Differential and Gonad Stage-Dependent Roles of Kisspeptin1 and Kisspeptin2 in Reproduction in the Modern Teleosts, *Morone* Species

Short title: Kiss1 and Kiss2 regulate reproduction in modern fish

Summary sentence: Kiss1 and Kiss2 can alternate between stimulation and inhibition of the reproductive axis in different gonadal stages.

Key words: fish, kisspeptin, kisspeptin receptor, reproduction

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Abstract

Kisspeptin is an important regulator of reproduction in many vertebrates. The involvement of the two kisspeptins, Kiss1 and Kiss2, and their receptors, Gpr54-1 and Gpr54-2, in controlling reproduction was studied in the brains of the modern teleosts, striped and hybrid basses. In situ hybridization and laser capture microscopy followed by QRT-PCR detected co-expression of *kiss1* and *kiss2* in the hypothalamic nucleus of the lateral recess (NRL). *gpr54-1* and *gpr54-2* expressing neurons were detected in several brain regions. In the preoptic area, *gpr54-2* was co-localized in GnRH1 neurons while *gpr54-1* was expressed in cells attached to GnRH1 fibers, indicating two different modes of GnRH1 regulation. The expression of all four genes was measured in the brains of males and females at different life stages using QRT-PCR. *kiss1* and *gpr54-1* mRNA levels, the latter expressed in minute levels, were consistently lower than *kiss2* and *gpr54-2*. While neither gene’s expression increased at prepuberty, all were dramatically elevated in mature females. *kiss2* mRNA levels increased also in mature males. Kiss1 peptide was less potent than Kiss2 in elevating plasma Lh levels, and in up-regulating *gnrh1* and *gpr54-2* expression in prepubertal hybrid bass in vivo. In contrast, during recrudescence, Kiss1 was more potent than Kiss2 in inducing Lh release, and Kiss2 down-regulated *gnrh1* and *gpr54-2* expression. This is the first report in fish to demonstrate the alternating actions and the importance of both neuropeptides for reproduction. The organization of the kisspeptin system suggests a transitional evolutionary state between early to late evolving vertebrates.
Introduction
The kisspeptin system's importance in the control of reproduction has been established in recent years in many vertebrate species [1, 2], excluding avian species that lack kisspeptin [3]. Kisspeptin is a conserved RFamide neuropeptide that elicits an increase in circulating gonadotropins via GnRH neurons [4-6], most likely through its receptor expressed in GnRH preoptic neurons [4, 7, 8].

Kiss1 transcript and KiSS1 peptide have been consistently mapped in two major diencephalic sites in several mammalian species: 1) the preoptic region (POA), which specifically in rodents is the anteroventral periventricular nucleus (AVPV) and 2) the arcuate nucleus (ARC) [9-15]. In rodents, the AVPV KISS1 neurons exhibit a sexual dimorphism, with greater abundance in female mice and rats [11, 16]. The AVPV neurons are involved in mediating the positive feedback action of estradiol to the HPG axis [17-20], while ARC kisspeptin neurons likely modulate the negative feedback actions of steroids [17, 18, 21, 22].

Two isoforms of kisspeptin, Kiss1 and Kiss2, have recently been found in fish, with the exception of puffer fishes, Senegalese sole and the three spined stickleback that possess only Kiss2 [23-25]. Both kisspeptins were potent in stimulating the release of gonadotropins and up-regulating GnRH/gonadotropin gene expression in teleosts [24, 26-30]. kiss1 in medaka and kiss2 in zebrafish are influenced by steroids [31-33], suggesting a role for kisspeptin in the gonadal steroid feedback loop of fish as well.

Temporal expression patterns of kiss1 and kiss2 generated for zebrafish exhibited correlation to the onset of puberty in males and females [26, 28]. Histological localization of kiss1- and kiss2-expressing neurons in the brain of fish display some species-specific differences. kiss1 mRNA was found in the hypothalamic nucleus ventral tuberal (NVT), nucleus posterior periventricular (NPPv) and in the extra-hypothalamic region habenula [28, 31], either exclusively in the habenula [28] or also in the nucleus of the lateral recess (NRL) [34]. kiss2-expressing neurons were found more caudally in and around the NRL [28, 31, 34]. The NVT, NPPv and the NRL in fish brain are parts of the tuberal hypothalamus [35] that corresponds to the mammalian hypothalamic tuberal region where the arcuate nucleus is located.

Biological activity of Kiss1 and Kiss2, tested in vivo by administration of the core decapeptides, has also revealed variable results. In female goldfish, only Kiss1 injection elicited an increase in Lh plasma levels [30]. In the European sea bass, systemic administration of Kiss2 peptide increased Lh and Fsh plasma levels more efficiently than Kiss1 and the effect was more pronounced in immature males than in adults [24]. Kiss2 injected into zebrafish was more potent than Kiss1 in up-regulating pituitary lhb and fshb expression [28]. A growing body of data suggests that the Kiss2-Gpr54-2 pathway is more relevant to reproduction in many fish species, however this presumption must still be verified and may not be true for all teleosts [36].

The isolation of kisspeptin receptor cDNA from Nile tilapia (Oreochromis niloticus) proved to be the first demonstration of the existence of a kisspeptin system in fish [37], which has since been found in several other teleosts from various classes [26, 27, 38, 39]. Two receptor forms have been reported in some fish such as zebrafish, goldfish and sea bass [26, 30, 34], but in most fish species only one form was reported. These forms were classified as: 1) the common and
most abundant form Gpr54-2 (with highest affinity to Kiss2), and 2) Gpr54-1 (with highest affinity to Kiss1), in zebrafish [26], medaka (Oryzias latipes), goldfish [30] and the amphibian Xenopus (Silurana) tropicalis [29]. Expression of gpr54-2 in the brain of several fish species peaked during the onset of puberty [26, 27, 40]. In the fathead minnow (Pimephales promelas), gpr54-2 transcript reached maximal levels when spermatogonia type B appeared in the testis and in females the increase coincided with the appearance of perinucleolar stage oocytes [27].

Altogether, the above information supports the idea of the involvement of the kisspeptin system in the neuroendocrine regulation of puberty and reproduction in teleosts. However, it also emphasizes the variability among fish species and the gap in our understanding of the system, including the functional roles of Kiss1 and Kiss2. Therefore, the aim of the current study was to characterize the kisspeptin system, with respect to reproduction, in basses that belong to the family of modern teleosts, the Perciformes. We have studied the bass Kiss1 and Kiss2 potencies in activating the HPG axis in vivo, generated temporal expression profiles, compared gene expression levels and localized the kisspeptins and their receptors in the brain. We show that kiss1 and kiss2 are co-expressed in the hypothalamus, and that their receptors are expressed in association with GnRH1 neurons. Importantly, we found that the effect of Kiss1 and Kiss2 on the HPG axis varies from stimulatory to inhibitory at different reproductive stages, reinforcing the widely held view that kisspeptin, in addition to being an important regulator of reproduction, also has a role as a central processor of an array of inputs like steroid feedback, photoperiod and energy balance signals [17; 41, 42; 43].

Materials and Methods

Animals
Striped bass (Morone saxatilis), used for the gene expression profiling and neuroanatomical studies, were obtained as juveniles from Maryland Department of Natural Resources (Annapolis, MD) and maintained at ambient conditions in a 20 m³ tank, supplied with constant exchange of recirculated artificial 8-10 ppt seawater, until reaching sexual maturity. Hybrid bass {Morone saxatilis X M. chrysops [44]}, used in the kisspeptin induction experiments were obtained from Susquehanna Aquaculture (York Haven, PA). Fish were acclimated to ambient conditions in recirculated four foot diameter tanks (1 m³ volume) for a week. Hybrid bass were used because both males and females reach maturity simultaneously during their first year of age and at a smaller size compared to striped bass (~500 g vs. 2-3 Kg body weight). Animal maintenance and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Isolation of kiss1, kiss2, gpr54-1 and gpr54-2 cDNAs from the brain of the striped bass
Total RNA was extracted from brain of a mature female striped bass using Trizol reagent (Invitrogen). First-strand cDNA was generated from 1 μg total RNA using 5’- and 3’- RACE (SMART RACE cDNA Amplification Kit; BD Biosciences). All primers used for cloning are listed in Table 1. The initial degenerate primers were designed according to conserved amino acid domains of fish. In all cases, degenerate primers were used in first and nested PCR to obtain initial amplicons, followed by amplification of the 5’ and 3’ cDNAs using gene-specific primers. kiss1 cDNA first amplicon was obtained with degenerate primers kiss1f and kiss1rev1 and the Advantage cDNA polymerase mix (BD Biosciences). kiss1 5’- and 3’ RACE fragments were amplified with kiss1rev1 and the universal adapter primer UPM (from the kit) followed by the nested primers kiss1rev2 or the forward primers gskskiss1f1 and gskskiss1f2 from a 5’- and 3’ RACE
brain cDNA library, respectively. 5’ kiss2 cDNA was amplified using degenerate primer kiss2rev1 plus UPM, followed by a nested PCR using kiss2rev2 and NUP. kiss2 3’cDNA was amplified with gskiss2f and UPM, followed by gskiss2f and NUP amplification. gpr54-2 first amplicon was obtained using GPR54f1 and GPR54r, then using GPR54f2 and GPR54r. For obtaining 3’ and 5’amplicons, gsGPR54r2 or gsGPR54f2 were combined with UPM and NUP as described above. An initial fragment of gpr54-1 cDNA was obtained with GPRbf and kissrbr. The next amplification to amplify 5’cDNA used gskissrbr1f and gskissr1br2, and to amplify 3’ cDNA gskissrbr1 and gskissrbr2 was used in combination with UPM and NUP, respectively. All fragments were inserted into pGEM-T vector (Promega) for further analysis and sequencing.

**Hormones and gene transcript measurements**

Lh levels in the plasma were measured using Lh ELISA as previously described [45]. For the measurement of gnrh1, 2 and 3 transcript levels, brain total RNA (1 µg) was reverse-transcribed using Quantitect RT kit (Qiagen). Real-time PCR was performed on 50 ng cDNA using SYBR Green PCR mix (Applied Biosystems) in duplicate for each sample, with 0.1 µM gene-specific primers for each GNRH, as previously described [46], in an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Ct values of each sample were normalized against the levels of 18S RNA amplified from 0.2 ng cDNA [47] and then converted to the fold-change of the mean Ct value.

Striped bass kiss1, kiss2, gpr54-1 and gpr54-2 transcript levels were measured similarly using gene-specific primers: TAQKiss1, TAQKiss2, TAQgpr54-1 and TAQgpr54-2 primer sets (Table 1). The efficiency of each gene primer set, as obtained from the standard curve slope values, was: kiss1 = 0.948, kiss2 = 0.949, gpr54-1 = 1.17 and gpr54-2 = 1.11.

A standard curve for each gene, ranging from 10^2 to 10^7 gene copies, was generated using RNA transcribed in vitro by T7 or SP6 RNA polymerase to synthesize the sense RNA from the cDNA cloned in pGEM-T vector. The standard curve, included in each QPCR plate was used to convert the Ct values of the unknown samples to a copy number of mRNA [47]. Amplification reactions were carried out at 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melting points for all primers ranged between 59-61°C. Proper and specific amplification of each gene was verified using gel electrophoresis and by the dissociation curve of the primer sets. In each run, two negative water controls and a reference control, added to each plate, were included in addition to the standard curve.

**In situ hybridization of kiss1, kiss2, gpr54-1 and gpr54-2 in the brain of the striped bass**

Brains of striped bass were removed immediately after decapitation, fixed in buffered 4% paraformaldehyde for 6 h at 4°C, cryoprotected in 15% sucrose overnight at 4°C and embedded in Tissue Tek OCT (Electron Microscopy Sciences). Coronal sections of 12 µm were mounted onto Plus glass slides and stored at -80°C. Sense and antisense hapten-labeled riboprobes were synthesized from the full cDNA clones of kiss1, kiss2 and gpr54-2 using RNA polymerase (Roche Diagnostics). gpr54-1 probes were synthesized from a 425 bp coding region upstream of the stop codon. Sections were air-dried, post-fixed in 4% PFA for 10 min then treated with 5 µg/ml proteinase K for 5 min at 37°C and re-fixed. After incubation with 0.1 M triethanolamine 0.25% acetic anhydride for 10 min, the sections were pre-hybridized at 58°C for 2h and hybridized with the labeled riboprobe at 58°C overnight. The sections were washed with 2, 0.4
and 0.1xSSC each for 30 min at 58°C. The signal was detected using Tyramide Signal Amplification kit (TSA, Perkin Elmer), according to the manufacturer’s protocol, using streptavidin-HRP to detect biotinylated probe and anti-DIG HRP (Roche) to detect DIG-labeled probe. Fluorescence was obtained via Cy3 or fluorescein from the kit. Double-label staining of kiss1 and kiss2 used biotin labeled kiss1 riboprobe at a concentration of 500 ng/ml and kiss2 at 300 ng/ml. Double-labeling of gpr54-2 or gpr54-1 with GnRH1 was performed using a combination of in situ hybridization (ISH), using a TSA kit and Cy3, and immunohistochemistry (IHC) using anti-GnRH1-associated peptide (GAP) of the European sea bass (93% identity), as described previously [48, 49], and FITC-conjugated goat anti-rabbit secondary antibody (Sigma). Negative control slides were similarly treated with sense riboprobes, except that all probes were DIG-labeled and signal was developed with streptavidin-HRP followed by Cy3 staining.

Laser capture microscopy and QRT-PCR
Positively ISH-stained neurons were further stained with NBT-BCIP using anti-fluorescein-AP (Roche). The slides were dehydrated through 75%, 95% and 100% ethanol for 30 sec each and xylene for 5 min, then air dried. Neurons were captured using an Arcturus II infrared laser beam at 65 mW for 25 ms. Approximately 10 positively stained cells from the desired region were collected into a CapSure HS cap (Applied Biosystems). Total RNA was obtained using a Micro-RNeasy kit (Qiagen) according to the manufacturer’s fixed sample protocol. cDNA was transcribed using a Quantitect RT kit (Qiagen) and 5% of the transcribed amount was used for Q-PCR amplification for 60 cycles as described above. The resulting PCR products were resolved on a 2% agarose gel stained with ethidium bromide.

Expression levels of kiss1, kiss2, gpr54-1 and gpr54-2 in the brains of juvenile, prepubertal and mature males and females
Juvenile striped bass were obtained from Horn Point Laboratory, University of Maryland Center of Environmental Sciences in Cambridge, MD at the age of 4 months. Fish were maintained in 6 and 12 foot tanks at 8 ppt salinity under natural thermo- and photo-period regimes. Juvenile fish were sampled in May (~ 1 year old; N=6 for males, N=7 for females; GSI=0.5%), prepubertal fish were sampled in August of the second year of age for males (N=10, GSI = 0.3%) and the third year of age for females (N=10, GSI = 0.6%), respectively. Mature males (N=8, GSI = 4.2%) and females (N=10, GSI = 2%) were sampled in January of their 4th year of age at their mid-gonadal development stage. More detailed information about gonadal stages and seasonality has been provided in a prior publication [50].

Whole brains were used to extract total RNA. mRNA levels of kiss1, kiss2, gpr54-1 and gpr54-2 in the brains were determined using QRT-PCR as described above.

Induction of the HPG axis by kiss1 and kiss2 in vivo
Kiss1(15 aa, QVSSYNLNSFGLRY-NH2) and Kiss2 (12aa, SKFNFNPFLRF-NH2) peptides were used to test kisspeptin activity in hybrid bass. The bioactivities of the two peptides were studied in two separate experiments carried out in May and November of 2010. The first experiment included 7 groups of six pubertal males and females with 0.4% GSI. The different groups received a single injection of Kiss1 or Kiss2 at 5, 25 and 100 nmol/Kg body weight (BW; equivalent to 8.8, 44 and 176.1 µg/Kg BW Kiss1 and 7.36, 36.8 and 147.2 µg/Kg BW Kiss2), prepared in saline, and the control group was treated with saline. One ml blood was sampled
immediately before and at 1, 2, 4 and 24 hr post-injection. The second experiment included 7 similar groups of mature males and females with an average GSI of 1.68% and 1.35%, respectively, that received 50, 100 and 150 nmol/Kg BW Kiss1-15 (44, 176.1 and 220.1 µg/Kg BW) or 5, 25 and 50 nmol/Kg BW Kiss2-12 (7.36, 36.8 and 73.6 µg/Kg BW). The peptide doses in the second experiment were adjusted based on the results of the first experiment. Blood samples were collected immediately before, and at 4 and 24 hr post-injection.

In both experiments, the tested peptide was injected intramuscularly in a volume of 0.1 ml/Kg BW. Plasma was collected at the designated times. At the end of the experiment fish were sacrificed, gonad stage was determined microscopically, and brains and pituitaries were snap-frozen and stored at -80°C.

**Statistical analysis**
Statistical analyses were performed by one-way ANOVA and Tukey’s post hoc test for multiple comparisons using Instat (GraphPad). For Lh plasma level analyses, statistical significance was accepted only when the tested group showed significant difference to the saline-treated group at the same time point as well as to the zero point of the same treatment. In the case of the temporal expression analysis, all three life stages of both males and females were analyzed together using the same analysis comparing all pairs of columns.

**Results**

**Characterization of the cDNAs encoding kiss1, kiss2, gpr54-1 and gpr54-2**
Striped bass kiss1 cDNA (Genbank accession # GU351864) is most similar to that of the European sea bass (96% identity at the nucleotide and 92% at the deduced amino acid levels). The precursor contains a predicted cleavage site for prohormone convertase (PC) [51] with conserved dibasic amino acids (KR) and a Glutamine residue at positions 101-103, resulting in a 15 aa Kiss1 predicted peptide (QDVSSYNLNSFGLRY-NH2).

Striped bass kiss2 cDNA sequence (Genbank accession # GU351865) is 97% and 96% identical to that of the European sea bass at the nucleotide and amino acid levels, respectively. A PC cleavage amino acid motif (RR) [51] at position 87-90 of the pre-pro-precursor is predicted to give rise to a 12 aa peptide (SKFNFNPGLRF-NH2). Kiss1 contains three additional aa (QDV) at the N-terminus of the peptide, compared to Kiss2, and the downstream sequence differs by five aa at positions 2, 3, 4, 6 and 12.

The full size cDNA of gpr54-2 was cloned from mature female brain (Genbank accession # GU351869). gpr54-1 partial cDNA was amplified from cDNA of a prepubertal male brain. It spans 927 bp encompassing 429 bp of the coding sequence for trans-membrane domains 6 and 7 and outer loops 5, 6 and carboxy-terminus tail, and 498 bp 3’ UTR, and is highly homologous to gpr54-2 with the exception of the cytoplasmic C-terminus tail (Supplemental Figure S1, all supplemental data are available online at www.biolreprod.org).

**Localization of kiss1, kiss2, gpr54-1 and gpr54-2 neurons in the brain**
The localization of neurons expressing the two kisspeptins and the two kisspeptin receptors was determined in the brain of spermiating male striped bass using ISH. kiss1-expressing neurons are found in the dorsal and ventral subdivisions of the lateral nucleus of the recess (NRLd, NRLv)
and the posterior tuberal nucleus (NPT) from the hypothalamus (Figs. 1A, a-c). kiss2 neuron staining yielded a prominent signal in neurons of the NRLd and NRLv (Figs. 1A, d-f). These cells also stained positive for kiss1, as is apparent from merging the two sets of images (Figs. 1A, g-i). Sections treated with sense kiss1 and kiss2 riboprobes yielded no signal (Supplemental Figure S2a). Laser capture microscopy isolation of cell bodies in the NRLd and NRLv that stained positively for kiss1 and kiss2, followed by mRNA isolation and quantitative PCR, confirmed the co-expression of kiss1 and kiss2 in these two neuronal subsets (Fig. 1B). Kisspeptins were co-expressed with gpr54-2 in the NRLd cells but not in the NRLv (Fig. 1B). No kiss1 labeling was evident in the habenula. gpr54-1 neurons were located in the preoptic area, the habenula and the inferior subdivision of the lateral tuberal nucleus (NLTi) (Figs. 2a-c). gpr54-1 preoptic cell bodies were observed in close association with GnRH1 fibers present in the preoptic region (Fig. 2a, inset). gpr54-2-expressing neurons are widely expressed in the preoptic area and the dorsal hypothalamus including the anteroventral (NPOav) and parvocellular (NPOpc) regions of the parvocellular preoptic nucleus (Fig. 2d,e), the parvocellular (PMpc) and magnocellular (PMmc) regions of the magnocellular preoptic nucleus (Figs. 2f, the dorsal (NLTd), ventral (NLTv) and inferior (NLTI) subdivisions of the lateral tuberal nucleus (Figs. 2g,h), as well as the dorsal (NRLd), lateral (NRLi) and ventral (NRLv) regions of the lateral nucleus of the recess (Fig. 2h,i). They are also expressed in the preglomerular tertiary gustatory nucleus (NGT, Fig. 2g), the post-commissural nucleus of the ventral telencephalon (Vp) and, to a lesser extent, in the thalamic ventrolateral (VL) and ventromedial (VM) nuclei (not shown). gpr54-2 was co-localized with preoptic GnRH1 neurons in the NPOav and NPOpc (Fig. 2e, inset). Slides carrying consecutive sections double-labeled with gpr54-1 and gpr54-2 sense riboprobes yielded no staining (Supplemental Figure S2b-e).

The same mRNAs were similarly localized in the brains of juvenile male and female striped bass at their first year of life (average GSI=0.27% and 0.13%, respectively) and in vitellogenic mature females in January of their fourth year of life (GSI=5.8%). Selected regions are presented for comparison in Figure 3. kiss1, kiss2 and gpr54-2 were detected in the same brain regions in juvenile and mature males, although the number of expressing cells and staining intensity were markedly lower than in mature males (Fig. 3a-d). kiss1 and kiss2 were co-localized in the NRLd and NRLv (Fig. 3a) and in the NRLi (not shown), and gpr54-2 was localized in the NRLd, NRLv and NLTi (Fig. 3b) and in the NLTv (Fig. 3c). kpr54-1 was undetectable in juvenile striped bass brain and gpr54-2 was not detected in the preoptic area (not shown).

Mature vitellogenic females exhibited the same expression distribution observed in mature males. kiss1 and kiss2 were expressed and co-localized in neurons in the NRLd, NRLv (Fig 3 d,e) and NRLi (not shown). gpr54-2 was localized in the preoptic NPOpc, NPOav and PMpc (Fig. 3f) and in the NRLv and NLTi (Fig. 3g). gpr54-1 was localized in the NLTv (Fig. 3h) and in the NPOav (not shown).

**Temporal expression profiles in the brain**

In order to test the reproductive relevance of Kiss1 and Kiss2 and their receptors in the striped bass, brain transcript levels of all four genes were measured in males and females at three time points during reproductive development. kiss1 and kiss2 transcript levels were similar in juvenile and prepubertal fish (ranging from ~2000 to ~4000 copies/µg total RNA), however kiss1 transcript levels were 6.5 and 3.7 times lower than the levels of Kiss2 in mature males and
females, respectively (Fig. 4 A,C). gpr54-1 transcript levels were ~27-40 times lower than those of gpr54-2 (Fig. 4 B,D). It should be noted that despite the very low levels of gpr54-1 amplicons, similar to the other genes, a clear band at the expected size was clearly visible following gel electrophoresis. The mRNA levels of all four genes did not change significantly between juvenile and prepubertal fish of both genders, but increased significantly and dramatically in mature mid-vitellogenic females (Fig. 4). The levels of these genes in females were also significantly higher than the corresponding genes in mature males (Fig. 4). Only the levels of kiss2 mRNA increased significantly (~5 times) in mature males (Fig. 4C).

**Activation of the HPG axis by Kiss1 and Kiss2 peptides**

**Kisspeptin effect on Lh levels in the plasma**

Both Kiss1-15 and Kiss2-12 peptides were potent in inducing the release of Lh, however the response showed dose-dependent and reproductive stage differences. In the first experiment, which was conducted in May, the fish were at the prepubertal stage. We observed a continuous increase in plasma Lh levels during the 24 hr course of the experiment, including the control saline-injected group. Therefore all results were compared to both basal levels and the control (saline-injected) group of the same sampling time point. Kiss1-15 injection yielded no significant increase when compared to the saline-injected control fish of the same time point (Fig. 5A). Kiss2-12 injection, however, increased Lh blood levels by 4.5 to 6-fold (from 2ng/ml at time 0 to 9-14 ng/ml) at doses of 5 and 25 nmole/Kg BW, while 100 nmole/Kg BW induced Lh blood levels by only 2.5 and 3.5-fold at 4 and 24 hr post-injection, respectively (Fig. 5B).

In the experiment conducted in November, where fish were in their mid-gonadal development phase with GSI levels of ~1.5%, the response was less prominent compared to results obtained at prepuberty. Based on the results of May, the injected doses of Kiss1-15 were changed to 50, 100 and 150 nmole/Kg BW, while those of Kiss2-12 were changed to 5, 25 and 50 nmole/Kg BW and Lh levels were measured at 0, 4 and 24 hr post-injection. Kiss1-15 at doses of 50 and 100 nmole/Kg BW induced Lh plasma levels 24 hr post-injection, 1.7 and 2.5-fold over their respective basal levels, while a dose of 150 nmole/Kg BW had no effect (Fig. 5C). Kiss2 at doses of 5 and 25 nmole/Kg BW increased Lh plasma levels significantly at 24 hr post-injection (76% over basal level and 56% over the saline-injected group at 24 hr), while a dose of 50 nmole/Kg BW was ineffective at all times (Fig. 5D). It is noteworthy that unlike the May trial, where Lh levels were increased starting at 4 hr post-injection, Lh levels in the November trial increased only at 24 hr post-injection.

No differences in Lh levels were noted between males and females with regard to dose or time in either experiment. For example, in the second experiment Lh levels at 24 hr post-injection were: control 13.61±4.95 and 20.7 ± 11.26 ng/ml plasma, Kiss1-15 (50 nmole/Kg) 27±8 and 27±1.7 ng/ml plasma, and Kiss2 (50 nmole/Kg) 28.8±10.77 and 25.7±2.18 ng/ml plasma for males and females, respectively.

**Kisspeptin effect on gpr54-2 and gnrh1 transcript levels**

gpr54-2 transcript levels were up-regulated only by the highest dose of Kiss2-12 (150 nmol/Kg BW) at prepuberty (Fig. 6A), while at recrudescence a significant decrease in mRNA levels was obtained with the two higher doses of kiss1-15 and all doses of kiss2-12 (Fig. 6B). gnrh1 mRNA levels in the brains of prepubertal fish, sampled at 24 hr post-injection, increased only in
response to 100 nmol/Kg BW Kiss1-15 by 2.5 fold, while 25 and 100 nmol/Kg BW Kiss2-12 stimulated the same response (Fig. 6C). A different scenario was observed in recrudescent fish: Kiss1-15 at all doses had no effect on \textit{gnrh1} mRNA levels, but Kiss2-12 caused a decrease of \(~40\%\) at doses of 25 and 50 nmol/Kg BW (Fig. 6D). \textit{gnrh2} and \textit{gnrh3} brain levels in both experiments were not affected by any treatment (not shown).

**Discussion**

The current study describes the two kisspeptin systems in the brain and their relationships with the reproductive HPG axis in two representative \textit{Morone} species. The combined results obtained from the neuroanatomical, molecular and functional studies suggest that both kisspeptin systems have the capacity to control the HPG axis via GnRH and Lh in the striped bass and its hybrid. The deduced pre-pro-precursors of the striped bass kisspeptins possess several predicted dibasic cleavage sites that give rise to a 15 aa mature Kiss1 peptide and a 12 aa Kiss2 peptide, congruent with what is found in many teleosts [24, 36] and \textit{X. tropicalis} [29]. All Kiss2 precursors reported thus far possess PC sites of either RR or RXXR motifs, with the exception of the grass puffer and sockeye salmon (Genebank accession \# BAJ15497 and BAG80689, respectively). Moreover, the presence of Kiss2 mature dodecapeptide (SKFNPNFGLRF-amide) was confirmed in the brain of \textit{X. tropicalis} using HPLC purification and MALDI-TOF-MS [29]. This is different from mammalian kisspeptin, which contains no similar cleavage sites but is naturally found as 10-14 aa RF-amide peptides [52]. The deduced amino acid sequences of the two receptors, \textit{gpr54-1} and \textit{gpr54-2}, show high homology to other fish and mammalian kisspeptin receptors and are consistent with the two receptor paralogues found in teleost species [36].

Localization of the neurons expressing kisspeptins and their cognate receptors can contribute to the understanding of their potential roles. Using ISH, \textit{kiss1}-expressing neurons were found in the NRL and in the NPT of spermatiating mature male (Fig.1) and female (Fig. 3) brain. This finding is different from the habenular localization of \textit{kiss1} reported in the brain of zebrafish and medaka [28, 33]. The fact that the NVT \textit{kiss1} neurons in medaka are sexually dimorphic and sensitive to steroids [31, 32] suggests a role for Kiss1 in reproductive activity, albeit the function of the habenular kiss1 is not known [33]. Interestingly, \textit{kiss1} habenular expression seems to decrease in more evolved fish such as the European sea bass [34].

\textit{kiss2} neurons were localized in the NRLd and NRLv, and are co-expressing \textit{kiss1} (Figs. 1A, 1C, 3a, 3d). The NPT and the NRL are areas of the tuberal hypothalamus [35], which corresponds to the mammalian hypothalamic tuberal region that includes the arcuate nucleus [17, 18, 21, 22], thus suggesting that this kisspeptin expressing region is common to fish and mammals.

The co-expression of \textit{kiss1} and \textit{kiss2} indicates that Kiss1 and Kiss2 share some common neuronal targets in the striped bass. This is also supported by the ability of the two peptides to induce Lh release \textit{in vivo} (Fig. 6A). This scenario requires the expression of both receptors on the same target neurons or, alternatively, activation of one receptor form by both peptides. While our results do not support the co-expression of \textit{gpr54-1} and \textit{gpr54-2}, some studies, including our own results for \textit{gpr54-2} (not shown), have shown that the two receptors are promiscuous and are activated by both peptides [26, 53]. Tracking the neuronal projections using Kiss1 and Kiss2 antibodies, via immunohistochemistry, is necessary to verify this supposition.
In addition to confirming the co-localization of kiss1 and kiss2 mRNA in NRLv and NRLd neurons, quantitation of their transcript levels using QRT-PCR performed on LCM-isolated neurons, revealed kiss1 levels that were 8-16 times lower than kiss2 mRNA levels, consistent with the differences in the levels in the mature male whole brains (Fig. 4). The picture arising from the above information may indicate a convergence of Kiss1 and Kiss2 neurons associated with a decrease in the levels of kiss1 while kiss2 levels remain high. Thus, the kisspeptin system in the basses may represent a transitional state towards the loss of Kiss1, as seen in higher vertebrate species. This hypothesis could explain the lack of kiss1-labeled cells in the habenula of the striped bass.

The expression of gpr54-2 in GnRH1 neurons has been demonstrated in other perciform species, such as tilapia via LCM and QPCR [38] and the European sea bass via ISH [34], but not in GnRH3 neurons in the POA of zebrafish, a cypriniform [33]. So far, cyprinid and salmonid species are believed to not possess the GnRH1 form, whose role is fulfilled by GnRH3 [55]. In case that GnRH1 is found in these species in the future, gpr54-2 expression in its expressing neurons should be tested. The current study did not examine whether kisspeptin receptors are co-expressed in GnRH2 and/or GnRH3 neurons. The distribution of gpr54-1 and gpr54-2 expressing neurons in the striped bass brain, however, is not consistent with that of the previously published locations of GnRH2 and GnRH3 neurons in the European sea bass and gilthead seabream [55, 56] and our unpublished information in the striped bass. We have not observed gpr54-1 or gpr54-2 staining in the nucleus of the medial longitudinal fasciculus of the dorsal midbrain tegmentum (GnRH2 neurons), gpr54-2 expression was indeed observed in the telencephalic Vp, Vm and Vl, but not in the known GnRH3 neuronal location in the ganglion cells of the terminal nerve (TNgc) and the ventral telencephalon (Vv) [55]. Double-label ISH for gnrh2, gnrh3 and kisspeptin receptors should be performed in order to provide a conclusive picture. Nonetheless, the presence of kisspeptin receptor in GnRH1 neurons strongly suggests a role for both kisspeptin forms in the control of reproduction.

Interestingly, gpr54-1-expressing neurons were found in the habenula of the striped bass, congruent with zebrafish [33] and medaka [28]. It has been suggested in zebrafish that Kiss1 autoregulates its neurons in the habenula via its specific receptors co-expressed in the neurons [33]. The significance of this finding or whether the NRL/NPT Kiss1 neuronal projections reach the habenula is unknown as of yet. Unlike in zebrafish, gpr54-1 expression was found in neurons in the NLTv and in the rostral POA attached to GnRH1 fibers (Fig. 2a), suggesting a role for Kiss1 in the control of GnRH1. To our knowledge, this is the first demonstration of physical contact of gpr54-1-expressing neurons with GnRH1 fibers in fish, which indicates that Kiss1 or both Kiss1 and Kiss2 can regulate GnRH1 via non-GnRH1 neurons expressing gpr54-1.

kiss1, kiss2 and gpr54-2 expressing neurons of juvenile males were detected in the NRL, similar to what was observed in the adult male albeit with lower staining intensity and number of expressing cells (Fig. 3a,b). As in mature males, kiss1 and kiss2 were co-localized in the NRL (Fig.3a). gpr54-1 was undetectable in all juvenile brains tested, probably due to its low expression levels (Fig. 4). Interestingly, while detected in the NRL region, gpr54-2 was not observed in the preoptic region. This may be due to the low expression levels that are under the detection limit of the ISH procedure. Alternatively, gpr54-2 genuinely may be not expressed in this region at juvenile stages and it presence may mark the difference between juvenile and
Pubertal/mature stages in the striped bass. This, in any case, is different from the situation in mice, in which GPR54 expression in GnRH neurons is unchanged between juveniles and adults and the only difference between these life stages is their Kiss1 expression level [7].

No apparent difference was obtained in the expression distribution of all four genes between spermiating male and vitellogenic female, although their levels in females are higher than in males (Fig. 4). Sexual dimorphism of the kisspeptin system in fish was reported only in medaka [31, 32], specifically in kiss1 neurons, and not in other teleosts. Nevertheless, sexual dimorphism cannot be ruled out, as differences and neuronal plasticity of the kisspeptin system may occur at other stages of gonadal development. For example, a difference may occur at prepuberty, where fish showed a remarkable sensitivity to the administration of Kiss1 and Kiss2 peptides (Fig. 5) or at pre-ovulation. This aspect will require a thorough histological examination comparing key stages within the reproductive cycle and may be related to the nature of gonadal steroid feedback relayed through kisspeptin [57].

Expression profiles generated for each gene at three major developmental stages of male and female striped bass are characterized by remarkably high levels in mature females, which are higher than those of males (Fig. 4). The expression of these genes did not increase significantly throughout prepubescence, putting their role in the control of puberty in the striped bass in question. As reported above, one of the signature roles of kisspeptin(s) in mammals and other vertebrates is the control of puberty, and the lack of kisspeptin increase at this stage, as described for other vertebrate species [26, 27, 40], was unexpected. On the other hand, the results obtained from the in vivo induction study shows a higher and positive responsiveness of gnrh1 and Lh to both kisspeptins at prepuberty (Fig. 5 & 6), indicating that a distinct window of time may exist during which the kisspeptins are more potent. Thus, the control of puberty by kisspeptins in the striped bass may lie within the receptiveness of the reproductive HPG axis at this stage. Interestingly, only the levels of kiss2 mRNA increased significantly in adult males at the mid-gonadal stage suggesting a role for Kiss2 in male reproductive activities. The constant levels of the three other genes in males, particularly those of gpr54-2, are unclear. However, the relatively high mRNA levels of gpr54-2 throughout development may be sufficient for the execution of the action of any level of Kiss2.

In order to more clearly define the differential role of Kiss1 and Kiss2 in the control of reproduction, we compared their effect on the HPG axis in vivo, including the possibility that the function is exerted through GnRH1. We were able to compare the bioactivity of the two kisspeptins at different gonadal stages that are characterized by different gonadal steroid composition/levels [58]. In the experiment conducted on prepubescent animals (May), the effect of Kiss2-12 on Lh release was prominent, eliciting a release 4-6 times higher than basal levels within 4 hr post-injection (Fig.5B). In contrast, in recrudescent fish (November), Lh blood levels did not change in response to Kiss2-12 (Fig. 5D), whereas gpr54-2 and gnrh1 mRNAs were down-regulated (Figs. 6B and D). Kiss1-15 came into play at recrudescence (November), where it increased blood Lh levels by 2.5-fold at 24 hr post-injection (Fig. 5A). The difference in the response time of Lh release between the two trials, 4 vs. 24 hr, may be explained by the utilization of a differential control of GnRH1 neurons by kisspeptin. An indication that this is a feasible mechanism may be drawn from the localization of gpr54-1-expressing neurons along and outside GnRH1 neurons versus gpr54-2 expression in GnRH1 neurons. During the course of
the experiment, we observed a gradual increase in plasma Lh levels also in the control group. This phenomenon may be explained by diurnal rhythms of Lh as reported in mammals [59, 60] and fish [61, 62] or may be due to acute stress imposed by the handling and treatments [63]. Kiss1-15 also up-regulated gnrh1 and gpr54-2 mRNA levels in prepubertal fish, although higher levels than Kiss2-12 were needed to obtain the same effect (Fig.6). Indeed, the initial experiment was conducted on prepubertal fish, at the stage preceding first onset of gonadal development, which showed a higher and positive response to kisspeptin. Since these fish are also seasonal and there are notable similarities between puberty and seasonal onset of gonadal development [64], this exact phenomenon may reoccur each year at the onset of gonadal development. The latter scenario is supported by the observation that KISS1 expression in the ARC is elevated during the onset of the breeding season in the ewe [65].

The mechanism by which Kiss1 and Kiss2 induce different effects on gnrh1 and gpr54-2 expression is not yet known, but these results are corroborated by the positive steroidal feedback on GnRH1 at puberty and the negative feedback in recrudescent striped bass [58] as well as other vertebrates, including mammals [66-68]. A negative effect of kisspeptin on the HPG axis was also reported in the eel where kisspeptin directly inhibited lhb expression in vitro [69]. Although it is possible that kisspeptin acts at the level of the pituitary also in the bass, the down-regulation of gnrh1 and gpr54-2 at this stage suggests that the inhibitory action involves, at least in part, the kisspeptin-GnRH pathway. In mammals, in contrast, kisspeptin seems to display only a stimulatory effect on the HPG axis [70], directly on the GnRH1 neurons and indirectly by suppressing the inhibitory action of Gaba [71]. An inhibitory effect of kisspeptin, due to desensitization, is reported in mammals only following chronic administration of kisspeptin [70]. In that sense, the stimulatory and inhibitory feedback effects of gonadal steroids, acting through kisspeptin in mammals are thought to be mediated by up- or down-regulation of kisspeptin expression, respectively [70]. Intriguingly, our results in basses indicate that kisspeptin, regardless of its levels, can employ different mechanisms on GnRH1 that can result in excitatory or inhibitory effects depending on the gonadal stage. This differential action of kisspeptin may be explained by the emerging notion that kisspeptin acts also in fish as a central processor and mediator of several inputs not only by gonadal steroids but also by photoperiod and energy balance status of the animal [21]. Thus, the plastic nature of the kisspeptin function, as demonstrated in the two in vivo trials, meets the fundamental requirement of versatility of such a central integrator/modulator.

It was suggested that the evolutionary transition between multiple forms of kisspeptin, present in evolutionarily older vertebrates such as frogs and some fish, to a single form, as evident in higher vertebrates, is exemplified in the kisspeptin systems of the various fish species studied thus far [3, 33]. The new information provided herein suggests an evolutionary continuum of functional relocation and decreasing levels of Kiss1 from zebrafish [33] through modern teleosts such as the striped bass and the sea bass [34], and further to its complete disappearance in the puffer fish [23]. Based on these data, an evolutionary lineage for the kisspeptins as they evolved, from the gene duplication that formed the two paralogues through their convergence to one form, is suggested as such: 1) zebrafish (Ostariophysi, Cypriniformes), where kiss1 and kiss2 and their cognate receptors are expressed in different regions and probably carry out different functions, with reproductive pertinence to the Kiss2-Gpr54-2 system, 2) medaka (Acanthopterygii, Beloniformes), kiss1 and kiss2 are distinctly expressed in different neuronal subsets and engaged
in reproductive functions, 3) striped bass (Acanthopterygii, Perciformes), kiss1 is no longer expressed in the habenula; kiss1 and kiss2 are co-expressed in the NRL. Both fulfill reproductive functions but kiss1 expression is much lower than kiss2. gpr54-1 and gpr54-2 are expressed in the POA in association with GnRH1. gpr54-1 is expressed in minute levels, 4) fugu (Acanthopterygii, Acanthopterygii), kiss1 and gpr54-1 genes are lost and Kiss2 alone functions to control reproduction, 5) mammals, only the KISS1/ GPR54 system is present and this system regulates both puberty and reproduction. Characterization of the kisspeptin system in additional species, representative of different evolutionary classes, is required to further establish the proposed time line.

In summary, we have cloned and localized the four genes (kiss1, kiss2, gpr54-1 and gpr54-2) comprising the kisspeptin system in the striped and hybrid basses. The major findings were the co-expression of kiss1 and kiss2 in neurons in the NRL, and the evidence that both gpr54-1 and gpr54-2 neurons are associated with the regulation of GnRH1 neurons. The kisspeptin induction studies demonstrate, in addition to the differential potencies of Kiss1-15 and Kiss2-12, that their effect on the HPG axis can be either stimulatory or inhibitory, depending on the gonadal stage. Therefore, the gonadal stage, and particularly the hormonal milieu, should be taken into consideration when the kisspeptin system is studied. This study further establishes the kisspeptin system as a strong potential regulator of the HPG axis in vertebrates.

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References


38. Mechaly AS, Vinas J, Piferrer F. Identification of two isoforms of kisspeptin-1 receptor (Kiss1r) generated by alternative splicing in a modern teleost, the Senegalese sole (Solea senegalensis). Biol Reprod 2009; 80:60-69.


**Figure legends**

**Figure 1:** *kiss1* and *kiss2* mRNAs are co-localized in the NRL neurons. A, Double-labeling *in situ* hybridization, *kiss1* is expressed in the NPT (a), NRLv (b) and NRLd (c). *kiss2*-expressing neurons are found in the NRLv (e) and NRLd (f) but not in the NPT (d). Overlay of a and d, b and e, and c and f shows an exact match of the cells stained for *kiss1* and *kiss2* in the NRLv (h) and NRLd (i), but not in the NPT (g). *kiss1*-green fluorescence, *kiss2* - red fluorescence, Bar = 50 μm. Several chosen areas are magnified in insets to demonstrate neuronal structures or double staining. B, Quantitative RT-PCR of laser-capture microscopy isolated neurons confirmed the co-expression of *kiss1* and *kiss2* in the NRL. Amplification products, resolved by ethidium bromide-stained agarose gel electrophoresis, of RNA extracted from neurons stained positive for either *kiss1* or *kiss2* and isolated using LCM from NRLv (A) and NRLv (B) of two different animals and from neurons of the NRLd. *gpr54-2* is co-expressed with *kiss1* and *kiss2* only in the NRLd neurons. *gnrh1* amplification served as a negative control. K1-*kiss1*, K2- *kiss2*, R2-*gpr54-2*, G-*gnrh1*, 18S-18S RNA.

**Figure 2:** Localization of *gpr54-1* and *gpr54-2*-expressing neurons in the brain of adult male as revealed by *in situ* hybridization. *gpr54-1* is expressed in the preoptic area (NPOav, a), in the habenula (Hab, b), and the caudal hypothalamus (NLTi, c). *gpr54-2*-expressing neurons are particularly abundant in the preoptic area (d-f) and the hypothalamus (g-i), and were found within the NPOav and NPOpc (d,e), PMpc and PMmc (f), NLTv, NLTd and NGT (g), NRLI (h) and NRLv (h,i). Double-labeling *in situ* hybridization for *gpr54-1* or *gpr54-2* (in red) and immunohistochemistry for GnRH1 (in green) revealed that in the NPOav *gpr54-1* cell bodies lie in tandem along GnRH1-stained fibers (a) and *gpr54-2* is co-expressed with GnRH1, where double-stained cell bodies are marked with arrowheads (e). Scale bar = 50 μm. Several chosen areas are magnified in insets to demonstrate neuronal structures or double staining.

**Figure 3:** Localization of *kiss1*, *kiss2*, *gpr54-1* and *gpr54-2* expressing neurons in the brain of a juvenile male and a mature female. (a-c) Juvenile male: (a) Double-label of *kiss1* (green) and *kiss2* (red) reveals co-expression of the two genes in the NRLd (grey inset), NRLI and NRLv, *kiss1* is also expressed in the NPT (yellow inset). (b) *gpr54-2* is expressed NRLd, NRLv and NLTi (c), and in the NLTv. (d-h) Vitellogenic mature female: (d) *kiss2* expressing neurons (red) are located in the NRLd and NRLv (e) and are co-expressed with *kiss1* (green) as presented in the NRLd and magnified in the inset. (f) *gpr54-2* is expressed in the preoptic regions NPOpc, NPOav and PMpc (g) as well as in the NRLv and NLTi. (h), *gpr54-1* is expressed in the NLTv.
Figure 4: Temporal expression patterns of kiss1 and kiss2 systems of male and female striped bass. (A) kiss1, (B) gpr54-1, (C) kiss2, (D) gpr54-2. Absolute mRNA levels, presented as mean±SEM, were measured in brains of male (closed black bars) and female (grey bars) striped bass sampled at juvenile, prepuberty and mid-gonadal stages using Real Time Quantitative PCR. GSI values were statistically analyzed using one-way ANOVA followed by a multiple pairs comparison. Statistical difference was considered significant when p≤0.05. * p≤0.05, ** p≤0.01, *** p≤0.005

Figure 5: Kiss1 and Kiss2 induce plasma Lh in a stage-dependent manner. Hybrid bass at puberty (A, B) or gonadal recrudescence (D), received a single injection of Kiss1-15 (A, C), or Kiss2-12 (B, D) peptide at different doses in nm/Kg BW, as specified in the legend of each graph. Blood was sampled immediately before and at different times up to 24 hr post-injection. Lh levels are presented as mean±SEM of the percentage of basal 0 time sampling point, N = 6. Statistical difference was accepted only when it was significant (p≤0.05) compared to both the 0 point and the saline control levels at the same timepoint. * p≤0.05, ** p≤0.01, *** p≤0.005.

Figure 6: Kiss1-15 and Kiss2-12 effect on gpr54-2 and gnrh1 transcript levels is gonadal stage-dependent. Brain mRNA levels of gpr54-2 (A, B) and gnrh1 (C, D) in hybrid bass at prepuberty (A, B) and recrudescence (C, D). Fish were sampled 24 hr post-injection and mRNA levels were determined using Real-Time PCR. Transcript levels are presented as mean±SEM of % of saline-injected control, N = 6. * p≤0.05, ** p≤0.01, *** p≤0.005.
**TABLE 1.** Degenerate and gene-specific primers used for RT-PCR and real-time quantitative PCR.

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Zmora et al. Figure 1.

A

B

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Zmora et al. Figure 2:
Zmora et al. Figure 3
Figure 5

A and B: LH in plasma (% of 0 point) over time after kiss1 injection (hr) for different concentrations (con, 5, 25, 100).

C and D: LH in plasma (% of 0 point) over time after kiss2 injection (hr) for different concentrations (con, 50, 100, 150).
Zmora et al. Figure 6

A B C D

Kisspeptin (nmole/Kg BW)

Kiss1  Kiss2

Kisspeptin (nmole/Kg BW)