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Alejandro S. Mechaly, Jordi Viñas, Francesc Piferrer. Gene structure analysis of kisspeptin-2 () in the Senegalese sole (): Characterization of two splice variants of , and novel evidence for metabolic regulation of kisspeptin signaling in non-mammalian species. *Molecular and Cellular Endocrinology*, 2011, 10.1016/j.mce.2011.03.004 . hal-00708532

HAL Id: hal-00708532

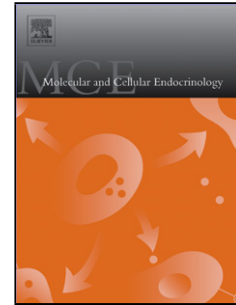
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Accepted Manuscript



Title: 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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PII: S0303-7207(11)00163-8
DOI: doi:10.1016/j.mce.2011.03.004
Reference: MCE 7789

To appear in: *Molecular and Cellular Endocrinology*

Received date: 19-11-2010
Revised date: 11-3-2011
Accepted date: 13-3-2011

Please cite this article as: Mechaly, A.S., 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, *Molecular and Cellular Endocrinology* (2008), doi:10.1016/j.mce.2011.03.004

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

5
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13 **Running title:** Kisspeptin splice variants in fish and energy balance

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18
19 **Funding information:** A.S.M. was supported by a predoctoral scholarship from the Spanish Ministry
20 of Education and Science (MEC). This study was carried out with the financial help of projects
21 “Pleurogene” and “Aquagenomics” (CDS-2007-0002) to F.P.

22
23 **Disclosure statement:** The authors have nothing to disclose.

24

25 **Abstract**

26 Kisspeptin signaling in the brain is involved in the control of the onset of puberty in
27 vertebrates. In this study, we present novel evidence indicating that kisspeptin may link
28 energy balance and reproduction. For that purpose, we determined the complete gene
29 structure of kisspeptin in a teleost fish, the Senegalese sole (Ss). In contrast to the situation
30 evident in several fish, in this species only Kiss2 was found. Yet, two Ss *Kiss2* isoforms
31 generated by alternative splicing through intronic retention were detected: Ss *Kiss2_v1*,
32 producing the functional protein, and Ss *Kiss2_v2*, coding for a truncated, non-functional
33 protein. Specific qPCRs showed that the expression of these two isoforms varied differently
34 in brain and gonads throughout maturation. In addition, and in contrast to what has been
35 observed in mammals, fasting increased hypothalamic mRNA levels of Ss *Kiss2_v1*, which
36 also caused a concomitant rise in pituitary Ss *LH* and Ss *FSH* mRNA. Together, these data
37 indicate the impact of the nutritional status on *Kiss* mRNA expression as a potential
38 regulatory mechanism for the metabolic control of reproduction in non-mammalian species,
39 albeit with some significant differences with respect to the situation described in mammals.

40

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

42

43 1. Introduction

44

45 In vertebrates, reproduction is subject to a complex regulation within the
46 hypothalamic-pituitary-gonadal (HPG) axis (for reviews see Gottsch et al., 2004; Aparicio et
47 al., 2005; Tena-Sempere, 2006; Roa et al., 2008; Oakley et al., 2009). In the last years, it has
48 become established that the peptide kisspeptin has an important role in the control of
49 reproduction, particularly relating to the onset of puberty as evidenced mostly by studies in
50 mammals (Gottsch et al., 2004; Tena-Sempere, 2006; Roa et al., 2008; Oakley et al., 2009;
51 Roseweir and Millar, 2009) and fishes (Parhar et al., 2004; Elizur et al., 2009; Carrillo et al.,
52 2009; Zohar et al., 2009; Taranger et al., 2009). Hence, mRNA levels of kisspeptin and its
53 receptor are highest at the time of the onset of puberty in rats (*Rattus norvegicus*) and rhesus
54 macaque (*Macaca mulatta*), where an increase in expression patterns for both genes were
55 observed in juveniles entering puberty (Navarro et al., 2004; Shahab et al., 2005). Moreover,
56 exogenous administration of kisspeptin activates the secretion of follicle-stimulating hormone
57 (FSH) and luteinizing hormone (LH) in mammals (Funes et al., 2003; Matsui et al., 2004;
58 Messenger et al., 2005), amphibians (Moon et al., 2009) and teleost fishes (Felip et al., 2009;
59 Kitahashi et al., 2009).

60

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

67 originated through one or two rounds of gene or chromosome duplication and named *Kiss1r*,
68 *Kiss2rA* and *Kiss2rB* (Mechaly et al., 2010), are also observed in medaka, zebrafish and
69 goldfish (Kitahashi et al., 2009; Li et al., 2009; Biran et al., 2008). However, this situation is
70 not universal, since only one kisspeptin gene, *Kiss2*, and one receptor, *Kiss2rA*, are present in
71 the orange-spotted grouper (*Epinephelus coioides*) (Shi et al., 2010), grass puffer (*Takifugu*
72 *niphobles*) (Shahjahan et al., 2010), Senegalese sole (*Solea senegalensis*) (Mechaly et al.,
73 2009) and Atlantic halibut (*Hippoglossus hippoglossus*) (Mechaly et al., 2010), indicating
74 that the paralogous gene has been lost during evolution (Mechaly et al., 2010). Furthermore,
75 in the Senegalese sole, a splice variant isoform of the *Kiss2rA* gene was observed in the
76 transcript (Mechaly et al., 2009), and in amphibian clawed frog (*Xenopus tropicalis*), a third
77 paralogous for both the ligand (*Kiss1b*) and the receptor (*Kiss1rb*) (Lee et al., 2009) have
78 been reported. Thus, the evolution of kisspeptin and its receptor in vertebrates involves gene
79 duplications, appearance of alternative splicing variants as well as gene loss, although the
80 course of events and their relationships are still not completely clear. Therefore, a more
81 exhaustive analysis is necessary to understand the phylogenetic relationships among these
82 gene duplications and isoforms in the kisspeptin family.

83

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

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

98

99 In addition to its role in the central control of reproduction, there is also increasing
100 evidence showing that kisspeptin signaling is involved in other processes (Roa et al., 2008;
101 Roseweir and Millar, 2009). These include a potentially important autocrine and/or paracrine
102 effects in the ovary (Gaytan et al., 2007), placenta (Hiden et al., 2007), and immune system
103 (Cardon et al., 2009), as well as the participation of kisspeptin in metastasis (Makri et al.,
104 2008), vasoconstriction (Mead et al., 2007) and stimulation of insulin secretion (Bowe et al.,
105 2009). Of particular interest is the possible role of kisspeptin linking energy balance and
106 reproduction (Popa et al., 2008), since recent observations indicate that the impacts of
107 negative energy balance towards reproduction can be modulated by kisspeptins (Tena-
108 Sempere, 2006). Hence, in rats, *Kiss1* is sensitive to nutritional conditions, with a decrease in
109 expression during negative energy balance status, thus contributing to reproduction
110 suppression (Fernández-Fernández et al., 2006). Furthermore, Kiss1 neurons are direct
111 targets of leptin, suggesting that reproductive deficits associated with leptin-deficient states
112 may be attributable, in part, to diminished expression of *Kiss1* (Smith et al., 2006). However,
113 the regulation of the metabolic condition and its subsequent impact on reproductive function
114 through kisspeptin and its receptor is far from being well understood. Nothing is known about

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

117

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

124

125 **2. Experimental Procedures**

126

127 **2.1. Animals and tissue sample collection**

128 Senegalese sole were obtained from the IFAPA research center in El Puerto de Santa
129 María (Cádiz, SW Spain). Fish stocks were maintained at the Experimental Aquarium
130 Facilities of the Institute of Marine Sciences, under natural conditions of temperature and
131 photoperiod and fed two times a day with a commercial diet (Skretting, Spain). Animals were
132 treated according to the approved institutional guidelines on the use of animals for research
133 purposes and in agreement with the European regulations of animals' welfare (ETS No.
134 123,01/01/91). Fish were anesthetized with an overdose of MS-222 (Sigma-Aldrich, St.
135 Louis, MO, USA) and sacrificed by decapitation. Tissues were quickly removed under
136 RNase-free conditions, frozen in liquid nitrogen and stored at -80°C until used. Biometric

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

139

140 2.2. RNA isolation and cDNA synthesis

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

148

149 2.3. Molecular cloning of kisspeptin in the Senegalese sole

150 Degenerated PCR primers based on the polypeptide sequences YNLNSFGLRY for
151 *Kiss1* and FNL/Y/FNPFGLRF for *Kiss2* were designed to discriminate between the two
152 genes. Degenerated PCR primers were designed based on the sequence of the conserved
153 region of Kp10 of zebrafish-1, medaka-1, sea bass-1 and goldfish-1 for the *Kiss1*, and *fugu-2*,
154 *pufferfish-2*, *sea bass-2*, *goldfish-2*, *stickleback-2* for the *Kiss2* (Supplementary Table 1).
155 PCR amplifications were carried out from four Senegalese sole genomic DNA libraries
156 constructed with the Universal Genome Walker Kit (Clontech Laboratories Inc, CA, USA.).
157 Briefly, two rounds of PCR were performed: first, PCR amplification was carried out using a
158 primer (AP1) provided in the kit and degenerate for *Kiss2* (1PCR_S_Kiss2 and
159 1PCR_AS_Kiss2). Second, a nested PCR was performed by the primer combination AP2 and

160 degenerate and nested Kiss2 PCR primers, 2PCR_S_Kiss2 and 2PCR_AS_Kiss2, using a
161 1/50 dilution of the product of the primary PCR. In all cases, PCR conditions were as
162 follows: initial denaturation at 94°C for 25 s, followed by 7 cycles at 94°C for 25 s and 26°C
163 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
164 final cycle. The full-length cDNAs of *Kiss2* was obtained from a Senegalese sole brain cDNA
165 library constructed in the UNI ZAP-XR vector. PCR reactions using gene-specific primers in
166 combination with primers annealing in the UNI Zap-XR vector were performed to obtain the
167 cDNA ends.

168

169 2.4. Gene structure, sequence alignment and phylogenetic analysis

170 To assess the genomic gene structure, the coding of the gene was amplified by the
171 primer combination Kiss2_Ex1_F1 and Kiss2_Ex2_R2 using genomic DNA as template.
172 Genomic DNA was extracted using a biopsy of muscle (~50 mg of tissue) incubated
173 overnight at 55°C in 1 ml of lysis solution (10 mM Tris-HCl, pH 7.5; 25 mM EDTA, pH 8;
174 0.5% SDS; 100 mM NaCl), and 10 µl of proteinase K (10 mg/ml). DNA was cleaned by a
175 wash of equal volume of phenol/chloroform and a wash of chloroform/ isoamyl alcohol.
176 Then, 5 µl of RNase A (10 mg/ml) were added and incubated at 55°C for 30 min. Finally,
177 DNA was precipitated with 95% ethanol, washed with 70% ethanol and eluted in 50 µl of
178 sterile water.

179

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

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

186

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

193

194 2.5. Tissue expression pattern of *Ss Kiss2*

195 The tissue distribution of *Ss Kiss2* in adult animals was analyzed by RT-PCR in
196 different tissues. Total RNA and cDNA synthesis were carried out following the protocol
197 described in section 2.2. The resulting cDNA was used to amplify *Ss Kiss2* by RT-PCR using
198 the Platinum Taq DNA Polymerase (Invitrogen). The PCR cycling conditions for *Ss Kiss2*
199 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
200 extension of 5 min at 72°C. Genomic DNA contamination in cDNA was checked with a
201 primer combination encompassing the Intron I (*Kiss2_Ex1_F1* and *Kiss2_Ex2_R2*)
202 (supplementary Table 1). Amplification of the *beta-actin* gene was used as RNA quality
203 control. No template control (NTC) was included to ensure that no contamination had
204 occurred. PCR products were run on 1.5% agarose gel and subsequently sequenced to verify
205 their authenticity.

206 2.6. Expression mRNA levels of two *Ss Kiss2* isoforms (*Ss Kiss2_v1* and *Ss Kiss2_v2*) in
207 brain and gonads in two developmental stages.

208

209 The expression pattern of *Ss Kiss2_v1* and *Ss Kiss2_v2* was analyzed in whole brain
210 of pubertal and mature fish (as classified in Mechaly et al., 2009) by real-time quantitative
211 PCR (qPCR) ($n = 4 - 6$). RNA and cDNA were obtained following the protocol described
212 above. The *Ss Kiss2* primers used for qPCR were designed to amplify each *Ss Kiss2* isoform
213 (see results and Supplementary Table 2). The qPCR amplification reaction mixture contained
214 2 μ l of diluted cDNA (1:3) (freshly synthesized from 500 ng of RNA), 4 μ M of each primer,
215 and 10 μ l of Power SYBRs Green PCR Master Mix (Applied Biosystems, Foster City, CA)
216 in a final volume of 20 μ l. Thermal cycling conditions comprised 95°C for 10 min, 40 cycles
217 at 95°C for 10 s, and 60°C for 30 s. At the end of the PCR cycles, the qPCR products were
218 analyzed using a dissociation curve step to confirm that only a single PCR product was
219 amplified. NTC reactions for every primer pair were also included on each reaction plate to
220 check for external DNA contamination. The amplification efficiency (E) of each primer set/
221 target gene was assessed as $E = 10^{-1/\text{slope}}$ as determined by linear regression of a series of
222 dilutions of the input RNA. The qPCR reactions were performed with an ABI Prism 7900
223 Sequence Detection Systems (Applied Biosystems).

224

225 Data were analyzed using the comparative cycle threshold (Ct) method (Bustin, 2000)
226 (DDCt method) to calculate relative changes in gene expression. Fold change (the relative
227 quantification, RQ) was calculated from the DDCt and normalized to the reference gene *beta-*
228 *actin*. Determinations were carried out in technical triplicates for the *Ss Kiss2_v1* *Ss*

229 *Kiss2_v2* and *beta-actin* genes. The RQ values for each sample were averaged and the
230 standard error of the mean (S.E.M.) was calculated, yielding the average fold change of the
231 target gene.

232

233 2.7. Effect of fasting and feeding conditions on the expression of kisspeptin and its receptor
234 in adult Senegalese sole

235 Adult male and female Senegalese sole were randomly distributed into two 600-liter
236 tanks. The nutritional study was conducted for a period of 15 days after a 5-day period of
237 acclimation, with seawater temperature at $20 \pm 1^\circ\text{C}$. One group of fish ($n = 8$) was fed *ad*
238 *libitum* (5 times a day) and used as the control group (“Fed”). Another group ($n = 7$) was
239 maintained under food deprivation conditions (“Fasting”). The initial mean BW (g) and SL
240 (cm) were similar in both groups: Fed; $885,71 \pm 140,53$ g and $33,14 \pm 1,99$ cm; Fasting;
241 $871,43 \pm 121,85$ g $32,57 \pm 1,97$ cm with non-significant differences (BW, $P = 0.68$; SL, $P =$
242 0.59). After 15 days of differential feeding all fish were sacrificed and the hypothalamus,
243 stomach and pituitary were quickly removed and keep at -80°C under. Biometric information,
244 SL (cm), BW (g), gonad and liver weight (g) were obtained for all the fishes. The
245 gonadosomatic (GSI) and hepatosomatic (HSI) indices were calculated as the percent of the
246 corresponding organ weight with respect to the total body weight. Total RNA isolation and
247 DNA treatment and cDNA synthesis were carried out as described in section 2.2. qPCR
248 amplification reaction was performed for each cDNA sample in triplicate in 10 μl of the
249 reaction mixture containing 5 μl of 10x master mix (Express SyBr green, Invitrogen), 0.2 μl
250 of each primer (10 μM), and 2 μl of a 1/10 diluted sample from the cDNA synthesis. Reaction
251 mixtures without a template were run as controls. The cycling conditions were as: 95°C for 10

252 min, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s. The expression
253 pattern was analyzed by qPCR using the Ss *Kiss2_v1* and Ss *Kiss2_v2* and Ss *Kiss2r_v1* and
254 Ss *Kiss2r_v2* in hypothalamus and stomach, whereas the Ss *LH* and Ss *FSH* (taken from
255 Cerdà et al., 2008) were assessed in pituitary for both experimental conditions (for full list of
256 primers see Supplementary Table 2).

257

258 2. 8. Statistical analyses

259 Data are shown as mean \pm standard error of the mean (S.E.M.). All statistical analyses
260 were performed using the SPSS v16 software. The significance of pairwise comparisons of
261 the mRNA levels of pubertal and mature fishes in brain and gonads of Ss *Kiss2_v1* and Ss
262 *Kiss2_v2* were determined using a one-way ANOVA followed by Fisher's least significant
263 difference (LSD) test for both isoforms. Statistical differences between fed and fasting fish
264 were analyzed by a Student *t*-test for the biometric data as well as the mRNA levels of Ss
265 *Kiss2_v1* and Ss *Kiss2_v2*, Ss *Kiss2r_v1* and Ss *Kiss2r_v2* in the hypothalamus and stomach
266 and Ss *FSH* and Ss *LH* in the pituitary. Differences were accepted as significant when
267 $P < 0.05$.

268

269 3. Results

270 3.1. Cloning and sequence analysis of Ss *Kiss2* gene

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

274 1A and B). The sequence of *Ss Kiss2* has been deposited into GenBank with accession
275 number HM116743. Sequence analysis identified a N-terminal with 19 aa residue of the
276 signal peptide and the ten conserved aa corresponding to the Kp10 region (Fig. 1B). A high
277 level of aa sequence identity was observed only in the decapeptide region Kp10 between *Ss*
278 *Kiss2* and zebrafish *Kiss2* (90%), zebrafish *Kiss1* (60%) and human *Kiss1* (70%), while the
279 degree of nucleotide conservation was reduced to 20% in adjacent regions of the gene (Table
280 1).

281

282 3.2. Genomic structure of *Ss Kiss2* gene

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

293

294 3.3. Detection of two transcript variants in the *mRNA* of the *Kiss2* of Senegalese sole

295 Specific RT-PCR with a primer set that amplifies a region that encompasses the intron
296 generates two different products of 390 and 426 bp (Fig. 3A) named hereafter *Ss Kiss2_v1*

297 and *Ss Kiss2_v2*, respectively. The presence of genomic contamination in the RNA extraction
298 was discarded with DNase treatment of all RNAs. Furthermore, A PCR with primers
299 flanking intron 1 were designed to amplify both *Ss Kiss2* isoforms and used to evaluate the
300 expression of *Ss Kiss2* in several tissues. The *Ss Kiss2_v2* transcript was detected in all
301 tissues analyzed, whereas the *Ss Kiss2_v1* was only detected in the ovaries, testis, stomach
302 and brain, with a higher expression in this latter tissue (Fig. 3A). Negative controls with non-
303 reverse transcribed RNA samples (no-template control, NTC) did not yield positive signals.
304 Sequence analysis revealed that *Ss Kiss2_v2* retained the entire intron 1 (Fig. 3B) and
305 revealed the presence of all the features characteristic of an alternative splicing mechanism in
306 the intron 1, including a 5' splice donor (5') splice site, consensus sequence (5') splice site (CS
307 5'ss), branch point (BP), polypyrimidine track (PT), 3' splice acceptor (3') splice site, and
308 consensus sequence (3') splice site (CS 3'ss) (Fig. 3C). The putative translation to aa of the
309 *Ss Kiss2_v2* isoform revealed the presence of two premature termination codons (PTCs) in
310 the intron.

311

312 3.4. Sequence comparison and phylogenetic analysis of *Ss Kiss2* in vertebrates

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

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

324

325 3.5. Comparison of Ss *Kiss2_v1* and Ss *Kiss2_v2* expression levels during development

326 The mRNA levels of the Ss *Kiss2_v1* and Ss *Kiss2_v2* isoforms were analyzed
327 separately by a specific qPCR in Senegalese sole male and female in brain and gonads (see
328 supplementary table 1 for specific primers used in the qPCRs). In the brain, both isoforms
329 were detected in both pubertal and mature fish of both sexes, with an overall higher and
330 significant ($P < 0.05$) difference in expression in the mature females with respect to males for
331 each of the isoforms (Fig. 5A). In the gonads, the two isoforms were also detected in both
332 sexes and stages of development. However, and also contrary to the situation in the brain, the
333 differences were stage-related, with a significantly ($P < 0.001$) high expression in pubertal
334 females (Fig. 5B).

335

336 3.6. Fasting effects on key genes of the reproductive axis

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

343

344 In the hypothalamus, induced fasting increased mRNA levels of *Ss Kiss2_v1* ($P =$
345 0.016), *Ss Kiss2_v2* ($P = 0.018$), *Ss Kiss2r_v1* ($P = 0.03$) and *Ss Kiss2r_v2* ($P = 0.044$) (Fig.
346 7A). Similarly, expression levels of *Ss LH* and *Ss FSH* in the pituitary increased up to ~ 4
347 fold ($P = 0.035$ and $P = 0.007$, respectively) (Fig. 7B). Although fasting increased the levels
348 of *Ss Kiss2_v1* and *Ss Kiss2_v2*, no statistically significant changes were detected for the *Ss*
349 *Kiss2_v1* ($P = 0.362$), *Ss Kiss2_v2* ($P = 0.245$), *Ss Kiss2r_v1* ($P = 0.324$) and *Ss Kiss2r_v2*
350 ($P = 0.790$) in the stomach (Fig. 7C).

351

352 4. Discussion

353

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

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

371

372 The results of our phylogenetic analyses (Fig. 4B and C) supports the overall
373 classification of kisspeptin into *Kiss1* and *Kiss2* as previously proposed by Kitahashi et al.
374 (2009), and are also in accordance with the classification of kisspeptins into the YY, YF and
375 FF forms, with a total correspondence of the FF form with *Kiss2*. Our analysis also reveals
376 two *Kiss* gene duplication events, the first occurring early in vertebrate evolution, giving rise
377 to *Kiss1* and *Kiss2*, and before the appearance of jawed fish since both genes are already
378 found in sea lampreys. However, our interpretation of the second duplication is contrary to
379 that of Um et al. (2010), which involved only *Kiss1* and gave rise to the *Kiss1A* and *Kiss1B*
380 ortholog genes. Um et al. (2010) considered that this duplication occurred prior to the
381 appearance of teleosts. Our analysis, in contrast, indicates that these paralogous genes are
382 never found in teleosts. Thus, the second duplication most likely occurred coinciding with the
383 colonization of land, appearing either already in the Sarcopterygians (lungfishes and
384 relatives) or later with the rise of the amphibians, as is evidenced in the clawed frog. Since
385 *Kiss1b* has never been found in reptiles, birds or mammals, we opted to place this duplication
386 in the amphibians as the most plausible possibility (Fig. 4C). However, future studies with
387 more Sarcopterygian and amphibian species sequences should clarify the precise timeline of
388 the appearance of the second gene duplication. Thereafter, *Kiss2* invariably was lost in
389 mammals (Um et al., 2010), with only one exception: a monotreme, Platypus
390 (*Ornithorhynchus anatinus*) (Lee et al., 2009). In fish, the opposite occurred, since while

391 some species retained both genes (Kanda et al., 2008; Felip et al., 2009; Kitahashi et al.,
392 2009; Um et al., 2010; Akasome et al., 2010), others apparently lost *Kiss1*, but never *Kiss2*,
393 as observed in the present study.

394

395 Sequence comparison of the *Ss Kiss2* with other kisspeptin orthologs indicated that
396 this prepropeptide contains a 19 aa signal peptide, followed by a 78 aa (Kp), which is longer
397 than the corresponding 54 aa peptide described in humans (Lee et al., 1996). Proteolytic
398 processing of the full-length 145 aa of the kisspeptin protein in humans results in shorter
399 peptides, with 54 (Kp54; previously known as metastin), 14 (Kp14), 13 (Kp13) or 10 (Kp10)
400 aas (Kotani et al., 2001; Ohtaki et al., 2001). Thus, a similar structure of kisspeptins appears
401 to be conserved from fish to mammals. For instance, in goldfish, a Kp56, Kp27, Kp16, Kp13
402 and Kp10 were also observed (Yang et al., 2010). However, and similar to was observed in
403 mammals, a sequence analysis of the *Ss Kiss2* showed a lack of the cleavage sites that would
404 yield shorter peptides. Nonetheless, the possibility of producing shorter peptides cannot be
405 completely disregarded since they can be generated by degradation (Popa et al., 2008). The
406 last 10 aa of Kp78 corresponding to Kp10 are followed by three aa (GKR) that correspond to
407 a putative site of terminal cleave and amidation, which is well conserved in all mammalian
408 (Kotani et al., 2001; Ohtaki et al., 2001) and fish *Kiss* genes (Biran et al., 2008) (Fig. 1B).
409 Interestingly, the last 29 aa resulting from the cleavage of *Ss Kiss2* indicate a kisspeptin-
410 associated peptide. Although a kisspeptin-associated peptide has never been explicitly
411 described for kisspeptin and its function would have to be confirmed, it is important to recall
412 a similar situation with GnRH, where the existence of a GnRH-associated peptide (GAP) is
413 required for proper processing of the precursor and production of the mature GnRH peptide
414 (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

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

446

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

460

461 The reproductive axis in animals is inhibited by negative energy balance produced,
462 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

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

508

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

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

518

519 **Acknowledgements**

520 Thanks are due to Dr. José Pedro Cañavate (IFAPA) for kindly donating the fish used
521 in this study; Dr. A. Gómez (Institute of Aquaculture, Castellón) for helpful comments on the
522 cloning; and Dr. F. Prat (Institute of Marine Science, Andalucía) for kindly supplying the
523 brain Senegalese sole cDNA library used in this study. We thank Drs. M. Tena-Sempere and
524 J.N. Nocillado for helpful comments on the manuscript. This study was carried out with the
525 financial help of the Genoma España project “Pleurogene”, and Ministry of Science and
526 Innovation Consolider project “Aquagenomics” (CDS-2007-0002) to F.P.

527

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- 723

724 **Figure Legends**

725

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

738

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

742

743 **Figure 3. A)** Tissue distribution of *Ss Kiss2* of mature fish analyzed by RT-PCR. Agarose gel
744 showing RT-PCR products obtained from brain, ovary, testis, liver, muscle, stomach, heart,
745 intestine and kidney. *beta-actin* was included for verifying the presence of mRNAs in the
746 sample tissues. No template control (NTC) was used as a negative control. **B)** Schematic
747 representation of the two *Kiss2* isoforms. The *Ss Kiss2_v2* isoform retains the intron. **C)**

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

752

753 **Figure 4. A)** Kisspeptin-10 (Kp10) aa sequence comparison among several vertebrates.
 754 Seven Kp10 variants are identified in vertebrates and are grouped as kisspeptin 1 and 2
 755 (separated by an horizontal solid line in the Figure). The last column depicts the kisspeptin
 756 classification as suggested by Yang et al., 2010. **B)** The phylogenetic tree comprising 38 *Kiss*
 757 sequences of vertebrates was constructed taking into account the 30 nucleotide of the Kp10
 758 region inferred from the Neighbor-Joining method using the Kimura 2-parameters distance.
 759 The continuous line encloses *Kiss1* or *Kiss2* sequences, whereas the dotted lines enclose
 760 kisspeptin according the classification of Yang et al., 2010. Accession numbers for the
 761 sequences: Human (*Homo sapiens*) (NM_002256), sheep (*Ovis aries*) (DQ059506), Rhesus
 762 macaque (*Macaca mulatta*) (XM_001098284), mouse (*Mus musculus*) (NM_178260), rat
 763 (*Rattus norvegicus*) (NM_181692), pig (*Sus scrofa*) (AB466320), Siberian hamsters
 764 (*Phodopus sungurus*) (EU365872), goat (*Capra hircus*) (AB433789), cattle (*Bos Taurus*)
 765 (XM_867473), chimpanzee (*Pan troglodytes*) (XM_514123) opossum (*Monodelphis*
 766 *domestica*) (BK006296), platypus (platypus-1, AAPN01239752, 1688-1735; platypus-2,
 767 AAPN01043280, -5154), African clawed frog (*Xenopus laevis*) (African clawed frog-1,
 768 AB455251; African clawed frog-2, AB435389) and western clawed frog (*Xenopus tropicalis*)
 769 (clawed frog-1a, EU853681; clawed frog-1b, EU853682; clawed frog-2, EU853683), grass
 770 lizard (*Takydromus sexlineatus*) (AB435432), sea lamprey (*Petromyzon marinus*) (sea
 771 lamprey-1, EB722290 and EB722291; sea lamprey-2; Ensembl Contig3941, 12551-18595)

772 elephant shark (*Callorhynchus milii*) (elephant shark-1, AAVX01162971, 1-922; elephant
 773 shark-2; AAVX01172388, 1-2587), medaka (medaka-1, NM_001122921; medaka-2,
 774 NM_001160441); zebrafish (zebrafish-1, EF690279; zebrafish-2, AB439561); goldfish
 775 (goldfish-1, FJ465137; goldfish-2, FJ465138), sea bass (sea bass-1, FJ008914; sea bass-2,
 776 FJ008915), chub mackerel (chub mackerel-1, GU731672; chub mackerel-2 GU731673),
 777 grass puffer (*Takifugu niphobles*) (AB548304), stickleback (*Gasterosteus aculeatus*)
 778 (assembly BROAD S1), sockeye salmon (*Oncorhynchus nerka*) (AB435387), orange-spotted
 779 grouper (*Epinephelus coioides*) (GQ258777) and *Solea senegalensis* (HM116743). **C**) *Kiss*
 780 gene evolution in vertebrates inferred from the information available. D = gene duplication, L
 781 = gene lost, S = gene lost in some species, N = gene not searched.

782

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

786

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

792

793 **Figure 7.** Gene expression during feeding and fasting conditions. **A**) Expression levels of *Ss*
 794 *Kiss2* and *Ss Kiss2r* isoforms in the hypothalamus. **B**) Expression levels of *Ss FSH* and *Ss*
 795 *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).

799

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

802

	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%

803

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.

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A

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 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 *

B

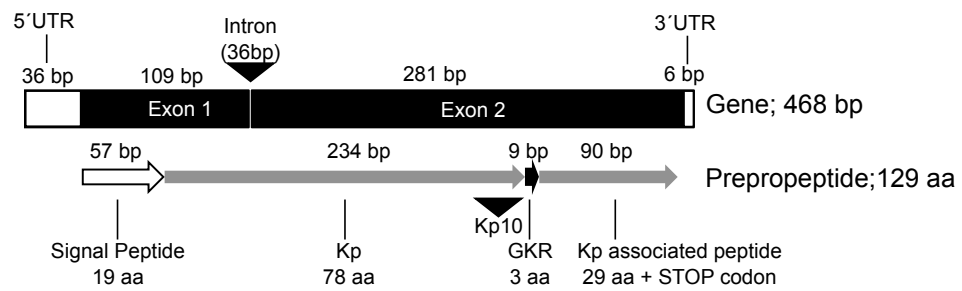
Signal peptide

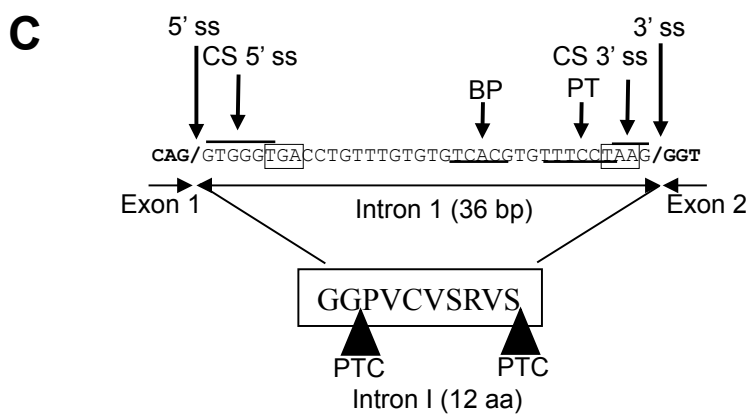
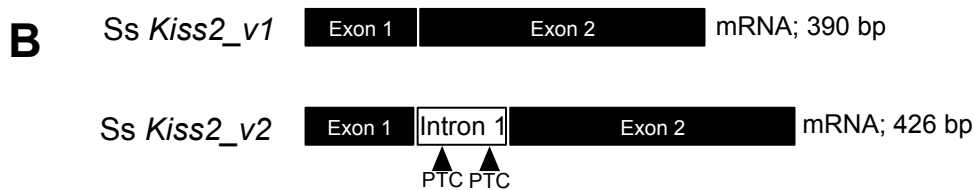
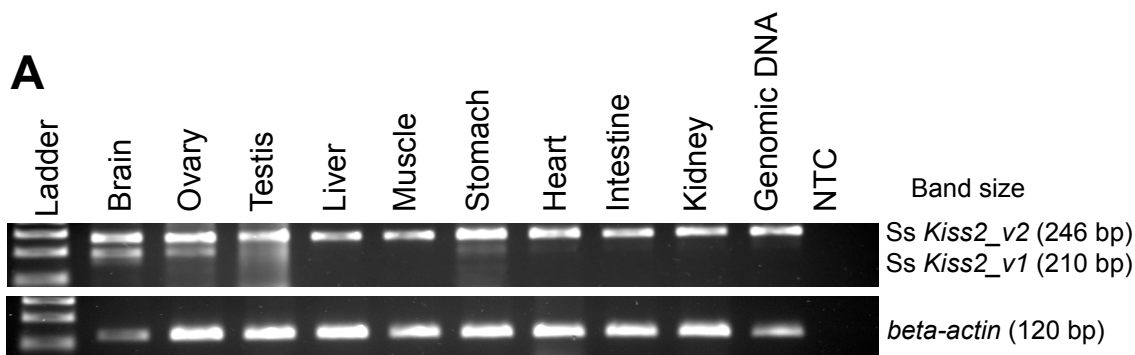
Medaka-2 MTRAVVVLVLCALIAAODGGRAAAGLAARDSGRGTHATGV---LWILRR-----SED 48
Goldfish-2 MKIKALILFMSAMICOS-TALRASFTDMDISDSEVPDQHYLSVERR-----QFDE 52
Zebrafish-2 MNTRALILFMSAMVSOS-TAMRAILTDMDTP--EPMPDPKPRFLSMERR-----QFEE 50
O.s.grouper-2 MRLVTLVVVCGLIVGQDGSVGAALPGFDSAQRTHATES---ILSALRR-----RSTG 50
Sea bass-2 MRLVALVIVCGLILGQDGGSVGAALPELDSAQRTGATGS---LLSALRR-----RTAG 50
Solea senegalensis-2 MRLVALAVVCGLMVGDGASVGVLDLPRYD-TQRTRATGSD--LLSAHRRGGAAAAA 57
* .: :. .: * . .: * . * **

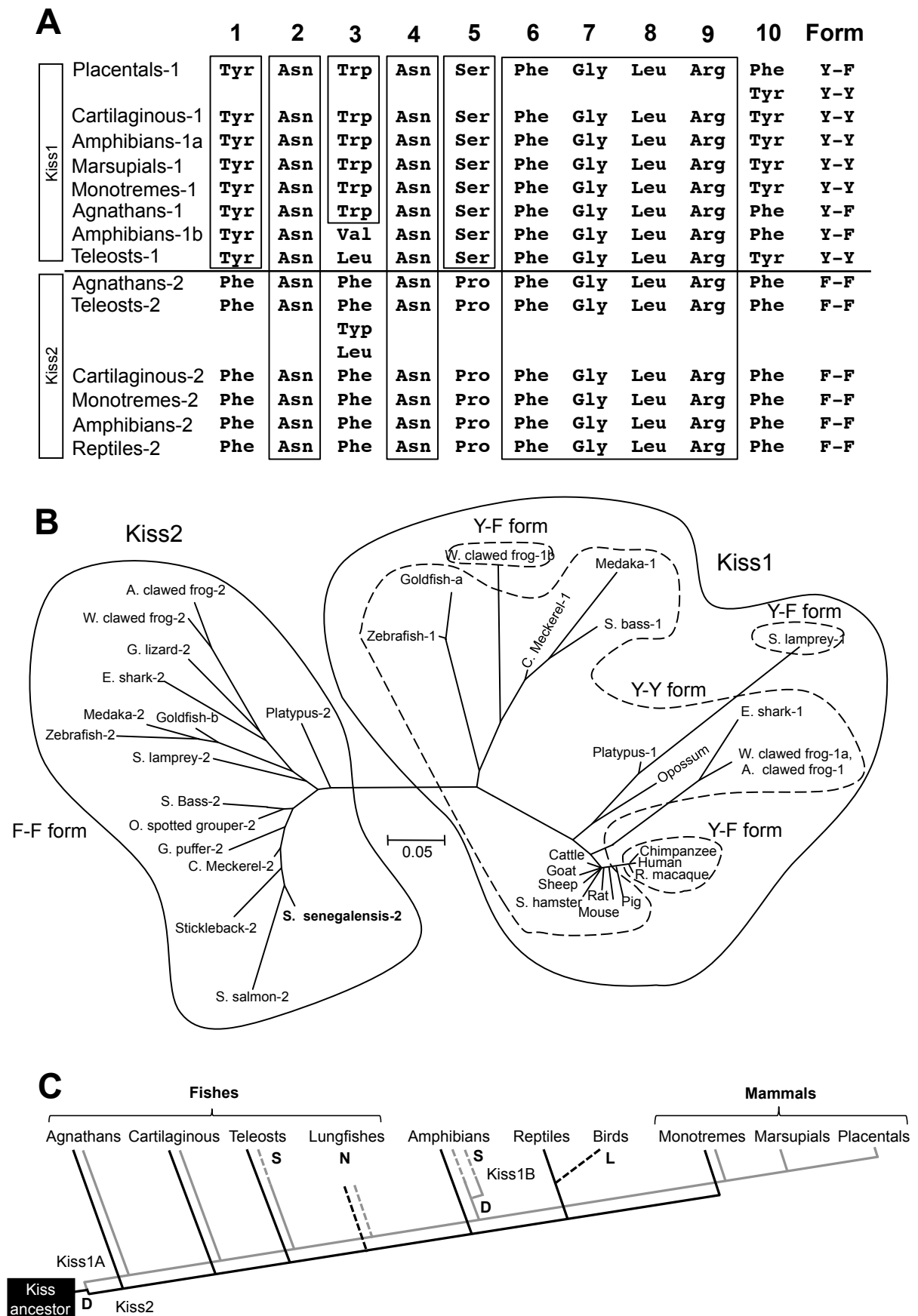
Kisspeptin-10

Medaka-2 DSAAGGAGLCSLREDDE--QLLCADR--RSKFNYNPFGLRFGKRAP--PPRG-AHRARA 101
Goldfish-2 PSSDDASLCFFFQEKDESTHISQHRLPRGKFNYNPFGLRFGKRNE--APT---DRPKH 107
Zebrafish-2 PSASDDASLCFFIQEKDETSQISCKHRLARSKFNYNPFGLRFGKRNE--ATTSDDRDLKH 108
O.s.grouper-2 EFVAEDTSPCLSLRENEEQRQLLCNDR--RSKFNFNPFGLRFGKRYNGYIYRAVKTART 108
Sea bass-2 EFFGEDSSPCFSLRENEEQRQLLCNDR--RSKFNFNPFGLRFGKRY---IYRRALKRART 105
Solea senegalensis-2 DFVAEEPSLCFSLRENEEPRQLLCNDR--RSKFNFNPFGLRFGKRF---TYRRAVTRART 112
. . . * :*:*: * : * . * * .***:*****: *

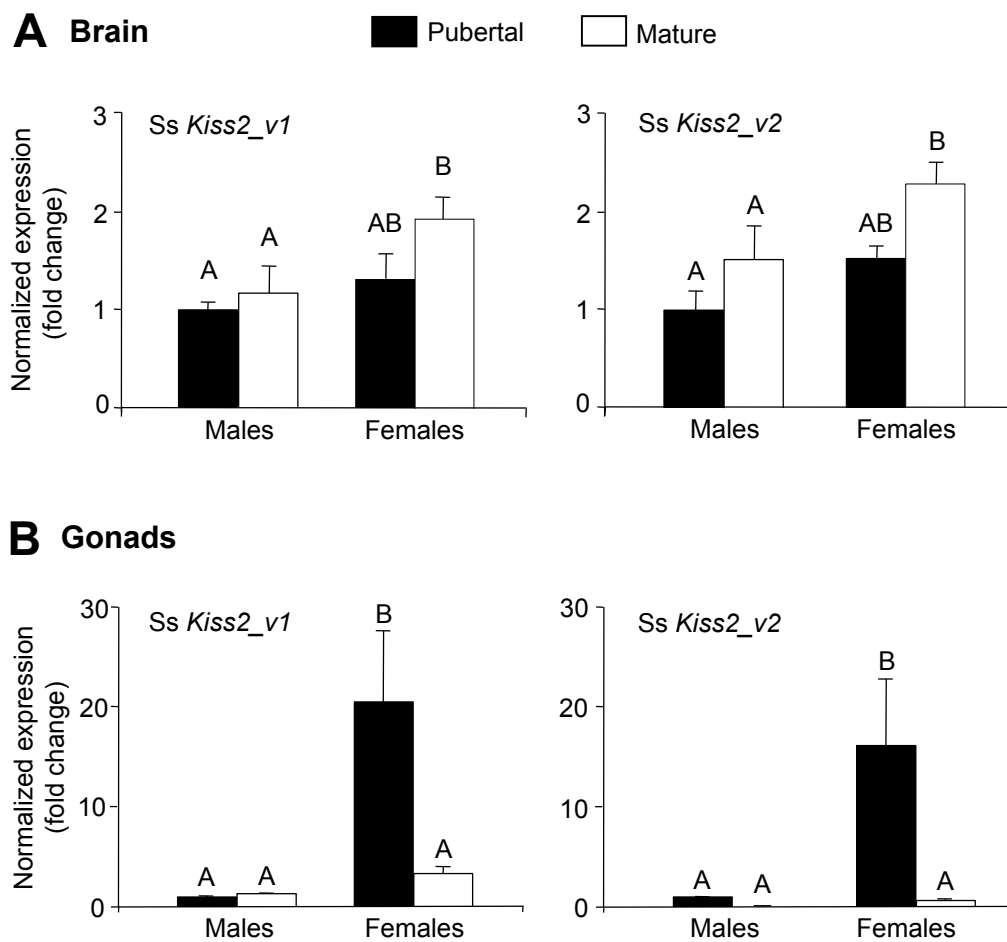
Orange-spotted-2 NKFSPFLSFSRELEVPS 125
Medaka-2 MKLPLMSLFQ---EVPT 115
Goldfish-2 KHLPLMMIYLRKQSETT 124
Zebrafish-2 KHLPLMMLYLRKQLETS 125
O.s.grouper-2 NKFSPFLSFSRELEVPS 125
Sea bass-2 NKFSPFLSFSRELEVPT 122
Solea senegalensis-2 NKFSPFLSFSRELEVPT 129
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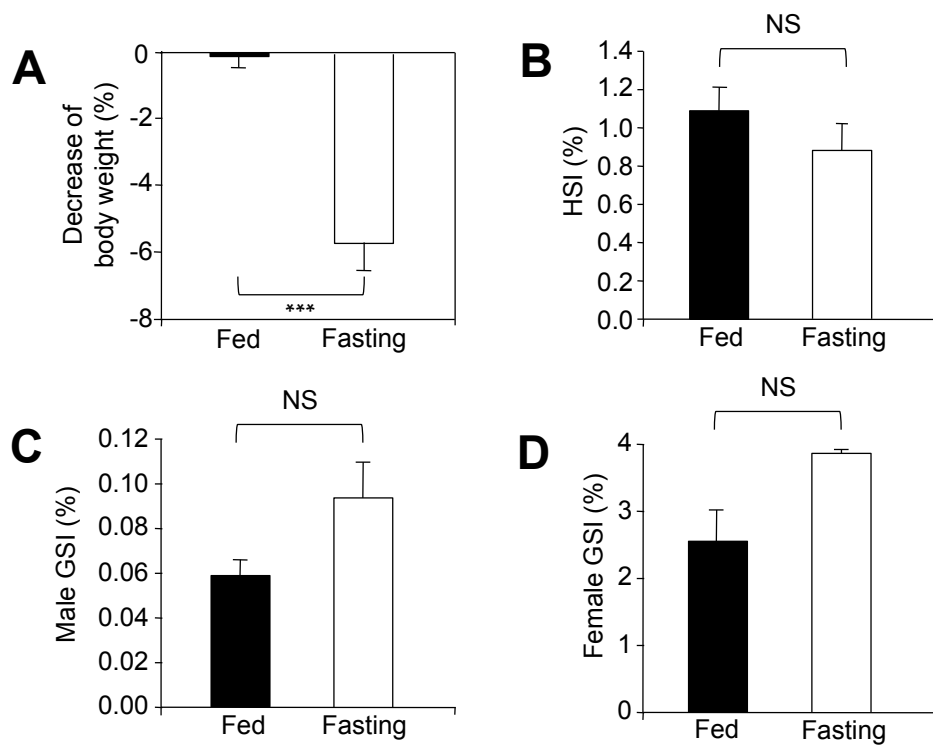


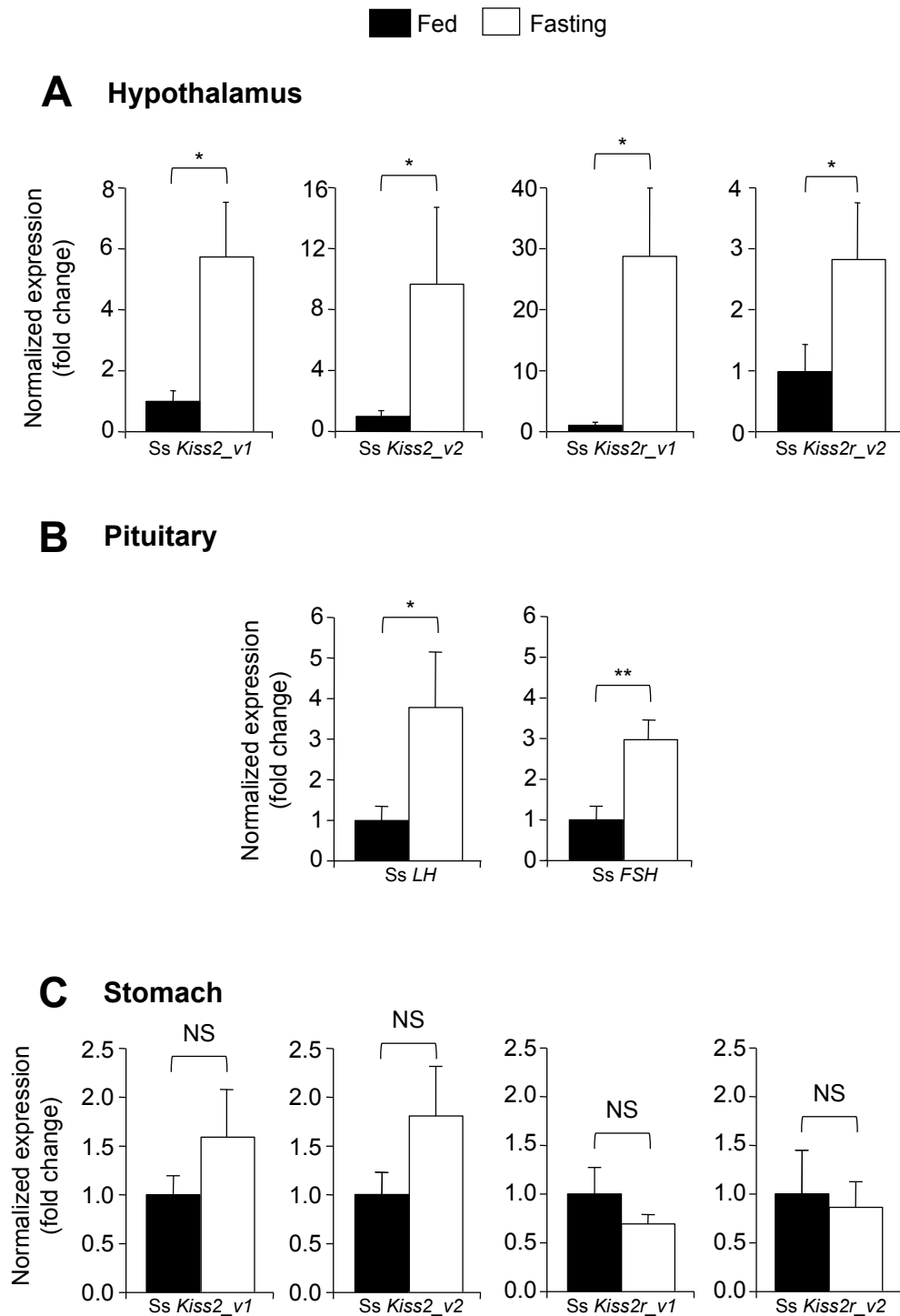




Mechaly, et al., 2010. Figure 4







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