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A novel mechanism regulating a sexual signal: The testosterone-based inhibition of female sex pheromone expression in garter snakes

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ABSTRACT

Vertebrates communicate their sex to conspecifics through the use of sexually dimorphic signals, such as ornaments, behaviors and scents. Furthermore, the physiological connection between hormones and secondary sexual signal expression is key to understanding their dimorphism, seasonality and evolution. The red-sided garter snake (*Thamnophis sirtalis parietalis*) is the only reptile for which a described pheromone currently exists, and because garter snakes rely completely on the sexual attractiveness pheromone for species identification and mate choice, they constitute a unique model species for exploring the relationship between pheromones and the endocrine system. We recently demonstrated that estrogen can activate female pheromone production in male garter snakes. The purpose of this study was to determine the mechanism(s) acting to prevent female pheromone production in males. We found that castrated males (GX) are courted by wild males in the field and produce appreciable amounts of female sex pheromone. Furthermore, pheromone production is inhibited in castrates given testosterone implants (GX + T), suggesting that pheromone production is actively inhibited by the presence of testosterone. Lastly, testosterone supplementation alone (T) increased the production of several saturated methyl ketones in the pheromone but not the unsaturated ketones; this may indicate that saturated ketones are testosterone-activated components of the garter snake's skin lipid milieu. Collectively, our research has shown that pheromone expression in snakes results from two processes: activation by the feminizing steroid estradiol and inhibition by testosterone. We suggest that basal birds and garter snakes share common pathways of activation that modulate crucial intraspecific signals that originate from skin.

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Introduction

Intraspecific communication is enabled by a variety of signals, some of which are overt (e.g., bright plumage, elaborate displays; Andersson, 1994) and others, private (e.g., electric waveforms, sex pheromones). These signals function as secondary sexual characteristics that can be seasonally activated by sex steroid hormones (e.g., Owens and Short, 1995). The removal of the activational hormone(s) via gonadectomy disables the expression of the sexual signals and functionally eliminates this dimorphism (e.g., Cox et al., 2008; Stokkan, 1979; Van Oordt and Junge, 1934).

Although the activation of sexually dimorphic signals by a single hormone is common, a suite of hormones is often involved, especially in the regulation of complex signals. For instance, sexual coloration in tree lizards requires activation and organization by two different androgens, testosterone and dihydrotestosterone, even though each hormone has specific effects on separate dimensions of the sexual coloration (Hews

and Moore, 1995). Furthermore, masculine sexual signals in sauropsids (birds and squamate reptiles) are often not activated by the presence of androgens; instead, the absence of a female hormone (typically estradiol) prevents the expression of the female sexual signals in males. For example, the castration of adult mallards has no effect on their bright breeding plumage, which suggests that androgens are not required for the expression of this signal (e.g., Haase and Schmedemann, 1992). Rather, ovariectomies in females induce the expression of male plumage (e.g., domestic fowl, Greenwood and Blyth, 1938; blue-winged teals, Greij, 1973). The absence of estrogen signaling, therefore, initiates a mechanism in the female's skin that activates the expression of male breeding plumage (first noted in female domestic fowl with pathological, non-functioning ovaries; Owens and Short, 1995).

Castration in vertebrates has long been known to suppress or reduce the expression of male sexual signals. Furthermore, males can modulate female behavior with chemical signals, the classic examples of which include the Bruce and Whitten effects in rodents, which are abolished by castration (e.g., Bronson and Whitten, 1968; Bruce, 1959, 1965; Whitten, 1956). These studies have demonstrated the activational capacity of androgens, especially testosterone, in relation to sexual signals. However, it may also be true that the removal of testosterone alone without the addition of a feminizing steroid, such as estrogen, may

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constitute a sufficient stimulus to reverse sexually dimorphic signals via relaxed inhibition.

Female red-sided garter snakes use a sexual attractiveness pheromone to signal their sex and condition to conspecifics (e.g., Mason and Parker, 2010; Mason et al., 1989, 1990; Shine et al., 2003b). This pheromone plays a major role as a species barrier in this system (e.g., LeMaster and Mason, 2003; Shine et al., 2004). The female sexual attractiveness pheromone is composed of a series of long-chain, saturated and monounsaturated methyl ketones (Mason et al., 1989), and the ratio of molecular abundance between the unsaturated and saturated ketones within a pheromone blend (U:S) is a predictor of a garter snake's attractiveness (e.g., LeMaster and Mason, 2002; Parker and Mason, 2009). As pheromone profiles become dominated by longer, unsaturated ketones, they become more attractive to males (LeMaster and Mason, 2002; Parker and Mason, 2012). Previously, we established that estrogen implantation activates female pheromone production in male red-sided garter snakes, specifically the secretion of only the longest, unsaturated methyl ketones (Parker and Mason, 2012). The removal of estrogen abolished the expression of the female trait, suggestive of a purely activational effect, and we revealed that the skin's lipid production and composition in male garter snakes are responsive to steroidal manipulation.

In the current study, we sought to further determine the role of sex steroids in the pheromone expression of garter snakes. To address our research questions, we designed two different experiments. The first experiment was a castration experiment where we wanted to determine if gonadectomy affected the attractiveness and skin lipid composition of male red-sided garter snakes. Following the results of that experiment, we then designed a second experiment to determine the reproducibility of the first experiment and also assess whether hormone replacement with testosterone in castrates was sufficient to abolish the effects of gonadectomy. Thus, the goals of the current experiments were to determine whether an absence of testosterone alone can activate the expression of female traits in males; whether testosterone treatment is able to reinstate pheromone inhibition; and whether the testosterone supplementation of intact males can alter their skin lipid compositions.

Methods

Collection of animals and laboratory conditions

We collected male red-sided garter snakes (*Thamnophis sirtalis parietalis*) in the spring (May) of 2007 for the first experiment ($n = 24$) and in the spring (May) of 2008 for the second experiment ($n = 60$). In the second experiment, several samples were contaminated prior to pheromone analysis, and the final number of animals in each group is given in the legend for each figure. All animals were collected from the same den (Inwood, Manitoba, CA). The snakes were transported back to the laboratory at Oregon State University (Corvallis, OR) and kept under simulated field conditions in environmental chambers (summer, 14 h L:10 h D, 26 °C:16 °C; fall, 10 h:14 h, 16 °C:8 °C; winter, 24 h D, 4 °C). Experimental animals were housed in groups of 3–4, but only with individuals from the same experimental group. Following artificial hibernation in the laboratory, all experimental males were transported back to Manitoba for bioassays. Each year, all snakes were sacrificed for pheromone collection in Manitoba (see below) at the conclusion of the bioassays, and the whole extracts were brought back to Corvallis, OR for processing and analysis.

All procedures involving the use of live animals were approved by the Institutional Animal Care and Use Committee at Oregon State University (ACUP 3120) and were in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The collection and use of these animals was approved by Manitoba Conservation (Manitoba Wildlife Scientific Permit WB02024).

Surgical procedures

For the first experiment, two groups of males were used, the SHAM (control surgery) and GX (castration surgery) groups ($n = 12$ in each group). Both groups underwent surgery and had their testes exposed, but the GX animals had their testes removed. The surgeries were conducted in September of 2007. All snakes shed ~1 month after the surgeries and did not shed again before their hibernation and subsequent transportation to Manitoba for the bioassays in May 2008.

For the second experiment, four groups of males were used, as follows: SHAM, GX, GX + T and T. All snakes in the second experiment underwent two surgeries: one for gonadectomy (sham or castration) in August 2008 and one for implantation (blank or testosterone) in September 2008. In addition to the surgical procedure described in the first experiment, the SHAM and GX animals in the second experiment received blank silastic implants (see below). GX + T animals were castrated and given a silastic implant containing crystalline testosterone (T) a month later. Animals in the T group were given a sham surgery followed by a T implant a month later. Implants were given a month after the castration surgeries so that the effects of the castration could take effect before testosterone was reinstated via supplementation (GX + T). All snakes shed ~1 month after the surgeries and did not shed again before their hibernation and subsequent transportation to Manitoba for bioassays in May 2009.

For surgery, the snakes were anesthetized with a subcutaneous injection of brevilat sodium (0.003 mL of 0.5% solution per 1 g body mass) until their righting reflex was abolished (~15 min.; see Wang et al., 1977). Sterile corneoscleral scissors were used to make an incision between the second and third dorsal scale rows to insert the implants into the peritoneal cavity. Silastic implants (1.67 mm i.d. \times 2.41 mm o.d. \times 10 mm length; Dow Corning, Midland, MI, USA) were created from silastic tubing sealed with medical adhesive (silicone) at both ends after being either filled with crystalline testosterone (T implant; Sigma-Aldrich, St. Louis, MO, USA) or left empty (blank implant). In castration surgeries, the testes were exposed and excised using sterile forceps, corneoscleral scissors and a cauterizer (see Camazine et al., 1980, for methods). Sham surgeries (SHAM, T) only exposed the testes. For implant surgeries, the incision site was made just anterior to the previous incision site, and the implant (blank or T) was inserted into the intraperitoneal cavity. Incision sites were sutured using 4–0 silk sutures fitted to a small cutting needle immediately after each surgery. Following all surgeries, the snakes were allowed time (6 h) to recover in a sterile cage before being placed back in their home cage.

Bioassays

We previously developed a bioassay during the spring mating season in Manitoba in which we initiated mating balls in the den by placing a single, newly emerged female on the ground until ~20 males began courting her (Parker and Mason, 2012). The female's cloaca was taped to prevent copulation, because mated females rapidly become unattractive (Devine, 1977). Using a clear medical adhesive bandage, a small piece of tape (7–10 cm) was wrapped completely around the cloacal region perpendicular to the body axis to prevent the cloaca from opening. This taping procedure does not affect male or female behavior (LeMaster and Mason, 2002; Lutterschmidt et al., 2004). Once the mating ball had formed, we introduced the experimental male within 20 cm of the mating ball. We counted the number of males that came off the mating ball to begin courting the experimental male over a 1 min period without replacement (Parker and Mason, 2012). Males were removed from the experimental male as soon as they exhibited chin rubbing behavior, which is an unequivocal mating behavior in this system (a score of 3 in the ethogram of Moore et al. (2000)).

Castration and testosterone implant validation via direct radioimmunoassay

We followed the same general radioimmunoassay methods from our previous paper to validate the experimental procedures (Parker and Mason, 2012). Blood (0.3 mL) was taken from all males via the caudal vein within 1 min using heparinized syringes (1 cm³, 25-g). Samples were stored on ice (<4 h) until centrifugation to separate out the red blood cells (1500 rpm, 5 min), and the plasma was frozen at –20 °C and stored at –80 °C until used in direct radioimmunoassays following modified procedures of Lutterschmidt et al. (2004), similar to previous hormone studies (e.g., Whittier et al., 1987). All males were bled in the fall 1 month after surgery to validate the castration surgeries. To validate hormone implantation, the males in the second experiment were bled again 1 month after implantation (2 months after the castration surgeries). Because previous research has shown that similar steroid implants (e.g., testosterone, melatonin, estradiol) maintain plasma steroid levels during artificial hibernation in red-sided garter snakes (e.g., Camazine et al., 1980; Mendonça et al., 1996; Parker and Mason, 2012), the steroids were only measured in the plasma at 1 month after implantation.

Briefly, we extracted steroids from plasma aliquots (20–40 µL) with anhydrous ethyl ether. The ether phase was isolated and dried under nitrogen gas in a water bath (35 °C). Hormone extracts were then resuspended in phosphate-buffered saline and incubated with tritiated testosterone (1,2,6,7-[3H]testosterone, GE Healthcare/Amersham Biosciences, Piscataway, NJ, USA) and testosterone antiserum (Wein Laboratories, Inc., Succasunna, NJ, USA) at 4 °C for 12–24 h. The cross-reactivity of this antiserum with 5- α -dihydrotestosterone is high (63.2%), and so the results are reported as total androgen concentration, not testosterone concentration. Unbound steroids were separated from bound hormones using dextran-coated charcoal, and the radioactivity of each sample was quantified in a Beckman SC 6000 scintillation counter. The samples were assayed in duplicate and corrected for individual recovery variation. The mean extraction efficiency for testosterone was 99%, as determined by the recovery of the tritiated testosterone added to samples before extraction with ethyl ether. All samples (i.e., treatment groups) were randomly distributed across the steroid assays (n = 3). The mean intraassay variation was 9.6%, and the interassay variation was 17.6%. The limit of detection for total androgen was 0.11 ng/mL, and this value was assigned to samples with non-detectable total androgen levels.

Pheromone extraction and analysis

The garter snake sex pheromone is composed of a series of long-chain, saturated and mono-unsaturated methyl ketones that are embedded in a matrix of lipids secreted throughout the dorsal and lateral surfaces of the skin. This lipid matrix is readily extracted by immersion in nonpolar solvents, such as hexane (Mason et al., 1987, 1989, 1990). All snakes were sacrificed for pheromone collection following the methods of Mason et al. (1989). The snakes were euthanized with a lethal overdose of brevitall sodium (6 mg/kg) prior to the individual collection of skin lipids by immersion in hexane for 12 h. The snakes were then removed from the solvent, and the snake mass (g), snout-vent length (SVL, cm) and midbody circumference (cm) were recorded. The volume of the skin lipid extracts was reduced under a vacuum using a rotary evaporator, and the total skin lipid yield of the dry product was determined (mg) before fractionation. The pheromone was isolated using alumina columns (activity III [Sigma-Aldrich; St. Louis, MO, USA]; pooled fractions 4–6 [2% diethyl ether:98% hexane as mobile phase]). The pooled fractions containing the pheromone were reduced by using a rotary evaporator and weighed to determine the mass (mg; termed “pheromone fraction mass” hereafter). The combined pheromone fractions are composed almost solely of the methyl ketones that compose the pheromone (>99% of fraction mass). The pooled pheromone fraction was

resuspended in a pheromone:hexane mixture (1 mg:1 mL) before analysis with gas chromatography/mass spectrometry.

Individual pheromone samples were analyzed with an Agilent 6890 N gas chromatograph fitted with a split injector (280 °C) and an Agilent 5973 mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA). Aliquots (1 µL) of the 1:1 samples (1 mg pheromone:1 mL hexane) were injected onto the fused-silica capillary column (RTX-1; 15 m \times 0.25 mm ID; Restek Corporation, Bellefonte, PA, USA) with helium as the carrier gas (5 cm/s). All injections were made in the splitless mode with the split valve closed for 60 s. The oven temperature was held initially 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 held at 310 °C for 5 min. Individual compounds were identified using the mass spectral data and ion chromatograms comparing our spectra to published data and authentic standards (Mason et al., 1990). By using the peak integration function in ChemStation software (Agilent Technologies Inc., Santa Clara, CA, USA) interfaced with the GC–MS, we determined the relative contributions of each component of the pheromone to the overall profile of each snake.

Data processing and statistical analysis

We tested for global differences in total pheromone mass, unsaturated to saturated component ratio and individual unsaturated and saturated component masses using one-way ANOVAs and two-way repeated measures ANOVAs (treatment, methyl ketone mass [Da]) followed by pairwise comparisons (Tukey tests or Student–Neuman–Keuls multiple comparisons; SigmaPlot 12). Effect sizes for significant ($p < 0.05$) relationships from pairwise comparisons and ANOVAs were estimated using Cohen's d (pairwise comparisons) and either η^2 (ANOVA) or partial η^2 (two way ANOVA), using online tools (<http://www.cognitiveflexibility.org/EffectSize/> and <http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD-main.php>). When the assumptions of normality (distribution, equal variance) were not met, the data were log-transformed before statistical analyses or appropriate nonparametric equivalent analyses were used. Pheromone mass and individual component mass were derived using previously published methods (LeMaster and Mason, 2002; Mason et al., 1990). By using an internal standard (methyl stearate, 20 µg/mL hexane; LeMaster et al., 2008), we were able to derive the individual component mass (µg) for all of the 17 long-chain methyl ketones composing the pheromone.

Global differences in the pheromone's composition were analyzed using the Multi-Response Permutation Procedure (MRPP) in the vegan package for R (v.1.8-8; McCune et al., 2002), as previously published (Parker and Mason, 2009, 2012). Pairwise comparisons for pheromone composition were run using the same procedure but excluding a new group each time. When the exclusion of a group resulted in a non-significant separation among the remaining groups, pairwise comparisons were then made between the excluded group and each remaining group, as in Parker and Mason (2009, 2012). Coordinates for a non-metric scaling plot to visually represent the differences in individual pheromone profiles were generated using the vegan package, and all graphics were created in SigmaPlot 12 (Systat Software Inc., San Jose, CA, USA).

Results

Radioimmunoassay

In the first experiment, castration (GX) completely reduced the total androgen levels of males compared to SHAM males (mean = 0.14 \pm 0.34 ng/mL and 2.01 \pm 0.66 ng/mL, respectively; $t = 2.91$, $p = 0.008$, $d = 1.21$).

In the second experiment, castration again completely reduced the total androgen levels in GX males compared to SHAM, GX + T and T males ($F_{3,59} = 139.53$, $p < 0.001$, $\eta^2 = 0.59$). The total androgen levels

for each group in the second experiment were as follows: SHAM (10.16 ± 3.53 ng/mL), GX (0.13 ± 0.01), GX + T (27.44 ± 2.32) and T (25.78 ± 2.03). SHAM male androgen levels were higher than those of GX males ($q = 3.99, p = 0.007, d = 0.99$). The total androgen levels for SHAM males were different between the two experiments, but the levels observed are within the normal fall range of male red-sided garter snakes in the laboratory (e.g., Crews et al., 1984). GX + T male androgen levels were higher than the levels in either GX ($q = 11.03, p < 0.001, d = 4.00$) or SHAM males ($q = 7.25, p < 0.001, d = 0.88$). T male androgen levels were higher than those in either GX ($q = 10.36, p < 0.001, d = 1.94$) or SHAM males ($q = 15.61, p < 0.001, d = 0.94$). The total androgen levels were not different between GX + T and T males ($q = 0.70, p = 0.618$). Although the implants significantly increased the total androgen levels in males compared to SHAM males, the levels were within the physiological range for this species in both the breeding and non-breeding seasons (e.g., Lutterschmidt and Mason, 2009; Moore et al., 2000, 2001).

Mating ball trial – experiment one

More wild males were attracted per minute to GX males (4.45 ± 0.96) than to SHAM males (1.23 ± 0.12) in the mating ball tests ($U = 9.50; p < 0.001, d = 1.35$) (Fig. 1). All SHAM males were courted in these mating ball tests by at least one wild male (three were courted by two males), but the level of attention the SHAM males received was significantly less than that received by GX males. This background level of attractiveness in SHAM males was also seen in our previous study (Parker and Mason, 2012).

Pheromone analyses – experiment one

The total skin lipid mass (mg/cm of length) of males was unaffected by castration (SHAM = 0.22 ± 0.09 , GX = 0.25 ± 0.07 ; $t = 0.71, p = 0.480, d = 0.30$) (Supplemental Fig. 1A). However, GX males produced significantly more total pheromone ($233.69 \mu\text{g} \pm 51.67$) than did SHAM males ($105.79 \mu\text{g} \pm 41.59$; $t = 3.04, p = 0.006, d = 1.24$) (Supplemental Fig. 2A). Furthermore, the composition of the pheromone was significantly altered in GX males. Based on MRPP analysis and NMS plotting, SHAM and GX snakes had significantly different pheromone profiles using the total abundance of methyl ketones obtained from the integration of chromatograms ($A = 0.14, p = 0.001$) (Fig. 2A; stress = 8.1). GC traces from the isolated pheromones of GX males revealed the expression of several long-chain, unsaturated methyl ketones of the same type observed in female garter snakes (Fig. 2B) (Mason et al.,

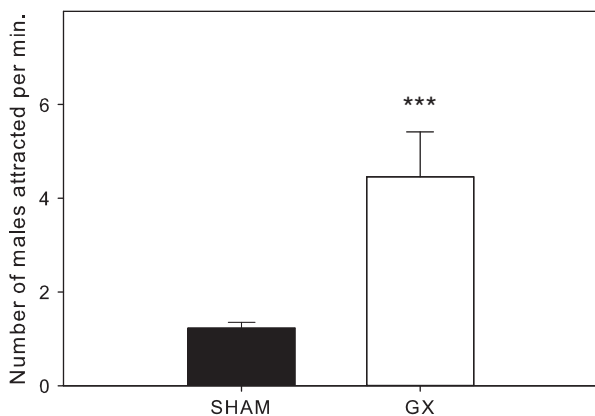


Fig. 1. Results from a mating ball bioassay with experimentally manipulated male red-sided garter snakes. Wild males engaged in a mating ball with a female in the den were counted as they came off the female to court single experimental animals over a period of 1 min (mean ± SEM). The asterisk represents a significant difference ($p < 0.05$) in attractiveness. SHAM = intact male garter snakes ($n = 12$), GX = castrated ($n = 12$).

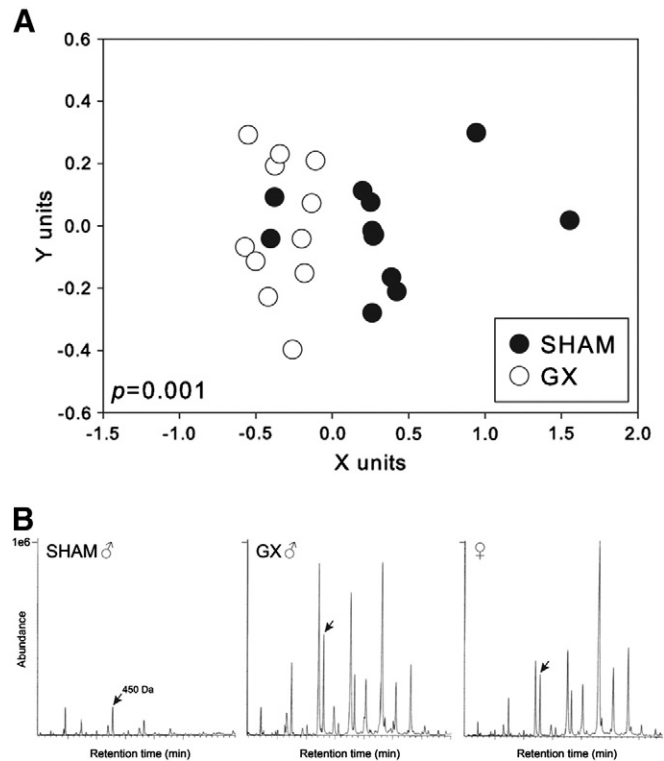


Fig. 2. Differences in pheromone profile compositions between SHAM (intact; $n = 12$) and GX (castrated; $n = 12$) male red-sided garter snakes. A. Non-metric multidimensional scaling plot showing variations in the pheromone compositions of the two groups. Points close to one another represent pheromone profiles from individual snakes that were similar in their expression patterns of the 17 individual methyl ketones composing the pheromone. Arrows indicate the individual snakes chosen for the representative GC traces. Stress = 8.1. Two points (one SHAM, one GX) are hidden in the plot due to overlap. B. Representative GC traces of pheromone profiles from SHAM, GX and female red-sided garter snakes. SHAM males have negligible methyl ketone expression. GX male and female snakes both express abundant amounts of long-chain, saturated and unsaturated methyl ketones. The arrows indicate that the same molecule (450 Da saturated methyl ketone) differs in its abundance across the profiles. GC traces are scaled to the same molecular abundance ($1e6$).

1989). The pheromone profiles of the castrated males resembled those of small females (e.g., LeMaster and Mason, 2002).

After analyzing the ratio of abundances of unsaturated to saturated components within a pheromone blend (U:S) that acts as a predictor of the attractiveness of a snake's pheromone profile (e.g., LeMaster and Mason, 2002; Parker and Mason, 2009), we concluded that the gonadectomy of males led to a near four-fold increase in the U:S ratio in GX males over SHAM animals (4.38 ± 0.89 vs. 1.18 ± 0.30 ; $U = 15.00, p = 0.001, d = 1.38$).

Mating ball trial – experiment two

More wild males were attracted to GX males than to the males in the other groups ($F_{3,58} = 18.24, p < 0.001, \eta^2 = 0.49$). GX animals attracted more males per minute (3.26 ± 0.52) than did the SHAM (0.47 ± 0.15 ; $q = 9.19, p < 0.001, d = 1.92$), GX + T (0.58 ± 0.22 ; $q = 8.07, p < 0.001, d = 1.67$) or T males (0.73 ± 0.22 ; $q = 8.08, p < 0.001, d = 1.62$) (Fig. 3). No other comparisons were significantly different (SHAM vs. GX + T: $q = 0.34, p = 0.806$; SHAM vs. T: $q = 0.86, p = 0.815$; GX + T vs. T: $q = 0.45, p = 0.751$).

Pheromone analyses – experiment two

Because testosterone can alter the total skin lipid production of terrestrial vertebrates (e.g., Abalain et al., 1984a; Ebling, 1974; Lindzey and Crews, 1993), we first analyzed the effects of testosterone treatment on

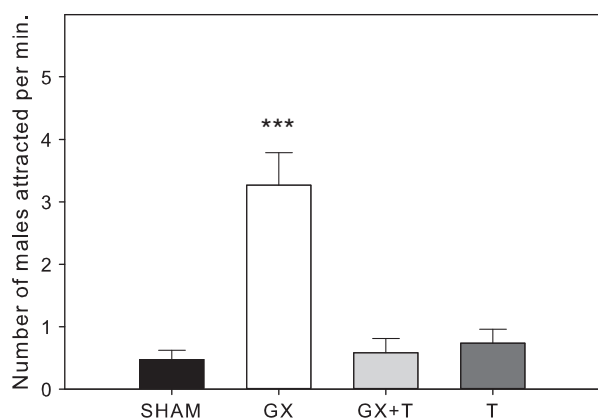


Fig. 3. Results from the mating ball bioassay using wild male red-sided garter snakes from the second experiment (mean \pm SEM). Wild males were significantly more attracted to castrated males (GX) and showed significantly less interest in control males (SHAM), castrated males implanted with testosterone (GX + T) or intact males implanted with testosterone (T). The asterisk represents statistical significance ($p < 0.05$). $N = 15$ for each group.

skin lipid mass corrected for body length (mg/cm of length). Testosterone treatment changed the total skin lipid mass among the groups: SHAM ($0.22 \text{ mg/cm} \pm 0.01$), GX (0.20 ± 0.01), GX + T (0.27 ± 0.03) and T (0.28 ± 0.01) ($F_{3,57} = 3.94$, $p = 0.013$, $\eta^2 = 0.17$) (Supplemental Fig. 2B). Males in the GX + T group produced more total skin lipids than did GX males ($q = 3.63$, $p = 0.034$, $d = 0.80$). The same was true for T vs. GX males ($q = 4.06$, $p = 0.029$, $d = 1.20$). T implantation marginally increased the total skin lipid production in GX + T and T males when compared to the SHAM group ($q = 2.66$, $p = 0.065$; $q = 3.08$, $p = 0.084$, respectively). No other comparisons were significantly different (SHAM vs. GX: $q = 1.03$, $p = 0.468$; GX + T vs. T: $q = 0.36$, $p = 0.797$).

Total pheromone mass (μg) was also affected by treatment (SHAM [$197.40 \mu\text{g} \pm 38.88$], GX [898.94 ± 181.29], GX + T [293.67 ± 55.10] and T [456.16 ± 95.07]) ($F_{3,53} = 5.73$, $p = 0.002$, $\eta^2 = 0.32$) (Supplemental Fig. 2B). Males in the GX group produced more pheromones than SHAM ($q = 5.60$, $p = 0.001$, $d = 1.36$) or GX + T males ($q = 3.79$, $p = 0.026$, $d = 1.13$). There was a marginal difference between SHAM and T males, with T males producing more pheromone ($q = 3.37$, $p = 0.054$, $d = 1.04$). However, the marginal increase in total pheromones seen in T animals was not due to increases in the same pheromone components as in the GX group (see individual component masses below). No other comparisons were significantly different (SHAM vs. GX + T: $q = 1.67$, $p = 0.243$; GX vs. T: $q = 1.94$, $p = 0.175$; GX + T vs. T: $q = 1.70$, $p = 0.233$).

Global differences in the pheromone composition were found among the groups using total abundance values from GC–MS analysis ($A = 0.06$, $\delta = 0.46$, $p = 0.001$) (Fig. 4; stress = 11.2). When the GX group was excluded from the MRPP analysis, there was no longer a significant difference among the groups ($A = 0.01$, $\delta = 0.44$, $p = 0.176$); therefore, the only post hoc comparisons conducted were between GX and the other groups. In pairwise comparisons, GX males had significantly different pheromone blends compared with SHAM ($A = 0.12$, $\delta = 0.48$, $p < 0.001$) and GX + T males ($A = 0.07$, $\delta = 0.48$, $p = 0.007$), but their pheromone blends only marginally differed from those of T males ($A = 0.030$, $\delta = 0.48$, $p = 0.07$).

There were differences in the U:S ratios among the groups ($F_{4,65} = 4.81$, $p = 0.002$, $\eta^2 = 0.30$). GX males had higher U:S ratios (5.26 ± 0.97) than SHAM (1.69 ± 0.17 ; $q = 5.62$, $p = 0.002$, $d = 1.29$), GX + T (2.19 ± 0.35 ; $q = 4.74$, $p = 0.007$, $d = 1.05$) or T males (2.70 ± 0.43 ; $q = 3.866$, $p = 0.022$, $d = 0.85$) (Fig. 5B). No other comparisons were significantly different (SHAM vs. GX + T: $q = 1.18$, $p = 0.407$; SHAM vs. T: $q = 2.36$, $p = 0.226$; GX + T vs. T: $q = 1.18$, $p = 0.406$).

The pheromone is composed of a series of individual methyl ketones, and so we next analyzed how the mass of each methyl ketone (μg)

changed across the four groups. There were significant main effects of the experimental group ($F_{3,48} = 4.24$, $p = 0.01$, $\eta^2_{\text{partial}} = 0.09$) and methyl ketone molecular mass [Da] ($F_{16,816} = 75.14$, $p < 0.001$, $\eta^2_{\text{partial}} = 0.27$) on ketone mass, as well as a significant interaction between group and ketone molecular mass ($F_{48,816} = 14.97$, $p < 0.001$, $\eta^2_{\text{partial}} = 0.21$). Several unsaturated methyl ketones showed a marked upregulation in response to castration, specifically the unsaturated ketones weighing 448, 476, 490, 504, 518 and 532 Da (Fig. 5A) (Supplemental Table 1). Five of these same unsaturated methyl ketones (476, 490, 504, 518 and 532 Da) were the only ones that significantly increased in amount following estrogen treatment in males, suggesting their major function as sex-specific components of the pheromone (Parker and Mason, 2012). One saturated methyl ketone was also expressed in higher quantities in GX than in SHAM animals (478).

An unexpected finding was that testosterone treatment elicited an increased expression of certain methyl ketones, mostly the longer, saturated ketones (Fig. 5A) (Supplemental Table 1). Specifically, saturated methyl ketones weighing 464, 478, 492 and 506 Da were expressed in higher amounts in the T-implanted males than in most other groups (Fig. 5A). The unsaturated methyl ketones 462 and 490 also showed an increase in T males. Although these changes were statistically significant, they failed to render T males attractive in our mating ball bioassays.

Please note that, prior to the GC–MS analysis in the second experiment, several pheromone extracts were contaminated during the fractionation process. The final number of males in each group is provided in the figure legends.

Discussion

Our results show that testosterone suppresses female pheromone production in male garter snakes. In the absence of testosterone, adult male garter snakes produce female pheromone blends that are attractive to males and can elicit courtship behaviors from wild males engaged in mating balls in the field. However, when castrated males are given testosterone implants (GX + T) they are rendered unattractive, and their pheromone blends return to the typical male blend. Both the testosterone and blank implants were given 1 month following castration to allow for depletion of any circulating testosterone via clearance and to permit the actions of the castration-induced effects before administering the subsequent experimental treatment. Thus, we suggest that testosterone is a key inhibitory endocrine signal mediating chemical dimorphism in garter snakes.

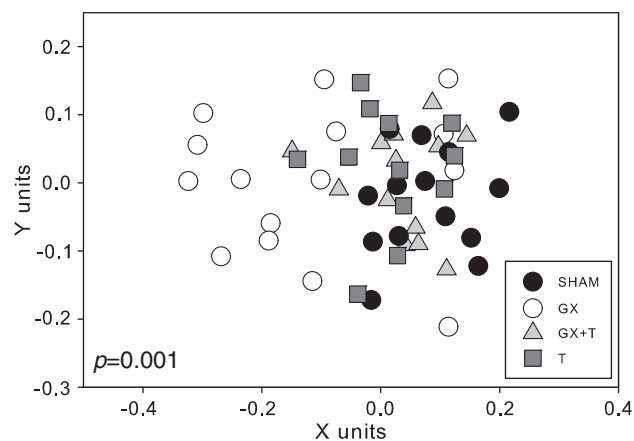


Fig. 4. Non-metric multidimensional scaling plot showing the variations in pheromone profiles among experimental red-sided garter snakes. GX males had significantly different pheromone profiles compared to the other groups based on the total abundance of the 17 methyl ketones that compose the pheromone. Stress = 11.2. Several points are hidden due to overlap in the NMS plot. The final numbers of individuals per group were as follows: SHAM ($n = 14$), GX ($n = 15$), GX + T ($n = 13$), and T ($n = 12$). Abbreviations are explained in the legend of Fig. 3.

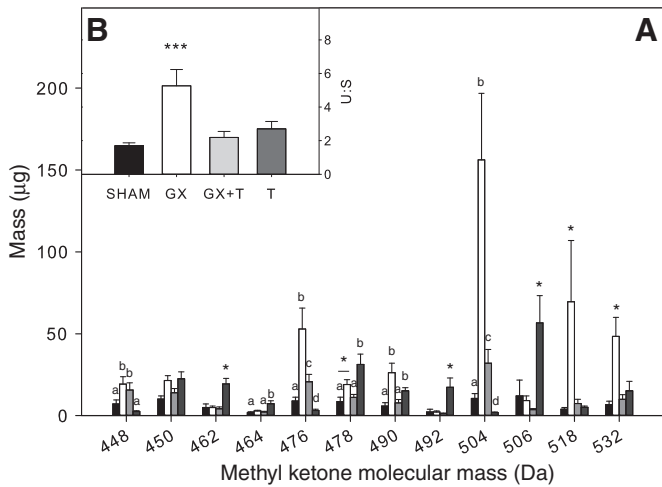


Fig. 5. Quantitative differences in pheromone composition across the experimental groups of red-sided garter snakes. **A.** Mass (μg ; mean \pm SEM) of individual methyl ketones (grouped by ketone molecular mass, Da) composing the sex pheromone blend. Asterisks represent the individual groups with significantly ($p < 0.05$) higher levels of that specific methyl ketone than any of the other groups. Lower-case letters are used when more complicated statistical relationships existed between the groups, with different letters representing significantly different ($p < 0.05$) experimental groups for that specific methyl ketone. The mass (μg) per methyl ketone was derived using an internal standard (methyl stearate). **B.** The ratio (mean \pm SEM) of unsaturated (U) to saturated (S) components in the pheromone profiles of each group. Asterisks represent statistical significance ($p < 0.05$). The final numbers of individuals per group were as follows: SHAM ($n = 14$), GX ($n = 15$), GX + T ($n = 13$), and T ($n = 12$). Abbreviations are explained in the legend of Fig. 3.

When testosterone is present in males, it has a consistent effect on pheromone composition; SHAM and GX + T males produced very similar pheromone blends (Fig. 4). Previously, we showed that the composition of the pheromone blend induced by estrogen implantation is relatively invariable, dominated by high molecular weight, unsaturated methyl ketones (Parker and Mason, 2012). Because GX males lack a major source of sex steroid hormone, unlike estrogen-implanted and intact males, variation in the composition of their pheromone blends is expected (i.e., different males may have different clearance rates post-gonadectomy). Extragonadal production of sex steroids (e.g., adrenal glands) could contribute to this variation further, although adrenal hypertrophy has not been noted in castrated male red-sided garter snakes in previous studies (e.g., Camazine et al., 1980; Crews et al., 1984).

We have shown that testosterone increases skin lipid production in red-sided garter snakes and drives the expression of at least some methyl ketones in the sex pheromone blend. In mammals, birds and lizards, androgen treatment (implantation, injection) stimulates skin lipid production (e.g., Abalain et al., 1984a; Abell, 1998; Ebling, 1974; Lindzey and Crews, 1993; Nikkari and Valavaara, 1970). Our results show that the total skin lipid production in garter snakes is affected by T supplementation (GX + T and T groups) compared to gonadectomy alone (GX). The lack of a difference between SHAM and GX males in total skin lipid production may result from the decrease in the amplitude of maximum seasonal total androgen levels in intact males in the laboratory compared to the field (laboratory: ~ 10 ng/mL, Parker, unpubl. obs.; field: ~ 70 ng/mL; Moore et al., 2001). The males in our study were in captivity for 11 months, which may have affected the magnitude of their androgen cyclicity. The silastic implants packed with testosterone elevated the males' circulating androgen levels to ~ 26 ng/mL, which may establish an increasing dose–response effect on skin lipid production among GX, SHAM and T/GX + T males. Our implants were validated in the fall after only 1 month, although previous work has shown that this type of implant persists in its maintenance of steroid delivery in red-sided garter snakes (e.g., Crews et al., 1984; Mendonça et al., 1996; Parker and Mason, 2012). In terms of pheromone composition changes, we observed peculiar alterations in the

methyl ketone expression of T males. The saturated methyl ketones that were upregulated in T males (464, 478, 492 and 506 Da) constitute a small proportion of a female's natural pheromone profile (e.g., less than 20% combined of all methyl ketones; LeMaster and Mason, 2003; Parker and Mason, 2009). Furthermore, they failed to elicit courtship behaviors from wild males in our experiment, as opposed to the unsaturated methyl ketones of the same length that constitute a larger proportion of the total pheromone profile of females (Mason et al., 1989, 1990). These saturated ketones may, instead, play a role in species recognition. Recent work comparing red-sided garter snakes to other species of *Thamnophis* has suggested that, instead of expressing novel ketones, different species have altered proportions of methyl ketone expression (Uhrig et al., 2014). Testosterone may thus play an important role in sculpting the specificity of sex pheromone composition if such saturated ketones inform the receiver about species identity.

The sex hormones testosterone and estrogen work in opposition to regulate the skin's lipid production in vertebrates. In the early stages of puberty, the wax esters and squalene in sebaceous secretions from humans increase in correlation with urinary androgen levels (e.g., Pochi et al., 1977; Yamamoto and Ito, 1992). In rats, lipid production in the sebaceous glands is sexually dimorphic, with males secreting more than females (Toh, 1980). This dimorphism relies on testosterone signaling, whereas castration abolishes the dimorphism, even more so if the rats are castrated before sexual maturity (Toh, 1980). Testosterone also has specific activating effects on lipogenesis in rodent (hamster) skin and suppresses the synthesis of polar lipids (e.g., Cabeza and Diaz de Leon, 1993; Hall et al., 1983). The activational effects of testosterone on the skin are counteracted by estrogen and progesterone, which inhibit the skin's lipid production in mammals (e.g., Cabeza and Diaz de Leon, 1993; Ebling and Skinner, 1967; Girard et al., 1980). Furthermore, female-typical lipid secretion cannot be induced in the sebaceous-like glands (e.g., the preputial glands of rats) by estrogen treatment (Alves et al., 1986). The uropygial gland of birds, a comparative skin lipid organ used as a model for studying sebaceous gland physiology, produces sex-specific blends of skin lipids that are affected by sex hormone treatment (e.g., Abalain et al., 1984a,b; Bohnet et al., 1991). More closely related to snakes, several lizard species show marked changes in skin lipid production in response to natural and/or supplemental sex steroids, although the chemical quantitation and analysis of the composition of these secretions has only been pursued more recently (e.g., Abell, 1998; Alberts et al., 1992; Fergusson et al., 1985; Martin et al., 2007; Mason and Parker, 2010). Our previous work revealed that estrogen overrides the inhibitory signal generated by testosterone and activates female-typical methyl ketone expression in intact male garter snakes (Parker and Mason, 2012). The data in our current paper show that testosterone activates total skin lipid production in garter snakes but suppresses long-chain, unsaturated methyl ketone expression. Although our work provides evidence that circulating sex hormones target the skin, it may also be that snake skin, much like human skin, is capable of synthesizing and metabolizing its own sex steroids to regulate the local production of activational hormones (e.g., Inoue et al., 2012).

Some male red-sided garter snakes in the Manitoba system naturally smell like females ("she-males"; Mason and Crews, 1985). It was originally proposed that these she-males represented an alternative reproductive strategy; however, further research discovered that most males express female pheromones transiently upon emergence when they are cold, are ignored in mating balls in the presence of females and, instead, stand to benefit from the friction generated by the courtship ritual, warming up quickly once they reach the surface (LeMaster et al., 2008; Shine et al., 2001, 2003a). Red-sided garter snakes exhibit a dissociated reproductive strategy in which their maximal mating behavior does not overlap temporally with their maximal androgen synthesis (Crews, 1984; Crews et al., 1984). Thus, in the spring, males have the lowest androgen levels of the annual cycle, although spring males can also sometimes have elevated androgens that quickly decline during the first weeks of spring (e.g., Krohmer et al., 1987; Moore et al.,

2000; Weil, 1985; summarized in Woolley et al., 2004). Lutterschmidt and Mason (2009) demonstrated that spring androgen levels in male garter snakes are determined by metabolic clearance before and during hibernation, as evidenced by the fact that warmer hibernation temperatures deplete the males' total androgen levels more than cold hibernation temperatures. This seasonal depletion of testosterone in red-sided garter snakes may therefore alter their expression of methyl ketones, although other studies would be needed to test this idea.

A specific question in our study centers on the mechanism activated by castration. In addition to the transient female mimics mentioned above, some she-males in our study system continually express female pheromones, especially unsaturated ketones, beyond the mating season (Mason and Crews, 1985). These "original" she-males also have higher circulating testosterone levels than normal males, although the results of our current study suggest that these males should have a significantly suppressed production of unsaturated methyl ketones. It was originally postulated that these high levels of testosterone may be constitutively metabolized to estrogens in the skin via dysfunctional aromatase activity, resulting in a localized, feminizing effect on the expression of pheromones (Mason and Crews, 1985). Such a scenario mirrors the findings from Sebright bantams, which showed that hen-type feathering in castrated males persists due to expression of a mutated, constitutively active form of the aromatase gene in their skin (George et al., 1981). Further work in mallards revealed that castration activated the expression of eclipse ("female") plumage in males, but only when the castrates were given high doses of testosterone; this result suggests that aromatase expression in the skin may be induced by castration (Haase and Schmedemann, 1992). Perhaps the female pheromone expression in castrated male garter snakes results from activation of aromatase in the skin, despite aromatase's direct inhibition by testosterone. Testosterone replacement in our study (GX + T) reverted castrates to male-typical pheromone expression, suggesting that T is a master regulator of this peripheral mechanism. Future work using aromatase inhibitors should better answer this question.

Castration is a routine procedure that is undertaken with livestock and companion animals primarily to prevent reproduction and stave off undesirable social behaviors. However, the concept of castration's having unintentional effects on channels of private communication, such as chemical signaling, has not been addressed previously. Several putative pheromonal compounds in rats are negatively affected by castration, although these were not clearly identified as pheromones (Gawienowski et al., 1976; Zhang et al., 2008). Furthermore, the relationship between testosterone and chemical signals in mammals is, at best, unresolved (reviewed in Baum and Bakker, 2013; Petrucci, 2013). However, in at least one species of lizard, the attractive component in male skin secretions, dehydrocholesterol, decreased with testosterone supplementation, even though this had no effect on the attraction of the females to the said cues (Martín et al., 2007). Our work suggests that endogenous testosterone may inhibit the secretion of some female-specific unsaturated methyl ketones in male garter snakes, whereas castration relaxes this inhibition and makes males attractive. These findings constitute the first evidence of testosterone actively inhibiting female pheromone production in a vertebrate. Furthermore, pheromones are cryptic sexual signals and may be carried in hormonal tandem with overt multimodal signals that are sculpted by testosterone in (all?) other vertebrates (e.g., plumage badges, skin colorations).

The garter snake sex pheromone serves as an interesting model for exploring the evolution of skin-based secondary sexual signals that are hormonally regulated. Furthermore, the pheromone shares common hormonal control processes with the mechanisms regulating dimorphic bird plumage. Female Paleognathid birds, the most basal bird group, exhibit dull, cryptic coloration that reflects the derived character state (Kimball, 2006). The female's plumage is estrogen-dependent in these birds, and estrogen can organize the skin of developing male birds to permanently express female plumage (Mueller, 1970). Male birds and male squamate reptiles are the homogametic sex (ZZ); thus,

male secondary sexual traits are thought to represent the ancestral character state while females (ZW) express hormone-dependent, derived character states (Kimball, 2006). Our collective data support this idea. In garter snakes, the derived state is implicated to be the estrogen activation of long-chain methyl ketone expression in females (Parker and Mason, 2012; Uhrig et al., 2012). The study of males, however, may provide a new window into the evolution of the hormonal regulation of secondary sexual signals: active inhibition by testosterone.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yhbeh.2014.07.007>.

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