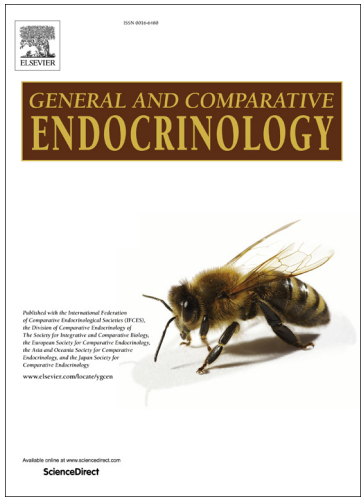

Research paper

Kisspeptins and their receptors in the brain-pituitary-gonadal axis of *Odonthestes bonariensis*: Their relationship with gametogenesis along the reproductive cycle.

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Kisspeptins and their receptors in the brain-pituitary-gonadal axis of *Odonthestes bonariensis*: Their relationship with gametogenesis along the reproductive cycle.

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Abstract

In vertebrates, the reproduction is controlled by the brain-pituitary-gonadal (BPG) axis and kisspeptin has emerged as a key player of this axis. In this study, we analyzed changes in the expression levels of *kiss1*, *kiss2*, and their receptors, *kissr2* and *kissr3* during gametogenesis in the BPG axis of feral *Odontesthes bonariensis*. In females, levels of brain *kiss1* showed an increase at final maturation (Fm), while *kiss2* levels were shown to be high at primary growth (Pg) stage, with no differences in the expression of their receptors. In the pituitary, *kiss1* and *kiss2* peaked at the cortical alveoli (Ca) stage, and *kissr3* at initial vitellogenesis. In parallel, there was an increase of *kiss1*, *kissr2* and *kissr3* in the ovary during the Ca stage and both receptors again at Fm stage. In males, the four genes were highly expressed in the brain at the arrested (A) stage. In the pituitary, *kiss2* peaked at spermatogonial (SG) and spermatocytary (SC) stages; while *kissr3* reached a peak at the spermiogenic stage (SP). In testes, *kiss1* and *kiss2* significantly increased during the SG and SC stages; meanwhile, *kissr2* increased at SG and SC, whereas *kissr3* levels were significantly high at SC and SP stages. Taken together these results showed that the kisspeptin system in pejerrey is expressed in the three levels of the BPG axis with different expression profiles during the gonadal cycle. These findings pointed that kisspeptins have different roles in gametogenesis

in this species.

Keywords: *kiss1; kiss2; kissr2; kissr3*; reproductive cycle; pejerrey; teleosts.

1. Introduction

Kisspeptin is a key component of the brain-pituitary-gonadal (BPG) axis and an important regulator of reproduction in mammals, where it stimulates GnRH secretion from the hypothalamus (Kauffman, 2010). Furthermore, kisspeptin directly induces LH secretion in the pituitary (Gutiérrez-Pascual et al., 2007), stimulates ovulation (Castellano et al., 2006; Matsui et al., 2004), spermiation (Meccariello et al., 2014) and plays a role in controlling gonadal steroidogenesis (Patterson et al., 2006).

In teleost fish, two kisspeptin genes known as *kiss1* and *kiss2* and three genes coding for kisspeptin receptors (*kissr1*, *kissr2* and *kissr3*) have been described (Pasquier et al., 2014). The new nomenclature for kisspeptin receptors established that *kissr1* is the orthologous form of the mammalian receptor and is only found in the European eel. Meanwhile, *kissr2* and *kissr3* correspond to the previously known *kiss1r* and *kiss2r* (Pasquier et al., 2014).

In bony fish, both kisspeptins induce *gnrh1* expression depending on gonadal stages, type of administration and dose of the different forms of the mature peptides employed (Kim et al., 2014; Park et al., 2016; Zmora et al., 2012; 2014; 2015). Although kisspeptins are considered as positive regulators of fish reproduction, there are conflicting results on their role on pituitary gonadotropin secretion and reproduction. For example, it has been reported

that in adult *Carassius auratus*, Kiss1 directly induced Lh secretion (Chang et al., 2012; Yang et al., 2010). However, in mature *Dicentrarchus labrax* males only Kiss2 stimulated Lh and Fsh *in vitro* secretion and no effects were reported for Kiss1 (Espigares et al., 2015). In mature *Morone saxatilis* males, Kiss1 and Kiss2 induced *in vitro* Fsh, and Kiss2 also induced Lh secretion (Zmora et al., 2015). On the other hand, Kiss1 and Kiss2 directly inhibited *lhb* expression in the pituitary gland of pre-pubertal *Anguilla anguilla* (Pasquier et al., 2011). In addition, recent results challenged the relevance of kisspeptin regulating reproduction in fish, since *kiss1*$^{-}$/*kiss2*$^{-}$ and kissr1^{-}/kissr2^{-} zebrafish can normally reproduce (Tang et al., 2015; Liu et al., 2017).

Besides, both Kiss1 and Kiss2 have been demonstrated to be expressed by pituitary cells (Alvarado et al., 2013; Li et al., 2009; Saha et al., 2016; Servili et al., 2011). Currently, the function of pituitary kisspeptins is far from clear. Nevertheless, it is believed that they are involved in an autocrine/paracrine regulation of gonadotropins and other pituitary hormones (Espigares et al., 2015; Jiang et al., 2014; Ohga et al., 2017; Zmora et al., 2015).

At the gonadal level, kisspeptin ligands and receptors were detected in the ovary of several teleost species, and the relative expression of some of these genes varied according to different gonadal stages (Saha et al., 2016; Selvaraj et al., 2010; Song et al., 2016). In addition, all the components of the

kisspeptin system were described in testes with variations during spermatogenesis (Fairgrieve et al., 2016; Marín-Juez et al., 2013; Mechaly et al., 2009; 2011; Song et al., 2016).

Kisspeptins have also been implicated in brain-pituitary feedback by sex steroids in fish. Kanda et al. (2008) reported that in the hypothalamus of medaka, *kiss1* gene expression responded to gonadal steroids. This is the case of Kiss2 neurons in the ventral and caudal hypothalamus of *Danio rerio* as well (Servili et al., 2011). However, Kiss2 producing-neurons in the lateral recess of *Oryzias latipes* (Kanda et al., 2012; Mitani et al., 2010), *C. auratus* (Kanda et al., 2012), *M. saxatilis* (Zmora et al., 2012) and *D. labrax* (Escobar et al., 2013), did not respond to E_2, or express estrogen receptors (ERs).

The relative abundance of kisspeptins/kisspeptin receptors transcripts during fish gonadal maturation, has been studied in few species belonging to 6 different orders: Scombriformes (Selvaraj et al., 2010; Ohga et al., 2013); Tetraodontiformes (Shahjahan et al., 2010), Spariformes (Shimizu et al., 2012), Pleuronectiformes (Mechaly et al., 2012), Perciformes (Alvarado et al., 2013; Migaud et al., 2012) and Cypriniformes (Saha et al., 2016). In general, all these studies found that kisspeptin(s) increased during or previous to ovulation and/or spermiation.

The pejerrey, *Odontesthes bonariensis* (Atheriniformes), is a multiple and

asynchronous spawner fish native of inland waters of Argentina with potential for intensive aquaculture (Somoza et al., 2008). During the spawning season females move from previtellogenic to maturation in a short period of time and males have, although in different degrees, sperm in their ducts (Strüssmann, 1989). The gonadal developmental stages of pejerrey females and males were recently studied in the wild (Elisio et al., 2014; 2015). The ovarian growth was related to the increase of day length and water temperatures from late winter to the beginning of spring; then gonadal regression normally occurs in summer, when water temperature surpasses 21 °C (Elisio et al., 2014). In males, spermiation was observed at spring and autumn where the light phase of photoperiod is longer than 11 h with water temperature ranging from 13-23 °C and regression occurred in summer when water temperatures reached over 23 °C (Elisio et al., 2015).

Recently, the genes coding for two kisspeptin ligands and receptors were characterized in pejerrey (Tovar Bohórquez et al., 2017). Hence, the aim of the present study was to analyze the expression profiles of *kiss1*, *kiss2*, *kissr2*, *kissr3* at the three levels of the BPG axis during the reproductive cycle of wild caught pejerrey fish. We expect with these results to expand the knowledge on the potential involvement of kisspeptins on reproduction in this species.

2. Materials and Methods

2.1Tissue collection and sample processing

All animals used in this study were previously captured in the Chascomús Lagoon (35˚36´S 58˚02´ W). The cDNA from these samples previously obtained by Elisio et al. (2014; 2015). Briefly, cDNA from brain (only including telencephalon, diencephalon and mesencephalon), pituitary and gonads were obtained and total RNA was extracted with TRIzol® Reagent (Thermo Fisher Scientific, Waltham, USA). Then RNA samples were treated with DNase I Amplification grade (Invitrogen™, Carlsbad, USA) and reverse transcribed using SuperScript III RNase H (Invitrogen™,) and oligo(dT)$_{12–18}$ (Invitrogen™). The characterization of the gonadal stages was performed by histology. A portion of each gonad was fixed in Bouin's fixative, processed and embedded in Paraplast Plus® (Sigma-Aldrich Co., Saint Louis, USA) as already described in Elisio et al. (2014). Pejerrey ovaries were histologically classified into six gonadal stages: primary growth (Pg), cortical alveoli (Ca), initial vitellogenesis (VtgA), final vitellogenesis (VtgB), final maturation (Fm) and atretic (At) according to Elisio et al. (2014). Adult testes were classified into four gonadal stages: arrested (A), spermatogonial stage (SG), spermatocytary stage (SP) and spermiogenic stage (SP) according to Elisio et al. (2015).

2.2Gene expression

Gene expression was determined by Real-time quantitative PCR (qPCR). Standard curves for each tissue were constructed by five points of four-fold serial dilutions for *kiss1*, *kiss2*, *kissr2*, *kissr3*, *b-actin* and elongation factor 1 alpha (*ef1a*). Triplicate reactions were run for each standard and duplicates for targets and references genes. The minimum coefficient of variation of each reference gene for each tissue was calculated and this was the criterion to select the reference gene used for each analysis. The reaction mixture (10 µl) for the qPCR was prepared with 5 µl of Fast Start Universal Master SYBR (Roche Diagnostics, Mannheim, Germany), approximately 50 ng of cDNA, 300 nM of each primer and ultrapure water. All primers used in this study are specified in Table 1. For negative controls, cDNA was replaced by ultrapure water (Invitrogen™, Carlsbad, CA, USA). All the qPCR reactions were performed in a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, USA), and the cycle conditions were as follows: 10 min at 95 °C, 40 cycles 95 °C for 15 s and 1 min at 60 °C. The melting curve analysis was always included. All data were analyzed by the relative standard curve method.

2.3Statistical analysis

All values are presented as mean ± standard error. In order to detect significant differences in the relative expression levels, the data were analyzed by one-way ANOVA with a p value <0.05, and Least Significant Difference (LSD) as *post-hoc* analysis. Data not following the assumptions of homocedacy and normality were subjected to logarithmic transformation. Correlation analysis was performed in order to detect potential interactions between kisspeptin transcript levels and key endocrine components regulating reproduction previously reported by Elisio et al. (2014; 2015). The following correlations were considered: kisspeptin transcripts in brain, pituitary and gonads against brain *gnrh1*, pituitary *fshb* and *lhb* and gonadal *fshr* and *lhcgr* relative abundance; and finally kisspeptin transcripts in the whole brain-pituitary-gonadal axis against plasma levels of 11-ketotestosterone (11-KT, in males) and testosterone (T) and estradiol (E2, -in females). Pearson´s correlation coefficient was calculated and the Student's t-test was used to assessed its statistical significance (p <0.05). All statistical analyzes were performed using SPSS v2.0 and the results were plotted with the GraphPad Prism v5 (GraphPad Software, Inc., La Jolla, CA, USA) program.

3. Results

3.1. Variations in kisspeptins transcripts in pejerrey brain

In female brains, *kiss1* transcript levels remained stable during previtellogenesis and vitellogenesis, increasing approximately 50% at final maturation (Fm) (Figure 1A). On the other hand, the expression of *kiss2* was found to be higher during primary growth (Pg) when compared to other stages (Figure 1B). The relative abundance of kisspeptin receptors did not vary significantly during the gonad annual reproductive cycle (Figure 1C and 1D). In male brains, the expression of the four transcripts was higher during the arrested (A) stage (Figure 2). Then the transcripts of the ligands decreased to approximately 50% during the other stages (Figures 2A and 2B). In parallel, the relative expression of *kissr2* and *kissr3* decreased from the SG to the spermiogenic (SP) stages (Figures 2C and 2D).

3.2. Variations in kisspeptins transcripts in pejerrey pituitary

In the female pituitary, the expression of *kiss1* increased around 2 times during the cortical alveoli (Ca) stage, and gradually decreased during the initial (VtgA) and final vitellogenesis (VtgB), reaching minimum values at Fm (Figure 3A). In the case of *kiss2*, its expression levels reached a peak (3 times compared to Pg stage) at Ca and remained low in all other stages (Figure 3B). While *kissr2* levels remained constant during all the process (Figure 3C),

kissr3 transcript levels increased 100 % from Pg to VtgA, slightly decreased during VtgB and being undetectable (UD) at Fm and AT stages (Figure 3D). In males, the relative expression of *kiss1* did not show any change during the cycle, while *kiss2* levels were low at A, and increased and kept constant during spermiogenic (SG and SC) stages (Figures 4A and 4B). Regarding receptor levels, the relative expression of *kissr2* did not change during the spermatogenesis (Figure 4C), while *kissr3* mRNA levels were minimum at SC and increased 3 times compared to the A stage at SP (Figure 4D).

3.3. Variations in kisspeptins transcripts in pejerrey gonads

In the ovaries, *kiss1* expression decreased 2 times at VtgA and VtgB from Ca (Figure 5A); while *kiss2* transcript levels did not change throughout oogenesis (Figure 5B). Regarding kisspeptin receptors, *kissr2* reached a peak at Ca (around 20 times compared to Pg), decreased during VtgA and then gradually increased during VtgB and Fm (Figure 5C); while *kissr3* increased 2 times at Ca, decreased at VtgA to then increased at VtgB, reaching the highest levels at Fm (Figure 5D). All transcripts were undetectable at the At stage.

In testes, *kiss1* and *kiss2* mRNA levels had similar expression patterns; with minimum expression at A, reaching maximum levels at SG and SC to then decrease at SP (Figure 6A and 6B). Relative expression of *kissr2* had 1-fold

increase at SG stage to then kept stable at SC and SP stages (Figure 6C); while *kissr3* remained at low levels at A and SG stages increasing 5 times at SC and SP stages (Figure 6D).

3.4. Correlations between kisspeptins transcripts and key endocrine components regulating reproduction

Correlation analysis among relative abundance of kisspeptin transcripts and reproduction-related components in pejerrey females are shown in Table 2. In brain, *kiss1* and *kissr3* relative abundance positively correlated with brain *gnrh1* expression levels. Furthermore, brain *kiss1* positively correlated with pituitary *lhb* levels and *kiss2* levels negatively correlated with gonadal *fshr* levels.

In the pituitary, a positive correlation between *kiss2* and *fshb* relative expression levels was observed. Besides, pituitary *kissr3* relative abundance positively correlated with brain *gnrh1* and both pituitary gonadotropins.

In the ovary, no significant correlations were observed for the ligands, while relative abundance of both kisspeptin receptors positively correlated with pituitary *fshb* expression levels. In addition, *kissr3* relative abundance positively correlated with brain *gnrh1* expression levels.

Correlation analysis among relative abundance of kisspeptin transcripts and

reproduction-related components in pejerrey males are shown in Table 3. The transcript relative abundance of all kisspeptin genes in the brain presented a negative correlation with brain *gnrh1* expression and 11-KT plasma levels. At the pituitary, only *kiss2* expression levels showed a positive correlation with brain *gnrh1*, pituitary *fshb* relative abundance and 11-KT plasma levels. In testes, the relative expression of *kiss1*, *kiss2* and *kissr3* positively correlated with brain *gnrh1*, pituitary *fshb* and 11-KT plasma levels.

4. Discussion

In pejerrey, as well as in other teleosts, the kisspeptin system (*kiss1*, *kiss2*, *kissr2* and *kissr3*) was expressed in brain, pituitary and gonads of both sexes (Escobar et al., 2013; Fairgrieve et al., 2016). Additionally, the relative abundance of their respective mRNAs varied at the three levels of the BPG axis in different gonadal stages, suggesting that all these components could have a role in pejerrey gametogenesis.

In the brain of female pejerrey, only *kiss1* reached a peak at the Fm stage. This is in agreement with results obtained in *S. japonicus* showing that *kiss1* mRNA levels increased at late vitellogenesis and ovulation (Matsuyama et al., 2013). A similar effect was observed in *Labeo rohita*, where *kiss1* levels were increased during the spawning period with no variations for *kiss2* levels (Saha

et al., 2016). Moreover, in *S. japonicus* it has recently been reported that Gnrh1-producing neurons expressed the orthologous of pejerrey *kissr2*, whereas these neurons did not express the orthologous of pejerrey *kissr3* (Ohga et al., 2017). Likewise, it was demonstrated that administration of Kiss1 induced *gnrh1* expression in recrudescent *M. saxatilis* (Zmora et al., 2012). These data, together with the positive correlations of *kiss1* expression in pejerrey brain with brain *gnrh1* and pituitary *lhb* suggested that *kiss1* is also involved in the Fm progression in pejerrey, probably by stimulating the Gnrh-Lh-LhcgR axis as previously reported by Elisio et al. (2014). In pejerrey female brain, *kiss2* transcript levels were higher at the Pg stage. This observation is in agreement with results obtained in *S. japonicus*, (Matsuyama et al., 2013). In teleosts, it is known that the transition from Pg to the secondary growth stages (early vitellogenesis) depends of pituitary signaling by Fsh (Lubzens et al., 2010) as it has also been described that Kiss2 was able to induce Fsh secretion in *D. labrax* (Felip et al., 2009) and *S. japonicus* (Ohga et al., 2014). In our fish model, brain *gnrh1*, pituitary *fshb* and gonadal *fshr* levels increase from Ca stage onwards (Elisio et al., 2014). However, in this work no positive correlation was found between brain *kiss2* and pituitary *fshb* levels and a negative correlation with ovarian *fshr* was observed. Taken together, the rise of *kiss2* levels in the female pejerrey brain could be related

to the onset of the ovarian development.

In the present study, no changes were observed in *kissr2* and *kissr3* brain expression levels in contrast with those reported in *S. japonicus* (Ohga et al. 2013), *D. labrax* (Alvarado et al., 2013) and *Chrysiptera cyanea* (Imamura et al., 2017). The differences could be due to these species having a different receptor expression pattern, thus suggesting that their involvement in the gonadal cycle is far from being understood.

In pejerrey male brain, all components of the kisspeptin system showed a high expression level at the A stage. Similar results were already reported in different species at initial stages of spermatogenesis: *S. japonicus* (Selvaraj et al., 2010), *L. rohita* (Saha et al., 2016) and *D. labrax* (Alvarado et al., 2013). It is important to highlight that in the present study all components of the kisspeptin system negatively correlated with brain *gnrh1* and 11-KT plasma levels. It was reported that the administration of Kiss peptides induced pituitary *fshb* expression and Fsh release in *M. saxatilis* (Zmora et al., 2015), pituitary *fshb* expression in *D. rerio* (Kitahashi et al., 2009) and *S. japonicus* (Ohga et al., 2014), as well as testicular development in *Seriola lalandi* (Nocillado et al., 2013). Pituitary *fshb* expression was reported to be increased at gonadal development in pejerrey (Elisio et al., 2015). However in the present study, we did not observed a correlation between brain *kiss*/*kissr*

system and pituitary *fshb*.

In pejerrey brain, females showed less variation in kisspeptin and kisspeptin receptors expression levels than males. This is probably due to the asynchronic ovarian development of this species (Strüssmann, 1989); then pejerrey ovary presents different oocytes stages in a particular moment with an overlap of the corresponding endocrine regulators.

Taken together, these results showed that brain *kiss1* and *kiss2* exhibited different expression profiles at different gonadal stages in both sexes, suggesting that the expression of these two peptides may reflect differences in gonadal development of female and male pejerrey at the brain level. These differences could be mediated, at least in part, through the interaction of steroid hormones on the promoter region of kisspeptin genes, as already demonstrated in other teleost species (Wang et al., 2013; Guo et al., 2017). Besides, our results revealed the expression of all the kisspeptin system components in the pituitary gland. In females, both ligands showed a peak at the Ca stage, when pituitary *fshb* started to increase as observed by Elisio et al. (2014) in the same samples. Regarding the receptors, only *kissr3* showed an increase at the beginning of vitellogenesis. In addition, a positive correlation was only observed between *kiss2* and *fshb*. Then, although the mechanism is not clear yet, it is possible that in pejerrey, pituitary Kiss2 may participate in

an autocrine/paracrine manner in the control of the beginning of vitellogenesis. Nevertheless, kisspeptin ligands and receptors were reported to vary in the pituitary gland at different stages of the ovarian cycle in other species, *Takifugu niphobles* (Shahjahan et al., 2010), *S. japonicus* (Selvaraj et al., 2010), *D. labrax* (Alvarado et al., 2013) and, *L. rohita* (Saha et al., 2016) a common expression pattern could not be found in females.

In pejerrey males, pituitary *kiss2* expression increased at spermiogenesis stages. This result was consistent with those observed in *D. labrax* (Alvarado et al., 2013), *T. niphobles* (Shahjahan et al., 2010) and *L. rohita* (Saha et al., 2016). In the present study, no variations were observed in pituitary *kiss1* as already reported in *S. japonicus* (Selvaraj et al., 2010) and *D. labrax* (Alvarado et al., 2013) suggesting that pituitary Kiss1 is not involved in the regulation of spermatogenesis. Our results showed that *kissr3* expression was downregulated at SC stage, however, if this is related to the concomitant rise in *kiss2* expression is not known at present. For example, in *T. niphobles* kiss receptor expression followed the expression of *kiss2* at the pituitary (Shahjahan et al., 2010). Pituitary *kiss2* expression was positively correlated with brain *gnrh1* and pituitary *fshb* expression together with plasma 11-KT levels (Elisio et al., 2015). Moreover, it is known that Fsh participates in testicular physiology since it is a potent stimulator of 11-KT synthesis (Schulz

et al., 2010). All these results suggest that, in pejerrey, pituitary Kiss2 could be controlling spermatogenesis.

In pejerrey, both kisspeptin ligands and receptors were expressed in female and male gonads. In females, *kiss1*, *kissr2* and *kissr3* showed a similar expression pattern with increases at Ca and Fm stages; meanwhile, *kiss2* expression was unchanged. Notably all components of the kisspeptin system were undetectable at the atretic stage. However, this is different from findings in *L. rohita*, where *kiss1* was up-regulated at spawning (Saha et al., 2016). In pejerrey, ovarian *fshr* and gonadal aromatase (*cyp19a1a*) started to increase from the Ca stage (Elisio et al., 2014). These facts were generally associated with pituitary *fshb* expression and plasma Fsh levels (Levavi-Sivan et al., 2010; Lubzens et al., 2010). The positive correlation between *fshb* and ovarian kisspeptin receptors expression suggested that Fsh up-regulated kisspeptin receptors. In testes, *kiss1* and *kiss2* increased at maturing stages, suggesting that these two ligands could be related with the meiotic phase of spermatogenesis. Similar results were found in the testes of *S. japonicus* (Selvaraj et al., 2010; Ohga et al., 2013) and *L. rohita* (Saha et al., 2016). Due to the positive correlations observed among *kiss1*, *kiss2* and *kissr3* with brain *gnrh1*, pituitary *fshb* expression and 11-KT plasma levels, it is possible to hypothesize that the testicular *kiss*/*kissr* system can participate in controlling

spermatogenesis.

It is important to take into account that in pejerrey gonads, key elements of the reproductive axis such as *gnrh1*, *gnrh3*, *gnrhr2*, *fshb* and *lhb*, were identified (Guilgur et al., 2007; 2009; Elisio et al., 2012), so it is likely that the kisspeptin system interacted locally with these components.

It is important to mention that different ligand selectivity have been shown in *D. rerio* (Onuma and Duan, 2012), *D. labrax* (Felip et al., 2015) and *M. saxatilis* (Zmora et al., 2015). In this context, future studies are needed to elucidate pejerrey Kiss1 and Kiss2 selectivity and affinity to get a better understanding of the differences in the interaction of the kisspeptin ligands and receptors in pejerrey.

Overall, it is clear from our results that the kisspeptin system is regulated along the gonadal cycle, suggesting that it is involved in the endocrine control of gonadotropin hormones and gonadal development. In this regard, recent reports showing that kisspeptins-knockout zebrafish normally reproduce (Liu et al., 2017; Tang et al., 2015) have to be taken with caution, since as fish reproduction is under multifactorial regulation, there could be compensatory mechanisms operating to ensure reproductive success in the absence of kisspeptin genes (Liu et al., 2017; Trudeau, 2015).

5. Conclusion

In conclusion, all components of the kisspeptin system are expressed in the BPG axis of pejerrey of both sexes with different expression profiles during gametogenesis. These results suggested that kisspeptins and their receptors may have different roles during reproduction probably acting on the regulation of Gnrh-Gths in the hypothalamus-pituitary system or directly within the gonads.

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Figure legends

Figure 1: Relative expression of *kiss1* (A), *kiss2* (B), *kissr2* (C) and *kissr3* (D) in pejerrey female brain at different gonadal stages. The bars represent the mean ± SEM. Different letters indicate significant differences between means (ANOVA, post-hoc LSD, $p < 0.05$). Pg: primary growth, Ca: cortical alveolus, VtgA: initial vitellogenesis, VtgB: mean vitellogenesis, FM: final maturation, At: atretic. All values were relativized to the expression of each gene at Pg stage. Reference gene for this analysis: *ef1a*. Samples size is indicated between parentheses below the x-axes.

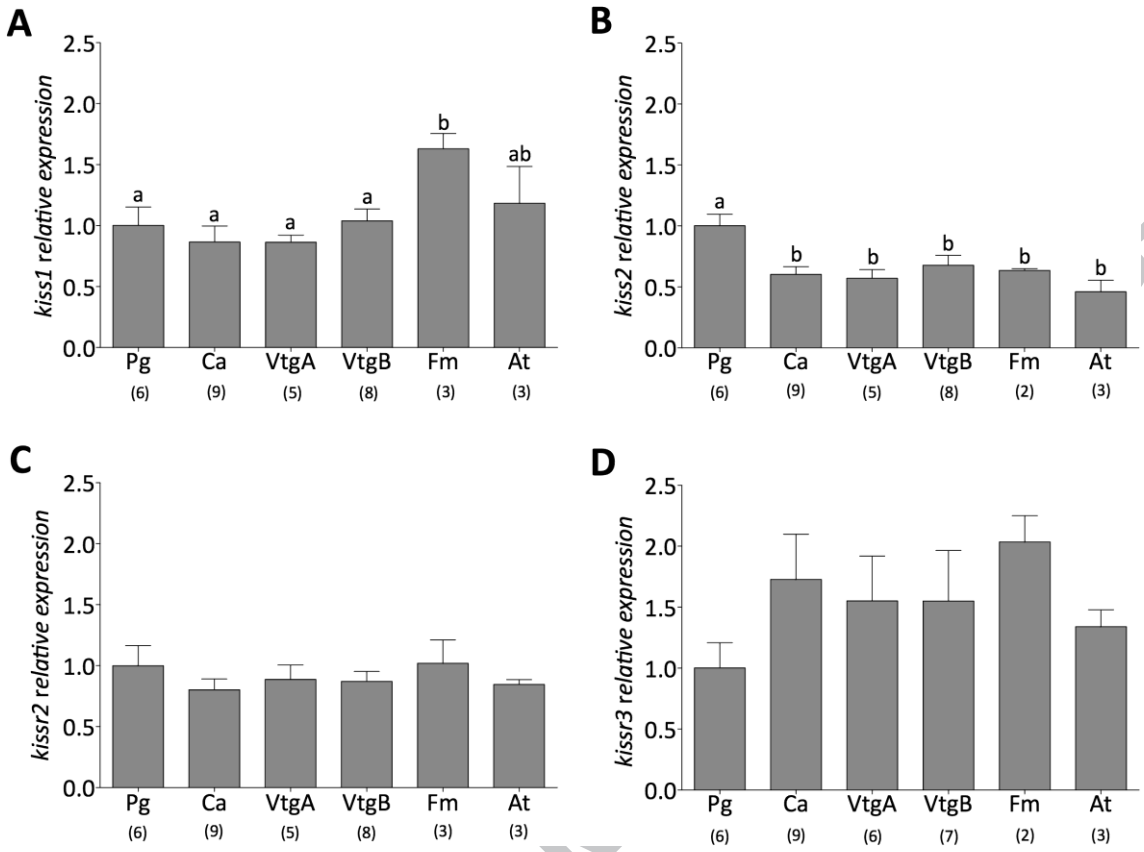

Figure 2: Relative expression of *kiss1* (A), *kiss2* (B), *kissr2* (C) and *kissr3* (D) in the pejerrey male brain at different gonadal stages. The bars represent the mean ± SEM. Different letters indicate significant differences between the means (ANOVA, post-hoc LSD, $p < 0.05$). A: arrested, SG: spermatogonial stage, SC: spermatocytary stage, SP: spermiogenic stage. All values were relativized to the expression of each gene at A stage. Reference gene for this analysis: *ef1a*. Samples size is indicated between parentheses below the x-axes.

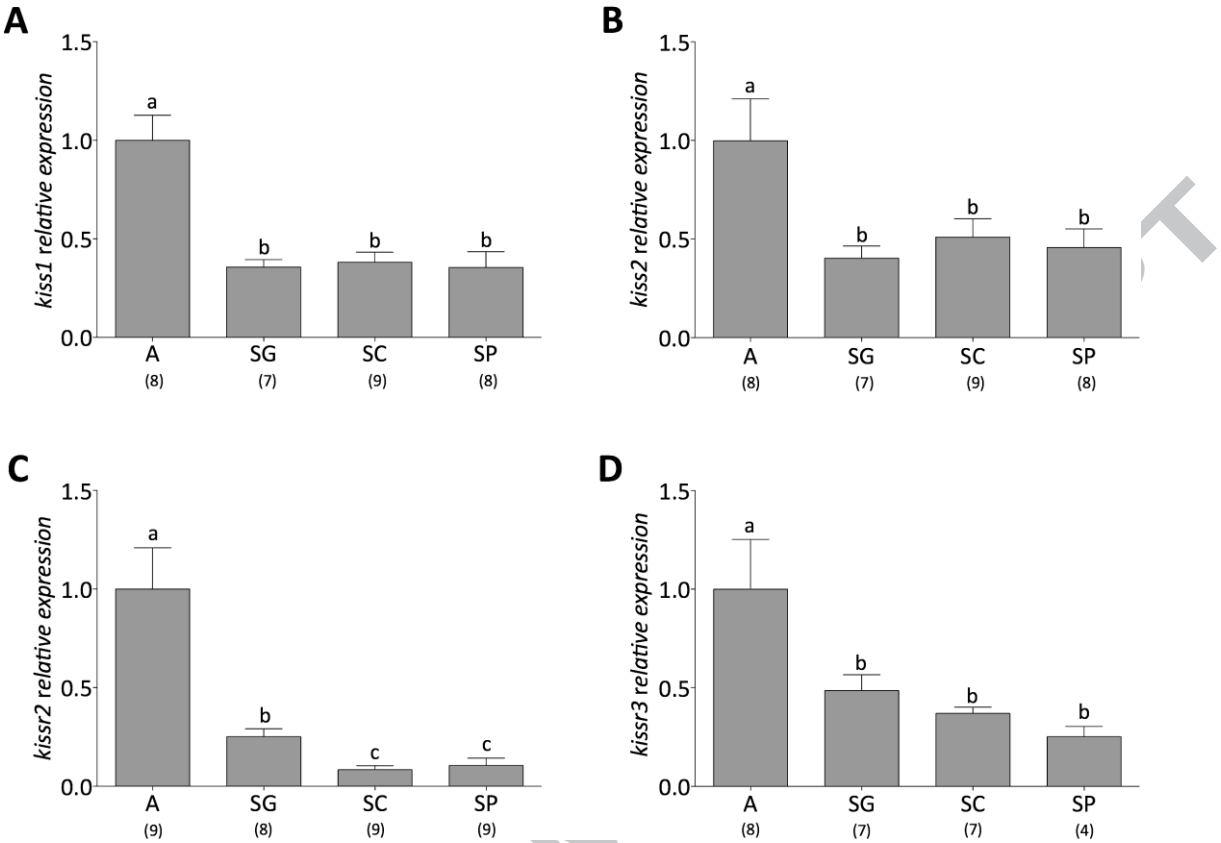

Figure 3: Relative expression of *kiss1* (A), *kiss2* (B), *kissr2* (C) and *kissr3* (D) in the pejerrey female pituitary gland at different gonadal stages. The bars represent the mean ± SEM. Different letters indicate significant differences between the means (ANOVA, post-hoc LSD, $p < 0.05$). Pg: primary growth, Ca: cortical alveolus, VtgA: initial vitellogenesis, VtgB: mean vitellogenesis, Fm: final maturation, At: atretic, UD: Undetectable. All values were relativized to the expression of each gene at Pg stage. Reference gene for this analysis: *βactin*. Samples size is indicated between parentheses below the x-axes.

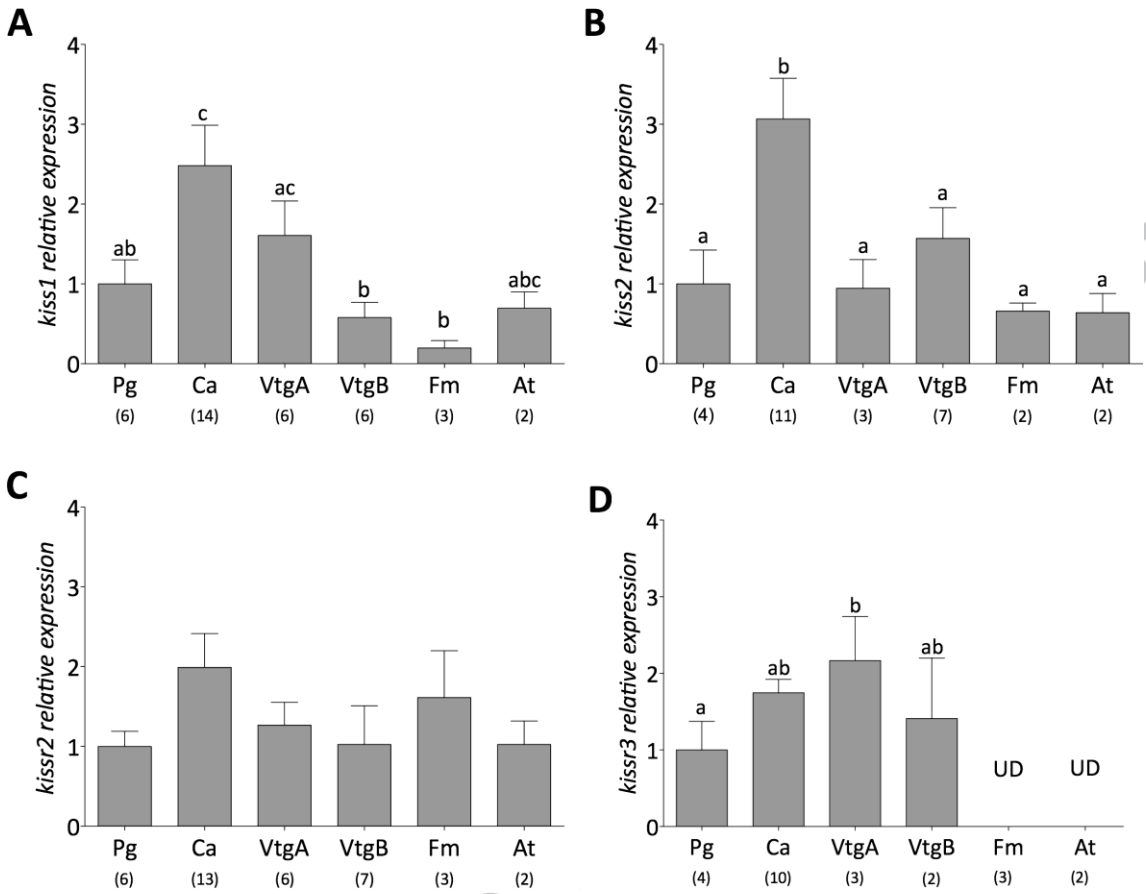

Figure 4: Relative expression of *kiss1* (A), *kiss2* (B), *kissr2* (C) and *kissr3* (D) in the pejerrey male pituitary gland at different gonadal stages. The bars represent the mean ± SEM. Different letters indicate significant differences between the means (ANOVA, post-hoc LSD, $p < 0.05$). A: arrested, SG: spermatogonial stage, SC: spermatocytary stage, SP: spermiogenic stage. All values were relativized to the expression of each gene at A stage. Reference gene for this analysis: *ef1a*. Samples size is indicated between parentheses below the x-axes.

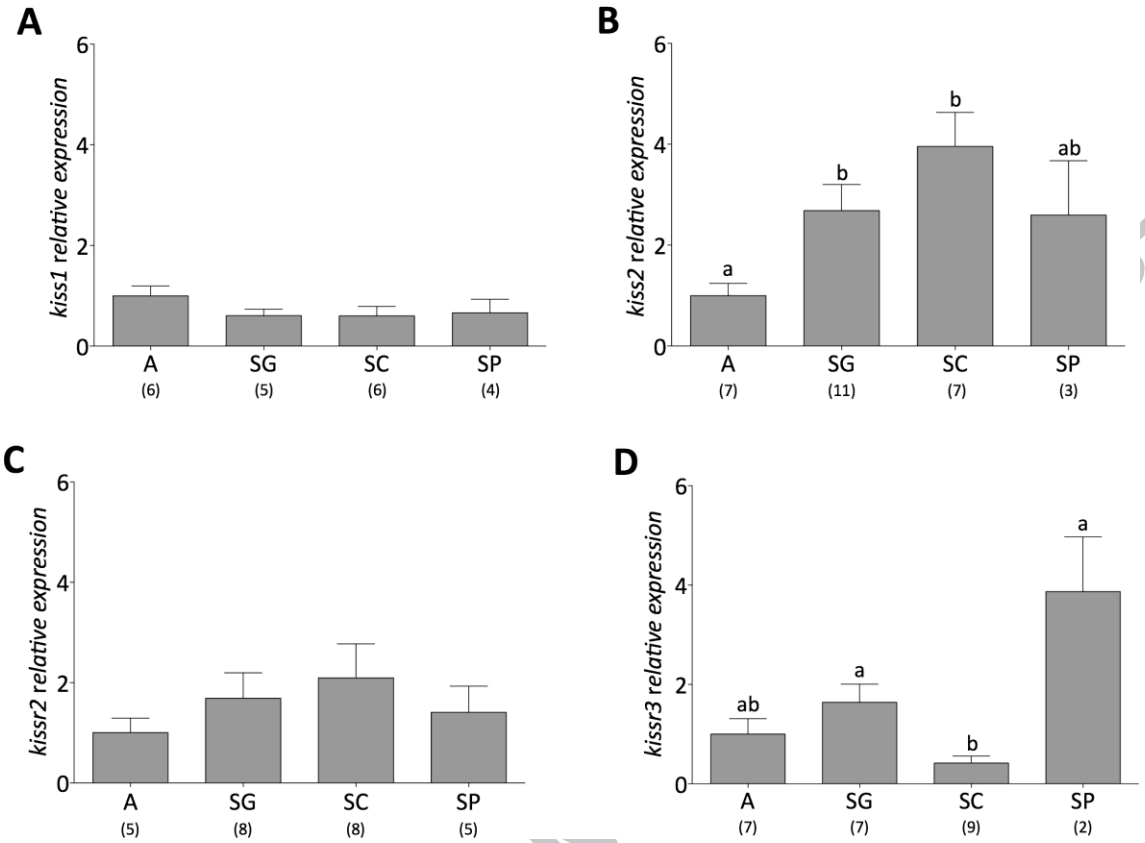

Figure 5: Relative expression of *kiss1* (A), *kiss2* (B), *kissr2* (C) and *kissr3* (D) in the pejerrey ovary at different gonadal stages. The bars represent the mean ± SEM. Different letters indicate significant differences between the means (ANOVA, post-hoc LSD, $p < 0.05$). Pg: primary growth, CA: cortical alveolus, VtgA: initial vitellogenesis, VtgB: mean vitellogenesis, FM: final maturation. All values were relativized to the expression of each gene at Pg stage. Reference gene for this analysis: □-*actin*. Samples size is indicated between parentheses below the x-axes.

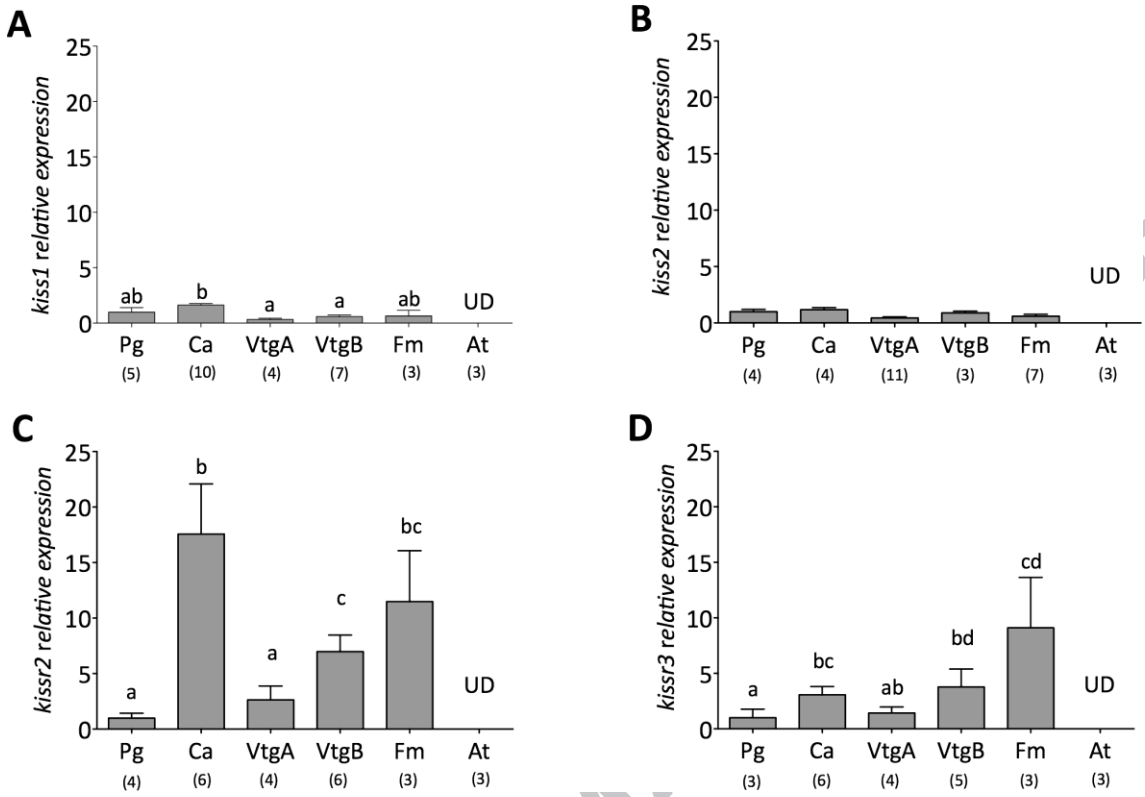

Figure 6: Relative expression of *kiss1* (A), *kiss2* (B), *kissr2* (C) and *kissr3* (D) in the pejerrey testes at different gonadal stages. The bars represent the mean ± SEM. Different letters indicate significant differences between the means (ANOVA, post-hoc LSD-, $p < 0.05$). A: arrested, SG: spermatogonial stage, SC: spermatocytary stage, SP: spermiogenic stage. All values were relativized to the expression of each gene at A stage. Reference gene for this analysis: *ef1a*. Samples size is indicated between parentheses below the x-axes.

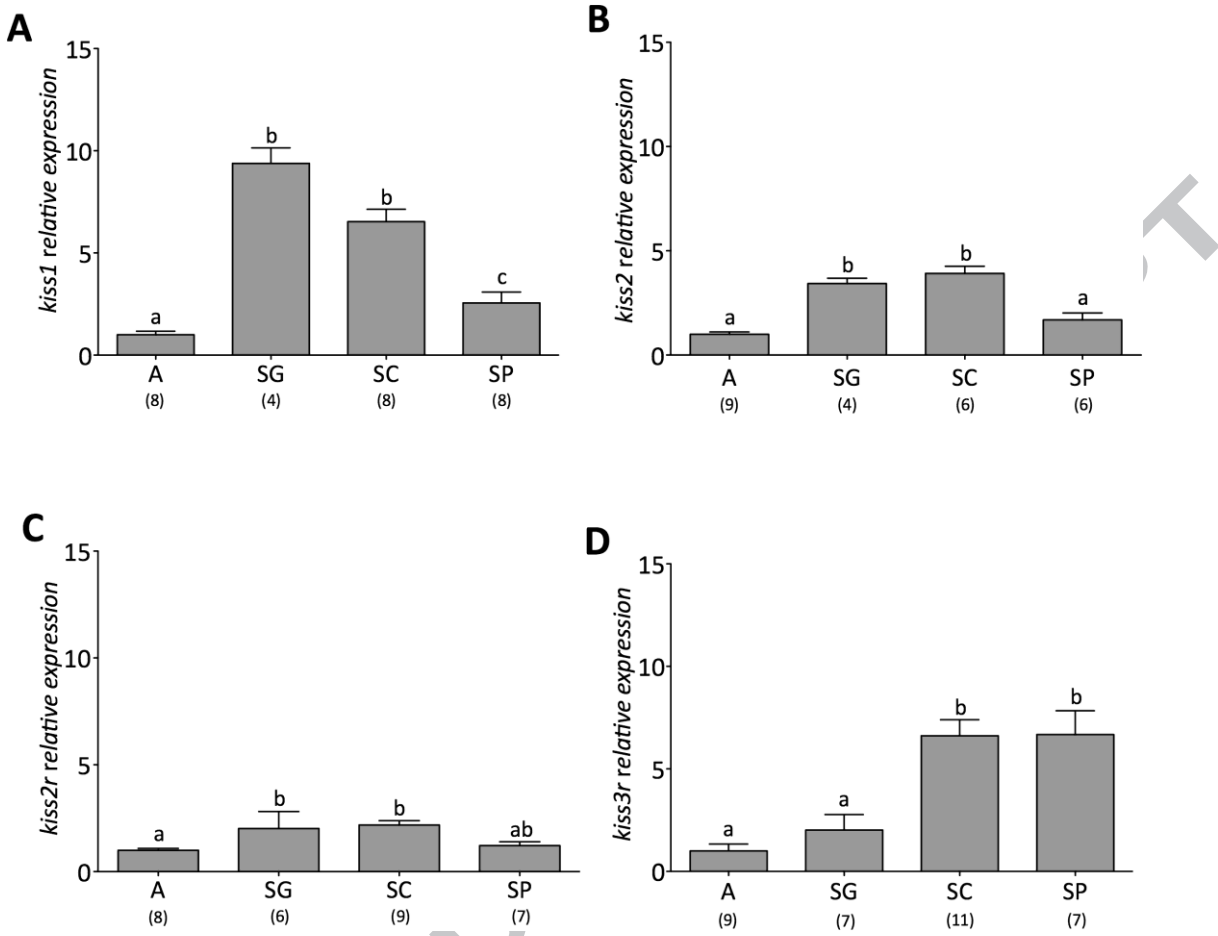

Table 1. Gene-specific primers used for qPCR analysis.

cDNA	Forward primer	Reverse primer	Efficiency	Slope	r^2	Ct value +/- sd	Product size bp	Tm °C
kiss1	AAGGCGTTGGTCAGCACTAC	CGGGAAGACCACCTTTGTAA	99%	-3.33	0.99	26.52+/-1.41	100	60
kiss2	CAGAGAGAGCGACGACCAG	AGAGAAAGAGGGGCGAAAAC	90%	-3.63	1	26.91+/-1.65	161	60
kissr2	TTTGGATTGGCTCCTTCATC	GCCGTACCAGTAACCCTCCT	93%	-3.51	1	28.21+/-2.12	74	60
kissr3	GTCTACCTCCTGCCCCTGCTTACC	GCCTGAGCCTGCAGTTGGTAGC	103%	-2.77	0.998	30.23+/-1.51	113	60
b-actin	GCTGTCCCTGTACGCCTCTGG	GCTCGGCTGTGGTGGTGAAGC	98%	-3.36	1	18.16+/-1.49	200	60
EF1α	AGAAATCCGTCGTGGATACG	TGATGACCTGAGCGTTGAAG	86%	-3.74	0.99	17.58+/-2.85	83	60

Table 2. Correlation analysis between the relative expression levels of *kiss*/*kissr* genes and the main endocrine components regulating reproduction in pejerrey females.

	Brain				Pituitary				Ovary			
	kiss1 n=34	*kiss2* n=33	*kissr2* n=34	*kissr3* n=33	*kiss1* n=37	*kiss2* n=33	*kissr2* n=37	*kissr3* n=19	*kiss1* n=30	*kiss2* n=34	*kissr2* n=24	*kissr3* n=21
gnrh1	0.362	-0.114	0.055	0.522	-0.232	0.313	-0.138	0.598	-0.110	-0.030	0.390	0.623
	0.035	0.526	0.759	0.002	0.168	0.077	0.414	0.007	0.564	0.868	0.060	0.003
fshb	0.064	-0.162	-0.202	0.284	0.014	0.434	0.085	0.709	-0.342	-0.284	0.794	0.555
	0.721	0.369	0.252	0.110	0.935	0.012	0.619	0.001	0.064	0.103	<0.001	0.009
lhb	0.404	-0.186	-0.120	-0.134	0.008	0.266	0.223	0.491	0.039	0.173	0.369	0.277
	0.018	0.301	0.500	0.456	0.960	0.134	0.184	0.033	0.840	0.329	0.076	0.225
fshr	-0.213	-0.358	-0.337	-0.069	0.129	0.153	-0.080	0.411	0.075	-0.011	0.161	0.266
	0.227	0.041	0.051	0.703	0.446	0.396	0.636	0.081	0.693	0.953	0.452	0.245
lhcgr	0.234	-0.108	-0.045	0.281	-0.279	0.195	-0.319	0.089	-0.249	-0.074	0.193	0.230
	0.183	0.550	0.802	0.113	0.095	0.277	0.055	0.716	0.185	0.676	0.365	0.316
T	0.022	-0.166	-0.101	0.186	0.037	0.120	0.015	0.134	-0.016	-0.141	0.058	0.163
	0.901	0.355	0.571	0.299	0.828	0.507	0.930	0.586	0.933	0.427	0.788	0.480
E2	0.019	-0.122	-0.037	0.263	-0.198	0.072	-0.104	0.238	-0.270	-0.306	0.331	0.325
	0.917	0.498	0.836	0.139	0.241	0.690	0.539	0.327	0.149	0.078	0.114	0.151

Shaded values are considered statistically significant ($p < 0.05$). Top line: Pearson's correlation coefficient. Bottom line: p values. Expression levels of *gnrh1* (brain), *lhb* (pituitary), *fshb* (pituitary), *fshr* (gonad), *lhcgr* (gonad) and serum levels of T and E2 were taken from Elisio et al 2014.

Table 3. Correlation analysis between the relative expression levels of *kiss*/*kissr* genes and the main endocrine components regulating reproduction in pejerrey males.

	Brain				Pituitary				Testes			
	kiss1 n=33	*kiss2* n=34	*kissr2* n=37	*kissr3* n=27	*kiss1* n=28	*kiss2* n=32	*kissr2* n=33	*kissr3* n=33	*kiss1* n=38	*kiss2* n=27	*kissr2* n=32	*kissr3* n=34
gnrh1	-0.415	-0.445	-0.685	-0.497	-0.260	0.460	-0.096	-0.163	0.535	0.643	0.341	0.797
	0.016	0.008	<0.001	0.008	0.181	0.008	0.595	0.366	0.001	<0.001	0.056	<0.001
fshb	-0.264	-0.210	-0.167	-0.213	0.045	0.655	-0.102	-0.284	0.529	0.689	0.228	0.368
	0.137	0.234	0.325	0.286	0.819	<0.001	0.571	0.109	0.001	<0.001	0.209	0.032
lhb	-0.089	0.006	-0.240	-0.200	0.007	0.112	0.145	0.233	0.048	0.096	0.000	-0.169
	0.621	0.973	0.153	0.318	0.974	0.540	0.422	0.192	0.775	0.632	0.998	0.340
fshr	-0.044	0.077	-0.112	-0.027	0.058	-0.231	0.130	0.229	-0.184	-0.185	0.092	0.166
	0.810	0.666	0.508	0.896	0.771	0.203	0.471	0.199	0.268	0.354	0.618	0.348
lhcgr	-0.102	-0.011	-0.254	-0.173	0.110	-0.086	0.138	0.142	0.036	0.062	0.243	0.210
	0.572	0.950	0.129	0.388	0.576	0.639	0.443	0.431	0.831	0.757	0.181	0.234
11-KT	-0.368	-0.368	-0.498	-0.405	-0.245	0.546	-0.065	-0.234	0.592	0.648	0.242	0.543
	0.035	0.032	0.002	0.036	0.209	0.001	0.720	0.190	<0.001	<0.001	0.182	0.001

Shaded values are considered statistically significant ($p < 0.05$). Top line: Pearson's correlation coefficient. Bottom line: p values. Expression levels of *gnrh1* (brain), *lhb* (pituitary), *fshb* (pituitary), *fshr* (gonad), *lhcgr* (gonad) and serum levels of 11kT and T were taken from Elisio et al 2015.

Highlights:

- Components of the kisspeptin system are expressed at the BPG axis in both sexes.
- *kiss*/kissrs had different expression profiles during the gonadal cycle in both sexes.
- *kiss1* was associated to high at final maturation in female´s brains.
- The four genes were highly expressed in males´ brains at the arrested stage.
- In the female pituitary *kiss1* and *kiss2* peaked at cortical alveoli stage.