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Dermal Application of Courtship Pheromones Does Not Influence Receptivity in Female Red-Legged Salamanders (Plethodon shermani)

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Abstract.—During courtship, adult male plethodontid salamanders transfer protein pheromones that augment female receptivity. The majority of plethodontid species apply pheromones transdermally (to the female’s dorsal skin). These pheromones apparently diffuse through the skin and enter into circulation, a unique type of pheromone delivery in vertebrates. In contrast, a behavioral and physiological transition occurred in the Plethodontidae, resulting in one clade of species (Plethodon glutinosus group) that uses a different pheromone delivery mode. Males within this clade apply pheromones directly to the nares of a female, and the pheromones subsequently are detected by the vomeronasal organ. We tested whether female Red-Legged Salamanders (Plethodon shermani), despite normally detecting pheromones via the vomeronasal organ, have retained the ancestral ability to respond to pheromones delivered transdermally. We staged courtship encounters and recorded the behavior of females given either pheromones or control solutions on the dorsal skin. Female receptivity (as inferred from courtship duration) in P. shermani was not affected by dermal application. Also, we used immunocytochemistry on female vomeronasal neurons to show that (1) females responded normally to pheromones delivered to the nares and (2) dermal application of pheromones did not activate vomeronasal neurons. We conclude that female P. shermani are not stimulated by pheromones delivered dermally and infer that this condition may characterize all the members of the P. glutinosus group, which use olfactory pheromone delivery.

Communication between animals requires the successful production and transmission of signals, as well as the receiver’s ability to respond via functional neural pathways. As signaling systems evolve, receivers and their neural pathways may become either more or less attuned to certain signals (Johnstone, 1997). Over time, an existing signal pathway may become redundant, or a new pathway may replace the existing one, depending on whether animals extract information from one or both pathways (Endler and Basolo, 1998). Multiple signaling pathways may be a unique feature of pheromone delivery in some plethodontid salamanders. These salamanders communicate with a potential mate via protein pheromones produced in the male’s mental gland, a specialized gland located on the chin (Arnold, 1977; Houck and Arnold, 2003). Among plethodontids, two dramatically different behaviors are used by males to deliver these mental gland pheromones during courtship. Depending on the species, a male will use one of two types of behaviors to deliver pheromones: either via “transdermal” or “olfactory” delivery (cf. Arnold, 1972, 1977), each of which appears to stimulate different sensory pathways in females.

In the majority of plethodontid species, a male rubs his mental gland over the female’s dorsum during courtship, often simultaneously exhibiting “snapping,” “biting,” or “pulling” behaviors (Noble, 1929; Arnold, 1977). These behaviors all entail a male using his elongated premaxillary teeth to scratch the female’s dorsum immediately after he rubs his gland on her skin; thus, this transdermal delivery mode has earlier been termed “scratching” (Arnold, 1977). Scratching behaviors have been observed in many genera of plethodontids (e.g., Desmognathus, Eurycea, Plethodon; reviewed in Salthe, 1967), and this transdermal delivery is clearly the ancestral mode of pheromone delivery in this family (Fig. 1; Houck and Sever, 1994; Houck and Arnold, 2003; Dyal, 2006). Experimental application of pheromones to the dorsum of females of species that deliver pheromones transdermally has shown that (1) courtship duration was significantly shorter for pairs in which the female received the pheromone (compared to those that received saline controls) and (2) the experimental protocol of simply mimicking pheromone delivery to the dorsum (without scratching) was sufficient to increase female receptivity (Houck and Reagan, 1990; Houck et al., 2008b). This result supports the premise that the pheromones diffuse through the skin and enter the female’s superficial circulatory system (Organ, 1961; Houck and Reagan, 1990).

In contrast, one monophyletic group of plethodontids (the Plethodon glutinosus group; Fig. 1) has evolved a second type of pheromone delivery behavior. In this olfactory delivery mode, courtship pheromones are delivered by the application of the male’s mental gland to the female’s nares (Arnold, 1976). From the nares, the pheromones enter the female’s nasal cavity, stimulate sensory neurons of the vomeronasal organ, and activate the accessory olfactory system (Wirsig-Wiewmann et al., 2002; Laberge et al., 2008), resulting in courtships that are more rapid (Rollmann et al., 1999, 2003; Houck et al., 2008a).

The plethodontid courtship pheromone system provides an avenue to test multiple questions regarding signaling system evolution. This study investigated whether (1) male plethodontid salamanders in the olfactory clade use mental gland secretions as redundant signals (which act on a female via both transdermal and olfactory stimulatory pathways) or (2) the olfactory group of plethodontids has lost the function of the transdermal pathway. Behavioral evidence suggests that transdermal delivery of pheromones may be used by males in the olfactory clade as a redundant signal. Males throughout the family Plethodontidae exhibit a “head rubbing” behavior (distinct from scratching behaviors) in which the mental gland is rubbed on various areas of the female body, including the dorsum (Arnold, 1972). Head rubbing has been observed in species in the olfactory clade of Plethodon that do not show any scratching behaviors characteristic of transdermal delivery (Organ, 1958; Arnold, 1976; Marvin and Hutchison, 1996) and may be a type of pheromone delivery behavior.

Also, the presence of the same pheromone proteins in both transdermal and olfactory delivery species suggests that males of olfactory delivery species may be able to stimulate females via diffusion through the skin. In the Red-Legged Salamander (Plethodon shermani), an olfactory species, the pheromones are multicomponent; at least two proteins act to affect female receptivity: Plethodontid Receptivity Factor (PRF) and Plethodontid Modulating Factor (PMF) (Rollmann et al., 1999; Houck et al., 2007). Preliminary proteomic analyses indicate the
FIG. 1. Cladogram showing the relationships of particular clades of plethodontid salamanders and their general types of pheromone delivery. Representative taxa are listed for each species group (High-ton, 1962); others species have been omitted for simplicity. Phylogenetic relationships are based on those described by Weins et al. (2006).

The presence of appreciable quantities of PRF in the gland secretions of a Plethodon species that uses transdermal pheromone delivery (Eastern Redbacked Salamander, Plethodon cinerius; PWF and RCF, unpubl. data). Because pheromones are often highly expressed in mental glands (Kiemnec-Tyburczy et al., 2009), the presence of PRF suggests that PRF may act as a transdermal pheromone, although behavioral data are needed to confirm these effects. These data suggest that head rubbing may be another way for species to deliver mental gland pheromones during courtship. To test this hypothesis, we experimentally manipulated pheromone delivery in an olfactory species, P. shermani.

MATERIALS AND METHODS

Behavioral Trials.—Adult female and male P. shermani were collected from Macon County, North Carolina (035°10′48″N 083°33′38″W) during August 2006. The salamanders were housed at Oregon State University for the duration of the behavioral trials, which were staged on 10 nights during 7–24 September 2006. Each trial night, 35–40 male–female pairs were given the opportunity to mate. Animal care followed the protocols of Houck et al. (1998). Animals were housed individually, experienced a natural photoperiod, and fed waxworm larvae (Galleria mellonella) weekly. In addition, to ensure that females only received pheromones experimentally delivered by the researchers, we anesthetized each male to be used in the courtship trials and surgically ablated its mental gland (for methods, see Houck et al. 1998). Deglanded males were given at least two weeks to recover before being used in behavioral trials. Males fully recover and court normally after this procedure (LDH, unpubl. data).

Before the behavioral trials began, males and females were prescreened to determine their propensity to mate in the laboratory, as described in Houck et al. (2008a). Once reproductively active animals had been identified, they were assigned randomly to different male–female pairs. Pairs remained matched in the trials until they had mated a single time. Thus, individual salamanders were used only once in the data set analyzed for behavioral trials.

The experimental protocol used in the behavioral trials was a slightly modified version of previously described protocols that revealed a female response to pheromones in Desmognathus ocoee, a species in which males use transdermal delivery of courtship pheromones (Houck et al., 2008b). Each female was placed in a courtship box (9 × 17 × 30 cm) with a damp paper towel substrate before the male was introduced. A 2 × 4 mm piece of low-protein-binding filter paper (Whatman glass microfiber filter) was held with fine forceps and 5 μl of one of two treatments was pipetted onto the paper. This paper “patch” was then placed on the female’s dorsum, between the forelimbs (for a more detailed description, see fig. 20.2 in Houck et al., 2008b). A female received a filter paper patch containing either (1) 6 μg/μl male pheromone (in 0.5 × PBS = phosphate buffered saline) extracted from the mental gland using standard procedures (Houck et al., 1998), or (2) a control saline solution (0.5 × PBS). Each small filter paper patch held the treatment solutions in place during the entire observation period. The exact diffusion rate of the pheromones is not known, but previous assays suggest that the patches allow the solutions to diffuse slowly into the female’s circulatory system in an effective manner (Houck et al., 2008b). We chose not to experimentally mimic the typical scratching behaviors associated with transdermal delivery because Houck et al. (2008b) showed that scratching was not necessary to elicit a behavioral response to patch-delivered pheromones in D. ocoee (and might be disruptive to the courting animals). The observers of male and female courtship behaviors were blind as to which treatment was placed on a given female. The treatments were randomized, except that half of the females received pheromone and half received the saline control during each night of courtship observations.

The filter paper rectangle remained on a female’s back for 15–20 min before a single male was introduced to that female’s box. The rectangles then remained in place for a 3-h observation period. The 3-h courtship periods were used to standardize the duration of male–female interactions across trial nights and to provide the animals enough time to court to completion. The trials were staged during the time of night when the animals normally would be found courting in the field (2200–0100 h EST; LDH, unpubl. data). Behavioral observations took place under dim red light and commenced as soon as a male had been placed in each box. During each trial night, we recorded courtship behaviors using instantaneous scan sampling and focal animal sampling (Altmann, 1974). For all pairs (pheromone- and saline-treated), we recorded (1) the time when courtship was initiated, defined as when the female first entered a tail-straddling walk (a distinct courtship behavior characteristic of all plethodontids; Arnold, 1972); (2) each attempt by a male to deliver pheromones to the female by touching his chin to the female’s nares; (3) the completion of courtship, defined as the time when the male deposited a spermatophore; and (4) whether the female was inseminated. We chose these behaviors (described in detail by Arnold, 1976) because these actions were unambiguous, scored by all observers, and closely reflect the duration of each courtship.

Data on the mean duration of courtship were analyzed using a one-tailed t-test with $z = 0.05$. We used a one-tailed test based on results from several prior behavioral experiments showing that pheromones reduced courtship duration significantly (Rollmann et al., 1999, 2003; Houck et al., 2008a). Given these past results, our prediction was that pheromone delivery would decrease average courtship duration by 15–20% for pairs in which the female was treated with pheromones. Data on the average number of times the male attempted to apply pheromone to the female’s nares (males attempted pheromone delivery even though each had been deglanded) were analyzed using a two-tailed t-test with $z = 0.05$ considered significant. In P. shermani, males slap almost exclusively during tail-straddling walk; thus, our counts during tail-straddling walk capture almost all of the male delivery attempts during the entire courtship. Normalities of the courtship duration and pheromone delivery attempt data were assessed by examining frequency histograms, variance, and residuals before the analyses were conducted. An association between insemination (yes or no) and pheromone treatment (pheromone or saline) was tested using a 2 × 2 contingency table with Chi-square test with the Yates correction. We did not expect number of pheromone delivery attempts or insemination success to vary between treatment groups (based on previous experiments) but
considered these variables to be indicators of normal courtships across treatment groups.

**Immunocytochemistry.**—Our experimental delivery of pheromones to the female’s dorsal skin might have resulted in the undesired flow of pheromones across the surface of the skin to the female’s head and then into the nasal cavities. Also, our goal was to ensure that male pheromones applied transdermally to a female did not result in activation of the same sensory target as olfactory delivery mode: the sensory neurons of the vomeronasal organ (e.g., by spreading across the skin from the dorsum to the nares). Thus, the immunocytochemistry would determine whether the method of experimental delivery truly delivered the pheromones only to the transdermal pathway in females. For the immunocytochemistry, we used 15 adult female *P. shermani* that were collected from the same locality as were the females in the behavioral trials. Each treatment group experimentally received one of three different combinations of male pheromones or saline control (0.5 × PBS) to the dorsal skin or to the nares. The three treatments were (1) saline to the nares and saline to the dorsal skin (N = 5), (2) saline to the nares and male pheromones to the dorsal skin (N = 5), and (3) male pheromones to the nares and saline to the dorsal skin (N = 5).

The method of agmatine uptake was used to examine whether pheromones stimulated neurons in the vomeronasal organ. Agmatine is a guanidine analogue that, when codelivered to the vomeronasal organ with a chemosensory stimulus, enters activated sensory neurons. Vomeronasal sensory neurons that have taken up agmatine can be visualized with standard immunocytochemical methods. The method of agmatine uptake has been described previously and was used to show that male pheromones from the mental gland activated vomeronasal sensory neurons of female *P. shermani* (Wirsig-Wiechmann et al., 2002, 2006; Schubert et al., 2008).

To deliver chemosensory stimuli to a female via diffusion through the dorsal skin, a 2 × 4 mm piece of the low-protein-binding filter paper was placed on the dorsum of each female salamander at the level of the forelimbs, as in the behavioral trials. Either 1 μl of male pheromone (10 μg/μl concentration) or a control saline solution was applied to the filter paper three times with an interval of 10 min between applications. This amount (30 μg total) equaled the total amount placed on each patch during the behavioral experiment.

Chemosensory stimuli (pheromones and saline control) were mixed 1:1 with a 6 mM solution of agmatine (dissolved in PBS) and applied to the nares. To deliver pheromones to the nares, 2 μl of either male pheromone (1.5 μg/μl concentration) or saline were delivered to the nares of the female using a micropipette every 2 min for a total of 21 applications per female. The pheromone and control (PBS) solutions contained 3 mM of agmatine. After the final application of the agmatine solution, an application of 3 × 5 μl PBS followed to rinse away any excess agmatine.

A female was sacrificed via decapitation 45 min after the first application of the chemosensory stimulus to her back. The upper jaw was fixed overnight in 4% paraformaldehyde-2.5% glutaraldehyde, pH 7.4, decalcified in DeCal (DeCal Corporation) for 2 days, and cryoprotected in 30% sucrose in PBS. Upper jaws were embedded in Tissue-Tek Optical Cutting Temperature Compound (Fisher Scientific) and sectioned at 20 μm using a cryostat and mounted on polylysine-coated superfrost plus slides. Every fourth section underwent immunocytochemistry for agmatine (following the methods of Schubert et al., 2006, 2008). Cells with darkly stained cytoplasms were considered to be agmatine-immunoreactive (AGB-IR) and were counted. The numbers of AGB-IR cells in the vomeronasal organ in both the left and right nasal cavities were summed to give the total number of AGB-IR cells. The person counting the cells was blind to the treatment of each animal.

The number of AGB-IR cells was log transformed such that error variances were homogeneous and the data were distributed normally. The number of AGB-IR cells were analyzed with a one-way ANOVA followed by Student-Newman-Keuls post hoc pairwise comparison tests.

**RESULTS**

We obtained behavioral data for 89 courtship encounters (of ~360 male–female encounters) in which each female had a treatment delivered to her dorsum: 47 in which the female was treated with pheromone, and 42 in which the female was treated with the saline control. Although we predicted a decrease in courtship duration in the pheromone-treated behavioral group, no difference in courtship duration was found between the two treatment groups (t<sub>WS</sub> = 2.02, P = 0.13; Fig. 2). The mean duration was 38.7 ± 3.4 min for the saline-treated group and 36.2 ± 3.6 min for the pheromone-treated group. For both groups combined, the overall mean insemination success was 88% and did not differ between groups (χ²<sub>2</sub> = 0.04, P = 0.84), nor did the number of times a male attempted the olfactory delivery of pheromones (t<sub>WS</sub> = 0.91, P = 0.36).

In the immunocytochemistry experiment, treatment groups differed significantly in the number of activated vomeronasal sensory neurons (F<sub>2,14</sub> = 17.2, P < 0.001). More vomeronasal cells were activated by delivery of male pheromones to the nares than by delivery of the saline control to the nares (Fig. 3). Application of male pheromone to a female’s dorsum did not activate significantly more vomeronasal sensory neurons than did the application of the saline control to the dorsum (Fig. 3). The immunocytochemistry results confirmed that the females we collected that season could respond normally to stimulation of the vomeronasal organ.

![Figure 2](image-url)
female receptivity or at smaller cost to male fitness (Palmer et al., 2007).

Another explanation for the lack of female response is that there has been a change in the female receivers. Pheromones delivered via transdermal delivery presumably target the brain via different mechanisms than do those that stimulate through the accessory olfactory system. However, the pheromones that diffuse into the skin may target organs other than the brain. Whatever the target organ, females may have lost their sensitivity to pheromones in those particular tissues. Male P. shermani (and males of other species) may have retained the head rubbing behavior because this contact provides tactile stimulation during courtship (Beachy, 1997).

Our work illustrates that signaling pathways can be dynamic over evolutionary time. Interplay between the multicomponent courtship pheromones, variable male delivery behaviors and female physiological targets likely provided many opportunities for species-specific signals to evolve. In fact, changes in chemical signals may have played a role in speciation and sexual isolation within the genus Plethodon (Weins et al., 2006). Further characterization of the chemical signals used in other social interactions may indicate whether major transitions—such as those we just described for the courtship pheromones—are a common feature of salamander chemical communication.

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LITERATURE CITED


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