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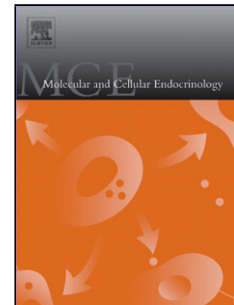
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**Gene structure analysis of kisspeptin-2 (*Kiss2*) in the Senegalese sole (*Solea senegalensis*): Characterization of two splice variants of *Kiss2*, and novel evidence for metabolic regulation of kisspeptin signaling in non-mammalian species**

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**Running title:** Kisspeptin splice variants in fish and energy balance

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## Abstract

Kisspeptin signaling in the brain is involved in the control of the onset of puberty in vertebrates. In this study, we present novel evidence indicating that kisspeptin may link energy balance and reproduction. For that purpose, we determined the complete gene structure of kisspeptin in a teleost fish, the Senegalese sole (Ss). In contrast to the situation evident in several fish, in this species only Kiss2 was found. Yet, two Ss *Kiss2* isoforms generated by alternative splicing through intronic retention were detected: Ss *Kiss2\_v1*, producing the functional protein, and Ss *Kiss2\_v2*, coding for a truncated, non-functional protein. Specific qPCRs showed that the expression of these two isoforms varied differently in brain and gonads throughout maturation. In addition, and in contrast to what has been observed in mammals, fasting increased hypothalamic mRNA levels of Ss *Kiss2\_v1*, which also caused a concomitant rise in pituitary Ss *LH* and Ss *FSH* mRNA. Together, these data indicate the impact of the nutritional status on *Kiss* mRNA expression as a potential regulatory mechanism for the metabolic control of reproduction in non-mammalian species, albeit with some significant differences with respect to the situation described in mammals.

**Keywords:** *Kiss2*, *kisspeptin-2*, *puberty*, *fasting*, *alternative splicing*, *Solea senegalensis*

## 1. Introduction

In vertebrates, reproduction is subject to a complex regulation within the hypothalamic-pituitary-gonadal (HPG) axis (for reviews see Gottsch et al., 2004; Aparicio et al., 2005; Tena-Sempere, 2006; Roa et al., 2008; Oakley et al., 2009). In the last years, it has become established that the peptide kisspeptin has an important role in the control of reproduction, particularly relating to the onset of puberty as evidenced mostly by studies in mammals (Gottsch et al., 2004; Tena-Sempere, 2006; Roa et al., 2008; Oakley et al., 2009; Roseweir and Millar, 2009) and fishes (Parhar et al., 2004; Elizur et al., 2009; Carrillo et al., 2009; Zohar et al., 2009; Taranger et al., 2009). Hence, mRNA levels of kisspeptin and its receptor are highest at the time of the onset of puberty in rats (*Rattus norvegicus*) and rhesus macaque (*Macaca mulatta*), where an increase in expression patterns for both genes were observed in juveniles entering puberty (Navarro et al., 2004; Shahab et al., 2005). Moreover, exogenous administration of kisspeptin activates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in mammals (Funes et al., 2003; Matsui et al., 2004; Messenger et al., 2005), amphibians (Moon et al., 2009) and teleost fishes (Felip et al., 2009; Kitahashi et al., 2009).

In mammals, there is only one kisspeptin gene (*Kiss1*), with the exception of the platypus, a mammalian monotreme, which has two genes (*Kiss1* and *Kiss2*) (Lee et al., 2009). The presence of two kisspeptin genes is common in fishes, as observed in medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), European sea bass (*Dicentrarchus labrax*) and chub mackerel (*Scomber japonicus*) (Felip et al., 2009; Kitahashi et al., 2009; Li et al., 2009; Selvaraj et al., 2010). As for the receptor, two *Kissr* genes,

originated through one or two rounds of gene or chromosome duplication and named *Kiss1r*, *Kiss2rA* and *Kiss2rB* (Mechaly et al., 2010), are also observed in medaka, zebrafish and goldfish (Kitahashi et al., 2009; Li et al., 2009; Biran et al., 2008). However, this situation is not universal, since only one kisspeptin gene, *Kiss2*, and one receptor, *Kiss2rA*, are present in the orange-spotted grouper (*Epinephelus coioides*) (Shi et al., 2010), grass puffer (*Takifugu niphobles*) (Shahjahan et al., 2010), Senegalese sole (*Solea senegalensis*) (Mechaly et al., 2009) and Atlantic halibut (*Hippoglossus hippoglossus*) (Mechaly et al., 2010), indicating that the paralogous gene has been lost during evolution (Mechaly et al., 2010). Furthermore, in the Senegalese sole, a splice variant isoform of the *Kiss2rA* gene was observed in the transcript (Mechaly et al., 2009), and in amphibian clawed frog (*Xenopus tropicalis*), a third paralogous for both the ligand (*Kiss1b*) and the receptor (*Kiss1rb*) (Lee et al., 2009) have been reported. Thus, the evolution of kisspeptin and its receptor in vertebrates involves gene duplications, appearance of alternative splicing variants as well as gene loss, although the course of events and their relationships are still not completely clear. Therefore, a more exhaustive analysis is necessary to understand the phylogenetic relationships among these gene duplications and isoforms in the kisspeptin family.

The existence of paralogous genes and isoforms in fish poses the interesting question as to what is the precise function of each gene. In the European sea bass, *Kiss2* was found to be the most potent elicitor of gonadotropin (LH and FSH) secretion (Felip et al., 2009). In zebrafish, habenular *Kiss1* and hypothalamic *Kiss2* regulate reproduction but *Kiss2* is more potent in stimulating gonadotropin synthesis and secretion (Kitahashi et al., 2009). In goldfish, *Kiss1* acts directly at the pituitary, inducing the secretion of LH, prolactin and growth hormone (Yang et al., 2010). However, administration of the Kp10 peptide failed to

elicit differences in *LH* mRNA levels (Li et al., 2009). In medaka, only hypothalamic *Kiss1* neurons of the nucleus ventralis tuberis (NVT) were involved in estrogen feedback (Kanda et al., 2008), and it was suggested that *Kiss2* neurons play a role in non-reproductive functions (Mitani et al., 2010). Intraperitoneal injection of *Kiss2-10* in mature orange-spotted grouper resulted in increased *GnRH1* mRNA levels in the hypothalamus and *FSHb* in the pituitary (Shahjahan et al., 2010). Together, these results provide ample evidence of the participation of kisspeptin in fish reproduction although the roles of *Kiss1* and *Kiss2* vary across species.

In addition to its role in the central control of reproduction, there is also increasing evidence showing that kisspeptin signaling is involved in other processes (Roa et al., 2008; Roseweir and Millar, 2009). These include a potentially important autocrine and/or paracrine effects in the ovary (Gaytan et al., 2007), placenta (Hiden et al., 2007), and immune system (Cardon et al., 2009), as well as the participation of kisspeptin in metastasis (Makri et al., 2008), vasoconstriction (Mead et al., 2007) and stimulation of insulin secretion (Bowe et al., 2009). Of particular interest is the possible role of kisspeptin linking energy balance and reproduction (Popa et al., 2008), since recent observations indicate that the impacts of negative energy balance towards reproduction can be modulated by kisspeptins (Tena-Sempere, 2006). Hence, in rats, *Kiss1* is sensitive to nutritional conditions, with a decrease in expression during negative energy balance status, thus contributing to reproduction suppression (Fernández-Fernández et al., 2006). Furthermore, *Kiss1* neurons are direct targets of leptin, suggesting that reproductive deficits associated with leptin-deficient states may be attributable, in part, to diminished expression of *Kiss1* (Smith et al., 2006). However, the regulation of the metabolic condition and its subsequent impact on reproductive function through kisspeptin and its receptor is far from being well understood. Nothing is known about

the possible kisspeptin-mediated connection between energy balance and reproduction in non-mammalian models.

The aim of this study was to evaluate the possible role of kisspeptin signaling in situations of negative energy balance in a non-mammalian model. For this purpose, we first cloned, characterized and analyzed the gene structure of *Kiss* in the Senegalese sole and the levels of expression in the brain and gonads of pubertal and mature fish. This species has only one *Kissr* gene, *Kiss2r*, and thus we also wanted to search for the possible existence of paralogous *Kiss* genes in this species.

## 2. Experimental Procedures

### 2.1. Animals and tissue sample collection

Senegalese sole were obtained from the IFAPA research center in El Puerto de Santa María (Cádiz, SW Spain). Fish stocks were maintained at the Experimental Aquarium Facilities of the Institute of Marine Sciences, under natural conditions of temperature and photoperiod and fed two times a day with a commercial diet (Skretting, Spain). 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 animals' welfare (ETS No. 123,01/01/91). 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, frozen in liquid nitrogen and stored at -80°C until used. Biometric



information, standard length (SL) (cm) and body weight (BW) (g) were obtained for all animals.

## 2.2. RNA isolation and cDNA synthesis

Total RNA was isolated with TRIZOL Reagent (Invitrogen, Carlsbad USA), its quality was checked in a 1.5% agarose gel and quantity measured in a Nanodrop® ND-1000 spectrophotometer (Nanodrop® Technologies Inc, Wilmington, DE, USA). All RNAs were treated with Dnase I (Invitrogen, Carlsbad, CA, USA) to remove any possible genomic DNA contamination. cDNA synthesis was performed following the manufacturer's instructions using 500 ng of RNA with Superscript III Reverse Transcriptase and random hexamer primers (Invitrogen, CA, USA) in a 20 µl reaction volume.

## 2.3. Molecular cloning of kisspeptin in the Senegalese sole

Degenerated PCR primers based on the polypeptide sequences YNLNSFGLRY for *Kiss1* and FNL/Y/FNPFGLRF for *Kiss2* were designed to discriminate between the two genes. Degenerated PCR primers were designed based on the sequence of the conserved region of Kp10 of zebrafish-1, medaka-1, sea bass-1 and goldfish-1 for the *Kiss1*, and *fugu-2*, *pufferfish-2*, *sea bass-2*, *goldfish-2*, *stickleback-2* for the *Kiss2* (Supplementary Table 1). PCR amplifications were carried out from four Senegalese sole genomic DNA libraries constructed with the Universal Genome Walker Kit (Clontech Laboratories Inc, CA, USA.). Briefly, two rounds of PCR were performed: first, PCR amplification was carried out using a primer (AP1) provided in the kit and degenerate for *Kiss2* (1PCR\_S\_Kiss2 and 1PCR\_AS\_Kiss2). Second, a nested PCR was performed by the primer combination AP2 and

degenerate and nested Kiss2 PCR primers, 2PCR\_S\_Kiss2 and 2PCR\_AS\_Kiss2, using a 1/50 dilution of the product of the primary PCR. In all cases, PCR conditions were as follows: initial denaturation at 94°C for 25 s, followed by 7 cycles at 94°C for 25 s and 26°C for 3 min, and 32 cycles at 94°C for 25 s and 67°C for 3 min, and 67°C for 7 min after the final cycle. The full-length cDNAs of *Kiss2* was obtained from a Senegalese sole brain cDNA library constructed in the UNI ZAP-XR vector. PCR reactions using gene-specific primers in combination with primers annealing in the UNI Zap-XR vector were performed to obtain the cDNA ends.

#### 2.4. Gene structure, sequence alignment and phylogenetic analysis

To assess the genomic gene structure, the coding of the gene was amplified by the primer combination Kiss2\_Ex1\_F1 and Kiss2\_Ex2\_R2 using genomic DNA as template. Genomic DNA was extracted using a biopsy of muscle (~50 mg of tissue) incubated overnight at 55°C in 1 ml of lysis solution (10 mM Tris-HCl, pH 7.5; 25 mM EDTA, pH 8; 0.5% SDS; 100 mM NaCl), and 10 µl of proteinase K (10 mg/ml). DNA was cleaned by a wash of equal volume of phenol/chloroform and a wash of chloroform/ isoamyl alcohol. Then, 5 µl of RNase A (10 mg/ml) were added and incubated at 55°C for 30 min. Finally, DNA was precipitated with 95% ethanol, washed with 70% ethanol and eluted in 50 µl of sterile water.

The gene structure was constructed using the nucleotide and predicted peptide sequence of *Ss Kiss2* compared with known *Kiss2* sequences available through GenBank. The presence and location of the putative signal peptide cleavage site of the *Kiss2* of fishes

were assessed using the prediction protein servers of the Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk/services/>). The 5'UTR and 3'UTR regions were localized upstream and downstream of the start codon (ATG) and stop codon (TGA), respectively.

Phylogenetic analysis was performed using multiple alignments of the DNA sequence corresponding to the 30 nucleotides that codified the 10 aa of Kp10. A total of 38 sequences were aligned using Clustal W (Thompson et al., 1994) and edited in Bioedit (Hall, 1999). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using the Kimura two parameters distance (Kimura, 1980). All phylogenetic analyses were conducted on MEGA version 4 (Tamura et al., 2007).

## 2.5. Tissue expression pattern of *Ss Kiss2*

The tissue distribution of *Ss Kiss2* in adult animals was analyzed by RT-PCR in different tissues. Total RNA and cDNA synthesis were carried out following the protocol described in section 2.2. The resulting cDNA was used to amplify *Ss Kiss2* by RT-PCR using the Platinum Taq DNA Polymerase (Invitrogen). The PCR cycling conditions for *Ss Kiss2* were: 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C and a final extension of 5 min at 72°C. Genomic DNA contamination in cDNA was checked with a primer combination encompassing the Intron I (*Kiss2\_Ex1\_F1* and *Kiss2\_Ex2\_R2*) (supplementary Table 1). Amplification of the *beta-actin* gene was used as RNA quality control. No template control (NTC) was included to ensure that no contamination had occurred. PCR products were run on 1.5% agarose gel and subsequently sequenced to verify their authenticity.

2.6. Expression mRNA levels of two *Ss Kiss2* isoforms (*Ss Kiss2\_v1* and *Ss Kiss2\_v2*) in brain and gonads in two developmental stages.

The expression pattern of *Ss Kiss2\_v1* and *Ss Kiss2\_v2* was analyzed in whole brain of pubertal and mature fish (as classified in Mechaly et al., 2009) by real-time quantitative PCR (qPCR) ( $n = 4 - 6$ ). RNA and cDNA were obtained following the protocol described above. The *Ss Kiss2* primers used for qPCR were designed to amplify each *Ss Kiss2* isoform (see results and Supplementary Table 2). The qPCR amplification reaction mixture contained 2  $\mu$ l of diluted cDNA (1:3) (freshly synthesized from 500 ng of RNA), 4  $\mu$ M of each primer, and 10  $\mu$ l of Power SYBRs Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a final volume of 20  $\mu$ l. Thermal cycling conditions comprised 95°C for 10 min, 40 cycles at 95°C for 10 s, and 60°C for 30 s. At the end of the PCR cycles, the qPCR products were analyzed using a dissociation curve step to confirm that only a single PCR product was amplified. NTC 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/target gene was assessed as  $E = 10^{-1/\text{slope}}$  as determined by linear regression of a series of dilutions of the input RNA. The qPCR reactions were performed with an ABI Prism 7900 Sequence Detection Systems (Applied Biosystems).

Data were analyzed using the comparative cycle threshold (Ct) method (Bustin, 2000) (DDCt method) to calculate relative changes in gene expression. Fold change (the relative quantification, RQ) was calculated from the DDCt and normalized to the reference gene *beta-actin*. Determinations were carried out in technical triplicates for the *Ss Kiss2\_v1* *Ss*

*Kiss2\_v2* and *beta-actin* genes. 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.

## 2.7. Effect of fasting and feeding conditions on the expression of kisspeptin and its receptor in adult Senegalese sole

Adult male and female Senegalese sole were randomly distributed into two 600-liter tanks. The nutritional study was conducted for a period of 15 days after a 5-day period of acclimation, with seawater temperature at  $20 \pm 1^\circ\text{C}$ . One group of fish ( $n = 8$ ) was fed *ad libitum* (5 times a day) and used as the control group ("Fed"). Another group ( $n = 7$ ) was maintained under food deprivation conditions ("Fasting"). The initial mean BW (g) and SL (cm) were similar in both groups: Fed;  $885,71 \pm 140,53$  g and  $33,14 \pm 1,99$  cm; Fasting;  $871,43 \pm 121,85$  g  $32,57 \pm 1,97$  cm with non-significant differences (BW,  $P = 0.68$ ; SL,  $P = 0.59$ ). After 15 days of differential feeding all fish were sacrificed and the hypothalamus, stomach and pituitary were quickly removed and kept at  $-80^\circ\text{C}$  under. Biometric information, SL (cm), BW (g), gonad and liver weight (g) were obtained for all the fishes. The gonadosomatic (GSI) and hepatosomatic (HSI) indices were calculated as the percent of the corresponding organ weight with respect to the total body weight. Total RNA isolation and DNA treatment and cDNA synthesis were carried out as described in section 2.2. qPCR amplification reaction was performed for each cDNA sample in triplicate in 10  $\mu\text{l}$  of the reaction mixture containing 5  $\mu\text{l}$  of 10x master mix (Express SyBr green, Invitrogen), 0.2  $\mu\text{l}$  of each primer (10  $\mu\text{M}$ ), and 2  $\mu\text{l}$  of a 1/10 diluted sample from the cDNA synthesis. Reaction mixtures without a template were run as controls. The cycling conditions were as:  $95^\circ\text{C}$  for 10

min, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s. The expression pattern was analyzed by qPCR using the *Ss Kiss2\_v1* and *Ss Kiss2\_v2* and *Ss Kiss2r\_v1* and *Ss Kiss2r\_v2* in hypothalamus and stomach, whereas the *Ss LH* and *Ss FSH* (taken from Cerdà et al., 2008) were assessed in pituitary for both experimental conditions (for full list of primers see Supplementary Table 2).

## 2. 8. Statistical analyses

Data are shown as mean  $\pm$  standard error of the mean (S.E.M.). All statistical analyses were performed using the SPSS v16 software. The significance of pairwise comparisons of the mRNA levels of pubertal and mature fishes in brain and gonads of *Ss Kiss2\_v1* and *Ss Kiss2\_v2* were determined using a one-way ANOVA followed by Fisher's least significant difference (LSD) test for both isoforms. Statistical differences between fed and fasting fish were analyzed by a Student *t*-test for the biometric data as well as the mRNA levels of *Ss Kiss2\_v1* and *Ss Kiss2\_v2*, *Ss Kiss2r\_v1* and *Ss Kiss2r\_v2* in the hypothalamus and stomach and *Ss FSH* and *Ss LH* in the pituitary. Differences were accepted as significant when  $P < 0.05$ .

## 3. Results

### 3.1. Cloning and sequence analysis of *Ss Kiss2* gene

Although PCRs were carried out to specifically find the two *Kiss* genes (*Kiss1* and *Kiss2*) in the Senegalese sole (*Ss*), only *Ss Kiss2* was detected. The complete coding region of fully spliced *Ss Kiss2* comprised 390 bp that predicts a 129 amino acid (aa) protein (Fig.

1A and B). The sequence of *Ss Kiss2* has been deposited into GenBank with accession number HM116743. Sequence analysis identified a N-terminal with 19 aa residue of the signal peptide and the ten conserved aa corresponding to the Kp10 region (Fig. 1B). A high level of aa sequence identity was observed only in the decapeptide region Kp10 between *Ss Kiss2* and zebrafish *Kiss2* (90%), zebrafish *Kiss1* (60%) and human *Kiss1* (70%), while the degree of nucleotide conservation was reduced to 20% in adjacent regions of the gene (Table 1).

### 3.2. Genomic structure of *Ss Kiss2* gene

In addition to the coding region, the genomic sequence of the *Ss Kiss2* was also obtained. *Ss Kiss2* is organized into 2 exons, exon 1 with 109 bp and exon 2 with 281 bp, and one intron of 36 bp (Fig. 2). The sequence obtained comprises a prepro *Ss Kiss2* cDNA that spans 432 bp and includes a 36 bp 5'-untranslated region (5'-UTR), a 390 bp open reading frame (ORF) encoding a putative preprohormone of 129 a, and a 6 bp 3' UTR (Fig. 2). The consensus ATTAAG polyadenylation signal and the poly (A) tail were not found. The molecular architecture of prepro *Ss Kiss2* includes a 19 aa peptide, the *Kiss2* peptide, a putative processing site, and a kisspeptin-associated peptide. The deduced sequence of *Ss Kiss2* peptide Kp10 consists of 10 aa. The proteolytic cleavage site (GKR) is conserved in all *Kiss2* genes analyzed so far (Fig. 1B and Fig. 2).

### 3.3. Detection of two transcript variants in the *mRNA* of the *Kiss2* of Senegalese sole

Specific RT-PCR with a primer set that amplifies a region that encompasses the intron generates two different products of 390 and 426 bp (Fig. 3A) named hereafter *Ss Kiss2\_v1*

and *Ss Kiss2\_v2*, respectively. The presence of genomic contamination in the RNA extraction was discarded with DNase treatment of all RNAs. Furthermore, A PCR with primers flanking intron 1 were designed to amplify both *Ss Kiss2* isoforms and used to evaluate the expression of *Ss Kiss2* in several tissues. The *Ss Kiss2\_v2* transcript was detected in all tissues analyzed, whereas the *Ss Kiss2\_v1* was only detected in the ovaries, testis, stomach and brain, with a higher expression in this latter tissue (Fig. 3A). Negative controls with non-reverse transcribed RNA samples (no-template control, NTC) did not yield positive signals. Sequence analysis revealed that *Ss Kiss2\_v2* retained the entire intron 1 (Fig. 3B) and revealed the presence of all the features characteristic of an alternative splicing mechanism in the intron 1, including a 5'ss donor (5') splice site, consensus sequence (5') splice site (CS 5'ss), branch point (BP), polypirimidine track (PT), 3'ss, acceptor (3') splice site, and consensus sequence (3') splice site (CS 3'ss) (Fig. 3C). The putative translation to aa of the *Ss Kiss2\_v2* isoform revealed the presence of two premature termination codons (PTCs) in the intron.

#### 3.4. Sequence comparison and phylogenetic analysis of *Ss Kiss2* in vertebrates

Amino acid sequence comparison of the peptide Kp10 among several vertebrate groups showed a complete fixation of aas 2 (Asn), 6 (Phe), 7 (Gly), 8 (Leu) and 9 (Arg). Nevertheless, the first and last positions (1 and 10) are considered the most important in the Kp10 classification (Roa et al., 2008). The Kiss1 form presented either a tyrosine (Y) in the position one and either a Phenylalanine (F) or Tyrosine in the last position corresponding to the Y-Y and Y-F forms, respectively. On the contrary, Kiss2 corresponded only to the F-F form (Fig. 4A). Since the *Kiss* gene presents very low levels of conservation among



vertebrates, which make it difficult for correct alignment of the gene, the phylogenetic analysis of a total of 27 species (38 sequences in total) was restricted to 30 nucleotides of the Kp10 region. The phylogenetic analysis (Fig. 4B) clustered two different groups corresponding to Kiss1 and Kiss2.

### 3.5. Comparison of Ss *Kiss2\_v1* and Ss *Kiss2\_v2* expression levels during development

The mRNA levels of the Ss *Kiss2\_v1* and Ss *Kiss2\_v2* isoforms were analyzed separately by a specific qPCR in Senegalese sole male and female in brain and gonads (see supplementary table 1 for specific primers used in the qPCRs). In the brain, both isoforms were detected in both pubertal and mature fish of both sexes, with an overall higher and significant ( $P < 0.05$ ) difference in expression in the mature females with respect to males for each of the isoforms (Fig. 5A). In the gonads, the two isoforms were also detected in both sexes and stages of development. However, and also contrary to the situation in the brain, the differences were stage-related, with a significantly ( $P < 0.001$ ) high expression in pubertal females (Fig. 5B).

### 3.6. Fasting effects on key genes of the reproductive axis

Fifteen days of fasting resulted in a significant reduction in BW (-5.63%) while control fish maintained their BW ( $P < 0.001$ ) (Fig. 6A). The HSI was reduced in the fasted fish although did not show statistical differences ( $P = 0.278$ ) (Fig. 6B). Similarly, the GSI did not show statistical differences between the two different nutritional conditions, although a slight increase in GSI could be detected for males and females, respectively (Figs. 6C and D). Changes in these indices indicate the effects of fasting in somatic vs. gonadal tissues.

In the hypothalamus, induced fasting increased mRNA levels of *Ss Kiss2\_v1* ( $P = 0.016$ ), *Ss Kiss2\_v2* ( $P = 0.018$ ), *Ss Kiss2r\_v1* ( $P = 0.03$ ) and *Ss Kiss2r\_v2* ( $P = 0.044$ ) (Fig. 7A). Similarly, expression levels of *Ss LH* and *Ss FSH* in the pituitary increased up to ~ 4 fold ( $P = 0.035$  and  $P = 0.007$ , respectively) (Fig. 7B). Although fasting increased the levels of *Ss Kiss2\_v1* and *Ss Kiss2\_v2*, no statistically significant changes were detected for the *Ss Kiss2\_v1* ( $P = 0.362$ ), *Ss Kiss2\_v2* ( $P = 0.245$ ), *Ss Kiss2r\_v1* ( $P = 0.324$ ) and *Ss Kiss2r\_v2* ( $P = 0.790$ ) in the stomach (Fig. 7C).

#### 4. Discussion

The presence of both *Kiss* paralogous genes (*Kiss1* and *Kiss2*) was thoroughly examined in the Senegalese sole, although only *Ss Kiss2* was found indicating that *Kiss1* most probably was lost in this species. Additional studies including synteny analysis of *KISS1* and cognate neighboring genes such as *golt1a* identified in other species (van Aerle et al., 2008; Kanda et al., 2008; Felip et al., 2009; Um et al., 2010), or searching for possible pseudogenes for *Kiss1*, would ultimately confirm the loss of *Kiss1* in this species. The absence of some genes of the kisspeptin system has been in fact observed in other species: stickleback (*Gasterosteus aculeatus*), sockeye salmon (*Oncorhynchus nerka*), orange-spotted grouper and grass puffer. Regarding the receptor, it should be noted that in the Senegalese sole only one paralogous gene, *Ss Kiss2r*, was found (Mechaly et al., 2009). The presence of only one gene for the ligand and the receptor was also recently reported in an another teleost fish, the orange-spotted grouper (Shi et al., 2010). However, it is still premature to conclude that the loss of a variant of the ligand implies a loss of a variant of the receptor or vice versa;

we simply point out these two situations. In any case, the loss of the second paralogous gene in these species is probably a consequence of the genome reduction that occurred during evolution (Smith and Gregory, 2009) in the absence of stable adaptive advantages due to subfunctionalization of the two genes.

The results of our phylogenetic analyses (Fig. 4B and C) supports the overall classification of kisspeptin into *Kiss1* and *Kiss2* as previously proposed by Kitahashi et al. (2009), and are also in accordance with the classification of kisspeptins into the YY, YF and FF forms, with a total correspondence of the FF form with *Kiss2*. Our analysis also reveals two *Kiss* gene duplication events, the first occurring early in vertebrate evolution, giving rise to *Kiss1* and *Kiss2*, and before the appearance of jawed fish since both genes are already found in sea lampreys. However, our interpretation of the second duplication is contrary to that of Um et al. (2010), which involved only *Kiss1* and gave rise to the *Kiss1A* and *Kiss1B* ortholog genes. Um et al. (2010) considered that this duplication occurred prior to the appearance of teleosts. Our analysis, in contrast, indicates that these paralogous genes are never found in teleosts. Thus, the second duplication most likely occurred coinciding with the colonization of land, appearing either already in the Sarcopterygians (lungfishes and relatives) or later with the rise of the amphibians, as is evidenced in the clawed frog. Since *Kiss1b* has never been found in reptiles, birds or mammals, we opted to place this duplication in the amphibians as the most plausible possibility (Fig. 4C). However, future studies with more Sarcopterygian and amphibian species sequences should clarify the precise timeline of the appearance of the second gene duplication. Thereafter, *Kiss2* invariably was lost in mammals (Um et al., 2010), with only one exception: a monotreme, Platypus (*Ornithorhynchus anatinus*) (Lee et al., 2009). In fish, the opposite occurred, since while

some species retained both genes (Kanda et al., 2008; Felip et al., 2009; Kitahashi et al., 2009; Um et al., 2010; Akasome et al., 2010), others apparently lost *Kiss1*, but never *Kiss2*, as observed in the present study.

Sequence comparison of the *Ss Kiss2* with other kisspeptin orthologs indicated that this prepropeptide contains a 19 aa signal peptide, followed by a 78 aa (Kp), which is longer than the corresponding 54 aa peptide described in humans (Lee et al., 1996). Proteolytic processing of the full-length 145 aa of the kisspeptin protein in humans results in shorter peptides, with 54 (Kp54; previously known as metastin), 14 (Kp14), 13 (Kp13) or 10 (Kp10) aas (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 was observed in mammals, a sequence analysis of the *Ss Kiss2* showed a lack of the cleavage sites that would yield shorter peptides. Nonetheless, the possibility of producing shorter peptides cannot be completely disregarded since they can be generated by degradation (Popa et al., 2008). The last 10 aa of Kp78 corresponding to Kp10 are followed by three aa (GKR) that correspond to a putative site of terminal cleave 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) (Fig. 1B). Interestingly, the last 29 aa resulting from the cleavage of *Ss Kiss2* indicate a kisspeptin-associated peptide. Although a kisspeptin-associated peptide has never been explicitly described for kisspeptin and its function would have to be confirmed, it is important to recall a similar situation with GnRH, where the existence of a GnRH-associated peptide (GAP) is required for proper processing of the precursor and production of the mature GnRH peptide (de Roux, 2006).

415

416 Two splice variants of the *Kiss2* ligand were observed in this study and named Ss  
417 *Kiss2\_v1* and Ss *Kiss2\_v2* for the shorter and longer isoform, respectively. Ss *Kiss2\_v2*  
418 retained the 36 bp of the intron. Sequence analysis of Ss *Kiss2\_v2* revealed the presence of  
419 several features characteristic of an alternative splicing mechanism (Fig. 3C). The deduced aa  
420 sequence of the intron shows 10 residues with two PTC. Thus, *Kiss2\_v2* most likely leads to  
421 the generation of a putative truncated product that lacks the Kp10 region, which would result  
422 in a completely non-functional peptide. The generation of mRNA variants containing PTCs is  
423 viewed as a characteristic result of alternative splicing (Ben-Dov, 2008). Furthermore, the  
424 occurrence of alternative splicing is expected to be higher in species with compact genomes,  
425 as in the Senegalese sole (Smith and Gregory, 2009) as compared to the lowest frequency  
426 observed in species (*e.g.*, zebrafish) in which duplication of the genomes occurred (Lu et al.,  
427 2010). At present, the biological significance of Ss *Kiss2\_v2* is not known, but based on the  
428 fact that the active peptide Kp10 is localized in exon 2 in the Senegalese sole as well as in the  
429 other species examined so far (Oakley et al., 2010), the retention of intron 1 could serve as a  
430 way to control the relative expression amounts of the functional Ss *Kiss2* isoform. A similar  
431 situation was found regarding the receptor in the Senegalese sole. In this case, intron 3 could  
432 be retained due to an alternative splicing mechanism to form truncated and non-truncated  
433 isoforms coded by the same gene (Mechaly et al., 2009). However, in the receptor, a  
434 hypothetical non-functional protein could be generated due the presence of two PTCs in  
435 intron 3, located in the middle of transmembrane domain 4. Future studies at the protein level  
436 are required to analyze the physiological significance of the two *Kiss2* isoforms. Alternative  
437 splicing is emerging as an important mechanism to control gene expression (Izquierdo and  
438 Valcárcel, 2006). Thus, it is surprising that splice variants of the *Kiss* genes have been

detected so far only in Pleuronectiformes (Mechaly et al., 2010), but not in any other vertebrate species. Therefore, and to the best of our knowledge, the Senegalese sole is the only species in which the machinery necessary for the occurrence of two splice variants in both the *Kiss* ligand and *Kiss1* receptor genes has been identified. This observation, together with the fact that in this species we detected only one paralogous gene for both the ligand and the receptor provides an interesting opportunity for the study of gene regulation in the kisspeptin system.

Although information is still scarce and more research is needed to determine the distribution of kisspeptin in different areas of the fish brain, studies in medaka indicate high expression in two specific hypothalamic nuclei: the nucleus posterior periventricularis (NPPv) and the nucleus ventral tuberis (NVT) (Kanda et al., 2008). In the present study, using whole brains but focusing on changes in expression according to sex and maturity stage, overall higher expression levels for both isoforms was detected in mature females. On the other hand, in the gonads, significantly higher mRNA levels of both isoforms were observed in the ovaries of pubertal females. This is in accordance with the observation that *Kiss2* is abundantly expressed in fish ovaries (Felip et al., 2009; Kitahashi et al., 2009) and suggests a role for kisspeptin in ovarian development. Thus, in the Senegalese sole, the results conform what one would expect, *i.e.*, higher levels of the mRNA in pubertal as compared to mature fish, but particularly in the ovaries rather than in the brain, where changes in expression levels were less evident.

The reproductive axis in animals is inhibited by negative energy balance produced, among other factors, by fasting (Popa et al., 2008). Studies relating energy balance and

463 reproduction via kisspeptin signaling are limited. In the mouse and rhesus macaque, fasting  
464 decreased mRNA levels of both kisspeptin and its receptor (Luque et al., 2007; Wahab et al.,  
465 2008), while it increased hypothalamic mRNA levels of *Kissr*, possibly through the apparent  
466 increase in sensibility to *Kissr* in starved animals (Castellano et al., 2005). There is no  
467 previous work regarding energy balance and the kisspeptin signaling in non-mammalian  
468 models. In this context, it is worth noting that key components of the system controlling  
469 energy balance (Volkoff et al., 2005) may have opposite effects in fish and mammals. Thus,  
470 for example, ghrelin appears to have a stimulatory role in the regulation of non-mammalian,  
471 but not mammalian, reproduction (Unniappan, 2010). In recent years, an increasing number  
472 of mammalian appetite-regulating peptide homologs have been characterized in fish  
473 (Volkoff et al., 2005), suggesting that, at least, the major components involved in the  
474 regulation of food intake have been relatively well conserved through vertebrate evolution.  
475 Fish occupy a wide range of habitats, which pose different environmental challenges and may  
476 have led to different adaptive feeding strategies (Volkoff et al., 2009). Thus, there is the  
477 opportunity for variation across fish species and while it is true that fasting can suppress  
478 reproduction in a manner similar to what has been described in mammals, it is also possible  
479 that in other species the reverse may apply. The Senegalese sole alternates periods of normal  
480 feeding and fasting during the annual cycle in nature, as already observed for many fish  
481 species (Costas et al., 2010). This would support our observations, which, in contrast to what  
482 has been observed in the mouse and rhesus macaque, show that fasting enhanced the  
483 expression of *Kiss2* and its receptor, along with *LH* and *FSH* expression. An effect of fasting  
484 on the reproductive axis has been found in sea bass, where a stimulatory effect of  
485 neuropeptide Y (NPY) on *LH* secretion was observed only in fasted animals (Cerdá-Reverter  
486 et al., 1999). Thus, in fasted Senegalese sole the increase in pituitary *LH* and *FSH* mRNA

levels most likely correlates with increased brain *Kiss2* expression. Many fish exhibit periods of sustained negative energy balance due to reduced or suppressed food intake just prior to reproduction as a normal part of their physiology (Milla et al., 2009). Our results indicate the possible existence of a link between nutritional status and reproduction in which hypothalamic kisspeptin and hypophysary gonadotropin participate. Therefore, it would be interesting in future studies to examine changes in kisspeptin genes together with changes in other genes known to be involved in feeding and energy balance, e.g., ghrelin, NPY, leptin, insulin, pro-opiomelanocortin (POMC), corticotropin-releasing hormone (CRH), among others. Furthermore, it is well established that in fish an increase in plasma cortisol levels was observed during the pre-spawning or spawning period (Milla et al., 2009). A similar situation was observed in the Senegalese sole after a prolonged period of feed deprivation (21 days), where an increase in plasma cortisol levels was observed (Costas et al., 2010). For that reason, the metabolic stress suffered at 15-days of food deprivation in our study could stimulate the HPG axis in a similar way. However, it should be mentioned that these are preliminary results that need further confirmation regarding whether fasting could inhibit the HPG axis and indirectly decrease the levels of sex steroids, producing a secondary increase in *Kiss2* mRNA expression levels, similar to the situation described in male rats after gonadectomy (Arai et al., 2008). Hence, it is tempting to speculate that kisspeptin signaling may participate in the integration of nutritional information within the reproductive axis, hence contributing to the control of reproduction in fish in response to changes in environmental conditions.

In conclusion, we have found in the Senegalese sole the *Kiss2* gene, but not the *Kiss1* gene, characterized its structure and defined the existence of a kisspeptin-associated peptide.



We have also analyzed all the available kisspeptin sequences in vertebrates and updated the phylogenetic tree, with new information regarding the evolution of the different kisspeptin genes. We found the novel existence of two isoforms of *Kiss2* and determined that they are more expressed in the female mature brain and in the ovaries of pubertal females. Finally, we present the first evidence of a relationship between food intake and kisspeptin signaling in a non-mammalian vertebrate, suggesting conserved involvement of kisspeptin in mediating energy balance effects on the reproductive axis during evolution.

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## References

- Akazome, Y., Kanda, S., Okubo, K., Oka, Y., 2010. Functional and evolutionary insights into vertebrate kisspeptin systems from studies of fish brain. *J. Fish. Biol.* 76, 161–182.
- Aparicio, S., 2005. Kisspeptins and GPR54 – the new biology of the mammalian GnRH axis. *Cell. Metab.* 1, 293–296.
- Arai, A.C., Orwig, N., 2008. Factors that regulate *Kiss1* gene expression in the hippocampus. *Brain Res.* 1243, 10–18.
- Ben-Dov, C., Hartmann, B., Lundgren, J., Valcárcel, J.A., 2008. Genome-wide analysis of alternative Pre-mRNA splicing. *J. Biol. Chem.* 283, 1229–1233.

- 537 Biran, J., Ben-Dor, S., Levavi-Sivan, B., 2008. Molecular identification and functional  
538 characterization of the kisspeptin/kisspeptin receptor system in lower vertebrates.  
539 Biol. Reprod. 79, 776-786.
- 540 Bowe, J.E., King, A.J., Kinsey-Jones, J.S., Foot, V.L., Li, X.F., O'Byrne, K.T., Persaud, S.J.,  
541 Jones, P.M., 2009. Kisspeptin stimulation of insulin secretion: mechanisms of action  
542 in mouse islets and rats. Diabetologia 52, 855-862.
- 543 Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription  
544 polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169-193.
- 545 Cardon, M., Ron-Harel, N., Cohen, H., Lewitus, G.M., Schwartz, M., 2009. Dysregulation of  
546 kisspeptin and neurogenesis at adolescence link inborn immune deficits to the late  
547 onset of abnormal sensorimotor gating in congenital psychological disorders. Mol.  
548 Psychiatry 15, 415-425.
- 549 Carrillo, M., Zanuy, S., Felip, A., Bayarri, M.J., Moles, G., Gomez, A., 2009. Hormonal and  
550 environmental control of puberty in perciform fish: the case of sea bass. Ann. NY.  
551 Acad. Sci. 1163, 49-59.
- 552 Castellano, J.M., Navarro, V.M., Fernández-Fernández, R., Nogueiras, R., Tovar, S., Roa, J.,  
553 Vazquez, M.J., Vigo, E., Casanueva, F.F., Aguilar, E., Pinilla, L., Dieguez, C., Tena-  
554 Sempere, M., 2005. Changes in hypothalamic KiSS-1 system and restoration of  
555 pubertal activation of the reproductive axis by kisspeptin in undernutrition.  
556 Endocrinology 146, 3917-3925.
- 557 Cerdà, J., Chauvigné, F., Agulleiro, M.J., Marin, E., Halm, S., Martínez-Rodríguez, G., Prat,  
558 F., 2008. Molecular cloning of Senegalese sole (*Solea senegalensis*) follicle-  
559 stimulating hormone and luteinizing hormone subunits and expression pattern during  
560 spermatogenesis. Gen. Comp. Endocrinol. 156, 470-481.
- 561 Cerdà-Reverter, J.M., Sorbera, L.A., Carrillo, M., Zanuy, S., 1999. Energetic dependence of  
562 NPY-induced LH secretion in a teleost fish (*Dicentrarchus labrax*). Am. J. Physiol.  
563 227, 1627-1634.
- 564 Costas, B., Aragão, C., Ruiz-Jarabo, I., Vargas-Chacoff, L., Arjona, F.J., Dinis, M.T.,  
565 Mancera, J.M., Conceição, L.E., 2010. Feed deprivation in Senegalese sole (*Solea*

- 566 *senegalensis* Kaup, 1858) juveniles: effects on blood plasma metabolites and free  
567 amino acid levels. *Fish Physiol. Biochem.* DOI 10.1007/s10695-010-9451-2.
- 568 de Roux, N., 2006. GnRH receptor and GPR54 inactivation in isolated gonadotropic  
569 deficiency Horm. Res. Syn. 20, 515–528.
- 570 Elizur, A., 2009. The KiSS1/GPR54 system in fish. *Peptides* 30, 164–170.
- 571 Felip, A., Zanuy, S., Pineda, R., Pinilla, L., Carrillo, M., Tena-Sempere, M., Gómez, A.,  
572 2009. Evidence for two distinct *KiSS* genes in non-placental vertebrates that encode  
573 kisspeptins with different gonadotropin-releasing activities in fish and mammals. *Mol.*  
574 *Cell. Endocrinol.* 312, 61–71.
- 575 Fernández-Fernández, R., Martini, A.C., Navarro, V.M., Castellano, J.M., Dieguez, C.,  
576 Aguilar, E., Pinilla, L., Tena-Sempere, M., 2006. Novel signals for the integration of  
577 energy balance and reproduction. *Mol. Cell. Endocrinol.* 254–255, 127–132.
- 578 Funes, S., Hedrick, J.A., Vassileva, G., Markowitz, L., Abbondanzo, S., Golovko, A., Yang,  
579 S.J., Monsma, F.J., Gustafson, E.L., 2003. The KiSS-1 receptor GPR54 is essential  
580 for the development of the murine reproductive system. *Biochem. Biophys. Res.*  
581 *Commun.* 312, 1357–1363.
- 582 Gaytan, M., Castellano, J.M., Roa, J., Sanchez-Criado, J.E., Tena-Sempere, M., Gaytan, F.,  
583 2007. Expression of KiSS-1 in rat oviduct: possible involvement in prevention of  
584 ectopic implantation?. *Cell. Tissue. Res.* 329, 571–579.
- 585 Gottsch, M.L., Cunningham, M.J., Smith, J.T., Popa, S.M., Acohido, B.V., Crowley, W.F.,  
586 Seminara, S., Clifton, D.K., Steiner, R.A., 2004. A role for kisspeptins in the  
587 regulation of gonadotropin secretion in the mouse. *Endocrinology* 145, 4073–4077.
- 588 Hall, T., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis  
589 program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41, 95–98.
- 590 Hiden, U., Bilban, M., Knöfler, M., Desoye, G., 2007. Kisspeptins and the placenta:  
591 regulation of trophoblast invasion. *Rev. Endocr. Metab. Disord.* 8, 31–39.
- 592 Izquierdo, J.M., Valcárcel, J.A., 2006. Simple principle to explain the evolution of pre-  
593 mRNA splicing. *Genes Dev.* 20, 1679–1684.

- 594 Kanda, S., Akazome, Y., Matsunaga, T., Yamamoto, N., Yamada, S., Tsukamura, H., Maeda,  
595 K., Oka, Y., 2008. Identification of KiSS-1 product kisspeptin and steroid-sensitive  
596 sexually dimorphic kisspeptin neurons in medaka (*Oryzias latipes*). *Endocrinology*  
597 149, 2467–76.
- 598 Kimura, M.A., 1980. Simple model for estimating evolutionary rates of base substitutions  
599 through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16,111–120.
- 600 Kitahashi, T., Ogawa, S., Parhar, I.S., 2009. Cloning and expression of kiss2 in the zebrafish  
601 and medaka. *Endocrinology* 150, 821–31.
- 602 Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwinden, J.M., Le  
603 Poul, E., Brezillon, S., Tyldesley, R., Suarez-Huerta, N., Vandeput, F., Blanpain, C.,  
604 Schiffmann, S.N., Vassart, G., Parmentier, M., 2001. The metastasis suppressor gene  
605 KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled  
606 receptor GPR54. *J. Biol. Chem.* 276, 34631–34636.
- 607 Lee, J.H., Miele, M.E., Hicks, D.J., Phillips, K.K., Trent, J.M., Weissman, B.E., Welch, D.R.,  
608 1996. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *J.*  
609 *Natl. Cancer Inst.* 88, 1731–1737.
- 610 Lee, Y.R., Tsunekawa, K., Moon, M.J., Um, H.N., Hwang, J.I., Osugi, T., Otaki, N.,  
611 Sunakawa, Y., Kim, K., Vaudry, H., Kwon, H.B., Seong, J.Y., Tsutsui, K., 2009.  
612 Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in  
613 vertebrates. *Endocrinology* 150, 2837–2846.
- 614 Li, S., Zhang, Y., Liu, Y., Huang, X., Huang, W., Lu, D., Zhu, P., Shi, Y., Cheng, C.H., Liu,  
615 X., Lin, H., 2009. Structural and functional multiplicity of the kisspeptin/GPR54  
616 system in goldfish (*Carassius auratus*). *J. Endocrinol.* 201, 407–18.
- 617 Lu, J.G., Peatman, E., Wang, W.Q., Yang, Q., Abernathy, J., Wang, S.L., Kucuktas, H., Liu,  
618 Z.J., 2010. Alternative splicing in teleost fish genomes: same-species and cross-  
619 species analysis and comparisons. *Mol. Genet. Genomics* 283, 531–539.
- 620 Luque, R.M., Kineman, D., Tena-Sempere, M., 2007. Regulation of hypothalamic expression  
621 of *KiSS-1* and *GPR54* genes by metabolic factors: Analysis using mouse models and a  
622 cell line. *Endocrinology* 148, 4601–4611.

- 623 Makri, A., Pissimissis, N., Lembessis, P., Polychronakos, C., Koutsilieris, M., 2008. The  
624 kisspeptin (KiSS1-1)/Gpr54 system in cancer biology. *Cancer Treat. Rev.* 34, 682–  
625 692.
- 626 Matsui, H., Takatsu, Y., Kumano, S., Matsumoto, H., Ohtaki, T., 2004. Peripheral  
627 administration of metastin induces marked gonadotropin release and ovulation in the  
628 rat. *Biochem. Biophys. Res. Comm.* 320, 383–388.
- 629 Mead, E.J., Maguire, J.J., Kuc, R.E., Davenport, A.P., 2007. Kisspeptins are novel potent  
630 vasoconstrictors in humans, with a discrete localization of their receptor, G protein-  
631 coupled receptor 54, to atherosclerosis-prone vessels. *Endocrinology* 148, 140–147.
- 632 Mechaly, A.S., Viñas, J., Piferrer, F., 2009. Identification of two isoforms of the kisspeptin-1  
633 receptor (*kiss1r*) generated by alternative splicing in a modern teleost, the Senegalese  
634 Sole (*Solea senegalensis*). *Biol. Reprod.* 80, 60–69.
- 635 Mechaly, A.S., Viñas, J., Murphy, C., Reith, M., Piferrer, F., 2010. Gene structure and  
636 regulation of the Kiss-1 receptor-2 (*Kiss1r-2*) in the Atlantic halibut: Insights into the  
637 evolution and regulation of *Kiss1r* genes. *Mol. Cell. Endocrinol.* 317, 78–89.
- 638 Messenger, S., Chatzidaki, E.E., Ma, D., Hendrick, A.G., Zahn, D., Dixon, J., Thresher, R.R.,  
639 Malinge, I., Lomet, D., Carlton, M.B.L., Colledge, W.H., Caraty, A., Aparicio, S.A.,  
640 2005. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G  
641 protein-coupled receptor 54. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1761–1766.
- 642 Milla, S., Wang, N., Mandiki, S.N.M., Kestemont, P., 2009. Corticosteroids: Friends or foes  
643 of teleost fish reproduction?. *Comp. Biochem. Physiol. Part A* 153, 242–251.
- 644 Mitani, Y., Kanda, S., Akazome, Y., Zempo, B., Oka, Y., 2010. Hypothalamic *Kiss1* but not  
645 *Kiss2* neurons are involved in estrogen feedback in medaka (*Oryzias latipes*).  
646 *Endocrinology* 151, 1751–1759.
- 647 Moon, J.S., Lee, Y.R., Oh, D.Y., Hwang, J.I., Lee, J.Y., Kim, J., Vaudry, H., Kwon, H.B.,  
648 Seong, Y., 2009. Molecular cloning of the bullfrog kisspeptin receptor Gpr54 with  
649 high sensitivity to *Xenopus* kisspeptin. *Peptides* 30, 171–179.
- 650 Navarro, V.M., Fernández-Fernández, R., Castellano, J.M., Roa, J., Mayen, A., Barreiro,  
651 M.L., Gaytan, F., Aguilar, E., Pinilla, L., Dieguez, C., Tena-Sempere, M., 2004.

- Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. *J. Physiol.* 561, 379–386.
- Oakley, A.E., Clifton, D.K., Steiner, R.A., 2009. Kisspeptin signaling in the brain. *Endocr. Rev.* 30, 713–743.
- Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., Ishibashi, Y., Watanabe, T., Asada, M., Yamada, T., Suenaga, M., Kitada, C., Usuki, S., Kurokawa, T., Onda, H., Nishimura, O., Fujino, M., 2001. Metastasis suppressor gene *KiSS-1* encodes peptide ligand of a G-protein-coupled receptor. *Nature* 411, 613–617.
- Parhar, I.S., Ogawa, S., Sakuma, Y., 2004. Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. *Endocrinology* 145, 3613–3618.
- Popa, S.M., Clifton, D.K., Steiner, R.A., 2008. The role of kisspeptins and GPR54 in the neuroendocrine regulation of reproduction. *Annu. Rev. Physiol.* 70, 213–238.
- Roa, J., Aguilar, E., Dieguez, C., Pinilla, L., Tena-Sempere, M., 2008. New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. *Front. Neuroendocrinol.* 29, 48–69.
- Roseweir, A.K., Millar, R.P., 2009. The role of kisspeptin in the control of gonadotrophin secretion. *Hum. Reprod. Update* 15, 203–212.
- Saitou, N., Nei, M., 1987. The Neighbor-Joining method - a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.
- Selvaraj, S., Kitano, H., Fujinaga, Y., Ohga, H., Yoneda, M., Yamaguchi, A., Shimizu, A., Matsuyama, M., 2010. Molecular characterization, tissue distribution, and mRNA expression profiles of two *Kiss* genes in the adult male and female chub mackerel (*Scomber japonicus*) during different gonadal stages. *Gen. Comp. Endocrinol.* 169, 28–38.
- Shahab, M., Mastronardi, C., Seminara, S.B., Crowley, W.F., Ojeda, S.R., Plant, T.M., 2005. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. *Proc. Natl. Acad. Sci. U.S.A.* 102, 2129–2134.

- 681 Shahjahan, M., Motohashi, E., Doi, H., Ando, H., 2010. Elevation of *Kiss2* and its receptor  
682 gene expression in the brain and pituitary of grass puffer during the spawning season.  
683 Gen. Comp. Endocrinol. 169, 48–57.
- 684 Shi, Y., Zhang, Y., Li, S., Liu, Q., Lu, D., Liu, M., Meng, Z., Cheng, C.H.K., Liu, X., Lin,  
685 H., 2010. Molecular identification of the *Kiss2/Kiss1ra* system and its potential  
686 function during 17 $\alpha$ -methyltestosterone-induced sex reversal in the orange-  
687 spotted grouper, *Epinephelus coloides*. Biol. Reprod. 83, 63–74.
- 688 Smith, J.T., Acohido, B.V., Clifton, D.K., Steiner, R.A., 2006. *KiSS-1* neurons are direct  
689 targets for leptin in the ob/ob mouse. J. Neuroendocrinol. 18, 298–303.
- 690 Smith, E.M., Gregory, T.R., 2009. Patterns of genome size diversity in the ray-finned fishes.  
691 Hydrobiologia 625, 1–25.
- 692 Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary  
693 Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol. 24, 1596–1599.
- 694 Taranger, G.L., Carrillo, M., Schulz, R.W., Fontaine, P., Zanuy, S., Felip, A., Weltzien, F.A.,  
695 Dufour, S., Karlsen, O., Norberg, B., Andersson, E., Hansen, T., 2009. Control of  
696 puberty in farmed fish. Gen. Comp. Endocrinol. 165, 483–515.
- 697 Tena-Sempere, M., 2006. GPR54 and kisspeptin in reproduction. Hum. Reprod. Update 12,  
698 631–639.
- 699 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity  
700 of progressive multiple sequence alignment through sequence weighting, position-  
701 specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.
- 702 Unniappan, S., 2010. Ghrelin: An emerging player in the regulation of reproduction in non-  
703 mammalian vertebrates. Gen. Comp. Endocrinol. 167, 340–343.
- 704 Um, H.N., Han, J.M., Hwang, J.I., Hong, S.I., Vaudry, H., Seong, J.Y., 2010. Molecular  
705 coevolution of kisspeptins and their receptors from fish to mammals. Ann. NY. Acad.  
706 Sci. 1200, 67–74.
- 707 Volkoff, H., Canosa, L.F., Unniappan, S., Cerdá-Reverter, J.M., Bernier, N.J., Kelly, S.P.,  
708 Peter, R.E., 2005. Neuropeptides and the control of food intake in fish. Gen. Comp.  
709 Endocrinol. 142, 3–19.

- Volkoff, H., Xu, M., MacDonald, E., Hoskins, L., 2009. Aspects of the hormonal regulation of appetite in fish with emphasis on goldfish, Atlantic cod and winter flounder: notes on actions and responses to nutritional, environmental and reproductive changes. *Comp. Biochem. Physiol. A* 153, 8–12.
- Wahab, F., Aziz, F., Irfan, S., Zaman, W., Shahab, M., 2008. Short-term fasting attenuates the response of the HPG axis to kisspeptin challenge in the adult male rhesus monkey (*Macaca mulatta*). *Life Sci.* 83, 633–637.
- Yang, B., Jiang, Q., Chan, T., Ko, W.K., Wong, A.O., 2010. Goldfish kisspeptin: Molecular cloning, tissue distribution of transcript expression, and stimulatory effects on prolactin, growth hormone and luteinizing hormone secretion and gene expression via direct actions at the pituitary level. *Gen. Comp. Endocrinol.* 165, 60–71.
- Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2009. Neuroendocrinology of reproduction in teleost fish. *Gen. Comp. Endocrinol.* 165, 438–455.



## Figure Legends

**Figure 1. A)** Nucleotide (top line) and deduced aa (bottom line) sequence of the Senegalese sole *Kiss2* (*Ss Kiss2*). The numbers at the end of the line indicate the nucleotide position. The DNA and aa sequence corresponding to kisspeptin-10 (Kp10) is underlined. The arrowhead indicates the location of the intron-exon boundary. The initiation codon and the proteolytic cleavage site (GKR) are indicated in bold, whereas the termination codon is indicated in bold and marked by an asterisk. **B)** Amino acid alignment of *Ss Kiss2* (HM116743) with selected fish *Kiss2* sequences: Medaka-2 (AB435386), Goldfish-2 (FJ465138), Zebrafish-2 (AB439561), Orange-spotted (O.s.) grouper-2 (ACT65993) and Sea bass-2 (ABG76790). Asterisks indicate a perfect match among all aligned sequences, whereas dots represent conservative replacements: (.) conserved; (:) highly conserved. Numbers at the end of each line refer to the aa position. The signal peptide appears underlined and the kisspeptin-10 region is boxed.

**Figure 2.** Genomic structure of the *Ss Kiss2* gene and the correspondence of the transcribed with the post-translation functional domains of the prepropeptide. UTR, untranslated region. GKR, amino acids of the proteolytic cleavage site.

**Figure 3. A)** Tissue distribution of *Ss Kiss2* of mature fish analyzed by RT-PCR. Agarose gel showing RT-PCR products obtained from brain, ovary, testis, liver, muscle, stomach, heart, intestine and kidney. *beta-actin* was included for verifying the presence of mRNAs in the sample tissues. No template control (NTC) was used as a negative control. **B)** Schematic representation of the two *Kiss2* isoforms. The *Ss Kiss2\_v2* isoform retains the intron. **C)**

Consensus sequences of the alternative splicing mechanism observed in Senegalese sole intron 1. Abbreviations: 5'ss, donor 5' splice site; CS 5'ss, consensus sequence 5' splice site; BP, branch point; PT, polypirimidine track; 3'ss, acceptor 3' splice site; CS 3'ss, consensus sequence 3' splice site. The premature termination codons (PTC) are indicated in the insert.

**Figure 4. A)** Kisspeptin-10 (Kp10) aa sequence comparison among several vertebrates. Seven Kp10 variants are identified in vertebrates and are grouped as kisspeptin 1 and 2 (separated by an horizontal solid line in the Figure). The last column depicts the kisspeptin classification as suggested by Yang et al., 2010. **B)** The phylogenetic tree comprising 38 *Kiss* sequences of vertebrates was constructed taking into account the 30 nucleotide of the Kp10 region inferred from the Neighbor-Joining method using the Kimura 2-parameters distance. The continuous line encloses *Kiss1* or *Kiss2* sequences, whereas the dotted lines enclose kisspeptin according the classification of Yang et al., 2010. Accession numbers for the sequences: Human (*Homo sapiens*) (NM\_002256), sheep (*Ovis aries*) (DQ059506), Rhesus macaque (*Macaca mulatta*) (XM\_001098284), mouse (*Mus musculus*) (NM\_178260), rat (*Rattus norvegicus*) (NM\_181692), pig (*Sus scrofa*) (AB466320), Siberian hamsters (*Phodopus sungus*) (EU365872), goat (*Capra hircus*) (AB433789), cattle (*Bos Taurus*) (XM\_867473), chimpanzee (*Pan troglodytes*) (XM\_514123) opossum (*Monodelphis domestica*) (BK006296), platypus (platypus-1, AAPN01239752, 1688-1735; platypus-2, AAPN01043280, -5154), African clawed frog (*Xenopus laevis*) (African clawed frog-1, AB455251; African clawed frog-2, AB435389) and western clawed frog (*Xenopus tropicalis*) (clawed frog-1a, EU853681; clawed frog-1b, EU853682; clawed frog-2, EU853683), grass lizard (*Takydromus sexlineatus*) (AB435432), sea lamprey (*Petromyzon marinus*) (sea lamprey-1, EB722290 and EB722291; sea lamprey-2; Ensembl Contig3941, 12551-18595)

elephant shark (*Callorhinchus milii*) (elephant shark-1, AAVX01162971, 1-922; elephant shark-2; AAVX01172388, 1-2587), medaka (medaka-1, NM\_001122921; medaka-2, NM\_001160441); zebrafish (zebrafish-1, EF690279; zebrafish-2, AB439561); goldfish (goldfish-1, FJ465137; goldfish-2, FJ465138), sea bass (sea bass-1, FJ008914; sea bass-2, FJ008915), chub mackerel (chub mackerel-1, GU731672; chub mackerel-2 GU731673), grass puffer (*Takifugu niphobles*) (AB548304), stickleback (*Gasterosteus aculeatus*) (assembly BROAD S1), sockeye salmon (*Oncorhynchus nerka*) (AB435387), orange-spotted grouper (*Epinephelus coioides*) (GQ258777) and *Solea senegalensis* (HM116743). C) *Kiss* 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.

**Figure 5.** *Ss Kiss2* expression of the two isoforms in brain **A)** and gonads **B)** in pubertal and mature Senegalese sole males and females. Letters indicate statistically significant differences ( $P < 0.05$ ) after ANOVA followed by Fisher's LSD test. Data as mean  $\pm$  S.E.M. (n = 4-6).

**Figure 6.** Effects of fasting (15 day period) in adult Senegalese sole. **A)** Decrease of percentage in body weight, **B)** Hepatosomatic index (HSI), **C)** and **D)** Gonadosomatic index (GSI) variation in males and females during the experiment. Asterisks indicate statistically significant differences after the Student's *t*-test. \*\*\*,  $P < 0,001$ . N.S., not significant. Data as mean  $\pm$  S.E.M. (n = 7-8).

**Figure 7.** Gene expression during feeding and fasting conditions. **A)** Expression levels of *Ss Kiss2* and *Ss Kiss2r* isoforms in the hypothalamus. **B)** Expression levels of *Ss FSH* and *Ss LH* in the pituitary. **C)** Expression levels of *Ss Kiss2* and *Ss Kiss2r* isoforms in the stomach.

796 Results are represented as fold change after data normalization against *beta-actin* levels

797 Asterisks indicate statistically significant differences after the Student's *t*-test. \*,  $P < 0,05$ ;

798 \*\*,  $P < 0,01$ . N.S., not significant. Data shown are expressed as mean  $\pm$  S.E.M. (n =7-8).

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**Table 1.** Homology of the kisspeptin (Kp) and kisspeptin-10 (Kp10) region of Ss *Kiss2* in comparison with human *KISS1* and zebrafish *Kiss1* and *Kiss2*.

	Kp nucleotide Homology	Kp10 nucleotide Homology	Kp aa Homology	Kp10 aa Homology
Human <i>KISS1</i>	< 20%	33%	< 20%	70%
Zebrafish <i>Kiss1</i>	< 20%	57%	< 20%	60%
Zebrafish <i>Kiss2</i>	< 20%	73%	33%	90%

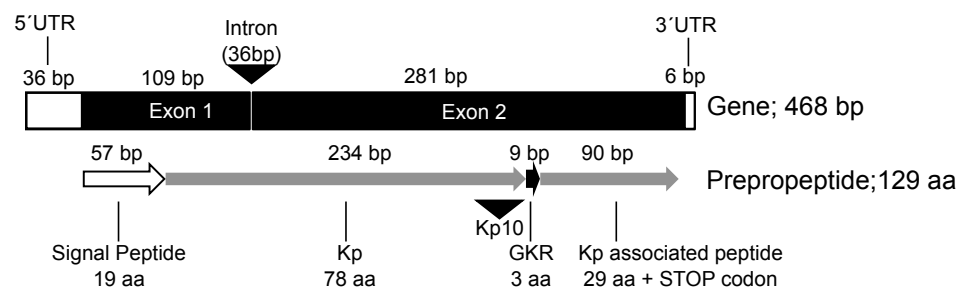
The *Kiss2* gene, but not the *Kiss1* gene, was found in the Senegalese sole, a modern flatfish with only one type of kisspeptin receptor. However, two isoforms of *Kiss2* are generated by alternative splicing in this species, with different levels of expression according to tissue and maturity status. We also present the first evidence of a relationship between food intake and kisspeptin signaling in a non-mammalian vertebrate, indicating that kisspeptin may link energy balance and reproduction.

A

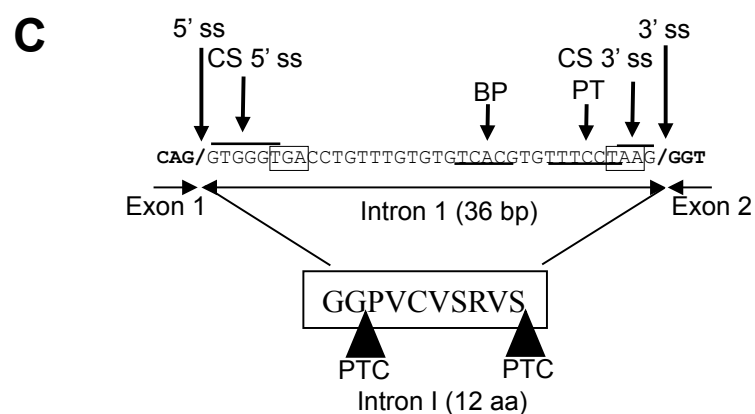
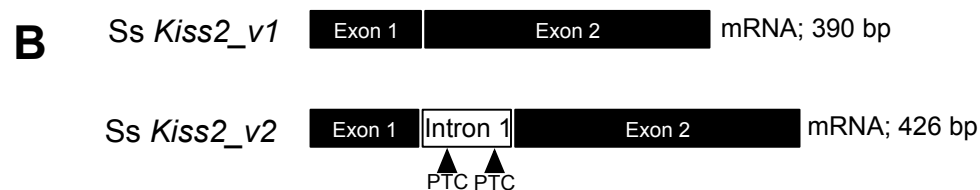
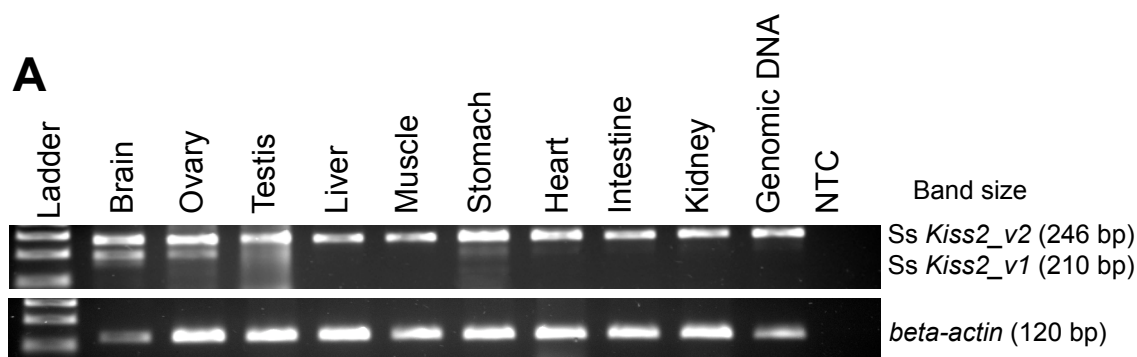
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TCA CAC AGA CGG ACG ATC TGA ATG TTG TGG TCA AAC ATG AGG CTC GTG GCT CTG GCT GTG 60
                                     M   R   L   V   A   L   A   V
GTG TGT GGC CTG ATG GTG GGT CAG GAC GGA GCG AGC GTG GGA GTG GAT CTG CCA CGA TAT 120
V   C   G   L   M   V   G   Q   D   G   A   S   V   G   V   D   L   P   R   Y
GAC ACG CAG AGG ACG CGA GCA ACA GGG TCA GAC CTC CTG TCC GCT CAC CGG AGA GGC GGC 180
D   T   Q   R   T   R   A   T   G   S   D   L   L   S   A   H   R   R   G   G
GCA GCG GCA GCG GCG GCG GCG GCG GCA GAC TTC GTG GCG GAG GAA CCG AGC CTC TGT TTC 240
A   A   A   A   A   A   A   A   A   D   F   V   A   E   E   P   S   L   C   F
TCC CTG AGA GAG AAC GAG GAG CCG CGG CAG CTG CTG TGT AAC GAC CGA CGG AGC AAG TTC 300
S   L   R   E   N   E   E   P   R   Q   L   L   C   N   D   R   R   S   K   F
AAC TTC AAC CCG TTT GGT CTC CGC TTT GGC AAA CGC TTC ACT TAC CGG AGA GCC GTG ACA 360
N   F   N   P   F   G   L   R   F   G   K   R F   T   Y   R   R   A   V   T
AGA GCG AGG ACA AAT AAG TTC TCA CCT CTG TCT CTC TTC TCG CGA GAA CTG GAG GTG CCT 420
R   A   R   T   N   K   F   S   P   L   S   L   F   S   R   E   L   E   V   P
ACC TGA GGC AGA 432
T   *
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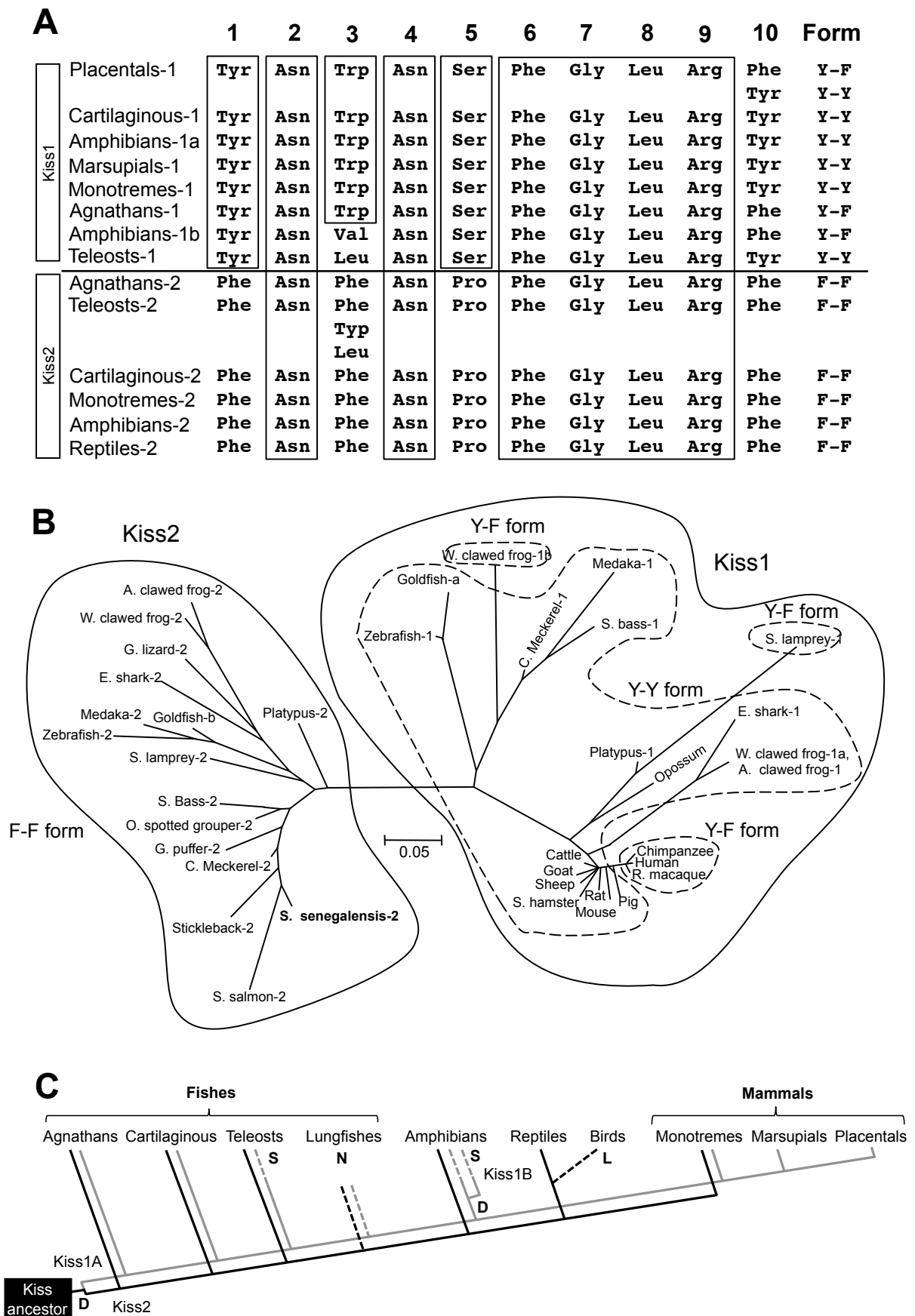
B

	<b>Signal peptide</b>	
Medaka-2	<u>MTRAVVILVLCALIAAODGGRAAAGLAARDSGRGTHATGV----</u> LWILRR-----SED 48	
Goldfish-2	<u>MKIKALILEMSAMICOS-TALRASFTDMDISDSEVPDQHYLSVERR-----</u> QFDE 52	
Zebrafish-2	<u>MNTRALILEMSAMVSOS-TAMRAILTDMDTP--EPMPDPKPRFLSMERR-----</u> QFEE 50	
O.s.grouper-2	<u>MRLVTLVVVCGGLIVGQDGDSVGAALPGFDSAQRTHATES---</u> ILSALRR-----RSTG 50	
Sea bass-2	<u>MRLVALVVVCGGLILGQDGGSVGAALPELDSAQRTGATGS---</u> LLSALRR-----RTAG 50	
Solea senegalensis-2	<u>MRLVALAVVCGLMVGODGASVGVDLPRYD-TQRTTRATGSD--</u> LLSAHRRGGAAAAAAA 57	
	* .: :. .: *. .: *	* **
	<b>Kisspeptin-10</b>	
Medaka-2	DSAAGGAGLCSSLREDDE--QLLCADR--RSKFNYNPFGLRF	GKRAP--PPRG-AHRARA 101
Goldfish-2	PSSDDASLCFFFQEKDESTHISQHRLPRGKFNYNPFGLRF	GKRNE--APT---DRPKH 107
Zebrafish-2	PSASDDASLCFFIQEKDETSQISCKHRLARSKFNYNPFGLRF	GKRNE--ATTSDSRLKH 108
O.s.grouper-2	EFVAEDTSPCLSLRENEEQRQLLCNDR--RSKFNFNPFGLRF	GKRYNGYIYRAVKTART 108
Sea bass-2	EFFGEDSSPCFSLRENEEQRQLLCNDR--RSKFNFNPFGLRF	GKRY---IYRRALKRART 105
Solea senegalensis-2	DFVAEEPSLCFSLRENEEPQQLLCNDR--RSKFNFNPFGLRF	GKRF---TYRRAVTRART 112
	. .. * :*:.* :: *.* *.***:*****	:
Orange-spotted-2	NKFSPFSLFSRELEVPS	125
Medaka-2	MKLPLMSLFQ---EVPT	115
Goldfish-2	KHLLPMMIYLRKQSETT	124
Zebrafish-2	KHLLPMMLYLRKQLETS	125
O.s.grouper-2	NKFSPFSLFSRELEVPS	125
Sea bass-2	NRFSPLFLFSRELEVPT	122
Solea senegalensis-2	NKFSPLSLFSRELEVPT	129
	:: ::: .:	

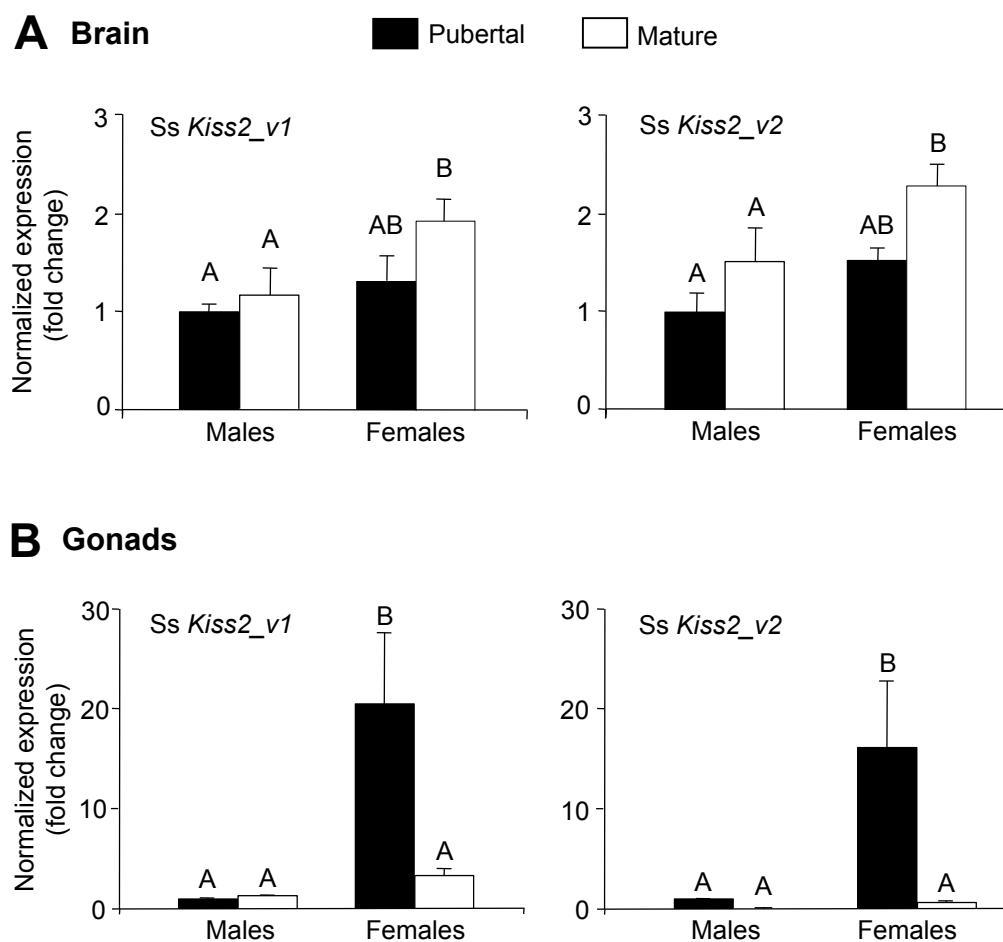


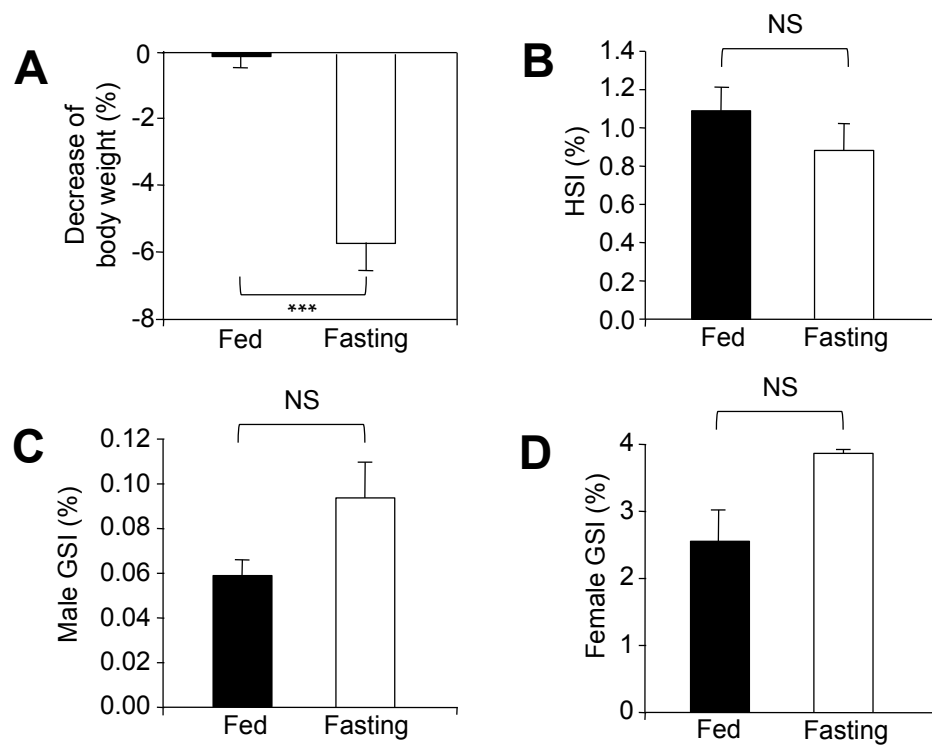


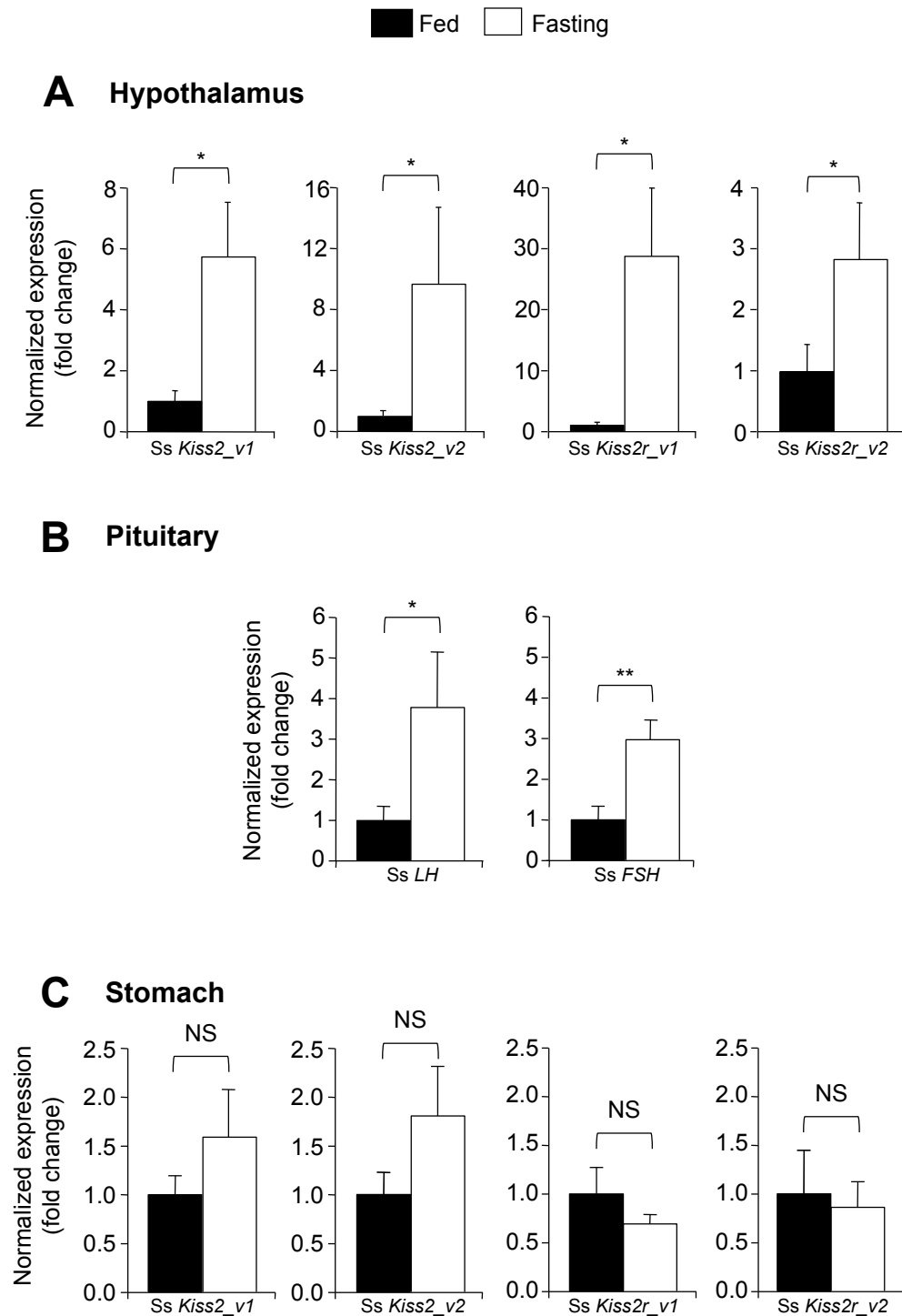




Mechaly, et al., 2010. Figure 4







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