A pheromone mechanism for swaying female mate choice: enhanced affinity for a sexual stimulus in a woodland salamander

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Traditionally, male signals have been thought to function in satisfying female choice by conveying meaningful information about a potential mate. However, the male signal, rather than merely providing raw material for female evaluation, may actively modulate a female’s intrinsic preferences or decision-making capabilities in favour of a given male. We propose two broad mechanisms by which male signals could modulate female behaviour: (1) specific augmentation of sexual motivation or (2) heightened general arousal. Specifically, we investigated the ability of a generic male pheromone mix to elicit changes in general activity or affinity for different classes of stimuli in female terrestrial salamanders (Plethodon shermani). Attraction to male olfactory stimuli was significantly increased by pheromones, but attraction to visual stimuli and nonsexual olfactory stimuli remained unaffected, as did locomotor activity. These results are consistent with the hypothesis that sex pheromones activate specific behavioural subsystems associated with augmented sexual motivation. This pheromone action may still function within the context of information-transfer signalling, for example, if pheromones influence female choice by affecting (1) sensory processing of relevant stimuli, (2) the value assigned to a set of sexual stimuli, or (3) the criteria used to decide whether to mate.

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female behavioural response to male stimuli. Thus, the male signal, rather than being merely the raw material for female evaluation, may actively modulate a female’s intrinsic preferences or decision-making criteria in favour of a given male.

By definition, pheromones have held unique status among animal communication signals. Karlson & Luscher (1959, page 55) characterized pheromones as ‘substances which... release a specific reaction, for example, a definite behavior or a developmental process’. Thus, these signals were defined as immediate modifiers of recipient behaviour or eventual modulators of recipient physiology rather than information to be evaluated by the receiver. In this context, sex pheromones and their potential role influencing mating decisions are of particular interest. Plethodontid salamander pheromones, like many other sex pheromones, are chemical signals that can coordinate or otherwise influence male–female mating interactions. However, plethodontid pheromones belong to a distinct subclass of ‘courtship pheromones’ that are delivered by the male only after initial contact with a female, and only if the female is not immediately responsive to the male’s overtures (Arnold 1976; Houck & Sever 1994). These pheromones activate distinct neural pathways to areas of the brain known to regulate mating behaviour (Schmidt et al. 1988; Wirsig-Wuthmann et al. 2002, 2006; Laberge et al. 2008). Behavioural studies of plethodontid salamanders have demonstrated pheromone-induced enhancement of sexual receptivity, as measured by decreased courtship duration (Houck & Reagan 1990). The effect of courtship pheromones in reducing courtship duration has been well documented (Houck et al. 1998, 2008a, b; Rollmann et al. 1999). However, we still lack a mechanistic understanding of the forces underlying a female’s tendency to respond to, and cooperate with, a pheromone-producing partner.

Here, we explore two broad mechanisms by which a female may be swayed in favour of a courting male: a specific mechanism of direct augmentation of sexual motivation and a general mechanism of central nervous system arousal. In the case of the specific mechanism, pheromones would activate behavioural subsystems affecting the tendency of a female to approach or affiliate with functionally related stimuli (in this case sexual stimuli) (Giraldi et al. 2004). In particular, we would expect a sexually motivated female to show (1) unaffected locomotor activity, (2) a greater interest in sexual stimuli (i.e. scent or sight of a reproductive male) and (3) unaffected or diminished interest in nonsexual stimuli. Alternately, in the case of the general mechanism, sexual receptivity would be heightened as a secondary consequence of broad central nervous system arousal, previously known as ‘general excitement’ (Tinbergen 1952). This internal state of general arousal has been operationally defined by Pfaff (2006) as being more alert to sensory stimuli in all sensory modalities, engaging in more voluntary motor activity, and being more reactive emotionally (as measured by patterns of change in autonomic activity). Thus, we would expect a generally aroused female to demonstrate (1) increased locomotor activity and (2) a broad tendency to approach attractive stimuli, regardless of the class or sensory modality of the stimulus.

We investigated these specific and general mechanisms of swaying female mate choice in plethodontid salamanders. Specifically, we examined whether pheromones altered female general activity or attraction to stimuli in two classes (sexual, ingestive) and two sensory modalities (olfactory, visual). We used a generic or ‘every male’ signal (pheromones pooled from ca. 200 males) that prevented the transmission of individual-specific information. Our results revealed that male pheromones increased female affinity for olfactory sexual stimuli alone, consistent with the mechanism of augmented sexual motivation. This pheromone action may still function within the context of information-transfer signalling if a male signal affects the recipient’s sensory processing, stimulus evaluation standards or decision-making criteria.

STUDY SPECIES

We studied the mechanism of pheromone-enhanced receptivity in the red-legged salamander, Plethodon shermani. Courtship in P. shermani is highly stereotyped and has been well described (Arnold 1977), and is summarized here. First, the male approaches and solicits the female with an array of behaviours such as physical contact, foot dancing and tail arching. If the female is amenable, she steps over the male’s tail and positions herself with her head resting on the base of the male’s tail. A ‘tail-straddling walk’ ensues in which the female roughly matches the male step for step. During the tail-straddling walk, the male pauses periodically to deliver pheromones by tapping his mental gland (pheromone-producing chin gland) to the female’s nares. The male lacks an intromittent organ, and insemination occurs via the deposition of a spermatophore. Immediately following deposition, sperm transfer occurs when the male guides the female over the spermatophore and the female draws the apical sperm mass into her cloaca.

Courtship for P. shermani can be considered a period of reciprocal assessment. The female may appraise the male’s desirability through multiple modes of input (e.g. visual, pheromonal, somatosensory), while the male simultaneously evaluates the moment-to-moment level of female receptivity by her willingness to match his pace during the tail-straddling walk (Arnold 1976). Thus, a reluctant female can prolong courtship duration and will likely receive a greater quantity of pheromone.

METHODS

Study Species Collection, Maintenance, Gland Removal and Prescreening

Methods followed those of Vaccaro et al. (2009) and are summarized here. Male and female P. shermani in reproductive condition were collected during the August 2008 mating season from Macon County, NC, U.S.A. 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 fed ad libitum and were offered 10 fly larvae weekly (Calliphora vomi
toria, GrubCo, Hamilton, OH, U.S.A.). Shortly following salamander collection, we removed the pheromone-producing mental glands from anaesthetized males. Each male recovered from anaesthesia with his chin resting on a pillow made from moistened surgical gauze treated with antibiotics. Because of the superficial location of the mental gland, the excision site usually healed within 1 week. Males were allowed to recover fully in the laboratory before being released at the collection site. Pheromones were extracted from the gland tissue following established protocols (Houck et al. 1998). Each female was prescreened for a willingness to mate under laboratory conditions. Reproductively active females 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 Institutional Animal Care and Use Committee at OSU (IAR 3549 to D.D.H.).

Observational Arena

All behavioural trials were conducted in an arena consisting of an array of eight observation boxes (245 × 245 × 20 mm, Square
BioAssay, Corning Inc., Corning, NY, 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. Low-light illumination provided by four 60 W red incandescent light bulbs (pointed away from the experimental arena) was sufficient for video recordings. Digital video footage was routed to a pair of laptop computers for recording and later review (WiLife Indoor Master System, Logitech).

Visual Response Trials

Pheromone effect on responsiveness to visual stimuli was assessed by comparing the time spent in proximity to visual stimuli (both sexual and nonsexual) after administration of treatment substances. Treatment substances consisted of either pheromone (extract of pooled male mental glands, 2 mg/ml in 1/2 × phosphate-buffered saline) or control (1/2 × phosphate-buffered saline). Treatments were administered to the female’s nares in 4 μl portions via micropipette (Pipetman P10, Gilson, Inc., Middleton, WI, U.S.A.). Observations were conducted during the normal nocturnal activity period for P. shermani females: from 2100 to 0000 hours Eastern Standard Time (EST). Before the observation period, each female was allowed to acclimate for 120 min within an observation box. The boxes were lined with moistened paper bases that had been printed with horizontal numbered lines spaced 2 cm apart. During the acclimation period, camera placement and focus were adjusted.

Each of the eight trial nights was divided into two stimulus sessions. For the first session of the trial, each female was repositioned along the centre line of the observation box and presented with two boxes placed along opposite flanking sides of her observation box (Fig. 1a). Flanking boxes were the same dimension as the observation boxes, but each contained a cardboard insert that created a 3 cm wide containment space along the side abutting the female’s box. Following treatment, we placed a visual stimulus in one of the containment spaces: either a reproductively active male P. shermani (sexual stimulus) or six live fly larvae (food stimulus). The containment space in the other flanking box was left empty (control). Female location within the observation box was monitored for 60 min by digital video camera. For the second session of the trial, each female was given a refresher 4 μl treatment of the same initial solution. The other visual stimulus (fly larvae or live male) was placed in one flanking box and the opposite box served as the empty control. Female location was monitored for an additional 60 min by video camera. Each female was tested 6 days after the original trial using the other treatment substance (control or pheromone) and the same male as before. We randomized the order of treatment substance administration (between trial nights) and the order and location of stimulus presentation (within a trial, between sessions). Using video data, we manually tallied the duration of time spent within proximity (head within 4 cm) of each stimulus or control container. Measurements from the video records were taken 3–4 weeks after the experiment was conducted. All of the recordings for a given female (i.e. all of the treatment conditions tested) were scored and reviewed by a single observer to eliminate possible interobserver differences in computing the difference between control and treatment conditions. The person making the measurements was blind to the treatment order for each female.

Olfactory Response Trials

Pheromone effect on responsiveness to olfactory stimuli was assessed by comparing the time spent in proximity to olfactory stimuli (both sexual and nonsexual) after administration of treatment substances (both pheromone and control). Protocols followed those of the visual experiment (described above), with the following modifications: (1) the moistened paper bases lining the observation boxes were printed with numbered diagonal lines spaced at 2 cm intervals (Fig. 1b); (2) the olfactory stimulus consisted of a triangular cotton pad soaked in 3 ml of either male P. shermani scent or fly larvae scent (methods for obtaining these stimuli are described below); (3) this stimulus was placed in one corner of the observation box, with the control stimulus (a triangular cotton pad soaked in dechlorinated water) placed in the opposite corner; (4) for the second session, each female was placed in a fresh observation box to eliminate any lingering scents from the previous session. Using video data, we manually tallied the time spent in contact (head or abdomen) with each stimulus or control pad.

Methods for obtaining olfactory stimuli were modified from Thompson & Moore (2000) and are summarized here. To obtain the

Figure 1. Experimental design for (a) visual and (b) olfactory response experiments. Each female subject was exposed to all treatment (pheromone extract or control) and stimulus (male or food) combinations over successive trials. Four teams of eight females each were tested over successive nights (with a 2-night break each week) such that each team was tested once every 6 days. Team assignment, treatment order, stimulus presentation order and stimulus presentation location were randomized.
olfactory sexual stimulus, 30 reproductively active males were placed in each of two plastic containers (36 × 22 × 14 cm) with 125 ml of distilled water. The males sat overnight (ca. 12 h) in the water before being returned to their home boxes. The water from the two containers was combined, filtered, separated into 25 ml aliquots and stored at −20 °C. To obtain the olfactory food stimulus, about 400 fly larvae were placed in a container with 250 ml of distilled water and left overnight. Larvae were removed from the container, and the water from the container was filtered, separated into 25 ml aliquots and stored as above. Aliquots were thawed to room temperature before use in the experiment.

**Locomotor Activity Trials**

Pheromone effect on locomotor activity was assessed by comparing the number and duration of movement bouts (detected by video camera) following experimental treatment (pheromone or control, described above), from approximately 0000 to 0030 hours EST. The next week, each female repeated the trial using the other treatment substance. Observations were recorded by video cameras set to high sensitivity motion detection capture (100%, WiLife Command Center software interface). The motion detection settings recorded abrupt motions but not slow permutations. Using video metadata, we tallied the number and duration of movement bouts in the first 30 min following treatment.

**Experimental Design and Data Analysis**

These experiments employed a crossover design such that each treatment was applied to each subject over successive testing periods. The rationale for this design was to enable greater precision in our estimates by reducing error due to variation between subjects, thereby maximizing efficiency and permitting the use of a relatively small sample size (N = 32 females). One major concern arises when conducting a crossover experiment: carryover (residual) effects from previous treatments on subsequent treatments. To minimize the possibility of carryover effects, trials for each team (a set of eight females) were scheduled for a 6-day ‘wash-out’ period between trials. Trials were staggered by team to allow the experiment to run over successive nights. Balanced treatment order assignments were used to counterbalance sequence effects (e.g. the effect of pheromone before control versus the effect of control before pheromone).

The olfactory and visual response experiments each employed a split-plot-in-time design (Jones & Kenward 2003). In this design, a first experimental factor is applied over a period of time, and each period is then subdivided into subperiods over which a second experimental factor is applied. In our experiment, each level of treatment (pheromone or control) was applied during a weekly trial, and each trial was subdivided into two sessions such that both levels of stimulus (male and food) were applied during a given trial. This crossover split-plot-in-time design (Raghavarao & Xie 2003) had multiple levels of grouping (i.e. session within trial within subject), so our analysis had to employ a multilevel formulation to account for interdependence (correlations) among observations within the same grouping (Pinheiro & Bates 2000). Mixed models enabled us to represent the covariance structure associated with this multilevel, grouped data. These powerful models incorporated a nested random-effects structure to model multiple sources of random variation by associating common random effects to observations sharing the same level of a classification factor (Pinheiro & Bates 2000), including the effect of subject (blocking factor) and the effect of trial (whole period) within subject. Regression coefficients and variance components were estimated using restricted maximum likelihood (REML) (Patterson & Thompson 1971; Harville 1977). Statistical tests were performed using S-PLUS, version 8.1 (TIBCO Spotfire, Somerville, MA, U.S.A.).

Visual and olfactory experiments were analysed separately because the response variable was different between the two experiments: visual experiments measured time spent in proximity to a stimulus located outside of the observation box, while olfactory experiments measured time spent in contact with a stimulus located within the observation box. The time spent with the stimulus was adjusted by calculating ‘stimulus affinity’ as the difference in the time spent at the experimental stimulus versus the control stimulus. We tested the effect of treatment and stimulus combinations on stimulus affinity using linear mixed-effects models. Model assessment used likelihood ratio tests. For the olfactory data set, assessment retained random error terms for the subject (both intercept and slope). For the visual stimuli data set, assessment retained error terms for subject and period (slope only). For both data sets, testing for treatment-by-stimulus interactions would have required replication within each treatment—stimulus combination for each individual. Instead, we applied contrasts for three specific treatment combinations of interest: pheromone effect with male stimulus ((pheromone + male) – (control + male)), pheromone effect with food stimulus ((pheromone + food) – (control + food)), and general pheromone effect ((pheromone/male + food) – control (male + food)). Treatment contrasts and associated P values were calculated following Kuehl (1999).

For the locomotor activity experiment, we assessed the effect of pheromone using two summary response measures: (1) mean number of movement bouts and (2) average duration per movement bout. For each response variable, we assessed linear mixed-effects models, taking into account the following factors: (1) a random (blocking) effect for subject, as is appropriate for a crossover study with repeated measures on individuals; (2) a fixed effect covariate designating the number of recently consumed larvae, as females that ate more larvae may have moved less; and (3) an outer factor corresponding to the individual camera, because light from the incandescent bulbs cast heterogeneous illumination across the recording arena, which could affect the motion detection capabilities of cameras in different arena locations. Model assessment using likelihood ratio tests revealed that neither camera illumination nor recent food consumption should be retained in the final model.

**RESULTS**

**Visual Affinity**

Estimated time spent at food stimulus when treated with pheromone was significantly longer than time spent at the control stimulus (REML estimate: 14.0 min; t_{61} = 2.83, P = 0.0063). Treatment contrasts revealed that this increase was not attributable to a pheromone-induced affinity for the visual food stimulus (pheromone + food versus control + food: estimate = 5.0 min; t_{61} = 0.93, two-sided P = 0.35; Fig. 2a). Instead, contrasts revealed a general preference for the visual food stimulus (food versus male, regardless of treatment: estimate = 22.3 min, t_{61} = 2.92, two-sided P = 0.005).

**Olfactory Affinity**

Analysis revealed a significant response to pheromone when administered with a male scent: females on average spent 9.5 min/h longer in contact with a male-scented cotton pad than a water moistened control pad (REML: t_{52} = 2.82, two-sided P = 0.006). Females without pheromone treatment showed no preference for male-scented pads (mean = −0.8 min; REML: t_{52} = −0.18, two-sided P = 0.86). The treatment contrast investigating the effect of
pheromone treatment on female affinity for male scent revealed a significant increase over control treatment, with a given female increasing time spent at male scent by 5.2–15.3 min (95% confidence interval, pheromone + male scent versus control + male scent: mean = 10.3; t0.92 = 4.05, two-sided P = 0.0001). Females generally were disinclined to associate with the food-scented cotton pad. This disinclination was significant when females were treated with control (mean = −8.3 min; REML: t02 = −2.38, two-sided P = 0.02) but not pheromone (mean = −4.75 min; REML: t02 = −1.07, two-sided P = 0.29). The contrast investigating the effect of pheromone treatment on female affinity for food scent revealed a nonsignificant increase over control treatment, with a given female modifying time spent at food scent by −1.5–8.6 min (95% confidence interval, pheromone + food scent versus control + food scent: mean = 3.6; t0.92 = 1.40, two-sided P = 0.16; Fig. 2b).

Locomotor Activity

For the final night of the locomotor activity experiment, incorrect video camera settings resulted in incomplete records for eight females, so their data were removed from the analysis. Analysis revealed no evidence of a significant difference in locomotor activity between control and pheromone conditions in the 30 min trial following treatment. This lack of significance was evident in both the number of movement bouts (REML estimate: −0.93 movement bouts; f23 = 0.52, P = 0.61) and the average duration of movement bouts (REML estimate: −0.03 min/bout; f23 = 0.36, P = 0.72).

DISCUSSION

We investigated both specific and general mechanisms by which female mate choice might be influenced by male courtship pheromones in red-legged salamanders. The specific mechanism of direct augmentation of sexual motivation was highly significant: females were much more likely to respond to olfactory stimuli from males when treated with pheromones than when treated with a control. In contrast, our experimental results did not support a general mechanism of central nervous system arousal (Pfaff 2006); pheromone treatment did not increase female tendency to approach attractive stimuli (either ingestive or sexual) regardless of sensory modality (visual or olfactory), nor did pheromone treatment increase the amount of voluntary motor activity. Our findings of unaffected locomotor activity, however, may indirectly add support to the specific mechanism of augmented sexual motivation if we consider typical courtship behaviours in red-legged salamanders. Unlike other salamanders, the male does not clasp the female during the lengthy courtship (56 min on average, Arnold 1976) and must rely solely upon his persuasive capabilities for active female cooperation. In this situation, a mechanism that greatly altered female general activity could derail male–female interactions that are critical for successful sperm transfer.

The mechanism of enhanced sexual motivation still may operate within the paradigm of information-transfer signalling. Johansson & Jones (2007) highlighted the potential for sex pheromones to function as the input for female mate choice evaluation, given that these signals vary by individual, are heritable, and are costly to produce (honest signals). While production costs have yet to be established, previous research on P. shermani pheromones have established (1) the existence of individual male profiles, with variation in both the expression and relative concentration of different proteinaceous isoforms (Rollmann et al. 2000) and (2) the genetic basis of isoform variation (Watts et al. 2004; Kiemnec-Tyburczy et al. 2009). Furthermore, if sexual motivation activates specific behavioural subsystems that heighten the female’s neural ‘tone’ or responsiveness to sexual incentives, this could affect the detection and assessment of sexual stimuli (Rose & Moore 2002). In particular, we discuss three informational mechanisms by which pheromones might affect female choice: (1) through the female’s ability to discriminate between different stimuli (sensory processing), (2) through the value she assigns to a set of stimuli (mate attractiveness), or (3) through the criteria she uses to choose whether to mate (decision rules).

Sensory Processing

In the broadest sense, pheromones could help a female distinguish between sexual and nonsexual stimuli by enhancing the neural processing of sexually relevant stimuli (Thompson & Moore 2000). As these pheromones rendered sexual olfactory stimuli, but not sexual visual stimuli, more attractive to the female, this mechanism may be limited to the sensory modality in which pheromonal information is normally transmitted. While we have not determined explicitly whether female attraction is intensified by conspecific male stimuli in particular, previous work has
revealed that *Plethodon* females can distinguish between conspecific and heterospecific male stimuli (Dawley 1986, 1987) and that male *P. shermani* can distinguish between the scents of same- and opposite-sex conspecifics, although females displayed no similar interest in detecting and assessing conspecific scents (Palmer & Houck 2005). In a narrower sense, pheromones could help females distinguish among the olfactory stimuli provided by different males. The potential for individual olfactory signatures is evident in both the substantial interpopulation variation in pheromone composition (Rollmann et al. 2000) and the diversity of chemical signals secreted from the skin (Largen & Woodley 2008).

**Mate Attractiveness**

Previous research has established that a female’s evaluation of a mate can receive inputs from internal influences (e.g. experience and memory, reproductive state), so it is within reason that these preferences could be susceptible to internal modulation by pheromones. Our research has revealed that pheromones increase female attraction to certain aspects of a male stimulus (i.e. scent but not sight), but has not determined whether this attraction is individually specific: the enhanced attraction shown by the females was to the scent of a skin wash derived from 60 males, and was caused by pheromones extracted from the pooled glands of approximately 200 other males. Thus, it remains unclear whether this enhanced attraction would be for male stimuli in general or the individual delivering the pheromone in particular. However, pheromones are delivered only after a male has succeeded in isolating a female, and are likely to have an ephemeral effect. Thus, female response effectiveness will be augmented in the presence of the delivering male’s olfactory stimulus alone. What is remarkable, however, is the possibility that the value that a female assigns to a suite of male stimuli can be actively modified by the male being evaluated.

**Decision Rules**

Internal physiology and environmental inputs are generally accepted (and experimentally controlled for) as a ‘major source of variability in translating a preference to a choice’ (Ryan et al. 2007, page 314). Our previous research has demonstrated that pheromones can modify elements of female physiology that influence mate choice, for example, by suppressing internal hunger drive (Vaccaro et al. 2009). Here, we define ‘decision rules’ as the function that describes female choosiness, or, the probability that a given male will be chosen (Kirkpatrick et al. 2006). Decision rules can be thought of as a cost–benefit analysis of mating with a given male. These decision rules primarily are a function of stimulus values (female preference), but also take into account a female’s internal states, previous experience, social context and ecological context. As of yet, our ability to gain a strong mechanistic understanding of mate choice dynamics is obscured by the cryptic nature of the processes underlying decision rules: these processes may be complex, stochastic and temporally variable.

This study supports a further examination of the mechanisms underlying the role of signals in animal communication, with attention to the dynamics of mate choice. Our study revealed that a generic male pheromone can increase female affinity for an olfactory sexual stimulus, and this response is robust in the absence of a displaying male. Further investigation is merited to determine whether this pheromone action functions within the context of information-transfer signalling (e.g. if the male signal affects the recipient’s sensory processing, stimulus evaluation standards or decision-making criteria). Thus, the process of evaluating a stimulus is not necessarily autonomous from influence by the stimulus itself.

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