A new vertebrate courtship pheromone, PMF, affects female receptivity in a terrestrial salamander


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Vertebrate pheromones that affect female receptivity have been documented only in salamanders. These courtship pheromones have been investigated most intensively in plethodontid salamanders. The source of the plethodontid courtship pheromone is the male's submandibular (mental) gland, which produces a multiprotein secretion. In earlier work with our main study species, *Plethodon shermani* (the red-legged salamander), an extract of protein secretions obtained from male mental glands acted to increase sexual receptivity in females. In addition, one particular protein in the gland secretion, plethodontid receptivity factor (PRF), could act alone to increase female receptivity. We now report that a second protein, termed 'plethodontid modulating factor' (PMF), acts oppositely to reduce female receptivity. The natural courtship pheromone blend thus includes two proteins that separately produce opposing messages, even though the combined effect of both proteins is to increase female receptivity.

Keywords: *Plethodon shermani*; plethodontid courtship pheromone; PMF; plethodontid receptivity/modulating factors; red-legged salamander; vertebrate chemical communication during courtship and mating

Pheromones are chemical signals that can mediate interactions between individuals of the same species. Reproductive pheromones can allow an animal to identify the species and sex of another individual, or help to coordinate a male and female during mating (Birch & Haynes 1982; Albone 1984; Johnston 2000; Luo et al. 2003). Reproductive pheromones also can accomplish sexual persuasion. In particular, courtship pheromones are male chemical signals that increase the likelihood that a given male will persuade a female to mate (Houck & Reagan 1990; Rollmann et al. 1999, 2003). These chemical signals differ from sex attractants in that courtship pheromones function only after a potential mate has been located and identified, and after preliminary courtship interactions have been initiated (Houck 1986).

Among vertebrates, the use of courtship pheromones to increase male insemination success is unique to salamanders. In aquatically breeding salamanders, male courtship pheromones often are released from the cloaca and delivered to the female via a water current that the male creates by tail fanning (Halliday 1975; Houck & Arnold 2003). In this type of delivery, courtship pheromones may be detected by nontarget receivers (cf. Kikuyama et al. 1995), including rival males. In contrast, courtship pheromones in plethodontid salamanders essentially are private signals: the male delivers the pheromone by bringing his submandibular (mental) gland in direct contact with the female. In these species, secretions from the mental gland are not delivered to any other animal and are never placed on the substrate (L. D. Houck, personal observations). Thus, the only function known for these salamander courtship pheromones is to increase female receptivity (cf. Houck & Reagan 1990; Houck et al. 1998).

One of the best documented cases of courtship pheromone effects is for *Plethodon shermani*, the red-legged salamander. The mental gland secretion in *P. shermani* (as in other *Plethodon* spp.) contains several different proteins that are relatively easy to isolate and purify (Feldhoff et al. 1999; Rollmann et al. 2000). Behavioural tests showed that this multiprotein secretion is capable of increasing female receptivity (Houck et al. 1998): females...
treated with a purified extract of the mental gland secretions completed courtship significantly faster than did control females given a saline solution (Rollmann et al. 1999). In fact, a single protein produced by the male mental gland alone can elicit the same female response. This protein was termed plethodontid receptivity factor (PRF) by Rollmann et al. (1999) following behavioural trials involving courtship encounters between male–female pairs. In these staged courtship encounters, the mental gland had been removed from each male so that pheromone delivery could be controlled by the experimenters. For pairs in which the female received a solution containing purified PRF, the duration of courtship was shorter than that of pairs in which the female received only a saline control solution (Rollmann et al. 1999). Thus, PRF increased female receptivity, as judged by courtships that were completed more quickly.

The demonstrated effect of male courtship pheromones on female receptivity suggests that these pheromones can be powerfully influenced by sexual selection. Biochemical and molecular analyses have identified PRF as a protein (molecular weight = 22 kDa) structurally related to IL-6 type cytokines (Feldhoff et al. 1999; Rollmann et al. 1999; Watts et al. 2004). Moreover, PRF is prone to rapid evolution driven by positive selection, which corresponds with expectations of strong sexual selection acting on this protein pheromone (Watts et al. 2004; Palmer et al. 2005). This level of diversification is unusual compared with the great many protein families that are highly conserved.

We examined the secretions from P. shermani mental glands to see whether other common proteins also might function as courtship pheromones. Approximately 85% of the pheromone extract is made up of only two proteins (Feldhoff et al. 1999). One of these two proteins is PRF; the other differs considerably in that (1) it is much smaller than PRF, having a molecular weight of only 7 kDa (see Figure 2 in Feldhoff et al. 1999; Rollmann et al. 2000) and (2) it is structurally related to proteins of the snake toxin-like superfamily, whereas PRF is related to cytokines. In purified extracts of mental gland secretions obtained from males collected in different years, the 7 kDa protein always was expressed in a 2:1 ratio with PRF (Feldhoff et al. 1999). The stability of this ratio suggests that both molecules are important in chemical communication.

We focus here on the second common protein (7 kDa) of the P. shermani courtship signal. We staged courtship encounters between male–female pairs, and delivered a purified extract of the 7 kDa protein to one group of females while a second group of females received a saline control. We recorded courtship duration for all pairs to test the hypothesis that the 7 kDa protein, acting alone, also would increase female receptivity in P. shermani.

**METHODS**

**Choreography of Plethodon shermani Courtship**

Courtship of P. shermani occurs in several stages: (1) orientation: a courtship is initiated when a male locates and orients to a female; (2) contact: the male approaches and makes contact with the female, rubbing his head along her body and head; (3) tail-straddling walk: the female straddles the male’s tail and places her chin on the dorsal surface of the male’s tail. In this position, the pair moves forward in a tail-straddling walk. This is the persuasion phase of the courtship in that, if a female is not highly receptive, the male will turn back and deliver courtship pheromones to the female by slapping his mental gland directly across her nares (Arnold 1976); (4) sperm transfer: still in the tail-straddling position, the pair halts and the male deposits a spermatophore. A receptive female will stay with the male, straddling his tail, during the approximately 7 min required for the male to complete spermatophore deposition. The male then lifts his cloaca, leaving the spermatophore on the substrate, and the pair moves forward until the female’s cloaca is over the spermatophore. The female lowers her cloaca and lodges the sperm mass into the cloaca. The female then lifts her cloaca (leaving the gelatinous spermatophore base behind) and moves away from the male.

**Study Species: Collection, Maintenance, Prescreening**

Adult P. shermani (120 males, 120 females) were collected during the breeding season in 2002 from a single locality in Macon Co., North Carolina, U.S.A. (35°10′48″ N, 83°33′38″W). Some animals will not court under laboratory conditions, so we first prescreened the salamanders for courtship propensity. Males and females were transferred into clean plastic boxes (17 × 9 × 13 cm) lined with damp paper towels (one pair per box). Pairs were left together overnight. In the morning, we examined and scored each box for the presence or absence of an intact spermatophore (gelatinous base plus apical sperm mass) or a spermatophore base. The presence of a spermatophore or base indicated that the pair had courted during the night. Each salamander was returned to its maintenance box after scoring. Each male and female used for experimental behavioural trials had mated at least once during prescreening trials. Following the prescreening, animals were shipped to Oregon State University (OSU), Corvallis, U.S.A. where behavioural experiments were conducted. Each salamander was housed in a plastic shoebox (17 × 9 × 13 cm) with damp paper towels as the substrate and damp crumpled paper towels for refuges. Animals were kept at 15–18°C on a late-August North Carolina photoperiod and were fed waxworms (lepidopteran larvae) weekly. A North Carolina scientific collecting permit was obtained, and animals were cared for using a protocol approved by the Animal Care and Use Committee at OSU.

**Gland Removal and Pheromone Preparation**

Each male had its mental gland removed prior to experimental trials so that we could control pheromone delivery (cf. Rollmann et al. 1999). Males were anaesthetized in a mixture of 7% ether in water. Each male’s gland
was excised and placed in a solution of acetylcholine chloride (AChCl) for approximately 60 min. The gland solution was processed by centrifuging for 10 min (at 14,000×g), removing the supernatant and centrifuging the supernatant again for 10 min, then removing the supernatant and freezing at −80°C. The frozen pheromone solution represented gland secretions pooled from approximately 120 males. The frozen gland extract was shipped to R.C.F. and P.W.F. at the University of Louisville, Kentucky, U.S.A. for processing to obtain our test solution of purified 7 kDa proteins. Pooled gland extracts were filtered (0.2-μm non-protein-binding filter), and then applied to a Mono-Q column (FPLC HR 5/5; Pharmacia, Piscataway, New Jersey, U.S.A.) equilibrated at 50 mM Tris-HCl, pH 8.0. The column was then eluted (same buffer) at 1 ml/min using a NaCl gradient (5.0 mM of NaCl/min). The protein content of the solution was standardized to 0.5 μg/μl in 0.5× PBS so that protein concentration was consistent for all pheromone trials. This concentration was selected because it elicited female behavioural responses for each behavioural trial were thawed just before use. Aliquots were shipped to OSU for behavioural trials. Behavioural Experiments

Behavioural effects of the pheromone solution on female receptivity were investigated by applying either the pheromone solution or a saline control solution to the nares of each female that engaged in courtship. We then measured courtship duration for each pair in which the female received treatment (cf. Houck & Reagan 1990; Rollmann et al. 1999). For our purposes, courtship duration was defined as the time a pair spent in the characteristic tail-straddling walk prior to spermatophore deposition. We selected tail-straddling walk (as defined above) as the onset of our courtship measure because (1) there is no ambiguity in scoring the precise time when a female enters tail-straddling walk and (2) this is the primary time when the male delivers courtship pheromones to the female. Similarly, we defined the end of courtship as the moment when the male lifted his cloaca to reveal a deposited spermatophore (also a behaviour that can be scored unambiguously). During spermatophore deposition, a process that can take 5–10 min, the male remains stationary and is fully committed to the task. The male does not usually enter this phase of courtship unless the female is highly receptive, since a nonreceptive female is likely to walk away uninseminated. Therefore, the time that the pair spends in the persuasion phase of courtship is ultimately determined by the female’s level of receptivity (Arnold 1976).

We staged courtship encounters between male–female pairs (as described above for the prescreening trials) during nine trials between 11 September and 9 October. Pairs that did not court during initial trials were used again in subsequent trials. Pairs set up for courtship encounters multiple times had at least 3 days off between trial nights to maintain natural levels of receptivity. Mental glands had been removed from all males, so pheromone delivery was restricted to experimental presentation. Males easily recover from this simple surgery within a matter of days, and they were given at least 2 weeks to recover before they entered into courtship trials. Gland removal does not affect subsequent male courtship behaviour (L. D. Houck, personal observations). On a given trial night, 40 male–female pairs were placed in courtship boxes for observation, one pair per box. Once a pair was engaged in the tail-straddling walk, we observed that pair continuously. The first time the male turned back and contacted the female’s nares with his chin (a behaviour that normally would result in pheromone delivery; Arnold 1976), the female was given either the pheromone solution or the 0.5× phosphate-buffered saline control. We used a 10-μl pipette to deliver the first of three 5-μl drops to the female’s nares. We delivered a second drop after 10 min and a third drop after 20 min from the first delivery, for a total of 1.5 μg of the 7 kDa protein delivered over this time span. We recorded the duration of courtship for all pairs in which a female had received three drops. We also noted insemination success for each pair. Individuals in each treated pair were used only once.

RESULTS

In staged encounters between P. shermani females and deglanded males, there were 32 completed courtships (i.e. courtships in which the female received three treatment drops and the male deposited a spermatophore). In these 32 courtships, females in 16 pairs had been treated with the 7 kDa pheromone solution and females in 16 pairs had been treated with the saline control. Average courtship duration for pairs in which females received the pheromone solution was 59 min (range 27–91 min), and the average duration for pairs having saline-treated females was 48 min (range 31–70 min; see Fig. 1). Compared with the control, treatment with the pheromone solution resulted in a decrease in female receptivity, as judged by a longer courtship duration (t test: t = 2.31, P < 0.028). Thus, the 7 kDa pheromone treatment increased the duration of courtship by an average of 18%.

Insemination success did not vary between groups. In most pairs, the female was inseminated regardless of
treatment. This result is consistent with past observations of insemination success in similar courtship experiments in which some females received a pheromone solution and other females received a saline control (L. D. Houck, personal observations).

**DISCUSSION**

In normal courtship encounters, the proteinaceous pheromone delivered by male *P. shermani* during the persuasion phase of courtship significantly enhances female receptivity (Houck et al. 1998). A single component of the multiprotein mental gland secretion, PRF, also is capable of increasing female receptivity when delivered to the female (Rollmann et al. 1999). However, we now show that a second abundant pheromone protein (7 kDa), acting alone, actually reduces female receptivity. The females that received the 7 kDa pheromone protein spent significantly longer in the persuasion phase of courtship than did females given a saline (control) solution. We termed this 7 kDa protein ‘plethodontid modulating factor’ (PMF) to reflect its adjustment of female behavioural response (in comparison to responses to the complete gland extract and to PRF). We know of no other instance in which signal components (PRF and PMF) acting within the same sensory mode produce opposing behavioural effects when delivered individually.

The modulatory effects of PMF were unexpected, particularly since the protein concentration of PMF was the same as that of PRF when used in behavioural tests that demonstrated increased female response. Although some signalling molecules can have an inhibitory effect at high doses, the decrease in female receptivity following treatment with PMF is unlikely to be a result of overdose inhibition. Recent studies indicate that both PRF and PMF are capable of stimulating the female’s accessory olfactory system via the vomeronasal organ (VNO): receptor cells in the VNO of female *P. shermani* substantially and equivalently responded to both proteins when PRF and PMF were delivered individually (Wirsig-Wiechmann et al. 2002, 2006). The protein dose that the female received in these studies was much higher (40 μg of protein delivered over 45 min) than the amount delivered to females in the current behavioural trials. Furthermore, while VNO receptors are finely tuned to distinguish only a small subset of pheromone components, there is no evidence that VNO response is inhibited at high concentrations of natural compounds (Leinders-Zufall et al. 2000). Also, the VNO and accessory olfactory system are known to initiate reproductive responses in vertebrates (Halpern & Martinez-Marcos 2003; Luo et al. 2003). Thus, the accessory olfactory pathway of salamanders apparently mediates pheromone reception of nonvolatile proteinaceous cues. Given that PMF reduced female receptivity to the male, the accessory olfactory pathway probably transmits negative, as well as positive, stimuli.

The ‘redundant-signal’ hypothesis predicts that individual components of a multicomponent signal are similar in function and should elicit the same response in the female (Zuk et al. 1992; Møller & Pomiankowski 1993). Such redundancy clearly is not the case for PRF and PMF because the two pheromone proteins produce opposite effects on female behaviour. Instead, PMF normally may have an accessory role to the primary chemical cue, PRF (cf. Taylor et al. 2000; Hebets & Uetz 2000). If this were the case, however, PMF alone should not affect female behaviour and we would expect no difference in courtship duration between females receiving PMF and females given saline. Despite producing opposite effects when PRF and PMF act alone, response to the whole extract (which includes both PRF and PMF) results in an increase in female receptivity (Houck et al. 1998). This combined effect suggests some form of interaction between PRF and PMF, such that the effect of PMF alone (decreased female receptivity) is muted or eliminated in the presence of PRF. We speculate that PMF may have some sort of calming effect on the female, and that this effect both reduces the chance that the female will leave the male and also facilitates the effects of PRF. Whatever the interaction between PMF and PRF, the significance of this interaction is indicated by a fixed 2:1 molecular ratio of PMF to PRF that is consistent in all extracts from mental gland secretions (Feldhoff et al. 1999).

Having established differential effects of PMF and PRF, we ideally would explore the nature of this difference by staging additional tests that directly compare female behavioural response to PRF alone versus female response to the natural combination of PRF and PMF versus female response to the control. Unfortunately, the propensity of females to mate under laboratory conditions is not sufficiently high to allow more than two treatments per experiment (L. D. Houck, personal observations). We are investigating neural responses (e.g. stimulation of vomeronasal cells; Wirsig-Wiechmann et al. 2002) as a possible way to discern the roles of PMF when delivered alone versus in the presence of PRF.

The functional significance of courtship pheromone delivery in *P. shermani* is related to the mating system of this species. This system is non-resource-based, in that females only obtain sperm from the male. The mating season for *P. shermani* is lengthy (lasting several months), and a given female most likely courts and is inseminated only a few times (cf. Adams 2004). Moreover, during each season approximately half of the females do not have sufficient resources to yolk a clutch of ova. Thus, on any particular night during the mating season, highly receptive females are few. A male encountering a reluctant female may be successful, however, by delivering a courtship pheromone that can increase her receptivity (Houck 1986; Houck & Sever 1994). One might expect that differences in female receptivity also would lead to differences in insemination success, such that females treated with pheromones would be more likely to be inseminated. Our experimental protocol precludes an examination of this effect, however, as we chose to limit potential sources of variance by comparing only courtships that proceeded to the stage when the male deposited a spermatophore. At this point, the male presumably has judged that the female is highly receptive (cf. Arnold 1976) and, therefore, no difference in insemination success is expected. This similarity in insemination success underscores the fact
that courtship pheromones reduce courtship duration, but are not essential in persuading a female to mate.

Our behavioural investigation of a salamander pheromone has been complemented by evolutionary studies of how these pheromone proteins vary across the genus *Plethodon* (Watts et al. 2004; Palmer et al. 2005). Sexual selection appears to be a major force guiding evolutionary changes in this pheromone system. Environmental effects on signal transmission are minimized, for example, because the male brings the pheromone in direct contact with the female’s nares. Also, the pheromone is never deposited on the substrate or used in male–male interactions, so there are no unintended receivers in this system. One possible effect of courtship pheromones might be to stimulate oviposition (and ultimately use the current male’s sperm). This effect can be ruled out, however, because fertilization and oviposition occur weeks or months after the mating season has ended, and thus are decoupled from insemination. The evolution of salamander courtship pheromones, therefore, presumably is guided by female behavioural response (as mediated through the accessory olfactory pathway) that increases the possibility of insemination success.

The two behaviourally active protein components of the salamander male courtship pheromone, PRF and PMF, may be the only functional components of this pheromone. These two components account for the majority of the protein content of the gland extract. Also, many of the proteins in the crude pheromone extract that were identified using protein electrophoresis (feldhoff et al. 1999) are likely to be blood products or common dermal secretions that were included inadvertently during the extraction process. Aquatic-breeding amphibian species produce simple messages (single proteins) that function as potential sex attractants (kolbe et al. 1993; kikuyama et al. 1995; yamamoto et al. 2000). Plethodontid salamander courtship pheromones may also be substantially simpler in their basic composition than are the complex pheromones found in other vertebrates and in insects. Although extracts of insect pheromones typically contain multiple components, the actual pheromone signal usually is a strict combination of a very particular subset of these chemical components (birch & haynes 1982). In fact, pheromones from related insect species can be made up of identical compounds and effectively differ only in the precise ratio of particular components (greenfield 2002; wyatt 2003). A similar situation is found for mice (*Mus musculus*) in that two compounds (combined together in urine) both were essential for a functional male signal (novotny et al. 1999). The sex attractant pheromone produced by female garter snakes (*Thamnophis sirtalis*) also is composed of a blend of compounds (mason et al. 1990). Here, we show that the proteinaceous courtship pheromone of plethodontid salamanders, unlike the single-component sex attractants of other amphibian species, contains at least two active components. We plan to test whether additional proteins in the *Plethodon* pheromone extract also act as signal components. In the meantime, however, the interactions between PRF and PMF remain a novel example of the independent effect of PMF being altered in the presence of PRF.

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