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# Brain nuclei in actively courting red-sided garter snakes: A paradigm of neural trimorphism

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ABSTRACT

During the breeding season, two distinct male phenotypes are exhibited by red-sided garter snakes (*Thamnophis sirtalis parietalis*), with courtship behavior being directed not only toward females, but also toward a sub-population of males called she-males. She-males are morphologically identical to other males except for a circulating androgen level three times that of normal males and their ability to produce a female-like pheromone. As in other vertebrates, limbic nuclei in the red-sided garter snake brain are involved in the control of sexual behaviors. For example, an intact anterior hypothalamus pre-optic area (AHPOA) is essential for the initiation and maintenance of reproduction. To determine if brain morphology varies among the three behavioral phenotypes (i.e., males, she-males, and females) during the breeding season, we examined the volume, cell size and cell density of the AHPOA as well as a control region, the external nucleus of the optic tract (ENOT). We used Luxol Fast Blue and Ziehl's Fuchsin to visualize neurons and glial cells, respectively. No significant differences were observed among the three behavioral phenotypes in the volume, cell size or density in the control region. In contrast, the volume, cell size and density of the AHPOA of she-males were significantly greater than those of both male and female snakes. While the volume of the AHPOA was significantly greater in females compared to males, no differences were observed in cell size or density. These differences in brain morphology suggest a possible underlying mechanism for phenotypic-specific behavioral patterns.



## 1. Introduction

At the northern limit of the species' range, male red-sided garter snakes (*Thamnophis sirtalis parietalis*) emerge from low temperature dormancy (LTD) en masse, while females emerge singly or in small groups. As a result, females are actively pursued by a large number of males, creating a pile of snakes referred to as a "mating ball". Upon emergence, attractive females possess a non-volatile, lipid-based pheromone on their dorsal and lateral surfaces. Newly emerged adult males utilize this pheromonal cue to determine a female's attractivity, which initiates courtship behavior and mating [1–3]. Courtship in the red-side garter snake is a collection of stereotypic behaviors that can be easily quantified to analyze intensity and duration [4]. Briefly, males recognize females using visual and olfactory cues. As a male detects an attractive female, its tongue-flick rate increases. Once recognized, males exhibit chin rubbing behavior, a behavior exhibited only in the context of courtship and mating. As courtship proceeds, the male travels along the female's body in an attempt to align his body parallel to the female. After alignment, the male initiates muscle contractions and tail searching behavior, in which the male attempts to maneuver his tail under the female to achieve intromission [4].

These robust behaviors have been used successfully to define the parameters of the neural pathways controlling reproductive behaviors in the red-sided garter snake. Similar to other vertebrate species, the integrity of the anterior hypothalamus pre-optic area (AHPOA) in the male red-sided garter snake is critical for the activation and maintenance of courtship and mating [5,6]. Lesions placed in the AHPOA following emergence from [5] or prior to LTD [6] extinguished all courtship behavior, while bilateral lesions in the septum or nucleus sphericus prior to LTD facilitated courtship behavior [7].

Interestingly, male courtship behavior is sometimes directed toward a subset of the male population that appears to mimic sexually attractive females [1,2]. These female mimics, or "she-males", have been described as morphologically and physiologically identical to other males except for two rather unique exceptions. First, she-males have been found to produce a female-like pheromone that

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attracts normal males, resulting in misdirected courtship efforts. Second, the level of circulating androgens in she-males has been reported to be as much as three times greater than levels found in normal males [8].

Differences in plasma androgen concentrations between male and she-male snakes are particularly intriguing because red-sided garter snakes exhibit a dissociated reproductive pattern, mating at a time when spermatogenesis and steroidogenesis are inactive and sex steroid hormones are reportedly low [9,10]. Although subsequent research has shown that sex steroid hormone levels can be elevated at the beginning of the breeding season [11–13], initiation of courtship behavior and mating in this species appears to be independent of sex steroid hormones [14–16].

Moore [17] proposed the relative plasticity hypothesis as a basis for understanding differences in behavioral and morphological characteristics observed among alternate male phenotypes. This hypothesis proposes that fixed differences between alternate phenotypes are due to organizational actions of steroid hormones, whereas more plastic differences are due to activational influences of these hormones. Subsequently, several model systems exhibiting specific, quantifiable examples of dimorphism linked to a behavior or behaviors that differ either between or within the sexes have been used to better understand sex differences in the brain [18–20].

The existence of two distinct male behavioral phenotypes in the red-sided garter snake offers a unique opportunity to further examine the biological principles that cause natural variation in brain structure and function within the same sex. Therefore, the purpose of this study was to examine the forebrains of male, female and she-male red-sided garter snakes during the breeding season to determine possible morphological differences within the pathways regulating courtship behavior and mating. Of specific interest is the AHPOA, a known sex steroid concentrating region [21] and critical component of the pathway regulating courtship behavior and mating [5,6]. Halpern et al. [21] describes this region as consisting of a cell-dense POA, confluent with the anterior portion of the hypothalamus that is composed of the dorsal, intermediate and ventral nuclei. In addition, the volume of the external nucleus of the optic tract (ENOT), an area not associated with reproduction in the red-sided garter snake, was evaluated as a control region.

## 2. Materials and methods

### 2.1. Animals and tissue collection

Animals were collected during the breeding season in the spring of 2001 from dens located in the Interlake Region of Manitoba, Canada. On days when animals were actively courting, mating balls were examined to determine if the animal being courted was an attractive female or male. Suspected she-males, actively courting males and attractive females collected on the same day were returned to the field lab in Chatfield, Manitoba. Snakes were placed in outdoor testing arenas [12,13], maintained under natural conditions and tested for courtship behavior (males) and attractivity (females and she-males) daily for three consecutive days. Only males that continued to exhibit intense courtship behavior and females and she-males that remained attractive to courting males for all three testing days were used in this study.

All brains (n = 8/group) were collected within 2 h of the final courtship trial. Briefly, animals were weighed, measured, and then deeply anesthetized with an overdose of 1% Brevital Sodium (0.0015 μl/kg body weight) [22]. Once anesthetized, the heart was exposed and 0.5 ml of 1% heparin (Sigma) was injected into the ventricle. Animals were perfused through the heart with cold saline until the return flow was clear (~100 ml) followed by approximately 150 ml cold 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Brains were removed from the cranium, cryoprotected in phosphate buffer containing 20% sucrose overnight at 4 °C, snap frozen on dry ice and stored at −70 °C until sectioned.

This study was conducted in accordance with the guidelines adopted by the Saint Xavier University Institutional Animal Care and Use Committee (IACUC). The Saint Xavier University IACUC adheres to the principles set forth by NIH and the PHS policy on Humane Care and Use of Laboratory Animals.

### 2.2. Histology and data collection

Brains were cut coronally at a thickness of 30 μm on a Leitz 1720 cryostat. All sections were collected directly onto gelatin-coated slides in the order of sectioning (anterior to posterior) and allowed to dry overnight. Tissues were stained with Luxol fast blue, resulting in myelinated nerve fibers and the phospholipids in the cell membrane appearing blue [23,24]. The tissues were then counter stained with Ziehl's Fuchsin, rendering glial and other support cells a reddish-purple. Tissues were then dehydrated in progressive alcohols, cleared in xylene and cover slipped using the Permount (Fisher) covering medium.

It should be noted that the stains we used to differentiate between neurons and glial cells do not provide absolute specificity. Therefore, in addition to differential staining, morphological criteria were used to distinguish neurons from glial cells. Thus in the most conservative approach, the results presented here represent the morphological analysis of cells with an apparent neuronal phenotype. For ease of communication, we simply refer to these cells as neurons throughout the manuscript. Future studies using immunohistochemistry for specific cell markers (i.e., NeuN) would be helpful in confirming the phenotype of these cells.

In the garter snake brain, the anterior boundary of the POA begins as the optic tracts extend laterally from the optic chiasm, corresponding with the initial appearance of the third ventricle (Fig. 1). As the optic tracts cut dorsally into the brain the interface of the POA and anterior hypothalamus (AH) is indicated by the appearance of the stria medullaris at the dorsal end of the optic tracts. At its caudal boundary the AHPOA becomes continuous with the bed nucleus of the stria terminalis (Bst) [25,26] and terminates at the anterior aspect of the supraoptic nucleus and rostrolateral hypothalamic area [21].

Similar to animals exhibiting an associated reproductive pattern, specific regions (nuclei) within the brain of red-sided garter snakes have been shown to concentrate sex steroid hormones [21]. These regions can be distinguished visually by morphological differences in the size of the region of interest as well as the number and density of neurons within these regions [27, Fig. 2]. Therefore, the AHPOA and external nucleus of the optic tract (ENOT) were assessed visually by comparison with the surrounding tissues. All sections comprising the rostrocaudal extent of the AHPOA from each brain were examined with a Nikon Labphot-2 light microscope fitted with a camera lucida. Using the camera lucida, the volume of each of the two regions was estimated by tracing the areas of interest in both hemispheres. All slides were coded to circumvent possible bias and each animal was drawn independently by two observers.

The area of each outlined region was determined by overlaying a grid containing units of area standardized to the magnification of the drawings (40×) and summing the number of squares contained within the outline. Using standard stereological procedures, partial boxes were not counted on the upper and left extent of the outline while partial boxes were counted on the lower and right side [28]. The volume of each section was computed by multiplying the area outlined by the thickness of the section (30 μm). The total volume of each brain nucleus could then be calculated by summing the volumes of all sections [6,27].

For each animal, the diameters of 25 and 10 neuronal cell bodies within the AHPOA and ENOT, respectively, were measured using a