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

M. Oswaldo Tovar Bohórquez[1], Alejandro S. Mechaly[1], Lily Hughes[2], Daniela Campanella[2], Guillermo Ortí[2], Luis F. Canosa[1], Gustavo M. Somoza[1*].

[1]Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH). CONICET-UNSAM, Chascomús, Argentina.

[2]Department of Biological Sciences, The George Washington University, Washington, DC, USA.

***Corresponding author**: Gustavo M. Somoza. IIB-INTECH (CONICET-UNSAM).

Av. Intendente Marino Km. 8.2 (B7130IWA). Chascomus, Buenos Aires. Argentina.

TE: +54-2241-430323 ext 111. FAX: +54-2241-424048. Email: somoza@intech.gov.ar

ABSTRACT

In vertebrates, kisspeptins and their receptors are known to be related to puberty onset and gonadal maturation; however there are few studies concerning their role in early development. In this context, the aims of this work were to characterize the kisspeptin system in the pejerrey, *Odontesthes bonariensis*, in order to construct the phylogenetic history based on the actual classification of the tree of life, determine their tissue distribution and study the early expression pattern in a species with a strong temperature dependent sex determination. In pejerrey, this system is composed by two ligands (*kiss1* and *kiss2*) and to receptors (*kissr2* and *kissr3*). Phylogenetic analysis clearly resolved the Percomorph clade and grouped the pejerrey with Beloniformes; however, the teleost-specific genome duplication event (3R) was not detected. Kisspentins and their receptors showed a wide tissue distribution in adult pejerrey, including tissues not related to reproduction. In larvae reared at 24º C, the four kisspeptins elements are expressed from week 1 to week 8 of life, with no differences in transcript levels. Larvae kept at a female producing temperature (17 ºC) did not show statistically significances in the transcript levels of all analyzed genes during the sex determination/differentiation period; however in those larvae raised at male producing temperature (29 ºC), *kiss2* levels were increased at week 4 after hatching. These results showed that all members of the kisspeptin system are expressed at this early period, and *kiss2* is probably related to male brain sex differentiation.

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

1. Introduction

Kisspeptins are neuropeptides that share a C-terminal motif characteristic of the RFamide peptide family (Pasquier et al., 2014). The first kisspeptin transcript was reported after isolation and characterization of cDNA from malignant melanoma cells (Lee et al., 1996). A few years later, a G protein-coupled orphan receptor was described and named GPR54 (Lee et al., 1999); subsequently, peptides that bind to it were identified as kisspeptins (Kotani et al., 2001; Ohtaki et al., 2001). This receptor is currently recognized as Kissr1 (Pasquier et al., 2012b; 2014). Kisspeptin and Kissr1 are generally considered key factors in the onset of puberty and reproduction in mammals (Lee et al., 2009; Seminara et al., 2003) and it has been demonstrated that either mutations in the ligand or the receptor cause hypogonadotrophic hypogonadism (Funes et al., 2003; Seminara et al., 2003). Furthermore, there is evidence that kisspeptin immunoreactive fibers end on or close to GnRH neurons (Clarkson and Herbison, 2006; Kinoshita et al., 2005;) that also present Kissr1 on their surface (Irwig et al., 2004; Koemeter-Cox et al., 2014). Kisspeptin stimulates hypophysiotropic GnRH secretion in the hypothalamus (d'Anglemont de Tassigny et al., 2008, Herbison et al., 2010), as well as gonadotropins, acting on the central nervous system (Gottsch et al., 2004; Thompson et al., 2004) or directly on the pituitary gland (Luque et al., 2011).

In teleost fish, two paralogous kisspeptin genes and three genes codifying for kisspeptin receptors have been reported (Pasquier et al., 2012a; 2012b; 2014). Most teleost species have two ligands, known as Kiss1 and Kiss2, and two receptors, currently named as Kissr2 and Kissr3 according to the comparative studies performed by Pasquier and collaborators (2014). However, in some percomorph species such as *Gasterosteus aculeatus* (Tena-Sempere et al., 2012), *Solea senegalensis* (Mechaly et al., 2011) and *Takifugu niphobles* (Shahjahan et al., 2010) only one ligand, Kiss2 has been detected until now. In contrast, Kissr2 and Kissr3 receptors have been found in most teleost species examined (Mechaly et al., 2013; Pasquier et al., 2014; Tena-Sempere et al., 2012), all of them carry Kissr2, and a few species such as spotted gar (*Lepisosteus oculatus*) and European eel (*Anguilla anguilla*) carry Kissr1 and, only spotted gar carries Kissr4 (Pasquier et al., 2014).

Most studies on the vertebrate kisspeptin system have been devoted to understand its role in puberty and reproduction (Gopurappilly et al., 2013; Mechaly et al., 2013; Oakley et al., 2009; Tena-Sempere et al., 2012;). More recently, however, it has been shown that kisspeptin is expressed early in the mammalian development and it plays a role in brain sex differentiation (Campbell and Herbison, 2014; Clarkson et al., 2014; Franceschini and Desroziers, 2013). Furthermore, in some teleost fish species kisspeptin mRNAs are differentially expressed early in development, during the sex differentiation process. For example, in the cichlid (*Oreochromis niloticus*), *kiss2* and *kissr2* mRNA levels are increased during the first 2-4 weeks after hatching, coinciding with the sex differentiation period (Park et al., 2012); in the mackerel (*Scomber japonicus*), expression of *kiss1*, *kiss2* and their receptors, *kissr2* and *kissr3* also is elevated during this critical period (Selvaraj et al., 2015).

It is also known that water temperature may affect kisspeptin expression in fishes. In adult zebrafish (*Danio rerio*), low temperatures (15 ºC) increase *kiss1*/*kissr3* mRNA levels in the brain while high temperatures (35 ºC) induce no changes when compared to control individuals kept at intermediate temperatures (27 ºC). Concomitantly, *kiss2*/*kissr2* mRNAs brain levels decrease at both low and high temperatures (Shahjahan et al., 2013). These studies show that the kisspeptin brain systems respond differentially to water temperature.

Our experimental species, the pejerrey (*Odontesthes bonariensis*), has become a useful model to study the influence of temperature on sex determination and gonadal differentiation in fishes (Fernandino et al., 2015; Yamamoto et al., 2014). In this species, phenotypic sex is contingent upon water temperature after hatching, during a critical period of larval development. Low temperatures (17-19 ºC) can produce 100% female larvae, high temperatures (29 ºC) induce 100% males and "sexually neutral" temperatures (24-25 ºC) favor a balanced sex ratio (Strüssmann et al., 1997). Also in pejerrey, previous studies demonstrated that both Fsh- and Lh-expressing cells can be visualized by immunocytochemistry just previous to the 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). In this context, the aims of the present study are to characterize the *kiss/kissr* system in *Odontesthes bonariensis*, to study its gene expression profile during the critical period of sex determination/differentiation. Additionally we wanted to place the newly described genes for pejerrey in a phylogenetic framework consistent with a recent classification of bony fishes (Betancur-R et al., 2013), and to test current hypotheses of the evolution of these genes in fishes (Pasquier et al., 2014).

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 and females were obtained from the IIB-INTECH stock for cDNA characterization. The samples were taken at the reproductive season when males were spermiating and females have already completed vitellogenesis. After dissection, the brain was divided into three different regions: rostral region: including the olfactory bulbs, telencephalon and preoptic area; medial region: optic tectum, thalamus, pineal gland and hypothalamus; and the caudal region containing the cerebellar body, vagal lobe and the *medulla oblongata*. The following tissues/organs were also sampled: pituitary gland, gonads, olfactory epithelium, retina, lateral line, liver, gills, muscle, heart, foregut, midgut, hindgut, kidney and spleen from males and females. All samples were quickly frozen in liquid nitrogen and then stored at -80 °C until mRNA extraction.

2.2. RNA extraction and reverse transcription

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

2.3. Primer design and cDNA characterization

Different consensus primers (CP) were designed using the PrimerSelect (DNASTAR®, Madison, Wisconsin, USA) program based on conserved regions of the open reading frames (ORF) of kisspeptin and kisspeptin receptor genes from different teleost species (Table 1). The different regions were amplified using nested PCR strategies with CPs. Both, *kiss1* and *kissr2* were amplified from whole brain cDNA while *kiss2* was amplified from pituitary cDNA. PCR reactions were 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 QUIAEX II Gel Extraction Kit (QIAGEN, Chatsworth, CA, USA). Partial fragments were directly sequenced (Macrogen, USA). Once a 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 was obtained by blasting the pejerrey genome database (Campanella et al., 2013).

In the case of *kissr3*, due to the low number of species in which this receptor had been characterized at the moment we did this study, the sequence was obtained from the pejerrey genome database. This database was blasted using *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 OligoAnalizer 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, *ß-actin* (GenBank accession no. EF044319). 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). Finally, these sequences were confirmed with the pejerrey genome database.

2.4. Sequence analysis

Identity of all cDNA products was confirmed with BLAST and multiple sequence alignments conducted with ClustalW. Analysis of identity and similarity was calculated with the default settings (http://imed.med.ucm.es/Tools/sias.html). The putative signal peptides for either ligands or receptors were predicted with SignalP 4.1 program based on neuronal network and hidden Markov model algorithms (http://www.cbs.dtu.dk/services/SignalP/) and receptor's transmembrane domains were predicted using either (http://www.cbs.dtu.dk/services/TMHMM/) based in hidden Markov method (https://www.predictprotein.org).

2.5. Phylogenetic analysis

Public databases with genomic and transcriptomic data were searched to obtain existing sequences for kisspeptin and kisspeptin receptor transcripts in fishes. The resulting list of genes and species with their GenBank accession numbers are in Suppl. Tables 1 and 2. All kisspeptin and kisspeptin receptor DNA sequences were aligned with MAFFT v. 5 using default parameters and the L-INS-I algorithm (Katoh et al., 2005). Phylogenetic analysis of aligned sequences was conducted under maximum likelihood with RAxML (Stamatakis, 2014), using the Cipres Science Gateway (www.phylo.org). Ten independent searches were performed for each alignment under the GTR+gamma model of DNA substitution, with 100 bootstrap replicates to obtain the maximum likelihood estimate. The resulting tree was compared with alternative topologies consistent with previous hypotheses. Topological constraints were enforced to test phylogenetic hypotheses advanced by Pasquier and colleagues (2014). The current hypothesis of gene evolution for the kisspeptin and kisspeptin receptor genes was based on synteny analysis and proposes a single ancestral gene that duplicated through 1R and 2R that occurred in early steps of vertebrate evolution, with no effect due to the 3R duplication at the base of the teleosts (Nakatani et al., 2007; Pasquier et al., 2012a; 2012b). We also compared trees that are consistent with the current tree of life of fishes (Betancur-R et al., 2013), and with an evolutionary scenario where the gene duplication occurred at the base of the teleosts (3R). Analyses under these constraints were ran under the same model in RAxML, and the alternative topologies obtained were compared to the unconstrained tree for statistical significance using the AU test with the package Consel v0.1 (Shimodaira and Hasegawa, 2001).

2.6. Pejerrey larval rearing and sample collection

Odontesthes bonariensis fertilized eggs were obtained from the IIB-INTECH brood-stock and maintained until hatching in flow-through incubators at 18 ± 0.5 ºC. Approximately 1000 larvae were transferred after hatching to 140 L tanks and reared at different temperatures for 12 weeks (W1 to W12). In all cases, fish were reared in brackish water under a constant photoperiod (16 h

light-8 h dark) as previously described (Fernandino et al., 2008a). Larval growth parameters are shown in Suppl. Fig. 4. A first experiment was performed during 12 weeks of rearing at 24 ºC, a neutral temperature for sex determination where a 1:1 female/male sex ratio (MixPT) is generally obtained (Strüssmann et al., 1997). A second experiment was then performed to analyze the effects of temperature on kisspeptins and kisspeptin receptors mRNA abundance. In this case the larvae were raised during 8 weeks either at female producing temperature (FPT, 17 ºC) or male producing temperature (MPT, 29 ºC); temperature was then gradually changed to 24 ºC until week 12. In both experiments, the larvae were exclusively fed with *Artemia spp. nauplii* 4 times a day until week 6; then *Artemia nauplii* were supplemented with commercial fish food until the end of the experiment. Samples were taken once a week, until week 8 after hatching. Fish were anesthetized on ice-cold water until death, then total length (TL) and body weight (BW) were obtained and the entire heads were dissected, frozen in liquid nitrogen and stored at -80 ºC until processed for RNA extraction. At the end of the experiment, 20 specimens were processed for histology to be sexed following criteria published by Ito et al. (2005).

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

RTqPCR assays were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA), using FastStart Universal SYBR Green Master (Rox, Roche Diagnostics, Mannheim, Germany). The reaction mix contained: 5 µl of SYBR, 2 µl of diluted (1:20) cDNA, 1 µl (3 mM) of each forward and reverse primer (Table 1) and 1 µl of ultrapure water (Invitrogen, Carlsbad, CA) in a 10 µl of final volume. Primer specificity was assessed with melting curve to confirm the presence of only one product. In addition, electrophoresis in 1 % agarose gels was performed to ensure a single band and to purify some representative products for sequencing. Primer efficiency was validated by 4-fold serial dilutions standard curves for *kiss1*, *kiss2*, *kissr2*, *kissr3* and *ß-actin* as reference gene, using a cDNA pool of whole heads for each temperature. A non-template control was always included for each pair of primers. RTqPCR cycle for all genes were performed as follows: 10 min at

95 °C for polymerase activation, and 40 cycles of 95 °C for 15 sec, 1 min at 60 °C for annealing/elongation, melting curve analysis was also included. The RTqPCR reactions were performed in duplicates or triplicates for samples and standard curve respectively. In the case of tissue distribution all values were relativized to medium region of the brain, in the case of early expression experiments values were relativized to week 1.

2.8. Statistical analysis

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

3. Results

3.1 Molecular characterization of Odontesthes bonariensis kiss1 and kiss2

The cDNA of *kiss1* containing the complete open reading frame (ORF) is 535 bp in length (Suppl. Fig. 1A). This sequence includes 64 bp and 177 bp corresponding to the 5' and 3' UTR respectively. The ORF consist of 294 bp coding for 98 amino acid (aa) residues with a deduced molecular weight (MW) of 10734.54 Da, including the N-terminal signal peptide (1-19 aa). The core sequence of Kiss1 (82-96 aa) is QDVSSYNLNSFGLRY, and contains a cleavage/amidation site at 97-98 aa.

The *kiss2* full length is 573 bp, including 68 bp and 133 bp from the 5' and 3' UTR, respectively. The ORF is 372 bp with a coding region of 124 aa residues with a deduced MW of 13607.41 Da. Sequence analysis with SignalP program revealed that N-terminal 1-19 aa correspond to the signal

peptide and the core sequence of Kiss2 (77-88 aa) is SKFNYNPFGLRF, and contains a cleavage/amidation site at 89-91 aa (Suppl. Fig. 1).

The amino acid alignments of pejerrey Kiss1 and Kiss2 together with sequences from other teleost fish are shown in Supp. Figs. 5A and 5B respectively. In both alignments, the signal peptides are underlined and the Kiss1 and Kiss2 mature peptides are shown in the open box, being the core peptides highly conserved among teleost species. The predicted *O. bonariensis* Kiss1 and Kiss2 amino acid precursor sequences have low identity (7.14 %) and similarity (20.4 %) between them. The analysis of these sequences revealed that the mature Kiss1 peptide has between 66.7 % to 100 % of identity and similarity, when compared to other teleost species. While the mature Kiss2 peptide shows between 75 % to 100 % of identity and similarity when compared to other fish. The identity analysis and expected values (e-value) by BLATP of *O. bonariensis* Kiss1 precursor shows the higher values with Perciformes, Scorpaeniformes and Beloniformes and Kiss2 precursor with Perciformes, Scorpaeniformes, Pleuronectiformes and Beloniformes. Kiss1 precursor has an identity from 7.2 % to 37 % and similarity from 17.4 % to 52.2 %; while Kiss2 precursor values are: identity from 3.2 % to 29.6 % and similarity from 7.9 % to 33.3 %. Moreover, Kiss2 presents lower e-values ($2e^{-52}$ to $7e^{-7}$) than Kiss1 ($3e^{-39}$ to 0.029).

3.2. Molecular characterization of Odontesthes bonariensis kissr2 and kissr3

A partial sequence of *kissr2* was amplified with consensus primers from brain cDNA, and then a 3' RACE was performed with GSP primers (Table 1). The obtained *kissr2* partial fragment (1081 bp, HM755975) was used to search the full-length sequence by BLAST 2.2.29 in the genome of *O. bonariensis*. The structure of the *kissr2* (Suppl. Fig. 2A) is as follows: 5' UTR composed by 64 bp, then ORF, 1134 bp, and the 3' UTR with 254 bp including the polyadenylation signal (aataaa). The *kissr2* ORF codifies for a 378 aa residues protein with a deduced MW of 43218.3 Da containing a signal peptide between amino acids 1 and 26.

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

Alignments of the deduced amino acid residues of *O. bonariensis* Kissr2 and Kissr3 with fish orthologous sequences are shown in Suppl. Figs. 6 and 7, respectively. The analysis of the predicted amino acid sequence revealed seven putative transmembrane domains (TMD), a typical characteristic of GPCRs. The TMDs' positions for Kissr2 are: TMD1 (47-69 aa), TMD2 (82-104 aa), TMD3 (121-140 aa), TMD4 (161-183 aa), TMD5 (209-233 aa), TMD6 (270-289 aa) and TMD7 (318-344 aa, Suppl. Fig. 6). The positions for Kissr3 are: TMD1 (39-59 aa), TMD2 (69-91), TMD3 (111-131 aa), TMD4 (150-170 aa), TMD5 (199-218 aa), TMD6 (255-276 aa) and TMD7 (291-312 aa, Suppl. Fig. 7).

The analysis revealed high identity values for Kissr2 (from 5.4% to 96.2%) and Kissr3 (from 6.1% to 54.6%) and similarity from 12.7% to 98.1% and 13.4% to 61.5% for Kissr2 and Kissr3 respectively. The e-value for Kissr2 is very close to 0.0 in all analyzed species, however for Kissr3, e-value varies from 0.0 and $1e^{-149}$. These low expectation values indicate a high similarity of *O. bonariensis* kisspeptin receptors with other teleosts species.

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

The maximum likelihood trees obtained with RAxML are shown in Figures 1 and 2 for kisspeptins and kisspeptin receptors, respectively. The newly described pejerrey sequences of *kiss1*, *kiss2*, *kissr2* and *kissr3* always grouped with orthologues of medaka (*Oryzias latipes*), as predicted by the currently accepted taxonomy. In spite of poor conservation of *Kiss* gene sequences among species (except for the sequence encoding the signal peptide), our analysis resolved the reciprocal monophyly of *kiss1* and *kiss2* orthogroups with high support using *kiss3* from elephant shark

312 (*Callorhinchus milli*) as an outgroup (Fig. 1). Within each group, a clade of percomorph fishes
313 (Subdivision Percomorphaceae) is clearly resolved, but rooting of each orthogroup produced
314 species trees that are not consistent with the tree of life of fishes (Betancur-R et al., 2013); most
315 notably, the expected relationship among chondrichthyans, sarcopterygians and actinopterygians is
316 not resolved. Enforcing a topology that reflects the constraint (Chondrichthys (Sarcopterygii
317 (Holostei (Teleostei)))) for each orthogroup results in a tree that is marginally less likely (AU test,
318 p=0.03) than the unconstrained topology shown in Figure 1. In contrast, a topology that is
319 consistent with a duplication event giving rise to *kiss1* and *kiss2* at the base of the teleosts (3R) is
320 highly unlikely (AU test, p<0.01).
321 The gene tree for Kiss receptors (Fig. 2) is more consistent with the tree of life of fishes than the
322 kisspeptin phylogeny, possibly due to higher conservation and a longer sequence alignment with
323 more phylogenetic signal. Two well-supported clades group *kissr2* with *kissr4* and *kissr1* with *kissr3*,
324 but the monophyly of *kissr3* is not supported in the unconstrained tree. Enforcing a topology that is
325 consistent with the monophyly of each orthogroup as proposed by Pasquier et al. (2012b) results in
326 a tree that cannot be rejected by the data (AU test, p=0.11), but the evolutionary scenario involving
327 3R as the origin of *kissr* diversity is significantly rejected (AU test, p<0.001).
328
329 *3.4. Tissues/organs distribution*
330 The tissues/organs distribution of *kiss1*, *kiss2*, *kissr2* and *kissr3* mRNA was studied by RTqPCR using
331 the primers specified in Table 2 in males and females. As shown in Figure 3, kisspeptin mRNAs have
332 a broad distribution in different tissues/organs and some of them show sex differences. In males,
333 *kiss1* is expressed in all sections of the brain and testes and it is significantly more mRNA levels in
334 gills and heart, than in females. Furthermore *kiss1* is highly expressed in the olfactory epithelium,
335 retina and gut sections. Females also have a wide tissue distribution for this gene, with high
336 transcript levels in the brain, olfactory epithelium and retina and faint levels in gills and gut
337 sections (Fig. 3A).

The expression of *kiss2* mRNA also has a widespread distribution in both sexes with very high levels at the pituitary gland. Messenger levels of *kiss2* were detected in almost all tissues examined with no significant differences between sexes (Fig. 3B).

Receptors, *kissr2* and *kissr3* (Fig. 3C and 3D), also show 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 ºC)

In order to evaluate the expression levels of all components of the kisspeptin system during early development, pejerrey larvae were kept at 24 ºC 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.

Nevertheless all transcripts were detected during this period, as early as week one after hatching, 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 ºC) and MPT (29 ºC)

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 ºC) or MPT (29 ºC).

In this case, sex ratio, as determined by histology followed the normal pattern for these conditions: 100 % females at 17 ºC and 100% males at 29 ºC.

Transcripts levels of *kiss1*, *kiss2*, *kissr2* and *kissr3* were always detectable in both temperatures during the sex determination and gonad differentiation period from W1 to W8 (Fig. 5). At MPT an increase of *kiss2* was observed at W4 ($p < 0.05$), meanwhile no variations were observed at FPT.

4. Discussion

In the present study, two kisspeptin ligands (*kiss1* and *kiss2*) and their receptors (*kissr2* and *kissr3*) were characterized in an atherinopsid species, *Odontesthes bonariensis*. The evolutionary history of these genes was tested; their tissues/organs gene distribution pattern and their gene expression pattern during early development were described.

As previously reported, in most teleost species, the kisspeptin system is composed by two ligands and two receptors (Pasquier et al., 2014). This conservation in a wide range of teleost orders such as Anguilliformes (Pasquier et al., 2012a), Cypriniformes (Biran et al., 2008; Kitahashi et al., 2009; Li et al., 2009), Beloniformes (Kitahashi et al., 2009), Scorpaeniformes (Fairgrieve et al., 2016) and Perciformes (Ohga et al., 2013; Selvaraj et al., 2010; Zmora et al., 2012;). However, as stated previously, the Kiss1 ligand has not been discovered in several teleosts (Felip et al., 2009; Kim et al., 2012; Kitahashi et al., 2009; Li et al., 2009; Mechaly et al, 2011).

The predicted precursors from *Odontesthes bonariensis* kisspeptins presented low identity and similarity when compared to kisspeptins of other teleost species. However, the mature peptides of Kiss1 and Kiss2 have highly conserved characteristics. *O. bonariensis* Kiss1 and Kiss2 are very similar in sequence to their orthologs carried by percomorphs and especially Beloniformes (medaka). Also, basic amino acids at the sites for protein convertase cleavage are highly conserved (Zmora et al., 2012). In the mature peptides, the first amino acids Q (glutamine) for Kiss1 and S (serine) for Kiss2 are also conserved. Nevertheless Kiss1 and Kiss2 from pejerrey are predicted from the prohormone

389 structure, similar peptides have already been purified from other vertebrate species indicating that
390 they are naturally present (Kotani et al., 2001; Lee et al., 2009; Osugi et al., 2013).
391 The *in silico* analysis of the predicted amino acid sequences of *Odonthestes bonariensis* Kissr2 and
392 Kissr3 revealed the typical structure of the rhodopsin superfamily of G protein-coupled receptors,
393 composed by seven transmembrane domains, common to all vertebrates (Muir et al., 2001;
394 Pasquier et al., 2014). Both receptors presented high identity and similarity when compared to
395 other receptors from teleost fish mainly because of TMDs are highly conserve among teleost
396 species (Tena-Sempere et al., 2012).
397 The pejerrey *kiss1*, *kiss2*, *kissr2* and *kissr3* sequences always group with orthologues of medaka,
398 and are grouped, in clades of percomorph fishes (Subdivision Percomorphaceae). Phylogenetic
399 analyses of Kiss ligands and receptors confirmed previous hypotheses proposed by Pasquier et al.
400 (2012a, 2012b, 2014) suggesting that the diversity of these genes can be traced back to the base of
401 the vertebrates, arising from the putative 1R and 2R whole genome duplication events (Nakatani et
402 al., 2007). As already reported (Kim et al., 2012; Pasquier et al., 2014), the effect of the teleost-
403 specific genome duplication event (3R) is not detected among these genes, possibly implying a
404 rapid loss of gene copies arising from such event. On this respect, it is important to note that in a
405 previous work from our research group, the 3R was weakly supported in the phylogenetic history
406 of GnRH precursors and the fish specific genome duplication was not the preferred hypothesis
407 (Guilgur et al., 2007), and a similar situation was reported for fish gonadotropins (Levavi-Sivan et
408 al., 2010). In view of the solid evidence that the 3R event took place in the evolutionary lineage
409 ending in the modern teleosts (Sato and Nishida, 2010; Glasauer and Neuhauss, 2014) it is then
410 possible that non-functionalization of one duplicated gene was the consequence in genes directly
411 related to reproduction.
412 As in almost all teleosts, *kiss1*, *kiss2*, *kissr2* and *kissr3* mRNA were detected in the pejerrey brain-
413 pituitary-gonadal (BPG) axis. This conserved characteristic suggests important roles of the
414 kisspeptin system in reproductive functions in teleosts (Pasquier et al., 2012a). Both, ligands and

415 receptors were detected with different abundance in the pejerrey brain, as it was reported in
416 different fish species (Biran et al., 2008; Fairgrieve et al., 2016; Kitahashi et al., 2009; Li et al., 2009;
417 Mechaly et al., 2011; Ohga et al., 2013). However, the neuroanatomical organization of this system
418 in teleosts is starting to be clarified in few species, *Danio rerio* (Servili et al., 2011), *Morone saxatilis*
419 (Zmora et al., 2012; 2014), *Dicentrarchus labrax* (Escobar et al., 2013) and *Oryzias latipes* (Kanda et
420 al., 2013).
421 Both forms of kisspeptin have also been detected in the pituitary gland of different teleosts (Li et
422 al., 2009; Alvarado et al., 2013; Saha et al., 2016), however it appears that some species only
423 express *kiss2* (Fairgrieve et al., 2016) and some others do not express either *kiss1* or *kiss2* at the
424 pituitary gland (Kanda et al., 2008; Kitahashi et al., 2009). Interestingly, in our case, *kiss2* but not
425 *kiss1* was found to be highly expressed in pejerrey pituitary. Nevertheless, this pattern should be
426 taken with caution because kisspeptin levels at the pituitary gland could varied according to the sex
427 cycle as already reported in *Dicentrarchus labrax* (Alvarado et al., 2013). Also, although Kanda et al.
428 (2008) reported that kisspeptin variants were not found to be expressed at the zebrafish pituitary,
429 the expression of Kiss2 was then confirmed using a specific antibody (Servili et al., 2011). Then,
430 further studies should be performed in order to analyze kisspeptin levels at the pituitary gland
431 along the reproductive cycle in pejerrey and other fish species.
432 Despite the fact that *kiss1*, *kiss2*, *kissr2* and *kissr3* levels were low, they were also detected at the
433 gonads of both sexes in pejerrey. Similar expression patterns, with differences in the relative mRNA
434 abundance, were also reported in other fish species and these levels varied according to the
435 gonadal cycle (Fairgrieve et al., 2016; Mechaly *et al*., 2012; Saha et al., 2016).
436 As already mentioned, pejerrey also expresses kisspeptins and their receptors in other tissues and
437 organs. It is interesting to note that in males there are higher levels of *kiss1* in pituitary, in the heart
438 *kiss1* and *kissr2* also are more abundant than in females. On the other hand, *kissr3* expression
439 levels are higher in the female olfactory epithelium than in the males. This wide tissue/organ
440 distribution of kisspeptin ligands and receptors suggests that kisspeptins may be involved in other

441 functions in addition to reproduction (Oakley et al., 2009). As an example, high expression of all
442 members of the kisspeptin system was detected in the olfactory epithelium and retina of both
443 sexes. In this context, Zhao and Wayne (2012) have reported that Kiss1 stimulates, through
444 synaptic regulation, the electrical activity of GnRH-III neurons located at the terminal nerve. Taken
445 together, these data suggest that kisspeptin is probably mediating, olfactory and visual signals.
446 The effect of water temperature on expression of kisspeptin brain systems is significant in zebrafish
447 (Shahjahan *et. al.*, 2013), but unknown in *Odontesthes bonariensis,* a fish model to study the
448 effects of temperature on sex determination (Fernandino et al., 2015; Yamamoto et al., 2014). In
449 this study, we quantified the expression of *kiss1*, *kiss2*, *kissr2* and *kissr3* during the critical period of
450 sex determination and gonad differentiation in relation to water temperature. At 24 °C (MixPT), all
451 these genes had detectable mRNA levels since the first week after hatching (WAH); however none
452 of them displayed significant variations during the first 5 WAH. It is important to note here that the
453 temperature sensitive window, when sex determination is established, was estimated to be
454 between 1-5 WAH in this species (Strüssmann et al., 1997).
455 A significant increase of transcript levels of kisspeptins and kisspeptin receptors during early
456 development had been reported in few teleost species (Mohamed et al., 2007; Ohga et al., 2014;
457 Park et al., 2012), but its functional implications are still not clear. Concerning to pejerrey, the four
458 kisspeptins genes were expressed at FPT and MPT during the first eight WAH. There is substantial
459 amount of data that support the idea that the first morphological signs of gonadal differentiation
460 appeared between 4-6 WAH (Fernandino et al., 2008b; Karube et al., 2007; Shinoda et al., 2010;
461 Strüssmann et al., 1997). On the other hand, the number of GnRH-I and gonadotropin
462 inmunoreactive cells was shown to be significantly increased at 3-4 WAH in the POA (Miranda et
463 al., 2001; 2003) before morphological gonad differentiation. Moreover, it was demonstrated that
464 *fsh*- and *lh-ß* mRNA levels are also significantly increased at 3-4 WAH (Shinoda et al., 2010). In the
465 present study, we could observe a slight but consistent increase of *kiss2/kissr2* at MFT in W4 that
466 coincided with the significant increase of GnRH and gonadotropin cells but also with *fsh*- and *lh-ß*

increase. Taken together, this temporal overlap between key players of the reproductive system, led us to hypothesize that *kiss2*/*kissr2* could have a role in brain sex differentiation inducing GnRH-I in the POA with the consequent gonadotropin response at MPT. This situation is similar to that reported in mammals, where there is early expression of KISS1/KISSR1 (Bellingham et al., 2009; Desroziers et al., 2012; Fiorini & Jasoni, 2010), and GnRH neurons responds to KISS1-10 in pre-natal mouse (Desroziers 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). Further studies are needed to verify this hypothesis using microdissected areas of pejerrey brain during the temperature sensitive window.

However kisspeptin could also be involved in other functions during early brain development, as suggested in other teleost fish (Hodne et al., 2013; Selvaraj et al., 2015).

In summary, the present study characterizes two ligands (*kiss1*/kiss2) and two receptors (*kissr2/kissr3*) for the first time in a species belonging to the order Atheriniformes. The four genes grouped with those of percomorph fishes and they form a clade with those of 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 brain sex differentiation.

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727

728

Figure legends

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

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

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

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

Figure 5: Figure 5: Fold changes in mRNA expression determined by RTqPCR on the whole head of *O. bonariensis* larvae keep at FPT (17ºC) or MPT (29ºC), *kiss1* at 17 ºC (A) and 29 ºC (B), *kiss2* at 17 ºC (C) and 29 ºC (D), *kissr2* at 17 ºC (E) and 29 ºC (F), *kiss3r* at 17 ºC (G) and 29 ºC (H). Black arrows indicate differences between weeks (P<0.05). All values were relativized to the expression of each gene at W1. Expression values were normalized to the ß-actin.

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

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

Supplementary files

Supplementary Figure 1: Nucleotide, deduced amino acid sequences of *O. bonariensis kiss1* (A) and the schematic representation of mRNA and protein structure (B). Nucleotide, deduced amino acid sequences of *kiss2* (C) and the schematic representation of mRNA and protein structure (D). Uppercase letters indicate the coding sequence. Start codon (ATG) is bold and stop codon (TGA) is indicated by asterisk (*). The putative signal peptide is underlined. The kisspeptin-15 (A) and kisspeptin-12 (B) amino acid sequences are bold and italic. The polyadenylation sequence (aataaa) is gray-shaded. Nucleotides are right numbered. GenBank Accession # for kiss1 (KF314719) and kiss2 (KF314720). Black arrows indicate primer position. In the mRNA 5' UTR, 3' UTR and exon position are indicated by boxes. ORF is indicated between brackets. The sites of Kiss1-15 and Kiss2-12 are indicated by dash arrows and its aminoacid sequence is in capital letters.

Supplementary Figure 2: Nucleotide and deduced amino acid sequences of *O. bonariensis kissr2* (A) and the schematic representation of mRNA and protein structure (B). Uppercase letters indicate the coding sequence. Start codon (ATG) is in bold and stop codon (TAA) is indicated by asterisk (*). The putative signal peptide is underlined. Nucleotides are right numbered. Black arrows indicate primer position. In the mRNA 5' UTR, 3' UTR and exon position are indicated by boxes. ORF is indicated between brackets. The sites of the transmembrane domains (TMDs) are indicated in boxes.

Supplementary Figure 3: Nucleotide and deduced amino acid sequences of *O. bonariensis kissr3* (A) and the schematic representation of mRNA and protein structure (B). Uppercase letters indicate the coding sequence. Start codon (ATG) is in bold and stop codon (TAA) is indicated by asterisk (*). The putative signal peptide is underlined. Nucleotides are right numbered. Black arrows indicate primer position. In the mRNA 5' UTR, 3' UTR and exon position are indicated by boxes. ORF is indicated between brackets. The sites of the transmembrane domains (TMDs) are indicated in boxes.

Supplementary Figure 4: Larval growth parameters.

Supplementary Figure 5: ClustalW putative amino acid sequence alignment of Kiss1 (A) and Kiss2 (B). Amino acid residues conserved in all species are indicated by asterisks (*) less conserved (: or .) and right numbered. The putative signal peptide is underlined. Amino acids different those from *O. bonariensis* kisspeptin-15 are gray shaded within the box.

Supplementary Figure 6: ClustalW alignment of Kissr2 deduced amino acid sequence. Amino acid residues conserved in all species are indicated by asterisks (*) and less conserved (: or .). Nucleotides are right numbered. The putative signal peptide is underlined and predicted transmembrane domains (TMD) are boxed.

Supplementary Figure 7: ClustalW alignment of Kissr3 deduced amino acid sequence. Amino acid residues conserved in all species are indicated by asterisks (*), less conserved (: or .) and right

numbered. The predicted signal peptide is underlined and predicted transmembrane domains (TMD) are boxed.

Supplementary Table 1: Species list and accession number used for the phylogenetic study of *kiss1* and *kiss2*.

Supplementary Table 2: Species list and accession number used for the phylogenetic study of *kissr2* and *kissr3*.

Table 1

Gene	*Sequence (5'-3')*	*Type*	*Primer name*	*Direction*
kiss1	TRGCTGCTTTGTCAACAGAG	CP	CPK1-Fw1	Forward
kiss1	GCYCTGAGAGATTTAAGCC	CP	CPK1-Fw2	Forward
kiss1	AAAGGAGTTGAGGTTGTATG	CP	CPK1-Rv1	Reverse
kiss1	GACACATCTTGAYGTTTCTTG	CP	CPK1-Rv2	Reverse
kiss1	GAGGGAGTGAGGGGGCGG	GSP	GSPK1-Fw1	Forward
kiss1	GCCCGGTCCTGCGTCTGA	GSP	GSPK1-Fw2	Forward
kiss2	ATGAGRCTNGTGRCTCTGGT	CP	CPK2-Fw3	Forward
kiss2	TYGACTCTGCACAGAGGA	CP	CPK2-Fw4	Forward
kiss2	AGGCACCTCCAGTTCTCG	CP	CPK2-Rv3	Reverse
kiss2	GCCCGGTCCTGCGTCTGA	GSP	GSPK2-Fw3	Forward
kiss2	TCAGGAGGACTGCGGGAGAC	GSP	GSPK2-Fw4	Forward
kissr2	CATGCTGGTCGGACTCGTGGG	CP	CPK2R-Fw5	Forward
kissr2	GCGTTGGCAGTCCTTGAAGC	CP	CPK2R-Rv4	Reverse
kissr2	TCTCCGCCACCGCACTCC	GSP	GSPK2R-Fw5	Forward
kissr2	CCAATTTATTgCTGCCTACCTGC	GSP	GSPk2R-Fw6	Forward
kissr2	AACAATCAGCATAAGGAGCAAAGT	GSP	GSPK2R-Fw7	Forward
kissr2	AAATGAGGACGGCCACCAACT	GSP	GSPK2R-Fw8	Forward
kissr3	CCAAGCATCAGCAGATGAAA	GSP	GSPK3RFw1	Forward
kissr3	ATAAACCAGCGGGTTGACAG	GSP	GSPK3RRv1	Reverse
ß-actin	GCTGTCCCTGTACGCCTCTGG	GSP	GSP*ß*a-Fw7	Forward
ß-actin	GCTCGGCTGTGGTGGTGAAGC	GSP	GSP*ß*a-Rv1	Reverse
AP d(T)	GGCCACGCGTCGACTAGTACT(15)G	UAP	UAP1	Reverse
AP	GGCCACGCGTCGACTAGTAC	UAP	UAP2	Reverse

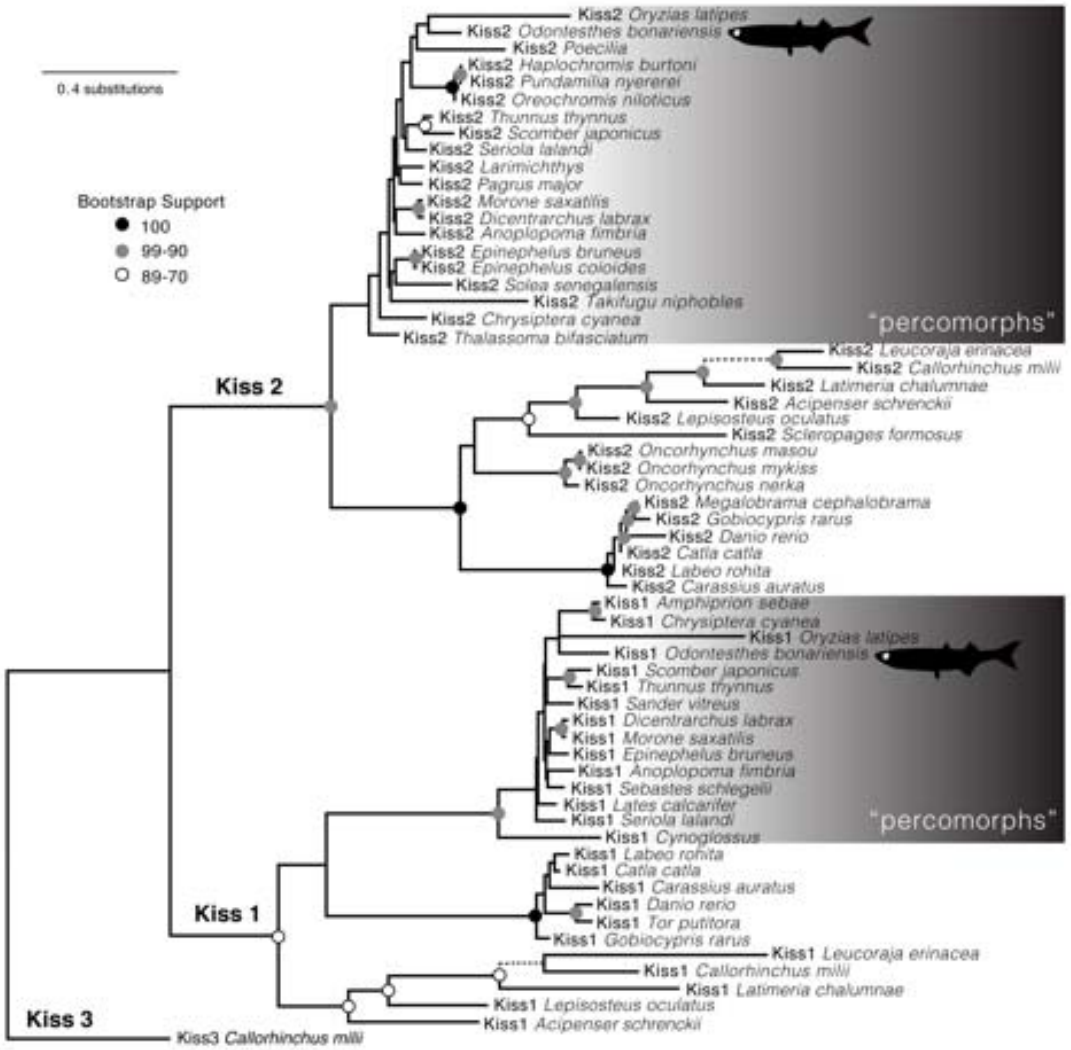
827 **Table 2**

828

Gene/ primer name	Forward primer (5' - 3')	Reverse primer (5' - 3')	Efficiency	Slope	r^2	Ct value +/- sd	Product size bp	Tm ºC
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
qß-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

829

Figure 1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Kiss2 Seriola lalandi
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Kiss2 Pagrus major
Kiss2 Morone saxatilis
Kiss2 Dicentrarchus labrax
Kiss2 Anoplopoma fimbria
Kiss2 Epinephelus bruneus
Kiss2 Epinephelus coioides
Kiss2 Solea senegalensis
Kiss2 Takifugu niphobles
Kiss2 Chrysiptera cyanea
Kiss2 Thalassoma bifasciatum
"percomorphs"
Kiss2 Leucoraja erinacea
Kiss2 Callorhinchus milii
Kiss2 Latimeria chalumnae
Kiss2 Acipenser schrenckii
Kiss2 Lepisosteus oculatus
Kiss2 Scleropages formosus
Kiss2 Oncorhynchus masou
Kiss2 Oncorhynchus mykiss
Kiss2 Oncorhynchus nerka
Kiss2 Megalobrama cephalobrama
Kiss2 Gobiocypris rarus
Kiss2 Danio rerio
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Kiss1 Thunnus thynnus
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"percomorphs"
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Kiss1 Tor putitora
Kiss1 Gobiocypris rarus
Kiss 1
Kiss1 Leucoraja erinacea
Kiss1 Callorhinchus milii
Kiss1 Latimeria chalumnae
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Kiss1 Acipenser schrenckii
Kiss 3
Kiss3 Callorhinchus milii

834 **Figure 2**

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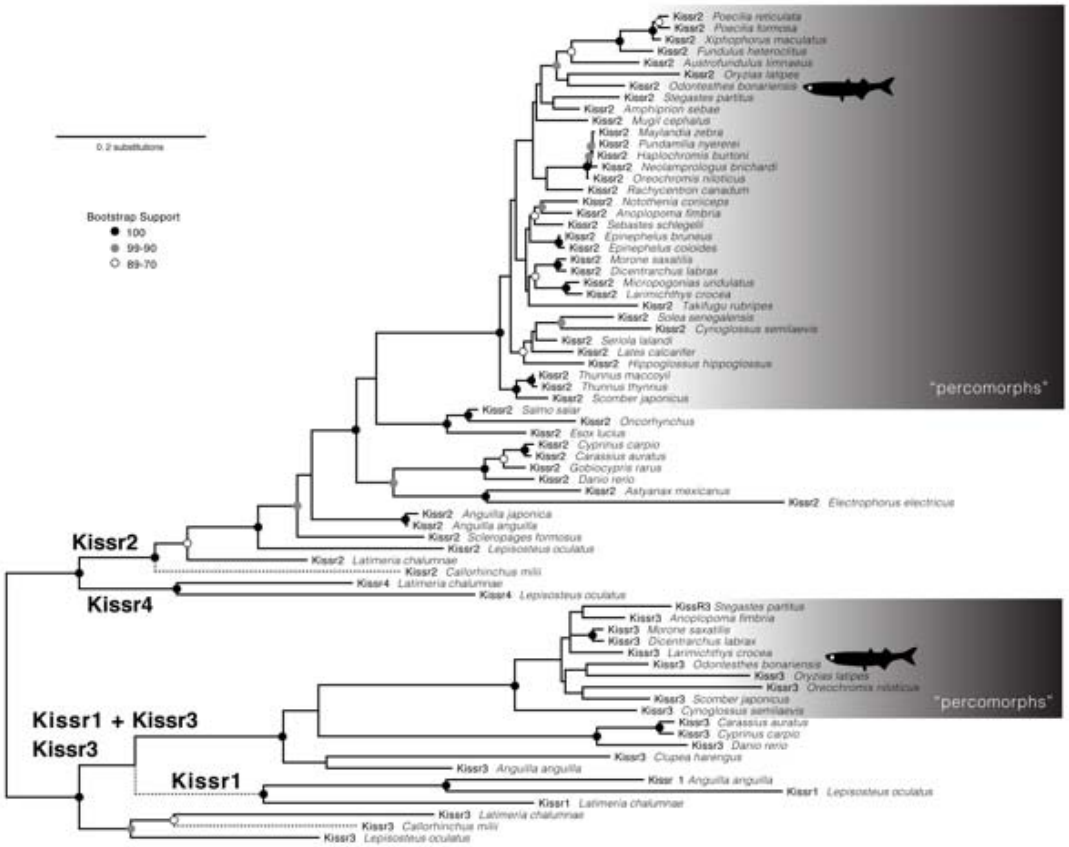

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838 **Figure 3**

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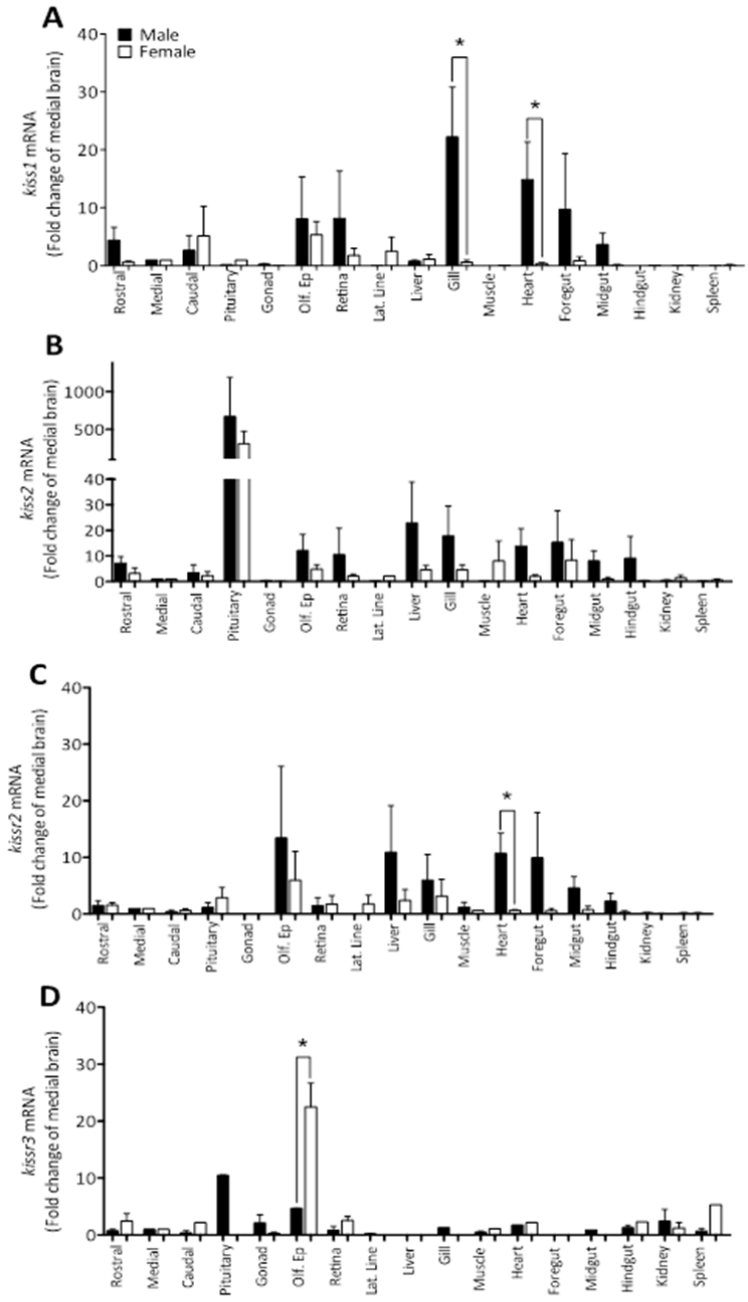

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841 **Figure 4**

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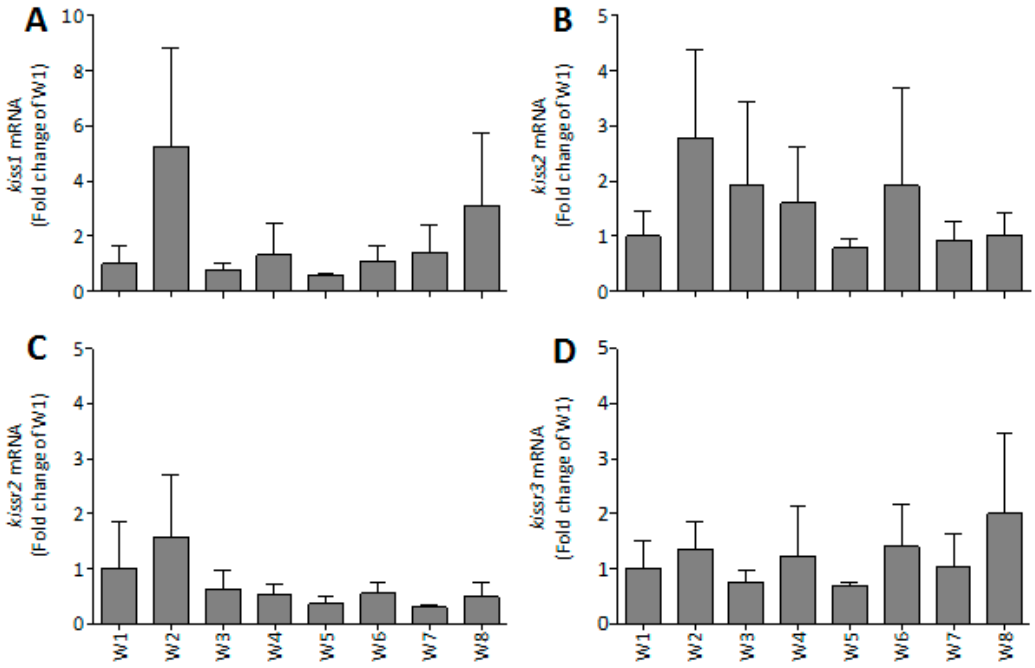

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845 **Figure 5**

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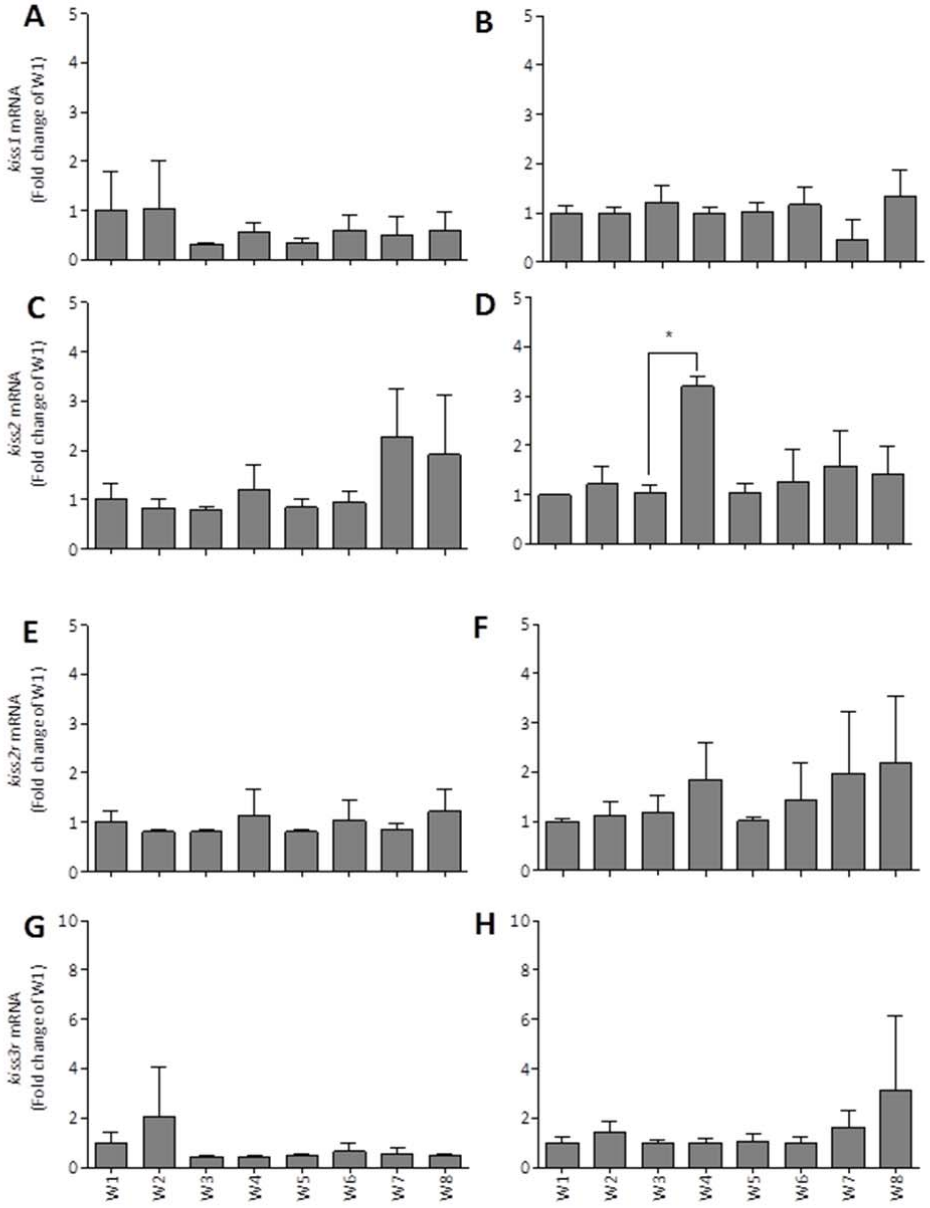

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Supplementary Table 1

Gene	*Species*	*Source*	*Accession #*	*Notes*
kiss1	*Odontesthes bonariensis*	This study		
kiss1	*Lepisosteus oculatus*	Genbank	XP_006628621.1	
kiss1	*Oryzias latipes*	Genbank	NM_001122921.1	
kiss1	*Danio rerio*	Genbank	EF690279.1	
kiss1	*Chrysiptera cyanea*	Genbank	AB894552.1	
kiss1	*Dicentrarchus labrax*	Genbank	FJ008914.1	
kiss1	*Epinephelus bruneus*	Genbank	GU984382.1	
kiss1	*Morone saxatilis*	Genbank	GU351864.1	
kiss1	*Scomber japonicus*	Genbank	GU731672.1	
kiss1	*Seriola lalandi*	Genbank	HQ449729.1	
kiss1	*Sander vitreus*	Genbank	JX524190.1	
kiss1	*Carassius auratus*	Genbank	FJ236327.1	
kiss1	*Catla catla*	Genbank	KM924445.1	
kiss1	*Gobiocypris rarus*	Genbank	KF837132.1	
kiss1	*Lates calcarifer*	Genbank	KR492513.1	
kiss1	*Anoplopoma fimbria*	Genbank	KP677561.1	
kiss1	*Labeo rohita*	Genbank	KF737179.1	
kiss1	*Amphiprion sebae*	Genbank	KP260915.1	
kiss1	*Sebastes schlegelii*	Genbank	KJ139960.1	
kiss1	*Thunnus thynnus*	Genbank	JX459926.1	
kiss1	*Tor putitora*	Genbank	KP710729.1	
kiss1	*Latimeria chalumnae*	Genbank	XM_005991685.1	
kiss1	*Cynoglossus semilaevis*	*Genbank:WGS*	*PRJNA251742*	*RefSeq Genome*
kiss1	*Callorhinchus milii*	Genbank:WGS	AAVX01162971.1	
kiss1	*Leucoraja erinacea*	Skatebase	LSb2-ctg630304	
kiss1	*Acipenser schrenckii*	Genbank	KT257658.1	

kiss2	*Odontesthes bonariensis*	This study		
kiss2	*Oryzias latipes*	Genbank	NM_001160441.1	
kiss2	*Danio rerio*	Genbank	EU853684.1	
kiss2	*Oreochromis niloticus*	Genbank	NM_001279468.1	
kiss2	*Chrysiptera cyanea*	Genbank	AB894852.1	
kiss2	*Dicentrarchus labrax*	Genbank	FJ008915.1	
kiss2	*Epinephelus bruneus*	Genbank	GU984383.1	
kiss2	*Morone saxatilis*	Genbank	GU351865.1	
kiss2	*Scomber japonicus*	Genbank	GU731673.1	
kiss2	*Seriola lalandi*	Genbank	HQ449730.1	
kiss2	*Epinephelus coioides*	Genbank	GQ258777.1	
kiss2	*Haplochromis burtoni*	Genbank	KM115576.1	
kiss2	*Pagrus major*	Genbank	AB632369.1	
kiss2	*Thalassoma bifasciatum*	Genbank	JX437963.1	
kiss2	*Carassisus auratus*	Genbank	GQ141877.1	
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kiss2	*Megalobrama amblycephala*	Genbank	KC146705.1	
kiss2	*Gobiocypris rarus*	Genbank	KF837133.1	
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kiss2	*Takifugu niphobles*	Genbank	AB548304.1	
kiss2	*Anoplopoma fimbria*	Genbank	KP677562.1	
kiss2	*Labeo rohita*	Genbank	KF695115.1	
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kiss2	*Oncorhynchus nerka*	Genbank	AB435387	
kiss2	*Thunnus thynnus*	Genbank	JX459927.1	
kiss2	*Scleropages formosus*	Genbank:WGS	PRJNA290065	Genome assembly
kiss2	*Larimichthys*	Genbank:WGS	PRJNA309464	Genome sequencing
kiss2	*Pundamilia nyererei*	Genbank:WGS	PRJNA220167	RefSeq Genome
kiss2	*Poecilia mexicana*	Genbank:WGS	PRJNA305619	RefSeq Genome
kiss2	*Latimeria chalumnae*	Ensembl	ENSLACT00000010278	
kiss2	*Callorhinchus milii*	Genbank:WGS	AAVX01172388	

kiss2	*Leucoraja erinacea*	Skatebase	LSb2-ctg804270
kiss2	*Acipenser schrenckii*	Genbank	KT257657.1
kiss2	*Lepisosteus oculatus*	Genbank	KT202355.1
kiss3	*Callorhinchus milii*	*Genbank:WGS*	*AAVX01250489.1*

850

851

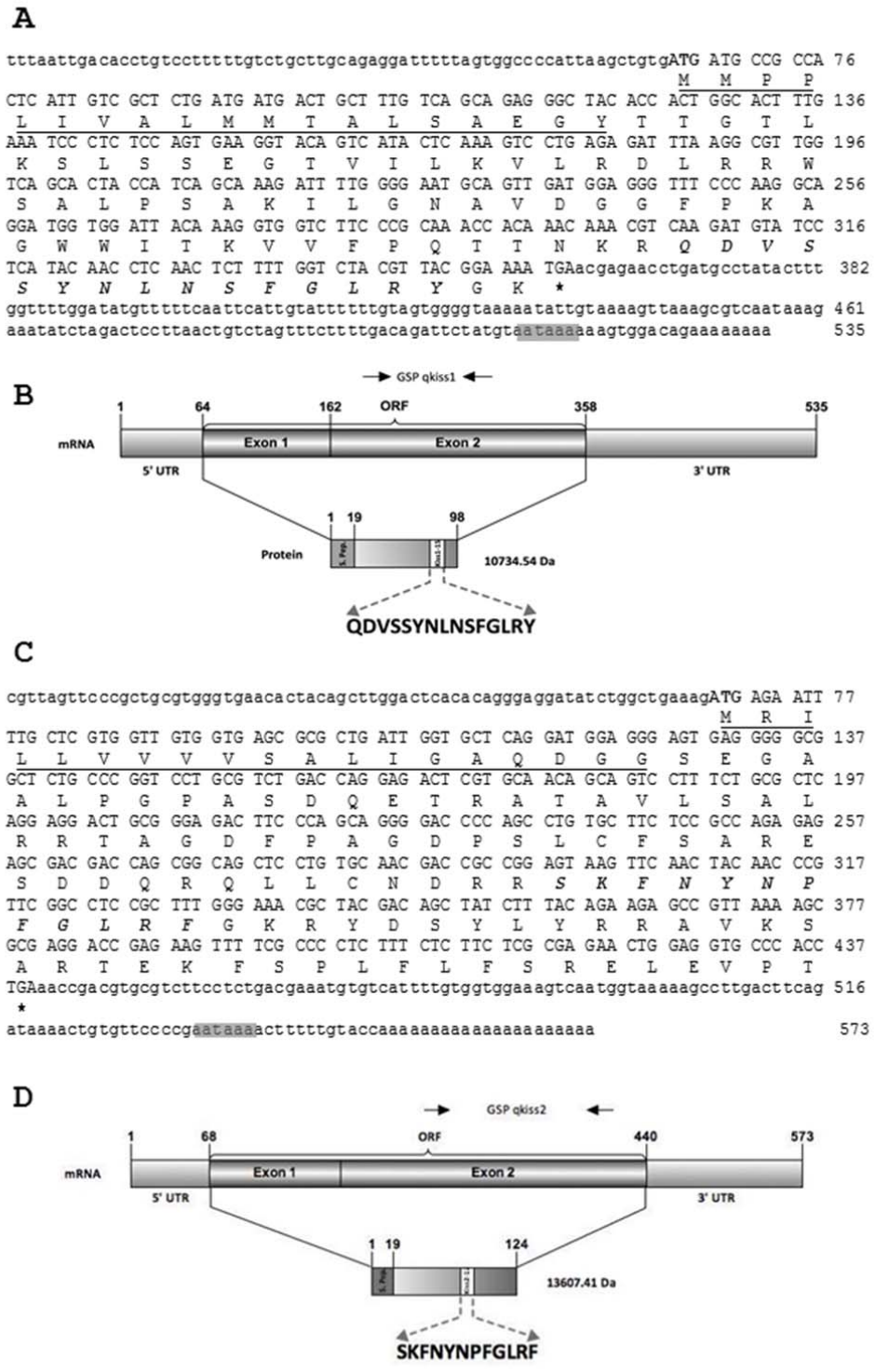
852 **Supplementary Table2**

853

854

Gene/ primer name	Forward primer (5' - 3')	Reverse primer (5' - 3')	Efficiency	Slope	r^2	Ct value +/- sd	Product size bp	Tm ºC
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
qß-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

Supplementary Figure 1



Supplementary Figure 2

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 L   V   I   Y   V   I   S   K   H   R   Q   M   R   T   A   T   N   F   Y   I
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 V   S   I   C   I   W   I   G   S   F   I   L   S   T   P   I   R   F   P   S
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 L   M   Y   Q   R   I   E   E   G   Y   W   Y   G   P   R   Q   Y   C   V   E
AAA ACA CAT GAG AGG GCG TTC ATC CTC TAC CAA TTT ATT GCT GCC TAC CTG CTG CCA GTG 726
 K   T   H   E   R   A   F   I   L   Y   Q   F   I   A   A   Y   L   L   P   V
CTC ACA ATC TCT TTT TGC TAC ACT CTG ATG GTG AAA AGG GTT GGC CAG CCC ACA GTT GAA 786
 L   T   I   S   F   C   Y   T   L   M   V   K   R   V   G   Q   P   T   V   E
CCT GTA GAC AAC AAT TAC CAG GTC AAC CTC CTG TCT GAG AGA ACA ATC AGC ATA AGG AGC 846
 P   V   D   N   N   Y   Q   V   N   L   L   S   E   R   T   I   S   I   R   S
AAA GTC TCC AAA ATG GTG GTC GTG ATA GTC CTC CTT TTT GCC ATC TGT TGG GGT CCC ATC 906
 K   V   S   K   M   V   V   V   I   V   L   L   F   A   I   C   W   G   P   I
CAG ATC TTT GTG CTT TTC CAA TCC TTC TAT CCA AAT TAT AGA CCC AAC TAC GCA ACA TAC 966
 Q   I   F   V   L   F   Q   S   F   Y   P   N   Y   R   P   N   Y   A   T   Y
AAG ATC AAG ACG TGG GCC AAC TGC ATG TCC TAT GCC AAC TCC TCA GTC AAC CCC GTA GTT 1026
 K   I   K   T   W   A   N   C   M   S   Y   A   N   S   S   V   N   P   V   V
TAT GGC TTC ATG GGA GCC AGT TTT CAA AAG TCT TTC AGG AAA ACA TTT CCC TTC CTG TTC 1086
 Y   G   F   M   G   A   S   F   Q   K   S   F   R   K   T   F   P   F   L   F
AAG CGC AAG GTC AGG GAT AGC AGC AGG GCT TCA AGG ACT GCC AAC GCT GAG ATC AAG TTT 1146
 K   R   K   V   R   D   S   S   R   A   S   R   T   A   N   A   E   I   K   F
GTT GCT GCT GAG GAA GGC AAC AAT AAT GAT AAT GGG GTA AAT TAAttcagactattaaagatagg 1211
 V   A   A   E   E   G   N   N   N   D   N   G   V   N   *
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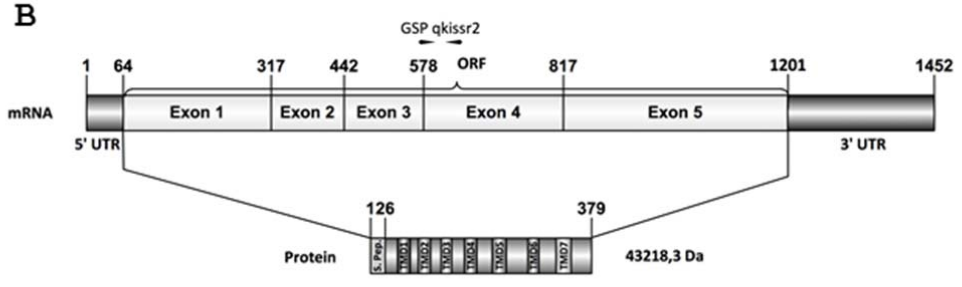
B



Supplementary Figure 3

A

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GCA GCT CTG GAA AGC CAA GGC CCG CCG CTG CTG GTC GAC GCT TGG CTG GTC CCC ACA TTC 120
 A   A   L   E   S   Q   G   P   P   L   L   V   D   A   W   L   V   P   T   F
TTC GGC CTC ATT ATG CTG GTC GGT CTG GTC GGG AAC TCG CTG GTC ATC CAT GTG GTC ACC 180
 F   G   L   I   M   L   V   G   L   V   G   N   S   L   V   I   H   V   V   T
AAG CAT CAG CAG ATG AAA ACT GTC ACC AAC TTT TAC ATT GTA AAC CTG GCC ACA ACT GAT 240
 K   H   Q   Q   M   K   T   V   T   N   F   Y   I   V   N   L   A   T   T   D
ATC TTG TTT CTG GTG TGC TGC GTG CCC TTC ACT GCC ACA CTG TAC CCA CTG CCC AGC TGG 300
 I   L   F   L   V   C   C   V   P   F   T   A   T   L   Y   P   L   P   S   W
ATC TTT GGA GAG TTC ATG TGC CGT CTG GTT AAC TAC CTT CAA CAA GTA ACG GCA CAG GCA 360
 I   F   G   E   F   M   C   R   L   V   N   Y   L   Q   Q   V   T   A   Q   A
ACG TGC ATC ACT CTG TCT GCT ATG AGT GTG GAC CGC TGC TAT GTG ACC GTC TAC CCT CTG 420
 T   C   I   T   L   S   A   M   S   V   D   R   C   Y   V   T   V   Y   P   L
CAG TCA CTC CGG AAC CGC ACC CCC CGC ATG GCT CTG CTT GTC TCA GTG TCC ATC TGG ATC 480
 Q   S   L   R   N   R   T   P   R   M   A   L   L   V   S   V   S   I   W   I
TCC TTG CTC CTG TCC ATC CCC GTA GCT GTG TAT CAG CGT CTG GAG GCA GGA TAC TGG TTT 540
 S   L   L   L   S   I   P   V   A   V   Y   Q   R   L   E   A   G   Y   W   F
GGC CCA CAG ACG TAC TGC ACC GAA GTC TTC CCC TCT CCC CAC CTC CAG AGA GTC TTC ATC 600
 G   P   Q   T   Y   C   T   E   V   F   P   S   P   H   L   Q   R   V   F   I
ATC TAC AGC TTC CTC GCA GTC TAC CTC CTG CCC CTG CTT ACC ATT ATC GCC TGT TAC GCC 660
 I   Y   S   F   L   A   V   Y   L   L   P   L   L   T   I   I   A   C   Y   A
TTC ATG CTC AAA CGC ATG GGC CAG CCC AGC GTG AAT CCC ATT GAC AGT AGC TAC CAA CTG 720
 F   M   L   K   R   M   G   Q   P   S   V   N   P   I   D   S   S   Y   Q   L
CAG GCT CAG GCA AAG CGA GCA ACA GCA ATG CGC GCG CGA GTC TCC CGA ATG GTG GTG GTG 780
 Q   A   Q   A   K   R   A   T   A   M   R   A   R   V   S   R   M   V   V   V
ATG GTG GCC CTG TTC CTC ATC TGC TGG GGT CCC ATC CAG GTC TGC ATC ATG CTG CAA GCT 840
 M   V   A   L   F   L   I   C   W   G   P   I   Q   V   C   I   M   L   Q   A
TTT GGC CTT CGC AGT TAC GTT TTA TAC AAG CTG AAG ATA TGG GGT CAC TGC ATG TCT TAC 900
 F   G   L   R   S   Y   V   L   Y   K   L   K   I   W   G   H   C   M   S   Y
TCC AAC TCC TCT GTC AAC CCG CTG GTT TAT GCA TTC ATG GGC AAT AAC TTT AGA AAA GCC 960
 S   N   S   S   V   N   P   L   V   Y   A   F   M   G   N   N   F   R   K   A
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 F   K   H   A   F   P   A   I   F   L   W   H   K   R   G   S   A   R   V   G
AAT ATG GAT GCA GAG GAC GGG GGA GAT ATG GAT CGC CGA GCA CCC AAA GGA GAA GCA GAG 1080
 N   M   D   A   E   D   G   G   D   M   D   R   R   A   P   K   G   E   A   E
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 M   H   F   L   S   S   G   S   *
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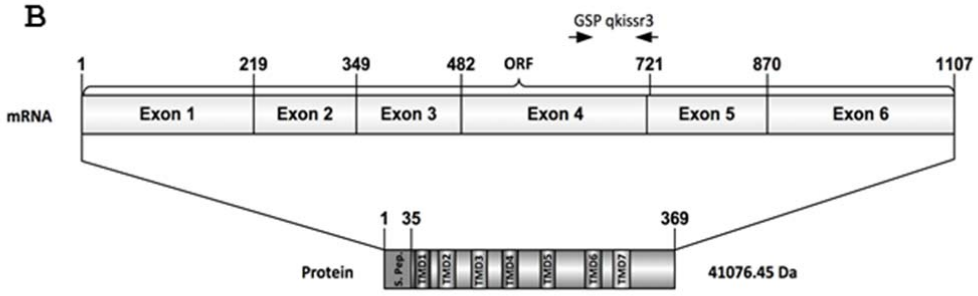
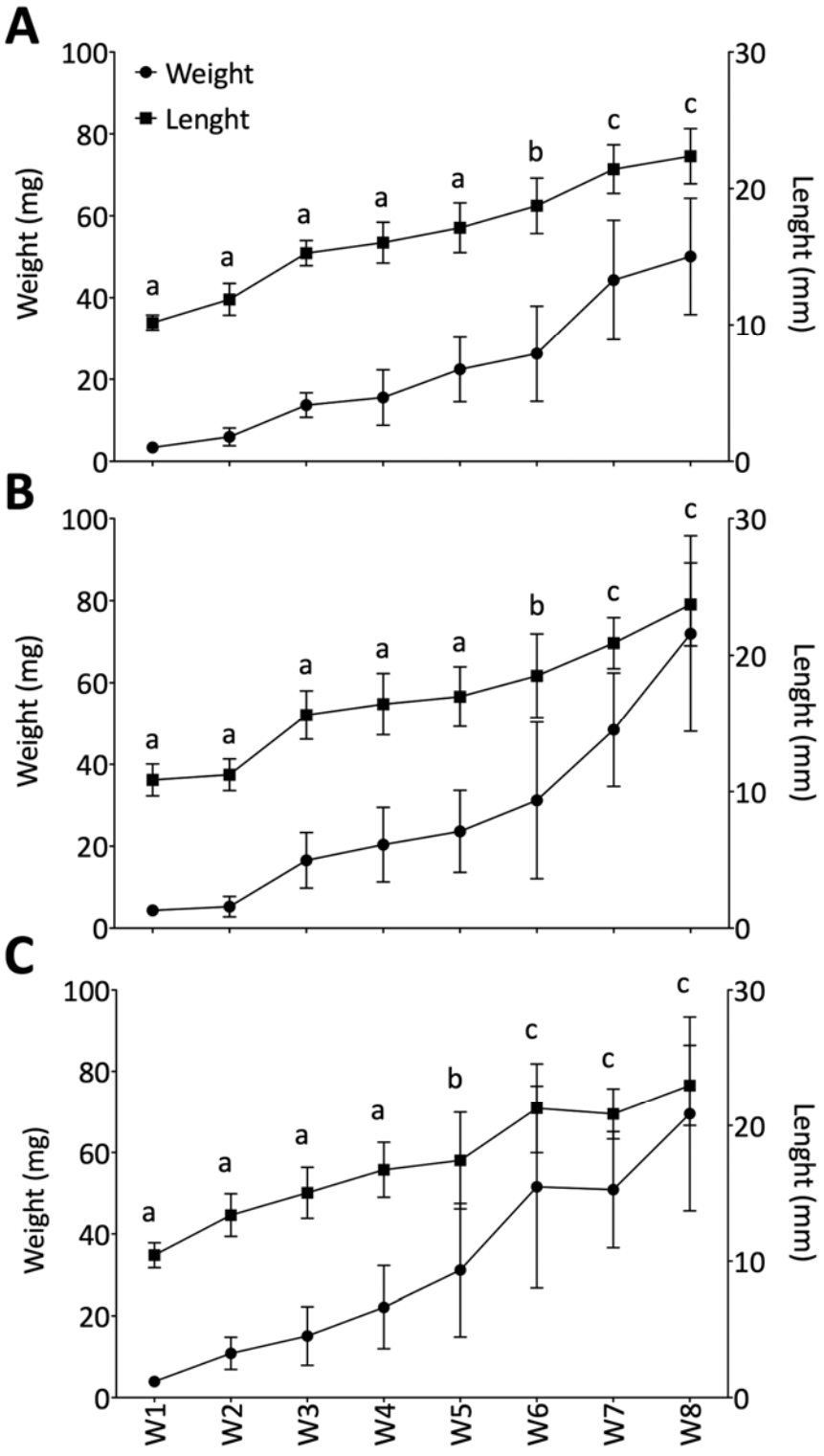
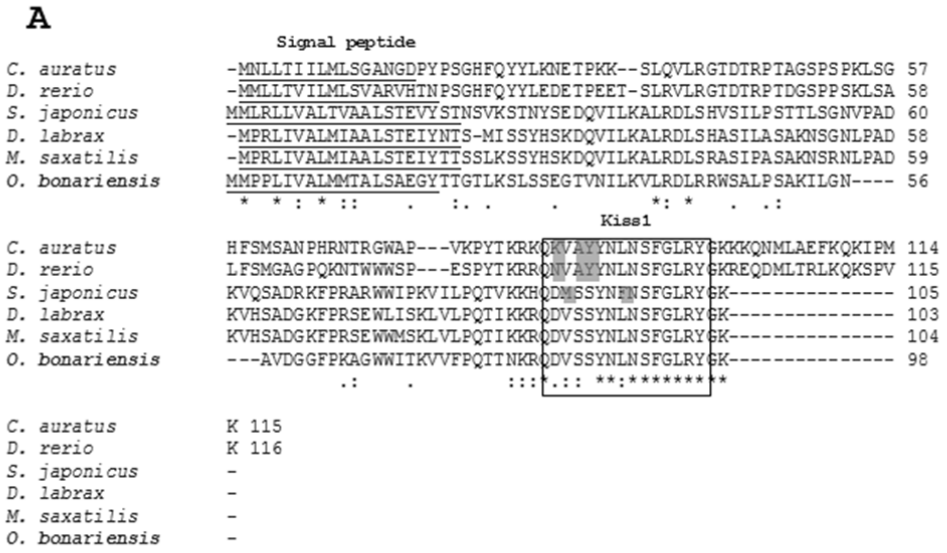
B



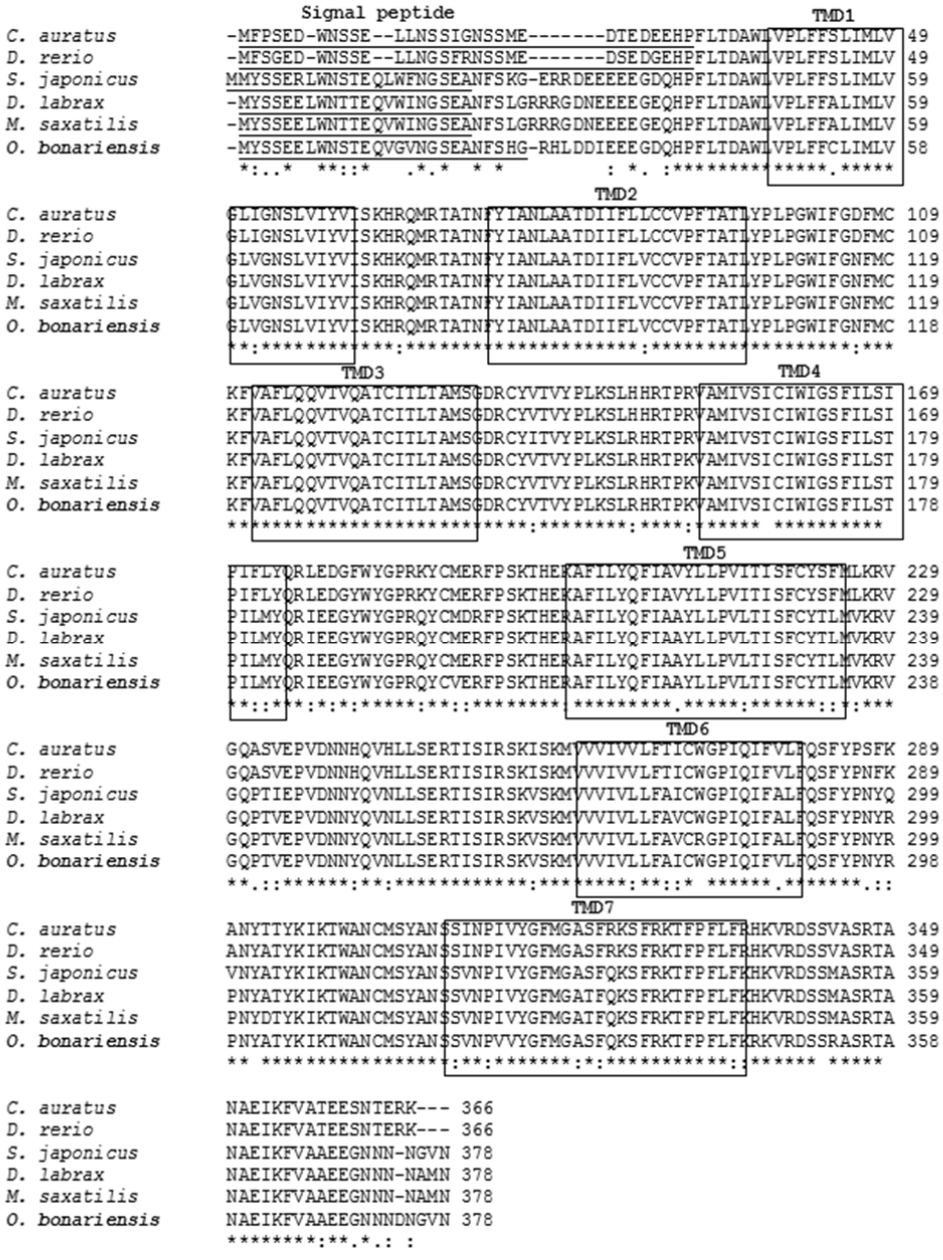
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7