

Effects of androgens on behavioral and vomeronasal responses to chemosensory cues in male terrestrial salamanders (*Plethodon shermani*)

Stephanie N. Schubert^a, Lynne D. Houck^b, Pamela W. Feldhoff^c,
Richard C. Feldhoff^c, Sarah K. Woodley^{a,*}

^a Department of Biological Sciences, Duquesne University, 600 Forbes Avenue, Pittsburgh, PA 15217, USA

^b Department of Zoology, Oregon State University, Corvallis, OR 97331-2914, USA

^c Department of Biochemistry and Molecular Biology, University of Louisville Health Sciences Center, Louisville, KY 40292, USA

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Abstract

Chemosensory stimuli and sex steroid hormones are both required for the full expression of social behaviors in many species. The terrestrial salamander, *Plethodon shermani*, is an emerging nonmammalian system for investigating the nature and evolution of pheromonal communication, yet little is known regarding the role of sex steroid hormones. We hypothesized that increased circulating androgen levels in male *P. shermani* enhance chemoreception through morphological, behavioral, and physiological mechanisms. Experimental elevation of plasma androgens increased development of cirri, morphological structures thought to enhance the transfer of chemosensory cues from the substrate to the vomeronasal organ (VNO). Elevated plasma androgens also increased expression of a chemo-investigatory behavior (nose tapping) and increased preference for some female-derived chemosensory cues. Male-produced courtship pheromones activated a large number of cells in the VNO as measured by the method of agmatine uptake. However, androgen levels did not affect the total number of vomeronasal cells activated by male-produced courtship pheromones. Future studies will determine whether androgens potentially modulate responsiveness of the VNO to female-derived (as opposed to male-derived) chemosensory cues.

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Introduction

Chemosensory stimuli are important to the full expression of reproductive and social behaviors in many vertebrate species (Johnston, 1983; Sachs and Meisel, 1988; Vandenberg, 1988; Wingfield et al., 1994; Wood and Newman, 1995). Nonvolatile chemosensory stimuli are typically detected by receptors expressed by sensory neurons of the vomeronasal organ (VNO) (Halpern and Martinez-Marcos, 2003). Sensory neurons of the VNO project axons to the accessory olfactory bulb. In turn, neurons of the accessory olfactory bulb project to areas of the brain involved in reproductive behaviors and physiology, such as the medial amygdala, bed nucleus of the stria terminalis, the preoptic area, and the ventromedial hypothalamus (Scalia and Winans, 1975; Schmidt and Roth, 1990). Sex steroid

hormone receptors are found in these brain areas, and the integration of hormonal and chemosensory information in the brain is required for the expression of male mating behavior (Wood, 1998; Wood and Newman, 1995).

Much information concerning chemosensory signals is available for mammals. We focus here, however, on a more basal tetrapod group: terrestrial salamanders. The nature of chemosensory signals involved in reproduction has been examined in this group, in particular for Shermani's salamander, *Plethodon shermani*, and related *Plethodon* species. These animals are nocturnal and rely on chemical communication to mediate social interactions. Information about species, sex, and individuality are conveyed via chemosensory cues (Dawley, 1984; Gillette et al., 2000; Jaeger and Gergits, 1979; Mathis, 1990; Palmer, 2004). In particular, a combination of studies at multiple levels indicates that *P. shermani* is an excellent model for understanding the nature and evolution of vertebrate pheromonal communication (Feldhoff et al., 1999; Houck and

* Corresponding author. Fax: +1 412 396 5907.

E-mail address: woodleys@duq.edu (S.K. Woodley).

Arnold, 2003; Palmer et al., 2005; Rollmann et al., 1999; Watts et al., 2004; Wirsig-Wiechmann et al., 2002). In particular, a blend of proteinaceous pheromones is produced by an exocrine gland (mental gland) on a male's chin and is applied to the nose of a female during courtship. These courtship pheromones activate VNO cells as indexed by agmatine labeling and increase female receptivity. Several pheromones in the blend have been biochemically characterized and genetic analyses reveal that the pheromones have experienced rapid, selection-driven evolution.

Despite the wealth of information about chemosensory communication in *P. shermani* and related *Plethodon*, almost nothing is known about the role of sex steroid hormones in chemical communication. We hypothesized that androgens enhance the ability of males to respond to conspecific chemosensory cues. Our hypothesis was based on the observation that androgens – both testosterone and dihydrotestosterone (DHT) – are highest during the breeding season when males are searching for female mating partners and interacting with rival males (Woodley, 1994). We predicted that elevation of androgen levels in adult males would (1) increase the size of cirri (protuberances that surround each nasolabial groove and extend below the upper lip), which transport nonvolatile chemosensory cues from the substrate into the lumen of the VNO (Dawley and Bass, 1989); (2) increase “nose tapping”, a chemo-investigatory behavior that brings the cirri in contact with the substrate so that cues from the substrate are drawn into the lumen of the VNO; (3) increase preferences for conspecific chemosensory cues over neutral cues; and (4) increase the number of VNO cells that physiologically respond to chemosensory cues.

Methods

We used chemosensory stimuli derived from the whole body of conspecific stimulus animals to measure behavioral responses to chemosensory cues. In contrast, we used extract from male mental glands (a source of pheromones used in courtship) to measure VNO responsiveness to chemosensory cues. Ideally, we would have used the same types of chemosensory stimuli for testing both behavioral and VNO responsiveness. However, for tests of VNO responsiveness, we decided to use male mental gland extract because, at the time, it was the only chemosensory stimulus demonstrated to activate the VNO (Wirsig-Wiechmann et al., 2002). Also, male mental gland extract was chemically characterized and could be prepared with a known concentration and purity (Feldhoff et al., 1999; Rollmann et al., 1999). This extract was available in amounts sufficient for VNO tests, but not in the quantity needed for chemosensory stimuli in behavioral tests. Thus, for behavioral tests, we used chemosensory stimuli derived from whole-body rinses of stimulus animals because large amounts could be prepared easily.

Animals

All methods were approved by Duquesne University's Institutional Animal Care and Use Committee. Animals were collected with the appropriate permits from North Carolina Department of Wildlife. Test subjects were collected from a single location (Wayah Bald, Macon County, NC, 83° 39' 30" N longitude; 35° 19' 49" W latitude) in August 2003 and August 2004. Shortly after capture, animals were anesthetized and mental glands were surgically removed (see below; procedure approved by OSU ACUP to LDH) to ensure that an animal's own mental gland secretions did not contribute to responses to chemosensory stimuli. In the laboratory, subjects were individually housed at 16°C on a 14L:10D photoperiod in 16×16×5 cm plastic boxes lined with moist paper towels and fed wax worm larvae. Average body length (snout-vent length) did not differ between animals in the 2 treatment groups.

Implants and surgery

Implants were made from Dow Corning Silastic Laboratory Grade Tubing (1.02 mm ID, 2.16 mm OD). Implants were 13 mm long, of which 10 mm were packed with crystalline testosterone propionate (TP) (Sigma #T1875). Ends were sealed with Sylgard 184 silicon elastomer. Control (BLANK) implants were similar, but contained no TP.

Animals were randomly assigned to treatment groups although we distributed animals caught in different years equally between the two treatment groups. Before surgery, animals were anesthetized in 0.5% MS222. The implant was inserted into the body cavity via a single 3-mm long incision in the abdominal wall and the incision was closed with a suture. A surgical adhesive and topical antibiotic were applied to the incision. Surgeries were performed in May 2005 and animals were allowed 3 weeks to recover before further testing.

Cirrus size

Six weeks after surgeries, we assessed cirrus size by taking digital images of the right-lateral view of each animal's head at 3× magnification. The length of the cirrus from the ventral margin of the external naris to the ventral tip of the cirrus along the nasolabial groove was measured with Image-Pro Plus imaging software. In addition, images were sorted into 4 groups based on overall size of the cirrus: not visible, small, medium, or large. The investigator was blind to treatment group when assessing cirrus size.

Behavioral tests

General

Scan sampling methods (Martin and Bateson, 1993) were used to quantify behavioral responses to chemosensory stimuli. All behavioral tests were conducted at 25°C under dim incandescent light in the evening during the dark period of the photoperiod when animals normally are most active. A single investigator performed all the testing and was blind to the experimental treatments of the subjects.

Chemosensory stimuli used in behavioral tests

For behavioral tests, we used (1) whole-body rinses and (2) substrates that were scent-marked by females in reproductive condition because previous studies showed that these sources of chemosensory cues elicited behavioral responses in *P. shermani* and *P. cinereus* (a congeneric of *P. shermani*) (Palmer, 2004; Sullivan et al., 2003).

We tested whole-body rinses derived from nonreproductive males ($n=5$), reproductive females ($n=5$), and nonreproductive females ($n=5$). To obtain body rinses, individual animals were placed in 50 ml of ddH₂O in glass containers for 48 h at 16°C. Body rinses from the same category (e.g., males) were pooled and diluted with ddH₂O to a volume of 520 ml. Body rinses were frozen at -20°C in 4 aliquots until use in behavioral tests. Control stimuli were prepared in an identical manner as for body rinses except that an animal was not placed in the ddH₂O. Aliquots were coded so the investigator was blind to the nature of the different chemosensory stimuli. Body rinses were used in behavioral tests within 7 days of collection. Animals used to obtain body rinses were not used as test subjects.

Substrates scent-marked by reproductive females were prepared by placing moistened paper towels on the bottom of females' home boxes for 48 h. The scent-marked substrates were then immediately used in behavioral tests. Control substrates were prepared by lining clean home boxes (without a female) with moistened paper towels for 48 h.

Response to chemosensory stimuli: nose tapping and locomotor activity

In plethodontid salamanders, nonvolatile substances gain access to the VNO via two nasolabial grooves running from the upper lip to the external nares (Dawley and Bass, 1989). Each nasolabial groove is contained within a cirrus, a fleshy protuberance that is thought to increase transport of nonvolatile substances via the nasolabial groove. Animals behaviorally control access of nonvolatile substances to the VNO by tapping their noses (and thereby the nasolabial cirri and grooves) to the substrate. The behavior is termed “nose tapping” and is an unambiguous and easily scored behavior. We measured nose tapping and as well

as movement on substrates containing chemosensory cues derived from males, reproductive females, nonreproductive females, and water. Each subject was tested individually in a $23 \times 23 \times 2$ cm testing chamber lined with a paper towel to which 10 ml (enough to fully moisten) of a chemosensory stimulus was applied. After a 10-min habituation period, the chamber was scanned for 2 s once every minute for 60 min. During each scan, data recorded for each animal were (a) the location, defined as the quadrant in which the salamander's head was positioned, and (b) the occurrence of nose-tapping behavior. The final score for occurrence of nose tapping was the total number of scans in which this behavior was observed (maximum possible=60). Activity was estimated by counting the number of times the subject was located in a quadrant that was different from the quadrant noted in the previous scan (maximum possible=60).

Subjects were tested during 4 trials for responses to body rinses from males, reproductive females, nonreproductive females, and to water. Each subject was tested every other night with a single type of chemosensory stimulus per night in a randomized order. On a given trial, an aliquot of each of the 4 different stimuli was thawed and used as a chemosensory stimulus. In this way, some subjects were tested on each type of chemosensory stimulus every night. Aliquots were coded so that the observer was blind to the nature of the chemosensory stimuli.

Behavioral preferences for female chemosensory stimuli

Testing chambers were prepared in which one half was lined with a paper towel that was scent-marked by a reproductive female or moistened with reproductive female body rinse and the other half was lined with a paper towel moistened with clean ddH₂O (with a gap of 1 cm between the substrates). After each male was placed in a testing chamber, data scored were (1) the location of the male's head (side with the chemosensory cue vs. water) and (2) the occurrence of nose tapping. Each subject was scanned and scored once every 2 min for 2 h (60 observations per male). Subjects that were on the side containing female chemosensory cues for more than 50% of the observations were considered to show a preference for that substrate over water.

VNO responses to pheromones

Method of agmatine uptake

We used the method of agmatine (AGB) uptake to identify vomeronasal cells that were activated after application of mental gland extract. Unlike electrophysiological methods and calcium imaging, AGB uptake samples activation throughout the entire extent of the chemosensory epithelium and can be used *in vivo* in nonsedated animals. The method was developed for use in lobsters and zebrafish (Michel et al., 1999) and adapted to plethodontid salamanders (Wirsig-Wiechmann et al., 2002, 2006). AGB is a guanidinium analog that, when co-delivered with a chemosensory stimulus, enters nonspecific open cation channels. AGB immunocytochemistry visualizes cells that have taken up agmatine and we infer that these cells were activated in some way by the chemosensory stimulus. In an earlier study, the method of AGB uptake provided evidence that male mental gland extract activates vomeronasal cells in female *P. shermani* (Wirsig-Wiechmann et al., 2002).

Mental gland extract

We used male mental gland extract to test responsiveness of the male VNO to chemosensory stimuli. Mental gland extract was obtained from approximately 100 reproductively active males collected from Wayah Bald, Macon County, NC, in August 2004 as described previously (Wirsig-Wiechmann et al., 2002). A concentration of 2.0 mg/ml of mental gland extract was used because it increased receptivity and activated vomeronasal cells in female *P. shermani* in previous studies (Wirsig-Wiechmann et al., 2002; Rollmann et al., 1999). Control animals received 0.5× PBS (vehicle) without mental gland extract.

Shortly before use, mental gland extract was mixed 1:1 with 6 mM AGB dissolved in 0.1 M PBS. The PBS control was prepared by mixing 0.5× PBS 1:1 with 6 mM AGB. Subjects were placed individually in clean boxes. A micropipet was used to deliver 2 μl of either mental gland extract or PBS control (both mixed with AGB) onto the anterior tip of the nose (to cover the nares) of each subject every 2 min for 44 min. Immediately after the final stimulus application, 3×5 μl of PBS was applied to the nose of each subject to rinse away excess AGB. Following this rinse, animals were sacrificed by decapitation, snouts were placed in fixative, and a trunk blood sample was collected.

Because of logistical constraints, time from onset of stimulus application to sacrifice ranged from 63 to 123 min, with a mean of 89 min. Average times from onset of stimulus application to sacrifice were the same for all four combinations of implant treatment (TP or BLANK)×chemosensory stimulus (mental gland or PBS control). We verified that time from onset of stimulus application to sacrifice did not contribute to variation in AGB immunoreactivity or hormone levels by determining that it was not a significant covariate in statistical tests.

Tissue processing

After sacrifice, upper jaws were processed and embedded in OCT for cryosectioning as described in Wirsig-Wiechmann et al. (2002). Four upper jaws, one from each of the 4 treatment groups, were embedded in each block to ensure that all treatment groups were processed similarly. Tissues were sectioned at a thickness of 20 μm using a cryostat and sections were thaw mounted onto slides. Slides were frozen at -80°C and underwent immunocytochemistry within 1 week of sectioning.

To visualize cells that took up AGB, every 4th section (i.e., 80 μm apart) underwent immunocytochemistry for AGB following a previously published protocol (Wirsig-Wiechmann et al., 2002). The primary antibody was a rabbit polyclonal anti-AGB antibody (Chemicon AB1568-2000T) diluted 1:400. The chromagen was DAB. Every 4th section of an alternate series of slides was stained with cresyl violet using standard histochemistry.

Image analysis

Slides were coded so the investigator was blind to the experimental treatments. For tissue that underwent AGB immunocytochemistry, cells with darkly staining cytoplasm were considered AGB-IR and counted with the aid of an Olympus Brightfield microscope. AGB-IR cells were counted in both the left and right VNOs on each section (i.e., every 4th section) and summed to give a total number of AGB-IR cells per animal. For cresyl-violet-stained tissue, the cross-sectional areas of both the right and left VNOs were measured on each section (i.e., every 4th section), summed, and multiplied by 80 μm to give an estimate of VNO volume.

Hormone measurement

Plasma steroid hormone levels were measured in trunk blood collected within 3 min of decapitation in order to verify our androgen manipulations. Samples were processed and assayed following established methods (Gruenewald et al., 1992; Resko et al., 1980). Briefly, for measurement of testosterone (T) and DHT, plasma was ether extracted, DHT and T were separated from each other with Sephadex LH-20 column chromatography, and fractions were subjected to radioimmunoassay. We also measured the stress hormone, corticosterone (CORT), to determine if implants affected plasma CORT. To measure CORT, ether extracted plasma was subjected directly to a radioimmunoassay. For DHT, T, and CORT, percent recoveries were 71.6%, 66.3%, and 81.7%, intraassay coefficients of variation were 9.6%, 9.9%, and 4.4%, and sensitivities were 0.4 ng/ml, 0.4 ng/ml, and 2 ng/ml, respectively.

Statistics

Plasma hormone levels were log-transformed and analyzed with 1-way ANOVA with implant treatment (TP or BLANK) and chemosensory stimulus (PBS control or mental gland extract) as between-subjects factors.

Cirrus sizes were analyzed with a Mann-Whitney *U* test. Cirrus length was analyzed with a one-tailed *t* test because we had an *a priori* prediction that testosterone would increase cirrus length (Sever, 1976).

Nose tapping and activity levels were analyzed with a 2-way repeated measures ANOVA with substrate (water, male body rinse, reproductive female body rinse, and nonreproductive female body rinse) as the within-subjects factor and implant treatment as the between-subjects factor. Significant overall effects were followed up using within-subjects contrast tests and SNK post hoc tests. Preferences for female chemosensory cues versus water were analyzed with 2-tailed binomial tests.

The volume of the VNO was analyzed with a 1-way ANOVA with implant treatment as a factor. The number of AGB-IR cells was log-transformed and analyzed with a 2-way ANOVA; between-subjects factors were implant treatment and chemosensory stimulus. To determine if the volume of the VNO contributed to the number of AGB-IR cells, VNO volume was included as a covariate. To analyze the rostral-caudal distribution of AGB-IR cells in males

exposed to mental gland extract, we conducted a repeated measures ANOVA on the log-transformed number of AGB-IR cells, with section number as a repeated measure and implant treatment as the between-subjects factor.

Results

Hormone levels

Plasma T and DHT levels were significantly elevated in TP-IMP males (Fig. 1), as compared to levels in BLANK-IMP males (T: $F(1,24)=181.9$, $P<0.001$; DHT: $F(1,24)=45.4$, $P<0.001$). Additionally, males that received mental gland extract (delivered to their nares) had significantly lower plasma testosterone ($F(1,24)=4.2$, $P=0.051$). In contrast, there were no significant differences in mean \pm SE plasma CORT between the treatment groups (TP-IMP: 30.9 ± 4.9 ng/ml; BLANK-IMP: 27.2 ± 4.13 ng/ml).

Cirrus size

Cirri of TP-IMP males were significantly longer and larger compared to those of BLANK-IMP males ($t(25)=1.96$, $P=0.03$; $U=9.5$, $N_1=14$, $N_2=13$, $P<0.001$). Mean (\pm SE) cirrus length was 1.178 ± 0.03 mm in TP-IMP males and 1.101 ± 0.03 mm in BLANK-IMP males. Median cirrus size was “medium-large” for TP-IMP males and “small” for BLANK-IMP males.

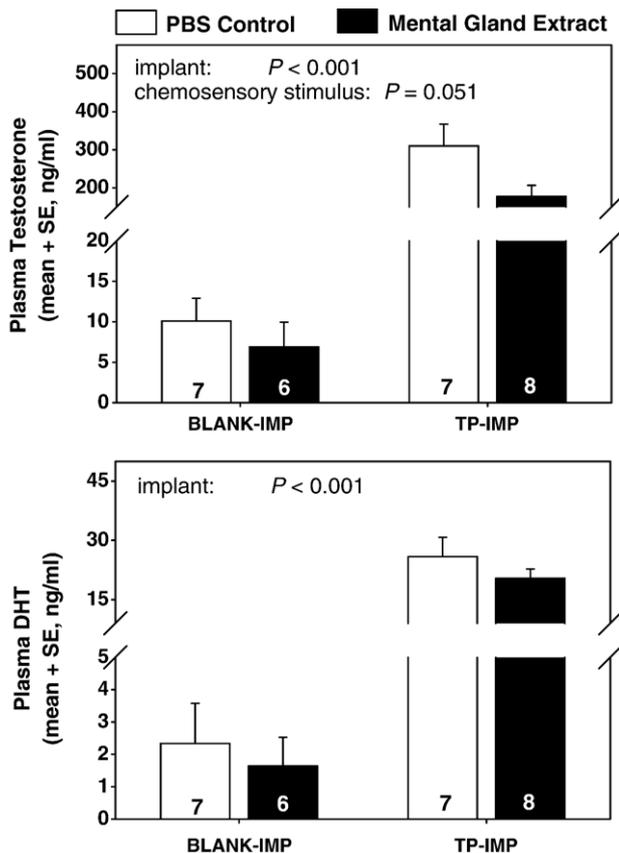


Fig. 1. Plasma androgen levels in BLANK-IMP and TP-IMP males in which PBS control or mental gland extract was applied to the nares. Significant main effects are indicated in panels. Sample sizes are indicated in bars.

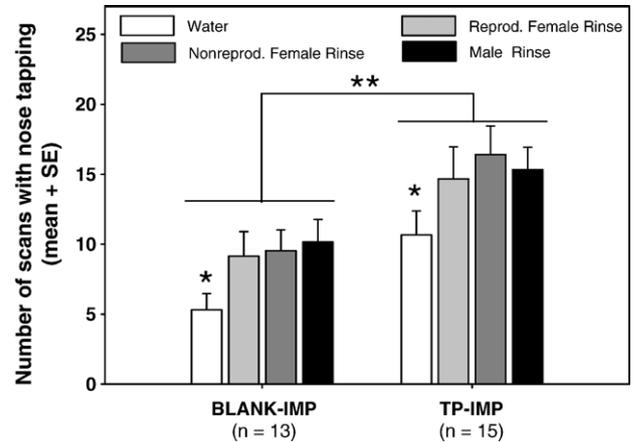


Fig. 2. The number of scans in which nose tapping was observed during a 1-h observation period in BLANK-IMP and TP-IMP males in response to substrates moistened with water or body rinses. **Significant overall effect of implant on nose tapping, $P=0.004$. *Significantly different from other chemosensory stimuli as determined by within subjects contrasts ($P \leq 0.005$).

Behavior

All males nose-tapped the substrates moistened with body rinses significantly more than the water substrate (Fig. 2; $F(3,78)=5.5$, $P=0.002$). There were no differences in responses to the three different body rinses. TP-IMP males nose-tapped significantly more than BLANK-IMP males on all substrates, including the water substrate (Fig. 2; $F(1,26)=9.7$, $P=0.004$). Males were equally active on body rinses and water moistened substrates (Table 1; $F(3,78)=0.64$, $P=0.59$) and there was no difference in activity between TP-IMP and BLANK-IMP males (Table 1; $F(1,26)=1.8$, $P=0.19$).

When given a simultaneous choice, TP-IMP males (but not BLANK-IMP males) preferred the substrate marked by a reproductive female over the water substrate (Table 2; binomial test, $P=0.035$). When given a choice between a substrate moistened with reproductive female body rinse versus water, neither TP- nor BLANK-IMP males showed a preference within the first 2 h of exposure.

Vomerinal responses to androgens

Immunocytochemical control procedures in which the primary or secondary antibodies were omitted showed no labeling for AGB, indicating that our immunocytochemistry for AGB was specific (Figs. 3A–C). Furthermore, the VNO

Table 1

The average number of scan observations in which the animal moved from one quadrant to another when tested on substrates moistened with water or body rinses, \pm SE

	Water	Male rinse	Nonreproductive female rinse	Reproductive female rinse
TP-IMP (n=15)	32.4 \pm 3.26	33.2 \pm 3.3	35.9 \pm 2.9	28.9 \pm 3.9
BLANK-IMP (n=13)	29.9 \pm 4.6	26.9 \pm 2.8	26.5 \pm 3.7	27.1 \pm 3.7

Table 2
Percent of animals on substrates containing reproductive female chemosensory cues

	Female-marked substrate versus water	Female rinse versus water
	% during 2 h observation	% during 2 h observation
TP-IMP (<i>n</i> = 15)	80 *	47
BLANK-IMP (<i>n</i> = 13)	54	46

* Significantly different from 50% (2-tailed binomial test, $P=0.035$).

of *P. shermani* does not endogenously express AGB (Fig. 3D). Controls in which AGB was co-delivered with vehicle (PBS control) showed relatively low levels of activation (Figs. 3G and H), indicating that AGB was not a chemosensory stimulus of vomeronasal cells in *P. shermani* (Michel et al., 1999).

The total number (mean \pm SE) of AGB-IR cells in each group were 57.7 ± 9.3 (TP-IMP, PBS control), 58.9 ± 12.3 (BLANK-IMP, PBS control), 321.1 ± 33.2 (TP-IMP, mental gland extract), and 326.3 ± 53 (BLANK-IMP, mental gland extract). Within each male group (TP-IMP and BLANK-IMP), more vomeronasal cells were immunoreactive for AGB in males that received male mental gland extract (Figs. 3 and 4) as compared to males that received PBS control ($F(1,23)=106.5$, $P<0.001$). Also, there was no effect of implant treatment on the total number of AGB-IR cells ($F(1,23)=0.08$, $P=0.78$), and VNO volume was not a significant covariate ($F(1,23)=0.18$, $P=0.68$). Overall volume (mean \pm SE) of the VNO did not differ between TP-IMP males (0.21 ± 0.01 mm³) and BLANK-IMP males (0.22 ± 0.01 mm³) ($F(1,26)=0.50$, $P=0.50$).

There was no difference in the rostral–caudal distribution of AGB-IR between TP-IMP and BLANK-IMP males that received mental gland extract (Fig. 4; interaction between rostral–caudal section and implant treatment: $F(14,168)=0.8$, $P=0.64$).

Discussion

The role of androgens in chemoreception was investigated for the first time in a male terrestrial salamander (*P. shermani*). Males with TP implants had circulating levels of T and DHT that were substantially higher than in blank implanted males but were similar to levels measured in field-caught male *P. shermani* during the natural mating season (Woodley, 1994). Levels of corticosterone were equivalent for both groups of males. Males with higher androgen levels also had increased cirrus size and increased nose tapping, a chemoinvestigatory behavior. Androgens also increased preferences for some female-derived chemosensory cues. Androgens had no effect on the volume of the VNO, or the ability of male courtship pheromones (i.e., mental gland extract) to activate cells in the VNO. Finally, an unexpected finding was that males that received male mental gland extract had decreased plasma T levels.

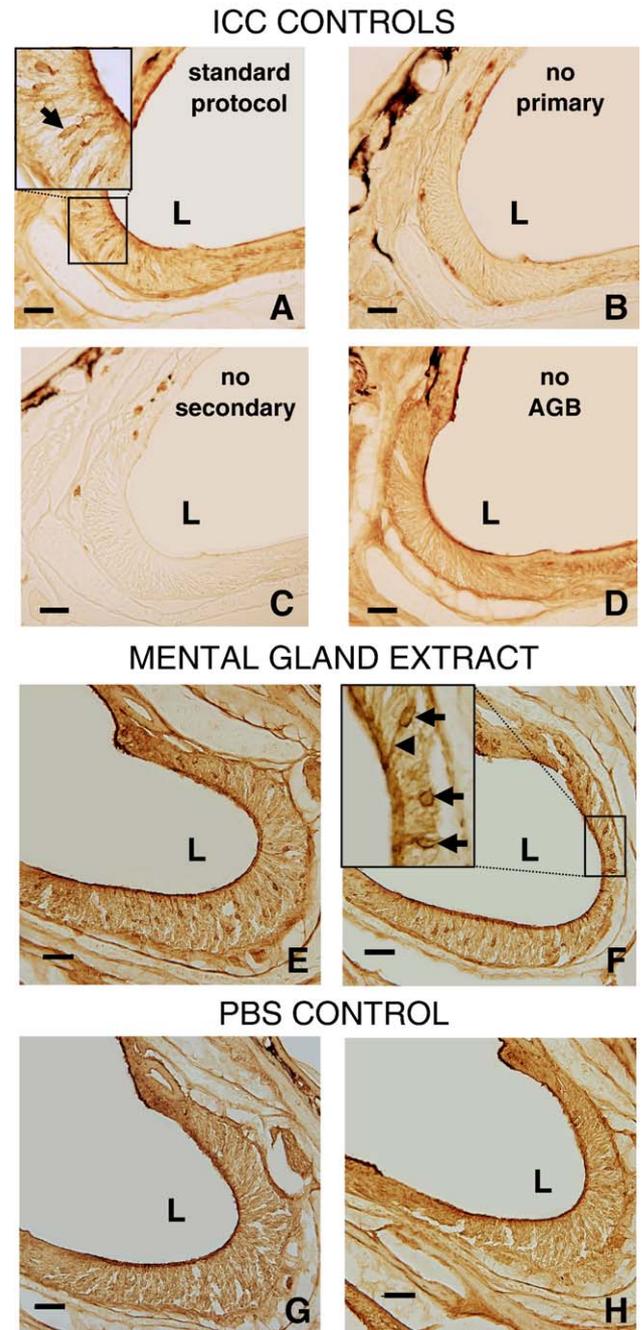


Fig. 3. Representative photomicrographs of 20 μ m coronal sections of the VNO that underwent immunocytochemistry (ICC) for AGB. (A) Many AGB-IR cells are present after application of mental gland extract to the nares of a male (inset shows boxed area at a larger magnification; an arrow indicates one of the AGB-IR cell bodies). (B and C) Sections incubated in the absence of the primary or secondary antibody possessed no AGB-IR, indicating that the immunohistochemistry was specific for AGB. (D) No AGB-IR is seen in the VNO of a female not exposed to exogenous AGB. (E) Many AGB-IR cells are seen in a BLANK-IMP male that received mental gland extract. (F) Many AGB-IR cells are seen in a TP-IMP male that received mental gland extract (inset shows boxed area in larger magnification, arrows label AGB-IR cell bodies, arrow head labels AGB-IR dendrite). (G) Very few AGB-IR cells are seen in a BLANK-IMP male that received PBS control. (H) Very few AGB-IR cells are seen in a TP-IMP male that received PBS control. L: lumen. Scale bars = 50 μ m.

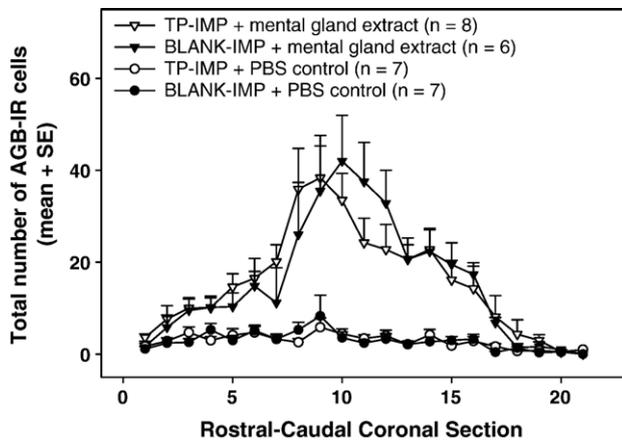


Fig. 4. The number of AGB-IR cells per coronal section of VNO, from the rostral to caudal end. Animals that received mental gland extract had more AGB-IR than animals that received PBS control.

Androgen modulation of morphology: cirrus size

Androgens increased length and overall size of male cirri, which are protuberances on the upper lip associated with the external nares. Exogenous androgens also triggered development of cirri in female *Eurycea quadridigitata*, a plethodontid salamander distantly related to *P. shermani* (Sever, 1976). The function of cirri has not been tested, but when the animal taps its nose to the substrate, the cirri presumably increase delivery of substrate-bound chemosensory cues to the nasal cavity (via capillary action of the nasolabial groove that is surrounded by the cirri). Once the water-borne chemosensory cues are delivered to the nasal cavity, these chemosensory cues are diverted to the VNO (Dawley and Bass, 1988, 1989). If so, the cirri provide a morphological mechanism by which androgens can enhance delivery of chemosensory cues to the VNO.

Androgen modulation of chemosensory behaviors

In both groups (TP and BLANK), males were more likely to nose tap substrates containing rinses of conspecifics (adult males, reproductive females, or nonreproductive adult females) than a water control. The expression of nose-tapping behavior also was modulated by androgens: TP-IMP males nose-tapped significantly more in response to all chemosensory stimuli, even water. This increased nose tapping suggests increased motivation by TP-IMP males to sample the chemosensory environment and also indicates that males with elevated androgens experience an overall increase in chemoreception by the VNO. Measures of general activity levels did not differ between the male groups, however, suggesting that increases in nose-tapping behavior were not simply a reflection of increased overall activity. In a related example, increased androgen levels in felids also resulted in increased flehmen behavior (which delivers urinary chemosensory cues to the VNO) (Hart and Leedy, 1987). Androgen enhancement of salamander nose tapping suggests that sampling substrate chemosensory cues that stimulate the VNO is especially important during the breeding season (a time when androgens normally are maximal).

TP-IMP male salamanders were more likely to be found on a substrate that had been scent-marked by a reproductive conspecific female than a substrate containing water only. A previous study showed that male *P. shermani* (recently collected from the field and presumably in breeding condition with seasonally elevated androgen levels) also preferred substrates marked by reproductive females over water (Palmer, 2004). Thus, the current study extends the results of Palmer (2004) by showing that the male preference for a female-marked substrate was androgen modulated. However, our androgen treatment did not increase preference for female body rinses. This result was unexpected because nose-tapping tests indicated that they could detect female body rinses. Possible reasons for the differential responses to female-marked substrate and female body rinses include potential differences in concentration, chemical composition, and informational content.

Effects of androgens on the VNO

Application of a solution of male mental gland extract (combined with AGB) to the VNO produced significantly more AGB-IR vomeronasal cells than did the application of a PBS (combined with AGB) control solution in both TP- and BLANK-IMP males. However, there were no differences between TP- and BLANK-IMP males in the total number of VNO cells activated by mental gland extract. Male groups also did not differ in the rostral–caudal distribution of cells activated by mental gland extract. Furthermore, there was no difference between TP- and BLANK-IMP males in the overall volume of the VNO. These data do not support the hypothesis that androgens increase the ability of the VNO to detect pheromones. This result differs from work in mice, in which androgens increased immediate early gene expression in the VNO of male mice exposed to female chemosensory cues (Halem et al., 2001). Our result also contrasts with work in fish, in which the sensitivity and magnitude of electroolfactogram responses to putative female sex pheromones was enhanced in juveniles by testosterone treatment (Cardwell et al., 1995). However, the cited studies examined male responses to female-derived pheromones, whereas our study examined male responses to mental gland extract, a male-derived chemosensory cue. Thus, before we can conclude much about the role of androgens in VNO processing, we need to examine responses to additional chemosensory cues. A better test of the hypothesis that androgens increase sensitivity of sensory neurons of the VNO would test responsiveness of the male VNO to a female-derived chemosensory cue. Additionally, because the VNO may respond to a range of different chemosensory cues (Halpern and Martinez-Marcos, 2003), it is important to examine responses to chemosensory cues not involved in reproduction such as those derived from prey or predators to determine if androgens increase sensitivity selectively. Alternatively, androgens might not act at the level of the VNO to increase sensitivity to pheromones. A number of behavioral studies comparing intact and castrated male mammals failed to find a difference in the sensory thresholds to chemosensory cues (Carr and Caul, 1962; Carr et al., 1962; Dhong et al., 1999; Dorries et al., 1995; Peters et al., 2004).

VNO responses to male mental gland extract

Application of male mental gland extract to males produced a greater VNO response (i.e., many more AGB-IR cells) than did the PBS control, but the biological relevance of this response is unclear. This level of activation was similar to that seen in a comparable study using female *P. shermani* exposed to male mental gland extract (Wirsig-Wiechmann et al., 2002). The response to mental gland pheromones by *P. shermani* females is easily interpreted as a normal consequence of typical courtship behavior between a male and female (Houck et al., 1998): the male turns toward the female, she lifts her head, and then the male brings his mental gland in contact with her nares. In thousands of observations of courtship behavior, this pheromone delivery has been observed only in the context of male-female courtship; furthermore, the mental gland secretions have never been observed being used in any other context (Houck, personal observation). Thus, the significance of the strong activation of the male VNO by male mental gland extract does not have an obvious social interpretation. The chance that a male would be exposed to the delivery of pheromone from another male's mental gland is exceedingly low, as this delivery only has been observed during courtship between a male and a female. Infrequently, a rival male might insinuate itself into an on-going male-female courtship (Arnold, 1976) but the rival need not lift its head to receive secretions from another male. The significance of male mental gland pheromone delivery to another male also is puzzling when considering that the recipient male may experience a reduction in levels of androgens (discussed below). We speculate that responses in higher brain areas downstream from the VNO may show a very different pattern of response when compared to the neural response of a female.

Males treated with mental gland extract had marginally ($P=0.051$) decreased testosterone and this effect was most evident in males with TP implants. The mechanism by which male mental gland extract decreases plasma testosterone levels is unclear. Because testosterone in TP-IMP males was exogenously determined by these implants, suppression of the hypothalamic–pituitary–gonadal axis cannot be a mechanism for the decreased testosterone. Possible mechanisms include an effect of male mental gland extract on uptake of testosterone from the circulation or clearance from the body. Future studies will attempt to replicate this result by measuring testosterone levels in intact males treated with male mental gland extract.

To conclude, this study indicates that androgens, normally elevated during the mating season, enhance chemoreception in two different ways: (a) by effecting morphological change (cirrus size) and (b) by altering behavior (chemo-investigatory behavior and preferences for chemosensory cues). Presumably, these androgen-based changes result in increased chemoreception of stimuli important for mating. Androgen treatment had no effect on the responsiveness of the VNO to male mental gland extract. Future studies will determine whether androgens modulate VNO responsiveness to other chemosensory cues, particularly to female-derived chemosensory cues. In addition, this study shows that male mental gland secretions can activate the VNO of males. Future studies will determine whether a male

response to male mental gland extract is biologically relevant. Thus, the study of pheromone signals in plethodontid salamanders will shed light on sex steroid effects related to reception and response to vertebrate chemical signals.

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