

AN ABSTRACT OF THE DISSERTATION OF

Sarah L. Eddy for the degree of Doctor of Philosophy in Zoology presented on April 17, 2012.

Title: Mutual Mate Choice in a Terrestrial Salamander, *Plethodon shermani*, with Long-Term Sperm Storage.

Abstract approved:

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Sexual selection can influence the mating system of an organism through multiple mechanisms. These mechanisms result in variation in reproductive success among individuals, and include scramble competition, endurance rivalries, contests, mate choice and cryptic choice, and sperm competition. Understanding the mating system of a species requires the identification of which processes are occurring, and to what degree. In this thesis, I explored the influence of mate choice mechanisms on the mating system of the terrestrial red-legged salamander, *Plethodon shermani*. I also documented the potential for post-copulatory processes (such as sperm competition and cryptic choice) to influence mating system dynamics.

The evolution of mate choice requires (among other factors) variation in the reproductive value of potential mates. This variation is made apparent to choosy individuals through cues. Most animals use multiples cues incorporating many modalities to assess the reproductive quality of potential mates. In Chapter 2, I tested the contribution of two cues (chemical and visual) to mate choice by female *P*.

shermani. I found that a male visual cue (“foot-dancing”) increased courtship success. In contrast, delivery of non-volatile pheromones during courtship did not influence courtship success in the laboratory setting, but did affect the duration of one of the courtship stages. In Chapter 4, I identified a tactile cue that was significantly correlated with male reproductive success. Thus, *P. shermani* females could use at least three modalities to assess the reproductive quality of potential mates.

Mate choice can also evolve in males. In Chapter 3, I tested this possibility in *P. shermani*. I found that males vary the reproductive effort they invest in a particular courtship based on the reproductive value of their partner, indicating male mate choice is occurring. A male invested most when paired with a female with large, well developed ova, and invested less with females that were non-gravid or had small ova. In addition to documenting male mate choice, I showed that the male visual display (“foot-dancing”) that affected female mate choice was correlated with male condition, implying foot-dancing may be an honest indicator of male quality.

Finally, in Chapter 5, I explored the potential for post-copulatory processes to influence the *P. shermani* mating system. The opportunity for sperm from multiple males to overlap in the female reproductive tract (i.e., the opportunity for females to mate multiply) is necessary for post-copulatory processes such as cryptic choice and sperm competition. The capacity for long-term sperm storage by females can increase the likelihood that this overlap in sperm from multiple males will occur. I found that females can store viable sperm for at least 9 months and in some cases beyond

oviposition. In addition, I documented one female with sperm in her sperm storage organ from a mating that occurred 17 months earlier. Such lengthy sperm storage allows the possibility of sperm from one breeding season to interact with sperm from a subsequent season. Thus, the potential for post-copulatory sexual selection within this salamander system is high.

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Mutual Mate Choice in a Terrestrial Salamander, *Plethodon shermani*, with Long-Term Sperm Storage.

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Sarah L. Eddy

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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.

Sarah L. Eddy, Author

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Mutual Mate Choice in a Terrestrial Salamander, *Plethodon shermani*, with Long-Term Sperm Storage.

Chapter 1 – General Introduction

SEXUAL SELECTION AND MATING SYSTEMS

Sexual selection involves the selection of traits associated with competition for mates and opportunities for fertilizations (Andersson 1994; Jones & Ratterman 2009). This type of selection occurs when individuals vary in their success at competing for mates and thus vary in the number of offspring they produce (Falconer & Mackay 1981). Competition for mates can occur both before and after copulation, and the type of competitive interactions that occur can vary. Some interactions involve direct competition between members of the same sex (such as when female Black Grouse, *Tetrao tetrix*, combat each other for access to mates; Karvonen et al. 2000) or indirect competitive actions as when a male Satin Bowerbird competes for mates via elaborate morphological and behavioral displays as well as physically constructing a bower all intended to influence females to choose him over other potential mates (Doucet & Montgomerie 2003). As illustrated by these examples, the types of traits under sexual selection can vary depending on the kind of competitive interactions that constitute the mating system of a particular species.

Post-copulatory sexual selection occurs if females mate multiply. Post-copulatory sexual selection determines how paternity is allocated among a female's mates (Eberhard 2009). It can take the form of sperm competition or cryptic choice. Sperm competition describes the situation in which sperm from multiple males

compete to fertilize a set of ova within one female (Parker 1970). Sperm competition can involve a diversity of competitive interactions, and these interactions can be indirect (including copulatory plugs or frequent copulations with a partner) or direct, including mate-guarding and gamete competition (Andersson 1994). Post-copulatory competition can also involve cryptic female choice in which female processes bias which sperm fertilize her ova (Eberhard 2009).

Competitive interactions that occur before copulation can be divided into four categories: contests, scrambles, endurance rivalries and mate choice (Andersson 1994). Contests involve individuals directly fighting over access to mates or access to resources (such as territories) necessary to gain mates (Ryan 1997). This form of competition selects for traits (such as weapons and larger size) that improve an individual's ability to win the contests. In contrast, a scramble involve indirect competition between individuals that vary in their abilities to rapidly find mates, and promotes traits that make an individual more likely to locate mates early and rapidly. For example, male red-spotted newts (*Notophthalmus viridescens*) with larger tail-fins experience a higher reproductive success than do newts with smaller fins. The advantage of the large tail-fin seems to be an increased acceleration ability which allows these males to reach a detected female more rapidly than other males (Able 1999). Endurance rivalries involve individuals varying in the duration they are able to remain reproductively active. For example, bull frogs (*Lithobates catesbeiana*) that are in better physical condition remain in the breeding chorus longer and so have more

mating opportunities (Judge & Brooks 2001). Finally, mate choice involves individuals of one sex varying in their attractiveness to potential mates and, thus, varying in the number of mates to which they have access. This type of indirect competition selects for behaviors and morphologies that attract mates, such as the elaborate display behaviors and feathers of the Sharp-Tailed Grouse, *Tympanuchus phasianellus* (Gibson 1996).

For mate choice to be a driving force in a mating system, the cost of losing opportunities to mate because one is choosy must be balanced by the benefits received by the choice (Barry & Kokko 2010). Factors that can lead to the evolution of mate choice are those that make offspring production costly and mate searching cheap. Specific conditions that promote mate choice include high parental investment in offspring, limited ability to produce gametes, high variation in the reproductive value of potential mates, high population densities and a prolonged breeding season (Edward & Chapman 2011).

One of the key factors that facilitates the evolution of mate choice is the presence of substantial variation in the reproductive value of potential mates (Barry & Kokko 2010). The reproductive value of mates can be conceptualized in multiple ways, but generally falls into two categories: direct and indirect benefits. Direct benefits have an immediate impact on the choosy individual's reproductive success (Ryan 1997). These benefits can include gaining access to a territory, assistance with

care of offspring, nuptial feeding, reduced risk of harassment and increased fecundity (Andersson 1994).

Hypotheses invoking indirect benefits involve increases in reproductive success enjoyed by the choosy individual's offspring, but not by the choosy individual itself (Ryan 1997). Indirect benefit models include the good genes models and compatibility selection. In both of these models, cues from potential mates are correlated with a heritable element of the mate's fitness. Choosy individuals can ensure the higher fitness of their offspring by mating with individuals having a particular expression level of a cue (Pomiankowski & Iwasa 1998). These indirect models invoke either additive (good genes) or non-additive (compatibility) genetic benefits, which can lead to different expected patterns of mate choice in a population (Mays & Hill 2004). Good genes models generally predict that choosy individuals will converge on the same mating preferences (because variation in the chooser's own genetic background does not change the value of the genes of the potential mate). Compatibility selection, on the other hand, could lead to variation in mating preferences across the population because the reproductive value of potential mates depends on the choosy individual's own genetic make-up (Mays & Hill 2004; Jennions & Petrie 1997). Either way, a genetic benefit for the choosy individual's offspring is obtained.

Additional models for the evolution of mate choice include circumstances under which preferred traits may not currently confer benefits to the choosy

individual, such as Fisherian runaway selection and sensory bias. Sensory bias is a physiological explanation for mate choice. The sensory systems of choosy individuals are tuned to detect cues that increase an individual's ability to survive (such as cues involved in food recognition or predator avoidance). If a trait evolves in a signaler that exploits these pathways, then the signaler may enjoy the benefits of increased attention from the receiver. Again this increased attention is not necessarily correlated with any benefits for the choosy individual, but simply occurs because the receiver's sensory systems are attuned to perceive the signal (Arnqvist 2006). Fisherian selection, on the other hand, requires indirect benefits exist for the choosy individuals at the evolutionary start of the process. In this case, the preference for a trait becomes genetically correlated with the expression of the trait, so individuals pass on to their offspring both the trait and the preference for this trait (Fisher 1930). Once the initial genetic correlation is established, the process is self-reinforcing and, thus, does not require further benefits to the choosy individuals (Lande 1981).

In species with two sexes, the variation in reproductive value (be it due to indirect or direct benefits) of potential mates can facilitate mate choice in either or both of the sexes (Edward & Chapman 2011). Mate choice can be assessed most directly via mating trials in which the binary decisions to mate or not to mate are recorded (*cf.* Verrell 1986). Mate preference (the underlying sensory and behavioral propensities that influence what phenotypes an individual selects as a mate; Jennions & Petrie 1997) can be assessed in many different ways including: latency to mate (*cf.*

Rollmann et al. 1999); the amount of time an individual spends associated with potential mates differing in some characteristic or characteristics (*cf.* Rosenthal 2004); and variation in effort dedicated to individual courtship bouts with potential mates that differ in various ways (*cf.* Houde 1997; Guevara-Fiore et al. 2010). In this thesis, I explore whether mate choice (by the male or female) occurs in an amphibian model system, the red-legged salamander, *Plethodon shermani*. In addition, I explore the potential for post-copulatory sexual selection processes to play a role in this system.

WHY STUDY MATE CHOICE IN *PLETHODON SHERMANI*?

Pre-copulatory mate choice and the use of cues to assess mates have been studied extensively in the animal kingdom. However, previous studies of courtship and multi-modal signals have largely focused on birds, insects, spiders, fishes and anurans (reviewed in Coleman 2009). One clade that has received less attention is urodele amphibians. Salamanders are well known for their use of chemical cues, but some species also have visual and tactile components to their courtship displays (Houck & Arnold 2003; Secondi et al. 2010). In particular, plethodontid salamanders (the clade containing *P. shermani*) are an ideal clade for studying mate choice for multiple reasons including: the high *a priori* expectation that mate choice exists for both sexes (based on the life history of the species), the tractability of the system, and the evolutionary history of the clade which involves shifts in the perception of sexual cues by females in different lineages.

Likelihood of male and female mate choice

In general, females are thought to be the choosy sex because they often invest more energy in the production of gametes, and they are more limited in the number of gametes they can produce (Andersson 1994). If females encounter more males than they can mate with, then mate choice is expected as long as other interactions (such as coercive mating or male-male contests) do not limit the expression of a female's preferences. *Plethodon shermani* males do not have coercive abilities; these males do not have an intromittent organ, nor do they physically constrain a female. Male-male contests do occur in this species (Hutchinson 1959), but whether these contests preclude female mate choice is not known. Further suggestive evidence for female mate choice is the complexity of the male salamander's courtship display; males employ tactile, visual and chemical cues extensively during courtship (Arnold 1976). Complex multi-modal courtship displays are often driven by mate choice (Candolin 2003). Indeed, the chemical cue employed by male *P. shermani* has been shown to increase female receptivity (measured as a reduction in courtship duration, Rollmann et al. 1999; Houck et al. 1998, Houck et al. 2008a), so it seems likely a female may also express preferences for other components of this display as well.

Male mate choice should evolve in systems where (1) the number of mates available exceeds a male's capacity to mate, (2) mates vary in their reproductive quality, and (3) the benefit of choosing a mate exceeds the cost of assessing a mate (Edward & Chapman 2011). Because males typically have higher mating capacities,

the variability in mate quality and the ease of access to additional mates must be high for mate choice to evolve, especially if mate choice is occurring sequentially rather than simultaneously (Barry & Kokko 2010). These conditions seem to be met in *Plethodon shermani*. First, females vary greatly in their reproductive value to males. Female *P. shermani* may only come into reproductive condition every other year, because the multi-month period they spend defending their eggs prevents the female from foraging extensively (Hairston 1983). Therefore, at the beginning of the mating season, some females will not have any reproductive value (non-gravid), while others will only have a few or poorly developed ova (low reproductive value), and still others will have a high reproductive value (strongly gravid). Second, reproductively mature females seems locally abundant. Population density estimates for the species are as high as 0.86 *P. shermani*/m² (Ash 1988). In addition, on warm rainy nights, 8 person hours (2 hrs/ person) is sufficient to collect over 100 gravid females at our field site (Eddy, pers. obs.) which implies a high density of reproductively active females. Together, these conditions of high variation in mate quality and a large number of potential mates make it likely that male mate choice could evolve in this system.

The variation in reproductive value of potential mates is often indicated through variation in some sort of cue or ornament (Zahavi 1975; Jones & Ratterman 2009). Thus, mate choice studies often focus on the assessment of these cues. Many species of salamanders are known to use size (for example: Verrell 1994; Verrell 1995; Verrell 1986), reproductive condition (Verrell 1989) and chemical cues

(Rollmann 1999; Houck et al. 1998; Houck & Regan 1990; Hock 2008) to assess potential mates. Another obvious set of cues intended to influence potential mates are those involved in courtship displays (Vinnedge and Verrell 1998). Courtship displays frequently are composed of multiple cues that may or may not provide unique information to members of the opposite sex (Candolin 2003). During *P. shermani* courtship, a male performs a series of behaviors that provide tactile cues (touching the female), visual cues (including foot-dancing which involves a male raising and lowering its limbs while holding its trunk still), and chemical cues (courtship pheromones) (Arnold 1976). Any or all of these cues could be involved in mate choice.

Tractability of the system

Sexual selection studies on *P. shermani* have been conducted for over 10 years. These animals are easily collected, and mate readily and multiple times in the lab. These features facilitate the collection of a large number of courtship observations for our experiments. An additional useful aspect of the system is the well characterized distinctive five-stage courtship (Houck & Arnold 2003), which allows researchers to explore the finer scale influences of different courtship cues on mate choice (for example, by documenting changes in the likelihood of a courting pair transitioning between stages).

Evolutionary history of the clade

The evolution of chemical communication during courtship has been the focus of much work on the clade of plethodontid salamanders (Watts et al. 2004; Palmer et al. 2005; Palmer et al. 2007a; Palmer et al. 2007b; Palmer et al. 2010). As in other comparative studies of signal evolution, this work has explored the origin, loss, and diversification of signals. In particular, diversification of the chemical cues (and the behaviors necessary to transmit the chemical signal to a female) has occurred in plethodontids via a unique form of signal co-option: an evolutionary shift in the sensory mode by which females perceive the chemical cue. In this system, proteinaceous pheromones (chemical cues) produced by courting males are delivered to females with the result that the duration of courtship is significantly reduced (Houck & Reagan 1990; Rollmann et al. 1999; Houck et al. 2008a). Plethodontid salamanders exhibit two dominant forms of pheromone delivery, and these forms differ in the sensory pathways by which females perceive these signals. These sensory pathways are termed “transdermal” and “olfactory”, and are described below (reviewed in Woodley 2010; Houck & Arnold 2003; Houck 2009). The evolutionary shift in how female plethodontids perceive courtship pheromones is coupled with a correlated change in a functional complex of traits that include male morphology, courtship behaviors, and the molecular make-up of the pheromone itself (Watts et al. 2004).

Transdermal delivery of courtship pheromones is the ancestral mode of pheromone delivery in plethodontid salamanders (Houck & Arnold 2003).

Transdermal delivery can occur via two methods: biting or scratching the skin of the female. Both methods involve a multi-modal cue combining the instantaneous delivery of tactile and pheromonal stimulation. Scratching is the dominant method used by transdermal species. Males using scratching delivery have small submandibular (mental) glands that open to the skin surface and produce courtship pheromones (Houck & Sever 1994), as well as enlarged premaxillary teeth, which a male uses to abrade a female's skin through tactile behaviors termed "snapping," "pulling," "sliding" or "rubbing" (Organ 1961; Arnold 1972; Arnold 1977). These behaviors also involve the male sliding his mental gland along the female's body and thus applying the chemical cue to the abraded areas of the female's skin. Transdermal modes of pheromone delivery are presumed to allow the diffusion of courtship pheromones across the skin into the female's blood stream (Woodley 2010). The effect of this type of pheromone delivery has been evaluated in one species with scratching pheromone delivery, the Ocoee salamander, *Desmognathus ocoee*. In laboratory tests involving staged courtships encounters, the application of male courtship pheromone reduced the duration of the early stages of courtship (Houck & Reagan 1990; Houck et al. 2008a). The chemical composition of these courtship pheromones has been identified in *D. ocoee* and related species. The majority of the male mental gland secretion is composed of a 23 kDalton protein. This protein is structurally similar to a courtship pheromone, sodefrin, found in a distantly related group of aquatic newts in the genus *Cynops* (Kikuyama et al. 1995; Palmer et al.

2007b). Thus, this courtship pheromone has been labeled “sodefrin precursor-like factor” (SPF) (Palmer et al. 2007b; Houck et al. 2008b).

Olfactory delivery of courtship pheromones evolved in one clade of large eastern plethodontids approximately 19 mya (Larson et al. 2003; Watts et al. 2004). The mental gland of these *Plethodon* species opens to the surface of the skin, and is much larger and pad-like than that of the scratching species (Houck & Sever 1994). To deliver courtship pheromones, a male using olfactory delivery brings his mental gland in contact with the female’s nares through a behavior termed “slapping” (Arnold 1976). The chemical cue is shunted into the female’s VNO and stimulates neurons there (Wirsig-Wiechmann et al. 2002). Eighty-five percent (85%) of the mental gland product in these species is composed of two proteins: Plethodontid Receptivity Factor (PRF) and Plethodontid Modulating Factor (PMF) (Feldhoff et al. 1999). In one species of *Plethodon* with olfactory delivery, *Plethodon shermani*, courtship pheromone delivery is known to influence the duration of one of the late stages of courtship (Rollmann et al. 1999; Houck et al. 2008b; Houck et al. 2007; Chapter 2).

In summary, the clade of plethodontid salamanders has experienced an evolutionary transition in how chemical cues used in courtship are delivered by the male and processed by the female. This sort of transition of courtship cues from one receiver sensory pathway to another has not been documented in other systems, although the potential for it to occur is present in species such as *Drosophila* in which cues are perceived by both gustatory and olfactory receptors (Ferveur 2005).

The novel evolutionary shift in perception of chemical cues by receivers, the tractability of the system and the evidence that mate choice may be occurring in *P. shermani* are ample reasons to examine mate choice in this system. In chapters 2, 3 and 4, I explore whether mate choice occurs in this species and, if so, which of the many potential cues are used to assess mates. Specifically, I determine whether females use foot-dancing, courtship pheromones and/or tactile cues to make mate choice decisions. For males, I focus on whether female size or reproductive condition influenced the amount of effort they allocated to a mating bout. In good gene models, the cues by which mating decisions are made are indicators of signaler quality (Andersson 1996). In chapter 3, to identify male traits that may be indicator traits, I test whether the traits used for mate choice by females are statistically associated with male condition. In chapter 4, I attempt to place what we've learned about the *P. shermani* system into the larger context of plethodontid courtship evolution.

WHAT ABOUT POST-COPULATORY SEXUAL SELECTION?

The presence of post-copulatory sexual selection (both sperm competition and cryptic female choice) is largely unknown in plethodontid salamanders (but see Adams 2004), but there are reasons to expect that it is also a driving force in the evolution of this clade. First, in most salamander species studied to date, females mate with multiple males during the breeding season (Adams et al. 2005; Jones et al. 2002; Halliday & Verrell 1984; Myers & Zamudio 2004). In addition, female salamanders

have specialized sperm storage organs that range in their ability to retain sperm from 2 days up to 2 years (Sever 1995; Baylis 1940). Sperm storage increases the likelihood that the sperm of multiple males will be present at the time of ova fertilization. In chapter 4, I examine the potential for post-copulatory sexual selection in a plethodontid salamander, *Plethodon shermani*, by documenting how long females can store viable sperm and comparing my results to those found in other salamanders.

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Chapter 2 – The influence of sequential male courtship behaviors on courtship success and duration in a terrestrial salamander, *Plethodon shermani*

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ABSTRACT

Most organisms use multiples cues across many modalities to assess potential mates. In this study, we tested the contribution of two cues (chemical and visual) to courtship success and duration in the red-legged salamander, *Plethodon shermani*. We found that a male visual cue increased the likelihood that a male and female would progress to subsequent courtship stages. In contrast, delivery of a non-volatile pheromone during courtship did not influence courtship success, but did affect the duration of one of the courtship stages. Early use of visual cues early in courtship, combined with the use of a non-volatile chemical cue primarily later in courtship, may (a) increase the detectability of the male message by increasing the range at which the male can be perceived by the female, (b) indicate the context of the male's approach and subsequent behaviors (i.e., aggression vs. courtship), and (c) provide additional information about male quality.

INTRODUCTION

Mate choice studies often focus on receiver preference for a single signal, yet most courtship displays involve multiple signals targeting different sensory modalities (Candolin 2003). Even species with highly conspicuous primary signals often have additional signals in other modalities that are involved in mate choice (Caprona & Ryan 1990; McLennan 2003; Richardson et al. 2010; Rosenthal, et al. 2004; Taylor et al. 2007; Verzijden et al. 2010). On an evolutionary timescale, multiple cues can

persist in a courtship display for many reasons including: acting as a backup signal, providing information on different aspects of quality, and increasing the detectability of the sender's message (Candolin 2003).

In the context of courtship, increased detectability of the sender's message could occur because signals presented across varied modalities provide information assessable at different distances or during different stages of the courtship encounter (Candolin 2003). For example, male Iberian wall lizards (*Podarcis hispanica*) use visual cues for mate choice at longer ranges, but then use pheromones at short ranges (Lopez & Martin 2001). Three-spined sticklebacks (*Gasterosteus aculeatus*) also employ different signals at different distances: first, olfactory cues are used to assess mates at a distance, and then visual cues are employed at closer ranges (McLennan 2003). In garter snakes (*Thamnophis sirtalis*), males at different stages of courtship attune to different cues from the female. Males searching for mates use visual and thermal cues, but when males progress to the courtship stage they assess females via pheromones (Shine & Mason 2001).

In addition to increasing the receiver's ability to detect the signaler, signals used in earlier stages of courtship could influence subsequent courtship signals and courtship stage durations. For example, mate assessment based on signals employed during one stage could influence the amount of assessment needed in subsequent stages (i.e., if a male is particularly convincing during an early courtship stage, then he may not need to signal as intensely later on). Thus, studies designed to explain the

variation in courtship success ideally should include the impact of cues used during different courtship stages and/or at varying distances (*cf* Scheffer et al. 1996).

Previous studies of courtship and multi-modal signals have largely focused on birds, insects, spiders, fishes and anurans (reviewed in Coleman 2009). Urodele amphibians (salamanders) have received less attention. Salamanders are well known for their use of chemical cues, but some also have visual and tactile components to their courtship displays (Arnold 1976; Secondi et al. 2010). In particular, salamanders in the genus *Plethodon* have elaborate courtship interactions that are likely to involve multiple cues across many modalities. In many *Plethodon* species (such as *P. cinereus* and *P. jordani*) volatile cues are initially used for sex determination and mate quality evaluation before the onset of courtship (Dantzer & Jaeger 2007a; Dantzer & Jaeger 2007b; Dawley 1984). *Plethodon* salamanders also employ non-volatile pheromones produced by the male's mental (chin) gland during courtship. Reception of these non-volatile cues by the female reduces courtship duration in the red-legged salamander, *P. shermani* (Houck et al. 1998, Rollmann et al. 1999; Houck et al. 2007). In addition to these chemical signals, *Plethodon* males typically employ visual and tactile stimuli during courtship. A *P. shermani* male, for example, will rub his chin on the female's tail and dorsum during the early stages of courtship, a form of tactile stimulation (Arnold 1976). In addition, males of at least nine species of large eastern *Plethodon* (Highton et al. 2012) also engage in a distinctive visual display ("foot-dancing") during the early persuasive stages of courtship (Organ 1958; Arnold 1976; Dyal 2006;

Marvin & Hutchison 1996; Petranka 1998). This foot-dancing display involves a male lifting and lowering his front and hind limbs while his torso remains stationary, although in some species just the hindlimbs are employed (Arnold 1976; Dyal 2006). The frequency and length of foot-dancing during courtship varies among *Plethodon* species.

Plethodon salamanders are ideal for studying the importance of signals employed at different stages of courtship because their courtship stages are distinctive and highly stereotyped (Arnold 1976; Houck & Arnold 2003). During the first stage of courtship (orientation) a male orients towards the female and approaches her. The second stage involves the male attempting to “persuade” the female to enter into the third stage with him. The third stage of courtship is defined by a characteristic behavior called “tail-straddling walk”. During tail-straddling walk, a female straddles the male’s tail and moves forward with him in tandem (Stebbins 1949; Arnold 1976). While a pair is in tail-straddling walk, the male typically delivers non-volatile courtship pheromones to the female (Houck 2009). In the clade of large eastern *Plethodon*, the pheromone delivery behavior (termed “slapping”) involves the male making direct contact with his mental gland to the female’s nares (Arnold 1976). This behavior is preformed occasionally during the persuasion stage, but primarily during tail-straddling walk. In laboratory-staged courtships, the number of “slaps” delivered to the female in tail-straddling walk can range from 0 to 40 (S. Eddy, personal observations). A successful tail-straddling walk ends with spermatophore deposition

(stage 4) and sperm transfer to the female (stage 5). During deposition, the male lowers his vent and deposits a spermatophore on the substrate. A spermatophore consists of a gelatinous base that supports a sperm cap. Insemination occurs when a female lowers her cloaca over the spermatophore and lodges the sperm cap in her cloaca before lifting it off the base. These distinct stages provide the opportunity to identify the roles that courtship signals might play, including (a) increasing the probability that a pair will transition to the next stage of courtship, or (b) influencing events and timing of subsequent stages of courtship. Note that the progression through sequential courtship stages can be halted at any time if the female chooses to disengage from the male.

In this study, we used the red-legged salamander, *P. shermani* to test whether courtship success was affected by two cues: visual (foot-dancing) and olfactory (non-volatile courtship pheromones). We viewed courtship success as a combination of two factors: (a) initiation of tail-straddling walk and (b) insemination success. Tail-straddling walk initiation is crucial for courtship success because insemination does not occur if a pair does not enter this stage of courtship. In our first experiment, we tested the contribution of the visual cue, foot-dancing, to the likelihood of tail-straddling walk initiation (as foot-dancing only occurs during the persuasion stage of courtship). In the second experiment, we measured the contribution of the non-volatile courtship pheromone to (a) insemination success once a pair was in tail-straddling

walk (as pheromone delivery primarily occurs during tail-straddling walk) and (b) tail-straddling walk duration.

METHODS

Animal Collection and Maintenance

We collected a total of 238 adult male and 241 adult female *Plethodon shermani* in Macon County, North Carolina (035°10'48"N 083°33'38"W) during the breeding season in late July and August of 2007 and 2008. Animals were shipped to Oregon State University (OSU), Corvallis, USA where the experiments were conducted. Each animal was maintained individually in a plastic box (17 cm x 9 cm x 13 cm) with substrate and refugia made of moist paper towels. Animals were kept at 15-18° C on a late-August North Carolina photoperiod and each fed two waxworms (*Galleria mellonella*) weekly.

Gland Removal and Sham Surgeries

To distinguish the contributions of foot-dancing and pheromone delivery, we experimentally excised the mental gland from each male collected in 2007 (n=77), so that no male could deliver courtship pheromones. Males collected in 2008 (n = 161) were used in behavioral trials to investigate the influence of pheromone delivery on insemination. These 2008 males were randomly assigned to one of two surgical treatment groups: (1) sham surgery (n=76) or (2) gland removal (n=85).

Each male in the gland removal treatment was anesthetized by submersion in a mixture of 7% ether in water. Its gland was then surgically excised using iridectomy scissors, (*cf.* Rollmann et al. 1999). For sham surgeries, each male was anesthetized and a small incision was made on the submandibular area to mimic the cut made to remove the mental gland. In both years, all males were given at least two weeks to recover before use in courtship trials; males recovered completely during this interval.

Behavioral Assays

Courtship encounters were staged in September and early October of 2007 and 2008, a period within the *P. shermani* annual breeding season (typically, late July to early October, Arnold 1976). Each evening (between 2030-2100 EST), we set up 30-60 male-female pairs (15 pairs per observer) by placing a single male with a randomly chosen female in a plastic box (17 cm x 9 cm x 13 cm) that had a damp paper towel substrate. Each male-female pair remained together for 3 hours.

Behavioral observations were conducted under low light conditions using red lights that mimicked nocturnal light levels in the field. Observers were responsible for recording particular behaviors (described below) specific to each experiment. In Experiment 1 (testing the visual cue), each individual salamander was used in only one trial. In Experiment 2 (testing the chemical cue), each individual was included in courtship trials until it participated in a trial in which tail-straddling walk was initiated. After that trial, the pair was removed from the remaining experimental observations.

An equal number of sham-operated and de-glanded males were observed each trial night.

In addition to courtship cues, we recorded two additional variables that could influence the results of courtship encounters: individual size and number of days since an individual last encountered a potential mate. We expected a correlation between male foot-dancing duration --and/or number of pheromone delivery attempts-- and how recently the male had encountered a female. A longer duration since a male had last encountered a female might increase his motivation to mate with the current female (*cf.* Janetos 1980: fixed threshold with a last chance option). A similar pattern of increased motivation might also hold for females. To account for the influence of days since the last courtship encounter, we began the experiment with initial courtship encounters that were not observed. Thus, for every experimental courtship trial, we knew the number of days since each salamander's previous encounter. From these data, we calculated the number of days since each male or female had last been used in a staged courtship encounter.

Individuals were randomized with respect to size. However, as body size has been shown to be important for mate choice in other salamander species (Verrell 1989), we included size as a potential variable in our statistical analyses. Our measure of size was snout-vent length (SVL: tip of snout to posterior end of the cloaca). We measured the SVL of each animal using calipers after the behavioral trials were completed.

Experiment 1: Do males with a surgically excised mental gland behave differently in courtship than males that have a mental gland?

In our two main experiments, we used males that had ablated glands. We conducted a preliminary statistical analysis to assess whether the deglanding process subsequently altered male behavior in courtship trials. We compared the number of slaps delivered during courtship between deglanded and sham-surgery males using a Welch's two-sample *t*-test. A difference in number of slaps delivered by glanded and deglanded males would indicate that males modified their pheromone delivery behavior as a consequence of gland presence or absence. Such an affect would preclude us from being able to identify the effect of pheromone presence alone on courtship.

Experiment 2: Does foot-dancing influence tail-straddling walk initiation?

In this experiment, we recorded the behaviors employed by males during the orientation and persuasion stages of courtship. Observers scanned each box containing a salamander pair once every five minutes for the first hour that a male-female pair spent in the same box. Each observer recorded whether a male was (1) foot-dancing, (2) not foot-dancing, or (3) in tail-straddling walk. The initiation of tail-straddling walk indicated a successful attempt at persuasion and the transition to the next stage of courtship. Time of tail-straddling walk initiation and duration of tail-straddling walk were also recorded. We used time of tail-straddling walk initiation to determine the end of the orientation and persuasion stages.

For this experiment, only males with ablated glands were used. This restriction prevented any attempted pheromone delivery that might have occurred during the persuasion stage of courtship from confounding the effect of foot-dancing.

We used a powerful multi-model inference technique using AIC (Akaike 1973) to explore which variables best represent the data collected from Experiment 1 rather than null hypothesis testing. Several authors have argued that multi-model inference is a better approach to model selection and variable selection in regression analyses than the more common method of significance testing (Akaike 1974; McQuarrie & Tsai 1998; Anderson et al. 2000; Johnson and Omland 2004; Burnham et al. 2011; Symonds & Moussalli 2011; Garamzegi 2011). Some of the arguments against null hypothesis testing most germane to our study design include the following. First, we did not have a meaningful null hypothesis. When working with multiple variables, nearly all null hypotheses are false *a priori* (Savage 1957; Johnson 1999; Anderson et al. 2000). Second, many of our explanatory variables (individual SVL and days since last courtship encounter) could not be randomized across our experimental groups because we could not control or predict how much a male would foot-dance. In observational studies such as this, p-values are hard to interpret because we cannot accurately calculate the null distribution from our data (Anderson et al. 2000). In addition, the information theoretical methods that we used avoid some of the common issues of stepwise model selection methods, including the inconsistencies in model

selection that result from different stepwise methods and criteria (reviewed in Hegyi & Garamszegi 2011).

We employed multi-model selection using the Akaike's Information Criterion corrected for small sample sizes (AICc) on regression models. For our study, we fit logistic regression models with a binomial response variable: either the pair entered tail-straddling walk or did not. AICc generate estimates of the likelihood that each possible model is the best model given our sample size (Akaike 1973; Anderson 2008). These AICc values can be used to rank the models. From these AICc values we can calculate AICc differences (Δ_i), and Akaike weights (ω_i). Δ_i represents the evidence in support of each model as the best model. The larger the Δ_i the less likely the model. Models with an $\Delta_i > 10$ have essentially no support from the data and, thus, are not included in our analyses (Burnham and Anderson 2004). Akaike weights are a calculation of the likelihood of the observed data given the model used that have been standardized to add up to one. They can be used to compare models as the likelihood is approximately the probability that the model is actually the best model. Finally, we used information from all possible models to generate regression coefficients through model averaging (Anderson 2008; Garamszegi 2011). This method of calculating regression coefficients (β) and 95% confidence intervals (CI) accounts for the underlying uncertainty about which model actually best fit our data. We can also use the Akaike weights to calculate a measure of the relative importance of the variable. This involves summing the Akaike weights across all the models that include a

particular explanatory variable. The relative variable importance gives us a measure of how important the variable is likely to be (% probability that variable is important for explaining observed variation in foot-dancing).

AICc analyses were implemented in R using the MuMIn package (Barton). Five potential variables were initially considered to contribute to the initiation of tail-straddling walk: proportion of time spent foot-dancing during orientation and persuasion stages, days since a female last encountered a male, days since a male last encountered a female, male SVL, and female SVL. Combinations of these variables produced a total of 32 potential models to describe our data. The total number of models tested was substantially lower than our number of observations (n=77), which justified fully exploring this dataset using multi-model selection.

Experiment 3: Does pheromone delivery influence insemination success and tail-straddling walk duration?

For this experiment, we tested whether the delivery of courtship pheromones influenced the likelihood of insemination and timing of events after a pair had entered tail-straddling walk. Observers recorded the timing and incidence of the following behaviors: slapping, spermatophore deposition and insemination. The slapping behavior was of particular importance to this study because, by contacting the female with his mental gland, the male attempts to deliver courtship pheromones to her nares (Arnold 1976). Half of the males in this study had their glands ablated, so they could

not deliver courtship pheromones, and half of the males were able to deliver courtship pheromones. Spermatophore deposition typically occurs at the end of courtship. Time of tail-straddling walk initiation and duration of tail-straddling walk were also recorded.

We identified six variables that could influence the likelihood of female insemination after tail-straddling walk was initiated: mental gland presence or absence, number of slaps, days since a female last encountered a male, days since a male last encountered a female, male SVL and female SVL. Using AICc, we examined 63 models. A total of 109 observations allowed us enough degrees of freedom to explore these models using the same techniques described for Experiment 1 with a binomial response variable (whether the female was inseminated or not).

In addition, we asked whether persuasion stage influenced the relationship between gland and TSW duration using AICc. We combined data across both years and identified 4 potential variables that could influence tail-straddling walk duration: gland presence or absence; duration of persuasion stage; an interaction between duration of persuasion stage and gland; and year. We had a total of 190 trials which allowed us to examine all 10 models using the technique described in Statistical Analyses for Experiment 1. We used a general linear model with a gamma distribution to model our data.

RESULTS

Experiment 1: The behavior of surgically de-glanded males vs. glanded males

Males that had been de-glanded (n=61) did not differ from sham-surgery males (n=60) in the number of slaps delivered during tail-straddling walk (n= 120, Welch's two sample *t*-test: $t = -0.88$, $p = 0.38$). De-glanded males delivered on average 10.9 ± 11.3 slaps (mean \pm standard deviation) during tail-straddling walk, and glanded males delivered 13.0 ± 14.9 slaps (Figure 1). Based on this result we assumed that differences in courtship success were not due to differences in male slapping behavior between sham surgery and de-glanded males.

Experiment 2: The influence of foot-dancing on tail-straddling walk initiation

We staged 77 courtship trials using de-glanded males paired with novel females to identify whether the visual cue of foot-dancing increased the likelihood of tail-straddling walk initiation. In these trials, tail-straddling walk was initiated 16 times (22% of the trials). Our initial full model included: proportion of time spent foot-dancing, days since last courtship encounter for the male and female, and the SVL of the male and female. Using model selection, we found 16 reasonable ($\Delta i < 10$) regression models for the initiation of tail-straddling walk (Table 1). The best model ($R^2 = 0.317$) included the proportion of time spent foot-dancing and the intercept as the only parameters. The main affect of foot-dancing was to increase the likelihood of transitioning into tail-straddling walk (Table 2). The time spent foot-dancing was the

variable with the highest relative importance across all potential models and was present in all the reasonable models (Table 2). Male SVL was the second most important variable (present in 42% of the models). The main affect of male SVL was to increase the likelihood of tail-straddling walk initiation (Table 2). Averaging across all models, the proportion of time spent foot-dancing is the only variable in which the 95% confidence interval was bounded away from zero (Table 2). Using evidence from the set of reasonable models, R^2 values, Δ_i , relative variable importance and the 95% confidence intervals (Tables 1 and 2), we conclude that foot-dancing was positively correlated with the likelihood of tail-straddling walk initiation. Using β s calculated from model averages, males that spent half of the first hour of the courtship experiments foot-dancing were 2.7 times more likely to transition into the tail-straddling walk than were males that did not foot-dance at all. It is important to note that foot-dancing is not always necessary for tail-straddling walk initiation. In three courtship trials, pairs entered this stage of courtship without foot-dancing.

Experiment 3: The influence of pheromone delivery on insemination and tail-straddling walk duration

We observed 109 trials in which tail-straddling walk was initiated. Of these trials, insemination occurred in 99 (90.8% transition probability). We used AICc to analyze the data. The initial full model included: the number of pheromone delivery attempts (slaps), whether a male was glanded or not, days since a male last encountered a female, days since a female last encountered a male, and SVL for male

and female. All the models resulting from this analysis were nearly equally supported (i.e., model weights all were low, and Δi between models were low) by our data (Table 3). In addition, even the best model (which did not include gland as a factor) only had an R^2 of 0.092, indicating this model did not explain much of the variability in insemination success. The first model that included gland as an explanatory variable (Table 3, model 3) explained only 8.8% of the variance in the data. The single parameter with the highest relative importance in these models was the the number of times the male attempted pheromone delivery to the female (present in over half the models, Table 4). The more pheromone delivery attempts, the less likely insemination was to occur. Gland presence or absence had the second highest relative importance and seemed to have a slightly negative impact on the likelihood of insemination (Table 4). However, the 95% confidence interval for gland presence or absence (and all the explanatory variables proposed) included zero. From this evidence, we could not conclude which variables (if any) were correlated with insemination success once tail-straddling walk had been initiated.

We also examined the relationship between the length of time spent in the orientation and persuasion stages, gland presence or absence, and the length of the third stage of courtship: tail-straddling walk. We used AICc and a data set combining both years of courtship data to explore this relationship ($n = 191$). The initial full model included: gland presence or absence, duration of the combined orientation and persuasion stages, year, and an interaction between duration of persuasion stage and

gland. We found 4 reasonable models for the duration of tail-straddling walk. The best model included all the variables except year (Table 5, Figure 2). This model was 2.5 times more plausible given our data and the possible models than the second best model.

Across all potential models, the parameter with the highest relative importance was the duration of orientation and persuasion stage (present in all models; Table 5). Duration was the only variable that had a 95% confidence interval bounded away from zero (Table 6). The main effect of duration was to decrease the length of the tail-straddling walk stage (Table 6, Figure 2). Gland presence or absence was the second most important variable (present in 4 out of 4 models; Table 5, Table 6). The main affect of gland presence was to lengthen tail-straddling walk duration, but the interaction between gland and persuasion stage (present in 2 out of 4 models) had the opposite effect of decreasing tail-straddling walk duration. Gland and the interaction between persuasion stage duration and gland were present in the best two models which had a combined weight of 0.7 (meaning that these models were 2.3 times as likely as the models that did not include these two variables). Thus, glanded males with long persuasion stages had shorter tail-straddling walk stages than did de-glanded males with the same persuasion stage duration. Using the best model and the average duration of persuasion stage across all trials (88 min), glanded males had a 5.5 minute reduction in tail-straddling walk duration compared to de-glanded males (34.5 min vs. 39.9 min). At 139 min of persuasion (the third quartile of persuasion stage durations),

tail-straddling walk was 9.7 min shorter for glanded than de-glanded males (24.9 min vs 34.6 min).

DISCUSSION

Understanding the relationship between courtship signals and courtship success in taxa with multiple courtship cues is challenging. Nevertheless this relationship can be elucidated in systems in which it is possible to investigate the relative contributions of each signal to courtship success. Male *Plethodon shermani* perform a series of sequential courtship displays, and we were able to determine the contribution of two components of the courtship display to courtship success. Foot-dancing, a visual cue provided by males during the orientation and persuasion stages of courtship, increased the likelihood that a female would enter into the next stage of courtship with the male. The transition from the persuasion stages of courtship to the tail-straddling walk stage is the most critical for mating success. Overall, the transition probability between persuasion and tail-straddling walk is relatively low. Successful transitions occurred in only 22% of the staged encounters. Thus, the visual display of foot-dancing clearly is a key component of male courtship behaviors. Moreover, the outcome of the persuasion phase seems to be critically important for male insemination success, because if a male can progress to the tail-straddling walk stage there is a 90% chance of courtship success (i.e., female insemination).

In contrast to the relationship between the visual signal and courtship success, males with intact mental glands did *not* have higher insemination success than males

without a mental gland, although we did find an affect of gland on tail-straddling walk duration. Several factors could explain this lack of courtship pheromone effect on insemination. First, despite our large sample size, we may not have had the power to detect differences in courtship success due to courtship pheromones. In our laboratory setting, we had relatively few failures once a pair was in tail-straddling walk: insemination occurred in over 90% of these courtships. The transition probabilities in the field may be lower than those observed in the laboratory for both the transition from persuasion to tail-straddling walk and from tail-straddling walk to insemination. Second, there may be a complex relationship between courtship pheromone and insemination success. Although we did not find that courtship pheromones increased insemination success, they did influence courtship duration. At the average duration of persuasion stage pheromone delivery reduced tail-straddling walk duration by 14% (comparable to the range of values observed in previous courtship experiments that used pheromone treatment with de-glanded males, Rollmann et al. 1999; Rollmann et al. 2003; Houck et al. 2008). This difference between courtship duration of glanded and deglanded males increased with longer persuasion stages, but was inverted when males had very short persuasion stages. The study design of previous experiments on *P. shermani* avoided this complicated interaction between pheromone delivery and duration of orientation and persuasion stage by only delivering pheromone after TSW initiation. In this study, glanded males could also deliver pheromone during the persuasion stage.

Our working hypothesis is that *P. shermani* employ signals from multiple modalities during courtship because of the difference in range at which each signal is detectable. The initial use of visual signals that can be perceived at a distance is common and often combined with other displays that are employed once the receiver is in closer proximity to the sender (Hebets & Papaj 2005). When *P. shermani* males are within the visual range of the female, the visual cue of foot-dancing is the primary component of the multi-modal courtship display. Unlike the non-volatile courtship pheromone which requires contact with the female to be perceived, this signal can be detected by the female during the male's approach. Another function of foot-dancing may be to establish the context for the male's approach enabling her to distinguish between aggression and courtship (*cf* Patricelli et al. 2003; Hughes 1996). In many *Plethodon* salamanders the behaviors leading up to aggression include "turning towards" (i.e., orienting the head and body towards an individual) and "approach"ing a rival (Thurrow 1975; Nishikawa 1985; Anthony and Wicknick 1993; Marvin 1998). These same behaviors precede courtship. In addition, to deliver pheromone to the female's nares, a male must raise his head above the female, a motion that could be perceived as an aggressive behavior. In other plethodontid salamanders, lifting the head and body off the substrate ("all trunk raised" position) is considered an aggressive displays (Jaeger 1984). Thus, foot-dancing may allow the male to get closer to the female even when he behaves in ways that might otherwise be considered aggressive. The concept that components of multi-modal displays may provide a

context for each other is supported by observations in snapping shrimp (*Alpheus heterochaelis*): the receiver responds aggressively to the visual display alone but, in the presence of a chemical cue indicating sex, the receiver responds in a sex-dependent manner (either courting or threatening the sender) (Hughes 1996). A third possible role of foot-dancing is sex determination of potential partners (Organ 1960). This strategy is proposed to be employed when males do not distinguish sex by chemical cue. Instead sex is thought to be determined via the differential response of males and females to the approaching male's foot-dance display. Whether this is a strategy on the part of a male or a side effect of insufficient information on the approached individual's sex still needs to be tested.

In addition to exploring the influence of multiple signals on the transition probabilities between courtship stages, we investigated whether information transfer in one stage could influence subsequent stages. We compared the duration of the orientation and persuasion stages (when foot-dancing primarily occurs) with the duration of the tail-straddling walk (when pheromone delivery occurs). We found that for courtships involving both deglanded and glanded males, the more time a pair spent in the orientation and persuasion stages of courtship, the less time they spent in tail-straddling walk. This is interesting because the duration of tail-straddling walk is highly correlated with the number of pheromone delivery attempts. Thus, females in courtship with long persuasion and orientation stages receive less chemical cues than females in courtships with short persuasion and orientation stages. One explanation

for this pattern is that a female that receives relatively more information during an initial stage of courtship may need less information in subsequent stages. With the additional information received from the male in a longer persuasion stage, she may become more certain of her choice and require less information from the tail-straddling walk stage. If so, the pair may transition to the spermatophore deposition stage more rapidly. Inversely, a reduction in persuasion time may mean that a female has less information and so requires more informative signals from the male, thus lengthening the tail-straddling walk stage.

One alternative to this scenario of information transfer is that overall courtship time (including persuasion and tail straddling walk) is determined by physiological constraints on males. It is possible that males need a set amount of time to mobilize all the secretions in their cloacal glands to form the spermatophore. If a pair transitions to tail-straddling walk rapidly, males may need more time during that stage to create the spermatophore. Whereas, if the male has been courting the female for a long time during the persuasion stages, the spermatophore may already be created and the male will be ready to deposit it sooner, leading to a reduced tail-straddling walk stage. However, this physiological limitation hypothesis is less plausible given that we observed several courtships with both a short persuasion stage and a short tail-straddling walk that ended with spermatophore deposition (Figure 2). In addition, spermatophores are sometimes deposited when males shed, implying the

spermatophore is at least partially formed before courtship (Organ & Lowenthal 1963).

In conclusion, courtships involving multi-modal cues are complex, and investigating the relative roles of individual signals is critical to determine their individual contributions to courtship success. Our study, coupled with previous research in this system, suggests that female red-legged salamanders incorporate information from both visual and chemical signals when interacting with a potential mate, a phenomenon that is increasingly documented in animal courtships.

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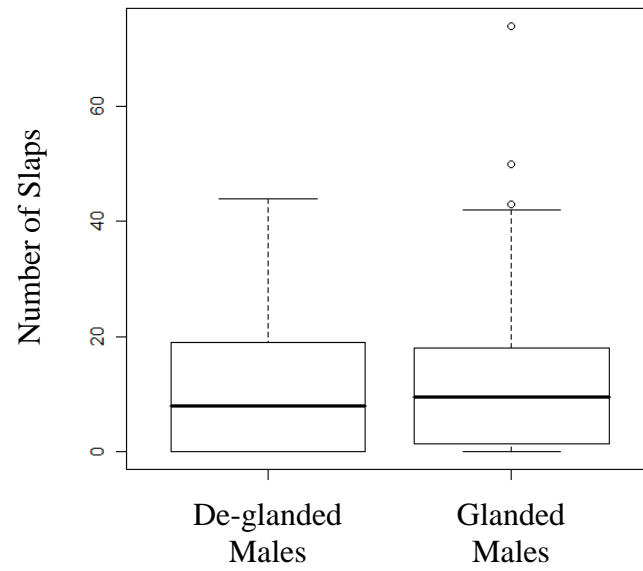


Figure 2.1 Number of slaps delivered by de-glanded (N= 61) and glanded males(N= 60) during tail-straddling walk.

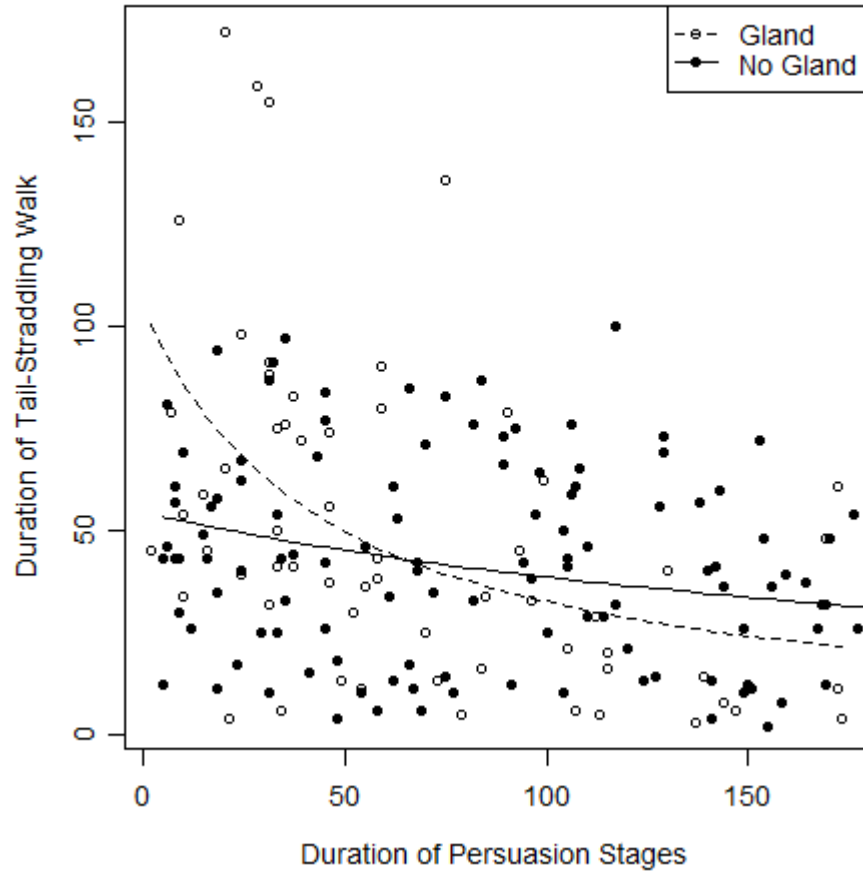


Figure 2.2 Relationship between duration of Persuasion stages (minutes) and duration of Tail-Straddling Walk stage (minutes) in *P. shermani*. Open circles are trials using de-glanded males. Closed circles are trials using glanded males. Lines are predicated responses from the best model identified using AICc ($TSW \text{ duration} = 1/(0.01836 - 0.008856*(\text{gland}) + 0.00007593*(\text{persuasion stage}) + 0.000371*(\text{persuasion stage})(\text{gland}))$).

Table 2.1 Model selection results for factors influencing tail-straddling walk initiation in *P. shermani*. Only models that are informative ($\Delta_i < 10$) are shown. Models are ranked from those with the most support to those with the least. Abbreviations are: Pi.Foot-dancing = proportion of time in persuasion and orientation a male spent foot-dancing, M.SVL = male SVL, F.SVL = female SVL, M.sincecourt = number of days since a male last encountered a female, F.sincecourt = number of days since a female last encountered a male. For an explanation of the values reported in the table refer to the Statistical Analyses section for Experiment 1 in the Methods section.

Rank	Model	k	AICc	Δ_i	ω_i
1	Pi.Foot-dancing	2	65.42	0.00	0.23
2	Pi.Foot-dancing +M.SVL	3	66.10	0.68	0.16
3	Pi.Foot-dancing +M.sincecourt	3	67.23	1.81	0.09
4	Pi.Foot-dancing + F.SVL	3	67.52	2.10	0.08
5	Pi.Foot-dancing + F.sincecourt	3	67.53	2.11	0.08
6	Pi.Foot-dancing +F.sincecourt + M.SVL	4	67.94	2.52	0.06
7	Pi.Foot-dancing + M.sincecourt + M.SVL	4	68.09	2.67	0.06
8	Pi.Foot-dancing + F.SVL + M.SVL	4	68.19	2.77	0.06
9	Pi.Foot-dancing + F.SVL + M.sincecourt	4	69.38	3.96	0.03
10	Pi.Foot-dancing + F.sincecourt + M.sincecourt	4	69.43	4.01	0.03
11	Pi.Foot-dancing + F.SVL + F.sincecourt	4	69.67	4.25	0.03
12	Pi.Foot-dancing +F.SVL + F.sincecourt+ M.SVL	5	70.07	4.65	0.02
13	Pi.Footdnacing + F.sincecourt+M.sincecourt + M.SVL	5	70.11	4.69	0.02
14	Pi.Foot-dancing + F.SVL +M.sincecourt + M.SVL	5	70.25	4.83	0.02
15	Pi.Foot-dancing + F.SVL +F.sincecourt+M.sincecourt	5	71.64	6.22	0.01
16	Pi.Foot-dancing + F.SVL + F.sincecourt + M.sincecourt +M.SVL	6	72.31	6.89	0.01

Table 2.2 Relative parameter importance for predicting tail-straddling walk initiation in *P. shermani* weighted across all models with a $\Delta_i < 10$ as well as averaged model parameters. For an explanation of the values reported in the table refer to the Statistical Analyses section for Experiment 1 in the Methods section.

Parameters	Relative Variable Importance	Model Averaged β	Unconditional 95% CI
Intercept	N/A	-4.71	-13.8 to 4.33
Proportion of first hour male spent foot-dancing	1	5.40	2.50 to 8.30
Male SVL (mm)	0.42	0.0458	-0.0971 to 0.189
Male's days since last courtship	0.28	0.0337	-0.149 to 0.217
Female SVL (mm)	0.26	-0.00606	-0.0548 to 0.427
Female's days since last courtship	0.26	-0.0282	-0.220 to 0.163

Table 2.3 Model selection results for factors influencing insemination success in *P. shermani* courtship. Only models that are informative ($\Delta i < 10$) are shown. Models are ranked from those with the most support to those with the least. Abbreviations are: Slaps = number of times the male delivered pheromone to the female's nares, gland = presence (1) or absence (0) of mental gland, M.SVL = male SVL, F.SVL = female SVL, M.sincecourt = number of days since a male last encountered a female, F.sincecourt = number of days since a female last encountered a male. For an explanation of the values reported in the table refer to the Statistical Analyses section for Experiment 1 in the Methods section.

Rank	Model	k	AICc	Δi	ω_i
1	Slaps + F.SVL	3	68.35	0.00	0.04
2	Slaps	2	68.38	0.03	0.04
3	Slaps + gland	3	68.58	0.23	0.04
4	gland	2	68.71	0.35	0.04
5	Slaps + gland + F.sincecourt	4	68.81	0.45	0.03
6		1	68.87	0.51	0.03
7	gland + M.sincecourt	3	69.17	0.82	0.03
8	gland + F.sincecourt	3	69.19	0.83	0.03
9	Slaps + F.sincecourt + F.SVL	4	69.34	0.99	0.03
10	Slaps + F.sincecourt	3	69.39	1.03	0.03
...					
60	Slaps + gland + F.sincecourt + M.sincecourt + F.SVL + M.SVL	7	72.72	4.37	0.00
61	Slaps + F.sincecourt + M.sincecourt + M.SVL	5	72.73	4.38	0.00
62	Slaps + F.sincecourt + M.sincecourt + F.SVL + M.SVL	6	72.74	4.38	0.00
63	F.sincecourt + M.sincecourt + M.SVL	4	72.79	4.43	0.00
64	F.sincecourt + M.sincecourt + F.SVL + M.SVL	5	73.50	5.15	0.00

Table 2.4 Relative parameter importance for predicting insemination success in *P. shermani* courtship weighted across all models with a $\Delta_i < 10$ as well as average model parameters. For an explanation of the values reported in the table refer to the Statistical Analyses section for Experiment 1 in the Methods section.

Parameters	Relative Variable Importance	Model Averaged β	Unconditional 95% CI
Intercept	N/A	6.75	-4.55 to 18.00
Slaps	0.55	-0.26	-0.92 to 0.40
Gland	0.52	-0.57	-2.06 to 0.92
Female SVL (mm)	0.42	-0.05	-0.21 to 0.11
F.sincecourt	0.42	0.17	-0.36 to 0.69
M.sincecourt	0.39	-0.20	-0.85 to 0.46
M.SVL (mm)	0.27	-0.01	-0.07 to 0.06

Table 2.5 Model selection results for factors influencing tail-straddling walk duration in *P. shermani* courtship. Only models that are informative ($\Delta_i < 10$) are shown. Models are ranked from those with the most support to those with the least. Abbreviations are: Persuasion duration = number of minutes pair spent in orientation and persuasion stages, gland = presence (1) or absence (0) of mental gland, year = year of study (either 2007 or 2008). For an explanation of the values reported in the table refer to the Statistical Analyses section for Experiment 1 in the Methods section.

Model Rank	Model	k	AICc	Δ_i	ω_i
1	Persuasion Duration + Gland + Gland x Persuasion	5	1771	0.00	0.502
2	Persuasion Duration + Gland + Gland x Persuasion + Year	6	1772	1.81	0.203
3	Persuasion Duration	3	1773	2.58	0.138
4	Persuasion Duration + Gland	4	1774	3.82	0.074
5	Persuasion Duration + Year	4	1775	4.64	0.049
6	Persuasion Duration + Gland + Year	5	1776	5.37	0.034

Table 2.6 Relative parameter importance for predicting tail-straddling walk duration in *P. shermani* courtship weighted across all models with a $\Delta_i < 10$ as well as average model parameters. For an explanation of the values reported in the table refer to the Statistical Analyses section for Experiment 1 in the Methods section.

Parameters	Relative Variable Importance	Model Averaged β	Unconditional 95% CI
Intercept	N/A	-0.747	-4.66 to 3.17
Persuasion Stage	1	0.0000843	0.0000306 to 0.000138
Gland	0.85	-0.00714	-0.0159 to 0.001
Gland x Persuasion	0.77	0.000105	-0.00000371 to 0.000247
Year	0.22	0.000381	0.00157 to 0.00233

Chapter 3— Sequential male mate choice in a terrestrial salamander, *Plethodon shermani*

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ABSTRACT

Male mate choice can be documented by identifying variation in male reproductive effort when paired with females that differ in some aspect. We explored male mate choice in a plethodontid salamander, *Plethodon shermani*, by measuring male investment in a visual display correlated with courtship success. We also assessed whether males used female size as a proxy for female fecundity. In the first experiment, we observed males paired with females of varying fecundity (non-gravid, weakly gravid and strongly gravid). Males foot-danced most frequently for females with high reproductive value (strongly gravid females) and less for non-gravid and weakly gravid females. When paired with females of varying levels of fecundity, males did not use female size to determine how much effort to allocate to courtship. However, when paired with females of similar reproductive value (strongly gravid females), males increased their effort in correlation with female size. Finally, male foot-dancing effort was correlated with male condition.

INTRODUCTION

Mate choice has traditionally been documented in a female-centric manner, with male mate choice only explored in sex-role reversed species (Andersson 1994). More recently, however, male mate choice has been observed across many different types of mating systems, including polygynous species with no paternal care. These polygynous systems are found in a diversity of taxa ranging from insects

(Bouduriansky 2001) to fish (Amundsen & Forsgren 2001) and birds (Amundsen 2000), and are expanding our understanding of the factors that influence mate choice decisions.

Male mate choice can be defined as a modified sexual response to different reproductively mature conspecific females (Bouduriansky 2001). Pre-copulatory male mate choice can be expressed as either: (a) discrete choices by males between females of differing quality (*cf.* Verrell 1986), or (b) as continuous variation in the amount of effort invested by males in individual courtship encounters (*cf.* Guevara-Fiore et al. 2010). Post-copulatory mate choice involves differential donation of sperm or seminal proteins to females of varying quality (Wedell et al. 2002).

Models predict that male mate choice will evolve in species that meet three criteria: (1) the number of mates available exceeds a male's capacity to mate, (2) females are variable in their reproductive quality, and (3) the benefits of choosing females exceeds the cost of assessing them (Edward & Chapman 2011). One group of organisms that meets all three of these requirements is the clade of Caudate amphibians, and, in particular, salamanders of the genus *Plethodon*.

The first criterion for male mate choice (possible mates exceed male mating capacity) requires demonstrating that males have limited resources to expend on mating. Among other factors, limitations on male mating capacity can come from energetically costly or prolonged courtship displays: the time and energy a male spends courting one female reduces his ability or opportunities to court others (Edward & Chapman 2011). Animals with elaborate and prolonged courtship that

seem to limit male mating capacity include many species of birds, insects, fish and amphibians (Bouduriansky 2001). Among Urodeles, salamanders in the genus *Plethodon* have some of the most complex and prolonged courtships that involve visual, tactile and olfactory cues. One of the best studied of these species is the red-legged salamander (*Plethodon shermani*), a large terrestrial species found in the forested mountains of western North Carolina.

Red-legged salamanders have a highly stereotyped five-stage courtship (Houck & Arnold 2003). During the first stage of courtship the male orients and moves towards the female. In the second stage (persuasion), a male attempts to “convince” a female to enter into the third stage by performing a visual display (“foot-dancing”). The foot-dancing behavior involves the male raising and lowering his legs while his trunk rests on the ground and remains still (Organ 1958; Arnold 1976). Males will consistently foot-dance for multiple hours, with short breaks. This prolonged courtship effort seems to be preferred by females in the sense that the proportion of time the male spends foot-dancing increases the likelihood that a pair will transition to the next stage of courtship (Chapter 2). The third stage of courtship is defined by a distinctive behavior termed “tail-straddling walk”. During this stage, a female straddles the male’s tail while moving forward in tandem with him (Noble 1929; Stebbins 1949; Arnold 1976). While the pair is in tail-straddling walk, the male typically delivers proteinaceous courtship pheromones to the female by bringing his mental gland in direct contact with the female’s nares (a behavior termed “slapping”). A successful tail-straddling walk leads to the deposition of a spermatophore (stage 4), which

consists of an apical sperm mass on a gelatinous base, and transfer of the sperm mass to the female's cloaca (stage 5). The overall duration of courtship (as observed in male-female pairs in the laboratory) varies wildly between pairs from as short as 17 minutes up to as long as 4.5 hours, with a mean length of 2 hours (Chapter 2). Thus, courtship duration and energy expenditure may limit male mating capacity in this species, setting a precedent for males to be choosier in the assessment of potential mates.

The second criterion that promotes the evolution of male mate choice is variation in female quality, defined as variation in fecundity or viability (Servidio & Lande 2006). As with most salamanders (Kaplan & Salthe 1979), larger females have higher fecundity among the *Plethodon* and thus have more reproductive value to males. In addition to the variation in body size, female *P. shermani* may only come into reproductive condition every other year. The multi-month period that a female must spend defending her eggs prevents her from foraging extensively (Petranka 1998). Therefore, at the beginning of the mating season, some large females will not have any reproductive value to a male (non-gravid), while others will only have a few or poorly developed ova (weakly gravid), and still others will have a high reproductive value to a male (strongly gravid). This variation in gravidity would additionally place a strong selective pressure on males to be choosy in investing time and energy into courting particular females.

In addition to variation in female quality, males must be able to detect this variation if choice is to evolve. Multiple solutions are possible to this detection

problem, including the assessment of females by different sensory systems, such as olfactory, visual and tactile cues (Bounduriansky 2001). In many oviparous species, the most common cue for variation in female fitness is her size: which is often directly correlated with fecundity (Servidio and Lande 2006). Male salamanders of several species distinguish among conspecifics by the visual cue of size (Verrell 1985; Verrell 1986). Other species detect differences between gravid and non-gravid females through olfactory cues (Dantzer & Jaeger 2007; Marco et al. 1998). This assessment has not been documented for *P. shermani*, but *P. shermani* males are known to be able to identify the sex and species of an individual based on substrate-borne chemical cues (Palmer and Houck 2005).

The third criterion for male mate choice to evolve is a low cost of searching for and assessing mates. For *P. shermani*, females are locally abundant: one estimate of density was as high as 0.86 *P. shermani*/m² (Ash 1988). In addition, individuals tend to encounter each other only on rainy or moist nights (Petranka 1998). Hence, although these animals have a prolonged breeding season (from late July to early October), males and females may actually have a limited number of nights when the temperature and ground conditions are appropriate for surface activity that includes courting and mating. Given the density of the available females and the limited time a male has to court, the number of females encountered by males could exceed his capacity for mating. This circumstance is especially likely considering how protracted and potentially energetically costly an individual courtship is. Thus, in these high

population densities with sporadic mating opportunities, the costs of searching for and assessing mates, in terms of mating opportunities lost, could be low.

In this study, we explored whether male *Plethodon shermani* engage in mate choice through modification of the foot-dancing display. We predicted that males would foot-dance most often for strongly gravid females, less for weakly gravid, and with the least investment in non-gravid females. We chose to examine this aspect of courtship because foot-dancing is easily quantified and is the only courtship component that has been directly correlated with courtship success (Chapter 2). We examined the potential for male mate choice between females of different levels of gravidity (non-gravid, weakly gravid and strongly gravid). We also explored whether males used cues other than gravidity (such as body size) to determine female reproductive fitness by assessing male foot-dancing effort during staged courtships, each with a differently sized strongly gravid female. Finally, we investigated whether selected physical characteristics of males (condition, mental gland diameter and height, and tail length) were related to male courtship effort.

METHODS

Animal Collection and Maintenance

We collected 60 adult male and 100 adult female *Plethodon shermani* in Macon County, North Carolina (035°10'48"N 083°33'38"W) during early August 2011. Each animal was maintained individually in a clear plastic box (17 cm x 9 cm x 13 cm) with a substrate of moist paper towels and crumpled damp towels for refugia.

For Experiment 1 (described below), animals were housed at Highlands Biological Station (HBS; Highlands, NC, USA). Females were not fed for the duration of the experiment to prevent their condition from changing between trials. Males were fed two waxworms (*Galleria mellonella*) weekly. Females were fed at the end of the experiment (approximately 2 weeks after collection). For Experiment 2 (described below), animals were shipped to Oregon State University (OSU; Corvallis, OR, USA). *P. shermani* were maintained at 15 – 18°C on a late-August North Carolina photoperiod and each fed two waxworms (*Galleria mellonella*) weekly.

Gland Removal

Mental gland removal was necessary to control for the delivery of male courtship pheromones in our experiments. To remove a gland, we anesthetized each male in a mixture of 7% aqueous ether. Each gland was surgically excised using iridectomy scissors (after Houck et al. 1998). All males were given at least two weeks to recover before use in trials, during which time all males recovered completely.

Experiment 1: Do males vary the proportion of orientation and perussation they foot-dance based on female fecundity?

Courtship trials were initially set up at HBS to identify deglanded males willing to mate in the lab. Of the males that would initiate courtship, 20 were randomly chosen to participate in the behavioral trials. The 60 females collected for this experiment were not used in any courtship trials before the start of the experiment.

We measured the snout-vent length (SVL; mm) and degree of gravidity of each female. To quantify gravidity, we placed females into one of three categories: non-gravid (NG; no ova present in either ovary), weakly gravid (WG; small, poorly developed ova present), or strongly gravid (G; both ovaries full of large well-developed ova or a mix of small and well developed ova). Females measuring less than 46 mm in SVL were not considered reproductively mature and were not used in our study (*cf.* Hairston 1983). For the trials, one female from each category was randomly assigned to each male.

Three nights of courtship trials were staged in mid-August 2011 (August 10, 12 and 16th) from 9 – 10:30 pm EST. Each male was placed in a clear plastic box (17 cm x 9 cm x 13 cm) with a damp paper towel lining the bottom. He was paired with either a non-gravid, weakly gravid or strongly gravid female. The order in which each male was exposed to the three different types of females was randomized. The pairs were observed for 90 minutes. Each observer watched and scored the same 5 males each night to eliminate inter-observer variation within males. Data were collected by scan sampling (Altmann 1974), each minute (for a total of 90 minutes), an observer recorded whether or not the male was foot-dancing.

Experiment 1: Statistical Analyses

We used general linear mixed modeling (GLMM) with the nlme and lme4 libraries in R to determine whether female reproductive condition influenced male foot-dancing behavior. We chose linear mixed effect models because: (1) our analyses

incorporated repeated measures, and (2) our response variable was a proportion. General linear mixed effect models are more robust against deviation from normality than are repeated measures ANOVAs (Bolker et al. 2009). General linear mixed models can also account for non-independence of observations by incorporating a clustering variable (in this case male ID) into the model as a random effect (Bolker et al. 2009). Because our response variable was a proportion, we used a binomial distribution with the probit linking function to model our data. The full model included the proportion of observations that a male spent foot-dancing as the response variable, female reproductive condition as a fixed effect, and male ID as a random effect. We used the χ^2 -squared goodness of fit test to compare the fit of this full model and a model that did not include female reproductive condition. A full model that yielded a significantly better fit to our data would indicate that female reproductive condition is an important variable influencing male foot-dancing effort.

Experiment 2: Do males vary foot-dancing effort based on female size?

Forty (40) deglanded males and 40 strongly gravid females were chosen for behavioral trials from a pool of animals that mated under laboratory conditions. Before the start of the trial, morphometric data were collected on the males and females, including: snout-vent length (SVL in mm), tail-length (mm), and body mass (g). Each male also received a score for the initial height (1: flush with chin; 2: slight bulge; 3: greatly swollen beyond surface of chin) of his mental gland, and its diameter (mm). Females were scored for gravidity (NG, WG, G). Only strongly gravid females were

used in this experiment. The chosen animals were randomly placed into 4 blocks (10 males and 10 females in each). Over the course of the experiment, each male was paired with every female in his block; consequently, each female was also paired with every male in her block. Trials were conducted from mid-September to early October, 2011. Animals had one to two nights off between each trial night.

On each trial night, beginning at 8:30 pm EST, 40 male-female pairs were placed into individual plastic containers (17 cm x 9 cm x 13 cm) that were lined with a damp paper towel substrate. Behavioral observations were performed under low light conditions using dim white light that mimicked nocturnal light levels in the field. Each trial lasted 3 hours, and each block of 10 animals was watched by the same observer across all 10 trial nights. Observers scanned each pair every 3 minutes and recorded whether the male was foot-dancing, or if the pair had progressed successfully to the next stage of courtship (tail-straddling walk). If a pair was observed in tail-straddling walk for 2 consecutive scans (6 minutes) scoring was terminated. The observer then disrupted the courtship to prevent the female from becoming inseminated. This precaution was necessary because each female needed to be paired with all the males in her block. Inseminated females will not re-mate for at least 3 days (Chapter 5) and thus would not be reusable. The number of times a male or female entered into tail-straddling was also tracked throughout the experiment.

Experiment 2: Statistical Analyses

We again used GLMM in R to determine whether males varied their foot-dancing efforts across the strongly gravid females that varied in SVL. We addressed two questions through this analysis. First, we determined whether female SVL influenced male foot-dancing effort. In these models, we included female SVL as a fixed factor and male ID as a random factor in our full model. We used a χ^2 goodness-of-fit test to compare this model to a model that did not include female SVL.

In addition to assessing whether male responsiveness varied according to female size, our secondary goal was to identify whether any characteristics of males themselves could predict the proportion of observations that each male spent foot-dancing. We tested for correlations among male morphological characters that could be related to reproductive effort, including: male condition (residuals from a regression of mass on SVL), male mental gland diameter and height (measured before each male was deglanded for these trials) and tail length. We explored the relationship between these variables and male foot-dancing effort using general linear regression with a binomial response.

RESULTS

Experiment 1: Influence of female reproductive condition on male visual displays

Tail-straddling walk was not initiated in any of the trials, so every male had 90 observations per trial. The χ^2 -squared goodness of fit test revealed that female reproductive condition accounted for a significant amount of the variance in the

proportion of time a male spent foot-dancing ($\chi^2 = 125.25$, $p = <0.0001$; Figure 1A). When paired with strongly gravid females, males foot-danced, on average, for 29.4% of the trial period (Figure 1B). Males paired with non-gravid females were observed foot-dancing on average 1.6 times less often ($z = -10.89$, $p < 0.0001$), and 2.3 times less often when with weakly gravid females ($z = -6.514$, $p < 0.0001$). We used a post-hoc randomization test to compare whether males foot-danced significantly more or less for weakly gravid versus non-gravid females and found no significant difference between these groups ($p = 0.2877$).

To determine whether fecundity was assessed via female size, we compared two models with and without female SVL. The model using female SVL did not significantly explain our data better than a model without it ($\chi^2 = 0.80$, $p = 0.37$).

Experiment 2: Influence of female size and male morphology on male visual displays

Not every female was paired with 10 males for two reasons: (1) two females were inseminated in our trials and were subsequently removed from the experiment; these females were replaced with two new females, and (2) one male became sick during the trials and was removed, but not replaced. The average proportion of persuasion and orientation stages spent foot-dancing in these trials was 17.47% (ranging from 0 to 46%).

A χ^2 test comparing a full model containing female SVL and male identity (as a random factor) to a model with only male identity (as a random factor) was significant, indicating that female SVL influenced male foot-dancing effort ($\chi^2 =$

62.55, $p < 0.0001$, Figure 2a). The difference in foot-dancing effort expended between the smallest (54 mm) and largest (71 mm) females was estimated to be 6%.

Our final analyses explored the connections between foot-dancing and male morphological variables, including male condition, tail length, and the mental gland diameter and thickness. An initial check for correlations between morphological variables revealed that male condition was positively correlated with tail length (Table 1). Mental gland height was also positively correlated with mental gland size. Consequently, we chose to pursue only male condition and mental gland size our analyses. We found both male condition ($\beta = 0.214 \pm 0.029$, $p < 0.00001$) and mental gland diameter ($\beta = 0.214 \pm 0.012$, $p < 0.00001$) were significantly positively correlated with foot-dancing. Males with higher than average condition foot-danced 1.3x more often during orientation and persuasion than those with lower than average condition (median values of 18.37% vs. 14.12%). Males with a mental gland diameter greater than average foot-danced 2.25x more often in orientation and persuasion than those with smaller than average mental glands (22.5% vs. 10%).

DISCUSSION

Plethodon shermani males clearly exhibit mate choice, as demonstrated by a differential allocation of courtship signaling effort (amount of foot-dancing) to females in different reproductive conditions. When paired with females with large differences in reproductive value (non-gravid, weakly gravid and strongly gravid), males did not use female size as a cue for allocating foot-dancing effort. In contrast,

when males were paired with females of similar reproductive value (all strongly gravid) size was used by males to determine how much effort to allocate to courtship. In addition to female characteristics, male condition also contributed to how much a male foot-danced. As foot-dancing influences courtship success (Chapter 2), the observed reduction in foot-dancing by males in poor condition could have reproductive fitness consequences. Collectively, these data reveal that: (a) female and male factors affect the courtship behavior and decisions of this species and (b) male condition can influence mate-assessment behaviors through differential male signaling investment.

Foot-dancing is a visual cue employed during the early persuasion stages of courtship. The more time a male spent foot-dancing, the more likely a female was to engage in the next stage of courtship (tail-straddling walk; Chapter 2). However, investment in foot-dancing may be energetically expensive, placing a cost on males to be factored into the trade-offs associated with courtship. In the laboratory, males would foot-dance on average during 20% of a courtship trial. If a female did not enter into tail-straddling walk within the 3 hour period allotted, the male would display (on average) for 36 minutes. These salamanders are ectothermic, and also are relatively sedentary: *P. shermani* move only short distances on average (e.g., 2.6 m over 17 days), and also have relatively small home ranges (e.g., 5.04 m²; Nishikawa 1990). Given these conditions, a 36 minute foot-dancing duration may be a substantial investment of energy. In addition, *P. shermani* only are active on the surface for about 2 – 4 hours on rainy or humid nights (Petranka 1998). Courtship duration in this

species is lengthy ($\bar{x} = 120$ minutes in the laboratory), so investment in a single courtship for a male is also costly because the lengthy courtship could limit the opportunity for additional courtships. An additional cost of foot-dancing may be the attention it brings from rival males. Both Organ (1960) and Arnold (1972) noted that rival males attack courting males. For all these reasons, we expect males to exhibit mate choice, and also expect that differential investment should be made in the early stages of courtship (via foot-dancing effort) to further reduce the cost of courting females of lesser reproductive value. Males foot-dance more intensely for strongly gravid females than for non-gravid or weakly gravid females. Males may not differentiate between non-gravid and weakly gravid females because their reproductive value is equally low or because the cues given off by females in these categories are indistinguishable. Female *P. shermani* store sperm obtained during the late summer and fall, with oviposition and fertilization occurring the following late winter or early spring (Hairston 1983). Eggs must be sufficiently large to produce viable offspring, and a female yolking her ova may require up to two seasons (Highton 1962). Thus, neither non-gravid nor weakly gravid females (with their small eggs) are as likely to oviposit in a given year as are strongly gravid females. Non-gravid and weakly gravid females therefore may have similarly low reproductive values for a male. In this context, males receive the greatest reproductive pay-off in courtships with strongly gravid females, and males seem to modify their signaling investments accordingly.

Unlike most other taxa in which males engage in mate choice, female size does not seem to be the definitive cue that *P. shermani* males use to assess potential mates. When presented with females of different reproductive value, males did not use the visual cue of size to select a mating partner. In addition, when presented with only strongly gravid females, although foot-dancing was significantly correlated with female size (after controlling for male identity), there was only an average 6% increase in foot-dancing effort across the entire range of females sizes. This finding seems to set *P. shermani* apart from several other salamander systems in which female size affects male effort, but additional factors such as how males are presented potential mates need to be considered.

In mate choice studies potential males are presented sequentially or simultaneously. The differences in how mates are presented can influence the whether or not mate choice occurs (Werner & Lotem 2006). Sequential choice is a more restrictive test than simultaneous choice because it requires a male to reject a courtship opportunity without knowing whether another opportunity will appear. Rejecting a mate under these circumstances is much riskier than rejecting a potential mate when another is immediately available (simultaneous choice). Thus, sequential mate choice is expected to evolve less frequently and under more restrictive conditions than simultaneous choice (Barry & Kokko 2010). However, sequential encounters with potential mates may be the condition most species encounter in nature (Barry & Kokko 2010).

Our study used sequential choice trials. Previous male mate choice studies on salamanders have primarily used simultaneous presentation of potential mates. These studies uniformly demonstrated male choice based on female size (*Notophthalmus viridescens*, Verrell 1982; *Triturus vulgaris*, Verrell 1986; *Desmognathus ochrophaeus*, Verrell 1989; *D. santeelah*, Verrell 1995; *Plethodon vehiculum* and *P. dunni*, Marco et al. 1998; *N. viridescens* subspecies, Takahasi et al. 2010). In the few experiments that used sequential choice, this preference for larger females was not demonstrated (*D. ochrophaeus*, Verrell 1989; *D. santeelah*, Verrell 1995). Thus, *P. shermani* is the first salamander species to show male preference for large female size in a sequential mate choice study. But, this preference is slight, and size is not used to assess large differences in female reproductive value.

Two alternative cues could be involved in male mate assessment across different levels of female gravidity: female behavioral feedback and female chemical cues. In many systems, the male is attuned to the female's behavioral responses and modifies his courtship displays accordingly. In birds, for instance, a Whitethroat male (*Sylvia communis*) will reduce his courtship displays when the female does not respond to his displays with an appropriate vocalization (Balsby & Dabelsteen 2002). Thus, by assessing female behavioral cues, males lessen their investment in courtship with less responsive (and likely less receptive) females. Unfortunately, female *P. shermani* exhibit minimal receptivity cues during the orientation and persuasion stages of courtship. Receptive females simply either do not move away or do not move as far away when a male approaches her (Arnold 1976).

In addition to potential behavioral cues of receptivity, chemical cues may be likely indicators of female reproductive condition. In many salamander species, including other plethodontids, males can distinguish between gravid and non-gravid females using only olfactory cues (*P. vehiculum* and *P. dunni*, Marco et al. 1998; *P. cinereus*, Dantzer & Jaeger 2007; *N. viridescens*, Verrell 1985; *T. vulgaris*, Verrell 1986). *P. shermani* males presumably have this same ability, as other members of the *P. glutinosus* complex can use volatile cues to identify the sex, species and identity of conspecifics (Dawley 1984; Palmer & Houck 2005). In fact, the sophisticated chemical signaling system in this family of salamanders (including the olfactory detection of gravidity), strongly supports the conclusion that chemical cues likely serve as an indicator of female reproductive condition, and thus can facilitate male mate choice.

Finally, we found that male condition was a significant predictor of the amount of effort a male would invest in a courtship. As the amount of foot-dancing was positively correlated with courtship success, males with higher condition are likely to be more successful in courtship. In addition, foot-dancing was correlated with other measures of reproductive effort (mental gland diameter and height), although condition was not directly correlated with mental gland diameter and height.

Overall, this study showed that male *P. shermani* demonstrated mate choice through differential investment in their persuasive visual display in the earliest stages of courtship. Males invested the most in strongly gravid females, and did not show differential investment between non-gravid and weakly gravid females. Unlike many

other instances of male mate choice, males do not seem to use female size as the primary cue of female reproductive value, but may be assessing direct cues of female gravidity through olfactory mechanisms or behavioral cues.

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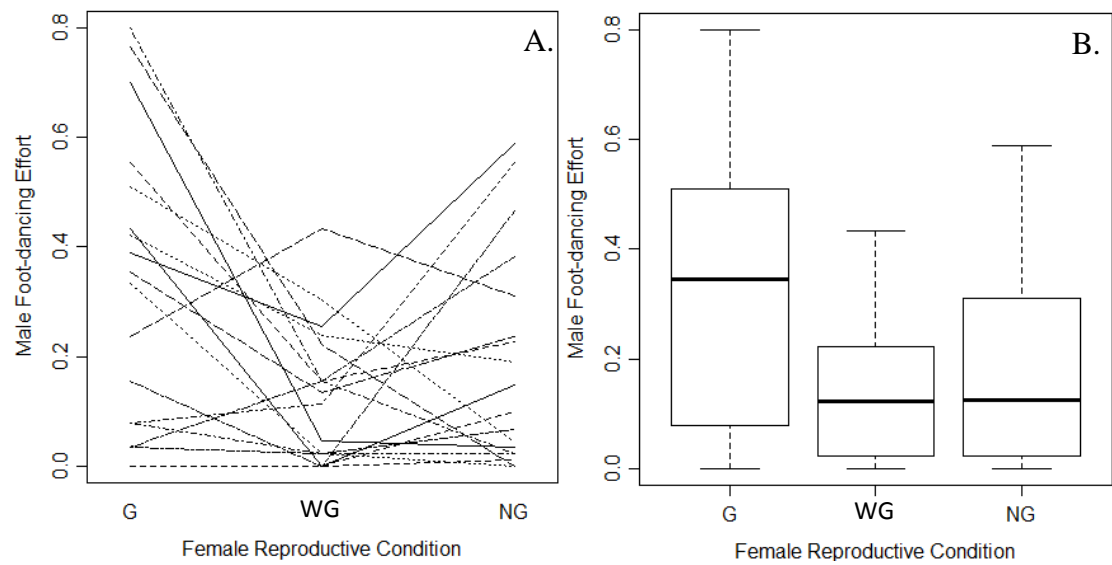


Figure 3.1 (A) Variation in individual male foot-dancing effort across three female reproductive condition categories (G = strongly gravid, WG = weakly gravid, NG = nongravid). Each line represents one male. (B) Summary of male foot-dancing effort across 3 female reproductive conditions. The same 20 males were assessed for each boxplot. Boxes represent interquartile range and middle line represents median.

Table 3.1 Correlation matrix for male *P. shermani* (n=40) morphological variables. Correlations significant at the 0.01 level are indicated with a *.

	Condition	Tail Length	Mental Gland Height	Mental Gland Diameter
Tail Length (mm)	0.626*	1		
Mental Gland Height	0.0520	0.280	1	
Mental Gland Diameter (mm)	0.169	0.244	0.823*	1

Chapter 4 – The role of courtship pheromone delivery in the early stages of plethodontid courtship and a review of the timing and delivery mode across the family.

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ABSTRACT:

Comparative studies of signal evolution seek to examine the origin, maintenance, loss and diversification of signals. A novel form of signal co-option that leads to signal diversification may have occurred in plethodontid salamanders. Some species in this family have experienced a shift in the sensory mode by which females perceive a chemical signal (proteinaceous courtship pheromones). This shift involved the transition from transdermal delivery of courtship pheromones to olfactory delivery. We examined whether the functional significance of courtship pheromones may have changed with the shift in delivery modes. In addition to surveying the literature on plethodontid courtship behavior, we conducted courtship experiments on *Plethodon shermani*, a species with olfactory pheromone delivery. In *P. shermani*, we found no affect of pheromone delivery on duration of the early stages of courtship, or on the probability of transitioning to subsequent stages of courtship. These negative results are in contrast with species with transdermal pheromone delivery. In these species the early courtship stages are reduced in duration when courtship pheromone is delivered to a female. In addition, compared to species with transdermal pheromone delivery, *P. shermani* are more likely to: (a) deliver pheromone during later stages of courtship, and (b) have more prolonged later stages (similar to other species using olfactory delivery). Overall, these data suggest the functional significance of courtship pheromones in olfactory species (like *P. shermani*) occurs in the later stages of courtship. This is a shift in timing of the pheromone affects from the ancestral mode of pheromone delivery (transdermal).

INTRODUCTION

The study of signal evolution commonly focuses on the origin, maintenance, loss, or diversification of signals across lineages (Bradbury & Vehrencamp 2011). Diversification of a signal can occur via elaboration or co-option of a signal from one social context to another. An example of signal co-option can be observed in female Magpie-Jays (*Calocitta formosa*) that employ juvenile begging calls during courtship to elicit courtship feeding from males (Tobias & Seddon 2002). Co-option of a signal potentially could also involve the evolutionary transition of perception of a cue from one sensory pathway to another. This transition would be difficult for visual or auditory cues because the receptors involved in the detection of these signal have such different inputs. Chemical cues, however, have the potential to transition between different chemosensory pathways. For example, both sexes of *Drosophila* employ cuticular hydrocarbons for chemical communication during courtship. These chemicals can be perceived by both gustatory and olfactory organs on the fly's body and, thus, can be processed through either (or both) sensory pathways (Ferveur 2005). This rare ability of receptors from different sensory pathways to recognize the same signals could facilitate the transition of perception between distinct sensory pathways.

Such a transition in signal preception has been documented in plethodontid salamanders (Figure 1). In this system, protein pheromones (chemical signals) produced by courting males are presented to the female via either transdermal or olfactory delivery, depending on the lineage (Arnold 1977; Houck & Arnold 2003). Transdermal delivery of courtship pheromones is the ancestral mode that later shifted

to olfactory delivery in one clade of *Plethodon* salamanders, the *P. glutinosus* group (Houck & Arnold 2003; Highton et al. 2012). Both modes of pheromone reception involve a multi-modal cue combining the instantaneous delivery of tactile and pheromonal stimulation. The change in the dominant sensory pathway for pheromone perception in females is coupled with correlated changes in a functional complex of traits that includes male morphology, courtship behaviors, and the molecular make-up of the signal itself (Watts et al. 2004).

Transdermal delivery can occur via two methods: biting or scratching the skin of the female. Pheromone delivery via biting occurs in at least two species of small desmognathine salamanders (Promislow 1987; Verrell 1999). Courtship pheromones in these species are produced in a submandibular (mental) gland that releases secretions into a male's mouth (Houck & Sever 1994). The tactile component of the pheromone delivery behavior occurs when a male closes his jaws on the female's body during courtship, and then tugs on the female's body. The tugging is thought to release the courtship pheromone, which is directly "injected" into the female (Houck & Sever 1994).

The gland morphology and pheromone delivery behavior of scratching species are very different from biting species. Male of these species have small mental glands that open to the skin surface of the chin (Houck & Sever 1994). Correlated with this change in gland morphology is the presence of enlarged premaxillary teeth, which the male uses to abrade the female's skin through tactile behaviors, known as "snapping," "pulling," "sliding" or "rubbing" (Organ 1961; Arnold 1977). These behaviors also

involve the male sliding his mental gland along the female's body and thus applying courtship pheromone to the abrasions in the female's skin. In one species using the scratching mode of pheromone delivery, *Desmognathus ocoee*, courtship pheromone delivery reduces the duration of the early stages of courtship (Houck & Reagan 1990; Houck et al. 2008a). Both biting and scratching modes of pheromone delivery apparently result in the courtship pheromone diffusing through the skin into the female's blood stream (Woodley 2010).

Olfactory delivery of courtship pheromones evolved in the *P. glutinosus* species group approximately 19 mya (Larson et al. 2003; Highton et al. 2012). As in species with scratching delivery, the mental gland of olfactory species opens to the surface of the skin, but is much larger and pad-like (Houck & Sever 1994). To deliver courtship pheromones in the olfactory manner, a male raises his head and then touches his mental gland to the female's nares. This behavior is termed "slapping" (Arnold 1976). Following the "slapping" delivery, the courtship pheromone is shunted (by capillary action) into the female's nasal cavity, and to her vomeronasal organ (VNO). When the VNO is stimulated, neurons relay signals to the accessory olfactory bulb (Wirsig-Wiechmann et al. 2002) and ultimately to other regions of the female's brain (Laberge et al. 2008). In one species with olfactory delivery, *Plethodon shermani*, courtship pheromone delivery influences the duration of one of the late stages of courtship (Rollmann et al. 1999; Houck et al. 2008b; Houck et al. 2007; Chapter 2). However, an earlier study on the same species presents contradictory data with

pheromone delivery shortening the early stages of courtship and not the later (Houck et al. 1998).

Overall, the emergence of the olfactory pheromone delivery mode in one clade of *Plethodon* salamanders represents a diversification in the sensory pathways by which courtship pheromones can be perceived within plethodontids: from acting on a female solely through transport via the bloodstream (transdermal delivery), to acting via stimulation of sensory neurons in the vomeronasal organ of olfactory delivery species. As reviewed above, this signal diversification across the plethodontid lineage has affected at least three aspects of pheromone delivery: (a) the chemical composition of the pheromone signal, (b) male secondary sexual characteristics and (c) the behavior by which the chemical signal is delivered, the tactile component (Watts et al. 2004). However, additional changes to the structure of plethodontid courtship itself may have occurred in tandem. These changes can be assessed across the entire plethodontid lineage because the same five stages of courtship are present in all plethodontid salamanders (Houck & Arnold 2003).

Plethodontids have a stereotyped five-staged courtship (Houck & Arnold 2003). The first stage (orientation) involves the male identifying the female and approaching her. The male then initiates contact with the female (stage 2, persuasion). If the female is receptive she will straddle his tail and enter into the third stage of courtship (tail-straddling walk, TSW). During the fourth stage of courtship the male deposits a spermatophore (a gelatinous base supporting an apical sperm mass) in front of the female and, in the fifth stage, the female is inseminated. These stages are

always present, but can vary across species in terms of the timing of pheromone delivery and the duration of each stage (Marvin & Hutchinson 1996; Verrell 1999). A shift in the timing of pheromone delivery during courtship could imply a change in the functional role of the pheromone in courtship.

To address this hypothesis of a functional shift in the role of courtship pheromones across the plethodontid lineage requires comparative data on: (a) the timing of pheromone delivery across plethodontids and (b) the impact of pheromone delivery on the different stages of courtship. Although the behaviors and temporal organization of courtship have been described for many species (Houck & Arnold 2003; Marvin & Hutchinson 1996; Verrell 1999), few quantitative data are available on the frequency of pheromone delivery during different stages of courtship, or the relative duration of each courtship stage. In particular, quantitative data are lacking for species with olfactory delivery. Thus, in this study we first gathered relevant experimental courtship data for a species employing olfactory delivery, *P. shermani*. We then compared data from this species to data reported in the literature for other plethodontid species having olfactory and transdermal delivery of courtship pheromones.

With *P. shermani*, we addressed the following questions: (1) how frequently does courtship pheromone delivery occur during the persuasion stage of courtship, and (2) does pheromone delivery influence the duration of the persuasion stage (as found in species that deliver pheromones transdermally), or has the role of the pheromone changed to influence only the TSW stage. We then compared the data obtained from

P. shermani with data from the primary literature to test the hypothesis that the timing of pheromone delivery and relative duration of individual courtship stages has changed in association with the transitions in delivery mode in the plethodontid lineage. These analyses will begin to assess possible changes in the functional context of courtship pheromones.

METHODS

Behavioral Analyses of an olfactory delivery species, P. shermani

Animal Collection and Maintenance

We collected 80 adult male and 80 adult female *P. shermani* in Macon County, North Carolina (035°10'48"N 083°33'38"W) during late August 2009. Animals were shipped to Oregon State University (OSU), Corvallis, USA where courtship trials were conducted. Each animal was maintained individually in a plastic box (17 cm x 9 cm x 13 cm) with substrate and refugia made of moist paper towels. Animals were kept at 15-18°C on a late-August North Carolina photoperiod and each fed two waxworms (*Galleria mellonella*) weekly.

Gland Removal and Sham Surgeries

Mental gland removal was necessary to control for the delivery of courtship pheromones in our experiments. Males were anesthetized in a mixture of 7% aqueous

ether, and each gland was surgically excised using iridectomy scissors (as in Rollmann et al. 1999). All males were given at least two weeks to recover before use in courtship trials; males recovered completely during this interval.

Behavioral Assays

Courtship encounters were staged across five nights in late August – early September 2009. Between 2030-2100 EST on each of the five trial nights, 12-36 male-female pairs (12 pairs per observer) were formed by placing a single deglanded male with a randomly chosen female in each plastic observation box (17 cm x 9 cm x 13 cm) that was lined with a damp paper towel substrate. Each male-female pair remained together for three hours. Each individual salamander was used in only one trial. Behavioral observations were conducted under low light conditions using red lights that mimic nocturnal light levels in the field.

Observers scanned each assigned box every two minutes. Instances of the following three behaviors were recorded at each scan sample: (1) male foot-dancing, (2) initiation of TSW, and (3) attempted pheromone delivery outside of TSW. Foot-dancing is a male visual display performed during the orientation and persuasion stages of courtship (Organ 1958; Arnold 1976). While foot-dancing, a male raises and lowers his legs, but keeps his trunk still and pressed to the substrate. This display is correlated with male motivation to mate (see Chapter 3). We assumed that males that did not foot-dance were not motivated to mate, and therefore we did not include these males in the study. Attempted pheromone delivery involved a deglanded male

slapping the female and, thus, providing tactile stimulation, but no pheromone. For the sake of clarity, slapping by deglanded males will be classified as tactile stimulation to distinguish this behavior from artificial pheromone delivery (a chemical cue) for the rest of the paper.

Pheromone delivery

Once a male was observed foot-dancing during 3 out of 5 consecutive observations, we delivered a treatment to the female's nares: either (a) 5 μ l of whole pheromone extract (2 mg/ml) collected from the mental gland using standard procedures (Houck et al., 1998), or (b) a vehicle control (PBS only). Additional 5 μ l treatments were delivered to the female after 3 and 6 minutes had elapsed, for a total of 15 μ l (30 μ g) of mental gland extract or PBS. The timing of these applications was recorded.

Statistical analyses

We explored the influence of courtship pheromone delivery behaviors (tactile stimulation) and courtship pheromones on the persuasion stage of courtship in *P. shermani*, a species with olfactory pheromone delivery. We identified: (1) the proportion of trials in which slapping was attempted by a male during the persuasion stage, and (2) the influence of slapping on the probability of transitioning to the next stage of courtship using a Fisher's exact test. We also explored (3) whether the performance of slapping was correlated with foot-dancing using a Point Biserial

Correlation (Glass & Hopkins 1995) using the ltm library in R and whether slapping remained significantly correlated with transitioning into TSW when foot-dancing was included as a predictor variable in a logistic regression.

Finally, (4) we addressed the question of whether the chemical signal (courtship pheromones) influenced the duration of the early stages of courtship (persuasion and orientation) as seen in species with transdermal delivery. We performed a linear regression with pheromone treatment and foot-dancing as explanatory variables and the duration of the combined orientation and persuasion stages as the response variable. To test if pheromone delivery influenced the likelihood of initiating TSW, we performed a Fisher's exact test comparing the probability of TSW initiation across the pheromone and control groups.

Comparative Analyses of Courtship among Plethodontids

To answer our main question concerning how the functional context of courtship pheromones may have changed with the shift from transdermal to olfactory pheromone perception, we compared our results from the *P. shermani* experiments to a survey of the published literature on salamander courtship. We focused on two aspects of courtship behavior: (1) the duration of courtship stages across the different pheromone delivery modes in plethodontids and (2) the timing (in terms of courtship stage) of pheromone delivery. Two methods were employed to find courtship data for plethodontid salamanders. First, we searched the references in previously published reviews on plethodontid courtship behavior (including: Arnold 1972; Marvin &

Hutchinson 1996; Petranka 1998; Verrell 1999; Houck & Arnold 2003; Houck 2009). We also searched available literature databases using various combinations of key words, such as: plethodontid, urodele, courtship and the various genera contained within the plethodontids (as identified by Vieites et al. 2011). To be included in the first analysis on courtship stage duration across the plethodontids, studies must have provided the type of pheromone delivery that occurred in the species, as well as a quantitative description of the duration of at least two of the first three stages of courtship (orientation, persuasion and TSW). From each study that met these criteria, we only included courtships that concluded with spermatophore deposition. For the second question on timing of pheromone delivery, we recorded if and how frequently (when available) pheromone delivery occurred before or during TSW. A final constraint we placed on the data analysis was to operationally define TSW in a way that was comparable across all the plethodontids, because there are some species specific variations of TSW, including female-first and circular TSW (Dyal 2006; Sapp & Kiemnec-Tyburczy 2011). Thus, to be consistent, we constrained our working definition of TSW to the linear movement with the male leading the female immediately prior to spermatophore deposition.

As the available quantitative data were limited, and because of the variable way in which these data were reported (mean duration, median duration, distance traveled during the stage, or percentage of total courtship represented by stage), no statistical analyses were appropriate for this data set. Instead, we identified gross-scale differences among plethodontid species that used olfactory or transdermal

delivery. We defined a courtship stage as long if it was either (a) over 15% of the total courtship time, (b) more than 20 minutes, or (c) the distance traveled during the stage was at least 10 body lengths. We focused our comparison on the two following measures: (1) the duration of TSW relative to the total duration of orientation and persuasion, and (2) the frequency of pheromone delivery during orientation and persuasion vs. TSW.

RESULTS

Behavioral analyses for a species with olfactory delivery, P. shermani

How often are courtship pheromones delivered during the persuasion stage? Does the tactile stimulation of the pheromone delivery behavior influence the duration of persuasion or the likelihood of transitioning to TSW?

Deglandened males performed slapping during the persuasion stage of courtship in 16 out of 72 trials (22.2% of trials). Males who provided this tactile cue entered into TSW with a female in 9 out of these 16 trials (56.2%). This percentage of TSW transitions was significantly higher than in trials where males did not perform slapping (28.6%; Fisher's exact test $n = 72$, $p = 0.012$, odds ratio = 4.59, 95% CI: 1.24-18.0, Figure 2a). The latency to TSW was on average 45% shorter in trials where slapping occurred than in those trials where it did not, but this was not a significant difference (Figure 2b, $\beta = -30.84 \pm 19.25$, $t = -1.602$, $p = 0.129$).

Does the occurrence of slapping during persuasion correlated with other persuasive behaviors?

We found a moderately-valued, positive correlation between slapping occurring during persuasion and the proportion of the persuasion stage that a male spent foot-dancing (Figure 3, $r_{pb} = 0.39$, $t = 3.53$, $df = 70$, $p = 0.000739$; Pett 1997). When foot-dancing and slapping were both included as explanatory variables in a logistic regression model, slapping was no longer significantly correlated with TSW initiation ($\beta = 0.173 \pm 0.124$, $t = 1.41$, $p = 0.164$), but foot-dancing was ($\beta = 0.718 \pm 0.20$, $t = 3.59$, $p = 0.0006$). This result implies that the significant relationship between slapping and TSW initiation may be an indirect effect mediated by the positive correlation between slapping and foot-dancing effort.

Does pheromone delivery during the persuasion stage influence persuasion stage duration and the probability of transitioning to the next stage of courtship?

Out of 124 trials, 78 males actively courted the female and, TSW initiations occurred in 27 of these courtships, (34.6% of trials). In the 35 control trials (in which saline vehicle was delivered to the female), 10 instances of TSW occurred (28.6% of trials, Figure 4a). In the 43 trials in which courtship pheromone was delivered, 17 instances of TSW (39.5%) occurred. Despite the approximately 11% difference in the probability of transitioning to TSW between the two groups, the number of successful transitions into TSW did not differ significantly (Fisher's exact test, $n = 78$, $p = 0.3472$, odds ratio = 1.62, 95% CI = 0.57 - 4.80).

Linear regression analysis revealed that pheromone treatment did not correlated with the latency to TSW ($\beta = -2.2 \pm 16.2$, $p = 0.893$). The average latency to TSW for the control group was 72.2 minutes vs. 70.0 minutes for the pheromone treatment group (see Figure 4b).

Comparative Analyses of Courtship among the Plethodontids

Has the timing of pheromone delivery (i.e. stage) changed across the plethodontid lineage in correlation with pheromone delivery mode?

We found 30 studies reporting information on timing of pheromone delivery for transdermal ($n = 24$), and olfactory species ($n = 6$). Interestingly, all species with mental glands attempt pheromone delivery during the persuasion stage at least occasionally, but only one species using transdermal delivery (*Plethodon vandykei*) and three olfactory (out of five) species attempt pheromone delivery during TSW (Table 2). Overall, pheromone delivery during TSW was predominately performed by species with olfactory delivery.

The frequency with which species delivered courtship pheromone during persuasion varied (Table 2). Species using transdermal delivery delivered pheromone in the persuasion phase in 25% to 100% of observed trials. Species using olfactory delivery also varied in the frequency of pheromone delivery in each stage. One species in the transitional group (*P. doralis*) resembled transdermal species in that it delivered pheromone exclusively in the persuasion stage. In the olfactory delivery group, *P. kentucki* delivered courtship pheromone more often during persuasion than

in TSW (53% vs. 43%). *P. shermani* is the only species to deliver pheromone predominately during TSW (22% during persuasion vs. 72% during TSW).

Have the durations of courtship stages changed across plethodontids in association with mode of pheromone delivery?

Quantitative data on courtship stages was found for 24 plethodontid species that did not employ courtship pheromones (n=3) or used transdermal delivery (n=17) or olfactory delivery (n=4) of courtship pheromones.

Plethodontid species that do not employ courtship pheromones had prolonged TSW stages ranging from 20 minutes to over 2 hours (Table 1). *Ensatina eschscholtzii* has the longest reported TSW at 300 minutes (Stebbins 1949). For species that deliver courtship pheromones, TSW duration was generally longer for species using olfactory delivery than for species employing transdermal delivery modes (Table 1). This pattern apparently was driven by the short duration of TSW for those species using the scratching mode to deliver pheromones (median duration of 5 minutes or 3 - 9% of total courtship duration). Biting species, on the other hand, have fairly prolonged TSW stages that are more similar in duration or percentage of total courtship duration to species with olfactory delivery. For species employing olfactory delivery, TSW averaged 28.6 minutes (representing roughly 40% of the total courtship duration).

There are several exceptions to this general trend of olfactory species exhibiting a longer TSW than transdermal species and these exceptions illustrate a

potential mechanism to explain the differences in TSW duration. We suggest this pattern is driven by an association between duration of a courtship stage and whether pheromone is delivered during it. In the one transdermal species (*Plethodon vandykei*) that delivers pheromone during TSW, TSW was prolonged (Table 2). A parallel pattern is seen in an olfactory species that only delivers pheromone during the persuasion stage (*P. doraslis*): TSW has a short duration (similar to scratching species). Additional support for this mechanism comes from *P. shermani* courtships. We have more extensive and detailed accounts of pheromone delivery in TSW for *P. shermani* than we have in any other species. Male *P. shermani* attempt pheromone delivery in 72% of staged courtship trials (Eddy, *unpublished data*). In staged courtship encounters where males do not attempt pheromone delivery during TSW, TSW is as short as it generally is for scratching species (ranging from 4 – 11 minutes). When males did deliver courtship pheromones, however, TSW was much longer (ranging from 14 – 92 minutes) (Eddy, *unpublished data*).

In contrast to the apparent differences in TSW duration between species with transdermal and olfactory delivery, the two stages before TSW (orientation and persuasion) do not differ in duration in relation to delivery mode (Table 1).

DISCUSSION

In this study we explored whether the functional significance of pheromone delivery had changed with the evolutionary shift in courtship pheromone perception among plethodontid salamanders. Pheromone delivery data first were collected for *P.*

shermani, a species with olfactory delivery. This species primarily delivered pheromone during the TSW phase of courtship. When the pheromone was delivered to the female during the earlier (persuasion) stage of courtship, there was no observable effect of the pheromone on the duration of that stage. This result contrasts with those from species with transdermal delivery: these species almost exclusively deliver pheromone during the persuasion stage, and this pheromone delivery affects the duration of that stage (Table 2; Houck & Reagan 1990). Thus, the functional significance of the courtship pheromone may have shifted from influencing the duration of persuasion to influence primarily TSW in species with olfactory delivery. In the following section, the findings in *P. shermani* are discussed, and then evaluated in the context of pheromone delivery among other plethodontid salamanders.

Behavioral Analyses of an Olfactory Delivery Species, P. shermani

Earlier work had documented that male *P. shermani* could deliver pheromone to the female well before the TSW stage of courtship (Arnold 1976; L. Houck, unpub. data). The frequency of this behavior, however, had not been quantified until now. We found that male *P. shermani* perform the slapping behavior during the persuasion stage in approximately 22% of the 72 staged courtship trials. For trials in which deglanded males were used, we were able to assess the effects of the physical contact of slapping without the confounding effect of simultaneous pheromone delivery. The

effect of the tactile stimulation was an increased probability of initiation of the next stage of courtship, TSW.

The effects of this purely tactile aspect of slapping could be explained in multiple ways. First, the tactile cue itself could influence female receptivity. Tactile stimuli are used by most animals during courtship and are known to affect mating success in many species (Bradbury & Vehrencamp 2011). For example, in some insects, such as the medfly (*Ceratitis capitata*), the male taps the bristles of its antennae against the bristles of the female's antennae during courtship. This tactile behavior significantly increases the probability of mating (Briceno & Eberhard 2002). A second possible explanation is that a male may slap during the persuasion stage only when he is highly motivated to court the female. It is known from previous studies that males allocating more effort to a courtship encounter (performing behaviors such as foot movements, scratching, and rubbing more often) have higher courtship success (Eddy, Chapter 2; Vinnedge & Verrell 1998; Gershman & Verrell 2002). In a different plethodontid salamander, *Desmognathus ocoee*, an increase in male pheromone delivery attempts is correlated with increases in a visual display (Gersham & Verrell 2002). We found a similar pattern in *P. shermani*: foot-dancing effort and pheromone delivery during persuasion are correlated. Altogether, this evidence supports the idea that a suite of correlated behaviors are performed by highly motivated males and it is this complete suite of traits that is important, not just the single tactile component (Vinnedge & Verrell 1998).

Although the tactile aspect of the male's slapping behavior had an obvious affect on courtship success in *P. shermani*, delivery of the actual courtship pheromones did not alter courtship success. While there are multiple competing hypotheses that may explain the lack of pheromone effect on persuasion (outlined below), we hypothesize that the lack of effect is simply due to pheromone not acting during the persuasion stage. This hypothesis is supported by a previous study (Chapter 2), in which males with intact mental glands did not have significantly different durations of persuasion nor were they more likely to transition into TSW from those with surgically excised glands. Instead, gland presence influenced TSW duration via a curvilinear relationship with duration of persuasion. This effect meant that males with longer persuasion stages, and an intact mental gland, had a shorter TSW stage than did males without a gland. From this study it seems possible that pheromone delivered during the early persuasion stage does not have an immediate effect during the persuasion stage, but rather the affect occurs later during TSW.

The results of the current study and the study referenced above conflict with the results from Houck et al. 1998 which found pheromone delivery shortened the persuasion stage. The Houck et al. 1998 study differed from this study and Chapter 2 in terms of when pheromone was delivered to the female. In these two studies pheromone was delivered during persuasion, whereas in Houck et al. 1998 females first received pheromone before they were even paired with a male. The earlier timing of pheromone delivery may have led to the observed early effect of the pheromone. In natural courtships, females do not receive pheromonal stimulus before persuasion

because a male must be present to deliver it. The timing of delivery in natural courtships would, thus, be more similar to this current study and Chapter 2. So, the results we observe here may be more reflective of those natural courtships.

A second possible explanation for the lack of pheromone effect on the persuasion stage is that, without the tactile stimulus of the slap, a female may not have a context for interpreting the chemical cue (Candolin 2003). This loss of effect without a tactile context has been shown in *Drosophila* in which chemical and gustatory cues from rival males are typically delivered simultaneously with male tactile cues (Ferveur 2005). The tactile and chemical contact with rivals causes the male to increase his courtship duration with a given female. Without the tactile contact with other males, male gustatory and olfactory cues do not stimulate rival males to increase their courtship durations (Bretman 2011). We do not believe such a lack of context for the chemical cues is as viable a hypothesis for *P. shermani*, because artificial delivery of pheromone during TSW does elicit a response (Houck et al. 1998; Rollmann et al. 1999; Houck et al. 2007; Houck et al. 2008). Exogenous pheromone application in these trials was not timed to correspond with slapping events, so females received the pheromone outside the context of the slapping behavior. In addition, artificial pheromone application before courtship trials elicited a reduction in duration of the subsequent courtship for another plethodontid, *D. ocoee* (Houck et al. 2008a; Houck & Regan 1990). Overall, these results imply that the coupling of tactile cue and pheromone delivery is not necessary to elicit a behavior response in the female.

A third possible explanation for the lack of pheromone effects in the persuasion stage is that courtship pheromone delivery during persuasion influences the sexual arousal of the female in a way that is not captured by our metrics. For example, female *P. shermani* showed a reduced interest in food when given male courtship pheromones (Vaccaro et al. 2009). Our experiments did not offer an array of alternative stimuli for females to experience, however, and this restriction might have masked possible pheromone effects.

Comparative analyses of courtship among plethodontids

If courtship pheromones truly do not have an effect on the persuasion stage in *P. shermani*, then this finding, in combination with the low frequency with which pheromone delivery is attempted before TSW, could represent a shift in the function of courtship pheromones: from shortening persuasion stage to shortening TSW. With our limited comparative data set, this shift in function seems associated with the change in pheromone delivery mode. The transition from the ancestral scratching delivery to olfactory delivery is already known to be associated with changes in pheromone delivery behaviors, pheromone chemical composition, and male morphology (Watts et al. 2004). Our study expands this set of correlated characters to include three additional traits: relative duration of the courtship stages, frequency of pheromone delivery in each stage and, potentially, the dominant courtship stage affected by pheromones.

A transition in timing of pheromone delivery, a time consuming behavior, could lead to the observed patterns of courtship stage durations in plethodontids salamanders. In scratching species, pheromone is delivered during persuasion and, very rarely, during TSW (1 out of 22 species) and thus, TSW is generally short. In the one scratching species (*P. vandykei*) that delivers pheromone during TSW this stage is prolonged (Table 1). A similar situation occurs for olfactory delivery species.

Olfactory species generally deliver pheromones during TSW and have an increased duration of that stage relative to persuasion and orientation. In the one species that delivers pheromone exclusively during persuasion (*P. dorsalis*), TSW is short as in transdermal delivery species. Overall, across both types of delivery, it seems that the stage in which pheromone is predominately delivered is the longest stage of courtship.

The timing of pheromone delivery also seems to be correlated with the stage or stages of courtship influenced by the pheromone. In at least one species of scratcher (*Desmognathus ocoee*), courtship pheromone delivery influenced the duration of the persuasion stage (Houck & Reagan 1990; Houck et al. 2008a). In contrast, in the olfactory species *P. shermani*, pheromone delivery seems to affect the duration of the TSW stage, which is the more dominant stage in this species (Rollmann et al. 1999; Eddy Chapter 2; Houck et al. 2007; Houck et al. 2008b). Interestingly, *P. shermani* attempt pheromone delivery during the persuasion stage less frequently than any other examined plethodontid species. These findings support a potential shift in pheromone function (affecting the duration of TSW vs. persuasion) in this species as compared to the scratchers. To determine if this shift in pheromone effects is generally

characteristic of species using olfactory delivery during TSW or if it is specific to *P. shermani*, much additional data is needed from other members of the *Plethodon glutinosus* complex.

In summary, the current study supports the paradigm of a functional shift for male courtship pheromones from shortening the persuasion stage of courtship to shortening the TSW stage. This shift is also correlated with the switch in mode by which the signal is perceived. This finding further elaborates on the behavioral, chemical and morphological correlates of the diversification of chemical courtship signals that have occurred in plethodontid salamanders. Evolutionary diversification of a signal by evolutionary shifts in sensory modes is rare, but it illustrates an alternative way for the co-option of a signal to lead to signal evolution and diversification across a lineage.

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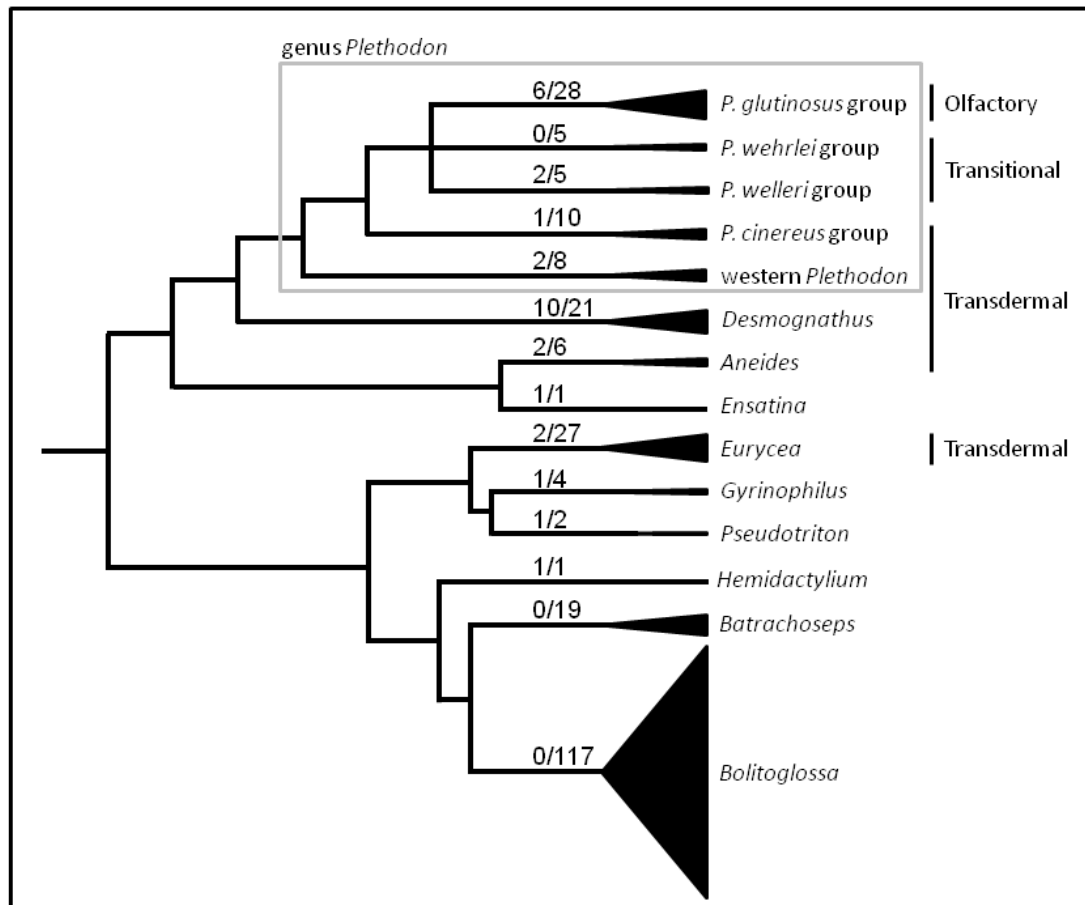


Figure 4.1 Phylogeny of the plethodontids showing genera in which quantitative courtship data is available (Adapted from Highton et al. 2012, Vieites et al. 2011 and Fisher-Reid & Wiens 2011). On the right side, each clade is labeled with its proposed pheromone delivery mode (Watts et al. 2004). Transitional species vary in whether they use olfactory or transdermal pheromone delivery (see Table 2 for a more detailed breakdown of pheromone delivery in some of these species). On the branch leading to each clade is the proportion of species in the clade for which quantitative observations on complete courtships (i.e., through spermatophore deposition) have been recorded.

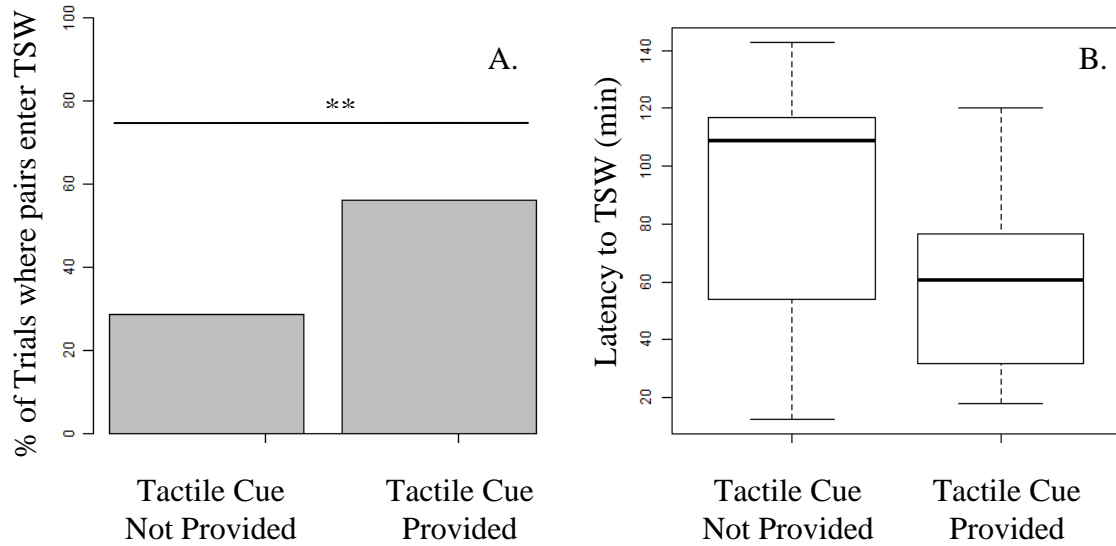


Figure 4.2 (A) Probability of transitioning from the persuasion stage into the next stage of courtship, TSW, in *P. shermani* trials with and without the tactile cue of slapping during the persuasion stage. (B) Latency (in minutes) to TSW in trials with and without the tactile cue of slapping during persuasion. A significant difference ($p < 0.05$) between treatments is indicated by **.

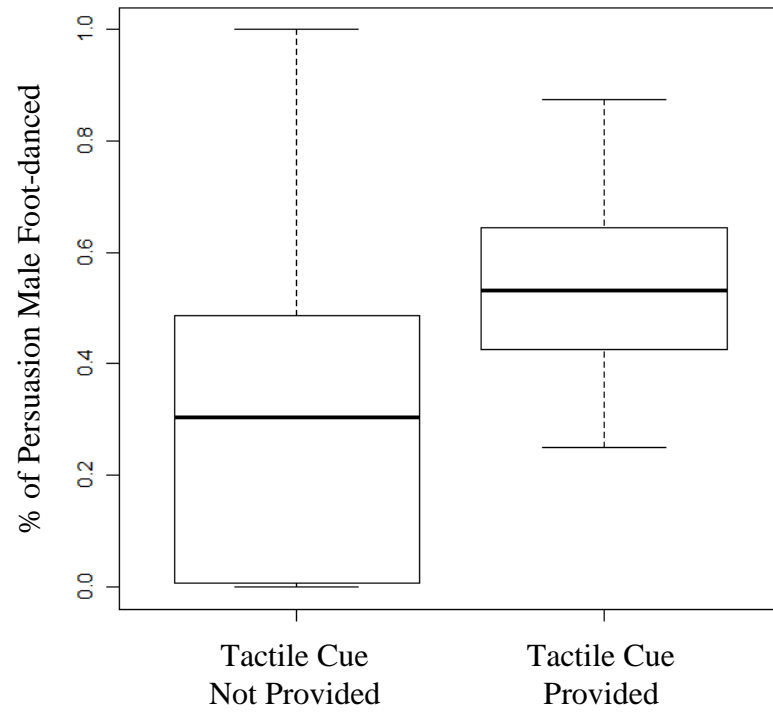


Figure 4.3. Relationship between the proportion of orientation and persuasion stages a male *P. shermani* was observed foot-dancing and whether or not he provided tactile stimulation by slapping during persuasion.

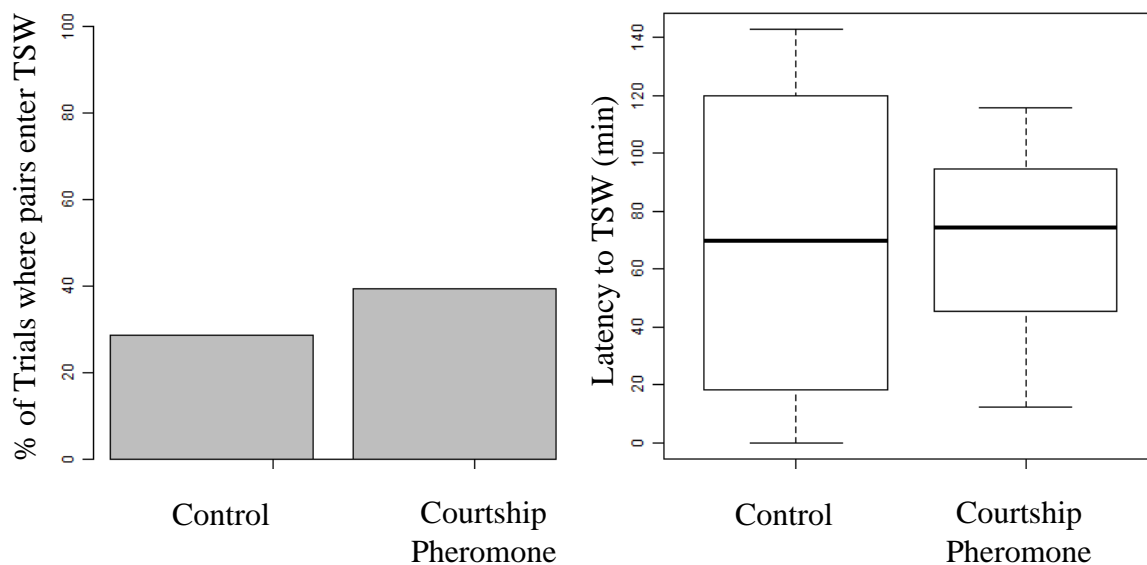


Figure 4.4. (A) Probability of transitioning from the persuasion stage into the next stage of courtship, tail-straddling walk (TSW), in trials in which whole pheromone extract or a saline control was delivered to the female during the persuasion stage of courtship. (B) Latency (in minutes) to tail-straddling walk in trials where whole pheromone extract or a saline control was delivered during persuasion. A significant difference between treatments is indicated by **.

Table 4.1 Duration of orientation, persuasion and TSW stages across the plethodontids. Typically mean and standard error reported. Numbers in () are ranges. * indicate that the median rather than average was reported. A “?” denotes that the duration was not reported in the paper.

Species:	Relative Length of TSW:	Type of Pheromone Delivery:	Duration of Orientation and Persuasion (minutes):	Duration of TSW (minutes):	Distance traveled during TSW (inches):	Proportion of Courtship spent in TSW:	References:
<i>Gyrinophilus porphyriticus</i>	Long	N/A	80.5±20.5	29.8±9.2	?	32.3%	Beachy 1997
<i>Hemidactylium scutatum</i>	Long	N/A	?	>20	?	?	Branin 1935
<i>Pseudotriton ruber</i>	Short	Scratch	?	2	?	?	Organ & Organ 1968
<i>Ensatina eschscholtzii</i>	Long	N/A	?	(120-300)	>120	?	Stebbins 1949
<i>Aneides ferreus</i>	Short	Scratch	205.9±38.5	11.2±3.9	?	9.0%	Sapp & Kiemnec-Tyburczy 2011
<i>A. aeneus</i>	Short	Scratch	?	?	5.9	?	Cupp 1971
<i>Desmognathus aeneus</i>	Long	Bite	38.8±19.9	5.1±2.2	?	33.9%	Promislow 1987
<i>D. apalachicola</i>	Short	Scratch	15*(6-30)+51*(37-117)	5* (4-7.5)	1.6	?	Verrell 1994a
<i>D. monticola</i>	Short	Scratch	15*(6-30)+157*(98-458)	5.5* (4-6)	?	?	Brock & Verrell 1994
<i>D. imitator</i>	Short	Scratch	17*(3-69)+165*(18-215)	?	?	?	Verrell 1994b
<i>D. fuscus</i>	Short	Scratch	?	?	1.4	?	Verrell 1995

<i>D.ochrophaeus</i>	Short	Scratch	103.7 ± 19.4	?	?	?	Mead & Verrell 2002
<i>D. ocoee</i>	Short	Scratch	?	?	?	3.1%	Gershman & Verrell 2002
<i>D. santeetlah</i>	Short	Scratch	321.8 ± 30.3	?	1.2	?	Maksymovitch & Verrell 1992
<i>Eurycea rathbuni</i>	Short	Scratch	?	11.5	?	?	Bechler 1988
<i>Plethodon angusticlavius</i>	Short	Scratch	100 ± 32	5.5 ± 2.5	?	5.0%	Dyal 2006
<i>P. welleri</i>	Short	Scratch	?	?	3	7.5%	Organ 1960a
<i>P. cinereus</i>	Short	Scratch	59 ± 26.3	2.5 ± 0.29	?	9.9%	Dyal 2006
<i>P. vandykei</i>	Long	Scratch	?	?	118.1	?	Lynch & Wallace 1987
<i>P. vehiculum</i>	Long	Scratch?	?	>65	39.4	?	Lawrence et al. 2001
<i>P. kentucki</i>	Long	Slap	95 ± 14.7	20.4 ± 2.5	?	17.6%	Marvin & Hutchison 1996
<i>P. shermani</i>	Long	Slap	68.1±6.3	36.8±3.25	?	39.5%	Eddy, Chapter 2
<i>P. glutinosus</i>	Long	Slap	?	?	up to 204 inches	?	Organ 1960b
<i>P. dorsalis</i>	Short	Slap	128.3 ± 112.4	3.8 ± 3.3	?	3.4%	Picard 2006

Table 4.2. When and where courtship pheromone is delivered to the female in plethodontid salamanders. Pheromone delivery was scored as the occurrence of scratching, biting or olfactory delivery during courtships where tail-straddling walk occurred. A “> 0” denotes that the behavior occurs but at undocumented frequency; 0 = does not occur; ** indicates scores from multiple populations.

Species	Pheromone Delivery Type	% of courtships where courtship pheromone delivered:		Where does the male touch his mental gland to the female?	References:
		During Persuasion	During TSW		
<i>Plethodon glutinosus</i>	Olfactory	> 0	> 0	Body & Nares	Organ 1960b
<i>P. kentucki</i>	Olfactory	53%	43.3%	Body & Nares	Marvin & Hutchinson 1996
<i>P. jordani metcalfi</i>	Olfactory	> 0	0	Body & Nares	Organ 1958
<i>P. yonahlossee</i>	Olfactory	> 0	0	Body & Nares	Arnold 1972
<i>P. shermani</i>	Olfactory	22.2%	72.2%	Body & Nares	Eddy, this study and Chapter 2
<i>P. ouachitae</i>	Scratch	> 0	0	Body	Arnold 1972
<i>P. vandykei</i>	Scratch	> 0	> 0	Body	Lynch & Wallace 1987
<i>P. vehiculum</i>	Scratch?	?	0	?	Lawrence et al. 2001
<i>P. welleri</i>	Scratch	> 0	0	Body	Organ 1960a
<i>P. dorsalis</i>	Olfactory	60%	0	Body & Nares	Picard 2006
<i>P. cinereus</i>	Scratch	> 0	0	Body	Gergits & Jaeger 1990; Dyal 2006; Arnold 1972
<i>Aneides ferreus</i>	Scratch	100%	0	Body	Sapp & Kiemnec-Tyburczy 2011
<i>A. aeneus</i>	Scratch	> 0	0	Body	Cupp 1971
<i>Eurycea cirrigera</i>	Scratch	> 0	0	Body	Kozak 2003
<i>E. wilderae</i>	Scratch	> 0	0		Kozak 2003

<i>E. rathbuni</i>	Scratch	0	0	N/A	Bechler 1998
<i>Pseudotriton ruber</i>	Scratch	> 0	0	Body	Organ & Organ 1968
<i>Desmognathus apalachicolae</i>	Scratch	> 0	0	Body	Verrell 1994a
<i>D. brimleyorum</i>	Scratch	68.7%	0	Body	Verrell 1997
<i>D. monticola</i>	Scratch	50-90% **	0	Body	Brock & Verrell 1994; Organ 1961
<i>D. santeetlah</i>	Scratch	> 0	0	Body	Maksymovitch & Verrell 1992
<i>D. fuscus</i>	Scratch	77%	0	Body	Arnold 1972; Organ 1961; Verrell 1995; Uzendoski & Verrell 1993
<i>D. ochrophaeus</i>	Scratch	85-100% **	0	Body	Organ 1961; Uzendoski & Verrell 1993
<i>D. imitator</i>	Scratch	> 0	0	Body	Verrell 1994b
<i>D. welteri</i>	Scratch	>25%	0	Body	Verrell & Mabry 2003
<i>D. quadrimaculatus</i>	Scratch	100%	0	Body	Verrell 1999
<i>D. wright</i>	Bite	100%	0	Body	Verrell 1999
<i>D. aeneus</i>	Bite	80%	0	Body	Promislow 1987

**Chapter 5 – Sperm and sperm mass longevity in the female reproductive tract of
a terrestrial salamander, *Plethodon shermani***

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ABSTRACT

The risk of or presence of sperm from multiple males within the female reproductive tract is necessary for post-copulatory sexual selection. Sperm storage by females can facilitate this condition. We assess the likelihood of post-copulatory sexual selection in a plethodontid salamander, *Plethodon shermani*, by exploring the ability of males to prevent female re-mating via a form of post-copulatory mate guarding and of females to retain viable sperm for long periods of time. We found that inhibition of female re-mating by retention of the male sperm mass can last at most 4 days. Given the prolonged breeding season exhibited by this species (> 10 weeks) it seems unlikely that the sperm mass functions efficiently to prevent female re-mating. In addition, females can store viable sperm in their sperm storage organs for at least 9 months. In some cases, females also retain sperm beyond oviposition. Finally, we documented one female with sperm in her sperm storage organ from a mating 17 months prior. Taken together, these experiments indicate the potential for post-copulatory sexual selection in this system is high. Furthermore, sperm from one breeding season may interact with sperm from the subsequent season.

INTRODUCTION

Post-copulatory sexual selection is an important selective force in many taxa (Eberhard 2009). Like pre-copulatory sexual selection, it can take two forms. The most well documented form is sperm competition in which sperm from multiple males

compete to fertilize a set of ova (Parker 1970). Less documented is cryptic female choice, in which a female biases paternity towards a particular male (Eberhard 1996). Both forms of post-copulatory sexual selection require that sperm from multiple males be present in the female reproductive tract or at least the risk of this state. The opportunity for post-copulatory sexual selection is higher when a female is able to retain sperm in her reproductive tract for long periods of time (Parker 1970). Many invertebrates and vertebrates have organs dedicated to long-term sperm storage (Pizzari et al. 2008). In vertebrates, some urodeles and some sauropsids have organs dedicated to storing sperm (Holt & Lloyd 2010). The presence of sperm storage organs implies that post-copulatory processes probably occur in urodeles. Post-copulatory processes have been studied in several species, but have not been explored in terrestrial salamanders in the genus *Plethodon*, which possess a uniquely shaped sperm storage organ.

Salamander species vary in the structure of their respective sperm storage organs. Some salamanders have simple spermathecae formed by of multiple blind-ended chambers extending dorsally from the cloacal chamber (Sever & Brizzi 1998). Species in which the female has a simple spermatheca have either: (a) first-male sperm precedence or sperm mixing (Adams 2004; Jones et al. 2002; Gabor et al. 2000; Rafinski & Osilkowski 2002; Tennessen & Zamudio 2003), and (b) a duration of sperm storage ranging from 2 days to 1 year (Table 1).

The Plethodontidae have a novel sperm storage organ: a complex spermatheca. This spermatheca initially has a single duct leading dorsally from the top of the cloacal chamber. Multiple smaller blind-ended ducts (called “bulbs”) branch off the larger initial duct (Sever & Brizzi 1998). To date, there has only been one study on sperm precedence in salamanders with a complex spermatheca. In that study, *Desmognathus ocoee* exhibited first male sperm precedence (Adams 2004). In addition, the duration of sperm storage has been documented in only a few speices. From the studies that have been conducted, however, we know that sperm storage can range from 2 to 8 months (Table 1).

In this study, we explored the potential for post-copulatory sexual selection in another plethodontid salamander, *Plethodon shermani*. As discussed above, the opportunity for post-copulatory processes is enhanced in species in which females have evolved prolonged sperm storage. These processes can be limited by events such as post-copulatory mate guarding. In some species, mate guarding can take the form of a copulatory plug (Shine et al. 2000). Salamanders do not produce copulatory plugs, but the presence of a sperm mass in the female’s cloaca is known to inhibit re-mating in a similar way (Verrell 1991). If the sperm mass is retained in the female cloaca for long periods it could limit the potential for sperm competition or cryptic choice.

The *P. shermani* system has several advantages for the study of sperm storage and the exploration of the potential for post-copulatory sexual selection. First, both

males and females will mate under laboratory conditions. This behavior allows us to document the date of insemination and later determine the number of days sperm were actually stored in the spermatheca. Second, many previous behavioral studies have revealed that females will mate with multiple males in one breeding season, at least in the laboratory (Houck, pers. comm.). Third, we can retain females in the laboratory for multiple years and these females will come back into reproductive condition each year. This cyclic receptivity allowed us to conclude with confidence that the sperm present in a female's spermatheca is from staged male-female inseminations in the laboratory. A fourth advantage of the *P. shermani* system is that we can induce oviposition in the laboratory. Many urodeles fertilize their eggs at the time of oviposition (Watanabe & Onitake 2003), so inducing oviposition allowed us to control the duration between insemination and fertilization. The laboratory-control of the duration of sperm storage is a more accurate method than those previously employed to investigate sperm storage duration (e.g., sampling females in the field each month). A fifth benefit of this system is that we are able to rear viable eggs in the laboratory. Egg rearing allows us to determine if the sperm present in the spermatheca was actually viable and able to fertilize ova. The viability of sperm is crucial, as sperm senescence is known to reduce embryo viability (Lodge et al. 1971; Wagner et al. 2004). This method of testing the viability of embryos via rearing the eggs also alleviates the problem raised in some previous studies reporting long-term sperm

storage wherein sperm was present in the spermatheca but may have been too degraded to be viable (e.g., Sever & Brizzi 1998).

We used *P. shermani* females to examine both (a) the potential for sperm competition within a breeding season and (b) the ability to retain sperm from one breeding season to the next. First, we documented how long the sperm mass remains in the female reproductive tract to determine if sperm mass retention reduces the possibility of sperm competition. Second, we documented how long females retain viable sperm in their spermatheca. Finally, we tested whether a female could retain sperm in her spermatheca after oviposition and for up to 17 months. If females retain sperm after 17 months or oviposition, sperm competition might span multiple seasons.

METHODS

Animal Collection and Maintenance

We collected adult male (n= 60) and female (n= 70) *Plethodon shermani* in Macon County, North Carolina (035°10'48"N 083°33'38"W) during late July and August 2008. Animals were shipped to Oregon State University (OSU), Corvallis, USA where they were maintained individually, each in a plastic box (17 cm x 9 cm x 13 cm) with a substrate of moist paper towels and crumpled damp towels for refugia. Animals were kept at 15-18° C on a late-August North Carolina photoperiod and were fed two waxworms (*Galleria mellonella*) weekly.

Insemination Protocol

In September and October 2008, each female was paired nightly with a male in a plastic box (17 cm x 9 cm x 13 cm) with a paper towel substrate. Each pair was left overnight. Male salamanders do not have an intromittent organ. Instead, they deposit a spermatophore, a sperm mass held above the substrate by a gelatinous base (Organ 1960; Arnold 1976). A female becomes inseminated by wedging the sperm mass into her cloaca. After a successful insemination, the spermatophore base is generally left attached to the substrate. Thus, observers can determine if a female has been inseminated by searching the observation box for a spermatophore base. The female was considered inseminated if a base was present and no sperm mass could be seen. Females were used in as many trials as necessary to facilitate insemination, but each female was inseminated by a unique male. We recorded the date of insemination and male identity for each inseminated female.

Experiment 1: Sperm mass longevity in the cloacal chamber

Ten (10) females were randomly chosen from the pool of inseminated females to evaluate the duration that a sperm mass remained in the female cloaca following insemination. Each female was first immobilized using benzocaine. A researcher then examined a female's cloaca under a Leica MZ6 light microscope. If the folds on the dorsal cloacal chamber were visible, the cloaca was considered empty. If white or yellow material could be observed in the cloacal chamber, then the sperm mass was

considered to be present in the cloaca. Females were checked daily until each had an empty cloacal chamber.

Experiment 2: Sperm longevity within the spermatheca

Injection Protocol

Female *P. shermani* will not consistently oviposit naturally in the laboratory setting. To induce oviposition, we applied a hormone treatment modified from Crespi & Lessig (2004). We applied this hormone treatment to 10 females each month for 7 months from January (at least 3 months post-insemination) to July 2009. Each female was injected first with pregnant mare serum gonadotropin (50 IU in 50 μ l phosphate buffered water, PBS), and two days later with human chorionic gonadotropin (100 IU in 50 μ l PBS). Females were left in their home boxes. Most females oviposited a full clutch within 2 -3 days of the final injection. We checked home boxes for eggs twice daily and, if eggs were found, they were immediately collected for rearing. This procedure allowed us to standardize the conditions under which the eggs were reared across all clutches.

Egg Rearing Protocol

The number of ova in each clutch was counted and each clutch was placed into a 135 ml jar filled with chilled water. Each jar was placed under a water drip system to circulate the water and increase oxygen concentration. Although the temperature

varied slightly during the experiment, the average water temperature was 12.2 °C. Eggs were checked daily and non-fertilized eggs were removed. Eggs were reared under these conditions for approximately 2 months until embryos were easily observable by the naked eye. The embryos were morphologically similar to Stage 10 of embryonic development for *Plethodon cinereus* (see Kerney 2011). At this time, surviving embryos were counted and we recorded the proportion of the total clutch that was fertilized.

Statistical Analyses

We used a general linear regression that included the number of days that sperm were retained in the spermatheca as an explanatory variable. We used a binomial distribution with a probit linking function (Long 1997) to model with the proportion of each clutch that was fertilized. We analyzed both the full data set and also a reduced data set that excluded clutches that did not contain a single fertilized ovum. We performed this second analysis because, in the instances of no fertilized ova, we thought it was possible that our methodology (hormone injections rather than natural births) might have caused the females to lay unfertilized eggs. Means and standard errors for explanatory variables are reported.

Experiment 3: Sperm retention in the spermatheca after oviposition and into the next breeding season

Spermathecal Sectioning

We sacrificed 14 total females (6 that had oviposited, 5 that had not oviposited, and 3 that had been inseminated 17 months earlier) in 5% MS-222 (Tricaine methanesulfonate). The 11 oviposition animals were sacrificed in the same month (end of April 2010), so each had sperm of approximately the same age in her spermatheca. Animals were fixed in formaline and then preserved in 70% ethanol until sectioning. Just before sectioning, the spermathecal area was excised. We followed standard histological protocols to embed and section the samples (Presnell & Schreibman 1997). Each sample was dehydrated in graded ethanol, cleared in Citrisolv, embedded in paraffin and sectioned at 12 μm with a rotary microtome. Sections were mounted on microscope slides. The presence of sperm in the sections was determined by staining samples with haematoxylin and eosin.

Sperm Counts

We chose 3 sections from each female and determined the lumen area of the spermathecal bulbs occupied by sperm. We only included sections of the spermatheca that had bulbs present. One section with bulbs was randomly chosen from the first third of the spermatheca, another section from the middle of the spermatheca, and the final section from the last third. This sampling method allowed us to explore the length of the spermatheca, but avoided the possibility that we would recount the same

sperm. We photographed the chosen sections using an Olympus CX31 light microscope with an Olympus camera at 20x magnification. For each section, we overlaid a grid with $10\ \mu\text{m}^2$ squares. We scored each square based on whether sperm was present or absent. We totaled these scores across all three sections to create a measure of sperm density across the spermatheca.

Statistical Analyses

For each female, we calculated the total area of the spermatheca sampled that was occupied by sperm. We used a one-tailed t-test to determine whether there was a reduction in sperm present in the spermatheca after oviposition and after 17 months. A one tailed test was appropriate because we had no biologically relevant reason to expect the amount of sperm in the spermatheca would increase after oviposition or through time after insemination.

RESULTS

*Experiment 1: Sperm mass longevity in the *P. shermani* female cloacal chamber*

The average longevity of a sperm mass in the female reproductive tract was 2.8 days (Figure 1). In only one case did the sperm mass come out of the female reproductive tract (i.e., was observed on the floor of the female's box). Instead, the sperm mass seems to become fluid through time, and is either absorbed into or eliminated from the female reproductive tract.

Experiment 2: Sperm longevity within the spermatheca

Across all 7 months, 35 of 70 females were successfully induced to oviposit (50%). The months with the highest oviposition rates were February (80% oviposited), April (80%) and May (100%). The months with the lowest oviposition rate were March (0% oviposited), June (20% oviposited) and July (20% oviposited). Viable offspring were produced from sperm stored in a female's spermatheca for the duration of the study (February through July), except for the period between days 125 and 155 (Figure 2). The average clutch size for the females induced to oviposit was 15.6 ± 4.8 (mean \pm standard deviation) eggs (ranging from 1 to 29 eggs). Four (4) females oviposited without hormone injection during the experiment. These females had an average clutch size of 20.25 ± 7.6 eggs (ranging from 14 to 24 eggs).

The number of days sperm were stored in the spermatheca before fertilization was not significantly related to the number of viable eggs produced when all data were included ($\beta = 0.001213 \pm 0.0007$, $p=0.06$, Figure 2A). There was still no significant relationship between duration sperm stored in the spermatheca and proportion of clutch fertilized, when we excluded females that oviposited but did not have any fertilized eggs ($\beta = 0.00205 \pm 0.0014$, $p = 0.17$, Figure 2B).

Experiment 3: Sperm retention in the spermatheca after oviposition and into the next breeding season

The proportion of spermathecal bulbs occupied by sperm in females who mated 8 months prior averaged $57.9 \pm 5.7\%$. Females who oviposited had 1.6 times

less sperm present ($34.4 \pm 9.2\%$ of lumen area occupied) than female who had not oviposited. Of the females who had mated 17 months prior, only one contained any sperm. This female had 1.2 times less sperm than females at 8 months (47.6% of lumen area occupied). Overall, the area of the spermatheca occupied by sperm was significantly less for both females after oviposition ($t = 2.16$, $p = 0.031$) and 17 months ($t = 2.58$, $p = 0.041$) compared to control females (Figure 3).

DISCUSSION

We found that the sperm mass is retained in the female cloaca for on average 3 days and that females are able to retain viable sperm in their sperm storage organ for at least 9 months without a decline in sperm viability. Once a female has oviposited she retained significantly less sperm in her spermatheca than prior to oviposition. These observations led us to conclude that the potential for post-copulatory sexual selection, at least within a breeding season, is high.

The risk of post-copulatory sexual selection processes can lead to various strategies to limit female re-mating (Parker 1970). In salamanders, one proposed strategy is the retention of the sperm mass in the female reproductive tract (Verrell 1991). In a previous study on *Desmognathus ochrophaeus*, Verrell (1991) demonstrated that a physical blockage of the cloaca decreased female receptivity to male courtship displays by 64.2%, but this affect only lasts as long as the sperm mass was retained in the cloaca. In *P. shermani* the sperm mass remains in the cloaca at

most for four days. Compared to the >10 week breeding season (Arnold 1976), this period is brief. Thus, the sperm mass is unlikely to prevent females from re-mating during the breeding season, but it is possible that it prevents females from re-mating during a critical window for sperm movement from the spermatophore into the female spermatheca.

The time necessary for sperm to move between the spermatophore and the spermatheca is unknown in species with a complex spermatheca, but in two species with simple spermathecae (*Lissotriton vulgaris* and *Notophthalmus viridescens*) sperm are found in the spermathecae within 24 hours of insemination (Hardy & Dent 1986; Sever et al. 1999). All female *P. shermani* retained sperm masses in their cloacas for at least that long and, thus, it seems the sperm mass could prevent the female from re-mating until the first male's sperm has moved into the sperm storage organ. In desmognathine species and *Plethodon cinereus* (which both exhibit a complex spermatheca), sperm in the spermatheca form dense tangles in the common tubule and necks of the bulbs (Sever and Hamlett 1998; Sever and Siegel 2006). It's likely these sperm tangles occur in *P. shermani* as well. Sperm from subsequent matings collide with these tangles. Although the results of these collisions are currently unknown, there are many possible outcomes including (a) sperm mixing, (b) pushing the sperm from the initial mating further into the spermatheca and (c) preventing the majority of sperm from current mating from entering the spermathecal bulbs or common tubule.

This third option could explain the finding of first male sperm precedence in *Desmognathus ocoee* (Adams 2004).

The retention of the sperm mass in the female reproductive tract for several days could have roles other than mate guarding that also contribute to male reproductive success. First, the sperm mass could prevent passive sperm loss during the critical period before sperm has moved into the spermatheca. All female *P. shermani* retained a sperm mass in their cloacas for at least that long and, thus, it seems that the sperm mass could physically prevent sperm from leaving the cloaca. We believe this hypothesis is unlikely, because of the way sperm must leave the sperm mass. In *Plethodon jordani*, a closely related species to *P. shermani*, the sperm are oriented so the heads are towards the base of the sperm mass (Zalisko et al 1984). This implies the sperm exit the sperm mass through the bottom of the mass rather than the top. They, thus, have to swim around the outside of the sperm mass to reach the cloaca. So, there must be a gap between the wall of the cloacal chamber and the sperm mass that sperm could be lost from.

A second means by which retention of the sperm mass could influence male courtship success is the potential affect of sperm mass proteins on female reproductive physiology. In many taxa, seminal proteins affect female physiology by manipulating, among other things, re-mating rate, the amount of resources a female dedicates to a clutch, and when a female oviposits (Wolfner 2002; Pitnick et al. 2009). Seminal

proteins are also known to enhance sperm motility and longevity (Schoneck et al. 1996; Simmons & Beveridge 2011). Either of these alternative roles for the sperm mass could increase male reproductive success.

Interestingly, the duration the sperm mass remained in the female cloaca in *P. shermani* is almost 3xs longer than the sperm mass is retained in *Desmognathus ochrophaeus* (an average of 24 hours; Verrell 1991). This difference in duration could be due to differences in the structure of the spermatophore. Salamanders in the genus *Plethodon* have spermatophore with a series of capsules surrounding the sperm mass (Organ & Lowenthal 1960; Zalisko et al. 1984). The original function proposed for these capsules was to prevent dessication of the sperm mass in these highly terrestrial salamanders (Organ and Lowenthal 1960). We propose an alternative function: the rigid capsules help the sperm mass retain its shape in the female cloaca, which inturn could increase the duration the sperm mass remains blocking the female cloaca compared to species without these capsules.

In addition to the high likelihood of female re-mating, the opportunity for post-copulatory sexual selection in *P. shermani* is also high because of the long duration of sperm storage. Females can retain viable sperm for at least 9 months. This period is longer than the length of the breeding season, so the sperm from males encountered early in the breeding season is still present and viable when a female re-mates. Re-mating rate in the field is unknown for this species, but in laboratory staged trials

females are known to mate up to 8 times (Eddy, unpublished data). The re-mating rates of two other plethodontid species, *Desmognathus ocoee* and *P. cinereus*, has been recorded in the field (Adams et al. 2005; Leibgold et al. 2006). Using paternity analysis, a minimum of 2-3 partners per female was estimated for both species. In other salamander species the number of mating partners ranges from a minimum estimate of 1.4 partners to 8 from paternity analyses on wild populations (Peico 1992; Myers & Zamudio 2004). In laboratory studies *D. ocoee* females were inseminated up to 29 times (out of 36 opportunities) during one study with an average of 19 inseminations per female (Houck et al. 1985). Taken together, these results demonstrate there is a high probability that the sperm from multiple males interact in the sperm storage organ of *P. shermani* females.

The variability in fertilization success within each month was high. For instance, in our final month of observations two clutches had embryo viability < 10%, whereas in the third clutch 75% of the embryos were viable. This variability could have multiple causes. First, variation could be the result of the hormone treatment necessary for inducing oviposition: the exogenous hormones could have interacted differently with the female's reproductive physiology. We do not think this is the best explanation for the phenomenon of variation in viability, because even in the 4 females who oviposited naturally there was large variation in embryo survival between clutches. A second biologically significant explanation is that males differ in the ability of their sperm to survive through time. Variability in sperm longevity has been

observed in many fish species (reviewed in Stockley et al. 1997) and in field crickets (*Teleogryllus oceanicus*; Garcia-Gonzalez & Simmons 2005). Even if sperm remain alive in the spermatheca, it's possible that it ages and degrades leading to infertility or zygote death (Lodge et al. 1971; Wagner et al. 2004). A final possible explanation for this pattern is that females vary in their ability to keep sperm alive in their spermatheca.

In contrast to the high probability of post-copulatory sexual selection within a season, the probability that sperm from one season could be retained to a second season is much lower. First, to fertilize eggs from a second reproductive cycle sperm would have to be retained for at least 15 – 19 months. We sectioned the spermatheca of females who had mated 17 months earlier. Only one of three females still had sperm in her spermatheca and we do not know whether the sperm observed was still viable.

Second, females in reproductive condition in one year oviposit their ova before the start of the next courtship season. In the majority of cases in our study most of the stored sperm was lost within 2 weeks of oviposition (although 2 of 6 females had sperm levels indistinguishable from those who had not oviposited) either by release during oviposition or potential active destruction by the female. In other plethodontid salamanders, sperm retained after oviposition is frequently associated with the walls of the spermatheca unlike the situation in females who have not oviposited (Sever &

Hamlett 1998; Sever & Brunette 1993). It is thought that the fluids secreted by the walls of the spermatheca degrade sperm (Sever & Kloepfer 1993). So, it would seem the remaining sperm in the spermatheca may be in the process of being destroyed by the female.

Overall, this study found *P. shermani* to have one of the longest sperm storage durations of any plethodontid salamander: 9 months for viable sperm (with potential for 17 months). This results plus the short duration that the sperm mass is retained in the cloaca relative to the breeding seasons suggests that post-copulatory sexual selection is important in this system.

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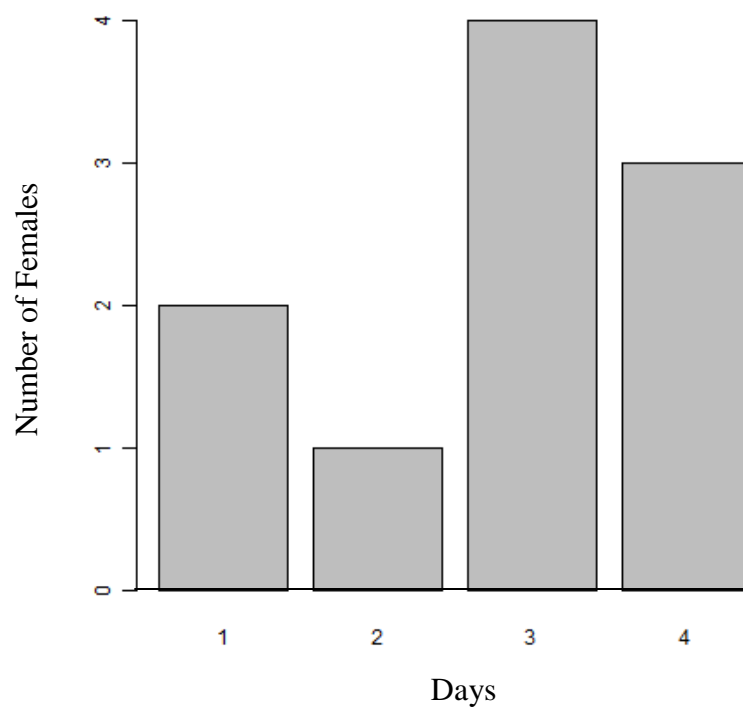
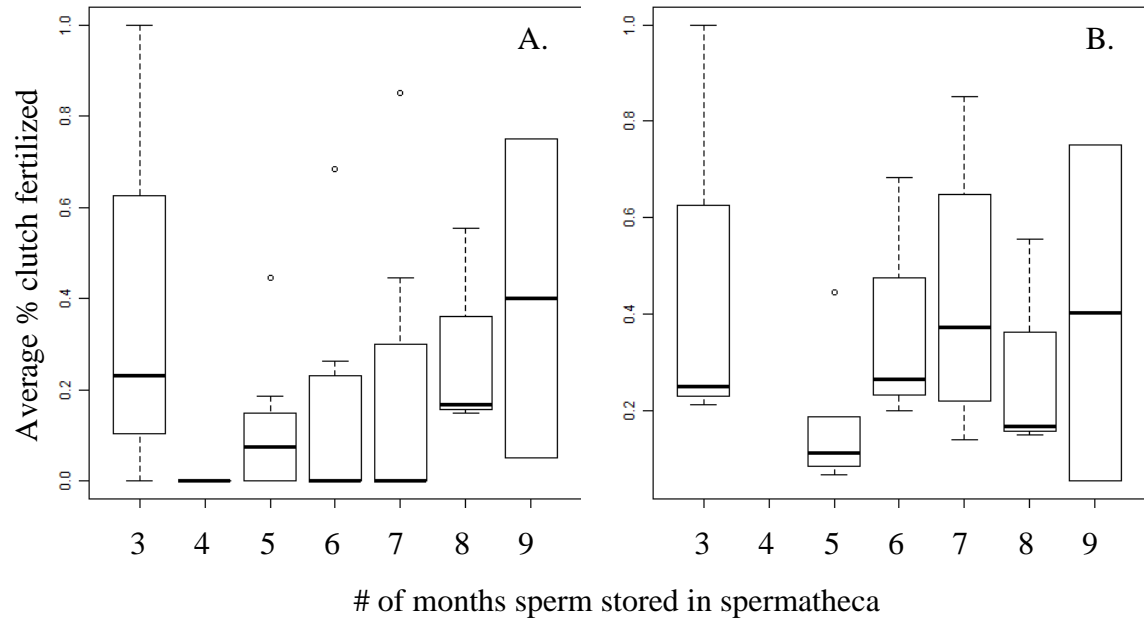


Figure 5.1. Days since insemination that the sperm mass remained visible in the female *P. shermani* cloacal chamber.



5.2. Proportion of a clutch fertilized as a function of the number of months sperm were stored in the spermatheca of *P. shermani* (A) including clutches where 0 eggs were fertilized and (B) excluding clutches where 0 eggs were fertilized.

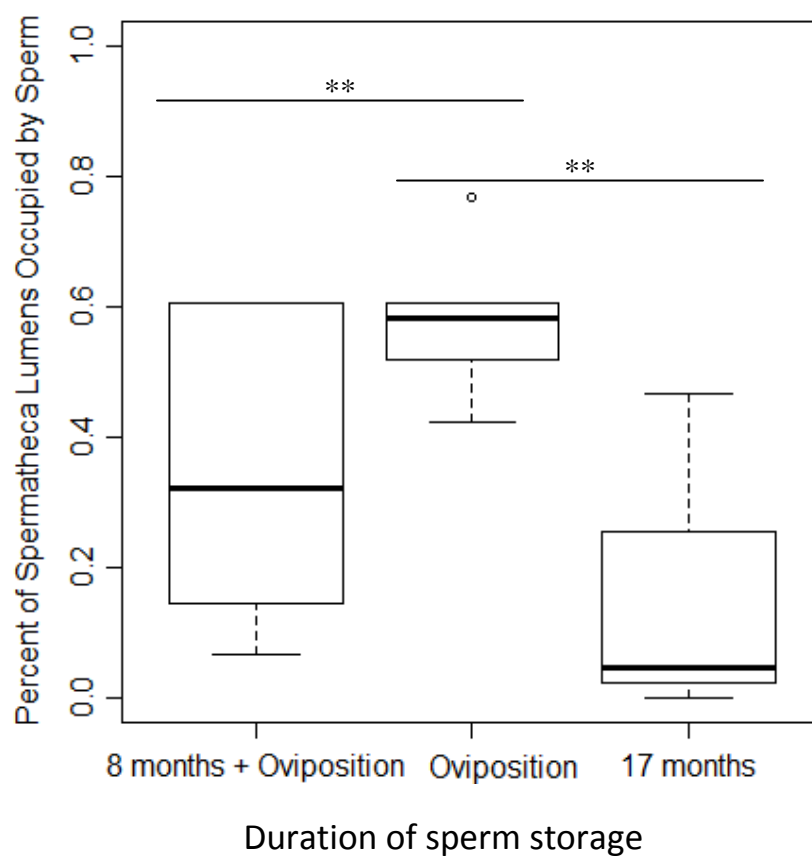


Figure 5.3. Proportion of the lumen of spermathecal bulbs filled with sperm as a function of elapsed time or whether or not oviposition occurred. ** indicates a significant difference in proportion of lumen occupied.

Table 5.1 Sperm storage durations across the Urodeles. Superscripts indicate which citation is being referenced if there is more than one sperm storage account for a particular species.

Species	Family	Estimated Length of Sperm Storage	Method of Sampling	Citations:
<i>Ambystoma tigrinum</i>	Ambystomatidae	2 days ¹ ; between seasons (or at least more than 2 days) ^{2,3}	Sampled females throughout the breeding cycle ¹ ; paternity analysis ³	¹ Sever 1995a; ² Rose & Armentrout 1976; ³ Bos et al. 2009
<i>A. mexicanum</i>	Ambystomatidae	12 days	Induced ovulations	Humphrey 1977
<i>A. talpoideum</i>	Ambystomatidae	up to 5 months	Sampled females throughout the year	Trauth et al. 1994
<i>A. opacum</i>	Ambystomatidae	1-2 month	Sampled females throughout the breeding cycle	Sever et al. 1995b; Sever & Kloepfer 1993
<i>A. maculatum</i>	Ambystomatidae	Days ¹ ; 1 year ² ; 2 years ³	Paternity analysis ² ; Observation of captive individuals ³	¹ Petranka 1998 ² Tennessen & Zamudio 2003 ³ Baylis 1940
<i>Amphiuma tridactylum</i>	Amphiumidae	4-5 months	Sampled females throughout the breeding cycle	Sever et al. 1996a
<i>Plethodon cinereus</i>	Plethodontidae	4-5 months	Sampled females throughout the breeding cycle	Sever 1997
<i>Eurycea quadridigitata</i>	Plethodontidae	8 months	Sampled females from Jan to April	Pool & Hoage 1973;
<i>E. cirrigera</i>	Plethodontidae	2-3 months	Sampled females throughout the breeding cycle	Sever 1991; Sever 1992; Sever & Brunette 1993

<i>Desmognathus fuscus</i>	Plethodontidae	3-4 months	Sampled females from May to December	Marynick 1971
<i>Necturus beyeri</i>	Proteidae	6 months	Sampled females throughout the breeding cycle	Sever & Bart 1996
<i>Salamandra salamandra</i>	Salamandridae	sperm from previous year not used by wild caught females	Paternity Analysis	Steinfartz et al. 2006
<i>Notophthalmus viridescens</i>	Salamandridae	up to 6 months	Sampled females throughout the breeding cycle	Sever et al. 1996b
<i>Salamandrina terdigitata</i>	Salamandridae	up to 6 months	Sampled females throughout the breeding cycle	Brizzi et al. 1995
<i>Cynops pyrrhogaster</i>	Salamandridae	6 months	Captive bred	Tsutsui 1931

Chapter 6 – Conclusion

6.1 GENERAL CONCLUSIONS

In this thesis, I investigated the potential role of mate choice and, at an exploratory level, post-copulatory sexual selection in the evolution of courtship in an emerging amphibian model, *Plethodon shermani*. In particular, I identified the cues correlated with increased courtship success via both male and female mate choice, as well as the potential for post-copulatory sexual selection via sperm storage. My results indicated that (a) courtship success in *P. shermani* is mediated by multiple cues across different sensory modalities, and (b) mutual mate choice occurs in this species. In addition, (c) *P. shermani* females were capable of storing viable sperm for longer than any other plethodontid salamander that has been investigated.

In species with two sexes, mate choice can evolve in either sex as long as the cost of being choosy (in terms of energy spent searching for mates and lost opportunities to mate) are balanced by the benefits gained (Andersson 1994). In Chapters 2 and 3, I argue that these conditions must met in both sexes of *P. shermani* because neither mates indiscriminately. The benefit of being choosy (i.e., the higher reproductive value of the chosen mate) can be is indicated by some cue discernable by a choosey individual. In fact, most organisms use multiples cues across many modalities to assess their mates (Candolin 2003). Unfortunately, compared with studies in other clades (e.g., birds), relatively little work has been conducted on multi-

modal cues in salamanders (Coleman 2009). In Chapters 2 and 4, I found that female *P. shermani* preferred males that: (a) engage more frequently in a visual display (foot-dancing), and (b) provide tactile stimuli (contact of the male's chin to the female's nares) during the persuasion stage of courtship. In addition, male courtship pheromones influenced the duration of later stages of courtship, but not courtship success (at least in the laboratory) (Chapter 2). Thus, at least three modalities (visual, tactile and chemical) seem involved in female mate assessment. I propose that the early use of a visual cue may provide the female with the context for a male's approach of a female (courting vs. aggression), and thus prime her for the subsequent tactile and pheromonal cues she receives during courtship.

Interestingly, in Chapter 3, I found that male foot-dancing effort, and other measures of energy devoted to reproduction (such as size of the courtship pheromone producing gland), were all correlated with male condition. These correlations indicate that these traits may be honest indicators of male fitness. I also found that foot-dancing effort and occurrence of attempted pheromone delivery (the tactile cue) during persuasion were correlated (Chapter 4). Thus, all three traits that females seem to use to assess males are correlated with a measure of male fitness (male condition). This correlation indicates that a reasonable explanation for the evolution of female choice in this system may be the "good genes" models (Andersson 1994). These models propose that choice evolves because the offspring of choosy individuals have

higher fitness due to the higher fitness of the chosen partner, and often involve an indicator trait linked with male fitness.

P. shermani males demonstrated mate choice through differential performance of courtship behaviors. In particular, I showed in Chapter 3 that males varied the effort they devoted to the visual cue (foot-dancing), depending on the reproductive value of the presented female. Males foot-danced most frequently for females having the highest reproductive value (highly gravid females), and less for non-gravid and weakly gravid females. Unlike other salamander species, male *P. shermani* did not seem to use the visual cue of female size as the primary indicator of reproductive value. Instead, I propose that they can detect differences in female gravidity via chemical cues as demonstrated in several other species of plethodontids (Marco et al. 1998; Dantzer & Jaeger 2007). Because choosy males benefit via an increased number of offspring by mating with females having more developed ova, the evolution of male mate choice in this system is likely due to direct benefits.

In addition to pre-copulatory processes of sexual selection, post-copulatory processes also have the potential to influence the fitness of an individual. For post-copulatory processes such as cryptic choice and sperm competition to occur, the opportunity for sperm from multiple males to be present in the female's reproductive tract is necessary (Eberhard 2009). The opportunity for post-copulatory processes to occur is enhanced in species in which females have evolved prolonged sperm storage

(Parker 1970). Post-copulatory processes can be limited by events such as post-copulatory mate guarding or the production of a copulatory plug (e.g. Shine et al. 2000). In another salamander species (*Desmognathus ochropheaus*), the male sperm mass may act as a copulation deterrent for the duration of its retention in the female's cloaca (Verrell 1991). The opportunity for post-copulatory processes to influence reproductive success thus needed to be investigated, both in terms of duration of sperm storage and the extent to which post-copulatory mate guarding (via the sperm mass) limits a female's opportunity to re-mate.

In Chapter 4, I showed that, compared to the duration of the breeding season, the 3 days in which the average sperm mass remained in the female's spermatheca would not be sufficient to prevent her from mating multiply. Thus, the sperm mass is not an effective at preventing female re-mating, but it may prevent re-mating during the critical window for sperm transport into the spermatheca. This could provide an advantage to a male if it prevents the sperm from rival males from entering the sperm storage organ or displacing his sperm.

The presence of a sperm storage organ increases the likelihood of long-term sperm storage. Previous studies of salamanders have proposed that sperm can be stored for multiple years in the sperm storage organs. These claims, however, have been challenged based on the grounds that (a) sperm are probably too degraded to be viable over the long term, and (b) no sperm are retained in the sperm storage organ

after oviposition (Sever & Brizzi 1998). In Chapter 5, I show that females can retain viable sperm for at least 9 months, the longest period documented for any plethodontid salamander. I also observed that females retained varying amounts of sperm in their sperm storage organs after oviposition, and that sperm could be retained until the next reproductive bout (approximately 17 months later). Thus, sperm from one breeding season potentially could interact with sperm from the previous season. Unfortunately, sperm in the oviposition and 17 month treatments could not be tested for viability.

Overall, my thesis investigations highlight the complex interactions of different components of sexual selection that have shaped the *P. shermani* mating system. Mate choice was employed by both males and females, thus the outcome of a reproductive bout is not simply due to a male's motivation to mate, but also the female's. In addition, mate choice is not in response to one cue, but rather the evaluation of multiple traits in multiple modalities. Finally, post-copulatory sexual selection has a high potential to occur in this system due to the capability of long-term sperm storage by females.

6. 2 FUTURE DIRECTIONS

The findings in my dissertations suggest many additional questions. These questions can be organized into two categories mirroring the organization of the forces of sexual selection: pre-copulatory and post-copulatory processes in *P. shermani*.

Pre-copulatory Processes

How do males determine female gravidity? From my current work, males apparently can evaluate the reproductive state of a female, but do not use female size as the primary evaluation factor. This inattention to female size makes sense in terms of the ecology of the species: females typically come into reproductive condition every other year (Hairston 1983). Thus, large females may or may not be gravid in a given year. I propose that males use chemical cues instead of size to assess female reproductive condition, but future behavioral trials are necessary to evaluate this hypothesis.

Is foot-dancing an honest indicator of heritable male quality? Based on the results in Chapters 2 and 3, I propose that female mate choice may have evolved via “good-gene” mechanisms, and that foot-dancing (correlated with male condition) may be an indicator trait. Testing this hypothesis will be challenging. A first step would be to document a cost to males of intensive foot-dancing. A cost would indicate that not all males are capable of sustaining this behavior, and thus indicate that foot-dancing is an honest indicator of male quality (Andersson 1994). One way to measure this cost would be to identify and quantify a metabolic cost to male foot-dancing. Another approach would be to examine heritable variation in the trait and the correlation with offspring fitness.

Post-copulatory Processes

What proteins are contained in the sperm mass? Even if the sperm mass does not act as a form of post-copulatory mate guarding, males may still use it to manipulate their reproductive success. For example, accessory gland proteins found in the seminal fluid of *Drosophila*, bumble bees and rats can reduce female re-mating rate (reviewed in Poiani 2006). In plethodontids, the components of the spermatophore are produced by four types of glands: the Kingsbury glands, anterior ventral glands, dorsal pelvic glands and caudal pelvic glands (Sever and Houck 1985). The products of these glands are complexes of carbohydrates and proteins, as well as lipids (Sever 2003). Further chemical characterization of the substances found in the spermatophore could determine whether proteins in the sperm mass could: (a) modify female physiological processes in a way that benefits a male, and (b) identify additional post-copulatory processes influencing the mating system of *P. shermani* male.

What pattern of sperm precedence is present in P. shermani? Salamander species differ in their patterns of sperm precedence. Possible patterns include: (a) first male (where the first male to mate with a female garners the majority of the paternity of her ova), (b) last male, or (c) mixing (all males share paternity equally). These patterns of sperm use can lead to divergent selective pressures and thus the employment of divergent reproductive strategies. Sperm precedence studies on salamanders with a

complex spermatheca have only been performed in one species so far (*D. ocoee*, Adams 2004). Thus exploring the precedence pattern in *P. shermani* could be informative for comparative studies on how the shape of sperm storage organs influences paternity.

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