Investigating the kisspeptin system in the hermaphrodite teleost gilthead seabream (*Sparus aurata*)

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Abstract. The kisspeptin system, a known regulator of reproduction in fish, was investigated during two key phases within the gilthead seabream (*Sparus aurata*) life cycle: protandrous sex change and larval ontogeny. Seabream specific partial cDNA sequences were identified for two key targets, *kissr4* and *kiss2*, which were subsequently cloned and qPCR assays developed. Thereafter, to examine association in expression with sex change, a group of adult seabream (2+ years old) undergoing sex change were sampled for gene expression at two different periods of the annual cycle. To study the kisspeptin system ontogeny during early life stages, transcript levels were monitored in larvae (till 30 days-post-hatch, DPH) and post-larvae (from 30 till 140 DPH). During sex change, higher expression of *kissr4* and *kiss2* was observed in males when compared to females or individual undergoing sex change, this is suggestive of differential actions of the kisspeptin system during protandrous sex change. Equally, variable expression of the kisspeptin system during early ontogenic development was observed. The higher expression of *kissr4* and *kiss2* observed from 5 DPH, with elevations at 5-20 and 90 DPH for *kissr4* and at 5, 10, 20, and 60 DPH for *kiss2*, is coincident with the early ontogeny of *gnrh* genes previously reported for seabream, and possibly related with early development of the reproductive axis in this species.

Additional keywords: sex change, *kissr4*, *kiss2*, protandric hermaphroditism, ontogeny

Introduction

The discovery of kisspeptin as a key regulator system of puberty and reproduction in mammals has been a major breakthrough in the field (Terasawa et al., 2013). This system has been reported as part of the seasonal control of reproduction, apparently being the missing link between the major photo transducer structure (pineal/melatonin system) and the Brain-Pituitary-Gonad (BPG) axis (Li et al., 2015; Revel et al., 2007). It is known that kisspeptin, acting centrally via the kisspeptin receptor, stimulates GnRH neurons in the hypothalamus to release GnRH, causing the release of gonadotropins from the pituitary (Clarke et al., 2015; Zohar et al., 2010). Research in this field is far more advanced in mammals, nevertheless, several studies have recently emerged in fish, suggesting a major role of the kisspeptin system in the regulation of the gonadotropic axis, especially in timing of puberty and control of gonadotropin secretion (Cowan et al., 2017a; Cowan et al., 2012; Filby et al., 2008; Zmora et al., 2015), with two paralogous genes (*kiss1* and *kiss2*) identified (Mechaly et al., 2013; Migaud et al., 2012). Kisspeptins are ligands for the receptor Kissr (previously called GPR54), with four paralogous genes identified in vertebrates, but only two encountered in teleosts: *kissr2* and *kissr4* (Migaud et al., 2012; Zohar et al., 2010). Among these two, k*issr4* is apparently the most predominant and functionally active form, being present in many fish species (Akazome et al., 2010).

The gene *kiss2* appears to have a predominant role in the control of fish reproduction (Akazome et al., 2010; Felip et al., 2009). Nevertheless, due to the variety in reproductive strategies seen in teleosts, the reported reproductive roles and distributions of the two kisspeptin forms and their receptors can vary (Kitahashi et al., 2009; Li et al., 2009; Selvaraj et al., 2013; Yang et al., 2010; Zmora et al., 2015). However, a clear relationship between the kisspeptin system and the annual reproductive cycle has been reported both in Senegalese sole (*Solea senegalensis*) and in European seabass (*Dicentrarchus labrax*) (Cowan et al., 2017b; Mechaly et al., 2012; Migaud et al., 2012), suggesting conservation of its role in the seasonal control of reproduction, as reported in mammals. Indeed, this system has been suggested to integrate both environmental cues and metabolic signals in fish, as well as in mammals, transducing this information onto the reproductive axis (Zohar et al., 2010). With respect to the integration of environmental signals, there is evidence in both seasonal species like European sea bass (Alvarado et al., 2015; Cowan et al., 2017b; Espigares et

al., 2017), and Atlantic salmon (*Salmo salar*) (Chi et al., 2017) as well as tropical species like Nile Tilapia (*Oreochromis niloticus*), (Martinez-Chavez et al., 2008)

Recent studies have also proposed a role of the kisspeptin system in early development and gonadal sex differentiation in some fish species (e.g. cobia, *Rachycentron canadum*, Mohamed et al. (2007); Nile Tilapia, Park et al. (2012); pejerrey, *Odontesthes bonariensis*, Bohórquez et al. (2017); Chub mackerel, Selvaraj et al. (2015)). During cobia ontogeny, *kissr4* was highly expressed very early in larvae, in parallel with *gnrh* expression (Mohamed et al., 2007). In the other three species, expression of the kisspeptin system was observed to be elevated in periods coinciding with sex differentiation, indicating a potential role of these genes in such process, though similar information regarding species with sequential hermaphroditism is very limited and requires further research (Todd et al., 2016). Interestingly, in the pejerrey, a pleiotropic effect has even been proposed, related with mediation of olfactory and visual signals (Bohórquez et al., 2017). All of these results eludes to a significant central role of the kisspeptin system in early fish development, however the functional mechanisms are still unclear.

The gilthead seabream, *Sparus aurata*, is one of the most important species for Mediterranean aquaculture. It is a protandric hermaphrodite species, maturing first as male (during the first or second reproductive cycles) before undergoing sex change so that after the second or third reproductive cycles, almost all individuals will be functional mature females (Liarte et al., 2007; Zohar et al., 1978). It has been proposed that the kiss system is likely to be involved in fish sex change processes, based on the example of the orange-spotted grouper (*Epinephelus coioides*) (Shi et al., 2010; Todd et al., 2016), but it remains to be investigated in seabream. Equally, while the early ontogeny of the GnRH system and reproductive axis has been described, with expression of related genes being detected very early in development (Wong et al., 2004), no information is available about the kisspeptin system during early ontogeny. With all this in mind, this study intends to identify in gilthead seabream *kissr4* and *kiss2*, the two forms which have been suggested to be functionally important in fish, and further investigate a possible involvement in sex change and early life stages of development: larvae and post-larvae.

Materials and methods

To fulfil the objectives of this study, two experiments were performed. In order to investigate a possible role of the kiss system in the sex change process in gilthead seabream, *kissr4* and *kiss2* expression were measured in brain and gonad tissues from broodstock individuals undergoing sex change (experiment 1). Experiment 2 studied the ontogeny of this system in the same species. A first trial described a detailed profile of *kissr4* and *kiss2* transcript levels until 30 DPH (days post-hatch) while in a second, gene expression was monitored from 30 until 140 DPH to expand the previous results.

Ethical statement

Experimental procedures were conducted in accordance with ARRIVE guidelines (Kilkenny et al., 2010), with directives 86/609/EU and 2010/63/EU of the European Parliament and Council, and Portuguese legislation for the use of laboratory animals (PORT 1005/92) of the Portuguese direction for veterinary and food services (Direção-geral de alimentação e veterinária, DGAV). All persons involved in the animal trials have a FELASA class C permit for animal experimentation and CCMAR facilities are authorized by DGAV for animal experimentation (permit number 0421/000/000/2013).

Animals and housing

For the first experiment, forty farmed adult gilthead seabream (2+ years old and mean body mass of 920 ± 136 g) were reared outdoors at CCMAR, in four 1000 L tanks under ambient photoperiod and temperature conditions. Over the study duration (August till January) water temperature averaged 19.2 ± 4.7ºC, mean dissolved oxygen saturation was 86.6 ± 6.5% and salinity averaged 35.4 ± 1.5‰. Individuals were fed daily at the rate of 1% of tank biomass using a commercial feed (Sparos Lda.).

In the first trial of experiment 2, gilthead seabream larvae were reared at CCMAR experimental facilities until the age of 30 DPH. Standard rearing protocols for this species were used in accordance with Moretti (1999). Eggs were incubated in a 100 L fibreglass cylindroconical tank for 48 hours. Newly hatched larvae were transferred to 3 similar tanks (100 L) at a density of approximately 100 larvae L^{-1}. Larvae were fed with enriched rotifers (*Brachionus plicatilis* enriched with Easy DHA Selco, INVE, Belgium) from the onset of exogenous feeding (3 DPH) until 11 DPH. From 12 until 21 DPH they were co-fed with rotifers and *Artemia* nauplii and from 22 to 30 DPH, with solely *Artemia* nauplii. Fish were kept at 19 ± 1ºC, 35‰ salinity, dissolved oxygen

above 90% saturation and under a 14 h light, 10 h dark photoperiod (lights on at 08:00 h).

The second trial took place in CULMAREX aquaculture facilities from 30 till 140 DPH and under standard commercial rearing conditions. Larvae were weaned at the age of 80 DPH using a commercial diet (Gemma Micro, Skretting, Norway). Larvae were reared at 20ºC and exposed to a photoperiod of 13 h light and 11 h of darkness.

Experimental design

Experiment 1: Investigating the kiss system during sex change in gilthead seabream

The study group consisted of males, females, and males during sex change. Samples of brain and gonads were collected at two different stages of the reproductive season (n=20 total animals at each sampling); in October, during full spawning and January, at the beginning of the resting period. Seabream were individually sacrificed with an overdose of 2-phenoxyethanol (1000 ppm) and immediately dissected. Sex was firstly determined by striping the fish and identifying the presence of sperm or oocytes. When this was not possible, namely during the resting period, the functional gonad was determined by macroscopic or microscopic observation, depending on developmental stage during dissection. After this evaluation, gonads were excised and a small piece was cut in half, one part for total RNA extraction and the other for histological confirmation of gonadal development in accordance with Brusléa-Sicard and Fourcault (1997) and Somarakis et al. (2013). Haematoxylin and eosin staining technique was used in 5-mm sections to determine the maturation status of testis and ovaries, according to Pacchiarini et al. (2013). Individuals were subsequently classified as males or females. When both testis and ovary were present in the same fish and at equal stage of development (no predominant functional gonad could be recognised) individuals were identified as sex changing (Zohar et al., 1978). The whole brain and 300 mg gonad were collected from male and female individuals. For individuals undergoing sex change, a combination of both testis and ovary was collected at a proportion of 1:1 (150 mg for each). Dissection was performed under RNase-free conditions to avoid contamination of the samples. Tissue samples were immediately frozen in liquid nitrogen and stored in -80ºC to avoid RNA degradation.

Experiment 2: Kiss system ontogeny during larvae and post-larvae stages in gilthead seabream

In the first ontogeny trial developed at CCMAR facilities, samples were periodically taken to further assess *kissr4 and kiss2* transcript levels. Egg samples (circa 100 per aliquot) were collected in the morning after the spawning event (gastrula stage), and also prior to hatching (embryo stage). Larvae samples were collected at 0, 5, 10, 20 and 30 DPH at 11:00 am, to avoid temporal differences in gene expression. Samples were rinsed with Milli-Q water and immediately frozen in liquid nitrogen. All steps were carried out in RNase-free conditions. For 0 and 5 DPH ca. 20 larvae were pooled per Eppendorf, while from 10 to 30 DPH, this number was reduced to 10/15 larvae per aliquot.

To assess ontogeny of the kiss system including post-larval stages a second batch of larvae was monitored from 30 till 140 DPH in the facilities of CULMAREX company. Larvae samples were collected from 30 to 140 DPH (30, 45, 60, 75, 90, 105, 120 and 140 DPH), always in the morning. From 30 to 60 DPH full larvae were pooled in the same sample (10 per aliquot), while from 75 until 140 DPH, only heads were collected and pooled in cryovials containing RNA-later® (5 heads per aliquot). Post-larvae previously anesthetised with MS-222 (100 mg/L) and sacrificed by decapitation. Once more, all steps were carried out in RNase-free conditions and samples were immediately frozen.

Molecular biology analyses

RNA extraction, DNase treatment and cDNA synthesis

All RNA extractions were carried out at a ratio of 100 mg tissue per ml TRI reagent (Sigma-Aldrich, St Louis, MO USA) according to manufacturer's instructions. Larger tissue samples were homogenised using Yellow line D125 Basic homogeniser (SLS – Scientific Laboratory Supplies Ltd) while smaller samples under 150 mg were disrupted using a mini bead beater-24 (Biospec, Bartlesville, OK, USA). The total RNA pellet was dissolved in appropriate volume of DNA and RNA free nanopure H_20 to a concentration of 1000 - 1500 ng total RNA/µl. For all samples, concentration and quality of total RNA was checked by spectrophotometery (ND-1000 Nanodrop, Labtech Int., East Sussex, UK) and gel electrophoresis. For each sample, 5 µg of total RNA was treated with a DNase enzyme (DNA-free™: Applied biosystems, UK) according to manufacturer's instructions. cDNA was then reverse transcribed from 1 µg DNase treated RNA in a 20µl total reaction volume, using a high capacity reverse transcription

kit without RNase inhibiter (Applied biosystems, UK) according to manufactures instructions. All reactions were subsequently diluted 1/5 prior to qPCR.

Primer design and molecular cloning of gilthead seabream *kissr4* and *kiss2*

For both genes qPCR primer pairs were designed using Primer Select (Lasergene® DNASTAR) (Table 1) and tested by PCR Using Klear Taq polymerase with supplied buffer (Kbiosciences, UK), and 50 mM $MgCl_2$ as detailed in manufactures protocol. Cycling conditions were as follows: 15 min 95°C followed by 30 cycles of 95°C 20 s, X°C 20 s, 72°C 1 min, where X equates to the primer pair specific melting temperature, T_m (Table 1). All primer pairs generated a single PCR product. In order to generate qPCR standards for absolute quantification PCR products were cloned into a pGEM-T Easy vector (Promega, UK) and sequenced using a Beckman 8800 autosequencer (CEQ-8800 Beckman Coulter Inc., Fullerton, USA). Lasergene SEQman software (DNASTAR, www.dnastar.com) was used to edit and assemble DNA sequences. Products identities were verified using BLASTn http://www.ncbi.nlm.nih.gov/BLAST/) and showed 100% nucleotide identity.

Table 1

Primer name	Sequence (5' → 3')	Product size (bp)	T_m (°c)	Genebank ID
Teleost kissr4 F	TATGAGTGGAGACCGCTGTTACG	556	59	**JQ839286**
Teleost kissr4 R	CTATGGGGTTGACAGAGGAGTTG			
SBream kissr4 3out	TAATCGTCCTCCTCTTCGCCATCT	N/A	56	
SBream kissr4 3in	GCCCAACTACGCCACATACAAGA			
SBream kiss2 F	CTCTGGTCGTGGTGTGCGGG	310	58	
SBream kiss2 R	TCCTGGCTGTTTTAACTGCYCTYCT			
SBream kiss2 qPCR1F	TCAGGAGGAGCAGCGCAGGAGAGTT	91	66	
SBream kiss2 qPCR1R	CACAGGAGCTGCCGCTGGTCTTCAT			
SBream kissr4 qPCR1F	ATTGCTGCGTACCTGCTGCCTGTCC	95	66	
SBream kissr4 qPCR1R	TTGTCTACGGGCTCTACGGTG GGCT			
SBream βActin qPCR F	GACCCAACTGGGATGACATGG	171	60	**X89920**
SBream βActin qPCR R	GCATACAGGGACAGCACAGC			
SBream Gapdh qPCR F	TGCCCAGTACGTTGTTGACTCCAC	250	60	**DQ641630**
SBream Gapdh qPCR R	CAGACCCTCAATGATGCCGAAGTT			

Sequence identification and extension with RACE protocol

A 720bp *Sparus aurata kissr4* was cloned from brain tissue as follows: A 556 base pair sequence was obtained by PCR on seabream cDNA using generic *Teleost kissr4* primers (**JQ839286**, table 1) previously used in a variety of teleost species including Cod (Cowan et al., 2012) and seabass (Migaud et al., 2012) and MyTaq™ Mix (Bioline reagents ltd, London, UK) according to manufactures instructions. 3′ ends from the sequence generated were amplified using Rapid Amplification of cDNA Ends (RACE)-PCR as described by Betancor et al. (2014). RACE cDNAs were generated from 1 µg of seabream total RNA (mixed tissue origin) using the SMART RACE kit as described in the user manual (Clontech, Mountain View, CA). The 3′ RACE amplicons were generated by two rounds of PCR using *SBream kissr4 3out* and *3in* primer sets (table 1). The final 720bp sequence was confirmed by Blast (NCBI blastN).

RACE protocol for seabream *kissr4* 5' and *kiss2* was attempted, however no product was obtained. A 308 bp fragment for *kiss2* was generated from primers designed (*SBream kiss2 F* and *R* - table1) on *Sparus aurata* est *(***AM962676**). All PCRs were run at annealing temperatures as listed in table 1 with an extension time of 1 min/Kb of predicted PCR product, with 3 min applied for unpredictable RACE PCR products. All primers were designed using Primer Select Ver. 6.1 program (DNASTAR, www.dnastar.com). Sequencing was performed using a Beckman 8800 autosequencer and Lasergene SEQman software (DNASTAR) used to edit and assemble DNA sequences.

Phylogenetic trees and protein alignment

Phylogenetic trees were generated from a Clustal W alignment of deduced amino acid alignments of similar species and appropriate outliers using the neighbour joining method on in MEGA (Ver. 6) (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Kumar et al., 2004) and are in the units of the number of base substitutions per site. Protein alignments were generated using Kissr4 and Kiss2 translated protein sequences from a number of teleost species aligned by Clustal W in Bioedit sequence alignment editor (Ver.7.2.5).

kissr4 and *kiss2* Quantitative PCR (QPCR) assays

Expression of the target genes was measured by absolute quantification. In experiment 1 and in the first trial of experiment 2 (larvae ontogeny) *ß-actin* was used as

a reference gene while *Gapdh* was proven to be the most stable in the post-larvae samples. Both these genes have previously been verified as reliable and stable reference genes in seabream (Minghetti et al., 2010; Minghetti et al., 2011). The decision to use different reference genes was justified by the absence of significant differences between any points in each group of samples during the stability tests performed prior to qPCR analysis. All cDNAs for qPCR were synthesised as described previously and qPCR primers (Table 1) were used at a concentration of 0.7 pM, with 5µl cDNA synthesis reaction (at a concentration of 10 ng Total RNA / µl) and 10 µl ABsolute™ QPCR Mix, SYBR green (Thermo scientific, Leon-Rot, Germany). Additionally, 3 µl DNA/RNA free H_2O was added to each reaction to a total reaction volume of 20 µl. All qPCR assays were carried out in a Techne Quantica Realtime qPCR thermocycler (Bibby Scientific Ltd, Cambridge, UK) in a thermo cycling programme consisting of a 15 minute hot start at 95°C, followed by 45 cycles of 3 temperature steps: melt at 95ºC for 15 s, anneal at X°C (see Table 1 for target specific melting temperatures, T_m) for 15 s and extensions at 72ºC for 30 s. This was followed by a temperature ramp from 70 – 90°C for melt-curve analysis. Quantification was achieved by translating cycle threshold (CT) values of unknown samples from a parallel set of reactions containing a serial dilution of spectrophotometrically determined linearized plasmid containing partial cDNA sequences generated as described above. All samples were run in duplicate and each qPCR plate included non-template controls.

Data analysis

Statistical analysis and data plotting were performed using Microsoft Excel®, SPSS® and GraphPad®. Transcript levels of each target gene were normalised against the appropriate reference gene and absolute quantification results were expressed as means ± standard error of the means (SEM). All data sets were tested for normal distribution using the Shapiro-Wilk test (Zar, 1999). Normalised gene expression was then tested for significant differences among sampling points or groups using a one-way ANOVA, or a Kruskal-Wallis test when data did not follow a normal distribution. Analysis of variance were followed by Tuckey HSD or Dunn post-hoc tests, respectively. In the sex change trial, also a Student's *t* test was applied for comparisons between sexes in January sampling and between samplings within each sex. In all cases statistical significance was taken at $p<0.05$.

Results

Gene identification and sequencing of gilthead seabream *kissr4* and *kiss2*

A 720bp fragment was generated showing a high degree of identity with *kissr4* in other teleost species, having 97% identity with blackhead seabream (*Acanthopagrus schlegelii*) and 93% with Atlantic striped bass (*Morone saxatilis*) and European seabass (*Dicentrarchus labrax*) and 70% nucleotide identity to zebrafish (*Danio rerio*) (Fig. 1). The translated partial protein fragment contains 242 amino acids and importantly 41 amino acids of the predicted transmembrane protein domains 3-7 of 7 (Fig. 1). With regard to *kiss2* a 308 bp fragment was identified and as with *kissr4* it displayed the highest percentage of nucleotide identity with the blackhead seabream (97%) and only 62% with zebrafish and is distinct from the teleost *kiss1* clade (Fig. 2). The predicted translated protein sequence shows notable identity with red seabream, striped bass and European seabass and importantly also contains the decapeptide core *kiss-10* sequence that defines the gene *kiss2* (Fig. 2).

Experiment 1: Investigating the Kiss system during sex change in gilthead seabream

From the 20 animals used for the first sampling (October), 7 were identified as being males (with clear mature and functional testis), 5 as females (with clear mature and functional ovary) and 8 individuals possessed both female and male gonads, at similar proportion. In January only 3 males were identified, while the other 15 (out of 18 in total) were females, with there being no sex changing individuals present in the sample. Transcript levels for both targets was an order of magnitude higher in brain in contrast to gonad tissues (Fig. 3 and 4). The receptor *kissr4* (Fig. 3) showed comparable transcript levels in the brain irrespective of gender state in October (Fig. 3A), while in gonad, values were significantly higher in males when compared to females with individuals undergoing sex change being intermediate and statistically comparable to both (Fig. 3B, Kruskall-Wallis test, Dunn's post-hoc test $p<0.05$). Expression of this same gene was generally lower in January (reducing in the region of 13.96 – 93.81 %), with this decrease being significant only in between the female brain samples (59.58 %, Student's *t* test, $p<0.01$). In this second sampling, coincident with the beginning of the resting period, no statistical differences between males and females in either the brain or the gonad samples were found (Fig. 3C, D). With respect to *kiss2* expression, in October, male expression level was significantly higher when compared to females in both tissues studied with individuals undergoing sex change being intermediate and

statistically comparable to both males and females (brain, Fig. 4A, and gonad, Fig. 4B). While in January, transcript levels in brain were greater for females when compared to males (Fig. 4C), and comparable between sexes when measured in the gonad (Fig. 4D). When expression levels between the spawning season (October) and the beginning of the resting period (January) were compared, transcript number decreased in the region of 31.02 to 89.98 %. This reduction was significant in brains of both males and females as well as in male gonads (Student's *t* test, $p<0.05$).

Experiment 2: Kiss system ontogeny during larvae and post-larvae stages in gilthead seabream

The results of the first trial (larvae ontogeny, until 30 DPH) revealed that during larval development there was a similar profile of expression for both *kissr4* and *kiss2* genes: very low expression in eggs, embryos and post hatch larvae (0 DPH), and increasing significantly from 5 DPH onwards (Fig. 5). For *kissr4* the surge at 5 DPH was almost a twenty-fold increase, which was maintained during 10 and 20 DPH and then expression levels significantly decreased by *circa* 50% at 30 DPH (Fig. 5A). In the case of *kiss2*, the expression not only increased from 0 to 5 DPH (64-fold), it further doubled between 5 and 10 DPH. Thereafter, at 20 and 30 DPH, transcript level reduced to levels comparable to those observed at 5 DPH (One-way ANOVA, Tuckey HSD post-hoc test, $p<0.05$, Fig. 5B).

There was a differential response of both genes observed in the second ontogeny trial where samples extending to the post-larval stage (30 – 140 DPH). For *kissr4* and *kiss2* a significantly elevated peak in transcript level was observed at 90 and 60 DPH respectively (Fig. 6). For the receptor, transcript levels were similar during all sampling points from 30 to 75 DPH, significantly increasing at 90 DPH in relation to the first point (3-fold increase compared) before returning to levels comparable to the earlier stages. For *kiss2*, all sampling points showed similar levels of transcripts, with only the peak at 60 DPH (26-fold increase compared to the average abundance from all other points) being significantly higher (One-way ANOVA, Tuckey HSD post-hoc test, $p<0.05$, Fig. 6B).

Discussion

This research provides the first insight on the kisspeptin system in gilthead seabream, providing partial cDNA sequences which code for the isoforms of signal

peptide (*kiss2*) and its receptor (*kissr4*) that are widely considered to be the forms responsible for regulation of reproduction in teleosts (Akazome et al., 2010). Thereafter, the expression studies allude to an association in expression of this system with both early ontogenetic development as well as sex change in the species. As a whole, this work broadens our understanding of the role that the kisspeptin system plays in reproductive physiology in fish with the interaction in sex change in particular being largely un-investigated (Todd et al., 2016). Seabream are an important aquaculture species, with there being a considerable interest in controlling sex ratios therefore a better understanding of sex change and the neurochemical regulation has both scientific and significant commercial value.

Prior to this study the lack of seabream gene sequences represented a barrier to investigating the kisspeptin system in the species. For *kissr4* a 720bp product was detected showing high structural similarity with other teleost species including blackhead seabream, striped bass and European seabass and *in silico* analysis of the predicted translated protein sequence revealed the presence of highly conserved transmembrane domains (5 of 7 total), which are characteristic of Kissr4 (Cowan et al., 2012; Parhar et al., 2004). Similarly, the 308bp *kiss2* fragment identified for seabream displays a high level of nucleotide identity to red seabream, striped bass and European seabass. The translated protein sequence contains the highly conserved kisspeptin core sequence which had a 100% aa identity with that reported in striped bass and European seabass (Felip et al., 2009; Zmora et al., 2012). Out with the kisspeptin core sequence a lesser degree of conservation was observed with goldfish and zebrafish sequences, such a pattern is not uncommon with this gene as was previously reported in Atlantic cod (Cowan et al., 2012). While there is a lack of genome sequence information available in the public domain for the gilthead seabream, which negates our ability to identify additional kisspeptin transcripts or preform synteny analysis, the levels of sequence identity and structural conservation observed with other teleost species provides compelling evidence that these are the key transcripts for the species and as such represent a valuable resource to support subsequent research. In teleosts, various kisspeptin (*kiss1* and *kiss2*) and kisspeptin receptors (*kissr1*, *kissr2*, *kissr3*, *kissr4*) gene forms have been encountered, with variations among species (Ohga et al., 2018), but always with *kiss2* and *kissr4* having a functional significance in the control of reproduction (Akazome et al., 2010). This is in agreement with the results of the present study, however, the presence of other forms in seabream should not be ruled out.

Gilthead seabream are sequential protandrous hermaphrodites and during the sex change process, expression of both *kissr4* and *kiss2* was generally higher in October (spawning period), coinciding with a high number of changing individuals and thus at the climax of sex change. The low number of males and the absence of reverting fish observed in January (beginning of resting period), indicated that the sex change process had already finished for that breeding season, in agreement with the 80% of males Zohar et al. (1978) described as changing to females during the second year of life.

Transcript levels of both *kissr4* and *kiss2* in the brain were always higher in comparison to the gonads, as seen in other teleost species (Bohórquez et al., 2017; Felip et al., 2009; Shi et al., 2010), stressing an important signalling role of kisspeptin in this region, where the BPG axis activation begins (Zohar et al., 2010). This is in line with the presence of kisspeptin receptors in GnRH neurons in fish (Parhar et al., 2004; Servili et al., 2011). Also, the stability in *kissr4* expression between sexes in the brain, suggested the kisspeptin receptor to be equally active in all genders during sex change. In the gonad, in contrast, *kissr4* expression was higher in males in relation to females in October. In the same sampling, *kiss2* transcript levels were also higher in males in comparison to females in both tissues. Such elevated expression of *kissr4* and *kiss2* in males at the beginning of sex change might suggest that the kiss system has a participation in the induction of sex change in seabream. A similar role has already been proposed in another sequential hermaphrodite, the protogynous orange-spotted grouper (Shi et al., 2010), bringing about the idea that due to its control over GnRH, the kisspeptin signalling could have a regulatory role during sex change in fish, both for protandrous and protogynous species. The cues inducing sex changes are likely to be species-specific, however the underlying physiology has received little attention (Guiguen et al., 2010). Recent findings have associated estrogens (estradiol) and aromatase with the activation of natural sex change, as their decrease or increase triggers protogynous or protandrous sex change, respectively, the opposite being true for 11keto-testosterone (Guiguen et al., 2010; Liu et al., 2017). In relation to this, a regulatory effect of sex steroids over the kisspeptin system has also been proposed, as a gonadal steroid positive feedback control of reproduction (Alvarado et al., 2016). Considering that in our results, higher number of transcripts observed in October in males, corresponded mostly to spermiating specimens, could also indicate a steroid sensitivity of kisspeptin expression. All the above highlights the complexity of the mechanisms driving sex change in this hermaphroditic species, particularly considering

the overlap with the reproductive season, that makes it difficult to disentangle both effects. We believe that the kisspeptin system is very likely to play a role in the signalling of this process, along with other key players. However, to confirm such hypotheses, more research would be needed, focusing on the influence of blocking kisspeptin receptors using appropriate antagonists in relation to different developmental stages.

Mechaly et al. (2013) and more recently Ohga et al. (2018) reviewed the role of the kisspeptin system on pubertal development in fish, reporting high interspecies variation. However both reviews suggest that typically in teleosts *kissr2* expression was more elevated at early stages than in advanced stages of pubertal development. This process presents similarity with sex change if we consider that in both cases a new gonad is differentiating and maturing for the first time. Both processes are often accompanied by drastic morphological, physiological and even behavioural changes, leading to species-specific secondary sexual characters (Rousseau and Dufour, 2012; Todd et al., 2016), very likely using similar physiological pathways. In fact, as seen for puberty, GnRH signalling was suggested to be involved in sex change in gilthead seabream, as *gnrh-3* mRNA expression was increased around the time the gonad began to differentiate (Reyes-Tomassini, 2013), which aligns with the elevated *kissr4* and *kiss2* expression in the gonad samples of males in the current study, at the beginning of gonad differentiation. In view of the known role of the kisspeptin system in controlling puberty, it is also reasonable to suggest a similar role over sex change. In January, the results of *kiss2* expression slightly differed and expression was now higher in female's brain when compared to males, which could be related with species and gender specific differences in kisspeptin reproduction patterns, as seen in other species such as Senegalese sole or Atlantic cod (Cowan et al., 2012; Mechaly et al., 2012).

During larvae and post-larvae stages of the ontogeny study, both *kissr4 and kiss2* presented clear temporal patterns in expression, which helped pinpoint potentially significant developmental periods in gilthead seabream. The elevated peak of expression observed between 5 and 20 DPH for *kissr4* and at 10 DPH for *kiss2,* could be related with specific events of the early development of the reproductive axis in agreement with the conclusions of Ohga et al. (2018). Wong and co-authors (2004) proposed that the ontogeny and organisation of gilthead seabream reproductive axis, measured through the mRNA expression of *gnrhs* (*cgnrh-II*, *sgnrh* and *sbgnrh*) and other reproduction-related genes (*gnrhr*, *fshβ*, *lhr*, *fshr* and *vasa*), may start as early as 5

days post fertilization (DPF) (equivalent to 3 DPH), although transcripts of these genes were detected as early as 1 or 1.5 DPF, likely from maternal origin. According to these same authors (Wong et al., 2004), at least four concomitant increases in the level of some of the transcripts above mentioned (*gnrhs and gnrhr*, *fshβ*, *lhr*, *fshr* and *vasa)* were observed at 5, 8, 14, and 28 DPF (3, 6, 12 and 26 DPH), which is compelling evidence of synchronised events in the early ontogeny and organization of the reproductive axis. After 28 DPF gene expression remained elevated, showing a more stable development. These authors observed paired developing gonads (with few primordial germ cells) at 14 DPF, which grow but remained undifferentiated until 59 DPF (57 DPH). Comparisons should be made carefully between these data and our results since rearing conditions among trials are not exactly the same, yet, the temperature range used by Wong and colleagues (18-20ºC) is similar to the present study. The transcript level increases in reproduction related genes (Wong et al., 2004) appear to be coincident with the time range at which both *kissr4* and *kiss2* were highly expressed in the present work, which could indicate a parallel ontogeny of the kisspeptin system and the early development of the reproductive axis in gilthead seabream. Furthermore, the evident elevations in expression in post larval stages at 60 and 90 DPH, for *kissr4* and *kiss2* respectively are coincident with gonadal differentiation and/or germ cell proliferation in the species suggesting that there are multiple possible roles for the kisspeptin system within early ontogeny as suggested by Ohga *et al.* (2018). There is scarce information available on the timing of specific developmental events in early ontogeny of seabream to corroborate such hypothesis but this work provides a new aspect to such research that should be further explored. For example, an association between gonadal development and the kisspeptin system has been reported in fathead minnow, *Pimephales promelas* (Filb*y et al.* 2008). In this species, a peak in *kissr4* (referred to as *kiss1r* by the authors) expression in the brain at 60 DPF was associated to the onset of meiosis and the formation of the lobules in the testis (Filby et al., 2008). In other teleosts (e.g. cobia, chub mackerel or tilapia), the early expression of *kissr* and *kiss* transcripts was seen to be parallel with rises in expression of GnRH genes, at earlier or later stages of ontogeny, pointing to a close association between kisspeptin genes and multiple GnRHs during reproductive development (Martinez-Chavez et al., 2008; Mohamed et al., 2007; Ohga et al., 2015; Park et al., 2012; Selvaraj et al., 2015). On the other hand, in model species like medaka or zebrafish, gene knockout and knockdown trials during early development have

suggested alternative functional roles. Hodne et al. (2013) suggested a critical role of kisspeptin and the respective receptors in neurulation, morphogenesis and embryonic survival in medaka, while Tang et al. (2015) described the *kiss/kissr* signaling as not absolutely required for zebrafish reproduction. Though, Zhao et al. (2014) described that Kiss1(but not Kiss2) stimulated proliferation of terminal nerve and hypothalamic populations of GnRH3 neurons in the central nervous system. These opposing results in relation to the roles of the kisspeptin system in teleosts ontogeny suggests this research to be still its infancy, and reflects the complexity of the neuroendocrine interactions occurring during early development of organs and structures in larvae. Based on all the above, we may state that the seabream kisspeptin system could be a useful biomarker to explore the regulation of larval stages ontogeny, namely of the reproductive axis, given its very prompt signalling response. Future localization studies utilising the sequence data generated by the current work could help to define other roles of the kisspeptin system and confirm such hypotheses.

Conclusions

The present work represents the first investigation aiming to identify and explore the functional role of the kisspeptin system in physiological pathways in gilthead seabream, during two distinct periods of its life cycle in captivity. The results could be indicative of a participation of the kisspeptin system, along with other key players, in the complex mechanisms driving the protandrous sex change, in a similar but opposing manner to that reported in protogynous teleosts. Although more research is required *e.g.* localisation of neurons & pharmacological studies of the receptor, the current sequence information and expression data provides a new perspective that could improve our understanding of sex change in gilthead seabream. The kisspeptin system is also thought to be involved in early ontogeny of the reproductive axis in teleosts. The current results allude to a similar role in seabream, pointing to the potential to use the kisspeptin system as a biomarker for larval development in future studies.

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Figure Captions

Table 1. List of primers used for cDNA cloning and quantitative real-time PCR standards with sequence and melting temperature (T_m) of studied genes.

Figure 1a. 720bp nucleotide and deduced amino acid sequence of gilthead seabream *kissr4* 3' RACE product containing predicted transmembrane domains, 3-7 of 7, as described by Cowan et al. (2012) and Parhar et al. (2004) and shown in bold and underlined.

Figure 1b. ClustalW alignment of teleost KissR4 protein sequences including the following Kissr4 protein sequences:

Common Name	**Scientific Name**	**Accession number**
blackhead seabream	*Acanthopagrus schlegelii*	**ALQ81855.1**
striped bass	*Morone saxatilis*	**ADU54205.1**
European seabass	*Dicentrarchus labrax*	**AFK84356.1**
goldfish	*Carassius auratus*	**ACK77792.1**
zebrafish	*Danio rerio*	**NP_001099149.2**
Xenopus	*Xenopus tropicalis*	**NP_001165296.1**

Transmembrane domains numbers 3-7 are shown in boxed regions and conserved amino acid regions are shaded.

Figure 1c. Phylogenetic Tree of teleost Kissr 4 proteins

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 14 amino acid sequences from Kissr4 proteins in a number of teleost's and relevant outliers. In addition to species described in **fig.1b** analysis also included the following Kissr4 / Kissr1 proteins:

Common Name	**Scientific Name**	**Accession number**
Atlantic croaker	*Micropogonias undulatus*	**ABC75101.1**
long tooth grouper	*Epinephelus bruneus*	**AEN14599.1**
Korean rockfish	*Sebastes schlegelii*	**AIZ68244.1**
yellowtail amberjack	*Seriola lalandi*	**ACT78955.2**

human	*Homo sapiens*	**NP_115940.2**
mouse	*Mus musculus*	**NP_444474.1**

All positions containing gaps and missing data were eliminated. There were a total of 231 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Figure 2a. 308bp nucleotide and deduced amino acid sequence of gilthead seabream *kiss2*. The DNA sequence displayed 100% nucleotide identity to gilthead seabream EST (**AM962676**). Kisspeptin – core sequence is underlined and in bold in figure.

Figure 2b. Kiss2 protein sequences aligned by ClustalW for gilthead seabream along with the following species:

Common Name	**Accession number**
striped bass	**ADU54201.1**
European seabass	**ACM07423.1**
goldfish	**ACS34769.1**
zebrafish Kiss1	**ABV03802.1**
zebrafish Kiss2	**NP_001136057.1**

Conserved amino acid regions are shaded and Kisspeptin-10 epitope boxed within the in figure.

Figure 2c. Phylogenetic Tree of teleost Kiss 1 and 2 proteins.

The evolutionary history of Kiss2 was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 17 amino acid sequences from Kiss1 and Kiss2 proteins in a number of teleost's and relevant outliers. In addition to species described in **fig. 2b** analysis also included the following sequences for Kiss2 and Kiss1:

Common Name	**Scientific Name**	**Accession number**
Red seabream Kiss2	*Pagrus major*	**BAL44206.1**
medaka Kiss1		**NP_001116393.1**
medaka Kiss2		**BAG86623.1**

western clawed frog Kiss1A	*Xenopus tropicalis*	**ACJ50538.1**
western clawed frog Kiss1B		**NP_001163986.1**
western clawed frog Kiss2		**NP_001156332.2**
European seabass Kiss1		**ACM07422.1**
European seabass Kiss2		**ACM07423.1**
striped bass Kiss1		**ADU54200.1**
striped bass Kiss2		**ADU54201.1**
zebrafish Kiss1		**NP_001106961**
zebrafish Kiss2		**NP_001136057**
goldfish Kiss1a		**ACK77790.1**
goldfish Kiss1b		**ACK77791.1**
mouse Kiss1		**NP_839991.2**
human Kiss1		**NP_002247.3**

All positions containing gaps and missing data were eliminated. There were a total of 73 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Kumar et al., 2004).

Figure 3. *kissr4* transcript levels in brain (A and C) and gonad (B and D) in a group of gilthead seabream with males, females and individuals undergoing sex change at two different moments of the reproduction season: (A and B) in October, full spawning, and (C and D) in January, beginning of the resting period (values expressed as mean ± S.E.M.). Different letters indicate groups with statistical significant differences (Kruskal-Wallis test, $p<0.05$).

Figure 4. *kiss2* transcript levels in brain (A and C) and gonad (B and D) in a groups of gilthead seabream with males, females and individuals undergoing sex change at two different moments of the reproduction season: (A and B) in October, full spawning, and (C and D) in January, beginning of the resting period (values expressed as mean ± S.E.M.). Different letters indicate groups with statistical significant differences (ANOVA, Tuckey HSD, October sampling and Student's t test, January sampling, $p<0.05$).

Figure 5. Ontogeny of *kissr4* (A) and *kiss2* (B) expression in gilthead seabream eggs at gastrula and embryo stage (pre-hatch), and larvae during early development until 30

DPH (values expressed as mean ± S.E.M.). Letters a, b, c indicate groups with statistical significant differences between development stages (ANOVA, Tuckey HSD, $p < 0.05$).

Figure 6. Ontogeny of *kissr4* (A) and *kiss2* (B) expression in gilthead seabream post-larvae during development from 30 till 140 DPH (values expressed as mean ± S.E.M.). Letters a, b indicate groups with statistical significant differences between development stages (ANOVA, Tuckey HSD, $p < 0.05$).

776 Figure 1a

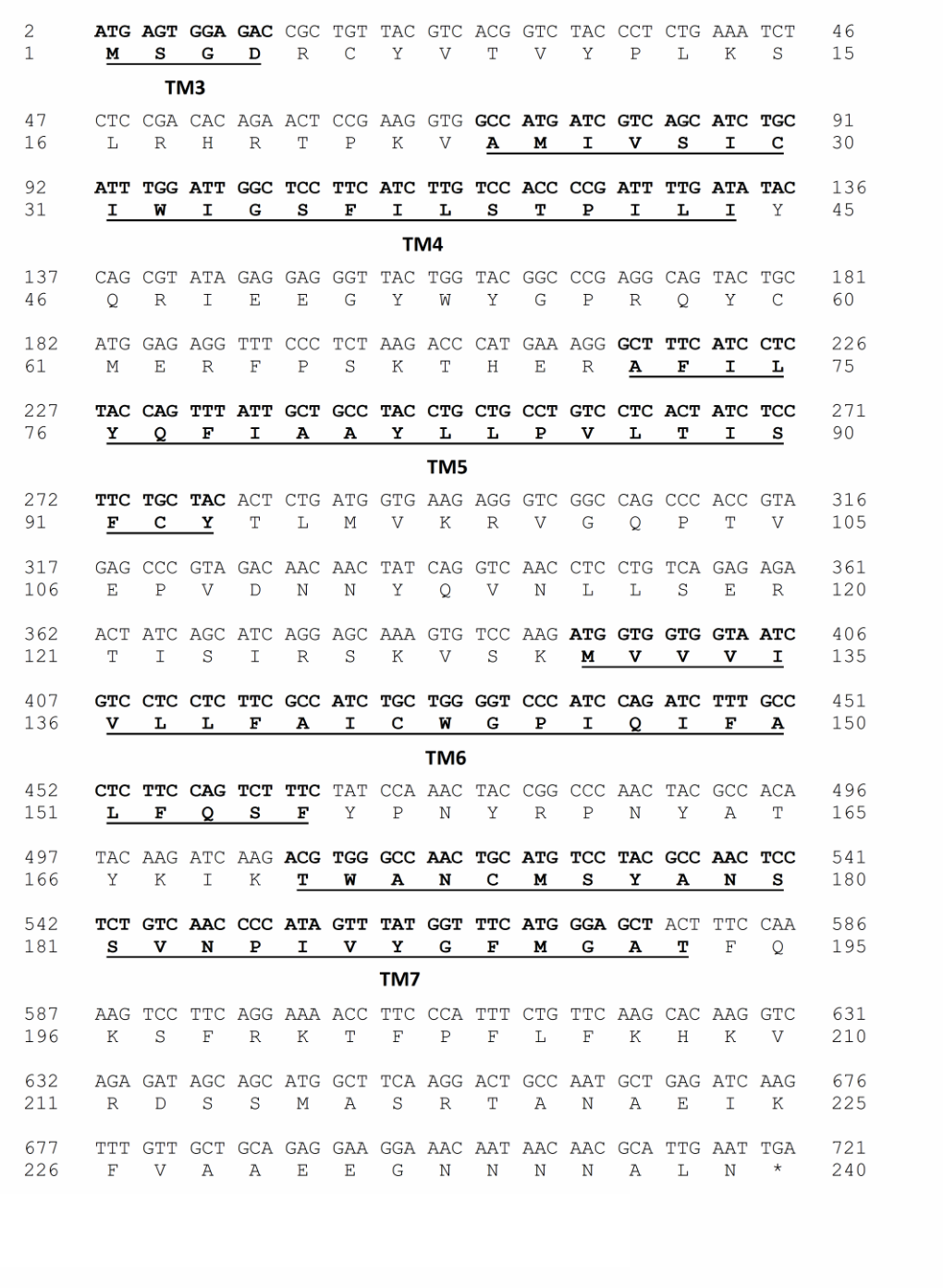

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Figure 1b

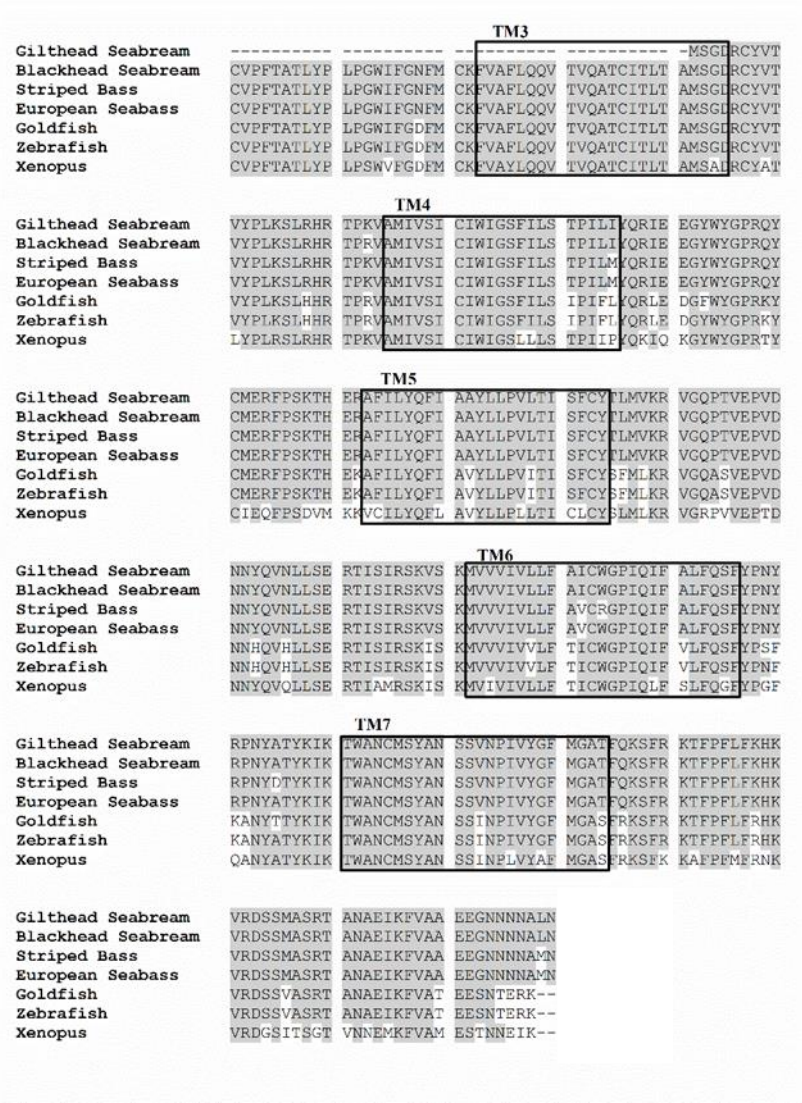

TM3
TM4
TM5
TM6
TM7
Gilthead Seabream
Blackhead Seabream
Striped Bass
European Seabass
Goldfish
Zebrafish
Xenopus

Figure 1c

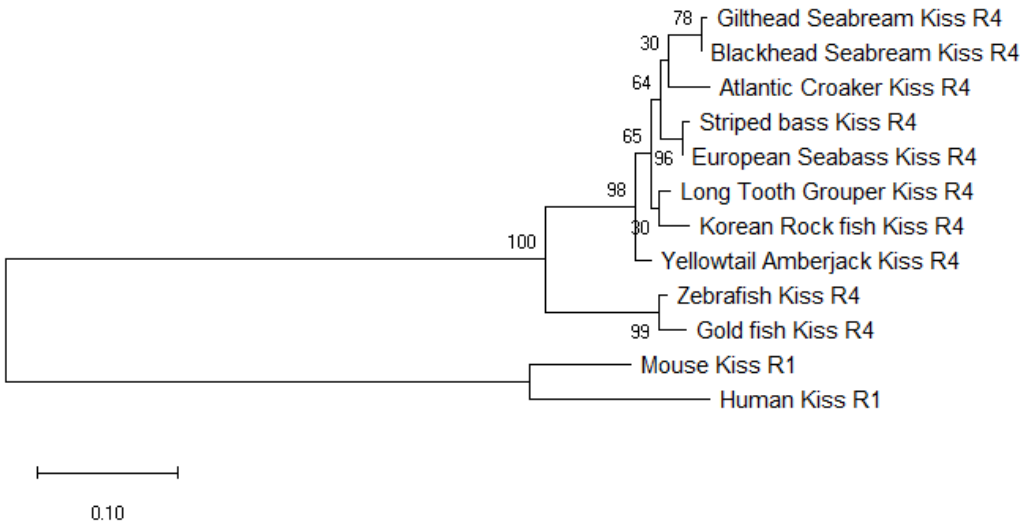


Figure 2a

```
3     CTG GTC GTG GTG TGC GGG CTG ATT GTT GGT GAG GAT GGA GGA AGC   47
1      L   V   V   V   C   G   L   I   V   G   E   D   G   G   S    15

48    GTG GGA GCA GCT CTG CCA GGA TTT GAC TCT GCA CAG AGG ACA CAT   92
16     V   G   A   A   L   P   G   F   D   S   A   Q   R   T   H    30

93    GTG ACA GGA TCA GTC CTC TCA GCA CTC AGG AGG AGC AGC GCA GGA   137
31     V   T   G   S   V   L   S   A   L   R   R   S   S   A   G    45

138   GAG TTT TTG GCA GAG GAT TCC AAC CCC TGT TTC TCC CTG AGA GAG   182
46     E   F   L   A   E   D   S   N   P   C   F   S   L   R   E    60

183   AAT GAA GAC CAG CGG CAG CTC CTG TGC AAC GAC CGC AGG AGT AAA   227
61     N   E   D   Q   R   Q   L   L   C   N   D   R   R   S   K    75

228   TTC AAC TTC AAC CCG TTC GGC CTC CGC TTT GGG AAA CGC TAC AAC   272
76     F   N   F   N   P   F   G   L   R   F   G   K   R   Y   N    90

273   GGC TAC ATT TAC AGR AGA GCA GTT AAA ACA GCC AGG AA-    311
91     G   Y   I   Y   X   R   A   V   K   T   A   R   X    103
```

795 Figure 2b

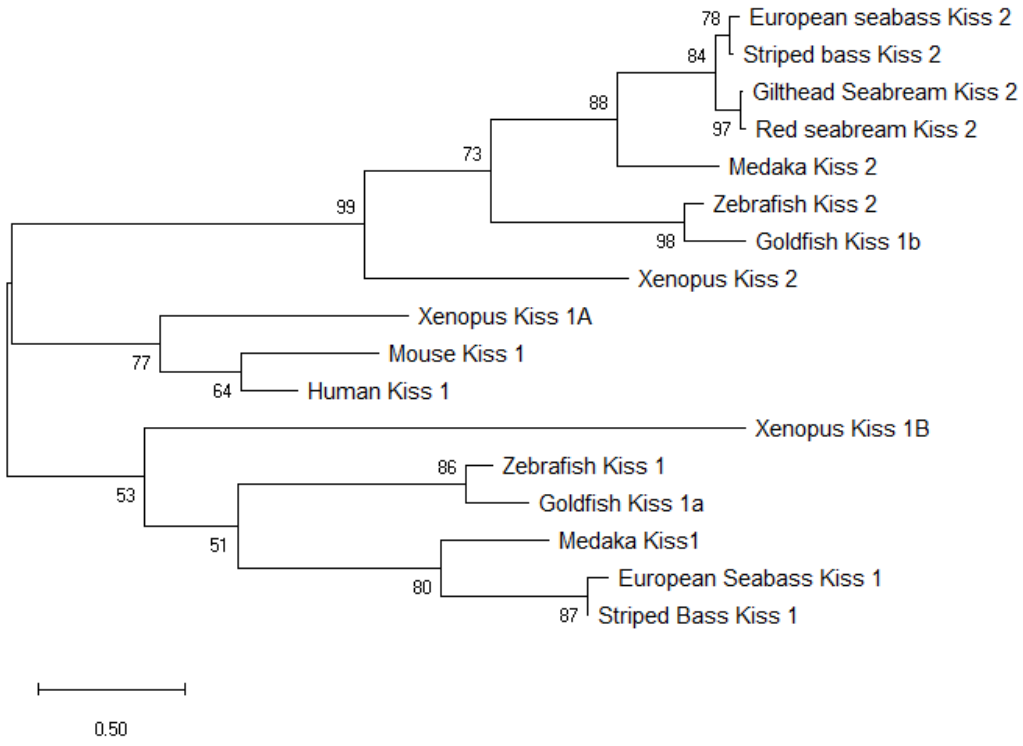
```
Gilthead Seabream     -----LVVVC GLIVGEDGGS VGAALPGFDS AQRTHVTGS- --VLSALRRS
Striped Bass          MRLVALVVVC GLIVGQDGGS MGAALPGLDS AQRTGATGS- --LLSALRRR
European Seabass      MRLVALVVVC GLILGQDGGS VGAALPELDS AQRTGATGS- --LLSALRRR
Goldfish              MKIKALILFM SAMICQS-TA LRASFTDMDI SDSEPVPDSK QHYLSVERRQ
Zebrafish Kiss2       MNTRALILFM SAMVSQS-TA MRAILTDMDT P--EPMPDPK PRFLSMERRQ
Zebrafish Kiss1       MMLLTVMLML SVVRVHT-NP SGHFQYYLED ETPEETSLR- --VLRGTDTR

Gilthead Seabream     SAGEFLAEDS NPCFSLRENE DQ-------R QLLCNDR--R SKFNFNPFGL
Striped Bass          TAGEFFGEDS SPCFSLRENE EQ-------R QLLCNDR--R SKFNFNPFGL
European Seabass      TAGEFFGEDS SPCFSLRENE EQ-------R QLLCNDR--R SKFNFNPFGL
Goldfish              FDEPSSSDDA SLCFFFQEKD ES-------T HISCQHRLPR SKFNYNPFGL
Zebrafish Kiss2       FEEPSASDDA SLCFFIQEKD ET-------S QISCKHRLAR SKFNYNPFGL
Zebrafish Kiss1       PTDGSPPSKL SALFSMGAGH QKNTWWWSPE SPYTKRRQNV AYYNLNSFGL

Gilthead Seabream     RFGKRYNGYI YXRAVKTARX ---------- -------
Striped Bass          RFGKRY---I YRRALKRART NKFSPLSLFS RELEVPI
European Seabass      RFGKRY---I YRRALKRART NRFSPLFLFS RELEVPT
Goldfish              RFGKRNE--A PTD-----RP KHLLPMMIYL RKQSETS
Zebrafish Kiss2       RFGKRNE--A TTSDSDRLKH KHLLPMMLYL RKQLETS
Zebrafish Kiss1       RYGKREQD-M LTRLIQKSPV K--------- -------
```

796

797 Figure 2c



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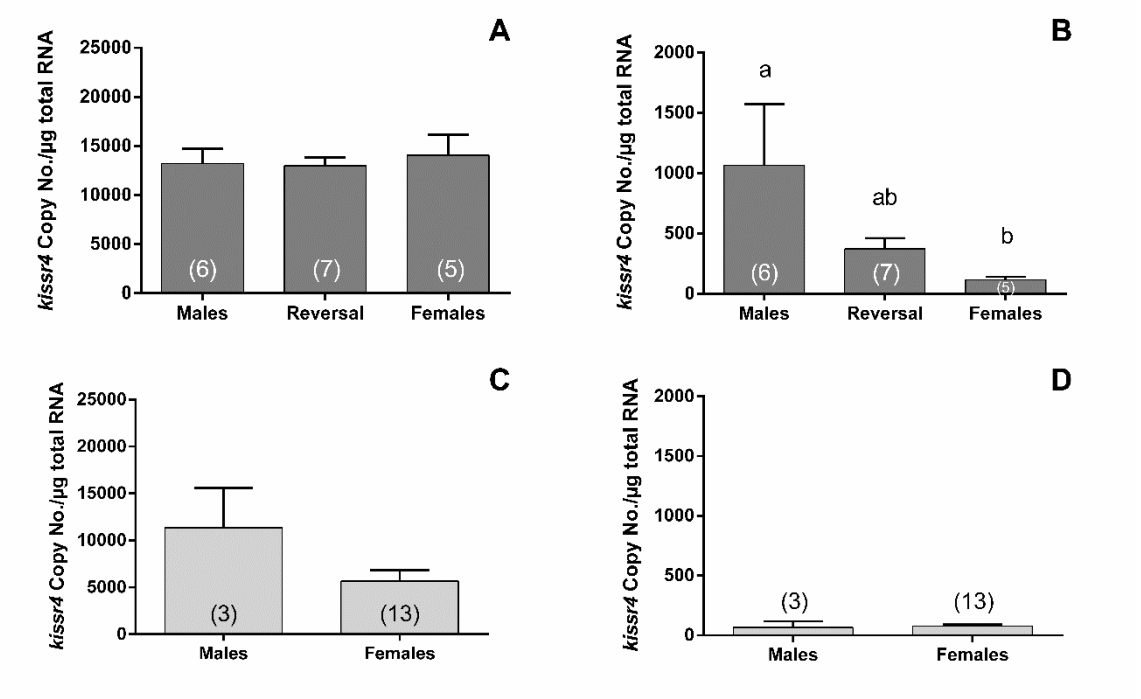
799 Figure 3



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801 Figure 4

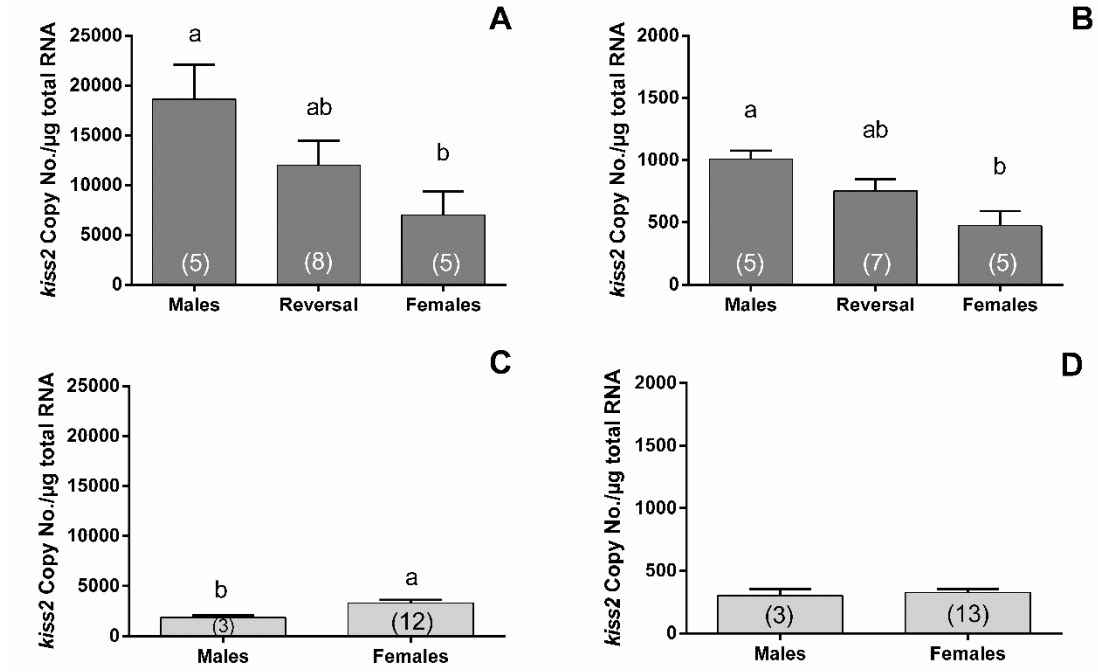

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Figure 5

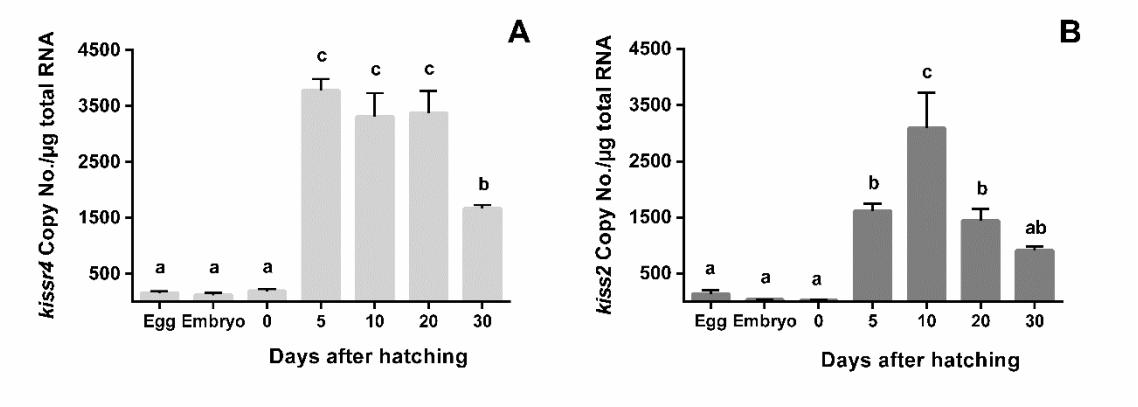

Figure 6