1 **Kisspeptin system in pejerrey fish (*Odontesthes bonariensis*).**
2 **Characterization and gene expression pattern during early developmental**
3 **stages.**

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24 **ABSTRACT**

25

26 In vertebrates, kisspeptins and their receptors are known to be related to puberty onset and
27 gonadal maturation, however, there are few studies concerning their role in early development.
28 Here, we characterize the kisspeptin system in the pejerrey, *Odontesthes bonariensis*, a fish with
29 strong temperature-dependent sex determination. We reconstructed the phylogenetic history of
30 the two ligands (*kiss1* and *kiss 2*) and two receptors (*kissr2* and *kissr3*) in pejerrey in the context of
31 recent classifications of bony fishes, determined their tissue distribution and documented the early
32 expression pattern of these ligands and receptors. Phylogenetic analysis of these gene families
33 clearly resolved the Percomorph clade and grouped pejerrey with Beloniformes. Paralogous sets of
34 genes putatively arising from the teleost-specific genome duplication event (3R) were not
35 detected. Kisspeptins and their receptors showed a wide tissue distribution in adult pejerrey,
36 including tissues not related to reproduction. In larvae reared at 24 °C, the four kisspeptin
37 elements were expressed in the head from week 1 to week 8 of life, with no differences in
38 transcript levels. Larvae kept at a female-producing temperature (17 °C) did not show statistically
39 significant differences in the transcript levels of all analyzed genes during the sex
40 determination/differentiation period; however, in those larvae raised at male producing
41 temperature (29 °C), *kiss2* levels were increased at week 4 after hatching. These results showed
42 that all members of the kisspeptin system are expressed at this early period, and the increase of
43 *kiss2* transcripts at week 4 could be interpreted as it would be related to the differentiation of the
44 brain-pituitary axis in male development.

45

46 **Keywords:** kisspeptin, kisspeptin receptor, pejerrey fish, phylogeny, sex differentiation.

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48 **1. Introduction**
49

50 Kisspeptins are neuropeptides that share a C-terminal motif characteristic of the RFamide peptide
51 family (Pasquier et al., 2014). The first kisspeptin transcript was reported after isolation and
52 characterization of cDNA from malignant melanoma cells (Lee et al., 1996). A few years later, a G
53 protein-coupled orphan receptor was described and named GPR54 (Lee et al., 1999); subsequently,
54 peptides that bind to it were identified as kisspeptins (Kotani et al., 2001; Ohtaki et al., 2001) and
55 GPR54 was renamed KISSR1 (Pasquier et al., 2012b; 2014). Kisspeptin (KISS) and KISSR1 are
56 generally considered key factors in the onset of puberty and reproduction in mammals (Lee et al.,
57 2009; Seminara et al., 2003) and it has been shown that mutations in the ligand or the receptor
58 may cause hypogonadotropic hypogonadism (Funes et al., 2003; Seminara et al., 2003).
59 Association of kisspeptin with gonadotropic function also was suggested by the presence of
60 kisspeptin immunoreactive fibers ending on or close to GnRH neurons (Clarkson and Herbison,
61 2006; Kinoshita et al., 2005) that present KISSR1 on their surface (Irwig et al., 2004; Koemeter-Cox
62 et al., 2014). Kisspeptin stimulates hypophysiotropic GnRH secretion in the hypothalamus
63 (d'Anglemont de Tassigny et al., 2008, Herbison et al., 2010), as well as gonadotropins, acting on
64 the central nervous system (Gottsch et al., 2004; Thompson et al., 2004) or directly on the pituitary
65 gland (Luque et al., 2011). Most recent studies on the vertebrate KISS/KISSR system have been
66 devoted to understanding its role in puberty and reproduction (Gopurappilly et al., 2013; Mechaly
67 et al., 2013; Oakley et al., 2009; Tena-Sempere et al., 2012;). But, it has been shown that kisspeptin
68 is expressed early in mammalian development and plays a role in brain sex differentiation
69 (Campbell and Herbison, 2014; Clarkson et al., 2014; Franceschini and Desroziere, 2013).
70
71 Surveys of the KISS/KISSR system among vertebrate genomes revealed the presence of up to three
72 copies of KISS and four copies of KISSR in different species. The current phylogenetic hypothesis for
73 the origin of these genes in vertebrates is based mostly on synteny analysis and proposes that a
74 single ancestral gene duplicated during the so-called 1R and 2R genome duplications in early

75 vertebrate evolution (Nakatani et al., 2007; Pasquier et al., 2012a; 2012b; 2014). Teleost fishes,
76 known to have gone through a third genome duplication event (3R), still have two paralogous
77 kisspeptin genes (*Kiss1* and *Kiss2*) but three genes coding for kisspeptin receptors have been
78 reported, although most fishes only have two receptors, *Kissr2* and *Kissr3* (Pasquier et al., 2012a;
79 2012b; 2014). However, in a few percomorph species such as *Gasterosteus aculeatus* (Tena-
80 Sempere et al., 2012), *Solea senegalensis* (Mechaly et al., 2011) and *Takifugu niphobles* (Shahjahan
81 et al., 2010) only one ligand, *Kiss2*, has been detected.

82

83 The potential role of *kiss/kissr* genes in sex differentiation among fishes has been investigated in
84 some teleost species by measuring expression of kisspeptin mRNAs in early development. In the
85 cichlid fish *Oreochromis niloticus* *kiss2* and *kissr2* mRNA levels increase during the first 2-4 weeks
86 after hatching, coinciding with the sex differentiation period for this species (Park et al., 2012). In
87 the mackerel (*Scomber japonicus*), expression of *kiss1*, *kiss2* and their receptors, *kissr2* and *kissr3*
88 are also elevated during this critical sex-differentiation period (Selvaraj et al., 2015). Other studies
89 showed that water temperature may also alter kisspeptin expression in fishes. In adult zebrafish
90 (*Danio rerio*), low temperatures (15 °C) increase *kiss1/kissr3* mRNA levels in the brain, while high
91 temperatures (35 °C) induce no changes when compared to control individuals at intermediate
92 temperatures (27 °C). Concomitantly, *kiss2/kissr2* mRNAs brain levels decrease at both low and
93 high temperatures (Shahjahan et al., 2013).

94

95 Observed variation of *kiss/kissr* expression levels during early development and the effects of
96 temperature prompted us to explore this system in the pejerrey, (*Odontesthes bonariensis*), a
97 useful model for studying the influence of temperature on sex determination and gonadal
98 differentiation in fishes (Fernandino et al., 2015; Yamamoto et al., 2014). In this species,
99 phenotypic sex is contingent upon water temperature after hatching, during a critical period of
100 larval development. Low temperatures (17-19 °C) can produce 100% female larvae, high

temperatures (29 °C) induce 100% males and “sexually neutral” temperatures (24-25 °C) favor a balanced sex ratio (Strüssmann et al., 1997). Previous studies demonstrated that both Fsh- and Lh-expressing cells can be visualized by immunocytochemistry before morphological differentiation of the gonads and that GnRH-I cells could be the physiological transducers of temperature (Miranda et al., 2001; 2003). Moreover, *fsh*, *lh* and *fshr* show an increase in their mRNA abundance before histological differentiation of the gonads regardless of temperature (Shinoda et al., 2010).

Here we characterize the *kiss/kissr* system in *Odontesthes bonariensis* by identifying kisspeptin ligand and receptor sequences for this species and placing the newly described genes for pejerrey in a phylogenetic framework (Betancur-R et al., 2013). We use phylogenetic hypothesis testing to assess the effect of teleost-specific and vertebrate genome duplications on the history of this gene family, which have previously been explored in vertebrates through synteny analysis (Pasquier et al., 2014). We also document expression levels of kisspeptins and their receptors in different tissues and organs in the pejerrey, and test the effect of temperature on kisspeptin gene expression during the critical period of sex differentiation.

2. Materials and methods

2.1. Animals and tissues/organs sampling

All procedures involving fish were performed following guidelines in the UFAW Handbook on the Care and Management of Laboratory Animals (<http://www.ufaw.org.uk>) and internal IIB-INTECH Institutional regulations. Fish were anesthetized with benzocaine solution and then sacrificed by rapid cervical transection. Three-year-old adult males (n=3; BW, 379.7 ± 72.06 gr; TL, 37,333 ± 2.32 cm) and females (n=3; BW, 405.7 ± 77.17 gr; TL 39.37 ± 1.33 cm) were obtained from the IIB-INTECH stock for cDNA characterization. Individuals were collected during the reproductive season when males were spermiating and females have already completed vitellogenesis. Sampling was conducted between 09.00 and 11.00 during the morning in order to avoid possible circadian

129 fluctuations. After dissection, the adult brains were divided in three different regions: rostral
130 region, including the olfactory bulbs, telencephalon and preoptic area; medial region, including the
131 optic tectum, thalamus, pineal gland and hypothalamus; and the caudal region containing the
132 cerebellar body, vagal lobe and the *medulla oblongata*. The following tissues/organs were also
133 sampled: pituitary gland, gonads, olfactory epithelium, retina, lateral line, liver, gills, muscle, heart,
134 foregut, midgut, hindgut, kidney and spleen from males and females. All samples were quickly
135 frozen in liquid nitrogen and then stored at -80 °C until mRNA extraction.

136

137 2.2. RNA extraction and reverse transcription

138

139 Total RNA from all tissues was extracted using a Trizol reagent kit (Invitrogen™, Carlsbad, CA, USA)
140 according to manufacturer's recommendations. RNA was dissolved in RNAase-free water
141 (Invitrogen) and stored at -80°C. Total RNA concentration was measured with a Sinergy H1
142 spectrophotometer (BioTek Instruments Inc, Winooski, Vermont USA). Subsequently, one
143 microgram of total RNA was treated with DNAase I (Invitrogen™) and used as template for reverse
144 transcription reactions with SuperScript™ II first strand cDNA synthesis kit (Invitrogen), containing
145 RNAase inhibitor (RNAse OUT, Invitrogen™) in a final volume of 20 µL. In all cases, a universal
146 adaptor primer (UAP) was used (Table 1).

147

148 2.3. Primer design and cDNA characterization

149

150 Different consensus primers (CP) were designed using the PrimerSelect (DNASTAR®, Madison,
151 Wisconsin, USA) program based on conserved regions of the open reading frames (ORF) of
152 kisspeptin and kisspeptin receptor genes from different teleost species (Table 1). The different
153 regions were amplified using nested PCR strategies with CPs. Both, *kiss1* and *kissr2* were amplified
154 from whole brain cDNA while *kiss2* was amplified from pituitary cDNA. PCR reactions were
155 performed in an Eppendorf MasterCycler gradient (Eppendorf, Germany) using the following

program: 94 °C for 5 min, then 40 cycles of 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec and a final step of 72 °C for 5 min. PCR products were electrophoresed in 1% agarose gels and the bands of expected sizes were purified with a QUIAEX II Gel Extraction Kit (QIAGEN, Chatsworth, CA, USA). Partial fragments were directly sequenced (Macrogen, USA). Once partial sequences were obtained, 3' rapid amplifications of cDNA ends (RACE) were performed with gene-specific primers (GSP, Table 1) designed with PrimerSelect based on previously obtained partial sequences of *O. bonariensis kiss1*, *kiss2* and *kissr2*, combined with the UAP. At least 5 different plasmid DNA products were sequenced in both directions (Macrogen, USA). Finally, the full length cDNA was obtained by blasting the pejerrey genome database (Campanella et al., 2013). Due to the low number of species in which *kissr3* had been characterized at the time of this study, the sequence was obtained directly from the pejerrey genome database by blasting *Scomber japonicus* G-protein coupled receptor *gpr54-1* mRNA (GenBank accession no. JX982322) and *Dicentrarchus labrax* kisspeptin receptor *gpr54-2b* mRNA (GenBank accession no. JN202447).

GSPs for real-time quantitative PCR (RTqPCR) were designed with Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and checked with OligoAnalyzer 3.1 (<https://www.idtdna.com/calc/analyzer>) based on partial sequences of pejerrey *kiss1* (GenBank accession no. KF314719), *kiss2* (GenBank accession no. KF314720), *kissr2* (GenBank accession no. HM755975), *kissr3* (this manuscript) and, β -actin (GenBank accession no. EF044319), then confirmed with the pejerrey genome database. In those genes where alternative splicing has been detected (unpublished results), and in order to avoid genomic contamination, the primers were designed on exon-exon boundaries (See Suppl. Figs. 1, 2, and 3).

2.4. Sequence analysis

Identity of all cDNA products was confirmed with BLAST and sequences were aligned with ClustalW. Analysis of identity and similarity were calculated with the default settings in SIAS

183 (<http://imed.med.ucm.es/Tools/sias.html>). The putative signal peptides for either ligands or
184 receptors were predicted with SignalP 4.1, based on neuronal network and hidden Markov model
185 algorithms (<http://www.cbs.dtu.dk/services/SignalP/>) and each receptor's transmembrane
186 domains were predicted using TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) based on a
187 hidden Markov model.

188

189 *2.5. Phylogenetic analysis*

190

191 Public databases with genomic and transcriptomic data were searched to obtain existing sequences
192 for kisspeptin and kisspeptin receptor transcripts in fishes. The resulting list of genes and species
193 with their GenBank accession numbers are in Suppl. Tables 1 and 2. All kisspeptin and kisspeptin
194 receptor DNA sequences were aligned with MAFFT v. 5 using default parameters and the L-INS-I
195 algorithm (Katoh et al., 2005). Phylogenetic analysis of aligned sequences was conducted under
196 maximum likelihood with RAxML (Stamatakis, 2014), using the Cipres Science Gateway
197 (www.phylo.org). Ten independent searches were performed for each alignment under the
198 GTR+gamma model of DNA substitution, with 100 bootstrap replicates to obtain the maximum
199 likelihood estimate. The resulting tree was compared with alternative topologies consistent with
200 previous hypotheses. Topological constraints were enforced to test phylogenetic hypotheses
201 advanced by Pasquier and colleagues (2014) suggesting that multiple copies were generated during
202 the 1R and 2R duplications, while the 3R (teleost-specific) duplication has had no effect on copy
203 number. We also compared phylogenetic hypotheses that are consistent with the current tree of
204 life of fishes (Betancur-R et al., 2013), and with an evolutionary scenario where the gene
205 duplication occurred at the base of the teleosts (3R). Analyses under these constraints were ran
206 under the same model in RAxML, and the alternative topologies obtained were compared to the
207 unconstrained tree for statistical significance using the AU test with the package Consel v0.1
208 (Shimodaira and Hasegawa, 2001).

209

210 2.6. *Pejerrey larval rearing and sample collection*

211

212 *Odontesthes bonariensis* fertilized eggs were obtained from the IIB-INTECH brood-stock and
213 maintained until hatching in flow-through incubators at 18 ± 0.5 °C. Approximately 1000 larvae
214 were transferred after hatching to 140 L tanks and reared at different temperatures for 12 weeks
215 (W1 to W12). In all cases, fish were reared in brackish water under a constant photoperiod (16 h
216 light-8 h dark) as previously described (Fernandino et al., 2008a). Larval growth parameters are
217 shown in Suppl. Fig. 4. A first experiment was performed during 12 weeks of rearing at 24 °C, a
218 neutral temperature for sex determination where a 1:1 female/male sex ratio (MixPT) is generally
219 obtained (Strüssmann et al., 1997). A second experiment was then performed to analyze the
220 effects of temperature on kisspeptins and kisspeptin receptors mRNA abundance. In this case the
221 larvae were raised during 8 weeks either at female producing temperature (FPT, 17 °C) or male
222 producing temperature (MPT, 29 °C); temperature was then gradually changed to 24 °C until week
223 12. In both experiments, the larvae were exclusively fed with *Artemia spp. nauplii* 4 times a day
224 until week 6; then *Artemia nauplii* were supplemented with commercial fish food until the end of
225 the experiment. Samples (n=5) were taken once a week, until week 8 after hatching. Fish were
226 anesthetized in ice-cold water until death, then total length (TL) and body weight (BW) were
227 obtained and the entire heads were dissected (with no gills, head, kidney and heart), frozen in
228 liquid nitrogen and stored at -80 °C until processed for RNA extraction. At the end of the
229 experiment, 20 specimens were processed for histology to be sexed following criteria published by
230 Ito et al. (2005). As above, the sampling procedure was carried out between 9:00 and 11:00 AM.

231

232 2.7. *Real-time quantitative PCR (RTqPCR) of kiss1, kiss2, kissr2 and kissr3 mRNAs*

233

234 Real-time quantitative PCR (RTqPCR) assays were performed on a StepOnePlus Real-Time PCR
235 System (Applied Biosystems, CA, USA), using FastStart Universal SYBR Green Master (Rox, Roche
236 Diagnostics, Mannheim, Germany). The reaction mix contained: 5 µl of SYBR, 2 µl of diluted (1:20)

237 cDNA, 1 µl (3 mM) of each forward and reverse primer (Table 1) and 1 µl of ultrapure water
 238 (Invitrogen, Carlsbad, CA) in a 10 µl of final volume. Primer specificity was assessed with a melting
 239 curve to confirm the presence of only one product. In addition, electrophoresis in 1 % agarose gels
 240 was performed to ensure a single band and to purify some representative products for sequencing.
 241 Primer efficiency was validated by 4-fold serial dilutions standard curves for *kiss1*, *kiss2*, *kissr2*,
 242 *kissr3* and *β-actin* as reference gene, using a cDNA pool of whole heads for each temperature. A
 243 non-template control was always included for each pair of primers. RTqPCR cycle for all genes were
 244 performed as follows: 10 min at 95 °C for polymerase activation, and 40 cycles of 95 °C for 15 sec, 1
 245 min at 60 °C for annealing/elongation. A melting curve analysis was also included. The RTqPCR
 246 reactions were performed in duplicates or triplicates for samples and standard curve respectively.
 247 For assessing how expression is distributed in different tissues, all values are relative to the medial
 248 region of the brain. For assessing how expression changes in early development in response to
 249 temperature, all values are relative to week 1 samples.

250

251 2.8. Statistical analysis

252

253 All values are presented as mean ± standard error of the mean (SEM). In order to detect significant
 254 differences in growth parameters and expression levels of kisspeptin transcripts, we used a one-
 255 way Analysis of Variance (ANOVA), followed by a Tukey's multiple comparison test or unpaired t-
 256 test where appropriate. Before the analysis, values were appropriately transformed when
 257 necessary to meet normality and homogeneity of variance requirements. All statistical analyzes
 258 were performed using SPSS v20 program, differences were accepted as significant when $p < 0.05$.

259

260 3. Results

261

262 3.1 Molecular characterization of *kiss1* and *kiss2* in *Odontesthes bonariensis*

263 The cDNA of *kiss1* containing the complete open reading frame (ORF) is 535 bp in length (Suppl.
 264 Fig. 1A). This sequence includes 64 bp and 177 bp corresponding to the 5' and 3' UTR respectively.
 265 The ORF consists of 294 bp coding for 98 amino acid (aa) residues with a deduced molecular weight
 266 (MW) of 10734.54 Da, including the N-terminal signal peptide (1-19 aa). The core sequence of Kiss1
 267 (82-96 aa) is QDVSSYNLNSFGLRY, and contains a cleavage/amidation site at 97-98 aa (Suppl. Fig.
 268 1B).
 269 The *kiss2* full length sequence is 573 bp, including a 68 bp 5' UTR, and a 133 bp 3' UTR. The ORF is
 270 372 bp with a coding region of 124 aa residues, and a MW of 13607.41 Da (Suppl. Fig. 1C).
 271 Sequence analysis with SignalP revealed that the N-terminal 1-19 aa corresponds to the signal
 272 peptide. The core sequence of Kiss2 (77-88 aa) is SKFNYNPFGGLRF, and contains a
 273 cleavage/amidation site at 89-91 aa (Suppl. Fig. 1D).
 274 The amino acid alignments of *pejerrey* Kiss1 and Kiss2 together with sequences from other teleost
 275 fish are shown in Supplementary Figures 5A and 5B respectively. In both alignments, the signal
 276 peptides are underlined and the Kiss1 and Kiss2 mature peptides are shown in the open box, which
 277 are both highly conserved among teleost species. The predicted *O. bonariensis* Kiss1 and Kiss2
 278 amino acid precursor sequences have low identity (7.14 %) and similarity (20.4 %) between them.
 279 The mature Kiss1 peptide from *O. bonariensis* has between 66.7 % to 100 % identity with Kiss1
 280 from other teleost species, while mature the Kiss2 peptide has between 75 % to 100 % identity
 281 when compared to sequences from other fish.

282 283 3.2. Molecular characterization of *Odontesthes bonariensis* *kissr2* and *kissr3*

284 A partial sequence of *kissr2* was amplified with consensus primers from brain cDNA, and then a 3'
 285 RACE was performed with GSP primers (Table 1). The obtained *kissr2* partial fragment (1081 bp,
 286 HM755975) was used to search the full-length sequence by BLAST 2.2.29 in the genome of *O.*
 287 *bonariensis*. The structure of the *kissr2* (Suppl. Fig. 2A) is as follows: 5' UTR composed by 64 bp,
 288 then ORF, 1134 bp, and the 3' UTR with 254 bp including the polyadenylation signal (aataaa). The

289 *kissr2* ORF codes for a 378 aa residue protein with a predicted MW of 43218.3 Da. It contains a
290 signal peptide between amino acids 1 and 26.

291

292 The presence of *kissr3* ORF in the *O. bonariensis* genome database was detected using *kissr3* from
293 *Dicentrarchus labrax* (JN202446) and *Scomber japonicus* (JX982322.1) as templates. Subsequently,
294 GSPs were designed using the obtained sequence in order to confirm its sequence experimentally.
295 The *kissr3* ORF consists in 1107 bp with a coding region of 368 aa, a MW of 41076.45 Da, and the
296 N-terminal signal peptide falling between 1 and 35 aa (Suppl. Fig. 3).

297

298 Alignments of the amino acid residues of *O. bonariensis* Kissr2 and Kissr3 with orthologous fish
299 sequences are shown in Supplementary Figures 6 and 7, respectively. The analysis of the predicted
300 amino acid sequence revealed seven putative transmembrane domains (TMD) for both receptors, a
301 typical characteristic of GPCRs (see Suppl. Fig. 7 for positions). Our analysis revealed high identity
302 values for Kissr2 (from 5.4% to 96.2%) and Kissr3 (from 6.1% to 54.6%) and similarity from 12.7% to
303 98.1% and 13.4% to 61.5% for Kissr2 and Kissr3 respectively.

304

305 3.3. Phylogenetic analysis of *Odontesthes bonariensis* *kiss1*, *kiss2*, *kissr2* and *kissr3*

306 The maximum likelihood trees obtained with RAxML are shown in Figures 1 and 2 for Kiss and
307 KissR, respectively. The newly described pejerrey sequences of *kiss1*, *kiss2*, *kissr2* and *kissr3*
308 consistently grouped with orthologues of medaka (*Oryzias latipes*), as predicted by the currently
309 accepted taxonomy. Despite poor conservation of *kiss* gene sequences among species (except for
310 the sequence encoding the signal peptide), our analysis resolved the reciprocal monophyly of *kiss1*
311 and *kiss2* orthogroups with high support using *kiss3* from elephant shark (*Callorhynchus milli*) as an
312 outgroup (Fig. 1). Within each group, a clade of percomorph fishes (Subdivision Percomorphaceae)
313 is clearly resolved, but rooting of each orthogroup produced gene trees that are not consistent
314 with relationships among species indicated by the tree of life of fishes (Betancur-R et al., 2013);

most notably, the expected relationship among chondrichthyans, sarcopterygians and actinopterygians is not resolved. Enforcing a topology that reflects the constraint (Chondrichthys (Sarcopterygii (Holostei (Teleostei)))) for each orthogroup results in a tree that is marginally less likely (AU test, $p=0.03$) than the unconstrained topology shown in Figure 1. In contrast, a topology that is consistent with a duplication event giving rise to *kiss1* and *kiss2* at the base of the teleosts (3R) is highly unlikely (AU test, $p<0.01$).

321

The gene tree for *kiss* receptors (Fig. 2) shows relationships among species that are more consistent with the tree of life of fishes than the kisspeptin phylogeny. *Kissr* genes are more evolutionarily conserved and longer than *Kiss* genes, providing more robust phylogenetic signal. Two well-supported clades group *kissr2* with *kissr4* and *kissr1* with *kissr3*, but the monophyly of *kissr3* is not supported in the unconstrained tree. Enforcing a topology that is consistent with the monophyly of each orthogroup as proposed by Pasquier et al. (2012b) results in a tree that cannot be rejected by the data (AU test, $p=0.11$), but the evolutionary scenario involving 3R as the origin of *kissr* diversity is significantly rejected (AU test, $p<0.001$).

330

3.4. Tissue distribution

The tissue distribution of *kiss1*, *kiss2*, *kissr2* and *kissr3* mRNA was assayed by RTqPCR using primers specified in Table 2 in males and females. As shown in Figure 3, kisspeptin mRNAs have a broad distribution in different tissues and organs, some of which showed sex differences. In males, *kiss1* is expressed in all sections of the brain and testes and its mRNA levels in the gills and heart were significantly higher compared with females.

337

Expression levels of *kiss2* were detected in almost all tissues examined with no significant differences between sexes, and were particularly highly expressed in the pituitary gland (Fig. 3B).

The receptors, *kissr2* and *kissr3* (Fig. 3C and 3D), also have a broad gene expression distribution. In

both sexes, they are expressed in the brain and the olfactory epithelium. Males had significantly more transcript levels in the heart than females. It is important to note that *kissr3* mRNA is expressed at high levels in the male pituitary, however in females this receptor is significantly more expressed in the olfactory epithelium (Fig. 3D).

3.5. Early developmental expression of kiss1, kiss2, kissr2 and kissr3 mRNAs in larval heads at MixPT (24 °C)

In order to evaluate the expression levels of all components of the kisspeptin system during early development, pejerrey larvae were kept at 24 °C during the first eight weeks after hatching and mRNA levels were measured in whole head extracts. Sex ratio, as determined by histology was as follows: 62% females and 38% males. All transcripts were detected during this period, as early as week one after hatching, but no differences in mRNA levels were detected from W1 to W8 (Fig. 4).

3.6. Early developmental expression of kiss1, kiss2, kissr2 and kissr3 mRNAs in larval heads at FPT (17 °C) and MPT (29 °C)

As temperature is a key factor involved in sex determination/differentiation in pejerrey, and because the expression at MixPT could hide dimorphic expression differences, a second experiment was performed in order to evaluate the effects of temperature during the sex determination and gonadal differentiation period in larvae exposed either at FPT (17 °C) or MPT (29 °C). In order to confirm the effect of temperature in sex determination, sex ratio, as determined by histology, followed the reported pattern for these conditions: 100 % females at 17 °C and 100% males at 29 °C. Transcripts levels of *kiss1*, *kiss2*, *kissr2* and *kissr3* were always detectable in both temperatures during the sex determination and gonad differentiation period

366 from W1 to W8 (Fig. 5). At MPT an increase of *kiss2* was observed in the head at W4 ($p < 0.05$),
367 meanwhile no variations were observed at FPT.

368

369 **4. Discussion**

370

371 We characterized two kisspeptin ligands (*kiss1* and *kiss2*) and their receptors (*kissr2* and *kissr3*) in
372 the atherinopsid species, *Odontesthes bonariensis*. As previously reported, in most teleost species,
373 the kisspeptin system is composed of two ligands and two receptors (Pasquier et al., 2014),
374 conserved in a wide range of teleost orders such as Anguilliformes (Pasquier et al., 2012a),
375 Cypriniformes (Biran et al., 2008; Kitahashi et al., 2009; Li et al., 2009), Beloniformes (Kitahashi et
376 al., 2009), Scorpaeniformes (Fairgrieve et al., 2016) and Perciformes (Ohga et al., 2013; Selvaraj et
377 al., 2010; Zmora et al., 2012;). The predicted precursors from *Odontesthes bonariensis* kisspeptins
378 share low similarity with kisspeptins of other teleost species. However, the mature peptides of
379 Kiss1 and Kiss2 have highly conserved characteristics. *O. bonariensis* Kiss1 and Kiss2 are very similar
380 in sequence to orthologs carried by other percomorphs, particularly with Beloniformes
381 (represented here by *Oryzias latipes*). Amino acids at the sites for protein convertase cleavage are
382 highly conserved (Zmora et al., 2012). Nevertheless Kiss1 and Kiss2 from pejerrey are predicted
383 from the prohormone structure, similar peptides have already been purified from other vertebrate
384 species indicating that they are naturally present (Kotani et al., 2001; Lee et al., 2009; Osugi et al.,
385 2013).

386

387 The *in silico* analysis of the predicted amino acid sequences of *Odonthestes bonariensis* Kissr2 and
388 Kissr3 revealed the typical structure of the rhodopsin superfamily of G protein-coupled receptors,
389 composed of seven transmembrane domains. Both receptors presented high identity and similarity
390 when compared to other receptors from teleost fish mainly because of TMDs are highly conserved
391 among teleost species (Tena-Sempere et al., 2012).

392 The pejerrey *kiss1*, *kiss2*, *kissr2* and *kissr3* sequences always group with orthologues of medaka,
393 and are included in a clade with genes of other percomorph fishes (Subdivision Percomorphaceae).
394 Phylogenetic analyses of Kiss ligands and receptors confirmed previous hypotheses proposed by
395 Pasquier et al. (2012a, 2012b, 2014) suggesting that the diversity of these genes can be traced back
396 to the putative 1R and 2R whole genome duplication at the base of the vertebrates (Nakatani et al.,
397 2007). As reported earlier (Kim et al., 2012; Pasquier et al., 2014), putative paralogs arising from
398 the teleost-specific genome duplication event (3R) are not detected among these genes, implying
399 rapid loss of duplicate gene copies shortly after the 3R event. A similar pattern of gene loss after
400 the fish-specific genome duplication was suggested for the phylogenetic history of GnRH
401 precursors (Guilgur et al., 2007) and for gonadotropins (Levavi-Sivan et al., 2010). Although the
402 effect of the 3R duplication at the base of teleosts has been documented extensively for many
403 autosomal genes (e.g., Sato and Nishida, 2010; Glasauer and Neuhauss, 2014), our results suggest
404 that retention of duplicated genes with reproductive function in teleosts has not been favored by
405 selection. Most species of fishes carry one or at most two Kiss genes and up to four Kissr genes,
406 with no evidence for a second set of paralogs originating from the 3R event.

407

408 Expression of *kiss/kissR* mRNAs detected in the pejerrey brain-pituitary-gonadal (BPG) axis is
409 consistent with results of previous studies in other teleosts species (Biran et al., 2008; Fairgrieve et
410 al., 2016; Kitahashi et al., 2009; Li et al., 2009; Mechaly et al., 2011; Ohga et al., 2013). This
411 conserved characteristic suggests important roles of the kisspeptin system in reproductive
412 functions in teleosts (Pasquier et al., 2012a). The neuroanatomical organization of this system in
413 teleosts, however, is only starting to be revealed in a few species, namely *Danio rerio* (Servili et al.,
414 2011), *Morone saxatilis* (Zmora et al., 2012; 2014), *Dicentrarchus labrax* (Escobar et al., 2013) and
415 *Oryzias latipes* (Kanda et al., 2013).

416

Both forms of kisspeptin have also been detected in the pituitary gland of different teleosts (Li et al., 2009; Alvarado et al., 2013; Saha et al., 2016), however, it appears that some species only express *kiss2* (Fairgrieve et al., 2016) and some express neither (Kanda et al., 2008; Kitahashi et al., 2009). Interestingly, *kiss2*, but not *kiss1*, was found to be highly expressed in the pejerrey pituitary gland. However, this pattern should be interpreted with caution because kisspeptin levels in the pituitary gland are known to vary during the sex cycle in *Dicentrarchus labrax* (Alvarado et al., 2013). Additionally, although Kanda et al. (2008) reported that kisspeptin variants were not expressed at the zebrafish pituitary, the expression of Kiss2 was then confirmed using a specific antibody (Servili et al., 2011). Further studies should be performed in order to characterize kisspeptin levels in the pituitary gland during the reproductive cycle in pejerrey and other fish species.

As mentioned above, we found that pejerrey also expresses kisspeptins and their receptors in other tissues and organs. Low levels of expression of *kiss1*, *kiss2*, *kissr2* and *kissr3* were detected in the gonads of both male and female pejerrey. Similar expression patterns, with differences in the relative mRNA abundance, were also reported in other fish species and these levels varied according to the gonadal cycle (Fairgrieve et al., 2016; Mechaly et al., 2012; Saha et al., 2016). It is interesting to note that males express higher levels of *kiss1* in pituitary and *kiss1* and *kissr2* in the heart than females. On the other hand, *kissr3* expression levels are higher in the olfactory epithelium of females than in males. This wide tissue/organ distribution of kisspeptin ligands and receptors suggests that kisspeptins may be involved in other functions in addition to reproduction (Oakley et al., 2009). As an example, high expression of all members of the kisspeptin system was detected in the olfactory epithelium and retina of both sexes. In this context, Zhao and Wayne (2012) have reported that Kiss1 stimulates, through synaptic regulation, the electrical activity of GnRH-III neurons located at the terminal nerve. Taken together, these data suggest that kisspeptin is probably mediating olfactory and visual signals.

443

444 The effect of water temperature on expression of kisspeptin brain systems is significant in zebrafish
445 (Shahjahan *et al.*, 2013), but unknown in *Odontesthes bonariensis*, a model species for studying
446 temperature-dependent sex determination (Fernandino *et al.*, 2015; Yamamoto *et al.*, 2014). We
447 quantified the expression of *kiss1*, *kiss2*, *kissr2* and *kissr3* during the critical period of sex
448 determination and gonad differentiation in relation to water temperature. At 24 °C (MixPT), all
449 these genes had detectable mRNA levels since the first week after hatching (WAH); however none
450 of them displayed significant variations during the first 5 WAH. It is important to note here that the
451 temperature sensitive window, when sex determination is established, was estimated to be
452 between 1-5 WAH in this species (Strüssmann *et al.*, 1997). However, it must be emphasized that
453 the measurements were done in the whole brain because the size of brain in pejerrey larvae
454 precluded region specific analysis at this stage.

455

456 A significant increase of transcript levels of kisspeptins and kisspeptin receptors during early
457 development has already been reported in few teleost species (Mohamed *et al.*, 2007; Ohga *et al.*,
458 2014; Park *et al.*, 2012), but their functional implications are still not clear. In pejerrey, the four
459 kisspeptins genes were expressed at FPT and MPT during the first eight WAH. There is a substantial
460 amount of data to support the idea that the first morphological signs of gonadal differentiation in
461 this species appear between 4-6 WAH (Fernandino *et al.*, 2008b; Karube *et al.*, 2007; Shinoda *et al.*,
462 2010; Strüssmann *et al.*, 1997). On the other hand, the number of GnRH-I and gonadotropin
463 immunoreactive cells was shown to be significantly increased at 3-4 WAH in the POA (Miranda *et*
464 *al.*, 2001; 2003) before morphological differentiation of the gonads. Moreover, it has been
465 demonstrated that *fsh*- and *lh-β* mRNA levels are significantly increased at 3-4 WAH in larval heads
466 (Shinoda *et al.*, 2010). We observed a slight, but consistent increase of *kiss2* at MFT in W4 that
467 coincided with the significant increase of GnRH-I and gonadotropin cells and also with *fsh*- and *lh-β*
468 increase. Taken together, these temporal overlaps between key players of the reproductive system

led us to hypothesize that *kiss2* could have a role in sex differentiation inducing GnRH-I in the POA with the consequent gonadotropin response at MPT. This pattern is similar to that reported in mammals, where there is early expression of KISS1/KISSR1 (Bellingham et al., 2009; Desrozier et al., 2012; Fiorini & Jasoni, 2010), and GnRH neurons responds to KISS1-10 in pre-natal mouse (Desrozier et al., 2012). Subsequent studies demonstrated that KISS1/KISSR1 signaling is necessary for the normal sex differentiation mediated by GnRH induction with the consequent testosterone surge necessary for mouse-male brain sex differentiation (Clarkson et al., 2014). However, we have to take into consideration that *kiss2* is also expressed in the pituitary gland, olfactory epithelium and retina, we cannot rule out the participation of *kiss2* from these sources in this process. Although methodologically difficult, further studies are needed to verify this hypothesis using micro dissected areas of pejerrey brain and pituitary during the temperature sensitive window. Kisspeptin could also be involved in other functions during early brain development, as has been suggested in other teleost fish (Hodne et al., 2013; Selvaraj et al., 2015).

482

In summary, we characterized two ligands (*kiss1/kiss2*) and two receptors (*kissr2/kissr3*) for the first time in an atheriniform fish. The four genes grouped with those of percomorph fishes and they form a clade with medaka (Beloniformes). These genes were shown to be expressed not only in the brain, pituitary, and gonads but also in peripheral tissues. The early expression of all components of the kisspeptin system suggests that this system is may be important in brain development and could participate in the participation of the brain and pituitary in the sex differentiation process.

489

490 **Acknowledgments**

The authors wish to thank the Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina (CONICET) and grants from ANPCyT (PICTs 2008-1383 and 2012-2619) to GMS. Partial support from the National Science Foundation of the USA (grant DEB-1541554 to G.O.) is also acknowledged.

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727

728

Figure legends

730

731 **Figure 1:** Maximum likelihood tree of fish kisspeptin genes based on analysis of DNA sequences of
 732 the coding regions using RAxML. The *kiss1* and *kiss2* orthogroups are well supported (bootstrap
 733 values shown at nodes) but rooting of each group produced inconsistent organismal phylogeny.
 734 The dotted branches leading to chondrichthyan genes mark the correct rooting for each clade that
 735 would result in the predicted phylogenetic results. Percomorph fishes (Subdivision
 736 Percomorphaceae) are highlighted, and the fish outline marks the positions of new pejerrey
 737 sequences.

738 **Figure 2:** Maximum likelihood tree of fish kisspeptin receptor genes based on analysis of DNA
 739 sequences of the coding regions using RAxML. There is strong support for grouping *kissr2* and
 740 *kissr4* (100% bootstrap) and *kissr1* and *kissr3* (100% bootstrap), but monophyly of *kissr3* genes is not
 741 obtained. The organismal phylogeny implied by the *kissr2* gene tree is consistent with the tree of
 742 life of fishes (Betancur-R et al., 2013), rooted with the *kissr2* elephant shark (*Callorhinchus*)
 743 sequence (dotted line). Relationships among *kissr1* and *kissr3* genes are not well resolved
 744 (bootstrap support <70%); the dotted lines leading to *kissr1* genes and to *kissr3* *Callorhinchus*
 745 should be joined together at the base of this clade to produce a phylogenetically consistent result.
 746 Enforcing this change does not result in a significantly worse likelihood score (AU test, $p=0.11$).

747 **Figure 3:** *kiss1* (A), *kiss2* (B), *kissr2* (C) and *kissr3* (D) mRNA tissue distribution in *O. bonariensis*
 748 males (black bars) and females (white bars) analyzed by RTqPCR. Values are shown as the mean $\pm$
 749 SEM. Black arrows represent significant differences between sexes ($P<0.05$ t-test). All values were
 750 relativized to the expression of the different genes in the medium region of the brain. Expression
 751 values were normalized to the β -actin.

752 **Figure 4:** Fold changes in mRNA expression determined by RTqPCR of *kiss1* (A), *kiss2* (B), *kissr2* (C)
 753 and *kissr3* (D) on the whole head of *O. bonariensis* larvae from W1 to W8 keep at 24 °C MixPT.
 754 Values are shown as the mean $\pm$ SEM. All values were relativized to the expression of each gene at
 755 W1. Expression values were normalized to the β -actin.

756 **Figure 5:** Figure 5: Fold changes in mRNA expression determined by RTqPCR on the whole head of
 757 *O. bonariensis* larvae keep at FPT (17°C) or MPT (29°C), *kiss1* at 17 °C (A) and 29 °C (B), *kiss2* at 17
 758 °C (C) and 29 °C (D), *kissr2* at 17 °C (E) and 29 °C (F), *kissr3* at 17 °C (G) and 29 °C (H). Black arrows
 759 indicate differences between weeks ($P<0.05$). All values were relativized to the expression of each
 760 gene at W1. Expression values were normalized to the β -actin.

761

762 **Table 1:** Primer list used for characterization of pejerrey *kiss1*, *kiss2*, *kissr2*, *kissr3* mRNAs.

763 **Table 2:** Primer list used for RTqPCR of pejerrey *kiss1*, *kiss2*, *kissr2*, *kissr3* mRNAs.

764

765

0.4 substitutions

Bootstrap Support

- 100
- 99-90
- 89-70

0.2 substitutions

Bootstrap Support

- 100
- 99-90
- 89-70

Kissr2

Kissr4

Kissr1 + Kissr3

Kissr3

Kissr1

Kissr2 *Poecilia reticulata*
 Kissr2 *Poecilia formosa*
 Kissr2 *Xiphophorus maculatus*
 Kissr2 *Fundulus heteroclitus*
 Kissr2 *Austrofundulus limnaeus*
 Kissr2 *Oryzias latipes*
 Kissr2 *Odontesthes bonariensis*
 Kissr2 *Stegastes partitus*
 Kissr2 *Amphiprion sebae*
 Kissr2 *Mugil cephalus*
 Kissr2 *Maylandia zebra*
 Kissr2 *Pundamilia nyerelei*
 Kissr2 *Haplochromis burtoni*
 Kissr2 *Neolamprologus brichardi*
 Kissr2 *Oreochromis niloticus*
 Kissr2 *Rachycentron canadum*
 Kissr2 *Notemnia coniceps*
 Kissr2 *Anoplopoma fimbria*
 Kissr2 *Sebastes schlegelii*
 Kissr2 *Epinephelus bruneus*
 Kissr2 *Epinephelus coioides*
 Kissr2 *Morone saxatilis*
 Kissr2 *Dicentrarchus labrax*
 Kissr2 *Micropogonias undulatus*
 Kissr2 *Larimichthys crocea*
 Kissr2 *Takifugu rubripes*
 Kissr2 *Solea senegalensis*
 Kissr2 *Cynoglossus semilaevis*
 Kissr2 *Seriola lalandi*
 Kissr2 *Latea calcarifer*
 Kissr2 *Hippoglossus hippoglossus*
 Kissr2 *Thunnus maccoyi*
 Kissr2 *Thunnus thynnus*
 Kissr2 *Scomber japonicus*
 Kissr2 *Salmo salar*
 Kissr2 *Oncorhynchus*
 Kissr2 *Esox lucius*
 Kissr2 *Cyprinus carpio*
 Kissr2 *Carassius auratus*
 Kissr2 *Gobiocypris rarus*
 Kissr2 *Danio rerio*
 Kissr2 *Astyanax mexicanus*
 Kissr2 *Electrophorus electricus*

"percomorphs"

Kissr3 *Stegastes partitus*
 Kissr3 *Anoplopoma fimbria*
 Kissr3 *Morone saxatilis*
 Kissr3 *Dicentrarchus labrax*
 Kissr3 *Larimichthys crocea*
 Kissr3 *Odontesthes bonariensis*
 Kissr3 *Oryzias latipes*
 Kissr3 *Oreochromis niloticus*
 Kissr3 *Scomber japonicus*
 Kissr3 *Cynoglossus semilaevis*
 Kissr3 *Carassius auratus*
 Kissr3 *Cyprinus carpio*
 Kissr3 *Danio rerio*
 Kissr3 *Clupea harengus*
 Kissr1 *Anguilla anguilla*
 Kissr1 *Latimeria chalumnae*
 Kissr3 *Latimeria chalumnae*
 Kissr3 *Callorhynchus milii*
 Kissr3 *Lepisosteus oculatus*

"percomorphs"

Kissr1 *Lepisosteus oculatus*

<i>Gene</i>	<i>Sequence (5'-3')</i>	<i>Type</i>	<i>Primer name</i>	<i>Direction</i>
<i>kiss1</i>	TRGCTGCTTTGTCAACAGAG	CP	CPK1-Fw1	Forward
<i>kiss1</i>	GCYCTGAGAGATTTAAGCC	CP	CPK1-Fw2	Forward
<i>kiss1</i>	AAAGGAGTTGAGGTTGTATG	CP	CPK1-Rv1	Reverse
<i>kiss1</i>	GACACATCTTGAYGTTTCTTG	CP	CPK1-Rv2	Reverse
<i>kiss1</i>	GAGGGAGTGAGGGGGCGG	GSP	GSPK1-Fw1	Forward
<i>kiss1</i>	GCCCGGTCCTGCGTCTGA	GSP	GSPK1-Fw2	Forward
<i>kiss2</i>	ATGAGRCTNGTGRCTCTGGT	CP	CPK2-Fw3	Forward
<i>kiss2</i>	TYGACTCTGCACAGAGGA	CP	CPK2-Fw4	Forward
<i>kiss2</i>	AGGCACCTCCAGTTCTCG	CP	CPK2-Rv3	Reverse
<i>kiss2</i>	GCCCGGTCCTGCGTCTGA	GSP	GSPK2-Fw3	Forward
<i>kiss2</i>	TCAGGAGGACTGCGGGAGAC	GSP	GSPK2-Fw4	Forward
<i>kissr2</i>	CATGCTGGTCGGACTCGTGGG	CP	CPK2R-Fw5	Forward
<i>kissr2</i>	GCGTTGGCAGTCCTGAAGC	CP	CPK2R-Rv4	Reverse
<i>kissr2</i>	TCTCCGCCACCGCACTCC	GSP	GSPK2R-Fw5	Forward
<i>kissr2</i>	CCAATTTATTgCTGCCTACCTGC	GSP	GSPk2R-Fw6	Forward
<i>kissr2</i>	AACAATCAGCATAAGGAGCAAAGT	GSP	GSPK2R-Fw7	Forward
<i>kissr2</i>	AAATGAGGACGGCCACCAACT	GSP	GSPK2R-Fw8	Forward
<i>kissr3</i>	CCAAGCATCAGCAGATGAAA	GSP	GSPK3RfW1	Forward
<i>kissr3</i>	ATAAACCAGCGGGTTGACAG	GSP	GSPK3RRv1	Reverse
<i>β-actin</i>	GCTGTCCCTGTACGCCTCTGG	GSP	GSPβa-Fw7	Forward
<i>β-actin</i>	GCTCGGCTGTGGTGGTGAAGC	GSP	GSPβa-Rv1	Reverse
<i>AP d(T)</i>	GGCCACGCGTCGACTAGTACT(15)G	UAP	UAP1	Reverse
<i>AP</i>	GGCCACGCGTCGACTAGTAC	UAP	UAP2	Reverse

Gene/ primer name	Forward primer (5' - 3')	Reverse primer (5' - 3')	Efficiency	Slope	r ²	Ct value +/- sd	Product size bp	Tm °C
qkiss1	AAGGCGTTGGTCAGCACTAC	CGGGAAGACCACCTTTGTAA	97%	-3,39	0,87	30,18+/-0,65	100	60
qkiss2	CAGAGAGAGCGACGACCAG	AGAGAAAGAGGGGCGAAAAC	102%	-3,29	0,91	28,01+/-0,27	161	60
qkissr2	TTTGGATTGGCTCCTTCATC	GCCGTACCAGTAACCCTCCT	96%	-3,43	0,90	30,49+/-0,43	74	60
qkissr3	GTCTACCTCCTGCCCCTGCTTACC	GCCTGAGCCTGCAGTTGGTAGC	142%	-2,6	0,97	28,76+/-0,83	113	60
qβ-actin	GCTGTCCCTGTACGCCTCTGG	GCTCGGCTGTGGTGGTGAAGC	111%	-3,10	0,98	24,50+/-0,34	200	60
qEF1α	AGAAATCCGTCGTGGATACG	TGATGACCTGAGCGTTGAAG	110%	-3,11	0,97	23,25+/-0,30	83	60