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Kisspeptin Antagonists Reveal Kisspeptin 1 and Kisspeptin 2 Differential Regulation of Reproduction in the Teleost, *Morone saxatilis*[1]

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ABSTRACT

The importance of kisspeptin in regulating vertebrate reproduction has been well established, but the exact mechanism continues to unfold. Unlike mammals, many lower vertebrates possess a dual kisspeptin system, Kiss1 and Kiss2. To decipher the roles of the kisspeptins in fish, we identified two potential kisspeptin antagonists, pep 234 and pep 359, by screening analogs for their ability to inactivate striped bass Kiss1 and Kiss2 receptors expressed in COS7 cells. Pep 234 (a mammalian KISS1 antagonist) antagonizes Kiss1r signaling activated by Kiss1 and Kiss2, and pep 359 (a novel analog) antagonizes Kiss2 activation of both receptors. In vitro studies using brain slices demonstrated that only Kiss2 can upregulate the expression of the hypophysiotropic *gnrh1*, which was subsequently diminished by pep 234 and pep 359. In primary pituitary cell cultures, the two antagonists revealed a complex network of putative endogenous and exogenous regulation by kisspeptin. While both kisspeptins stimulate Fsh expression and secretion, Kiss2 predominately induces Lh secretion. Pep 234 and 359 treatment of spawning males hindered sperm production. This effect was accompanied with decreased brain *gnrh1* and *gnrh2* mRNA levels and peptide content in the pituitary, and increased levels of pituitary Lh, probably due to attenuation of Lh release. Strikingly, the mRNA levels of *arginine-vasotocin*, the neurons of which in the preoptic area coexpress *kiss2r*, were dramatically reduced by the antagonists. Our results demonstrate differential actions of Kiss1 and Kiss2 systems along the hypothalamic-pituitary axis and interactions with other neuropeptides, and further reinforce the importance of kisspeptin in the execution of spawning.

gonadotropes, neuroanatomy, neuropeptides, reproductive axis, spawning

INTRODUCTION

The involvement of kisspeptin in the control of the reproductive hypothalamic-pituitary-gonadal (HPG) axis has been demonstrated in most vertebrates, including numerous fish species [1–7]. Kisspeptin mainly acts as an afferent controller of GnRH neurons, inducing both GnRH secretion and expression in a dose-dependent manner [8]. Recently, it has been shown that kisspeptin can also act at the pituitary level at critical time points [9, 10].

Unlike most mammals that possess only one kisspeptin system (KISS1) [11], the majority of teleosts have two kisspeptin systems comprised of Kiss1 and Kiss2 and two cognate receptors, Kiss1r and Kiss2r [12]. The kisspeptin system has a similar role to that found in mammals, but the precise function of each kisspeptin form is only beginning to emerge.

The neuroanatomical distribution of kisspeptin neurons and their two cognate receptors in the brain and pituitary of the modern teleost striped bass, *Morone saxatilis* (stb) is similar to that of mammals, in that kisspeptin neurons are found in the preoptic area (equivalent to the AVPV in mammals) and the hypothalamus/mediobasal hypothalamus (equivalent to the mammalian arcuate nucleus). In general, kisspeptin receptors are widely distributed in the brain, pituitary, and other peripheral tissues [13–15], consistent with the diverse roles of kisspeptin. Nevertheless, because teleosts exhibit varied reproductive strategies, there are differences in the reported reproductive roles and distributions of the two kisspeptin forms and their receptors. For example, in the medaka and the European sea bass, kiss1 neurons express estrogen receptor [14] and are sexually dimorphic [16, 17], whereas, in the zebrafish, kiss2 neurons respond to estrogen [18]. Likewise, kiss1r is expressed in the pituitary of the European sea bass [19], while kiss2r is reported in the pituitary of the chub mackerel [20]. It is now clear that, in *M. saxatilis*, both Kiss1 and Kiss2 neurons are involved in the control of reproduction, and, as in the rat [21], they show marked plasticity in the mediobasal hypothalamus (in the nucleus lateralis tuberis [NLT]) at critical reproductive stages, such as spawning [10]. These kisspeptin neurons exert dose-, sex-, and reproductive stage-dependent inhibitory or stimulatory effects on Gnrh and gonadotropins [10, 13]. However, it is not clear yet how these functions are split/shared between the two kisspeptin systems.

We have therefore taken the route of identifying antagonists for each kisspeptin receptor. The development of kisspeptin antagonists for mammalian KISS1 is initiated to elucidate the role of kisspeptin in the control of different reproductive

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TABLE 1. Gene-specific primers used for real-time quantitative PCR.

Primer	Sequence 5′ to 3′	Direction	GenBank accession no.
TAQgnrh1F	GGAACGGACGGCCTCTCA	Forward	AF056314
TAQgnrh1R	GTGGGAAGCCCCCGACTA	Reverse	
TAQgnrh2f	AAGCAGCCCAAACAGTAAAACC	Forward	AF056313
TAQgnrh2R	CTGACCAACTGAAGGACAAGCA	Reverse	
TAQgnrh3F	TGGAGTCATCATTAATTACATTGTATGG	Forward	AF224280
TAQgnrh3R	GAAGAGAAGTGTGGGAGAGCTAGAG	Reverse	
TAQFSHbF	TGGCCTCACCGAGGTCAT	Forward	L35070
TAQFSHbR	CAGTCTCCGTTACAGATTCTCTGTTC	Reverse	
TAQLHbF	CTTGGGACAGCCCTCCTTCT	Forward	L35096
TAQLHbR	CTGGGAGCCACATCTGACAT	Reverse	
TAQAVTF	GAAAACTACCTGCTCACCCC	Forward	KP294907
TAQAVTR	GCTGGATCTGAAACCTCGG	Reverse	
TAQITF	GCTGGTCACACTTCTCATCG	Forward	KP294906
TAQITR	ACAGCTCAAGACTTTCCCAC	Reverse	
TAQEF1αF	GGAGTGAAGCAGCTCATCGT	Forward	AJ866727
TAQEF1αR	GCGGGCCTGGCTGTAAG	Reverse	

processes, including reproductive development, puberty, positive/negative gonadal feedback, as well as related metabolic and stress effects on the reproductive system, all with a potential therapeutic application [22, 23]. By systematic substitution of amino acids along the core kisspeptin peptide, KISS1-10, an array of peptides have been designed and tested [24–26]. The most potent antagonists have already shed light on specific reproductive processes and are increasingly integrated in basic and applied studies [22, 26–28].

Through methodical screening of numerous potential antagonistic peptides, we identified two antagonists: one that inhibits Kiss1r signaling activated by the two kisspeptin forms and another that specifically antagonizes Kiss2 activation of both receptors. Using the selected antagonists in vitro and in vivo, we established the importance of the kisspeptin system in striped bass reproduction, the differential roles of the two kisspeptins, its critical role in inducing Gnrh and intricate regulation of pituitary gonadotropins, and, finally, the involvement of a kisspeptin:arginine-vasotocin pathway in spawning.

MATERIALS AND METHODS

Animals

Striped bass were obtained as juveniles from the Maryland Department of Natural Resources and maintained at ambient conditions in a 20-m^3 tank, supplied with constant exchange of artificial 8–10 ppt sea water. Animal maintenance and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University Of Maryland School of Medicine. Treatments were carried out in water containing 200 mg/L tricaine methanesulfonate (MS-222). Post-treatment fish were then either killed or transferred to 90-gallon tanks maintained under the same ambient conditions for experimental purposes.

Peptides

Striped bass pyro-gluKiss1-15 (Kiss1) or Kiss2-12 (Kiss2) peptides were synthesized at >95% purity by Genscript. Peptide analogs 186–191, 200–203, 206–213, and 228–248 were custom synthesized by EZBiolabs. Peptide analogs for preliminary studies were synthesized at a purity of 80% (range, 83–99%, measured by HPLC), and peptides 234 and 359, which were selected for detailed studies, were synthesized at a purity of >95%. The authenticity of peptides was confirmed by mass spectrometry.

Antagonist Selection Using In Vitro Luciferase Reporter Gene Assays

In order to generate activation profiles of the two kisspeptin receptors by Kiss1 and Kiss2, we employed a sensitive luciferase (LUC) reporter gene assay using LUC transcriptionally regulated by either a serum response element (SRE; Invitrogen) or a cAMP response element (CRE; Invitrogen). Striped bass Kiss1r and Kiss 2r (GenBank accession nos. KJ725183 and GU351869, respectively) were cloned in pcDNA3.1 expression vector (Zeo; Invitrogen) driven by CMV promoter. Transient transfection, cell procedures, and stimulation protocols followed the procedures previously developed by the coauthors [1, 29–32]. COS7 cells were grown in 96-well plates in DMEM supplemented with 10% FBS, 1% glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco BRL) under 5% CO_2 until 90% confluent. Cotransfection of either kiss receptor (30 ng/well), a reporter plasmid (30 ng/well), and pCMV-Renilla LUC as transfection control (0.03 ng/well; Promega) was carried out with FuGENE 6.0 reagent (Promega) for 24–48 h. The cells were serum deprived for 18 h, stimulated with different concentrations of either Kiss1or Kiss2 (with or without the tested potential antagonists) for 6 h in a Hepes-modified DMEM supplemented with 0.5% BSA, and then harvested and analyzed. Lysates prepared from the harvested cells were assayed for both firefly and renilla LUC activity using the dual LUC reporter assay kit following the manufacturer's protocol (Promega). Transfection experiments were performed in triplicate in up to 10 independent experiments. Kiss peptides were used to generate an initial dose response and to determine the signal transduction pathway utilized in 10× serial dilutions from 1 femto-M to 10 μM. EC_{50} (half maximal effective concentration) values were calculated via analysis by a three-parameter response curve formula using GraphPad Prism software.

A total of 23 potential antagonists that were previously designed to target mammalian KISS1R [24] were selected based on their chemical characteristics as candidates to bind striped bass Kiss receptors: 234, 273, 276, 311, 318, 343–346, 348, 349, and 351–362.

Antagonist testing was performed on either Kiss1r or Kiss2r stimulated with a fixed dose of Kiss1 or Kiss2 combined with different doses of the analogs. In all cases, the analogs were added ~30 min prior to the addition of Kiss peptide. IC_{50} (half maximal inhibitory concentration) values were extrapolated by Sigmoidal 4PL analysis using GraphPad Prism software.

Brain and Pituitary Incubation Assays In Vitro

The two selected peptides, 234 and 359, were further examined for their Kiss antagonistic abilities in striped bass brains and pituitaries using brain slices and primary pituitary culture assays, respectively [33]. Brains and pituitaries of precociously spermiating mature male striped bass were collected during the reproductive season (March and April) and placed into the appropriate medium on ice.

Brain slices. Brains were divided into identical hemispheres. Each hemisphere was then immediately sliced to 300-μm slices using a McIlwain Tissue Slicer. Slices were placed in 74-μm mesh inserts in a six-well plate, containing 4 ml high glucose DMEM at pH 7.44 supplemented with 25 mM Hepes, 0.1% BSA, 50 μM ascorbic acid, and 50 μM bacitracin (Sigma). The brain slices were washed for 3 h, with hourly medium exchanges, and then incubated with medium containing the tested peptides for 6 h at 20°C with gentle agitation. Tissues and medium were then frozen immediately and stored until further analysis. RNA was extracted from the brains, and its high quality and integrity was verified in about 25% of the samples on a denaturing paraformaldehyde agarose gel electrophoresis. Complimentary DNAs were prepared and followed by quantitative RT-PCR as described below.

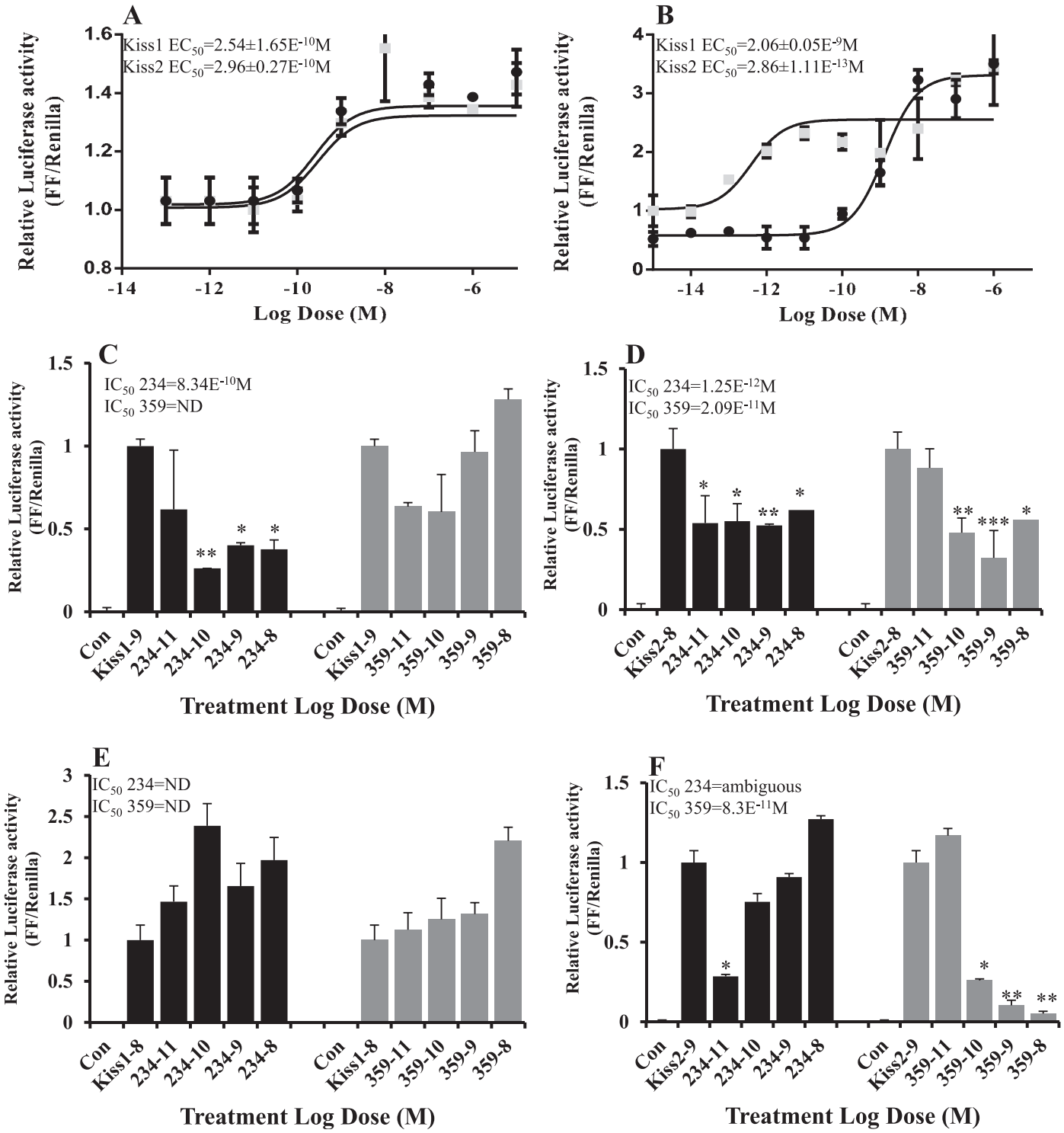

FIG. 1. Activation of Kiss1r and Kiss2r by Kiss1 or Kiss2 is selectively inhibited by pep 234 and pep 359. **A** and **B**) Activation of striped bass Kiss1r or Kiss2r expressed in COS7 cells via the PKC/SRE transduction pathway. **A**) Activation of Kiss1r by serial concentrations of Kiss1 (black circles) and Kiss2 (grey squares). **B**) Activation of Kiss2r by serial concentrations of Kiss1 (black circles) and Kiss2 (grey squares). **C**–**F**) The effect of pep 234 (black bars) and pep 359 (grey bars) at concentrations from 0.1 pM to 10 nM on the activation of Kiss1r by 1 nM Kiss1 (**C**), Kiss1r by 1 nM Kiss2 (**D**), Kiss2r by 10 nM Kiss1 (**E**), and Kiss2r by 1 nM Kiss2 (**F**) ($n \geq 5$). All treatments contained the specified concentration of either Kiss1 or Kiss2, as noted in the second bar of each graph (controls had no added peptides). The antagonist and its concentration in M appear in the next columns. Results are presented as mean ± SEM of the relative activity of firefly LUC (FF) vs. Renilla LUC (renilla). Calculated EC_{50} values for each peptide are specified in **A** and **B**, and IC_{50} values are specified for each antagonist in **C**–**F**. Statistical difference was accepted at $P \leq 0.05$, compared to a kisspeptin-only treatment. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.005$.

Primary pituitary culture. The culture procedure generally followed Levavi-Sivan et al. [34] and was carried out under aseptic conditions. Pituitaries were grossly chopped using a scalpel and cells were dispersed with 1 ml 0.25% trypsin-EDTA in DMEM (Gibco) for 5 min at room temperature, during which the medium was run through a 1-ml syringe equipped with an 18-gauge needle followed by a 21-gauge needle ~10 times each. The reaction was stopped by

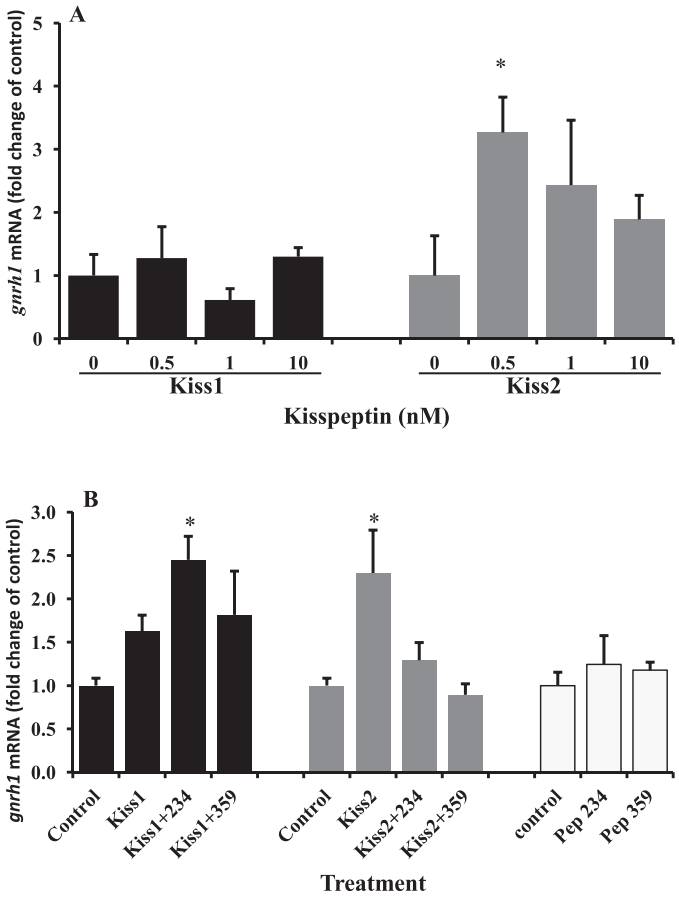

FIG. 2. Peptides 234 and 359 diminish the stimulation of *gnrh1* by Kiss2 in brain slices, in vitro. **A**) The effect of different concentrations of Kiss1 (black bars) and Kiss2 (grey bars) on the expression of *gnrh1*. Doses of kisspeptin in nanomoles appear next to the kisspeptin form in the x axis. **B**) The effect of pep 234 and pep 359 (20 nM) on *gnrh1* expression in the presence of 0.5 nM Kiss1 (black bars) or Kiss2 (dark gray bars). Light gray bars represent antagonist treatments without Kiss peptides. Results are presented as mean ± SEM of fold change of control levels obtained from the absolute copy number of mRNA. Statistical difference was accepted at $P \leq 0.05$ compared to control levels. *$P \leq 0.05$.

the addition of 1 ml fetal calf serum, and the cells were counted in the presence of methylene blue using a hemocytometer and populated at a density of 50 000 or 25 000 per well in 24- or 48-well plates, respectively. The cells were incubated for 3 days at 20°C in DMEM (pH 7.44) supplemented with 20 mM Hepes, 9 mM sodium bicarbonate, 0.15% BSA, penicillin/streptomycin, and 10% FCS for recovery and attachment. The wells were then washed and medium was replaced with serum-free medium (with 0.5% BSA) and the tested peptides for 18 h. The cells and medium from each well was collected separately and kept frozen until analysis.

Hormones and Gene Transcript Measurements

Lh levels in the plasma were measured using ELISA as previously described [35]. Levels of Fsh were measured using a specific ELISA developed for tilapia, *Oreochromis niloticus*, Fsh [36] and validated for use in *M. saxatilis* [10].

For the measurement of *gnrh 1*, *2*, *3*, *arginine-vasotocin* (*avt*; Genbank accession no. KP294907), *isotocin* (*it*; Genbank accession no. KP294906), *lhβ*, and *fshβ* transcript levels, brains and pituitary total RNA (1 μg) was extracted using Trizol reagent (Invitrogen), and then reverse transcribed by Quantitect RT kit (Qiagen). Real-time PCR was performed on cDNA (50 ng for *gnrh* and *kiss1* and *kiss2*, 10 ng for *fshβ*, and 5 ng for *lhβ, avt* and *it*) using SYBR Green PCR mix (Applied Biosystems) in duplicate, with 0.1 μM gene-specific primers (primers appear with the preface TAQ in Table 1), as previously described [13, 37], in an ABI-Prism 7500 Detection System (Applied Biosystems). Ct values of each sample were normalized against the levels of *ef1α* RNA amplified from 5 ng cDNA [38] and then converted to the fold change of the mean Ct value. Amplification reactions were carried out at 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 55°C–60°C for 1 min. Proper and specific amplification 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. The duplicate reactions were repeated when the standard deviation between them exceeded 7% to ensure accurate reading.

Treatment of Male Striped Bass with Peptides 234 and 359 In Vivo

Spermiating males at the time of spawning (mid-April), five per group, were injected intramuscularly with poly (D,L-lactide-co-glycolide) 50:50 (PLGA; Sigma) [39] slow-release microspheres containing pep 234 or pep 359 at doses of 5 or 30 μg/kg body weight (BW). The control group received microspheres containing no peptide. The experiment was concluded 9 days after implantation. Blood was sampled immediately prior to and at 9 days postimplantation for Lh and Fsh plasma level measurements. Sperm from each fish was collected at the end of the experiment, immediately before decapitation, and placed into preweighed tubes to determine milt volume and weight [40]. Brains and pituitaries were collected and stored frozen for measurement of g*nrh*, *avt*, *it* and *gonadotropin* (g*th*) transcript and hormone levels, as described above.

In Situ Hybridization to Localize Kisspeptin Receptors, avt *and* it

Brains were removed immediately after decapitation, fixed in buffered 4% paraformaldehyde overnight 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. In situ hybridization was applied using Tyramide Signal Amplification kit (Perkin Elmer), according to the manufacturer's protocol. Anti-DIG HRP and anti-FITC HRP (Roche) were used to detect antisense DIG-labeled probes (kisspeptin receptors) and fluorescein-labeled probes (*avt* and *it*). Fluorescence was obtained via Cy3 (red) or fluorescein (green) from the kit.

Statistical Analysis

Statistical analyses were performed by one-way ANOVA and Tukey post hoc test for multiple comparisons, using Instat3 (GraphPad). Statistical difference was accepted at $P \leq 0.05$.

RESULTS

Kisspeptin Receptor Activation Assays and Screening for Antagonists

Kiss1 and Kiss2 binding assays. As a first step, dose-response activation curves were generated for Kiss1 and Kiss2 with the two receptors via SRE, a responsive element activated by PKC, or CRE activated by PKA. Both response elements drive the translation of the firefly LUC gene. No obvious dose response was obtained with the PKA/CRE reporter system (Supplemental Fig. S1; Supplemental Data are available online at www.biolreprod.org), whereas the SRE-reporter system generated dose-response curves, albeit with different activation capacities for each receptor and ligand. Kiss1r was activated almost equally by both Kiss1 and Kiss2, having an average EC_{50} of 2.54 ± 1.65 E^{-10} M for Kiss1, and an EC_{50} value of 2.96 ± 0.27 E^{-10} M for Kiss2 (obtained from three independent experiments, n = 3) (Fig. 1A). Kiss2r, however, was activated approximately four orders more efficiently by Kiss2 (EC_{50} = 2.86 ± 1.11 E^{-13} M) than by Kiss1 (EC_{50} = 2.06 ± 0.05 E^{-9} M), and displayed a clear preference for Kiss2 (n = 5; Fig. 1B). The core peptides Kiss2-10 and Kiss1-10 were significantly less efficient in activating their cognate receptors: Kiss2-10 had an EC_{50} of 5.09E^{-8} M and Kiss1-10 had an EC_{50} of 1.922E^{-9} M (Supplemental Fig. S2).

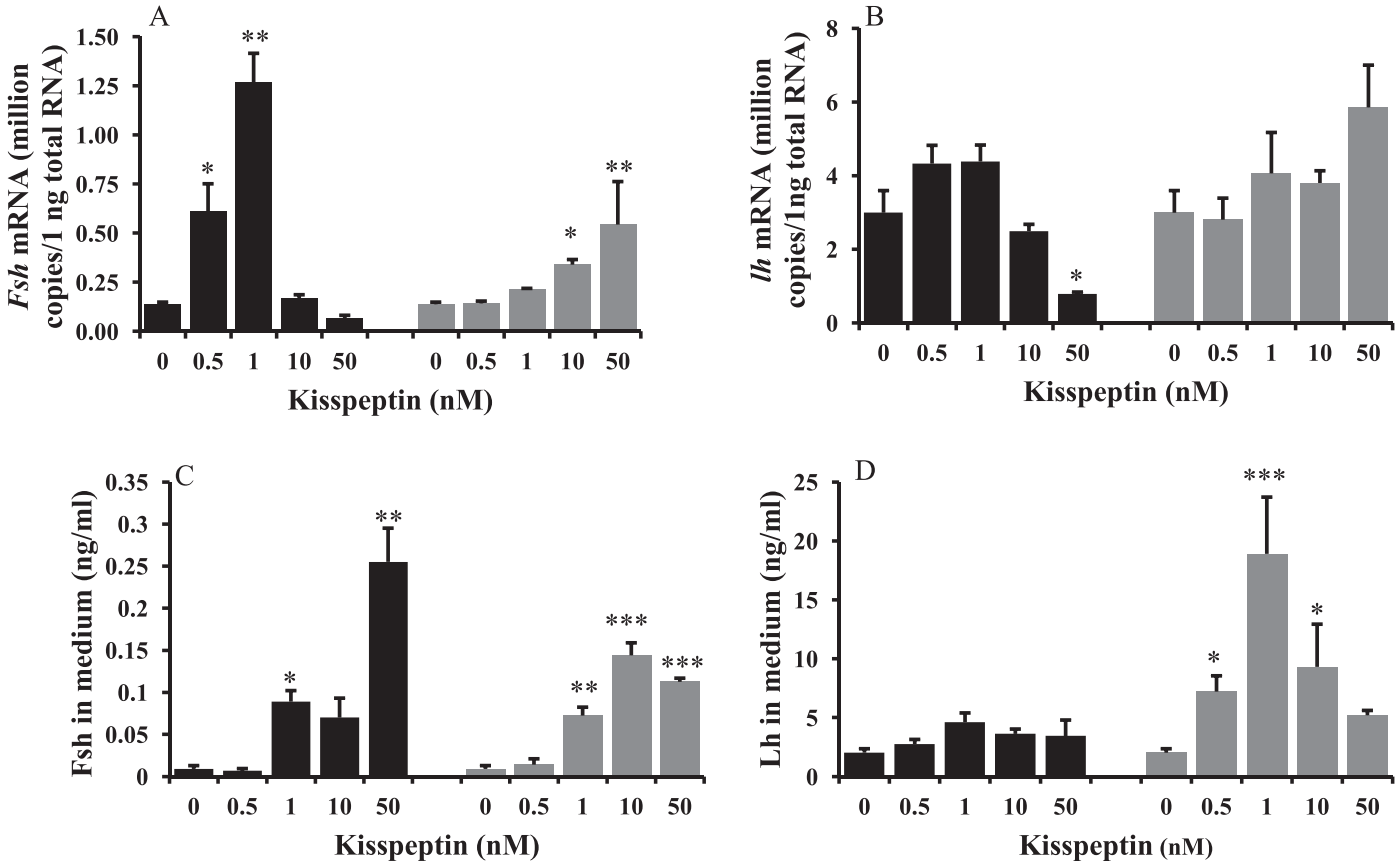

FIG. 3. The effects of Kiss1 and Kiss2 on the expression and secretion of Lh and Fsh in pituitary cells in vitro. Primary pituitary cells of recrudescent males were treated with 0.5, 1, 10, or 50 nM Kiss1 (black bars) or Kiss2 (grey bars) for 18 h. **A**) *fshβ* mRNA levels. **B**) *lhβ* mRNA levels. **C**) Fsh levels in medium. **D**) Lh levels in medium. Results are presented as mean ± SEM of absolute copy number of mRNA/1 ng total RNA for gene expression and ng/ml for protein levels in the medium. Statistical difference was accepted at $P \leq 0.05$ compared to control levels. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$.

Antagonist screening by binding competition with Kiss1 and Kiss2. The antagonistic ability of all 23 peptides was tested by combining serial doses of each antagonist with Kiss1 or Kiss2 at a fixed dose, selected based on the EC_{50} values obtained in Figure 1 as follows: Kiss1r was induced with 10 nM Kiss1 or Kiss2, and Kiss2r was induced with 10 nM Kiss1 or 1 nM/1 pM Kiss2; Kiss1r/Kiss1—pep 359, added at doses of 10^{-11} to 10^{-8} M, had no significant effect on the activation by Kiss1 having nondetected IC_{50}, while Pep 234 reduced the activation level by 70% having an IC_{50} of $8.34*E^{-10}$ M (Fig. 1C); Kiss1r/Kiss2—pep 359 significantly inhibited the activation of Kiss1r by Kiss2 peptide with an IC_{50} of $2.09*E^{-11}$ M (Fig. 1D) and pep 234 reduced the activation by Kiss2 down to ~30% with an IC_{50} of $1.25*E^{-12}$ M (Fig. 1D); Kiss2r/Kiss1—neither pep 234 nor pep 359 had an effect on the activation of Kiss2r by Kiss1 (Fig. 1E); Kiss2r/Kiss2—unlike the Kiss2r/Kiss1 results, pep 359 at doses of 10^{-10} and 10^{-8} M reduced the activation (up to ~90%) by Kiss2 ($IC_{50} = 8.3*E^{-11}$ M), and pep 234 significantly inhibited Kiss2 activation only at a concentration of 10^{-11} M, yielding an ambiguous IC_{50} value (Fig. 1F; n = 5). Similar results were obtained when 1 pM Kiss2 was used with Kiss2r. These results suggest that pep 359 specifically and potently antagonizes the activation of Kiss1r and Kiss2r by Kiss2 (with similar IC_{50} values), while pep 234 antagonizes both Kiss1 and Kiss2 activation of Kiss1r. The IC_{50} value of kiss1r activation by Kiss2 is 15 times lower than kiss1r activation by Kiss1, indicating a higher potency in inhibiting Kiss2 activation.

The Effect of the Selected Antagonists on the Hypothalamic-Pituitary Axis: In Vitro Studies

Kisspeptin and antagonists effects on the expression of Gnrhs in the brain. An in vitro brain slice assay was established to test the effects of Kiss1 and Kiss2 on the expression levels of all three *gnrhs* and to test the antagonistic effect of pep 234 and 359.

Kiss1 and Kiss2 were tested at doses of 0.5, 1, and 10 nM. While *gnrh1* mRNA levels were not changed significantly in response to Kiss1, Kiss2 showed a dose-dependent stimulatory effect, being highest at the lower dose (Fig. 2A). When pep 234 was added at a dose of 20 nM to Kiss1, *gnrh1* transcript levels were significantly increased, while a 20-nM pep 359 dose had no effect. When pep 234 or pep 359 was added to 0.5 nM Kiss2, the stimulatory effect of Kiss2 was abolished by ~50% (Fig. 2B); pep 234 or pep 359 alone had no effect on *gnrh1* mRNA levels. Kiss1 and Kiss2 had no significant effect on the expression levels of *gnrh2* and *gnrh3* (data not shown).

The effect of kisspeptin and its antagonists on the expression and secretion of Fsh and Lh. Primary pituitary cell cultures of recrudescent males were used to test the effect of Kiss1 and Kiss2 on Fsh and Lh and to determine the effect of the selected antagonists (pep 234 and 359). In terms of expression, *fshβ* expression was upregulated ~5 and 10 times by 0.5 and 1 nM Kiss1, respectively, compared to control levels (but unchanged with the higher doses), and was less efficiently upregulated by 10 and 50 nM Kiss2 (~2.5 and ~5 times, respectively; Fig. 3A). A high dose of 50 nM Kiss1

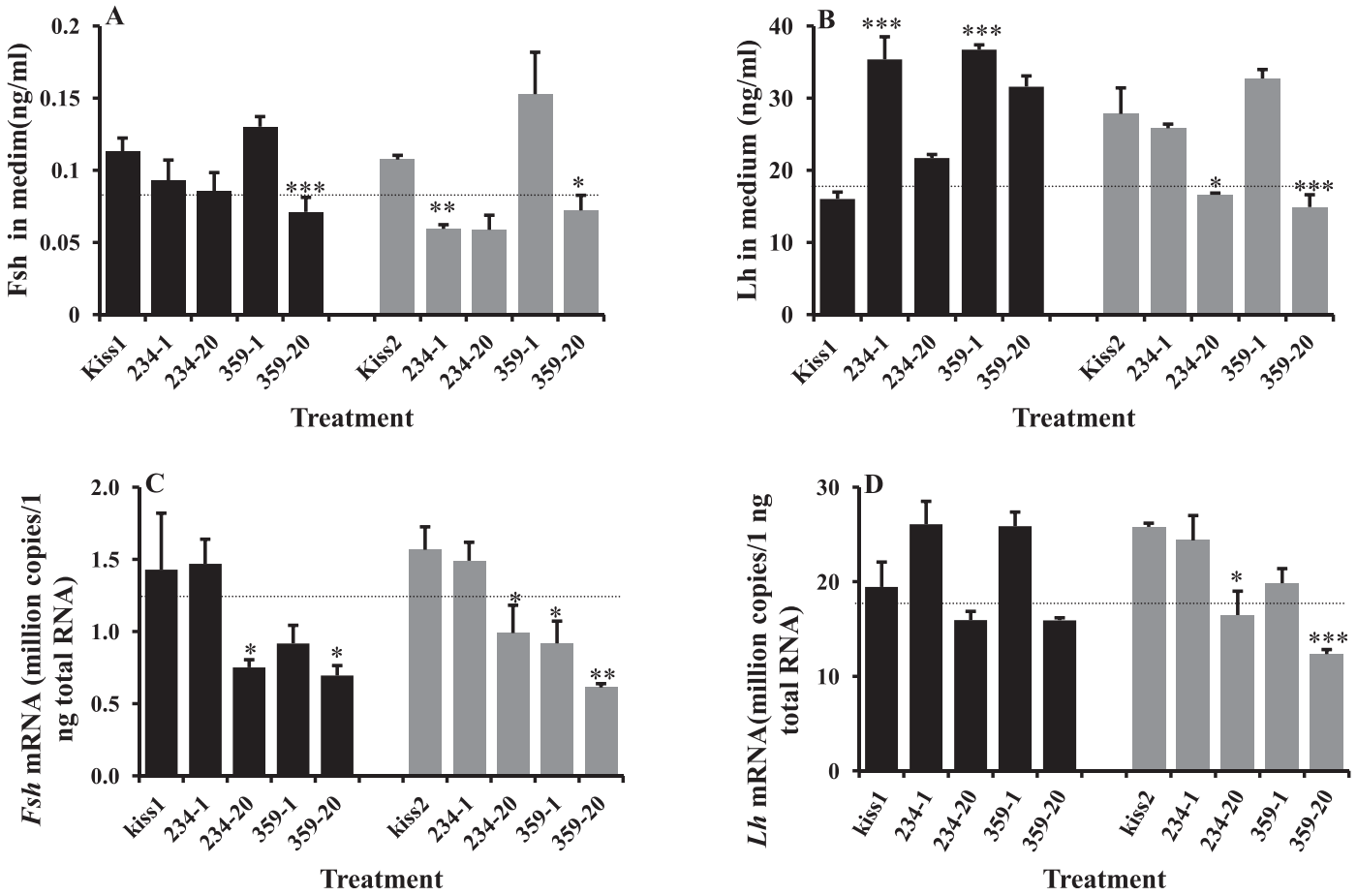

FIG. 4. The effect of pep 234 and pep 359 on the expression and secretion of Lh and Fsh from pituitary cells in vitro. Primary cultures of pituitary cells were treated for 24 h with 1 nM Kiss1 (black bars) or Kiss2 (grey bars), with the addition of 0–1 or 20 nM pep 234 and pep 359 (denoted by the peptide name and the concentration in the x axis). **A**) FSH levels in medium. **B**) Lh levels in medium. **C**) *fshβ* mRNA levels. **D**) *lhβ* mRNA levels. Basal levels of the negative control are presented with a horizontal dashed line in each graph. Results are presented as mean ± SEM of absolute copy number of mRNA/1 ng total RNA for gene expression and ng/ml for protein levels in the medium. Statistical difference was accepted at $P \leq 0.05$ compared to Kiss peptide alone treatment levels. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.005$.

downregulated *lhβ* transcript levels, but levels were unaffected by a similar dose of Kiss2 (Fig. 3B).

Both kiss1 and Kiss2 significantly increased the levels of Fsh in the medium in a dose range of 1–50 nM (Fig. 3C). Lh secretion levels were significantly induced by 0.5–10 nM Kiss2, but were unaffected by Kiss1 (Fig. 3D). Therefore, it appears that: 1) only Kiss2 stimulates Lh, and this effect is restricted to its secretion, 2) Kiss1 is generally ineffective, but a high dose can inhibit Lh expression, and 3) both Kiss1 and Kiss2 induce Fsh at both the gene transcription and secretion levels.

When Kiss1 or Kiss2 (1 nM) was coincubated with pep 234 or pep 359 at doses of 1 or 20 nM, several trends were apparent. First, a 1-nM level of antagonist together with Kiss1, but not with Kiss2, stimulated Lh secretion by ~2-fold (Fig. 4B). Second, at a level of 20 nM, both antagonists abolished the stimulatory effect of Kiss2 on Lh (Fig. 4B) and Fsh secretion (Fig. 4A).

Although, in this experiment, the stimulating effects of Kiss1 and/or Kiss2 on *fshβ* and *lhβ* mRNA were apparent, but not statistically significant, the addition of pep 234 or pep 359 decreased *fshβ* (by 50%) and *lhβ* below basal levels (Fig. 4, C and D). This phenomenon was observed also when the antagonists were added individually (data not shown; n = 2).

The Effect of the Selected Antagonists on the Hypothalamic-Pituitary Axis: In Vivo Studies

Spermiating males were used to test the antagonistic effect of pep 234 and pep 359, based upon previous indications that Kiss1 and Kiss2 play a role in the spawning process [10]. The peptides were administered via microspheric slow-release polymeric preparations that release the peptides for 3 wk postimplantation, with well-documented release kinetics [39, 41]. Fully spermiating males were injected with microspheres containing pep 234 or pep 359 at doses of 0, 5, and 30 μg/kg BW (referred to as control, pep 234/pep 359-5, or pep 234/pep 359-30, respectively). The effect of the treatment on sperm volume and other factors along the HPG axis was determined 9 days after implantation based on previous observations that maximal induction of sperm density and relevant hormones occur 7–14 days after implantation with GnRHa [40].

The effect of pep 234 and pep 359 on sperm production. Because of the high viscosity of striped bass milt, we measured total weight of the milt in addition to spermatocrit. The differences in milt volumes between the control and treated groups were visually apparent. Four of the five males in the group treated with pep 234-5 had lower milt volumes compared to controls, and all fish in the pep 359-30 group had lower milt volumes than the controls (Fig. 5A). The milt weight of these two groups was significantly lower (~50%

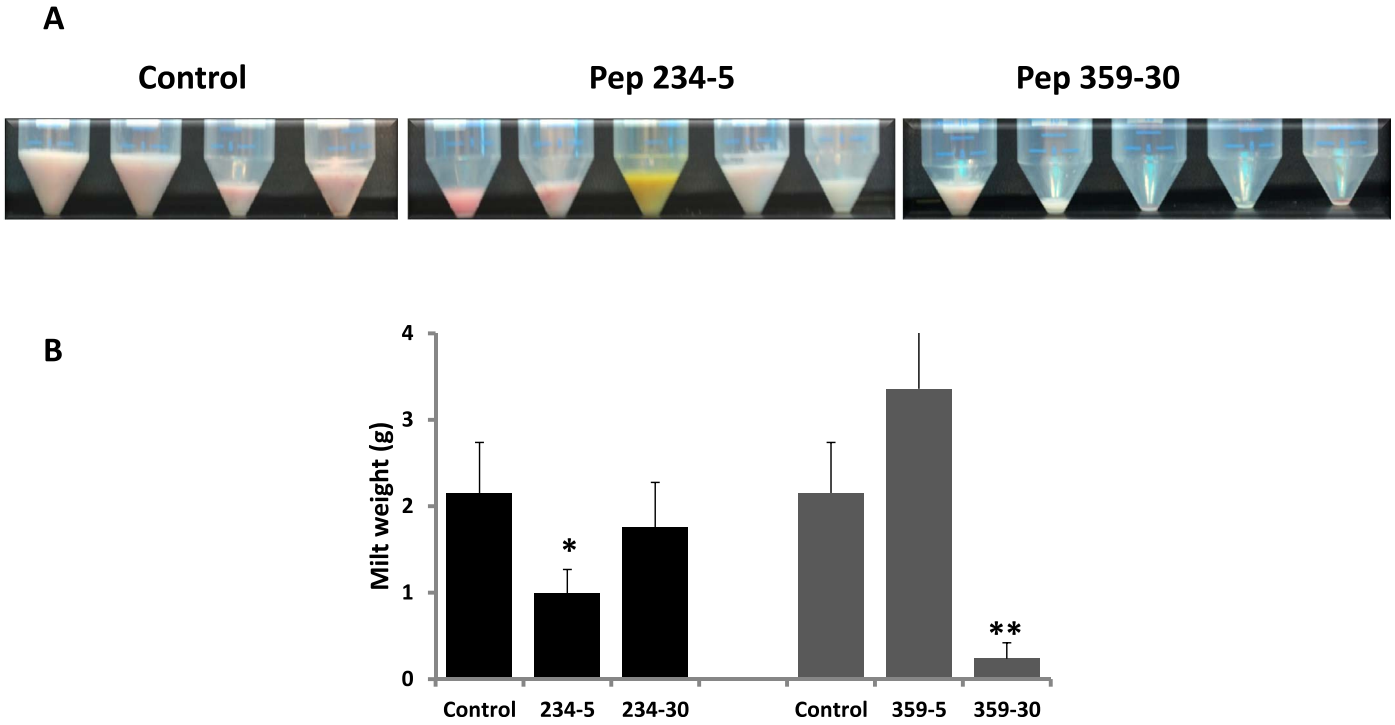

FIG. 5. Peptides 234 and 359 hindered sperm production in spermiating males. Spermiating male striped bass in the spawning season were treated with implants releasing either pep 234 or pep 359 at sustained doses of 5 or 30 μg/kg BW (n = 5 per group). Milt was stripped from each fish and milt volume (**A**) and net weight (**B**) were recorded after 9 days and compared to the control fish (n = 4) treated with implants containing no peptides. **A**) Photographic images demonstrating the differences in sperm volumes obtained from fish treated with control implants (left panel, n = 4), 5 μg/kg BW pep 234 (middle panel, n = 5), and 30 μg/kg BW pep 359 (right panel, n = 5). **B**) Net weights of the sperm of all five treatment groups presented as mean ± SD. Numbers in the x axis correspond to the antagonist peptide and the dose in the implants. Statistical difference was accepted at $P \leq 0.05$ compared to control treatment levels. *$P \leq 0.05$, **$P \leq 0.01$.

and ~90% of control, respectively), although pep 234-30 and pep 359-5 had no effect on sperm volume (Fig. 5B).

The effect of pep 234 and pep 359 on brain gnrh*,* arginine-vasotocin*, and* isotocin *expression*. Peptides 234 and 359 downregulated the expression of *gnrh1* and *gnrh2* in the pepe 234-5 and pep 359-30 groups (Fig. 6A, a and b), in correlation to milt weight. The transcript levels of *gnrh3* were not affected by any of the treatments (data not shown). While *isotocin* was not affected (Fig. 6A, c), transcript levels of *avt* were downregulated by both pep 234 and pep 359 at the two doses (Fig. 5A, d). Double-labeled *ish* on brains of pubertal male striped bass colocalized *kiss2r* and *avt* in magnocellular neurons in the preoptic area, PMmc (Fig. 6B, a–c), while neurons in the anterior periventricular nucleus, NAPv, coexpress *kiss1r* and *it* (Fig. 6B, d–f). Negative control *ish* on adjacent sections using sense *avt* and *it* combined with antisense *kissr* resulted in only *kissr* signal (Supplemental Fig. S3, A and B).

The effect of pep 234 and pep 359 on Gnrhs and gonadotropin content in the pituitary. Both pep 234 and pep 359 at the two doses considerably reduced Gnrh1 peptide content (~50% of control; Fig. 7A). Gnrh2 levels were only slightly decreased and only by the higher dose of pep 234-30 and pep 359-30 (Fig. 7B). With regard to Lh and Fsh content in the pituitary, only the treatment with pep 359-30 resulted in a significantly higher Lh level than controls (Fig. 7C). Fsh levels were also higher in this treatment, but were not statistically significant (Fig. 7D).

No significant difference was detected in Lh and Fsh plasma levels between the treatments or between the two sampling points of the same treatment (Fig. 8).

DISCUSSION

We have identified two antagonists that efficiently and differentially blocked the activation of stb Kiss1r and Kiss2r. One is the known mammalian KISS1 antagonist, pep 234 [24], and the second, pep 359, is novel. Kisspeptin activity on Gnrh neurons and gonadotropes was blocked by selected doses of these two antagonists, hindering physiological processes, such as sperm production.

As in mammals, activation of Kiss1r and Kiss2r expressed in COS7 cells by both Kiss1 and Kiss2 showed clear selectivity to the Ca^{2+}/PKC transduction pathway, although the cAMP/PKA pathway was also active in a few other fish species [1, 42]. Kiss1r is equally activated by both kisspeptins, while Kiss2r is ~10 000 times more selective to Kiss2 than to Kiss1. Stb Kiss2R activation by Kiss2 requires significantly lower doses (EC_{50} ~10^{-13} M) than the nanomolar range reported for other fish [20, 42] and mammalian [11] receptors. The difference may be due to the use of the less-potent core decapeptide and/or because doses below 1 pM were not tested. Nevertheless, low doses of KISS1 efficiently stimulated circulating LH/FSH and testosterone levels in mammals in vivo [43, 44] and icv treatment at doses as low as 1 fmol were effective [45]. The effectiveness of low doses of Kiss2 was also evident in our in vitro studies, in which *gnrh1* expression and Lh secretion were stimulated by a dose of less than 1 nM.

The two antagonists, pep 234 and pep 359, that were selected from a battery of modified KISS1, using Kissr expressed in COS7 cells, revealed that while pep 234 preferentially antagonizes Kiss1r signaling, pep 359 seems to antagonize Kiss2 activation of both receptors, and therefore it is a more specific antagonist for Kiss2. Peptide 234 is the first

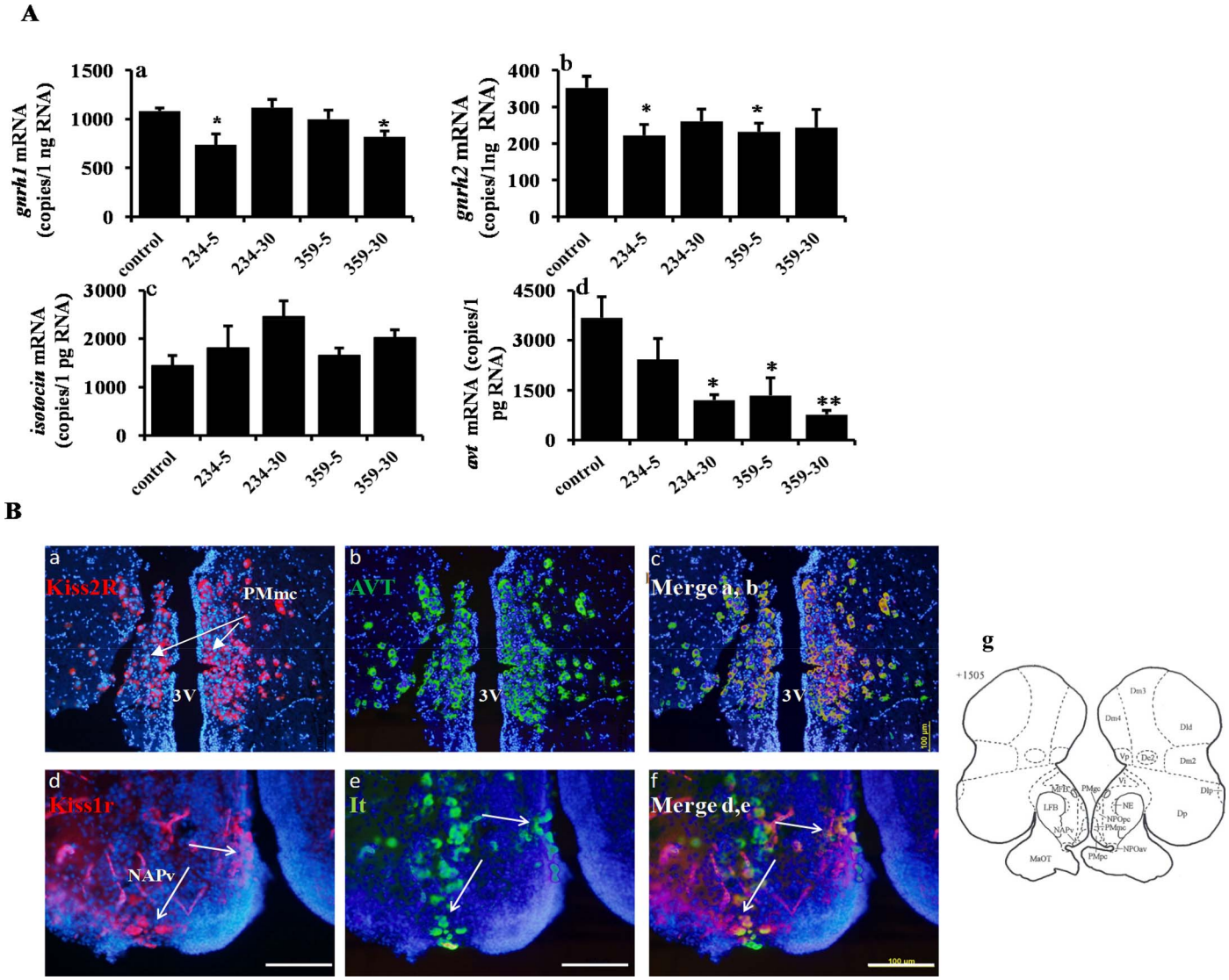

FIG. 6. Peptides 234 and 359 downregulated *gnrh* and *avt* expression in the brain in vivo. Spermiating male striped bass in the spawning season were treated with implants releasing either pep 234 or pep 359 at sustained doses of 5 or 30 μg/kg BW (n = 5 per group). **A**) The effect of the treatment on the expression of *gnrh1*, *gnrh2*, *avt*, and *isotocin* was measured using quantitative RT-PCR after 9 days and compared to the control fish (n = 4) treated with implants devoid of peptides. **a**) *gnrh1* mRNA levels. **b**) *gnrh2* mRNA levels. **c**) *it* mRNA levels. **d**) *avt* mRNA levels. Numbers in the x axis correspond to the antagonist peptide and the dose in the implants. Results are presented as mean ± SEM of absolute copy number of mRNA/1 ng or 1 pg total RNA. Statistical difference was accepted at $P \leq 0.05$ compared to control treatment levels. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. **B**) Double in situ hybridization demonstrating colocalization of *avt* (plate **b**, green) and kiss2r (plate **a**, red) in magnocellular neurons (PMmc, plates **a**–**c**), and of *it* (plate **e**, green) in kiss1r neurons (plate **d**, red) in the anterior periventricular nucleus in the preoptic area (plates **d**–**f**). **g**) A schematic drawing of the relevant coronal section in the preoptic area adopted from Muñoz-Cueto et al. [67] with permission from Maryland Sea Grant.

potent mammalian KISS1 antagonist to be reported [24]. It carries a substitution of Leu^{8} with D-Trp in combination with a Ser^{5} substitution with Gly and a substitution of Tyr^{1} with D-Ala: (viz. ac[D-A]NWNGFG[D-W]RF-NH_2). Peptide 234 blocked KISS1-10 effects on GnRH and LH in a variety of mammalian species [24, 25, 27, 46]. Peptide 359 is based on pep 234 with sarcosine substitutions.

Next, we tested the antagonists in their tissues of origin, in vitro and in vivo: Kisspeptin dose response in vitro studies in the brain of the stb, that possesses three Gnrh forms [47, 48], demonstrated that the low level of 0.5 nM Kiss2 stimulated the expression of the hypophysiotropic *gnrh1*, whereas Kiss1 had no effect. This result is consistent with the presence of Kiss2 neurons in close proximity to Gnrh1 neurons and axons in the preoptic area and in the basal hypothalamus of stb, and the expression of Kiss2r in Gnrh1 neurons [10, 13]. Again, kisspeptin seems to be stimulatory only at low levels, congruent with the low EC_{50} determined for Kiss2 activation of Kiss2r. Higher doses are either noneffective or even inhibitory in fish [10, 13, 49] and mammals [43]. One possible explanation is that high levels desensitize the receptor. Another is that high levels of Kiss1 or Kiss2 are able to activate GnIH receptor (GPR147). Interestingly, pep 234 does not activate the GnIH receptor, but activity of pep 359 has not been tested at the GnIH receptor (Gallant and Millar, unpublished results). The two antagonists diminished the stimulation of *gnrh1* by Kiss2. Indeed, neuroanatomical localization implicates the two receptors in the regulation of Gnrh1 neurons in the POA: Kiss2r is expressed in Gnrh1 neurons and Kiss1r is expressed in neurons attached to Gnrh1 fibers [13], representing direct and indirect kisspeptin regulation of Gnrh1 neurons, as reported in mammals [50]. Surprisingly, coincubation of a

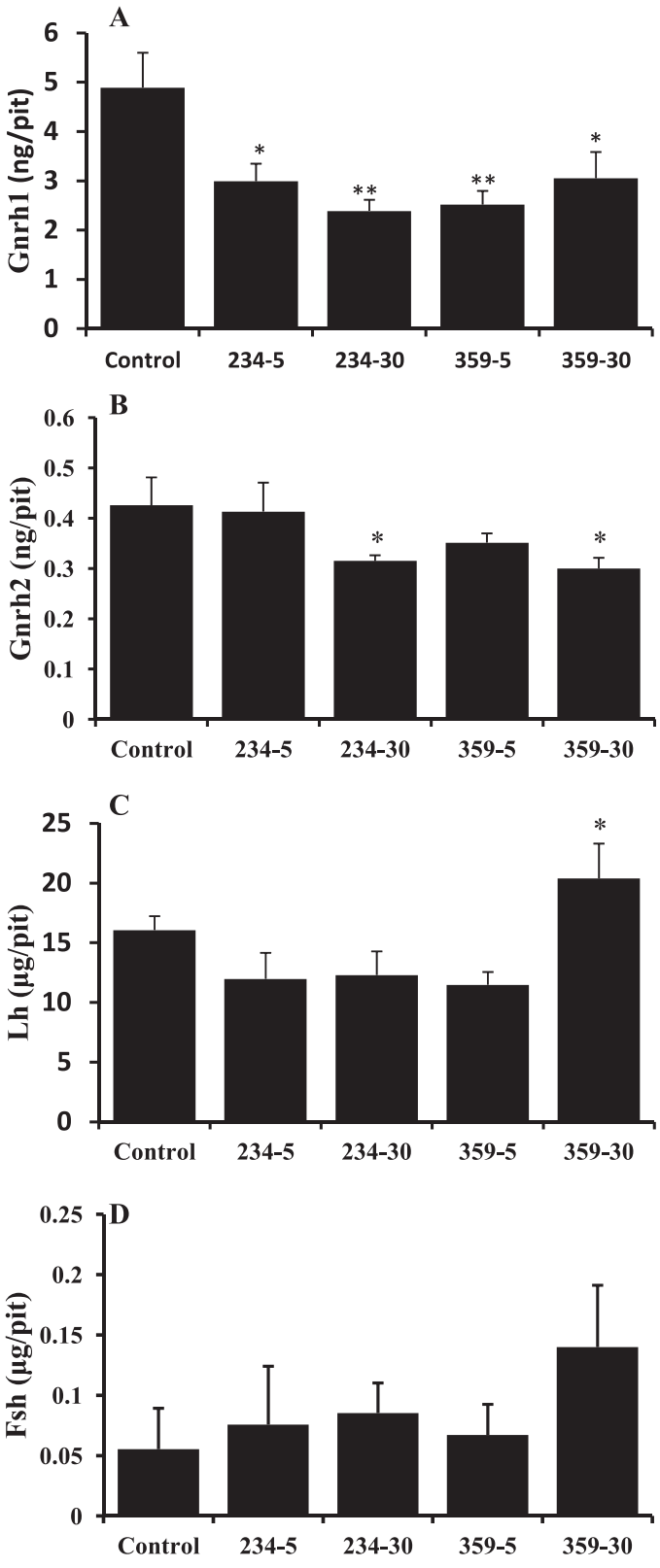

FIG. 7. The effect of pep 234 and pep 359 on Gnrh and gonadotropin content in the pituitary in vivo. Spermiating male striped bass in the spawning season were treated with implants releasing either pep 234 or pep 359 at sustained doses of 5 or 30 µg/kg BW (n = 5 per group). The effect of the treatment on the amount of Gnrh1, Gnrh2, Lh, and Fsh were measured using specific ELISA assays after 9 days and compared to the control fish (n = 4) treated with implants devoid of peptides. **A**) Gnrh1. **B**) Gnrh2. **C**) Lh. **D**) Fsh. Numbers in the x axis correspond to the antagonist peptide and the dose in the implants. Results are presented as mean ± SEM of protein in one pituitary. Statistical difference was accepted at $P \leq 0.05$ compared to control treatment levels. *$P \leq 0.05$, **$P \leq 0.01$.

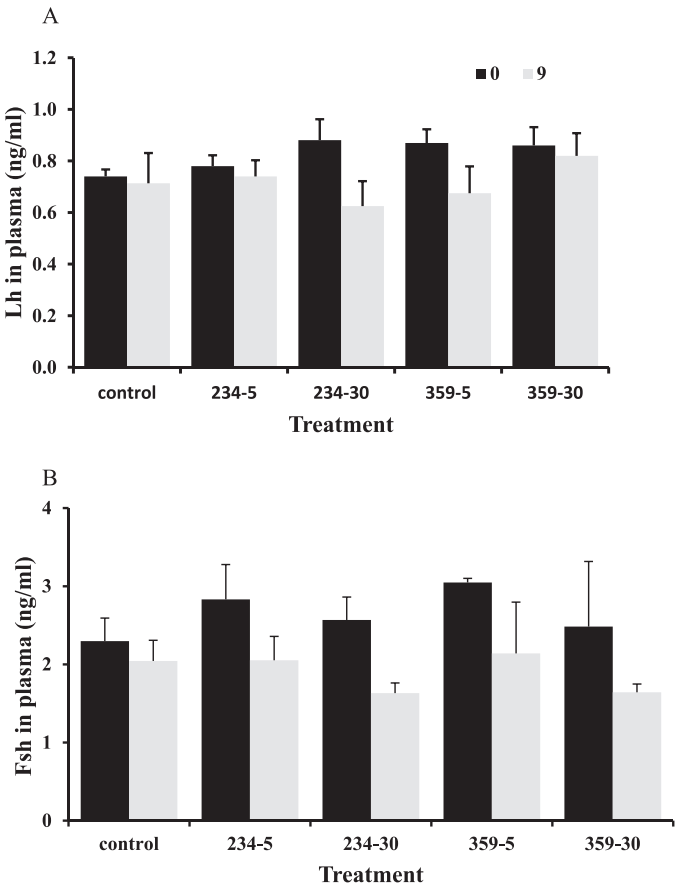

FIG. 8. The effect of pep 234 and pep 359 on plasma Lh and Fsh levels. Spermiating male striped bass in the spawning season were treated with implants releasing either pep 234 or pep 359 at sustained doses of 5 or 30 µg/kg BW (n = 5 per group). Lh and Fsh levels in the plasma were measured using specific ELISA for each hormone prior to (0) and 9 days after implantation (9). **A**) Lh. **B**) Fsh. Statistical difference was accepted at $P \leq 0.05$ compared to control treatment levels.

low level of pep 234 with Kiss1 stimulated *gnrh1* expression and Lh secretion in vitro, and is noted also in the kiss1r activation by kiss1 + pep 234. This consistency suggests that pep 234 at a low dose acts as a stimulator of the kiss1/kiss1r action.

This may be caused by a cooperative binding process resulting from receptor dimerization [51], or perhaps Kiss1 can be inhibitory under certain yet-unknown circumstances. The latter scenario is supported by our results (Fig. 3B); however, further experiments are needed to clarify this phenomenon.

Kisspeptin, in addition to its action on GnRH, can act directly on the pituitary in mammals [9, 52–54], albeit only at supraphysiological doses. This was shown in stb as well by detecting specific NLT populations that innervate the pituitary [10]. Studies in primary pituitary cells from recrudescent males indicate that both Kisspeptins stimulate Fsh and that only Kiss2 stimulates Lh. These results are in line with the fact that Kiss1r, which is activated by both Kisspeptins, is widely expressed throughout the proximal pars distalis in the pituitary (Supplemental Fig. S3C; [14]). The results reinforce the seemingly stimulatory direct effect of Kiss1 on *fsh* expression and Fsh

release in vivo [10]. The observation that Kiss2 induces the release of Lh fits previous findings in stb and in *Dicentrarchus labrax* [2, 13]. However, at the time, it was solely attributed to the stimulation of Gnrh1. We suggest that a direct action of Kiss2 on Lh can take place as well. Nonetheless, the interpretation of the present set of experiments is somewhat complex, because kisspeptins are expressed at minute levels in the pituitary (a few hundred mRNA copies/ng total RNA), and may act in an autocrinic/paracrinic fashion. As a support, coexpression of *kiss1* in Fsh gonadotropes was demonstrated in male *D. labrax* [19]. The endogenous presence of kisspeptin and Kiss1r in the pituitary may explain the lower than basal levels of Lh and Fsh caused by pep 234 and pep 359, which also occurred in the absence of exogenous Kiss1/Kiss2. This phenomenon is probably more relevant to Kiss1 than to Kiss2, because exogenous Kiss2 induced *lhβ* expression and release that was further blocked by the two antagonists. Remarkably, despite the complexity of the kisspeptin system in the pituitary, pep 234 and pep 359 were able to inhibit the effects of Kiss1 and Kiss2 whether they were exogenous or endogenous.

We then determined whether these antagonists can interfere with reproductively relevant processes in vivo. To test this, we used spermiating male stb, because of the transient expression of Kiss1 and Kiss2 in the NLT at spawning (assumed to regulate spawning via Gnrh1 axon terminals in the neurohypophysis and gonadotropes) [10]. Treatment with implants sustainably releasing pep 234 or pep 359 attenuated sperm production (more profoundly by pep 359 than by pep 234), suggesting that the normal flow of the HPG axis was disrupted. The effect was dose specific: a low level of pep 234 (5 μg/kg BW) and a high level of pep 359 (30 μg/kg BW) was needed to inflict this effect. The discrepancy between their efficacy in vivo and the in vitro IC_{50} values may be caused by various factors that differ between the conditions in whole body and the heterologous cell line that can affect clearance rates, transport, and blood-brain barrier penetration. Nonetheless, at this point, we have no explanation as to why a higher level of pep 234 was not inhibitory in vivo. The exact same doses of pep 234 and pep 359 also downregulated the expression of *gnrh1*, whereas all doses reduced the levels of Gnrh1 peptide in the pituitaries. This result indicates that, as seen in the in vitro studies, both antagonists acted on Gnrh1 neurons to inhibit its expression and protein synthesis/release, probably via the inhibition of both Kiss1r and Kiss2r. Gnrh2 was also affected by the two antagonists, but its involvement in regulating the HPG axis is not yet clear.

The most dramatic inhibitory effect of the antagonists was on *arginine-vasotocin*. Avt (the fish arginine-vasopressin [55]) is implicated in inducing spawning reflex, courtship behavior, and hierarchical status [56–60]. Kiss2 probably acts via Kiss2r expressed in these neurons, thus explaining the strong inhibition of *avt* expression by pep 359. Kiss1r may be also indirectly involved, since, despite the lack of Kiss1r in *avt* neurons, the higher dose of pep 234 downregulated *avt* expression. A possible route could involve direct Gnrh modulation of *avt* expression (rainbow trout [61]). Although isotocin (the fish oxytocin) is also implicated in sexual behavior in fish [62, 63] and, despite our finding that *kiss1r* is expressed in *isotocin* neurons, the two antagonists did not affect *it* expression in these spawning males, suggesting that the effect may be reproductive stage dependent. Taken together, these results suggest that Kiss2 employs multiple simultaneous routes to regulate reproductive behavior and physiology via Gnrh, Avt and Lh.

At the same time, Lh was retained in the pituitary in the pep 359-30 microgram treatment. Because this was not seen with pep 234, which inhibited *gnrh1* equally well, it can be postulated that Lh retention is due to a direct inhibition of Kiss2 induction of Lh release that probably originates from the spawning-stage NLT Kiss2 neurons. We did not find any difference in gonadotropin levels in the plasma at the end of the experiment; however, differences occurring during the 9 days of the experiment cannot be ruled out. Indeed, Lh levels gradually increase during testicular development, peaking in spermiating males at the time of spawning [64]. It is very likely that this continuous, gradual increase in plasma Lh was temporarily disrupted during the first days of the treatment, when the levels of the antagonists were higher, before decreasing in a matter of days and then remaining constant [65]. The higher Lh and Fsh content in the pituitary in the pep 359 treatment supports the idea that the release of these hormones was attenuated at some point during the course of the experiment.

Although the exact role is not yet clear, kisspeptins and kisspeptin receptors are also expressed in the gonads [66], where the antagonists may have interrupted their function. The profound sperm reduction resulting from the treatment may, therefore, be caused by the disruption of upstream events in the brain and pituitary and/or in the gonads. Nonetheless, it underscores the importance of the kisspeptin system in the execution of final gametogenesis and spawning, perfectly matching the appearance of Kiss1 and Kiss2 neurons in the NLT at spawning.

In summary, the two kisspeptin antagonists were instrumental in better defining each kisspeptin's specific roles and reinforced previous observations that both are important in the regulation of the reproductive HPG axis. These antagonists may help us to understand if and how each kisspeptin system regulates different reproductive stages, including the mechanism that drives reproductive cycles.

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