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Influence of testosterone on male-male competition in the red-sided garter snake, *Thamnophis
sirtalis parietalis*

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Math and Science
James Madison University

by Isabella Marie Gething Bukovich

December 2020

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors College.

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PUBLIC PRESENTATION

This work was accepted for presentation, in part or in full, at the Annual Meeting for the Society of Integrative and Comparative Biology for January 2021.

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Acknowledgments

I would like to thank the Honors College for funding my research through the generosity of the Hillcrest Scholarship. E. Thompson, L. Nazarian, J. Lincoln, and S. Jayamohan for animal care assistance. C. Friesen for guidance in sperm collection, imaging, and counting methodology. A. Kahrl for advisement in sperm morphology technique and methodology. This research will be presented at the Annual Meeting for the Society of Integrative and Comparative Biology in January 2021.

I. Abstract

Female mimicry in the red-sided garter snake, *Thamnophis sirtalis parietalis*, is described in several well-studied evolutionary advantages, but an aspect that remains elusive is how reproductive traits are linked to the 3-fold higher circulating androgens in males with this phenotype. In this study, we implanted male garter snakes (n=15 per group) with either a blank implant (SHAM; control) or a T implant, the latter simulating the female mimic phenotype. Following simulated low-temperature dormancy, males were scored for courtship intensity over three days of behavioral trials with females housed in the same facility. Males were sacrificed, and sperm were collected from the ductus deferens for analysis of quantity and morphology. Increased testosterone (T) provides more substrate to activate sexual behavior in this species, thus I predict that males supplemented with T will have a competitive advantage by initiating courtship at a greater intensity or sustaining courtship behavior for longer than controls. I further predict T will promote spermatogenesis to manifest as higher sperm concentration and quality. For my results, T males exemplified greater courtship intensity via an immediate effect; courtship scores were higher 1-minute following trial initiation on day 3. Sperm count was marginally higher for T males, and the sperm head region may be smaller for T males, though no other region (midpiece, tail, total length) was affected. These findings provide evidence for formerly unknown added advantages of female mimicry in snakes via possibly enhanced courtship and sperm competitiveness.

II. Introduction

i. Female mimicry and evolutionary advantages

Intense mate competition poses many challenges to the evolutionary fitness of all vertebrate species. As a result, selective pressures enable the adoption of various reproductive strategies as a means of attaining the highest reproductive output. One such tactic—female mimicry—involves the expression of female phenotypes (i.e., coloration, plumage, pheromones) by males and occurs in a variety of species (Andersson 1994). In bluegill sunfish (*Lepomis macrochirus*), some males are female mimics and adopt the size and coloration of females in order to discretely enter the nesting grounds of a larger, territorial male and fertilize female eggs (i.e., “sneaker” males) without having to expend effort to make a nest or defend fertilized eggs post-spawn (Dominey 1980). Female mimic pied flycatchers (*Ficedula hypoleuca*) disguise their sex in order to initiate attacks against rival males first, thus allowing for a competitive advantage in winning over and mating with females (Saetre and Slagsvold 1996). In vertebrates, the only known example of chemical female mimicry, the production of female pheromones by males, is found in the red-sided garter snake, *Thamnophis sirtalis parietalis* (Mason and Crews 1985).

ii. The red-sided garter snake

Red-sided garter snakes emerge from limestone hibernacula in Manitoba, Canada, every spring to participate in a scramble mating system in which males form mating balls around singly emerging females (Gregory 1974; Crews and Garstka 1982). Such intense male-male competition encourages alternative reproductive strategies like female mimicry to arise. Mimics in the garter snake system exemplify typical male morphology and behavior but produce female pheromones which ultimately confer several advantages by eliciting attraction of other males;

these include aiding in thermoregulation as well as protection from avian predators (Mason et al., 1989; Shine et al., 2001). Pheromones in the garter snake system consist of a combination of long-chain saturated and unsaturated methyl ketones (Mason et al., 1989; Mason and Parker, 2010). The methyl ketones produced in the skin lipids of the garter snakes are predominately unsaturated for females, mostly saturated for males, and an intermediate mix for female mimics (Mason 1993; Parker and Mason 2011). In addition to distinctive pheromone profiles, female mimics maintain 3-fold higher circulating androgen concentrations, masculinizing sex hormones like testosterone, when compared to normal males (Mason and Crews 1985). Although female mimics are not common in populations of this species, they are still maintained in the system (Mason and Crews 1985). Given that androgens directly regulate multiple, crucial reproductive functions, it is possible that additional, cryptic benefits to female mimicry relating to higher testosterone levels have yet to be unveiled, and yet are necessary in order to identify how this alternative strategy is maintained in the population.

iii. Testosterone effects on courtship behavior

Early studies of androgen replacement in adult male *T. sirtalis parietalis* suggest that courtship behavior operates independently of circulating testosterone in a given year: the male garter snake reproductive cycle is governed by a dissociated reproductive pattern in which peak androgen levels and gonadal hypertrophy occur mid- to late summer and regress before entering hibernation, then remain low during the breeding season (Camazine et al., 1980; Crews et al., 1984). By contrast, testosterone (T) directly restores courtship behavior in castrates in species following an associated reproductive pattern (*Anolis carolinesis*, Crews et al. 1978; Licht 1984). Courtship behavior declines in garter snakes across the breeding season following emergence from hibernation, and this occurs similarly in castrated, SHAM-implanted, and castrates with a T

implant (Camazine et al., 1980; Crews et al., 1984). However, both castrated and intact male neonates and yearlings exhibit courtship behavior when supplemented with T prior to hibernation (Crews 1985). These findings suggest that although androgens do not directly influence courtship behavior, they instead influence specialized neural mechanisms and prepare the brain for eventual reproductive behavior (Crews 1991).

Preparatory increase of circulating androgens likely primes sexual brain regions for postponed sex behavior eventually activated by environmental cues (e.g., temperature) (Crews 1991). In garter snakes, the anterior hypothalamus-preoptic area (AHPOA) contains sex steroid-concentrating regions and serves as an important neural center for regulating sexual behavior (Friedman and Crews 1985a; Krohmer and Crews 1987). Aromatase—an enzyme that converts testosterone to estradiol—exhibits greatest activity in these sex-steroid concentrating regions of the brain (Krohmer and Baleckaitis 2000), and estrogens are critical in controlling reproductive behavior in males of the majority of vertebrate species studied (quail, Panzica, Viglietti-Panzica & Balthazart 1996; rats, Vagell and MiGinnis 1997). Given that the volume, cell size, and density of the AHPOA is greater in female mimics than in males or females (Krohmer et al., 2011), mimics may maintain larger sex steroid-concentrating regions in addition to higher levels of T, which, when coupled, provide an optimal scenario for aromatization and its ultimate control of male reproductive behavior. This combination could result in more vigorous courtship or courtship behavior that endures for a longer window of time across the breeding season.

A recent study demonstrated that an aromatase inhibitor abolishes courtship behavior in male garter snakes, while those receiving an aromatase inhibitor + estradiol exhibit normal courtship (Krohmer 2020). Likewise, sex steroid hormones were found to be important in influencing neuroplasticity in the brain pathways controlling reproductive behavior (Krohmer &

Jurkovic 2020). These findings indicate that the effects of both low temperature dormancy and sex steroid hormones are crucial in mediating male reproductive behavior. Since intact males fail to court when T is implanted in the AHPOA during non-breeding seasons (Friedman & Crews 1985b), it is further supported that careful consideration of the reproductive cycle and necessity for low-temperature dormancy in this species must be taken into account in follow-up studies.

When competing against normal males, female mimics mate with more females in courtship trials (Mason & Crews 1985). This preference implicates high T level as a potential mechanism in which the hormone profile associated with the mimic phenotype constitutes a greater behavioral advantage in the form of outcompeting normal male courtship. I therefore predict that T-implanted males will either sustain courtship with greater intensity or for a longer duration than control males. Such a finding would identify a competitive advantage for female mimics in the system related to naturally higher androgen levels.

Most studies using androgen replacement in garter snakes focus on the abolition or renewal of courtship behavior, executed by methods of gonadectomy and T replacement. However, my investigation serves to clarify whether supplementation of exogenous testosterone to intact adult male garter snakes prior to hibernation provides more neural substrate for aromatization in the AHPOA region of the brain, thereby orchestrating a competitive advantage in courtship rigor following hibernation compared to males with a blank implant. Courtship in the red-sided garter snake system has been exhaustively studied and a well-refined courtship intensity scoring matrix has been developed previously (Lutterschmidt & Maine 2014). Utilizing this scoring matrix, I can accurately score courtship behavior to assess intensity and then quantify intensity over time.

iv. Testosterone effects on sperm

Higher circulating levels of testosterone may constitute greater quality and quantity of sperm produced by female mimics. In a study examining the lizard *Lacerta vivipara*, sperm motility is maximized when circulating levels of testosterone are highest (Courty and Dufaure 1980). In some reptiles, testosterone implantation induces spermatogenesis (*Sceloporous spinosus*, Forbes 1941; crocodiles, Ramaswami and Jacob 1965), yet reduces, terminates, or has no effect on spermatogenesis in others (*Lacerta sicula* and *Lacterta muralis*, Lich et al. 1969; *Uromastyx*, Ramaswami 1963). In Lich et al., testosterone implants also resulted in testicular regression (1969). In general, the effects of testosterone on reptile testes and sperm may be species-specific.

The testes of garter snakes implanted with testosterone in the anterior-hypothalamus pre-optic area (AHPOA) of the brain contained more mature sperm and a greater density of sperm in the epididymis (Friedman and Crews 1985b). In the same experiment, control animals and animals with implants placed outside of the AHPOA did not have the same effects. By contrast, a separate study claims intact males with intraperitoneal T implants contain more immature sperm compared to intact males without an implant (Ross and Crews 1978). In my experiment, I aim to clarify the effects of testosterone on sperm abundance and morphology in the red-sided garter snake. Sperm in red-sided garter snakes is well-studied; reliable sperm-counting methods have been developed and applied in this species (Friesen et al., 2013; Friesen et al., 2014a; Friesen et al., 2014b). Likewise, there are established and dependable methods for analyzing sperm morphology in garter snakes (Friesen et al., 2017) and other snake species (Tourmente et al., 2006; Tourmente et al., 2009). I predict that testosterone supplementation will enhance sperm concentration/maturity in red-sided garter snakes, thereby elucidating a possible evolutionary advantage of female mimics via promotion of testicular processes.

III. Methods

i. Snake collection and animal husbandry

In spring 2019, male garter snakes (n=30) were collected in Manitoba, Canada, at the Inwood snake dens during the mating season. This is a very well-studied population which lends stronger inference to these results. Snakes were brought back to James Madison University and housed in glass aquariums in groups of n=4 per tank. They were fed fish and worms weekly and provided water *ad libitum*.

All procedures involving the use of vertebrate animals were approved by Manitoba Conservation and the IACUC of James Madison University.

ii. Implantation and bleeding

In July 2019, snakes were sorted evenly into two groups (n=15 per group) based on body condition (mass[g]/snout-to-vent length[cm]). Individuals were rank-ordered this way to create equal mean body condition in both groups, then alternately assigned to either SHAM or T. Snakes were then bled via caudal vein puncture (25G heparinized needle), and bleed time was recorded to account for effects of handling stress. Blood samples were stored on ice and then centrifuged to separate plasma from blood cells. Plasma samples are currently stored at -80°C until sent for radioimmunoassay at Virginia Tech (via collaborators Ignacio Moore and Ben Vernasco) to validate T dose per snake at the time of sacrifice (see below).

For implant surgeries, snakes were anesthetized with a subcutaneous injection of sodium brevitall (1.0 mg/kg) until elimination of the righting reflex. Implants were made from dialysis tubing sealed with medical grade silicone; the implant was either sealed at both ends and blank (SHAM) or filled with 1 cm of crystalline testosterone before sealing. Implants were placed intraperitoneally in proximity to the anterior testis and directly in the fat body to ensure

solubilizing of the steroids inside the implant. The incision was sealed with cyanoacrylate (NuSkin) and allowed to dry. Following the procedure, the snakes were placed into a recovery tank on top of a heating pad and monitored for a regain of righting reflex, tongue-flicking, and movement.

Blood samples were taken at regular intervals (monthly) from the day before surgery until November (5 sampling periods). Snakes were transitioned to fall and then hibernation following Ashton et al. (2018). To induce courtship behavior via simulating spring conditions at the hibernaculum, the snakes were warmed according to Lutterschmidt and Mason (2009). This pattern was a 10°C hold for 15 hours, then establishing a daily 16:8 h light:dark cycle at 24°C (during the light phase) : 8°C thereafter. Heat lamps were on 5hrs/day in the middle of the light cycle to enable basking. This warming regime resulted in robust courtship behavior from all males.

iii. Behavioral Trials

Behavioral trials commenced one hour after heat lamps turned on each morning after the snakes had been on the L:D warming:cooling cycle for 2 days. Tanks (n=7) containing only bedding and a water bowl were configured with males from each treatment (n=2 SHAM, n=2 T) sharing a tank for the behavioral trials (n=4 per tank). The same body condition-based sorting technique was used to determine which snakes went into which tank. This created equal individual sizes between the experimental groups given that courtship behavior is correlated with male size in garter snakes (Shine et al. 2001). Two males died during hibernation (n=1 T and n=1 SHAM) resulting in a total of n=28 males. Males were then coded by different colors randomly marked on the neck to ensure the observer (me) was blind to the treatment group. For each trial, one unmated stimulus female with a taped cloaca was placed into a tank and a timer started;

taping does not affect female behavior (Lemaster & Mason 2002; Lutterschmidt et al. 2004). There were $n=7$ stimulus females and $n=7$ test tanks. Male courtship scores were recorded at the 5- and 10-minute interval following placement of female in the tank. Trials were run for 3 consecutive days and the order of female testing was randomized such that the largest, most attractive females were not the first tested in the tanks containing the largest males. Immediate courtship score upon placing the female in the tank was also recorded on the third day of trials based on observations made across the duration of individual trials where courtship was most intense as soon as the female was presented then dissipated. The previously described and modified version of the courtship scoring rubric for red-sided garter snakes was utilized (Lutterschmidt & Maine 2014). Briefly, a score of 0 = no courtship behavior; 1 = increased tongue flicks on/near female; 2 = chin rubbing; 3 = body alignment; 4 = tail searching/caudocephalic waves. At the conclusion of the 10-minute interval, females were moved to a new tank in a round-robin style until all tanks had visited with every female. Males were then bled on day three following the conclusion of behavioral trials. Female body condition (mass/SVL) was recorded following the trials to standardize male courtship rigor against female body size/attractiveness.

iv. Animal euthanization and organ collection

Males were sacrificed via overdose of sodium brevital (10 mg/kg) followed by decapitation. Snout-vent-length (SVL) and mass were taken immediately after injection and corrected for injection mass (1 ml = 1 g). Specific organs were weighed to validate the success of the T implants: kidneys, liver, heart, and testes. These organs were then snap frozen in liquid nitrogen for preservation and later gene expression analyses. Condition of the implant in each snake was also visually confirmed.

v. Sperm collection

The portion of the ductus deferens posterior to the kidney was removed, placed into Hamm's buffer, and allowed to rest overnight at 4°C so that sperm could be collected after acclimating to the buffer solution (Mattson et al. 2007; Friesen et al. 2013). Following this, the ductus deferens was strained using forceps to gently palpate the sperm bolus into the buffer. The length of ductus deferens for each side (right, left) was standardized as the length immediately posterior to each respective kidney until it meets the cloaca. After n=10 palpations were done on each length of ductus deferens, the volume of buffer was recorded. This volume is used to standardize concentration after conducting sperm counts.

Using the methods of Friesen et al. (2013) a set volume of sperm was put onto a hemocytometer for counting after sperm were lightly fixed in 0.1% paraformaldehyde and smeared onto slides; each sample was visualized at 4X on a compound microscope with an HDMI-enabled camera, pictures were taken of each sample, and the hemocytometer was set up in triplicate to take n=3 unique pictures per sample for sperm counting. Sperm imaging was captured on a 5×5 grid, and sperm were manually counted within each of the 25 grid blocks, executed strategically as not to double count a single sperm. Sperm outside of the grid were not included in the count; sperm were designated as within the grid/block based upon the location of the sperm's head. Incomplete sperm/fragments were not included in the analysis.

vi. Sperm morphology

Slide smears of 200 ul of each sperm sample were placed onto positively charged slides following the methods of Kahrl and Cox (2015). Sperm were lightly fixed in 0.1% paraformaldehyde before smears were created. Sperm quality was analyzed by measuring length (µm) of head, body, and tail, and total length of individual sperm (40X magnification, n=20

sperm per snake) following previously established methods in garter snakes and other snake species (Friesen et al., 2017; Tourmente et al., 2008; Tourmente et al., 2009) (Fig. 1).

Measurements were taken using ImageJ software.

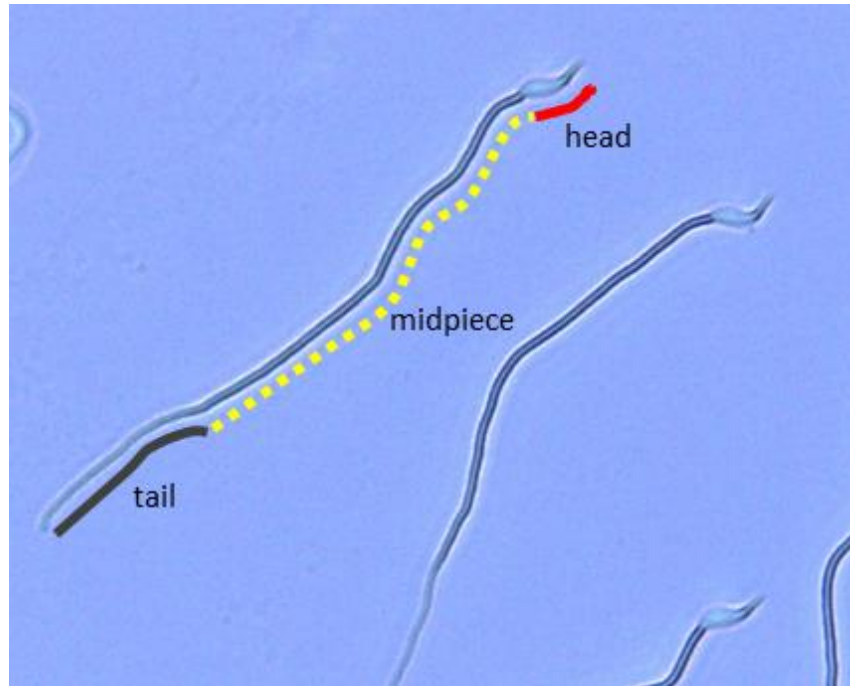


Figure 1. Sperm regions (head, midpiece, tail) in a sperm sample collected from a red-sided garter snake in my study. Total length of sperm was also measured in the analyses.

vii. Statistics

Courtship data were multi-factorial (time period, day, experimental group); therefore, analysis of variance was used. For all other statistical comparisons (organ indices, body condition, sperm counts, sperm morphology), two-sample t tests were used to compare T vs. SHAM males after assessing data for normality and equal variance. Alpha was set at 0.05 and both significant ($p < 0.05$) and marginal differences ($0.05 < p < 0.10$) were reported. All other results were described as non-significant ($p > 0.1$).

IV. **Results**

Body condition was marginally affected by the T treatment; males in the T group had marginally higher body conditions based on both the standard index (mass/SVL; $t_{28}=-1.32$, $p=0.098$) and Quetelet's index (mass/SVL²; $t_{28}=-1.61$, $p=0.058$; both one-tailed t tests). The T males had a significantly increased kidney somatic index (kidney mass/body mass) compared to SHAM males ($t_{28}=1.87$, $p=0.035$, one-tailed t test) (Fig. 2). The kidney is known to be responsive to T in garter snakes, and this result validates that the T implants delivered a biologically-relevant dose of T to the males in that group. Due to COVID-19 restrictions at Virginia Tech, the androgen levels from my blood samples were not analyzed in time for inclusion in my thesis. The other organ somatic indices were not significantly different between the groups (liver, heart, testis), which was expected.

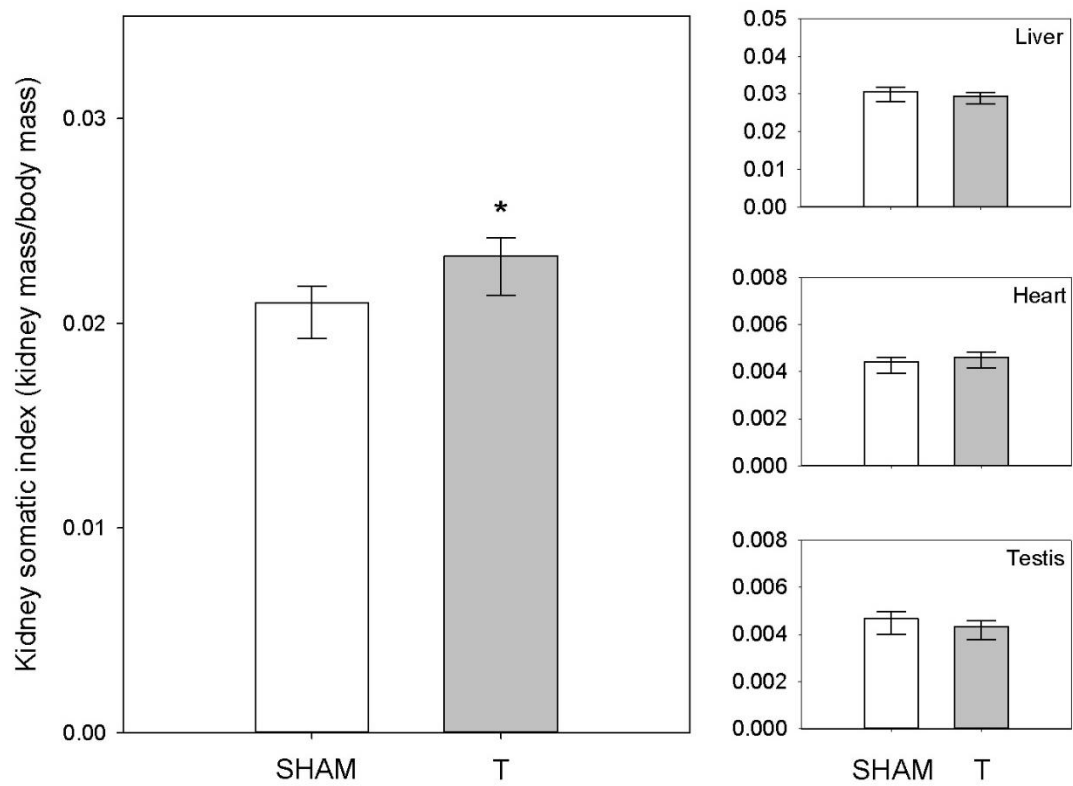


Figure 2. Organ somatic indices (organ mass/body mass) between the T and SHAM groups. T-implanted males had significantly higher kidney indices than SHAM males. Bars are means (+SEM, -95% C.I.).

When assessing courtship scores across the three days where males had access to females, there was a main effect for day ($F_{2,83}=11.78$, $p<0.001$) but not treatment ($F_{1,83}=2.29$, $p=0.14$), and courtship scores were higher on day 1 and day 2 than day 3 ($q=6.45$, $p<0.001$; $q=5.25$, $p<0.001$, respectively) (Fig. 3). On day 3, immediate courtship scores were assessed after 1 min of access to a female, and T males had higher courtship scores than SHAM males ($t_{26}=1.95$, $p=0.035$) (Fig. 4).

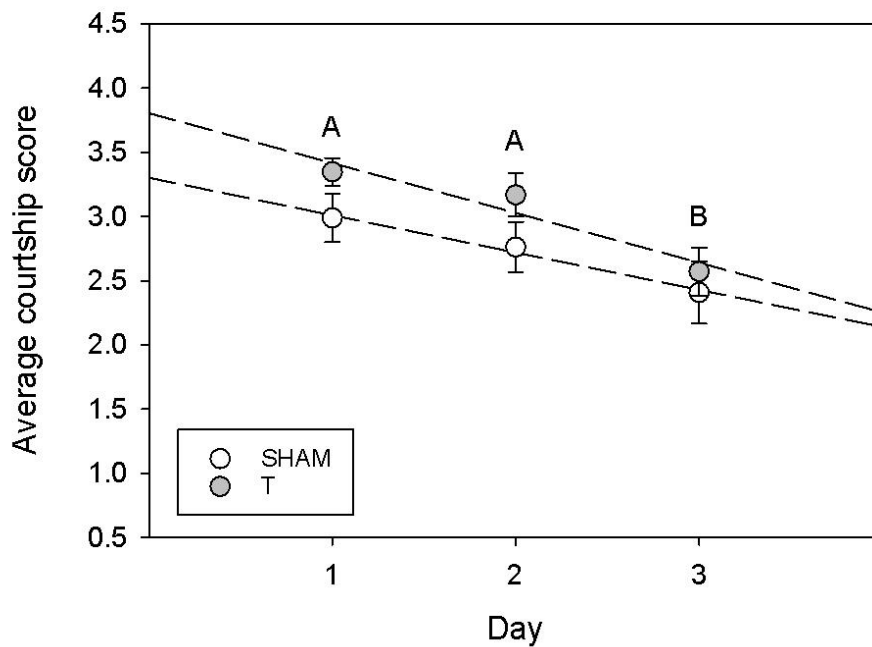


Figure 3. Courtship scores from T- and SHAM-implanted male red-sided garter snakes across three days. Courtship significantly declined over time but was not affected by treatment. Different letters represent statistical differences between testing days. Points are means (\pm SEM).

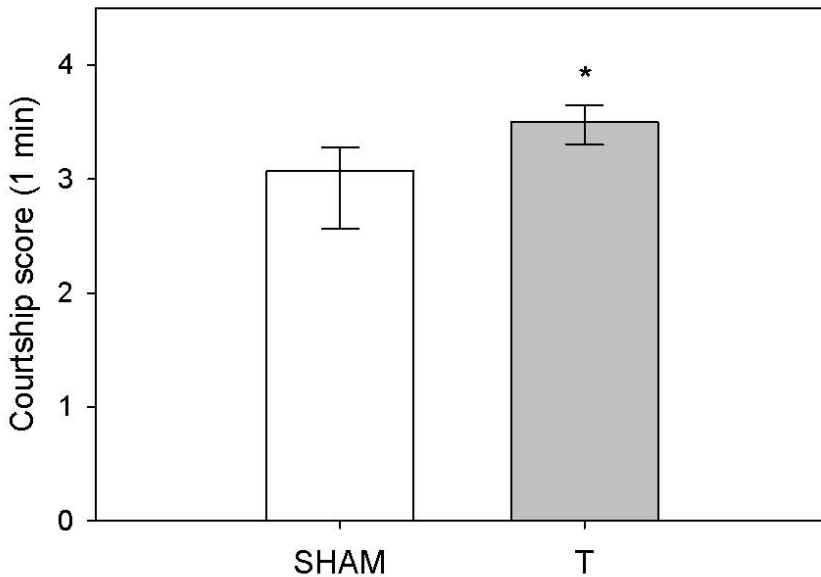


Figure 4. Immediate courtship scores from T and SHAM males from day 3 of having access to females. T males had significantly higher courtship scores than SHAM males. Bars are means (+SEM, -95% C.I.).

Male garter snakes implanted with testosterone averaged marginally higher sperm counts than SHAM males ($t_{22}=1.76$, $p=0.091$, two-tail p-value) (Fig. 5). When looking at sperm quality across multiple dimensions of sperm morphology (midpiece, flagellum, and total length), there were no differences between the groups (Fig. 6). My sperm morphology measurements (head, midpiece, tail) averaged nearly identical to those of male garter snakes in Friesen et al. 2017. However, there may be a difference in head length; median head length ($10.72\ \mu\text{m}$) was marginally shorter in the T group than the SHAM group (median= $11.08\ \mu\text{m}$; $t_{25}=1.70$, $p=0.10$). This difference was not the case, though, for mean head length (T= $10.77\ \mu\text{m}$, SHAM= $10.98\ \mu\text{m}$; $t_{25}=1.37$, $p=0.18$).

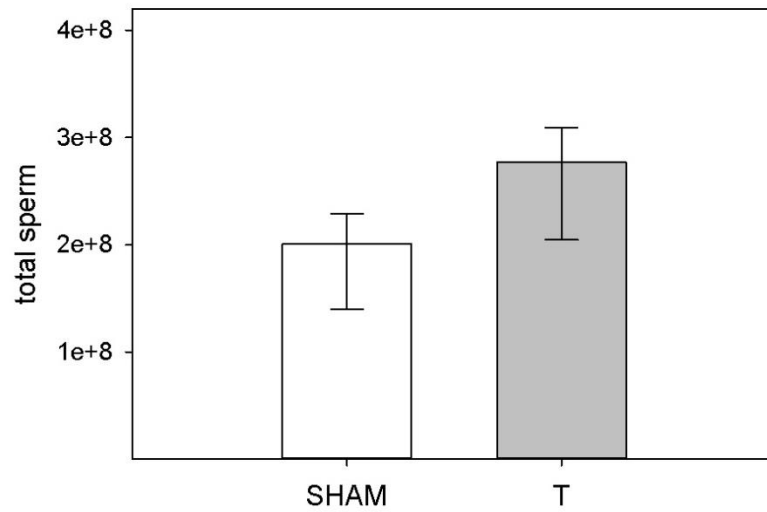


Figure 5. Total sperm differences between the T and SHAM groups. T-implanted males had marginally higher sperm counts than SHAM males. Bars are means (+SEM, -95% C.I).

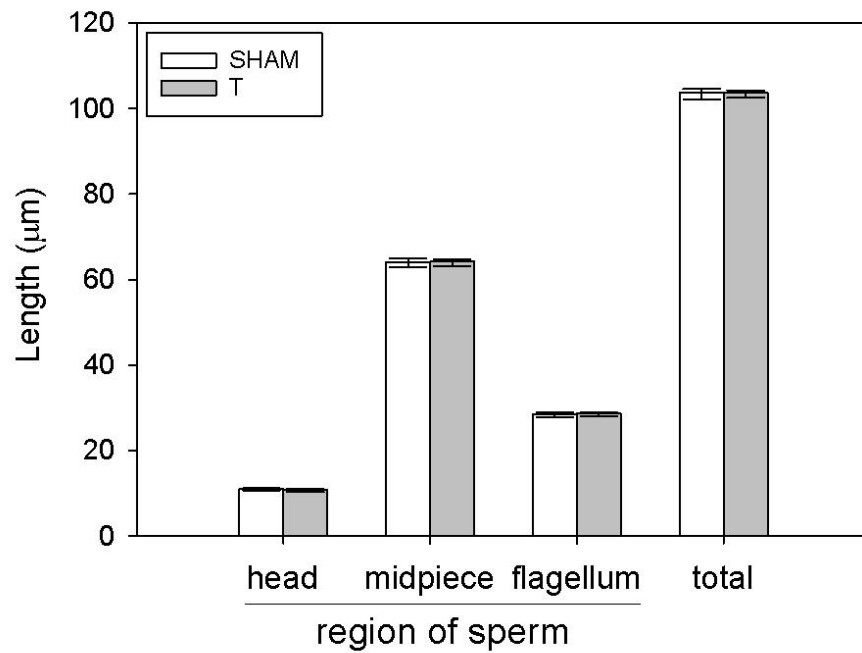


Figure 6. Comparisons of different sperm dimensions between the T and SHAM groups. No significant differences were detected. Bars are means (+SEM, -95% C.I).

V. Discussion

Increased circulating androgens may pose a greater pre-copulatory advantage in male garter snakes via enhanced initial courtship intensity and possibly a post-copulatory advantage via greater sperm quantity. Female mimics, a subset of the red-sided garter snake population that maintain as much as 3-fold higher circulating androgens (Mason and Crews 1985), possibly experience these advantages during the mating season. This indicates that development of alternative reproductive strategies, such as female mimicry, can be critical to the reproductive success of individuals in a highly competitive system such as that of red-sided garter snakes.

Courtship intensity was higher for both treatments for Days 1 and 2 than Day 3 of courtship trials; this pattern recapitulates the natural decline of male courtship activity across the breeding season (Aleksiuk & Gregory 1974). Although testosterone supplementation failed to enable greater persistence of courtship over the three-day period, I observed sex behavior in these snakes, and there was an immediate courtship effect for day 3 trials. Whether the same immediate courtship effect would persist on additional trial days warrants further investigation. However, this result could underly the mechanism responsible for the ability of female mimics to outcompete normal males in copulation success with females (Mason & Crews 1985): supplemental testosterone provides greater neural enrichment for aromatization in sex-steroid concentrating regions of the brain (AHPOA), allowing for immediate, enhanced courtship rigor following low-temperature dormancy. My finding of enhanced immediate courtship is bolstered by established understanding of the AHPOA in facilitating courtship behavior of male garter snakes and the importance of estrogens in regulating courtship behavior (Friedman & Crews 1985a; Krohmer & Crews 1987). Further, recent findings demonstrated that both low temperature dormancy and sex steroid hormones are crucial in activating eventual courtship

behavior (Krohmer 2020; Krohmer & Jurkovic 2020). Although previous studies failed to demonstrate differences in courtship between groups of castrates, SHAMs, and castrates with a T implant (Camazine et al., 1980; Crews et al., 1984), it is important to note that these studies differed from mine in that they 1) involved castration followed by androgen replacement and 2) did not examine the immediate courtship score 1 minute after placement of the female into the mating enclosure. My study offers novel insights into replication of the female mimic phenotype in that it involves supplementation of normal, intact males with testosterone to test for courtship enhancement, as opposed to gonadectomy followed by androgen replacement with the intent of courtship restoration.

It is further notable that the short, 3-day duration of my experiment possibly did not allow enough time for observation of courtship differences between the groups over time; most courtship studies in the garter snake system maintain several weeks of courtship trials to mimic the natural duration of courtship in the field (Camazine et al., 1980; Crews et al., 1984; Crews 1985). Whether or not the rate of decline of courtship intensity between testosterone and SHAM males tapers differentially cannot be confidently extrapolated from my data due to the limited window of courtship trials. However, due to the limitations of COVID-19, we could not allocate more than three days to courtship trials, and thus a follow-up experiment expanding the duration of courtship trials may be necessary to observe male courtship trends over a more realistic timeline.

The marginally higher sperm counts observed in testosterone-implanted males corroborates findings in a few reptile species given differential amounts of testosterone (Crocodiles, Ramaswama & Jacob 1965; Lizards, Forbes 1941). Given the higher testosterone levels in female garter snake mimics, my result suggests that this phenotype may reap in the post-

copulatory benefits of enhanced sperm competitiveness when compared to normal male competitors. Friedman and Crews demonstrate that T implants in the AHPOA portion of the garter snake brain generated greater sperm density in the testis (Friedman and Crews 1985b). My data therefore suggest that direct intraperitoneal testosterone supplementation can also enhance spermatogenesis in the red-sided garter snake, specifically contributing to more sperm in the ductus deferens. Further, the T-implanted males still produced sperm and did not downregulate spermatogenesis, a consequence seen in many vertebrates where excess T supplementation suppresses spermatogenesis. Production of testosterone in the testes by Leydig cells is crucial in inducing spermatogenesis in the seminiferous tubule, and thus exogenous testosterone, at an effective dose, may enhance the process of spermatogenesis. The mechanism underlying the influence of exogenous testosterone over spermatogenesis has received much attention in rodents and primates (Boccabella 1963; Cunningham & Huckins 1979; Buhl et al., 1982); however, decrypting the means by which differential amounts of testosterone influence and restore spermatogenesis is complicated and not well understood (Sharpe 1987). Steroid synthesis via Leydig cells is required for spermatogenesis in reptiles (Aldridge & Sever 2011), but published studies focused on the natural steroid cycle with respect to these testicular cell types as opposed to androgen supplementation (Chinese soft-shell turtle, Tarique et al., 2019; Snapping turtle, Mesner et al., 1993). My data offer innovative routes for exploration of the effects of differential amounts of exogenous testosterone on spermatogenesis in a reptile species.

Most aspects of sperm morphology (midpiece, tail, total length) were not influenced by implantation of testosterone in male garter snakes. It is likely, then, that sperm quantity is the most robust post-copulatory trait influenced by testosterone. However, the marginally shorter median head lengths in T males might maintain some hidden structural advantage. In mammals,

an elongated head and tail is associated with greater sperm velocity (Malo et al., 2006), while instead a longer midpiece is associated with faster sperm in birds (Lupold et al., 2009).

Structurally, snake sperm are relatively unique among the aforementioned taxa in that the midpiece makes up approximately half of the cell length, and the length of the midpiece is found to be positively correlated with the strength of sperm competition across different snake species (Tourmente et al., 2009). If testosterone indeed reduces the length of the head region of the sperm in red-sided garter snakes, then this effect might offer a structural advantage in relation to greater swimming velocity. However, due to the onset of COVID-19, I was unable to conduct analyses involving sperm motility with Dr. Christopher Friesen at our field site in Manitoba, Canada. My findings warrant further investigation in deriving concrete conclusions and mechanisms by which testosterone affects sperm morphology and motility.

My findings have unveiled previously unidentified potential evolutionary advantages of male garter snakes exhibiting higher circulating testosterone levels exemplary of the female mimic phenotype. Future research should investigate courtship intensity in males with differential testosterone over a several-week period of trials as well as observation of courtship over 1, 5, and 10-minute durations across all trials. Additionally, the ease of studying reproduction in red-sided garter snakes make them a model organism for delving deeper into the testicular mechanisms responding to differential testosterone that influence the rate of spermatogenesis as well as sperm structure. Ultimately, the male traits I studied may provide cryptic-yet-powerful advantages to female mimics favored by sexual selection: enhanced immediate courtship rigor and greater competitiveness through higher sperm quantity.

VI. References

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