The Male Red-sided Garter Snake (\textit{Thamnophis sirtalis parietalis}): Reproductive Pattern and Behavior

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Abstract

Among the small group of species (e.g., some temperate zone turtles, snakes, and bats) that exhibit a dissociated reproductive pattern, the red-sided garter snake (\textit{Thamnophis sirtalis parietalis}) is probably the most well studied. For these species, courtship and mating occur immediately upon emergence from winter dormancy; the gonads remain essentially inactive. Male red-sided garter snakes are a particularly informative animal model for examining the role of neuroendocrine factors associated with reproductive physiology and behavior because unlike species that exhibit an associated reproductive pattern, in which sex steroids initiate and control sexual behavior, reproductive behavior in the male garter snake appears to be independent of circulating sex hormone control. In fact, the only factor associated with the initiation of courtship behavior and mating in the male garter snake appears to be an extended period of low temperature dormancy followed by exposure to warm temperatures. Yet the presence of sex steroid-concentrating neurons within the pathways regulating courtship and mating suggests that sex hormones may be involved in the activation of sexual behavior. Although circulating androgens are elevated upon emergence from hibernation, the initiation of courtship behavior and mating appears to be independent of direct androgen control. Thus steroid hormones may have indirect effects on mating behavior in animals that display “dissociated” reproductive behaviors.

Key Words: behavior; natural history; neuroendocrinology; red-sided garter snake; reproductive physiology; \textit{Thamnophis sirtalis parietalis}

Patterns of Reproduction

In the majority of seasonally breeding vertebrates, the expression of courtship behavior and mating is directly correlated with the activation and hypertrophy of the testes, accompanied by release and elevation of circulating sex steroid levels (Crews et al. 1984; Licht 1984). In species that exhibit an “associated reproductive pattern” (Figure 1), castration extinguishes sexual activity. Reimplantation of the testes into the abdominal cavity or the administration of exogenous sex steroids by injection or implantation directly into the brain has been shown to extend the mating season and will initiate sexual activity in noncourting animals (Crews 1982). The initiation and maintenance of sexual behavior in animals exhibiting an associated reproductive pattern thus appears to be directly dependent on the presence and concentration of circulating sex steroids.

In contrast, a small number of vertebrate species including some turtles, snakes, and bats have been shown to mate at a time when their gonads are quiescent and circulating levels of sex steroids were reported to be low (Garstka et al. 1982). This pattern of reproduction has been termed a “dissociated reproductive pattern” (Crews 1976; Licht 1984; Lofts 1977; Volsøe 1944; Figure 1). In species that exhibit this reproductive pattern, castration does not extinguish sexual activity. Unlike the effects of hormones on species exhibiting associated reproduction, administration of exogenous sex steroids or pituitary hormones or the implantation of sex steroids directly into the brain does not initiate courtship behavior and mating in noncourting individuals (Camazine et al. 1980; Friedman and Crews 1985a). Therefore it appears that control and regulation of sexual behavior in species that exhibit a dissociated reproductive pattern are independent of direct effects of steroid hormones.

The red-sided garter snake (\textit{Thamnophis sirtalis parietalis}) is the most well-studied species that exhibits a dissociated reproductive pattern. Male red-sided garter snakes emerge from winter dormancy and immediately exhibit intense breeding activity that can last as long as 4 to 6 wk. At emergence, the testes are completely regressed and do not initiate recrudescence until the breeding season has ended (Crews et al. 1984). Many studies have attempted to delineate the ultimate and proximate cues that control and regulate courtship behavior and mating in the male red-sided garter snake. Although it has now been demonstrated that circulating androgen levels are elevated upon emergence (Krohmer et al. 1987; Moore et al. 2000, 2001), initiation of courtship behavior still appears to be independent of direct hormonal regulation. After almost 30 yr of investigating numerous aspects of red-sided garter snake reproduction, researchers have linked the initiation of courtship behavior and mating only to environmental factors (Aleksiuk and Gregory 1974; Camazine et al. 1980). The initiation of sex-
ual behavior in the red-red-sided garter snake requires a prolonged period of low temperature dormancy followed by exposure to warm temperatures. The intensity at which males exhibit courtship behavior appears to be directly correlated with the length of time males remain in low temperature dormancy (Garstka et al. 1982).

Natural History

The range of the red-sided garter snake extends farther north than any other reptile in the western hemisphere (Logier and Toner 1961). The extremely large population of red-sided garter snakes (Figure 2) located in central Manitoba, Canada, is one of the most adaptable reptile populations existing under extreme climatic conditions that border on the subarctic. This population is able to survive winter temperatures that reach −40°C and snow cover that may be continuous for up to 9 mo. The extreme seasonal variations in this region present a major evolutionary force that dictates the characteristics of the population (Aleksiuk 1970, 1971; Aleksiuk and Stewart 1971). Therefore it is not surprising that the entire life history of the red-sided garter snake in Manitoba, including reproduction, must be extremely seasonal in nature.

The red-sided garter snake has a very restricted period of activity each year that lasts approximately 3 mo. During this brief period of activity, individual snakes are widely dispersed throughout the summer habitat. Migration from the summer feeding areas to hibernacula begins in early to mid-August. It has been reported that individual snakes return to and hibernate in the same den during successive years (Gregory 1971). Migration, estimated to cover distances as great as 17.7 km (Gregory 1974), intensifies throughout August and early September; and by mid-September, several thousand to more than 8000 animals can be found around the entrance of the dens. Once they reach the hibernaculum, snakes remain in or near the mouth of the den, are active on clear sunny days, and retreat underground during cloudy weather and at night. Snakes finally disappear underground when the ambient daytime temperature remains below 0°C.

Although the cues responsible for spring emergence are not fully understood, snakes reappear on the surface in late April or early May. Due to the highly unpredictable nature of the weather during the breeding season, snakes must actively thermoregulate to increase their body temperature. However, Shine et al. (2000a) recently reported that warm snakes were not more successful in mating or avoiding predation than cooler snakes. In fact, courting snakes spent little time actively thermoregulating, and body temperatures were quite variable despite opportunities for more precise control. Garter snakes were found to cool rapidly, and any benefit of higher body temperatures would only be temporary. Because of the high cost of precise thermoregulation to snakes, the males must be concerned with courtship rather than taking the time to bask and increase body temperature (Shine 2000a).

In the spring, male red-sided garter snakes emerge essentially en masse from the dens and wait for females to emerge individually or in small groups over the next several weeks (Crews and Garstka 1982). Once mated, the female leaves for the summer feeding areas while males remain at or near the den until all courtship has ended before they disperse in late May or early June. From dispersal in late spring to the return migration in late summer, life history observations are minimal due to the difficulty of locating animals in their summer habitat. However, available data indicate that snakes spend the summer around marshes, and
stomach contents collected during this period have contained primarily anurans (Aleksiuk and Stewart 1971).

After emergence, males compete for females and will copulate numerous times during the 4- to 6-wk courtship season. Upon emergence from low temperature dormancy, female red-sided garter snakes can be identified by courting males through the detection of a nonvolatile attractiveness pheromone that is present on the dorsum of the female. Noble (1937) reported that female common garter snakes (Thamnophis s. sirtalis) are sexually attractive upon emergence from winter dormancy and that this attractiveness appears to be associated with chemical cues exuding through the skin. Vagvolgyi and Halpern (1983) suggest that males, in addition to recognizing females by a specific attractiveness pheromone, produce a male-identifying pheromone that communicates their male sex and acts as a signal to dissuade other males from courting the respective females. A male-identifying pheromone would have a beneficial role by reducing male-male courtship, which might limit their exposure to potential mates (Mason 1992).

She-Male Snakes

The existence of a subpopulation of male red-sided garter snakes with female-like skin lipids—"she-males"—indirectly supports the male-identifying pheromone hypothesis. Males court the she-males as they would court attractive females (Mason and Crews 1985, 1986). Except for the presence of a female-like pheromone, which attracts courting males, and circulating testosterone levels three times greater than normal males, she-males are anatomically, morphologically, and behaviorally male; and they court and mate with attractive females (Mason and Crews 1985, 1986). In competitive mating trials, she-males were reported to mate twice as often as normal males, indicating that she-maleness offers a selective advantage (Mason 1992).

Behavioral Characteristics

Beginning in 1998, a group of scientists studying the red-sided garter snake began to reinvestigate the concept of the she-male. These new studies propose that she-maleness is not a characteristic of a subset of males as reported in earlier work, but rather is a transitory phase that essentially all males exhibit upon emergence from winter dormancy (Shine et al. 2000b, 2001). It appears that newly emerged males may need as much as 2 days to "recover" from the effects of the long winter dormancy. During this time, the snakes are cold, relatively inactive, and weak (Shine et al. 2001). Actively courting males have an average body temperature that exceeds 25°C, whereas newly emerged animals are essentially the temperature of the substrate (≈10°C). The newly emerged animals may benefit from the existence of she-maleness as it may provide protection from predators, because they are hidden beneath other males, and receive transferred heat from courting males (Shine et al. 2001).

The new information generated by the studies described above provides an alternative explanation of she-male characteristics immediately after emergence. Female mimicry could have evolved through natural selection rather than sexual selection (Shine et al. 2001). Newly emerged male garter snakes may manipulate actively courting snakes, not to gain an advantage in mating but rather to seek warmth and reduce their exposure to predators. However, these new data suggesting a transitory period of she-maleness in all (or most) newly emerged males do not explain the ability of a male red-sided garter snake to continue exhibiting she-male activity for 4 wk or more after emergence (Mason and Crews 1985, 1986; R. W. K., G. Demarchi, and D. D. Baleckaitis, unpublished data).

Neural Characteristics

Recently, colleagues and I1 examined the volume of sex steroid-concentrating nuclei and the average diameter of individual neuron perikarya located within the anterior hypothalamus—preoptic area (AHPOA2) of male, female, and she-male red-sided garter snakes collected during the courtship season. The AHPOA has been shown to be a critical link in the neural pathway(s) controlling sexual behavior (Friedman and Crews 1985b, 1987a). Our data indicate that animals designated “long-term she-males” (exhibiting she-maleness >10 days) had significantly greater hypertorophy in the AHPOA than normal males and, although also larger than females, there was no significant difference in volume. The average diameter of the perikarya was also statistically greater in she-males than in males or females (Krohmer and DeMarchi, unpublished data).

Captive Maintenance and Behavior

The specific environmental conditions and physiological manipulations used in laboratory studies on red-sided garter snakes vary, depending on the study requirements and available facilities. However, since the early 1980s, many laboratories that conduct garter snake research have typically maintained animals that are not involved in studies as well as the study animals. In general, maintenance begins with weighing animals taken from the field to a laboratory, recording their snout to vent length (SVL2), and giving each

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1 In this article, subsequent discussions of the author’s studies refer to work with colleagues in the Department of Biological Sciences, Saint Xavier University, Chicago, Illinois.

2 Abbreviations used in this article: AHPOA, anterior hypothalamus preoptic area; ARO-ir, aromatase immunoreactive cells; POA, preoptic area; RSS, renal sexual segment; SVL, snout to vent length.
animal a unique clipping pattern on the ventral scutes for identification. Although animals can be housed in large groups, we believe that it is easier to assess their health when they are housed in small groups (e.g., 10-15 animals), in 10-gal glass aquaria covered with screen tops. Since about 1995, we have used bedding that is composed of paper pulp (e.g., Carefresh™ bedding, Absorption Corp., Bellingham, WA), instead of the formerly standard wood chips, to cover the floor of the aquaria.

Fresh water has always been a high priority, and it is important to provide it ad libitum for drinking and soaking. We feed fresh, canned, or frozen fish to all of the animals twice a week, and we supplement the diet with vitamins and minerals (e.g., Chirp vitamin, mineral, and protein powder, Carter-Wallace, Inc., Cranbury, NJ; and Rep-Cal, Rep-Cal Research Labs, Los Gatos, CA). We maintain the general population under a photic cycle and temperature range that approximates the natural environment (e.g., 14:10 light:dark photic cycle at 28 ± 4°C from May to August; 8:16 light: dark photic cycle at 20 ± 4°C from mid-August; low temperature dormancy 0:24 light:dark at 4°C from mid-September to April), or as specified by protocol. When an environmental chamber is available, it is possible to vary temperatures between day and night. As stated above, many variations of these general environmental conditions can be implemented according to the particular study protocol.

Finally, snakes depend on olfaction to assess their surroundings. It has been observed that snakes can appear agitated for a period of time when placed in unfamiliar surroundings. Animals appear much more comfortable in an aquarium they can recognize as containing a familiar odor. Therefore it is not beneficial to the animals to clean and sanitize aquaria while housing particular populations. Obviously, aquaria need to be cleaned periodically. Therefore, for snakes it is best to remove and replace bedding while only wiping down the aquarium with plain water. The cage will appear clean but to the snake, it will retain all the familiar odors. It is important to clean and sanitize only between populations (N. Ford, University of Texas, Tyler, TX, personal communication, 1995).

Courtship in red-sided garter snakes comprises an unmistakable set of innate behavior patterns (Table 1), which are easily quantified to analyze intensity and duration (Camazine et al. 1980). Briefly, males recognize females by using visual and olfactory cues. As a male approaches an attractive female, his investigatory tongue flicks increase (Kubie et al. 1978a,b). Once the female has been identified, the male begins to exhibit chin rubbing behavior, a behavior seen only in the context of courtship and mating. The courtship proceeds with the male traversing the female’s body and then attempting to align his body so that it is parallel to the female’s body. After alignment, the male exhibits a series of body contractions and attempts to maneuver his tail under the female in an effort to intromit (Camazine et al. 1980). Such quantifiable behaviors, the ease of maintaining a captive population, and the reproducibility of results in the laboratory and/or field make the red-sided garter snake an excellent model system for investigating the intricacies of the dissociated reproductive pattern.

### Reproductive Physiology

When the red-sided garter snake emerges from winter dormancy, the testes appear regressed. However, histological examination of the seminiferous tubule germinal epithelia revealed that hypertrophy had been initiated shortly after emergence and that primary spermatocytes comprised the dominant cell type (Krohmer et al. 1987). By mid-summer, the germinal epithelia had increased to 10 to 15 cell layers, predominately with spermatids and mature sperm, but spermatogenesis had not yet been initiated. In late summer to early fall, a few residual sperm were still being shed although the germinal epithelia had been repressed to three to four cell layers, indicating that active spermatogenesis had ended (Krohmer et al. 1987). Sperm are stored throughout the winter in the vas deferens in preparation for spring mating.

The concentration of circulating androgens increases as spermatogenesis and subsequent hormone synthesis and release progress. Testosterone and dihydrotestosterone, col-

<p>| Table 1 Graduated scale of male courtship in the garter snake, <em>Thamnophis sirtalis parietalis</em>&lt;sup&gt;a&lt;/sup&gt; |</p>
<table>
<thead>
<tr>
<th>Courtship score</th>
<th>Behavioral description</th>
</tr>
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<tbody>
<tr>
<td>0.0</td>
<td>Male either fails to investigate female or investigates female only briefly.</td>
</tr>
<tr>
<td>0.5</td>
<td>Male tongue-flicks and/or chin-rubs female after initial investigation.</td>
</tr>
<tr>
<td>1.0</td>
<td>Male’s chin is in contact with female for entire testing period or male courts female at a higher level but becomes distracted from courting.</td>
</tr>
<tr>
<td>1.5</td>
<td>Male is aligned with and covers the female’s back while moving slowly with female; male can not generally be distracted from courting.</td>
</tr>
<tr>
<td>2.0</td>
<td>Male presents all of the characteristics of 1.5 but also rapidly and repeatedly traverses the length of the female’s body and/or contractile waves along the length of his body.</td>
</tr>
<tr>
<td>2.5</td>
<td>Male behaves as 2.0 but also attempts cloacal apposition by maneuvering his body and/or tail.</td>
</tr>
<tr>
<td>3.0</td>
<td>Male achieves intromission.</td>
</tr>
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</table>

lectively, are known as androgens. However, DHT comprises such a small percentage of the total circulating androgens in reptiles that in many cases, measurement of circulating androgens directly reflects variations in testosterone synthesis and release. Initial studies that established the concept of the dissociated reproductive pattern reported that circulating androgen levels in the male red-sided garter snake declined to low or basal levels before or during winter dormancy (Camazine et al. 1980; Crews 1984; Crews et al. 1984). However, later studies demonstrated that circulating androgens elevated in the fall before entering winter dormancy remain elevated during dormancy and upon emergence (Krohmer et al. 1987; Moore et al. 2000, 2001). Krohmer et al. (1987) reported that the concentration of plasma androgens decreased rapidly in the initial 10 to 14 days after emergence from winter dormancy (Figure 3).

It is suspected that the initial finding of low or basal levels of circulating androgens were based on blood samples collected throughout the courtship season averaging greater than 10 to 14 days after emergence, which would imply that androgen levels were low. In addition, circulating androgen levels in captive maintained animals appeared to drop dramatically between completion of spermatogenesis (androgenesis) and placement into artificial dormancy. This rapid reduction of circulating androgens is most likely a product of temperatures warmer that the normal habitat at that time of the season resulting in the metabolization of androgens before hibernation (Krohmer et al. 1987). Therefore, these findings suggest that time after emergence plays a significant role in the spring androgen profile (Krohmer et al. 1987).

The renal sexual segment (RSS2) is an accessory sexual structure found in the kidneys of male squamate reptiles (Gampert 1866; Reguard and Policard 1903). To date, the function of the RSS is not completely understood. It has been suggested that secretions from the RSS may sustain and activate sperm (Bishop 1959; Cuellar 1966), form copulatory plugs (Devine 1975), or have other functions associated with seminal fluid (Prasad and Reddy 1972). The RSS exhibits distinct seasonal changes in response to variations in the concentration of circulating androgens (Bishop 1959). In the male red-sided garter snake, the RSS tubule diameter and epithelial height were hypertrophied, and the density of sexual granules within the epithelial cells was hypertrophied upon emergence. Regression of the RSS was evident only after the mating season had been completed and circulating androgens were significantly reduced. The RSS tubular diameter and epithelial height remained regressed as did the density of sexual granules during the beginning of the brief summer. By mid-summer, as spermatogenesis and the subsequent increase in circulating androgens reached a peak, the RSS began to hypertrophy, and sexual granule density increased. Hypertrophy of the RSS and production of sexual granules continued until late summer and reached maximal development when fall migration was initiated (Krohmer et al. 1987).

Neural and Hormonal Pathways That Regulate Courtship Behavior

Examinations of the neural network regulating sexual behavior in male vertebrates revealed that the AHPOA is a major integrative region for the coordination of both external and internal stimuli (Crews and Silver 1985; Ingle and Crews 1985). The AHPOA contains sex steroid-concentrating neurons and is involved in receiving and coordinating stimuli from afferent pathways of the major sensory systems. In all vertebrates that exhibit an associated reproductive pattern, lesions in the AHPOA result in the extinction of courtship behavior in adult males. This result suggests that the AHPOA plays an important role in the control of motor patterns associated with the display of courtship behavior and mating (Satinoff 1978, 1983).

In the male red-sided garter snake, the integrity of the AHPOA is critical for the activation and maintenance of reproductive behaviors (Friedman and Crews 1985b; Krohmer and Crews 1987a). Actively courting males that received lesions in the AHPOA ceased to exhibit courtship behavior (Friedman and Crews 1985b). Animals that received lesions before they entered low temperature dormancy failed to exhibit any courtship behavior upon emergence (Krohmer and Crews 1987a). Animals that received lesions primarily confined to the anterior portion of the preoptic area (POA2) exhibited atypical thermoregulatory behavior. Unlike the other animals in this study that moved toward and remained under the heat source supplied for several hours each day, animals with POA lesions regularly failed to approach the heat source (Figure 4). Initially, animals in this group also did not court attractive females; however, after a period of forced warming, most of these animals exhibited normal courtship behavior (Krohmer and Crews 1987a). Studies that have investigated the effect of

![Circulating Androgens](image)

Figure 3 Circulating androgen profiles from four distinct populations emerging at different times. Androgens, elevated upon emergence, appear to decrease rapidly reaching basal level by 10 to 14 days after emergence. Redrawn from Krohmer RW, Grassman M, Crews D. 1987. Annual reproductive cycle in the male red-sided garter snake, *Thamnophis sirtalis parietalis*: Field and laboratory studies. *Gen Comp Endo* 68:64-75.
various temperature regimens on the initiation and maintenance of courtship behavior have demonstrated that the sensitivity of the neural pathways critical for the integration of cues used to regulate courtship behavior may be increased by extending the time maintained in low temperature dormancy (Garstka et al. 1982; Krohmer and Crews 1989; Figure 5). It also appears that this increase in sensitivity may be “reset” by exposure to short periods of low temperature after the end of all courtship behavior (Krohmer and Crews 1989).

During the past decade, there have been a number of investigations on the role or roles of melatonin in the timing of reproductive events in reptiles. Although many investigations have identified the distribution of melatonin receptors in fish, amphibians, and lizards, there appears to be no information on the distribution of melatonin in the snake brain. However, in the lizard, Anolis carolinensis, melatonin receptors were observed in areas receiving primary, secondary, and tertiary visual input. These areas include the superficial layers of the optic tectum, lateral geniculate nucleus, nucleus rotundus, dorsal ventricular ridge, and the striatum (Wiechmann and Wirsig-Wiechmann 1994). Other brain regions exhibiting melatonin binding included the left medial habenular nucleus, the interpeduncular nucleus, medial and dorsal cortex, mammillary nucleus, and septum. These results suggest that melatonin receptor binding sites are widely distributed in areas of the forebrain and midbrain associated with visual processing. The highest degree of melatonin binding appeared in the left medial habenular nucleus, interpeduncular nucleus, and dorsal ventricular ridge, areas that are important targets for the actions of melatonin, affecting circadian rhythmicity, thermoregulation, and photoperiodic reproduction (Wiechmann and Wirsig-Wiechmann 1992, 1994).

Male red-sided garter snakes whose pineal glands were surgically removed before they entered an extended period of low temperature dormancy exhibited significantly impaired expression of courtship behavior upon emergence (Nelson et al. 1987). After pinealectomy, animals that exhibited a disrupted melatonin pattern did not court, whereas animals with a more typical pattern exhibited normal courtship behavior (Mendonca et al. 1996a, b). When animals were pinealectomized after courtship had ensued, the pineal gland no longer played a critical role. In addition, intact males that courted upon emergence exhibited a typical pattern of melatonin secretion, whereas noncourting animals displayed the opposite pattern of secretion (Mendonca et al. 1996a). These results demonstrated the critical nature of melatonin in the stimulation of courtship behavior and mating (Mendonca et al. 1995).

As discussed above, the red-sided garter snake requires exposure to warm temperatures after a prolonged period of cold temperature dormancy for the initiation of courtship behavior. In addition, an intact POA is essential for the integration of temperature information in the red-sided garter snake (Krohmer and Crews 1987a). For these reasons, removal of the pineal gland could possibly disrupt specific components of a temperature detection system and prevent the snake from recognizing warm temperatures upon emergence (Mendonca et al. 1996a, b; Nelson et al. 1987). Therefore, the pineal gland may mediate courtship behavior by forming a direct neural link with the POA, independent of environmental factors (Nelson et al. 1987).

In contrast, bilateral lesions in the nucleus sphericus (homolog to the mammalian amygdala) or septum before low temperature dormancy facilitates courtship behavior.
upon emergence (Krohmer and Crews 1987b; Table 2). These results suggest a central inhibitory control of sexual behavior in the male red-sided garter snake. Lesions in the nucleus sphericus or septum also had a significant effect on reproductive physiology. Animals receiving lesions in the nucleus sphericus had significantly elevated levels of circulating androgens compared with the controls. Although lesions in the septum elevated the level of circulating androgens, they were not significantly different from controls. The quantity of sexual granules (Bishop 1959) present in the RSS epithelia of either lesioned group was significantly greater than controls. There was, however, no significant difference in sexual granule density between the groups receiving lesions in either the nucleus sphericus or septum (Krohmer and Crews 1987b). To date, only lesions placed in the anterior hypothalamus preoptic area have resulted in extinguishing courtship behavior and mating (Friedman and Crews 1985; Krohmer and Crews 1987a).

The brains of all vertebrates that exhibit an associated reproductive pattern contain a similar distribution of sex steroid-concentrating neurons, which appear to be closely associated with the neural pathway that regulates courtship behavior (Crews and Silver 1985). Sex steroid-concentrating neurons that respond to both testosterone and estrogen have also been identified in the brain of the garter snake, *Thamnophis sirtalis* (Halpern et al. 1982). The locations of the sex steroid-concentrating regions in the male red-sided garter snake are similar to those described in other vertebrates that exhibit the associated reproductive pattern (Crews and Silver 1985; Krohmer et al. 2002; Pfaff et al. 1994; Rhen and Crews 1999, Skipper et al. 1993). However, in a species that exhibits a dissociated reproductive pattern, in which sex steroids do not appear to have an active role in the initiation and regulation of courtship behavior (Camazine et al. 1980; Crews et al. 1984; Friedman and Crews 1985a), the presence of sex steroid-concentrating neurons appears to be redundant.

Although several studies have demonstrated that androgens are elevated upon emergence (Krohmer et al. 1987; Moore et al. 2000, 2001; Figure 3), there is still no evidence to suggest that androgens have any direct role on the initiation of courtship behavior and mating. Nevertheless, the existence of sex steroid-concentrating neurons in the brain of male red-sided garter snakes, in association with elevated levels of circulating androgens upon emergence, may indicate that androgens perform a critical, albeit indirect, role in the control of reproductive behavior.

Castrated or hypophysectomized adult male red-sided garter snakes continue to exhibit courtship behavior and mating for at least 3 yr provided they are placed into an extended period of low temperature dormancy (Crews 1991; Crews et al. 1984). However, castrated individuals continue to have measurable concentrations of testosterone in their blood (Camazine et al. 1980). This evidence suggests that androgens of either a neural or adrenal source may be important in the initiation of sexual behavior. In water snakes (*Nerodia* spp.), adrenal glands of castrated individuals increased by an average of 57% during the active season. After castration, hypertrophy of the adrenal glands is most likely influenced by a negative feedback. The response to the dramatic reduction in circulating testosterone increases in gonadotropin-releasing, follicle-stimulating, and luteinizing hormones stimulates the adrenals to increase the production of androgens, which results in the observed hypertrophy (Krohmer 1985).

The organization and embryonic development of neural pathways in rats and ferrets depends predominantly on the action of estrogenic metabolites of circulating testosterone (George and Ojeda 1982; Krohmer and Baum 1989; MacClusky et al. 1985; Weaver and Baum 1991), rather than on a direct action of testosterone. Subsequently, in adult animals, aromatization of testosterone appears to play a key role in the activation of copulatory behavior (Vagell and McGinnis 1997). Additional behavioral studies have shown that neural aromatase activity is critical for the activation and sexual differentiation of numerous neuroendocrine and behavioral responses in a variety of species (Balthazart 1989; Maclusky and Naftolin 1981; Maclusky et al. 1984; McEwen 1981; McEwen and Krey 1984; Roselli et al. 1984).

Recently, we investigated the possible effect(s) of environmental factors in combination with sex steroids (either testosterone or estrogen) on areas identified as containing sex steroid-concentrating neurons in the red-sided garter snake (R. W. K. and D. D. Baleckaitis, unpublished data). Adult male red-sided garter snakes were castrated and implanted with a 15-mg timed release tablet of testosterone, estrogen, or a placebo (Innovative Research of America, Sarasota, FL) and maintained for 12 wk in conditions that were either warm and spring-like (25±1.5°C, 14:10 light: dark photic cycle) or of low temperature dormancy (4±2°C,

<table>
<thead>
<tr>
<th>Days</th>
<th>Sham lesion (%)</th>
<th>Septum (%)</th>
<th>Nucleus sphericus (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>0</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>4-6</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
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<td>7-9</td>
<td>13</td>
<td>40</td>
<td>27</td>
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<tr>
<td>10-12</td>
<td>42</td>
<td>93</td>
<td>87</td>
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<td>13-15</td>
<td>67</td>
<td>80</td>
<td>87</td>
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<td>16-18</td>
<td>59</td>
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<td>60</td>
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<tr>
<td>19-21</td>
<td>17</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>22-24</td>
<td>0</td>
<td>27</td>
<td>47</td>
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Table 2. Percentage of testing trials in which courtship intensity exceeded 1.0, grouped by time and treatment.
Data revealed that sex steroid-concentrating neurons in the POA exhibited greater hypertrophy when treated with estrogen than with testosterone or the placebo. In addition, all treatment groups maintained in low temperature dormancy exhibited greater hypertrophy in the sex steroid-concentrating neurons in the POA compared with their counterparts maintained under warm conditions (Figure 6).

Aromatase activity has been identified in the brain of the male red-sided garter snake, and the greatest activity is in areas containing sex steroid-concentrating neurons (Krohmer and Baleckaitis 2000). However, the specific role of the aromatase enzyme in the neural tissues of the garter snake remains unclear because sex steroids appear not to activate courtship behavior in noncourting individuals.

The exact location of aromatase activity has been difficult to evaluate before the development of antibodies, which are a very useful tool for the identification of aromatase immunoreactive cells (ARO-ir) (Foidart et al. 1995; Krohmer et al. 2002). In animals collected in the fall, we found ARO-ir cells in all regions of the garter snake forebrain, with specific concentrations in areas previously suspected of controlling reproductive behavior (Krohmer et al. 2002). Two immunochemically different ARO-ir cells were identified in all regions of the garter snake forebrain (Figure 7. Intense immunostaining of the perikarya and processes characterized the type I immunoreactive cells, whereas type II immunoreactive cells exhibited only weak to moderate immunostaining in the perikarya with little or no staining of the processes. Type II cells were more numerous than type I and were scattered throughout the entire forebrain. The distribution of type I cells was, for the most part, restricted to regions that had high levels of aromatase activity and/or areas described above as containing a dense population of ARO-ir cells. Although a few scattered type I cells were identified in all regions of the garter snake brain, a larger proportion occurred in the AHPOA, nucleus sphericus, and septum (Krohmer et al. 2002).

**Summary of Current Knowledge**

Delineation of the neural pathway(s) that control courtship and mating in the red-sided garter snake has introduced a wealth of information and a new way to investigate reproduction in a species that exhibits a dissociated reproductive pattern. The increased response of steroid-concentrating neurons in the AHPOA to sex steroids during a period of low temperature dormancy adds to our understanding of what may be occurring during low temperature dormancy, which ultimately sets up the pathway(s) critical for the initiation and maintenance of reproductive behavior. However, the discovery that estrogen appears to be more effective in stimulating hypertrophy of the sex steroid-concentrating neurons and the identification of dense populations of type

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**Figure 6** Photomicrographs of cross-sections through the POA of male red-sided garter snakes after various hormonal and temperature regimens. Regions outlined in white indicate the edge of the hypertrophy that occurred. (A) Animals implanted with timed release testosterone tablets and maintained under warm, spring-like conditions for 12 wk. (B) Animals implanted with timed release testosterone tablets and maintained under conditions of low temperature dormancy for 12 wk. Although some hypertrophy is apparent in animals maintained under ambient conditions, a prolonged period of low temperature dormancy appears to intensify the response of the sex steroid concentrating neurons to testosterone. (C) Animals implanted with timed release tablets of 17β estradiol and maintained under warm, spring-like conditions for 12 wk. (D) Animals implanted with timed release tablets of 17β estradiol and maintained under conditions of low temperature dormancy for 12 wk. The amount of hypertrophy exhibited by the animals maintained under warm conditions was not significantly different from either of the testosterone implanted group. However, animals receiving estradiol in combination with low temperature dormancy exhibited greatly hypertrophied regions of sex steroid concentrating neurons within the POA. AH, anterior hypothalamus; ot, optic tract; POA, preoptic area; III, third ventricle.

**Figure 7** Photomicrographs of the two types of aromatase immunoreactive (ARO-ir) cells in the neural tissues of the male red-sided garter snake. (A and B) Sections through the preoptic area, which contain both types of immunoreactive cells. Type I ARO-ir cells (arrows) appear to have larger, darker staining perikarya and visible processes. Type II ARO-ir cells (arrowhead) appear to have smaller perikarya and few (or no) visible processes.
I ARO-ir cells in specific regions before entering winter dormancy suggests a possible relation between aromatase activity in the garter snake brain and courtship behavior. These data further suggest that aromatase activity may remain active throughout the fall and possibly well into winter dormancy (Krohmer and Baleckaitis 2000). Estrogens produced by aromatase activity in the neural tissues associated with courtship behavior may somehow be involved in the activation of sexual behavior when the animals emerge in the spring. These findings offer a possible (albeit indirect) role for hormone involvement in the initiation and control of reproduction in the male red-sided garter snake.

Despite much progress after 3 decades of active research, the dissociated reproductive pattern of the red-sided garter snake is still a relatively complicated neuroendocrine puzzle. Future studies should examine possible changes in the seasonal distribution of ARO-ir cells. A comparative study should also be conducted to determine whether the distribution of ARO-ir cells found in the garter snake is a common feature of reptiles or a specialization in a species that exhibits a dissociated reproductive pattern. Finally, if estrogen indeed plays a role in regulating male sexual behavior, it would be very interesting and informative to examine the seasonal distribution of estrogen receptors. These suggested studies comprise some of the research necessary to delineate the ultimate control of courtship behavior and mating in the male red-sided garter snake.

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