^{13}C del músculo blanco de las doradas con natación voluntaria (controles) refleja una mayor reserva lipídica en este grupo respecto a las doradas con natación sostenida (ejercitadas). Además, el incremento del δ^{13}C-lípido y δ^{13}C-glucógeno en el músculo blanco de las doradas ejercitadas indicaría un mayor uso de estas reservas debido al enriquecimiento en ^{13}C de los tejidos durante los procesos de descarboxilación (Gannes et al 1998). El incremento del uso de estas reservas en el músculo blanco de doradas ejercitadas se ve reflejado en un aumento de la ratio COX/CS, la cual expresa la capacidad oxidativa relativa (Capkova et al. 2002), además de la sobreexpresión de proteínas implicadas en el catabolismo de los carbohidratos como la glucógeno fosforilasa, la piruvato quinasa y la lactato deshidrogenasa. Esta última también estaría indicando un aumento de la glucólisis anaeróbica.

Todos estos resultados demuestran que el uso de δ^{13}C como indicador de cambios fisiológicos debe ser empleado con cautela, ya que fenómenos como el '*isotopic routing*' o variaciones tisulares en el contenido graso pueden enmascarar los cambios en la composición isotópica debidos a cambios metabólicos que se producen en el músculo de peces bajo diferentes condiciones de cultivo. Sin embargo, los cambios de los valores de δ^{13}C de la fracción proteica se comportan generalmente en el mismo sentido que sus homólogos para el δ^{15}N. Por ejemplo, la relación inversa entre el $\Delta\delta^{13}$C de la fracción proteica del músculo y el contenido proteico de la dieta observada en el **Capítulo I**, muestra una pendiente idéntica a la encontrada para el $\Delta\delta^{15}$N de la proteína. De igual forma, en el **Capítulo III**, el δ^{13}C-proteína de los músculos blanco y rojo de las doradas ejercitadas y control no mostraba diferencias, como se observó en el δ^{15}N. Por tanto estos resultados sugieren un uso factible del δ^{13}C de la fracción proteica como indicador de cambios en el metabolismo proteico.

3. Los isótopos estables como trazadores

De acuerdo a lo establecido por DeNiro y Epstein (1978, 1981), la composición isotópica de un animal refleja la composición isotópica de su dieta de una manera predecible: "*We are what we eat, also isotopically*". Por tanto, los isotopos estables son una herramienta muy valiosa a la hora de asociar a un animal con una fuente específica de alimento, la cual es característica de los sistemas de producción y de la intensidad de alimentación. Esto ha permitido la discriminación entre peces de distintos orígenes y sistemas de producción (Molketin et al. 2007, Bell et al 2007). En el segundo experimento (**Capítulo II**), los alevines del Mediterráneo presentaban en origen el mayor $\delta^{15}N$ tanto en el pez entero como en el músculo, lo que concuerda con el mayor $\delta^{15}N$ de su dieta. Sin embargo, los alevines del Cantábrico, cuya dieta presentaba el $\delta^{15}N$ más bajo, no mostraban los menores valores de $\delta^{15}N$ en origen, siendo incluso en músculo significativamente superiores a los del Atlántico (AC). Focken (2001) observó en tilapias alimentadas con dietas con un elevado contenido vegetal un incremento del $\delta^{15}N$ a medida que aumentaba la ración, sugiriendo una mayor transformación de aminoácidos de origen vegetal en proteína animal. Por tanto, el mayor contenido vegetal de la dieta CC y la mayor ración dada a este grupo en origen explicarían porque presenta un $\delta^{15}N$ en músculo superior al del grupo AC. Esto se refleja también en el fraccionamiento ($\Delta\delta^{15}N$) del grupo CC que muestra la mayor diferencia entre la composición isotópica del músculo y la de la dieta. Además este grupo presentaba valores más altos de las ratios RNA:DNA y COX:CS, lo que estaría relacionado con la mayor ración dada a este grupo, ya que en peces se sabe que el nivel de ingesta está positivamente relacionado con la síntesis proteica y la demanda energética (Millward, 1989; Mathers et al. 1992; Houlihan et al. 1995). Sin embargo, a pesar del aumento de tamaño y de la unificación a una misma dieta y condiciones de cultivo, las diferencias en $\delta^{15}N$ observadas al inicio se mantuvieron tras el periodo experimental, especialmente en el músculo. Sweeting et al. (2007) observaron que, en lubinas alimentadas con una dieta constante en condiciones controladas, el $\delta^{15}N$ del músculo alcanzaba el equilibrio con la dieta al doblar de peso el animal (de 8 a 17 g) y posteriormente se mantenía estable siguiendo una función sinusoidal (± 0.15‰) a lo largo de los 823 días que duró el estudio. De acuerdo con estos resultados, todos los alevines de nuestro experimento se encontrarían en equilibrio con la dieta, aunque las diferencias observadas entre los grupos en el $\delta^{15}N$ del músculo indicarían que habrían alcanzado un equilibrio distinto. Estas diferencias son el resultado de una 'imprenta o huella isotópica' característica de las condiciones de cultivo de cada *hatchery*, las cuales afectan la fisiología y el metabolismo de los alevines y hacen que respondan de manera diferente frente a un cambio de condición, como se ha observado en los distintos parámetros metabólicos analizados. En este sentido Tieszen et al. (1983) especularon que los tejidos pueden tener una "memoria isotópica distinta". Con respecto al ^{13}C, las diferencias iniciales en el $\delta^{13}C$-proteína,

tanto en el pez entero como en el músculo de los alevines de distinto origen, reflejaban las diferencias en el δ^{13}C-proteína entre las dietas. Estas diferencias, además, se mantuvieron tras el periodo de homogenización, mostrando las doradas del grupo AC valores significativamente más bajos a las de los grupos MC y CC, lo cual evidencia un comportamiento del δ^{13}C de la fracción proteica similar de al del δ^{15}N, como se ha mencionado anteriormente.

Estos resultados del estudio muestran que la signatura isotópica del músculo, especialmente el δ^{15}N, es un indicador más adecuado que la del pez entero a efectos discriminatorios, ya que es menos variable debido a su menor actividad metabólica comparada con otros tejidos como el hígado (Pinnegar y Polounin 1999). No obstante se necesita información fisiológica adicional para determinar aquellos alevines con mayor potencial de crecimiento.

4. Cambios en el proteoma del músculo en respuesta al ejercicio

En el estudio descrito en esta Tesis (**Capítulo III**), los juveniles de dorada fueron sometidos a una natación sostenida de 1.5 longitudes corporales/s (BL/s) durante 4 semanas. Este protocolo de ejercicio moderado, que representa aproximadamente el 35% de la velocidad crítica de natación para esta especie (Basaran et al. 2007), causó una expresión diferencial significativa de casi el 15% de las proteínas sarcoplasmáticas del músculo blanco y rojo. La mayor parte de las proteínas que modificaron su expresión, estaban incrementadas en el músculo blanco mientras que en músculo rojo estaban reducidas. A continuación, describiremos los cambios proteómicos experimentados en cada tipo de músculo por separado. En esta discusión se incluyen algunas proteínas que a pesar de tener una expresión diferencial (p<0.05), no aparecen en el artículo publicado por presentar un cambio inferior a dos veces el nivel de expresión (< 2-*fold*).

4.1 Efectos de la natación sostenida en el músculo blanco

El aumento de la maquinaria de síntesis proteica en el músculo blanco de dorada (RNA:proteína) con el ejercicio moderado y sostenido, como ya observaron Houlihan et al. (1987) en trucha arcoíris, concuerda con la sobreexpresión de proteínas implicadas en los procesos relacionados con la síntesis proteica, como la traducción (glicil-tRNA sintetasa). La reducción de la expresión de las cadenas ligeras de la miosina (MLCs), lo cual ya se ha observado en el músculo del besugo (Silva et al. 2010) a medida que el tamaño celular aumenta, indicaría por tanto una hipertrofia de las fibras del músculo blanco en doradas ejercitadas. En

este sentido, el aumento de tamaño de los peces, respondería al aumento del área y del perímetro de las fibras de musculo blanco de dorada que se ha observado con el ejercicio moderado (Ibarz et al. 2011). Además, tanto la transferrina como la proteína DJ1, ambas promotoras del crecimiento en mamíferos (Ozawa 1989; Shinbo et al. 2006), estaban sobreexpresadas en respuesta al ejercicio sostenido.

La natación sostenida también causó un aumento de la maquinaria contráctil en el músculo blanco de la dorada. Así, varias formas de miomesina, una proteína implicada en la estabilización del sarcómero durante la extensión continua (Schoenauer et al. 2005), estaban sobreexpresadas en el músculo blanco. Además, una isoforma de parvalbumina, una proteína secuestrante del Ca^{2+} que presenta una mayor expresión durante prolongados estados de relajación en el músculo de carpa (Brownridge et al. 2009), disminuyó su expresión en el músculo blanco de doradas ejercitadas, al igual que las MLCs. De acuerdo con estos resultados, la parvalbumina y varias isoformas de MLCs son menos abundantes en el músculo de ratas sometidas a un ejercicio de resistencia, lo que es consistente con una cambio hacia un fenotipo aeróbico más resistente a la fatiga (Yamaguchi et al. 2010). Además, la sobreexpresión de DJ-1 en el músculo blanco de dorada ejercido sugiere una mejor capacidad para regular la homeostasis del Ca^{2+} durante el ejercicio ya que el músculo esquelético de ratones *knock out* para DJ-1 muestran un aumento de la concentración de Ca^{2+} en reposo y una reducción de la liberación de Ca^{2+} (Shtifman et al. 2011). Una mejora de la homeostasis de Ca^{2+} por un aumento de la expresión regulación de dihidropiridinas y receptores de rianodina, como consecuencia de la práctica de ejercicio moderado, también se encontró en el músculo blanco de salmón (Anttila et al. 2006). Por otro lado, dos fragmentos de creatina quinasa (CK) estaban sobreexpresados en el músculo blanco de las doradas ejercitadas, indicando una actividad más alta de esta enzima durante la natación sostenida para mantener la fuente de energía inmediata necesitada para la contracción en músculo. Apoyando esta idea, en condiciones de demanda energética creciente se observó la acumulación de productos parcialmente degradados de CK en el músculo de la carpa (McLean et al. 2007). Todos estos hallazgos señalan un incremento de la carga de trabajo del músculo blanco en dorada, lo que estaría de acuerdo con el reclutamiento de fibras blancas durante la natación moderada observado en otros peces (Johnston and Moon 1980, 1981).

Para cubrir los costes energéticos causados por el aumento en el esfuerzo contráctil del músculo blanco durante la natación continuada, las doradas, que estaban alimentadas con una dieta rica en carbohidratos y baja en proteína, exhibían un aumento de la expresión de proteínas implicadas en la oxidación de carbohidratos como la glucógeno fosforilasa, la piruvato quinasa y la lactato deshidrogenasa. Esta última indica un incremento de la glucólisis anaeróbica lo cual ha sido observado en diversas especies de teleósteos (Wokoma y Johnston 1981, Hinterleitner et al. 1992, Sänger y Pötscher 2000, McClelland et al 2006). La expresión más baja de la enzima

fructosa bisfosfatasa, como se ha observado en jureles sometidos a natación sostenida (Luschak et al. 2001), indicaría una reducción de la vía gluconeogénica en el músculo blanco debido al ejercicio. Así, el aumento de la glucólisis frente a la gluconeogénesis en el músculo blanco implica un cambio hacia un mayor uso de carbohidratos como combustible, que puede preservar los aminoácidos gluconeogénicos para la síntesis de la proteína. De acuerdo con esto, truchas arcoíris alimentadas con dietas ricas en carbohidratos y bajas en proteínas, como la de nuestro estudio, mostraban una menor actividad de enzimas que introducen aminoácidos al ciclo de Krebs (Walton 1986). El aumento de la glucólisis es consistente con el incremento de la actividad de la lanzadera malato-aspartato, indicada por la sobreexpresión de la malato deshidrogenasa citoplasmática, para desplazar los electrones producidos durante glucólisis a través de la membrana interna de la mitocondria con destino a la fosforilación oxidativa. De acuerdo con estos resultados, se observó una mayor ratio COX:CS en el musculo blanco de doradas ejercitadas, la cual indica la capacidad oxidativa relativa (Capkova et al. 2002). A pesar del aumento de la actividad COX, la subunidad de ATP-sintasa-β redujo su expresión en doradas ejercitadas, así como dos formas de AMP-desaminasa. Los cambios en la concentración de los pools de nucleótidos adenilados (ATP / ADP + AMP) puede explicar estos resultados. Por ejemplo, una mayor cantidad de ADP causado por un mayor deterioro de la ATP durante una elevada carga de trabajo da lugar a una mayor saturación por sustrato de la ATP-sintetasa (Das 2003). Sin embargo, la cantidad de ATP requerido para la actividad de natación continua en el músculo blanco debe ser satisfecho por un aumento de las actividades metabólicas mencionadas (glucólisis y creatina quinasa). Con respecto al metabolismo lipídico, la natación sostenida en dorada causó una reducción de la expresión de la apolipoproteina-14kDa en el músculo blanco, la cual es específica de peces y homóloga a la APOA2 de mamíferos (Choudhury et al. 2009), aunque su función biológica es desconocida.

Finalmente, se detectó un aumento de la defensa frente al estrés oxidativo en respuesta a ejercicio debido al aumento del metabolismo energético en el músculo blanco. Entre las enzimas antioxidantes sobreexpresadas en doradas ejercitadas están la peroxiredoxina-6, la glutatión-S-transferasa y la proteína DJ1. La primera tiene un papel importante en el metabolismo básico de la defensa frente a daños oxidativos en el músculo blanco de dorada (Perez-Sanchez et al. 2011), mientras que la glutatión-S-transferasa cataliza la desintoxicación de los productos de peroxidación y también ha sido descrita en dorada (Mourente et al. 2002). Por su parte, DJ-1 desempeña un papel primordial frente al estrés oxidativo en las neuronas (Bonifati et al. 2004). Esta última proteína también se ha encontrado en músculos glucolíticos de mamíferos (Hwang et al. 2004; Sayd et al. 2006) y en el músculo blanco de la trucha (Morzel et al. 2006). Por otra parte, un par de proteínas que unen hierro aumentaron su expresión en músculo blanco en respuesta a ejercicio: wap-65kDa, homóloga a la hemopexina de mamíferos en peces (Sha et al.

2008), y transferrina. Estas dos proteínas, así como otras que se unen a iones metálicos pro-oxidantes, pueden proteger contra el daño oxidativo inducido por radicales libres (Limon-Pacheco et al. 2009).

4.2 Efectos de la natación sostenida en el músculo rojo

El músculo rojo no demostró ninguna muestra de incremento anabólico por ejercicio. Este hecho se podría relacionar con una reducción de la actividad contráctil del músculo rojo en doradas ejercitadas, puesto que la hipertrofia de las fibras asociada al ejercicio moderado está causada por el continuo estímulo físico del músculo (Davison 1997). De hecho, la miomesina, la actina y la cofilina, esta última reguladora de la dinámica de la actina durante los ciclos de contracción y relajación en el músculo de mamíferos (Gunst et al. 2008), mostraban una expresión reducida en músculo rojo de doradas ejercitadas. Por otra parte, la reducción de una forma de parvalbumina y dos de CK, contrastaban con los resultados observados en el músculo blanco. En este sentido, a pesar de que las diferencias metabólicas entre el músculo rojo y blanco han conducido a diversos investigadores a considerar que las fibras blancas son reclutadas principalmente en la natación explosiva y la fibras rojas en la natación sostenida, estos resultados proporcionan la evidencia de una mayor intensificación de la maquinaria contráctil en el músculo blanco que en músculo rojo de las doradas sometidas a natación moderada y sostenida.

Además, en contraste con el músculo blanco, el metabolismo energético del músculo rojo no fue potenciado por el ejercicio. Así, el músculo rojo de doradas ejercitadas mostró una disminución de la expresión de varias proteínas implicadas en distintas vías relacionadas con la producción energética, como la glucólisis (enolasa, piruvato deshidrogenasa, fosfoglicerato quinasa y piruvato quinasa), el ciclo de Krebs (fumarato hidratasa e isocitrato deshidrogenasa), la beta oxidación (hidroxiacil-CoA deshidrogenasa, acil-CoA deshidrogenasa y dienoil-CoA isomerasa), el *shunt* de las pentosas (hidroxisobutirate deshidrogenasa), el ciclo de nucleótidos de purina (adenilosuccinato liasa) y síntesis del ATP (subunidad alfa de la ATP-sintetasa). La reducción en la expresión de la GBAS, una proteína '*nipsnap*' que se encuentra en mitocondria mamíferos y participa en la fosforilación oxidativa (Martherus et al. 2010), también evidenciaba la disminución del metabolismo del músculo rojo con el ejercicio. Como resultado de la caída del metabolismo energético en músculo rojo con el ejercicio, se observó una reducción en la expresión de varias proteínas implicadas en vías de desintoxicación, como son la antiquitina, una aldehído deshidrogenasa implicada en la desintoxicación del metabolismo lipídico y carbohidratado (Tang et al. 2005), y varias chaperones (grp-75, cdc-48 y disulfuro isomeras).

4.3 Observaciones finales

De este estudio podemos deducir que el ejercicio moderado y sostenido afecta profundamente a la expresión proteica en el músculo blanco y rojo, aunque aparentemente en dirección opuesta. Así, el ejercicio activó en el músculo blanco vías metabólicas relacionadas con la oxidación de los carbohidratos, la síntesis de la proteína, la contracción del músculo y la detoxificación. Estos resultados fueron corroborados por el aumento de la capacidad de síntesis proteica (RNA:proteína), el mayor uso de energía (estimado mediante el δ^{13}C-lipido y δ^{13}C-glucógeno) y la mayor capacidad aeróbica (COX:CS) junto con la no modificación del *turnover* proteico (δ^{15}N). Por el contrario, el ejercicio redujo en músculo rojo las vías relacionados con la producción energética, la contracción del músculo y los procesos homeostáticos, sin mostrar variaciones en los parámetros metabolicos e isotópicos analizados. Los resultados obtenidos ponen de manifiesto los mecanismos que subyacen a la respuesta fenotípica al ejercicio y a los procesos adaptativos que se dan en el músculo esquéletico de dorada. Este estudio apoya la noción de que el músculo blanco soporta gran parte de la natación moderada y sostenida en esta especie, reduciendo así la cantidad de trabajo del músculo rojo y mejorando la eficiencia natatoria.

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conclusions

CONCLUSIONS

1. The nitrogen isotopic composition ($\delta^{15}N$) of white muscle of gilthead sea bream, closely linked to the protein fraction, reflects dietary changes in the protein-to-lipid ratio, mainly as a result of higher protein turnover in fish fed on low protein diets than in those on high protein diets, because of the lower amino acid availability. This determines changes in the muscle amino acid profile.

2. The inverse relationship observed between ^{15}N-fractionation of muscle and growth rate indicates a higher protein gain in muscle as the dietary protein content increases, although no differences were found in either parameter above a dietary ratio of 44% protein / 22% lipid. This threshold determines the state where catabolism and recycling of protein significantly increases in muscle due to the decrease in dietary protein, indicating therefore that the protein sparing effect of dietary lipids may be primed by a slight decrease in the dietary protein-to-lipid ratio.

3. Stable isotope analysis (especially ^{15}N-fractionation, which responds to changes in protein metabolism) is a reliable indicator of nutritional status in fish, and, therefore, may be a valuable tool for determining the optimal nutritional requirements in diets for growing fish.

4. Specific rearing conditions, fundamentally diet and feeding level, of geographically distinct hatcheries affect the metabolism (COX:CS) and growth (RNA:DNA) of gilthead sea bream fingerlings, resulting in a specific isotopic composition of muscle and whole body.

5. After a one-month homogenization period under identical nutritional and rearing conditions, gilthead sea bream fingerlings can still be discriminated mainly on the basis of isotopic measures (especially $\delta^{15}N$) combined with metabolic parameters and proximal composition in muscle. This suggests that the specific rearing conditions and/or genetic divergence between fingerlings of different hatcheries, which affect their growth capacity, are defined mainly by the isotopic imprint.

6. Stable isotopic composition (especially $\delta^{15}N$), combined with metabolic and growth variables, can be used as a tool that goes beyond the discrimination of geographical origin, allowing the selection of fish seeds with high growth capacity. Moreover, the muscle isotopic signature is a better indicator than that of whole fish for discrimination purposes, since the whole fish contains soft tissues that are metabolically more active and isotopically more variable.

7. Carbon isotope composition ($\delta^{13}C$) of muscle, unlike $\delta^{15}N$, does not respond as well to changes in metabolic state. Although the $\delta^{13}C$ of the protein fraction seems to mirror the behavior of $\delta^{15}N$, the greater complexity of factors affecting $\delta^{13}C$ of tissue, such as isotopic routing or fatness, mean the physiological significance of its changes should be interpreted with caution.

8. Moderate and sustained exercise improve fish growth and feed efficiency by increasing protein synthesis (RNA:DNA), oxidative capacity (COX:CS), and energy use (estimated by $\delta^{13}C$ of lipid and glycogen) in white muscle, while protein turnover (expressed by ^{15}N-fractionation) do not change. In contrast, red muscle remains unchanged. Moreover, the high carbohydrate content of the experimental diet seems to contribute to improving the sparing effect of protein for enhancing growth and food conversion efficiency during continuous swimming in gilthead sea bream.

9. The profound involvement of the skeletal muscle proteome during moderate and sustained swimming supports these changes. White muscle shows an enhancement of several pathways (carbohydrate catabolism, protein synthesis, muscle contraction, and detoxification), whereas in red muscle many other processes (energy production, muscle contraction, and homeostatic processes) are reduced.

10. The proteomic study, combined with metabolic and isotopic parameters, reveals that, contrary to common belief, white muscle makes a major contribution to locomotion during sustained and moderate swimming in gilthead sea bream, whereas the work load of red muscle decreases. This suggests that neither muscle type acts as a closed system during continuous swimming but instead they form a complex relationship, complementing the work done by the other and allowing more efficient swimming.

11. Overall, in combination with other metabolic parameters, stable isotope analysis provides useful information for understanding biological processes underlying changes in physiological conditions, thereby supplementing and supporting the information provided by new integrative techniques such as proteomics.

conclusiones

CONCLUSIONES

1. La composición isotópica de nitrógeno ($\delta^{15}N$) del músculo blanco de dorada, íntimamente ligada a la fracción proteica, refleja los cambios en la proporción proteína/lípido de la dieta, principalmente como resultado de un mayor *turnover* proteico en los peces alimentados con dietas bajas en proteína que en aquellos alimentados con dietas altamente proteicas, debido a la menor disponibilidad de aminoácidos. Este hecho produce cambios en el perfil aminoacídico del músculo.

2. La relación inversa observada entre el fraccionamiento de ^{15}N del músculo y la tasa de crecimiento indica una mayor ganancia de proteína en músculo a medida que el contenido proteico de la dieta aumenta, aunque no se encontraron diferencias en ninguno de los dos parámetros por encima de una proporción de 44% proteína / 22% lípido en dieta. Este umbral determina el estado donde el catabolismo y el reciclaje de la proteína en el músculo aumentan significativamente debido a la disminución de la proteína dietética, indicando así que el efecto de ahorro proteico por parte de los lípidos de la dieta puede ser promovido por una ligera disminución de la proporción proteína/lípido en dieta.

3. El análisis de isótopos estables (sobre todo el fraccionamiento del ^{15}N, el cual responde a cambios en el metabolismo proteico) es un indicador fiable del estado nutricional en peces, y por lo tanto puede ser una herramienta útil para la determinación de los requerimientos nutricionales óptimos en las dietas para peces cultivados.

4. Las condiciones de cultivo específicas de los criaderos con un origen geográfico distinto, principalmente la dieta y la ración, afectan al metabolismo (COX:CS) y al crecimiento (ARN:ADN) de alevines de dorada, dando lugar a una composición isotópica muscular y corporal específica.

5. Tras un mes bajo idénticas condiciones nutricionales y de cultivo, los alevines de dorada todavía pueden ser discriminados principalmente en base a la composición isotópica muscular (especialmente $\delta 15N$), en combinación con parámetros metabólicos y con la composición

proximal del músculo. Esto sugiere que las condiciones específicas de cultivo y/o la divergencia genética entre los alevines de los distintos criaderos, las cuales afectan a su capacidad de crecimiento, son definidas principalmente por una 'huella o imprenta' isotópica.

6. La composición de isótopos estable (especialmente el $\delta^{15}N$), combinada con variables metabólicas y de crecimiento, puede ser utilizada como herramienta que vaya más allá de la discriminación del origen geográfico, permitiendo la selección de alevines con mejor capacidad de crecimiento. Por otra parte, la signatura isotópica del músculo es un indicador mejor que la de los peces enteros a efectos discriminatorios, puesto que el pez entero contiene los tejidos blandos que son metabólicamente más activos e isotópicamente más variables.

7. La composición isotópica del carbono ($\delta^{13}C$) del músculo, a diferencia del $\delta^{15}N$, no responde igual a los cambios en estado metabólico. Aunque el $\delta^{13}C$ de la fracción de la proteína parece reflejar el comportamiento del $\delta^{15}N$, la gran complejidad de los factores que afectan $\delta^{13}C$ de los tejidos, como el *'isotopic routing'* o el contenido graso, hace que la significación fisiológica de sus cambios se deba interpretar con precaución.

8. El ejercicio moderado y sostenido mejora el crecimiento y la eficiencia alimenticia de los peces, mediante el aumento de la síntesis de proteínas (ARN: ADN), la capacidad oxidativa (COX: CS), y el uso de energía (estimado por ^{13}C de los lípidos y glucógeno) en el músculo blanco, mientras que el *turnover* proteico no cambia (expresado por ^{15}N-fraccionamiento). El músculo rojo, por el contario, se mantiene sin cambios. Por otra parte, el alto contenido de carbohidratos de la dieta experimental parece contribuir a mejorar el efecto de preservación de la proteína para el crecimiento y la eficiencia de conversión alimenticia durante la natación continua en dorada.

9. La profunda afectación del proteoma del músculo esquelético durante la natación moderada y sostenida apoya estos cambios. El músculo blanco muestra una activación de varias vías metabólicas (catabolismo de los hidratos de carbono, la síntesis de proteínas, la contracción muscular, y la desintoxicación), mientras que en el músculo rojo se reducen muchos otros procesos (producción de energía, la contracción muscular, y los procesos homeostáticos).

10. El estudio proteómico, junto con los parámetros metabólicos e isotópicas, pone de manifiesto que, contrariamente a la creencia común, el músculo blanco contribuye de manera significativa a la locomoción durante la natación sostenida y moderada en dorada, mientras que la carga de trabajo del músculo rojo disminuye. Esto sugiere que ninguno de los dos tipos de músculo actúa como un sistema cerrado durante la natación continua, sino que forman una relación compleja, complementando el trabajo realizado por el otro lo que permite una natación más eficiente

11. En general, en combinación con otros parámetros metabólicos, el análisis de isótopos estables proporciona información útil para la comprensión de los procesos biológicos que subyacen a los cambios en las condiciones fisiológicas, lo que complementa y apoya la información proporcionada por nuevas técnicas integrativas como la proteómica.

annex

ANNEX CHAPTER I

Table S1. Table of eigenvalues for PCA of proximal and isotopic data of muscle from gilthead sea bream juveniles from the different dietary conditions.

factor	eigenvalue	variability (%)	cumulative eigenvalue	cumulative variability (%)
F1	4.33	39.36	4.63	39.36
F2	2.32	21.08	6.65	60.44
F3	1.36	12.36	8.01	72.79
F4	0.96	8.76	9.97	81.56
F5	0.71	6.41	10.68	87.97

Table S2. PCA factor loadings of variables measured from muscle of gilthead sea bream juveniles

variable	F1	F2
protein $\delta^{15}N$	**0.856**	0.017
muscle $\Delta\delta^{15}N$	**0.807**	0.404
protein $\Delta\delta^{15}N$	**0.796**	-0.077
muscle $\Delta\delta^{13}C$	**0.786**	-0.163
protein $\Delta\delta^{13}C$	**0.729**	-0.412
muscle $\delta^{15}N$	**0.726**	0.536
protein $\delta^{13}C$	0.531	-0.481
lipid $\delta^{13}C$	0.305	**0.835**
lipid $\Delta\delta^{13}C$	-0.181	**0.819**
glycogen $\delta^{13}C$	-0.016	-0.167
muscle $\delta^{13}C$	0.476	-0.196

Numbers in bold indicate loadings >0.7 and <-0.7

ANNEX CHAPTER III

Table S1. Eigenvalues for PCA analysis of non-proteomic data set from all the fishes of the study.

factor	eigenvalue	variability (%)	cumulative eigenvalue	cumulative %
F1	6.54	17.67	6.54	17.67
F2	5.61	15.15	12.14	32.82
F3	4.23	11.43	16.37	44.25
F4	3.29	8.88	19.66	53.13
F5	2.88	7.79	22.54	60.92
F6	2.34	6.31	24.88	67.23
F7	1.97	5.32	26.85	72.56
F8	1.57	4.25	28.42	76.81
F9	1.41	3.81	29.83	80.61
F10	1.24	3.34	31.06	83.95
F11	1.12	3.04	32.19	86.99

Table S2. Factor Loadings for the two main components (F1 and F2) of PCA analysis of non-proteomic data set from all the fishes of the study.

variables	F1	F2
glycogen (RM)	**.732**	-.037
moisture (RM)	**-.731**	.011
lipid (RM)	**.711**	.057
CF	**.632**	.212
moisture (WM)	**-.623**	.307
δ^{15}N (WM)	**.615**	.459
glycogen (WM)	**.610**	-.064
δ^{15}N-protein (RM)	.581	.015
lipid (WM)	.555	-.541
δ^{13}C-glycogen (WM)	-.540	.267
δ^{13}C-lipid (RM)	.484	.398
δ^{15}N-protein (WM)	.390	.313
δ^{13}C-protein (WM)	-.060	**.722**
fish weight	.402	**.678**
RNA:DNA (WM)	.241	**.665**
COX:CS (WM)	-.418	**.660**
δ^{13}C-lipid (WM)	-.196	**.630**
CS (WM)	.134	**-.607**
fish length	.060	**.605**
δ^{13}C (WM)	-.408	.599
RNA:protein (WM)	.140	.548
COX (WM)	-.437	.491
protein (RM)	.042	-.188
δ^{13}C (RM)	-.137	.045
COX (RM)	-.138	-.008
protein:DNA (RM)	-.415	-.393
RNA:DNA (RM)	-.366	-.216
protein (WM)	.091	.270
CS (RM)	.452	-.116
δ^{15}N (RM)	-.096	.363
COX:CS (RM)	-.375	.150
protein:DNA (WM)	.079	.138
RNA:protein (RM)	.211	-.069
δ^{13}C-protein (RM)	.250	.200
δ^{13}C-glycogen (RM)	-.413	-.310
MFI	-.117	.227
IHS	.510	.215

Bold type indicates loadings >0.6 and <0.6.

Table S3.1: 2-Dimensional gel electrophoresis protein identifications from WM gel spots of exercised sea bream (1.5 BL/s for 4 weeks)

Spot[a]	Instrument[b]	Accession no.[c]	Protein name[c]	Link[d]
3	Q-TOF	gi|348503713	PREDICTED: myomesin-1-like	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIrcaE.dat
5	Q-TOF	gi|348503713	PREDICTED: myomesin-1-like	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIraER.dat
7	Q-TOF	gi|348503713	PREDICTED: myomesin-1-like	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIraTE.dat
80	MALDI	gi|317419952	Glycyl-tRNA synthetase	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIrunm.dat
81	Q-TOF	gi|317419952	Glycyl-tRNA synthetase	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIrawL.dat
172	Q-TOF	gi|45501385	Pkm2a protein	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIratT.dat
230	Q-TOF	gi|47221527	unnamed protein product (fructose-1,6-bisphosphatase)	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIrsSt.dat
244	MALDI	gi|268315573	muscle-type creatine kinase CKM1	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIrswe.dat
255	Q-TOF	gi|348518580	PREDICTED: glycogen phosphorylase, muscle form-like	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIreET.dat
273	Q-TOF	gi|197631857	phosphoglycerate kinase 1	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaEwE.dat
371	MALDI	gi|226441997	phosphoglycerate mutase 2	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIretT.dat
373	Q-TOF	gi|348513753	PREDICTED: phosphoglycerate mutase 2-like	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIrTEE.dat
410	Q-TOF	gi|126211553	creatine kinase 1	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIrTaO.dat
424	Q-TOF	gi|31335193	lactate dehydrogenase-A	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIacTE.dat
457	Q-TOF	gi|48476449	parvalbumin-like protein	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaaOm.dat
466	Q-TOF	gi|5852838	myosin light chain 2	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaEne.dat
469	Q-TOF	gi|348506725	PREDICTED:ATP synthase subunit beta, mitochondrial-like	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIasSS.dat
477	Q-TOF	gi|7678732	myosin light chain 1	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaseh.dat
481	MALDI	gi|207898058	EM_EST:FM153890 (glutathione S-transferase)	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIastR.dat
519	MALDI	gi|51011067	pyruvate kinase, muscle, b	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120126/FtoTObESL.dat
547	MALDI	gi|298361180	peroxiredoxin 6	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaTOe.dat
671	Q-TOF	gi|224551742	warm temperature acclimation-related 65 kDa protein	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaTtR.dat
771	Q-TOF	gi|57547484	adenosine monophosphate deaminase	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaHEL.dat
772	Q-TOF	gi|57547484	adenosine monophosphate deaminase	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaHmO.dat

[a]Spot number from Figure 3 (WM) of the manuscript. [b]Instrument used for MS identifications: MALDI-TOF-TOF (MALDI) or ESI-QUAD-TOF (Q-TOF). [c]Accession number and Protein name from selected protein hit identification. [d]Click on the links to view the MS search information. Web links allow access to *online* MASCOT search; it should be read in *Protein Summary (deprecated)*.

Table S3.2: 2-Dimensional gel electrophoresis protein identifications from RM gel spots of exercised sea bream (1.5 BL/s for 4 weeks)

Spot[a]	Instrument[b]	Accession no.[c]	Protein name[c]	Link[d]
4	MALDI	gi|6653228	skeletal alpha-actin	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaSST.dat
16	MALDI	gi|50539866	pyruvate dehydrogenase E1 alpha 1	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaSmL.dat
24	Orbitrap	gi|146448775	cell division cycle 48	24
53	Orbitrap	gi|209153384	disulfide-isomerase A3 precursor	53
59	Orbitrap	gi|224551742	warm temperature acclimation-related 65 kDa protein	59
92	Orbitrap	gi|47225516	unnamed prot. prod. (3-hydroxyisobutyrate dehydrogenase)	92
138	Orbitrap	gi|47215116	unnamed protein product (adenylosuccinate lyase)	138
150	Orbitrap	gi|74096033	pyruvate kinase	150
160	MALDI	gi|225706838	Short chain 3-hydroxyacyl-CoA dehydrogenase, mitoc. prec.	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaStS.dat
170	Orbitrap	gi|14009437	mitochondrial ATP synthase alpha-subunit	170
171	Orbitrap	gi|47225614	unnamed protein product (fumarate hydratase)	171
172	Orbitrap	gi|41055718	fumarate hydratase, mitochondrial precursor	172
174	MALDI	gi|41054651	isocitrate dehydrogenase [NADP], mitochondrial	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIfcOe.dat
175	Orbitrap	gi|93115142	mitochondrial isocitrate dehydrogenase 2-like	175
176	Orbitrap	gi|213514668	medium-chain specific acyl-CoA dehydrogenase, mitoc.	176
184	MALDI	gi|31322103	creatine kinase mitochondrial isoform	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIfcae.dat
186	Orbitrap	gi|31322103	creatine kinase mitochondrial isoform	186
187	Orbitrap	gi|41388972	Pgk1 protein	187
212	Orbitrap	gi|209730966	Hydroxyacyl-coenzyme A dehydrogenase, mitoc. prec.	212
231	Orbitrap	gi|225706936	Delta3,5-delta2,4-dienoyl-CoA isomerase, mitoc. prec.	231
251	Orbitrap	gi|41055718	fumarate hydratase, mitochondrial precursor	251
259	Orbitrap	gi|328677135	hypothetical protein (cofilin-2)	259
271	MALDI	gi|261825915	parvalbumin	http://www.matrixscience.com/cgi/master_results.pl?file=../data/20120120/FtoTIaYmm.dat
308	Orbitrap	gi|93115142	mitochondrial isocitrate dehydrogenase 2-like	308
322	Orbitrap	gi|47221217	unnamed protein product (nipsnap-like 2)	322
336	Orbitrap	gi|119692141	glucose regulated protein 75	336

[a]Spot number from Figure 3 (RM) of the manuscript. [b]Instrument used for MS identifications: MALDI-TOF-TOF (MALDI) or Orbitrap. [c]Accession number and Protein name from selected protein hit identification. [d]Click on the links to view the MS search information. Web links allow access to *online* MASCOT search; it should be read in *Protein Summary (deprecated)*. Numbers links allow access to in-house MASCOT search identification..

Table S4.1: List of spots with multiple protein identifications from WM of exercised sea bream (1.5 BL/s for 4 weeks)

Spot no.[a]	Protein family groups[b]	Accession number	Protein Score[c]	Mass (Da)[d]	Matches (signif.)[e]	Sequences (signif.)[f]	Protein name
81	**1.1**	**gi|317419952**	**393**	**86293**	**12(8)**	**9(6)**	**Glycyl-tRNA synthetase [Dicentrarchus labrax]**
	1.2	gi|66910298	356	87107	11(5)	8(4)	Si:dkey-276i5.1 protein [Danio rerio]
	1.3	gi|47223387	296	85574	7(5)	6(4)	unnamed protein product [Tetraodon nigroviridis]
	1.4	gi|348503634	259	85673	9(5)	8(4)	PREDICTED: glycyl-tRNA synthetase-like [Oreochromis niloticus]
	2	gi|348541613	87	85188	3(1)	3(1)	PREDICTED: prolyl endopeptidase-like [Oreochromis niloticus]
172	**1.1**	**gi|45501385**	**184**	**58589**	**5(3)**	**4(2)**	**Pkm2a protein [Danio rerio]**
	1.2	gi|74096033	146	58572	6(2)	5(1)	pyruvate kinase [Takifugu rubripes]
	1.3	gi|213512270	67	58865	4(0)	4(0)	pyruvate kinase [Salmo salar]
	2	gi|41056111	62	61375	1(1)	1(1)	phosphoglucomutase-1 [Danio rerio]
230	**1.1**	**gi|47221527**	**160**	**38965**	**5(3)**	**3(2)**	**unnamed protein product [Tetraodon nigroviridis]**
	1.2	gi|348514003	122	36998	5(2)	3(1)	PREDICTED: fructose-1,6-bisphosphatase isozyme 2-like [Oreochromis niloticus]
	2	gi|4210819	129	32483	4(2)	3(1)	malate dehydrogenase [Oryzias latipes]
273	**1**	**gi|197631857**	**248**	**44917**	**4(4)**	**4(4)**	**phosphoglycerate kinase 1 [Salmo salar]**
	2	gi|5880679	53	43037	2(0)	2(0)	muscle creatine kinase [Danio rerio]

Selected hit for each spot identification is in **bold**

a. Spot number from Figure 3 (WM) of the manuscript (all these spots were analyzed by ESI-Q-TOF)

b. Protein hits are grouped by protein family

c. MASCOT protein score

d. theoretical molecular weight

e. peptide matches assigned to protein hits

f. peptide sequence matches assigned to protein hits

Table S4.2: List of spots with multiple protein identifications from RM of exercised sea bream (1.5 BL/s for 4 weeks)

This table is not included due to its large size but it available via the Internet at http://dx.doi.org/10.1021/pr3002832.

Table S5. Identified proteins differentially expressed during sustained exercise (1.5 BL/s, for 4 weeks) in white and red muscle types.

Spot no.[a]	Protein identity	GENE symbol[b]	GENE number[c]	UniProtKB[d]
	WHITE MUSCLE			
3, 5, 7	myomesin-1	MYOM1	8736	P52179
80, 81	glycyl-tRNA synthetase	GARS	2617	P41250
172, 519	pyruvate kinase	PKM2	5315	P14618
273	phosphoglycerate kinase 1	PGK1	5230	P00558
230	fructose-1,6-bisphosphatase	FBP1	2203	P09467
244, 410	muscle-type creatine kinase	CKM	1160	P06732
255	glycogen phosphorylase	PYGM	5837	P11217
371, 373	phosphoglycerate mutase 2	PGAM2	5224	P15259
424	lactate dehydrogenase-A	LDHA	3939	P00338
457	parvalbumin	PVALB	5816	P20472
466	myosin light chain 2	MYL2	4633	P10916
469	ATP synthase subunit beta	ATP5B	506	P06576
477	myosin light chain 1	MYL1	4632	P05976
481	glutathione S-transferase	GSTM2	2946	P28161
547	peroxiredoxin 6	PRDX6	9588	P30041
671	WT acclimation-rel. 65 kDa protein (*)	HPX	3263	P02790
771, 772	adenosine monophosphate deaminase	AMPD	270	P23109
	RED MUSCLE			
4	skeletal alpha-actin	ACTA1	58	P68133
16	pyruvate dehydrogenase E1 alpha 1	PDHA1	5160	P08559
24	cell division cycle 48	VCP	7415	P55072
53	disulfide-isomerase A3 precursor	PDIA3	2923	P30101
59	WT acclimation-rel. 65 kDa protein (*)	HPX	3263	P02790
92	3-hydroxyisobutyrate dehydrogenase	HIBADH	11112	P31937
138	adenylosuccinate lyase	ADSL	158	P30566
150	pyruvate kinase	PKM2	5315	P14618
160, 212	short chain 3-hydroxyacyl-CoA DHase	HADH	3033	Q16836
170	ATP synthase subunit alpha	ATP5A1	498	P25705
171, 172, 251	fumarate hydratase	FH	2271	P07954
174, 175, 308	isocitrate dehydrogenase 2-like	IDH2	3418	P48735
176	medium-chain acyl-CoA DHase	ACADM	34	P11310
184, 186	creatine kinase mitochondrial isoform	CKMT2	1160	P17540
187	phosphoglycerate kinase 1	PGK1	5230	P00558
231	Δ3,5-Δ2,4-dienoyl-CoA isomerase	ECH1	1891	Q13011
259	cofilin	CFL2	1073	Q9Y281
271	parvalbumin	PVALB	5816	P20472
322	nipsnap-2	GBAS	2631	O75323
336	glucose regulated protein 75	HSPA9	3313	P38646

[a] Spot number from Figure 3 (WM) and Figure 4 (RM) of the manuscript. Gene symbol[b], Gene number[c] (Entrez gene database from NCBI, http://www.ncbi.nlm.nih.gov/) and UniprotKB[d] (http://www.uniprot.org) of each protein was obtained from Genecards database search process (http://www.genecards.org). UniprotKB number was used for further gene ontology enrichment analysis showed in Supplementary Table S6. (*) homologous to mammalian hemopexin

Table S6. Functionality related proteins from Gene Ontology enrichment analyses performed using Batch Gene tool of Gene Ontology Enrichment Analysis Software Toolkit (GOEAST, http://omicslab.genetics.ac.cn/GOEAST/).

GOID[a]	Biological Process Term	n[b]	Symbols[c]	*p*-value[d]
			WHITE MUSCLE	
GO:0006091	generat of precursor metabolites and energy	8/24	PGK1 // PKM2 // PKM2 // PGAM2 // PGAM2 // LDHA // ATP5B // PYGM	$9.70 \cdot 10^{-8}$
GO:0005975	carbohydrate metabolic process	8/24	PGK1 // PKM2 // PKM2 // PGAM2 // PGAM2 // LDHA // FBP1 // PYGM	$4.15 \cdot 10^{-5}$
GO:0006936	muscle contraction	7/24	MYOM1 // MYOM1 // MYOM1 // PGAM2 // PGAM2 // MYL2 // MYL1	$6.94 \cdot 10^{-9}$
GO:0006096	glycolysis	6/24	PGK1 // PKM2 // PKM2 // PGAM2 // PGAM2 // LDHA	$1.31 \cdot 10^{-8}$
GO:0006094	gluconeogenesis	4/24	PGK1 // FBP1 // PGAM2 // PGAM2	$6.54 \cdot 10^{-6}$
			RED MUSCLE	
GO:0006091	generat of precursor metabolites and energy	11/26	PDHA1 // ADSL // PKM2 // ATP5A1 // FH // FH // FH // IDH2 // IDH2 // IDH2 // PGK1	$5.36 \cdot 10^{-12}$
GO:0005975	carbohydrate metabolic process	8/26	PDHA1 // HIBADH // PDIA3 // PKM2 // IDH2 // IDH2 // IDH2 // PGK1	$7.41 \cdot 10^{-5}$
GO:0006099	tricarboxylic acid cycle	6/26	FH // FH // FH // IDH2 // IDH2 //IDH2	$1.38 \cdot 10^{-9}$
GO:0006635	fatty acid beta-oxidation	4/26	HADH // HADH // ACADM // ECH1	$3.56 \cdot 10^{-4}$
GO:0006936	muscle contraction	3/26	ACTA1 // CKMT2 // CKMT2	0.013
GO:0006096	glycolysis	3/26	PDHA1 // PKM2 // PGK1	0.003

[a]Gene Ontology Identification number. [b]Number of gene products in the sample set that are annotated / number of gene products in the sample set. [c]List of gene products in the sample set that are annotataed to the GO term. [d]Related proteins are shown by *p*-values < 0.05 (Hypergeometric statistical test).

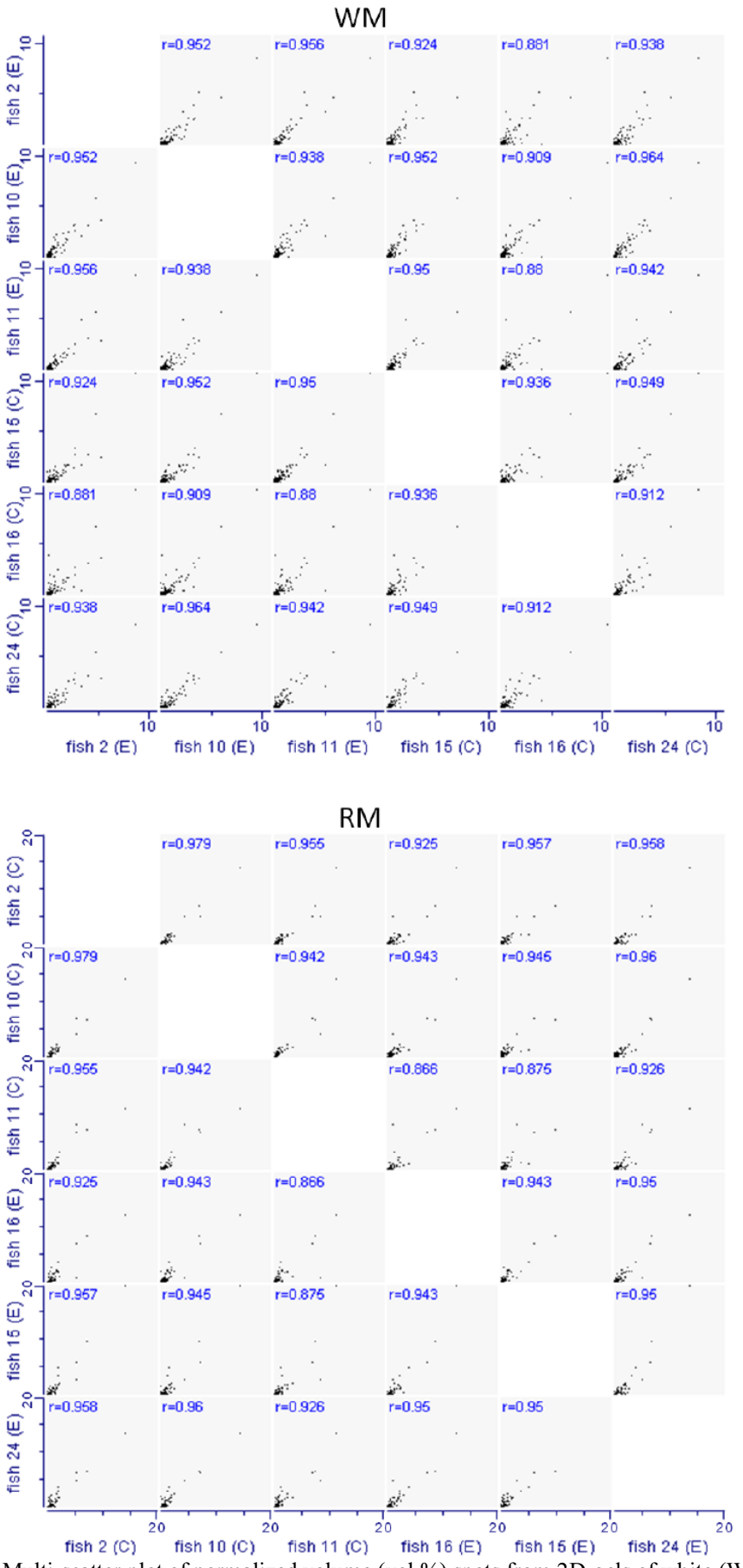


Figure S1. Multi-scatter plot of normalized volume (vol %) spots from 2D-gels of white (WM) and red (RM) muscle. Correlation coefficients (r) are indicated in each plot.

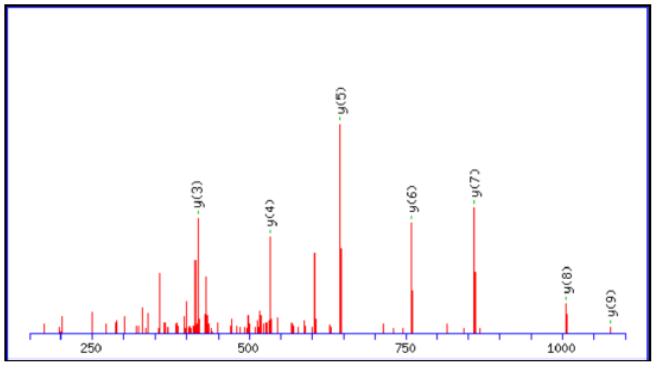
Mascot Search Results

Peptide View

MS/MS Fragmentation of EAFTIIDQNR
Found in gi|5852838, myosin light chain 2 [Sparus aurata]

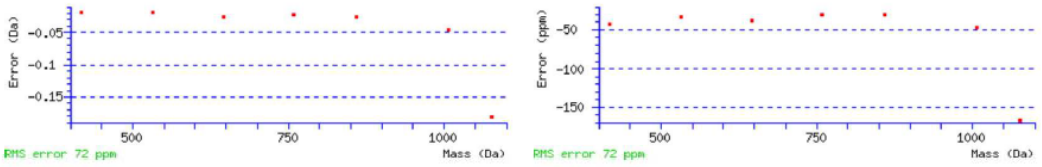
Match to Query 14: 1205.528048 from(603.771300,2+) intensity(2419.7888) index(2)
Data file 466.pkl

Click mouse within plot area to zoom in by factor of two about that point
Or, Plot from 150 to 1100 Da Full range
Label all possible matches ○ Label matches used for scoring ◉



Monoisotopic mass of neutral peptide Mr(calc): 1205.6040
Fixed modifications: Carbamidomethyl (C) (apply to specified residues or termini only)
Ions Score: 63 **Expect:** 0.00014
Matches : 7/88 fragment ions using 9 most intense peaks (help)

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	130.0499	65.5286			112.0393	56.5233	E							10
2	201.0870	101.0471			183.0764	92.0418	A	*1077.5687*	539.2880	1060.5422	530.7747	1059.5582	530.2827	9
3	348.1554	174.5813			330.1448	165.5761	F	*1006.5316*	503.7694	989.5051	495.2562	988.5211	494.7642	8
4	449.2031	225.1052			431.1925	216.0999	T	*859.4632*	430.2352	842.4367	421.7220	841.4526	421.2300	7
5	562.2871	281.6472			544.2766	272.6419	I	*758.4155*	379.7114	741.3890	371.1981	740.4050	370.7061	6
6	675.3712	338.1892			657.3606	329.1840	I	*645.3315*	323.1694	628.3049	314.6561	627.3209	314.1641	5
7	790.3981	395.7027			772.3876	386.6974	D	*532.2474*	266.6273	515.2209	258.1141	514.2368	257.6221	4
8	918.4567	459.7320	901.4302	451.2187	900.4462	450.7267	Q	*417.2205*	209.1139	400.1939	200.6006			3
9	1032.4997	516.7535	1015.4731	508.2402	1014.4891	507.7482	N	289.1619	145.0846	272.1353	136.5713			2
10							R	175.1190	88.0631	158.0924	79.5498			1



NCBI **BLAST** search of EAFTIIDQNR
(Parameters: blastp, nr protein database, expect=20000, no filter, PAM30)
Other BLAST web gateways

All matches to this query

Score	Mr(calc):	Delta	Sequence
63.5	1205.6040	-0.0760	EAFTIIDQNR
63.5	1205.6040	-0.0760	EAFTLIDQNR
21.1	1205.5461	-0.0180	EAMVPMEVWV
21.1	1205.5524	-0.0243	SLESDNEEKR
12.7	1205.5896	-0.0616	LSCSNLMLPR
9.6	1205.6404	-0.1124	KAFTNEQGLAK
8.4	1205.5160	0.0120	GDDEDLKDSGR
8.4	1205.6292	-0.1011	SFDIVLDEIR
7.9	1205.5461	-0.0180	EAMVPMEVWV
7.8	1205.6000	-0.0719	NRSSSLESQAK

Figure S2. Representative MS/MS spectrum of the single peptide used for identification of the protein myosin light chain 2 (spot 466 of WM).