Conspecific and heterospecific pheromone effects on female receptivity

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Courtship pheromones play an important role in salamander reproductive behaviour. In salamanders of the family Plethodontidae, males deliver specialized pheromones to females during courtship interactions. These courtship pheromones increase female receptivity and may be involved in mate discrimination. In order to test hypotheses related to mate discrimination, we staged courtship encounters between male–female *Plethodon shermani* pairs in which the female received pheromones obtained from either conspecific (*P. shermani*) or heterospecific (*P. yonahlossee* or *P. montanus*) males. Both conspecific and heterospecific pheromones increased female receptivity. Moreover, pheromones from both heterospecific species were as effective as the conspecific pheromone in increasing female receptivity in *P. shermani* females. Our results suggest that the courtship pheromone signal and function may be conserved across related species, with mate discrimination occurring before pheromone delivery.

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Organisms receive a wide range of sensory stimuli from their environment. These visual, auditory and olfactory signals can serve a variety of functions, such as species identification (Coyne & Oyama 1995; Inomata et al. 2000), predator avoidance (Brodie 1992, 1993) and mate attraction (Dussourd et al. 1991; Coyne et al. 1994). Signals that affect reproductive interactions, such as mate recognition or mate persuasion, may also be used in the assessment and selection of mates (Ryan & Rand 1993). Early studies focused either on the role of signals in species recognition (e.g. Lanier et al. 1980), or in mate choice (reviewed in Andersson 1994). More recently, however, studies have begun to investigate the continuum between species recognition and mate preference by looking at how a signal is used not only to discriminate among males within a species, but also in species recognition (Wiernasz & Kingsolver 1992; Boake et al. 1997; Ptacek 2000).

In salamanders, chemical signals play an important role in both species recognition and mate persuasion. Chemical signals are used, for example, in the family Plethodontidae for the identification of conspecifics (Dawley 1984, 1986; Uzendoski & Verrell 1993) and for the stimulation of females during courtship (Houck & Reagan 1990; Houck et al. 1998; Rollmann et al. 1999). The use of pheromones during courtship is common among plethodontid salamanders (Arnold 1977; Houck 1980; Houck & Sever 1994). In these terrestrial salamanders, the male produces courtship pheromones from a specialized submandibular (mental) gland that is hypertrophied only during the breeding season. Courtship pheromones are delivered almost exclusively during an intermediate courtship stage, termed tail-straddling walk (Arnold 1972, 1976). During the tail-straddling walk, the female straddles the male's tail and then moves forward in this position in tandem with the male (see Figure 1 in Rollmann et al. 2000). A pair must be in tail-straddling walk when a spermatophore is deposited to ensure sperm transfer. Following insemination, the pair typically separates and courtship interactions end.

Courtship pheromones increase female receptivity, as indicated by a decrease in courtship time (Houck & Reagan 1990; Houck et al. 1998; Rollmann et al. 1999). Increased female receptivity may increase the probability that the female will be inseminated by reducing the chances of the courtship being interrupted by rival males or predators. Furthermore, the level of female receptivity may be critical to successful sperm transfer. Following a period of tail-straddling walk, a female must remain

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stationary in the tail-straddling position during the 4–5 min required for spermatophore deposition. If a female is not sufficiently receptive and leaves tail-straddling walk, the male is committed to completing deposition even though the female will not obtain the sperm mass from this spermatophore (Houck & Reagan 1990). If courtship pheromones increase the probability of the female remaining with the male during spermatophore deposition, these pheromones would play an important role in increasing male mating success.

In plethodontid salamanders, courtship pheromones may also be used to discriminate between conspecific and heterospecific males. Courtship pheromones have the potential to be used for mate discrimination (both within and between species) even though these pheromones are delivered after male–female interactions are initiated (Arnold et al. 1993). Courtship in plethodontid salamanders progresses by stages and a female can disengage from a courtship at any time. Such female departures were observed for plethodontid species in which male–female encounters were staged between conspecifics from allopatric populations and between heterospecifics (*Desmognathus* spp., Verrell & Arnold 1989; *Plethodon* spp., Reagan 1992). Some of these departures occurred during the 'persuasion stage' of courtship when courtship pheromones were being delivered. This timing also would be consistent with a role for courtship pheromones in interspecific discrimination. Furthermore, intra- and interspecific chemical variability in courtship pheromones suggest that these chemical signals could be used by females to identify conspecifics. Populations of *P. shermani* and other *Plethodon* species differ significantly in the biochemical composition of their mental gland extracts, with glandular components varying in relative amount, as well as in the presence or absence of particular components (Rollmann et al. 2000). To the extent that courtship pheromones contribute to discrimination against males from different species, these pheromones may function in species recognition.

To investigate the role of courtship pheromones in mate discrimination, we observed behavioural responses of *P. shermani* females to courtship pheromones from conspecific males and from heterospecific males (*P. yonahlossee* or *P. montanus*). *Plethodon shermani* and *P. montanus* belong to the *P. jordani* complex, a group of seven species that are distributed as geographical isolates in the southern Appalachian mountains (Highton & Peabody 2000). These isolates are estimated to have been geographically separated for over 3 million years (R. Highton, personal communication). *Plethodon yonahlossee* is a member of the *P. glutinosus* complex and occurs sympatrically with *P. montanus* but with no interbreeding. Previous tests revealed sexual incompatibility (i.e. courtships breaking down prior to insemination) between *P. shermani* and the heterospecific *Plethodon* species tested (Arnold 1976; Reagan 1992). More specifically, behavioural tests of sexual isolation between the populations of *P. shermani* and *P. montanus* used in this study support the recent designation of these populations as separate species, in accordance with the biological species concept (Reagan 1992; Highton & Peabody 2000). In addition to their status as distinct species, *P. yonahlossee* and *P. montanus* were tested because these species are allopatric to *P. shermani* and represent different levels of phylogenetic divergence (Highton & Peabody 2000). Furthermore, significant differences in pheromone composition were observed between males in our study populations of *P. shermani* and *P. montanus* (Rollmann et al. 2000).

We staged courtship encounters between male–female pairs in which the male's mental gland had been ablated. We experimentally delivered pheromone or saline (control) treatments to females during courtship and recorded the time each pair spent in tail-straddling walk. We hypothesized that females would discriminate between the pheromonal cues of these plethodontid species and that conspecific pheromones would be more effective at reducing courtship duration. We also hypothesized that, for courtships that lead to spermatophore deposition, courtship duration would be longer for pairs in which no pheromonal stimulation was delivered to the female.

METHODS

Approximately 400 adult males and 400 gravid females of *P. shermani* were collected from a single locality in Macon County, North Carolina, U.S.A. (83°33′38″N, 35°10′49″W) for use in the behavioural experiment. *Plethodon yonahlossee* and *P. montanus* males were collected from McDowell County (82°11′35″N, 35°44′12″W) and Madison County, North Carolina (82°57′24″N, 35°47′50″W), respectively, to obtain heterospecific pheromone extract. These three species do not co-occur at any of the collection localities and no hybridization occurs between *P. yonahlossee* and *P. montanus* where these species are sympatric. Animals were housed individually in plastic boxes ($31 \times 17 \times 9$ cm) lined with damp paper towels and under a natural photoperiod.

Methods of gland removal, treatment solution preparation and experimental design follow Houck et al. (1998). These procedures are summarized below.

Following collection, we removed the mental gland from each male and prepared pheromone extracts. Within 1–2 days of gland removal, male *P. shermani* court (i.e. attempt to deliver pheromones; L. D. Houck, personal observation) and feed normally, but were allowed to recover for at least 1 week before being used in behavioural experiments.

We initially paired males and females in boxes to assess their tendency to mate in the laboratory. Salamanders were subsequently ranked according to their courtship propensities. Each group of salamanders used on a given experimental trial night was composed of individuals with a similar range of propensity ranks. We are aware that a bias might be introduced by selecting females having initially high levels of female receptivity. However, as courtship pheromones act to increase female receptivity, any bias would tend to minimize the probability of demonstrating courtship pheromone effects. Thus, this bioassay provides a conservative estimate of the effects of pheromones on female receptivity.

We tested the effects of heterospecific pheromones on female receptivity in two sequential experiments by staging encounters between male–female pairs of *P. shermani* on 16 trial nights during 9–27 September 1996. Experiment 1 tested the effects of either a pheromone solution (from *P. yonahlossee* or *P. shermani* males) or a saline (control) solution on courtship between male–female pairs of *P. shermani*. Similarly, experiment 2 tested the effects of *P. montanus* or *P. shermani* pheromones on courtship behaviour of *P. shermani* pairs. Because temporal differences may be associated with seasonal variation in female receptivity, we present data from each experiment separately. Male–female pairs were placed in individual courtship boxes at dusk. Each male lacked a mental gland and therefore pheromone delivery was controlled experimentally. For each pair, we administered a treatment solution immediately after the male's chin contacted the female's nares. We applied three 5-µl drops of pheromone or control solution 10 min apart to simulate natural pheromone delivery by the male during courtship. Although the quantity of pheromone delivered by the male during courtship is unknown, courtships lasting 30 min, as in our experiments, are within the normal range of courtship variation (Arnold 1972; L. D. Houck, personal observation). On a given trial night, we randomly determined treatment order (e.g. saline first, pheromone second), then repeated this order throughout the trial night (e.g. saline-treated pair first, pheromone-treated pair second, etc.).

We began focal animal sampling (Altmann 1974) when a pair began a tail-straddling walk. For pairs that courted, we defined courtship duration as beginning when the female first began the tail-straddling walk and ending with the completion of spermatophore deposition. We operationally defined an increase in female receptivity as a decrease in courtship time. Pairs that did not court on a given night were maintained in their individual boxes for 3 days before being used in a subsequent trial. The 3-day period between trial nights maintains the probability of courtship for plethodontid salamanders used for repeated observations (Verrell 1988; Houck & Verrell 1993). All subjects except those used for further molecular analyses ($N \approx 20$) were released at their capture sites following the experiment.

We made comparisons of courtship times between treatment and control groups using analysis of variance (ANOVA). Each salamander was used only in a single pair combination to ensure that the data were independent. A priori hypotheses (i.e. female behavioural response to saline differs from that of pheromone treatments; the behavioural response to the two pheromone treatments differ, and conspecific pheromones are more effective at altering female receptivity) were tested using a least significant difference post hoc test (Statistica 5.1; Statsoft 1996).

RESULTS

Experiment 1: *P. yonahlossee* Pheromone Effects

The experimental delivery of courtship pheromones decreased the average courtship time for *P. shermani* pairs
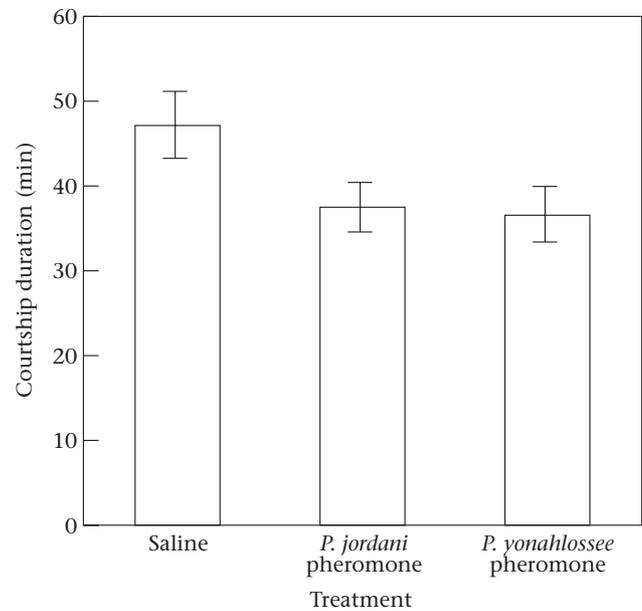
Figure 1. Differences in courtship duration (tail-straddling walk) for pairs in which a *P. shermani* female was treated with saline (control) solution, conspecific pheromone extract or heterospecific (*P. yonahlossee*) pheromone extract.

(i.e. time spent in tail-straddling walk; one-tailed ANOVA: $F_{2,59}=3.046$, $P \leq 0.028$). Pairs receiving either the conspecific or heterospecific pheromone showed a significant decrease of approximately 22% in average courtship time relative to the saline-treated pairs ($\bar{X} \pm SE$: saline: 47 ± 3.9 min, $N=21$; conspecific pheromone: 37 ± 2.9 min, $N=23$, $P \leq 0.040$; heterospecific pheromone: 36 ± 3.2 min, $N=18$, $P \leq 0.035$; Fig. 1). Pheromones from both conspecific and heterospecific males did not differ significantly in their effectiveness at reducing courtship time ($P \leq 0.850$; Fig. 1).

Experiment 2: *P. montanus* Pheromone Effects

There was no significant difference in mean $\pm$ SE courtship time for *P. shermani* pairs receiving either conspecific pheromone (42 ± 3.8 min, $N=12$) or *P. montanus* pheromone (41 ± 4.0 min, $N=18$; ANOVA: $F_{1,28}=0.03$, $P \leq 0.869$).

DISCUSSION

The courtship behaviour of female *P. shermani* was significantly affected by conspecific male pheromones. Female receptivity increased following exposure to an extract of mental gland pheromone from conspecific males, thus reducing overall courtship duration. Shorter courtships are assumed to be of benefit, because they reduce the probability that mating will be interrupted by rival males or predators. Furthermore, a female that is sufficiently receptive is also more likely to remain with a male until transfer of his sperm is complete, thus ensuring fertilization (cf. Houck & Reagan 1990).

Surprisingly, *P. shermani* females also were responsive to pheromones from congeneric *P. yonahlossee* and *P. montanus* males. This responsiveness occurred despite significant variation, not only in the courtship pheromone but in other extract components that may confer species-specific information. The extract of *Plethodon* mental glands is composed of approximately 20 protein components that differ in molecular weight (Feldhoff et al. 1999; Rollmann et al. 2000). We have identified at least one protein component, plethodontid receptivity factor (PRF), that alone can function to increase female receptivity (Rollmann et al. 1999). Multiple isoforms of PRF exist that differ in their amino acid sequences. In comparison to *P. shermani*, *P. montanus* glandular components (including PRF) differ significantly in their relative amounts, as well as in the presence and absence of components and PRF isoforms (Rollmann et al. 2000). Although mental gland extracts for *P. yonahlossee* have not yet been analysed as extensively, the relative amounts of individual components in mental gland extracts of *P. yonahlossee* do differ from those of *P. shermani* (unpublished data). In addition, the gene encoding PRF shows an average nucleotide sequence divergence of approximately 2.4% between *P. shermani* and *P. yonahlossee* (S. J. Arnold, personal communication). The relatively low divergence between these two *Plethodon* species does not, however, necessarily preclude significant functional differences in the effects of PRF.

Courtship pheromones represent only one of many concurrent signals provided by a male. In plethodontid salamanders, chemical cues that are most important in conveying information on species identity may be received before delivery of courtship pheromone. In several plethodontid species, chemical signals are used for sex discrimination (Jaeger & Gergits 1979) and species discrimination (Dawley 1984, 1986) and for advertising territory ownership (Jaeger et al. 1986; Mathis et al. 1995). Therefore, females may have multiple opportunities to determine species identity before delivery of male courtship pheromone. In addition to chemical stimulation, females also receive tactile and visual cues during courtship. If female *P. shermani* rely on multiple cues for mate discrimination, as has been observed in other systems (e.g. *Xiphophorus nigrensis* and *X. pygmaeus* swordtails: Crapon de Caprona & Ryan 1990; *Drosophila*: Greenspan & Ferveur 2000), a single incorrect cue may be insufficient to influence mate discrimination. Alternatively, females may discern differences between conspecific and heterospecific male courtship pheromones but effectively ignore these differences, given that females in the present study received all of the cues they would normally receive except for the courtship pheromones. In the *Plethodon* system, males not only differ in the chemical composition of the pheromone, but also in the rate and number of times pheromones are delivered to the female. These behaviours, separate from the pheromone itself, may be important in mate choice by the female.

Studies of female preferences for conspecific versus heterospecific signals have been documented for a variety of species. Few studies, however, have investigated behavioural responses to a heterospecific signal received in the absence of a corresponding conspecific signal (but see McLennan & Ryan 1997, 1999 and references therein). The response of *P. shermani* females to heterospecific pheromones suggests conservation of the male pheromone signal, as well as the female response. The conservation of signal and response suggests that courtship pheromones alone are not an effective mechanism for maintaining behavioural isolation in *Plethodon* species. Whether the behaviours associated with pheromone delivery play a role in discrimination against heterospecific males in the *Plethodon* system remains to be tested.

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