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Decrypting female attractivity in garter snakes

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Decrypting Female Attractivity in Garter Snakes

(*Thamnophis sirtalis parietalis)*

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By Holly Rose Rucker

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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

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Acknowledgments

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I. Abstract

Pheromones are utilized by many species as sexual signals driving mate choice, and pheromone production in vertebrates hinges on sex hormone action. Female red-sided garter snakes (*Thamnophis sirtalis parietalis*) produce a skin-based sex pheromone used by males for mate detection and selection. Estradiol is necessary for pheromone production, yet the specific mechanisms within the skin are unknown. Central to this is the metabolism of testosterone to estradiol via the enzyme aromatase. It is hypothesized that female garter snakes synthesize estradiol locally in the skin and maintain pheromone production via tissue-specific regulation of aromatase. Further, I hypothesize that female attractiveness, and therefore pheromone production, can be inhibited by targeting aromatase activity. Using qPCR, I detected sexually dimorphic expression of aromatase in the skin (3.5-fold increase in females; $t_{20}=2.30, P=0.032$). To inhibit aromatase activity, I treated females with a known aromatase inhibitor (fadrozole; FAD). Females received either FAD injections (100 μg/mL; n=10 females) or control injections (saline; SHAM; n=10) three times a week for six months. Pheromones were isolated from snake shed skins, and blood plasma was collected to determine circulating estradiol. In the den the next spring in Manitoba, Canada, SHAM and FAD females were differentially attractive based on bioassays with wild males. FAD females attracted ~50% less courtship than wild females in two different bioassays (competition: $F_{2,22}=6.54$, $P=0.007$; mating ball test: $F_{2,24}=22.454$, $P=<0.001$). Collectively, my results are the first to indicate a key role for tissue-specific aromatase expression invertebrate pheromone production

II. Introduction

i. Chemical signaling and sexual dimorphism

Animals communicate with a multitude of cues, including visual, auditory, tactile, and chemical. Chemical signaling can be used in territory marking, as seen in many big cat species, where urine spray is used to distinguish individual home ranges and territories held (Poddar-Sarkar & Brahmachary, 2014). Another use of chemical communication includes alarm signaling in many insect species, such as the odorants released by honeybees that function in alerting the hive of a possible threat (Reinhard and Srinivasan, 2009). Chemical signals can also be used for mate detection and selection. One example of reproductive chemical signaling is the salamander *Plethodon shermani*, where males present pheromones to females that increase female receptivity, prior to deposition of his spermatophore (Houck et al., 2008).

Chemical signals used by vertebrates in mate choice are often directly regulated by sex steroid hormones, such as testosterone and estradiol. This regulation is physiologically dimorphic and results in expression of sexually dimorphic secondary sexual signals. A classic example of such hormonally-mediated sexual dimorphism is in the mallard, *Anas platyrhynchos*, in which males have distinct coloration (Omland, 1996). Females, however, have "duller" coloration. Sexually dimorphic traits, in addition to indicated an individual's sex, are also valuable indicators of quality, and as seen in male peafowl, where the more ornate their tail coloration, the more competitive the individual and the greater their access to mates (Omland, 1996).

ii. Female mimicry

Female sexual mimicry, the expression of female phenotypes by males, is an alternative reproductive tactic that occurs in a wide range of species (Mason and Crews, 1985). The most

common form of female mimicry is via morphological traits, such as body pattern, coloration or plumage that act as visual signals. Male giant cuttlefish, *Sepia apama*, can mimic the mottled pattern of females and "sneak" past the courting, dominant male to mate with females (Norman et al., 1999). In birds, female mimicry is quite common and often the result of delayed morphological puberty. In other species, though, mimicry can be permanent. For example, male marsh harriers, *Circus aeriginosus*, can exhibit permanent female mimicry via female-like plumage and derive a benefit of less aggression from rival males (Sternalski, et al. 2011).

Other types of female mimicry utilize non-visual signals, such as chemical or auditory signal. Chemical cues, such as pheromones, are used by many species for mate choice or as a method of communication. For female green-veined butterflies, *Pieris napi*, the concentration of sex pheromone released by males helps females distinguish the flight activity of her suitor (Andersson et al., 2007). This aids female mate choice because males capable of producing concentrated pheromones father healthier, stronger offspring, increasing their fitness. While examples of males that mimic non-visual, female-typical signals (chemical or auditory) are rare, there is only one example of chemical female mimicry in vertebrates: the red-sided garter snake, *Thamnophis sirtalis parietalis* (Mason and Crews, 1985).

iii. *Thamnophis sirtalis parietalis*

Red-sided garter snakes use sex pheromones as essential components to their mating behaviors, which have been studied intensively at den sites in Narcisse, Manitoba, Canada for more than 40 years. What makes this area the locus for studying garter snakes is that every year in the spring, nearly 75,000 garter snakes emerge from hibernation and begin actively searching for mates in a competitive scramble mating system (Klein, 2016).

Male garter snakes emerge first from underground hibernacula but stay near the den as females emerge singly over the 4-6 week breeding season (Gregory, 1974). The males move instantly to court emerging females, resulting in the formation of mating balls, a tangled mass of males competing to mate with one female (Crews and Garstka, 1982). While female garter snakes typically have a larger snout-vent length (SVL) than males, the sex pheromone mixture she expresses is key to her sex identity and enables males to find her in the chaos of the mating ball.

Female garter snake sex pheromones are a blend of non-volatile, long-chain (C_{29}-C_{37}) saturated and monounsaturated methyl ketones, and the longer, unsaturated methyl ketones elicit the strongest male responses (Fig 1) (Mason et al., 1989). A pheromone trail alone can elicit male responses, a feature of this system that researchers manipulate by creating Y shaped mazes in which different trails are placed along the stem of the Y and into one branch (Parker and Mason, 2011). The sex pheromone blend also conveys significant information about species, sex, condition, and body length which is a representation of fecundity as larger females are able to yolk more follicles and give rise to larger numbers of offspring per reproductive episode (Crews and Garstka, 1982).

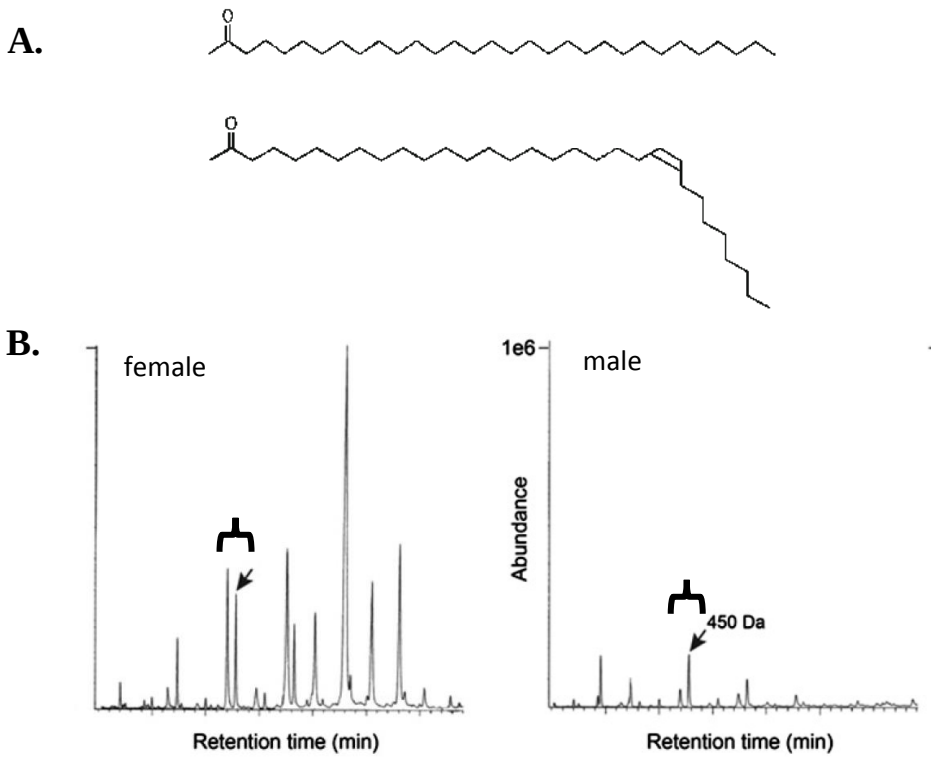

Figure 1. A) An example of a saturated methyl ketone (top) and an unsaturated methyl ketone (bottom), which contains a double bond in the ω-9 position. B) GC-MS traces from skin lipid extracts of female and male garter snakes (Parker and Mason, 2012). The methyl ketones are grouped in unsaturated-saturated doublets, as indicated by the bracket. The arrow is indicating a saturated methyl ketone of 450 Da in both the female and male trace. Larger compounds have a longer retention time and are represented by peaks on the right side of the trace.

Females are not the only ones producing pheromones; female mimics are morphologically and physiologically male yet produce female-typical pheromones and attract courtship from other males (Shine et al., 2011). There are two types of female mimics: newly emerged female mimics who lose their attractiveness (abundant every spring but transient) and those that are long-term mimics (rare but permanent) (LeMaster et al., 2008). Newly emerged mimics thermally benefit from their attractiveness. The mimic emerges with a very cold body temperature (2-4°C), slow to move and subject to possible avian predation. But because a mating ball forms around them as males court the pheromones on their skin, transient mimicry protects these males and enables them to warm quickly due to body contact from warmer males (Shine et al., 2006). Chemically, there is no significant difference in the amount of methyl ketones per

surface area or in the amount of saturated vs monounsaturated compounds found in newly emerged and long-term female mimics (LeMaster et al., 2008). However, the long-term mimics have a more intermediate ("intersexual") pheromone composition between females and males, with a higher relative abundance of mid-range carbon chain compounds.

Little is known about the mechanism by which female and long-term female mimics produce female pheromones. It is hypothesized that female and female mimic garter snakes have a higher amount of aromatase, an enzyme which catalyzes the conversion of androgens like testosterone into estrogens such as estradiol (Fig 2.A) (Elbrecht and Smith, 1992). Aromatase is expressed abundantly in ovary, liver and fat of vertebrates (George and Wilson, 1980). Aromatase in the skin specifically plays a significant role in regulating sexually dimorphic plumage, such as hen feathering in male Sebright chickens. Some male Sebrights naturally express female plumage yet physiologically and behaviorally are male (testes, crowing, high circulating testosterone). The reason for this trait is a dysfunctional aromatase gene in the skin that is constitutively active, leading elevated, local production of estrogens that feminize only the skin. Previous research in the Parker lab showed that skin in garter snakes is a potential target of estradiol signaling (Ashton et al. 2018). And while estradiol is normally made by females via ovarian aromatase activity, the skin itself may be a site of aromatase activity in garter snakes.

Aromatase activity in a tissue like skin would allow female and female mimic garter snakes to convert any androgen into estrogens, such as 17-β estradiol, to activate pheromone production (Fig 2.A). It is important to note that estradiol is rarely available in circulation for female garter snakes across their annual cycle but is made primarily in the ovaries, so aromatase in the skin is not the sole mechanism regulating pheromone production. It is hypothesized that long-term female mimics utilize a similar mechanism in order to produce estrogens. Testosterone

in normal male garter snakes is not metabolized to estradiol by aromatase in skin, presumably because males lack elevated aromatase expression in their skin and thus do not produce appreciable sex pheromone blends.

Using an aromatase inhibitor, the metabolism of testosterone to estradiol can be prevented, subsequently suppressing pheromone production (Fig 2.B) (Wade et al., 1994). Previous research primarily in birds, such as zebra finch (*Taeniopygia guttata)*, utilized fadrozole (FAD) hydrochloride ($C_{14}H_{13}N_3$) as an effective aromatase inhibitor (Wade et al., 1994). I predict that if aromatase conversion of androgens to estrogens is inhibited via FAD treatment, females and female mimics will be unable to synthesize estrogens and, thus, pheromones locally in the skin and will be unattractive.

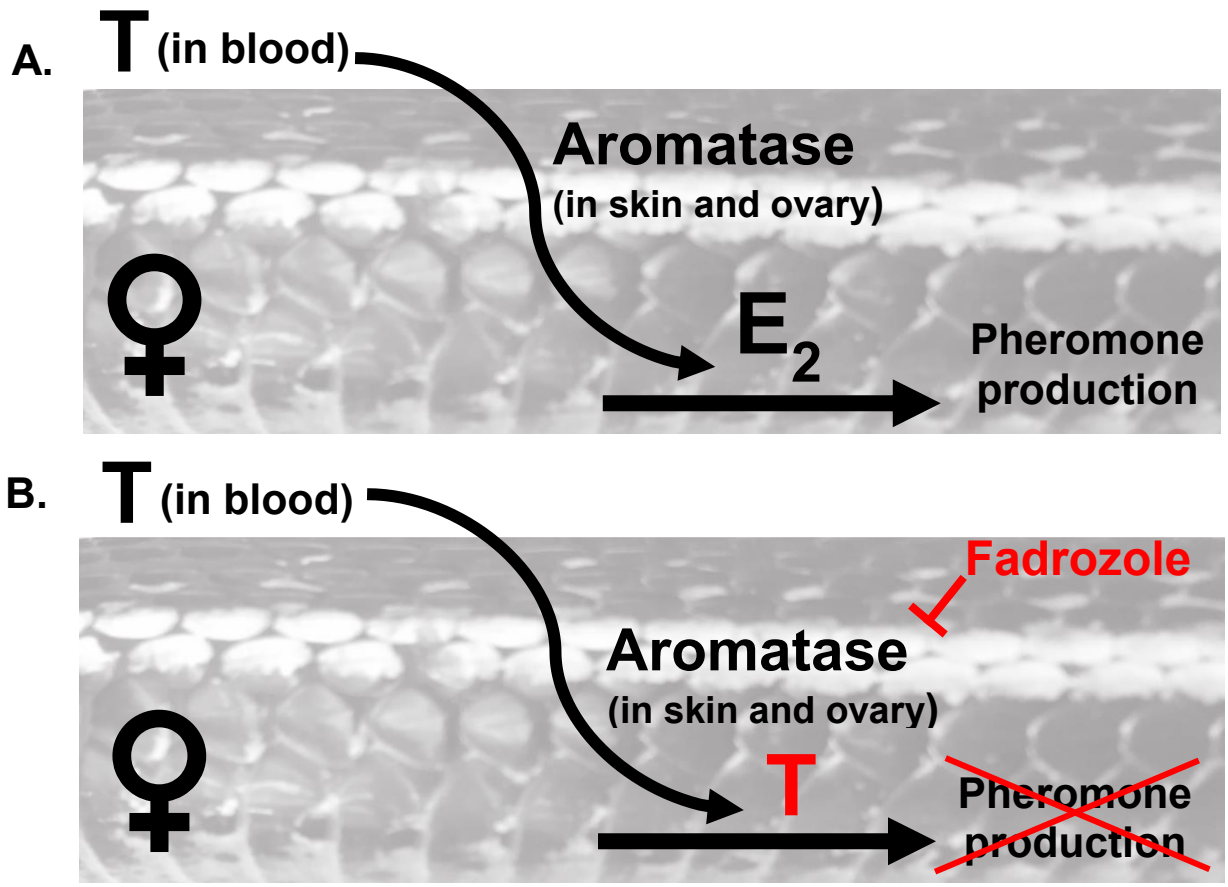

Figure 2. A) Female pheromone synthesis pathway, in which testosterone (T) from the blood is feminized by aromatase in the skin (and ovary) to form estradiol (E_2) which then signals for pheromone production by the skin. B) Inhibition of aromatase by the chemical fadrozole in females, which would prevent the aromatization of T into E_2 and therefore inhibit pheromone synthesis.

The purpose of this research project is to investigate if the production of female pheromones is mediated through the activity of aromatase. This experiment will provide a synthetic understanding of how females are able to produce estrogens in the skin, which have been shown in previous experiments to be necessarily for pheromone expression (Parker and Mason, 2012). If aromatase expression is sexually dimorphic between females and males, then inhibition of aromatase should decrease pheromone synthesis and overall attractiveness for both females and female mimics.

III. Experimental Methods

Animal Collection

For the FAD experiments, male (n=20) and female (n=20) red-sided garter snakes were collected from a den site in Inwood, Manitoba, Canada (N 50° 30’ 25.5416”, W 97° 29’ 49.7003”, 277m) in May 2017 during the mating season. The attractivity of selected female garter snakes was determined by a competition assay. In this assay, a stimulus female was lightly restrained by holding her tail and covering the cloaca to prevent mating, and a mating ball was allowed to form around her (Parker & Mason 2012; 2014). A focal female was then held parallel to this mating ball, and any male that began courting the focal female was counted and removed. Courting behavior was identified as chin rubbing along the female’s body (Parker & Mason 2012; 2014). All females were tested in this bioassay every three days for nine days, and females were selected for the study if they maintained high courtship scores. Males were tested in basic courtship assays where they were given access to a female and their behavior observed. Males were selected if they always courted the female (chin rubbing and body alignment) and were never attractive to other males. Prior to leaving the field site, all snakes were given an ID and their length (SVL; snout-to-vent length; cm) and mass (g) were recorded. This enables monitoring of individual body condition (mass/SVL) in captivity as experiments progress.

Blood samples were taken via caudal vein using heparinized syringes (25G x 1/2, 1mL capacity) to determine a baseline 17 β-estradiol levels for each subject animal. Plasma was separated via centrifugation (10,000xG; 5 minutes at 4°C) and then stored at -80°C. The plasma samples were sent to Ignacio Moore and Ben Vernasco at Virginia Tech for radioimmunoassay. Estradiol was undetectable in all samples, which is not surprising given the low circulating

concentrations of estrogens for female garter snakes across the majority of the annual cycle (Ashton et al., 2018).

The snakes were then driven to the Biology Department at James Madison University in mid-May and maintained in 20-gallon aquariums within an environmental chamber. The environmental chamber was set to temperature and photoperiod cycles to mimic the natural conditions of the Interlake region of Manitoba for summer (May-August) and then fall (September-November) before simulating hibernation (December-May) (Lutterschmidt & Mason, 2008; 2009). Following the breeding season, the snakes were fed a mixed diet of fish and earthworms once a week and water was provided *ad libitum* until fall.

Administration of fadrozole and saline injections

The treatments began at different times per individual, starting after their first shed in captivity. Four experimental groups were established where each individual received an injection every 2 days. All injections were given subcutaneously in the first half of the snake's body. The control groups (SHAMs) were females (N=9) and males (N=8) that received injections of physiological saline (reptile Ringer's solution) only. The treatment groups (FADs) were females (N=9) and males (N=9) that received injections of fadrozole HCl (1.0 mg/kg body mass) diluted in Ringer's. Injections continued until either the 3rd shed was collected or prior to hibernation. The original goal of the project was to collect shed skins to assess changes in pheromone production due to the treatments. However, because I could not be certain that the treatments did not negatively affect the snakes' abilities to reproduce naturally in the wild, Manitoba Conservation mandates that all animals be euthanized at the conclusion of the study. The skin lipids were therefore obtained from the study animals following euthanasia.

Behavior Assays

All groups were maintained in common conditions in the environmental chambers until May 2018, and they were then transported back to the field site in Manitoba for bioassays. Mating ball competition assays were performed using all of the experimental animals plus wild females (N=8) and males (N=10) at the den. Also, a second behavioral assay was developed to test if the focal animal could sustain a mating ball over time. In the "mating ball size test," a wild stimulus female was again used to generate a mating ball as described above, and the focal animal was placed parallel to the stimulus female. However, a two-minute timer was set and the mating ball allowed to form on the focal animal until whatever number of males accumulated. At the end of the two minutes, all males in the mating ball were collected and counted. Wild females (N=10) and males (N=10) were also tested in this assay.

Pheromone Analysis

Pheromone isolation followed the protocol outlined in Baedke et al. (2019), which can be accessed and followed in detail in the Journal of Visualized Experiments with a video demonstrating the techniques. After soaking in hexane for 24 hours, the hexane solution was evaporated using a rotary evaporator. This allows for separation of the solvent from the skin lipids. The extracted lipids were weighed to obtain total lipid mass (mg) and solubilized in hexane for storage (-20°C). The lipid samples were fractionated using alumina-packed columns, using 0 and 4% diethyl ether to elute methyl ketones from the column. The 7th and 8th fractions from each sample will be evaporated using a rotary evaporator and solubilized in hexane (1 mg/ml). The samples were sent to the Carver Biotechnology Center at the University of Illinois for analysis via gas chromatography-mass spectrometry, which determines the abundance of individual methyl ketones that I can compare to retention times to obtain a profile of the methyl

ketones present in the sample. The abundance of a given ketone was converted to a relative abundance value scaled to the highest peak per chromatogram. External standards (methyl stearate) were also analyzed to approximate peak area to a concentration per methyl ketone (LeMaster et al. 2008).

Molecular Analysis

Tissue collection and RNA extraction were performed by Sydney Ashton, a previous Honors student, as outlined in Ashton et al. (2018). Wild female and male snakes were euthanized via an injection with an overdose of sodium brevital followed by decapitation. Skin, ovary/testis, liver, kidney, muscle, duodenum, and heart samples were collected from each snake and placed in cyrotubes, snap frozen in liquid nitrogen and stored at -80°C.

RNA was extracted by grinding the sample in a Cyro-cup grinder and using PureLink RNA Mini Kit with on-column DNaseI treatment. Total RNA concentrations were calculated via NanoDrop (260nm/280nm ratio). cDNA was synthesized using a standardized amount of RNA (0.2µg per tissue). The quality of cDNA was checked using the NanoDrop and by performing RT-PCR with primers for GAPDH and gel electrophoresis on 2% agarose gels (140 V, 30 minutes). Aromatase primers used for all PCR experiments were designed specific to the *Thamnophis sirtalis* genome and verified by Sydney Ashton using Primer-BLAST (Ashton et al., 2018). GAPDH was used as a control gene.

Quantitative RT-PCR (qPCR) was performed on male and female skin, as well as ovary, using Fast SYBR Green. The reactions were carried out in a 96-well plate and ran with the following protocol: activation at 95 °C for 30s and 45 cycles at 95 °C (5s) and 60 °C (30s). This was followed by 31s at 65 °C and melt curve analysis from 65 °C to 95 °C in 0.5 °C increments for 5s" (Ashton et al., 2018). To analyze the qPCR data for an individual sample, the Δ-C_T the

method was used, in which the cycle threshold (C_T) of the gene of interest, aromatase, is subtracted from the C_T value for the reference gene, GAPDH: $\Delta C_T = C_{T\ GAPDH} - C_{T\ ARO.}$ C_T values have an inverse relationship with the amount of amplification; a lower cycle threshold value indicates a higher abundance of the gene of interest, as fewer cycles were needed to amplify the intercalated SYBR green and produce a detectable signal (Schmittgen et al. 2008).

Statistical Analyses

For analysis of behavioral and gene expression data, one-way ANOVAs were conducted followed by pairwise comparisons (Tukey tests). For the methyl ketone data, a two-way ANOVA was conducted (methyl ketone and group as factors) followed by pairwise comparisons (Student's t tests). When comparing single variables only (e.g., ratio of unsaturated to saturated methyl ketones), Student's t tests were used. For all statistical analyses, alpha was set at 0.05.

IV. Results

i. qPCR

Aromatase expression was significantly variable across the three tissues tested ($F_{2,30}$=15.07, P<0.001), with females having a 3.5 fold-change in aromatase expression relative to male skin (Fig 3). Female ovary had a 40 fold-change in expression relative to male skin.

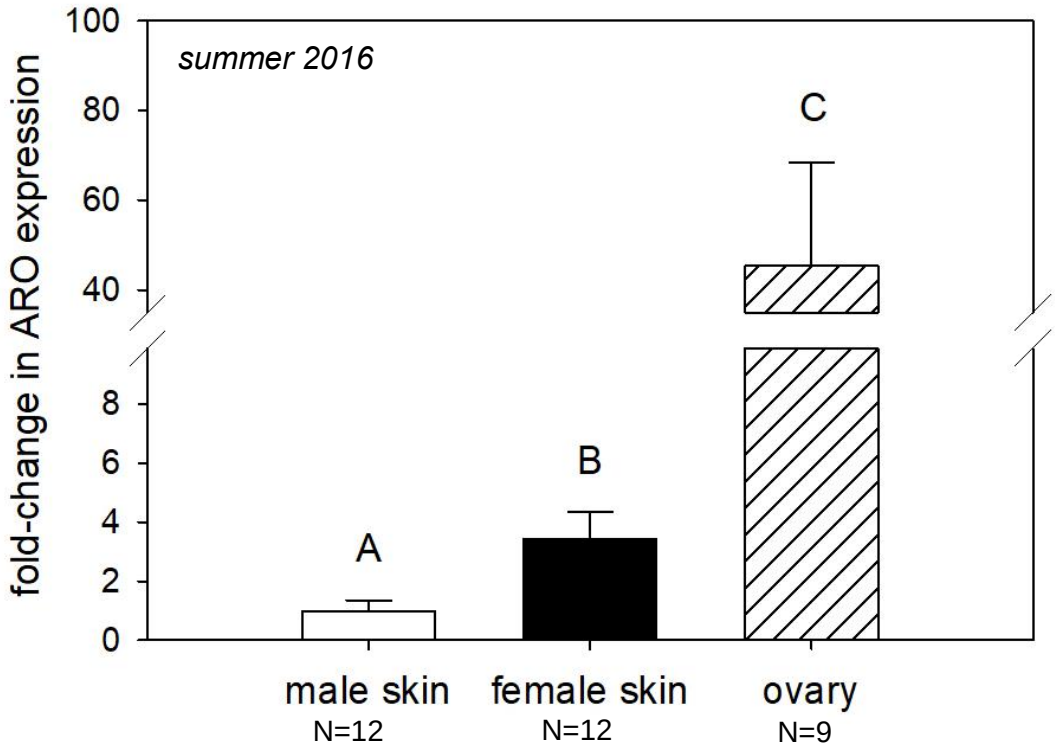

Figure 3. Fold-change expression of aromatase (ARO) in the skin of male and female garter snakes, standardized to male skin. Gene expression for ARO was normalized to GAPDH for each sample. Skin tissues were combined across spring and summer (June-Aug) for this assay. Letters indicate statistically significant differences in expression based on pairwise comparisons ($p<0.05$). The qPCR primer efficiencies were: ARO = 108.1% and GAPDH = 97.5%.

ii. Behavior

For the mating ball competition assay performed in the field the year after treatment, there was a statistically significant difference in the number of males that initiated courtship across the females tested ($F_{2,22}=6.54$, $p=0.007$) (Fig 4). Both wild and SHAM females received courtship more males on average than FAD females and there was no statistical difference between wild and SHAM females.

In the mating ball size test, there was also a statistical difference in the number of males attracted across the groups ($F_{2,24}=22.454$, $p<0.001$) (Fig 5). FAD treated females maintained courtship from approximately half the number of males that SHAM females maintained. There

was also a statistical difference between SHAM and wild females, with wild females maintaining a greater number of courting males. Collectively, these results demonstrate that FAD females were less attractive than both wild and SHAM females during the breeding season.

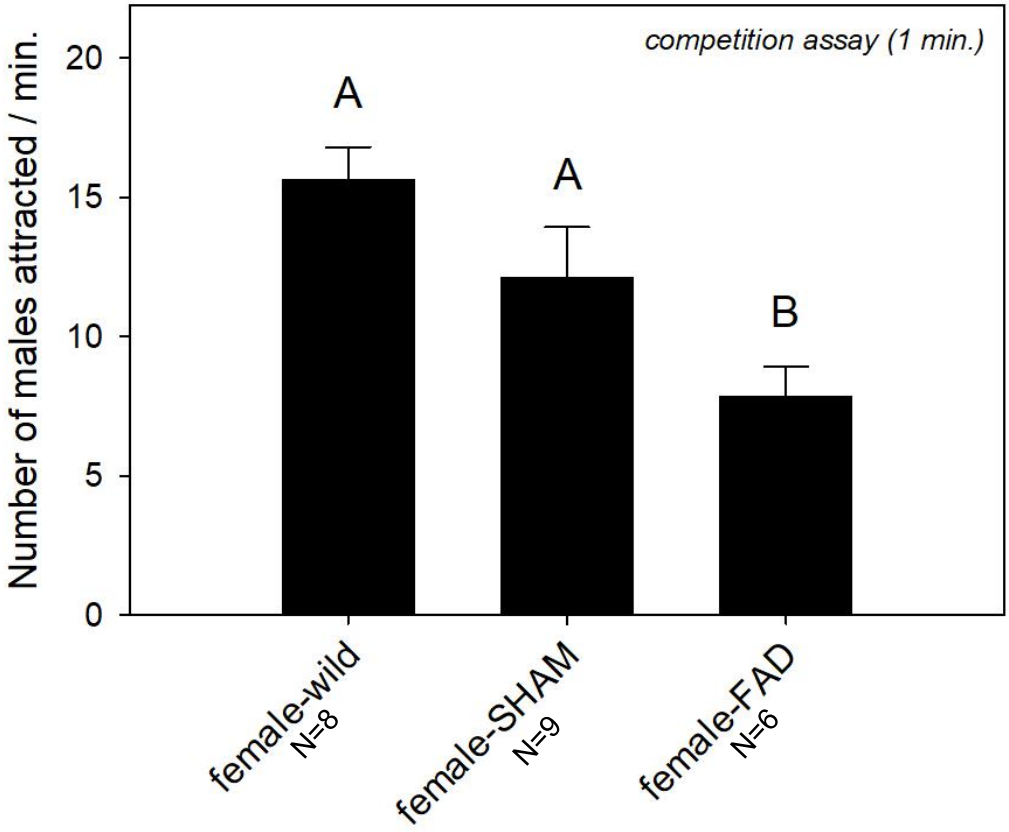

Figure 4. Competition assay of FAD, SHAM and wild females. FAD treated females attracted fewer males than both wild and SHAM females. Data were log-transformed prior to analysis. Letters indicate statistically significant differences ($p<0.05$) between groups.

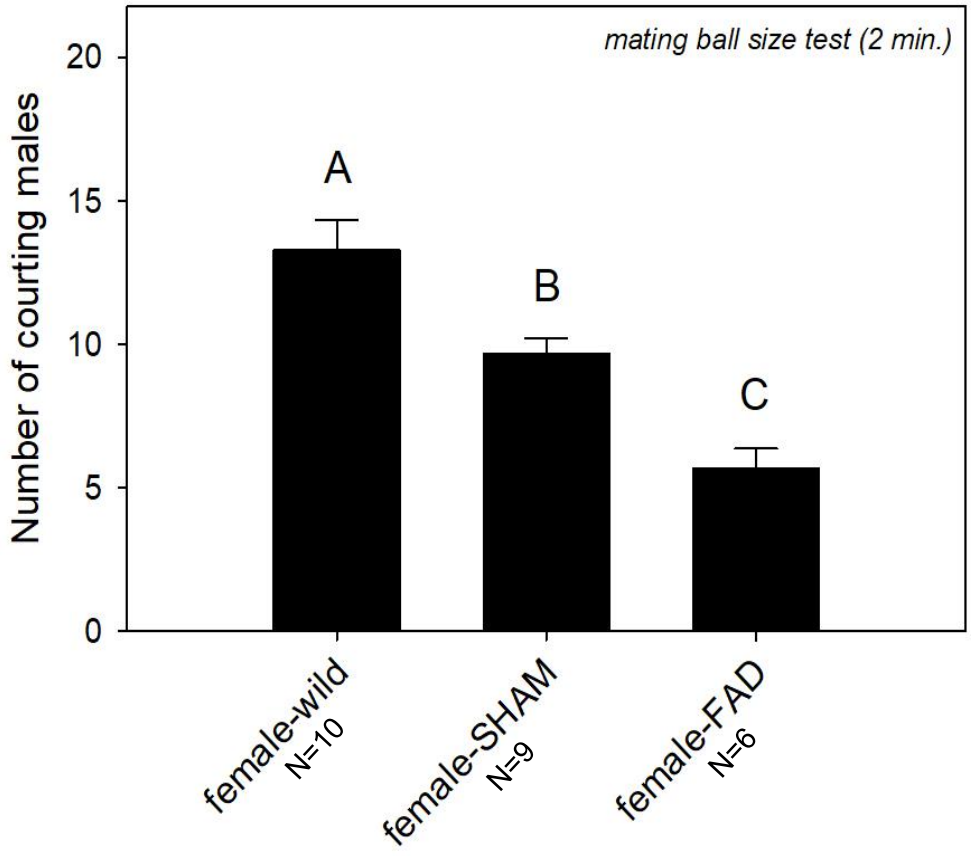

Figure 5. Mating ball size test for FAD, SHAM and wild females. FAD treated females maintained courtship from fewer males than wild and SHAM females. Data were log-transformed prior to analysis. Letters indicate statistically significant differences ($p<0.05$) between groups.

iii. Pheromones

Because the unsaturated methyl ketones elicit more vigorous courtship from male garter snakes, the ratio of unsaturated to saturated methyl ketones in a pheromone profile is a reliable indicator of an individual's attractiveness (LeMaster and Mason 2002). There was no significant difference between the ratio of unsaturated: saturated methyl ketones between SHAM and FAD females ($t_{13}=1.31$, $p=0.10$; Fig 6-A). There was also no statistically significant difference in total pheromone mass between treatments ($t_{13}=1.49$, $p=0.079$; Fig 6-B). However, when individual methyl ketone mass was determined, there was a marginal effect of treatment x methyl ketone

interaction ($F_{17,269}$=1.63, p=0.056). Specifically, four unsaturated methyl ketones were significantly higher in mass in SHAM females compared to FAD females after pairwise analysis: ketones 476 (q=3.66, p=0.011), 490 (q=2.99, p=0.037), 504 (q=5.14, p<0.001), and 532 Da (q=2.84, p=0.047) (Fig 7).

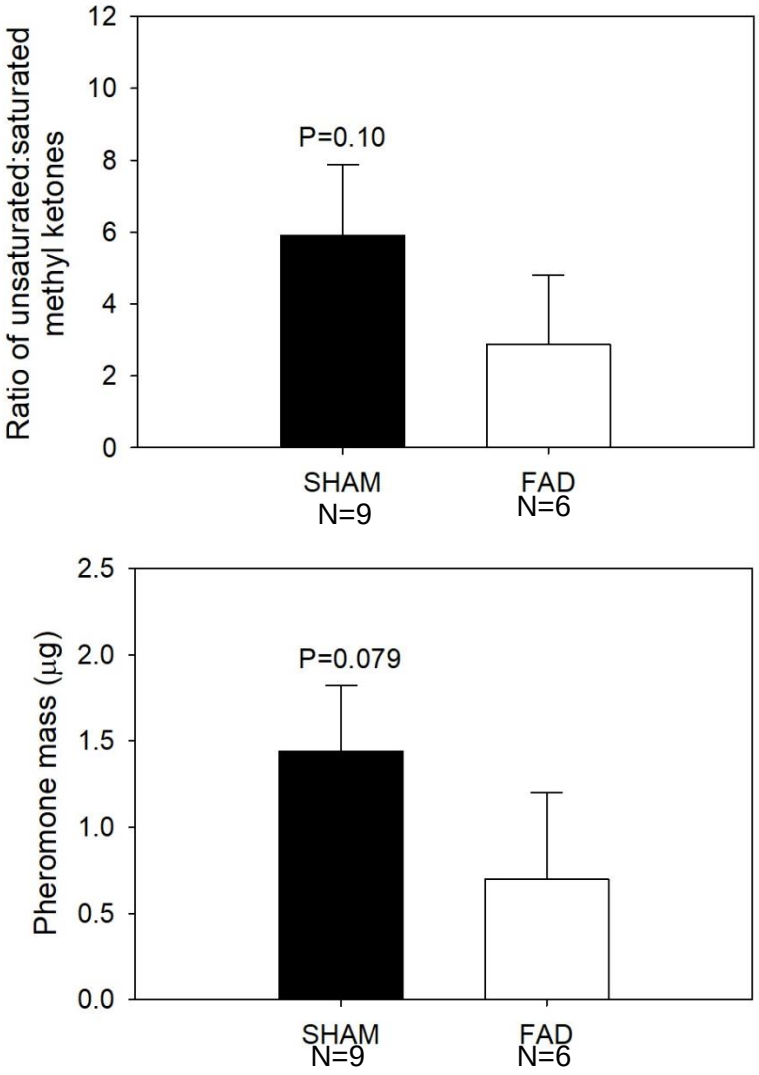

Figure 6. A) Ratio of the sum of unsaturated: saturated methyl ketones between treatment groups. B) Total pheromone mass extracted from each group. Wild females were not included in this assay due to conservation and permit restrictions.

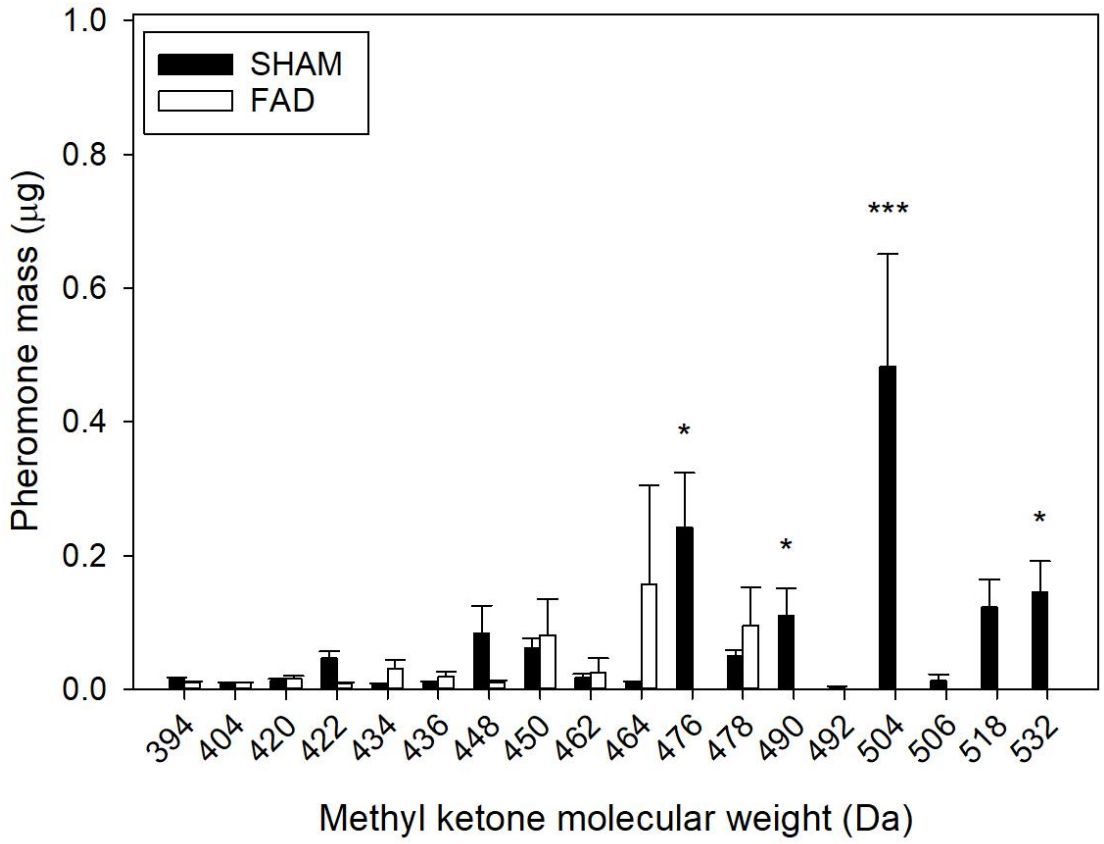

Figure 7. Mass of individual methyl ketones classified by molecular mass (Da) between SHAM (n=9) and FAD females (n=6). Asterisks indicated statistical differences between the groups. The methyl ketones 476, 490, 504 and 532 Da were significantly elevated (p=0.05) in SHAM females than the FAD treated females.

V. Discussion

The data from these experiments demonstrates that aromatase gene expression is key for pheromone synthesis and resulting female attractivity. My results showed that aromatase expression is sexually dimorphic between males and females. By using an aromatase inhibitor, I was able to make females significantly less attractive in two different mating ball assays as compared to our control females. The use of both mating ball assays allows us to show that FAD treated females received less courtship initiation (courtship assay) and were unable to maintain a large mating ball over time (mating ball size test).

The pheromone data supports the differential attractivity of the behavior assays as FAD treated females produced little to none of the four largest unsaturated methyl ketones: 476, 490, 504 and 532 Da. The unsaturated methyl ketone with a mass:charge of 518 Da was also found to be more abundant in SHAM females, although this was not statistically significant ($q=2.62$, $p=0.066$). These methyl ketones are the largest unsaturated ketones, making them the most attractive to males (Mason et al., 1989). In previous studies where males are given estradiol implants, a similar upregulation of these five unsaturated methyl ketones are found and these males are perceived as attractive by other males (Parker & Mason, 2012; Parker et al. 2018). My study, in addition to these previous studies, indicates that these five unsaturated methyl ketones are necessary in order for an individual to be actively courted and are directly regulated by estradiol.

Previous research on this species showed that ovariectomy of females renders them unattractive and unreceptive to males in the laboratory under field-simulated, but artificial, conditions (Mendonca and Crews, 1996). The present study, however, takes the relationship between estradiol and female attractiveness further by presenting chemical and behavioral data that indicates a decrease in pheromone quantity as well as quality. The pheromone analysis allowed for direct investigation of the pheromones present within the females' skin during the breeding season following experimental manipulation. My study, in addition to the Mendonca and Crews study, shows that estrogen plays a vital role in female attractivity, both in the ovary and in the skin.

The detection of pheromones by males serves as the single most important method of mate identification and selection in this system. The results of this study show that although circulating levels of sex hormones are typically low in females, they are capable of converting

testosterone into estradiol locally in their skin via aromatase to signal for pheromone production. Since males given estradiol implants produce female pheromones, the primary sexual dimorphism between normal males and females is not the ability to produce pheromones, but the abundance of aromatase in their skin making the estradiol needed to produce these pheromones. Estrogen receptors are also present in both male and female skin, which further supports that males are unable to produce female pheromones due to a lack of estradiol or a method of aromatizing testosterone into estradiol (Ashton et al., 2018). Understanding the role aromatase plays in pheromone production will allow researchers to further study the cryptic mechanism of pheromone synthesis in female skin.

Hormonal manipulation of vertebrates allows scientists to better understand the mechanisms that drive sexual signals, such as Dunlap et al. 1997, in which electric fish (*Stemopygus*) were given estradiol implants, resulting in a more potent electric organ discharge frequency and pulse duration. Removal of gonads that synthesize sex hormones can also provide information of sexual signaling. An example of gonad removal resulting in variable sexual signals is the decrease of male typical blue coloration in Yarrow's Spiny lizards (*Sceloporous jarrovii*) after castration, which disrupts testosterone circulation (Cox et al., 2008). In this same study, supplemental testosterone administered after castration reestablished the male typical coloration patterns (Cox et al., 2008). Manipulation of sex hormones allows researchers to study how hormonal signals directly regulate sexual signals and therefore the potential fitness of an organism. By inhibiting aromatase, I was able to indicate a relationship between testosterone, estradiol, aromatase and pheromone signaling in garter snakes. With the successful interference of aromatase via fadrozole in this experiment, future experiments targeting tissues with known

aromatase activity, such as the HPOA region of the brain or the ovary, can be conducted to study the importance of aromatized estradiol in these tissues (Krohmer et al., 2010).

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