SEX-SPECIFIC CHANGES IN THE EXPRESSION OF KISSPEPTIN, KISSPEPTIN RECEPTOR, GONADOTROPINS AND GONADOTROPIN RECEPTORS IN THE SENEGALESE SOLE (*Solea senegalensis*) DURING A FULL REPRODUCTIVE CYCLE

Alejandro S. Mechaly[1,2], *Jordi Viñas*[1,3] *and Francesc Piferrer*[1*]

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

[2]Present address: Brain and Mind Research Institute, Sydney Medical School, University of Sydney, Australia.

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

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

e-mail: piferrer@icm.csic.es

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ABSTRACT

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

1. Introduction

Kisspeptin has emerged as a key player in the neuroendocrine control of reproduction in vertebrates (Roa et al., 2008; Tena-Sempere, 2010), and is thought to be particularly implicated in the control of the onset of puberty in mammals (de Roux et al., 2003; Seminara et al., 2003) and teleost fish (Oaklet et al., 2009; Taranger et al., 2010). Kisspeptin is a neuropeptide product of the *KISS1* gene and forms a signaling system with its receptor, *KISSR* (Roa et al., 2008; Roseweir et al., 2009). In mammals, these genes are well conserved, with one ligand, *KISS1*, and its receptor, *KISS1R* (Oakley et al., 2009). However, several fish have two ligands, *kiss1* and *kiss2*, and two receptors, *kiss1r* and *kiss2r*, as a result of gene duplications (Akazome et al., 2010; Lee et al., 2009; Mechaly et al., 2010; Um et al., 2010; Tena-Sempere et al., 2012). In the Senegalese sole (*Solea senegalensis*), only *kiss2* and *kiss2r* have been detected (Mechaly et al., 2009, 2011) and thus this species appears to have lost *kiss1* and *kiss1r*, probably as a consequence of the genome reduction characteristic of Pleuronectiformes. However, in the Seenegalese sole each gene produces two splice variants but one of them results in putative non-functional products due to the presence of stop codons in the mRNA (Mechaly et al., 2009, 2011). Thus, some teleosts have lost one of the two paralogous genes, either of the ligand, the receptor or both (Mechaly et al., 2010, 2011).

Kisspeptin and its receptor (*kissr*) are expressed in several tissues, but due to their proposed role in reproduction (Tena-Sempere, 2010) the majority of studies have focused on the brain and gonads. In the medaka (*Oryzias latipes*), two populations of Kiss1 neurons were found in the hypothalamus, one in the nucleus posterioris periventricularis (NPPv), and another in the nucleus ventral tuberis (NVT) (Kanda et al., 2008; Kitahashi et al., 2009). In addition, in medaka and also in zebrafish (*Danio rerio*) the highest levels of *kiss1* mRNA were found in the ventromedial habenula, whereas *kiss2* mRNA was localized in the posterior tuberal nucleus and the periventricular hypothalamic nucleus (Kitahashi et al., 2009; Servili et al., 2011). Regarding

the kisspeptin receptor, expression profiles of *kiss2r* during development and sexual maturation have been determined in the brain of several fish species. In the Nile tilapia (*Oreochromis niloticus*), *kiss2r* mRNA levels were higher in gonadotropin-releasing hormone (GnRH) neurons of mature males when compared to those of immature males (Parhar et al., 2004). An increase of *kiss2r* expression before the onset or during early puberty was observed in the brain of cobia (*Rachycentron canadum*) (Mohamed et al., 2007), grey mullet (*Mugil cephalus*) (Nocillado et al., 2007), fathead minnow (*Pimephales promelas*) (Filby et al., 2008) and Atlantic halibut (*Hippoglossus hippoglossus*) (Mechaly et al., 2010). In zebrafish, *kiss2r* mRNA levels peaked coinciding with the onset of puberty in the female brain but those of *kiss1r* increased before the onset of puberty and remained high thereafter in both sexes (Biran et al., 2008). Thus, most studies have analyzed the expression of these genes either in whole brains or specifically in the hypothalamus due to its direct involvement in reproduction. Nevertheless, there is still limited knowledge on the neuroendocrine mechanism that controls puberty in teleost fish (Taranger et al., 2010).

The Senegalese sole is a highly prized fish with a great potential for aquaculture (Anguis et al., 2005; Imsland et al., 2003). Although in some cases cultured males complete spermatogenesis and sperm maturation with normal levels of androgen in plasma (García-López et al., 2006b; Cabrita et al., 2006), recent studies observed low sperm quality caused by some cellular damage in the spermatozoa of the F1 fish (Beirao et al., 2008), together with alterations in protease inhibition, iron and glucose metabolism. Further, protection against oxidative stress may cause the low production of the sperm and poor fertilization capacity by F1 males (Forné et al., 2009). In females, administration of GnRHa induced oocyte maturation and spawning. However, GnRHa administration was not completely effective in avoiding poor fertilization of the eggs produced by F1 females (Guzmán et al., 2009a). In addition, previous studies analyzed the influence of abiotic factors, particularly the lunar and daily changes of natural (Oliveira et al.,

2009) and artificial photoperiods (García-López et al., 2006a), on the spawning of this species. In fish, melatonin contributes to synchronize neuro-hormonal changes and behavior with daily and annual variations of photoperiod (Falcón et al., 2010). In the Senegalese sole, the relationship between the lunar cycle, melatonin, and sex steroids is thought to facilitate spawning during the darkest nights as an adaptation to escape predators and thus increase the chances of survival of the offspring (Dinis et al., 1999; Oliveira et al., 2010). Furthermore, another abiotic factor, water temperature, plays a crucial role in the reproductive cycle of this species by determining when gonadal maturation can take place (Anguis et al., 2005; García-López et al., 2006a; 2007). Additionally, injection of GnRHa during the spring induces multiple spawns but these treatments were ineffective in inducing sperm production in males (Agulleiro et al., 2006). Moreover, blockage of an endogenous dopamine (DA) inhibitory system stimulates spermatogenesis and sperm production in mature males (Guzmán et al., 2011). In the pituitary, follicle-stimulating hormone and luteinizing hormone β subunit (*fsh*β and *lh*β, respectively) gene expression increased in males during winter and spring, coinciding with a peak of androgens in plasma and development of testicular germ cells and spermatozoa, suggesting that these genes regulate spermatogenesis in the semi-cystic, asynchronous testis type characteristic of this species (Cerdà et al., 2008). In the gonads, mRNA levels of *fshr* and *lhr* during the reproductive cycle were consistent with earlier observations showing that *fshr* regulates ovarian growth and spermatogenesis, whereas *lhr* triggers gamete maturation, suggesting a role of the *lhr* in the differentiation of spermatids into spermatozoa (Chauvigné et al., 2010). Thus, Senegalese sole is an excellent model for the study of the expression patterns of several key genes related to the onset of puberty or at the beginning of the reproductive cycle, and these results can be related with situations where spermatogenesis progression is unaffected.

In fish, very few studies analyzed the expression pattern of the kisspeptin system genes during the different seasons of the year. In the grass puffer (*Takifugu niphobles*) *kiss2* and *kiss2r*

mRNA levels in the brain and pituitary of both sexes were higher during the spawning season when compared to the non-reproductive season, suggesting an important role of the kisspeptin system in the regulation of reproductive function (Shahjahan et al., 2010). A recently study in red seabream (*Pagrus major*) examined the influence of *kiss2* neurons on GnRH1 neurons and, similar to what was observed in grass puffeer, *kiss2* mRNA was higher during the spawning period (Shimizu et al., 2012). In a previous study with Senegalese sole, we found some differences in expression of *kiss2* and *kiss2r* between pubertal and mature fish (Mechaly et al., 2009, 2011). Furthermore, showed that fasting stimulated *kiss2* and *kiss2r* expression, which was followed by a concomitant increase in pituitary *fshβ* and *lhβ* gene expression, suggesting a link between nutritional status and reproduction mediated by hypothalamic kisspeptin and hypophysary gonadotropins (Mechaly et al., 2011). However, the expression pattern of *kiss2* and *kiss2r* in different parts of the brain-pituitary-gonad (BPG) axis and throughout a full reproductive cycle is not known in this and the vast majority of fish species.

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

2. Experimental Procedures

2.1. Source of the animals and sample collection

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

transported and maintained at the Experimental Aquarium Facilities of the Institute of Marine Sciences, Barcelona (41º23'13"N; 2º11'49"E) under simulated conditions of natural temperature and photoperiod and fed once a day with a commercial diet (Skretting, Spain). The animals were treated according to the approved institutional guidelines on the use of animals for research purposes, and in agreement with the European regulations of animal welfare (ETS No. 123,01/01/91). Fish were sampled during a full reproductive cycle during spring (SP1, 4 June 2008), summer (SM, 10 July 2008), fall (FL, 25 November 2008), winter (WT, 17 February 2009) and again the following spring (SP2, 4 May 2009). For sampling, fish (usually sample size was 7–9 fish per sampling with a maximum range of 3–11 depending upon sex and/or season) were anesthetized with an overdose of neutralized MS-222 (Sigma-Aldrich, St. Louis, MO, USA) and sacrificed by decapitation. Tissues were quickly removed under RNase-free conditions, flash frozen in liquid nitrogen and stored at -80ºC until used. For tissue distribution analysis, the pituitary was separated from the brain and the brains were dissected into six regions: Olfactory bulb, Telencephalon, Optic tectum, Cerebellum, Medulla oblongata and Hypothalamus. For gene expression analysis, and because of the low levels of the targeted genes, brain areas considered were the forebrain (including telencephalon and hypothalamus) and midbrain (optic tectum). Fragments of testis and ovary were fixed in 4% paraformaldehyde (PAF) for histological analysis. Biometric information, including standard length (SL) (precision 0,1 cm) body weight (BW) (precision 1 g) and gonad weight (GW) (precision 0,01 g) were assessed in all sampled fish. The gonadosomatic index (GSI) was determined according to the formula: GW (g)/BW (g)* 100.

2.2. Histological analyses

After fixation in 4% PAF for approximately 24 h at room temperature, gonads were washed for an additional 24 h in phosphate buffer (PB) (pH 7.4), dehydrated in a series of increasing alcohols, embedded in paraplast, sectioned at 7 μm, and stained with hematoxilin-eosin

following conventional histological procedures. Stages of spermatogenesis and oogenesis were determined according to the germ cell types present in the testes (García-López et al., 2006b) and ovaries (García-López et al., 2007), and the fish were classified as their sexually stages of gonadal development as immature, maturing or mature.

2.3. Determination of plasma levels of sex steroids

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

2.4. RNA isolation and cDNA synthesis

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

2.5. RT–PCR analysis of gene expression

mRNA levels of *kiss2* and *kiss2r* in different brain areas of male and female Senegalese sole in summer were assessed by previously validated Reverse Transcriptase PCR (RT-PCR) (Mechaly et al., 2009, 2011). Total RNA from six brain areas (olfactory bulb, telencephalon, optic tectum, cerebellum, medulla oblongata and hypothalamus) plus the pituitary were extracted as described above. One negative control (without cDNA sample) was included in each determination to ascertain that no cross-contamination took place. The PCR was carried out with 1 µl of the RT reaction in a total volume of 20 µl containing 1X PCR buffer plus, 3 mM $Mg2^{+}$, 0.2 mM dNTPs, 0.2 mM of each forward and reverse primers, and 1 IU of Platinum Taq DNA Polymerase (Invitrogen). The specific primers for amplification of *kiss2* and *kiss2r* cDNAs were designed according to the nucleotide sequences of the full-length cDNAs (Table 1). Amplification of the *ßactin* was used as RNA quality control using a combination of appropriate primers (Table 1). The PCR cycling conditions were: 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, 1 min at 72°C and a final extension of 7 min at 72°C. PCRs were performed with an initial cycle at 95°C for 5 min; then variable number of cycles was applied: 95°C for 30s; 65°C for 30s; 72°C for 1 min and a final extension cycle at 72°C for 7 min. An aliquot of the PCRs product was electrophoresed on 1.5% agarose gel containing ethidium bromide, and products were visualized and photographed.

2.6. Seasonal changes in mRNA levels of kiss2 and kiss2r in different brain areas and mRNA levels of fshβ, lhβ in pituitary and fshr and lhr in the gonads

The expression patterns of several genes were analyzed in males and females in the forebrain (hypothalamus plus telencephalon), midbrain (optic tectum), pituitary and gonads at the five different samplings stated above (SP1, SM, FL, WT and SP2) comprising a full reproductive cycle by quantitative real-time PCR (qRT-PCR). RNA and cDNA were obtained following the

protocol described above including the DNAse treatment step. The primers used for qRT-PCR were based on the sequences reported in previous studies (Cerdà et al., 2008; Chauvigné et al., 2010; Mechaly et al., 2009, 2011) and are summarized in Table 1.

The qRT-PCR amplification reaction mixture contained 2 µl of diluted cDNA (1:10) (freshly synthesized from 500 ng of RNA), 4 µM of each primer, and 10 µl of Power SYBRs Green PCR Master Mix (Applied Biosystems) in a final volume of 10 µl. Thermal cycling conditions comprised heating to 95°C for 10 min followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. The qRT-PCR products were immediately analyzed using a dissociation curve step to confirm that only a single product was amplified. No-template control reactions for every primer pair were also included on each reaction plate to check for external DNA contamination. The amplification efficiency (E) of each primer set/gene target was assessed as $E = 10^{(-1/slope)}$ as determined by linear regression of serial dilutions of the input RNA. To calculate relative changes in gene expression, we analyzed the data using the comparative Ct method (Schmittgen and Livak, 2008; also known as the ΔΔCt method). Fold change (the relative quantification, RQ) was calculated from the ΔΔCt and normalized by the endogenous reference gene *ßactin*. The RQ values for each sample were averaged and the standard error of the mean (S.E.M.) was calculated, yielding the average fold change of the target gene. Determinations were carried out in technical triplicates for all the genes studied.

2.7. Data representation and statistical analyses

Prior to analysis of data, GSI levels were arcsine-transformed to ensure homoscedasticity of variances. Normality of data was assessed by the Shapiro-Wilks W test. Differences in the GSI, plasma steroid and gene expression levels during the different seasons were analyzed by a one-way analysis of variance (ANOVA). Only in the case of significant differences, ANOVA was followed by the Fisher's least significant difference (LSD) test. Statistical analyses of data were

253 performed using the SPSS 15.0 package. Differences were accepted as statistically significant
254 when $P < 0.05$. Data are expressed as mean ± standard error of the mean (S.E.M).
255
256 **3. Results**
257 ***3.1 Biometric parameters and gonadosomatic indices of males and females (GSI)***
258 According to previously published studies, Senegalese sole reach first sexual maturity when
259 they attain an average SL of ≈ 30 cm, although there is a well known large interindividual
260 variability (Dinis et al., 1999; García-López et al., 2006b). The fish used in this study had a
261 similar range of length and weight for both sexes. However, maturing males were always ≥ 25
262 cm and ≥ 256 g while maturing females were always ≥ 30 cm and ≥ 489 g. Furthermore,
263 regardless of season, the GSI range of males was independent of the degree of gonadal
264 maturation while the GSI range of females increased with maturation. Changes in the GSI of
265 males ($P = 0.010$) and females ($P = 0.005$) were observed during the different seasons. The GSI
266 significantly increased ($P < 0.05$) after the fall, with maximum values observed in the winter in
267 males (Fig. 1A) and during following spring in females (Fig. 1B).
268
269 ***3.2 Plasma steroid hormone levels***
270 In males, plasma levels of the two major androgens (T and 11-KT) followed the same pattern as
271 the GSI, with a clear and significant ($P = 0.045$ and $P = 0.024$, respectively) peak in winter (Fig.
272 1C and Fig. 1E). In females, E_2 plasma levels remained low until the fall and then sharply
273 increased through winter and the following spring ($P = 0.005$) (Fig. 1D). Thus, in both sexes the
274 GSI and plasma levels of the major sex steroids shared a similar pattern, with maximum values
275 observed in winter for males and in the following spring for females (Fig. 1).
276
277 ***3.3 Gonadal development***

Based on microscopic evaluation, three developmental stages of spermatogenesis in males (Fig. 2A, C and E) and of oogenesis in females (Fig. 2B, D and F) were identified. Males with testis filled only with spermatogonia (Spg) were considered as immature (Fig. 2A); males which in addition had spermatocytes (Spc) and spermatids (Spd) were classified as maturing (Fig. 2C), whereas males with testis containing spermatozoa (Spz) were classified as sexually mature (Fig. 2E). Females with only previtellogenetic oocytes were considered immature (Fig. 2B); with early and intermediate vitellogenic oocytes were considering maturing (Fig. 2D), whereas mature females were characterized by the presence of fully developed oocytes (Fig. 2F). In accordance with previous observations showing that once Senegalese sole males reach a certain size can remain mature throughout the year, maturing males were found in all seasons, with a maximum in winter.

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

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

3.5 Seasonal changes on the expression of kiss2 and kiss2r in different brain areas, fshβ **and** *lhβ in the pituitary and fshr and lhr in the gonads of the Senegalese sole*

In the forebrain of males, *kiss2* (Fig. 4A) and *kiss2r* expression (Fig. 4C) increased after summer and peaked in winter ($P = 0.027$ and $P = 0.002$, respectively). In females, in contrast, *kiss2* (Fig. 4B) and *kiss2r* expression (Fig. 4D) progressive increased and the maximum mRNA levels were observed in the following spring ($P = 0.0040$ for both genes). In the midbrain of males, changes in both *kiss2* ($P = 0.004$) and *kiss2r* ($P = 0.016$) mRNA levels were observed, with a clear peak of expression in winter (Fig. 5A and C). In females, in contrast, *kiss2* mRNA levels started to increase in the fall and reached maximum levels in winter ($P = 0.002$), and then started to

slightly decrease, whereas *kiss2r* mRNA levels kept increasing until they reached maximum values in the following spring ($P = 0.008$) (Fig. 5B and D).

Regarding the expression levels of gonadotropin genes in the pituitary, *lhβ* in males peaked in winter ($P = 0.003$) (Fig. 6C) and *fshβ* of females during the following spring ($P = 0.001$) (Fig. 6B). No significant differences were observed in *fshβ* mRNA levels in males (Fig. 6A), as well as in *lhβ* mRNA levels in females (Fig. 6D), probably due to insufficient sample size in this case. In the gonads, mRNA levels of *fshr* and *lhr* remained low during most part of the study, but were consistently higher in winter. However, the inverse situation was found with respect to the levels of mRNA for *fshβ* and *lhβ* observed in the pituitary, *i.e.*, differences were observed for *fshr* in the testis ($P = 0.031$) (Fig. 7A) and *lhr* in the ovaries ($P = 0.001$) (Fig. 7D). The lack of differences in *fshβ* of levels in females (Fig. 7B) and *lhr* levels in males (Fig. 7C) is probably due to large interindividual variations and/or too small sample sizes in these cases. Nevertheless, maximum values were observed in winter, as seen for many of the variables analyzed in this study.

4. Discussion

The Senegalese sole is a multiple-spawning fish, with a main spawning period during spring and a secondary period during the fall according to studies based on captive breeders (Anguis et al., 2005; García-López et al., 2006a, 2007). In this study, we investigated the relationship between the expression profiles of several key genes of the BPG axis and maturation status during a full reproductive cycle in this species.

We actually measured the expression of each one of the two splice variants of each of the two genes of the kisspeptin system, the ligand and the receptor, previously characterized (Mechaly et al., 2009; 2011). However, analysis of the mRNAs leading to the truncated isoforms during the

annual cycle showed that, although changes could be measured between seasons, no defined pattern could be observed (data not shown). Thus, only the functional splice variant is considered in the present study. Whether changes in the transcription of these mRNAs through changes in the alternative splicing towards one or the other isoform contributes to control the abundance of the mRNA producing the functional protein has not been investigated.

In this study, the major brain areas implicated in the control of reproduction (Zhang et al., 2009; Zohar et al., 2010) were examined together with the pituitary and the gonads. In males, telltale signs of the initiation of reproduction could be observed in winter, as evidenced by the highest GSI, peak plasma levels of T and 11-KT and maximum number of observed maturing males. These changes were also evident at the gene expression level since the highest mRNA levels of *kiss2* and *kiss2r* in the forebrain and midbrain, *lhβ* in the pituitary and *fshr* in testis were also observed in winter (see data summarized in Fig. 8). This is probably related to the initiation of testicular meiosis that implies an increase of spermatocytes in winter (Anguis et al., 2005) and subsequent highest levels of spermatozoa production in spring (Cerdà et al., 2008). This situation is similar in Atlantic halibut, with an increase of testicular mass together with increased GSI and plasma levels of T and 11-KT during winter (Weltzien et al., 2002).

In females, the tight association observed in males between the winter and the maximum values of many of the measured variables was not evident. However, many measured variables exhibited a tendency to increase their values with time, peaking in the second spring, and thus in agreement with the fact that ovarian development reaches its maximum between the end of the winter and the beginning of spring, when the main spawning season begins in Senegalese sole (Anguis et al., 2005; García-López et al., 2006, 2007).

During the characterization of Senegalese sole *kiss2* and *kiss2r* we did measurements of these genes in maturing vs. mature animals, showing no differences in *kiss2* regardless of sex and only a decrease of *kiss2r* in mature females with respect to maturing females (Mechaly et al., 2009, 2011). Those preliminary results contrast with the ones presented here. A possible explanation of these discrepancies can be attributed to the fact that in the previous studies whole brains were used whereas in the present study different brain areas were examined separately. Furthermore, in previous studies fish were combined based on their reproductive status regardless of season of the year, whereas here samplings during specific seasons were carried out. Finally, the changes of *kiss2* and *kiss2r* observed during the different seasons in this study agree with the results observed in both sexes of grass puffer and read seabream, where higher levels of *kiss2* and *kiss2r* mRNAs in the whole brain (Shahjahan et al., 2010) and *kiss2* in the hypothalamus (Shimizu et al., 2012) where observed during the spawning season, although no differences between sexes were observed (Shahjahan et al., 2010) in contrast to the present study.

Like in the BPG axis of mammals, in fish GnRH is a major target of kisspeptin signaling (Parhar et al., 2004). Like in the rest of vertebrates, fish GnRHs are involved in gonadotropin secretion and gonad maturation (Amano et al., 2008). In the grass puffer, increased *GnRH1* expression resulted from *kiss2* and *kiss2r* increased expression (Shahjahan et al., 2010). Similar results were found concerning *GnRH3*, *kiss2* and *kiss2r* expression in the zebrafish brain (Kitahashi et al., 2009), where recently it was shown that kiss2 fibers innervate GnRH3 neurons (Servili et al., 2011). It is important to state that at present the Senegalese sole mRNAs of the GnRHs have not been characterized and therefore changes in their expression levels could not be correlated with *kiss2* and *kiss2r* expression. However, despite this shortcoming we could study kisspeptin signaling and gonadotropin expression in an effort to put kisspeptin effects into a more general context, as shown in the present study.

It is becoming well established that kisspeptins released in the pituitary induce gonadotropin secretion (Oakley et al., 2009), although *Kiss1r* might be involved in additional roles, *e.g.*, in the stimulation of growth hormone (GH) and prolactin (PRL) secretions via endocrine, and/or paracrine mechanisms (Richard et al 2009). In the goldfish, Kiss1 stimulated the synthesis and release of *lh*, *prl* and *gh* (Yang et al., 2010), although no effects on *lh* were detected in another study using the same species (Li et al., 2009). In the grass puffer, kisspeptin and its receptor expression peaked during the spawning season, in both brain and the pituitary (Shahjahan et al., 2010). Regarding the gonadotropins, our data show that in males *fshβ* and *lhβ* mRNA levels mirrored the expression changes of *kiss2* in the brain, although significant differences were observed only for *lhβ*, supporting the role of kisspeptin in triggering reproduction. In our study, *fshβ* levels were higher in winter and the second spring when compared to the previous seasons in agreement with a previous report showing increased levels of *fshβ* in the pituitary of Senegalese sole males in winter and spring (Cerdà et al., 2008). However, in that report *lhβ* levels paralleled those of *fshβ* (Cerdà et al., 2008), while in the present study *lhβ* levels in males dropped after winter. On the other hand, in females gonadotropins did not follow the expression pattern of *kiss2* or *kiss2r*, and *fshβ* did not increase until the second spring, which is in agreement with the role in the regulation of ovarian maturation as observed in other studies in this species (Guzmán et al., 2009b).

In the present study, mRNA levels of both *fshr* and *lhr* increased in winter, similar to the situation observed in the Atlantic salmon (*Salmo salar*) (Maugars et al., 2008), and probably in response to seasonal dynamics of their ligands, as described elsewhere (Mittelholzer et al., 2009). In any case, the role of kisspeptin signaling in fish gonads deserves further research.

In summary, the present study provides information on the changes in expression of kisspeptin and its receptor in the brain of the Senegalese sole, relating them with other histological,

biochemical and gene expression changes known to occur during the reproductive cycle. The major finding of this study is that, in males, *kiss2*, *kiss2r* and most variables analyzed changed synchronously and peaked in winter, coinciding with the highest number of maturing animals, and just before the spawning season, which took place the following spring. Thus, expression of *kiss2* in Senegalese sole males agrees with what one would expect according to its proposed role as a major regulator or trigger of the onset of reproduction. In females, such synchrony was not so evident and, furthermore, the highest levels of *kiss2* and *kiss2r* were observed in the spring, coinciding with the reproductive season, when all females were already fully mature. To the best of our knowledge, the present study is the first one in fish that considers kisspeptin signaling including several brain areas, accounts for sex differences and covers a full reproductive cycle. Thus, the origin and physiological significance of the observed sex-specific differences in kisspeptin signaling, which could also apply to other fish, deserve further investigation in order to contribute to firmly establish the role of kisspeptin in the control of reproduction. Also, and in the particular case of the Senegalese sole, whether these sex differences have any relationship with the recurring poor reproductive performance of captive F1s is at present unknown.

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

Gene	GenBank Acc. No.	Primer sequence (5'→3')	Amplicon size (bp)	Primer name	Reference
βactin	DQ485686	ACACCCAACAACTTCAGCTCTGT	120	Ss βactin-F1	Mechaly et al., 2009
		GAGTCAAGCGCCAAAATAATGA		Ss βactin-R1	
kiss2r	EU136710	TATGTGACAGTGTATCCTCTGAAATCC	89	Ss kiss2r_v1-F1	
		AAGGAGCCAATCCAAATGCA		Ss kiss2r_v1-R1	
kiss2	HM116743	TGGATCTGCACGATATGACA	50	Ss kiss2_v1-F1	Mechaly et al., 2011
		GTCTGACCCTGTTGCTCG		Ss kiss2_v1-R1	
fshβ	EU100409	TGATCTGTAACGGGGACTGG	153	Ss fsh*β*-F	Cerdà et al., 2008
		GACAGCTGGCAATCTCTCCA		Ss fsh*β*-R	
lhβ	EU100410	AGCATGTGTGCACGTACCAG	180	Ss lh*β*-F	
		TGTCGTTCATGCAGATGTCG		Ss lh*β*-R	
fshr	GQ472139	GGCGACTGGACTGAGTTTCG	186	Ss fshr-F	Chauvigné et al., 2010
		TCTTCACAACACGTGGGAGAG		Ss fshr-R	
lhr	GQ47140	GCTGTGCACTGCTGAACTGG	376	Ss lhr-F	
		GGCACCGTCATCTTGCTTCT		Ss lhr-R	

Figure legends

Fig. 1. Changes in the gonadosomatic index (GSI) arcsine-transformed of male (A) and female (B) Senegalese sole, and C, plasma levels of testosterone (T); E, 11-ketotestosterone (11-KT) in males, and D, plasma levels of estradiol−17β (E_2) in females during one full reproductive cycle. Data as mean ± S.E.M. (n= 3-7). Abbreviations: SP1, spring 1; SM, summer; FL, fall; WT, winter; SP2, spring 2. Different letters indicate statistically differences ($P < 0.05$).

Fig. 2. Photomicrographs of histological sections representing different stages of sexual maturation in Senegalese sole: immature (A), maturing (C) and mature (E) testis, and immature (B), maturing (D) and mature (F) ovaries. Abbreviations: Spg, spermatogonia; Spc, spermatocyte; Spd, spermatid; Spz, spermatozoa; Og, oogonia; Pno, perinucleolar oocyte; Voc, vitellogenic oocyte; Moc, mature oocyte; Oc, ovarian cavity. The scale bar, 100 μm, applies to all photomicrographs. N= 5-9.

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

Fig. 4. Changes in *kiss2* (A, B) and *kiss2r* (C, D) mRNA levels in the forebrain of Senegalese sole males (A, C) and females (B, D) during different seasons of the year as determined by quantitative real–time PCR (qRT-PCR). *βactin* was used as reference gene. Different letters indicate statistically differences ($P < 0.05$). Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3-9).

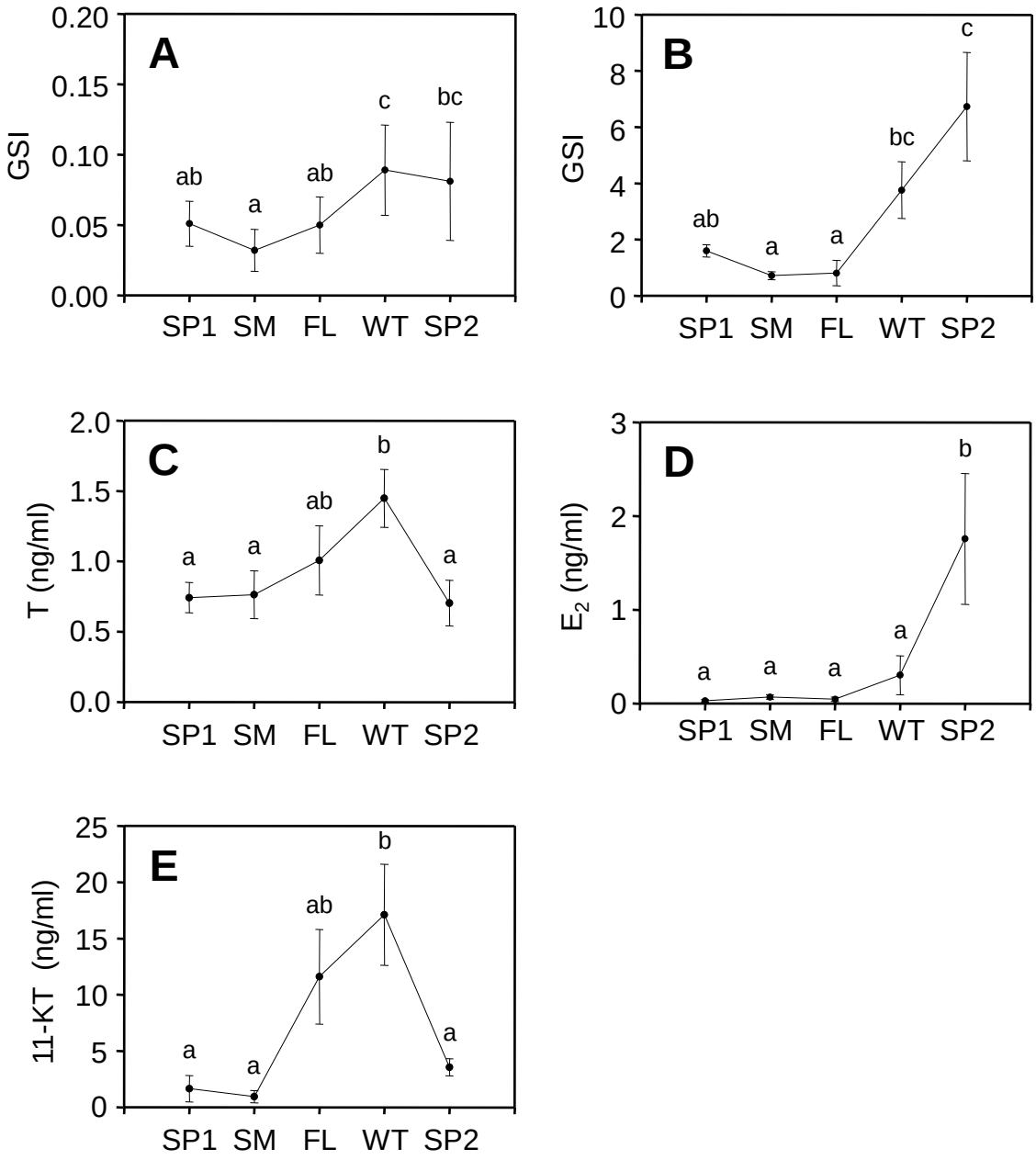
Fig. 5. Changes in *kiss2* (A, B) and *kiss2r* (C, D) mRNA levels in the midbrain (optic tectum) of

Senegalese sole males (A, C) and females (B, D) during different seasons of the year as determined by qRT-PCR. *βactin* was used as reference gene. Different letters indicate statistically differences ($P < 0.05$). Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3-9).

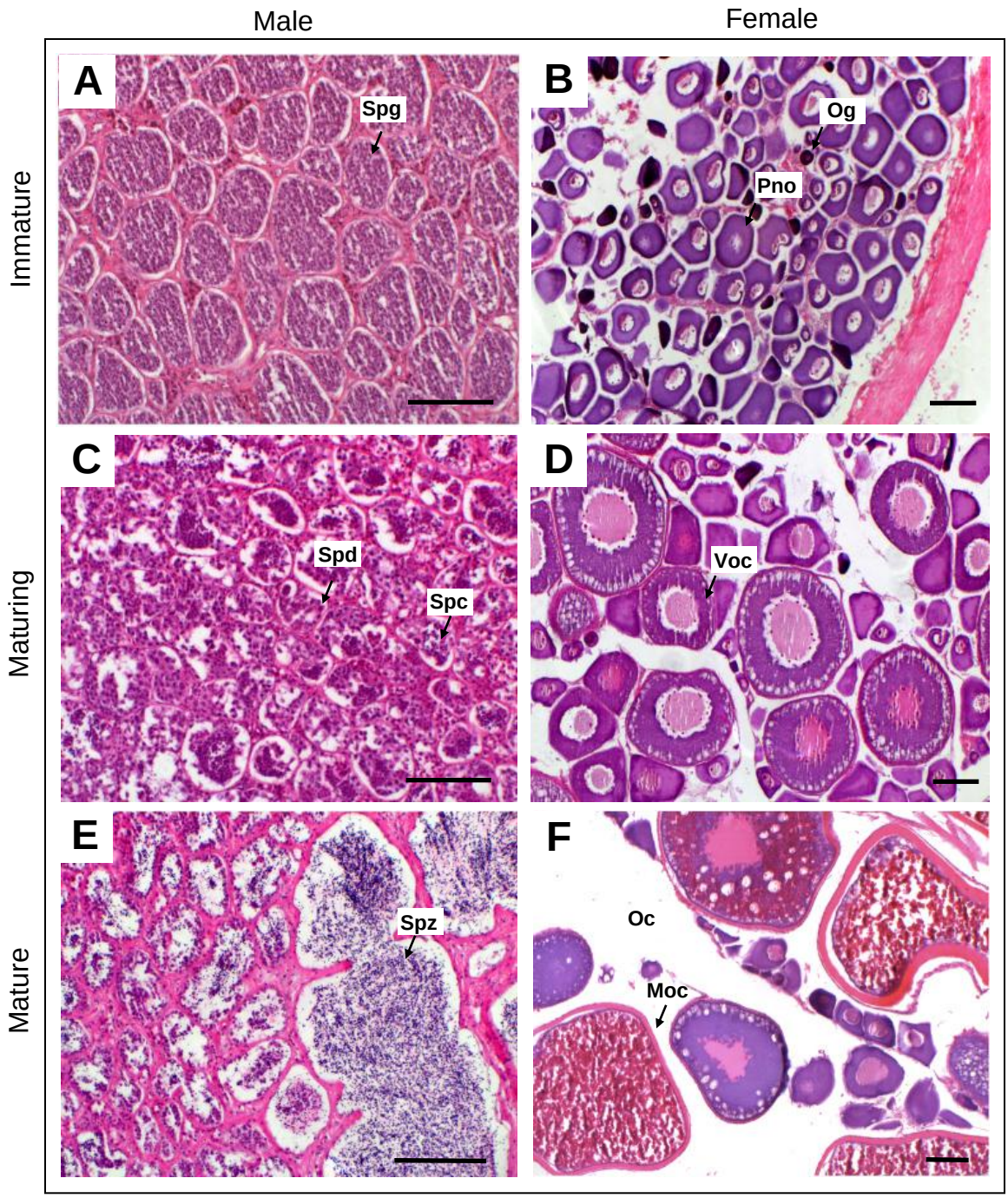
Fig. 6. Changes in *fshβ* (A, B) and *lhβ* (C, D) mRNA levels in the pituitary of Senegalese sole males (A, C) and females (B, D) during different seasons of the year as determined by qRT-PCR *βactin* was used as reference gene. Different letters indicate statistically differences ($P < 0.05$). Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3-9).

Fig. 7. Changes in *fshr* (A, B) and *lhr* (C, D) mRNA levels in the testis (A, C) and ovaries (B, D) of Senegalese sole during different seasons of the year as determined by qRT-PCR. *βactin* was used as reference gene. Different letters indicate statistically differences ($P < 0.05$). Abbreviations as in Fig. 1. Data as mean ± S.E.M. (n= 3-9).

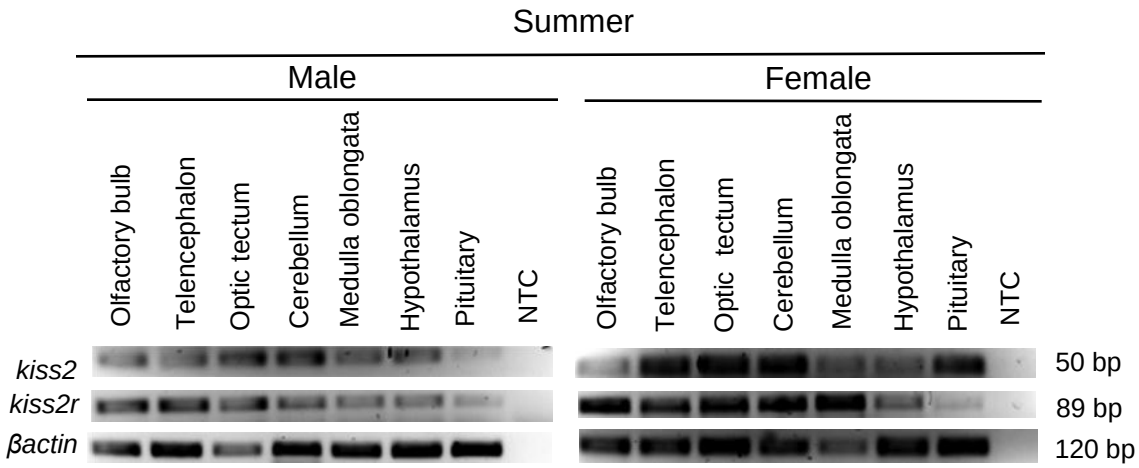
Fig. 8. Summary of the histological, biochemical and gene expression changes observed in the variables measured in the BPG axis of the Senegalese sole used in this study. Within each sex, the five boxes correspond, from left to right, to spring (SP1), summer (SM), fall (FL), winter (WT) and the following spring (SP2). In each box, the level of shading is related to the levels of the variable being considered: white, low or intermediate levels; grey, higher levels but without significant differences; black, significantly higher levels. Notice the evident differences between sexes.



Mechaly et al., Figure 1



Mechaly et al., Figure 2



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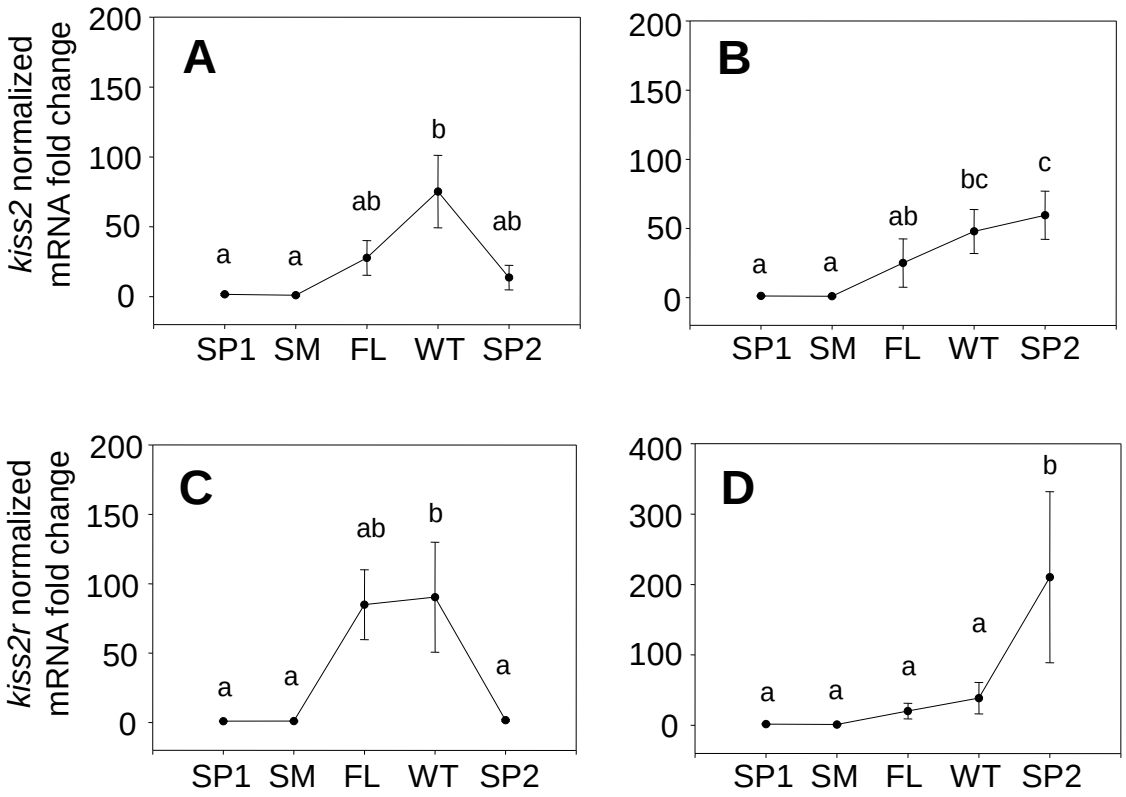

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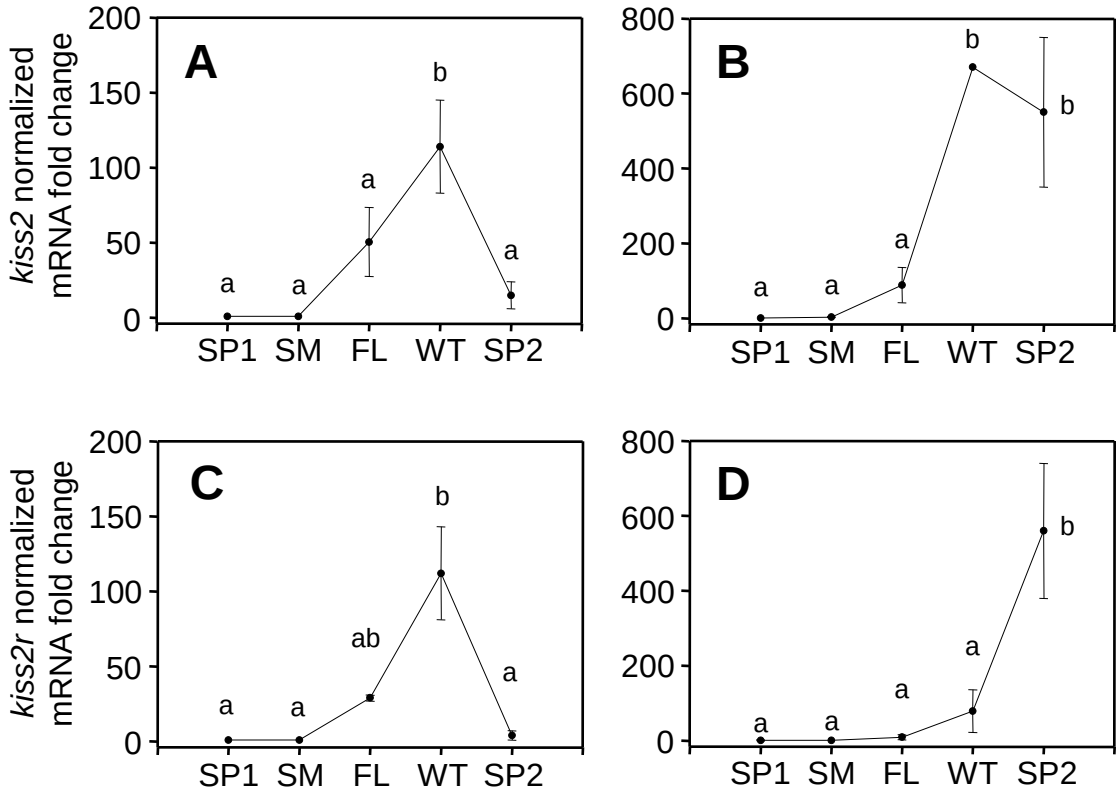

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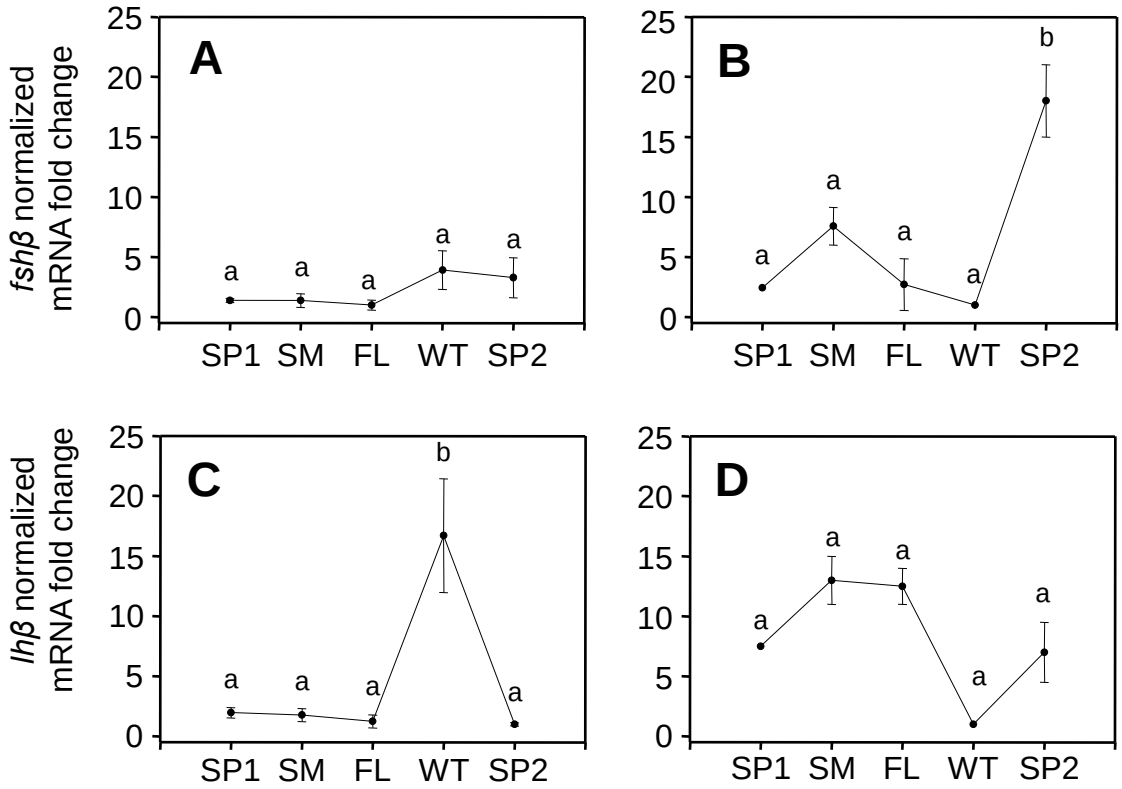

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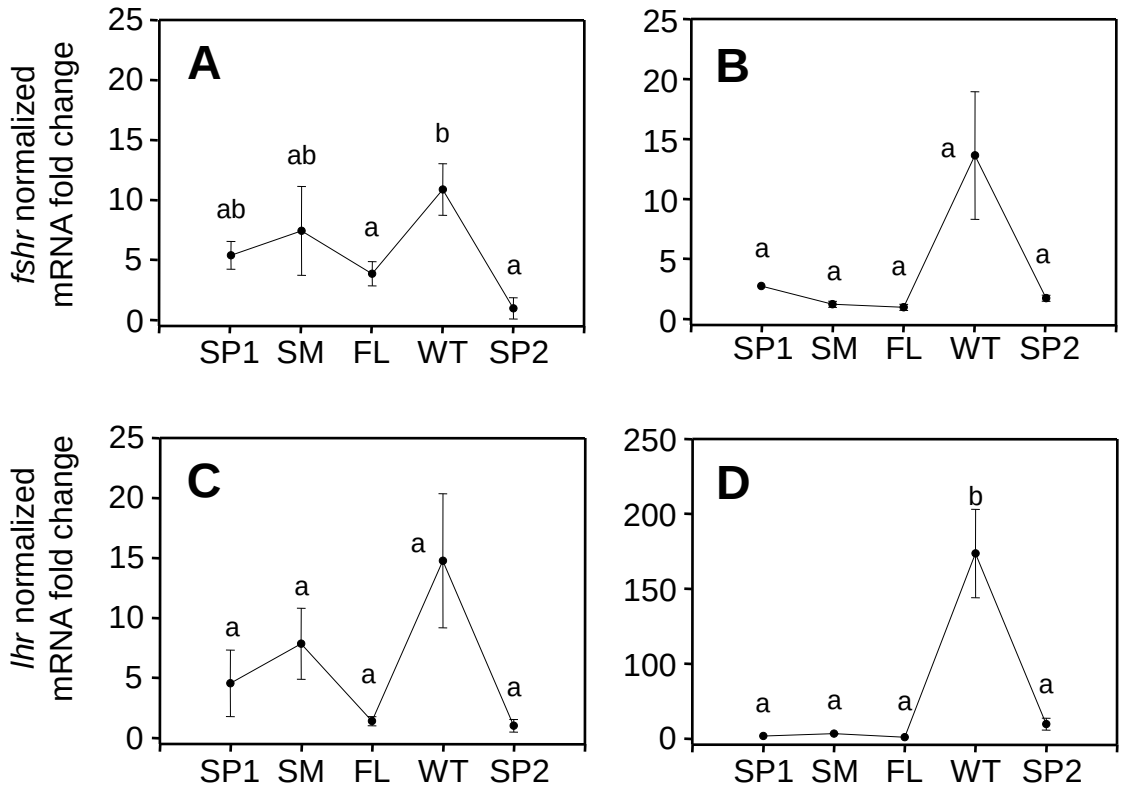

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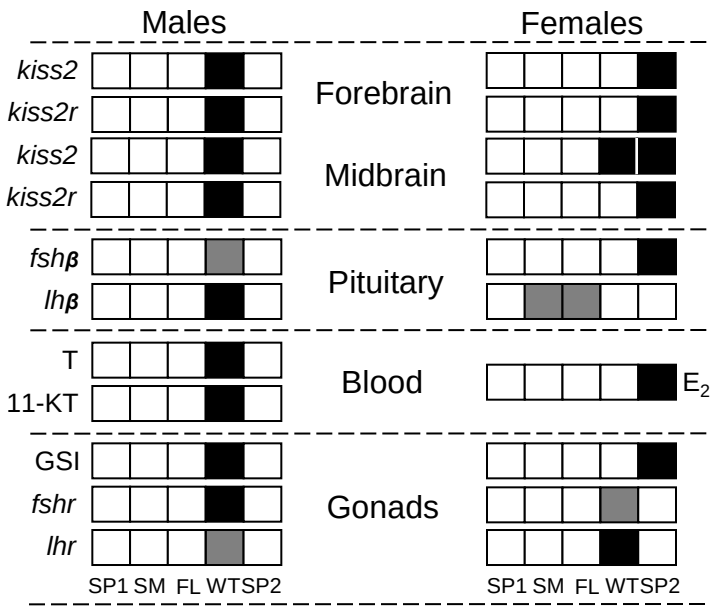

Mechaly et al., Figure 8