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# Seasonal aromatase activity in the brain of the male red-sided garter snake

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ABSTRACT

We investigated regional and seasonal variations in neural aromatase activity (AA), the enzyme that converts androgens into estrogens, to examine a possible indirect role of testosterone (T) in mediating spring reproductive behavior of red-sided garter snakes, a species exhibiting a dissociated reproductive pattern. Neural AA in male snakes varied significantly among brain regions. Additionally, there were significant interactions between brain region and season. In the spring, actively courting males had greater AA in the olfactory region (O) compared to the septum/anterior-hypothalamus preoptic area (S/AHPOA), nucleus sphericus (NS) and midbrain (Mb). Fall animals collected as they returned to the den prior to winter dormancy had significantly greater AA in the S/AHPOA compared to all other regions. These findings were consistent using either regional (gross) dissection or punch microdissection, which allowed us to separate the S and AHPOA. There were no significant differences in AA production between the S and AHPOA. This study provides the first documentation of seasonal and regional variations in AA in a snake brain and suggests that aromatization of androgens may play a role in regulating reproduction in red-sided garter snakes. During spring mating, elevated AA in the O may activate pathways essential for detection of courtship pheromones, while increased AA in the S and AHPOA of fall animals suggests that circulating androgens play an indirect role in programming critical neural pathways involved in reproduction. Thus, as in many other vertebrates, estrogenic metabolites of testosterone may be a critical hormonal component regulating reproductive behavior in this dissociated breeder.



## Introduction

The majority of seasonally breeding vertebrates display an associated reproductive pattern where gamete maturation and elevated levels of sex steroid hormones immediately precede or coincide with the breeding season (Crews et al., 1984; Licht, 1984). In species exhibiting this type of associated reproductive pattern, castration eliminates reproductive activity while administration of exogenous sex steroids restores sexual activity in castrates and will initiate courtship in non-breeding individuals (Crews, 1991). In contrast, a small number of vertebrate species including some turtles, snakes and bats exhibit a dissociated reproductive pattern, mating at a time when their gonads are inactive and circulating levels of sex steroid hormones are reported to be low (see reviews in Licht, 1984 and Woolley et al., 2004). In these species, castration does not eliminate sexual activity, nor does the administration of exogenous sex steroids initiate reproductive behavior in non-courting individuals (Woolley et al., 2004).

The red-sided garter snake (*Thamnophis sirtalis parietalis*) is a well-studied example of a species exhibiting a dissociated reproductive pattern, mating at a time when the gonads are quiescent. Subsequently, spermatogenesis and steroidogenesis are not initiated until the breeding season has ended, with sperm being stored during winter dormancy in the ductus deferens until the following spring mating period.

In the adult male red-sided garter snake, initiation of courtship behavior and mating has been reported to be independent of testicular or pituitary hormone control (Camazine et al., 1980). Systemic administration of sex steroids, hypothalamic or pituitary hormones, or implantation of sex steroid hormones directly into the hypothalamic region fails to induce reproductive behavior in non-courting individuals (Camazine, et al., 1980; Crews et al., 1984; Friedman and Crews, 1985a). Moreover, males continue to exhibit courtship behavior for up to 3 years following castration (Crews, 1991).

Initial studies reported the level of circulating androgens to be low or absent upon emergence from winter dormancy (Garstka et al., 1982). However, subsequent investigations found that circulating androgens, elevated in the fall prior to winter dormancy, remain elevated throughout low temperature dormancy (LTD; Krohmer et al., 1987; Lutterschmidt and Mason 2009) and are not basal upon emergence in the spring (Krohmer et al., 1987; Moore et al., 2000,

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2001). More recently, Lutterschmidt and Mason (2009) demonstrated that androgen concentrations are elevated during the fall and decline significantly faster in snakes hibernated at warmer temperatures (i.e., 10 versus 5 °C). Thus, androgen concentrations appear to decline during winter dormancy via metabolic clearance. These results indicate that the observed annual variation in spring androgen levels may be related to a variation in environmental conditions, particularly temperature profiles (Lutterschmidt and Mason, 2009). To date, the only known stimulus capable of initiating courtship behavior in the red-sided garter snake is a prolonged period of LTD (Camazine et al., 1980; Garstka et al., 1982; Bona-Gallo and Licht, 1983; Whittier et al., 1987). Similar to the suggestions of Crews (1991) and Saint Girons et al. (1993), our current hypothesis is that elevated androgen concentrations during the fall and winter dormancy period, in combination with low temperature exposure, induce the changes in neuroanatomy and neurophysiology necessary to elicit reproductive behavior in the spring (Krohmer et al., 1987; Lutterschmidt and Mason, 2009).

Studies of the neural pathways regulating sexual behavior in male vertebrates have shown that the anterior-hypothalamus preoptic area (AHPOA) is a major integrative region for the coordination of internal and external stimuli (Crews and Silver, 1985; Ingle and Crews, 1985). These neural pathways contain sexually dimorphic sex steroid concentrating nuclei (regions) in the AHPOA, bed nucleus of the stria terminalis (BNST), nucleus sphericus (NS), septum (S) and ventromedial nucleus of the amygdala (AMY) (Tobet et al., 1986; Cherry et al., 1990; Aste et al., 1993; O'Bryant and Wade, 2002; Beck et al., 2008). Moreover, both androgens and estrogens can cause hypertrophy of these sex steroid concentrating nuclei (Balthazart and Adkins-Regan, 2002; Panzica et al., 1996). Similar to species exhibiting an associated reproductive pattern, the AHPOA also plays an important role in regulating reproduction of male red-sided garter snakes. Lesions placed in the AHPOA of male snakes eliminates all courtship behavior (Friedman and Crews, 1985b; Krohmer and Crews, 1987a), while lesions limited to the anterior portion of the preoptic area (POA) affect thermoregulatory capabilities but do not automatically affect courtship behavior and mating (Krohmer and Crews, 1987a). In the male red-sided garter snake, the neural regions that comprise the pathways regulating reproductive behavior (i.e., POA, NS, S and hypothalamus) also contain sex steroid concentrating nuclei (Halpern et al., 1982) that are responsive to both androgens and estrogens (Baleckaitis and Krohmer, unpublished data). However, unlike the majority of seasonally breeding vertebrates, where sex steroids play a critical role in the initiation of courtship behavior, the role of sex steroid concentrating nuclei in an animal exhibiting a dissociated reproductive pattern remains unknown, as does the importance of sex steroid hormone metabolism within these neural regions.

One component of sex steroid hormone metabolism critical to regulating sexual behavior in associated breeders is aromatase, the enzyme that catalyzes the conversion of androgens to estrogens. Since the initial characterization of aromatase in the brains of several vertebrate species (Naftolin et al., 1975), aromatase activity has been found in all major vertebrate groups (Callard et al., 1978a,b). The importance of estrogens in the control of reproductive behavior has been investigated in great detail in both mammals and birds (e.g., Balthazart et al., 2009; Wallen and Baum, 2002). Specifically, it has been shown that aromatization of testosterone (T) in the POA can mediate the activation of many aspects of reproductive behavior in a variety of vertebrate species (e.g., Balthazart, 1989; Ball and Balthazart, 2002, 2004; Baum, 2003; Naftolin et al., 1997; Wallen and Baum, 2002). However, only a few studies have examined the role of aromatase in the control of courtship behavior and mating in reptiles (Callard, 1983; Beck and Wade, 2009a,b; Rosen and Wade, 2001; Winkler and Wade, 1998; Wade, 1997). Using an antibody developed for quail (QR1; Foidart et al., 1995), Krohmer et al. (2002) documented that aromatase enzyme is present in all regions of the male red-sided garter snake forebrain. This study identified aromatase enzyme in two morphologically distinct cell types. Type II neurons, characterized as small neurons with a weakly staining cell body and few, if any, visible processes are found scattered throughout the entire forebrain. In contrast, the large, deeply staining Type I neurons are concentrated in areas containing sex steroid concentrating nuclei that are associated with the regulation of courtship and mating (Halpern et al., 1982; Krohmer et al., 2002). Thus, elevated circulating levels of testosterone during fall and winter dormancy, in association with sex steroid concentrating nuclei within the pathways that control courtship behavior and mating, suggest that testosterone may play a role in regulating reproduction indirectly through its neural aromatization to estrogens. To better understand the importance of neural sex steroid hormone metabolism to dissociated breeders, we examined the seasonal variation in aromatase activity (AA) among different regions of the male red-sided garter snake forebrain.

## Material and methods

### *Animal and tissue collection*

Male red-sided garter snakes (*T. sirtalis parietalis*) were collected from dens located in the Interlake Region of Manitoba, Canada during the spring mating season and in the fall as animals were returning to the dens in preparation for winter dormancy (Experiment 1, $n = 10$/season; Experiment 2, $n = 24$/season). All animals were returned to the field lab in Chatfield, Manitoba and, when possible, processed within 4 h or maintained in outdoor testing arenas (Moore and Mason, 2001) under natural conditions for no more than 5 days. Briefly, the snout-vent length (SVL) and body mass of each animal was measured and a lethal dose of sodium brevital (methohexitol) was administered (Jones Pharma Inc., St. Louis, MO, Wang et al., 1977). Once anesthetized, the heart was exposed, 0.2 ml of 1% heparin (Sigma, St. Louis, MO) was injected into the ventricle and animals were perfused through the heart with cold buffered saline (pH 7.2) until the return flow was clear (approximately 100 ml). Following perfusion, the brain was removed from the cranium, cryoprotected in 20% sucrose in 0.1 M phosphate buffer solution (pH 7.2) at 4 °C overnight, snap frozen on dry ice and stored at −70 °C until processed. 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. This study was conducted in accordance with the guidelines adopted by the Saint Xavier University Institutional Animal Care and Use Committee (IACUC).

### *Brain dissections*

#### *Experiment 1. Regional dissection*

Brains ($n = 10$/season) were removed from −70 °C, placed on a glass plate seated over ice, allowed to warm slightly to prevent shattering and dissected into four regions: olfactory (O), NS, septum/anterior-hypothalamus preoptic area (S/AHPOA), and midbrain (Mb) (Fig. 1A). All neural tissue anterior to the optic chiasm was identified as O. Subsequently, the optic chiasm formed the anterior-most extent of the S/AHPOA while the blood sinus containing the pineal gland at the junction of the telencephalon and midbrain marked the posterior border (Halpern, 1980; Krohmer and Crews, 1987a,b; Krohmer et al., 2002). The border between the S/AHPOA and NS was distinguished by the lateral extent of the optic tracts. Each region was placed into a separate conical tube containing cold stabilizing medium (50 mM potassium phosphate, 0.1 mM EDTA, 20% glycerol and 1.0 mM dithiothreotol, all purchased from Sigma, St. Louis, MO, USA) and maintained on ice until assayed.

#### *Experiment 2. Specific area punch microdissection*

Specific area punch microdissection of the brains was performed using a modification of the procedure of Palkovitz (1973). The specific

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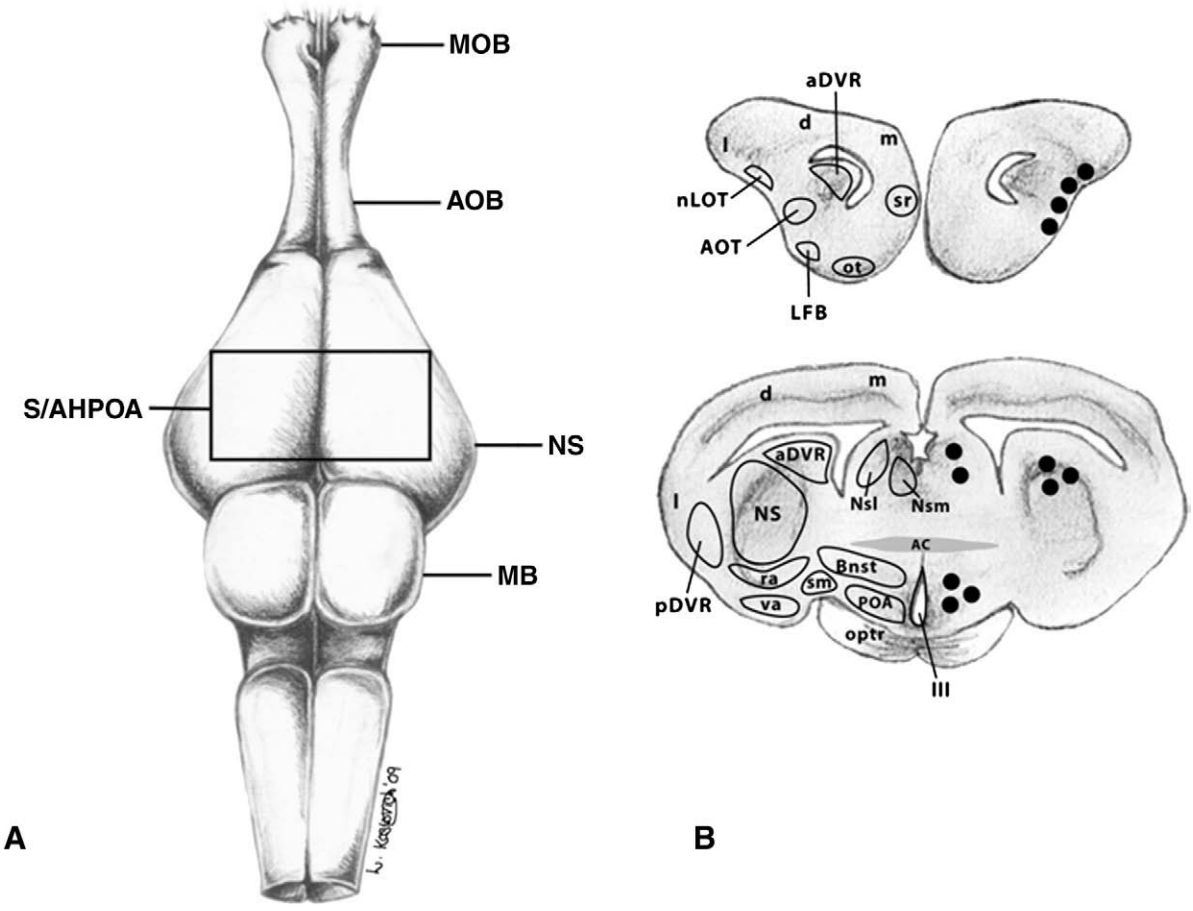


**Fig. 1.** Schematic drawings of the snake brain. (A) Dorsal view of red-sided garter snake brain depicting the regional dissections used in Experiment 1. (B) Coronal sections illustrating the specific area punch microdissections used in Experiment 2. Upper figure depicts a section at the level of the accessory olfactory area; lower figure depicts a section at the level depicting the AHPOA, S and NS. While only one punch was taken from each region/snake, the multiple circles show the variation of punch placement within each region. Drawing by L. Kostovich, Undergraduate Research Program in Biology. *Abbreviations*: III, third ventricle; aDVR, anterior dorsal ventricular ridge; AHPOA, anterior-hypothalamus preoptic area; AC, anterior commissure; AOB, accessory olfactory bulb; AOT, accessory olfactory tract; Bnst, bed nucleus of the stria terminalis; d, dorsal cortex; l, lateral cortex; LFB, lateral forebrain bundle; m, medial cortex; MOB, main olfactory bulb; nLOT, nucleus of the lateral olfactory tract; NS, nucleus sphericus; Nsl, lateral septal nucleus Nsm, medial septal nucleus; optr, optic tract; olfactory tubercle; pDVR, posterior dorsal ventricular ridge; POA, preoptic area; ra rostral amygdaloid nucleus; sm, strial medullaris; sr, rostral septal nucleus; va, ventral amygdaloid nucleus.

area punch microdissection has been found to minimize the inclusion of non-aromatase containing tissues (Palkovits and Brownstein, 1983; Roselli et al., 1985; Roselli, 2007) and was utilized in this study to assess AA within suspected sex steroid concentrating nuclei.

Brains ($n = 24$/season) were removed from −70 °C and mounted rostral side up on a cryostat chuck seated on dry ice using Histo Prep Frozen Tissue Embedding Media (Fisher Scientific, Fair Lawn, NJ). The mounted brains were transferred to a Leitz 1720 cryostat (Germany), allowed to warm to −15 °C, sectioned at 200 μm and placed on microscope slides. The slides were warmed slightly to affix the tissue sections, placed into a humidity controlled chamber and stored at −70 °C until tissue punches were collected.

Punches were collected from tissue sections placed on top of an ice chamber and viewed with a dissecting microscope that was placed inside the cryostat set at −30 °C. Punch microdissection allowed for the separation of the S and AHPOA. Tissue punches were taken from areas previously shown to contain high concentrations of aromatase-immunoreactive (ARO-ir) cells (Krohmer et al., 2002). Using the optic chiasm, optic tracts, third and lateral ventricles as landmarks, tissue punches were collected from the regions of the AHPOA, NS, O and S (Fig. 1B) using a modified 20-guage needle fitted with a stylet. No tissue was collected from the Mb. Once the punches were collected, all tissue sections were stained in luxol-fast blue and examined with a Nikon Labphot-2 light microscope to verify the accuracy of punch placement.

To ensure enough tissue was collected from each region to carry out the assay, punches from four animals were pooled to make up each of the regions assayed. Therefore, for each region $n = 6$ samples per season, with each sample comprised of a single brain punch collected and pooled from four independent snakes (for a total of 24 snakes collected per season for this part of the study). The combined tissues were placed into labeled conical vials containing a cold stabilizing medium (50 mM potassium phosphate, 0.1 mM EDTA, 20% glycerol, and 1.0 mM dithiothreotol) and stored at −70 °C until assayed.

### *Aromatase assay*

The aromatase assay developed for use in the red-sided garter snake is a modification of Canick and Ryan (1976), Tobet et al. (1985) and Krohmer and Baum (1989). 1,2,6,7-[$^3$H] androst-4-ene, 17-dione (graciously supplied by Amersham Pharmacia Biotech, Piscataway, NJ) was purified by thin layer chromatography immediately prior to use. Unlabeled steroids were purchased from Steraloids (Wilton, NH, USA).

Brain tissues were removed from the stabilizing medium and homogenized on ice in a Teflon-glass tissue grinder containing cold 0.1 M potassium phosphate buffer, pH 7.4. All homogenates were maintained on ice until assay initiation. Incubation was initiated by

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adding 200 µl of the homogenized tissue to glass tubes containing 200 picomoles (pmol) 1,2,6,7-[$^3$H] androst-4-ene, 17-dione (specific activity = 5 Ci/mmol, final concentration = 0.5 µM) and 200 µl of 0.1 M potassium phosphate buffer (pH 7.4). Two mM NADPH cofactor (Sigma, St. Louis, MO, USA) was added immediately prior to use. Background counts were determined by incubating tubes containing 200 pmol 1,2,6,7-[$^3$H] androst-4-ene, 17-dione, buffer and cofactors but no tissue. The tubes were incubated and shaken gently for 90 min at 37 °C in a water bath (determination of optimal assay conditions is described in the following section). The reaction was terminated by adding 400 µl of methanol to each tube.

The homogenized tissue was pelleted by centrifugation (10 min @ 4000 RPM) and the supernatant transferred to a clean tube. Proteins were solubilized with 1 N NaOH and stored at 4 °C until protein determination. Aliquots of the supernatant (50 µl) were spotted in duplicate onto the preabsorbent region of silica gel G thin layer chromatography (TLC) plates (Analtech, Newark, DE) with 50 µl of carrier steroids (25 µg each of 17β estradiol and estrone in 1 ml absolute ETOH) placed on top. The loaded TLC plates were run three times in acetone to advance the steroids to the edge of the preabsorbent region and two times in benzene:heptane:ethyl acetate (5:2:3). The plates were allowed to dry completely between successive runs. The carrier steroids and substrate products were visualized using iodine vapor. The bands containing estrone were scraped into 5 ml scintillation vials and the silica gel was inactivated with 400 µl of distilled water. Toluene-based liquid scintillation cocktail was added to each vial, which were then vortexed and counted for 20 min in a Beckman LS 1800 liquid scintillation counter. A representative number of bands containing the substrate ($^3$H-androstenedione) and estradiol product were also collected and counted. While a large number of counts remained in the substrate bands, no band containing 17β estradiol scrapings exceeded background counts.

Protein content of each sample was determined by the method of Lowry et al. (1951). Aromatase assay results are expressed as pmol product formed per milligram (mg) protein per 90 min. For each experiment, all samples were run in a single aromatase assay to eliminate the potential for interassay variation.

### *Determination of optimal aromatase assay conditions*

While published accounts of aromatase assays conducted in reptiles report varying incubation times, aromatase assays in snakes (Callard et al., 1977) and lizards (Wade, 1997) reported 37 °C as their incubation temperature. The optimal conditions for assaying neural tissues of the red-sided garter snake were determined by examining varying concentrations of whole brain homogenates over a wide range of incubation times and temperatures. The amount of brain tissue used in this assay was found to be optimized at 200 µl of homogenate. While estrone production could be detected at as low as 50 µl, estrone production reached a maximum level at 200 µl and remained level at 250 µl. We also measured estrone production at varying temperatures (i.e., 15, 20, 25, 37 °C) and incubation times (i.e., 10, 30, 45, 60, 90 and 120 min) to determine the optimal assay temperature and incubation time for red-sided garter snake tissue.

At temperatures below 20 °C, no labeled estrone, aromatized from the substrate (1,2,6,7-[$^3$H] androst-4-ene, 17-dione), could be measured after a 120 min incubation. At temperatures between 25 and 30 °C, estrone could be detected after 120 min, but at levels only slightly higher than blank controls. At 37 °C, estrone production could be measured even after a 10 min incubation (Fig. 2). At this incubation temperature, estrone production varied significantly with incubation time ($F = 5.78$; $df = 5$; $p = 0.0012$, results from a one-way ANOVA followed by a Tukey's multiple comparisons test). Estrone production was significantly greater ($p < 0.002$) at incubation times of 90 and 120 min compared to incubations ranging from 10–60 min. No significant difference ($p = 0.93$) was found between 90 and 120 min incubations, suggesting the assay reaches maximum production at approximately 90 min. Therefore, incubation of tissues at 37 °C for 90 min was determined as the optimal assay conditions, which were subsequently used for all assays conducted in this study. However, it should be noted that the body temperature set-point of red-sided garter snakes may vary seasonally, and it is therefore possible that some of the seasonal variations in aromatase activity reported here may be due to seasonal differences in body temperature preferences. Future studies are needed to determine whether the temperature response curve for aromatase activity varies seasonally.

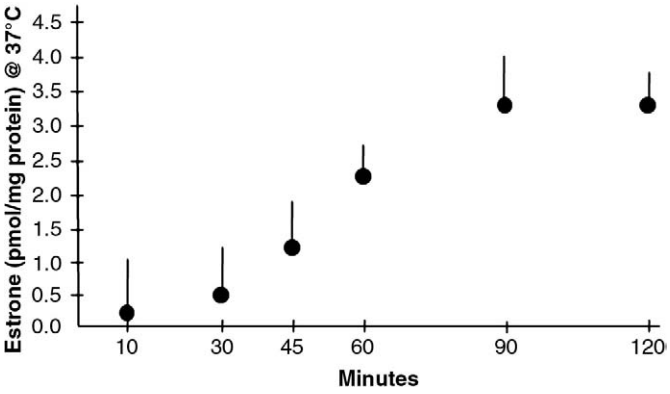


**Fig. 2.** Time course of aromatase enzyme activity in AHPOA homogenates incubated at 37 °C.

### *Assay verification*

The end product of the assay (estrone) was validated and confirmed by recrystallization and gas chromatography/mass spectrometry following methods described by LeMaster and Mason (2002), Mason et al. (1990), Rosen and Wade (2001), and Wade (1997). Recovery efficiency was determined by the addition of a known quantity of [$^3$H] estrone to tubes processed in parallel. Estrone obtained by TLC of tubes containing the known quantity of [$^3$H] estrone exhibited a level of recovery of 72.1%. Estrone obtained by TLC of our samples exhibited a level of purity greater than 90% as demonstrated by ether extraction and recrystallization of pooled samples to a constant specific activity using ethanol and water (Tobet et al., 1985; Rosen and Wade, 2001; Wade, 1997). The presence of estrone in the TLC extracts was further confirmed using a Hewlett Packard 5890 Series II gas chromatograph fitted with a split injector (280 °C) and a Hewlett Packard 5971 Series mass selective detector. Aliquots (1 µl) of the extract were injected onto a fused-silica capillary column (HP-1; 12 m × 0.22 mm ID; Hewlett Packard) with helium as the carrier gas (5 cm/s). Oven temperature was initially held at 70 °C for 1 min, increased to 210 °C at 30 °C/min, held at 210 °C for 1 min, increased to 310 °C at 5 °C/min, and finally held at 310 °C for 5 min. The candidate estrone peak was characterized by comparison to the estrone peak in the published database included in the ChemStation software (Version B.02.05; Hewlett Packard). In addition, the gas chromatographic retention time and mass spectrum of the candidate estrone peak were identical to those of a synthetic estrone sample (Steraloids).

### *Statistics*

For each experiment, we analyzed estrone concentrations using a two-way analysis of variance (ANOVA) with season (2 levels) and region (4 levels) as between-subjects factors. Both analyses were followed by a Tukey's pair-wise multiple comparisons procedure. Similar to the methods of Gonçalves et al. (2009), we used multifactor

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ANOVAs because these analyses permitted us 1) to compare statistically aromatase activity among the different brain regions examined and 2) to examine possible interactions between brain region and season. However, aromatase activity among the different brain regions examined may be related (i.e., not independent) within an individual. Thus, for comparison purposes we also performed multivariate analyses (MANOVA) for each experiment using brain region as the dependent variable and season as the between-subjects factor. We used SigmaStat® 3.5 (Systat, 2006) and SPSS® 17.0 (SPSS, 2008) for all statistical analyses. Data were natural log-transformed where necessary to correct for non-normality. The significance level for all statistical tests was set at $p<0.05$. All group data are reported as mean ± 1 standard error.

## Results

### *Experiment 1. Regional dissection*

There were significant main effects of brain region ($F_{3,72}=38.88$, $p<0.001$) and season ($F_{1,72}=24.73$, $p<0.001$) on AA (quantified as estrone production) in male red-sided garter snakes (results from a two-way ANOVA). In addition, we observed a significant interaction between brain region and season on estrone concentration ($F_{3,72}=87.14$, $p<0.001$; from a two-way ANOVA). Thus, we present here the results of statistical comparisons occurring within each level of a factor (i.e., the effect of brain region within each level of season and the effect of season within each brain region). In addition, the results of the multivariate ANOVA for the between-subjects factor season were identical to those of the two-way ANOVA. Thus, for consistency and completeness, all results reported here are from the two-way ANOVA followed by a Tukey's multiple comparisons procedure.

Within the fall season, the S/AHPOA had significantly higher aromatase activity (AA) than the other brain regions (O, $q=14.604$, $p<0.001$; Mb, $q=14.07$, $p<0.001$; NS, $q=15.12$, $p<0.001$; from a Tukey's test; Fig. 2). There were no significant differences in AA between the O, Mb, and NS during the fall. In the spring, the O had significantly higher estrone production than the other brain regions (S/AHPOA, $q=17.728$, $p<0.001$; NS, $q=17.12$, $p<0.001$; Mb, $q=16.09$, $p<0.001$; from a Tukey's test; Fig. 2). No other comparisons between regions within the spring season were significant.

Lastly, within each brain region, we observed significant differences in AA between the fall and spring seasons. The S/AHPOA was the only area with higher estrone concentrations in the fall than in the spring ($q=12.42$, $p<0.001$; from a Tukey's test; Fig. 2). All other regions had significantly higher estrone concentrations in the spring (O, $q=19.90$, $p<0.001$; Mb, $q=3.28$, $p=0.023$; NS, $q=3.30$, $p=0.022$; from a Tukey's test; asterisks in Fig. 2).

### *Experiment 2. Specific area punch microdissection*

Similar to the results of Experiment 1, there was a significant main effect of brain region on estrone concentration ($F_{3,47}=13.44$, $p<0.001$; results from a two-way ANOVA). The main effects of season were not statistically significant ($F_{1,47}=0.514$, $p=0.477$), but a significant interaction between season and brain region was present ($F_{3,47}=35.25$, $p<0.001$; results from a two-way ANOVA; Fig. 3).

In the fall, the AHPOA had higher concentrations of estrone than the O ($q=10.57$, $p<0.001$) and NS ($q=10.29$, $p<0.001$), but there was no difference in estrone production between the AHPOA and S ($q=2.87$, $p=0.193$; all results from a Tukey's multiple comparisons test). In the spring, the O had higher concentrations of estrone than the AHPOA ($q=8.69$, $p<0.001$), S ($q=8.00$, $p<0.001$), and NS ($q=17.12$, $p<0.001$).

Although the main effects of season were not statistically significant, the seasonal changes in AA varied significantly with region (i.e., there was a significant region × season interaction). Similar to Experiment 1, within the factor brain region we observed significantly higher AA in fall-collected animals in both the AHPOA ($q=8.474$, $p<0.001$) and S ($q=4.908$, $p=0.001$; results from a Tukey's test; Fig. 3). In contrast, the O had significantly higher AA in the spring ($q=10.757$, $p<0.001$). There was no significant seasonal difference in AA in the NS. The results reported above for the between-subjects factor season from the two-way ANOVA followed by a Tukey's multiple comparisons test are identical to those of the multivariate ANOVA (Fig. 4).

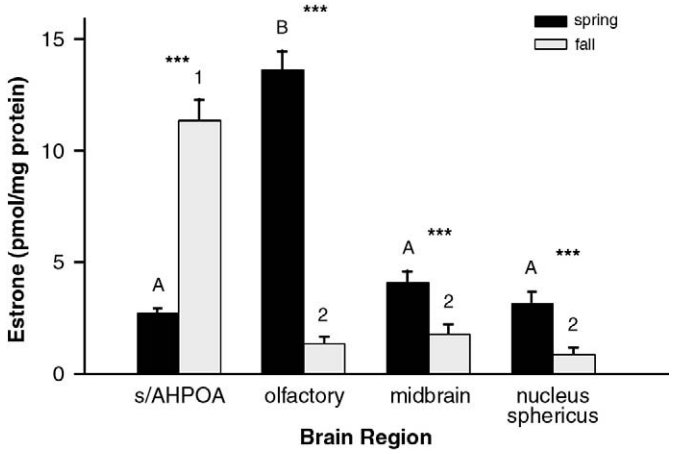


**Fig. 3.** Aromatase activity, as measured by estrone production (pmol/mg protein; mean + 1 SEM), of regionally dissected brain areas of male red-sided garter snakes in fall (grey bars; $n=10$) and spring (black; bars; $n=10$). Asterisks indicate significant differences between spring and fall estrone concentrations within each brain region. Numbers represent statistical differences in estrone production among regions within the fall, while capital letters represent statistical differences among regions during the spring. Estrone concentrations were significantly higher during the spring for all brain regions except the AHPOA, which had significantly higher estrone levels in the fall. In the fall, estrone levels were highest in the AHPOA. Within the spring, estrone levels were highest in the olfactory region. All statistical comparisons are from a two-way ANOVA followed by a Tukey's multiple comparisons test.

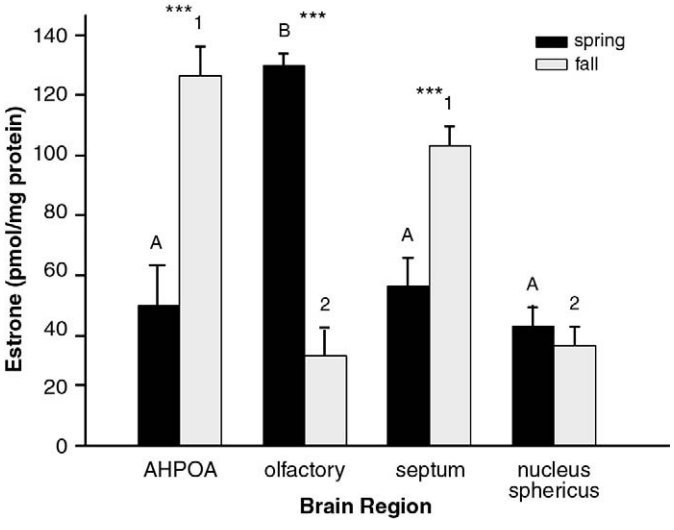


**Fig. 4.** Aromatase activity, as measured by estrone production (pmol/mg protein; mean + 1 SEM) of punch microdissected brain areas of male red-sided garter snakes in fall (grey bars) and spring (black bars). For each season, $n=6$ samples with each sample comprised of a single brain punch collected and pooled from 4 independent snakes ($n=24$/season). Asterisks indicate significant differences between spring and fall estrone concentrations within each brain region. Numbers represent statistical differences in estrone production among regions during the fall, while capital letters represent statistical differences among regions within the spring. Estrone concentrations were significantly higher during the spring in the olfactory region, while both the AHPOA and septum produced significantly more estrone in the fall. All statistical comparisons are from a two-way ANOVA followed by a Tukey's multiple comparisons test.

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## Discussion

The present study documents AA in the brain of the male red-sided garter snake, a species exhibiting a dissociated reproductive pattern. AA (quantified as estrone production) was detected in all regions of the male red-sided garter snake forebrain, with significant seasonal differences in estrone production among the brain regions examined. In the regional brain dissection (Experiment 1), brains from fall-collected animals exhibited significantly greater estrone production in the S/AHPOA compared to the Mb, O, or NS. In contrast, brains of spring-collected animals exhibited greater AA in the O compared to all other regions. Using specific area punch microdissection to separate the S and AHPOA (Experiment 2), we observed no significant differences in estrone production between the S and AHPOA, while both of these regions again had significantly greater estrone production than the NS or O during the fall. Seasonal differences in AA were also observed within each brain region. The only exception was the NS, in which no significant difference was observed between fall and spring AA using the punch microdissection technique. While a trend for a seasonal difference in aromatase activity in the NS is present in Experiment 2, differences in the dissection techniques between these two experiments must be considered. Further research is needed to determine if the observed seasonal difference in AA in the NS using the gross dissection technique is due to the inclusion of non-NS tissue. Our data provide the first documentation of seasonal and regional variation in AA in a snake brain and suggest that the aromatization of androgens may play a role in regulating reproduction in red-sided garter snakes.

Although AA has been documented in all classes of vertebrates (Callard et al., 1978a,b), relatively few studies have investigated AA in reptiles. In the only study to examine AA in a snake, Callard et al. (1978a) found AA in the forebrain of female brown watersnakes (*Natrix taxispilota*) but no activity was detected in the mid- or hindbrain. However, Callard et al. (1978a) did not examine specific regions within the forebrain of the brown watersnake or examine possible seasonal variation. Although no other regional examination of AA in snakes is available, several studies have investigated the regional distribution of AA in a reptilian brain. In the painted turtle, *Chrysemys picta*, AA occurred in all regions of the forebrain with the greatest activity occurring in the amygdala and dorsal cortex (Callard et al., 1977). In the green anole (*Anolis carolinensis*), AA was higher in the POA and hypothalamus (Wade, 1997).

The majority of studies of AA in reptiles have been conducted on lizards, animals exhibiting an associated reproductive pattern (Gobbetti et al., 1994; Wade, 1997). In the green anole, testosterone facilitates sexual behavior in the male (Winkler and Wade, 1998; O'Bryant and Wade, 2002) while the conversion of testosterone to estrogens aid receptivity in females (Winkler and Wade, 1998). Rosen and Wade (2001) found that courtship in male green anoles was partially mediated by 5α-reductase, the enzyme that converts testosterone to dihydrotestosterone. However, AA was greater in breeding males than non-breeding males while there was no sex or seasonal variation in 5α-reductase activity.

The significantly greater level of estrone production we observed in the S/AHPOA (experiment 1) and the AHPOA and S (experiment 2) of fall-collected animals indicates that AA is higher in these regions. These data correlate with the higher density of aromatase-immunoreactive (ARO-ir) cells in the S and AHPOA of fall-collected male red-sided garter snakes (Krohmer et al., 2002). Further, an increase in ARO-ir cells in the O of spring-collected animals (Krohmer, unpublished data) corroborates the elevated AA observed in spring brains during the present study.

In both experiments 1 and 2, spring AA was significantly greater in the O compared to the other regions examined. In the red-sided garter snake, courtship and mating is initiated in the spring upon emergence from winter dormancy. Seasonal production of a sex attractiveness pheromone by female red-sided garter snakes is used by males to identify sexually attractive females during the breeding season (Mason et al., 1989). Furthermore, detection of the female pheromone by male snakes is dependent on a functional vomeronasal system (Halpern, 1992). Therefore, elevated AA in the O during courtship and mating may indicate a fundamental role for estrogens, aromatized from circulating testosterone, in the male's ability to identify and respond to attractive females. Unlike studies dealing with olfactory sensitivity, little is known about sex differences in olfactory discrimination abilities. In one study with ArKO mice, wild-type male mice are significantly better than wild-type females at discriminating body odors from male versus female and male versus gonadectomized male. ArKO males were significantly poorer than wild-type males and were similar to wild-type females in their ability to discriminate the same odor pairings (Wesson et al., 2006). While the role of estrogens on olfaction in males is limited, our data agree with the comparative importance of estrogens on olfaction in females of other vertebrate systems. In mammals, sexually relevant pheromonal cues are detected by the vomeronasal system (Brennan, 2001). In mice lacking the aromatase enzyme (aromatase knockout, ArKO) sexual behavior is impaired (Aste et al., 1993). Female ArKO mice treated with testosterone spent less time than wild-type mice sniffing odors of either male or female mice. However, estradiol treated ArKO females did not differ from wild-type mice in time spent investigating odors (Bakker et al., 2002).

In the fall, as red-sided garter snakes are returning to the dens, AA in the O is at its lowest level. The seasonal variation in AA found in the O of the red-sided garter snake appears to offer support for the trailing studies conducted by Ford (1981, 1982). These studies demonstrate that olfaction in garter snakes appears to exhibit seasonal variations in the ability of male garter snakes to trail conspecific females changes throughout the active season (Ford, 1981, 1982; O'Donnell et al., 2004). While male garter snakes exhibit a strong trailing tendency during the spring mating season (Ford, 1981, 1982; LeMaster and Mason, 2001; O'Donnell et al., 2004), a period of high AA in the O, seasonal decreases in trailing behavior during the summer and fall (Ford, 1981, 1982) coincide with periods of low AA in the O. Thus, it is possible that seasonal changes in AA in the O mediate seasonal changes in trailing behavior. Future studies manipulating AA during spring trailing behavior would help clarify the role of aromatase in mediating seasonal changes in reproductive behaviors in red-sided garter snakes.

Similarly, the observed elevation in AA in the S and AHPOA in fall-collected snakes suggests that androgens, via aromatization to estrogens play an important although indirect role in the seasonal activation of the neural pathways controlling reproductive behavior. Male red-sided garter snakes are one of the most studied reptilian models of dissociated reproduction [reviewed in Woolley et al. (2004)]. Reproductive behavior of red-sided garter snakes occurs while plasma sex steroid concentrations are declining and gonads are regressed (Aleksiuk and Gregory, 1974; Crews, 1984; Crews et al., 1984; Krohmer et al., 1987). Although the mating behavior of red-sided garter snakes does not coincide with peak gonadal activity, this does not necessarily preclude a role for sex steroid hormones in regulating reproductive behavior. Because reproduction occurs immediately following spring emergence, the concomitant changes in neurophysiology and behavior that accompany reproduction are likely to occur during winter dormancy (Lutterschmidt and Mason, 2009). Thus, sex steroid hormones may play a role in regulating reproduction in dissociated breeders outside of the mating season.

Indeed, androgen concentrations of male snakes are elevated in the fall and remain elevated throughout winter dormancy (Krohmer et al., 1987; Lutterschmidt and Mason, 2009). The significantly higher AA we observed in the S and AHPOA of male snakes during the fall (this study), combined with the known importance of the AHPOA in regulating reproduction from lesion studies (Friedman and Crews,

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1985b; Krohmer and Crews, 1987a), suggests that aromatization of androgens to estrogens in the POA during the fall is important to reproduction. These results are particularly important because the only known stimulus capable of initiating courtship behavior in the red-sided garter snake is a prolonged period of low temperature exposure (Camazine et al., 1980; Garstka et al., 1982; Bona-Gallo and Licht, 1983; Whittier et al., 1987). Further research is necessary to examine how aromatization of androgens during the fall and winter dormancy period, in combination with low temperature exposure, function in inducing the changes in neuroanatomy and neurophysiology necessary to elicit spring reproductive behavior. Such studies would significantly advance our understanding of how reproduction is regulated in animals exhibiting a dissociated reproductive strategy.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yhbeh.2010.04.011.

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