AN ABSTRACT OF THE DISSERTATION OF

Elyse A. Vaccaro for the degree of Doctor of Philosophy in Zoology presented on June 30, 2011.

Title: Pheromone Mechanisms and Behavioral Strategies for Maximizing Courtship Success in a Terrestrial Salamander, *Plethodon shermani*

Abstract approved:

Lynne D. Houck

Darwin devised the evolutionary theory of sexual selection to account for the manifold extravagances of courtship behaviors and displays. Mating interactions represent a major evolutionary process driving the elaboration, vibrancy, and peculiarity of these courtship traits. For my dissertation research, I strived to elucidate the dynamics that constitute the complex and delicate interplay between the satisfaction of the male's and female's reproductive interests.

Recent work on the red-legged salamander, *Plethodon shermani*, has established that a pheromone blend from male plethodontid salamanders increases female sexual receptivity as measured by reduced time to insemination. These courtship pheromones work as "releasers," stimulating centers of the brain to produce near-immediate effects on behavior. Pheromones have the potential to bypass the more traditional methods of mate assessment and directly activate centers of the brain implicated in the control of motivated behavior. I hypothesized that there were three candidate mechanisms by which male pheromones may alter female receptivity and therefore influence mate choice: (1) increased sexual motivation, (2) suppressed competing motivations, and (3) enhanced general (non-specific) arousal.

Chapters 2 through 4 describe a series of behavioral experiments in *Plethodon* designed to uncover patterns in female responses to pheromones. Chapter 2 examines pheromone-induced modulation of female choice as an indirect consequence of suppressing competing motivational forces. These studies demonstrated that pheromones inhibit feeding activity, but not the tendency to flee from an alarming stimulus. Chapter 3 examines pheromone-induced modulation of female choice as a direct effect of enhanced sexual motivation, or as a secondary consequence of general nervous system arousal. The results supported a specific pheromone mechanism in which attraction to male scent is enhanced, but not attraction to other appealing stimuli. Chapter 4 examines the role of individual pheromone components. I hypothesized that these different pheromone components may work in tandem to increase female receptivity by activating or suppressing specific behavioral systems. My studies supported pheromone component-enhanced attraction to male olfactory stimuli as a mechanism to enhance sexual receptivity.

For Chapter 5, I conducted a meta-analysis designed to: (1) quantify variation in female willingness to engage in courtship, (2) quantify variation in male effort expended during courtship, and (3) determine whether these male and female characteristics interacted in a manner to shorten courtship or affect the probability of insemination. The meta-analysis uncovered associations between the time spent in the mutual participation phase of courtship and: female receptivity, male-female compatibility, and male effort. Furthermore, insemination success was associated with the interaction between male-female compatibility and male effort. In light of these results, a male would do well to be attuned to his potential partner's motivational states and intrinsic preferences, and to adjust his courtship behavior accordingly. I conclude my dissertation by discussing the findings from Chapters 2 – 5 within the larger contexts of the potential role of pheromones as the substrate for sexual selection, or as a form of manipulation when the reproductive interests of the courter and courted are not in perfect accordance, and finally, the implications for behavioral studies and interpretations of evolutionary processes.

Pheromone Mechanisms and Behavioral Strategies for Maximizing Courtship Success in a Terrestrial Salamander, *Plethodon shermani*

by

Elyse A. Vaccaro

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APPROVED:

Major Professor, representing Zoology

Chair of the Department of Zoology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Elyse A. Vaccaro, Author

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Oh, you mavens of modeling, masters of microsatellites,
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Oh, you duchesses of dune grass, disciples of dimensionality,
And doyennes of graphic design.
You prodigies of phylogenies, commodores of chromatography,
And eelgrass experts.
You fanatical fungiphiles, whizzes of pop quizzes
and ace TAs.
You spermatheca divas and
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Thank you, thank you, thank you,
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For showing me what to aspire to, and
How to handle adversity with grace, and
For demonstrating, every day, what it
Means to be a scientist.

CONTRIBUTION OF AUTHORS

Lynne Houck provided animals, experimental facilities, and feedback on the writing of manuscript Chapters 2 – 4. Pamela and/or Richard Feldhoff provided purified pheromones used for the behavioral trials in Chapters 2 – 4. Paul Sims assisted in the experimental trials and subsequent tallying of data in Chapter 4. Lynne Houck granted access to data from previous studies for the meta-analysis in Chapter 5.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF TABLES

DEDICATION

This dissertation is dedicated to my spouse,
Jeremy Andrew Noring

Pheromone Mechanisms and Behavioral Strategies for Maximizing Courtship Success in a Terrestrial Salamander, *Plethodon shermani*

CHAPTER 1. GENERAL INTRODUCTION

Darwin (1859; 1871) devised the evolutionary theory of sexual selection to account for the manifold extravagances of courtship behaviors and displays. Mating dynamics represent a major evolutionary process driving the elaboration, vibrancy, and peculiarity of these courtship traits. Mating interactions such as courtship constitute the complex and delicate interplay between the satisfaction of the male's and female's reproductive interests. Courtship is a diplomatic negotiation, as male and female interests do not necessarily align. In the quintessential case of the courting male and discriminating female, courtship is a period of mutual assessment during which the female assesses the male's attractiveness (i.e., how well do the male's attributes align with her intrinsic preference?) while the male assesses her readiness to mate with him (i.e., what is her moment-by-moment receptivity level?).

Courtship Strategies & the Inscrutable Nature of Female Preference

Mate choice is the discriminative selection of mating partners, as measured by the execution of fixed motor patterns related to courtship and mating. The material for this assessment is sensory input that the recipient evaluates from the signaler. In the archetypal courtship, the discriminating female evaluates the displaying male. A new generation of mechanistic studies has illuminated the tricky calculus of female mate choice: female preference is determined by genetics, previous experience (Lea et al. 2000; Kodric-Brown & Nicoletto 2001), and social context (e.g., cues from conspecifics: Alonzo & Sinervo 2001; Royle et al. 2008), while female receptivity is influenced by physiological condition and reproductive state (Aisenberg & Costa 2005; Lynch et al. 2005).

While the terms “female preference” and “female receptivity” are sometimes used in an overlapping or interchangeable manner, for the purposes of this dissertation, I distinguish between them. Preference is an intrinsic affinity for certain male characteristics. Preference exists independently of a given male that the female encounters. Receptivity is the moment-by-moment tendency to mate, and in contrast to preference, female receptivity is absolutely susceptible to the efforts of the particular male that presents himself. The operative differences between preference and receptivity can be difficult to distinguish. For example, a female could have a preference for male behaviors that increase receptivity, such as male persistence. Conversely, if a female’s preference is for a diversity of mates over successive courtships, then attributes that she once found attractive may lower her receptivity in subsequent courtships. Furthermore, the concepts of preference and receptivity are based on experimental inference rather than direct examination; thus, like any internal state, the ultimate behavioral outcome alone readily submits itself to experimental scrutiny. Finally, the factors affecting female preference and receptivity may be overlooked in experimental studies unless they are explicitly investigated: the laboratory setting controls for most relevant physiological and environmental variables, and therefore these variables are masked from observation.

In light of the enigmatic complexities of female mate choice, a male would do well to be attuned to his potential partner’s motivational states and intrinsic preferences, and to adjust his courtship displays accordingly. However, it may behoove a male to be both consistent and flexible in his behavior. While flexibility allows a male to cater his behavior to a female’s preferences, consistency is a potentially heritable trait that can convey reproductive fitness (Boake 1989). Additionally, flexibility is not without its downside: flexibility requires the resource investment of learning a behavior (Gershman & Verrell 2002) which may delay insemination while the male assesses the female’s receptivity. My dissertation research endeavored to elucidate the relative contributions of behavioral flexibility and consistency as dual strategies to address the complex and enigmatic dynamics of female mate choice.

For Chapter 5, I conducted a meta-analysis based on 17 studies compiled across 13 years of courtship experiments in the red-legged salamander, *Plethodon shermani.* I measure courtship success as latency to insemination (i.e., courtship duration) and probability of insemination. The analysis focused on how the following factors influenced courtship success: female receptivity, male persuasiveness, pair compatibility, the intensity of male courtship behavior (delivered or elicited), total male and female courtship experience, and experimental pheromone delivery. The aims of the meta-analysis were three-fold: (1) to identify variation in female willingness to engage in courtship, (2) to identify variation in male effort expended during courtship, and (3) determine whether these male and female characteristics interacted in a manner to shorten courtship or affect the probability of insemination.

The Ace in the Hole: Pheromone Mechanisms for Influencing Female Receptivity

While the female may receive input from sources both internal and external, the assumption underlying many studies is that female preference is intrinsic. By extension, it is assumed that the decision-making function is not subject to modulation by external influences, i.e., "the signaler cannot directly influence the processing of the signal by the receiver's nervous system" (Wiley 1983; see Chapter 6 for further discussion.) However, it has been demonstrated that certain classes of chemical signals can have profound effects on the probability that the receiver will engage in subsequent reproductive behaviors (Melrose et al. 1971; Ball & Balthazart 2009). These changes in behavior are likely achieved by altering the neuroendocrine physiology of the receiver, in particular, via modulation of: (1) sensory inputs, (2) central pattern generators, and/or (3) effector organs. In this way, signals would function to modify the female's current state of receptivity or circumvent her usual assessment capabilities in favor of a given male. The existence of such a potent mechanism for modifying female behavior would be highly advantageous to a male's potential reproductive success.

Such a mechanism of active manipulation would be distinctly different from the action of influencing female choice by priming pheromones, mate copying or sensory exploitation. Priming pheromones work via the endocrine system to modify reproductive development and cycling (Wyatt 2003; Wyatt 2009), and thus only affect mate choice via belated and indirect means. Mate copying occurs when an individual's choice is modified using social cues from other females (Dugatkin 1992; Dugatkin & Godin 1992), and thus is still an inherent cognitive process. Sensory exploitation takes advantage of pre-existing sensory biases in the female (e.g., photopigment sensitivity, auditory tuning; reviewed in Ryan 1990; Basolo & Endler 1995; Endler & Basolo 1998) and thus is a passive appeal to the female's intrinsic preferences.

In contrast to the belated effects of priming pheromones, the indirect effects of mate copying, and the passive appeal of sensory exploitation, sex pheromones are the chemosensory equivalent of the male's ace in the hole. These pheromones work as "releasers," stimulating centers of the brain to produce near-immediate effects on behavior (Wyatt 2003; Wyatt 2009). Pheromones have the potential to bypass the more traditional methods of mate assessment and directly activate centers of the brain implicated in the control of motivated behavior. Determining how the perception of such a chemical signal could induce a change in behavior is a challenging task. However, recent work addressing receptivity has established that a pheromone blend from male plethodontid salamanders increases female sexual receptivity: courtships in which the female receives pheromones versus saline control have a reduced time to insemination (Rollmann et al. 1999; Houck et al. 2007).

The vomeronasal system of the red-legged salamander, *Plethodon shermani*, can illuminate how sensory input is relayed, modulated and processed as it travels to brain regions implicated in the control of motivated behavior. The *Plethodon* vomeronasal system is hypothesized to have receptor mechanisms for each type of biologically relevant cue (Maerz et al. 2001; Madison et al. 2002; Placyk Jr. & Graves 2002). Thus, specific mechanisms would detect cues of prey, predators and conspecifics, as

well as individual pheromone components. Different vomeronasal pathways are further hypothesized to interact among each other in the central nervous system to prioritize the choice of behavioral options (Halpern 1987; Halpern & Martínez-Marcos 2003).

Chapters 2 through 4 describe a series of behavioral experiments in *Plethodon* salamanders designed to uncover patterns of female responses associated with pheromone-induced behavioral and physiological changes. In particular, I hypothesized that male pheromones act by three candidate mechanisms to alter female receptivity and therefore influence mate choice: 1) increased sexual motivation, 2) suppressed competing motivations, and 3) enhanced general (non-specific) arousal.

Chapter 2 describes studies that investigated this second candidate mechanism: pheromone-induced modulation of female choice as an indirect consequence of suppressing competing motivational forces. To the extent that the motivation to reproduce is inhibited by defensive (Moore & Miller 1984; Moore & Zoeller 1985) and ingestive (Dickerman et al. 1993; Jones & Lubbers 2001) motivational forces, any effect that subdues fear and hunger could indirectly enhance female sexual receptivity. The behavioral studies supported the inhibitory mechanism of altering female choice by demonstrating that *P. shermani* pheromones suppressed female motivation to feed, but not to flee. I concluded that salamander courtship pheromones may have an inhibitory effect on the processing of prey cues, but not predator or olfactory cues.

Chapter 3 examines the specific versus general candidate mechanism: pheromone-induced modulation of female choice as a direct effect of enhanced sexual motivation or as a secondary consequence of general nervous system arousal. To the extent that all motivated behavior is believed to spring from generalized arousal (Tinbergen 1952; Pfaff 2006), we would expect a generally aroused female to demonstrate increased locomotor activity and a broad tendency to approach any type of attractive stimulus, thereby increasing the chances that she will approach and engage in courtship with a male. In contrast, a sexually aroused female should demonstrate enhanced attraction to

specific sexual stimuli, perhaps in specific sensory modalities. My studies supported a specific pheromone mechanism of enhanced sexual motivation in which female attraction to male olfactory stimuli is enhanced, but not male visual stimuli or food (olfactory or visual) stimuli.

In Chapter 4, I investigated the effects of individual pheromone components in altering female receptivity. Previous studies have shown that Plethodontid Receptivity Factor (PRF) and Plethodontid Modulating Factor (PMF) may have contrasting or even conflicting effects on female receptivity as measured by courtship duration (Rollmann et al. 1999; Houck et al. 2007). I hypothesized that these different pheromone components may activate or suppress specific behavioral systems, e.g., activating the tendency to approach a mate while suppressing the tendency to feed. These complex behavioral effects could work in tandem to increase female receptivity. My studies supported PRF-enhanced attraction to male olfactory stimuli as a mechanism to enhance sexual receptivity. The behavioral effects of PMF remain shrouded from experimental observation.

SIGNIFICANCE

My dissertation research strived to link proximate and evolutionary aspects of a pheromone signal-response system by examining the dynamics underlying mate choice in a plethodontid salamander. I proposed that the primacy of satisfying female preference can drive the evolution of complex mating strategies and sexual signaling systems. Coadaptation between the male signal and female response is a central prediction for models of evolution by sexual selection (Fisher 1930). The pheromone-response pattern in *Plethodon* salamanders has been retained over the last 27 million years (Palmer et al. 2005). Comparative studies of the genus *Plethodon* reveal that both PRF and PMF have undergone rapid, selection-driven evolution (Watts et al. 2004; Palmer et al. 2007; Palmer et al. 2010) and it is likely that the response systems for processing pheromone information have evolved in tandem. By defining the mechanisms by which pheromones influence female receptivity, my dissertation

research strived to provide crucial insights into the workings of vertebrate pheromones as well as the processes and patterns that drive the coevolution of signal-response mechanisms.

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CHAPTER 2. MALE COURTSHIP PHEROMONES SUPPRESS FEMALE TENDENCY TO FEED BUT NOT TO FLEE IN A PLETHODONTID SALAMANDER

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ABSTRACT

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.

INTRODUCTION

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) 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 ºC 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 x 245 x 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 x 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 (REML *t* test : $t_{68} = -0.25$, $P = 0.80$) 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 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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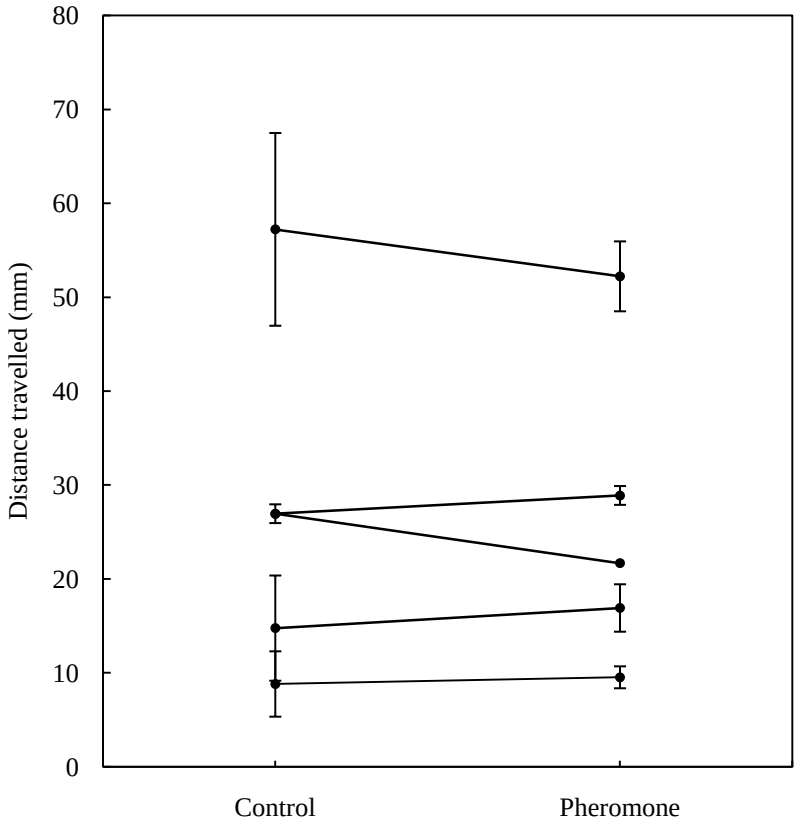

Figure 2.1. Distance rapidly travelled by a female salamander in response to a probe poke at the base of the tail following pheromone and saline control treatments (average of three pokes per treatment). Lines (+/- SE) represent paired data for females grouped by averaged startle response across treatments. Modified from Vaccaro et al. 2009.

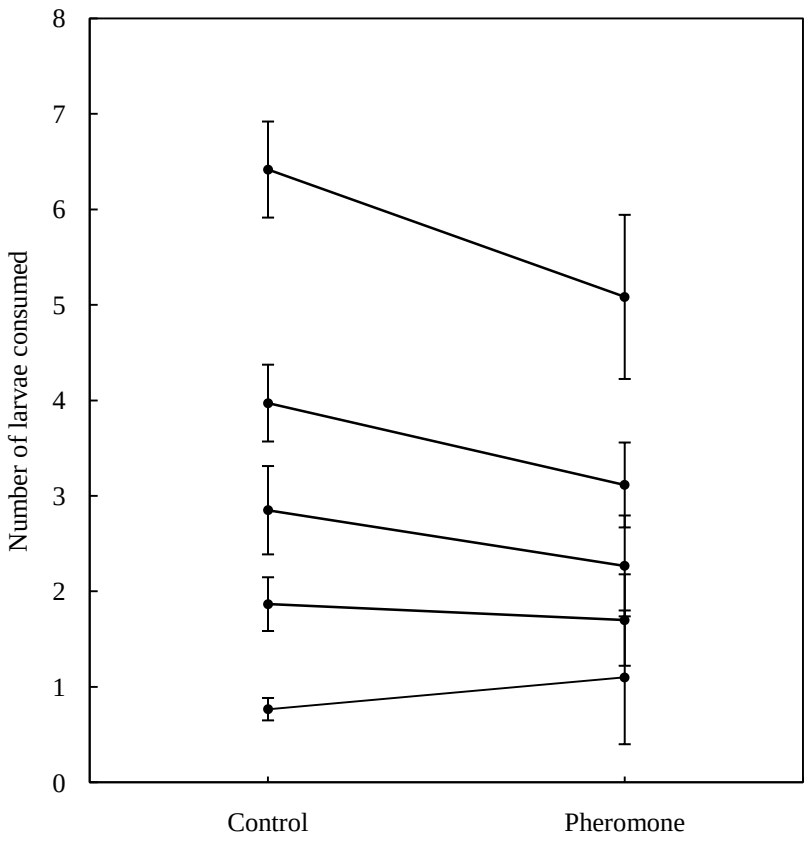

Figure 2.2. Number of fly larvae eaten by a female salamander following pheromone and saline control treatments (average of three replicates per treatment). Lines (+/- SE) represent paired data for females grouped by averaged larvae consumed across treatments. Modified from Vaccaro et al. 2009.

CHAPTER 3. A PHEROMONE MECHANISM FOR SWAYING FEMALE MATE CHOICE: ENHANCED AFFINITY FOR A SEXUAL STIMULUS IN A WOODLAND SALAMANDER

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ABSTRACT

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.

INTRODUCTION

Within the paradigm of the signalling male and the discriminating female, sexual selection is dictated by the primacy of satisfying female choice. Sexual signals have been researched in terms of the personal information (such as sex, strength, status, size, age) that a signaller can convey to a potential mate. This information is gathered from multiple senses and integrated over an array of male characteristics, then used to assess male attractiveness (Burley 1981; Zuk et al. 1992). This assessment, or female preference, dictates how mate quality is determined and therefore drives mate choice. However, can sexual signals influence mate choice by means other than conveying information for female assessment?

Ongoing debate over the definition of information has caused some researchers to call for an eschewal of the idea of a message or meaning in animal communication and instead focus on the effect of the signal on its receiver (Dawkins & Krebs 1978; Rendall et al. 2009). Regardless of whether semantic information (Krebs & Dawkins 1984) is conveyed by the male signal, many factors are known to influence female preference and mate choice without directly providing input for mate assessment. Female preference can vary both among individuals and within an individual, and is susceptible to the vicissitudes of (1) reproductive state (Lea et al. 2000; Lynch et al. 2005) (2) previous experience (Kodric-Brown & Nicoletto 2001; Aisenberg & Costa 2005) and (3) social context (e.g., cues from conspecifics: Alonzo & Sinervo 2001; Royle et al. 2008). Additionally, factors unrelated to mate preference can influence mate choice, including female physiological condition (e.g., food energy reserves: Dickerman et al. 1993; Jones & Lubbers 2001; Fisher & Rosenthal 2006) and ecological context (e.g., threat of predation: Moore & Miller 1984; Moore & Zoeller 1985). Finally, male signals can act as sensory modulators that activate female-specific behaviours. Recent studies on pheromone effects on female mice (Kimchi et al. 2007) and mating calls in túngara frogs (Hoke et al. 2008) indicate that male signals can trigger a sex-specific 'sensory switch' or neural 'gatekeeper nucleus' that regulates 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-Wiechmann 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 vomitoria*, 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 ºC 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 (LAR 3549 to L.D.H.).

Observational Arena

All behavioural trials were conducted in an arena consisting of an array of eight observation boxes (245 x 245 x 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 1/2x phosphate-buffered saline) or control (1/2x 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 to 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 olfactory sexual stimulus, 30 reproductively active males were placed in each of two plastic containers (36 x 22 x 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 perambulations. 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: $t_{92} = 2.82$, two-sided $P = 0.006$). Females without pheromone treatment showed no preference for male-scented pads (mean = -0.8 min; REML: $t_{92} = -0.18$, 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 to 15.3 min (95% confidence interval, pheromone + male scent versus control + male scent: mean = 10.3; $t_{92} = 4.05$, 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: t_{92} = -2.38, two-sided P = 0.02) but not pheromone (mean = -4.75 min; REML: t_{92} = -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 to 8.6 min (95% confidence interval, pheromone + food scent versus control + food scent: mean = 3.6; t_{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; t_{23} = 0.52, P = 0.61) and the average duration of movement bouts (REML estimate: -0.03 min/bout; t_{23} = 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 effectively 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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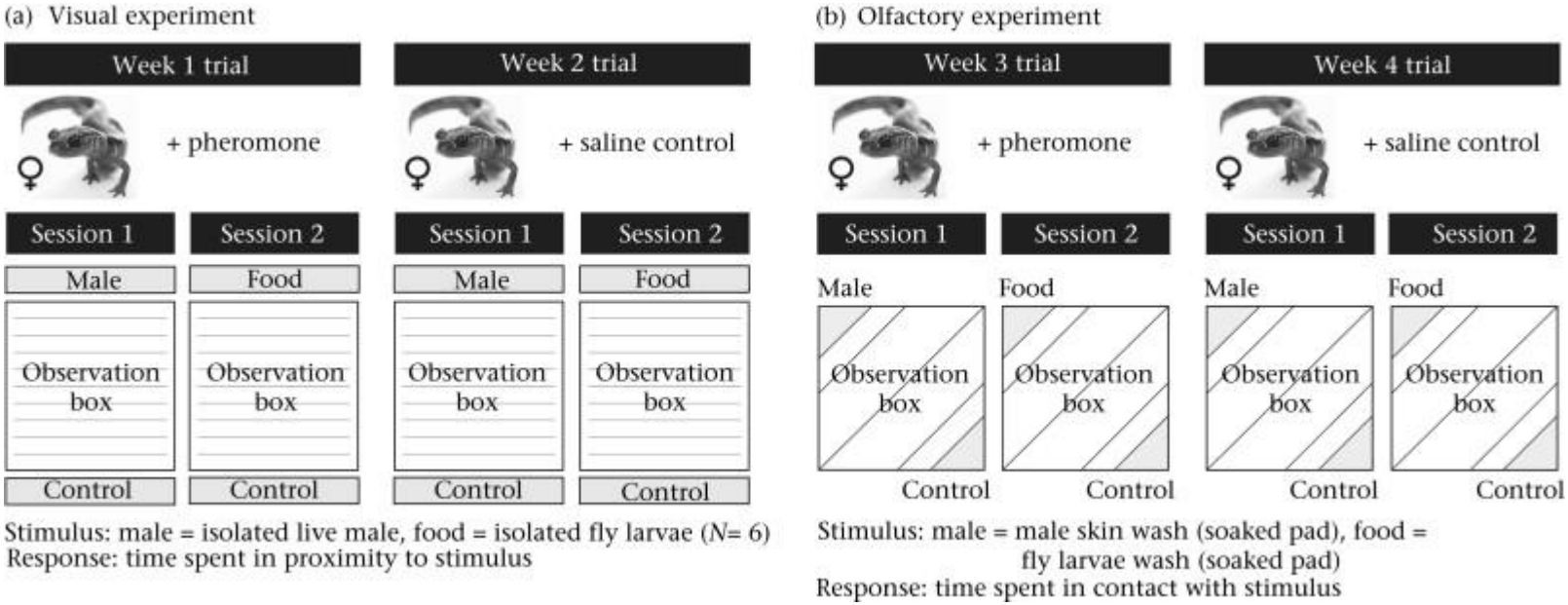

Figure 3.1. Experimental design for (a) visual and (b) olfactory response experiments. Each female subject was exposed to all treatment (pheromone extract or control) and stimulus (male or food) combinations over successive trials. Four teams of eight females each were tested over successive nights (with 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.

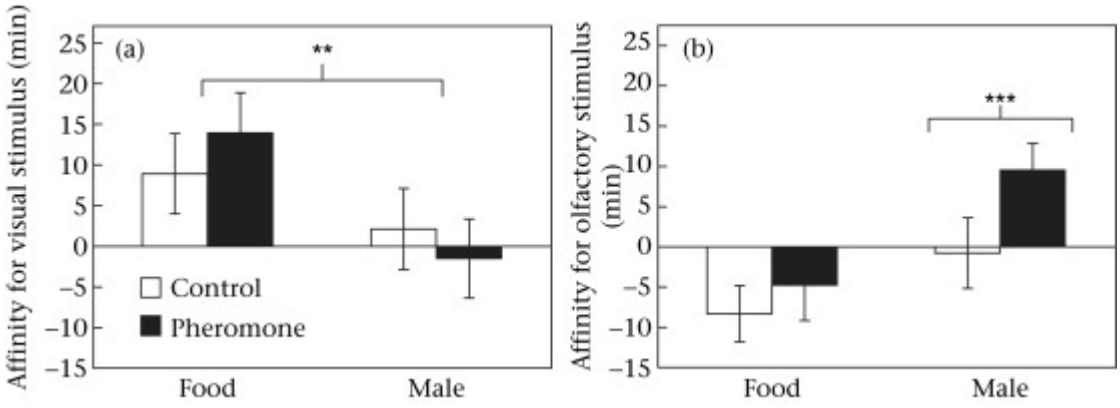

Figure 3.2. Female salamander affinity for visual and olfactory stimuli following control and pheromone treatments. Affinity was measured by time spent in proximity to the stimulus in the 60 min following treatment, adjusted by time spent in proximity to a control stimulus; negative affinity values indicate that the control stimulus was preferred over the experimental stimulus (food or male). (a) Visual stimuli: the food stimulus was represented by six fly larvae located in an isolated clear chamber flanking the female; the sexual stimulus was represented by a reproductively active male located in an isolated clear chamber flanking the female; the control stimulus was an empty flanking chamber. Values are means ± SE, REML estimates from linear mixed effects modeling with subject (regression intercept only) as a random effect. (b) Olfactory stimuli: the food stimulus was represented by a cotton pad soaked in fly larvae wash; the sexual stimulus was represented by a cotton pad soaked in a wash from reproductively active males; the control stimulus was a cotton pad soaked in dechlorinated water. Values are means ± SE, REML estimates from linear mixed effects modeling with subject (both regression intercept and slope) as a random effect.

CHAPTER 4. COMPONENTS OF A COURTSHIP PHEROMONE MIX DIFFERENTIALLY MODULATE PROCESSING OF SEXUAL AND NONSEXUAL STIMULI IN FEMALE SALAMANDERS

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INTRODUCTION

In plethodontid salamanders, female choice is the ultimate determinant of mating success. Female control of mating is conferred by a variety of factors: (1) male-male competition for mates is intense, as adult females only produce clutches in alternating years (Highton 1962), resulting in an operational male-female sex ratio of approx. 2:1; (2) exclusive insemination is not guaranteed, as a female may mate with several males during the multi-month reproductive season, and all sires may produce offspring; (3) forced insemination is impossible, as the male lacks an intromittent organ and does not clasp the female during mating; thus, (4) the female may abandon the male at any time during the lengthy courtship; and finally, (5) a female can afford to be exceptionally discriminating in her choice of mates, as she can store sperm for several weeks or even months before ovulation (Sever 2003; Houck & Schwenk 1984; unpublished research: E.A. Vaccaro and S.L. Eddy), thereby freeing her from the constraint of urgently seeking a mate on a given night.

Given the relative infrequency of encountering a highly receptive female, it behooves the male to invest considerable resources to enhance his persuasive capacities (Arnold 1976). In our study species, the red-legged salamander (*Plethodon shermani*), courtship is an intense and lengthy affair. First, the male solicits the female by: (1) rubbing his head along the female's head and flanks, (2) displays of prancing in place and flashing his fiery colored limbs, and (3) arching and undulating his tail. A sufficiently amenable female will step over the proffered tail and position herself astride with her chin resting near the base of the male's tail. Next, the pair enters into "tail-straddling walk" in which the female roughly matches the male step-for-step. A male that fails to pace himself or to map his path appropriately risks having his potential mate wander away. During the tail-straddling walk, the male pauses periodically to deliver pheromones by tapping his mental gland (pheromone-producing chin gland) on the female's nares (Fig. 4.1). A male that positions himself awkwardly to deliver pheromones risks losing his potential mate. As the male lacks an

intromittent organ, insemination occurs via the deposition of a spermatophore. A male that provides insufficient stimulation (via tail undulation on the female's chin and venter) during spermatophore deposition may again risk losing his potential mate. Sperm transfer occurs if the male successfully guides the female over the spermatophore and the female wedges the apical sperm mass into her cloaca.

From a sexual selection standpoint, communicating a sophisticated and powerfully persuasive signal would be highly advantageous to the male. The lengthy and stereotyped courtship for *Plethodon* salamanders functions as a period of reciprocal assessment. The female may use several sensory modalities (e.g., visual, olfactory, somatosensory) to appraise the male's desirability; simultaneously, the male can monitor the female's moment-by-moment level of receptivity by her willingness to match his pace during the tail-straddling walk (Arnold 1976). A reluctant female can slow the tail-straddling walk, prolong courtship duration, and presumably receive a greater quantity of pheromone from the male. *Plethodon* males in reproductive condition produce proteinaceous pheromones with a mental (chin) gland. A group of *Plethodon* species from the eastern United States (including our study species) possesses large mental glands and employ a specialized method of pheromone delivery: the male taps his mental gland on the female's nares (Highton 1962; Houck & Arnold 2003). These glandular secretions then stimulate the female's vomeronasal system, including areas of the brain implicated in motivated behavior (Wirsig-Wiechmann et al. 2002; Wirsig-Wiechmann et al. 2006; Laberge et al. 2008). While this method of pheromone delivery is highly conserved, research gives evidence of strong diversifying selection on the pheromone components themselves (Watts et al. 2004).

A diversity of signals is present in *P. shermani* pheromone secretions and the major components embody multiple protein isoforms. Biochemical investigations revealed that male pheromone secretions comprise a mixture of proteinaceous compounds. Two main protein components account for about 85% of the whole

extract: the 22 kDa Plethodontid Receptivity Factor (PRF) and the 7 kDa Plethodontid Modulating Factor (PMF) (Fig. 4.2; Rollmann et al. 1999). These components differ both structurally and functionally: PRF is structurally related to the IL-6 family of cytokines (Feldhoff et al. 1999; Rollmann et al. 1999; Watts et al. 2004), while PMF belongs to the three finger protein (TFP) superfamily that includes snake venom toxins and xenoxins (Palmer et al. 2007). PRF and PMF produce distinctly different female responses: PRF reduces courtship duration (Rollmann et al. 1999), but PMF isoforms lengthen courtship duration (Houck et al. 2007). The primary function of PMF is not yet known, but one possibility is that PMF facilitates a state of "relaxation" in the female. In this state, a female presumably would be less responsive to distracting stimuli that could cause the female to discontinue courtship activities. Indeed, PRF and PMF combined may produce specific stimulatory and sedative effects that facilitate courtship.

Female response to these seemingly conflicting signals is likely to be mediated via different neural pathways: PRF and PMF each bind to specific receptors and are believed to activate separate populations of female vomeronasal neurons (Wirsig-Wiechmann et al. 2006) and may elicit different response patterns in the female brain (Laberge et al. 2008). This vomeronasal pathway results in activation of areas of the brain known to mediate female sexual behavior and endocrine regulation in other vertebrates (Laberge & Roth 2005; Laberge 2008). Thus, this multi-component pheromone mix, capable of activating multiple brain regions, could produce complex behavioral outcomes in the female.

Our major goal has been to tease apart the respective and seemingly conflicting roles of PMF and PRF on female receptivity. Our approach indirectly addressed the neurophysiological mechanisms by which individual pheromone components may elicit specific behaviors by differentially activating areas of the brain responsible for sexual motivation (discussed below). Our previous research revealed that pheromones reduced competing motivations and increased sexual attraction in the female: whole

pheromone treatment suppressed hunger (Vaccaro et al. 2009) and enhanced attraction to male scent (Vaccaro et al. 2010). For the current investigation, we examined the individual and combined effects of PMF and PRF on female attraction to visual and olfactory stimuli from both sexual partners and prey.

METHODS

Experimental methods were adapted from Vaccaro et al. (2010) and are summarized here.

Study Species Collection, Maintenance, Gland Removal, and Prescreening

Male and female *P. shermani* in reproductive condition were collected during the August 2009 mating season from a single locality in Macon County, North Carolina, U.S.A. We examined all females for the presence of mature oocytes in the oviducts (visible through the ventral skin). Individuals were housed in plastic boxes (31 × 17 × 9 cm) lined with moist paper towels as substrate and crumpled damp paper towels as refuges. Animals were fed 10 fly larvae weekly (*Calliphora vomitoria*, GrubCo). Shortly following salamander collection, we removed the mental glands from anesthetized males and prepared pheromone extracts for experimental treatments. Methods of gland removal and preparation of the PRF, PMF and whole pheromone extract treatment solutions followed established protocols (Rollmann et al. 1999). Some animals will not court in the laboratory, so males and females were first prescreened to assess their willingness to mate under laboratory conditions. Following prescreening, animals that had courted one or more times were shipped to Oregon State University (OSU), Corvallis, U.S.A. where behavioral experiments were conducted. Animals were kept in conditions similar to the field: 15-18 ºC 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 and 4053 to LDH).

Observational Arena

All behavioral trials were conducted in an observational arena consisting of an array of twelve observation boxes (245 x 245 x 20 mm Square BioAssay, Corning). Each box was monitored by a dedicated high-resolution digital video camera (WiLife indoor surveillance camera, Logitech). The cameras were placed aperture downwards upon transparent glass shelves located approx. 20 cm above each observation box. Indirect illumination provided by four 60W red incandescent light bulbs (pointed away from the experimental arena) was sufficient for video recordings. Digital video footage was routed to a pair of desktop computers for recording and later review (WiLife Indoor Master System, Logitech, Fremont, CA, U.S.A.).

Visual Response Trials

Pheromone effect on responsiveness to visual stimuli was assessed by comparing the duration of time a female spent in proximity to visual stimuli (both sexual and nonsexual) after administration of a treatment substance. The treatment substance consisted of one of the following: whole pheromone extract (derived from the mental glands of multiple males, 2 mg/ml in ½x phosphate-buffered saline(PBS)), PRF (0.7 mg/ml in ½x PBS), PMF (0.5 mg/ml in ½x PBS), or control (½x PBS). Treatments were administered to a 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, corresponding to 2100 to 0000 hours Eastern Standard Time (EST). Each female was allowed to acclimate for 120 min within an observation box that was lined with a moistened paper base printed with horizontal numbered lines spaced 2 cm apart. During the acclimation period, camera placement and focus was adjusted.

Each of the 16 trial nights was divided into two stimulus sessions. For the first session of each trial, each female was positioned along the center line of the observation box and presented with two clear petri dishes placed on top of and at opposite sides of her observation box. Following treatment, a visual stimulus was

placed under one of the petri dishes: either a reproductively active male *P. shermani* (sexual stimulus) or six live fly larvae (food stimulus). The other petri dish was left empty as a control. Female location within the observation box was monitored for 60 minutes by digital video camera. For the second session of the trial, each female was given a 4 µl refresher of the original treatment (whole pheromone, PRF, PMF, or control) and positioned again in the center of the observation box. The other visual stimulus (fly larvae or male *P. shermani*) was placed in one of the petri dishes, leaving the remaining empty dish as a control. Female location was monitored for an additional 60 minutes by video camera. Each group of 12 females was tested once per week, for a total of four weeks until all treatment combinations had been administered. The initial order of treatment substance administration (between trial nights) and the order and location of stimulus presentation (within a trial, between sessions) were randomized. Each female was assigned the same male for each of her trials to control for variation in female preference for different males. Using the video records for each female, we manually tallied the duration of time a female spent in close proximity (head within 3 cm) to each stimulus or control. The person taking 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 duration of time spent in proximity to olfactory stimuli (both sexual and nonsexual) after administration of treatment substances. Protocols followed those of the visual experiment (described above), with the following modifications: (1) the moistened paper base lining each observation box was printed with numbered diagonal lines spaced at 2 cm intervals; (2) the olfactory stimulus consisted of a triangular cotton pad soaked in 3 ml of either male *P. shermani* scent or fly larvae scent; see Vaccaro et al. (2010) for methods for obtaining olfactory stimuli; (3) each 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.

Experimental Design & Statistical Analysis

Substantial intrinsic variability in the response to pheromone across subjects was expected, so we employed a crossover design such that each treatment was applied to each subject over successive testing periods. The rationale for this crossover design was to maximize efficiency by having each subject serve as her own control, thereby enabling greater precision and permitting the use of a relatively small sample size (n = 48 females). To minimize the possibility of carryover effects from previous treatments, trials for each group of twelve females were scheduled after a seven day wash-out period between trials. To further separate treatment effects from possible carryover effects, treatment assignments were ordered as digram-balanced Latin square designs (Wagenaar 1969; Ratkowsky et al. 1993) such that each treatment was preceded and followed by the other treatments with equal frequency.

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 (PRF, PMF, whole pheromone extract, or saline 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. Furthermore, each subject was exposed to each treatment by stimulus combination making this a crossover split-plot-in-time design (Raghavarao & Xie 2003) with multiple levels of grouping (i.e., session within trial within subject). Our analysis accounted for interdependence (correlations) among observations within the same grouping. Mixed models enabled us to represent the covariance structure associated the multiple sources of random variation intrinsic to multilevel, grouped data (Pinheiro & Bates 2000). Our analyses evaluated the effect of treatment and stimulus combinations on stimulus affinity, while accounting for

random variation attributable to subject, stimulus location, trial and session. For each experimental analysis, we applied the following contrasts for parameter estimation: (1) saline versus: PMF, PRF and whole pheromone for the male stimulus; (2) saline versus: PMF, PRF and whole pheromone for the food stimulus; and (3) food vs. male stimulus (across all treatments).

Statistical analyses were performed in the *R* statistical computing environment (version 2.12.1; R Development Core Team 2011). Model assessment used likelihood ratio tests, with parameter estimation by restricted maximum likelihood (REML) using *lme4a*, a linear mixed modeling package (version 0.999375-61; Bates et al. 2011). Highest posterior density (HPD) intervals and *P*-values were generated using Markov chain Monte Carlo (MCMC) simulations (n = 10,000) in the *language R* package (version 1.0, Baayen 2010). We also report probabilities based on the *t*-distributions with the number of observations minus the number of fixed effects coefficients as the degrees of freedom, and with the caveat that these *P*-values could be anti-conservative with smaller data sets.

RESULTS

Visual Response Trials

Analyses revealed suggestive but not significant evidence that whole pheromone extract increased the amount of time a female was interested in the sight of food by an average of 4.1 min over control treatment (95% HPD interval: -0.2 to +9.0 min, *pMCMC* = 0.09; see Table 4.1A). These results align with a previous study in which the estimated effect size and significance of pheromone treatment on the sight of food was similar (Vaccaro et al. 2010).

Tests revealed no evidence for a difference in affinity for food visual stimulus over control for PRF or PMF, nor a difference in affinity for male visual stimulus over control for PRF, PMF, or whole pheromone extract. In fact, the analysis established that attraction to male visual stimuli was immune to influence by PMF (null

hypothesis *pMCMC* = 0.03) and whole pheromone extract (null hypothesis *pMCMC* = 0.01). Finally, no evidence was found for a broad difference in affinity for male versus food visual stimulus (regardless of treatment); in fact, there was relatively strong support for equivalent affinity for food and male visual stimuli (null hypothesis *pMCMC* = 0.07). The estimated subject-to-subject (random) variation in the model intercept corresponded to a standard deviation of 8.4 min (95% HPD interval of 4.1 to 9.1 min).

Olfactory Response Trials

PRF significantly increased female affinity for male scent by an estimated 3.8 min over the control treatment (95% HPD interval: 0.7 to 6.8 min, *pMCMC* = 0.02; see Table 4.1B). There was no evidence for a difference in affinity for male scent caused by PMF (*pMCMC* = 0.73). In contrast to the Vaccaro et al. (2010) study, the analysis could not conclusively determine whether whole pheromone affected time spent at the male scent (estimate: -2.5 min, 95% HPD interval: -5.5 to +0.5 min, *pMCMC* = 0.10). While evidence suggested a broad difference in affinity for male versus food scent stimulus, with females spending 1.2 min longer at the male stimulus on average (95% HPD interval: -0.04 to +2.4 min,*pMCMC* = 0.06), this difference in preference does not appear to be attributable to the whole pheromone extract or either of its main components. The estimated subject-to-subject (random) variation in the model intercept corresponded to a standard deviation of 3.8 min (95% HPD interval of 0 to 4.4 min).

DISCUSSION

Our approach addressed indirectly the neurophysiological mechanisms by which individual pheromone components may differentially activate areas of the brain responsible for courtship and sperm transfer. These components, acting along dedicated neural routes, have the potential to modulate both the timing and sequence of: (1) the activation of relevant behaviors and (2) the suppression of irrelevant behaviors. This study provides significant evidence that: (1) a salamander pheromone

component enhances female affinity for sexual stimuli, but not ingestive stimuli, and (2) this enhanced attraction is evident in the olfactory modality but not in the visual modality, and (3) this pheromone-induced attraction to the male scent is mediated by the pheromone component, PRF.

These results concur with earlier work showing that the entire extract of these salamander pheromones enhances female affinity for the male scent (Vaccaro et al. 2010). It now appears that this enhanced affinity to male scent is attributable to the PRF component alone. In contrast, PMF does not appear to function in female attraction to male stimuli. Nonetheless, PMF may still function to enhance female receptivity, albeit through indirect means. PMF may increase female receptivity as either: (1) a secondary consequence of altered general central nervous system arousal (Pfaff 2006; Vaccaro et al. 2010) or, (2) an indirect consequence of suppressed competing motivations, e.g., hunger (Vaccaro et al. 2009). While this study finds no evidence that PMF inhibits the sensory processing of the olfactory or visual components of food (as measured by time spent associating with these stimuli), further investigation is warranted to determine whether PMF affects other relevant behaviors associated with hunger (as measured by actual food consumption). To this end, previous work has demonstrated that whole pheromone extract reduced consumption of fly larvae (Vaccaro et al. 2009), and it is hypothesized that this effect is mediated by PMF.

When a *P. shermani* male taps his mental gland on the female's nares during courtship, pheromones enter the female's nasal cavity and are shunted laterally to the vomeronasal organs (VNO) (Dawley & Bass 1989). The VNO is a component of the accessory olfactory system that is capable of responding to non-volatile proteins, in contrast to the main olfactory system which generally responds to volatile compounds (Halpern & Martínez-Marcos 2003). Plethodontid pheromones have been shown to bind to cognate receptors in the female's vomeronasal epithelium (Wirsig-Wiechmann et al. 2002), activating specific populations of vomeronasal neurons (Wirsig-

Wiechmann et al. 2006). As in most tetrapods, the vomeronasal sensory neurons of *Plethodon* salamanders project to the accessory olfactory bulb (Schmidt et al. 1988); from there, projection neurons extend to the amygdaloid complex (Laberge & Roth 2005; Laberge et al. 2006). The pathway from the vomeronasal sensory neurons to the vomeronasal amygdala is isolated from the influence of other brain regions, and the vomeronasal amygdala has abundant direct projections to the hypothalamus (Laberge & Roth 2005; Laberge 2008). In particular, *P. shermani* pheromone stimulation of the vomeronasal amygdala results in neural activation of the ventromedial hypothalamus (VMH) and preoptic area (POA) of the hypothalamus, and then the raphe median (Laberge et al. 2008). The amygdala, VMH and POA are necessary for normal female arousal and sexual behavior in mammals (Swanson 2000), and the raphe median is a principal serotonergic area of the brain. In general, activation of the POA is associated with sexual behavior in males (Woolley et al. 2004), while activation of the VMH (Pfaff & Sakuma 1979; Emery & Moss 1984; Kendrick et al. 1995; Floody 2002) and inhibition of the POA (Aou et al. 1988) are associated with normal receptive behavior in females.

These uniquely direct connections to the hypothalamic region suggest that vomeronasal information reaching the "behavioral brain" is processed distinctly from other sensory modalities. Furthermore, the timing of neural activation likely plays a critical role in female reproductive behavior. Detailed studies in mammals have shown that, although female appetitive (proceptive, courtship) behavior is characterized by the *inhibition* of the POA, female consummatory (receptive, mating) behavior is characterized by *activation* of the POA (Aou et al. 1988; Hoshina et al. 1994). Specifically, a particular set of POA neurons is associated with each facet of female sexual behavior (Kato & Sakuma 2000). As such, the particular timing and sequence of behaviors necessary for a successful courtship and insemination are mirrored by the particular timing and sequence of neural activation in affiliated brain regions.

Further research in plethodontid salamanders may elucidate how pheromone sensory input is relayed, modulated and processed en route to brain regions that control the motor elements of motivated behavior. By examining patterns of neural activation following stimulation by pheromone components, we may be able to establish whether these components act along dedicated neural routes to modulate the timing and sequence of relevant behaviors. Thus, a complex mixture of pheromone signals may be capable of eliciting a complex series of behaviors in the recipient.

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Figure 4.1. Courtship pheromone delivery. During tail-straddling walk, a male *Plethodon shermani* (on right) turns to deliver pheromones via quick taps with his pheromone-producing mental (chin) gland to the female's nares. Photo reproduced with permission from S.J. Arnold.

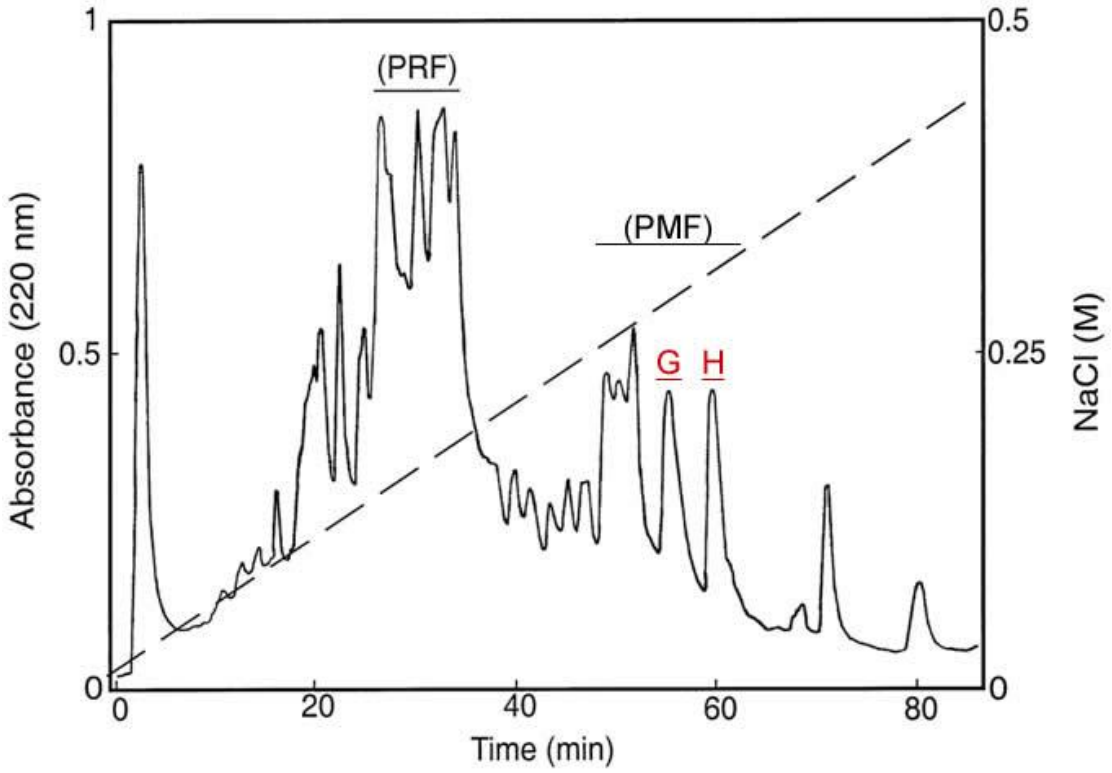

Figure 4.2. Example anion-exchange HPLC chromatogram of *P. shermani* pheromone extract. Samples of the mental gland extract (1.2 to 1.7 ml) were separated to reveal multiple isoforms of the proteinaceous components. The 22 kDa PRF fraction contains 3 major isoforms and the 7kDa PMF fraction contains ~50 isoforms. The PMF-G and -H isoforms are expressed at very high levels. Modified from Rollman (1999).

Table 4.1. Female salamander attraction to male or food stimuli following different pheromone treatments. Affinity was measured as the time spent in proximity to the stimulus in the 60 min following treatment. (A) Visual stimuli: the food stimulus was represented by six fly larvae visible to (but otherwise isolated from) the female; the sexual stimulus was represented by a reproductively active male visible to (but otherwise isolated from) the female. (B) Olfactory stimuli: the food stimulus was represented by a cotton pad soaked in fly larvae wash; the sexual stimulus was represented by a cotton pad soaked in a wash from reproductively active males. Parameters represent different treatment-by-stimulus contrasts. Estimates were made using linear mixed effects modeling with restricted maximum likelihood (REML) and Markov chain Monte Carlo (MCMC) simulations (n = 10,000). Probabilities based on the *t*-distributions are included below (in italics) with the caveat that these *P*-values tend to be anti-conservative, especially with small data sets. Significance codes: ** indicates ≤ 0.001 and * indicates ≤ 0.05.

Table 4.1

A. VISUAL CONTRASTS	MCMC mean *REML mean*	95% HPD *95% CI*		pMCMC *Pr(>|t|)*
(Intercept)				
	16.69	-54.88	85.80	0.313
	17.18	*4.59*	*29.78*	*0.001 ***
All Treatments (Food - Male)				
	-0.08	-2.10	1.76	0.932
	-0.09	*-1.96*	*1.77*	*0.922*
Food (PMF - Control)				
	-1.45	-5.99	3.38	0.554
	-1.46	*-6.04*	*3.09*	*0.534*
Food (PRF - Control)				
	-1.47	-6.12	3.35	0.542
	-1.53	*-6.12*	*3.02*	*0.514*
Food (Whole Extract - Control)				
	4.10	-0.58	8.98	0.089
	4.12	*-0.43*	*8.71*	*0.081*
Male (PMF - Control)				
	0.09	-4.59	4.78	0.967
	0.11	*-4.47*	*4.67*	*0.964*
Male (PRF - Control)				
	0.49	-4.08	5.26	0.841
	0.47	*-4.11*	*5.03*	*0.843*
Male (Whole Extract - Control)				
	0.01	-4.63	4.78	0.994
	0.00	*-4.57*	*4.56*	*1.000*

B. OLFACTORY CONTRASTS	MCMC mean *REML mean*	95% HPD *95% CI*		pMCMC *Pr(>|t|)*
(Intercept)				
	8.59	-28.30	43.75	0.321
	8.65	*2.39*	*14.91*	*0.001 ***
All Treatments (Food - Male)				
	-1.21	-2.44	0.04	0.055
	-1.19	*-2.38*	*0.03*	*0.054*
Food (PMF - Control)				
	-0.24	-3.28	2.75	0.867
	-0.24	*-3.13*	*2.74*	*0.873*
Food (PRF - Control)				
	-1.14	-4.15	1.84	0.450
	-1.14	*-4.15*	*1.77*	*0.453*
Food (Whole Extract - Control)				
	0.37	-2.57	3.45	0.810
	0.36	*-2.64*	*3.25*	*0.813*
Male (PMF - Control)				
	0.51	-2.57	3.39	0.730
	0.53	*-2.39*	*3.47*	*0.727*
Male (PRF - Control)				
	3.76	0.70	6.76	0.019 *
	3.77	*0.78*	*6.72*	*0.014 **
Male (Whole Extract - Control)				
	-2.49	-5.50	0.46	0.099
	-2.47	*-5.42*	*0.45*	*0.102*

CHAPTER 5. META-ANALYSIS OF 17 COURTSHIP EXPERIMENTS ON TERRESTRIAL SALAMANDERS PROVIDES NOVEL INSIGHTS INTO MATING STRATEGIES

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INTRODUCTION

Though courtship may be considered a complex negotiation between the sexes, courtship outcomes may be influenced more by one sex than the other. Within a system of female mate choice, a discriminating female evaluates a displaying male. Thus, courtship provides crucial input for mate choice criteria. Mate choice is the major dynamic by which a mating system is shaped by sexual selection. A female's decision is a function of both her preference and her receptivity. While the terms "female preference" and "female receptivity" are sometimes used in an overlapping or interchangeable manner, for the purposes of this chapter, I distinguish between them. Preference is an affinity for certain male characteristics (Ryan 1997; Ryan et al. 2009). Preference exists independently of a given male that the female encounters. Receptivity is the moment-by-moment tendency to mate, and in contrast to preference, female receptivity is absolutely susceptible to the efforts of the particular male that presents himself.

The operative differences between preference and receptivity can be difficult to distinguish. For example, a female could have a preference for male behaviors that increase receptivity, such as male persistence. Conversely, if a female's preference is for a diversity of mates over successive courtships, then attributes that she once found attractive may lower her receptivity in subsequent courtships. Furthermore, the factors affecting female preference and receptivity may be overlooked in experimental studies unless they are explicitly investigated: the laboratory setting controls for most relevant physiological and environmental variables, and therefore these variables are masked from observation. Finally, the concepts of preference and receptivity are based on experimental inference rather than direct examination; thus, like any internal state, the ultimate behavioral outcome alone readily submits itself to experimental scrutiny.

A new generation of mechanistic studies has illuminated the tricky calculus of female mate choice criteria. Female preference is determined by genetics, previous experience (Lea et al. 2000; Kodric-Brown & Nicoletto 2001), and social context (e.g.,

cues from conspecifics: Alonzo & Sinervo 2001; Royle et al. 2008), while female receptivity is influenced by physiological condition and reproductive state (Aisenberg & Costa 2005; Lynch et al. 2005).

These studies indicate that female mate choice is influenced by a complex cadre of innate and environmental factors. As such, how might a male best navigate these murky and impenetrable waters to ensure his mating success? In another plethodontid, the Ocoee salamander (*Desmognathus ocoee*), males that were highly persuasive mated more frequently than males of lower persuasiveness, irrespective of the females' level of responsiveness (Gershman & Verrell 2002). These highly persuasive males also exhibited more types of sexual behaviors than less persuasive males. This study indicates that a broad behavioral repertoire may aid male reproductive success when confronted with variation in female receptivity.

First, we consider a scenario in which preference for male traits varies among females. A male that is not in possession of sufficiently desirable environmental resources, physiological characteristics or social status could improve his reproductive fitness by catering to a given female's preferred behavioral traits in a suitor. Thus, sexual selection would reward a male capable of gathering feedback from a female regarding her intrinsic preferences and adapting his behavioral repertoire accordingly. In particular, a male encountering a novel potential mate would do well to: (1) be attuned to the individual female's preferences, and (2) cater his behavior to the greatest extent possible to these preferences. In contrast, a male encountering a familiar female might do well to remember that female's preferences and behave in an accordingly consistent manner.

Next, we consider a scenario in which receptivity also varies within a given female. A female's receptivity may decline over successive courtships with the same male. In other words, a female may have a higher receptivity threshold (be less receptive) when courted by a familiar male. How would the male respond in such a case? The feedback he receives from this female may be decidedly different than the

first time they mated. In this case, it could be to the male's advantage to determine whether the amount of persuasion necessary to court a recalcitrant female is worth the expenditure of effort and time. In any instance, a male should increase his reproductive success by: (1) becoming more efficient at learning a novel female's preferences, (2) remembering the preferences of an amenable familiar female, and (3) evaluating the amount of persuasive effort he is willing to dedicate to an obdurate familiar female.

Experimental studies of the influence of male behaviors and female preference or receptivity on courtship duration must account for substantial inter-individual variation and thus require an often prohibitively large sample size. Using a meta-analysis approach, we obtained a sufficiently large sample size by compiling 17 studies across 13 years of courtship experiments in terrestrial salamanders (*Plethodon shermani*). The courtship and mating behavior of these terrestrial salamanders is discussed comprehensively elsewhere (Arnold 1976) and is summarized here: these salamanders have a multi-month reproductive season during which a male encounters potential mates infrequently. Initially, a male woos a female using a wide array of behaviors, such as head-to-body contact, foot dancing, and tail arching. The male does not clasp the female during courtship and mating, but an amenable female will engage the male in a "tail-straddling walk." During this time, pheromones are delivered via quick taps of the male's chin gland to the female's nares (also called "slaps," cf. Arnold 1976). Males lack an intromittent organ and instead deposit a spermatophore on the ground. Insemination occurs only if a female retrieves the spermatophore's apical sperm mass with her cloaca. For each of the above steps, absolute female compliance is crucial for successful mating.

The studies that comprised our meta-analysis data set focused on the role of pheromones in modulating courtship behavior. These studies generated records of two measurable outcomes of a courtship interaction: (1) latency to insemination, measured as courtship duration beginning with the initiation of tail-straddling walk and ending

with spermatophore deposition; and (2) whether or not insemination was accomplished (see Table 5.2. for a summary of the meta-analysis variables). As yet, none of the individual studies has been able to determine factors affecting insemination rates. Our meta-analysis data set also included the experimental treatment (the variable of primary interest in the studies that comprise our data set, standardized to a saline control), and the year that the study was conducted (used to account for systematic differences from year to year). These studies also generated behavioral data that was the focus of our meta-analysis, including: the number of times a female had mated across experimental trials (a proxy for female receptivity), the number of times a male had mated across trials (a proxy for male persuasiveness), the number of times a given pair had mated across trials (a proxy for pair compatibility), and the number of pheromone delivery taps during a given courtship (a proxy for male courtship effort). While this meta-analysis served as a formalized synthesis of current information, the data incorporated were by no means complete. In particular, we were unable to re-create records for staged encounters in which a given male or female did not engage in courtship. As such, our inferences are limited to courtships that proceeded to completion (i.e., to spermatophore deposition).

We endeavored to elucidate the role of male strategies to address the dynamics of female mate choice. In order for these context-dependent mating strategies to exist, females first must demonstrate systematic differences in their mate preference or receptivity. As such, a female would prefer a type of courtship behavior that aligns with her preferences, or an intensity of courtship behavior that caters to her level of receptivity. For the purposes of this study, the number of times a female had engaged in courtship (regardless of whether insemination occurred) served a proxy for broad differences in female receptivity, although a higher number may also indicate less discriminate preferences. Second, the means for a strategy must exist: males must be able to both: (1) ascertain these differences among females, and (2) exploit behavioral flexibility to accommodate these differences. As such, the manner in which a male courted a female would be dependent on that female. For the purposes of this study,

the number of chin taps that a male delivered to a female serves as proxy for the male courtship effort. Male effort may be influenced by female receptivity, contribute to male persuasiveness, and/or be a response to pheromone efficacy. Finally, evidence of male strategies must exist: the context-dependent behaviors exhibited by males must be associated with increased mating success. For the purposes of this study, there must be an interaction between male and female characteristics affecting the duration of courtship or the probability of insemination.

In summary, the aims of meta-analysis were three-fold: (1) to quantify variation in female willingness to engage in courtship, (2) to quantify variation in male effort expended during courtship, and (3) to determine whether these male and female characteristics interacted in a manner to shorten courtship or affect the probability of insemination. Our results provided evidence that time spent in the mutual participation phase of courtship (tail-straddling walk) was associated with: (1) the female's *a priori* receptivity level, (2) male-female compatibility, (3) the intensity of male courtship efforts, and (4) pheromone treatment. Furthermore, insemination success was associated with the interaction between male-female compatibility and the intensity of male courtship efforts. Thus, we provide evidence that males can use context-dependent mating strategies to improve their reproductive success.

METHODS

Animal Collection and Maintenance

Each summer for the ten years of 1996 through 2007, adult males and gravid females of *P. shermani* were collected during the breeding season from a single locality in Macon County, North Carolina, U.S.A (35°10′48″ N, 083°33′38″ W). 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 waxworms (lepidoteran larvae) weekly. Shortly following salamander collection, the pheromone-delivery mental (chin) glands were surgically ablated from anesthetized males and pheromone extracts for experimental treatments were

prepared. Methods of gland removal and treatment solution preparation follow established protocols (Houck et al. 1998; Rollmann et al. 1999). Within 1-2 days of gland removal, males court and feed normally; however, to ensure complete recovery, they were not used in courtship trials for at least 1 week. Animals were prescreened to assess willingness to mate under laboratory conditions. Following prescreening, animals that had courted one or more times were shipped to Oregon State University (OSU), Corvallis, U.S.A. where behavioral experiments were conducted. Animals were kept in similar conditions, at 15-18ºC on a late August North Carolina photoperiod. A North Carolina scientific collecting permit was obtained each year, and animals were cared for using protocols approved by the Animal Care and Use Committee at OSU. Preserved vouchers were placed in the Oregon State University herpetological collection.

Experimental Protocols

Courtships were observed in the context of multiple laboratory experiments conducted from 1995 through 2007 (Table 5.1). Experiments tested the effects of various pheromone treatments on female receptivity using staged encounters between male-female pairs during September-November of the collection year. Pairs were placed in individual courtship boxes at approximately 9pm EST. Focal animal sampling (Altmann 1974) began when a pair began a tail-straddling walk. Once a pair was engaged in tail-straddling walk, that pair was observed continuously. Courtship duration was defined as beginning when the female first began tail-straddling walk and ending with the completion of spermatophore deposition (cf. Houck & Reagan 1990; Rollmann et al. 1999; Houck et al. 2007). During courtship, a male would tap his chin to the female's nares. This behavior normally would result in pheromone delivery; however, the male's pheromone-producing mental gland had been surgically removed. Pheromones instead were administered experimentally: for each pair, 4-5 µl of pheromone extract (specific pheromones varied by experiment) or a control solution (1/2X phosphate buffered saline) was applied immediately after the male's chin first

tapped the female's nares. An additional two applications of treatment solution were administered during courtship to simulate regular pheromone delivery by the male.

Data Collection

For this meta-analysis, data over the 13 years of courtship observations were collected, concatenated and standardized to assess the factors associated with courtship and insemination success. For each experiment, a male was given between one and ten opportunities to court a female. For certain experiments, each male-female combination was unique; in other experiments, the same individuals were paired throughout the duration of the experiment. Among the pairs that mated during these staged encounters, observers usually scored: (1) the time when the females received pheromone treatment, (2) the number of pheromone-delivery taps given by the male, (3) the duration of courtship (from the initiation of tail-straddling walk to the conclusion of spermatophore deposition), and (4) whether sperm transfer was accomplished (see Table 5.2. for a summary of the meta-analysis variables). Data were expected to vary between data sets by: (1) specific pheromone treatment, which varied by experiment and by year, (2) collection site, which varied by year within a 10 km radius, (3) observers, who varied over the years (though observation protocol remained fairly consistent), and (4) the completeness of the data recorded, which varied by experiment (e.g., records for tap and insemination data frequently were incomplete).

Statistical Analysis

Courtship duration and insemination each were modeled as a function of the following variables: (1) female receptivity index (FRI: the number of times a female had mated in the lab), (2) male persuasiveness index (MPI: the number of times a male had mated in the lab), (3) pair compatibility index (PCI: the number of times a given male-female pair had mated in the lab), and (4) the treatment administered (various pheromone treatments, saline control, or no treatment). Included as a covariate was the number of pheromone delivery taps administered during the courtship (a proxy for

male effort). Based on preliminary data exploration, tap number and female receptivity were modeled as both linear and quadratic terms. Interactions were modeled between tap number and: (1) female receptivity, and (2) male persuasiveness. Included as random effects were the grouping variables for individual male and female subjects, and the year the study was conducted.

Several data transformations were necessary to compensate for the peculiar nature of the data, e.g. non-normality, heteroscedascity, and unequal sample sizes among levels of grouping. Sampling was very sparse at higher PCI numbers, so we compared pairs that mated once versus pairs that courted repeatedly, i.e. ≥ 2 times. Courtship typically involves tapping behavior, thus in order to model a "typical" courtship, the transformed tap number was centered by its mean (Schielzeth 2010). Pheromone treatments were generalized to several broad categories (Table 5.1) and we compared courtships with saline control treatment to each of the other treatments and to courtships with no treatment. Furthermore, correlations were expected between the various male, female and pair indices and between treatment and study year, so we assessed collinearity using variance inflation factors.

The meta-analysis data set functioned as a very large partial factorial design between male and female subjects. This design also had unbalanced replications, its variables were both non-normally distributed and heteroscedastic, and its treatments had unequal sample sizes. Complex groupings among interdependent data, both nested and crossed, were intrinsic to the design. As such, the meta-analysis data were analyzed using mixed effect modeling in order to account for: unbalanced data (both in terms of treatment sample sizes and replications within a subject), and groupings among the response variables by year, male subject, and female subject. The courtship duration response variable (a zero-truncated integer) was modeled using generalized linear mixed models assuming either a Gaussian or log-linked Poisson distribution (Bolker et al. 2009). Additionally, and we assessed the appropriateness of fit for simple linear models and additive models. The insemination data (binary response

variable) were modeled as a logistic regression on a Bernoulli (binomial) distribution with generalized linear mixed effects modeling. Likelihood estimation methods varied by model type. Linear mixed models used restricted maximum likelihood (REML) and Markov chain Monte Carlo (MCMC) simulations (n = 10,000). For the more computationally intensive generalized linear mixed models, Laplace approximations were used (Pinheiro & Chao 2006). Model selection, where appropriate, was performed using backwards Akaike information criteria (AIC) and likelihood ratio tests on maximum likelihood (ML) estimates.

Statistical analyses were performed in the *R* statistical computing environment (version 2.13.1; R Development Core Team 2011). Model assessment and parameter estimation used *lme4*, a linear mixed modeling package (version 0.999375-40; Bates et al. 2011). The *language R* extension was used for MCMC sampling and *P*-value estimates (version 1.2, Baayen 2010).

RESULTS

Summary of the Meta-Analysis Data Set

Overall, we obtained records on 1,130 completed courtship encounters (i.e., that proceeded to the point of sperm transfer) involving 738 females and 715 males. Of these 1,130 courtship records, we obtained complete records (i.e. data for all of the meta-analysis variables) for 912 courtships when courtship duration was the measured response and complete records on 430 courtships when insemination was the measured response. The following summary pertains to the 912 records for which courtship duration was the response. We obtained records on repeated courtships for 291 females and 282 males (Fig. 5.1). Of these repeat courtships, 141 courtships were between familiar (rather than novel) male-female pairs, comprising 78 unique familiar pairs. Whether females were paired with the same or different males depended on each experiment's protocol. The maximum recorded number of completed courtships for a given female was eight, for a given male was five, and for a given pair was five. Note that inferences are inherently limited at higher index numbers as the sampling was

very sparse; e.g., only seven females mated five times, no females mated six or seven times, and one female mated eight times.

Overall, the mean courtship duration was 35.4 min (standard deviation: 21.4 min), the median was 33 min, the shortest was 3 min, and the longest courtship duration recorded was 131 min. The mean number of taps recorded was 9.6 (standard deviation: 11.1); the median was 6, the minimum was 0 and the maximum number of taps recorded was 69.

Factors Associated with Shortened Courtship Duration

A typical courtship, modeled with an average number of taps and the control treatment, lasted for 32.0 min (95% HPD = 27.2 to 38.1 min, t_{REML} = 41.32, P_{MCMC} = 0.000; see Table 5.3 for statistical summaries of model coefficients). Courtship duration covaried with the number of pheromone delivery taps (our proxy for male effort). This effect was curvilinear, peaking at 39 taps with an estimated 40.6 additional min spent in courtship. Additional taps were associated with increasingly shorter courtship durations (linear effect: a 6.6% increase with each additional tap, t_{REML} = 29.58, two-sided P_{MCMC}-value = 0.000; quadratic effect: a 0.11% decrease for each additional tap^2, t_{REML} = -15.08, two-sided P_{MCMC}-value = 0.000).

Female receptivity (the index for the number of times a female had mated in the laboratory) was associated with increased courtship duration, with twice-mating females taking an estimated 12% longer than singly-mating females. This effect peaked with females that had mated four times taking an additional 24% longer than singly-mating females (linear effect: HPD interval = 3.5 to 24.6%, t_{REML} = 2.66, two-sided P_{MCMC}-value = 0.006; quadratic effect: HPD interval = -3.1 to -0.5%, t_{REML} = -2.70, two-sided P_{MCMC}-value = 0.007).

Pair compatibility (the index of the number of times a given pair had mated in the laboratory) was associated with shortened courtship duration. Multiply-mating pairs

took 15% less time to complete their courtships as compared to singly-mating pairs (HPD interval = -23.0 to -5.2%, t_{REML} = -2.97, two-sided P_{MCMC}-value = 0.003).

We report pheromone treatment effects here although these effects were not of primary interest to our study aims. In particular, PMF was associated with a 15% increase in courtship duration as compared to the saline control (HPD interval = 0.8% to 30.9%, t_{REML} = 2.14, two-sided P_{MCMC}-value = 0.035). We found evidence to suggest that whole pheromone extract, whether delivered dorsally or to the nares, increased courtship duration, however this effect was not significant (dorsal delivery: t_{REML} = 1.77, two-sided P_{MCMC}-value = 0.073; nasal delivery: t_{REML} = 1.72, two-sided P_{MCMC}-value = 0.063). The following treatments gave no evidence of an effect on courtship duration: the PRF pheromone component, heterospecific pheromones from *Plethodon cinereus, Plethodon yonahlossee* or *Plethodon montanus*, and conspecific pheromones from a different population of *P. shermani* (treatments described in Table 5.1).

Standard deviation estimates for random effects of male subject, female subject, and experimental year were near zero, indicating that the degree of subject-to-subject and year-to-year variability was relatively low after accounting for fixed effects in the log-linear regression model (Bates et al. 2011). Residuals analysis indicated no particular violations of the linear mixed model, which has been presented here and in Table 5.3. Generalized (Poisson) distributions and additive (smoother) effects on the Taps covariate did not provide sufficient improvements on the goodness-of-fit to warrant their incorporation in the model.

Factors Associated with Insemination Success

As is customary with a binary logistic regression, results are reported as odds ratios. We were able to compile complete insemination records for only two pairs that had mated more than twice (one pair mated three times, one pair mated five times), so we compared only single-mating pairs to twice-mating pairs.

The probability of insemination was 3.8 times the probability of not being inseminated (95% CI: 2.7 times to 5.4 times, z = 7.65, two-sided *P*-value < 2 x 10^{-14}). The probability of insemination for twice-mating pairs was estimated to have been 3.2 times greater than that of single-mating pairs (95% CI: 1.1 times to 9.1 times, z = 2.11, two-sided *P*-value = 0.03). Tapping behavior affected the odds of insemination, but this effect depended on pair compatibility scores: for twice-mating pairs, male effort (as measured by greater numbers of taps) was associated with decreased odds of insemination (0.92 times less per tap, z = -2.86, two sided *P*-value = 0.002); however, for single-mating pairs, intense tapping behavior may have actually increased the odds of insemination, though this effect was not significant (1.02 times greater per tap, z = 1.26, two sided *P*-value = 0.10).

The random effects of female subject-to-subject variability corresponded to a standard deviation of 0.095. The level of male subject-to-subject variability and year-to-year variability were not sufficient to warrant incorporating these random effects in the model.

DISCUSSION

Compiling data from multiple studies often provides the opportunity to infer broad patterns not seen in individual studies. From our meta-analysis of 17 studies of behavioral data on salamander courtships, we found evidence that time spent in the mutual participation phase of courtship was associated with the female's *a priori* receptivity level, male-female compatibility, male effort (tapping behavior), and pheromone treatment. Furthermore, insemination was associated with the interaction between male-female compatibility and the intensity of male courtship behavior.

Our results bolstered findings in published studies, first by finding no evidence of inconsistencies from year to year, and second by providing expanded inference to multiple years of data. In particular, inferences about the actions of PMF from Houck et al. (2007), can be expanded from the 2002 experiment to experiments in 1997 and

2000. Furthermore, inferences about the actions of PRF from Rollmann et al. (1999) and rPRF from Houck et al. (2008) can be expanded tentatively from their representative experiments (in 1998 and 1995, respectively) to similar experiments in years 1995, 1996, 1997 and 2004. We have omitted discussion of the heterospecific pheromone experiments from 1996, which included all heterospecific pheromones investigated except for *P. cinereus*, as our analysis cannot provide a broader scope of inference than the original analysis conducted by Rollmann et al. (2003).

A quick caveat before proceeding: a meta-analysis is, by nature, an observational study, and therefore causal inferences cannot be made. Thus, inferences in the discussion that follows serve as hypotheses warranting further investigation.

Variation in Female Receptivity and the Required Amount of Male Persuasion

Systematic differences in the amount of assessment required by females of varying receptivity may indicate the existence of broad classes of female preferences. Our analysis revealed that females varied in the number of courtships a given female would engage in over numerous staged encounters. Many factors outside of the scope of our analysis could account for this variation in *a priori* receptivity. For instance, highly receptive females may have been in prime reproductive condition. Alternately, females may experience a post-refractory resurgence in sexual receptivity. While our analyses found no evidence of order effects (such as a sexually-refractive period) over successive courtships, this result may be a function of experimental protocol: females were generally given a recuperation period following staged courtship encounters. Therefore, subsequent encounters for mated females may have occurred during a resurgence in sexual receptivity following the refractory period. Our results demonstrate that higher female receptivity is associated with differences in female assessment during courtship, as measured by latency to insemination.

Courtships with single-mating females were the most common type of courtships. The durations of these courtships were the shortest across female receptivity levels.

Thus, single-mating females may both be highly discriminating and demonstrate clear preferences, as evidenced by courtships that were rarely entered into, but with alacrity. We presume that the role of assessment conducted by the female before engaging in the mutual participation phase of courtship may be of particular importance to these females, although evaluating this supposition is beyond the scope of this study. Among plethodontid salamanders, it has already been hypothesized that much of the species- and sex-recognition occurs before a pair has engaged in physical contact (Rollmann et al. 2003). It is possible that a female may evaluate much of a male's alignment with her preferences during the male display phase and before engaging in tail-straddling walk. Indeed, the female has ample time to assess male visual stimuli (foot dancing and tail undulations), olfactory stimuli (male odorants conveyed during physical contact) and somatosensory stimuli (head and flank rubbing) before deciding to advance to the tail-straddling walk phase of courtship.

Courtships for multiply-mating females, contrary to expectations, constituted some of the most prolonged assessments. These results may reflect a general tendency among highly receptive females to shift the primary assessment period from the earlier male display phase to the tail-straddling walk phase. Pre-courtship assessment may have been insufficient for these females, who instead demanded extra assessment time and the correlated extra male effort: more male pheromone delivery behavior. Furthermore, females with higher *a priori* receptivity could be more likely to engage in courtship with an unvetted male, and we speculate that this may coincide with a correlated shift of spending less time assessing visual and somatosensory displays in the earlier stages of courtship. Alternately, given their relative experience with courtship and mating, highly receptive females may be more efficient at communicating their preferences. As such, courtship may serve as a period of attrition for contending males. Of particular interest are the findings that: (1) the chances of insemination decreased with increasing tapping effort on the male's part, irrespective of female receptivity, (2) courtships in which the males delivered the most taps were also the most efficient, in that the rate of tapping increased with higher tap number,

and (3) the rate of tapping increased significantly over subsequent courtships for a given female (order effect on tap rate; unpublished analysis by E. Vaccaro). Together, these results suggest that multiply-mating females may elicit both the most efficient and most intense courtship behavior from their male counterparts.

Male- and Female-Specific Attributes Contributing to Insemination Success

Chances of insemination were associated with the interaction between male-female compatibility and the intensity of male courtship effort. The probability of insemination was over three-times higher for twice-mated pairs than for single-mated pairs. We had insufficient data on insemination to make inferences beyond male-female pairs that had mated twice. Our analysis demonstrated that it was not the persuasiveness of the male alone nor the responsiveness of the female alone, but the compatibility of a given pair that increased the chances of insemination. Furthermore, for twice-mated pairs, the extra time and effort a male spent tapping may not have been merely unproductive, but actually counterproductive. This effect was independent of the order of the courtships, thus we can provide no evidence of a learning type of effect for males mating with familiar females or vice versa.

Context-Dependent Strategies to Improve Male Mating Success

The measured differences in the chances of insemination were a function of the interaction between male effort and pair compatibility. This interaction dictated that male reproductive success can be maximized by employing two different strategies, depending on the dynamic between the male and the female. For less compatible pairs, the male should maximize his effort during courtship, i.e., the number of pheromone-delivery taps. For more compatible pairs, overly intense pheromone-delivery behavior is counterproductive. Interestingly, without a significant effect for the male persuasiveness index (number of completed courtships for a given male), there was no support for the hypothesis that females prefer the “fittest” males. However the fact that the pair compatibility effect on courtship duration was significant suggested that females may prefer compatible males in an assortative mating context.

Pair compatibility presumably emerges from the process of mutual assessment between the male and female during courtship. For a compatible pair, the female may be better at conveying her preferences during courtships and, conversely, the male may be better at gauging the female's preferences. Reciprocal assessment during courtship should enable the male to be attuned to the female's changing receptivity in response to his signals. As such, the male can cater his behavior to maximize his courtship success. As an experienced male increases his mating success across successive courtships, the duration of the courtship negotiation should decrease accordingly. As such, our study provided evidence of context-dependent male mating strategies based on the male's ability to: (1) gauge a given female's moment-by-moment receptivity, (2) infer her preferences for mating behavior based on her receptivity, and then (3) cater his courtship efforts accordingly.

Sexual selection should favor a male that is capable of honing this process of preference-attunement and behavioral-catering over successive encounters with potential mates. However, it may behoove a male to be consistent, as well as flexible, in his behavior. While flexibility may allow a male to cater his behavior to the female's needs, flexibility may delay insemination while the male engages in a lengthy reciprocal assessment with the female. In contrast, consistency may avoid the investment of resources necessary to alter one's own behavior (Houck et al. 2010). Perhaps most importantly, consistency is a potentially heritable trait that endows a male with a unique behavioral identity (Sih et al. 2004) and can promote response to selection (Boake 1989). Future studies on the meta-analysis data will investigate the relative contributions of behavioral flexibility and consistency over repeated courtships for males and females.

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Table 5.1. Summary of experimental studies comprising the meta-analysis data set. Year represents when the study was conducted; multiple studies may have been conducted in a single year. The pheromone of study was the experimental treatment and primary variable of interest for these studies. The origin of the pheromone designates the species from which the pheromone was extracted (and when relevant to study, the year of the pheromone extraction), or alternately, if recombinant pheromone components were used. Experimental treatment was most commonly delivered via pipette to the female's nares (first delivered following the male's first tap during courtship, then delivered again after 10 min and 20 min); however pheromones were delivered naturally in the 1998-I study, and experimentally via a dorsal patch in 2006. Courtship records were incomplete for several years; however, courtships for which we have even partial records are listed here. Animals may have been paired with novel or familiar partners over multiple courtships for a given study. Publications that were generated from a given study are listed.

Table 5.1

Year	Pheromone of Study	Origin of Pheromone	Experimental Delivery	Courtship	Pairing Type	Publication
1995 - I	Major component: PRF	*Plethodon shermani* (1995)	5 µl directly to nares X 3	38	Novel	
1995 - II	Major component: PRF	*Plethodon shermani* (1994)	5 µl directly to nares X 3	18	Novel	
1995 - III	Whole extract	*Plethodon shermani*	5 µl directly to nares X 3	[A]	Novel	
1995 - IV	Major component: PMF	*Plethodon shermani*	5 µl directly to nares X 3	[A]	Novel	
1996 - I	Major component: PRF	*Plethodon shermani* (1994)	5 µl directly to nares X 3	58	Novel	
1996 - II	Whole extract from: (1) conspecifics, other population (2) heterospecific *Plethodons*	*Plethodon shermani* *P. shermani ("Wayah")* *Plethodon yonahlossee* *Plethodon montanus*	5 µl directly to nares X 3	147	Novel	Rollmann et al. 2003
1997 - I	Whole extract; major components: PRF and PMF	*Plethodon shermani*	5 µl directly to nares X 3	123	Novel	
1997 - II	Major and minor components: inert loading peak, "enriched" PRF and PMF	*Plethodon shermani*	5 µl directly to nares X 3	65	Novel	
1997 - III	None[B]	N/A	N/A	95	Novel	
1998 - I	Whole extract, male delivery (versus deglanded male control)	*Plethodon shermani*	via glanded male	37	Novel	
1998 - II	Major component: PRF	*Plethodon shermani*	5 µl directly to nares X 3	102	Familiar	Rollmann et al. 1999
1998 - III	Unknown	N/A	N/A	70	Both	
2000	Whole extract; major component: PMF	*Plethodon shermani*	5 µl directly to nares X 3	55	Familiar	
2002	Major component: PMF[C]	*Plethodon shermani*	5 µl directly to nares X 3	64	Familiar	Houck et al. 2007
2003	Whole extract from heterospecific *Plethodon*	*Plethodon cinereus*	5 µl directly to nares X 3	42	Familiar	
2004	Major component: rPRF (single isoform)	bacterial expression system	5 µl directly to nares X 3	12	Novel	
2005	Major component: rPRF (single isoform)	bacterial expression system	5 µl directly to nares X 3	30	Both	Houck et al. 2008
2006	Whole extract, dorsal delivery	*Plethodon shermani*	5 µl applied via dorsal patch	101	Familiar	Kiemnec-Tyburczy et al. 2011
2007	None	N/A	N/A	73	Novel	

[A] Data unrecoverable; [B] Tapless courtships (as per experimental protocol, treatments can only be administered for treatments with 3 or more taps); [C] Most prevalent pheromone isoforms removed by Feldhoff lab

Table 5.2. Summary of meta-analysis model terms. Terms represent aspects of staged laboratory courtship encounters for *Plethodon shermani.*

Table 5.2

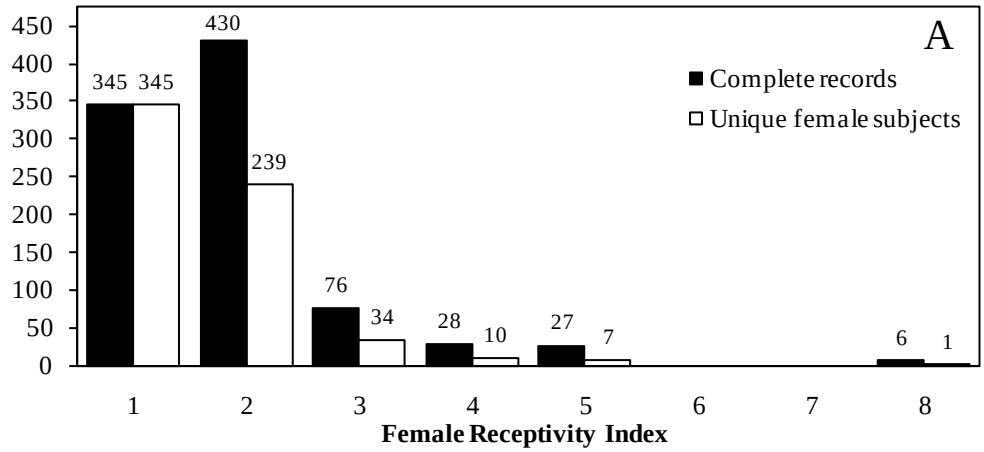
Model Term	Type of Data	Proxy Measure
Response variables		
Courtship duration (min)	Integer (positive)	Latency to insemination, result of male-female interaction
Insemination (yes/no)	Binary	Mating success
Explanatory variables		
Number of taps	Integer (count)	Male effort
Number of courtships, per male	Integer (count)	Male persuasiveness
Number of courtships, per female	Integer (count)	Female receptivity
Number of courtships, per pair	Integer (count)	Pair compatibility
Experimental treatment	Factor	Variable of interest in component studies
Taps x number of male courtships	Interaction	Male persuasiveness as a function of male effort, or vice versa
Taps x number of female courtships	Interaction	Female receptivity as a function of male effort, or vice versa
Taps x number of pair courtships	Interaction	Pair compatibility as a function of male effort, or vice versa
Taps x experimental treatment	Interaction	Male effort as a function of pheromone efficacy
Random effects		
Subject effect (male, female)	Factor	Variation attributable to differences among subjects
Year	Factor (ordered)	Variation attributable to differences across studies

Table 5.3 Meta-analysis regression coefficients at each level of the factors associated with courtship duration in *Plethodon shermani*. Model terms are as follows: tap number (male pheromone delivery behavior and a proxy for male effort); female receptivity index (the number of times a given female mated); pair compatibility index (the number of times a given pair mated); and experimental treatment (various pheromones, control, or no treatment, the primary variable of interest in the studies that comprised the meta-analysis data set). Estimates were made using linear mixed effects modeling with restricted maximum likelihood (REML) and Markov chain Monte Carlo (MCMC) simulations (n = 10,000). Probabilities based on the *t*-distributions are included below (in italics) with the caveat that these *P*-values tend to be anti-conservative, especially with small data sets. Significance codes: *** indicates ≤ 0.001, ** indicates ≤ 0.01, * indicates ≤ 0.05, and . (period) indicates ≤ 0.10.

Table 5.3

	REML Estimates				MCMC Estimates				
Fixed effects	**Mean**	**SE**	***t* value**	**Pr(>|*t*|)**	**Mean**	**95% HPD Interval**		***p* MCMC**	**exp(Mean)**
(Intercept)	3.47	0.08	41.32	0.000 ***	3.47	3.30	3.64	0.000 ***	32.05
Taps, linear	0.06	0.00	29.58	0.000 ***	0.06	0.06	0.07	0.000 ***	1.07
Taps, quadratic	0.00	0.00	-15.08	0.000 ***	0.00	0.00	0.00	0.000 ***	1.00
Female receptivity index, linear	0.13	0.05	2.66	0.008 **	0.13	0.03	0.22	0.006 **	1.14
Female receptivity index, quadratic	-0.02	0.01	-2.70	0.007 **	-0.02	-0.03	0.00	0.007 **	0.98
Pair compatibility index	-0.16	0.05	-2.97	0.003 **	-0.16	-0.26	-0.05	0.002 **	0.85
Treatments									
No treatment (X)	0.09	0.05	2.00	0.045 *	0.09	-0.01	0.19	0.063 .	1.10
Whole extract (WE)	0.15	0.08	1.72	0.086 .	0.15	-0.03	0.31	0.085 .	1.16
Whole extract, dorsal appl. (dWE)	0.15	0.09	1.77	0.076 .	0.15	-0.01	0.32	0.073 .	1.17
Component: PMF	0.14	0.07	2.14	0.033 *	0.14	0.01	0.27	0.035 *	1.15
Component: PRF	-0.01	0.05	-0.22	0.823	-0.01	-0.12	0.10	0.818	0.99
Heterospecific: *P. cinereus* (Pc)	0.14	0.14	1.03	0.304	0.14	-0.13	0.41	0.305	1.15
Heterospecific: *P. montanus* (Pm)	-0.14	0.13	-1.03	0.304	-0.14	-0.40	0.12	0.307	0.87
Heterospecific: *P. yonahlossee* (Py)	-0.10	0.14	-0.71	0.478	-0.10	-0.37	0.19	0.491	0.91
Population: Wayah (Pw)	-0.14	0.17	-0.81	0.415	-0.14	-0.49	0.19	0.416	0.87
Treatment Unknown (Unk)	-0.11	0.10	-1.08	0.281	-0.11	-0.31	0.09	0.284	0.90
Random effects	**Var.**	**SD**			**Mean**	**95% HPD Interval**		**Median**	
Male subject effect (Intercept)	0.00	0.00			0.01	0.00	0.03	0.01	
Female subject effect (Intercept)	0.00	0.00			0.01	0.00	0.03	0.01	
Year effect (Intercept)	0.01	0.09			0.10	0.03	0.19	0.09	
Residual	0.21	0.46			0.46	0.44	0.48	0.46	

Figure 5.1


A
Complete records
Unique female subjects
450
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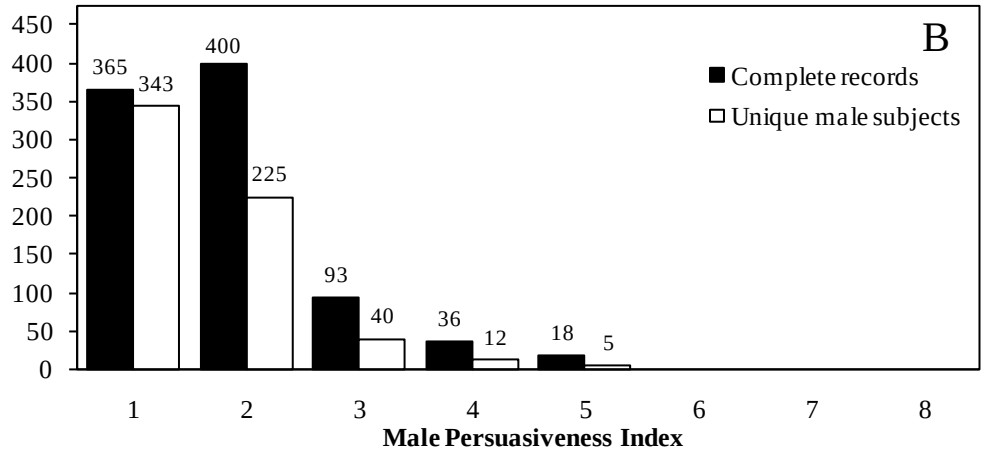
1 2 3 4 5 6 7 8
Female Receptivity Index


B
Complete records
Unique male subjects
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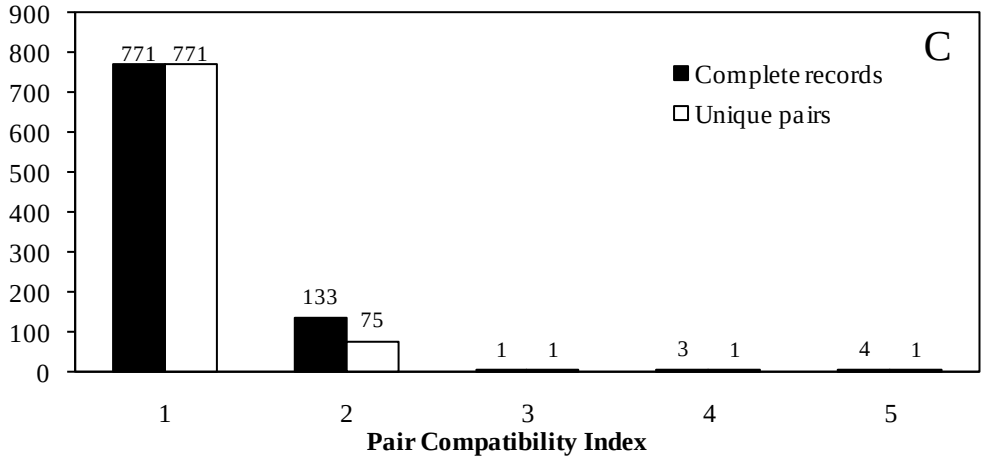
Male Persuasiveness Index


C
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Unique pairs
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800
700
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500
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100
0
771 771
133
75
1 1
3 1
4 1
1 2 3 4 5
Pair Compatibility Index

Figure 5.1. Number of recorded courtships or unique individuals by index. (A) Female receptivity index, as measured by the number of courtships completed by an individual within a season of staged laboratory encounters. The number of records that were incorporated into the meta-analysis, and the number of females that generated these records. (B) Male persuasiveness index, the male equivalent of the receptivity index. (C) Pair compatibility index, the number of repeated courtships completed by a given pair. The 771 courtships completed by pairs that mated only once involved 569 females and 555 males that either mated only once or mated multiply but with novel partners.

CHAPTER 6. CONCLUSION: INFORMATION AND MANIPULATION IN SEXUAL SIGNALING

The nature of signals in animal communication has been the subject of intense and sometimes heated debate within the behavioral sciences for decades. A prominent aspect of this debate focuses on the manner in which signal content and signal processing are conceptualized. In particular, the definition of signal information has profound ramifications for how we understand the dynamics between signaler and receiver. Herein, I summarize the debates covering the semantics of information terminology, and the theoretical basis of signaler-receiver dynamics. I discuss these subjects first as they pertain to animal communication in general, then as they pertain to pheromones as sexual signals. My aim is to examine my research findings within the larger contexts of: (1) the potential role of pheromone information as the substrate for sexual selection, (2) the concept of pheromone manipulation when the interests of the courter and courted are not in perfect accordance, and (3) some of the implications of (1) and (2) for behavioral studies and interpretations of evolutionary processes.

I have standardized some of the debate terminology to encompass all forms of communication. Much of the earlier literature discusses communication in terms of providing information about the subsequent behavior of the actor (e.g., Wiley 1983). This definition is pertinent to visual forms of communication, e.g., threat displays in a male-male competition context. I generalize this discussion to include communication in all sensory modalities, including pheromone semiochemicals in an intra-sexual selection context. For example, rather than using the term “displays” which implies visual communication, I use the term “signals” which can also include olfactory and somatosensory communication.

Signals as Information

There is a natural tendency to couch animal communication in terms of language-based constructs. However, a potential pitfall can occur with this conceptualization of a signal as a message that is transmitted by a sender and evaluated by a receiver. This pitfall is the implicit assumption that animal communication involves the transmission of meaning and that the receiver evaluates this meaning via cognitive function. The claim that signals need not convey any particular message or meaning *per se* has been echoed in a semantic call to arms that has spanned more than thirty years of debate within the ethology community (from Dawkins & Krebs 1978 to Rendall et al. 2009). Proponents claim that language-based terminology is an anthropomorphic carryover (e.g., Ownings & Morton 1997) and that we can avoid making any assumptions regarding internal processes in the receiver only if we abandon the concept of a signal as a transmitted message. These exhortations reiterate the basic ethological principle of avoiding assumptions about the internal states, motivations, and cogitations of the observed subject (see Ramifications for the Study of Communication section below).

The heated response has been that the concept of information is critical to studies of animal communication. Information relates animal communication to learning theory and the mechanisms of forming associations between signals and observed behavioral outcomes (Seyfarth et al. 2010). Information, when defined as reduction in uncertainty, enables the ethologist observer to make probabilistic predictions based on responses to a particular signal (Hinde 1981). Furthermore, a signal without informational content would be ignored by the receiver and the associated communication system could not be maintained by selection (Searcy & Nowicki 2009).

As tends to happen with long-running debates, much of the heuristic progress is eroded into obscurity by subsequent waves of researchers repeatedly disputing the same arguments. As for the role of signals in animal communication, the heart of the disagreement may have sprung from two very different concepts of information.

Broadcast information (Wiley 1983), also known as *Shannon information* (Shannon 1948; Krebs & Dawkins 1984), is a statistical measure of the decrease in uncertainty, or increase in predictability, of the signaler's identity or behavior. Thus, broadcast information is associated with the process of encoding internal states into external signals. Broadcast information is loosely analogous to the message of a signal (and our notion of information). In contrast, *transmitted information* (Wiley 1983) is the increase in predictability of the *receiver's* subsequent behavior as the result of a transmitted signal. As such, transmitted information is associated with the process of decoding a signal into change in the recipient's internal state. The concept of transmitted information is loosely correlated with the concept of *semantic information* (Krebs & Dawkins 1984), in which a message or meaning is conveyed to the receiver. Transmitted information thus is a function of the context, current state, and history of the recipient, in addition to the signaler's broadcast information.

The technical definitions provided by Wiley (1983) and Krebs & Dawkins (1984) both delineated and synthesized the various schools of thought in animal communication. However, the prescriptive aspect of this terminology is unclear, in particular, as it relates to the influence of information theory on studies of animal communication. These definitions of information (broadcast vs. transmitted, semantic vs. Shannon) have been confused further by the identity of the "observer," which may be either the ethologist or the animal recipient for which the signal was intended. Furthermore, ambiguous terms such as "encoding" and "decoding" have been used in relation to Shannon information (neuronal function: e.g., Wiley 1983) and semantic information (cognitive function: e.g., Rendall et al. 2009). It is worth noting that Dawkins & Krebs (1978) and Rendall et al. (2009) were referring to broadcast or semantic information during their call to abandon the concept of information, although neither specifically made this distinction.

Ramifications for the Study of Communication

Wiley (1983) cautioned that "transmitted information, not broadcast information, is fundamental in an objective analysis of communication, where the primary concern is the effect of a signal on a receiver." Wiley, like Dawkins & Krebs, was advocating for research that focused solely on the response evoked in the receiver by a particular signal or signaler. These exhortations reference the behavioral methodology of Watson's (1913) "Behaviorist Manifesto," which espoused observable behavior, rather than internal mental processes, as the only truly objective unit of study. The prescriptions of Wiley, Dawkins & Krebs also echo Skinner's radical behaviorism, the ultra-deterministic approach to behavioral studies in which mental processes such as thoughts, emotions or perceptions were invalid causal phenomena for behavior (Skinner 1974; 1976).

Yet, what if the dichotomy between these two approaches to the study of animal signals is entirely contrived? It is possible to reconcile the two opposing sides if we consider the ethologist as gleaning: 1) broadcast information from observations of the signaler, and 2) transmitted information from observations of the receiver. Thus, research that examines a signal's message aims to define common characteristics from the signalers, while research that examines a signal's ability to evoke a response aims to define characteristics shared by the receivers (Wiley 1983).

Furthermore, these views may provide two complementary perspectives that coincide with the "traditional ethological approach" to examining communication and predictions from game theory (Hinde 1981). To the extent that game theory can generate testable hypotheses to guide traditional ethological studies, these two approaches are *de facto* highly compatible. For instance, game theory can model stratagems based on (Shannon) information theory, such as the reduction in uncertainty about another "player" based upon its signal; this modeling in turn can generate predictions about the relative advantages of releasing or withholding information conveyed about the signaler's current state or subsequent behavior (Hinde

1981; Laidre 2009). Additionally, game theory can generate testable hypotheses about how animals in conflict (including in a mate choice context) should communicate (e.g., Caryl 1979; Caryl 1982). These hypotheses can foster an examination of how both signalers and receivers may take advantage of each other, as discussed in the meta-analysis (Chapter 5) which examined the presence of signaler-based strategies for evoking a response in the receiver.

Signals as Manipulation & Ramifications for Evolutionary Theory

Dawkins & Krebs (1978) criticized the traditional interpretation of communication "as a vehicle of inter-individual cooperation and its evolution [as] mutual co-evolution." These authors further claimed that the evolutionary basis of communication is not the exchange of information, but rather the manipulation of the receiver's response for the signaler's benefit. Many researchers took exception to this oversimplified portrayal of "traditional ethologists." Hinde (1981) argued that Dawkins & Krebs had worked to "produce a straw man which neither accurately nor adequately conveys the main stream of ethological studies." Indeed, decades previously, Tinbergen had outlined the basic tenets of communication as a non-mutualistic interaction. In his conflict hypothesis for the phylogeny of threat displays, Tinbergen (1953; 1959) had theorized that: (1) the selective forces working on signaler and receiver were not the same, (2) communication did not necessarily evolve to more effectively transmit information about the signaler, but rather (3) communication solely evolved based on the response elicited in the recipient.

Many of these discussions focused almost exclusively on the benefits of communication to the signaler and neglected the influence of the receiver on the evolution of animal signaling. Several researchers sought to rectify this omission, and elaborated on two opposing types of dishonesty in communication: manipulation by the signaler, known as "deceit" or "bluffing"; and manipulation by the receiver, known as "eavesdropping" or "mind-reading" (Wiley 1983; Krebs & Dawkins 1984).

These two opposing phenomena were described by Krebs & Dawkins (1984) as "intimately locked together in evolutionary arms races and feedback loops."

Ultimately, manipulation depended on the changes in relative fitness of signaler versus receiver. As such, manipulation could exist independently of the concepts of information transmittal and exchange. This manipulatory view was compatible with comparative evidence of how signals could have evolved through a process of exaggeration and ritualization in which "natural selection work[s] on the [signaler], making his signal more effective by exploiting the [receiver's] responsiveness to supernormal stimuli" (Hinde 1981).

Krebs & Dawkins took great pains to explain that manipulative communication need not, by definition, work to the receiver's detriment. Nonetheless, the term "manipulation" is burdened with the notion of communication as a relationship between perpetrator and victim. Furthermore, the terms "exploitation", "deceit", "bluffing", "eavesdropping" and "arms races" carry connotations of antagonism, coercion, and/or subjugation. Indeed, these terms may be both misleading and unconstructive, e.g., if a "deceitful" individual that gives a threat display while deciding whether to attack or flee may merely be indecisive (Hinde 1981).

The notion of signal and receiver coevolution (be it antagonistic or mutualistic) is central to models of evolution by sexual selection, which predict coadaptation between the male signal and female response (Fisher 1930). A signal that worked by manipulating the receiver's nervous system should be maintained by selection if it benefited the recipient (Seyfarth et al. 2010). However, this dynamic can destabilize honest communication (Johnstone 2009). Game theoretical models of signal dishonesty have posited that reliable signals can be maintained if there is a cost to signaling (the handicap principle: Zahavi 1975; Zahavi et al. 1999), although this cost may not always be necessary (e.g., van Rhijn & Vodegel 1980; Johnstone & Grafen 1993). Nonetheless, evolutionary conflicts should arise when signals that benefit the signaler also entail costs for the receiver. Selection should drive the receiver to resist a

signal if the manipulation is detrimental; within a sexual signaling context, this "evolutionary arms race" is known as sexual conflict (Arnqvist & Rowe 2005).

Information versus Manipulation in Sexual Signaling

Sexual selection has been extremely important in determining the evolution of animal communication (Searcy & Nowicki 2009). Within the paradigm of intra-sexual selection, female mate choice is the agent of sexual selection, and male signals comprise the substrate for female consideration. Sexual signals have traditionally have been viewed in terms of semantic information, i.e., what information can a male convey to a potential mate? The female is believed to receive and use this information to assess mate quality according to intrinsic criteria (see Chapter 1. General Introduction). However, the signal itself is generally considered a passive element, and while the transmission and processing of the signal may be subject to degradation (Endler & Basolo 1998), it is believed that "the signaler cannot directly influence the processing of the signal by the receiver's nervous system" (Wiley 1983).

Chemical signals used in courtship and mating hold a unique status among animal signals. Karlson & Luscher (1959) described pheromones as "substances which…release a specific reaction, for example, a definite behavior or a developmental process." As such, these chemical signals were defined as immediate modifiers of recipient behavior or eventual modulators of recipient physiology rather than information to be evaluated by the receiver. In (transmitted) information theory, mate choice would function as a form of pheromone decoding in which the reception of a male signal translates into changes in the receiver's internal state. This "translation" would also depend on external context and the receiver's current internal state, which in turn is influenced by prior experience.

I have proposed that sex pheromones can influence mate choice through means other than the conveyance of semantic information (Vaccaro et al. 2010). In particular, I have proposed three broad mechanisms by which pheromones function in mate

choice: (1) as a secondary consequence of increased general central nervous system arousal, (2) as a direct consequence of enhanced sexual motivation, and (3) as an indirect consequence of suppressed competing motivations. By using male pheromones pooled from over 200 individuals, my collaborators have created a generic "every male" signal capable of eliciting broad behavioral responses, i.e., not specific to particular individuals. Previous research has shown that female *Plethodon shermani* salamanders respond to this pooled pheromone via: (1) suppressed competing motivations, as measured by decreased hunger (Chapter 2), and (2) augmented sexual motivation, as measured by an increased attraction to male scent but not to the scent or sight of food (Chapters 3 & 4).

Conceptually, these proposed pheromone mechanisms are based upon the contrast between male signals as information transfer and male signals as "manipulation." By making this contrast, I do not wish to espouse a cynical view of communication, but rather a mechanistic one. It does not follow that the male signal must be to the detriment of the female. Nor do I claim these signals must not function in information transfer. Information could be conveyed in our salamander system, even within the context of enhanced sexual motivation, suppressed competing motivations, or general arousal. In fact, the capacity exists for salamander pheromones to function in information transfer (Johansson & Jones 2007); these pheromones are suitable substrates for mate choice evaluations to the extent that they: 1) are heritable (e.g., Watts et al. 2004; Palmer et al. 2005; Palmer et al. 2007), 2) vary among individuals (Rollmann et al. 2000), and 3) may be costly to produce (honest signals). In this paradigm, pheromones would function to convey information about an individual to be assessed by a potential mate. However, behavioral studies lack the sensitivity to detect differential female responses to individual male pheromone profiles.

Plethodontid salamanders, including my study species, *Plethodon shermani*, are the only known vertebrates in which male pheromone signals increase female receptivity (Houck et al. 1998; Rollmann et al. 1999). In *P. shermani*, as in other

terrestrial vertebrates, the vomeronasal system transmits chemosensory information to the brain. Previous research has shown that pheromone stimulation of the vomeronasal organ (VNO) activates specific populations of neurons. These neurons in turn activate dedicated neural routes to brain nuclei such as the vomeronasal amygdala, preoptic area and ventromedial hypothalamus (Wirsig-Wiechmann et al. 2002; Laberge et al. 2008). These brain regions are known to be responsible for courtship and copulatory behavior in other vertebrates (Keverne 1999; Firestein 2001; Devidze et al. 2006). In future research, I will use neurophysiological studies of the *Plethodon* vomeronasal system to examine differences in neural processing of individual male signals.

FUTURE RESEARCH: THE *PLETHODON* PHEROMONE-RESPONSE SYSTEM

For my postdoctoral research with Dr. Frédéric Laberge at the University of Guelph (Ontario, Canada), I will use the salamander vomeronasal system to understand how sensory input is relayed, modulated and processed as it travels to brain regions implicated in the control of motivated behavior. The vomeronasal system is hypothesized to have receptor mechanisms for each type of biologically relevant cue. Thus, specific mechanisms would detect cues of prey, predators, and conspecifics, as well as individual pheromone components. Different vomeronasal pathways are further hypothesized to interact in the central nervous system to prioritize the choice of behavioral options (Christensen & Sorensen 1996; Mustaparta 1996). For example, behavioral studies indicate that salamander courtship pheromones have an inhibitory effect on the processing of prey cues, but not predator or olfactory cues.

In this research, I will use *in vivo* electrophysiology to take simultaneous recordings from different stations along the pathway from the vomeronasal epithelium to the hypothalamus. I aim to elucidate patterns of neural activation associated with enhanced female receptivity. This elucidation will be accomplished by examining neural responses to individual components of the male courtship pheromone mixture. My second objective is to characterize interactions between competing afferents

dedicated to different behavioral outcomes. This characterization will be done by identifying the neural modulation that results from presenting pheromones in tandem with a broad panel of relevant olfactory cues.

The plethodontid salamander system presents a rare opportunity to study the mechanisms of pheromone action in a vertebrate. The direct nature of the salamander's vomeronasal pathway is ideal for examining how a sensory signal is shaped before communicating with centers of the brain that control motivated behavior. In addition to highlighting basic principles of sensory processing and mechanisms of behavioral choice, this research will help me develop multi-disciplinary collaborations with an international group of scientists working on the evolution of plethodontid salamander pheromones.

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