HOW CONSERVATIVE ARE SEXUAL PHEROMONES?: A CROSS-GENERA STUDY OF PLETHODONTID SALAMANDERS

A thesis presented to the faculty of the Graduate School of
Western Carolina University in partial fulfillment of the
requirements for the degree of Master of Science in Biology

By

Christopher Brannon Love

Director: Dr. Joseph H. K. Pechmann
Associate Professor of Biology
Biology Department

Committee Members: Dr. Jeremy Hyman, Biology
Dr. Seán O'Connell, Biology

April 2013

ACKNOWLEDGEMENTS

I would like to thank the Houck-Feldhoff research team for use of their pheromone extracts as well as their expertise in planning the experimental design of my study. Funding for their work would not be possible without awards from the National Science Foundation (Award #'s 1147271 & 1146899). With this, I would also like to deeply thank two students from Oregon State University: Christy Baggett and Robbie Blenk. Without these two students helping observe mating salamanders, my observational nights would have been impossible.

Moreover, I would also like to thank the Highlands Biological Station for receiving a Grant-in-Aid of Research. Providing monetary support as well a place to conduct my research was invaluable.

I would like to thank my thesis committee for their guidance and support. I would also like to especially thank the reader of my thesis, Dr. Pattie Foley, for her continued assistance throughout my graduate school experience and for always being there for me.

Lastly, I would like to thank my family for their endless and unyielding support and love. Without them, my thesis as well as many other things would not have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

HOW CONSERVATIVE ARE SEXUAL PHEROMONES?: A CROSS-GENERA STUDY OF PLETHODONTID SALAMANDERS

Christopher Brannon Love, M.S.

Western Carolina University (April 2013)

Director: Dr. Joseph Pechmann

Pheromones play a pivotal role in salamander reproductive behavior. Males deliver internally made pheromones from their mental gland to females during courtship interactions in the family Plethodontidae. These courtship pheromones typically increase female receptivity to mating by lowering courtship time. Differences in pheromone structure are thought to contribute to mating isolation among species. This experiment tested whether *Plethodon shermani* pheromones stimulated mating in a plethodontid salamander of a different genus. Mating trials were conducted with gravid female and deglanded male pairs of *Desmognathus ocoee*, while delivering *D. ocoee* pheromone, *P. shermani* pheromone, or saline control. Results showed that pairs receiving *P. shermani* pheromone spent twice as much time in tail-straddle walk when compared to the saline control, and 1.6 as much time in tail-straddle walk compared to native *D. ocoee* pheromone. These results indicate that pheromones are possibly specific to a given species and are not used as general mating stimulus, thereby possibly acting as a reproductive isolating mechanism among plethodontid salamanders.

INTRODUCTION

The ability to signal as well as perceive and interpret incoming signals is what allows organisms to communicate. Signals can be visual, auditory, olfactory, vibratory, or tactile in nature and can function in recognition, reproduction, alarm, finding food, and social actions (Drickamer et al. 2002). Many studies have illustrated the significance of chemical signals in both the sexual and social behavior of animals, and these chemical signals are loosely called pheromones (Wyatt 2003). For instance, in the honeybee *Apis mellifera*, worker bees can use a pheromone they produce to recruit other worker bees to help in taking care of certain larvae that they want to develop into potential queens of the colony (Al-Kahtani & Bienefeld 2012). Another social study of pheromones found that *Lasius niger* worker ants mark foraging pathways to food sources. The pheromones laid down by worker ants helped guide other worker ants to the food source. The laid down pheromones also facilitated the formation of that particular route to the food into the memory of the other worker ants, even though it was discovered by the previous worker ant (Czackes et al. 2012).

Reproductive pheromones allow animals to identify species and sex in another individual, coordinate male and female interactions during mating, and function in sexual persuasion of individual males and females (Houck et al. 2007, Rollmann et al. 2003). In *Drosophila melanogaster*, pheromones emitted by the male stimulate receptors in olfactory neuron regions of males and females. Researchers mutated this receptor in males and found that these mutated males tried to court other males, while mutated females were less receptive to courting males (Kurtovic et al. 2007). In this case, the

receptor in question plays an important role in coordinating male and female interactions. Pheromones that coordinate male and female interactions also exist in aquatic species. After ovulation in *Carassius auratus*, the female goldfish releases a pheromone from her ovary, which draws the male and elicits a persistent courtship that accompanies spawning. Males that have nares blocked or permanently severed nares, where their receptors for this ovarian pheromone reside, show less courtship behavior (Partridge et al. 1976). In this instance, not only are the pheromones being used in coordinating actions between male and female, but also are used to persuade males to potentially mate females.

My research focuses solely on pheromones in salamanders. In plethodontid salamanders, the males will deliver pheromones produced by his mental gland, which enlarges during the mating period, to a female during an active courtship (Houck et al. 1985). Sexual pheromones in salamanders can increase the likelihood that a given male will persuade a female to mate (Houck et al. 2007). Therefore, pheromones help coordinate male and female interactions after a courtship has already been initiated and are involved in female persuasion.

Species Of Interest

The focal species for this experiment was *Desmognathus ocoee*. *Desmognathus ocoee* is found in parts of the Southern Appalachian Mountains of Tennessee, North Carolina, South Carolina, and Georgia (Lannoo 2005). It is usually found in close proximity to flowing bodies of water but can range far into the uplands. *D. ocoee* also has a free-swimming larval stage, which means that females will make their way to water to oviposit their eggs, and the hatchlings will develop in the water where they were

deposited. A variety of colors and patterns of *D. ocoee* can be found. Its adult size ranges from 70-111mm (Beane et al. 2010).

I examined the response of *D. ocoee* females to courtship pheromones extracted from males of *Plethodon shermani*. *Plethodon shermani*, also known as the red-legged salamander, has a black body color with a grey belly. They are known as the red-legged salamander because the species has both front and hind legs colored red to varying degrees throughout most of its range (Lannoo 2005). *Plethodon shermani* has direct development in its terrestrial habitat, which means a female will oviposit her eggs on land and the larvae will develop exclusively on land. Its adult size ranges from 90-184mm (Beane et al. 2010). They are found in the Nantahala and Unicoi Mountains at high elevations between 850-1500m (Niemiller & Reynolds 2011). These two species live in the same space among the Southern Appalachian Mountains, since their habitat ranges can overlap, therefore it is likely that they do encounter each other.

Mating Ritual

The mating ritual of *Desmognathus ocoee* occurs in 3 distinct phases: orientation, persuasion, and sperm transfer (Herring & Verrell 1996). During orientation, the male approaches or follows the female, and continues to do so until the female slows or ceases her locomotor activity. The male may also display visual stimuli for the female to observe. An example is the butterfly movement, where the male moves his forelimbs backwards, raises them from the substratum, and then will sweep them in a circular movement, forward, and then downward and back again (Herring & Verrell 1996).

The persuasion phase happens when the female ceases to move away when approached or followed by the male (Verrell 1997). The male then engages in head

rubbing, tail undulation, pulling, and snapping, all of which are tactile stimuli for the female. Head rubbing is where the male rubs his head on the head of the female. Pulling is defined as when the male presses his chin against the dorsum of the female by bending his head downwards, drawing his head backwards in one or a few short strokes, while keeping his chin in contact with the female (Verrell 1997). The premaxillary teeth of the male will scrape along the dorsum of the female, resulting in delivery of his pheromone, from the scratching action (Houck et al. 1985). The male sometimes has the possibility of being able to slide his body, particularly the dorsum of his undulating tail, under the chin of the female. He then can curve his body around the female in the shape of a C. The male will then draw his head backward in a sudden and forceful movement and snap away from the female, possibly launching him several centimeters away from the female. This action is known as snapping (Verrell 1997). A male will also exhibit tail undulation in the persuasion phase of courtship. The male will move his entire tail laterally in tail undulation, during which the female will sometimes rest her head upon his tail. If tail undulation was successful, the last state of sperm transfer would commence, called tail-straddling walk (Vinnedge & Verrell 1997).

Tail-straddle walk is where the male slides his undulating tale under the chin of the female and her forelimbs straddle his tail. He begins to walk forward and the female follows, walking with the male. The male deposits his spermatophore on the substrate and continues walking forward, with the female eventually moving her cloaca over the spermatophore, such that it enters her cloaca (Herring & Verrell 1996).

Movements of the male can be visually stimulating during *D. ocoee* courtship, which is where the butterfly movement comes into effect (Verrell 1997). The goal of

these visual movements would be for the female to notice the individual male. Movements involved in the persuasion phase, pulling, snapping, and rubbing, can all together be correlated with males having a higher rate of courtship success. However, tail undulation was the only single act that the male commits that has been significantly correlated to mating success (Vinnedge & Verrell 1997). Courtship success is when a male manages to get a female to go through all of the stages of the courtship ritual. Mating success is when a male manages to get a female to go through spermatophore reception. Courtship success may not necessarily lead to mating success, as the two are distinct events. The female could go through the courtship ritual and then choose not to partake in the actual mating process (Vinnedge & Verrell 1997). Reasons that a female my chose to flee an active courtship is that something around her may have startled her, the male with whom she is mating startles her, or she is no longer interested in mating the male she is currently with (Verrell 1997).

Differences In Pheromone Delivery Among Plethodontidae

Evolutionary shifts in pheromone delivery have occurred over the span of time that plethodontid salamanders have existed. There are two main methods in which a male will deliver his pheromone to a potential mate within plethodontid salamanders (Kiemnec-Tyburczy et al. 2011). The one used by *D. ocoee* is the vaccination/scratching method and is the oldest method of pheromone delivery in the Plethodontida*e* family, originating about 100 million years ago (Kiemnec-Tyburczy et al. 2009). *Desmognathus ocoee* males have premaxillary teeth that are used to abrasively contact the skin of the female, and while the male drags these teeth across the female, he delivers his pheromone. This act is thought to diffuse the pheromone into the capillary system of the

female and activate receptors that can be targeted via the bloodstream (Houck et al. 1985). The other method of pheromone delivery is through the olfactory system, as *P. shermani* utilizes. Here the male will slap his chin on the nares of the females directly depositing his pheromones into her vomeronasal organ. Receptors reside here that are stimulated by the delivery of the pheromone (Palmer et al. 2005). Thus, the method of delivery of pheromone is different for the two species used in this experiment. The adaptation of olfactory delivery of pheromones occurred approximately 19 million years ago (Kiemnec-Tyburczy et al. 2009). It has been shown that methods of delivery of pheromones are species-specific, and that trying to use a method of pheromone delivery that is not natural to a species does not elicit a significant response (Kiemnec-Tyburczy et al. 2011).

Composition Differences Of Pheromones

The compositions of the pheromones for both species used in this study are also different. So far, there have been two distinctive roles that constituents of plethodontid pheromone solutions take. There are individual constituents that make up the reproductive pheromone part of the pheromone solution, and there are other individual constituents that so far, have not been shown to play a role in salamander reproduction (Kiemnec-Tyburczy et al. 2009). Only the pheromones constituents that make-up the reproductive constituents of the entire pheromone solution will be discussed in this experiment. The two principal constituents of mental gland secretions of *D. ocoee* are proteins from two distinct families (Houck & Reagan 1990). The main protein found in *D. ocoee* pheromones is Sodefrin Precursor-like Factor (SPF), with a small amount of Plethodon Modulating Factor (PMF). *Plethodon shermani* pheromones are mainly

composed of PMF, and smaller amounts of Plethodon Receptivity Factor (PRF), and even smaller amounts of SPF. Both SPF and PRF have both been shown to increase female receptivity to mating by a decrease in courtship time. However, PMF has been shown to decrease female receptivity due to courtship time lengthening when it is solely administered to a female (Kiemnec-Tyburczy et al. 2009). With this in mind, PMF is not considered to be an accessory constituent to any other part of the overall pheromone solution, since PMF on its own did cause a change in mating time. It is though that PMF may serve as some type of calming effect on females, acting to keep the female from fleeing from a mating that is already in progress (Houck et al. 2007). This is more applicable to *P. shermani* matings, since their courtships last longer than *D. ocoee* courtship rituals, especially the tail-straddle walk phase (personal communication, Dr. Lynne Houck). High performance liquid chromatography (HPLC) profiles of all of these proteins differ dramatically among populations. Thus, on biochemical grounds, these courtship pheromones are good candidates for being behavioral isolating mechanisms (Houck & Reagan 1990). SPF is noted as the most ancestral component of pheromones in plethodontid salamanders, with SPF's existence dating back to before plethodontids evolved (Fig. 1; Kiemnec-Tyburczy et al. 2009). PMF arose during the evolution of the Plethodontidae family of salamanders, and PRF evolved only within the genus of *Plethodon* (Fig. 1). This is seen with the presence of PRF in *P. shermani* and not in *D. ocoee* (Kiemnec-Tyburczy et al. 2009).

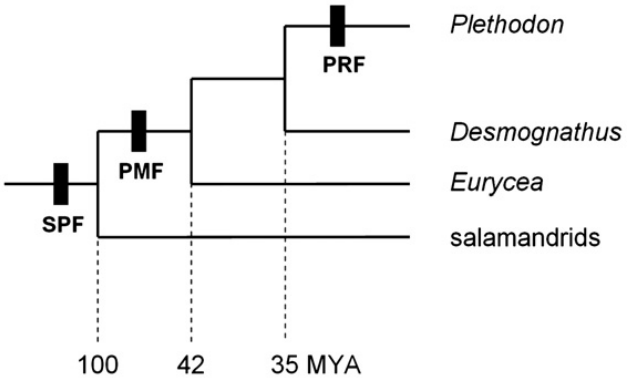

Figure 1. Cladogram Illustrating The Evolution Of The Main Constituents of Plethodontid Pheromones. Taken from Kiemnec-Tyburczy et al. 2009 with permission.

Pheromone Importance

Overall, the only role courtship pheromones play in plethodontid salamanders is to reduce time spent in courtship, excluding the one pheromone PMF (Rollmann 1999). This is likely the only purpose of salamander pheromones since these pheromones function only after a potential mate has been located and identified and the preliminary courtship has been initiated (Houck et al 2007). Courtship pheromones reduce courtship time in within-population matings as demonstrated in experiments with *D. ocoee* where deglanded males were used. Houck & Reagan (1990) & Houck et al. (1985) used secretions from the male mental gland to investigate the effects of the male pheromone on female receptivity. Females treated with whole pheromone extract showed a significant reduction in courtship duration, as compared to females given a saline control solution. The average time to tail-straddling walk was 26% faster and the average time to spermatophore deposition was 28% faster for females treated with pheromone extract (Houck & Reagan 1990).

Experiments have shown that pheromone stimulation of olfactory organs in females of Plethodons can augment sexual motivation significantly (Vaccaro et al. 2010). Additionally, plethodontid male courtship pheromones have been shown to suppress females' tendency to feed, and suppress fleeing from an ongoing courtship/mating session during the mating season (Vaccaro 2009). Since delivery of male pheromone can cause females to suppress their foraging habits, but yet still give females the ability to be to be selective with potential mates, this suggests that reproduction might be a greater motivation than ingestion during the mating season.

Plethodontid salamanders have been provided opportunities for matings where conspecific matings were staged but females were given either conspecific or heterospecific pheromones using deglanded males (Rollmann et al. 2003). The importance of this study is that the differing pheromones were congeneric, meaning that the two pheromones being used in the study belonged to the same genus of salamanders, but differed at the species level. No significant difference in courtship time was found between the groups in the mating trials (Rollmann et al. 2003). From this, it does not seem there is necessarily a significant difference in pheromones and their role in courtship from species within the same genus.

There are several conclusions that can be drawn about the evolutionary dynamics of the pheromone signaling system in plethodontid salamanders. There is stabilizing selection occurring on the behavioral and morphological aspects of signal delivery since both vaccination/scratching and olfactory delivery have been around for at least 19 million years (Watts et al. 2004, Kiemnec-Tyburczy et al. 2009). Moreover, there is heterogeneity of the pheromone signal itself present within populations, meaning that in

general, the sequencing of amino acids for these pheromones is changing among male salamanders (Watts et al. 2004, Houck & Regan 1990). Specific pheromone protein sequences can differ among individual males, therefore, the actual pheromone that is constructed by the individual male can differ. Lastly, there must be positive selection occurring on the pheromone signal itself (Watts et al. 2004). Females are selecting for males using these pheromones during courtship, and their continued use allows for females to be selective with potentially courting males. Therefore, female receptors for pheromones and male production of pheromones are thought to be in a "molecular tango" where both coevolve within a confined molecular space (Palmer et al. 2005). This confined molecular space is the space between the molecular male produced pheromones and the specific molecular receptors targeted by these pheromones that reside within the female.

Objective/Significance

This experiment addressed the importance of pheromones as an isolating mechanism for mating between salamander genera. Are courtship pheromones a mere general stimulus for mating in plethodontid salamanders, or do they act specifically on particular species or genera? The experiment tested whether delivering a male *P. shermani* pheromone solution to a family-related female salamander, *D. ocoee*, will cause a significant change in courtship mating time. It was a logical extension of the study that found that no significant difference in courtship time existed when experimentally delivering pheromones from different species of salamanders within the genus *Plethodon* (Rollmann et al. 2003).

The importance and benefits of this study relate to the high diversity of salamander species in the Southern Appalachians. The specificity of pheromones is believed to affect speciation mechanisms and therefore the mechanisms generating and maintaining plethodontid diversity (Palmer et al. 2005, Watts et al. 2004, Rollmann 2003). By understanding the diverse levels on which these pheromones work, we can understand how these species are evolving and changing in this unique geographic region.

METHODS

Collection

My assistants and I collected 111 male and 63 gravid female *D. ocoee* salamanders from Deep Gap, Macon County, NC on August 1, 2, and 5, 2012. The 63 gravid females and 63 randomly selected males were used for the experiment. An estimate of the *D. ocoee* population density at Deep Gap is 6.27 per square meter (Pursel 2012).

Animal Maintenance

Collected *D. ocoee* were housed in the basement of the Nature Center at Highlands Biological Station, equipped with an air conditioner and two humidifiers to maintain a constant temperature of 68°F and 55% humidity. Animals were individually housed in clear plastic shoeboxes (17 x 12 x 6 cm) containing crumpled moist paper towels as refugia. The animals were housed under red light to simulate their natural environment, as this color of light is the least disturbing to them. White lighting was only turned on during normal maintenance time. After 24 hours of acclimation in the initial shoebox, the salamanders were fed two wax worms. Salamanders were not fed again until right before they were released back into their habitats. However, once the experiment started, the salamanders were no longer fed. This is more important for the female salamander. Not feeding the female during the mating trials ensures that females will remain in a reproductive mood. When a female is satiated from feeding, she is not receptive to courtship mating. Since courtship trials lasted only nine days, this period of not being fed did not cause any harm to the animals being used in the experiment. All

males and females were fed after the experiment ended before they were released back into their native habitat.

Mental Gland Removal

Male salamanders were anesthetized by submersion in 4% ether solution for 10 minutes. After this period, animals no longer displayed any responses to physical contact and began to recover from anesthesia within an hour. Following anesthesia, male salamanders had their mental glands removed. Using a dissecting microscope, a small incision was made adjacent to the mental gland. Forceps were used to pull the mental gland from the chin, and the underlying connective tissue was severed using iridectomy scissors; consequently, the gland was removed without invading underlying tissue. Male salamanders were returned to a new, sterilized box, with their chins placed on a small cotton pad dabbed with penicillin gel in order to restrict the likelihood of infection. Male salamanders were allowed at least one full week to recover before being used in mating trials. Dr. Lynne Houck, from Oregon State University, completed all the surgery on the males. The pheromones used in my study did not come from these recently collected and deglanded males. Whole pheromone extracts were already created and provided, for both *D. ocoee* as well as *P. shermani*, by Dr. Richard Feldhoff from the University of Louisville from previous mental gland excisions/extractions.

Mating Trials

Once the males had fully recuperated, I began mating trials. Each of the males and females were given a randomly designated mating trial pair number. This randomization of male to female ensured no bias in mate pairings. Treatment outcomes were analyzed within each pair, since the same male and female would only mate with

each other. The 60 mating pairs were split into groups of 30 pairs, making 2 groups of 30 mating pairs. This was done so that only one set of 30 mating pairs would be observed each mating trial night. There were 3 observers for mating trial nights: Robbie Blenk, Christy Baggett (both undergraduates from Oregon State University), and myself. Each of the three observers observed 10 pairs each night. Each observer was given a predetermined set of mating pairs they observed each mating trial night, that did not change from night to night. The assigned observer scored only their assigned mating pairs. This set observer-mating pair relationship would reduce any effects of observer bias.

On a given mating trial night, each observer was responsible for administering an A, B, or C solution to a predetermined list of female *D. ocoee*. The A, B, and C stood for each of the 3 different treatments, *D. ocoee* pheromone solution, *P. shermani* pheromone solution, or saline control. This randomization of which person gave which solution ensured that no one treatment condition would be different than another due to the actions of the person applying the pheromone solution. The A, B, and C designations of the different treatments also allowed for a blind study to be conducted, disallowing observer bias during the mating trials as well as during data analysis. The order in which each pair received one of three possible treatments was randomized over three mating trial nights; each mating pair received all of the possible treatments of the experiment. Also randomized was the person who administered each of the three possible treatments each night. At the end of my experiment and data analysis, I was given the key to my blind treatment conditions. Treatment A was saline, treatment B was *P. shermani* pheromone, and C was *D. ocoee* pheromone.

On a mating trial night the female was placed into a larger box (31 x 17 x 9 cm) used for mating trials. She was prepped for application of the pheromone solution by rubbing a Q-tip on the designated spot on her back, which was directly behind her forelimbs. This rubbing was used in order to remove her mucous layer, allowing for maximum absorption of the treatment being applied. Next, a standardized punch-hole (approximately 6.5 mm) of Whatman glass microfiber filter paper was wetted with 15 microliters of the treatment solution. Immediately after this, the wetted paper was applied to the clean spot on the back of the female. The female was allowed to stay in the box for 30 minutes with her treatment patch; then her mating trial paired male was added to the box. The mating pair was observed for 5 hours, making note of when pairs would enter tail-straddle walk, as well as when spermatophore deposition occurred during this time. To do this, each observer had a spreadsheet with 60-minute intervals on each sheet; thereby making observation tallies over 5 sheets in all, totaling 300-one minute intervals. Each minute the observer would simply note if the pair was in tail-straddle walk or not. If the pair was not in tail-straddle walk, the observer put a 0 on the spreadsheet, and if the pair was in tail-straddle walk, a 1 was entered. When a pair entered into tail-straddle walk during the 5-hour observation period, the pair was also observed for spermatophore deposition. If this happened while being observed, the observer noted this action and the time. After 5 hours, the pairs were left over night in their boxes and in the following morning each box was checked for spermatophore deposition. A deposited spermatophore consists of a gel base and the sperm cap that contains the sperm the male produced. When only a gel base from a spermatophore was found, the female was observed to check for presence of a spermatophore in her cloaca. I noted presence as SD,

for spermatophore deposition inside of the female. If the male deposited his spermatophore but the female did not pick it up, this was noted as SC for sperm cap.

Statistical Analysis

A mixed-model analysis was performed using restricted maximum likelihood (RML), with pairs as random effects since this was a subset of the entire *D. ocoee* population. With this, the variation among the pairs I observed is assumed to be representative of the variation of the whole population. I performed a pair-wise comparison of treatment means using the Tukey's correction to protect the experiment-wise error rate. This was completed for analysis of time in tail-straddle walk and time to tail-straddle walk. A mixed-model analysis using RML was also performed on the number of spermatophore depositions. Spermatophore deposition was treated as a binomial response variable. For analysis, spermatophore deposition included when the sperm cap was not picked up, but was deposited on the substratum. This was included since a male does not deposit his spermatophore without being in tail-straddle walk, thereby showing the female was inclined to mate with her mating partner; therefore it is logical to include spermatophore deposition whenever it is picked up or not.

RESULTS

Desmognathus ocoee mating pairs treated with *P. shermani* pheromone spent significantly more time in tail-straddle walk than those treated with *D. ocoee* pheromone or saline (Tables 1 and 2, Figure 2).

Table 1. Comparisons Of Differences In Means Of Time In Tail-Straddle Walk Under Different Pheromone Treatments.

Treatment	Estimate	S.E.	z-value	p-value
Saline – *P. shermani*	5.232	1.696	3.085	0.00581
Saline – *D. ocoee*	1.375	1.638	0.839	0.67852
D. ocoee – P. shermani	-3.857	1.696	-2.274	0.05941

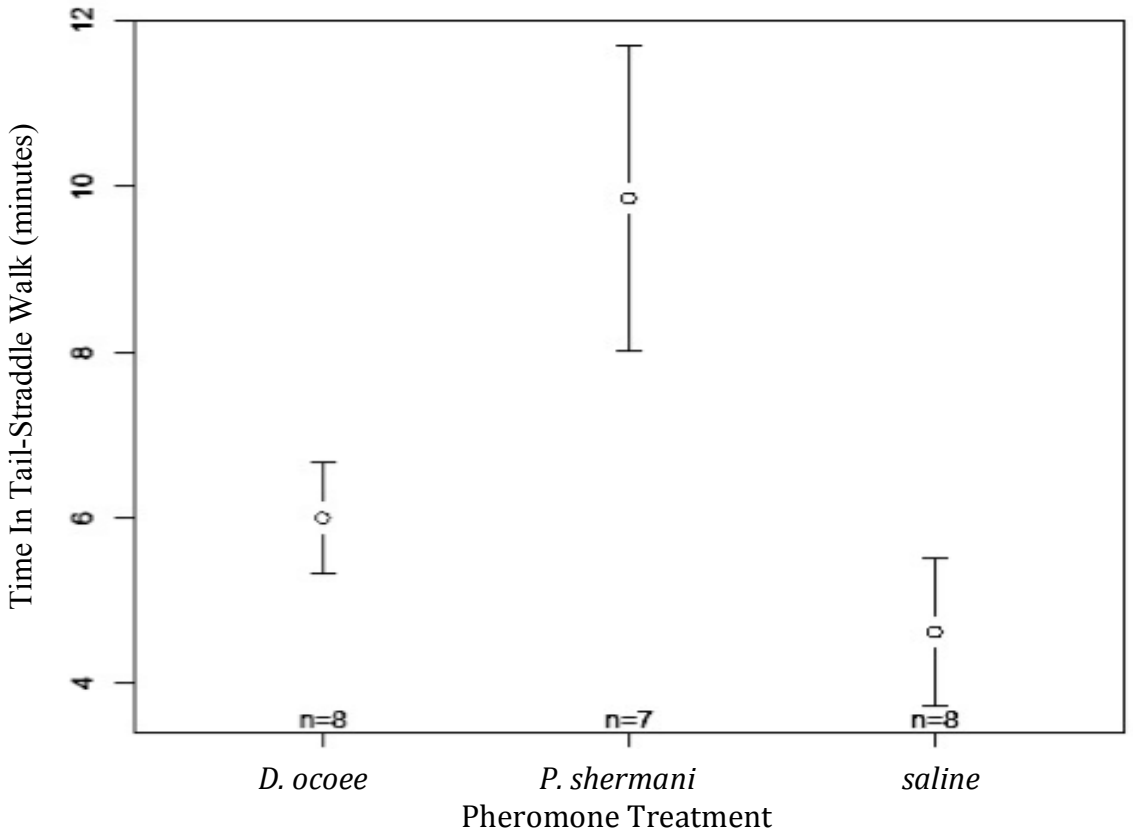

Figure 2. Means Of Time Spent In Tail-Straddle Walk For Each Pheromone Treatment.

Table 2. Summary Of Data Collected From The Experiment. Times Are In Minutes.

Treatment	Avg. time in TSW	Avg. time to TSW	Instances of TSW	Instances of SD
Saline	4.625	164.75	8	21
D. ocoee	6	186.875	8	16
P. shermani	9.857	142	7	20

There were no significant differences in the means of time to tail-straddle walk under different pheromone treatments (Tables 2 and 3).

Table 3. Comparisons Of Differences In Means Of Time To Tail-Straddle Walk Under Different Pheromone Treatments.

Treatment	Estimate	S.E.	z-value	p-value
Saline – *P. shermani*	22.75	37.77	0.572	0.835
Saline – *D. ocoee*	-22.12	38.42	-0.576	0.833
D. ocoee – P. shermani	-44.88	39.77	-1.128	0.497

There were no significant differences in the means of number of instances of spermatophore depositions. (Tables 4 and 2).

Table 4. Comparisons Of Differences In Means Of Spermatophores Deposited Under Different Pheromone Treatments.

Treatment	Estimate	S.E.	z-value	p-value
Saline – *P. shermani*	0.0167	0.0667	0.250	0.944
Saline – *D. ocoee*	0.0833	0.0667	1.249	0.425
D. ocoee – P. shermani	0.0667	0.0667	-0.999	0.577

DISCUSSION

My findings indicate that the *P. shermani* pheromone is too far removed from *D. ocoee* to elicit a response in courtship mating time that is similar to that of conspecific pheromones. Pairs spent about twice as much time in tail-straddle walk with *P. shermani* pheromone when compared to saline control, and about 1.6 times as much when compared to the *D. ocoee* pheromone treatment (Table 2).

These results help answer the original question of whether pheromones in plethodontid salamanders are used as a general mating stimulus or if they are truly specific for mating. It has been demonstrated that applying pheromones of differing congeneric species causes no significant difference in courtship time (Rollmann 2003). However, this same idea does not appear to extend to testing pheromones of members in different genera but still in the same family, as *D. ocoee* and *P. shermani* are. The time in tail-straddle walk is lengthened when using a pheromone from differing genera. The difference likely lies in the composition of the pheromones of these two species. As stated earlier, these two species do have reproductive pheromone constituents that are the same (SPF and PMF), but do differ in the amount of these two constituents present. *P. shermani* pheromone includes the addition of PRF, which is found in high concentrations, and only found within the genus *Plethodon*, not *Desmognathus*. Additionally, the PMF, which is found in much higher concentrations in *P. shermani* pheromones than *D. ocoee*, has been shown to lengthen courtship time while keeping the female interested (Kiemnec-Tyburczy et al. 2009). This is what my experiment showed, as the time in tail-straddle walk was lengthened when the *P. shermani* pheromone was used (Table 1).

Additionally, the olfactory method of delivery of pheromone evolved from the ancestral vaccination/scratching about 19 million years ago (Kiemnec-Tyburczy et al. 2009). This experiment did control for this behavioral difference since only *D. ocoee* males and females were used. Regardless of what pheromone was delivered, each male performed the same courtship ritual that is normal for the species. However, the evolution of these particular pheromones may have also been influenced by their method of delivery. *Desmognathus ocoee's* vaccination/scratching method is aimed at targeting receptors that would be found via the bloodstream after being scratched. *Plethodon shermani's* olfactory method is aimed at stimulating receptors in the vomeronasal organ found in the female's nares. Going back to the "molecular tango" idea, where both the male pheromone and the female receptor are coevolving (Palmer et al. 2005), the female selective pressure upon the male may be driving divergence in pheromones between the two species since their pheromones are probably targeting different receptors.

Some aspects of this experiment complicated interpretation. The pheromone that is applied to the female wears off and is no longer effective after 90 minutes (Dr. Lynne Houck, *personal communication*). Any data after these first 90 minutes is typically not used in data analysis. My data analysis included all data collected within the 5-hour observation time. If I had truncated my data, only 4 data points would have been used, since I only had 4 instances of tail-straddle walk occurring before the 90-minute cutoff. This would have made data analysis impractical. However, it can be argued that my data does illustrate female preference in terms of willingness to mate. There is a clear and significant result that the pheromones are different enough in *P. shermani* to extend

courting time. This could be due to a lingering effect of the pheromone on courtship and mating.

There also was not a difference in the number of instances spermatophore deposition among treatment conditions (Tables 2 and 4). The purpose of courtship pheromones in plethodontid salamanders is only to keep an already involved female interested during the actual courtship (Rollman et al. 2003). A male would deliver his pheromones to his potential mate after the female has already shown receptivity to his courtship actions (Houck & Reagan 1990, Rollmann et al. 2003). However, females are also using visual and tactile cues to identify and select mates. Given that speratmophores deposited or received over night were included in analysis, there should not be a difference in these numbers among the treatment conditions. After the pheromone wears off, a female likely mated with her partner, regardless of what pheromone condition was administered.

Mating pairs spent the least amount of time in tail-straddle walk when they received the saline control or the *D. ocoee* pheromone treatment, and the times for these two treatments were not distinguishable from one another. There should be a significant difference between the saline control and the *D. ocoee* pheromone treatment condition, with the *D. ocoee* treatment having the shortest time spent in, and to tail-straddle walk, since it is the native pheromone (Houck & Regan 1990). A possible explanation of this would be that the *D. ocoee* pheromone wore off too quickly, perhaps because I was using a malfunctioning or nonfunctional pheromone extract. Transportation of the pheromone many times could have caused the pheromone solution to become compromised, or when the pheromone extract was created, something may have gone wrong. I do not have any

factual evidence that the protein extract was nonfunctional. The proper way to test the functioning of the *D. ocoee* pheromone used in this experiment would be to use the same pheromone extract at a different time on a different group of mating *D. ocoee* salamanders. However this was not possible, as the experiment was done at the end of the mating season. Moreover, the patch delivery method might cause the pheromone to wear off quicker, since the pheromone is applied first to patch and then applied to the female. Finally, this patch may not allow for maximum absorption of the pheromone for the female, acting as a barrier in this case for pheromone absorption.

Were there other reasons why my experiment did not work ideally? One possibility was that a new lighting system was introduced in the basement where these matings took place, different from what was used in this same setting in previous mating studies. The additional light may have been too much or too bright for the mating salamanders, since these events normally happen at night under a moderate to heavy canopy.

Additionally, this is first time a patch has been used to deliver pheromone treatment in these types of mating trials. In all previous studies, a predetermined amount of pheromone solution was dripped onto the appropriate part of the female using a pipette at predetermined intervals. This extra patch that was allowed to stay on the female for up to the entire 5 hours could have played a role in her receptivity to mating and her behavior, since this patch is completely foreign to the female. As stated earlier, the patch could also have acted as a barrier to the actual absorption of the pheromone solution by the female.

The mating boxes used in this experiment were also not the mating boxes that were previously used in *D. ocoee* mating experiments. In previous studies, the smaller box (17 x 12 x 6 cm) that *D. ocoee* was housed in was the same box that is used for the mating trials (Houck & Regan 1990). I decided to use a larger box (31 x 17 x 9 cm) for mating trials since I thought with the added space, the observer would have a better chance to see the different parts of the courtship ritual more clearly. The female and male probably came into contact much less than if I had of used the smaller box for mating trials, thereby lowering the chances of courtship mating between the male and the female.

In conclusion, plethodontid salamanders are a model system for studying speciation processes and the development of mating isolation mechanisms. These salamanders are of great interest due to their use of pheromones in courtship rituals. Differences in reproductive pheromone composition, as well as differences in method of delivery of the pheromone, could potentially contribute to mating isolation among species from my experiment. The data presented by this experiment potentially shows that pheromones are at least genera specific and do not serve as a general stimulus for mating. The specificity of these pheromones affects speciation mechanisms, acting as a reproductive isolating mechanism, and therefore act as mechanisms generating and maintaining plethodontid diversity in the Southern Appalachian mountains. We better understand how these species are evolving and changing in this unique geographic region when we understand the actions of these pheromones.

LITERATURE CITED

AL-Kahtani, Saad Naser & Bienefeld, Kaspar. (2012). The nasonov gland pheromone is involved in recruiting honeybee workers for individual Larvae to be reared as queens. *Journal of Insect Behavior, 25*(4).

Beane, J. C., Braswell, A. L., Mitchell, J. C., & Palmer, W. M. (2010). *Amphibians and Reptiles of the Carolinas and Virginia* (2nd ed.). The University of North Carolina Press.

B.L. Partridge, N.R. Liley, N.E. Stacey. (1976). The role of pheromones in the sexual behaviour of the goldfish, *Animal Behaviour*, *24*(2).

Czaczkes, T. J., Grüter, C., Ellis, L., Wood, E., & Ratnieks, F. L. W. (2012). Ant foraging on complex trails: Route learning and the role of trail pheromones in *Lasius niger*. *The Journal of Experimental Biology, 216*(2).

Drickamer, L. C., Vessey, S. H., & Jakob, E. M. (2002). *Animal behavior: Mechanisms, ecology, evolution* (Fifth ed.). New York: McGraw-Hill Higher Education.

Herring, K., & Verrell, P. (1996). Sexual incompatibility and geographical variation in mate recognition systems: Tests in the salamander *Desmognathus ochrophaeus*. *Animal Behaviour, 52*(2), 279.

Houck, L.D., S.J. Arnold and R.A. Thisted. (1985). A statistical analysis of sexual selection in the plethodontid salamander *Desmognathus ochrophaeus*. *Evolution, 39*: 370-386.

Houck, L. D., & Reagan, N. L. (1990). Male courtship pheromones increase female receptivity in a plethodontid salamander. *Animal Behaviour, 39*(4), 729.

Houck, L. D., Palmer, C. A., Watts, R. A., Arnold, S. J., Feldhoff, P. W., & Feldhoff, R. C. (2007). A new vertebrate courtship pheromone, PMF, affects female receptivity in a terrestrial salamander. *Animal Behaviour, 73*(Part 2), 315-320.

Kiemnec-Tyburczy, K. M., Woodley, S. K., Feldhoff, P. W., Feldhoff, R. C., & Houck, L. D. (2011). Dermal application of courtship pheromones does not influence receptivity in female red-legged salamanders (*Plethodon shermani*). *Journal of Herpetology, 45*(2), 169.

Kiemnec-Tyburczy, K. M., Watts, R. A., Gregg, R. G., von Borstel, D., & Arnold, S. J. (2009). Evolutionary shifts in courtship pheromone composition revealed by EST analysis of plethodontid salamander mental glands. *Gene, 432*(1-2), 75-81.

Kurtovic, A., Widmer, A., & Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioral responses to a Drosophila sex pheromone. *Nature*, *446* (7135), 542-546.

Lannoo, Michael. (2005). *Amphibian Declines: The Conservation Status of United States Species* (1st ed.) University of California Press.

Niemiller, M. and Reynolds, R. G. (Ed.). (2011). *The Amphibians of Tennessee* (1st ed.) University of Tennessee Press.

Palmer, C., Watts, R., Gregg, R., McCall, M., Houck, L., Highton, R., & Arnold, S. (2005). Lineage-specific differences in evolutionary mode in a salamander courtship pheromone. *Molecular Biology and Evolution, 22* (11), 2243-2256.

Pursel, K. (2012). The effects of rhododendron on salamander communities in the Nantahala Mountains. (Masters of Science Thesis). Western Carolina University, Cullowhee, North Carolina.

Rollmann, S., Houck, L., & Feldhoff, R. (2003). Conspecific and heterospecific pheromone effects on female receptivity. *Animal Behaviour, 66*(Part 5), 857-861.

Rollmann, S. M., Houck, L. D., & Feldhoff, R. C. (1999) Proteinaceous pheromone affecting female receptivity in a terrestrial salamander. *Science, 285*(5435), 1907-1909.

Vaccaro, E. A., Feldhoff, P. W., Feldhoff, R. C., & Houck, L. D. (2009). Male courtship pheromones suppress female tendency to feed but not to flee in a plethodontid salamander. *Animal Behaviour, 78*(6), 1421-1425.

Vaccaro, E. A., Feldhoff, P. W., Feldhoff, R. C., & Houck, L. D. (2010). A pheromone mechanism for swaying female mate choice: Enhanced affinity for a sexual stimulus in a woodland salamander. *Animal Behaviour, 80*(6), 983.

Verrell, P. A. (1997) Courtship in desmognathine salamanders: The southern dusky salamander, *Desmognathus auriculatus*. *Journal of Herpetology, 31*(2), 273-277.

Vinnedge, B., & Verrell, P. (1997). Variance in male mating success and female choice for persuasive courtship displays. *Animal Behaviour, 56*(2), 443.

Watts, R., Palmer, C., Feldhoff, R., Feldhoff, P., Houck, L., Jones, A., Arnold, S. (2004). Stabilizing selection on behavior and morphology masks positive selection on the signal in a salamander pheromone signaling complex. *Molecular Biology and Evolution, 21*(6), 1032-1041.

Wyatt, T. D. (2003). *Pheromones and animal behaviour: Communication by smell and taste*. Cambridge, UK: Cambridge University Press.