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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.

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 brevital 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. × 2.41 mm o.d. × 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)).