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The Effects of Sex and Reproductive Condition on Chemosensory Communication in the Terrestrial Salamander, Plethodon shermani

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The Effects of Sex and Reproductive Condition on Chemosensory Communication in the Terrestrial Salamander, *Plethodon shermani*

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By

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ABSTRACT

Non-volatile chemosensory cues may be detected by sensory neurons of the vomeronasal organ (VNO) and elicit changes in reproductive behavior and physiology of conspecifics. In the terrestrial salamander, *Plethodon shermani*, the VNO of males is larger than that of females, despite males' smaller overall body size. I hypothesized that a larger VNO reflects an enhanced ability to detect chemosensory cues, and that animals with elevated levels of steroid levels would be more sensitive to cues from the opposite sex.

Chemo-investigatory behavior was both sexually dimorphic and hormonally modulated. In contrast, responsiveness of the VNO (as measured by agmatine uptake, a marker of sensory neuron activation) to chemosensory cues did not differ between the sexes or animals with different levels of sex steroid hormones. Differences in responsiveness to chemosensory cues between the sexes and reproductive conditions is occurring behaviorally, but not at the level of the VNO.

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ATTRIBUTIONS

I did all of the work described in this thesis, with the following exceptions:

1) Dr. Lynne Houck assisted in field collection of animals.

2) Mental gland extract was a gift from Drs. Lynne Houck, Pamela Feldhoff and Rick Feldhoff.

3) All hormone levels were measured by the Endocrine Services Laboratory at the Oregon State University Primate Research Center (Director, Dr. David Hess).

With respect to the writing of this thesis, Dr. Sarah Woodley wrote the results section for Study 2 and portions of the materials and methods section for Studies 1& 2.

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INTRODUCTION

I. Chemosensory cues & the vomeronasal organ

Chemosensory cues may be used for communication between members of the same species to relay information regarding mating and reproduction, as well as serve as markers for territories, act as alarm signals and may be important for interaction with young (Rodriguez, 2004; Jaeger et al., 1986; Mathis, 1990; Brown and Brown, 1992). In humans, chemosensory cues have been implicated in influencing menstrual cycle phase, LH surge and affecting moods (McClintock, 1998; Jacob et al., 2001; Preti et al., 2003). For vertebrates, chemosensory cues may be detected by sensory neurons of the vomeronasal organ (VNO), a specialized structure separate from the main olfactory epithelium in the nasal cavity (fig i) (Halpern and Martinez-Marcos, 2003).

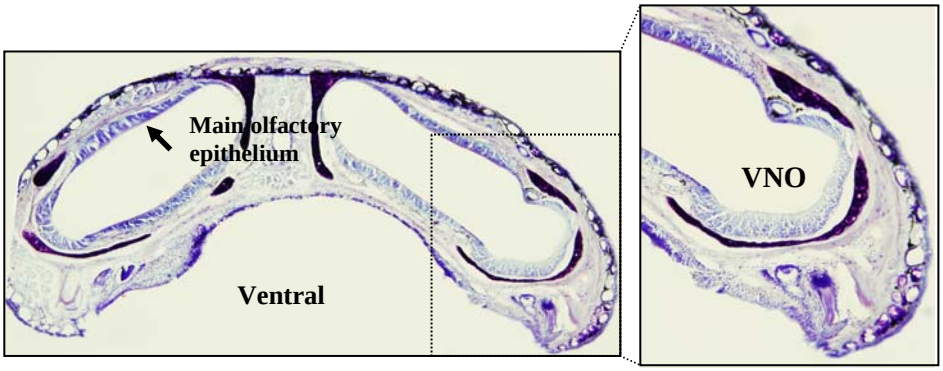

FIGURE i: Coronal section of salamander upper jaw at 10x magnification. Image courtesy of Woodley Lab.

Sensory neurons in the vomeronasal epithelium each possess one or two 7-transmembrane G-coupled receptors that are narrowly tuned and highly sensitive (Luo et al., 2003; Maritini et al., 2001; Leinders-Zufall et al., 2000). The neurons project axons to the accessory olfactory bulb and send sensory information to areas of the brain such as the medial amygdala, bed nucleus of the stria terminalis, the preoptic area and the ventromedial hypothalamus, which contain sex steroid hormone receptors and are key centers for regulation of reproductive physiology and behavior (Scalia and Winans, 1975; Schmidt and Roth, 1990).

II. Hormonal modulation of sensory signals

Chemosensory cues may elicit changes in reproductive behaviors and endocrine physiology of the recipient (Halpern and Martinez-Marcos, 2003). In turn, the endocrine status of an animal may affect how a cue is processed by the nervous system. Many studies indicate that processing of chemical signals by higher brain centers are sexually dimorphic and modulated by hormones; however, much less is known about similar effects on sensory neurons (Wood, 1993). Non-volatile chemosensory cues are detected by neurons of the VNO sensory epithelium and recent evidence suggests that sex steroid hormones modulate the responsiveness of the VNO to these signals. For example, castrated male mice exposed to female odors showed increased sensory neuron responsiveness in the VNO, as measured by c-fos expression, a marker of neuronal activation, when treated with testosterone propionate relative to treatment with estradiol benzoate (Halem et al., 2001). In Japanese newts, males produce a sex pheromone called sodefrin, which attracted reproductive females but not non-reproductive females or other males (Kikuyama and Toyoda, 1999). Treatment of ovariectomized females with estradiol alone, or in combination with the pituitary hormone prolactin, increased the sensitivity of the VNO to sodefrin when measured with electrophysiological methods. A smaller, but significant increase was seen in castrated males treated with estradiol, or estradiol in combination with prolactin (Toyoda et al., 1999; Toyoda and Kikuyama, 2000). It is unknown whether testosterone modulates sensitivity of VNO neurons to pheromones in the Japanese newt. There is also a report on the plainfin midshipman fish that estradiol increased sensitivity of the female auditory nerve fiber that responds to male vocalization (Sisneros et al., 2004). These studies provide evidence that sex steroid hormones can modulate the responsiveness of neurons in a sensory system responding to reproductive cues, although more work is necessary to understand these principles.

III. Sexual dimorphism of sensory structures

Even less is known regarding sexual dimorphism in sensory structures. The VNO of plethodontid salamanders *Plethodon cinereus* and *Plethodon shermani* is larger in males relative to females, despite male salamanders slightly smaller body size (Dawley 1993, Woodley, unpublished). This example of a sex difference in size of a sensory structure is rare. The sphinx moth, *Manduca sexta*, provides an example of sensory structure sexual dimorphism in an invertebrate system, with differences in antennae size and flagellum shape between the sexes (Shields and Hildebrand, 2001). In mammalian models, the rat VNO is larger in males than in females and is controlled by organizational effects of sex steroid hormones; however, male rats are larger than female rats in general body size as well (Segovia and Guillamon, 1993). For mice, vomeronasal receptor gene expression in the VNO is sexually dimorphic and regulated by gonadal sex steroids (Alekseyenko et al., 2006). There are also examples of sex dimorphism in the auditory system in amphibians. The male bullfrog, *Rana catesbeiana,* has a larger tympanic membrane than the female bullfrog. In the neo-tropical tree frog, *Eleutherodactylus coqui*, the call of the male elicits differences in auditory frequencies between males and females. These examples of sensory organ sexual dimorphism suggest that sex and sex steroid hormones may modulate the detection of biologically relevant reproductive cues.

IV: The model organism: *Plethodon shermani*

A. Chemical communication in plethodontid salamanders

Plethodontid salamanders serve as an excellent model system to evaluate the role of sexual dimorphism and hormone modulation of chemosensory processing. These terrestrial salamanders are characterized by their lunglessness and specialized structures called nasolabial grooves that

serve to draw non-volatile compounds into the VNO, via capillary action (fig ii, A) (Dawley and Bass, 1989). The nasolabial grooves run from the rostral tip of the upper lip into the nasal cavity and are contained within fleshy protuberances called cirri (fig ii, B). Cirri are thought to be androgen dependent and are hypothesized to increase transfer of chemical cues as they appear to be largest in males during the breeding season, when androgens are maximal; however, this has not been tested (Woodley, 1994).

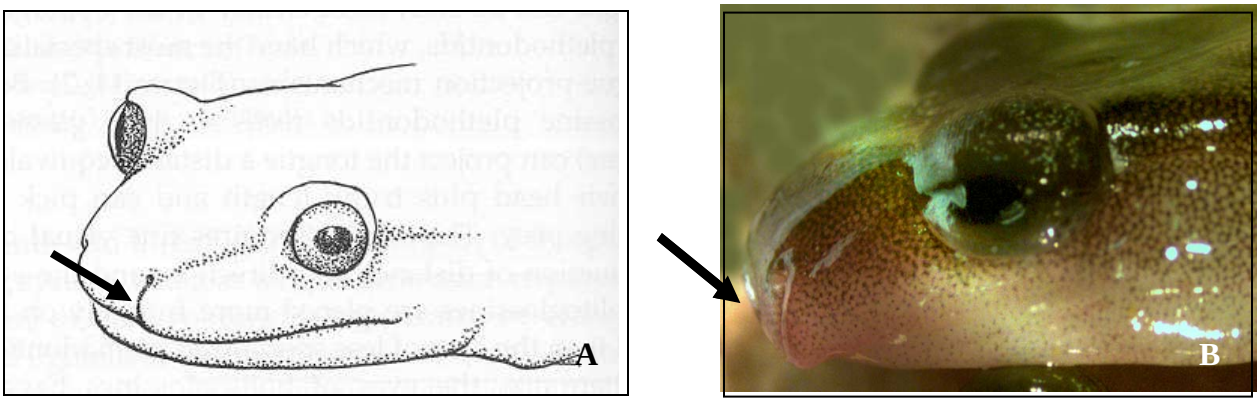

FIGURE ii: (A) arrow indicates nasolabial groove of a plethodontid salamander and (B) arrow indicates nasolabial groove within a cirrus of a male *P. shermani* salamander with enhanced androgen levels, 2x magnification. Illustration courtesy of fiu.edu; photograph by author.

Plethodontid salamanders are nocturnal and rely heavily on chemical signals for communication and have evolved two distinct modes of pheromone delivery that have undergone stabilizing selection and endured the approximately 100 million year span of their existence (Watts, et al. 2003). The vaccination mode of delivery, in which the male uses elongated premaxillary teeth to abrade the skin of the trunk of the female during courtship and inject secretions from a specialized glandular pad located beneath his chin, the mental gland, is considered the ancestral mode and persists in the majority of plethodontids. This method utilizes the lungless feature of these animals by which they rely on a capillary system located superficially beneath the skin to allow for oxygen to diffuse through the skin and into the bloodstream. By using the vaccination mode of delivery, chemical signals are injected directly into the bloodstream

of the female (Arnold, 1977). A subset of plethodontids including *P. shermani,* has evolved a derived olfactory mode of pheromone delivery. For male-female courtship interactions, the male applies courtship pheromones directly to the nose of the female by slapping the mental gland that produces chemical cues over her nares, for uptake of the compound into the VNO. This action is performed only during courtship (Organ, 1958; Arnold 1976; 1977; Houck and Arnold, 2003).

B. Reproductive behavior of *P. shermani*

Plethodontids have well characterized, stereotypical courtship and reproductive behaviors. Sperm transfer is external and delivery of courtship pheromones is thought to be critical in increasing the chances that a female is successfully inseminated. The highly organized series of movements leading to sperm deposition culminates with the tail-straddling walk when the female straddles the tail of the male. This is the key phase in courtship when the male applies courtship pheromones to the nose of the female which increases her receptivity as defined as a decrease in their overall courting time (Organ, 1958; Arnold, 1976; Houck, 1998; Verell and Mabry, 2000). The receptivity of the female is particularly important in these animals. Once tail-straddling walk is attained, the male will release his sperm mass in the form of a spermatophore. The spermatophore is deposited externally onto the ground and pair march forward, for the female to position herself above the spermatophore for uptake into her cloaca. She will store the sperm internally until fertilization and oviposition in the late spring. If the female leaves the tail straddling walk at any point, the spermatophore will be wasted. Spermatophore production is costly and males can only deposit one per evening (Arnold, 1977). In this way, the female is highly responsible for a reproductive success between an individual coupling; thus the importance of the courtship pheromone that the male administers to increase her receptivity.

C. The chemosensory cues of *P. shermani*

1. Mental Gland Extract

In recent years significant work has been done to identify and biochemically purify the courtship pheromones produced by the mental gland of *P. shermani*. The mental gland yields secretions containing about 20 glycoproteins. Two proteins, Plethodontid Receptivity Factor (PRF) and Plethodontid Modulating Factor (PMF) comprise approximately 85% of the total (Feldhoff, 1999). PRF is a 22kD protein member of the Il-6 cytokine family which increases female receptivity (Rollmann et al., 1999). PMF when applied with PRF, increases receptivity of the female; however, when applied alone decreases female receptivity (Wirsig-Wiechmann, et al., 2006). Purification of these proteins has allowed for known compounds of exact concentrations to be utilized in my examination of chemosensory communication and detection.

2. Female Skin Secretions

These salamanders utilize chemical signals not only for courtship but all social interaction. They frequently perform a regular and highly stereotyped behavior called nose-tapping, in which the animal taps the tip of its nose onto a substrate so that non-volatile cues may be taken up to the VNO through the nasolabial groove. Nose-tapping is a chemo-investigatory behavior that is used to search the environment for relevant chemical cues that give information about sex, species and individuality of other animals (Dawley, 1984, Gillette, Kolb, Smith and Jaeger, 2000; Jaeger and Gergits, 1979; Mathis, 1990; Palmer, 2004).

One potential signal that plethodontids may be detecting is a sticky skin secretion produced by both males and females when handled or by predators. These secretions have been shown to function as an anti-predator mechanism, as well as to activate sensory neurons of the VNO (Graves & Quinn, 2000; Schubert and Woodley, unpublished). The skin secretions elicit specific avoidance behaviors in conspecifics (Woodley, unpublished) and are easily collectible in large

volumes at consistent concentrations for use in behavioral and VNO activation experiments. Colleagues have begun the characterization of the cues present in these skin secretions. SDS-PAGE gel analysis indicates a combination of mucins and proteins including anti-microbial agents and those of unknown function (Fredericks & Dankert, 2000, Woodley, unpublished). Identification of chemosensory signals is difficult and there are only a handful of examples. This work serves not only to allow for continued exploration of the evolution of these chemical cues and reproductive behaviors but also the underlying physiological principles at work.

V. Rationale for thesis work

Sexual dimorphism in a sensory structure is rare. While the body size of male *P. shermani* is generally smaller than the female, the VNO is twice as large. I wanted to investigate whether this sex difference in size correlated to differences in functionality. Do males have more sensory neurons responding to relevant social cues, and are they more sensitive than females? Sex steroid hormones early in development may play a role in this sexual dimorphism of the sensory structure, so a further question is whether adult levels of sex steroid hormones are affecting responsiveness of the neurons.

By utilizing the supply of male mental gland extract collected during the mating season and collecting skin secretions from reproductively active females I have chemical signals from both sexes to test sex differences and hormonal modulation of responsiveness to chemosensory cues at the level of the VNO. Also, female *P. shermani* reproduce every other year, and so in a given mating season, both reproductive and non-reproductive females can be collected and used for experimentation in addition to artificially manipulating steroid hormone levels of males in the laboratory. These tools allow us to examine differences in responsiveness in reproductively active males and females as compared to non- reproductive condition males and females in response to chemosensory cues on both the behavioral level and the level of detection in the VNO.

VI. Thesis Objectives

A. Hypotheses

I investigated whether the sexual dimorphism of the VNO volume reflects an enhanced ability to detect and respond to chemosensory cues and whether differences in reproductive condition affects these processes. I hypothesized that a larger VNO in males provides a greater ability to detect and behaviorally respond to female derived chemosensory cues that may function to signal potential mates. Additionally I hypothesized that males and females in reproductive condition would better respond to cues than their non-reproductive counterparts.

B. Experimental design

1. *Study 1:* For Study 1 I compared reproductively active males and females, as well as females in reproductive condition to non-reproductive females. These animals were caught fresh from the field during the mating season and held in the lab for only 2 months before testing. The biennial breeding cycle of female *P. shermani* allowed us to directly compare females in different reproductive conditions that presumably had high versus low estradiol levels, without artificially manipulating hormone levels. This study directly addresses sex differences and is the first step in addressing estradiol modulation of responsiveness to chemosensory cues.

2. *Study 2:* In this study I used males that had been held in the laboratory for many months and were no longer reproductively active, with basal androgen levels. In half of the males I surgically altered androgen levels with testosterone propionate (TP) implants in order to produce males with plasma androgen levels typical of reproductively active, breeding males. I compared these TP-implanted males with the half of the males that received blank implants, containing no testosterone in order to directly investigate androgen modulation of responsiveness to chemosensory cues.

C. Predictions

I predicted that for ***Study 1:*** 1) males would chemo-investigate (nose-tap) female cues more than females; 2) behaviorally, males would prefer substrates moistened with female cues; 3) the VNO of males would be more responsive to female cues (in this case, female derived skin secretions) than females' VNO; 4) reproductive females would nose-tap male cues more than non-reproductive females due to differences in levels of estradiol; 5) the VNO of reproductive females would be more responsive to male cues (male mental gland extract) than non-reproductive females' VNO. For ***Study 2:*** 6) males with higher levels of testosterone would nose-tap female cues more than males with lower levels; 7) behaviorally, males with higher levels of testosterone would prefer substrates moistened with female cues more than males with lower levels; and 8) males with higher levels of testosterone would more responsive to female derived chemosensory cues at the level of the VNO than males with lower levels.

Study 1: The effects of sex and female reproductive condition on behavioral and VNO responses to chemosensory cues

In this study, reproductively active males and females, and females in both reproductive and non-reproductive condition were compared. Sex differences and the effects of female reproductive condition on behavioral and VNO sensory neuron responses to chemosensory cues were investigated. I used chemosensory stimuli derived from the whole body rinses of male and female stimulus animals and skin secretions collected from female stimulus animals to measure behavioral responses to chemosensory cues. In contrast, I used extract from male mental glands (a source of pheromones used in courtship) and female skin secretions to measure VNO responsiveness to chemosensory cues. Ideally, I would have used all of the same types of chemosensory stimuli for testing both behavioral and VNO responsiveness. However, for tests of VNO responsiveness, I decided to use male mental gland extract because it was the only male 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, I used chemosensory stimuli derived from whole body rinses and skin secretions of stimulus animals because large amounts could be prepared easily. Despite the difference in the male chemosensory stimuli used in the tests of vomeronasal function and the behavioral tests, some evidence suggests that sex steroid hormones nonspecifically increase responsiveness to all chemosensory stimuli (Pietras and Moulton, 1974). Thus I reasoned that mental gland extract would serve as a general male chemical signal for chemosensory function.

METHODS

I. Animals

All methods were approved by Duquesne University's Institutional Animal Care and Use Committee. Animals were collected from a single location (Wayah Bald, Macon County, NC, 83° 30' 30" N longitude; 35° 10' 49" W latitude) in August 2005 under appropriate permits from the North Carolina Department of Wildlife. All males used in this experiment had their mental glands surgically removed upon collection in North Carolina (see below; procedure approved by Oregon State University ACUP to L.D. Houck). Animals were housed temporarily, for two months, at Oregon State University and participated in behavior experiments that involved mating. Animals arrived at Duquesne University in October 2005 and were housed individually at 16°C on a 14L:10D photoperiod in 16 x 16 x 5 cm plastic boxes lined with moist brown paper towels. Animals were fed wax worm larvae. Animals were considered to be in reproductive condition based on being collected at the start of the mating season, participating in mating experiments and being housed on a long day photoperiod,. Sex and reproductive condition were determined for each animal by examining the ventral abdominal body wall for presence of yolky ova (REPRO female), absence of yolky ova (NON-REPRO female) or testes (MALE) and were confirmed later by dissection. Experiments were begun in early November and finished by mid December 2005. Sample sizes were 24 MALES, 24 REPRO females, and 24 NON-REPRO females. In this species, males are slightly smaller in average body length than females. The body lengths as determined by measuring the distance from tip of the snout to the anterior end of the cloacal vent, were: REPRO females: 59.3 ± .7mm; NON-REPRO female: 57.8 ± .6mm; MALES: 56.3 ± .7mm.

II. Behavioral tests

A. General

Scan sampling methods (Martin and Bateson, 1993) were used to quantify behavioral responses to chemosensory stimuli. Scan sampling is commonly used to measure behavior in plethodontid salamanders because this method provides accurate estimates of subjects' behavior and allows testing of multiple subjects at the same time. All behavioral tests were conducted in the evening during the dark period of the photoperiod when animals normally are most active. Tests were conducted at 25 °C under dim incandescent light. A single investigator performed all the testing and was blind to the experimental treatments of the subjects.

B. Chemosensory stimuli used in behavioral tests

For testing behavioral responses to chemosensory cues, two types of stimuli that were collected from animals caught fresh from the field at Wayah Bald, Macon County, NC in August 2005 were used. Whole-body rinses were used because they elicit behavioral responses in *P. shermani* as well as in a congeneric species, *P. cinereus* (Sullivan et al., 2003; Schubert et al., *in press*). In addition, female skin secretions were tested. Skin secretions are secreted from exocrine glands in the skin of the animal when handled.

Whole-body rinses were collected from reproductive females (n=10) and males (n=10). Reproductive condition of females used as stimulus animals was determined by examining the ventral abdominal body wall for presence of yolky ova and confirmed later by dissection. Body rinses were collected by placing a single animal in 75 ml of ddH20 in round glass containers at 16°C on a 14L: 10D photoperiod for 48h. Body rinses from each category (e.g. repro females) were pooled, a BCA protein assay performed to determine protein concentration and the pooled rinses were diluted to a volume of 750 ml. Rinses were frozen at -20°C in 4 aliquots until use in

behavioral testing. Protein concentrations of reproductive female whole body rinses were 0.01 ug/ul; male whole body rinses were 0.03 ug/ul.

Skin secretions were collected from reproductive females (n=8) by placing an individual animal in 100 ml of ddH20 in a round, glass container and gently pressing the animal's tail with blunt-nosed forceps. This procedure produces copious amounts of secretions. The secretions were pooled, a BCA protein assay performed to determine final concentration and then stored at -20°C in aliquots until use in testing. Protein concentration of skin secretions was 0.50 ug/ul. Control stimuli were prepared by placing 75 ml of ddH20 in 10 individual glass containers for 48hrs, then pooled, aliquotted and stored as described above. All aliquots were coded by an outside individual so that the investigator was blind to each stimulus group as it was administered. Body rinses were used within 7 days of collection and animals used to collect stimuli were not used as test subjects.

C. Response to chemosensory stimuli: Nose-tapping and locomotor activity

In the terrestrial salamander, *P. shermani*, non-volatile chemosensory cues are detected by sensory neurons of the VNO. These cues are transported into the VNO from the exterior via the nasolabial groove, which runs from the distal tip of the upper lip into the nasal cavity (Dawley and Bass, 1989). Animals perform the nose-tapping behavior in which they physically touch the tip of the nose to a substrate to sample their environment for chemosensory information. Nose-tapping is an easily scored and unambiguous behavior that provides an assay by which to evaluate levels of chemosensory investigation by the animal. Amount of movement on substrate was also recorded to determine if nose-tapping is correlated with general locomotor activity.

Each individual was placed in a 23 x 23 x 2 cm plastic test chamber with the bottom lined with brown paper towel moistened with 10ml of a chemosensory stimulus and scan sampling was used to observe behavioral responses. Each animal was scanned for approximately 2 seconds every 60 seconds for a period of 60 minutes. During each scan, the occurrence of nose-tapping

(1=nose-tapping observed; 0=no nose-tapping observed) was recorded as well as the general location of the animal in the chamber, as defined by which quadrant the animal's head was positioned. Upon completion of observations each animal was given two scores: 1) total number of nose-taps observed (maximum= 60) and 2) general activity, measured by the number of times the animal moved from one quadrant to a different quadrant than noted in the previous scan (maximum= 60).

Each animal was tested on one of the 4 prepared chemosensory stimuli on a given night over the period of one week with one night's rest between trials. The order of presentation of stimulus was randomized and a previously coded aliquot of each of the 4 stimuli were presented each night, so every animal was tested on one type of chemosensory cue each night and the investigator was blind to the treatment.

D. Behavioral avoidance of reproductive female skin secretions

Each individual was placed in a 23 x 23 x 2 cm plastic test chamber with chamber prepared as follows: half lined with brown paper towel moistened with water and the other half lined with paper towel moistened with skin secretions collected from reproductive females. A 1cm gap was left between the paper towel halves to prevent transfer between the substrates.

Each animal was placed in the chamber and given a 15 min habituation period. Following this period, each animal was scanned every 2 min for a period of 2 hours. At each scan the following scores were noted: 1) location of animal's head (on water or chemosensory cue side) and 2) the occurrence of nose-tapping (1= nose-tapping observed, 0= no nose-tapping). Animals that spent more than 50% of the 60 observations on the side moistened with water were considered to show an avoidance of reproductive female skin secretions. After the 2 hr observation, animals were left in their testing chambers overnight and location of the animal's head was recorded the next morning, (after lights turned on at 9am, approximately 12h after placing animals in

chambers). Animals with head positioned on the water side were considered to show an avoidance of the reproductive female skin secretions.

E. Behavioral preference for reproductive female body rinse

Testing chambers were prepared as described above but with reproductive female body rinse moistening one side and water on the other. Animals were placed in testing chambers and observed and scored as described above. Animals that spent more than 50% of the 60 total observations on the reproductive female body rinse were considered to show a preference for the rinse. Animals were again left in testing chambers overnight and next day location recorded. Animals with head positioned on the side moistened with reproductive female body rinse were considered to have a preference for the body rinse.

III. VNO responses to chemosensory cues

A. Method of agmatine uptake

In order to identify cells of the vomeronasal organ that respond to chemosensory cues, the method of agmatine (AGB) uptake was used. Agmatine is a guanidium analog that, when co-delivered with a chemosensory stimulus can enter the sensory cell through activated non-specific cation channels. AGB is sequestered within the cytoplasm of the cell and immunocytochemistry for AGB allows us to visualize cells that have taken up the AGB and presumably, were activated by the chemosensory cue. This method has been used in lobster and zebra fish models (Michel et al., 1999) and was adapted for use in plethodontid salamanders (fig iii) (Wirsig-Wiechmann et al., 2002; Wirsig-Wiechmann et al., 2006).

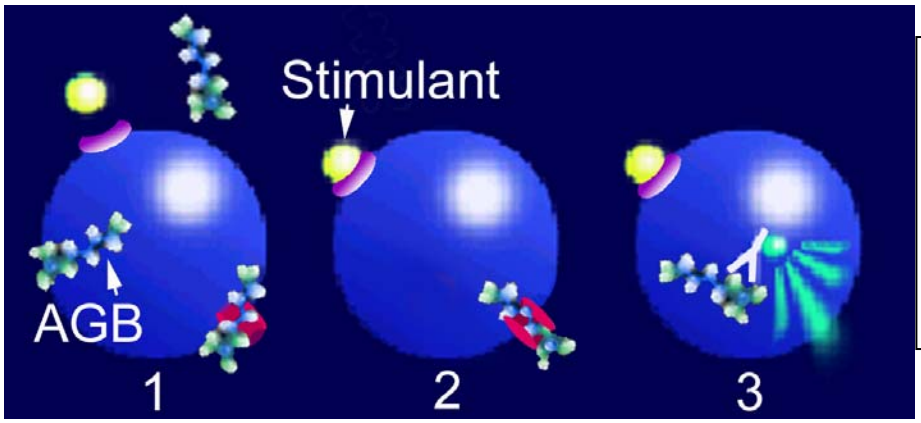

FIGURE iii: Method of AGB uptake. When a stimulant binds its receptor, AGB is able to pass through activated cation channels to be sequestered within the cell and immunocytochemistry for AGB can be performed. Illustration courtesy of Dr. Celeste Wirsig-Wiechmann

To compare VNO responses between sex and reproductive groups, two chemosensory cues were applied with agmatine in this study, male mental gland extract and reproductive female skin secretions.

B. Chemosensory stimuli used to measure VNO responsiveness

1. Male Mental Gland Extract

I used male mental gland extract to test responsiveness of the male VNO to chemosensory stimuli. Mental gland extract was a gift from Drs. Richard Feldhoff, Pamela Feldhoff and Lynne Houck. It was obtained from approximately 100 males collected from Wayah Bald, Macon County, NC, in August 2004. Mental glands were surgically excised from these animals and extracted in 0.8 mM acetylcholine chloride as described previously (Wirsig-Wiechmann et al., 2002). Acetylcholine chloride was removed by ultra-filtration with a 3KDa cutoff to ensure that levels of acetylcholine chloride were below the level of physiological responsiveness. Extracts were pooled, standardized to a concentration of 2.0 µg/ml in 0.5X Phosphate Buffered Saline (PBS), and frozen at -20°C until use. I used 0.5X PBS as the diluent in order to mimic natural osmotic concentrations of typical bodily secretions. A concentration of 2.0µg/ml 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).

2. Female Skin Secretions

A single reproductive female that was collected fresh from the field at Wayah Bald, Macon County, NC in August 2005 was placed in 100 ml of ddH20 and the tail gently pressed with blunt nosed forceps as previously described. The resulting secretions were determined to be at a protein concentration of 0.466 ug/ul via BCA assay was aliquotted and stored at -20°C until use.

C. Application of AGB + chemosensory stimulus

Just prior to use, both male mental gland extract (2µg/µl) and female skin secretions (.466µg/µl) were individually mixed 1:1 with 6mM AGB dissolved in 0.1 M PBS. A PBS control was prepared by mixing 0.5X PBS with 6mM AGB. Animals were placed singly in clean home boxes and a micropipette was used to deliver 2µl of male mental gland extract, female skin secretions or PBS control mixed with AGB to the anterior tip of the opening of the external nares every 2 min for a period of 44min (22 applications per animal). An application of 3 x 5µl of PBS followed to rinse away any excess AGB. Following the final rinse, animals were sacrificed via rapid decapitation, noses were placed in fixative and blood samples were collected from the trunk. Animals were processed in batches of 24 by two investigators. The mean time of final PBS rinse application to sacrifice was 42 minutes. The different treatment groups did not differ in the time from final PBS rinse to sacrifice (F (8, 58) = 1.07, P = 0.40). I verified that any variation in time from application to sacrifice did not contribute to variation in AGB-immunoreactivity by determining that it was not a significant covariate in statistical analyses.

D. Tissue processing

After sacrifice, carcasses were fixed in formalin and sex and reproductive condition were confirmed via dissection. The upper jaws containing the nasal cavity were placed in 4% paraformaldehyde-2.5% gluteraldehyde in PBS fixative, pH 7.4 overnight. The next day tissues were moved to decalcification solution (DeCal, Decal Corporation) for 3 days, then cryoprotected

in 30% sucrose for 2 nights and finally placed in 1:2 (v:v) solution of OCT (Fisher Scientific) embedding gel in 30% sucrose for 1 day. The nasal cavity was injected with, and entire upper jaw was embedded in OCT. Four upper jaws were embedded together per block of OCT. The reproductive condition and treatments were assigned across each block evenly to ensure that all groups were processed similarly. Tissue was sectioned at 20µm using a cryostat at -14°C. Sections containing VNO tissue were collected on poly-lysine coated superfrost plus slides and stored at -80°C and immunocytochemistry for AGB was performed within 3 weeks of sectioning.

E. AGB immunocytochemistry

Every 4th section underwent immunocytochemistry for AGB. The average diameter of an AGB-immunoreactive VNO cell body is 8µm (Schubert and Woodley, unpublished data), so by processing every 4th section I can avoid counting the same cell more than once. Tissue was rinsed 6 times for 5 min each with 0.1 M PBS then incubated for 30 min in a preincubation solution of 0.2% Triton-X, 1% normal goat serum, and 0.004% sodium azide in PBS. Tissue was incubated in rabbit polyclonal anti-AGB antibody (Chemicon AB1568-2000T) diluted 1:4000 in preincubation solution for 3 days in a humid chamber at room temperature. Tissue was rinsed again as described above in PBS, incubated 30 minutes with biotinylated goat anti-rabbit antibody, rinsed in PBS, incubated for 30 minutes with Avidin-Biotin Enzyme Complex (ABC elite, Vector) and rinsed again in PBS. Following a rinse in 0.05 M Tris-HCl, pH 7.4, tissue was incubated for approximately 5 min in 0.02% DAB-0.001% H202 and rinsed 5 more times for 5 min each in Tris-HCl. Slides were coated with Crystal Mount (Biomedia), allowed to dry overnight and coverslipped with Permount (Fisher Scientific).

An alternate series of slides containing every 4th section of tissue was stained with cresyl violet using standard histochemistry.

F. Image analysis

Tissue was analyzed using an Olympus Brightfield microscope at 20x. Slides were coded and the investigator was blind to the treatment, sex and reproductive condition of each animal. Each tissue section was evaluated and cells with darkly stained cytoplasm were considered to be AGB-immunoreactive (AGB-IR) and counted. AGB-IR cells were counted for the VNO located in both the left and right nasal cavities and both sides were summed to give the final AGB-IR count. For cresyl violet tissue, images of each section were captured using an Olympus DP70 digital camera and the cross sectional area of the VNO tissue located in both the left and right nasal cavities was measured using Image-Pro Plus software. The areas from each side were summed and multiplied by 80 μm to give an estimate of total VNO volume (due to every 4^{th} section being physically measured).

IV. Hormone measurement

All plasma hormone levels were measured by Endocrine Services Laboratory at the Oregon Primate Research Center (Dr. David Hess). Trunk blood was collected from each animal at the time of sacrifice to measure plasma hormone levels. Trunk blood was collected within 3 minutes of decapitation. Blood samples were centrifuged and plasma was frozen at -20°C until assaying. All samples were assayed in a single assay following established methods (Gruenewald, et al., 1992; Resko, et al., 1980). For testosterone (T) and dihydrotestosterone (DHT), plasma was ether extracted, separated from each other with Sephadex LH-20 column chromatography, and fractions subjected to radioimmunoassay. Corticosterone (CORT) and estradiol (E2) were measured by ether extracting plasma and directly subjecting it to radioimmunoassay. CORT, T and DHT were measured in males; CORT and E2 were measured in females. Intra-assay coefficients of variation were 8.6%, 12.3%, 9.4%, and 10.8% for CORT, E2, T, and DHT, respectively. Percent recoveries were 100%, 79%, 74%, and 59% for CORT, E2, T, and DHT, respectively.

V. Statistics

Statistical analyses were done using SPSS. Plasma hormone levels were log-transformed to obtain homogeneous variances and analyzed with 1-way ANOVA with chemosensory stimulus (male mental gland extract, female skin secretions or PBS control) and group (MALE, REPRO, NON-REPRO) and as between-subjects factors. Since estradiol levels for non-reproductive females were non-detectable, I compared estradiol levels between reproductive and non-reproductive females with a nonparametric Mann-Whitney U test.

Nose-tapping and activity were analyzed with 2-way repeated measures ANOVAs with substrate (female body rinse, male body rinse, female skin secretions and water) as within-subjects factor and group (MALE, REPRO, NON-REPRO) as between-subjects factor. Significant main effects were followed up with within-subjects contrasts and Student Newman-Keuls post hoc pairwise comparison tests. To specifically test whether sex and reproductive condition affected responses to specific chemosensory stimuli, I analyzed nose-tapping and activity with 1-way ANOVAs for each chemosensory stimulus with group as the between-subjects factor and followed up significant differences with Student Newman-Keuls pairwise comparisons To determine the contribution of locomotor activity to nose-tapping, I conducted 1-way ANOVAs for each chemosensory stimulus with group as the between-subjects factor and with activity as a covariate. Because locomotor activity was indeed as significant covariate, I adjusted mean levels of nose-tapping by subtracting the effects of activity using the estimated marginal mean function in SPSS. Significant differences were followed up with Bonferroni pairwise comparisons.

Simultaneous choice preference tests (repro female body rinse vs. water; repro female skin secretions vs. water) were analyzed using 2-tailed binomial tests.

VNO volume was analyzed with a 1-way ANOVA with group (MALE, REPRO, NON-REPRO) as a factor. The numbers of AGB-IR cells were log-transformed to create homogeneous

variances to satisfy assumptions of parametric statistics. The total numbers of ABG-IR cells were analyzed with a 2-way ANOVA with group (MALE, REPRO, NON-REPRO) and chemosensory stimulus (male mental gland extract, female skin secretions or PBS control) as between subjects factors. To determine if animals with larger VNOs also had more activated cells, a set of analyses included VNO volume as a covariate. To determine if groups differed in the rostral-caudal distribution of AGB-IR cells after application of female skin secretions or male mental gland extract, the numbers of AGB-IR on each coronal section (from rostral to caudal) were analyzed with a 2-way repeated measures ANOVA with section number as the repeated measure and group and chemosensory stimulus as between-subjects factors.

RESULTS

I. Hormone Levels

Plasma corticosterone levels were significantly different across groups (F (2, 57) = 11.53, P < 0.001), with MALES having the highest corticosterone, followed by NON-REPRO, followed by REPRO females (Fig 1; top panel). There was no effect of chemosensory stimulus on plasma corticosterone (Fig 1; top panel: F (2, 57) = 1.79, P = 0.18). In females, plasma estradiol levels were significantly higher in REPRO females than NON-REPRO females (Fig 1; bottom panel, left axis: U = 110.5, N_1 = 24, N_2 = 24, P < 0.001). Estradiol was non-detectable in most NON-REPRO females. There was no effect of chemosensory stimulus on estradiol levels (Fig 1; bottom panel, left axis: F (2, 21) = 0.11, P = 0.90). In MALES, there was no effect of chemosensory stimulus on plasma androgens (Fig 1; bottom panel, right axis: testosterone: F (2, 19) = 0.36, P = 0.70; DHT: F (2, 20) = 0.29, P = 0.75). Plasma testosterone levels in MALES were slightly lower than in fresh field-caught males during the breeding season, but were notably higher than males housed in the laboratory long-term (Woodley, 1994).

II. Behavior

A. Nose-tapping responses to chemosensory cues

All subjects nose-tapped chemosensory stimuli derived from conspecifics more than the water saturated stimulus (Fig 2, top panel; overall main effect of chemosensory stimulus: F (3, 207) = 12.96, $P < 0.001$; within-subjects contrasts comparing nose-tapping to water versus conspecific chemosensory cues, all P < 0.001). Furthermore, MALES nose-tapped in general more than females, and REPRO females nose-tapped in general more than NON-REPRO females (overall main effect of group: F (2, 69) = 29.65, $P < 0.001$; Student Newman-Keuls pairwise comparisons, $P < 0.05$).

Considering nose-tapping responses to each specific chemosensory stimulus separately, MALES, REPRO and NON-REPRO females differed in response to each stimulus (Fig. 2, top panel; water: F (2, 69) = 13.89, $P < 0.001$; female body rinse: F (2, 69) = 15.99, $P < 0.001$; male body rinse: F (2, 69) = 9.58, $P < 0.001$; female skin secretions: F (2, 69) = 13.09, $P < 0.001$). In particular, MALES nose-tapped water and female body rinse more than REPRO females. Also, REPRO females nose-tapped water, male body rinse, and female skin secretions more than NON-REPRO females.

All subjects were more active on substrates saturated with chemosensory stimuli derived from conspecifics more than the water (Fig 2, middle panel; overall main effect of chemosensory stimulus: F (3, 207) = 8.546, $P < 0.001$; within-subjects contrasts comparing activity on water versus conspecific chemosensory cues, all P < 0.01). Furthermore, MALES were overall more active than both groups of females, but there was no difference in activity between REPRO and

NON-REPRO females (overall main effect of group: F (2, 69) = 6.38, P = 0.003; Student Newman-Keuls pairwise comparisons, $P < 0.05$).

Considering activity on each specific chemosensory stimulus separately (see Fig 2, middle panel), MALES were significantly more active than females on substrates moistened with male body rinse (F (2, 69) = 5.74, P = 0.005) and water (F (2, 69) = 6.16, P = 0.003) but not on female body rinse (F (2, 69) = 1.7, P = 0.19) or female skin secretions (F (2, 69) = 0.20, P = 0.82).

Nose-tapping co-varied significantly with activity on female body rinse (F (1, 68) = 10.38, P = 0.002), male body rinse (F (1, 68) = 3.93, P = 0.051), and water (F (1, 68) = 9.09, P = 0.004) but not on female skin secretions (F (1, 68) = 0.15, P = 0.70). Analyses of nose-tapping in which activity was included as a covariate produced results similar to analyses without activity as a covariate (described above). Thus, analyses with activity as a covariate indicated that groups differed in the amount of nose- tapping in response to all chemosensory stimuli (Fig. 2, bottom panel: water: F (2, 68) = 10.71, $P < 0.001$; female body rinse: F (2, 68) = 13.86, $P < 0.001$; male body rinse: F (2, 68) = 7.67, $P < 0.001$; female skin secretions: F (2, 68) = 12.71, $P < 0.001$).

B. Simultaneous choice responses to chemosensory stimuli

When given a simultaneous choice for water or repro female skin secretions, REPRO females significantly avoided repro female skin secretions during 2 hours of observation; NON-REPRO females showed only a marginally significant avoidance and MALES did not avoid the secretions (Table 1). When the location of each animal was checked the next morning (12h later), only REPRO females continued to avoid the repro female skin secretions, NON-REPRO and MALES were found equally on water vs. skin secretions (Table 1).

When given a simultaneous choice between water and repro female body rinse, neither REPRO females, NON-REPRO females or MALES showed any preference or avoidance for the

substrate moistened with body rinse during the 2hr observation period, or when location was checked the next day (Table 1).

III. Vomeronasal responses according to sex and female reproductive condition

The VNO volume (mean +/- SE) of MALES was twice as large as the volume of the VNO volume of REPRO and NON-REPRO females (Fig 3) (F (2, 69) = 57.86, $P < 0.001$). There was no difference in VNO volume between REPRO and NON-REPRO females. VNO volume was not a significant covariate in analyses of AGB-IR either across all subjects, or within each group (MALE: F (1, 20) = 0.49, $P = 0.50$; REPRO female: F (1, 20) = 1.38, $P = 0.25$; NON-REPRO female: F (1, 20) = 1.409, $P = 0.25$).

The number of AGB-IR cells depended on chemosensory stimulus (F (2, 63) = 80.98, $P < 0.001$), but not group (Table 2, Fig 4) (F (2, 63) = 2.68, $P= 0.08$) and the interaction between chemosensory stimulus and group was not significant (F (4, 63) = 1.23, $P = 0.31$). Student-Newman-Keuls post hoc tests indicated that, overall, the number of AGB-IR cells differed significantly in response to all 3 chemosensory stimuli: PBS control < female skin secretions < male mental gland extract.

Considering the effects of each specific chemosensory stimulus on the number of AGB-IR cells separately, groups did not differ in responses to female skin secretions or male mental gland extract, although males had more AGB-IR cells in response to the PBS control than REPRO and NON-REPRO females (female skin secretions: F (2, 20) = 0.93, $P = 0.41$; male mental gland extract: F (2, 22) = 0.43, $P = 0.65$; PBS control: F (2, 21) = 4.68, $P = 0.02$).

The rostral-caudal distribution of AGB-IR cells activated by female skin secretions did not differ among MALES, REPRO females, and NON-REPRO females (Fig 5: non-significant interaction between section number and group: F (22,154) = 0.71 $P = 0.82$). The rostral-caudal

distribution of AGB-IR cells activated by male mental gland extract differed among MALES, REPRO females, and NON-REPRO females, as indicated by a significant interaction between section number and group (Fig 5: F (22,176) = 3.023 P = 0.001). I did not analyze the rostral-caudal distribution in response to PBS control because numbers of AGB-IR were too low for the analysis.

Study 2: The effects of androgens on behavioral and vomeronasal responses to chemosensory cues

Males with surgically elevated androgen levels were compared to males that had been housed in the laboratory long term with basal androgen levels. Androgen modulation of behavioral, morphological and VNO sensory neuron responsiveness to chemosensory cues was evaluated.

I used chemosensory stimuli derived from the whole body rinses of male and female stimulus animals to measure behavioral responses to chemosensory cues. In contrast, I used only extract from male mental glands (a source of pheromones used in courtship) to measure VNO responsiveness to chemosensory cues. Ideally, I would have used all of the same types of chemosensory stimuli for testing both behavioral and VNO responsiveness. However, for tests of VNO responsiveness, I decided to use male mental gland extract because it was the only chemosensory stimulus demonstrated to activate the VNO at the time of experimentation and for reasons previously described in Study 1 (Wirsig-Wiechmann et al., 2002).

METHODS

I. 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 Dept. of Wildlife. Test subjects were collected from a single location (Wayah Bald, Macon County, NC, 83° 30' 30" N longitude; 35° 10' 49" W latitude) in August 2003 and August 2004. Shortly after capture, animals were anesthetized and mental glands were surgically removed as previously

described (procedure approved by Oregon State University ACUP to L.D. Houck). Removal of mental glands from subjects ensured that an animal's own mental gland secretions did not contribute to behavioral and VNO responses to chemosensory stimuli. In the laboratory, subjects were individually housed at 16 °C on a 14L:10D photoperiod in 16 x 16 x 5 cm plastic boxes lined with moist paper towels. Animals were fed wax worm larvae. Average body length (distance from the tip of the snout to the anterior edge of the cloacal vent) did not differ between animals in the 2 treatment groups.

II. Implants and surgery

Testosterone implants were made from Dow Corning Silastic Laboratory Grade Tubing (1.02 mm ID, 2.16 mm OD). Implants were packed with crystalline testosterone propionate (TP) (Sigma #T1875) and sealed on both ends with Sylgard 184 silicon elastomer. Total length of the implants was 13 mm, of which 10 mm was packed with TP (approximately 0.012 g TP per implant). Blank implants were of a similar length, with sealed ends, but with no TP. Pilot studies determined appropriate implant size and experiment duration because these implants continued to deliver high levels of testosterone for at least three months when cirri length was observed to be maximal and androgen levels were measured (Schubert and Woodley, unpublished). Animals were randomly assigned to treatment groups although I distributed animals caught in different years equally between the two treatment groups. Before surgery, animals were anesthetized in 0.5 % MS222, pH 7.4. A single 3 mm long incision was made laterally in the ventral abdominal wall. The implant was inserted into the body cavity and the incision was closed with a 7-0 suture. A surgical adhesive and a topical antibiotic were applied to the incision. Surgeries were performed in June 2005 and animals were allowed 3 weeks to recover before further testing.

III. Cirrus size

Six weeks after surgeries, I assessed cirrus size by taking digital images of the right-lateral view of each animal's head at 3X magnification using an Olympus SZ61 stereomicroscope connected to an Olympus DP12 digital camera. The length of the cirrus from the ventral margin of the external nares to the ventral tip of the cirrus along the nasolabial groove was measured with Image-Pro Plus imaging software. Because cirrus length may not fully describe the increased overall size of the cirrus, 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.

IV. Behavioral tests

A. Chemosensory stimuli used in behavioral tests

Two types of chemosensory stimuli were collected for use in behavioral tests. First, I used whole-body rinses because similar rinses elicited behavioral responses in a congeneric species, *P. cinereus* (Sullivan, et al., 2003). Second, I used substrates that were scent-marked by females in reproductive condition, because an earlier study showed that male *P. shermani* preferred substrates marked by reproductive females over a substrate moistened with clean water (Palmer, 2004).

I tested chemosensory cues derived from males (n = 5), reproductive females (n = 5), and non- reproductive females (n = 5), reasoning that chemosensory cues from animals of different sexes and reproductive conditions might contain different chemosensory information. Reproductive condition in female donors was determined by externally examining the abdominal cavity for yolky ova and was confirmed later by dissection. To obtain body rinses, individual animals were placed in 50 ml of ddH2O in glass containers for 48 hours at 16 °C as previously

described. Body rinses from the same category (e.g. males) were pooled and diluted with ddH2O to a volume of 520 ml (approximately a 1:2 dilution). 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 hrs. The scent-marked substrates were then immediately used in behavioral tests. After 48 hrs, papers were clearly soiled with feces and presumably scent marked as well (Jaeger, et al., 1986). Control substrates were prepared by lining clean home boxes (without a female) with moistened paper towels for 48 hrs.

B. Response to chemosensory stimuli: Nose-tapping and locomotor activity

I measured both nose-tapping and locomotor activity on substrates containing chemosensory cues derived from males, reproductive females, non- reproductive females, and water. Each subject was tested individually in a 23 x 23 x 2 cm testing chamber lined with a paper towel to which 10 ml (enough to fully moisten) of a chemosensory stimulus was applied and the chamber was scanned for 2 seconds every minute for 60 min and scored as previously described. Subjects were tested during 4 trials for responses to body rinses from males, reproductive females, non-reproductive 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.

C. 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 female or moistened with female body rinse, and the other half was lined with a paper towel moistened with clean ddH2O and a gap of 1 cm was left in the middle of the testing chamber 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 minutes for 2 hours (= 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. After the 2 hr observation period, each male was left in its testing chamber overnight and its location was noted the next day (after lights were turned on, at about 9 am, approximately 12 hr after setting up the test chambers). Males found on the side with female chemosensory cues the next day were considered to prefer those substrates over water.

V. VNO responses to chemosensory cues

A. Method of agmatine uptake

I used the method of agmatine (AGB) uptake to identify vomeronasal cells that were activated after application of mental gland extract. The method was developed for use in lobsters and zebra fish (Michel, et al., 1999) and adapted to plethodontid salamanders as previously described in Study 1 (Wirsig-Wiechmann et al., 2002; Wirsig-Wiechmann, et al., 2006). I chose to use the method of AGB uptake because 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 non-sedated animals. To further validate the method, I (1) verified that my immunocytochemistry for AGB is specific (i.e. there is no immunoreactivity in the absence of

primary or secondary antibodies) (Fig. 6 A, B, and C) and (2) that the VNO in P. shermani does not endogenously express AGB (Fig.6 D). Finally, in case AGB itself is a chemosensory cue that activates sensory neurons (Michel et al., 1999), my

experiments included controls in which AGB was co-delivered with vehicle (i.e., 0.5X phosphate buffered saline (PBS)). These controls typically showed relatively low levels of activation, indicating that AGB was not a potent chemosensory stimulus of vomeronasal cells in *P. shermani.*

B. Mental Gland Extract

I used male mental gland extract to test responsiveness of the male VNO to chemosensory stimuli. Mental gland extract was a gift from Drs. Lynne Houck, Richard C. Feldhoff and Pamela Feldhoff. It was obtained from approximately 100 males collected from Wayah Bald, Macon County, NC, in August 2004. Mental gland extract was prepared as previously described. I used 0.5X PBS as the diluent in order to mimic natural osmotic concentrations of typical bodily secretions. 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 0.5X PBS without mental gland extract. Shortly before use, mental gland extract was mixed 1:1 with 6mM AGB dissolved in 0.1 M PBS. The PBS control was prepared by mixing 0.5X PBS 1:1 with 6 mM AGB. Subjects were placed individually in clean boxes and a micropipet was used to deliver 2 µl of either mental gland extract or PBS control (both mixed with AGB) as previously described in Study 1. Following a final PBS 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 minutes, with a mean of 89 minutes. Average times from onset of stimulus application to sacrifice were the same for all four combinations of implant treatment (TP or BLANK) x chemosensory

stimulus (mental gland or PBS control). Furthermore, I verified that time from onset of stimulus application to sacrifice did not contribute to variation in AGB-immunoreactivity or hormones levels by determining that it was not a significant covariate in statistical tests.

C. Tissue processing, AGB immunocytochemistry & Image analysis

After sacrifice, upper jaws were placed in fixative overnight (4% paraformaldehyde-2.5% glutaraldehyde in PBS, pH 7.4) and tissues were processed and sectioned as previously described. 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. Tissue was rinsed 6 times for 5 minutes each in 0.1 M PBS. Tissue was then incubated 30 minutes with a preincubation solution consisting of 0.2% Triton-X 100, 1% normal goat serum, and 0.004 % sodium azide in PBS. Tissue was incubated in for 3 days at room temperature with rabbit polyclonal anti-AGB antibody (Chemicon AB1568-2000T) diluted 1:400 in preincubation solution. Tissue was rinsed again (as described above) in PBS, incubated 30 minutes with biotinylated goat anti-rabbit antibody, rinsed in PBS, incubated for 30 minutes with Avidin-Biotin Enzyme Complex (ABC elite, Vector), and rinsed again in PBS. Following a rinse in 0.05 M Tris-HCl, pH 7.4, tissue was incubated approximately 5 minutes in 0.02%DAB-0.001% H2O2, and then rinsed 5 more times for 5 minutes each in Tris-HCl. Slides were coated with Crystal Mount (Biomedia), allowed to dry, and coverslipped with Permount (Fisher Scientific). Control slides in which either the primary or secondary antibody was omitted had no immunoreactive AGB (AGB-IR) (Fig. 6). Every 4th section of an alternate series of slides was stained with cresyl violet using standard histochemistry. Images were visualized and cells with AGB-IR were counted as previously described in Study 1.

VI. Hormone measurement

All plasma hormone levels were measured by Endocrine Services Laboratory at the Oregon Primate Research Center (Dr. David Hess). Plasma steroid hormone levels were measured in trunk blood collected at the time of sacrifice in order to verify the success of androgen manipulations. Trunk blood was collected within 3 minutes of decapitation. Blood samples were centrifuged and plasma was frozen at -20 °C until hormone assays were performed. All samples were assayed in a single assay following established methods (Gruenewald, et al., 1992; Resko, et al., 1980). Briefly, for measurement of testosterone (T) and its androgenic metabolite, dihydrotestosterone (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. I also measured the stress hormone, corticosterone (CORT), since a study in amphibians indicated that implants affected plasma CORT (Harvey and Propper, 1997). 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 %, intra-assay coefficients of variation were 9.6%, 9.9%, and 4.4%, and sensitivities were 0.4 ng/ml, 0.4 ng/ml and 2ng/ml, respectively.

VII. Statistics

Plasma hormone levels were log-transformed to generate homogeneous variances 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. Scores for cirrus size were ranked and analyzed with the nonparametric Mann-Whitney U-test. Cirrus length was analyzed with a one tailed t-test since I 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 non-reproductive female body rinse) as the within-subjects factor and implant treatment (TP or BLANK) as the between-subjects factor. Significant overall effects were followed up using within subjects contrast tests and Student Newman-Keuls 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 (TP or BLANK) as a factor. The number of AGB-IR cells was log-transformed to generate homogeneous variances and analyzed with a 2-way ANOVA; between-subjects factors were implant and chemosensory stimulus (PBS control or mental gland extract). To determine if the volume of the VNO contributed to the number of AGB-IR cells, VNO volume was included in models as a covariate. To determine if the rostral-caudal distribution of AGB-IR cells was different between TP- and BLANK-IMP males that were exposed to mental gland extract, I

conducted a repeated measures ANOVA on the log-transformed number of AGB-IR cells, with section number as a repeated measure and implant and chemosensory stimulus as between-subjects factors.

RESULTS

I. Hormone levels

Plasma T and DHT levels were significantly elevated in TP-IMP males (Fig. 7), 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 ± SE plasma CORT between the treatment groups (TP-

IMP: 30.9 ± 4.9; BLANK-IMP: 27.2 ± 4.13).

II. Cirrus size

Cirri of TP-IMP males were significantly longer and larger compared to those of BLANK-IMP males ($t(25) = 1.96$, $P = 0.03$; $U = 9.5$, $N1 = 14$, $N2 = 13$, $P < 0.001$). Mean (± SE) cirrus length was 1.178 ± 0.106 in TP-IMP males and 1.101 ± 0.098 in BLANK-IMP males. Median cirrus size was "medium-large" for TP-IMP males and "small" for BLANK-IMP males.

III. Behavior

All males nose-tapped the substrates moistened with body rinses significantly more than the water substrate (Fig. 8, $F(3, 78) = 5.5$, $P = 0.002$). 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. 8, $F(1, 26) = 9.7$, $P = 0.004$). Males were equally active on body rinse and water moistened substrates (Table 3, $F(3, 78) = 0.64$, $P = 0.59$) and there was no difference in activity between TP-IMP and BLANK-IMP males (Table 3: $F(1, 26) = 1.8$, $P = 0.19$). 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 4, binomial test, $P = 0.035$). When location on the substrate was checked the next day, there was no preference for one substrate over the other in either TP- or BLANK-IMP males. When given a choice between a substrate moistened with reproductive female body rinse versus water, neither TP- nor BLANK-IMP males showed a preference either within the first 2 hrs of exposure or the next day, although there was a marginal effect for BLANK-IMP males to avoid female body rinses on the next day (Table 4).

IV. Vomeronasal responses to androgens

Overall volume (mean ± 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) ($F(1, 26) = 0.50$, $P = 0.50$). 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. 9, 10, Table 5) 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 ($F(1, 23) = 0.18$, $P = 0.68$). 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. 9, repeated measures ANOVA (interaction between rostral-caudal section and hormone treatment): $F(14, 168) = 0.8$, $P = 0.64$).

DISCUSSION

I. Summary of major results:

This was the first investigation into the role of sexual dimorphism and sex steroid modulation of chemosensory detection in the terrestrial salamander, *P. shermani*. For male salamanders in Study 1 plasma androgen levels were lower than those found in the field during the mating season, but were notably higher than levels found in males housed in the laboratory long term (Woodley, 1994). The circulating estradiol levels in reproductively active females in this study were significantly higher than that of the non-reproductive females, which had mostly non-detectable levels. Plasma corticosterone levels were different between both groups of females as well as compared to the males. For males in Study 2, those that received TP-implants had androgen levels that were representative of levels found in the field during the mating season and were significantly higher than the males that received blank implants. There were no differences in corticosterone between the two groups.

The VNO of males was on average twice as large as in the females, despite a smaller overall body size. There was no difference in VNO volume between reproductively active females and non-reproductive females or males with elevated or basal levels of testosterone. Animals of both sexes and reproductive conditions nose-tapped chemosensory cues significantly more than water and males nose- tapped more than females. Females in reproductive condition and males with elevated testosterone nose- tapped in general more than their non-reproductive counterparts. Males were more active than females, but there were no differences in activity between reproductive and non-reproductive females or testosterone enhanced and not males. Males with elevated testosterone showed a preference for female marked substrates while males with lower

testosterone did not; females in reproductive condition showed a strong avoidance for conspecific reproductive female skin secretions and non-reproductive females (as well as males) did not.

All animals had significantly more AGB-immunoreactivity to chemosensory cues than to saline control; however, there were no differences in VNO responsiveness to the cues between the sexes or reproductive groups. Next I will discuss each major result in turn.

II. VNO volume between sexes and reproductive groups

A. Sexual dimorphism

The volume of the male *P. shermani* VNO was twice as large as that of female VNO in Study 1. This parallels results that the VNO of freshly field caught male *P. shermani* is larger in males than females (Woodley, in prep) as well as in the congeneric species *P. cinereus* (Dawley, 1994). This is one of only a handful of examples of sexual dimorphism in a sensory structure. There are many examples of sex differences in motor output relating to reproductive behaviors. The vocal motor pathway of the zebra finch, a species in which males sing and females do not, is sexually dimorphic (Wang et al., 1999). There are also sex differences in morphology and physiology of the motor output muscles involved in calling during the mating season (again a species in which males call and females do not) in the gray tree frog, *Hyla chrysoscelis* (Girgenrath & Marsh, 2003). The rat VNO provides the only other reported example of sexual dimorphism in a sensory structure in a vertebrate species, though in the rat while the VNO is larger, so is male body size (Segovia and Guillamon, 1993).

B. Sex steroid effects

There is no difference in salamander VNO volume between reproductively active females and non-reproductive females (Study 1) or males with elevated or basal levels of testosterone (Study 2), indicating that the sexual dimorphism of the salamander VNO is permanent in adults and not due to changes in levels of sex steroid hormones. Thus, the salamander example provides

a model in which to investigate how organizational differences in a sensory structure affect sex differences in sensory detection and processing, and in turn reproductive behaviors as sex differences in VNO volume appears to be permanent and does not change with sex hormone levels or reproductive condition.

III. Behavioral responses to chemosensory cues between sexes and reproductive groups

A. Nose-tapping

In Study 1, males chemo-investigated (i.e. nose-tapped) more than females. In particular they showed a greater response to water as well as to body rinse collected from reproductively active females. Males appeared to be more motivated to investigate substrates that may contain signals to identify potential mates. Reproductive females nose-tapped, more than non-reproductive females, particularly in response to male derived body rinses and reproductive female skin secretions. Male body rinses may very likely contain signals for reproductive females relevant to finding a mate. Based on the highly organized courtship behavior and external sperm transfer, females are highly responsible for mating success in this species. It has not been observed in the field whether it is males or females that are responsible for searching out and selecting potential mates. These data suggest that not only the male is responsible for mate searching but the female as well, particularly given the result that non-reproductive females nose-tapped male derived cues at a lesser rate.

The heightened response to reproductive female skin secretions by the reproductive female group is an interesting one. The secretions are white and sticky and they function as an anti-predator mechanism (Evans & Brodie, 1994). Based on this information I had originally hypothesized that this cue was functioning as an alarm signal to conspecifics; however, only

reproductively active females show a strong behavioral avoidance response and that reproductively active females chemo-investigated the signal more than non-reproductives. These data suggest that the cue is involved in territory maintenance between females during the mating season. Additional behavioral and molecular assays are required for this chemosensory cue to be more fully characterized.

In Study 2, males that received testosterone implants nose-tapped all cues more than males that received empty implants. This mirrors a result found in cats; the feline mode of chemo-investigatory behavior, the flehman response, is controlled by androgens (Hart & Leedy, 1987). In rough skin newts, clasping behavior in response to female scented newt-models is dependent on a combination of testosterone and vasotocin (Thompson & Moore, 2003). Biologically, it is a logical principle that chemo-investigatory behavior would be highest when androgens (and estrogens) are peaked, so that response to reproductively relevant social cues would be maximal.

In summary**,** males have a larger VNO and appear to "use" it more in nose-tapping and searching for biologically relevant social cues. Animals with higher levels of sex steroids nose-tap more than animals with basal levels, suggesting that chemo-investigatory behavior may be an important behavior relating to reproduction and may be modulated by activational levels of hormones.

B. Activity

For Study 1, males were more active than the females, even on a water soaked substrate, perhaps indicating a higher motivational level to search for chemosensory cues. Activity levels were positively correlated with nose-tapping. However, when general activity levels are corrected for, only nose-tapping responses to water substrate are altered. In this case, reproductive females nose-tap water at the same rate as males, above non-reproductive females. If activity is not corrected for, both female groups nose- tap water less than males. Responses to all other

chemosensory cues remain the same. In this Study 1, there were differences in activity between the female groups, but only in response to certain chemosensory cues. In overall analyses there are no differences in activity between reproductive females and non-reproductive females. This parallels results from Study 2, in which males with elevated androgens and basal androgens are equally active. These overall activity results from Studies 1 & 2 are interesting when compared to the nose-tapping results because activity level differences are only sexually dimorphic and not affected by activational levels of sex steroids as nose-tapping behaviors are.

C. Preferences for chemosensory cues

1. Female skin secretions

In Study 1, when presented with reproductive female derived skin secretions, only reproductive females significantly avoided the substance. Non-reproductive females marginally followed this trend and males showed no avoidance at all. This result was addressed above, in that reproductive females also nose-tapped the skin secretions more than non-reproductive females. This lends evidence to the thought that female skin secretions function in territory maintenance between females during the mating season.

It is unexpected that males did not show a preference for the female skin secretions. They did nose-tap the cue at the same rate as the reproductive females, indicating that they were able to detect it and were perhaps interested in the cue, as it was derived from reproductive females. Males do have a larger VNO. They may be able to determine that this signal is from female conspecifics but it may have little biological relevance to them, as it appears likely to be functioning in territoriality between females rather than as a signal in a reproductive context.

2. Female scent-marked substrate

In Study 2, males that received testosterone implants preferred substrate scent marked by reproductively active females, while males that received blank implants did not. This result adds

to work done at Oregon State University that showed that reproductively active male *P. shermani* preferred reproductive female marked substrate (Palmer, 2004). Previous studies using males housed in the laboratory long term, with basal levels of androgens showed no preference for substrate scent marked in this way (Schubert & Woodley, unpublished). The current study clarifies that this scent preference is androgen dependent. Androgen mediation of odor preference is a common theme in vertebrate model species. In rodents, males in mating condition or with enhanced testosterone showed preference for females in estrus above anestrus females or other males (Merkx, 1984; Moffatt, 2003; Sakuma et al., 2004; Stern, 1970). In summary, my data suggest that in *P. shermani*, preference for markings derived from reproductively active females follows the general principle of androgen modulation illustrated in rodent model systems.

3. Female body rinse

For Study 1 and Study 2, neither group of males nor females in either reproductive condition showed any preference or avoidance for reproductive female body rinses when given a choice with water. This result was puzzling given that all animals tested nose-tapped this substrate more than water control in the sequential presentation behavior assay, indicating that they were able to detect the cue. There was also a clear sex difference in response to this cue in Study 1, as males showed a stronger nose-tapping response than females. The concentration of body rinses is notably lower than that of skin secretions collected, and simply may be too low to elicit a preference response when given a choice even if the animals are clearly able to detect the cue. Also, there is a possibility of volatile signals being present in the rinse that may be traveling to both sides of the testing chamber and blurring the line between substrates.

The work to characterize the content of the body rinses is in the very early stages. I know that animals show a clear nose-tapping response and that sensory neurons of the VNO have been shown to be activated by the body rinses (Schubert & Woodley, unpublished data). BCA assay

reveals protein present in the rinses, but at low concentrations. At this point it is unclear whether behavioral responses to body rinses come from secretions of the exocrine glands of the salamander skin 1) in general or 2) in localized areas; if they are released 1) into the rinses or 2)if the signals are contained in fecal matter that end up in the rinses. Further investigation into the chemical content of these body rinses is necessary to determine if this is a reliable preparation with which to test behavioral responses to chemosensory cues.

IV. VNO responsiveness

A. Sex differences in VNO responses

All animals tested in Study 1 had more agmatine-immunoreactive (AGB-IR) cells in response to the chemosensory cues applied, above saline control. Further, all animals had more AGB-IR in response to male mental gland extract above female skin secretions. The fact that male mental gland extract elicited a stronger response in the VNO than female skin secretions may simply be due to concentration differences. Male mental gland extract applied was 2μg/μl and female skin secretions were only approximately 0.5 μg/μl. It is unknown if either of these are saturating concentrations, but there is clearly a difference between the two. Concentrations of both of the signals are strong enough for behavioral responses. Female skin secretions elicit both strong nose-tapping responses in males and females and avoidance behaviors in reproductive females. Male mental gland extract has been experimentally shown to increase female receptivity when applied to the nose of the female salamander during courtship by decreasing the latency of sperm transfer (Rollmann, et al. 1999). It is unknown if this male-derived cue has any biological relevance to other males.

In Study 1, there were no differences between males and females in numbers of AGB-IR cells to saline or the chemosensory cues presented. Both sexes responded equally to the cues. The result of no differences between the sexes in AGB-IR in the VNO to chemosensory cues is a

surprising one, given the size of the male VNO relative to the female. I had hypothesized that males would be more sensitive, with more sensory neurons responding to reproductively relevant chemosensory cues. These results do not support my hypothesis; however, I cannot conclusively say that the female derived cue presented here functions in a reproductive context for the male. In addition, there was a difference in rostral-caudal distribution of male mental gland extract between the groups.

This difference in distribution between the groups to mental gland extract was surprising, given that there were no distribution differences for the female skin secretions in Study 1 or in for male mental gland extract in Study 2. It appears that the difference is a later peak of AGB-IR cells for this cue in males than in the females, while the overall distribution shape looks very similar across the board. A logical explanation for this result is due simply to the larger VNO size in males. The largest volume of vomeronasal receptor cells per section of the organ may not directly correspond in the sexes. The concentration of female skin secretions is much less than the male mental gland extract and may not be saturating its receptors and so I am not seeing the same effect.

Based on the cues presented here I see little to no sexual dimorphism of detection of chemosensory cues by the VNO. This contrasts findings in mice in which certain urinary compounds collected from both males and females elicited VNO responses in females but not in males (Thompson et al., 2004; Halem et al., 2001).

It is entirely possible that a larger VNO in male salamanders does mean that the males have more vomeronasal receptors available for particular chemosensory cues than females do, particularly cues that may function to identify potential mates but I have not found the appropriate cue to test as of yet. Alternatively, males may have a wider variety of vomeronasal receptors that

are able to respond to a greater number of cues than females do, perhaps signals involved in territory maintenance or predator or prey cues.

B. Sex steroid modulation of VNO responses

Male mental gland extract has been shown to be a strong reproductive cue for the female, as it functions in courtship encounters, but in Study 1 reproductive females did not show a higher VNO response to the cue than the non-reproductive females. These results contrast findings in newts that sex steroids, particularly estradiol, function in modulation of sensory cues (Halem et al., 2001; Kikuyama and Toyoda, 1999; Toyoda et al., 1999; Toyoda and Kikuyama, 2000). It is possible that the cues presented in this study have no biological reason for reproductively active females to have higher responses non-reproductive females, as male mental gland extract is applied to a female's nose after courtship has already been initiated. A cue that contains signals from a conspecific male that may be involved in mate search may be a more appropriate cue to test, such as male derived body rinse.

It was surprising that reproductive females did not show higher numbers of AGB-IR in response to female skin secretions above non-reproductive females given that they showed stronger behavioral responses. It would seem that this is a biologically appropriate cue to test given these behavior data though it is not a mating signal for the female and the VNO is thought to function primarily in cues relating to reproduction.

In Study 2, males with higher levels of androgens showed no higher responses to any of the cues than males with lower levels. Again, it is not likely that female skin secretions function in a reproductive context for males and male mental gland extract has no known biological function for males. Female derived cues involved in mate search and identification would be more appropriate cues to test androgen modulation of chemosensory cues as the VNO is thought to

function primarily in reproductively relevant signals and androgens are maximal during the mating season (Woodley, 1994).

In conclusion, elevated levels of sex steroid hormones did not increase sensitivity of vomeronasal sensory neurons to chemosensory cues in either males or females. This does not support my hypothesis that sex steroids modulate the responsiveness of the VNO to chemosensory cues, though additional signals that are known to function more in reproductive behavior need to be tested.

V. Sex steroid modulation of morphology

A. Cirri of male salamanders

Cirri are fleshy protuberances that run from the tip of the upper lip to the external nares. Cirri contain nasolabial grooves that use capillary action to draw non-volatile substances up into the nasal cavity for detection by the VNO (Dawley & Bass, 1993). I have shown that males that received testosterone implants have significantly longer cirri than males that received empty implants, suggesting that cirri are androgen dependent secondary sex characteristics. As cirri contain the nasolabial grooves, elongation of these structures may be a form of morphological modulation by activational levels of androgens to enhance chemo-investigatory behavior during the mating season when androgen levels peak (Woodley, 1994). Female salamanders also have nasolabial grooves and cirri; however little is known about cirri size and possible elongation in females. Unfortunately in Study 1, female cirri length was not investigated.

In summary, these data suggest that cirri elongation is androgen dependent and may function in enhancing chemo-investigatory behavior for males during the mating season.

VI. The effects of chemosensory cues on hormones

Chemosensory cues can elicit changes in physiology and endocrinology of the recipient, referred to as primer effects (Halpern and Martinez-Marcos, 2003). In general there were no true primer effects seen in response to application of chemosensory cues in these studies. In Study 1 there was a marginal effect that males that received male mental gland extract had higher levels of corticosterone than females that received the same cue or other males that received female skin secretions or saline. In Study 2, males that received male mental gland extract had lower testosterone than males that received saline control.

Both of these results are only marginally significant and inconclusive as they were not replicated between male groups in the two studies but it was encouraging that in both responses males that received the male derived cue demonstrated possible primer effects that could be biologically interesting. An increase in corticosterone is a traditional stress response when detecting another male cue. Testosterone levels dropping in response to contact with a male cue is a particularly interesting response and may have implications in aggression, though the mechanism for this effect would be difficult to explain given the time frame of application of the chemosensory cue to time of sacrifice.

The study of primer effects was not the main focus of the experiments presented here. Variation in time between application and sacrifice was not controlled in a way that was conducive to investigating these types of effects. Hormone levels were measured to verify reproductive condition in Study 1 and hormone manipulations in Study 2, only when the data were fully analyzed did these potentially interesting results emerge. These data suggest that males that received a male derived chemical cue may have changes in steroid hormone levels in response to the cue though further studies are warranted in this area.

VII. Conclusion

Sex steroid hormones appear to be modulating responsiveness to chemosensory cues in the terrestrial salamander, *P. shermani*. Males have larger VNO and androgen dependent cirri that aid in sampling of cues on a morphological level. Behaviorally, males are more active than females and in general chemo-investigated their environment for chemosensory cues more, as do males and females with higher levels of sex steroid hormones, supporting my hypotheses. Behavioral responses to chemical signals do appear to be influenced by steroid hormones, but detection of the cues presented in this study by the VNO is not. There were no differences between sexes or reproductive groups in VNO responsiveness, which does not support my hypotheses. Sex dimorphism and sex steroid modulation appear to occur at the morphological and behavioral levels. Future studies may indicate higher processing centers in the brain, but not at the level of detection by the sensory neuron of the VNO.

VII. Future Directions

Males have a larger VNO and I have not yet determined if males are more sensitive to particular cues at this level or are perhaps able to respond to a wider variety of cues. Identification and examination of additional cues, such as predator, prey and reproductive cues involved in mate search may be helpful in answering this question.

The comparison of females in reproductive and non-reproductive conditions was the first step in assessing estradiol modulation of behavioral and VNO responsiveness to chemosensory cues. Future work would include ovariectomizing females +/- estradiol enhancement to determine that this sex steroid is indeed responsible for differences reported here.

Comparing cirri length in males and females and in different female reproductive groups, or with manipulated levels of estradiol is an important future study to determine if there are permanent sex differences in this morphological structure and if estradiol is involved in elongation in females.

For males, there were interesting potential primer effects found. Future studies that focus solely on these effects are warranted. Time course of application of chemosensory cue and time of sacrifice would be key to find the appropriate window for hormonal responses, rather than focusing on the time frame for ideal AGB-immunoreactivity.

Fig 1. Top Panel: Plasma corticosterone levels in REPRO, NON-REPRO females and MALES that received PBS control, female skin secretions or male mental gland extract. Bars that share a letter are not significantly different. There were no significant effects of chemosensory stimulus on corticosterone levels. Sample sizes are indicated on bars.

Bottom Panel, Left Axis: Plasma estradiol in females that received PBS control, female skin secretions or male mental gland extract. * Plasma estradiol levels were significantly higher in REPRO females than NON-REPRO females, $P < 0.001$. (Estradiol levels in NON-REPRO females were mostly non-detectable, with the exception of a few animals that received PBS control.) There was no effect of chemosensory stimulus on estradiol levels.

Bottom Panel, Right Axis: Plasma testosterone in MALES that received PBS control, female skin secretions or male mental gland extract. There was no effect of chemosensory stimulus on plasma testosterone. Sample sizes were 7-9 for each treatment group.

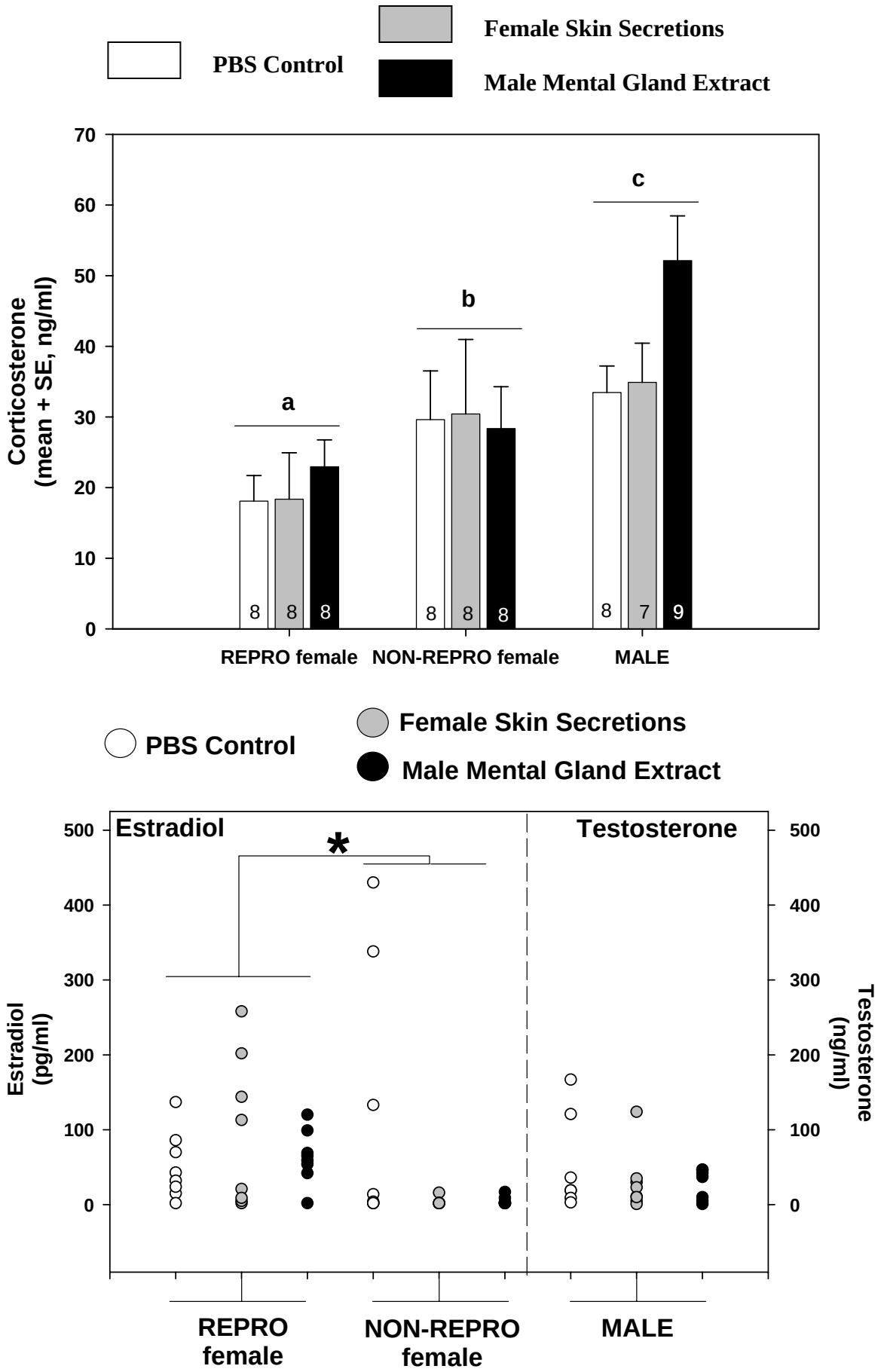
PBS Control
Female Skin Secretions
Male Mental Gland Extract
Corticosterone (mean + SE, ng/ml)
70
60
50
40
30
20
10
0
a
b
c
8
8
8
8
8
8
8
7
9
REPRO female
NON-REPRO female
MALE
PBS Control
Female Skin Secretions
Male Mental Gland Extract
Estradiol
Testosterone
*
500
400
300
200
100
0
Estradiol (pg/ml)
Testosterone (ng/ml)
REPRO female
NON-REPRO female
MALE

Fig 1.

Fig 2. Top Panel: The number of scans in which MALES, REPRO and NON-REPRO females were observed nose-tapping chemosensory cues (A.) repro female body rinse, B.) male body rinse, C.) repro female skin secretions or D.) water) during a 1-hour scan sampling observation. Asterisks indicate results of Student Newman Keuls pairwise comparisons following significant 1-way ANOVAs for each chemosensory stimulus, $P < 0.05$.

Middle Panel: The number of scans in which MALES, REPRO and NON-REPRO females were observed moving from one quadrant to another, noted as general activity on chemosensory cues (A.) repro female body rinse, B.) male body rinse, C.) repro female skin secretions or D.) water) during a 1-hour scan sampling observation. Asterisks indicate results of Student Newman Keuls pairwise comparisons following significant 1-way ANOVAs for each chemosensory stimulus, $P < 0.05$.

Bottom Panel: Nose-tapping after correcting for activity, as indicated by the estimated marginal means. Asterisks indicate results of Bonferroni pairwise comparisons following significant 1-way ANOVAs for each chemosensory stimulus, $P < 0.05$.

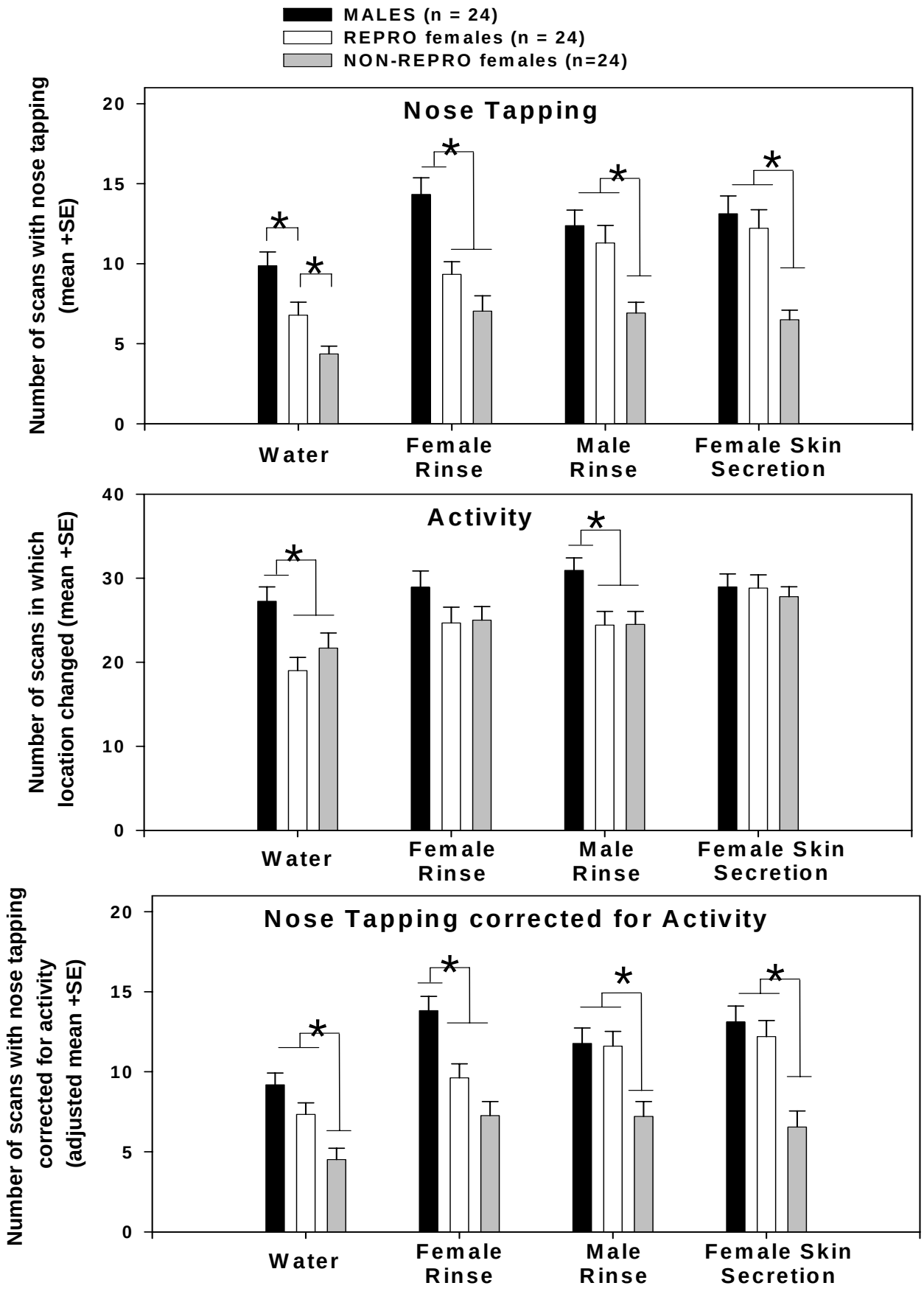
MALES (n = 24)
REPRO females (n = 24)
NON-REPRO females (n=24)
Nose Tapping
Number of scans with nose tapping (mean +SE)
0
5
10
15
20
Water
Female Rinse
Male Rinse
Female Skin Secretion
Activity
Number of scans in which location changed (mean +SE)
0
10
20
30
40
Water
Female Rinse
Male Rinse
Female Skin Secretion
Nose Tapping corrected for Activity
Number of scans with nose tapping corrected for activity (adjusted mean +SE)
0
5
10
15
20
Water
Female Rinse
Male Rinse
Female Skin Secretion

Fig 2.

Fig 3. The VNO volume (mean +/- SE) of MALES, REPRO and NON-REPRO females. * The volume of the MALE VNO is significantly greater than REPRO or NON-REPRO female VNO volumes. Sample sizes are indicated on bars.

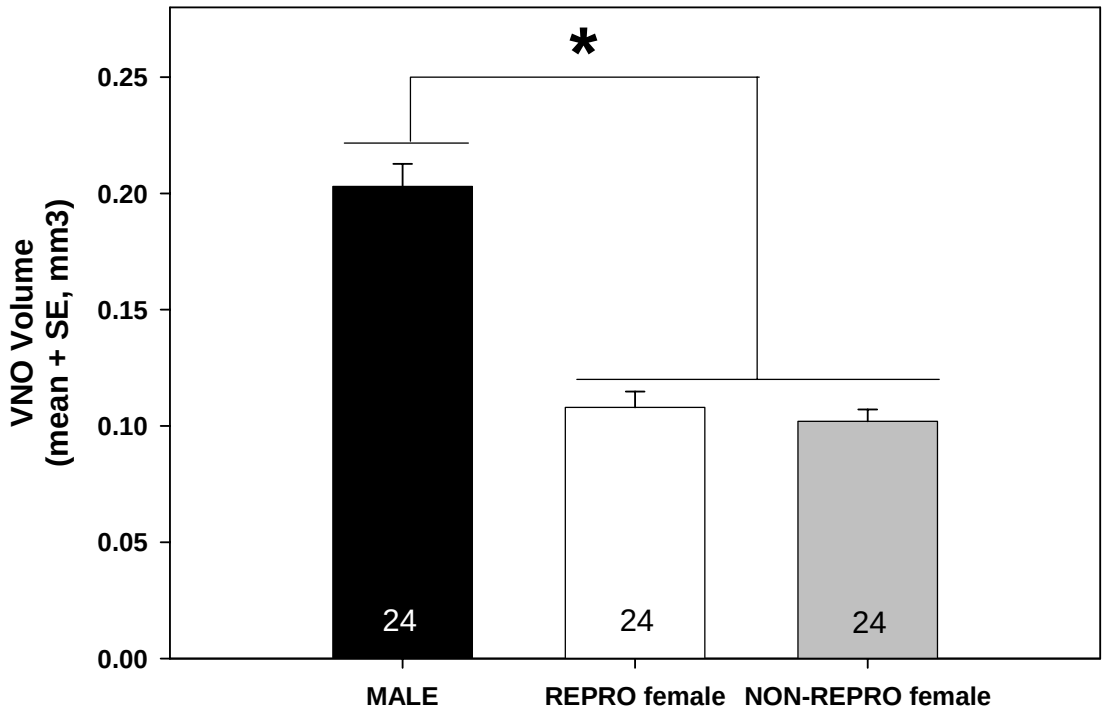
*
0.25
0.20
0.15
0.10
0.05
0.00
VNO Volume
(mean + SE, mm3)
24
24
24
MALE
REPRO female
NON-REPRO female

Fig 3.

Fig 4. Representative photomicrographs of 20μm coronal sections of the VNO. MALES (A), REPRO (D) and NON-REPRO (G) females that received male mental gland extract had many cells with AGB-IR; MALES (B), REPRO (E) and NON-REPRO (H) females that received female skin secretions had less AGB-IR; MALES (C), REPRO (F) and NON-REPRO (I) females that received PBS control had very few AGB-IR cells at all. Round bottom arrows indicate AGB-IR dendrites. Straight bottom arrows indicate AGB-IR cell bodies. Scale bars = 10μm.

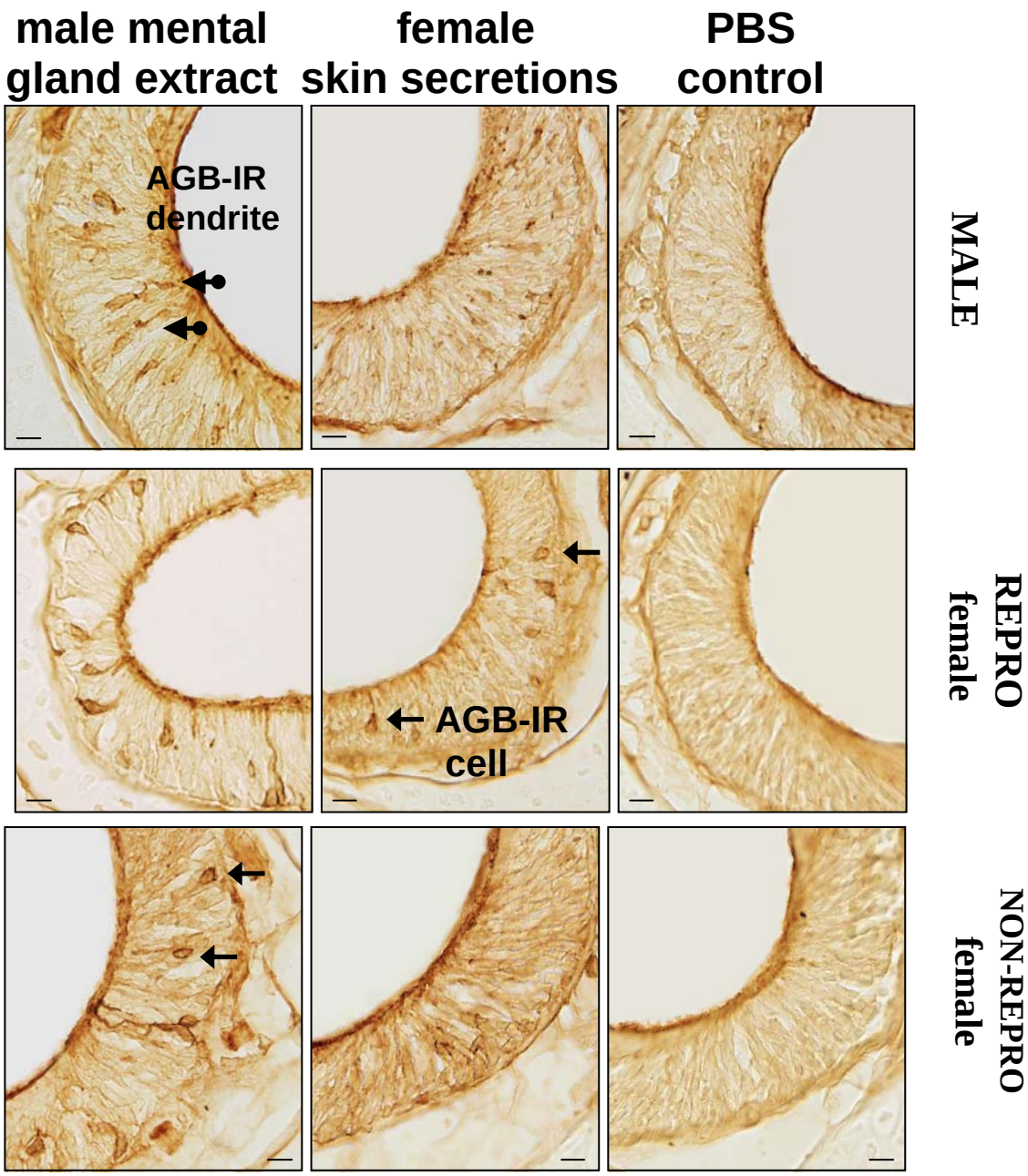
male mental gland extract
female skin secretions
PBS control
AGB-IR dendrite
AGB-IR cell
MALE
REPRO female
NON-REPRO female

Fig 4.

Fig 5. The number of AGB-IR cells per coronal section of the VNO from rostral to caudal end of the VNO. The rostral-caudal distribution of AGB-IR cells in response to male mental gland extract differed across animals (significant interaction between section number and group, $P = 0.001$).

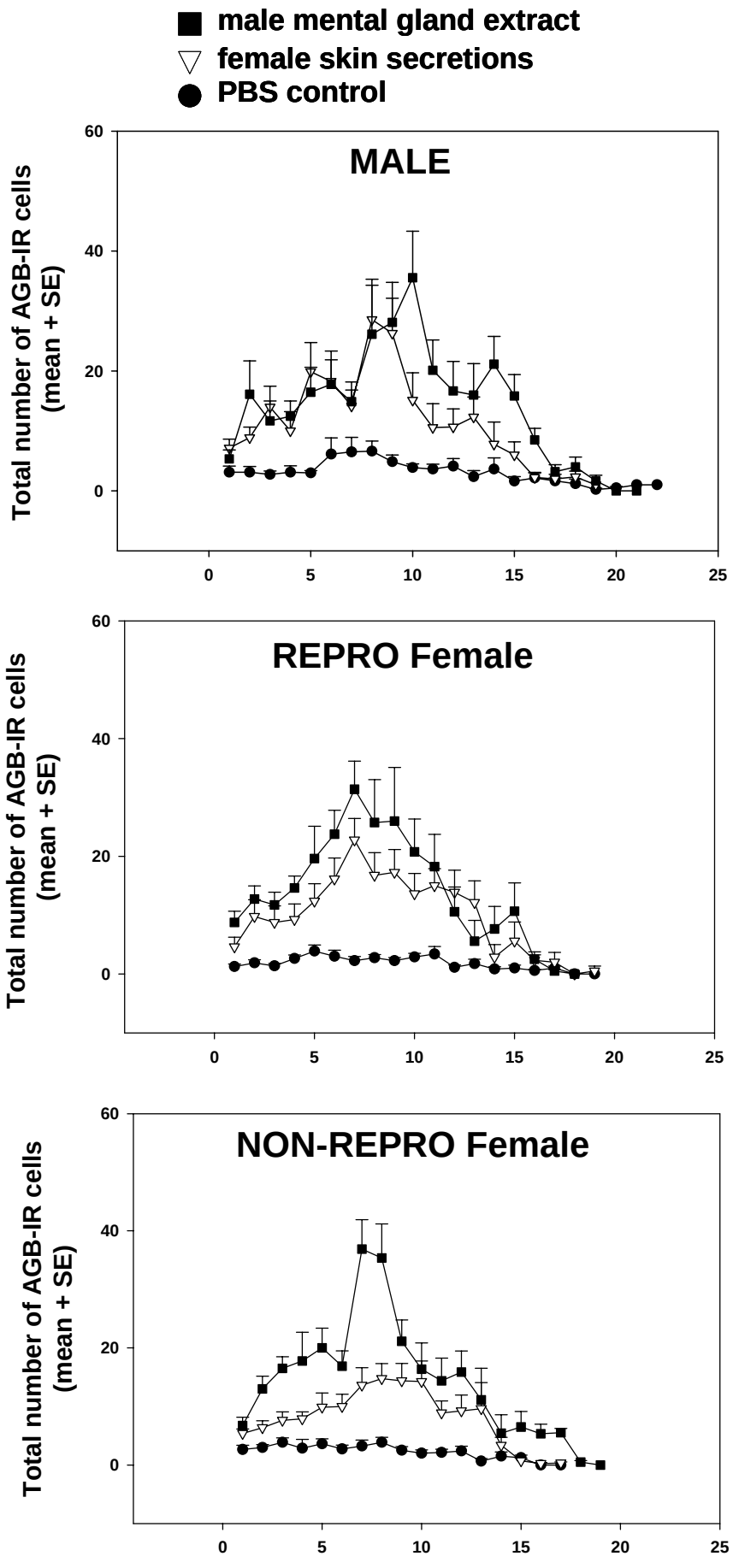
male mental gland extract
female skin secretions
PBS control
MALE
REPRO Female
NON-REPRO Female
Total number of AGB-IR cells (mean + SE)
Section Number
0
5
10
15
20
25
0
20
40
60

Fig 5.

Fig 6. Representative photomicrographs of 20 μm coronal sections of the VNO. Mental gland extract (A) applied to the VNO of a male activated VNO cells (inset shows boxed area at a larger magnification with an arrow indicating one of the AGB-IR cell bodies). Omission of the primary (B) or secondary (C) antibody on alternate sections revealed no AGB-IR, indicating that the immunohistochemistry was specific for AGB. No AGB-IR is seen in the VNO of a female that was not exposed to AGB (D). L: lumen. Scale bars = 50 μm.

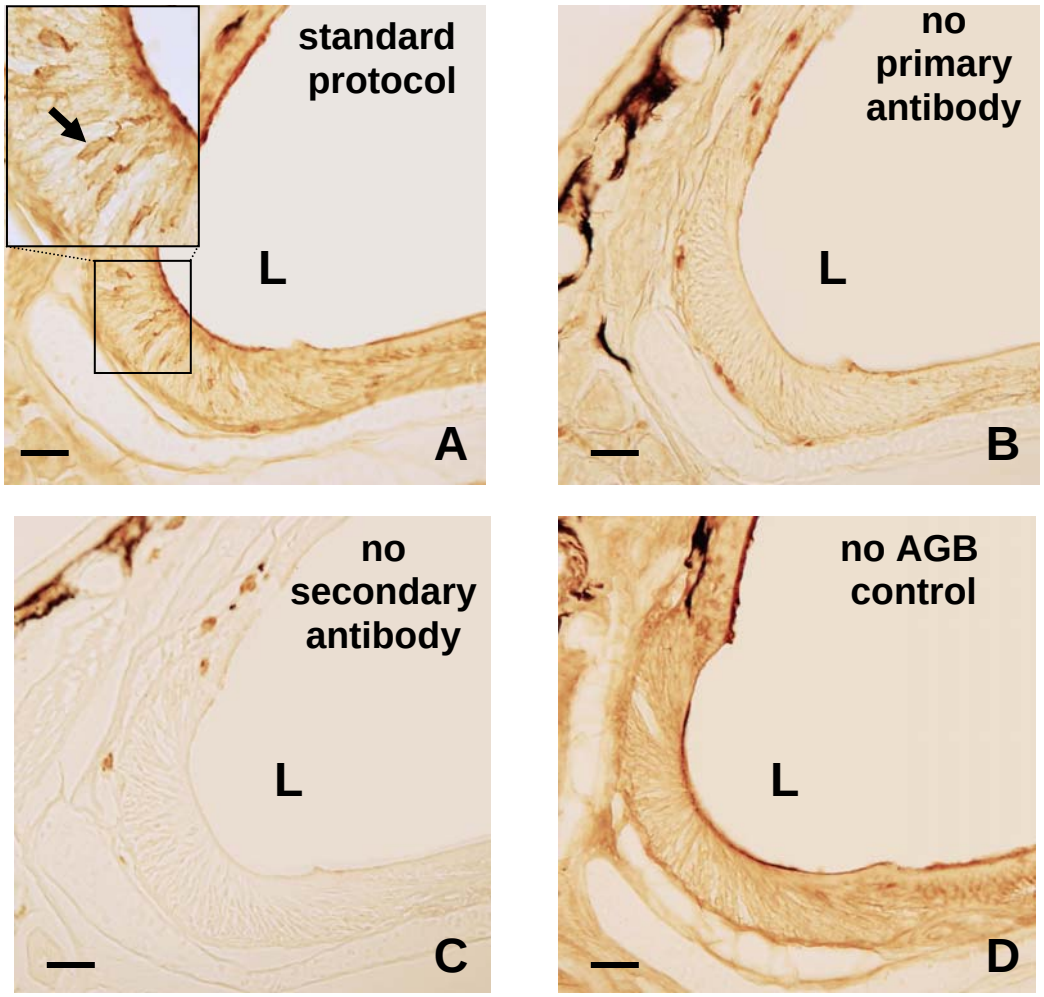
standard
protocol
L
A
no
primary
antibody
L
B
no
secondary
antibody
L
C
no AGB
control
L
D

Fig 6.

Fig 7. Plasma androgen levels in TP-IMP and BLANK-IMP males that received PBS control or mental gland extract. Significant main effects are indicated in panels. Sample sizes are indicated in bars.

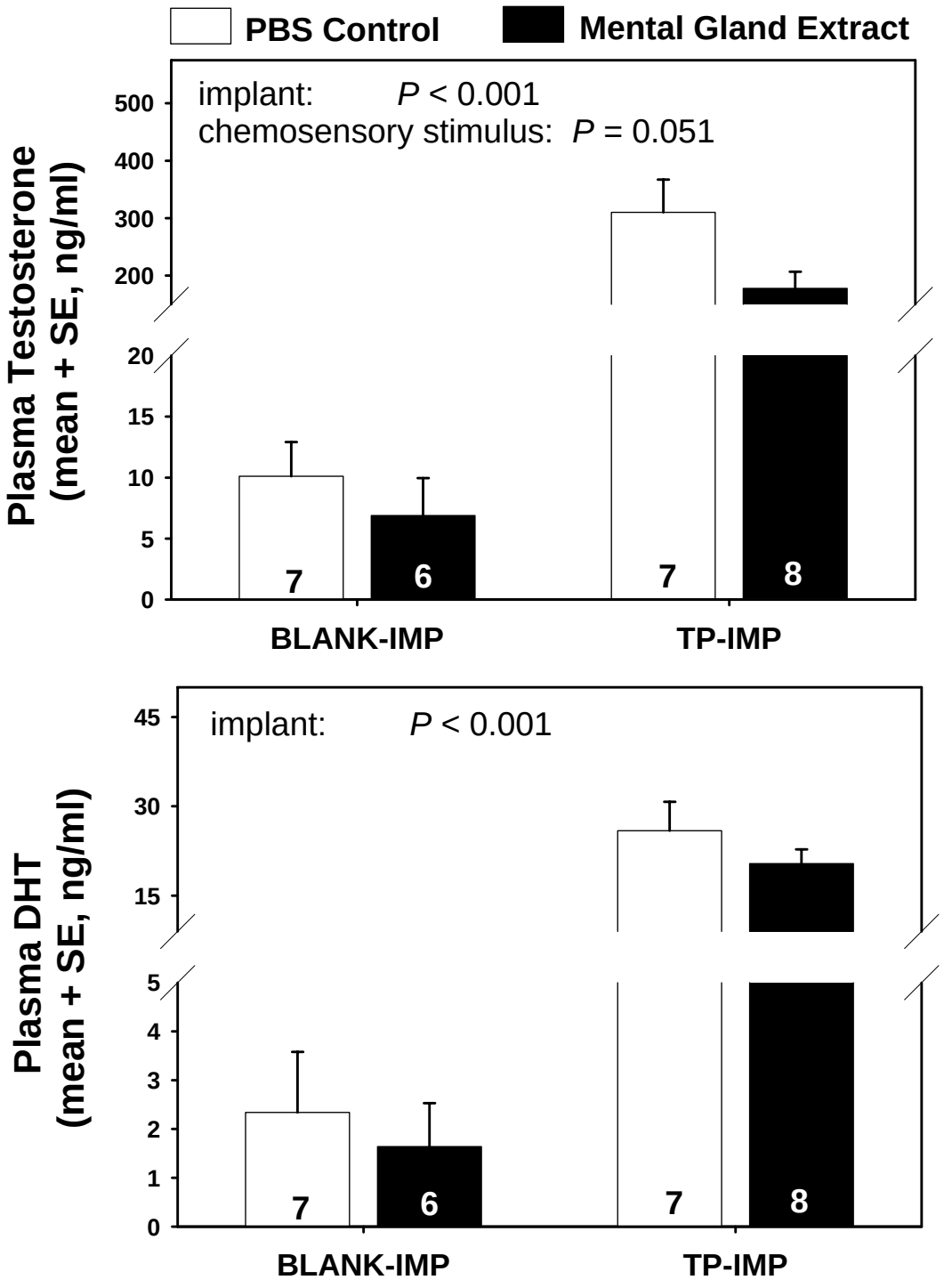
PBS Control
Mental Gland Extract
implant: P < 0.001
chemosensory stimulus: P = 0.051
Plasma Testosterone (mean + SE, ng/ml)
500
400
300
200
20
15
10
5
0
7
6
7
8
BLANK-IMP
TP-IMP
implant: P < 0.001
Plasma DHT (mean + SE, ng/ml)
45
30
15
5
4
3
2
1
0
7
6
7
8
BLANK-IMP
TP-IMP

Fig 7.

Fig 8. The number of scans in which nose-tapping was observed during a 1 hour observation period in TP-IMP males and BLANK-IMP males. Animals were tested sequentially on substrates saturated with water, body rinse from a non-reproductive female, body rinse from a reproductive female, and body rinse from a male. ** significant overall effect of implant on nose-tapping. * significantly different from other chemosensory stimuli, $P \leq 0.005$, with-in subjects contrasts.

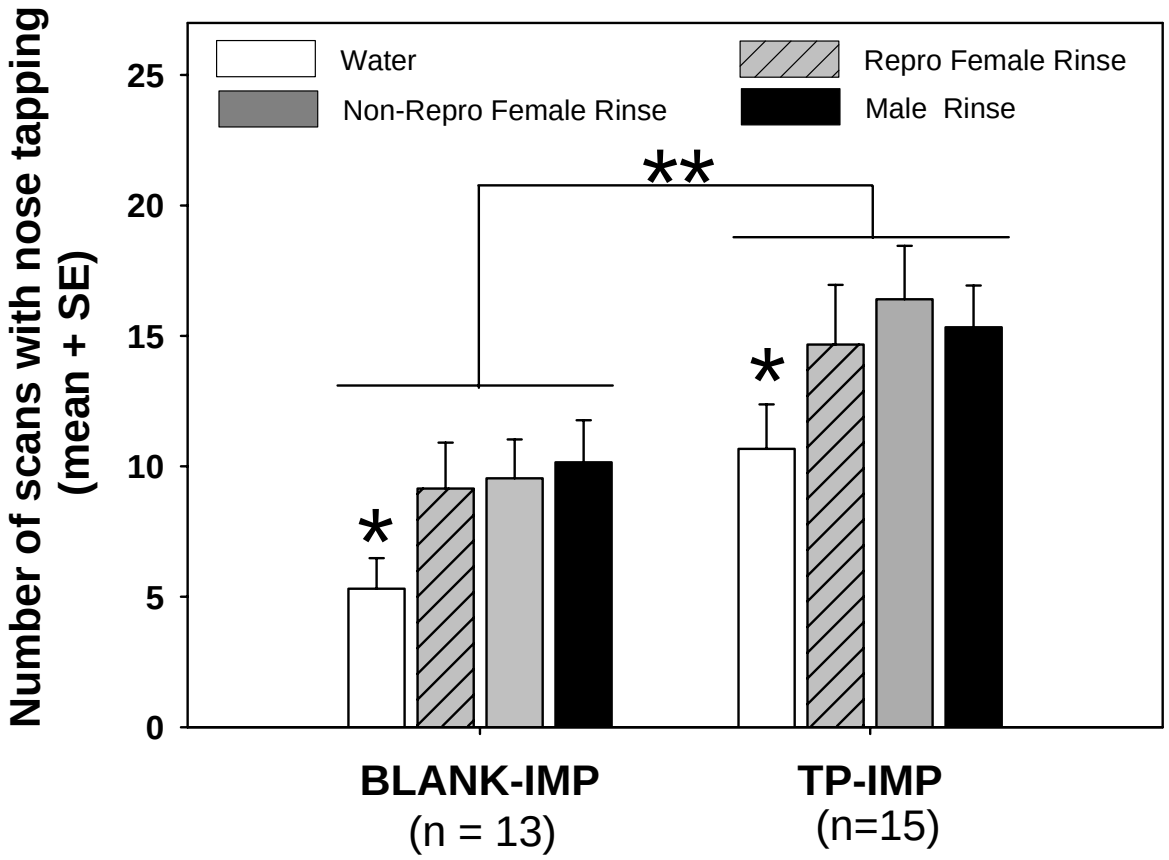
Number of scans with nose tapping
(mean + SE)
25
20
15
10
5
0
Water
Non-Repro Female Rinse
Repro Female Rinse
Male Rinse
**
*
*
BLANK-IMP
(n = 13)
TP-IMP
(n=15)

Fig 8.

Fig 9. The number of AGB-IR cells in each 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.

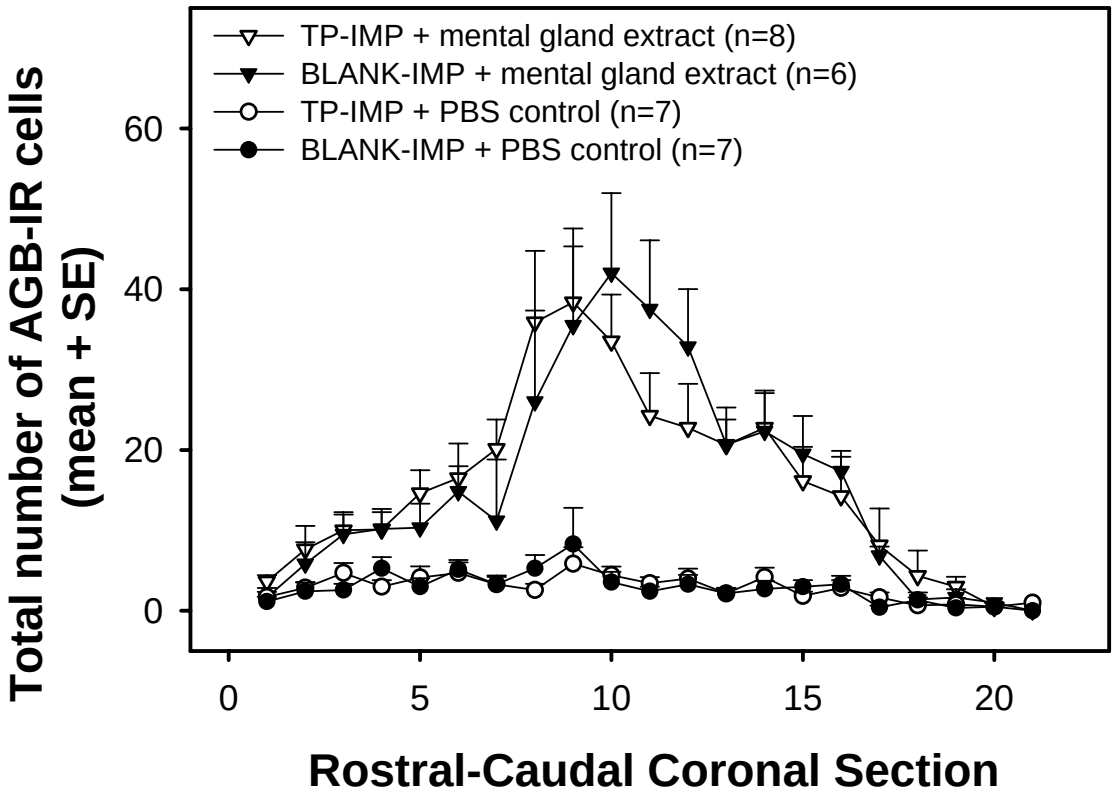
TP-IMP + mental gland extract (n=8)
BLANK-IMP + mental gland extract (n=6)
TP-IMP + PBS control (n=7)
BLANK-IMP + PBS control (n=7)
Total number of AGB-IR cells
(mean + SE)
0
20
40
60
0
5
10
15
20
Rostral-Caudal Coronal Section

Fig 9.

Fig 10. Representative photomicrographs illustrating AGB-IR in 20 μm coronal sections of the VNO. Males that received mental gland extract had many cells with AGB-IR (Panel A: BLANK-IMP, Panel B: TP-IMP) whereas males that received a PBS control had very few AGB-IR cells (Panel C: BLANK-IMP, Panel D: TP-IMP). In panel B, inset shows boxed area in larger magnification. Arrows label AGB-IR cell bodies. Arrow head labels an AGB-IR dendrite. L: lumen. Scale bars = 50 μm.

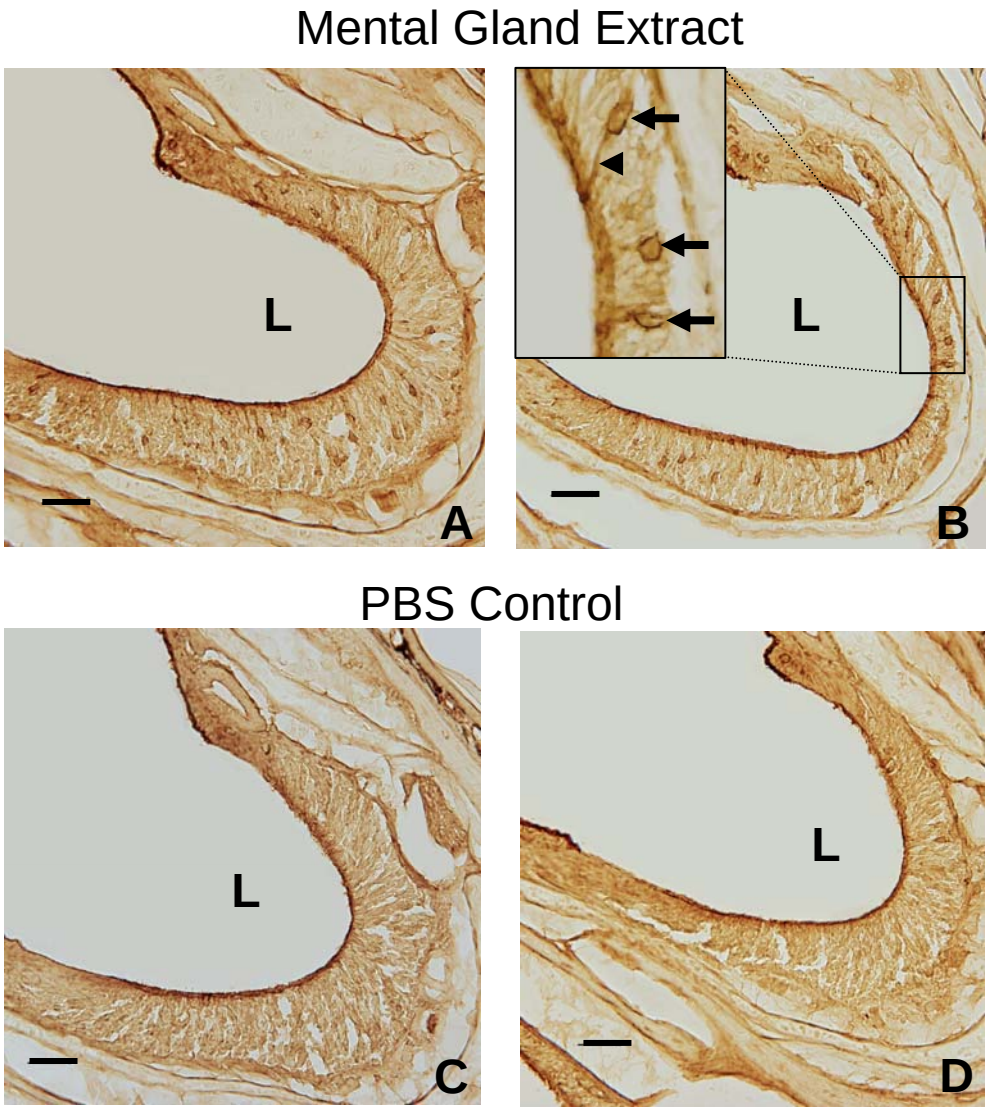

Fig 10.

Table 1
Study 1: Percent of animals on substrates containing reproductive female chemosensory cues.

	Female Skin Secretions versus Water		Female Body Rinse versus Water	
	% during 2 hr observation	% next day	% during 2 hr observation	% next day
REPRO female (n= 24)	17 *	21*	39	63
NON-REPRO female (n=24)	27†	33	43	38
MALE (n=24)	33	38	57	33

*** significantly different from 50% (2-tailed binomial test, *P*<0.01)**
† marginally different from 50% (2-tailed binomial test, *P*<0.10)

Table 2
Study 1: Total number of AGB-IR vomeronasal cells, mean ±SE.

	PBS Control	Female Skin Secretions	Male Mental Gland Extract
REPRO female	**33 ± 3 a (n=8)**	**175 ± 37 b (n=8)**	**234 ± 46 c (n=8)**
NON-REPRO female	**37± 5 a (n=8)**	**130 ± 16 b (n=8)**	**285 ± 44 c (n=8)**
MALE	**63 ± 10 a (n=8)**	**205± 34 b (n=7)**	**270± 50 c (n=9)**

Numbers that share a letter are not significantly different.

Table 3
Study 2: Activity (number of scan observations in which animals had moved to a different quadrant) on substrate containing water or different body rinses, mean ± SE.

	Water	Male Rinse	Non-reproductive Female Rinse	Reproductive Female Rinse
TP-IMP (n=15)	**32 ± 3**	**33 ± 3**	**36 ± 3**	**29 ± 4**
BLANK-IMP (n=13)	**30 ± 5**	**27 ± 3**	**27 ± 4**	**27 ± 4**

Table 4
Study 2: Percent of animals on substrates containing reproductive female chemosensory cues.

	Female Marked Substrate versus Water		Female Rinse versus Water	
	% during 2 hr observation	% next day	% during 2 hr observation	% next day
TP-IMP (n= 15)	80 †	67	47	60
BLANK-IMP (n=13)	54	46	46	23 ††

† different from 50% (2-tailed binomial test, $P < 0.05$)
†† marginally different from 50% (2-tailed binomial test, $P < 0.10$)

Table 5
Study 2: Total number of AGB-IR vomeronasal cells, mean ± SE.

	PBS Control	Mental Gland Extract
TP-IMP	**58 ± 9 (n=7)**	**321 ± 33 †** **(n=8)**
BLANK-IMP	**59 ± 12 (n=7)**	**326± 53 †** **(n=6)**

† significant main effect of chemosensory stimulus, 2-way ANOVA.

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