

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

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16 **Abstract.** The kisspeptin system, a known regulator of reproduction in fish, was
17 investigated during two key phases within the gilthead seabream (*Sparus aurata*) life
18 cycle: protandrous sex change and larval ontogeny. Seabream specific partial cDNA
19 sequences were identified for two key targets, *kissr4* and *kiss2*, which were
20 subsequently cloned and qPCR assays developed. Thereafter, to examine association in
21 expression with sex change, a group of adult seabream (2+ years old) undergoing sex
22 change were sampled for gene expression at two different periods of the annual cycle.
23 To study the kisspeptin system ontogeny during early life stages, transcript levels were
24 monitored in larvae (till 30 days-post-hatch, DPH) and post-larvae (from 30 till 140
25 DPH). During sex change, higher expression of *kissr4* and *kiss2* was observed in males
26 when compared to females or individual undergoing sex change, this is suggestive of
27 differential actions of the kisspeptin system during protandrous sex change. Equally,
28 variable expression of the kisspeptin system during early ontogenic development was
29 observed. The higher expression of *kissr4* and *kiss2* observed from 5 DPH, with
30 elevations at 5-20 and 90 DPH for *kissr4* and at 5, 10, 20, and 60 DPH for *kiss2*, is
31 coincident with the early ontogeny of *gnrh* genes previously reported for seabream, and
32 possibly related with early development of the reproductive axis in this species.
33
34 **Additional keywords:** sex change, *kissr4*, *kiss2*, protandric hermaphroditism, ontogeny

35 **Introduction**

36 The discovery of kisspeptin as a key regulator system of puberty and
37 reproduction in mammals has been a major breakthrough in the field (Terasawa et al.,
38 2013). This system has been reported as part of the seasonal control of reproduction,
39 apparently being the missing link between the major photo transducer structure
40 (pineal/melatonin system) and the Brain-Pituitary-Gonad (BPG) axis (Li et al., 2015;
41 Revel et al., 2007). It is known that kisspeptin, acting centrally via the kisspeptin
42 receptor, stimulates GnRH neurons in the hypothalamus to release GnRH, causing the
43 release of gonadotropins from the pituitary (Clarke et al., 2015; Zohar et al., 2010).
44 Research in this field is far more advanced in mammals, nevertheless, several studies
45 have recently emerged in fish, suggesting a major role of the kisspeptin system in the
46 regulation of the gonadotropic axis, especially in timing of puberty and control of
47 gonadotropin secretion (Cowan et al., 2017a; Cowan et al., 2012; Filby et al., 2008;
48 Zmora et al., 2015), with two paralogous genes (*kiss1* and *kiss2*) identified (Mechaly et
49 al., 2013; Migaud et al., 2012). Kisspeptins are ligands for the receptor Kissr
50 (previously called GPR54), with four paralogous genes identified in vertebrates, but
51 only two encountered in teleosts: *kissr2* and *kissr4* (Migaud et al., 2012; Zohar et al.,
52 2010). Among these two, *kissr4* is apparently the most predominant and functionally
53 active form, being present in many fish species (Akazome et al., 2010).

54 The gene *kiss2* appears to have a predominant role in the control of fish
55 reproduction (Akazome et al., 2010; Felip et al., 2009). Nevertheless, due to the variety
56 in reproductive strategies seen in teleosts, the reported reproductive roles and
57 distributions of the two kisspeptin forms and their receptors can vary (Kitahashi et al.,
58 2009; Li et al., 2009; Selvaraj et al., 2013; Yang et al., 2010; Zmora et al., 2015).
59 However, a clear relationship between the kisspeptin system and the annual
60 reproductive cycle has been reported both in Senegalese sole (*Solea senegalensis*) and
61 in European seabass (*Dicentrarchus labrax*) (Cowan et al., 2017b; Mechaly et al., 2012;
62 Migaud et al., 2012), suggesting conservation of its role in the seasonal control of
63 reproduction, as reported in mammals. Indeed, this system has been suggested to
64 integrate both environmental cues and metabolic signals in fish, as well as in mammals,
65 transducing this information onto the reproductive axis (Zohar et al., 2010). With
66 respect to the integration of environmental signals, there is evidence in both seasonal
67 species like European sea bass (Alvarado et al., 2015; Cowan et al., 2017b; Espigares et

68 al., 2017), and Atlantic salmon (*Salmo salar*) (Chi et al., 2017) as well as tropical
69 species like Nile Tilapia (*Oreochromis niloticus*), (Martinez-Chavez et al., 2008)
70 Recent studies have also proposed a role of the kisspeptin system in early
71 development and gonadal sex differentiation in some fish species (e.g. cobia,
72 *Rachycentron canadum*, Mohamed et al. (2007); Nile Tilapia, Park et al. (2012);
73 pejerrey, *Odontesthes bonariensis*, Bohórquez et al. (2017); Chub mackerel, Selvaraj et
74 al. (2015)). During cobia ontogeny, *kissr4* was highly expressed very early in larvae, in
75 parallel with *gnrh* expression (Mohamed et al., 2007). In the other three species,
76 expression of the kisspeptin system was observed to be elevated in periods coinciding
77 with sex differentiation, indicating a potential role of these genes in such process,
78 though similar information regarding species with sequential hermaphroditism is very
79 limited and requires further research (Todd et al., 2016). Interestingly, in the pejerrey, a
80 pleiotropic effect has even been proposed, related with mediation of olfactory and visual
81 signals (Bohórquez et al., 2017). All of these results eludes to a significant central role
82 of the kisspeptin system in early fish development, however the functional mechanisms
83 are still unclear.

84 The gilthead seabream, *Sparus aurata*, is one of the most important species for
85 Mediterranean aquaculture. It is a protandric hermaphrodite species, maturing first as
86 male (during the first or second reproductive cycles) before undergoing sex change so
87 that after the second or third reproductive cycles, almost all individuals will be
88 functional mature females (Liarte et al., 2007; Zohar et al., 1978). It has been proposed
89 that the kiss system is likely to be involved in fish sex change processes, based on the
90 example of the orange-spotted grouper (*Epinephelus coioides*) (Shi et al., 2010; Todd et
91 al., 2016), but it remains to be investigated in seabream. Equally, while the early
92 ontogeny of the GnRH system and reproductive axis has been described, with
93 expression of related genes being detected very early in development (Wong et al.,
94 2004), no information is available about the kisspeptin system during early ontogeny.
95 With all this in mind, this study intends to identify in gilthead seabream *kissr4* and
96 *kiss2*, the two forms which have been suggested to be functionally important in fish, and
97 further investigate a possible involvement in sex change and early life stages of
98 development: larvae and post-larvae.

99

100 **Materials and methods**

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

108

109 Ethical statement

110 Experimental procedures were conducted in accordance with ARRIVE
111 guidelines (Kilkenny et al., 2010), with directives 86/609/EU and 2010/63/EU of the
112 European Parliament and Council, and Portuguese legislation for the use of laboratory
113 animals (PORT 1005/92) of the Portuguese direction for veterinary and food services
114 (Direção-geral de alimentação e veterinária, DGAV). All persons involved in the animal
115 trials have a FELASA class C permit for animal experimentation and CCMAR facilities
116 are authorized by DGAV for animal experimentation (permit number
117 0421/000/000/2013).

118

119 Animals and housing

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

126 In the first trial of experiment 2, gilthead seabream larvae were reared at
127 CCMAR experimental facilities until the age of 30 DPH. Standard rearing protocols for
128 this species were used in accordance with Moretti (1999). Eggs were incubated in a 100
129 L fibreglass cylindroconical tank for 48 hours. Newly hatched larvae were transferred to
130 3 similar tanks (100 L) at a density of approximately $100 \text{ larvae L}^{-1}$. Larvae were fed
131 with enriched rotifers (*Brachionus plicatilis* enriched with Easy DHA Selco, INVE,
132 Belgium) from the onset of exogenous feeding (3 DPH) until 11 DPH. From 12 until 21
133 DPH they were co-fed with rotifers and *Artemia* nauplii and from 22 to 30 DPH, with
134 solely *Artemia* nauplii. Fish were kept at $19 \pm 1^\circ\text{C}$, 35‰ salinity, dissolved oxygen

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

137 The second trial took place in CULMAREX aquaculture facilities from 30 till
138 140 DPH and under standard commercial rearing conditions. Larvae were weaned at the
139 age of 80 DPH using a commercial diet (Gemma Micro, Skretting, Norway). Larvae
140 were reared at 20°C and exposed to a photoperiod of 13 h light and 11 h of darkness.

141

142 Experimental design

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

144 The study group consisted of males, females, and males during sex change.
145 Samples of brain and gonads were collected at two different stages of the reproductive
146 season (n=20 total animals at each sampling); in October, during full spawning and
147 January, at the beginning of the resting period. Seabream were individually sacrificed
148 with an overdose of 2-phenoxyethanol (1000 ppm) and immediately dissected. Sex was
149 firstly determined by striping the fish and identifying the presence of sperm or oocytes.
150 When this was not possible, namely during the resting period, the functional gonad was
151 determined by macroscopic or microscopic observation, depending on developmental
152 stage during dissection. After this evaluation, gonads were excised and a small piece
153 was cut in half, one part for total RNA extraction and the other for histological
154 confirmation of gonadal development in accordance with Brusléa-Sicard and Fourcault
155 (1997) and Somarakis et al. (2013). Haematoxylin and eosin staining technique was
156 used in 5-mm sections to determine the maturation status of testis and ovaries,
157 according to Pacchiarini et al. (2013). Individuals were subsequently classified as males
158 or females. When both testis and ovary were present in the same fish and at equal stage
159 of development (no predominant functional gonad could be recognised) individuals
160 were identified as sex changing (Zohar et al., 1978). The whole brain and 300 mg gonad
161 were collected from male and female individuals. For individuals undergoing sex
162 change, a combination of both testis and ovary was collected at a proportion of 1:1 (150
163 mg for each). Dissection was performed under RNase-free conditions to avoid
164 contamination of the samples. Tissue samples were immediately frozen in liquid
165 nitrogen and stored in -80°C to avoid RNA degradation.

166

167 Experiment 2: Kiss system ontogeny during larvae and post-larvae stages in gilthead 168 seabream

169 In the first ontogeny trial developed at CCMAR facilities, samples were
170 periodically taken to further assess *kissr4* and *kiss2* transcript levels. Egg samples (circa
171 100 per aliquot) were collected in the morning after the spawning event (gastrula stage),
172 and also prior to hatching (embryo stage). Larvae samples were collected at 0, 5, 10, 20
173 and 30 DPH at 11:00 am, to avoid temporal differences in gene expression. Samples
174 were rinsed with Milli-Q water and immediately frozen in liquid nitrogen. All steps
175 were carried out in RNase-free conditions. For 0 and 5 DPH ca. 20 larvae were pooled
176 per Eppendorf, while from 10 to 30 DPH, this number was reduced to 10/15 larvae per
177 aliquot.

178 To assess ontogeny of the kiss system including post-larval stages a second
179 batch of larvae was monitored from 30 till 140 DPH in the facilities of CULMAREX
180 company. Larvae samples were collected from 30 to 140 DPH (30, 45, 60, 75, 90, 105,
181 120 and 140 DPH), always in the morning. From 30 to 60 DPH full larvae were pooled
182 in the same sample (10 per aliquot), while from 75 until 140 DPH, only heads were
183 collected and pooled in cryovials containing RNA-later® (5 heads per aliquot). Post-
184 larvae previously anaesthetised with MS-222 (100 mg/L) and sacrificed by decapitation.
185 Once more, all steps were carried out in RNase-free conditions and samples were
186 immediately frozen.

187

188 Molecular biology analyses

189 RNA extraction, DNase treatment and cDNA synthesis

190 All RNA extractions were carried out at a ratio of 100 mg tissue per ml TRI
191 reagent (Sigma-Aldrich, St Louis, MO USA) according to manufacturer's instructions.
192 Larger tissue samples were homogenised using Yellow line D125 Basic homogeniser
193 (SLS – Scientific Laboratory Supplies Ltd) while smaller samples under 150 mg were
194 disrupted using a mini bead beater-24 (Biospec, Bartlesville, OK, USA). The total RNA
195 pellet was dissolved in appropriate volume of DNA and RNA free nanopure H₂O to a
196 concentration of 1000 - 1500 ng total RNA/μl. For all samples, concentration and
197 quality of total RNA was checked by spectrophotometry (ND-1000 Nanodrop, Labtech
198 Int., East Sussex, UK) and gel electrophoresis. For each sample, 5 μg of total RNA was
199 treated with a DNase enzyme (DNA-free™: Applied biosystems, UK) according to
200 manufacturer's instructions. cDNA was then reverse transcribed from 1 μg DNase
201 treated RNA in a 20μl total reaction volume, using a high capacity reverse transcription

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

204

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

206 For both genes qPCR primer pairs were designed using Primer Select
 207 (Lasergene® DNASTAR) (Table 1) and tested by PCR Using Klear Taq polymerase
 208 with supplied buffer (Kbiosciences, UK), and 50 mM MgCl₂ as detailed in
 209 manufactures protocol. Cycling conditions were as follows: 15 min 95°C followed by
 210 30 cycles of 95°C 20 s, X°C 20 s, 72°C 1 min, where X equates to the primer pair
 211 specific melting temperature, T_m (Table 1). All primer pairs generated a single PCR
 212 product. In order to generate qPCR standards for absolute quantification PCR products
 213 were cloned into a pGEM-T Easy vector (Promega, UK) and sequenced using a
 214 Beckman 8800 autosequencer (CEQ-8800 Beckman Coulter Inc., Fullerton, USA).
 215 Lasergene SEQman software (DNASTAR, www.dnastar.com) was used to edit and
 216 assemble DNA sequences. Products identities were verified using BLASTn
 217 (<http://www.ncbi.nlm.nih.gov/BLAST/>) and showed 100% nucleotide identity.

218

219 Table 1

Primer name	Sequence (5' → 3')	Product size (bp)	T _m (°C)	Genebank ID
<i>Teleost kissr4 F</i>	TATGAGTGGAGACCGCTGTTACG	556	59	<u>JQ839286</u>
<i>Teleost kissr4 R</i>	CTATGGGGTTGACAGAGGAGTTG			
<i>SBream kissr4 3out</i>	TAATCGTCCTCCTCTTCGCCATCT	N/A	56	
<i>SBream kissr4 3in</i>	GCCCAACTACGCCACATAACAAGA			
<i>SBream kiss2 F</i>	CTCTGGTCGTGGTGTGCGGG	310	58	
<i>SBream kiss2 R</i>	TCCTGGCTGTTTTAACTGCYCTYCT			
<i>SBream kiss2 qPCR1F</i>	TCAGGAGGAGCAGCGCAGGAGAGTT	91	66	
<i>SBream kiss2 qPCR1R</i>	CACAGGAGCTGCCGCTGGTCTTCAT			
<i>SBream kissr4 qPCR1F</i>	ATTGCTGCGTACCTGCTGCCTGTCC	95	66	
<i>SBream kissr4 qPCR1R</i>	TTGTCTACGGGCTCTACGGTG GGCT			
<i>SBream βActin qPCR F</i>	GACCCAACCTGGGATGACATGG	171	60	<u>X89920</u>
<i>SBream βActin qPCR R</i>	GCATACAGGGACAGCACAGC			
<i>SBream Gapdh qPCR F</i>	TGCCCAGTACGTTGTTGACTCCAC	250	60	<u>DQ641630</u>
<i>SBream Gapdh qPCR R</i>	CAGACCCTCAATGATGCCGAAGTT			

220

221

222 Sequence identification and extension with RACE protocol

223 A 720bp *Sparus aurata kissr4* was cloned from brain tissue as follows: A 556
224 base pair sequence was obtained by PCR on seabream cDNA using generic *Teleost*
225 *kissr4* primers (**JO839286**, table 1) previously used in a variety of teleost species
226 including Cod (Cowan et al., 2012) and seabass (Migaud et al., 2012) and MyTaq™
227 Mix (Bioline reagents ltd, London, UK) according to manufactures instructions. 3' ends
228 from the sequence generated were amplified using Rapid Amplification of cDNA Ends
229 (RACE)-PCR as described by Betancor et al. (2014). RACE cDNAs were generated
230 from 1 µg of seabream total RNA (mixed tissue origin) using the SMART RACE kit as
231 described in the user manual (Clontech, Mountain View, CA). The 3' RACE amplicons
232 were generated by two rounds of PCR using *SBream kissr4 3out* and *3in* primer sets
233 (table 1). The final 720bp sequence was confirmed by Blast (NCBI blastN).

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

243

244 Phylogenetic trees and protein alignment

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

253

254 *kissr4* and *kiss2* Quantitative PCR (QPCR) assays

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

257 a reference gene while *Gapdh* was proven to be the most stable in the post-larvae
258 samples. Both these genes have previously been verified as reliable and stable reference
259 genes in seabream (Minghetti et al., 2010; Minghetti et al., 2011). The decision to use
260 different reference genes was justified by the absence of significant differences between
261 any points in each group of samples during the stability tests performed prior to qPCR
262 analysis. All cDNAs for qPCR were synthesised as described previously and qPCR
263 primers (Table 1) were used at a concentration of 0.7 pM, with 5µl cDNA synthesis
264 reaction (at a concentration of 10 ng Total RNA / µl) and 10 µl ABsolute™ QPCR Mix,
265 SYBR green (Thermo scientific, Leon-Rot, Germany). Additionally, 3 µl DNA/RNA
266 free H₂O was added to each reaction to a total reaction volume of 20 µl. All qPCR
267 assays were carried out in a Techne Quantica Realtime qPCR thermocycler (Bibby
268 Scientific Ltd, Cambridge, UK) in a thermo cycling programme consisting of a 15
269 minute hot start at 95°C, followed by 45 cycles of 3 temperature steps: melt at 95°C for
270 15 s, anneal at X°C (see Table 1 for target specific melting temperatures, T_m) for 15 s
271 and extensions at 72°C for 30 s. This was followed by a temperature ramp from 70 –
272 90°C for melt-curve analysis. Quantification was achieved by translating cycle
273 threshold (CT) values of unknown samples from a parallel set of reactions containing a
274 serial dilution of spectrophotometrically determined linearized plasmid containing
275 partial cDNA sequences generated as described above. All samples were run in
276 duplicate and each qPCR plate included non-template controls.

277

278 Data analysis

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

290

291 **Results**

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

293 A 720bp fragment was generated showing a high degree of identity with *kissr4*
294 in other teleost species, having 97% identity with blackhead seabream (*Acanthopagrus*
295 *schlegelii*) and 93% with Atlantic striped bass (*Morone saxatilis*) and European seabass
296 (*Dicentrarchus labrax*) and 70% nucleotide identity to zebrafish (*Danio rerio*) (Fig. 1).
297 The translated partial protein fragment contains 242 amino acids and importantly 41
298 amino acids of the predicted transmembrane protein domains 3-7 of 7 (Fig. 1). With
299 regard to *kiss2* a 308 bp fragment was identified and as with *kissr4* it displayed the
300 highest percentage of nucleotide identity with the blackhead seabream (97%) and only
301 62% with zebrafish and is distinct from the teleost *kiss1* clade (Fig. 2). The predicted
302 translated protein sequence shows notable identity with red seabream, striped bass and
303 European seabass and importantly also contains the decapeptide core *kiss-10* sequence
304 that defines the gene *kiss2* (Fig. 2).

305

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

307 From the 20 animals used for the first sampling (October), 7 were identified as
308 being males (with clear mature and functional testis), 5 as females (with clear mature
309 and functional ovary) and 8 individuals possessed both female and male gonads, at
310 similar proportion. In January only 3 males were identified, while the other 15 (out of
311 18 in total) were females, with there being no sex changing individuals present in the
312 sample. Transcript levels for both targets was an order of magnitude higher in brain in
313 contrast to gonad tissues (Fig. 3 and 4). The receptor *kissr4* (Fig. 3) showed comparable
314 transcript levels in the brain irrespective of gender state in October (Fig. 3A), while in
315 gonad, values were significantly higher in males when compared to females with
316 individuals undergoing sex change being intermediate and statistically comparable to
317 both (Fig. 3B, Kruskal-Wallis test, Dunn's post-hoc test $p < 0.05$). Expression of this
318 same gene was generally lower in January (reducing in the region of 13.96 – 93.81 %),
319 with this decrease being significant only in between the female brain samples (59.58 %,
320 Student's *t* test, $p < 0.01$). In this second sampling, coincident with the beginning of the
321 resting period, no statistical differences between males and females in either the brain or
322 the gonad samples were found (Fig. 3C, D). With respect to *kiss2* expression, in
323 October, male expression level was significantly higher when compared to females in
324 both tissues studied with individuals undergoing sex change being intermediate and

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

332

333 Experiment 2: Kiss system ontogeny during larvae and post-larvae stages in gilthead 334 seabream

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

345 There was a differential response of both genes observed in the second ontogeny
346 trial where samples extending to the post-larval stage (30 – 140 DPH). For *kissr4* and
347 *kiss2* a significantly elevated peak in transcript level was observed at 90 and 60 DPH
348 respectively (Fig. 6). For the receptor, transcript levels were similar during all sampling
349 points from 30 to 75 DPH, significantly increasing at 90 DPH in relation to the first
350 point (3-fold increase compared) before returning to levels comparable to the earlier
351 stages. For *kiss2*, all sampling points showed similar levels of transcripts, with only the
352 peak at 60 DPH (26-fold increase compared to the average abundance from all other
353 points) being significantly higher (One-way ANOVA, Tuckey HSD post-hoc test,
354 $p < 0.05$, Fig. 6B).

355

356 **Discussion**

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

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

369 Prior to this study the lack of seabream gene sequences represented a barrier to
370 investigating the kisspeptin system in the species. For *kissr4* a 720bp product was
371 detected showing high structural similarity with other teleost species including
372 blackhead seabream, striped bass and European seabass and *in silico* analysis of the
373 predicted translated protein sequence revealed the presence of highly conserved
374 transmembrane domains (5 of 7 total), which are characteristic of Kissr4 (Cowan et al.,
375 2012; Parhar et al., 2004). Similarly, the 308bp *kiss2* fragment identified for seabream
376 displays a high level of nucleotide identity to red seabream, striped bass and European
377 seabass. The translated protein sequence contains the highly conserved kisspeptin core
378 sequence which had a 100% aa identity with that reported in striped bass and European
379 seabass (Felip et al., 2009; Zmora et al., 2012). Out with the kisspeptin core sequence a
380 lesser degree of conservation was observed with goldfish and zebrafish sequences, such
381 a pattern is not uncommon with this gene as was previously reported in Atlantic cod
382 (Cowan et al., 2012). While there is a lack of genome sequence information available in
383 the public domain for the gilthead seabream, which negates our ability to identify
384 additional kisspeptin transcripts or perform synteny analysis, the levels of sequence
385 identity and structural conservation observed with other teleost species provides
386 compelling evidence that these are the key transcripts for the species and as such
387 represent a valuable resource to support subsequent research. In teleosts, various
388 kisspeptin (*kiss1* and *kiss2*) and kisspeptin receptors (*kissr1*, *kissr2*, *kissr3*, *kissr4*) gene
389 forms have been encountered, with variations among species (Ohga et al., 2018), but
390 always with *kiss2* and *kissr4* having a functional significance in the control of
391 reproduction (Akazome et al., 2010). This is in agreement with the results of the present
392 study, however, the presence of other forms in seabream should not be ruled out.

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

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

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

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

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

461 days post fertilization (DPF) (equivalent to 3 DPH), although transcripts of these genes
462 were detected as early as 1 or 1.5 DPF, likely from maternal origin. According to these
463 same authors (Wong et al., 2004), at least four concomitant increases in the level of
464 some of the transcripts above mentioned (*gnrhs* and *gnrhr*, *fsh β* , *lhr*, *fshr* and *vasa*)
465 were observed at 5, 8, 14, and 28 DPF (3, 6, 12 and 26 DPH), which is compelling
466 evidence of synchronised events in the early ontogeny and organization of the
467 reproductive axis. After 28 DPF gene expression remained elevated, showing a more
468 stable development. These authors observed paired developing gonads (with few
469 primordial germ cells) at 14 DPF, which grow but remained undifferentiated until 59
470 DPF (57 DPH). Comparisons should be made carefully between these data and our
471 results since rearing conditions among trials are not exactly the same, yet, the
472 temperature range used by Wong and colleagues (18-20°C) is similar to the present
473 study. The transcript level increases in reproduction related genes (Wong et al., 2004)
474 appear to be coincident with the time range at which both *kissr4* and *kiss2* were highly
475 expressed in the present work, which could indicate a parallel ontogeny of the
476 kisspeptin system and the early development of the reproductive axis in gilthead
477 seabream. Furthermore, the evident elevations in expression in post larval stages at 60
478 and 90 DPH, for *kissr4* and *kiss2* respectively are coincident with gonadal
479 differentiation and/or germ cell proliferation in the species suggesting that there are
480 multiple possible roles for the kisspeptin system within early ontogeny as suggested by
481 Ohga *et al.* (2018). There is scarce information available on the timing of specific
482 developmental events in early ontogeny of seabream to corroborate such hypothesis but
483 this work provides a new aspect to such research that should be further explored. For
484 example, an association between gonadal development and the kisspeptin system has
485 been reported in fathead minnow, *Pimephales promelas* (Filby *et al.* 2008). In this
486 species, a peak in *kissr4* (referred to as *kiss1r* by the authors) expression in the brain at
487 60 DPF was associated to the onset of meiosis and the formation of the lobules in the
488 testis (Filby *et al.*, 2008). In other teleosts (e.g. cobia, chub mackerel or tilapia), the
489 early expression of *kissr* and *kiss* transcripts was seen to be parallel with rises in
490 expression of GnRH genes, at earlier or later stages of ontogeny, pointing to a close
491 association between kisspeptin genes and multiple GnRHs during reproductive
492 development (Martinez-Chavez *et al.*, 2008; Mohamed *et al.*, 2007; Ohga *et al.*, 2015;
493 Park *et al.*, 2012; Selvaraj *et al.*, 2015). On the other hand, in model species like medaka
494 or zebrafish, gene knockout and knockdown trials during early development have

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

509

510 **Conclusions**

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

523

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530

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534

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707

708 **Figure Captions**

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

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

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

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

717

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

720 **Figure 1c.** Phylogenetic Tree of teleost Kissr 4 proteins

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

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

human	<i>Homo sapiens</i>	<u>NP 115940.2</u>
mouse	<i>Mus musculus</i>	<u>NP 444474.1</u>

729

730 All positions containing gaps and missing data were eliminated. There were a total of
731 231 positions in the final dataset. Evolutionary analyses were conducted in MEGA6
732 (Tamura et al., 2013).

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

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

Common Name	Accession number
striped bass	<u>ADU54201.1</u>
European seabass	<u>ACM07423.1</u>
goldfish	<u>ACS34769.1</u>
zebrafish Kiss1	<u>ABV03802.1</u>
zebrafish Kiss2	<u>NP_001136057.1</u>

738

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

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

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

Common Name	Scientific Name	Accession number
Red seabream Kiss2	<i>Pagrus major</i>	<u>BAL44206.1</u>
medaka Kiss1		<u>NP_001116393.1</u>
medaka Kiss2		<u>BAG86623.1</u>

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

750

751 All positions containing gaps and missing data were eliminated. There were a total of
752 73 positions in the final dataset. Evolutionary analyses were conducted in MEGA6
753 (Kumar et al., 2004).

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

760 **Figure 4.** *kiss2* transcript levels in brain (A and C) and gonad (B and D) in a groups of
761 gilthead seabream with males, females and individuals undergoing sex change at two
762 different moments of the reproduction season: (A and B) in October, full spawning, and
763 (C and D) in January, beginning of the resting period (values expressed as mean \pm
764 S.E.M.). Different letters indicate groups with statistical significant differences
765 (ANOVA, Tuckey HSD, October sampling and Student's t test, January sampling,
766 $p < 0.05$).

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

769 DPH (values expressed as mean \pm S.E.M.). Letters a, b, c indicate groups with statistical
770 significant differences between development stages (ANOVA, Tuckey HSD, $p < 0.05$).
771 **Figure 6.** Ontogeny of *kissr4* (A) and *kiss2* (B) expression in gilthead seabream post-
772 larvae during development from 30 till 140 DPH (values expressed as mean \pm S.E.M.).
773 Letters a, b indicate groups with statistical significant differences between development
774 stages (ANOVA, Tuckey HSD, $p < 0.05$).
775

776 Figure 1a

2	ATG AGT GGA GAC	CGC TGT TAC GTC ACG GTC TAC CCT CTG AAA TCT	46
1	M S G D	R C Y V T V Y P L K S	15
TM3			
47	CTC CGA CAC AGA ACT CCG AAG GTG GCC ATG ATC GTC AGC ATC TGC	91	
16	L R H R T P K V A M I V S I C	30	
92	ATT TGG ATT GGC TCC TTC ATC TTG TCC ACC CCG ATT TTG ATA TAC	136	
31	I W I G S F I L S T P I L I Y	45	
TM4			
137	CAG CGT ATA GAG GAG GGT TAC TGG TAC GGC CCG AGG CAG TAC TGC	181	
46	Q R I E E G Y W Y G P R Q Y C	60	
182	ATG GAG AGG TTT CCC TCT AAG ACC CAT GAA AGG GCT TTC ATC CTC	226	
61	M E R F P S K T H E R A F I L	75	
227	TAC CAG TTT ATT GCT GCC TAC CTG CTG CCT GTC CTC ACT ATC TCC	271	
76	Y Q F I A A Y L L P V L T I S	90	
TM5			
272	TTC TGC TAC ACT CTG ATG GTG AAG AGG GTC GGC CAG CCC ACC GTA	316	
91	F C Y T L M V K R V G Q P T V	105	
317	GAG CCC GTA GAC AAC AAC TAT CAG GTC AAC CTC CTG TCA GAG AGA	361	
106	E P V D N N Y Q V N L L S E R	120	
362	ACT ATC AGC ATC AGG AGC AAA GTG TCC AAG ATG GTG GTG GTA ATC	406	
121	T I S I R S K V S K M V V V I	135	
407	GTC CTC CTC TTC GCC ATC TGC TGG GGT CCC ATC CAG ATC TTT GCC	451	
136	V L L F A I C W G P I Q I F A	150	
TM6			
452	CTC TTC CAG TCT TTC TAT CCA AAC TAC CGG CCC AAC TAC GCC ACA	496	
151	L F Q S F Y P N Y R P N Y A T	165	
497	TAC AAG ATC AAG ACG TGG GCC AAC TGC ATG TCC TAC GCC AAC TCC	541	
166	Y K I K T W A N C M S Y A N S	180	
542	TCT GTC AAC CCC ATA GTT TAT GGT TTC ATG GGA GCT ACT TTC CAA	586	
181	S V N P I V Y G F M G A T F Q	195	
TM7			
587	AAG TCC TTC AGG AAA ACC TTC CCA TTT CTG TTC AAG CAC AAG GTC	631	
196	K S F R K T F P F L F K H K V	210	
632	AGA GAT AGC AGC ATG GCT TCA AGG ACT GCC AAT GCT GAG ATC AAG	676	
211	R D S S M A S R T A N A E I K	225	
677	TTT GTT GCT GCA GAG GAA GGA AAC AAT AAC AAC GCA TTG AAT TGA	721	
226	F V A A E E G N N N N A L N *	240	

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	TM3				
Gilthead Seabream	CVPTATLYP	LPGWIFGNFM	CKFVAFLLQOV	TVQATCITLTL	-MSGLRRCYVT
Blackhead Seabream	CVPTATLYP	LPGWIFGNFM	CKFVAFLLQOV	TVQATCITLTL	AMSGLRRCYVT
Striped Bass	CVPTATLYP	LPGWIFGNFM	CKFVAFLLQOV	TVQATCITLTL	AMSGLRRCYVT
European Seabass	CVPTATLYP	LPGWIFGNFM	CKFVAFLLQOV	TVQATCITLTL	AMSGLRRCYVT
Goldfish	CVPTATLYP	LPGWIFGDFM	CKFVAFLLQOV	TVQATCITLTL	AMSGLRRCYVT
Zebrafish	CVPTATLYP	LPGWIFGDFM	CKFVAFLLQOV	TVQATCITLTL	AMSGLRRCYVT
Xenopus	CVPTATLYP	LPSWVFGDFM	CKFVAYLQOV	TVQATCITLTL	AMSA LRRCYAT
	TM4				
Gilthead Seabream	VYPLKSLRHR	TPKVAMIVSI	CIWIGSFILS	TPILMQRIE	EGYWYGPRQY
Blackhead Seabream	VYPLKSLRHR	TPRVAMIVSI	CIWIGSFILS	TPILMQRIE	EGYWYGPRQY
Striped Bass	VYPLKSLRHR	TPKVAMIVSI	CIWIGSFILS	TPILMQRIE	EGYWYGPRQY
European Seabass	VYPLKSLRHR	TPKVAMIVSI	CIWIGSFILS	TPILMQRIE	EGYWYGPRQY
Goldfish	VYPLKSLHHR	TPRVAMIVSI	CIWIGSFILS	IPIFLQRLE	DGFWYGPRKY
Zebrafish	VYPLKSLHHR	TPRVAMIVSI	CIWIGSFILS	IPIFLQRLE	DGFWYGPRKY
Xenopus	LYPLRSLRHR	TPKVAMIVSI	CIWIGSLLLS	TRILMQRIO	KGYWYGPRTY
	TM5				
Gilthead Seabream	CMERFPSKTH	EHAFILYQFI	AAYLLPVLLI	SFCYFLMVKR	VGQPTVEPVD
Blackhead Seabream	CMERFPSKTH	EHAFILYQFI	AAYLLPVLLI	SFCYFLMVKR	VGQPTVEPVD
Striped Bass	CMERFPSKTH	EHAFILYQFI	AAYLLPVLLI	SFCYFLMVKR	VGQPTVEPVD
European Seabass	CMERFPSKTH	EHAFILYQFI	AAYLLPVLLI	SFCYFLMVKR	VGQPTVEPVD
Goldfish	CMERFPSKTH	EHAFILYQFI	AVYLLPVITI	SFCYFLMLKR	VGQASVEPVD
Zebrafish	CMERFPSKTH	EHAFILYQFI	AVYLLPVITI	SFCYFLMLKR	VGQASVEPVD
Xenopus	CIEQFPSDVM	KHVCILYQFL	AVYLLPLLLI	CLCYBLMLKR	VGRPVVEPTD
	TM6				
Gilthead Seabream	NNYQVNLLE	RTISIRSKVS	KMVVVIVLLE	AICWGPQIQIF	ALFQSFYFNY
Blackhead Seabream	NNYQVNLLE	RTISIRSKVS	KMVVVIVLLE	AICWGPQIQIF	ALFQSFYFNY
Striped Bass	NNYQVNLLE	RTISIRSKVS	KMVVVIVLLE	AVCRGPIQIF	ALFQSFYFNY
European Seabass	NNYQVNLLE	RTISIRSKVS	KMVVVIVLLE	AVCRGPIQIF	ALFQSFYFNY
Goldfish	NNHQVHLLSE	RTISIRSKIS	KMVVVIVVLE	TICWGPQIQIF	VLFQSFYPSF
Zebrafish	NNHQVHLLSE	RTISIRSKIS	KMVVVIVVLE	TICWGPQIQIF	VLFQSFYPNF
Xenopus	NNYQVQLLE	RTIAMRSKIS	KMVIIVIVLLE	TICWGPQIQIF	SLFQGFYFPGF
	TM7				
Gilthead Seabream	RPNYATYKIK	TWANCMSYAN	SSVNPVYGF	MGATFQKSEF	KTFPPLFKHK
Blackhead Seabream	RPNYATYKIK	TWANCMSYAN	SSVNPVYGF	MGATFQKSEF	KTFPPLFKHK
Striped Bass	RPNYATYKIK	TWANCMSYAN	SSVNPVYGF	MGATFQKSEF	KTFPPLFKHK
European Seabass	RPNYATYKIK	TWANCMSYAN	SSVNPVYGF	MGATFQKSEF	KTFPPLFKHK
Goldfish	KANYTTYKIK	TWANCMSYAN	SSINPIVYGF	MGASFRKSEF	KTFPPLFRHK
Zebrafish	KANYATYKIK	TWANCMSYAN	SSINPIVYGF	MGASFRKSEF	KTFPPLFRHK
Xenopus	QANYATYKIK	TWANCMSYAN	SSINELVYAE	MGASFRKSEK	KATPPMERNK
Gilthead Seabream	VRDSSMASRT	ANAEIKFVAA	EEGNNNNALN		
Blackhead Seabream	VRDSSMASRT	ANAEIKFVAA	EEGNNNNALN		
Striped Bass	VRDSSMASRT	ANAEIKFVAA	EEGNNNNAMN		
European Seabass	VRDSSMASRT	ANAEIKFVAA	EEGNNNNAMN		
Goldfish	VRDSSVASRT	ANAEIKFVAT	EESNTERK--		
Zebrafish	VRDSSVASRT	ANAEIKFVAT	EESNTERK--		
Xenopus	VRDGSITSGT	VNNEMKEVAM	ESTNNEIK--		

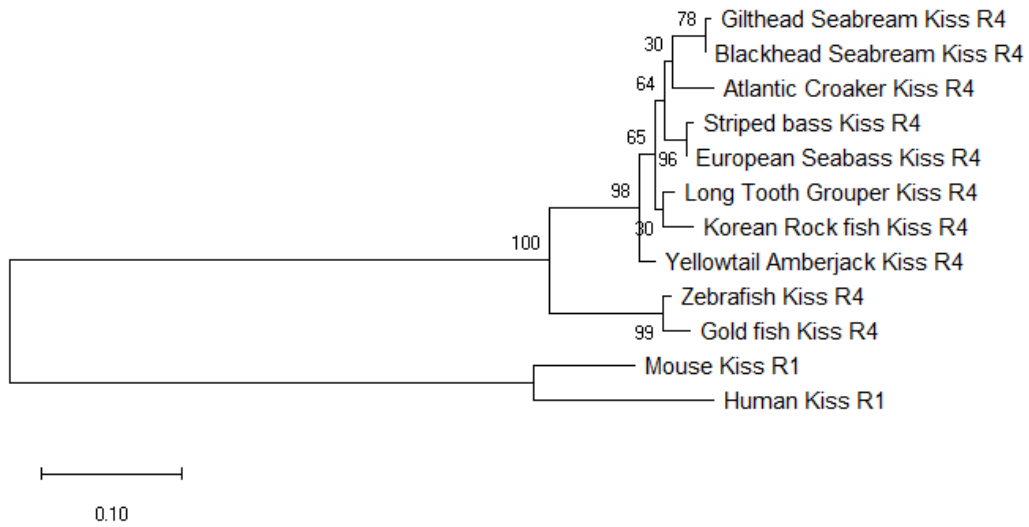
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783 Figure 1c



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785 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	

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795 Figure 2b

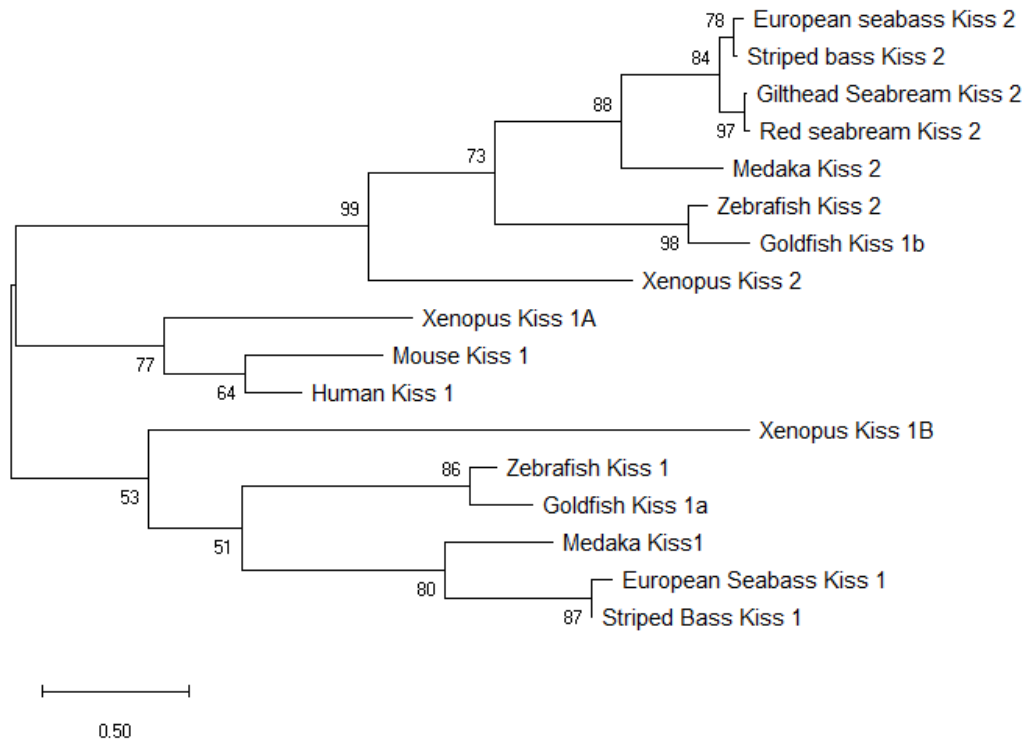
Gilthead Seabream	-----LVVVC	GLIVGEDGGS	VGAALPGFDS	AQRTHTVTS-	--VLSALRRS
Striped Bass	MRLVALVVVC	GLIVGQDGGG	MGAALPGLDS	AQRTGATGS-	--LLSALRRR
European Seabass	MRLVALVVVC	GLILGQDGGG	VGAALPELDS	AQRTGATGS-	--LLSALRRR
Goldfish	MKIKALILFM	SAMICQS-TA	LRASFTDMDI	SDSEVPVDSK	QHLYSVERRQ
Zebrafish Kiss2	MNTRALILFM	SAMVQS-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	FDEPSSDDA	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	NRFSPFLFLFS	RELEVPT
Goldfish	RFGKRNE--A	PTD-----RP	KHLLPMMIYL	RKQSETS
Zebrafish Kiss2	RFGKRNE--A	TTSDSDRLKH	KHLLPMMLYL	RKQLETS
Zebrafish Kiss1	RYGKREQD-M	LTRLIQKSPV	K-----	-----

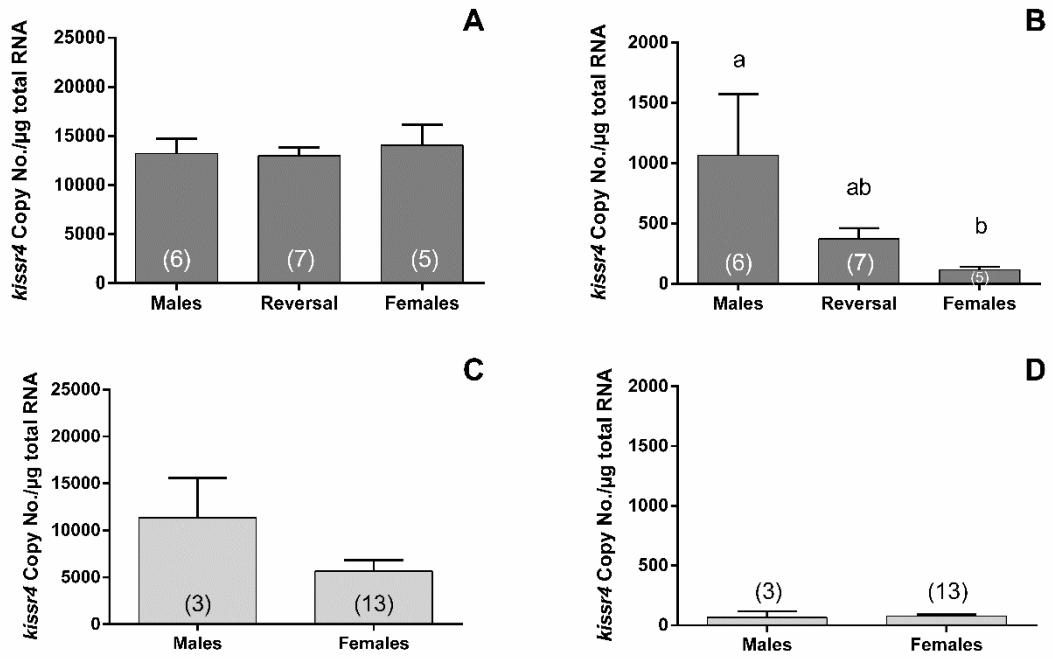
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797 Figure 2c



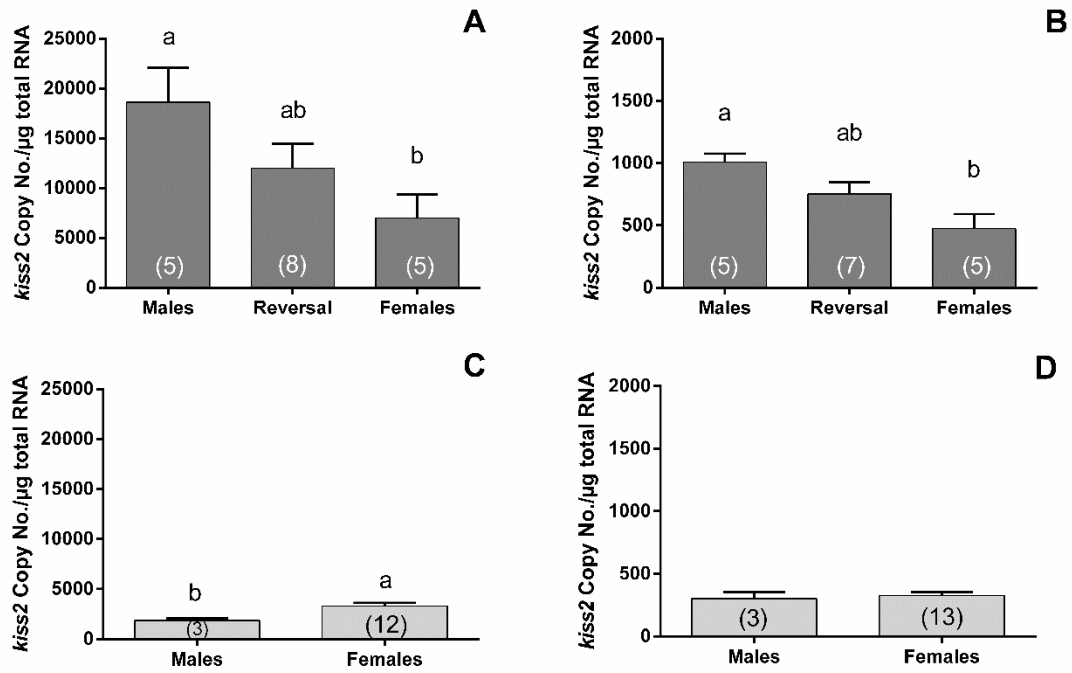
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799 Figure 3



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801 Figure 4



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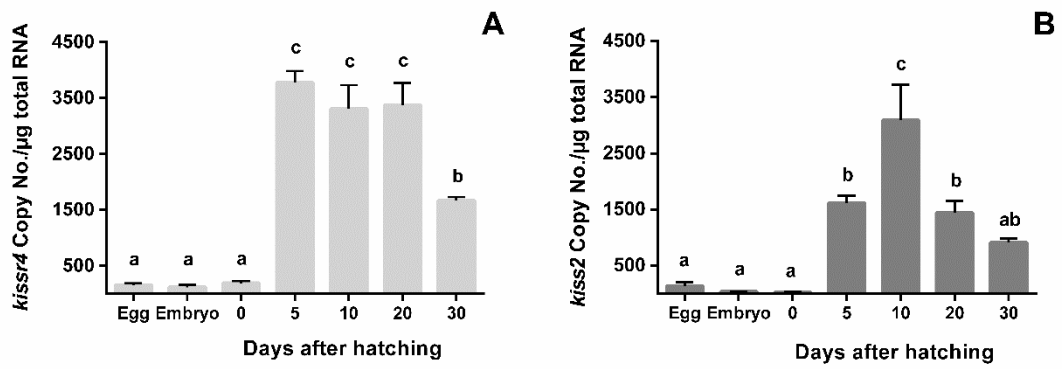
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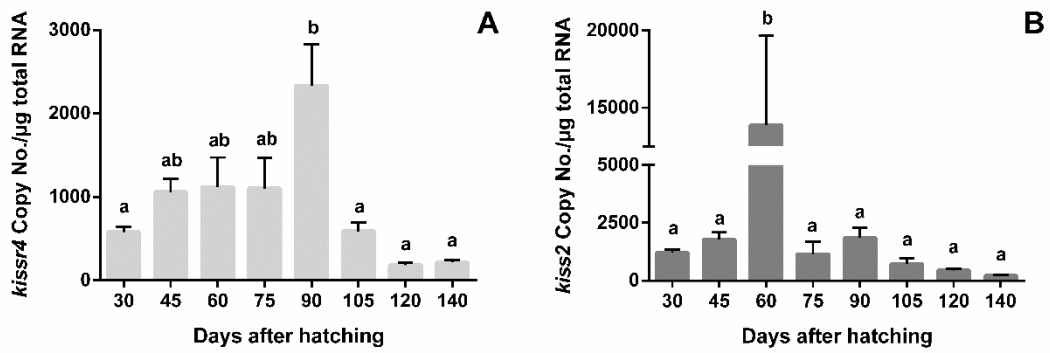
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808 Figure 5



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810 Figure 6



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