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Male courtship pheromones suppress female tendency to feed but not to flee in a plethodontid salamander

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Female sexual receptivity is a behaviour at the crux of mechanistic and evolutionary perspectives of reproductive behaviour. To gain insight into the general processes by which a male persuades a female to mate with him, we tested whether the courtship pheromones of the red-legged salamander, *Plethodon shermani*, dampened female defensive or ingestive behaviours. Females did not sprint significantly shorter distances to evade startling stimuli when experimentally treated with pheromone solution compared to a control. However, females did consume 25% fewer fly larvae when treated with pheromone compared to a control. The female's maintenance of normal defences suggests a behavioural state that is unresponsive or resistant to pheromone stimulation, but the change in feeding activity indicates that suppression of female hunger is beneficial to male mating success. Together, these results indicate that male courtship pheromones may augment female receptivity by modulating the expression of other competing or inhibitory motivated behaviours.

Sex pheromones are chemical signals that can draw potential mates together, coordinate the process of fertilization or insemination, or otherwise influence male–female mating interactions (reviewed in: Greenfield 2002; Wyatt 2003). In a marine polychaete (*Nereis succinea*), for example, the sexually mature female releases an aquatic pheromone along with her eggs and this pheromone results in the induction of sperm release by nearby males (Zeeck et al. 1998). In lepidopteran insects, the classic example of the silkworm moth *Bombyx mori* shows that bombykol, a volatile pheromone produced by a mature female, is exquisitely effective in attracting a male mate (reviewed in Agosta 1992). This coordination of male–female mating behaviours also is found in vertebrates, from amphibians to mammals. For instance, female aquatic newts (*Cynops pyrrhogaster*) that are ready to ovulate will urgently follow plumes of the male pheromone, sodefrin, to locate a nearby male in breeding condition (Kikuyama et al. 1995). Similarly, androgenic compounds in the frothy saliva of a sexually aroused male pig (*Sus scrofa*) emits a characteristic musky odour that facilitates the display of mating posture in a female pig in oestrus (Signoret 1970). What the above examples have in common is that mate attraction is occurring between females and males that are highly receptive and share a predisposition to mate.

In contrast with sex pheromones that function to coordinate individuals that already are inclined to mate, a distinct subset acts to augment sexual responsiveness in a female recipient. Since there is little advantage to making a female more receptive when a rival male could locate and sequester her, these pheromones are delivered during courtship so there is no general broadcast of this signal into the environment. These pheromones have been termed 'aphrodisiac pheromones' (Singer et al. 1986, 1987) or 'courtship pheromones' (Arnold & Houck 1982). Courtship pheromones are defined specifically as chemical signals that are (1) delivered by the male only after initial contact with a potential female mate, (2)

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delivered only if the female is not immediately responsive to the male's overtures and (3) produced by specialized glands that actively secrete during the breeding season (Arnold 1977; Houck & Sever 1994).

Behavioural responses to courtship pheromones have been well studied in terrestrial plethodontid (lungless) salamanders where enhancement of sexual receptivity has been measured by a shortened courtship duration (e.g. Houck & Reagan 1990; Rollmann et al. 1999; Houck et al. 2008). In our focal species, the red-legged salamander, *Plethodon shermani*, the courtship sequence is highly stereotyped: the male approaches and attempts to woo the female using an array of behaviours such as physical contact, foot dancing and tail arching (Arnold 1977). If the female is amenable, the pair enters into a 'tail-straddling walk' when the female steps over the male's tail and the pair walk together. During the tail-straddling walk, the male pauses periodically to deliver pheromones by tapping his mental (chin) gland on the female's nares (see Supplementary Material video, shermani slappi.avi). The male lacks an intromittent organ, and insemination occurs via the deposition of a spermatophore that an obliging female will straddle, lodging the sperm-filled cap in her cloaca (see Supplementary Material video, shermani transfer.avi). The effect of courtship pheromones in shortening courtship duration has been well documented; however, the behavioural mechanisms underlying a female's tendency to respond to and cooperate with a sexual partner are not yet known.

Given the importance of these pheromones in mediating courtship interactions, we turn from earlier behavioural studies to new experiments designed to elucidate the proximal mechanisms by which pheromones augment female receptivity. We propose that pheromones could enhance sexual receptivity indirectly by suppressing motivational forces that compete with or inhibit sexual motivation. Inherent in this notion is that while many motivations may be simultaneously manifested, an individual generally may not engage in simultaneous motivated activities (such as feeding and mating, except in the fortuitous case of nuptial gifts, e.g. Thornhill 1976). This incompatibility between mutually exclusive activities (Tinbergen 1952) is the basis for a situation in which behavioural subsystems (the combination of appetitive and executionary states that direct motivated behaviours) are in conflict for overt expression. This concept of incompatible behavioural subsystems is at the core of most theories of decision making in general (McFarland 1977; Enquist & Ghirlanda 2005) and for the theories of motivational competition and disinhibition in particular. These two theories are not mutually exclusive, and the scope of this study did not endeavour to distinguish between the two. In short, motivational competition posits that the behavioural subsystem with the strongest motivation is overtly expressed (Ludlow 1976); disinhibition posits that behavioural subsystems (mutually) inhibit each other, such that the expression of one behaviour is dependent upon the lack of suppression from the other behavioural subsystem(s) (McFarland 1969).

In the present study, we investigated this candidate mechanism to determine whether male reproductive pheromones could suppress the female's tendency to flee or feed. The three primary motivational forces are reproductive, defensive and ingestive, so any effect that subdues defence and ingestion could serve indirectly to enhance sexual receptivity (Swanson 2000). Since the male does not clasp the female during courtship interactions, she may leave the male at any time, and indeed, she frequently does. Often, this is attributable to the female being startled or distracted by environmental stimuli (L. D. Houck & E. A. Vaccaro, personal observations). Any mechanisms that dampen the female's aversion to alarming stimuli or weaken the potency of the female's drive to feed could focus female attention on the courting male, thereby increasing the chances for mating success. For this study, we compared the startle responses and feeding activity of female *P. shermani* salamanders with and without pheromone stimulation.

METHODS

Study Species Collection, Maintenance, Gland Removal and Prescreening

Male and female *P. shermani* were collected during the August 2008 mating season from a single locality in Macon County, North Carolina, U.S.A. We selected only females in reproductive condition as determined by the presence in the oviducts of mature oocytes (visible through the ventral skin). Animals were housed individually in plastic boxes ($31 \times 17 \times 9$ cm) lined with damp paper towels as substrate and crumpled moist paper towels as refuges. Animals were fed 10 fly larvae (*Calliphora vomitoria*, GrubCo, Hamilton, OH, U.S.A.) weekly. Shortly following salamander collection, we removed the mental glands from 8 to 13 anaesthetized males and prepared pheromone extracts for experimental treatments. Methods of gland removal and preparation of the treatment solution follow established protocols (Houck et al. 1998). Males were allowed to recover fully in the laboratory before being released at the collection site. Some animals will not court in the laboratory, so males and females were first prescreened to assess their tendency to mate under laboratory conditions. Each male–female pair was transferred to a clean plastic box lined with damp paper towels and left together overnight. In the morning we returned each animal to its home box, then examined and scored each box for the presence or absence of an intact spermatophore (gelatinous base plus a sperm mass) or a spermatophore base. The presence of an entire spermatophore or only the base indicated that the pair had courted during the night. Following prescreening, animals that had courted one or more times were shipped to Oregon State University (OSU), Corvallis, U.S.A. where behavioural experiments were conducted. Animals were kept in conditions similar to the field: 15–18 °C on a late August North Carolina photoperiod. North Carolina scientific collecting permits were obtained and animals were cared for using a protocol approved by the Animal Care and Use Committee at OSU (LAR 3549 to L.D.H.).

Experimental Design

Substantial intrinsic variability in the response to pheromone across subjects was expected, so we used a repeated measures design to enable greater precision and sensitivity in our estimates and to permit the use of a relatively small sample size ($N = 32 =$ four groups of eight females). We used a within-subjects crossover design in which each female salamander was observed under each treatment condition such that each female served as her own control. To greatly minimize the possibility of carryover effects from previous treatments, observations for each group of eight females were scheduled 6 days apart. The order of treatments was randomized.

Observational Arena

All behavioural trials were conducted in an observational arena consisting of an array of eight observation boxes ($245 \times 245 \times 20$ mm, Square BioAssay, Corning, Lowell, MA, U.S.A.). Each box was monitored by a dedicated high-resolution digital video camera (WiLife Indoor Surveillance Camera, Logitech, Fremont, CA, U.S.A.). Cameras were placed aperture downwards upon transparent glass shelves located about 20 cm above each observation box. Indirect illumination provided by four 60 W red

incandescent light bulbs (pointed away from the experimental arena) was sufficient to make video recordings. Digital video footage was routed to a pair of laptop computers for recording and later review (WiLife Indoor Master System, Logitech).

Startle Response

Pheromone effect on startle response was assessed by comparing the distance rapidly travelled in response to a probe poke to the base of the tail. Pretrials determined that poking elicited the most consistent evasive behaviour of the various alarming stimuli evaluated (e.g. vibrations, black cue cards presented in the visual field, compressed air puffs and pinches to the midsection or base of tail with forceps). Each female was tested (1) when treated with pheromone and (2) with control treatments. Observations were conducted during the normal nocturnal activity period for *P. shermani* females, from 2100 to 0000 hours Eastern Standard Time (EST). Observations were videorecorded for later scoring of the female response. One group of eight females was monitored per night, with two observers each monitoring four females at a time. Each female was allowed to acclimate for 120 min within an observation box lined with a moistened paper base printed with a 2×2 cm grid. Following acclimation, 4 μl of treatment substance (pheromone or saline control) was administered via pipette (Pipetman P10, Gilson, Inc., Middleton, WI, U.S.A.) in a randomized order: four females each received pheromone solution (extracted from pooled male mental glands) and four other females each received the saline control solution. To provide a startling stimulus, each female received a single tail poke with a dissecting probe every 20 min. After three pokes, a female was given 30 min of recovery time. Following the recovery period, we administered the alternate treatment (control or pheromone) to each female and repeated the series of tail pokes.

Using the video records for each female, we measured the distance the female travelled in response to probe pokes. For each poke, distance travelled was defined as the difference between the female's snout position before and immediately after receiving a poke. Distances travelled were usually short bursts in a straight line, so we measured the vector distance between the two points. Measurements from the video records were taken 3–4 weeks after the experiment was conducted. Thus, the person making the measurements was blind to the treatment order for each female.

Feeding Activity

Pheromone effect on feeding activity was assessed by comparing the number of fly larvae consumed following pheromone or control treatment. Observations were conducted from 2200 to 0730 hours EST. Four groups of eight females were tested each week for 4 weeks with a total of two replicates per treatment. Each female was placed in an observation box lined with a moistened paper substrate and allowed to acclimate for about 120 min. Following the acclimation period, 4 μl of treatment solution (pheromone or saline control) was administered as described above. Following treatment, 10 larvae were placed in the centre of the observation box. The next morning, each female was returned to her home box and given any remaining fly larvae from the previous night (to control for possible carryover effects on subsequent feeding activity trials). Preliminary experiments in which female feeding activity was monitored overnight by digital video camera showed that larvae were consumed only within the first hour of being placed in the observation box. During that hour, these prey were still moving and had not yet settled in the margins of the box. Accordingly, we tallied the number of the number of larvae consumed by the following morning as a measure of the amount eaten while under the effect of treatment (i.e. within the first hour following treatment).

Data Analysis

All statistical tests were performed using S-PLUS, version 8.0 (2007, TIBCO Spotfire, Palo Alto, CA, U.S.A.). We conducted our analyses using linear mixed effects (LME) models fitted by restricted maximum likelihood (REML) in which subject was treated as a random grouping (block) effect. Mixed modelling accounts for each subject as a potential source of asphericity (defined below) by including subject as a random effect (Hopkins 2000). Using mixed modelling thereby avoided potential issues in repeated measures ANOVA with violations of the sphericity assumption: sphericity, also known as circularity, is the condition of equality of variances for all pairwise differences between levels of the repeated measures factor (Crowder & Hand 1990; Davis 2002). The startle response analysis included a covariate designating the 'poke order' to account for changes in the response over successive pokes within a treatment period, and as an indicator for the order of treatment received (pheromone first or control first), to account for potential carryover pheromone effects since each female received both treatments on the same night. The feeding activity analysis included a covariate designating the week (1–4) to account for possible changes over the course of the 4-week experiment.

RESULTS

One of the female test subjects was later determined to be nongravid and so was removed from the study and subsequent analyses. The remaining sample size was 31 females.

Startle Response

Log transformation of the startle response measure was necessary. Pheromone treatment did not significantly affect female startle response, even after accounting for treatment order, poke number and subject random error (REML t test: $t_{158} = 1.47$, $P = 0.14$; Fig. 1). A linear time trend was evident: females sprinted significantly shorter distances with successive pokes ($t_{158} = -2.75$, $P = 0.007$). There was no evidence of a carryover effect of pheromone treatment ($t_{30} = 0.12$, $P = 0.90$) from the first session to the second session.

Feeding Activity

Some fly larvae avoided predation by seeking refuge in occasional wrinkles in the moistened paper base of the observation box; accordingly, we removed from the analysis individual trials for which four or more larvae hid under the paper base (15 observations removed, 104 observations remaining). Pheromone treatment affected female feeding activity (REML t test: $t_{68} = 2.36$, $P = 0.02$; Fig. 2). Females ate on average 25% fewer larvae when treated with pheromone (mean difference $\pm$ SE $= 0.73 \pm 0.31$) and these differences appeared to be driven by the groups of females demonstrating high levels of feeding activity in control conditions (Fig. 2, top two groups). There was no evidence of a week-to-week effect ($t_{68} = -0.25$, $P = 0.80$) during the 4 weeks of this experiment.

DISCUSSION

In *P. shermani* salamanders, male courtship pheromones did not significantly affect a measure of female defensive behaviour, but did suppress female feeding activity. This suppression was

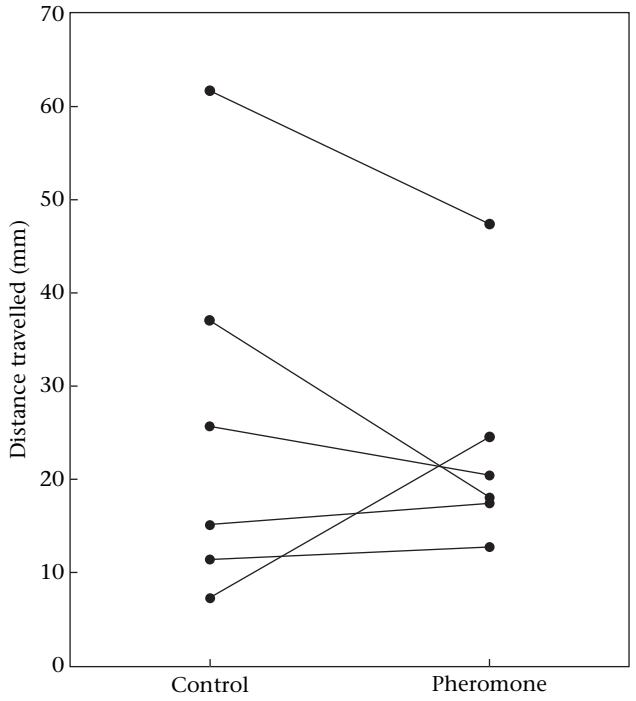

Figure 1. Distance rapidly travelled by a female salamander in response to a probe poke at the base of the tail following pheromone and saline control treatments (average of three pokes per treatment). Lines represent paired data for females grouped by base startle response (31 females divided into six quantiles ordered by the average individual responses during the control trial).

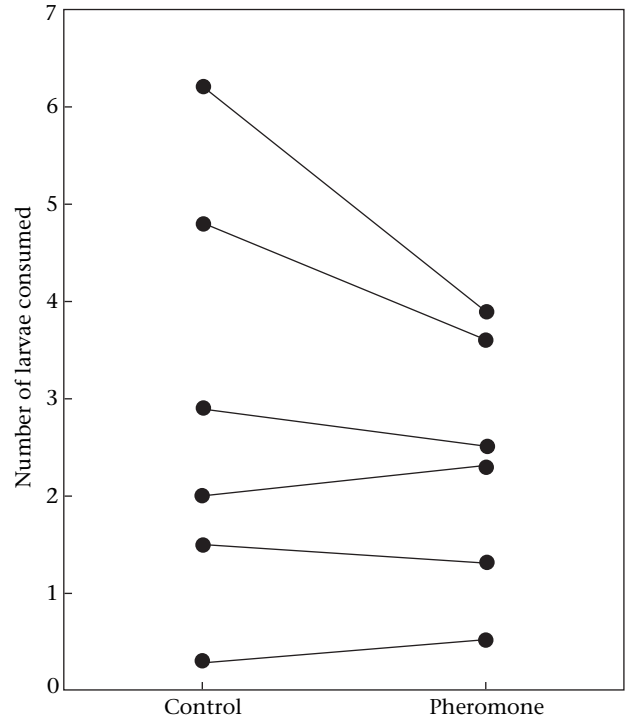

Figure 2. Number of fly larvae eaten by a female salamander following pheromone and saline control treatments (average of three replicates per treatment). Lines represent paired data for females grouped by base feeding activity (30 females divided into six quantiles ordered by the average number of larvae consumed by individuals during control trials).

demonstrated in an experiment in which the female did not encounter any sensory stimulation from the male other than a solution of pheromone that was experimentally delivered to nares. This suppression of feeding activity was evident among females that showed a strong tendency to feed under control conditions. Feeding is clearly a high priority for *Plethodon* females as nutritional and energetic constraints can restrict the frequency of their reproductive efforts (Highton 1962). During the multi-month mating season, females are in the process of rapidly yolking ova and typically are voracious. Thus, pheromones that weaken a female's drive to feed may increase a courting male's chance of insemination to the extent that the female becomes more focused on the male.

Successive nights of courtship and decreased feeding activity conceivably could have detrimental effects on the fitness of the female. However, not enough is yet known about the frequency of courtship and mating in a natural context to evaluate the magnitude of pheromone-induced appetite suppression. Paternity analyses in another plethodontid salamander (*Desmognathus ocoee*) revealed an average of two to three sires per clutch (Adams 2003), suggesting that a given female may mate with only a few males during the entire multi-month reproductive period. Paternity data, however, would not capture the number of courtships in which a given female participated that did not result in sperm transfer: in staged laboratory encounters, *P. shermani* females have participated in courtship to the point of spermatophore deposition with up to seven different mates; however, in 15–20% of all courtships, the female may still leave the male without accepting the sperm-filled cap (E. A. Vaccaro, personal observations). Staged courtship and insemination in the laboratory require an average of 35–45 min, and pheromone effects probably do not extend far beyond the courtship duration even with repeated pheromone administrations by the male.

Our experiment on the effect of male pheromone on female startle response did not detect a significant change in a measure of defensive behaviour. This may reflect the trade-off between insemination at a given moment versus the imperative to flee from a potential predator: given the lengthy nature of the courtship season, the availability of many potential male mates and the lack of urgency to be inseminated (oviposition typically occurs several months after mating), a female should do best to flee a potential predator rather than attempt to complete sperm transfer. In this case, the benefit to the female of maintaining a normal defensive behaviour (thereby avoiding potential harm) would outweigh the benefit of insemination on any given night. In the context of the nonsignificant pheromone effect on female defensive behaviour, the limited duration of pheromone-induced appetite suppression and the maximal frequency that a female would receive pheromones, courtship pheromones are unlikely to have a significant effect on overall female reproductive success.

At this initial stage, any inferences about the physiological mechanisms by which these pheromones alter behaviour are purely speculative. Current models of sensorimotor integration (e.g. Rose & Moore 2002) incorporate three basic stages at which behavioural subsystems can be modulated: (1) the processing of relevant inputs (i.e. the perception of sensory stimuli), (2) decision making (i.e. the moment-by-moment method of prioritizing the most salient motivational state, incorporating information from both internal physiological states and external stimuli), or (3) the control of relevant behaviours (i.e. motor outputs related to both appetitive and consummatory activities). Thus, until future research has established the physiological processes by which pheromones alter motivated behaviour, we continue to use overt and observable behaviours such as feeding activity and startle response as a proxy for implied motivational states such as hunger and fear.

The mechanism of increasing female receptivity by suppressing competing or inhibiting motivational states is only one of many

possible roles for male courtship pheromones. These pheromones can function at additional levels, including communicating sender-specific information to the female's accessory olfactory system and the activation of endogenous (neuroendocrine) signalling systems in the recipient: pheromones enter the female's nasal cavity and are shunted laterally (Dawley & Bass 1989) to the vomeronasal organ (VNO) where distinct populations of sensory neurons (Wirsig-Wiechmann et al. 2002) transmit pheromonal information to specific sites in the brain (Laberge 2008; Laberge et al. 2008) known to mediate endocrine function and sexual behaviour. Furthermore, since the pheromone is a mix of proteinaceous compounds encompassing many isoforms (Feldhoff et al. 1999; Rollmann et al. 1999; Watts et al. 2004; Palmer et al. 2007), different neural pathways are likely to mediate the response to individual pheromone components. Accordingly, this multicomponent signal can be capable of evoking a variety of behavioural responses critical to survival and reproduction.

Our research in the plethodontid system will continue to examine endogenous mechanisms by which male pheromones may affect female receptivity. Specifically, we are considering behavioural effects that may be promoted (1) as a secondary consequence of influences on the general state of central nervous system arousal (Pfaff 2006), previously known as 'general excitement' (Tinbergen 1952) and (2) by enhancing specific sensorimotor integration mechanisms involved in sexual motivation (Rose & Moore 2002; Thompson & Moore 2003), which may work as another form of motivational competition. By investigating the proximate aspects of a signal–response system, this and future studies may provide insights into how the perception of a chemical signal can induce a specific change in behaviour.

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Supplementary Material

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