NEUROENDOCRINE CONTROL OF PUBERTY IN VERTEBRATES: CHARACTERIZATION OF THE KISSPEPTIN SYSTEM IN FLATFISH

Alejandro S. Mechaly

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Thesis Supervisors:

Dr. Francesc Piferrer
Professor d'Investigació.
Institut de Ciències del Mar
(ICM-CSIC)

Dr. Jordi Viñas
Professor Lector.
Universitat de Girona
(UdG)

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Submitted in partial fulfillment of the requirements for the Ph.D. degree from the Universitat Pompeu Fabra (UPF). This work has been carried out at the Group of Biology of Reproduction (GBR), Department of Renewable Marine Resources, Institute of Marine Sciences (ICM-CSIC).

Thesis Supervisors:

Dr. Francesc Piferrer
Professor d'Investigació. Institut de Ciències del Mar (ICM-CSIC)

Dr. Jordi Viñas
Professor Lector. Universitat de Girona (UdG)

This Thesis is dedicated to
my family, specially to my mother, father and wife,
and to the memory of my grandfather Luis

“La verdadera ignorancia no es la ausencia de conocimientos,
sino el hecho de rehusarse a adquirirlos”

Karl Popper

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Abstract

The recently discovered decapeptide kisspeptin and its G-protein coupled receptor form a signaling system expressed ubiquitously and are implicated in a variety of still poorly characterized functions. In the brain, kisspeptin is secreted by specific neurons and its receptor is localized in GnRH neurons. Kisspeptin signaling has been fully established in the control of the onset of puberty in vertebrates, from fish to mammals. In this study, we characterized the kisspeptin gene in the Senegalese sole and characterized the kisspeptin receptor genes in both the Senegalese sole and in the Atlantic halibut. In contrast to other fish species, the two species analyzed here showed only the presence of one ligand and one receptor, probably as a consequence of the genome reduction characteristic of Pleuronectiformes. However, in both cases we found an alternative splicing mechanism based on intron retention that produces also non-functional isoforms, but whether this is part of a mechanism to control abundance of the active gene product is still not known. We document spatial and temporal changes of expression of kisspeptin and its receptor in the brain, pituitary and gonads related to the annual reproductive cycle. Finally, we present the first evidence of a possible link between energy balance and reproduction mediated by kisspeptin signaling in a non-mammalian vertebrate.

Resum

El recentment descobert decapèptid kisspeptina i el seu receptor associat a una proteïna G formen un sistema que s’expressa ubiqüitament i que està implicat en diverses funcions, moltes de les quals encara no estan ben caracteritzades. En el cervell, la kisspeptina és secretada per neurones específiques, mentre que el seu receptor es troba a les neurones GnRH. Aquest sistema s’ha relacionat amb el control de l’inici de la pubertat en diferents vertebrats, des de peixos fins a mamífers. En aquest estudi, hem caracteritzat el gen de la kisspeptina en el llenguado senegalès, i els gens del receptor de la kisspeptina tant a llenguado senegalès com en l’Halibut de l’Atlàntic. Al contrari del que ocorre en moltes altres espècies de peixos, aquestes dues espècies només presenten un gen pel lligand i un gen pel receptor. Aquest fet és probable que estigui relacionat amb la reducció de la mida del genoma que han sofert els Pleuronectiformes. Tot i així, en les dues espècies s’hi troba un mecanisme d’empalmament alternatiu conseqüència d’una retenció intrónica que produeix una isoforma no funcional. Ara bé, si aquest mecanisme està relacionat amb el control de l’abundància dels trànscrits de la isoforma funcional encara està per esbrinar. Per altra banda, hem trobat canvis en l’expressió gènica tant en l’espai com en el temps durant un cicle reproductiu dels gens de la kisspeptina i el seu receptor en el cervell, pituïtària i gònades. Finalment, també presentem la primera evidència, en un vertebrat no mamífer, d’una possible relació entre el balanç energètic i la reproducció controlada pel sistema kisspeptina.

Prologue

This thesis was carried out at the Group of Biology of the Reproduction (GBR), Department of Renewable Marine Resources, Institute of Marine Sciences (ICM-CSIC), Barcelona, and under the International Ph.D. Program of Basic Biomedical Research Health and Life Sciences in the Experimental and Health Sciences Department of the Pompeu Fabra University (UPF) during the period 2005-2010. The aim of this thesis was to study the mechanism that controls the onset of puberty in vertebrates, particularly in flatfish. For that purpose, we studied the kisspeptin system in the Senegalese sole (*Solea senegalensis*) and Atlantic halibut (*Hippoglossus hippoglossus*), which were used as model species. The results obtained not only apply in the context of teleost fish, but also some of them can be generalized to other vertebrates. However, and although these studies broadened our knowledge in this field, the results obtained posed new questions that should be resolved in future experiments.

The thesis presented here is structured into four papers:

Paper I. *Mechaly, A.S., Viñas, J., Piferrer, F., 2009. Identification of two isoforms of the Kisspeptin-1 receptor (kiss1r) generated by alternative splicing in a modern teleost, the Senegalese sole (Solea senegalensis). Biology of Reproduction. 80, 60–69.*

Paper II. *Mechaly, A.S., Viñas, J., Murphy, C., Reith, M., Piferrer, F., 2010. Gene structure of the Kiss1 receptor-2 (Kiss1r–2) in the Atlantic halibut: insights into the evolution and regulation of Kiss1r genes. Molecular and Cellular Endocrinology. 317, 78–89.*

Paper III. *Mechaly, A.S., Viñas, J., Piferrer, F., 2011. Gene structure analysis of kisspeptin-2 (Kiss2) in the Senegalese sole (Solea senegalensis): Characterization of two splice variants of Kiss2, and novel evidence for metabolic regulation of kisspeptin signaling in non-mammalian species. Molecular and Cellular Endocrinology. (doi: 10.1016/j.mce.2011.03.004).*

Paper IV. *Mechaly, A.S., Viñas, J., Piferrer, F., 2011. Seasonal and sex-specific changes in the expression of key genes of the reproductive axis in the Senegalese sole (Solea senegalensis): Kisspeptin signaling in different brain areas, pituitary and gonads. General and Comparative Endocrinology (submitted 26/04/2011, manuscript number: GCE-11-140).*

Also, during the realization of this thesis, a review focused on the kisspeptin system was published in a book chapter:

Mechaly, A.S., Viñas, J., Piferrer, F., 2008. Control neuroendocrí de l'inici de la maduresa sexual en els vertebrats mitjançant la kisspeptina i el seu receptor, amb especial èmfasi pel que fa a la situació en els peixos teleostis. Biologia de la Reproducció. Treballs de la Societat Catalana de Biologia, vol. 59, 95–107.

In addition, the results derived form this thesis have been presented at the following scientific meetings: IX International Symposium on Genetics in Aquaculture. Montpellier, France (June 2006); X Jornades de Biología de la Reproducció. Barcelona (May 2007); 8th International Symposium on the Reproductive Physiology of Fish. Saint Malo, France (June 2007); 11ème Journée Scientifique du réseau LARC-Neurosciences. Rennes, France (October 2007); 1er Simposi d'Aqüicultura de Catalunya. Barcelona (February 2008); 1st World Conference on Kisspeptin signaling in the brain. Córdoba (October 2008); XI Jornades de Biología de la Reproducció. Girona (May 2009); 7º Congreso de la Asociación Ibérica de Endocrinología Comparada. Porto, Portugal (September 2009); 9th International Congress on the Biology of fish. Barcelona (July 2010), and XII Jornades de Biología de la Reproducció. Barcelona (June 2011).

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Abbreviations

11-KT: 11-ketotestosterone
3'ss: 3' splice site
5'ss: 5' splice site
ANOVA: analysis of variance
ARC: arcuate nucleus
AVPV: Anteroventral periventricular nucleus
BLAST: basic local alignment search tool
bp: base-pairs
BP: branch point
BPG: brain-pituitary-gonadal
cAMP: cyclic adenosine monophosphate
cDNA: complementary deoxyribonucleic acid
CHO: chinese hamster ovary
Ct: threshold cycle
DAG: diacylglycerol
DNA: deoxyribonucleic acid
Dnase: deoxyribonuclease
E_2: 17β-estradiol
ELISA: enzyme-linked immunosorbent assay
ERE: estrogen response element
FSH: follicle stimulating hormone
FSHr: follicle stimulating hormone receptor
GnRH: gonadotropin releasing hormone
GPCR: G protein-coupled receptor
ICV: intracerebroventricular
IHC: inmunohistochemestry
IM: intramuscular
IP: intraperitoneally
IP_3: inositol triphosphate
ISH: in situ hybridization
Kb: kilobase
KISS: Kisspeptin
KISSR: Kisspeptin receptor
Kir: Inwardly rectifying potassium channel
KO: knock out
LH: lutenizing hormone
LHr: lutenizing hormone receptor
mPOA: medial preoptic nucleus
mRNA: messenger ribonucleic acid

PCR: polymerase chain reaction
PeN: periventricular nucleus
PIP2: phosphatidylinositol bisphosphate
PKA: protein kinase A
PKC: protein kinase C
PLC: phospholipase C
Ppy: polypyrimidine tract
pre-mRNA: pre-mature messenger ribonucleic acid
qPCR: quantitative real time PCR
RNA: ribonucleic acid
SC: subcutaneous
T: testosterone
TF: transcription factor
TRPC: transient receptor potential cation
WT: wild type

Introduction

I. Introduction

1. Reproduction

1.1. The brain-pituitary-gonadal (BPG) axis

It is well established that sexual reproduction in vertebrates relies on the activation of the brain-pituitary-gonadal (BPG) axis (Colledge, 2008). This axis integrates the central nervous system and the endocrine system from mammals (Dhillo *et al*., 2005; Ojeda *et al*., 2006) to fish (Weltzien *et al*., 2004). The gonadotropin-releasing hormones (GnRHs) are secreted from neurons located in different brain areas including the hypothalamus (Kumar *et al*., 2005). In the anterior pituitary gland, GnRH stimulates the synthesis and secretion of two gonadotropins, the luteinizing hormone (LH), and the follicle-stimulating hormone (FSH) (Colledge, 2008; Rainis and Ballestrazzi, 2005). These gonadotropins are transported through the bloodstream to the gonads, where they bind to specific receptors, the FSH receptor (FSHR) and the LH receptor (LHR), and induce, among others, the synthesis and release of sex steroid hormones, stimulating gametogenesis (Levavi-Sivan *et al*., 2010). In mammalian, avian, reptilian and amphibian species, pulsatile release of GnRH occurs via the hypothalamo-hypophyseal portal vessels. However, in teleost fish GnRH neurons directly innervate the pituitary (Amano, 2010; Kim *et al*., 2011). Sex steroids (testosterone and estradiol) exert both positive and negative feedback effects on gonadotropin synthesis and secretion at different levels of the BPG axis (Colledge, 2008; Dufour *et al*., 2010) (Figure 1). The precise regulation of hormone synthesis and secretion within the BPG axis is critical to the proper functioning of the reproductive system.

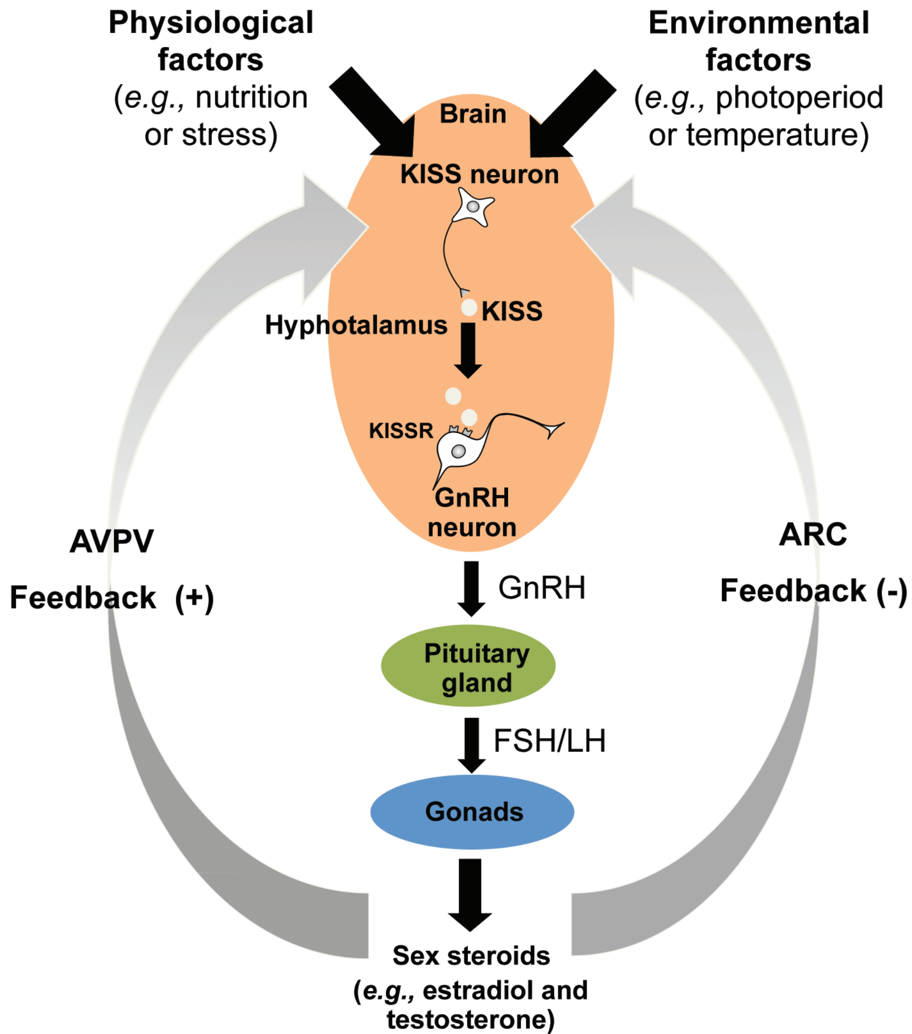

Figure 1. Diagram of the mechanism for the neuroendocrine control of gonadal maturation in vertebrates via the brain-pituitary-gonadal (BPG) axis. Abbreviations: KISS, kisspeptin; KISSR, kisspeptin receptor; GnRH, gonadotropin releasing hormone. FSH, follicle-stimulating hormone. LH, luteinizing hormone. AVPV, anteroventral periventricular nucleus. ARC, arcuate nucleus.

1.2. Puberty. Definition

Like in other vertebrates, in teleost fish puberty is defined as the period that covers the transition from a sexually immature juvenile to a reproductively mature adult (Schulz and Goss, 1999). During this period, a series of interactions occur between the organism and the environment, which ultimately lead to morphological, physiological and behavioral developmental changes (Ebling, 2005). The initiation of puberty and its physiological consequences represents one of the most important transformations that occur along the life of vertebrates and entails a great complexity of endocrine functions and regulations (Taranger *et al*., 2010). In teleost fish, puberty occurs after gonadal sex differentiation (Strussmann *et al*., 2002) and is characterized by the onset of spermatogenesis in males (Schulz and Miura, 2002; Schulz *et al*., 2010) and full vitellogenic ovarian development (cortical alveolar stage) in females (Patino and Sullivan, 2002) (Figure 2). The completion of puberty in fish is similar to what occurs in mammals leading to the first production of gametes (Okuzawa, 2002). However, this initial

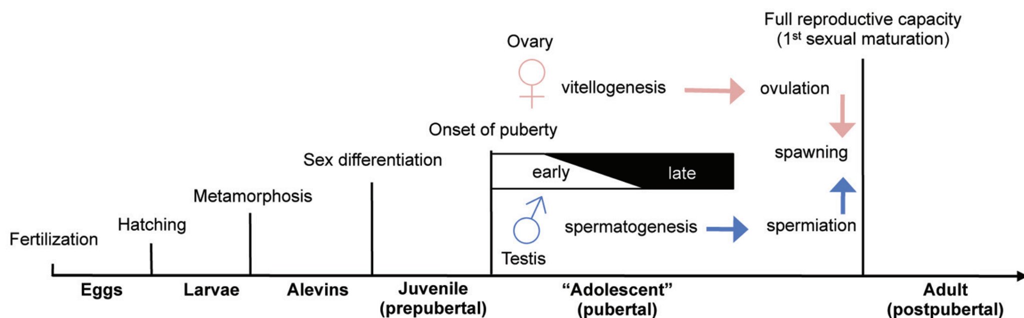

Figure 2. Schematic representation of the chronology of puberty in fish and terms commonly used in relation to gonadal maturation. The onset of puberty is characterized by the appearance of primary spermatocytes in males and oocyte maturation in females. The term "adolescent" is anthopogenic and has not been used in this thesis. Instead the term "pubertal" has been used. Notice that in females oogenesis starts right after or coinciding with sex differentiation but oocyte maturation does not take place until the onset of puberty. In males spermatogenesis starts with puberty (Modified from Carrillo *et al*., 2009).

maturation should not be mistaken with the annual re-activation of the reproductive cycle of seasonal breeders (Gerlach *et al*., 2000; Thièry *et al*., 2002). Although some physiological similarities may be found, these annual cycles of reproduction cannot be considered as puberty.

1.2.1. Puberty and maturation in teleost fish with different life history strategies

Teleost fish comprise a large number of species, close to 30,000 (Nelson, 2006), and are characterized by a high variability in the age and size at puberty (Taranger *et al*., 2010). Genetic, internal signals and environmental factors explain this variation (Dufour and Rousseau, 2007; Nocillado and Elizur, 2008). The onset of puberty in different species shows an extreme variability; from before the first year in species with short lifespan, such as the zebrafish (*Danio rerio*), to 8-9 years in species with longer lifespans, such as the Southern bluefin tuna (*Thunnus maccoyii*) (Figure 3A). However, when puberty is expressed relative to total lifespan, then it becomes evident that maturity is usually reached approximately at ≈10-25% of the total lifespan, always being less than one third of the maximum lifespan (Figure 3B). The two species studied in this thesis are phylogenetically closely related but some differences are evident in the time at first maturation (Figure 3A). In the Atlantic halibut (*Hippoglossus hippoglossus)*, the average age at sexual maturation in males was estimated to be at 4.5 years (≈12% of lifespan), while in females it was estimated at 6 years of age (≈18% of lifespan) (Jakupsstovu and Haug, 1988; Douglas and Ross, 2006). The onset of puberty in cultured Atlantic halibut juveniles with accelerated growth is advanced by about 1 year, although they attain a final body size similar to that of wild males (Norberg *et al*., 2001). In the Senegalese sole (*Solea senegalenesis*), males mature for the first time during the second year (≈10% lifespan) and females during the second or third year (≈15% lifespan), when the total length reaches ≈30 cm in males and ≈32 cm in females (Dinis *et al*., 1999; García-Lopez *et al*., 2006b). Thus, in both species the timing of first maturation follows the general rule of less that one third of total lifespan.

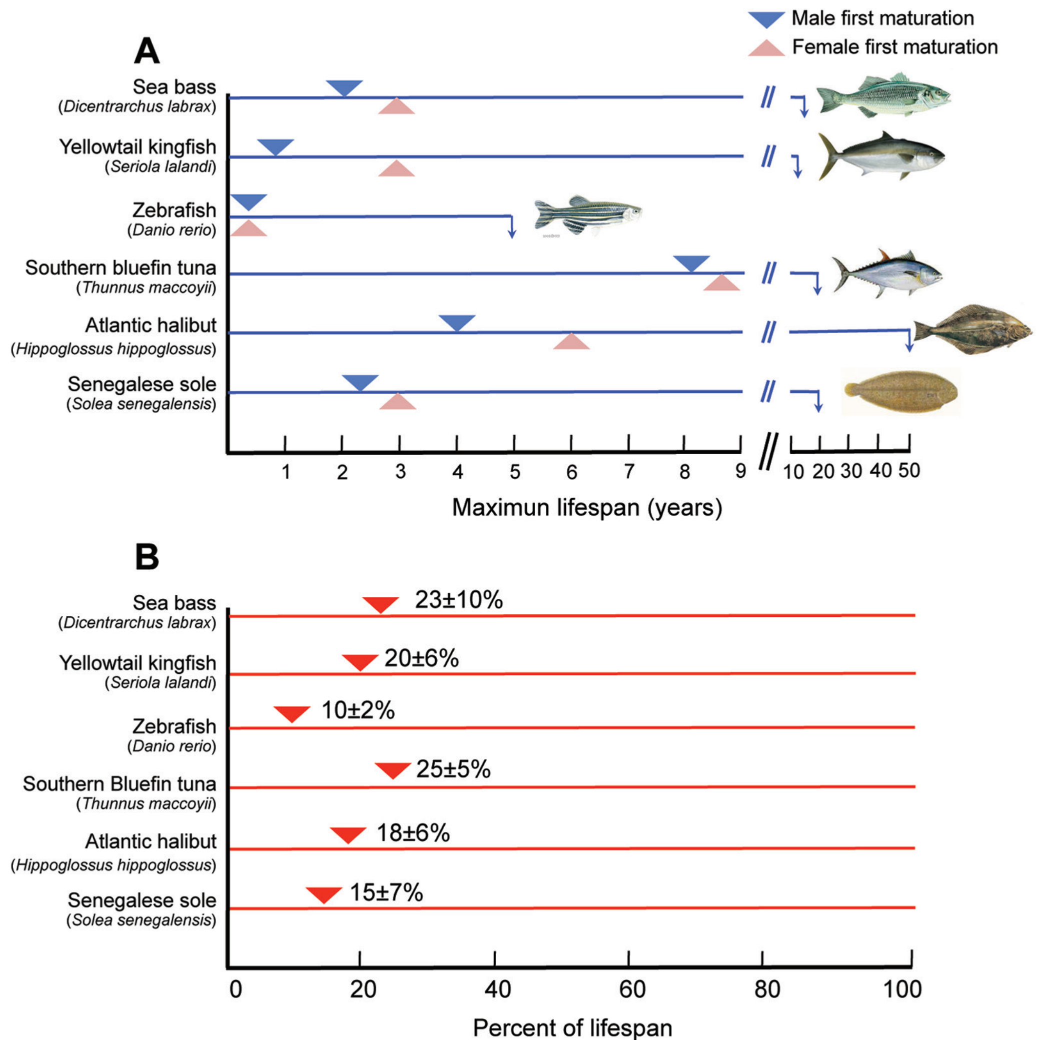

Figure 3. Onset of puberty and first sexual maturation in relation to age in teleost fish. **(A)** In relation to total lifespan. Notice that males usually mature before females. **(B)** In most cases puberty takes place approximately when 10 to 25% of total lifespan has passed. (Adapted from Bowering *et al*., 1986; Thorogooda, 1986; Moretti *et al*., 1999; Cargnelli *et al*., 1999; Gillanders *et al*., 1999; Dinis *et al*., 1999; Taranger *et al*., 2010; Lohr *et al*., 2011).

1.2.2. Abiotic factors that control puberty

A variety of abiotic environmental cues can influence the onset of puberty. These factors include, among others, photoperiod and temperature. These cues affect the neuroendocrine systems in the brain, leading to changes in the activity of the BPG axis (Schulz and Goos, 1999) (Figure 1). Nevertheless, in general, there is still limited knowledge on the neuroendocrine mechanism related to environmental control of puberty in fish (Taranger *et al*., 2010).

Photoperiod is a key environmental factor for initiation of puberty in fish species living at moderate to high latitudes, ensuring the appropriate seasonal timing of reproduction according to the most favorable conditions for the offspring (Bromage *et al*., 2001). In this process, melatonin plays a crucial role in the circadian and annual physiological rhythms (Falcón *et al*., 2010; Carrillo *et al*., 2010). Different structures by which light enters the organism and is transformed to a biological signal are involved: the pineal complex, retina, eye, and brain (Foster and Hankins, 2002). However, knowledge in this field is much more advanced in mammals and is still scarce in fish. The melatonin, a hormone produced by photoreceptors in the pineal gland and eyes of all vertebrates including fish (Bromage *et al*., 2001), regulates the transduction of photoperiodic information to the BGP axis (Amano *et al*., 2000). This hormone synchronizes behavior and neuroendocrine changes with the daily and annual photoperiod cycle (Falcón *et al*., 2010). Thus, the knowledge of the mechanisms by which the pineal gland and melatonin hormones transduce photoperiodic cues into neurochemical signals is crucial to understand seasonal reproduction in fish (Falcón *et al*., 2010; Carrillo *et al*., 2010).

Most teleost fish are poikilothermic, thus behavioral and physiological processes depend on the environmental water temperature (Lõhmus *et al*., 2010). Some tunas (Genus *Thunnus*) and related species are partially endothermic and can maintain their body temperatures above ambient temperature (Graham and

Dickson, 2001). Temperature affects puberty by modulating cellular differentiation, inhibiting or triggering gametogenesis (Taranger *et al*., 2010). Moreover, temperature can also affect the onset of puberty indirectly by its effects on somatic growth and energy stores (Bromage *et al*., 2001) and is often considered as a key factor in cyprinids (Peter and Yu, 1997), percids and moronids, where a decline in temperature favors reproduction (Migaud *et al*., 2002). In salmonids, temperature appears to play a minor role in the control of the reproductive cycle (Bromage *et al*., 2001). Nevertheless, some studies demonstrated that maturation in fish can be blocked when water temperature exceeds a certain threshold (Taranger *et al*., 2010), for example > 5 ºC in Atlantic halibut (Brown *et al*., 2006), > 11 ºC in Atlantic salmon (*Salmo salar*) (Taranger and Hansen, 1993), > 14 ºC in white sturgeon (*Acipenser transmontanus*) and rainbow trout (*Oncorhynchus mykiss*) (Pankhurst *et al*., 1996; Webb *et al*., 1999), > 16 ºC in sea bass (*Dicentrarchus labrax*) (Zanuy *et al*., 1986) and > 14 ºC in Senegalese sole (García-López *et al*., 2009). Thus, temperature also acts during the final stages of gonadal maturation and at spawning in teleost fish (Taranger *et al*., 2010; Wang *et al*., 2010).

Other factors that influence the onset of puberty of fish include: salinity (Saunders *et al*., 1994), pheromones (Burnard *et al*., 2008), pH, stress level, tides and rainfall (Pankhurts and Porter, 2003), but they have been far less studied.

1.2.3. Biotic factors: nutrition

The physiological mechanisms controlling food intake, energy balance and reproduction are intimately linked (Schneider, 2004; Castellano *et al*., 2009). In vertebrates, feeding is regulated by the equilibrium between appetite-stimulating (orexigenic) and appetite-inhibiting (anorexigenic) factors which act on the feeding centers in the brain (Volkoff *et al*., 2009). In fish, seasonal changes that normally occur with spawning migration and reproduction demonstrate the relationship between nutrition and reproduction (Volkoff *et al*., 2005). In mammals food deprivation inhibits the BPG axis (Schneider, 2004). On the other hand, fish

represent a vast phylogenetic group, with much variation in behaviors, suggesting that the endocrine control of feeding might also be extremely diverse. In goldfish (*Carassius auratus*) one week of fasting increased the mRNA level of the anorexigenic neuropeptide Y (NPY) in the hypothalamus (Narnaware and Peter, 2001). NPY is well established as a key regulator of the reproductive axis. In vivo and in vitro studies have demonstrated that NPY stimulates GnRH secretion (Advis *et al*., 2003). In catfish (*Clarias batrachus*), intracranial administration of NPY increased GnRH immunoreactivity, increasing the release of LH from the pituitary, suggesting that NPY may play a positive role in regulating reproduction (Subhedar *et al*., 2005). However, in the Atlantic cod (*Gadus morhua*), a similar time of food deprivation as the tested in the goldfish did not affect *NPY* mRNA expression in the forebrain (Kehoe and Volkoff, 2007), indicating that reproduction appeared not to be influenced by this fasting period. In general, the role of the biotic factors that are involved in the regulation of the initiation of puberty in fish is generally not well understood, probably due to the aforementioned diversity of evolutionary strategies of this large phylogenetic group (Okuzawa, 2002; Weltzien *et al*., 2004; Dufour and Rousseau, 2007; Taranger *et al*., 2010). The relationship between nutritional status and reproductive function should be investigated more extensively since in some species broodstock maturation can be controlled through nutrition under aquaculture conditions.

1.2.4. Other biotic factors

Another biotic factor known to influence reproduction is exercise. For instance, in the European eel (*Anguilla anguilla*) reproduction occurs only after a long migration, suggesting that this period of prolonged swimming might be the necessary stimulus for the onset of puberty (Sébert *et al*., 2007; van Ginneken *et al*., 2007). Furthermore, sexual maturation is also related to long-distance reproductive migration in rainbow trout (Palstra *et al*., 2010). Reproductive function in fish can also be affected by stress caused by physical disturbances, such as handling and transport (Milla *et al*., 2009).

2. The kisspeptin system

2.1. Discovery of the kisspeptin system

In 1996 Lee and coworkers discovered a new gene responsible for suppressing metastasis in malignant melanoma and breast cancers (Lee *et al*., 1996). The peptide product of this gene was originally known as metastin and is currently named kisspeptin (*KISS1*) (Lee *et al*, 1999). At the same time, studies carried out in rats discovered an orphan receptor and found that this receptor acted in association with a G-protein. This receptor with no known ligand was initially named as GPR54 (Lee *et al*., 1999). Two years later, three research groups almost simultaneously discovered that kisspeptin-54 (Kp54) of the human *KISS1* gene was responsible for the activation of the receptor GPR54 (Ohtaki *et al*., 2001; Kotani *et al*., 2001; Muir *et al*., 2001), and in consequence the initial nomenclature of GPR54 was changed to kisspeptin receptor (KISSR). Although kisspeptin and its receptor were initially linked in the regulation of cancer, independent reports from two groups showed the presence of inactivating mutations in KISS1R in humans (de Roux *et al*., 2003; Seminara *et al*., 2003), a condition that was later on replicated in mice (Funes *et al*., 2003; Seminara *et al*., 2003). The mutations in KISS1R were associated with the idiopathic hypothalamic hypogonadism (IHH) syndrome, which impairs puberty in humans (de Roux *et al*., 2003; Seminara *et al*., 2003). This syndrome is characterized by delayed or absent pubertal development secondary to gonadotropin deficiency. These initial discoveries brought unprecedented excitement among reproductive physiologists and endocrinologists. Further studies demonstrated that the ligand kisspeptin (KISS) and its receptor (KISSR) act as a principal positive regulator of the reproductive axis by directly stimulating GnRH neuron activity (Seminara *et al*., 2003; Tena-Sempere, 2006) (see below).

2.2. Nomenclature of genes and gene products of the kisspeptin system

The nomenclature used for the different kisspeptins (see below) and their receptors is very confusing. Recent reviews carried out in the laboratory of Robert Steiner, University of Washington, clarified the kisspeptin nomenclature and offered recommendations to unify the terminology among the kisspeptin gene, the mRNA, and the protein of different species (Gottsch *et al*., 2009; Oakley *et al*., 2009). *KISS1* should be used to represent the gene/mRNA in primates; *Kiss1* should be used for rodent and non-primate mammalian kisspeptin genes. The non-italicized versions of the gene nomenclature should be used to refer to the protein products *i.e.,* KISS1 for human and Kiss1 for other species, although spelling-out “kisspeptin” is also apwpropriate (Gottsch *et al*., 2009; Oakley *et al*., 2009). The terminology proposed agrees with the International Union of Basic and Clinical Pharmacology, recently reviewed (Kirby *et al*., 2010) and adapted to include also the teleost fish nomenclature (Akasome *et al*., 2010). The nomenclature referred in Table 1 will be used throughout this thesis.

Table 1. Summary of the terminology used for genes and proteins of the kisspeptin system [(1)]

Species	Kisspeptin	Previous terminology		Recommended terminology [(2)]	
		Gene/mRNA	Protein	Gene/mRNA	Protein
Primates	Ligand	KiSS-1 KiSS1[(3)]	Metastin KiSS-1 Kisspeptin (Kp)-145, -54, -14, -13, -10 Human metastin 45-54	*KISS1*	kisspeptin (abbreviated KISS1) (abbreviated peptides, Kp145, 54, 14, 13, 10)
	Receptor	AXOR12 HOT7T175 *GPR54* *KiSS1R* Metastin receptor	GPR54 KiSS-1	*KISS1R*	kisspeptin receptor (abbreviated KISS1R)
Rodent and non-primate mammals	Ligand	Kiss1 KiSS1[(3)]	Metastin Kisspeptin (Kp)-145, -54, -14, -13, -10	*Kiss1*	kisspeptin (abbreviated Kiss1)
	Receptor	GPR54	GPR54 Kiss1R	*Kiss1r*	kisspeptin receptor (abbreviated Kiss1r)
Non- mammalian species	Ligand	KiSS-1/KiSS-2 KiSS1/KiSS2 Kiss-1/Kiss-2 Kiss1/Kiss2 [(4)]	Kisspeptin-145, -	*kiss1/kiss2*	Kiss1/Kiss2
	Receptor	GPR54 Kiss1r/Kiss2r	GPR54 Kiss1R	*kiss1r/kiss2r*	Kiss1r/Kiss2r

(1) Modified from Gottsch *et al*., 2009; Oakley *et al*., 2009; Akasome *et al*., 2010.
(2) Gene and protein terminology adopted in this thesis.
(3) Typically italicized for the gene and not for mRNA.
(4) Usually a and b or A and B were used instead 1 or 2.

Kisspeptin owns its name to a chocolate factory located in Pennsylvania, very close to the laboratory where KISS1 was first discovered (Lee *et al*. 1996). Thus, the prefix of KISS1, “KI”, refers to a famous brand of chocolate, “Hershey’s Chocolate Kiss”; the letters “SS” make reference to the “suppressor sequence”, in relation to the first known function as metastasis suppressor of the gene; and finally the number “1” corresponds to a nomenclature convention (Smith *et al*., 2006a). Later, with the establishment of a relationship between *KISS1* gene and the onset of puberty, some studies started to refer to it as the “gene of the first kiss” (Smith *et al*., 2006a; Tena-Sempere, 2006).

2.3. The role of the kisspeptin system in the control of puberty

GnRH is a decapeptide considered the master hormone regulating reproductive function (Kakar and Jennes, 1995). Nowadays, a large body of evidence across several species provides strong proof that kisspeptin exerts its control of the reproductive axis through direct regulation of GnRH neurons, suggesting that the kisspeptin system activation is a critical point in the onset of puberty in vertebrates (Gottsch *et al*., 2009; Oakley *et al*., 2009). The importance of this finding is evidenced in the rapid increase in the number of publications in this area (Figure 4). The discovery of the role of kisspeptin was referred to as “one of the most exciting findings made in the field of reproductive biology since the discovery of GnRH in the 1970s” (Messager *et al*. 2005).

Expression studies of *KISS* and *KISSR* genes and their proteins have been reported in the brain of several fish species (Mitani *et al*., 2009; Servili *et al*., 2011), frogs (Moon *et al*., 2009), rats (Muir *et al*., 2001), mice (Gottsch *et al*., 2004), sheep (Smith *et al*., 2007) and primates (Shahab *et al*., 2005). Several studies reported the stimulatory effect of kisspeptin on LH secretion in the rat and mouse (Gottsch *et al*., 2004; Matsui *et al*., 2004; Navarro *et al*., 2004). These initial studies were replicated in the same species and other species (Table 2). Another line of interesting research is the response of the organism to administration of kisspeptin. Intracerebroventricular (ICV) injection of kisspeptin increased LH

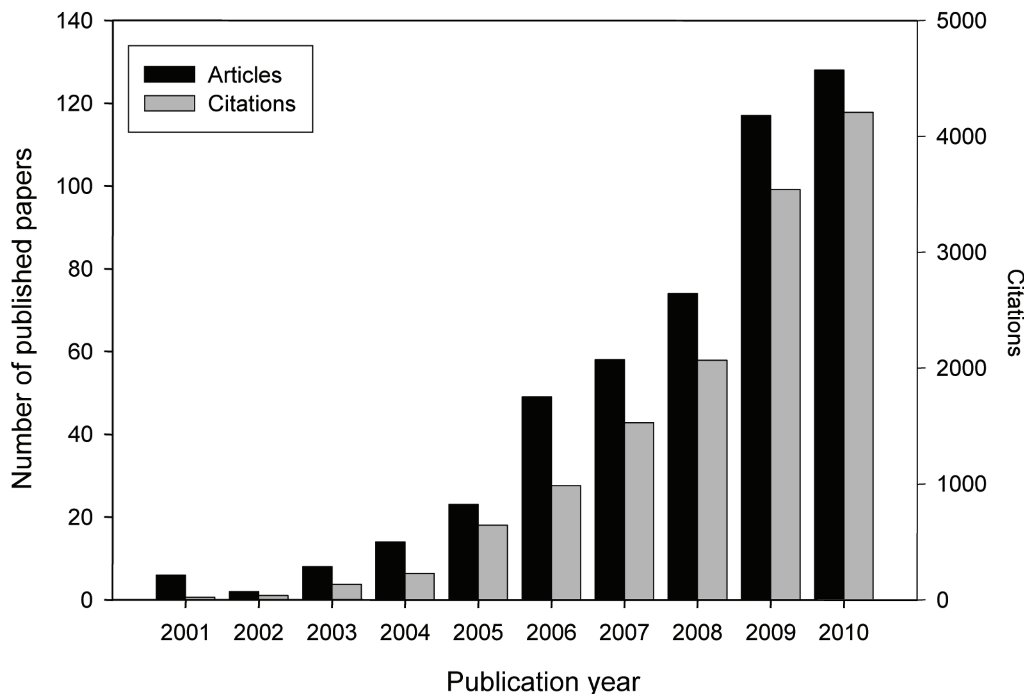

Figure 4. Evolution of the research output on the kisspeptin system and its impact as evidenced by the number of papers published on the subject since its discovery in 2001. Data obtained searching in the Web of Science (Thomson Reuters) using four keywords: *kisspeptin, metastin, GPR54, KISS1R, kiss2, kiss2r.*

secretion in rats (Matsui *et al*., 2004; Navarro *et al*., 2004; Navarro *et al*., 2005), mice (Gottsch *et al*., 2004), sheep (Messager *et al*., 2005), horses (Magee *et al*., 2009), pigs (Lents *et al*., 2008), and primates (Dhillo *et al*., 2005). Similar effects were also observed in fish. Intraperitoneal (IP) administration of kisspeptin in adult females goldfish (Li *et al*., 2009), intramuscular (IM) administration in sea bass (Felip *et al*., 2009) and ICV administration in females of orange-spotted grouper (*Epinephelus coioides*) (Shi *et al*., 2010) stimulated the secretion of FSH and LH (Table 2).

Table 2. Effects of kisspeptin administration in vertebrates

Species	Sex	Stage	Compound	Route	Effects	References
Mammals						
Rat	male	adult	Kp54	SC	Increased LH FSH	Matsui *et al*., 2004
	male female	adult pre-pubertal	Kp10	ICV	Increased LH	Navarro *et al*., 2004
Mouse	male	pre-pubertal	Kp10/Kp54	ICV	Stimulated LH secretion	Gottsch *et al*., 2004
Primates	male	pre-pubertal	Kp10	ICV	Stimulated LH secretion	Shahab *et al*., 2005
Teleost fish						
Sea bass	male female	pre-pubertal	Kp10 (Kiss1/ Kiss2)	IM	Stimulated LH secretion	Felip *et al*., 2009
	male	adult	Kp10 (Kiss2)	IM	Stimulated LH secretion	
Goldfish	female	adult	Kp10 (Kiss1)	IP	Stimulated LH secretion	Li *et al*., 2009
Orange-spotted grouper	female	adult	Kp10 (Kiss2)	ICV	Stimulated FSH secretion	Shi *et al*., 2010

ICV: Intracerebroventricular. SC: Subcutaneous. IM: Intramuscular. IP: Intraperitoneal. LH: luteinizing hormone. FSH: follicle-stimulating hormone.

Sex steroids play an important role in pubertal development, but the mechanism that regulates how this is achieved is poorly understood. With the emergence of kisspeptin as a critical regulator of GnRH neurons, it now seems likely that kisspeptin neurons integrate the signals exerted by the sex steroids to GnRH neurons (Oakley *et al*., 2009). Thus, some studies have recently suggested that estrogen regulation of puberty is mediated through kisspeptin. Unlike GnRH-producing neurons, kisspeptin-producing neurons express nuclear estrogen receptors (ER) (Franceschini *et al*., 2006; Kinoshita *et al*., 2005; Smith *et al*., 2005). Moreover, most of hypothalamic Kiss1 neurons also express progesterone receptors, as shown in the ewe (Smith *et al*., 2007). In rats, sheep, mice and monkey, castration in both sexes leads to increment levels of *Kiss1* mRNA in the hypothalamus (Navarro *et al*., 2004; Kinoshita *et al*., 2005; Shahab *et al*., 2005; Smith *et al*., 2005; Smith *et al*., 2007). In addition, several studies have demonstrated that kisspeptin neurons located in the anteroventral periventricular (AVPV) nucleus mediate the positive effects of estrogen, while kisspeptin neurons located in the arcuate nucleus (ARC) mediate the inhibitory effects of estrogen to ultimately control GnRH neurons (Smith *et al*., 2005; Oakley *et al*., 2009). For instance, steroid hormone reduced *Kiss1* mRNA expression in the ARC of

ovariectomized rats but increased expression in the ventromedial nuclei (VMN) of the hypothalamus (Smith *et al*., 2006b). Similar results were obtained in castrated mice after estrogen replacement (Smith *et al*., 2005). Studies in rats (Roa *et al*., 2006) and humans (Dhillo *et al*., 2007) indicate that maximal LH response to kisspeptin occurs during estrus or the preovulatory phase of the estrous cycle. Furthermore, when estrogen and progesterone levels were restored in ovariectomized rats, LH response to kisspeptin was maximized (Roa *et al*., 2006). Collectively, these studies can be interpreted to indicate that sex steroids play a critical role in regulating the ability of kisspeptin to affect the functionality of the BPG axis. Thus, the discovery of kisspeptin system provides new opportunities for the manipulation of reproduction in vertebrates, specifically by the activation-deactivation of GnRH and, in particular, for the control of puberty and maturation (Aparicio, 2005; Seminara and Kaiser, 2005; Gottsch *et al*., 2006; Kuohung and Kaiser, 2007; Roseweir and Millar, 2009; Tena-Sempere, 2010).

2.4. Structure and expression of kisspeptin genes and proteins in vertebrates

The first *KISS1* gene was characterized in humans with the description of a gene structure composed of four exons and three introns (West *et al*., 1998). The first two exons are not translated to protein, whereas the third and fourth are partially translated, making the entire coding region being comprised of only 438 base pairs (bp) (West *et al*., 1998). The human *KISS1* gene encodes a 145 amino acid protein that is predominantly hydrophilic in nature and belongs to the arginine-phenylalanine (RF)-amide family of peptides (Dhillo *et al*., 2006). This is an indefinite group of peptides all with an RF-amide structure at their carboxy-terminal (Dockray, 2004). The predicted KISS1 protein contains three dibasic motifs with their associated enzymatic cleavage sites (Luan *et al*., 2007). Furthermore, truncated forms of the KISS1 peptide of 13 and 14 amino acids with the same C-terminus as the 54-residue peptide can also occur naturally and maintain biological activity (Kotani *et al*., 2001). Although not naturally occurring, shorter synthetic peptides are biologically active as long as the minimum receptor-binding

motif of 10 amino acids (amino acids 112-121) are included in the design of the peptide (Kotani *et al*., 2001; Muir *et al*., 2001; Ohtaki *et al*., 2001). All these peptides can be collectively referred to as kisspeptins (Kp). The Kp145 is a preprotein that contains a sequence signal (amino acids 1 to 19), two possible sites of enzymatic cleavage (amino acids 57 and 67) and a site of terminal cleavage or amidation (amino acids 121 to 124) (Kotani *et al*., 2001; Muir *et al*., 2001; Ohtaki *et al*., 2001) (Figure 5). According to the site where the proteolytic modification of this

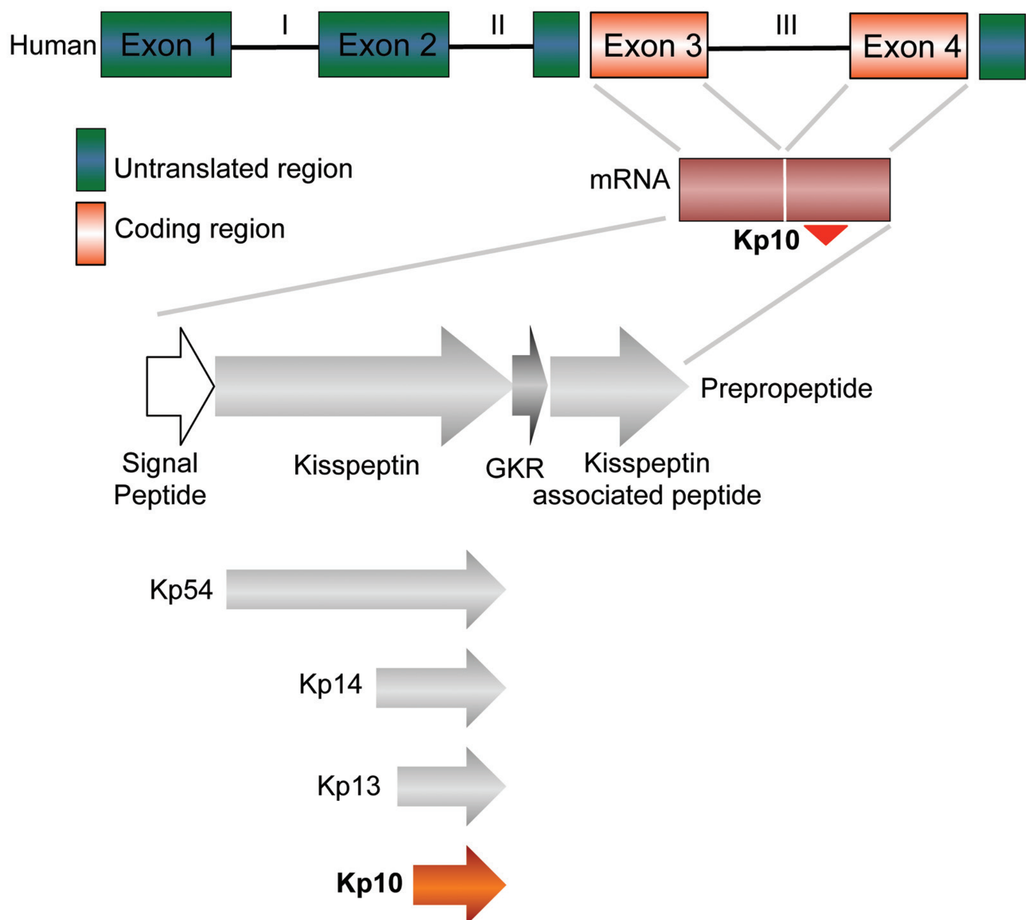

Figure 5. Gene and peptide structure of kisspeptin (KISS) in humans. Products of the *KISS1* gene in humans. *KISS1* mRNA is transcribed from the *KISS1* gene and translated to form a 145 amino acid prepropeptide named Kp145. The kisspeptin peptides, Kp54, Kp14, Kp13 and Kp10 are products of succesive cleavages. GKR, amino acids of the proteolytic cleavage site. Roman numerals indicate intron numbers.

preprotein is carried out, different kisspeptins are obtained. Other peptide products of modifications of the same precursor are kisspeptin-14 (Kp14), kisspeptin-13 (Kp13) and kisspeptin-10 (Kp10) (Kotani *et al*., 2001). Kp10 corresponds with the last 10 amino acids of the Kp54 and is the most important one in the activation of the receptor, KISS1R, as suggested by the high degree of conservation of these 10 amino acid residues along evolution (Stafford *et al*., 2002).

Currently, sequences of the *KISS1* gene are known in several mammalian species including placentals, marsupial and monotremes (Lee *et al*., 2009; Akasome *et al*., 2010). The cloning of sequences from an increasing number of species led to the discovery of KISS1 in an amphibian, the clawed frog (*Xenopus tropicalis)* (Lee *et al*., 2009). In addition, bioinformatic analysis and synteny studies have also helped in cloning and localizing the gene in two model species of teleost fish: the medaka (*Oryzias latipes*) and zebrafish (Kanda *et al*., 2008; van Aerle *et al*., 2008). In zebrafish, *kiss1* only has two exons and one intron. These two exons correspond with the exons 3 and 4 of the *KISS1* gene in humans (van Aerle *et al*., 2008; Biran *et al*., 2008) with a high degree of homology in the segment that encodes Kp10, the only peptide discovered in zebrafish so far (Biran *et al*., 2008). Recently, with the availability of the complete genome sequence of zebrafish and medaka and using bioinformatic tools, a second paralogous gene of kisspeptin was discovered and named *kiss2* (Kitahashi *et al*., 2009). This brought about the searches of all sequenced genomes for the two paralogous genes, resulting in identification of the two *Kiss* genes in only one ancestral mammal, the monotreme platypus (*Ornithorhynchus anatinus*), and three paralogous in the clawed frog (Lee *et al*., 2009; Kitahashi *et al*., 2009). In fish, two paralogous *kiss* genes, *kiss1* and *kiss2*, were found and cloned in medaka (Kitahashi *et al*., 2009), zebrafish (Kitahashi *et al*., 2009; Servili *et al*., 2011), sea bass (Felip *et al*., 2009), goldfish (Yang *et al*., 2010), and chub mackerel (*Scomber japonicus*) (Selvaraj *et al*., 2010). However, in grass puffer (*Takifugu niphobles*) (Shahjahan *et al*., 2010), orange-spotted grouper (Shi *et al*., 2010) and other species studied bioinformatically, such as the three-spined stickleback (*Gasterosteus aculeatus*), tiger puffer (*Takifugu*

rubripes), and green puffer (*Tetraodon nigroviridis*), the evidence so far obtained indicates the absence of the *kiss1* gene (Li *et al*., 2009; Kitahashi *et al*., 2009; Felip *et al*., 2009; Yang *et al*., 2010).

2.5. Structure and expression of KISSR genes and proteins in vertebrates

KISS1R was first partially isolated as an orphan G-protein receptor with 28-39% homology to human galanin receptors (GalR1, GalR2, GalR3). Nevertheless, this receptor was unresponsive to galanin ligands (Lee *et al*., 1999). The G protein-coupled receptors (GPCRs) constitute the largest superfamily of integral membrane receptors, containing all of them a well conserved structure of seven transmembrane domains (TMDs) (Clements *et al*., 2001). This degree of conservation facilitated the cloning in many species. From the first cloning back in 1999 in rats (Lee *et al*., 1999), this gene was successively cloned in human, mouse, and in many other mammalian species. Regarding gene structure, in mammals the gene has 5 exons and 4 introns and encodes a protein of 398 amino acids (Muir *et al*., 2001; Clements *et al*., 2001) (Figure 6). In 2004, the first non-mammalian *kissr* was reported in the Nile tilapia (*Oreochromis niloticus*) (Parhar *et al*., 2004). Later, and similar to the situation observed for the ligand, a second paralogue gene of *kissr* with a similar gene structure was found in goldfish (Li *et al*., 2009). The two paralogues *kissrs* of goldfish shared high amino acids sequence identity in the transmembrane regions, but showed rather low similarity in the extracellular N-terminus and the C-terminal tail (Li *et al*., 2009). Both receptors (*kiss1r* and *kiss2r*) contain the NPxxY motif (X represents any amino acid) in the 7 TMDs and the DRY motif, suggesting that they belong to the rhodopsin-like GPCR family (Schwartz *et al*. 2006).

2.6. Physiological mechanisms of action of kisspeptin

The activation of Kiss1r by Kisspeptin through phospholipase C (PLC) results in intracellular calcium release involving a pertussis toxin-insensitive

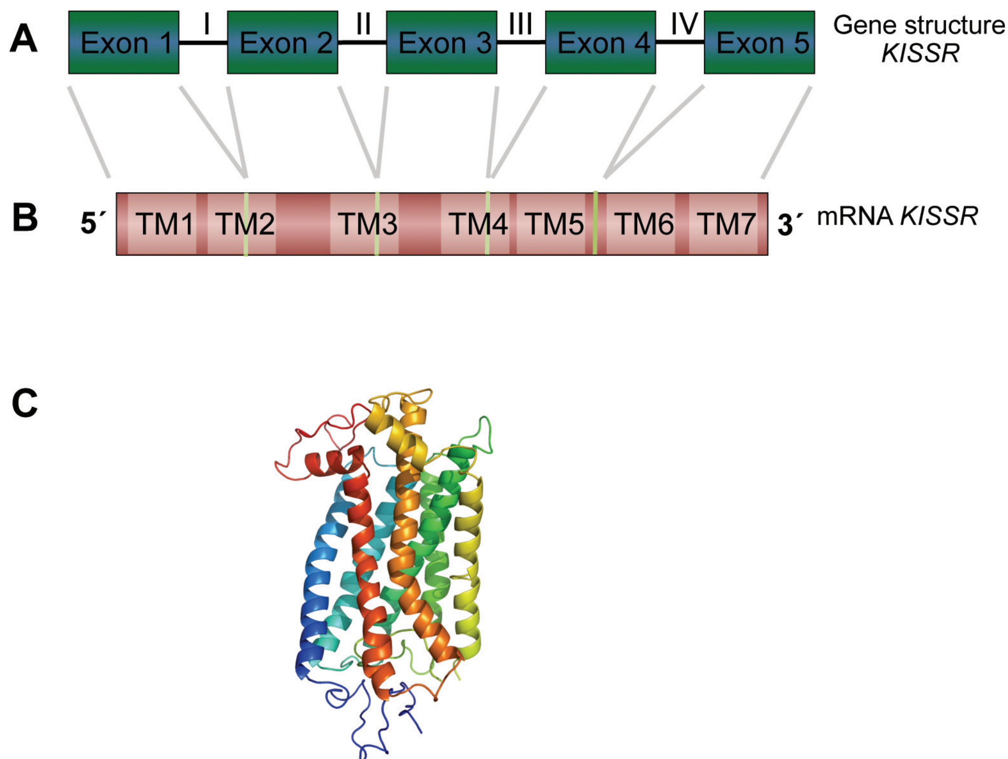

Figure 6. Gene and peptide structure of kisspeptin receptor (KISSR) in humans. **(A)**. Genomic DNA. **(B)**. *KISSR* mRNA. TM, transmembrane domain. Exons (1, 2, 3, 4 and 5) are displayed as green boxes and introns (I, II, III and IV) as thick dark lines. Thin horizontal lines represent the site of the TM **(C)**. Kissr structure prediction. Ribbon representation of an homology model of the Kiss2r from *Solea senegalensis* based on the X-ray structure of bovine rhodopsin (Okada *et al*., 2004) (PDB ID 1 1U19). The protein model has been generated by the Phyre web server (Kelley and Sternberg, 2009) from the Structural Bioinformatics Group, Imperial College London (http://www.sbg.bio.ic.ac.uk/phyre).

(presumably Gq/11) G protein (Castaño *et al*., 2009). In Chinese hamster ovary (CHO) cells transfected with *Kiss1r*, kisspeptin stimulated arachidonic acid release, phosphatidylinositol turnover, calcium mobilization, mitogen-activated protein (MAP) kinases increase, extracellular signal regulated kinase (ERK) 1 and 2, and p38 MAP kinase phosphorylation (Kotani *et al*., 2001; Castaño *et al*., 2009). Kisspeptin also increased inositol-triphosphate (IP_3) production and was shown to promote calcium mobilization and ERK1/2 phosporylation in a breast

cancer cell line (Matsui *et* al., 2004; Becker *et al*., 2005). In KISS1R-transfected NPA thyroid cancer cells, kisspeptin stimulated protein kinase C (PKC), ERK, and phosphatidylinositol-3-kinase pathways (Stathatos *et al*., 2005) (Figure 7). In fish, a similar mechanism of action was observed in the *kiss1r* of zebrafish, transducing

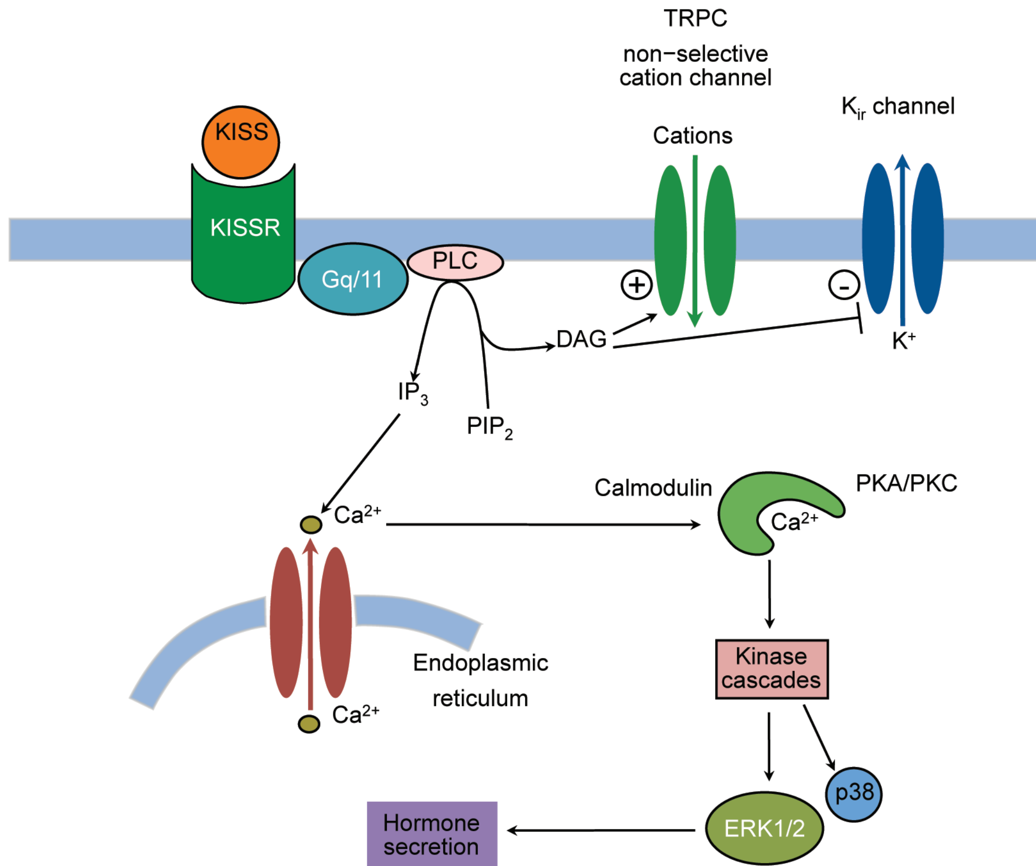

Figure 7. Mechanism of kisspeptin action in vertebrates. Proposed mechanism of action of GnRH neuronal depolarization by kisspeptin (KISS) in vertebrates. (i) KISS binding to its receptor (KISSR) activates the G-protein Gq/11 and phospholipase C (PLC) to cleave phosphatidylinositol bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). (ii) membrane depolarization is caused by activation (+) of non-selective transient receptor potential cation (TRPC) channels, possibly involving DAG and (iii) inhibition (−) of inwardly rectifying potassium (Kir) channels. IP_3 participate in the intracellular Ca^{2+} release from the endoplasmatic reticulum, wich activates protein kinase A (PKA) protein kinase C (PKC) an acts in the extracellular signal-related kinase 1/2 (ERK1/2) and p38 kinases (Adapted from Colledge *et al*., 2009; Oakley *et al*., 2009; Castaño *et al*., 2009; d'Anglemont de Tassigny and Colledge, 2010), with modifications.

its activity via PKC, whereas in its paralogous gene, *kiss2r*, transduces by two different ways: the PKC and protein kinase A (PKA) pathways (Biran *et al*., 2008). Thus, kisspeptin binding to its receptor is able to activate different signaling pathways.

2.7. Regulation of the kisspeptin system

Activation of the BPG axis by kisspeptin is manifested by GnRH release with accompanying LH and FSH increase upon kisspeptin stimulation in mice (Gottsch *et al*., 2004; Messager *et al*., 2005), rats (Castellano *et al*., 2006; Irwing *et al*., 2004; Matsui *et al*., 2004; Navarro *et al*., 2005), sheep (Arreguin-Arevalo *et al*., 2007), monkeys (Plant *et al*., 2006; Shahab *et al*., 2005) and humans (Dhillo *et al*., 2007). This activity of kisspeptin has been demonstrated to take place peripherally and centrally and is dependent upon activation of KISS1R (Messager *et al*., 2005; Dungan *et al*., 2007). KISS1R has been colocalized in GnRH neurons in the hypothalamus (Messager *et al*., 2003; Castellano *et al*., 2006), and subsequent activation of KISS1R by kisspeptin on these neurons results in GnRH neuronal activity (Matsui *et al*., 2004) and GnRH release (Messager *et al*., 2005) that can be inhibited by a GnRH antagonist (Gottsch *et al*., 2004; Irwing *et al*., 2004; Matsui *et al*., 2004; Navarro *et al*., 2005; Plant *et al*., 2006; Shahab *et al*., 2005).

In summary, kisspeptin is certainly expressed in the brain, particularly in hypothalamic neurons that are responsible for the secretion of GnRH (Muir et al., 2001) (Figure 8). The neuroendocrine control of the system is exerted via two nucleus of the forebrain, the AVPV and the ARC nucleus, where expression of kisspeptin stimulates the secretion of GnRH (Gottsch et al., 2006). This is completed with a feedback regulatory loop exerted by the sexual steroids (see section 2.3) with inhibition of KISS1 in the ARC or induction of the same gene in the AVPV (Figure 1) (Dungan et al., 2007).

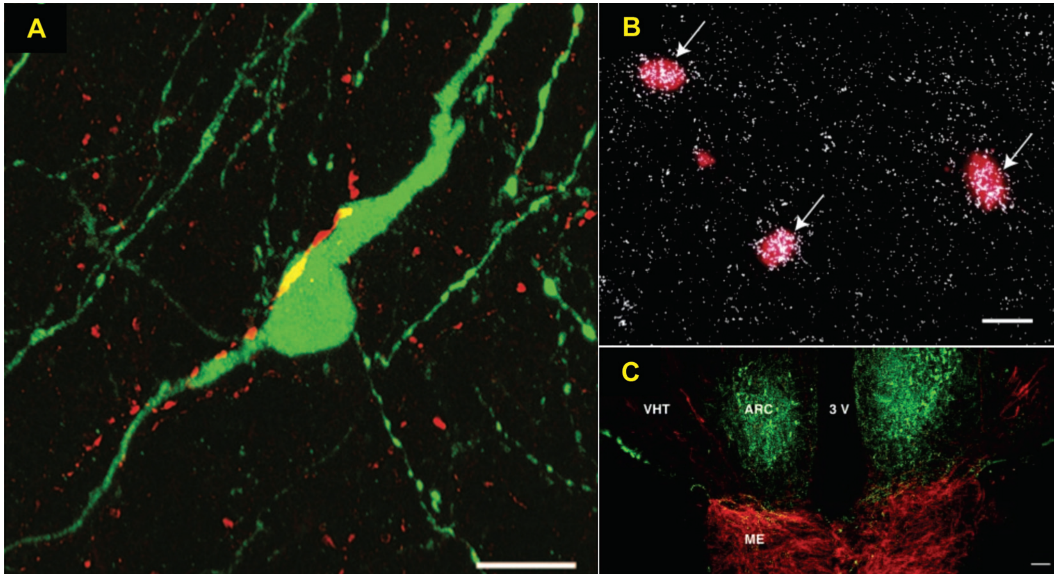

Figure 8. Kisspeptin system localization in the brain: **(A)** Kisspeptin projections to GnRH neurons in the adult female mice brain. Confocal image showing a single GnRH neuron (green) with kisspeptin (red) surrounding fibers (inmunohistochemistry). Scale bar, 10 *µm*. Taken from Clarkson and Herbison, 2006. **(B)** Coexpression of *GnRH* mRNA with *Kiss1* mRNA in rats. Representative photomicrograph from the medial preoptic area using double-label in situ hybridization. The arrows indicate GnRH neurons that coexpress *Kiss1r*. Scale bar, 20 *µm*. Taken from Irwing *et al*., 2004. **(C)** Structural interactions between KISS1 and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey. Confocal projection illustrating the distribution of kisspeptin neurons (green fluorescence) in relation to the GnRH neuronal network (red fluorescence) by inmunohistochemistry. VHT, ventral hypothalamic tract; ARC, arcuate nucleus; ME, median eminence; 3V, Third ventricle. Scale bar, 100 *µm*. Taken from Ramaswamy *et al*., 2008.

2.8. Evidences of pleiotropic effects of kisspeptin

Distinct spatial and temporal expression patterns of kisspeptin peptides and KISSR in non-reproductive tissues have been found in vertebrates. This suggests that the kisspeptin system may have pleiotropic effects besides its function in reproduction (Reynolds *et al*., 2009). Other reproductive roles include, stimulation of ovulation in female rats (Matsui *et al*., 2004; Kinoshita *et al*., 2005)

and in pony mares (Briant *et al*., 2006). Furthermore, high kisspeptin plasma level was observed in human during pregnancy (Roa *et al*., 2006; Reynolds *et al*., 2009), although the action has not been elucidated. Some recent studies indicate that the kisspeptin system may serve additional physiological functions in the nervous system and elsewhere (Oakley *et al*., 2009). Obviously, one of these additional functions is it effects on metastasis, thanks to which kisspeptin was initially discovered (Bilban *et al*., 2004; Hiden *et al*., 2007; Makri *et al*., 2008). Other functions related to kisspeptin include insulin secretion (Hauge-Evan *et al*., 2006; Bowe *et al*., 2009), vasoconstriction action (Mead *et al*., 2007) antioxidant function (Aydin *et al*., 2010), and anticoagulation (Qureshi *et al*., 2010) (Table 3). In fish very scarce information is available concerning other effects of kisspeptin besides reproduction (Elizur, 2009; Oakley *et al*., 2009). Recently, some functional studies in medaka, where two paralogous *kiss* genes are present, suggest that Kiss1, but not Kiss2, is associated with reproduction (Mitani *et al*., 2009). In contrast, a recent study in zebrafish indicated that only *kiss2* is implicated in reproductive events, while *kiss1* would play other functions that remain to be established (Servili *et al*., 2011).

Table 3. Kisspeptin system functions in mammals

Species	Function	References
Human	Vasoconstriction	Mead *et al*., 2007
Human	Tumor metastasis suppressor	Bilban *et al*., 2004: Hiden *et al*., 2007; Makri *et al*., 2008
Human	Pregnancy	Roa *et al*., 2006
Human/Rat	Anticoagulation	Qureshi *et al*., 2010
Rat	Antioxidative	Aydin *et al*., 2010
Rat	Insulin secretion	Hauge-Evans *et al*., 2006; Bowe *et al*., 2009
Rat/Pony mares	Stimulates ovulation	Matsui *et al*., 2004; Kinoshita *et al*., 2005; Briant *et al*., 2006

3. Reproductive-associated problems in cultured fish. The case of Senegalese sole and Atlantic halibut

Fish employ a wide range of reproductive strategies for successful propagation of their progeny (Schreck *et al*., 2010). However, the endocrine control of reproduction has been investigated only in a limited number of species. One of the main problems for the development of aquaculture is the failure of

cultured fish to reproduce predictably when raised in captivity (Zohar and Mylonas, 2001). Therefore, insights into the mechanisms of the neuroendocrine pathways in captive-raised compared to wild-caught fish is an important area for research (Zohar *et al*., 2010).

The Senegalese sole (Figure 9) and the Atlantic halibut (Figure 10) have been identified as strategic species for aquaculture. These two species of flatfish are members of the order Pleuronectiformes, a relatively large group of ray-finned fish consisting of about 570 species (Nelson, 2006). Flatfish are an interesting

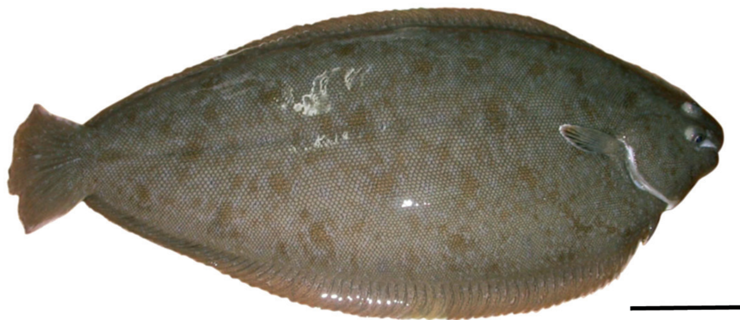

Figure 9. Adult Senegalese sole (*Solea senegalensis*). These fish are usually dextral, both eyes are on the right side of the body. Scale bar equals approx. 5 cm. Photograph from J.L. Rodríguez, B.F. Souto and J. Quintáns.

Figure 10. Adult Atlantic halibut (*Hippoglossus hippoglossus*). These fish are usually dextral, both eyes are on the right side of the body. Scale bar equals approx. 5 cm. Photograph from D. Andrey.

group of teleosts due to a distinct developmental process named metamorphosis, in which one eye migrates to the other side of the body (Okada *et al*., 2001). This is accompanied by drastic morphological and physiological changes (Power *et al*., 2008).

The Senegalese sole has nocturnal habits, spawning during the night and synchronizing its spawning capacity to light under different lighting conditions (Bayarri *et al*., 2004; Oliveira *et al*., 2010). The photoperiod (Isorna *et al*., 2008; Confente *et al*., 2010) and the lunar cycle (Oliveira *et al*., 2009a; Oliveira *et al*., 2009b; Oliveira *et al*., 2010) appear to influence their reproductive cycles. Furthermore, a decrease in water temperature stimulates gonadal maturation and sex steroid production (García-Lopez *et al*., 2009). Recently, some studies have addressed different aspects of its development and reproductive biology (Dinis *et al*., 1992; Dinis *et al*., 1999; Imsland *et al*., 2003). Thus, studies in Senegalese sole revealed that undergoes asynchronous spermatogenesis and that the testis is semi-cystic, an unusual characteristic among teleosts, in which spermatocytes and spermatids are released into the seminiferous lumen where they differentiate into spermatozoa (García-López *et al*., 2005; García-López *et al*., 2006b; Agulleiro *et al*., 2007a; Cerdà *et al*., 2008a; Guzmán *et al*., 2011). Although significant progress has been made regarding the basic biology and provided genomic resources for this species (Cerdà *et al*., 2008b; Cerdà *et al*., 2010), there are some problems related to reproductive performance and early development that are still unresolved. One of the main problems is that some captive males fail to successfully complete puberty and the lack of predictability in reproductive function (García-Lopez *et al*., 2005). Thus, the culture of Senegalese sole is seriously impaired primarily because of problems in reproduction in captivity and suboptimal rearing due to inadequate larval nutrition (Imsland *et al*., 2003). Consequently, Senegalese sole aquaculture needs to be optimized to allow a sustainable and profitable industrial production (Cerdà *et al*., 2010). The reduced growth rate appears to affect the overall quality of gametes. F1 Senegalese sole males often exhibit impaired sperm production and produce morphologically

abnormal spermatozoa, which consequently decrease fecundity rates unlike those observed in the F0 (Agulleiro *et al*., 2006) although in some cases cultured males complete spermatogenesis and sperm maturation with normal levels of androgen in plasma (García-López *et al*., 2006b; Cabrita *et al*., 2006). A recent study demonstrated some cellular damage in the spermatozoa of the F1 fish, which is causing low sperm quality (Beirao *et al*., 2008). A proteomic study comparing testis of F0 versus F1 animals at different stages of spermatogenesis concluded that alterations in protease inhibition, iron and glucose metabolism, and protection against oxidative stress may cause the low production of the sperm and poor fertilization capacity by F1 males (Forné *et al*., 2009). As in males, F1 females also show low oocyte production and poor quality gametes (Cabrita *et al*., 2006). Although vitellogenin (VTG) and steroid profiles of captive F1 females appear normal, egg fertilization is not always successful (Guzmán *et al*., 2008). Besides the dysfunctions observed in the F1 Senegalese sole generation, several studies with interesting results were obtained using those fish. For example, some studies analyzed gonadal development and sex steroid plasma profiles in males (García-López *et al*., 2006b) and females (García-López *et al*., 2007), the effect of gonadotropins and its receptors during spermatogenesis (Cerdà *et al*., 2008a; Chauvigné *et al*., 2010), or the effects of hormonal manipulation (Agulleiro *et al*., 2007b; Guzmán *et al*., 2009b).

The Atlantic halibut aquaculture is somewhat more advanced, with production occurring in Norway, Iceland, Scotland and Canada. The Atlantic halibut is one of the largest flatfish, growing in sizes over 220 cm and capable to live more than 50 years (Haug, 1990) reaching maturity at advanced age (8-9 years) (Douglas and Ross, 2006). As it has been observed in other teleost fish, photoperiod (Jonassen *et al*., 2000; Simensen *et al*., 2000; Norberg *et al*., 2001) and water temperature appear to be important factors that control the Atlantic halibut reproduction (Jonassen *et al*., 2000). Females are more appreciated for farming due to their delayed sexual maturation and larger body size (Douglas and Ross, 2006). However, there are still some culture problems to address, particularly with

regards to establishing the exact period of spawning for females, and the genetic selection of broodstock to avoid the deleterious effects of inbreeding that could affect disease resistance (Douglas *et al*., 2006). In addition, the early maturation and reduced growth rates in males are one of the major obstacles for commercial culture (Norberg *et al*., 2001; Weltzien et al., 2002). Consequently, the control of the initiation of puberty in both sexes is crucial for its aquaculture.

In summary, both flatfish yield high value market products, with Atlantic halibut farming being already an established industry, and the Senegalese sole a potential species for production in aquaculture due to their high market price, potential for reproduction in captivity and the reasonable advances made so far in their husbandry (Cañavate *et al*., 2006; Douglas and Ross, 2006; Cerdà *et al*., 2010). Understanding the kisspeptin system in these species would provide a comparative and evolutionary view about the system in this phylogenetic group, and knowledge could be used to enhance and further optimize the breeding technology of these two species.

Aims

II. Aims

The overall objective of this thesis was to contribute to the understanding of the neuroendocrine control of reproduction including the onset of puberty in teleost fish. To that end, we used Senegalese sole and Atlantic halibut, two important valuable flatfish species for aquaculture, as models for our research. In particular, we were interested in understanding the mechanisms associated with kisspeptin signaling within the reproductive axis in these species.

Towards the above overall objective, a combination of physiological, molecular, bioinformatics and genomic tools were used to address the following specific objectives:

1. To study kisspeptin and its receptor in two species of flatfish, the Senegalese sole and the Atlantic halibut, including the search for paralogous genes, study of the gene structure, their regulation, tissue distribution and to establish methods for quantifying their mRNA levels under a variety of physiological conditions.

2. To study the expression of kisspeptin and its receptor in the different components of the reproductive axis in relation to other important genes, according to sex and during a full reproductive cycle.

3. To study the possible participation of kisspeptin in the integration of conditions known to alter reproduction, such as the energetic balance, by looking at the effects of fasting on reproduction mediated by kisspeptin signaling.

Results

PAPER I

Identification of two isoforms of Kisspeptin-1 receptor (kiss1r) generated by alternative splicing in a modern teleost, the Senegalese sole (*Solea senegalensis*)

Mechaly, A.S., *Viñas, J. and F. Piferrer*

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PAPER II

Gene structure of the Kiss1 receptor-2 (Kiss1r-2) in the Atlantic halibut: Insights into the evolution and regulation of Kiss1r genes

Mechaly, A.S., *Viñas, J., Murphy, C., Reith, M., and F. Piferrer*

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PAPER III

Gene structure analysis of kisspeptin-2 (Kiss2) in the Senegalese sole (*Solea senegalensis*): Characterization of two splice variants of Kiss2, and novel evidence for metabolic regulation of kisspeptin signaling in non-mammalian species

Mechaly, A.S., *Viñas, J., and F. Piferrer*

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Mechaly AS, Viñas J, Piferrer F. Gene structure analysis of kisspeptin-2 (Kiss2) in the Senegalese sole (Solea senegalensis): characterization of two splice variants of Kiss2, and novel evidence for metabolic regulation of kisspeptin signaling in non-mammalian species. Mol Cell Endocrinol. 2011; 339(1-2): 14-24.

PAPER IV

Seasonal and sex-specific changes in the expression of key genes of the reproductive axis in the Senegalese sole (*Solea senegalensis*): Kisspeptin signaling in different brain areas, pituitary and gonads

Mechaly, A.S., *Viñas, J., and F. Piferrer*

General and Comparative Endocrinology *(Submitted 26/04/2011, manuscript number: GCE-11-140)*

SEASONAL AND SEX-SPECIFIC CHANGES IN THE EXPRESSION OF KEY GENES OF THE REPRODUCTIVE AXIS IN THE SENEGALESE SOLE (*Solea senegalensis*): KISSPEPTIN SIGNALING IN DIFFERENT BRAIN AREAS, PITUITARY AND GONADS

Alejandro S. Mechaly[1], Jordi Viñas[1,2] and Francesc Piferrer[1]*

[1]Institut de Ciències del Mar, Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain.

[2]Current address: Laboratori d'Ictiologia Genètica, Department of Biology, University of Girona, Girona, Spain.

*Corresponding author: Dr. Francesc Piferrer. Institut de Ciències del Mar, Consejo Superior de Investigaciones Científicas (CSIC), Passeig Marítim, 37-49, 08003 Barcelona, Spain.
e-mail: piferrer@icm.csic.es

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Short title: Kisspeptin signaling during the first reproductive cycle of the Senegalese sole

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ABSTRACT

Kisspeptin is thought to have a major role in the control of the onset of puberty in vertebrates. However, our current understanding of its function in fish and how integrates with other hormones is incomplete due to the high diversity of this group of animals and a still limited amount of available data. This study examined the temporal and spatial changes in expression of kisspeptin, gonadotropins and their respective receptors in the Senegalese sole during a full reproductive cycle. *Kiss2* and *kiss2r* expression was determined by qRT-PCR in the telencephalon, optic tectum and hypothalamus, the pituitary and gonads, while expression of *fsh* and *lh* was determined in the pituitary and *fshr* and *lhr* in the gonads. Plasma levels of testosterone (T), 11-ketotestosterone (11-KT) and estradiol-17β were measured by ELISA and gonadal maturation was assessed histologically. In males, *kiss2* and *kiss2r* expression in the brain areas examined and pituitary was highest towards the end of winter, just before the spawning season, which took place the following spring. This coincided with maximum levels of pituitary *fsh* and *lh*, plasma T and 11-KT and the highest number of maturing fish. However, these associations were not evident in females, since the highest expression of *kiss2*, *kiss2r* and gonadotropins were observed in the fall, winter or spring, depending upon the variable and tissue considered. Taken together, these data show not only temporal and spatial but also sex-specific differences in the expression of kisspeptin and its receptor. Thus, while expression of *kiss2* in Senegalese sole males agrees with what one would expect according to its proposed role as a major regulator of the onset of reproduction, in females the situation was not so clear, since *kiss2* and *kiss2r* expression was highest either before or during the reproductive season. The origin and physiological significance of these differences, which could also apply to other fish, deserve further investigation.

1. Introduction

Kisspeptin has emerged as a key player in the neuroendocrine control of reproduction in vertebrates [39,46], and is thought to be particularly implicated in the control of the onset of puberty in mammals [11,42] and teleost fish [34,45]. Kisspeptin is a neuropeptide product of the *KISS1* gene and forms a signaling system with its receptor, *KISSR* [39,40]. In mammals, these genes are well conserved, with one ligand, *KISS1*, and its receptor, *KISS1R* [34,51]. However, several fish have two ligands, *kiss1* and *kiss2*, and two receptors, *kiss1r* and *kiss2r*, as a result of gene duplications [2,25,28,47]. Furthermore, some teleosts have lost one of the two paralogous genes, either of the ligand, the receptor or both [28,29].

Kisspeptin and its receptor are expressed in several tissues, but due to their proposed role in reproduction [46] the majority of studies have focused on the brain and gonads. In the medaka fish (*Oryzias latipes*), two populations of Kiss1 neurons were found in the hypothalamus, one in the nucleus posterioris periventricularis (NPPv), and another in the nucleus ventral tuberis (NVT) [23,24]. In addition, in medaka and also in zebrafish (*Danio rerio*) the highest levels of *kiss1* mRNA were found in the ventromedial habenula, whereas *kiss2* mRNA was localized in the posterior tuberal nucleus and the periventricular hypothalamic nucleus [24,43]. Regarding the kisspeptin receptor, expression profiles of *kiss2r* during development and sexual maturation have been determined in the brain of several fish species. In the Nile tilapia (*Oreochromis niloticus*), *kiss2r* mRNA levels were significantly higher in gonadotropin-releasing hormone (GnRH) neurons of mature males when compared to those of immature males [37]. A temporal increase of *kiss2r* expression before the onset or during early puberty was observed in the brain of cobia (*Rachycentron canadum*) [32], grey mullet (*Mugil cephalus*) [33], fathead minnow (*Pimephales promelas*) [14] and Atlantic halibut (*Hippoglossus hippoglossus*) [28]. In zebrafish *kiss2r* mRNA levels peaked coinciding with the onset of puberty in the female brain but those of *kiss1r* significantly increased before the onset of puberty and remained high thereafter in both

sexes [5]. Thus, most studies have analyzed the expression of these genes either in whole brains or specifically in the hypothalamus due to its direct involvement in reproduction. Nevertheless, there is still limited knowledge on the neuroendocrine mechanism that controls puberty in teleost fish [45].

The Senegalese sole is a highly prized fish with a great potential for aquaculture but for some still unknown reason some individuals never mature in captivity [4,22]. Previous studies analyzed the influence of abiotic factors, particularly the lunar and daily changes of natural [35] and artificial photoperiods [15], on the spawning of this species. In fish, melatonin contributes to synchronize neuro-hormonal changes and behavior with daily and annual variations of photoperiod [12]. In the Senegalese sole, the relationship between the lunar cycle, melatonin, and sex steroids is thought to facilitate spawning during the darkest nights as an adaptation to escape predators and thus increase the chances of survival of the offspring [9,36]. Furthermore, another abiotic factor, water temperature, plays a crucial role in the reproductive cycle of this species by determining when gonadal maturation can take place [4,15,17]. Additionally, injection of GnRHa during the spring induces multiple spawns but these treatments were ineffective in inducing sperm production in males [1]. Moreover, blockage of an endogenous dopamine (DA) inhibitory system stimulates spermatogenesis and sperm production in mature males [21]. In the pituitary, follicle-stimulating hormone (*fsh*) and luteinizing hormone (*lh*) gene expression increased in males during winter and spring, coinciding with a peak of androgens in plasma and development of testicular germ cells and spermatozoa, suggesting that these genes regulate spermatogenesis in the semi-cystic, asynchronous testis type characteristic of this species [7]. In the gonads, mRNA levels of *fshr* and *lhr* during the reproductive cycle were consistent with earlier observations showing that *fshr* regulates ovarian growth and spermatogenesis, whereas *lhr* triggers gamete maturation, suggesting a role of the *lhr* in the differentiation of spermatids into spermatozoa [8].

To the best of our knowledge, only one study in fish analyzed the expression pattern of the kisspeptin system genes during the different seasons of the year. In the grass puffer (*Takifugu niphobles*) *kiss2* and *kiss2r* mRNA levels in the brain and pituitary of both sexes were higher during the spawning season when compared to the non-reproductive season, suggesting an important role of the kisspeptin system in the regulation of reproductive function [44]. In a previous study with Senegalese sole, we found some differences in expression of *kiss2* and *kiss2r* between pubertal and mature fish [27,29]. Furthermore, we have recently shown that fasting stimulated *kiss2* and *kiss2r* expression, which was followed by a concomitant increase in pituitary *fsh* and *lh* gene expression, suggesting a link between nutritional status and reproduction mediated by hypothalamic kisspeptin and hypophysary gonadotropins [29]. However, the expression pattern of *kiss2* and *kiss2r* in different parts of the brain-pituitary-gonad (BPG) axis and throughout a full reproductive season is not known in this and the vast majority of fish species.

The present study was undertaken to gain a better understanding of the spatial and temporal changes of kisspeptin and its receptor and their relationship with the gonadotropins in fish. With this purpose, biometric parameters, plasmatic sex steroids, and gene expression patterns of *kiss2* and *kiss2r* in different brain areas (including hypothalamus, telencephalon and optic tectum), pituitary and gonads, *lh* and *fsh* in the pituitary, and *lhr* and *fshr* in gonads, were determined in male and female Senegalese sole during a full reproductive cycle.

2. Experimental Procedures

2.1. Source of the animals and sample collection

Senegalese sole (F1 generation) were reared from eggs spawned by different stocks of wild fish (F0) and acclimated to captivity at the facilities of the IFAPA research center in El Puerto de Santa María (Cádiz, SW Spain). A group of those fish (range: 25–40 cm; 256–994 g) were

transported and maintained at the Experimental Aquarium Facilities of the Institute of Marine Sciences, Barcelona, (41º 23' 13" N; 2º 11' 49" E), under simulated conditions of natural temperature and photoperiod and fed once a day with a commercial diet (Skretting, Spain). The animals were treated according to the approved institutional guidelines on the use of animals for research purposes, and in agreement with the European regulations of animal welfare (ETS No. 123,01/01/91). Fish were sampled during a full reproductive cycle during spring (SP1, 4 June 2008), summer (SM, 10 July 2008), fall (FL, 25 November 2008), winter (WT, 17 February 2009) and again the following spring (SP2, 4 May 2009). For sampling, fish were anesthetized with an overdose of MS-222 (Sigma-Aldrich, St. Louis, MO, USA) and sacrificed by decapitation. Tissues were quickly removed under RNase-free conditions, flash frozen in liquid nitrogen and stored at -80ºC until used. Fragments of testis and ovary were fixed in 4% paraformaldehyde (PAF) for histological analysis. Biometric information, including standard length (SL) (precision 0,1 cm) body weight (BW) (precision 1 g) and gonad weight (GW) (precision 0,01 g) were assessed in all sampled fish. The gonadosomatic index (GSI) was determined according to the formula: GW (g)/BW (g)* 100.

2.2. Histological analyses

After fixation in 4% PAF for approximately 24 h at room temperature, gonads were washed for an additional 24 h in phosphate buffer (PB) (pH 7.4), dehydrated in a series of increasing alcohols, embedded in paraplast, sectioned at 7 μm, and stained with hematoxilin-eosin following conventional histological procedures. Stages of spermatogenesis and oogenesis were determined according to the germ cell types present in the testes [16] and ovaries [17], and the fish were classified as immature, maturing or mature.

7

2.3. Determination of plasma levels of sex steroids

At each sampling, approximately 1 ml of blood was withdrawn from the caudal vein with the aid of a heparinized syringe, centrifuged, and the plasma stored at -20°C until analysis. Plasma levels of testosterone (T) and 11-ketotestosterone (11-KT) were determined in males, whereas estradiol-17ß (E_2) plasma levels were determined in females, using commercially available enzyme immunoassay (EIA) kits (Cayman Chemical, Inc. Ann Arbor, Michigan, USA) following the manufacturer's instructions. Extra samples were spiked with known amounts of the corresponding tritiated steroid (New England Nuclear, Boston, MA) to calculate percent recovery, which typically was ≥ 90%, to adjust measured values. Plasma samples were assayed in duplicate using two 96-well plates. The assay coefficients of variation were 11.0 ± 1.8% for T, 6.4 ± 3.4% for 11-KT and 7.9 ± 2.7% for E_2.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from frozen brain and gonads with TRIZOL Reagent (Invitrogen, Carlsbad USA), its quality was checked in a 1.5% agarose gel stained with SYBR safe (Syber Safe™, Invitrogen, USA) and its quantity measured in a Nanodrop® ND-1000 spectrophotometer (Nanodrop® Technologies Inc, Wilmington, DE, USA). All RNAs were treated with DnaseI (Invitrogen, Carlsbad, USA) to remove any possible genomic DNA contamination. In all cases, 500 ng of RNA were used and reverse transcribed using SuperScript VILO cDNA synthesis kit (Invitrogen) and first strand cDNA was directly used for PCR into a 20 µl reaction volume.

2.5. RT–PCR analysis of gene expression

mRNA levels of *kiss2* and *kiss2r* in different brain areas of male and female Senegalese sole were assessed by previously validated Reverse Transcriptase PCR (RT-PCR) [27,29]. To account for seasonal variations, determinations were made at two different times (spring and

summer). Total RNA from six brain areas (olfactory bulb, telencephalon, optic tectum, cerebellum, medulla oblongata and hypothalamus) plus the pituitary were extracted as described above. One negative control (without cDNA sample) was included in each determination to ascertain that no cross-contamination took place. The PCR was carried out with 1 µl of the RT reaction in a total volume of 20 µl containing 1 PCR buffer plus 3 mM $Mg2^{+}$, 0.2 mM dNTPs, 0.2 mM of each forward and reverse primers, and 1 IU of Platinum Taq DNA Polymerase (Invitrogen). The specific primers for amplification of *kiss2* and *kiss2r* cDNAs were designed according to the nucleotide sequences of the full-length cDNAs (Table 1). Amplification of the *ßactin* was used as RNA quality control using a combination of appropriate primers (Table 1). The PCR cycling conditions were: 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, 1 min at 72°C and a final extension of 7 min at 72°C. PCRs were performed with an initial cycle at 95°C for 5 min; then variable number of cycles was applied: 95°C for 30s; 65°C for 30s; 72°C for 1 min and a final extension cycle at 72°C for 7 min. An aliquot of the PCRs product was electrophoresed on 1.5% agarose gel containing ethidium bromide, and products were visualized and photographed.

2.6. Seasonal changes in mRNA levels of kiss2 and kiss2r in different brain areas, pituitary and gonads; mRNA levels of lh and fsh in pituitary, and mRNA levels of lhr and fshr in gonads

The expression patterns of several genes were analyzed in males and females in the hypothalamus, telencephalon, optic tectum, pituitary and gonads at the five different samplings stated above (SP1, SM, FL, WT and SP2) comprising a full reproductive cycle by quantitative real-time PCR (qRT-PCR) (n = 3 - 9). RNA and cDNA were obtained following the protocol described above including the DNAse treatment step. The primers used for qRT-PCR were based on the sequences reported in previous studies [7,8,27,29] and are summarized in Table 1.

The qRT-PCR amplification reaction mixture contained 2 µl of diluted cDNA (1:10) (freshly synthesized from 500 ng of RNA), 4 µM of each primer, and 10 µl of Power SYBRs Green PCR Master Mix (Applied Biosystems) in a final volume of 10 µl. Thermal cycling conditions comprised heating to 95°C for 10 min followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. The qRT-PCR products were immediately analyzed using a dissociation curve step to confirm that only a single product was amplified. No-template control reactions for every primer pair were also included on each reaction plate to check for external DNA contamination. The amplification efficiency (E) of each primer set/gene target was assessed as E = 10 (-1/slope) as determined by linear regression of serial dilutions of the input RNA. To calculate relative changes in gene expression, we analyzed the data using the comparative Ct method [6] (also known as the DDCt method). Fold change (the relative quantification, RQ) was calculated from the DDCt and normalized by the endogenous reference gene *ßactin*. The RQ values for each sample were averaged and the standard error of the mean (S.E.M.) was calculated, yielding the average fold change of the target gene. Determinations were carried out in technical triplicates for the all the genes studied.

2.7. Data representation and statistical analyses

Prior to analysis of data, GSI levels were arcsine-transformed to ensure homoscedasticity of variances. Normality of data was assessed by the Shapiro-Wilks W test. Differences in the GSI, plasma steroid and gene expression levels during the different seasons were analyzed by a one-way analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) test. Statistical analyses of data were performed using the SPSS 15.0 package. Differences were accepted as statistically significant when $P < 0.05$. Data are expressed as mean ± standard error of the mean (S.E.M).

3. Results

3.1 Biometric parameters and gonadosomatic indices of males and females (GSI)

According to previously published studies, Senegalese sole reach first sexual maturity when they attain an average SL of ≈ 30 cm, although there is large interindividual variability [10,16]. The fish used in this study had a similar range of length and weight for both sexes (Table 2). However, maturing males were always ≥ 25 cm and ≥ 256 g while maturing females were always ≥ 30 cm and ≥ 489 g. Furthermore, regardless of season, the GSI range of males was independent of the degree of gonadal maturation while the GSI range of females increased with maturation (Table 2). Significant changes in the GSI of males ($P = 0.010$) and females ($P = 0.005$) were observed during the different seasons. GSI significantly increased ($P < 0.05$) after the fall, with maximum values observed in the winter in males (Fig. 1A) and during following spring in females (Fig. 1B).

3.2 Plasma steroid hormone levels

In males, plasma levels of the two major androgens (T and 11-KT) followed the same pattern as the GSI, with a clear and significant ($P = 0.045$ and $P = 0.024$, respectively) peak in winter (Fig. 1C and Fig. 1E). In females, E_2 plasma levels remained low until the fall and then sharply increased through winter and the following spring ($P = 0.005$) (Fig. 1D). Thus, in both sexes the GSI and plasma levels of the major sex steroids shared a similar pattern, with maximum values observed in winter for males and in the following spring for females (Fig. 1).

3.3 Gonadal development

Based on microscopic evaluation, three developmental stages of spermatogenesis in males (Fig. 2A, C and E) and of oogenesis in females (Fig. 2B, D and F) were identified. Males with testis filled only with spermatogonia (Spg) were considered as immature (Fig. 2A); males which in addition had spermatocytes (Spc) and spermatids (Spd) were classified as maturing (Fig. 2C),

whereas males with testis containing spermatozoa (Spz) were classified as sexually mature (Fig. 2E). Females with only previtellogenetic oocytes were considered immature (Fig. 2B); with early and intermediate vitellogenic oocytes maturing (Fig. 2D), whereas mature females were characterized by the presence of fully developed oocytes (Fig. 2F). In accordance with previous observations showing that once Senegalese sole males reach a certain size can remain mature throughout the year, maturing males were found in all seasons, with a maximum in winter. The highest number of mature males was found during spring of the second year of this study. On the other hand, the number of maturing females increased from 0% in spring to 20% in summer and to 33% in fall and winter. By the next spring (second year of the study) all sampled females were mature (Fig. 2).

3.4 Tissue distribution of kiss2 and kiss2r mRNA in adult Senegalese sole

The presence of *kiss2* and *kiss2r* mRNAs was investigated by specific RT–PCR in six different brain areas and in the pituitary of males and females in two different seasons. Sex- and seasonal-dependent changes were readily observed for both genes (Fig. 3).

3.5 Seasonal changes on the expression of kiss2 and kiss2r in different brain areas, pituitary and gonads; fsh and lh in the pituitary and fshr and lhr in the gonads of the Senegalese sole

In the telencephalon, *kiss2* increased after summer and peaked in winter in both males and females (Fig. 4A and B). In contrast, *kiss2r* mRNA levels were highest during the fall and winter in males but not until the following spring in females (Fig. 4 C and D). However, due to high individual variations differences were not statistically significant in any case. In the optic tectum of males, significant changes in both *kiss2* (P = 0.004) and *kiss2r* (P = 0.016) mRNA levels were observed, with a clear peak of expression in winter (Fig. 5A and C). In females, in contrast, *kiss2* mRNA levels started to increase in the fall and reached maximum levels in winter (P = 0.002), and then started to slightly decrease, whereas *kiss2r* mRNA levels kept increasing

until they reached maximum values in the following spring (P = 0.008) (Fig. 5B and D). In the hypothalamus, a similar pattern was observed, *i.e.,* low levels of both *kiss2* and *kiss2r* in males throughout except in winter, while a clear peak was observed in males (*kiss2; P* = 0.035) (Fig. 6A and C) and a progressive increase in females (Fig. 6B and D). However, significant differences were only seen in the changes of *kiss2* mRNA levels in the former (Fig. 6A). In general, no such marked changes in *kiss2* and *kiss2r* gene expression patterns were observed in the remaining tissues examined. *Kiss2* and *kiss2r* mRNA levels in the pituitary (Fig. 7) only exhibited a significant peak of expression for *kiss2* during the fall in females (Fig. 7B). In the gonads, both *kiss2* and *kiss2r* shared a similar expression pattern, with maximum mRNA levels in the winter, although no significantly differences were observed (P = 0.175; P = 0.158; P = 0.031; P = 0.190 for Fig. 8A, B, C and D, respectively).

Regarding the expression levels of gonadotropin genes in the pituitary, *lh* in males peaked in winter (P = 0.003) (Fig. 9C) and *fsh* of females in the second spring (P = 0.001) (Fig. 9B). Non-statistically significant changes were observed in *fsh* mRNA levels in males (Fig. 9A) and *lh* mRNA levels in females (Fig. 9D). In the gonads, mRNA levels of *fshr* and *lhr* remained low during most part of the study, but were consistently higher in winter. However, the inverse situation was found with respect to the levels of mRNA for *fsh* and *lh* observed in the pituitary, *i.e.,* significant differences were observed for *fshr* in the testis (P = 0.031) (Fig. 10A) and *lhr* in the ovaries (P = 0.001) (Fig. 10D).

4. Discussion

The Senegalese sole is a multiple-spawning fish, with a main spawning period during spring and a secondary period during the fall according to studies based on captive breeders [4,15,17]. In this study, we investigated the relationship between the expression profiles of several key genes of the BPG axis and maturation status during a complete reproductive cycle in this species.

In the Senegalese sole only *kiss2* and *kiss2r* have been detected [27,29] and thus it appears to have lost *kiss1* and *kiss1r*, probably as a consequence of the genome reduction characteristic of Pleuronectiformes. However, in this species each gene produces two splice variants but one of them results in putative non-functional products due to the presence of stop codons in the mRNA [27,29]. We actually measured the expression of the two variants of each of the two genes of the kisspeptin system. However, analysis of the mRNAs leading to the truncated isoforms during the annual cycle showed that although changes could be measured between seasons, no defined pattern could be observed (data not shown). Whether changes in the transcription of these mRNAs through changes in the alternative splicing towards one or the other isoform contributes to control the abundance of the mRNA producing the functional protein has not been investigated. Thus, from this point on only the functional splice variant will be considered.

In this study, the major brain areas implicated in the control of reproduction, *i.e*., telencephalon, optic tectum and hypothalamus [50,51], were examined together with the pituitary and the gonads. In males, telltale signs of the initiation of reproduction could be observed in winter, as evidenced by the highest GSI, peak plasma levels of T and 11-KT and maximum number of observed maturing males. These changes were also evident at the gene expression level since the highest mRNA levels of *kiss2* and *kiss2r* in the optic tectum, hypothalamus and testis, *lh* in the pituitary and *fshr* in testis were also observed in winter (Fig. 11). This is probably related to the initiation of testicular meiosis that implies an increase of spermatocytes in winter [4] and subsequent highest levels of spermatozoa production in spring [7]. This situation is similar in Atlantic halibut, with an increase of testicular mass together with increased GSI and plasma levels of T and 11-KT during winter [48].

In females, the tight association observed in males between the winter and the maximum values of many of the measured variables was not evident. However, many measured variables exhibited a tendency to increase their values with time, peaking in the second spring, and thus in agreement with the fact that ovarian development reaches its maximum between the end of the winter and the beginning of spring, when the main spawning period begins in Senegalese sole [4,15,17].

During the characterization of Senegalese sole *kiss2* and *kiss2r* we did measurements of these genes in maturing vs. mature animals, showing no differences in *kiss2* regardless of sex and only a significant decrease of *kiss2r* in mature females with respect to maturing females [27,29]. These preliminary results contrast with the ones presented here. A possible explanation of these discrepancies can be attributed to the fact that in the previous studies whole brains were used whereas in the present study different brain areas were examined separately. Furthermore, in previous studies fish were combined based on their reproductive status regardless of season of the year, whereas here samplings during different seasons were carried out. Finally, the changes of *kiss2* and *kiss2r* observed during the different seasons in this study agree with the results observed in both sexes of grass puffer, where significantly higher levels of *kiss2* and *kiss2r* mRNAs in the whole brain were found during the spawning period, although no differences between sexes were observed [44].

In the BPG axis, a major target of kisspeptin signaling is GnRH [37]. Like in the rest of vertebrates, in fish GnRHs are involved in gonadotropin secretion and gonad maturation [3]. In the grass puffer, increased *GnRH1* expression result from *kiss2* and *kiss2r* increased expression [44], whereas similar results were found concerning *GnRH3*, *kiss2* and *kiss2r* on the zebrafish [24], where recently it was shown that kiss2 fibers innervate GnRH3 neurons [43]. At present the Senegalese sole GnRHs have not been characterized, but despite this shortcoming we could

readily bring together kisspeptin signaling and the gonadotropins, in an effort to put kisspeptin effects into a more general context, as shown in the preent study.

It has been established that kisspeptins released in the pituitary induce gonadotropin secretion [34]. However, *Kiss1r* might be involved in additional roles, *e.g.,* in the stimulation of grow hormone (GH) and prolactin (PRL) secretions via endocrine, and/or paracrine mechanisms [38]. In the goldfish, Kiss1 stimulated the synthesis and release of *lh*, *prl* and *gh* [49], although no effects on *lh* were detected in another study using the same species [26]. In the grass puffer, kisspeptin and its receptor expression peaked during the spawning season, both in brain and the pituitary [44]. In our study, although *kiss2* in the pituitary peaked in winter in males, the only significant differences in expression were observed in females, with maximum levels in the fall. Regarding the gonadotropins, our data show that in males *fsh* and *lh* mRNA levels mirrored the expression changes of *kiss2*, both in the brain and the pituitary, although statistically significant differences were observed only for *lh*. This supports the role of kisspeptin in triggering reproduction. In our study, *fsh* levels were higher in winter and the second spring when compared to the previous seasons, in agreement with a previous report showing increased levels of *fsh* in the pituitary of Senegalese sole males in winter and spring [7]. However, in that report *lh* levels paralleled those of *fsh* [7], while in the present study *lh* levels in males dropped after winter. On the other hand, in females gonadotropins did not follow the expression pattern of *kiss2* or *kiss2r.* In addition, in females *fsh* did not increase until the second spring, which is in agreement with the role in the regulation of ovarian maturation as observed in other studies in this species [20].

Kispeptin is expressed in fish gonads [13,24] and changes have been detected with the reproductive cycle. Thus, for example, in the chub mackerel gonadal *kiss1* mRNA levels increased during late vitellogenesis in females and spermiation in males [41]. In our previous studies with the Senegalese sole, we observed significantly higher expression of *kiss2* in the

ovaries of pubertal females when compared to those mature females [29], but no changes in *kiss2r* expression [27]. Significant changes were observed neither in *kiss2* nor in *kiss2r* expression in males [27,29]. In the present study, although a peak of mRNA levels was observed for *kiss2* and *kiss2r* in females during winter, which could suggest higher expression prior to the reproductive season, the differences were not significantly different, probably due to higher interindividual variability and/or an insufficiently large sample size. On the other hand, mRNA levels of both *fshr* and *lhr* increased in winter, similar to the situation observed in the Atlantic salmon (*Salmo salar*) [30], and probably in response to seasonal dynamics of their ligands, as described elsewhere [31]. In any case, the role of kisspeptin signaling in fish gonads deserves further research.

In summary, the present study provides information on the changes in expression of kisspeptin and its receptor in the BPG axis of the Senegalese sole, relating them with other histological, biochemical and gene expression changes known to occur during the reproductive cycle. The major finding is that, in males, *kiss2*, *kiss2r* and most variables analyzed changed synchronously and peaked in winter, coinciding with the highest number of maturing animals, just before the spawning season, which took place the following spring. Thus, expression of *kiss2* in Senegalese sole males agrees with what one would expect according to its proposed role as a major regulator of the onset of reproduction. In females, such synchrony was not evident and, furthermore, the highest levels of *kiss2* and *kiss2r* were observed in the spring, coinciding with the spawning season, when all females were already fully mature. To the best of our knowledge, the present study is the first one in fish that considers the whole BPG, including several brain areas, and includes a full reproductive cycle. Thus, the origin and physiological significance of the observed sex-specific differences in kisspeptin signaling, which could also apply to other fish, deserve further investigation to firmly establish the role of kisspeptin in the control of reproduction. Also, and in the particular case of the Senegalese sole, whether these sex

differences are related to the recurring poor reproductive performance of captive F1s is at present unknown.

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Table 1. Gene-specific primers used for RT-PCR and qPCR in this study

Gene	GenBank Acc. No.	Primer sequence (5'→3')	Amplicon size (bp)	Primer name	Reference
βactin	DQ485686	ACACCCAACAACTTCAGCTCTGT	101	Ss βactin-F1	[27]
		GAGTCAAGCGCCAAAATAATGA		Ss βactin-R1	
kiss2r	EU136710	TATGTGACAGTGTATCCTCTGAAATCC	89	Ss kiss2r_v1-F1	
		AAGGAGCCAATCCAAATGCA		Ss kiss2r_v1-R1	
kiss2	HM116743	TGGATCTGCACGATATGACA	50	Ss kiss2_v1-F1	[29]
		GTCTGACCCTGTTGCTCG		Ss kiss2_v1-R1	
fsh	EU100409	TGATCTGTAACGGGGACTGG	153	Ss fsh-F	[7]
		GACAGCTGGCAATCTCTCCA		Ss fsh-R	
lh	EU100410	AGCATGTGTGCACGTACCAG	180	Ss lh-F	
		TGTCGTTCATGCAGATGTCG		Ss lh-R	
fshr	GQ472139	GGCGACTGGACTGAGTTTCG	186	Ss fshr-F	[8]
		TCTTCACAACACGTGGGAGAG		Ss fshr-R	
lhr	GQ47140	GCTGTGCACTGCTGAACTGG	376	Ss lhr-F	
		GGCACCGTCATCTTGCTTCT		Ss lhr-R	

Table 2. Biometric data of the fish used in this study

	Males	Females
All fish		
Standard length range (cm)	25–38	26–40
Weight range (g)	256–908	262–994
GSI range (%)	0.02–0.14	0.34–10.45
Immature fish		
Standard length range (cm)	25–33	26–40
Weight range (g)	278–613	262–994
GSI range (%)	0.04–0.11	0.34–1.38
Maturing fish		
Standard length range (cm)	25–38	30–36
Weight range (g)	256–908	489–962
GSI range (%)	0.02–0.14	0.36–2.01
Mature fish		
Standard length range (cm)	27–30	32–38
Weight range (g)	266–501	725–976
GSI range (%)	0.03–0.12	1.71–10.45

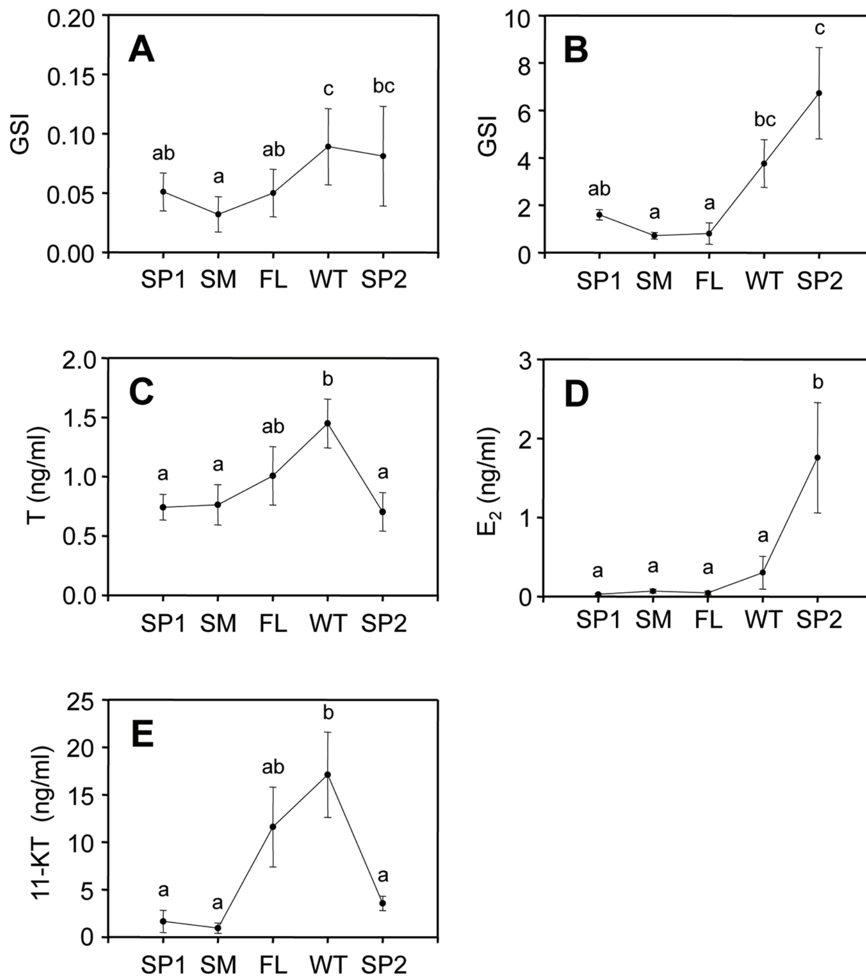


Fig. 1. Changes in the gonadosomatic index (GSI) of male (A) and female (B) Senegalese sole, and C, plasma levels of testosterone (T); E, 11-ketotestosterone (11-KT) in males, and D, plasma levels of estradiol−17β (E2) in females during one full reproductive cycle. Data as mean ± S.E.M. (n= 3-7). Abbreviations: SP1, spring 1; SM, summer; FL, fall; WT, winter; SP2, spring 2. Different letters indicate significant (*P* < 0.05) differences between the values corresponding to different sampling dates.

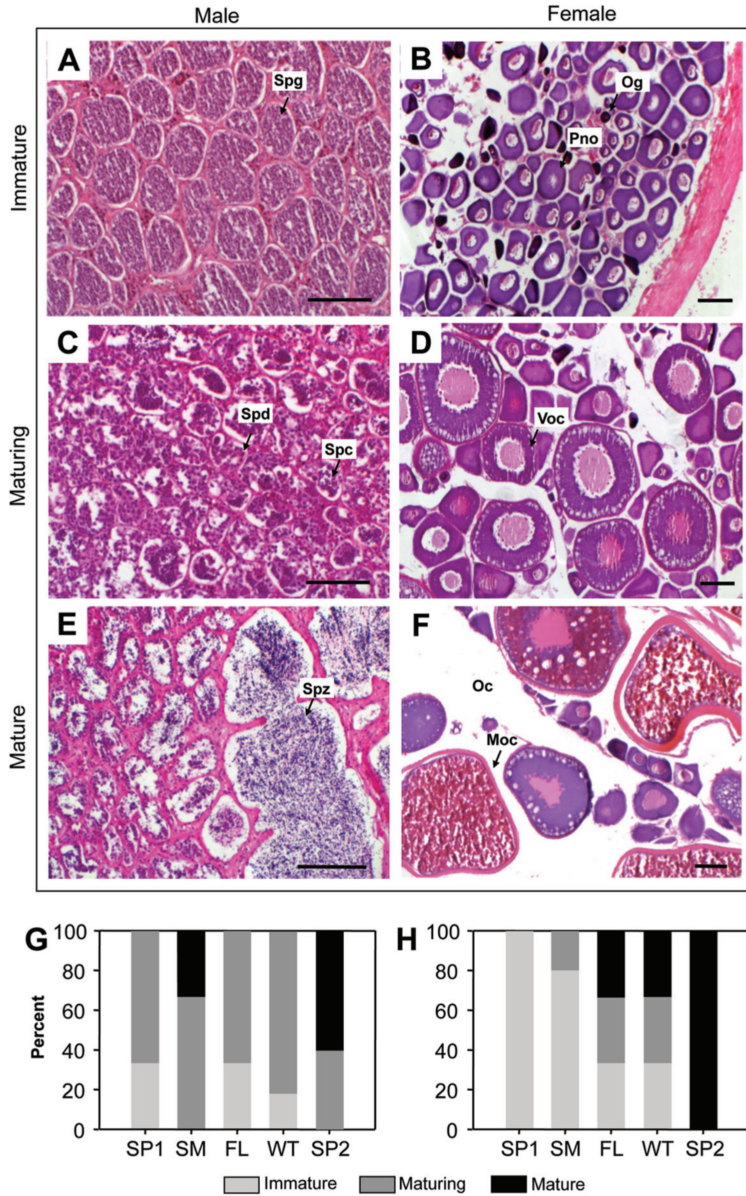


Fig. 2. Photomicrographs of histological sections representing different stages of sexual maturation in Senegalese sole: immature (A), maturing (C) and mature (E) testis, and immature (B), maturing (D) and mature (F) ovaries. Abbreviations: Spg, spermatogonia; Spc, spermatocyte; Spd, spermatid; Spz, spermatozoa; Og, oogonia; Pno, perinucleolar oocyte; Voc, vitellogenic oocyte; Moc, mature oocyte; Oc, ovarian cavity. The scale bar, 100 um, applies to all photomicrographs. Percent of immature, maturing and mature males (G) and females (H) according to sampling time. Abbreviations as in Fig. 1.

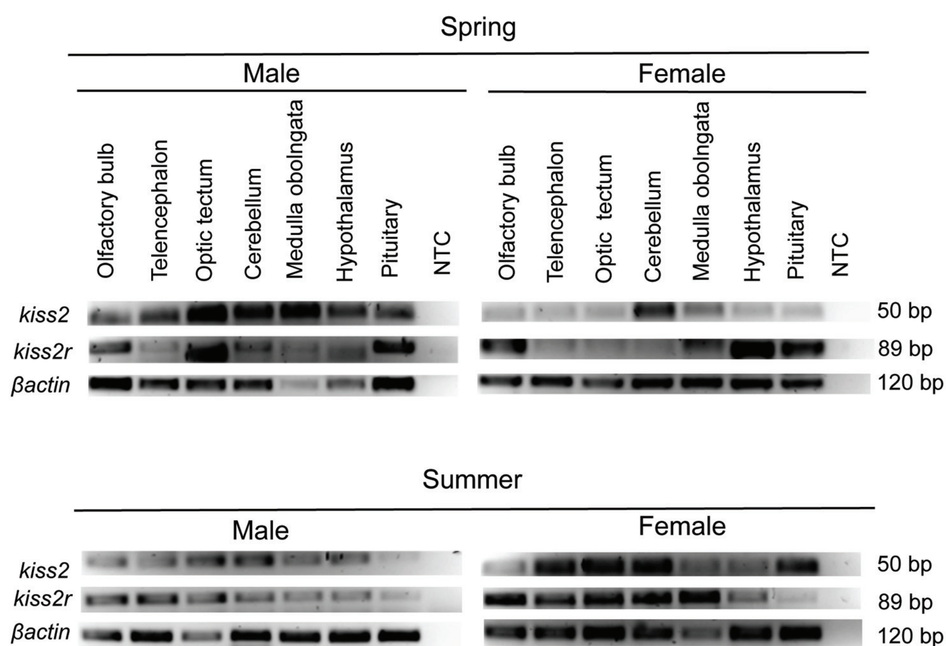


Fig. 3. Tissue distribution of *kiss2* and *kiss2r* in different brain areas in male and female Senegalese sole at two different seasons of the year. *βactin* was included as a reference gene to verify the presence of mRNA in each sample. No-template (NTC) was used as a negative control.

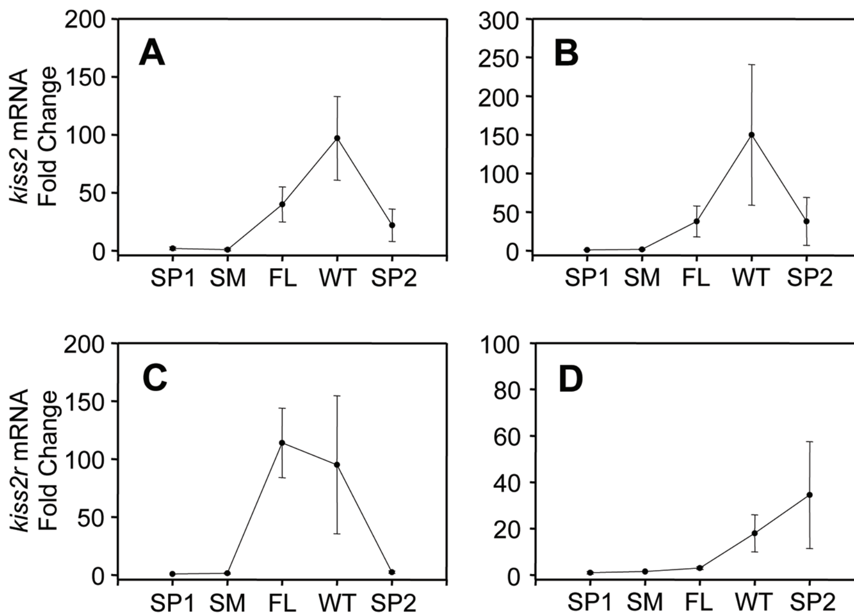


Fig. 4. Changes in *kiss2* (A, B) and *kiss2r* (C, D) mRNA levels in the telencephalon of Senegalese sole males (A, C) and females (B, D) during different seasons of the year as determined by quantitative real–time PCR (qRT-PCR) and normalized to *βactin*. Different letters indicate significant ($P < 0.05$) differences between the values corresponding to different sampling dates. Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3−9).

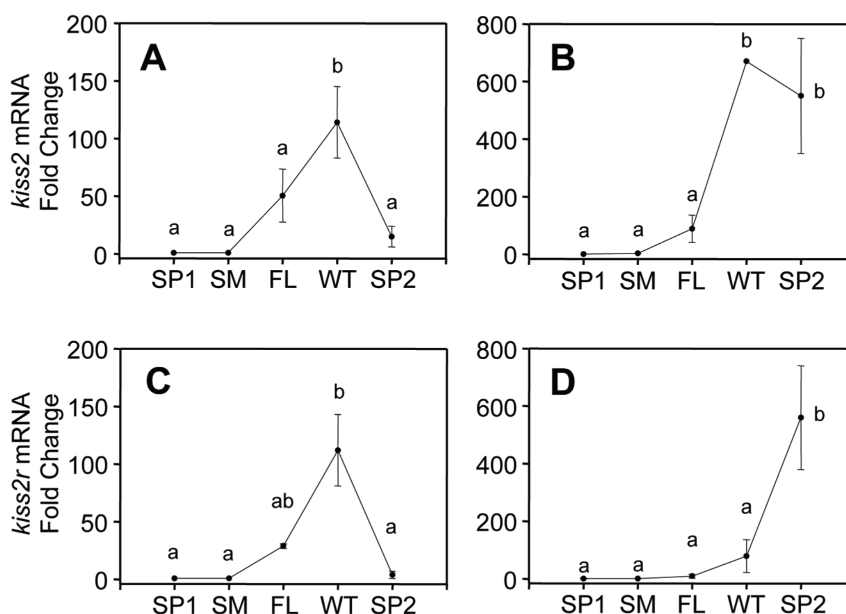


Fig. 5. Changes in *kiss2* (A, B) and *kiss2r* (C, D) mRNA levels in the optic tectum of Senegalese sole males (A, C) and females (B, D) during different seasons of the year as determined by quantitative real–time PCR (qRT-PCR) and normalized to *βactin*. Different letters indicate significant ($P < 0.05$) differences between the values corresponding to different sampling dates. Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3−9).

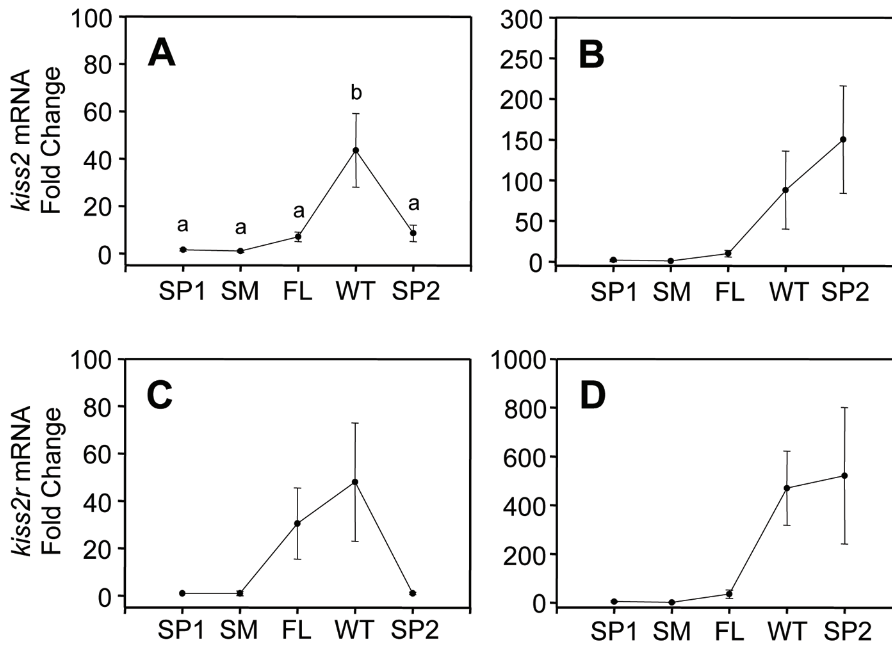


Fig. 6. Changes in *kiss2* (A, B) and *kiss2r* (C, D) mRNA levels in the hypothalamus of Senegalese sole males (A, C) and females (B, D) during different seasons of the year as determined by quantitative real–time PCR (qRT-PCR) and normalized to *βactin*. Different letters indicate significant ($P < 0.05$) differences between the values corresponding to different sampling dates. Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3−9).

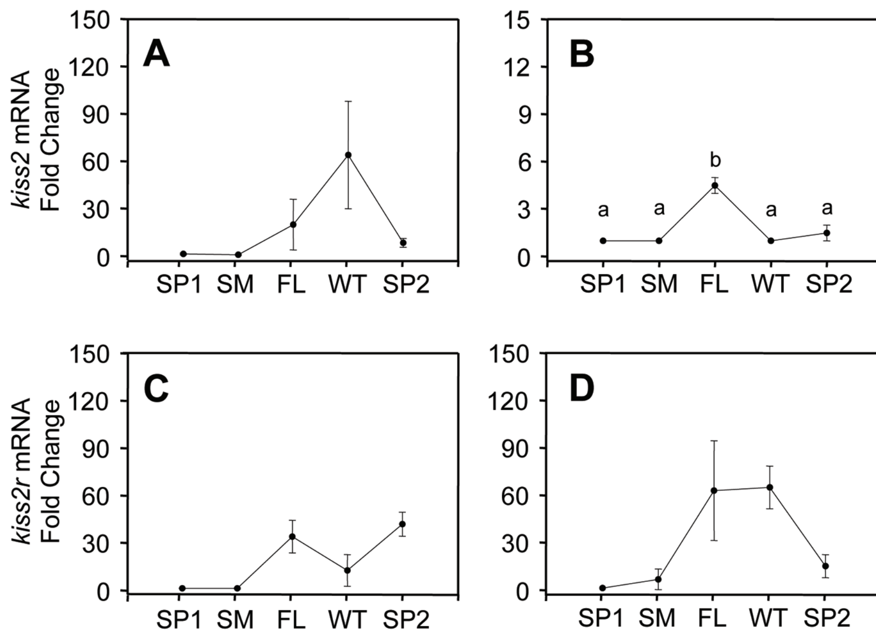


Fig. 7. Changes in *kiss2* (A, B) *kiss2r* (C, D) mRNA levels in the pituitary of Senegalese sole males (A, C) and females (B, D) during different seasons of the year as determined by quantitative real–time PCR (qRT-PCR) and normalized to *βactin*. Different letters indicate significant ($P < 0.05$) differences between the values corresponding to different sampling dates. Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3−9).

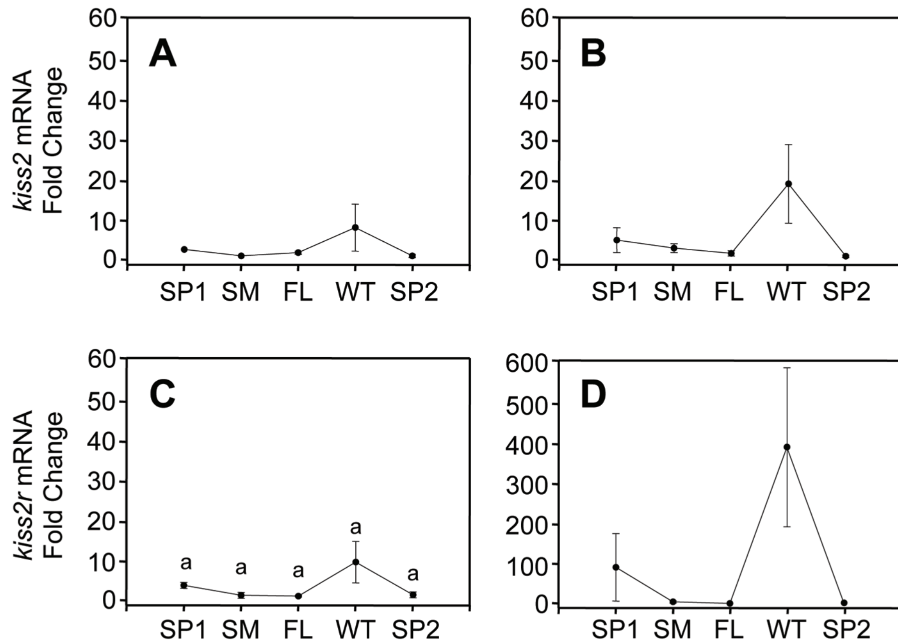


Fig. 8. Changes in *kiss2* (A, B) and *kiss2r* (C, D) mRNA levels in the testis (A, C) and ovaries (B, D) of Senegalese sole during different seasons of the year as determined by quantitative real–time PCR (qRT-PCR) and normalized to *βactin.* Different letters indicate significant ($P < 0.05$) differences between the values corresponding to different sampling dates. Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3−9).

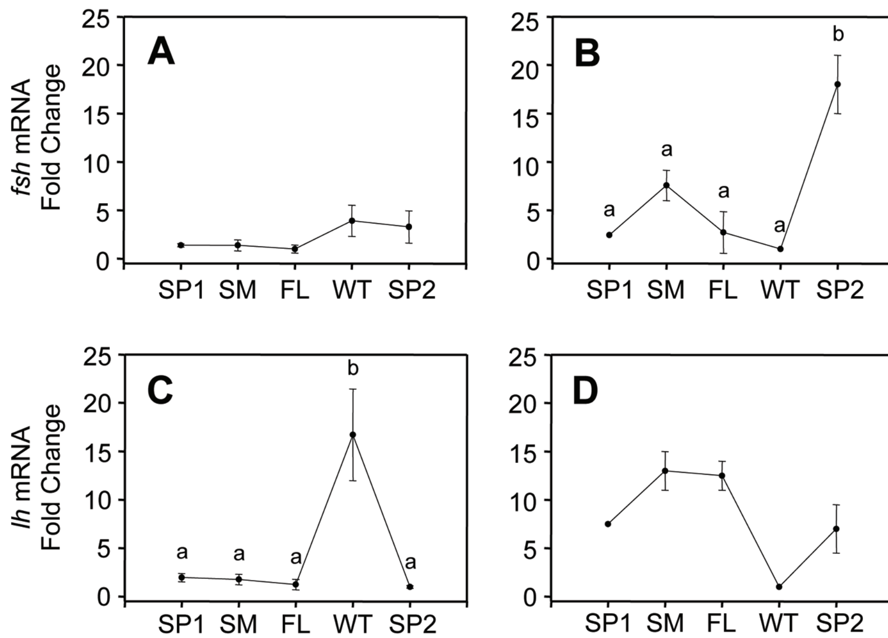


Fig. 9. Changes in *lh* (A, B) and *fsh* (C, D) mRNA levels in the pituitary of Senegalese sole males (A, C) and females (B, D) during different seasons of the year as determined by quantitative real–time PCR (qRT-PCR) and normalized to *βactin.* Different letters indicate significant ($P < 0.05$) differences between the values corresponding to different sampling dates. Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3−9).

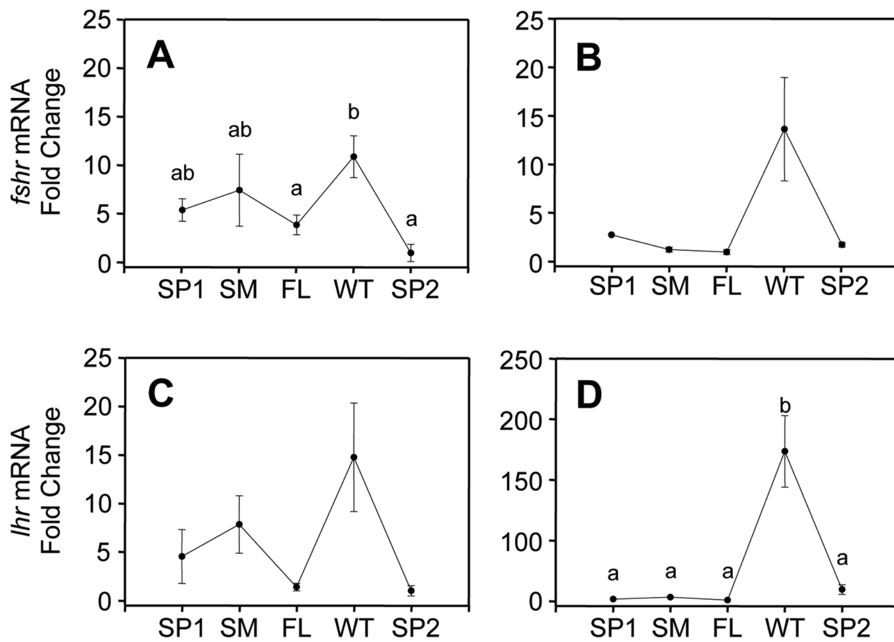


Fig. 10. Changes in *lhr* (A, B) and *fshr* (C, D) mRNA levels in the testis (A, C) and ovaries (B, D) of Senegalese sole during different seasons of the year as determined by quantitative real–time PCR (qRT-PCR) and normalized to *βactin*. Different letters indicate significant ($P < 0.05$) differences between the values corresponding to different sampling dates. Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3−9).

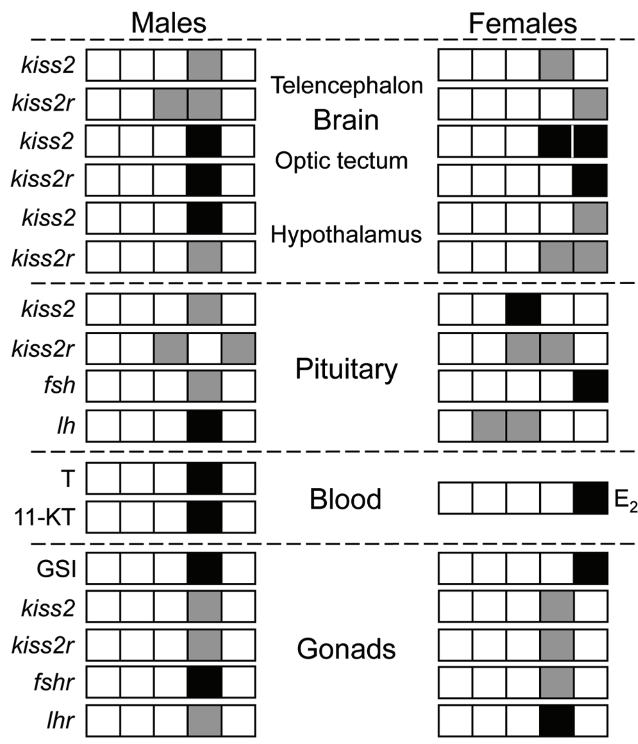


Fig. 11. Summary of the changes observed in the variables measured in this study. The five boxes correspond, from left to right, to: spring, summer, fall, winter and the following spring. The level of expression is related to the intensity of shadowing in each box: white, low expression; grey, high expression (but not significantly different); black, highest expression with significant differences.

Discussion

IV. Overall discussion and future directions

1. A note on the fish used in this thesis

The Senegalese sole is one of the most important species for the economy and diversification of aquaculture in Mediterranean countries (Imsland *et al*., 2003). Its breeding in captivity has been the focus of research, particularly in Spain and Portugal since the early 1980s (Anguis and Cañavate, 2005). The first fish bred in captivity (F0) were wild fish caught in the Bay of Cádiz. These fish were maintained in tanks at ambient photoperiod and temperature and adapted to captivity in the facilities of the Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica (IFAPA), Centro "*El Toruño*" (El Puerto de Santa María, Cádiz). The individuals used for this research were first generation (F1) fish, which were reared from eggs, spawned by the F0 broodstock and then were kept at ambient photoperiod and temperature at the aquarium facilities (ZAE) at the Institute of Marine Sciences (ICM) in Barcelona. About one hundred Senegalese sole were used (Papers I, III and IV).

Similar to the situation for the Senegalese sole, the development of Atlantic halibut aquaculture in Canada began in the late 1980s at the St. Andrews Biological Station (Fisheries and Oceans, Canada). The first commercial enterprise for Atlantic halibut production was established in the mid-1990s from a breeding stock (F0), which was collected from the Bay of Fundy and subsequently bred at the St. Andrews Biological Station (Jackson *et al*., 2003). The first efforts towards the establishment of a domestic broodstock began with the selection of candidate broodstock from F1 individuals resulting from the 1996-spawning cohort of the wild Bay of Fundy collection. These 1996 F1 fish were distributed to Atlantic halibut hatcheries, such as the Scotian Halibut, Ltd. (Woods Harbour, NS). Additional wild-caught fish were retained to ensure genetic diversity (Jackson *et al*., 2003). In our research, 50 F1 Atlantic halibut of different year classes at different stages of

development and of both sexes originated from the Scotian Halibut, Ltd. hatchery were used (Paper II).

Although we anticipate it would be rather similar, we do not know what the exact expression pattern of *kiss* and *kissr* would be in wild fish of the species used in this thesis when compared to captive fish such as the ones used here.

2. Some comments on the methods used and additional experiments

The main objective of this research was to understand the molecular endocrinology that controls the onset of puberty in teleost fish, specifically in the Senegalese sole and in the Atlantic halibut. For this purpose, several methodological approaches were used including immunological techniques (*i.e.,* ELISA), histology and several molecular techniques: reverse transcriptase polymerase chain reaction (RT-PCR), cloning and sub-cloning, BAC library screening, quantitative RT-PCR (QRT-PCR), and *in situ* hybridization (ISH). Additionally, appropriate bioinformatic tools were used to search for specific genes and perform *in silico* cloning, bioinformatics transcription motif searching and synteny analysis. These tools included, among others, BLAST, ClustalW, Expasy, browsing on-line genetic and genomic data bases such as EMBL, Genbank in NCBI, ENSEMBL, Multiple EM (Expectation Maximization) for Motif Elicitation (MEME) and the motif comparison tool (TOMTOM) software. In addition to sequence alignments, phylogenetic relationships were reconstructed (ClustalW, Bioedit, and MEGA) to infer the evolution of the genes studied. Furthermore, during the course of an experiment, culture and handling techniques were used for rearing the fish at different developmental stages. Dissection of different regions of the brain, visceral tissue extraction and collection of blood was also carried out. The methods of collecting, processing and storage of the extracted tissues were dependent on the specific aims of a particular experiment.

There are several techniques to analyze the mRNA expression of a sample. A common method of detection is the reverse transcriptase polymerase chain reaction (RT-PCR). This method can be used to detect the presence of a specific mRNA family but is deficient in quantification mRNA abundance. One of the first methodologies used for quantifying the abundance of a specific mRNA was by semi-quantitative RT-PCR. This methodology is based on the measure of the intensity of the band in the agarose gel stained with ethidium bromide. A more accurate method to measure gene expression is real-time PCR, with two different possibilities: relative quantitative PCR (RQ-PCR), where changes in gene expression are given relative to another reference sample, and the absolute quantitative PCR (AQ-PCR), where unknown amounts can be quantified by comparing then to an standard curve. Both real-time PCR methods can be performed using different chemistry approaches, the most common being SYBR Green or TaqMan. SYBR Green is an easy method that requires a double-stranded DNA dye in the PCR reaction, which binds to newly synthesized DNA. Detection of fluorescent signal occurs during the PCR cycle. The TaqMan requires a third primer labeled with a dye and a quencher. Except for the absolute qPCR quantification, one of the key points of relative quantification (either semiquantitave or real time) is the choice of the reference genes. Usually housekeeping genes, with highly and uniform expression during various stages of development, in different tissues and under different environmental conditions, are used as a reference. In our studies, we compared the expression levels of two frequently used housekeeping genes, *βactin* and *18S* by RQ-PCR over a set of three tissue/organ samples (brain, gonads, and muscle). Results revealed no significant changes in expression among the investigated tissues in both genes. However, *βactin* had better stability in the Senegalese sole (Papers I, III and IV), whereas *18S* had it in Atlantic halibut (Paper II). During the time that this thesis was in course, a study regarding housekeeping genes in larvae of flatfish suggested that *ribosomal protein S4* (*RPS4*), *ubiquitin* (*UBQ*) and *elongation factor I alpha* (*eEFIAI*) appeared more suitable than *βactin* and *18S* (Infante *et al*., 2008). However, this finding needs to be confirmed in juveniles and adult flatfish.

For the gene expression studies, we first started with tissues maintained at -80°C followed by total RNA extraction, always using DNAase treatment to avoid DNA contamination. The concentration of total RNA was determined spectrophotometrically, usually with a NanoDrop. Additionally, the quality of the extracted RNA was checked through an ethidium bromide agarose gel, and this was followed by a reverse transcription to generate cDNA. This cDNA was then used as a template in the RT-PCR and RQ-PCR. To assess gene expression levels we first used RT-PCR to detect the presence of the mRNA of interest. Secondly, more in deep studies were carried out using RQ-PCR, since our aim was to study the variation in mRNA expression levels of candidate genes under different conditions such as stages of development or different nutritional conditions. RQ-PCR was performed using SYBR Green, which presents the advantages of easier use and lower reaction cost compared to other fluorescent probes. The RQ-PCR analysis calculates changes in expression under different conditions. Initially (Papers I and II) expression values were not rescaled, but eventually we adopted the reference value of “1” for the value with the lowest expression (Papers III and IV). It should be mentioned that in a study which is currently underway in which we use laser capture microdissection (LCM) to study testicular germ cells at different stages of development we are using absolute real-time PCR.

After the cloning and characterization of the functional isoform of *kiss2r* (v1) in the Senegalese sole (Paper I), we intended to localize the mRNA expression of *kiss2r* in specific brain areas. For this purpose, a research stay for three months during the last quarter of the year 2007 at the laboratory of Dr. Olivier Kah (Unité Mixte de Recherche, UMR, Centre National de la Recherche Scientifique, CNRS, Rennes, France) was carried out. The objective of this stay was to learn ISH and then to implement it in our laboratory. Prior to visiting Dr. Kah’s laboratory, a visit at the laboratory of Dr. José Muñoz-Cueto (University of Cádiz, UCA, Cádiz) was undertaken to get a first glimpse of the ISH technique, but mainly to perfuse fish needed for ISH. For that purpose, six specimens of Senegalese sole (3 males and 3 females; about 30 cm SL) were anesthetized in MS-222 and perfused

transcardially with 60 ml of 0.65% physiological saline followed by a perfusion with the same volume of 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.4). Afterwards, fish were decapitated, whole brains removed and postfixed in 15 ml of the same fixative solution at 4°C. The following day, the brains were dehydrated and embedded in paraffin (Paraplast). Once in Rennes (at Dr. Kah's laboratory), ISH was carried out. This methodology first consisted of synthesizing the DIG–AP riboprobes corresponding to 450 bp of the *kiss2r_v1* cDNA, which were subcloned into the pCRII-TOPO vector (Invitrogen). The cRNAs were synthesized either with T7 or SP6 RNA polymerase using the DIG RNA Labeling Kit (Roche). Then, once the riboprobe was already tested, the histological sections from the perfunded fish were hybridized with antisense and sense (negative control) riboprobes. Non-radioactive ISH was performed in the Senegalese sole brain sections as described in Hanna et al. (2010). We observed a faint signal in some parts of the brain in some of the sections analyzed but definitive conclusions could not be made. Thus, no successful results were obtained. Later, in 2008, and with the knowledge and experience acquired at Dr. Olivier Kah lab, a second round of ISH was carried out in our laboratory in Barcelona. In this case, together with the *kiss2_v1* riboprobe, we used a riboprobe for the *fsh* gene, as a positive control for expression in the pituitary. The tissues and *fsh* riboprobe were kindly donated by Dr. Francois Chauvigné and Dr. Joan Cerdà (Reference Center in Aquaculture, IRTA, Barcelona). In this case, and in accordance with the previous results obtained in France, we observed a very weak *kiss2r_v1* signal in the brain. In contrast, a strong positive signal was obtained for *fsh* in the pituitary. These results indicated that the technique was correctly applied. Thus, it is likely that the difficulty to localize the expression of our candidate gene was related to the low expression in our tissue samples. Moreover, the big size of the Senegalese sole brain (20-25 mm) (Rodríguez-Gómez *et al*., 2000) in comparison with that of the zebrafish (5-6 mm) (Ullmann *et al*., 2010) confers a great deal of difficulty in the use of this technique. Thus, in the Senegalese sole we needed to use about 40-50 slides to scan the whole brain. In contrast, in zebrafish, only 3-4 slides were needed to have a good representation of the brain. The high number of sections

needed for the ISH of *kiss2r* in the brain of Senegalese sole together with the putative low level of expression in this species hampered the chances of finding the specific expression site of our gene of interest in the brain at different stages of pubertal development. It should be noted that at the moment that we carried out these studies, no previous study on *kiss2r* localization had been published in any teleost fish.

Another objective of the thesis was to clone and search for different isoforms of the *kissr* gene in other species such as the Atlantic halibut, studying the mRNA levels of *kissr* in fish with different ages and at different developmental stages. With this purpose, we first cloned a partial sequence of the Atlantic halibut *kiss2r* in our laboratory. The second part of this work was conducted during a four-month stay at the laboratory of Dr. Michael Reith at the Institute for Marine Biosciences (IMB) in Halifax, Canada. Using the partial *kiss2r* cloned previously we prepared a radiolabelled probe, which was used to hybridize an Atlantic halibut bacterial artificial chromosome (BAC) library. This eventually allowed to obtain the complete sequence of the gene of interest and all the additional genomic structures such as 5'UTR, intron, 3'UTR and other genomic features. Furthermore, gene structure analysis, phylogenetic relationships and synteny analysis was carried out with the *kiss2r* Atlantic halibut sequence. Finally, expression analysis of the *kiss2r* gene, comparing fish of different ages, was carried out. The results of this study were published in Paper II.

We attempted to characterize the Kiss2r protein of the Senegalese sole. With this aim, we proposed another stay at the laboratory of Dr. Silvia Zanuy, under the supervision of Dr. Ana Gómez (Instituto de Acuicultura de Torre de la Sal, IATS, Castellón). Unfortunately, after several attempts, we were unsuccessful in cloning the full-length *kiss2r_v1* cDNA and consequently could not do the stay in Castellón and follow this line of research.

There are two additional studies that although do not constitute part of this thesis are intimately related to it and thus a brief mention is warranted. In

one of these studies, LCM was used to analyze the expression level of several genes by Q-PCR, including *kiss2* and *kiss2r,* for their possible involvement during spermatogenesis in the Senegalese sole. This study benefited from: a) a previous study in which crucial genes implicated in the regulation of spermatogenesis in the sea bass were analyzed by this methodology, which allows the isolation of specific cells from cysts containing spermatogonia, spermatocytes, spermatids, or spermatozoa (Viñas and Piferrer, 2008), and b) from research carried out at the lab of Dr. Josep Planas (UB), in which gene expression in the testis of Senegalese sole was analyzed by microarray, identifying up to 90 genes involved in the regulation of steroid production and spermatogenesis (Marín-Juez *et al*., 2011). This work is currently in progress, and we are at the last steps of the analysis of the results.

Finally, in 2010 during a three and a half month research fellowship at the laboratory of Professor Abigail Elizur (University of Sunshine Coast (USC), Queensland, Australia) we conducted an *in vivo* experiment using the Yellowtail kingfish (*Seriola lalandi*) as a model to study the effect of chronic administration of kisspeptins (Kiss1 and Kiss2) on pre-pubertal fish. Gonadal histology, plasma sex steroid levels and expression levels of genes related to the reproductive function were used as indicators of the status of the reproductive axis. A manuscript with the results of the study is currently in preparation. Moreover, during the stay at Professor Elizur's laboratory, we isolated the promoter regions of the kisspeptin system genes in Yellowtail kingfish and in Southern Bluefin tuna. Putative transcription factor (TF) binding sites were identified using bioinformatic tools and compared them with those in the Atlantic halibut *kiss2r* genomic sequences.

3. Cloning of *kiss2* and *kiss2r* genes in flatfish. Detection of an alternative splicing mechanism and evolutionary speculations

A major effort of our work was directed towards the cloning and characterization of the elements of the kisspeptin system in flatfish and comparing

them with the kisspeptin system of other teleost species (Papers I, II and III). We first focused on cloning the *kissr* because at the moment when these studies started available information about *kissr* concerned only one teleost fish, the Nile tilapia (Parhar *et al*., 2004). At the same time, there was no information available about the ligand, *kiss*, in any species of fish. Also by that time, studies in other teleost fish showed a preliminary *kissr* gene structure, suggesting that this gene comprises five exons and four introns and also inferring their intron-exon boundaries (Parhar *et al*., 2004; Filby *et al*., 2007; Mohamed *et al*., 2007; Nocillado *et al*., 2007; Biran *et al*., 2008). However, to the best of our knowledge, our results in Senegalese sole were the first in elucidating the complete gene structure of a *kissr* gene in a non-mammalian species by cloning and analyzing the genomic gene sequence and confirming the previous deducted structure based on *in silico* analyses (Paper I). Furthermore, we found two transcripts of the receptor, the short transcript, *kiss2r_v1* of 1137 bp, and the long transcript, *kiss2r_v2* of 1218 bp. The long transcript was the consequence of complete retention of intron 3 containing 81 bp (Figure 11). Sequence analysis of intron 3 showed characteristics typical of alternative splicing, and thus it was concluded that both isoforms were generated by an alternative splicing mechanism. Furthermore, the presence of two premature termination codons (PTC) in the amino acid sequence of intron 3 has a correlation with alternative splicing, which is one of the most important mechanisms that regulates gene expression (Black, 2003; Izquierdo and Valcárcel, 2006). Therefore, it can be deduced that the long isoform, *kiss2r_v2*, is possibly translated into a truncated and non-functional protein, whereas the short isoform, *kiss2r_v1*, yields the functional protein. It should be mentioned that in Atlantic halibut, we were unable to confirm the existence of more than one gene isoform. However, analysis of the genomic sequence revealed that intron 3 was extremely short of about 0.9 kb and with several conserved features of an alternative splicing mechanism such as a branch point and 3´ and 5´consensus sites. In this species non PTC were detected within the intron 3, although in the hypothetical case of an intron 3 retention a change in the reading frame and then a PTC would appear within the exon 4 and, similar to the situation observed for

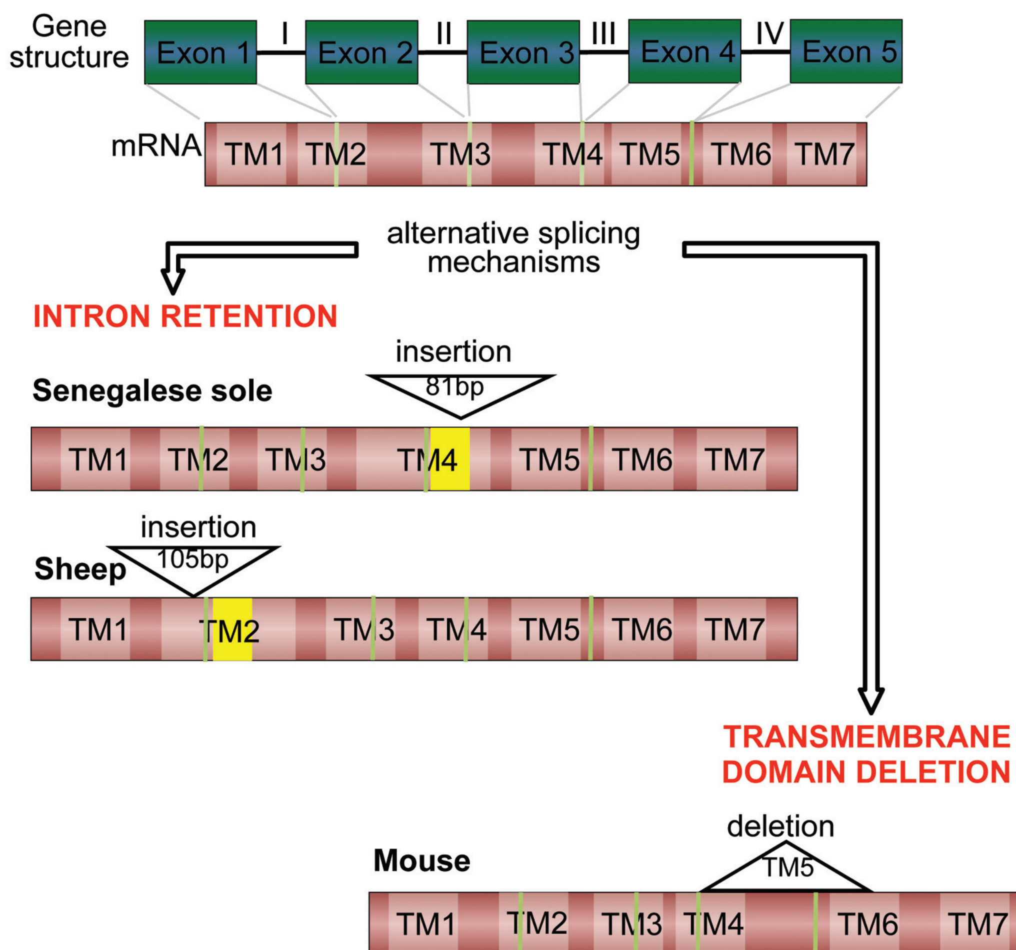


Figure 11. Summary of the alternative splicing mechanism of the *KISSR* gene in vertebrates: intron retention and transmembrane domain (TM) deletion. Exons (1, 2, 3, 4 and 5) are displayed as green boxes and introns (I, II, III and IV) as thick dark lines. Thin horizontal lines represent the site of the TM. The yellow boxes represent the retained intron.

the *kiss2r* in Senegalese sole, resulting in a non-functional isoform. Thus, if it is still unknown if these events of alternative splicing mechanism for the *kiss2r* gene with a short functional transcript and a long non-functional transcript observed in both species analyzed are conserved in other flatfish. Nevertheless, it can be hypothesized that these species share this alternative splicing as a mechanism

for gene expression regulation. Future research should be done to confirm this situation.

KISS1 was first cloned in mammals (Lee *et al*., 1996) and characterized in humans by West *et al*. (1998). In mammals, the gene was described consisting of four exons and three introns, with no translation of the first and second exons, whereas the third and fourth exons are partially translated into a 145 amino acid precursor peptide (West *et al*., 1998). Alternatively, other studies in humans described a different structure for the same gene, placing exons 1 and 2 in a single non-transcribed exon, thus rendering a gene organization of three exons and two introns (Luan *et al*., 2007) (Figure 12). In zebrafish, where two paralogous *kiss*

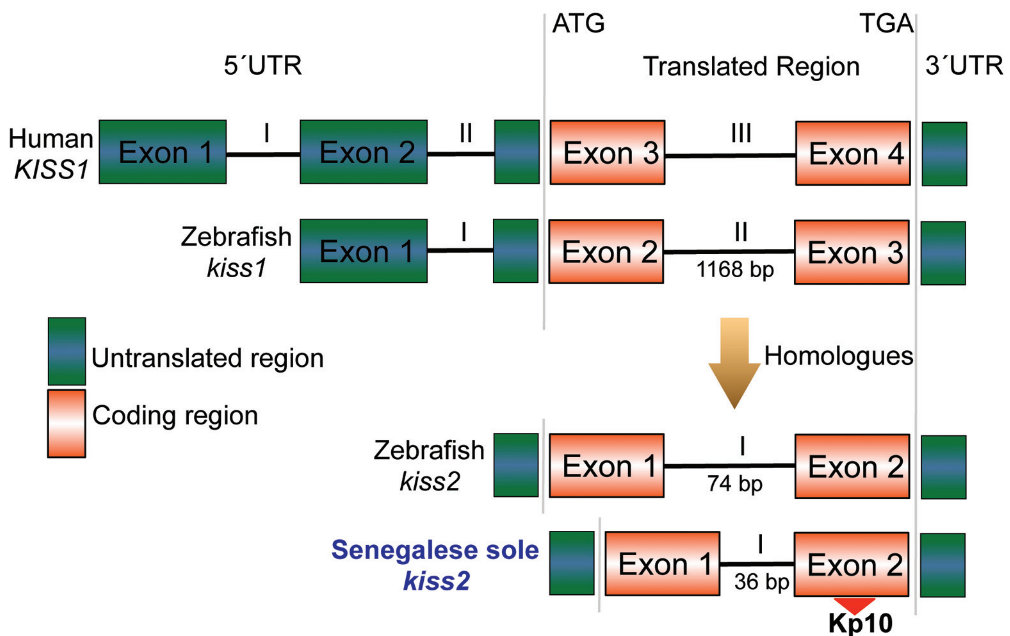


Figure 12. Comparison of the Senegalese sole *kiss2* gene structure with the human *KISS1* gene, zebrafish *kiss1* and zebrafish *kiss2*. Abbreviations: ATG, initiation codon; TGA, termination codon. UTR, untranslated region. Dotted lines indicate the boundaries between the translated and untranslated regions. White, non-translated exons; black, exons translated into protein. Exon and intron lengths are not to scale. Note that exons 1 and 2 of zebrafish and Senegalese sole kiss2 correspond to human and zebrafish kiss1 exons 3-4 and 2-3, respectively.

genes (*kiss1* and *kiss2*) were found, there also are some discrepancies about the gene structure of the *kiss1* gene. For instance, van Aerle *et al*. (2008) and Biran *et al*. (2008) proposed a gene organization of *kiss1* comprising two exons and one intron for the zebrafish. In contrast, Kitahashi *et al*. (2009) suggested a different organization, with three exons and two introns, and attributed these differences to incomplete sequencing of the 5`UTR region in earlier studies. On the contrary, the gene structure of the paralogous zebrafish *kiss2* was well confirmed, comprising two exons and a single intron of 74 bp (Kitahashi *et al*., 2009). In the Senegalese sole, only the *kiss2* gene was detected. Although more in-depth studies, including synteny analysis and searching for possible pseudogenes for *kiss1*, are required to fully confirm the loss of the *kiss1* gene in this species, our results strongly indicate that this species has the *kiss2* gene only. The *kiss2* in Senegalese sole is organized in two exons, with a single short intron of 36 bp (Figure 12). These two exons are putatively partially translated to a large polypeptide of 129 amino acids. Sequence comparison between other kisspeptin orthologous genes indicated that this prepropeptide contains a 19 amino acid signal peptide, followed by a 78 amino acid (Kp) peptide, longer than the corresponding 54 amino acid peptide described in humans (Lee *et al*., 1999). Proteolytic processing of the full–length 145 amino acid of the kisspeptin protein in humans results in shorter peptides, Kp54, Kp14, Kp13 or Kp10 (Kotani *et al*., 2001; Ohtaki *et al*., 2001). Thus, a similar structure of kisspeptins appears to be conserved from fish to mammals. For instance, in goldfish, a Kp56, Kp27, Kp16, Kp13 and Kp10 were also observed (Yang *et al*., 2010). However, and similar to that observed in mammals, sequence analysis of the Senegalese sole *kiss2* showed a lack of the cleavage sites that would yield shorter peptides. The possibility of producing shorter peptides cannot be fully discarded since they can be generated by degradation (Popa *et al*., 2008). The Kp10 appeared very well conserved with other *kiss2* paralagous genes in teleost fish and are located in the last 10 amino acids of the Kp78 followed by three amino acids (GKR) that coincide with a putative site of terminal cleavage and amidation, which is well conserved in all mammalian (Kotani *et al*., 2001; Ohtaki *et al*., 2001) and fish *kiss* genes (Biran *et al*., 2008). Interestingly, the last 29 amino acids of *kiss2* indicate the presence of a kisspeptin-associated peptide which has never

been explicitly described for kisspeptin before and whose functions need to be confirmed. It is important to recall a similar situation in GnRH, where a GnRH-associated peptide (GAP) is required for proper processing of the precursor and production of the mature GnRH peptide (de Roux, 2006) (Paper III).

One of the most surprising results of our studies is that the two genes studied (*kiss2* and *kiss2r*) presented alternative splicing variants, a result that has been observed for the first time in any species. The mechanism is strikingly similar for *kiss2* and *kiss2r* and in both cases related to intron retention. In addition, the isoforms that retained the intron, and again for both genes, most likely leads, at best, to a non-functional protein, since in both cases the retention of the intron incorporates a PTC, stopping transcription (Papers I and III). This mechanism seems to be conserved in Atlantic halibut. However, although the analysis of the sequence of intron 3 in the *kiss2r* of this species shares all the features of an alternative splicing mechanism no additional isoforms were actually found.

It is interesting to remark that in both the Senegalese sole and in the Atlantic halibut we found only one gene for the kisspeptin ligand and one gene for its receptor (Papers I, II and III). We postulate that the loss of the corresponding paralogous genes could be a consequence of genome reduction in pleuronectiformes when compared to the rest of teleosts (Brainerd *et al*., 2001). Thus, the presence of alternative splicing associated with the loss of paralogous genes can also be related to the genome size. Recent analysis of the genomes of teleost fish indicated that the rate of alternative splicing in genes is inversely correlated with genome size, suggesting that different splicing frequencies exist depending on the evolutionary strategies of a particular group of teleosts (Lu *et al*., 2010). For instance, the alternative splicing frequency in tiger puffer is 43%, in medaka 31% and in zebrafish 17% (Aparicio *et al*., 2002; Kasahara *et al*., 2007; Hukriede *et al*., 1999). These data clearly show that species with a more compact genome, such as the pleuronectiformes, have a higher alternative splicing frequency than species with larger and duplicated genomes (van der Aa *et al*., 2009). Thus,

we can postulate that the compact genome of the pleuronectiformes leads to the production of alternative splicing in genes for which one of the paralogous copies were lost. More complex genomic analysis with possible availability of the complete sequence of the genome together with high throughput transcriptional analysis will help to elucidate this hypothesis.

4. Phylogenetic and evolutionary relationships of the kisspeptin system

Vertebrates evolved ~450 million years ago (Mya) from a common ancestor (Holland *et al*., 2008) and are descendants of cephalochordates (*e.g*., *Amphioxus*) and urochordates (tunicates) (Sherwood and Adam, 2005), which originated ≥ 520 Mya. It is believed that vertebrate evolution included two rounds (R) of whole genome duplication (WGD) (Ohno, 1998). Although the timing of these duplications is controversial, the prevailing hypothesis (the “one-to-two-to-four” hypothesis) states that the first one (1R) occurred just before the appearance of agnathans (hagfish and lampreys) ~500 Mya (Furlong and Holland, 2002), the consequences of which (*i.e*., the paralogous genes originated) mostly and quickly disappeared (Kah *et al*., 2007), and the second one (2R) just before the appearance of gnathostomata, ~480 Mya. In addition, a fish-specific 3R occurred ~350 Mya between the appearance of Actinopterygii and before the basal teleosts evolved (“.. to-eight” hypothesis). It is argued that this last WGD may explain the abundance of paralogous genes in teleosts when compared to other vertebrates, which may have favored the great biological diversity of teleosts (Sato and Nishida, 2010). Further still, there is evidence of lineage-specific WGDs (LSWGD), which can affect one particular group of animals. Known examples are one of the piscine LSWGDs that occurred 25-100 Mya and affected the Salmonids (Mungpakdee *et al*., 2008), or the amphibian LSWGD that affected the genus *Xenopus*. However, many of the duplicated genes with these WGD have been lost (Meyer and Van de Peer, 2005) and thus it is always debatable to ascertain the evolution of a

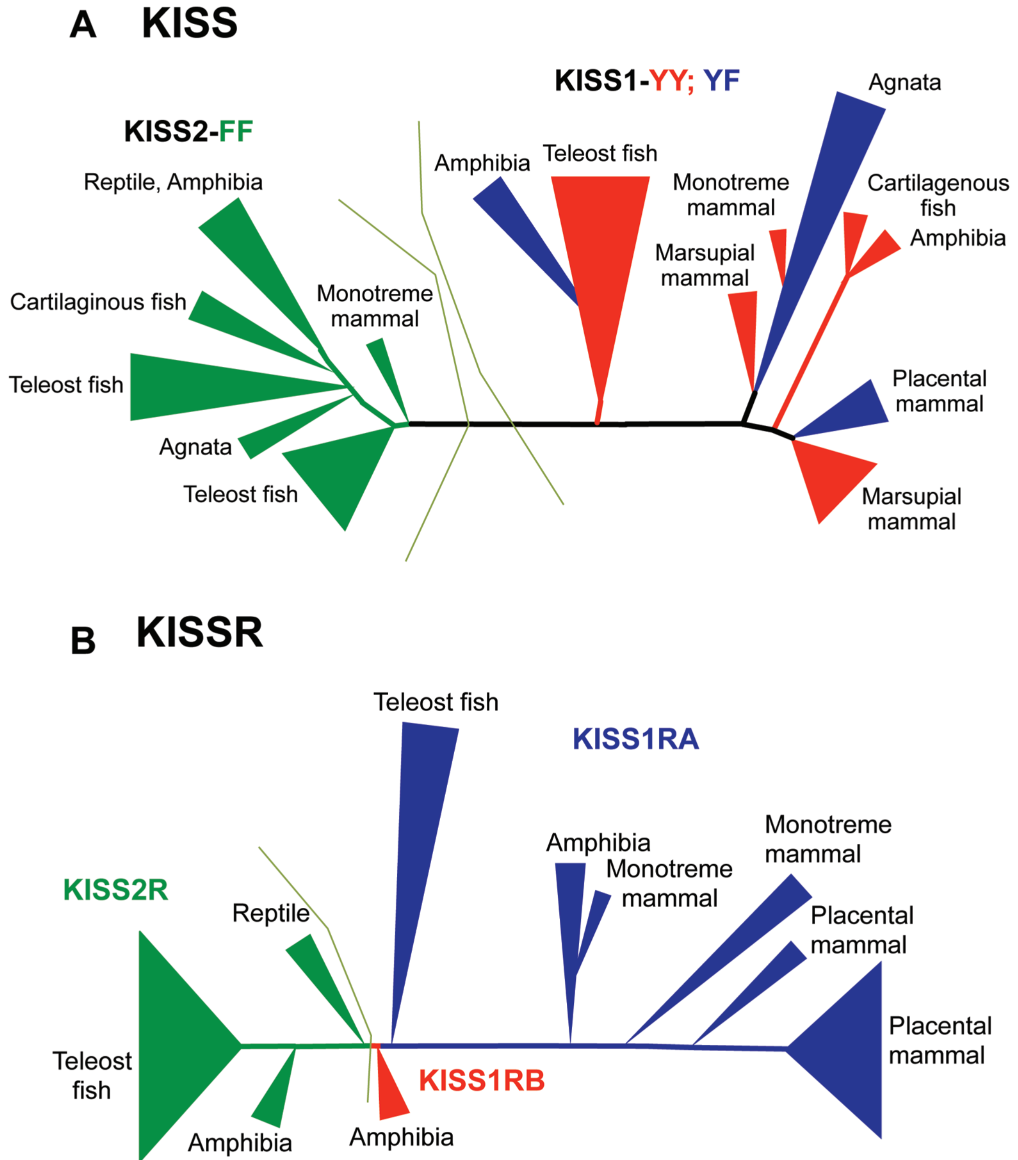
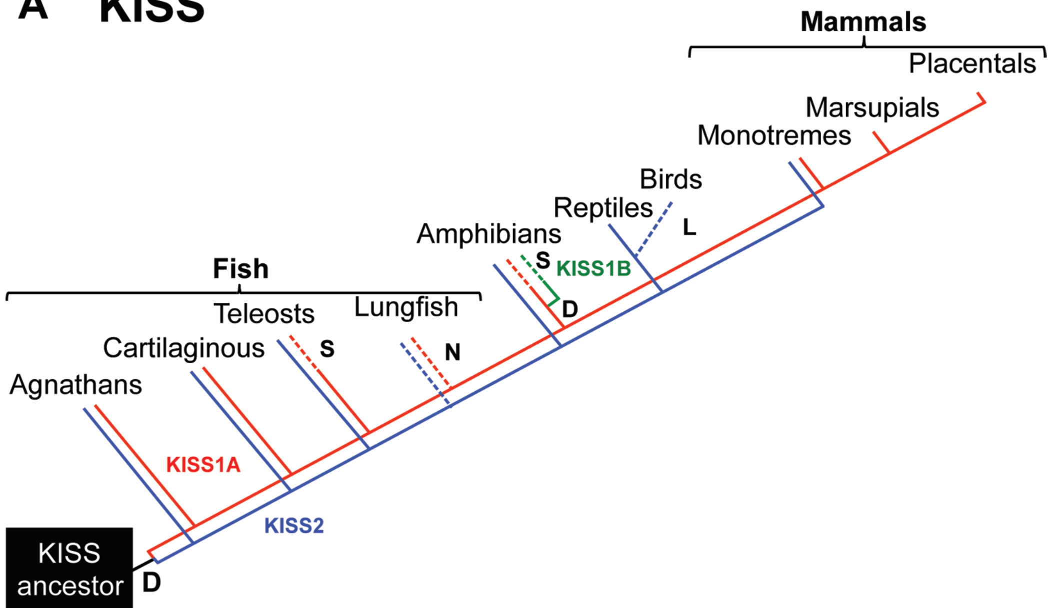


Figure 13. Phylogenetic tree of all sequences available of **(A)** kisspeptin genes and **(B)** the kisspeptin receptor. The width of the branches is approximately related to the number of sequences available while their length is proportional to the genetic distance.

particular set of genes. Therefore, our work, which was based on the analysis of available sequences, concerns the evolution by duplication (D) of the genes of the kisspeptin system.

So far, two paralogous *kiss* genes have been identified in one species of agnathans (sea lamprey, *Petromyzon marinus*) (Felip *et al*., 2009; Li *et al*., 2009) and in a cartilaginous fish (the elephant shark, *Callorhinchus milii*) (Lee *et al*., 2009). This observation agrees with our phylogenetic sequence analysis (Figure 13A) (Paper III). However, in the teleost lineage *kiss* genes depicts a more complicated history, with a proposed loss of one of the paralogous *kiss1* genes sometime during the late teleostean evolution (Shi *et al*., 2010; Shahjahan *et al*., 2010). This proposal is also supported by our results (Paper III). It remains to be determined, however, when the second genome duplication (2D) occurred. Recently, a phylogenetic study carried out in vertebrates proposed the emergence of *KISS1B* from a 2D in teleost fish (Um *et al*., 2010). However, our study leads to a different conclusion: that the 2D led to the emergence of *KISS1B* within the lungfish lineage or that *KISS1B* arose later during the divergence of amphibians (Paper III) (Figure 14A).

Regarding the receptor, a phylogenetic relationship was proposed in which the *KISSR* family was grouped into two main clusters, *KISS1R* and *KISS2R* (Biran *et al*., 2008). However, with the isolation and characterization of this gene in an increasing number of species, and with the detection of a third paralogous *KISSR* gene in amphibians, a more recent phylogenetic tree has been proposed (Lee *et al*., 2009). Many of the phylogenetic analyses that have been carried out have focused on showing the existence of two paralogous lineages of the receptor genes (Felip *et al*., 2009; Lee *et al*., 2009; Kitahashi *et al*., 2009; Akasome *et al*., 2010; Um *et al*., 2010), to which our phylogenetic studies agree (Papers I and II) (Figure 13B). The gene evolution observed in the receptor seems to be similar to the one of the ligand, but no further conclusions can be drawn due to the lack of data for the receptor in agnathans and cartilaginous fish. However, the concerted



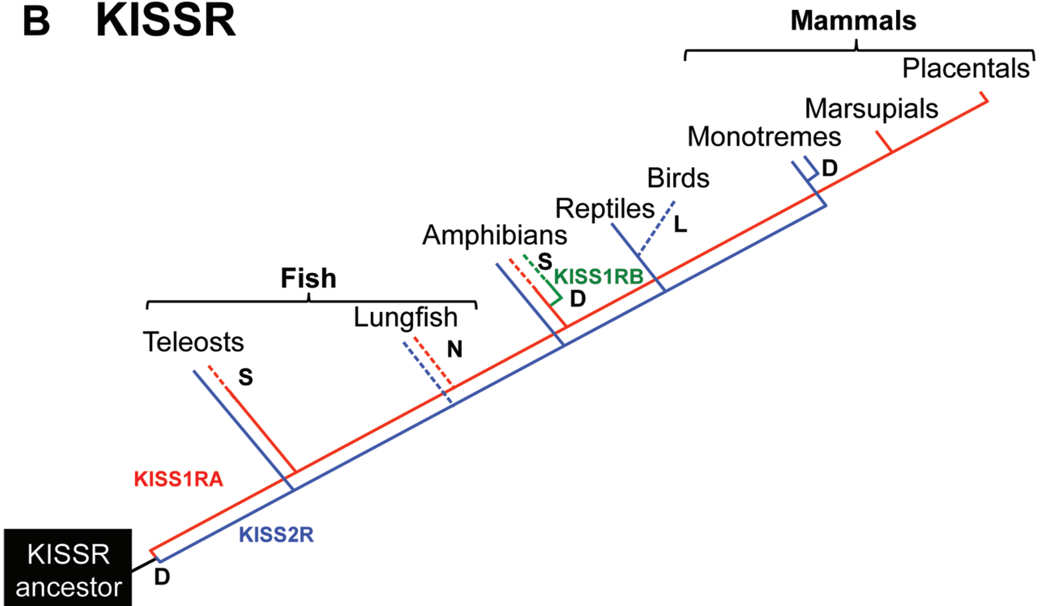


Figure 14. *KISS* **(A)** and *KISSR* **(B)** gene evolution in vertebrates inferred from the information available. D = gene duplication, L = gene lost, S = gene lost in some species, N = gene not searched for.

evolution of the genes from the ligand and receptor seems to be confirmed since the evolutive pathway with a second duplication that led to the appearance of another paralogous gene in amphibians and a loss of the second paralogous gene in some species is shared for both genes (Figure 14B).

In general, knowledge on gene evolution helps to get a better understanding of the functions of these genes and their particular physiological roles. Although there is a large evidence suggesting that the kisspeptin system has a pleiotropic effect and regulates several physiological functions, something particularly known in mammals (Oakley *et al*., 2009), kisspeptin research in teleost fish has mainly focused on reproduction and in particular in the control of the onset of puberty. It is probable that each paralogous gene in fish may also have pleiotropic functions. A recent study in zebrafish showed that *kiss2* is involved in the control of reproduction, while *kiss1* probably is implicated in perception of environmental and metabolic signals (Servili *et al*., 2011). However, future studies are needed to be carried out towards unraveling novel functions of the two *kiss* and *kissr* paralogous genes in other teleosts.

5. Variation in the expression of kisspeptin system genes at different stages of development and its implication for puberty in fish

Collectively, gene expression studies of the kisspeptin system in fish support their involvement in regulating puberty. For example, *kiss2r* levels are significantly higher in the brain of female grey mullet (*Mugil cephalus*) in the early stages than in the intermediate and advanced stages of pubertal development (Nocillado *et al*., 2007). Similarly, in cobia (*Rachycentron canadum*), increased *kiss2r* mRNAs occurred in early puberty (Mohamed *et al*., 2007). Studies in fathead minnow (*Pimephales promelas*) and zebrafish showed that *kiss2r* expression peaked at the onset of pubertal development and decreased with reproductive maturity (Biran *et al*., 2008; Filby *et al*., 2008). Our *kiss2r* gene expression analyses in Senegalese

sole (Paper I) and in Atlantic halibut (Paper II) in the whole brain are in agreement with these results. In contrast, *kiss2r* expressions in mature Nile tilapia males were higher than in immature males (Parhar *et al*., 2004; Martinez-Chavez *et al*., 2008).

For the kisspeptin ligand, results published so far regarding *kiss1* and *kiss2* expression patterns vary among the species studied. In zebrafish, mRNA levels of *kiss1* and *kiss2* increased during development, starting at mid-pubertal phase, and remaining high in adulthood (Kitahashi *et al*., 2009). In contrast, in chub mackerel, the highest expression levels were observed in pre-pubertal fish (Selvaraj *et al*., 2010). In the Senegalese sole, we found that *kiss2* mRNA peaked in mature fish but, in contrast with the zebrafish study, low levels were observed during pubertal stages (Paper III). Finally, in our last study we investigated the relationship between the expression profiles of several key genes of the reproductive axis, including *kiss2*, *kiss2r,* and maturation status during a complete reproductive cycle in this species, As a result we observed a close relationship between the expression levels of the genes of the kisspeptin system in the hypothalamus with the expression levels of gonadotropins in the pituitary and their receptors in the gonads during an annual reproductive cycle. The comparison of all these variables shows a differential behavior between sexes, with a reproduction peak in winter for males, which coincides with the onset of reproduction. On the other hand, in females, not all variables followed the same pattern, although for many a peak could be identified in spring, coinciding with the spawning season (Paper IV).

The discrepancies detected among studies from different laboratories studying different species are probably due to the different kisspeptin paralagous genes detected in the last few years (Lee *et al*., 2009; Um *et al*., 2010; Akasome *et al*., 2010), which were unknown at the time when previous studies were carried out. A second cause could be due to the presence of a second isoform still undetected in some species. A third cause can be attributed to the difficult determination of the precise stage of development in each sex and species. For example, some

studies referred to immature fish without precisely explaining whether the stage corresponded to juveniles or adults in an immature or regressed condition (Figure 15). Thus, for future research, and in order to avoid confusion and to establish a framework of reference, we recommend that in an experiment on gene expression during puberty several aspects should be consider: 1) Determine the precise stage of sexual maturation of each individual by analyzing gonadal development histologically, classifying the animals based on the presence of the different types of germ cells. 2) Clarify the existence of paralogous genes using phylogenetic

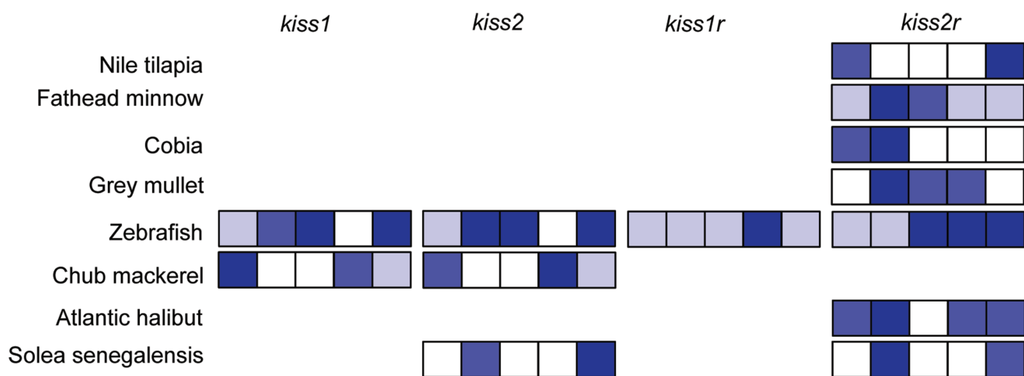


Figure 15. Expression of *kiss* and *kissr* in the teleost fish brain during development. Black boxes, high gene expression, grey boxes, moderate gene expression; light grey boxes, low gene expression, white boxes, not measured. The order of the five boxes from left to right correspond to; pre-pubertal (juvenile), onset, medium, and advanced puberty, and post-pubertal (adult) fish.

and synteny analysis and analyzing gene expression patterns. 3) Detect possible alternative splicing isoforms in the genes studied. Analyze gene structure, detecting intron-exons boundaries and by cloning and sequence the exons and introns. Search for isoforms using specific primers, particularly, when small introns (< 1 kb) are present. 4) Study the localization of genes and proteins using different tissues and, particularly for the brain, different areas. Include different stages of sexual maturation covering the different seasons of the year. Recent studies using

more accurate techniques of gene localization, such the ISH helped in elucidating the kisspeptin system in fish (Kanda *et al*., 2008; Lee *et al*., 2009; Kitahashi *et al*., 2009; Mitani *et al*., 2010; Grone *et al*., 2010; Servili *et al*., 2011).

This approach can contribute to a better understanding of the patterns of gene expression of the kisspeptin system during the puberty and it will facilitate the comparison among different studies, methodologies and across species for a further validation of the general pattern of expression of these genes.

6. Regulation of the kisspeptin system

To date there is scarce information regarding the factors and molecular mechanisms that regulate kisspeptin system genes in vertebrates. Estrogens, and particularly 17β-estradiol (E_2), play a key role in coordinating the neuroendocrine functions that control sexual development and reproduction (Wilson *et al*., 2008).

In mammals, one of the proposed mechanisms that regulate the kisspeptin system is through the effect of E_2, which differentially regulates KISS neurons in the ARC and AVPV regions of the brain, but the exact molecular pathway is still unknown (Oakley *et al*., 2009). Most estrogen actions occur via estrogen receptors (ERs). In mammals, two *ERs* have been identified: *ERα* and *ERβ*, generated by two distinct genes (Kuiper *et al*., 1998). However, in fish, the additional genome duplication gave rise to two *ERβ* genes: *ERβ1* and *ERβ2*, leading to a total of three *ER* genes (Steinke *et al*., 2006). The ER is a ligand-activated transcription factor that regulates gene expression (Weiss *et al*., 2008). The molecular mechanisms of ER action include the binding of estrogen to specific receptors in the nucleus, after which receptors dimerize and bind to the estrogen response elements (EREs), located in the promoter regions of target genes (Björnström and Sjöberg, 2005). This binding site is a palindromic sequence with a consensus sequence (AGGTCAnnnTGACCT) (Klein-Hitpass *et al*., 1986). Accordingly, in mammals,

most Kiss1 neurons express ERα (Smith *et al*., 2005; Smith *et al*., 2006c) and ERβ (Smith *et al*., 2006c). Studies in humans demonstrated that E_2-dependent transcriptional activation of *KISS1* is mediated by ER through the interaction with Sp1/Sp3 proteins in the GC-rich motifs of the *KISS1* promoter, giving a molecular interpretation of how steroid hormone feedback regulates *KISS1* expression (Li *et al*., 2007). It should be stated that in fish kisspeptin regulation by estrogen is more controversial. In medaka, Kiss1 neurons co-express ERα, whereas Kiss2 neurons do not, suggesting that Kiss1 neurons are regulated by estrogen and that *kiss1* is involved in the regulation of reproduction in this species (Mitani *et al*., 2009). In contrast, E_2 treatment of juvenile zebrafish caused a pronounced increase in *kiss2* expression (Servili *et al*., 2011). These discrepancies may be attributed to species-specific mechanisms, similar to what was has been observed with the three *GnRH* genes in fish (Lethimonier *et al*., 2004).

To the best of our knowledge, we were the first to clone the promoter region of *kiss2* and *kiss2r* genes in teleost fish, in the Atlantic halibut (Paper II) and in the Yellowtail kingfish (unpublished data). In Yellowtail kingfish, we detected a conserved ERE site within the first 100 bp upstream the *kiss2* gene that showed 75% homology to the accepted ERE consensus sequence. The Yellowtail kingfish ERE sequence is 5'-AGGTCAtggTGAAAA-3', which has 100% (6 of 6 in the first nucleotides) and 50% homology in the last 6 nucleotides in comparison with the consensus sequence of the ERE (unpublished data). This result confirms the possible control of the kisspeptin gene though E_2 signaling via the ER and in relation with the possible regulation of reproduction in this species. It should be noted that we made several attempts to clone the promoter region of *kiss1* gene in Yellowtail kingfish. However, our attempts were unsuccessful probably due to the complicated gene structure of the *kiss1* gene, where two non-transcribed exons are present. Nevertheless, further studies are required to functionally characterize the identified ERE, and to determine the regulatory role of estrogen on the kisspeptin system in fish.

There is very limited information regarding the *KISSR* gene promoter in vertebrates. It has been observed that in mouse repression of *Kiss1r* promoter activity by ERE resulted in an activation of *Kiss1r* expression by the action of the

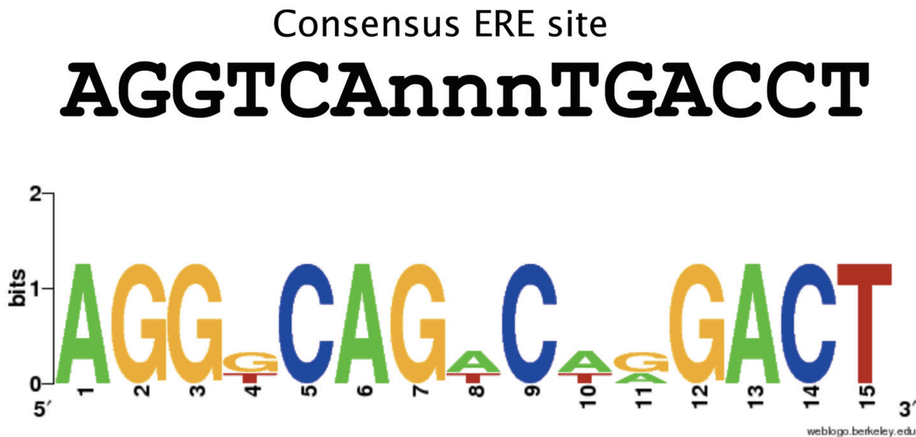


Figure 16. Estrogen response element (ERE), consensus site in the *kiss2r* promoter of Atlantic halibut, Yellowtail kingfish and Southern bluefin tuna generated using Weblogo sequences (http://weblogo.berkeley.edu).

transcription factor specificity protein-1 (SP1) (DeFino *et al*., 2010). In our study, carried out in the *kiss2r* gene promoter in the Atlantic halibut, we identified two regions that showed considerable similarity to the binding sites for the transcription factors, mini zinc finger 1 (MIF-1) and ERE (Paper II). Moreover, using genome sequences available for the *kiss2r* in five teleost species: zebrafish, medaka, three-spined stickleback, green pufferfish and tiger fugu (Paper II) we showed that there was a notable synteny or conserved location of the ERE sequences around 100 bp upstream of *kiss2r* genes (Figure 16). In the preliminary analysis of the *kiss2r* promoter sequences that we isolated from Yellowtail kingfish and Southern bluefin tuna, we identified homologous consensus ERE sites, suggesting that the putative role of estrogen regulation of the kisspeptin system is conserved at least among the teleosts (unpublished data). This opens another line of research in the future.

The control of gene expression in the kisspeptin system in flatfish according to the results derived from our studies could be related to, at least, two different factors: First, alternative splicing with the generation of at least two isoforms. Alternative splicing is accepted as one of the main mechanisms in generating diverse peptide products from a limited set of genes (Black, 2003). The question remains whether these transcripts are translated into significant amounts of protein and, if so, if this protein has any biological significance. Second, gene expression can also be controlled by estrogen through the ERE in the promoter of the genes. It has been observed that in some genes, the upstream region contains multiple promoters that each of them regulates the formation of different mRNA splice variants. Differential promoter usage could be important for the spatial or temporal regulation of transcription (Wilson *et al*., 2008). In fact, eukaryotic genes often contain multiple promoters that determine a different start site and in consequence generate different transcripts, suggesting a link to alternative splicing of internal exons and their physiological implications (Kornblihtt, 2005). Thus, alternative promoter usage with differential signaling mediated by the ERE could be postulated as a link of the two mechanism observed in this thesis. However, the interaction between the kisspeptin system genes in the promoter and the alternative splicing mechanism by intron retention is still unknown and represents an unexplored field of research. Moreover, additional studies on other physiological factors that may modulate the kisspeptin system, such as the nutritional condition, as we have demonstrated with the enhancement of the BPG axis with fasted Senegalese sole (Paper III), or the influence of different seasons of the year (Paper IV), would provide a more comprehensive understanding of the kisspeptin system in fish.

7. The kisspeptin system and its importance for fish aquaculture

A major goal of research for aquaculture is to synchronize natural spawning and induce spawning out of season (Munakata and Kobayashi, 2010). Another practical application is the manipulation of the BPG axis to generate reproductively sterile fish, which could diminish the genetic risk of possible escapees (Wong *et*

al., 2008; Piferrer *et al*., 2009). Moreover, the generation of sterile populations can be an important strategy in the culture of faster growing fish (Piferrer *et al*, 2009). One of the most typical reasons of failure of reproduction of cultured fish is the low secretion of LH from the pituitary (Mylonas *et al*., 2010). The first exogenous hormonal administration employed in fish to stimulate the maturation and spawning was pituitary homogenates (Houssay, 1930). However, actually the use of exogenous LH that acts directly in the gonads or GnRH agonist (GnRHa) that stimulates the release of the endogenous LH from pituitary are the most used to induce oocyte maturation and spermiation (Mylonas *et al*., 2010). Kisspeptin administration offers now a promising and efficient option, as observed in previous studies in fish, including the sea bass (Felip *et al*., 2009), medaka and zebrafish (Kitahashi *et al*., 2009), goldfish (Yang *et al*., 2010) and Yellowtail kingfish (unpublished data). Another area of interest is the development of methods for the non-invasive administration of compounds to fish on a large-scale basis. Hormone administration in fish aquaculture is labor intensive, time consuming, and therefore costly and not accepted by consumers (Mylonas and Zohar, 2001). Thus, work such the one carried out in this Ph.D. project regarding how nutritional conditions affect the kisspeptin system (Paper III) may hold promise.

8. Future work

There are important questions that need to be addressed in future studies. First, although the current established role of kisspeptin system concerns the regulation of reproduction, there likely are other functions that still need to be clarified, functions, which even may ultimately impact reproductive processes. Second, based on our findings regarding the presence of alternative isoforms with discrepant behavior in their expression patterns, new experiments should focus on the possible differential functions of the proteins or peptides derived from these isoforms even if they serve to conclude that there is no function for the splice variant. This line of research was attempted in this thesis, but due to the failure of the initial experimental procedures we decided to cancel it. Third, our study

has unraveled what seem to be conserved transcription factor binding sites on the promoter region of kisspeptin genes in teleost fish. Thus, the ERE should be studied in order to elucidate its role in the control and regulation of reproduction in non-mammalian species, as well as other putative regulators. In this regard, how biotic and abiotic factors influence kisspeptin signaling definitively needs to be studied, since research has so far centered on a very limited number of factors. Finally, some critical points in the aquaculture industry for many flatfish must be addressed. The Senegalese sole and Atlantic halibut are two promising species for the aquaculture industry and, given the resources available and the knowledge accumulated, they can be used as model species for flatfish in addressing current problems in their production, principally in the control of reproduction. Another objective will be to reduce the incidence of early maturation. This requires improved understanding of fish reproductive physiology. For future research, studies with in vivo assays using kisspeptin administration may help to control the precise moment of fish maturation in captivity of these species.

Conclusions

V. Conclusions

1. In contrast to the situation observed in several teleost fish, in the flatfish species used in this study only one paralagous gene of the kisspeptin system was identified for the ligand, *kiss2* (Senegalese sole), and for the receptor, *kiss2r* (Senegalese sole and Atlantic halibut). We conclude that the other paralagous gene was lost during evolution, as a consequence of the genome reduction characteristic of the Pleuronectiformes.

2. The organization of *kiss2* in the Senegalese sole revealed two exons separated by one intron, a gene structure similar to that of other *kiss2* genes found in fish. Its protein product is formed by a preprotein with the typical organization, including a signal peptide, followed by 78 aa of which the last 10 aa correspond to Kp10 plus a putative site of terminal cleavage and amidation. However, this is followed by a putative kisspeptin-associated peptide, the first one described in a kisspeptin gene in vertebrates.

3. The *kiss2r* genes of Senegalese sole and Atlantic halibut exhibit the same organization found in other vertebrates, with five exons and four introns. Their coding proteins contain the features of a G-protein coupled receptor, with the canonical structure of seven transmembrane domains (TMD).

4. *Kiss2* and *kiss2r* have two isoforms each originated by an alternative splicing mechanism based on intron retention. Such mechanism had never been described for kisspeptin genes in other species. The isoform with the retained intron codes in both cases for a truncated product, with preliminary termination codons that lead to non-functional proteins.

5. Gene expression analysis of the two isoforms of the kisspeptin system genes in the Senegalese sole revealed different expression patterns according to tissue and stage of development. Whether this constitutes a regulatory mechanism remains unknown.

6. Compilation of all vertebrate *KISS* and *KISSR* sequences available to date allowed the construction of an updated phylogenetic tree that brought light to the evolution of these genes. The new phylogenetic relationships suggest that both genes underwent a first duplication prior to the divergence of teleost fish, followed by a second duplication prior to the appearance of amphibians, the only lineage with three paralagous genes.

7. Analysis of the *kiss2r* flanking regions of the Atlantic halibut shows a synteny with the *kiss2r* locus of several other teleosts and suggests the likelihood of conservation of regulatory regions, with *atp6v0b* and *odf2L* as conserved motifs.

8. Comparative analysis of the *kiss2r* promoter in the Atlantic halibut against that of zebrafish, medaka, stickleback, fugu and pufferfish revealed the presence of a conserved estrogen response element (ERE) site, located less than 100 pb from the start codon of the *kiss2r* gene. This observation should lead to more research on the regulation of the kisspeptin system regulated by estrogen.

9. Fasting enhanced *kiss2* and *kiss2r* expression in the hypothalamus and caused a concomitant rise in pituitary *fsh* and *lh* expression in the Senegalese sole. These data indicate the impact of the nutritional status on *kiss2* mRNA expression as a potential regulatory mechanism for the metabolic control of reproduction in non-mammalian species.

10. Analysis of the temporal and spatial changes in expression of kisspeptin, gonadotropins and their respective receptors in the Senegalese sole during a full reproductive cycle shows that, in males, *kiss2* agrees with what one would expect according to its proposed role as a major regulator of the onset of reproduction. However, in females the situation is not so clear, since *kiss2* and *kiss2r* expression was highest either before or during the reproductive season. The origin and physiological significance of these differences, which could also apply to other fish, deserve further investigation.

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