Exposure to pheromones increases plasma corticosterone concentrations in a terrestrial salamander

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Abstract

Sensory cues involved in social interactions can influence plasma steroid hormone concentrations. Although pheromonal communication is common in amphibians, it is unknown whether pheromones can alter hormone levels in amphibians as they do in mammals. We tested whether courtship pheromones would alter steroid hormone concentrations in male and female terrestrial salamanders (Plethodon shermani). Plasma corticosterone concentrations were elevated in male salamanders exposed to mental gland courtship pheromones, as compared to males exposed to female skin secretions or a saline control. Chemosensory cues had no effect on testosterone levels in males or on corticosterone or estradiol levels in females. These results provide the first evidence that pheromones have priming effects on the endocrine system in amphibians.

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1. Introduction

Hormones act in the brain to influence the behavioral expression of social signals, and conversely, exposure to social signals can alter hormone levels. Primer pheromones are social signals that induce physiological effects such as changes in plasma hormone levels in conspecifics (Wyatt, 2003). For example, exposure to female urine increased plasma levels of luteinizing hormone (LH) in male mice after 30 min (Bronson and Maruniak, 1976; Maruniak and Bronson, 1976). Similar rapid hormonal responses to male urine were found in female mice (Bronson and Maruniak, 1976). Likewise, exposure to female vaginal secretions elicited increased plasma LH and plasma testosterone in male hamsters (Richardson et al., 2004; Romeo et al., 1998). In women, exposure to male axillary secretions altered the temporal dynamics of plasma LH (Preti et al., 2003), as well as reduced tension. Finally, in women, exposure to a putative human pheromone found in male axillary secretions maintained plasma cortisol at higher levels as compared to levels in women exposed to a control odor (Wyart et al., 2007). This effect was apparent within 15 min after exposure, and lasted up to 60 min. These examples of pheromonal effects on hormone levels have been found in many mammalian species (Koyama, 2004).

Chemosensory cues also are an important mode of social communication in many species of amphibians. Many amphibians possess sexually dimorphic breeding glands that potentially produce socially relevant chemosensory cues (Duellman and Trueb, 1986). Male dwarf African frogs produce female-attracting chemosensory cues (Pearl et al., 2000) and male Australian tree frogs produce splendiferin, a peptide pheromone that attracts females (Wabnitz et al., 2000). Male red-spotted newts are repelled by chemosensory cues emitted by other males (Park and Propper, 2001). Male rough-skinned newts are attracted to female-scented water (Thompson and Moore, 2000). In Japanese newts (Cynops), the peptide pheromone sodefrin attracts reproductive females, but not nonreproductive females or males (Kikuyama et al., 1995). In plethodontid salamanders, males possess a submandibular gland called the mental gland. Secretions from the mental gland are applied to a female during a lengthy courtship. Exposure to mental gland pheromones increased female receptivity to mating (Houck et al., 1998; Houck and Reagan, 1990; Rollmann et al., 1999), probably via activation of areas of the brain involved in reproduction (Laberge et al., 2008; Wirsig-Wiechmann et al., 2002, 2006).

Despite evidence for pheromonal signaling in amphibians, it is unknown whether chemosensory cues alter the endocrine state of amphibians. As modern representatives of basal tetrapods, it is
important to determine whether pheromones alter hormone levels in amphibians. We examined hormonal responses to mental gland pheromones in the red-legged salamander (Plethodon shermanii), a developing model for the evolution and physiology of pheromonal communication. First, we measured changes in plasma steroid hormones in males, reproductive females, and nonreproductive females after exposure to mental gland extract (male pheromone), female skin secretions, or a saline (PBS) control. A second experiment was conducted to confirm results of the first experiment. A third experiment measured the CORT response to a standard handling stressor in order to better understand changes in plasma CORT levels found in the first 2 experiments.

2. Materials and methods

2.1. General

All methods were approved by Duquesne University’s Institutional Animal Care and Use Committee. Adult animals were collected by hand from Macon County NC, (83°, 30°, 30° N longitude; 35° 10', 49° W latitude) using appropriate permits from the North Carolina Wildlife Resources Commission, Department of Fish and Wildlife. Mating begins in early August and continues into the late fall. Under laboratory conditions, these animals will mate until December. Males and females were easily identified because males had mental glands and gravid females had follicles visible through the body wall. In eastern large Plethodon salamanders like P. shermanii salamanders, females typically oviposit every other year (Highton, 1962). Thus, during the mating season, there are both reproductive females (having large, yolky follicles, and being receptive to mating) and nonreproductive females (having small, unyolked follicles, and not receptive to mating).

2.2. Preparation of chemosensory stimuli

Male mental gland extract was prepared as previously described (Schubert et al., 2006). Extracts from multiple males were pooled, purified, and standardized to a concentration of 2 μg protein/μL in 0.5X phosphate buffered saline (PBS), and frozen at −20°C until use. The diluent, 0.5X PBS, was designed to match osmolarity of typical bodily secretions. A concentration of 2 μg/μL of mental gland extract was used because this concentration had previously elicited behavioral responses in females and also had induced neural activity in the vomeronasal organ and brain (LaBerge et al., 2008; Rollmann et al., 1999; Schubert et al., 2006, 2008; Wirsig-Wiechmann et al., 2002). Males that provided mental gland extract were not used as test subjects.

Female skin secretions were used because these secretions are easily obtainable and have pheromonal properties. Skin glands of the dorsal tail release a sticky white secretion when animals are pressed with blunt-nosed forceps to induce the release of skin secretions. An aliquot of the skin secretions (approximately 0.5 μg protein/μL of ddH2O) was frozen at −20°C until used in experiments.

2.3. Experiment 1

Test subjects consisted of males, reproductive females and non-reproductive females collected in August 2005. Mental glands were removed from males (see Wirsig-Wiechmann et al., 2002, for details; procedure approved by Oregon State University ACUP to Dr. Lynne D. Houch) and animals were transported to Oregon State University where they participated in behavioral experiments that involved mating. In October 2005, animals were transferred to Duquesne University where they were housed individually in 16 × 16 × 5 cm plastic boxes lined with moistened paper towels at 16°C with a 14L:10D light cycle. At this time, the salamanders participated in additional behavioral experiments (Schubert et al., 2008).

In December 2005, animals were exposed to chemosensory stimuli. Each animal was placed in a clean 16 × 16 × 5 cm plastic box lined with a moistened paper towel. Using a micropipette, chemosensory stimuli were delivered to the nares of non-sedated, freely behaving animals. Animals were exposed to chemosensory stimuli in the early afternoon by applying 2 μl of chemosensory stimuli every 2 min for a total of 22 applications over 44 min. Chemosensory cues were mixed 1:1 with 6 mM agmatine (AGB) in PBS. A PBS control was prepared by mixing 0.5× PBS with 6 mM AGB in PBS. (AGB was mixed with the chemosensory cues in order to identify VNO cells activated by the chemosensory cues; see Schubert et al., 2008, for results of this procedure.) Animals were sacrificed via decapitation between 15 and 75 min after the final application of the chemosensory stimuli and within 2 min of decapitation, trunk blood was collected using a heparinized capillary tube. Blood samples were centrifuged and the plasma fraction was frozen at −20°C until hormone assays were performed. Plasma corticosterone (CORT) was measured for all animals, plasma testosterone was measured in males, and plasma estradiol was measured in females. We did not measure plasma testosterone in females because previous data indicated that plasma testosterone levels were non-detectable in females (Woodley, unpublished).

Hormone levels were assayed by the endocrine services laboratory at Oregon National Primate Research Center (Gruenewald et al., 1992; Resko et al., 1980). To measure corticosterone (CORT), approximately 5 μl of plasma was extracted and assayed. To measure testosterone and estradiol, samples were subjected to column chromatography before assaying. Intra-assay coefficients of variation were 8.6%, 12.3%, and 9.4% for CORT, estradiol, and testosterone, respectively. Percent recoveries were 100%, 79% and 74% for CORT, estradiol, and testosterone, respectively. Inter-assay coefficients of variation were less than 5%.

Plasma testosterone and CORT levels were analyzed using parametric statistics after confirming that data satisfied assumptions of the tests (when necessary, data were log-transformed before analysis). Plasma levels of estradiol were not normally distributed and were analyzed using nonparametric Kruskal–Wallis tests. CORT and testosterone data were analyzed with ANOVAs for each group (male, female, nonreproductive female). Because blood was collected from 15 to 75 min after the final application of the chemosensory stimuli, initial analysis included time from final application of chemosensory stimuli to blood collection as a covariate. When this was a significant covariate, analyzes were performed on the estimated marginal means. Significant main effects were followed by pairwise comparison tests using a Bonferroni correction for multiple tests.

2.4. Experiment 2

Test subjects consisted of reproductive males and reproductive females collected in late July, 2007, and housed individually in
plastic boxes at 16 °C with a natural photoperiod. After two weeks in captivity, animals were exposed to chemosensory stimuli in early August 2007 using methods similar to those described in Experiment 1. Animals were exposed to chemosensory stimuli in the early evening by applying 5 μl of chemosensory stimuli every 5 min for a total of 10 applications over 50 min. This regimen of delivery was used because we were simultaneously measuring fos-like immunoreactivity to identify central nervous system neurons activated by mental gland extract (data not shown). Chemosensory cues were mixed 1:1 with PBS that did not contain AGB. Each animal was sacrificed 120 min after the final chemosensory stimulus application. We sacrificed animals at 120 min after chemosensory stimulus application because fos-like immunoreactivity is greatest at this time (Laberge et al., 2008). Trunk blood was collected and plasma CORT was measured as described in Experiment 1. The intra-assay coefficient of variation for CORT was 13%. Recovery of CORT was 81%. CORT data were analyzed with two-tailed t-tests. Results were considered to be statistically significant when P ≤ 0.05.

2.5. Experiment 3

Test subjects consisted of males collected in August 2007. Mental glands were removed from males and males were housed in the laboratory under conditions as described above. In August 2008, trunk blood samples were collected from males either within 2 min of removal from their home boxes, or after 30 min of handling. Handling consisted of removing an animal from its home box, placing the animal in a ziplock bag, and manually palping the animal to ascertain gender. The above procedures are similar to the handling involved when capturing and handling animals in the field. A similar study in a different species of plethodontid salamander, Desmognathus ochrophaeus, found that CORT levels were increased (relative to baseline levels) after 30 min of handling, but returned to baseline after 60 min (Ricciardella and Woodley, unpublished data). Plasma CORT was measured in samples. The intra-assay coefficient of variation for CORT was 7%. CORT recovery was 82%. CORT data were analyzed with two-tailed t-tests. Results were considered to be statistically significant when P ≤ 0.05.

3. Results

3.1. Experiment 1

Time from the final application of chemosensory stimuli to blood collection was not a significant covariate for plasma testosterone levels (F1,18 = 0.054, P = 0.82). Plasma testosterone levels did not vary among groups of males exposed to mental gland extract, female skin secretions, or a PBS control (Table 1: F2,19 = 0.361, P = 0.70). Plasma estradiol levels did not vary among groups of reproductive females (Table 1: $\chi^2_{2} = 0.31, P = 0.86$) or nonreproductive females (Table 1: $\chi^2_{2} = 4.8, P = 0.09$) that had been exposed to mental gland extract, female skin secretions, or a PBS control.

In males, time from final application of chemosensory stimulus to blood collection was negatively correlated with plasma CORT ($r = -0.485, P = 0.019, n = 24$) and was a significant covariate when analyzing CORT in males (F1,19 = 11.8, P = 0.003). Therefore, an ANOVA was conducted that included time from final application to blood collection as a covariate in the ANOVA. Plasma CORT levels varied among males exposed to different chemosensory cues (Fig. 1; main effect of chemosensory cue: F2,19 = 5.18, P = 0.016). Males exposed to mental gland extract had significantly higher CORT (56% higher) as compared to males exposed to the PBS control (P = 0.019).

In females, time from the final application of chemosensory stimulus to blood collection was not a significant covariate in analyses of CORT in reproductive (F1,15 = 0.321, P = 0.58) or nonreproductive salamanders (F1,14 = 1.1, P = 0.31). Plasma levels of CORT did not vary in reproductive or nonreproductive females that were exposed to mental gland extract, female skin secretions, or a PBS control (Fig. 1; F2,19 = 0.39, P = 0.69 for reproductive females; F2,18 = 0.021, P = 0.98 for nonreproductive females).

3.2. Experiment 2

Plasma CORT was higher in males exposed to mental gland extract, as compared to males exposed to the PBS control (a 45% increase relative to the PBS control) (Fig. 2; t18 = −2.13, P = 0.047). Plasma CORT levels did not differ between reproductive females exposed to mental gland extract versus the PBS control (t15 = −2.03, P = 0.061).

3.3. Experiment 3

Males that were removed from their home boxes and handled for 30 min had significantly higher levels of plasma CORT (81% increase from baseline) than did males sampled immediately upon removal from their home boxes (Fig. 3; t16 = −3.82, P = 0.002).

Table 1

<table>
<thead>
<tr>
<th>Chemosensory stimulus</th>
<th>Males (testosterone)</th>
<th>Reprod. females (estradiol)</th>
<th>Nonreprod. females (estradiol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS control</td>
<td>53.6 ± 24.3</td>
<td>51.1 ± 15.7</td>
<td>115.6 ± 61.3</td>
</tr>
<tr>
<td>Female skin secretions</td>
<td>29.84 ± 14.0</td>
<td>94.2 ± 35.4</td>
<td>3.6 ± 1.8</td>
</tr>
<tr>
<td>Mental gland Extract</td>
<td>26.74 ± 7.8</td>
<td>63.7 ± 12.6</td>
<td>4.8 ± 1.8</td>
</tr>
</tbody>
</table>

Fig. 1. Plasma corticosterone concentrations in male and female salamanders after experimental application of a PBS control, female skin secretions, and male mental gland extract. Sample sizes are indicated in bars. * Indicates P = 0.019 between males exposed to the PBS control versus mental gland extract. Bonferroni-corrected multiple comparison tests.
In males, but not females, there was a negative correlation between the plasma CORT level and the time from final application of the chemosensory stimulus to blood collection. Thus, the application of chemosensory stimuli may have elicited a slight stress response that was waning by the time blood was collected. Interestingly, plasma CORT levels measured in the PBS controls in experiments 1 and 2 were higher than the baseline level of CORT measured in experiment 3, also suggesting that the application procedure may have elicited a slight stress response. However, after correcting for variation in the time to blood collection, a subsequent statistical analysis revealed that CORT was elevated in males that had received mental gland extract. This hormonal response to a natural pheromone has not previously been reported for amphibians.

Although previous studies demonstrated that mental gland pheromones elicited a behavioral response in females, it was not clear whether mental gland pheromones would have an effect in males. At the level of sensory detection, previous studies showed that mental gland pheromones activated sensory neurons of the vomeronasal organ in males (Schubert et al., 2006, 2008). Our current experimental results indicate that the VNO apparently provides the initial neural stimulus leading to increased plasma CORT in males exposed to mental gland extract (but not in males exposed to female skin secretions or to a PBS control). We propose two possibilities for how mental gland extract might function in males. First, feedback from a male’s own mental gland pheromones might contribute to persistent courtship behavior by a male. Second, the ventral location of the mental gland on the chin suggests that a male possibly could scent mark an area by touching its mental gland to the substrate.

Acute increases in CORT, such as those induced by stressors, are thought to mediate trade-offs between survival and reproduction (Breuner et al., 2008). Stress-induced elevation of CORT, for example, rapidly suppressed mating behavior in male rough-skinned newts (Moore and Miller, 1984). However, short-term elevations in CORT may support breeding activities via CORT’s ability to mobilize energy reserves. For example, plasma CORT was positively associated with courtship and mating in male toads and newts (Harvey et al., 1997; Orchinik et al., 1988; Zerani and Gobbetti, 1993). Thus, it is possible that the elevated CORT in male P. shermani exposed to mental gland pheromones might function to energetically sustain a male’s efforts during the relatively lengthy terrestrial courtship.

In some vertebrate species, CORT levels are correlated with increased activity (Breuner et al., 1998; Overli et al., 2002). In the P. shermani salamanders, expression of chemosensory and scent marking behaviors are correlated with activity (Schubert et al., 2008). Future studies should examine the effects of elevated plasma CORT on activity and other behaviors in P. shermani.

The amphibian hypothalamic–pituitary–interrenal axis appears to be sensitive to socially relevant sensory input. Although we focused on hormonal responses to chemosensory cues, other studies have shown that socially relevant acoustic stimuli altered plasma levels of CORT. In male treefrogs (Hyla cinerea), several days of exposure to the sounds of a mating chorus resulted in elevated CORT (and androgens) as compared to exposure to an array of tones or no tones at all (Burmeister and Wilczynski, 2000). Calling behavior and plasma CORT levels were not correlated within individuals, suggesting that the perception of social cues elicited the increase in steroid hormones, independent of the increase in calling behavior. The function of the elevated CORT in response to the sounds of the mating chorus is unclear. However, injections of CORT inhibited calling briefly in free-living bufonid toads (Leary et al., 2006), indicating that social modulation of CORT can have important behavioral outcomes.
In terms of female responses, some evidence indicates that estradiol levels contribute to female receptivity in amphibians (Kelley, 1982). In female rough-skinned newts, plasma estradiol increased within minutes to hours after mating with a male (Propper and Moore, 1991). We found no effect of mental gland pheromones on estradiol levels in female _P. shermani_, however, even though mental gland pheromones increased female receptivity to mating (Houck et al., 1998; Rollmann et al., 1999, 2003). Additional cues associated with mating, such as visual and tactile stimuli, may be required for mental gland pheromones to alter female receptivity. Future studies could test this possibility by measuring estradiol levels in females that have been courted by a male.

To conclude, we provide here the first evidence that chemosensory cues have a priming effect on the endocrine system in an amphibian. Specifically, stimulation from mental gland courtship pheromones resulted in elevated plasma CORT in male salamanders. These data, in combination with data from studies examining acoustic inputs in anurans, indicate that the amphibian endocrine axes are sensitive to social signals. The next challenge is to determine the functional consequences of social modulation of plasma CORT.

Acknowledgments

We thank the Directors of the Highlands Biological Field Station for use of their facilities. Financial support was provided by Duquesne University (SKW), the Highlands Foundation Bruce Fellowship (S.N.S. and C.L.W.), NSF I08 0416724 (L.D.H.), and NSF I08-0416834 (R.C.F., P.W.F.).

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