



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.

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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 × 17 × 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 × 245 × 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

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