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Gonadotropin Antagonist Modulates Courtship Behavior in Male Red-Sided Garter Snakes, *Thamnophis sirtalis parietalis*

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SMITH, M. T., AND R. T. MASON. *Exogenous gonadotropins modulate courtship behavior in male red-sided garter snakes, Thamnophis sirtalis parietalis.* PHYSIOL BEHAV **61** (1) 137–143, 1997.—Behavioral studies were used to investigate the central effects of chicken-I GnRH, chicken-II GnRH, and D-Phe^{2,6},Pro^{3}-GnRH, a GnRH antagonist, on the courtship behavior of male red-sided garter snakes, *Thamnophis sirtalis parietalis*. Intracerebroventricular (ICV) injections of chicken-I or chicken-II GnRH had no effect on time spent courting or latency to court when experimental males were exposed to unmated females, or when experimental males were exposed to the female sex attractiveness pheromone. ICV injections of D-Phe^{2,6},Pro^{3}-GnRH caused a significant decrease in latency to court when experimental males were exposed to unmated females. When males injected with D-Phe^{2,6},Pro^{3}-GnRH were exposed to the female sex attractiveness pheromone, it caused a significant increase in time spent courting compared to that in saline-injected controls. D-Phe^{2,6},Pro^{3}-GnRH was not able to initiate courtship behavior during the nonbreeding season, indicating that courtship behavior is dependent on the interaction of multiple components. This study does demonstrate that a hormone or neuropeptide can modulate sexual behavior in garter snakes. *Copyright © 1996 Elsevier Science Inc.*

Garter snake Thamnophis GnRH Sexual behavior Communication Pheromones Reproduction

RED-SIDED garter snakes, *Thamnophis sirtalis parietalis*, are the most northerly living reptile in North America and, perhaps, in the world (28). As a result of the extreme winter temperatures at these northerly latitudes, red-sided garter snakes are constrained to spend up to 8 months of the year in hibernation (2). In early May, the males of a given hibernaculum will emerge en masse and wait for the females to emerge (13). The females, who emerge singly or in small groups over the course of the next 3–4 weeks, are courted by anywhere from 10–100 males (9,12,13).

Courtship behavior in red-sided garter snakes is typified by chin-rubbing behavior that is coupled with an increased tongue-flicking rate (4,39). This tongue-flicking behavior transports nonvolatile sex pheromones sequestered on the female's dorsal surface to the male's vomeronasal organ (15,26). This sex attractiveness pheromone, a component of the integumental skin lipids, is the fundamental constituent by which males are attracted to females (29–33). This pheromone, a series of long chain, saturated, and monounsaturated methyl ketones, is present in the skin of females and elicits strong courtship behavior from males, even after it has been extracted from the skin of females and applied to a paper towel (30,31). If this pheromone is not present on the female's skin or if the male is unable to detect it, the males will not exhibit courtship behavior (16).

Many neurotransmitters and hormones have been investigated as possible regulators of courtship behavior in red-sided garter snakes but, to date, no hormone or neurotransmitter has been effective (7,12). Currently, only two parameters have been shown to be essential for reproduction. The pheromone system, mentioned previously, is essential for reproduction (30,31), as is a period of cold exposure prior to the mating season (8,12,17).

Members of the gonadotropin-releasing hormone (GnRH) family of decapeptides are found in the brains of vertebrates, frequently with more than one form in a given species (44,45). One function of GnRH is to regulate the hypothalamo–pituitary–gonad (HPG) axis by stimulating gonadotrophs of the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which, in turn, control a variety of functions including steroid secretion, gamete maturation, and ovulation (20).

Gonadotropin-releasing hormone, however, appears to have other functions in addition to regulating FSH and LH secretion. GnRH administration can enhance the sex behavior of newts (*T. granulosa*), voles (*Microtus canicaudus*), rats (*Rattus rattus*), frogs (*Xenopus laevis*), green anole lizards (*Anolis carolinensis*), and horses (1,6,24,34–36). In addition, GnRH levels change in specific brain areas in response to reproductive cues. GnRH concentration in the posterior olfactory bulb of female

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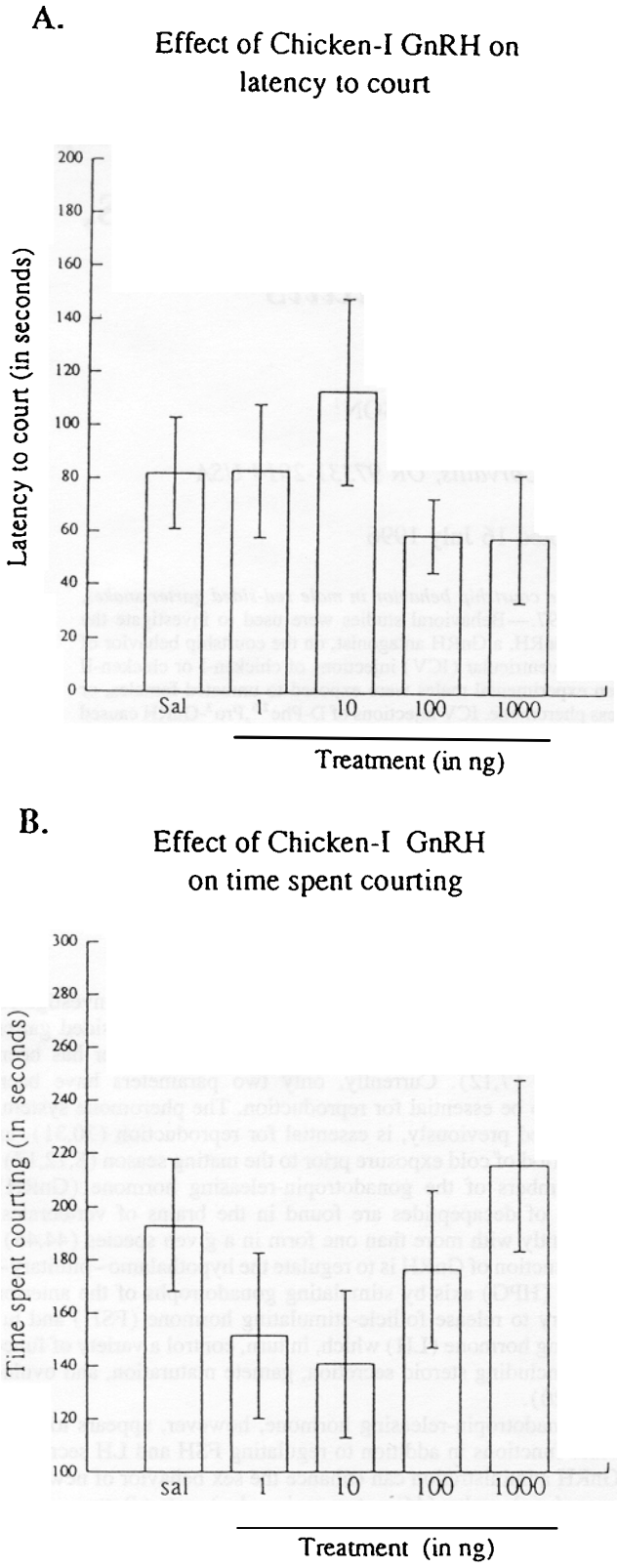

FIG. 1. Effect of chicken-I GnRH on latency to court and time spent courting an unmated female (mean ± standard deviation). (A). Effect of chicken-I GnRH on experimental male's latency to court an unmated female (n = 8 for each group). (B). Effect of chicken-I GnRH on the time spent courting an unmated female during a 5-min test period (n = 8 for each group).

voles (*Microtus ochrogaster*) increases in response to a male urine cue; the number of mast cells with GnRHir in the medial habenula of ring doves (*Streptopelia roseogrisea*) increases following two hours of courtship; and the GnRH concentration in the terminal nerve of *T. granulosa* increases in response to mating (10,11,41,51). These extra-hypothalamic changes in GnRH, as well as the direct effects of GnRH on behavior, indicate that GnRH may function as a neurotransmitter in the brain. Finally, in the sympathetic ganglia of the bullfrog, GnRH functions as a neurotransmitter, regulating potassium channels and the slow excitatory postsynaptic potential (21,22).

Given the precedents of the forementioned studies, the present study was designed to look at the direct effects, if any, of GnRH administration on the initiation and maintenance of sex behavior in male red-sided garter snakes. We are especially investigating those effects that do not involve activation of the HPG axis. We hypothesize that exogenous GnRH administration will affect courtship behavior in a time frame that indicates GnRH is acting as a neurotransmitter and not necessarily a trophic hormone.

MATERIALS AND METHODS

Reproductively active adult, male red-sided garter snakes, *Thamnophis sirtalis parietalis*, were collected in Manitoba, Canada during early May. Males were transported to a field station and housed in outdoor pens measuring 1.2 × 1.2 × 0.9 m. Each pen contained approximately 100 males and 3–5 unmated females. A courting male was removed from the back of an unmated female, a small hole was drilled into the skull, and 2 μl of test solution containing 1, 10, 100, 1000 ng of chicken-I GnRH, chicken-II GnRH, or D-Phe^{2,6},Pro^{3}-GnRH was injected over the course of 2 s into the third ventricle, using a Rainin peristaltic pump and a drawn-out glass micropipette. The third ventricle injection site was confirmed by sectioning the brains (n = 5) of animals that, after being anesthetized, had India ink injected into the same site. Many GnRH agonists and antagonists were tested in these studies, with the results of chicken-I GnRH, chicken-II GnRH, and D-Phe^{2,6},Pro^{3}-GnRH being presented here. Chicken-I GnRH is the major form of GnRH in the brain of *T. s. parietalis* found in a study that analyzed antibody cross-reactivity and HPLC retention times (46). D-Phe^{2,6},Pro^{3}-GnRH is a potent GnRH receptor antagonist for the mammalian form of the peptide, and chicken-II GnRH is the form of GnRH that appears to be localized in the midbrain of a variety of vertebrate species (37).

Experiment 1

The first testing regimen compared the responses of saline-injected control males (n = 8), males injected with chicken-I GnRH (n = 32, 8 animals/treatment), chicken-II GnRH (n = 32, 8 animals/treatment), or D-Phe^{2,6},Pro^{3}-GnRH (n = 32, 8 animals/treatment), in courting an unmated female. After injection, the test male was introduced into an arena that contained an unmated female and another courting male. The other male was present because of facilitated courtship, a phenomenon whereby one male will court more vigorously when another male is also courting (23). Latency to court and time spent courting were recorded over a 5-min test period. Test males had to exhibit both chin-rubbing behavior and caudocephalic waves to be scored as exhibiting sex behavior. The unmated female and auxiliary male were changed once after half of the males had been tested.

Experiment 2

In this testing regimen, males were tested in the absence of visual or tactile cues, and the behavior of males treated with

TABLE 1

RESULTS OF THE CHICKEN-II GnRH INJECTIONS

	Saline	Treatment Dose 1 ng	10 ng	100 ng	1000 ng
Latency to court (♂ courting ♀)	126 ± 35	127 ± 28	158 ± 19	142 ± 30	130 ± 28
Time spent courting (♂ courting ♀)	133 ± 32	99 ± 25	105 ± 21	138 ± 34	153 ± 31
Side preference (♂ courting ♀ pheromone)	66 ± 8	56 ± 10	78 ± 2	51 ± 11	64 ± 7
Time spent courting (♂ courting ♀ pheromone)	5 ± 3	4 ± 2	4 ± 4	3 ± 3	3 ± 3

All latency and time-spent data are expressed as mean ± standard error in s. Side-preference data are percentages of time spent on the pheromone side of the test arena in mean ± standard error.

chicken-I GnRH, chicken-II GnRH, or D-Phe^{2,6},Pro^{3}-GnRH was observed in response to female pheromone alone. Female sex attractiveness pheromone was obtained by extracting female skins with hexane (30). The hexane extracts from 5 females were reduced in volume by evaporating off excess solvent. Ten milliliters of concentrated pheromone was applied to a paper towel on one half of a testing arena, with 10 ml of hexane blank being applied to a paper towel on the other half of the testing arena. The solvent from both sides of the arena was allowed to evaporate prior to testing.

As in the previous experiment, saline-injected controls (n = 8), chicken-I GnRH (n = 32, 8 animals/treatment), chicken-II GnRH (n = 32, 8 animals/treatment), or D-Phe^{2,6},Pro^{3}-GnRH (n = 32, 8 animals/treatment) was injected into the third ventricle, with time spent courting and side preference being recorded over a 5-min test period.

Experiment 3

Male and female red-sided garter snakes were removed to the laboratory and tested to determine if chicken-I GnRH or D-Phe^{2,6},Pro^{3}-GnRH could reinstate mating behavior either immediately after cessation of courtship or during August, a month where normally no mating behavior is observed in this species. For the animals that were tested immediately after cessation of courtship behavior, a small hole was drilled in the skull and 2 μl of a test solution containing 10, 100, or 1000 ng of chicken-I GnRH (n = 24, 8 animals/group) or D-Phe^{2,6},Pro^{3}-GnRH (n = 24, 8 animals/group) was injected over the course of 2 s into the third ventricle using a Rainin peristaltic pump and a drawn-out glass micropipette. For the animals tested in August, the same injection procedure was used with test solutions containing 1 or 10 μg of chicken-I GnRH (n = 18, 9 animals/group) or D-Phe^{2,6},Pro^{3}-GnRH (n = 16, 8 animals/group). Females used in the August experiments were estrogen-primed to increase attractivity with 40 μg estradiol benzoate/75 g body weight/day for 3 days prior to testing with the males (26,42). After injection, test males were introduced into the testing arena containing the estrogen-primed female and another male. Latency to court and time spent courting were recorded over a 5-min test period.

Statistics

Data were analyzed by 1-way analysis of variance, followed by the Tukey multiple range test where appropriate. When variance was not equal between groups, data were natural-log transformed and analysis performed on the transformed data. The time spent courting data for D-Phe^{2,6},Pro^{3}-GnRH in Experiment 1 did not conform to the assumptions for parametric analysis and, thus, was analyzed using Kruskal–Wallis 1-way analysis by ranks. All of the data analyses were conducted with Statgrafics® software (Manugistics, Inc., Rockville, MD).

RESULTS

Experiment 1

Chicken-I GnRH had no effect on the latency to court an unmated female at any concentration (p = 0.54, Fig. 1A). There was no significant effect of any concentration of chicken-I GnRH on time spent courting (p = 0.37, Fig. 1B). Chicken-II GnRH had no effect at any concentration when looking at the latency to court an unmated female (p = 0.92, Table 1). There was also no significant effect of any concentration of chicken-II GnRH on time spent courting (p = 0.63, Table 1).

D-Phe^{2,6},Pro^{3}-GnRH significantly decreased the latency to court an unmated female (p = 0.002, Fig. 2A). The behavioral response to the 1000-ng antagonist injection significantly reduced the latency to court compared to that in the saline-injected controls ($p < 0.05$, Fig. 2A). The 100 ng injection also significantly decreased latency to court when compared to the 1-ng injection ($p < 0.05$, Fig. 2A). The time spent courting data for D-Phe^{2,6},Pro^{3}-GnRH showed no significant differences between any of the treatments and the saline-injected control animals. The time spent courting when 1000 ng of D-Phe^{2,6},Pro^{3}-GnRH was injected was statistically different from the males that were injected with 1 ng of D-Phe^{2,6},Pro^{3}-GnRH ($p < 0.05$, Fig. 2B).

Experiment 2

Chicken-I GnRH had no effect on side preference when tested with only the pheromone cues from females present (p = 0.47, Fig. 3A). All of the test males spent greater then 50% of their time on the pheromone side of the test arena, with the saline-injected animals spending 63% of tested time on the pheromone side of the test arena, compared to 86% for the 1000-ng injected animals. This was not statistically different at the 0.05 level. Time spent courting the female pheromone extract showed no significant differences in any of the groups injected with chicken-I GnRH when compared to that in saline-injected controls (p = 0.08, Fig 3B). In 3 of the groups, the 1, 10, and 100 ng of chicken-I GnRH, the test animals did not court at all. In 2 of the groups, the saline-injected control males and the 1000 ng antagonist-injected males, the animals spent 2% and 4%, respectively, of the total time courting.

Chicken-II GnRH had no effect on side preference when tested with the female pheromone (p = 0.20, Table 1), even though all of the animals spent greater than 50% of their time on the pheromone side of the test arena. Injection of chicken-II GnRH did not affect time spent courting (p = 0.74, Table 1).

D-Phe^{2,6},Pro^{3}-GnRH did not increase the amount of time spent on the pheromone side of the test arena (p = 0.30, Fig.

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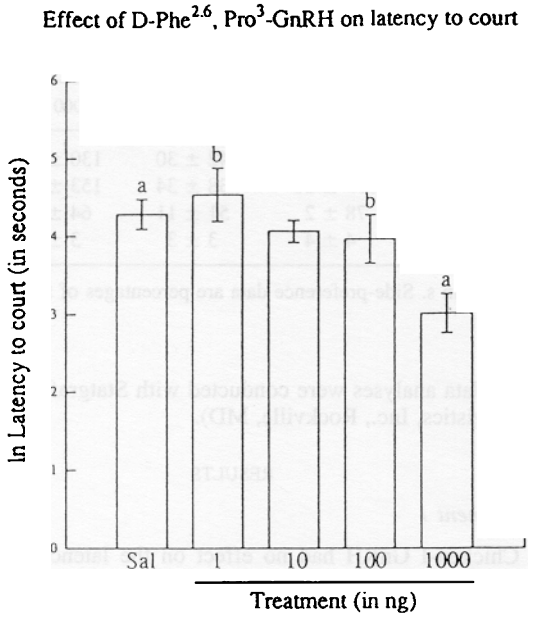

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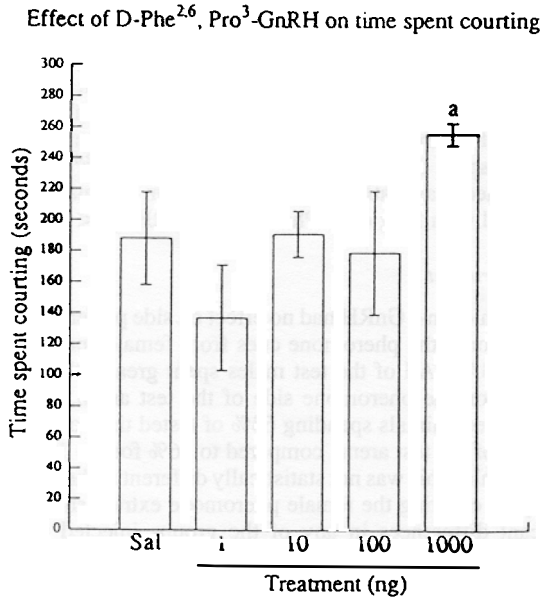
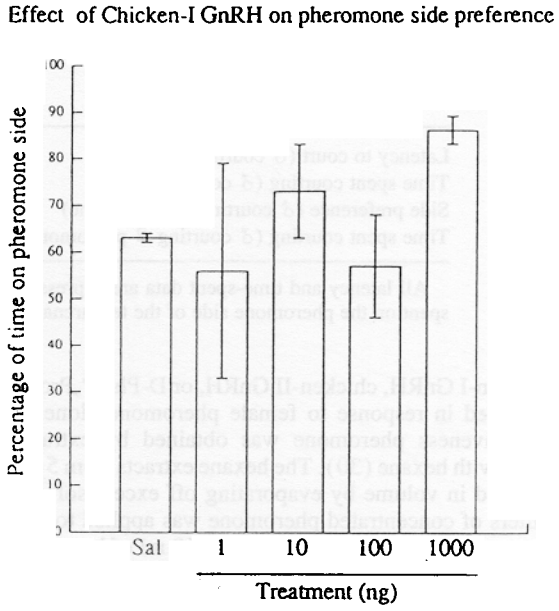


FIG. 2. Effect of D-Phe^{2,6},Pro^{3}-GnRH on latency to court and time spent courting an unmated female (mean ± standard deviation). (A). Effect of D-Phe^{2,6},Pro^{3}-GnRH on an experimental male's latency to court an unmated female (n = 8 for each group). a, different at the $p < 0.05$ level; b, different at the $p < 0.05$ level. (B). Effect of D-Phe^{2,6},Pro^{3}-GnRH on time spent courting an unmated female during a 5-min test period (n = 8 for each group). a, different at the $p < 0.05$ level.

A.



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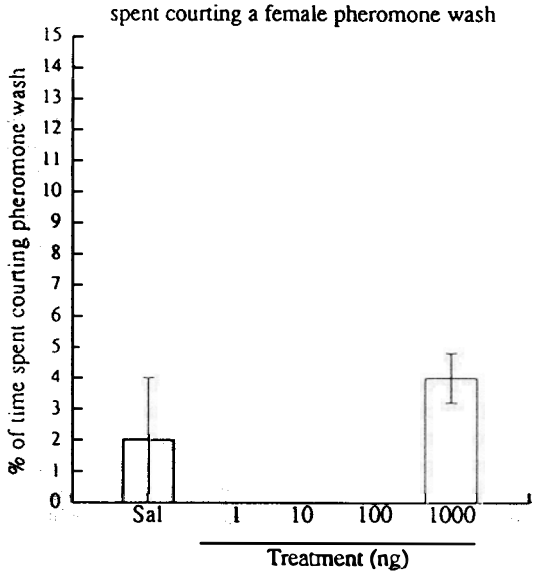
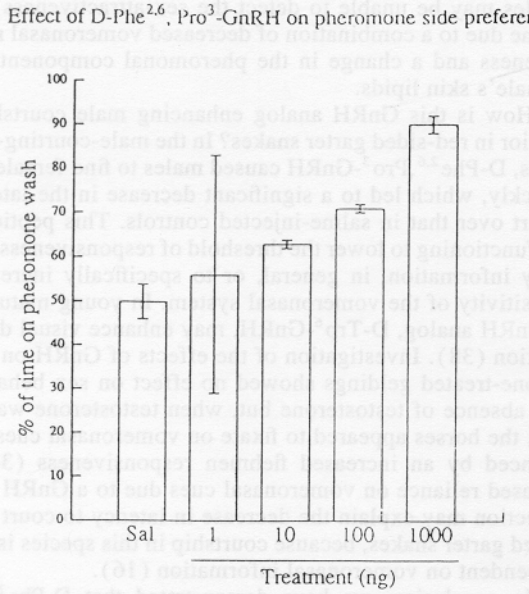


FIG. 3. Effect of chicken-I GnRH on side preference and time spent courting the female sex attractiveness pheromone (mean ± standard deviation). (A). Effect of chicken-I GnRH on the side preference of an experimental male to the side of the test arena with the female sex attractiveness pheromone vs. a hexane control (n = 8 for each group). (B). Effect of chicken-I GnRH on the time that the test male spent courting the female pheromone wash (n = 8 for each group).

A.



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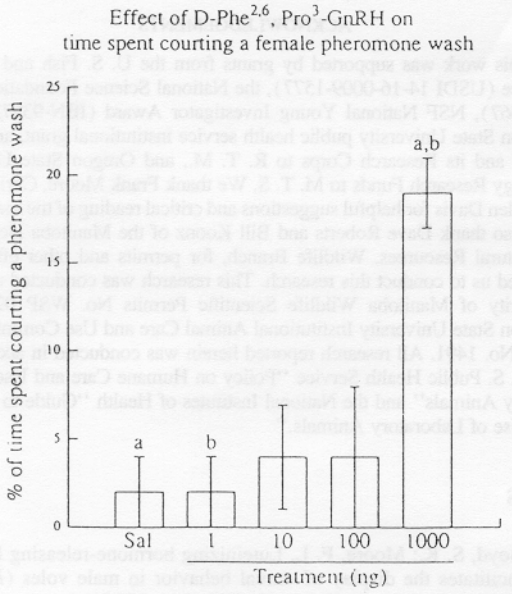

Fig. 4. Effect of D-Phe^{2,6},Pro^{3}-GnRH on side preference and time spent courting the female sex attractiveness pheromone (mean ± standard deviation). (A). Effect of D-Phe^{2,6},Pro^{3}-GnRH on the side preference of an experimental male to the side of the test arena with the female sex attractiveness pheromone vs. a hexane control (n = 8 for each group). (B). Effect of D-Phe^{2,6},Pro^{3}-GnRH on the time that the test male spent courting the female pheromone wash (n = 8 for each group). a, different at the $p < 0.05$ level; b, different at the $p < 0.05$ level.

4A). A large variance in the 1-ng group of this data set made the values not significantly different. The analog, D-Phe^{2,6}, Pro^{3}-GnRH, did cause an increase in courtship on the female pheromone side of the test arena (p = 0.04, Fig. 4B). The 1000-ng group spent significantly more time courting the female pheromone extract than the saline-injected controls or the 1-ng injected animals. Using these doses, there appeared to be a threshold effect with the saline-injected animals and the first three analog concentrations not being statistically different, but with a statistically significant increase in percent of time spent courting the female pheromone wash when 1000 ng of analog was injected.

Experiment 3

None of the males injected with either chicken-I GnRH or D-Phe^{2,6},Pro^{3}-GnRH immediately after cessation of courtship showed any significant differences in either time spent courting or latency to court (p = 0.46, p = 0.66, respectively, for chicken-I GnRH and p = 0.44, 0.48, respectively, for D-Phe^{2,6},Pro^{3}-GnRH; data not shown). None of the August males in the 1 or 10 μg chicken-I GnRH or the 1 or 10 μg D-Phe^{2,6},Pro^{3}-GnRH groups expressed courtship behavior to the estrogen-primed female (data not shown).

DISCUSSION

This study shows that the administration of a form of GnRH can alter the sex behavior of male red-sided garter snakes. D-Phe^{2,6},Pro^{3}-GnRH administration decreases the latency to court and increases the time males spend courting unmated females. In addition, D-Phe^{2,6},Pro^{3}-GnRH administration increases time spent courting when only pheromonal cues from females are available to the test male. Chicken-I or chicken-II GnRH administration has no effect on courtship behavior using these testing paradigms at these dosages.

Male sex behavior was not affected by administration of either chicken-I or chicken-II GnRH (cGnRH-I and cGnRH-II) in either the unmated female or pheromone-alone testing paradigms. GnRH modulates sex behavior in a variety of species, with the evidence for rapid effects of GnRH coming from work on rats (43). GnRH can potentiate lordosis behavior when systemically administered to estrogen-primed ovariectomized rats (36,40). The site of action for the rapid effects of GnRH on sex behavior appears to be the dorsal half of the midbrain central grey (43). GnRH administered to the midbrain central grey increases the lordosis reflex within 5 min, persisting for approximately 2 h; an antibody to GnRH eliminates the lordosis reflex in 90 min and this persists for up to 12 h (43). This effect was specific for the midbrain central grey; control injections to the superior colliculus were ineffective at eliciting a response (43). Immunocytochemical data suggests that the midbrain population of GnRH neurons in many vertebrates is immunoreactive to the cGnRH-II form of the GnRH molecule (for review, see 37).

Latency to court and time spent courting in male *T. s. parietalis* were significantly modulated with administration of D-Phe^{2,6},Pro^{3}-GnRH. This peptide increased the time males spent courting and decreased the latency to court when males were tested with unmated females. This peptide is an antagonist in mammals for mammalian GnRH and a partial antagonist in goldfish at 10^{-7} or 10^{-6} M, with a more pronounced ability to antagonize salmon GnRH than chicken-II GnRH (14). It is not known whether this peptide functions as an agonist or an antagonist for the receptor that is specific for chicken-I GnRH in red-sided garter snakes.

There is a small body of literature that describes the effect of GnRH antagonists on male sex behavior or physiology. Administration of a GnRH antagonist to adult male rhesus monkeys (*Macaca mulatta*) leads to decreased LH and testosterone secretion, decreased pituitary responsiveness to GnRH and, ultimately, to a decrease in male sexual behavior within 1 week (49). GnRH antagonist given to neonatal male rats evokes a transient infer-

tility in adults (25) and, if given to immature male rats, causes an inhibition of sexual development (48). In general, it appears that administration of GnRH antagonists elicits a depression in traits associated with male sexual behavior. To our knowledge, this is the first study that shows administration of a mammalian or fish GnRH antagonist increases sexual behavior in another class of vertebrate. This raises the possibility that D-Phe^{2,6},Pro^{3}-GnRH may function as an agonist in some nonmammalian vertebrates, including garter snakes.

Chicken-I GnRH (cGnRH-I) appears to be the major form of GnRH in the brain of *T. s. parietalis* (46) and cGnRH-I immunoreactivity has been localized to the median eminence and infundibulum in reproductively active males of this species (47). cGnRH-I would, presumably, control the release of the one ophidian gonadotropin molecule that appears to function like FSH (27) and, in turn, this gonadotropin would control testosterone release.

There is also evidence for other forms of GnRH in the brain of *T. s. parietalis* (46). It may be that the stimulatory effect of D-Phe^{2,6},Pro^{3}-GnRH that we observed occurs through another GnRH receptor subtype. It has been demonstrated that GnRH receptors with similar pharmacology are differentially regulated, depending on the tissue (3). It is also possible that D-Phe^{2,6},Pro^{3}-GnRH is able to induce the dimerization that is needed for typical agonist functionality, because some agonists and antagonists are able to induce the same responses of receptor clustering and internalization (19).

Although D-Phe^{2,6},Pro^{3}-GnRH was able to enhance male sex behavior during the breeding season, it failed to initiate courtship behavior during the nonbreeding season. It is important to note that, although there are seasonal differences in the skin lipids of female garter snakes, newly emerged males will court female pheromone extracts collected both during and after the breeding season (29; Mason, personal observation). The regulatory system by which D-Phe^{2,6},Pro^{3}-GnRH is enhancing courtship behavior during the breeding season is composed of more than one component and D-Phe^{2,6},Pro^{3}-GnRH alone is insufficient for the full expression of male sexual behavior during the nonbreeding season. Additional components can be either environmental factors or other neurohormones/peptides that, in concert, regulate the expression of male sex behavior. A strong possibility is that prior exposure to cold temperatures during hibernation is an essential factor that is missing during the nonbreeding months (5,8,12,18,50). Finally, there may be a seasonal change in the vomeronasal organ's responsiveness to the sex pheromone. Males may be unable to detect the sex-attractiveness pheromone due to a combination of decreased vomeronasal responsiveness and a change in the pheromonal component of the female's skin lipids.

How is this GnRH analog enhancing male courtship behavior in red-sided garter snakes? In the male-courting-female tests, D-Phe^{2,6},Pro^{3}-GnRH caused males to find females more quickly, which led to a significant decrease in the latency to court over that in saline-injected controls. This peptide may be functioning to lower the threshold of responsiveness to sensory information, in general, or to specifically increase the sensitivity of the vomeronasal system. In young mature rats, a GnRH analog, D-Trp^{6}-GnRH, may enhance visual discrimination (38). Investigation of the effects of GnRH on testosterone-treated geldings showed no effect on sex behavior in the absence of testosterone but, when testosterone was present, the horses appeared to fixate on vomeronasal cues as evidenced by an increased flehmen responsiveness (34). Increased reliance on vomeronasal cues due to a GnRH analog injection may explain the decrease in latency to court in red-sided garter snakes, because courtship in this species is highly dependent on vomeronasal information (16).

In conclusion, we have demonstrated that D-Phe^{2,6},Pro^{3}-GnRH administration is capable of enhancing male reproductive behavior in red-sided garter snakes. This study supports the hypothesis that the GnRH system is involved not only as a trophic hormone, but also as a neurohormone for the initiation of courtship behavior in red-sided garter snakes.

ACKNOWLEDGEMENTS

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