Preferential Mate Choice in *Plethodon cinereus* Based on Among-Population Variation in Chemical Cues

By
Lauren M. Durant
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Allegheny College
Department of Biology

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements
for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

_____
Pledge

Approval by the Senior Thesis Committee:

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Dr. Mumme

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Dr. Venesky

Abstract

Vertebrates utilize chemical cues during their daily social functions, and chemical cues are believed to play a large role in choosing a mate. In salamanders, males are primarily attracted to the scents of reproductively mature females over the scents of immature females and as well as other males. The eastern red-backed salamander (*Plethodon cinereus*) has two organs that are used to identify these reproductive chemicals. Because pheromones can differ in populations from different areas, I tested the hypothesis that the salamander *Plethodon cinereus* will show a preference for the chemical cues of potential mates from their local population rather than cues of potential mates collected from more distant populations. The local females were collected from Robertson Field woods. Other female salamanders were collected from Bousson located 11.3 km from the local population, State Game Lands 47 located 51.1 km from the local population, and State Game Lands 29 located 74.3 km from Robertson. I utilized a plastic container as a testing chamber. The chamber had each side lined with the paper towels of females from two different areas. To determine the preference of the salamander, I recorded what side of the container the salamander occupied every two minutes for a full hour as well as the number of nose taps, which function in detecting chemical signals or substrates, for each side of the chamber. Males showed a significant preference for the local females over females from 51.1 km and 74.3 km away in terms of both time and nose taps, but did not significantly prefer local females over those from 11.3 km away. Although this study suggests males prefer local females to distant ones, more research needs to be done to understand this topic.

Introduction

Chemical cues are important in the daily social functioning of many vertebrates. Responses to chemical cues have been studied in fish, amphibians, reptiles, and mammals. A chemical cue can be studied on a behavioral level as well as a physiological level (Park et al., 2004). Amphibians use chemical cues in predator avoidance, individual recognition, mate recognition, mate choice decisions, and choosing environmental conditions (Caspers and Steinfartz, 2011).

These key chemical cues have not been studied thoroughly when it comes to male sexual selection. Although chemical cues have been considered to be an important component of mate choice, there have been relatively few tests regarding chemically signaled mate choice (Marco et al., 1998). Reproductive chemical cues are primarily used to identify sex, species, and reproductive condition (Woodley 2010). In mammals, males will almost always associate with areas containing chemical cues from ovulating females over areas with chemical cues of non-ovulating females (Dantzer and Jaeger, 2007). Studies done on mice and frogs have shown that male chemical signals can sometimes activate reproductive behaviors from a female (Vaccaro et al., 2010).

Amphibian chemical cues can be studied in simple or very complex ways, which make them a great model to use. Many amphibian chemical signals have a close link to the genome because they are generally proteins (Woodley 2015). Chemical cues can be distributed several ways, one being through fecal pellets, which can provide dietary and reproductive cues (Reiter et al. 2014).

In salamanders, reproductive chemical communication is important to ensure the smooth transition of a spermatophore to a female during courtship (Woodley 2010). While long-range chemical cues given off provide information on location, sex, and reproductive condition. Males are primarily attracted to the scents of reproductively mature females over the scents of immature females and other males. Females show less attraction to male scent and show small preference when choosing between male and female scents (Woodley 2015).

Commonly known as the red-backed salamander, *Plethodon cinereus* is a terrestrial salamander species that gives off chemical cues and is most commonly found in eastern North America. The body of the salamander is a dark gray color with a distinguishing band that is a reddish-brown color. The average length of this type of salamander is around 8 cm. It can be found underneath stones and logs in both moist and dry areas (Cochran 1911). Red-backed salamanders are very abundant within Pennsylvania, because of the conditions found here. Red-backed salamanders prefer to eat things that move so they usually go after insects like flies and even an occasional worm (Cochran 1911). Red-backed salamanders begin to mate in autumn and usually lay their eggs in July (Cochran 1911).

Red-backed salamanders have two organs that are used to identify chemicals, the vomeronasal organ and the main olfactory epithelium. The vomeronasal organ is stimulated by "nose-tapping," which allows chemicals to move up nasal grooves into the organ. The main olfactory epithelium detects chemicals that are present through an increase in airflow (Dantzer and Jaeger 2007). *Plethodon cinereus* use chemical cues to mark their territory and for mating purposes (Mathis 1990).

Plethodon cinereus are a very territorial species. Both sexes defend territories on the forest floor under covered items. Their chemical cues and fecal pellets give off territorial information to other organisms (Mathis 1990). A salamander's territory is thought to influence diet, which means a higher-quality diet comes from a higher-quality territory (Reiter et al. 2014). Large body size is important when trying to obtain a territory (Mathis 1990). Female red-backed salamanders are generally going to favor a male with a better territory and diet (Mathis 1990).

In western red-backed salamanders (*Plethodon vehiculum*) both sexes prefer larger mates. Female western red-backed salamanders have shown a preference toward bigger males based on chemical cues alone (Marco et al. 1998). This relates back to the concept that bigger males have better territory. Eddy et al. (2016) showed that males spent more time trying to acquire a mate that was larger and had a greater reproductive value. Along with size, males also favor a female that is more gravid. Male red-backed salamanders are able to distinguish between gravid and non-gravid females through the females' chemical cues (Danzer and Jaeger 2007). These chemical cues have also been shown to vary between salamander populations.

Rollmann et al. (2000) examined plethodontid receptivity factor (PRF), one of the salamander courtship pheromones. The study looked at *Plethodon jordani* populations that have been geographically isolated from each other. Researchers took populations from two northern locations (Max Patch and Mt Mitchell) and two southwest populations (Cheoah and Wayah). The Max Patch sample was most similar to the Mt Mitchell sample and the Choah sample was most similar to the

Wayah sample. But the northern two sample populations RPF samples are not similar chemically to the southwestern RPF samples much at all. The analysis of the pheromone showed that each population had an individualized pheromone from the others.

As the distance between populations of a species increases, the total genetic variation also increases in a concept known as isolation-by-distance (Futuyma and Kirkpatrick 2018). One of the major factors of fragmentation is urban development (Nöel et al. 2007). Noel et al. (2007) studied four populations of eastern red-backed salamanders that were isolated by things like roads, graveyards, and buildings and compared them to four populations located in continuous habitats. The results showed that allelic richness and heterozygosity were lower in the urban populations. The isolated populations have a greater genetic variation between populations creating a more pronounced difference other than just spatial separation.

Storfer and Sih (1998) examined a streamside salamander (*Ambystoma barbouri*) whose larvae may need to survive predation from fish. Populations containing fish that were more isolated from fishless populations showed stronger antipredator responses and survived longer in a predation experiment than fishless populations. This suggests that the gene flow between populations of salamander limited local adaptation in some salamander populations (Storfer and Sih 1998). Different selecting pressures depending on spatial location may require a more distinct behavior or genetic component. This gives reason to believe that

salamanders should show a preference for a pheromone from their own area instead of another location.

To better understand the concept, more research on whether or not eastern red-backed salamanders have a preference for or against salamanders from their own area is required. This study aims to evaluate whether males from a local area favor females from their local area or others collected from distances of 11.3 km, 51.1 km, and 74.3 km away. I hypothesized that males will prefer local females to distant females and that their preference will become stronger as the distance between them increases. This provides insight as to whether male eastern red-backed salamanders prefer females from their own isolated area more than others.

Methods

I planned on collecting both male and female *Plethodon cinereus* in the early autumn; due to a lack of rain it was necessary to wait until the later fall months to do the collecting. I collected "local" salamanders from Robertson Field woods in Meadville located in Crawford County Pennsylvania on October 28, 2017. The other salamanders were collected from Bousson Environmental Research Reserve, State Game Lands 47, and State Game Lands 29. Their respective distances from my local Robertson collection site are 11.3 km, 51.1 km, and 74.3 km. I collected the salamanders from Bousson located in Crawford County Pennsylvania on October 29, 2017. The salamanders from both State Game Lands 47 located in Venango County Pennsylvania and State Game Lands 29 located in Warren County Pennsylvania were collected on November 4, 2017.

I housed the salamanders in individual labeled plastic containers with damp paper towels in the bottom. I changed the salamanders' paper towels and fed them once a week. I fed each salamander 6 wingless fruit flies per week. I allowed the salamanders to have a three-week adjustment period on the fruit fly diet, before I began testing. Throughout the week I would also check on them to make sure their paper towels stayed moist.

For the setup of the experiment I mirrored the study done by Marco et al. (1998). I conducted 4 experimental treatments of the experiment during the natural mating period of the salamander as in Marco et al. (1998). The paper towels at the bottom of the salamanders' storage containers were used as my source of a chemical cue. Local Robertson males were given the choice between the paper towels of local

females versus the paper towels of a distant female from Bousson or either of the state game lands. Each salamander was not fed for 6 days leading up to the experiment so there was no food debris on the paper towel. For the experiment, I used a plastic circular container as a testing chamber.

In the testing chamber each side was lined with the paper towels of the desired salamander. Actual placement of the paper towels with the females' chemical cues was determined randomly by a coin flip. I decided to test male response to female chemical cues because based on previous studies. To avoid cross contamination, paper towels were placed at least 2 cm apart. The paper towels were also both sprayed with water to avoid any moisture differences in the towels. The four experimental treatments are shown graphically in Figure 1.

To determine the preference of the salamander, I utilized its location within the testing chamber and nose taps. I recorded what side of the container the salamander occupied every two minutes for a full hour. If the salamander was located in the middle of the cage I used its snout position to assign the location. The testing chambers were rotated 180 degrees every 15 minutes to avoid any bias from orientation (Marco et al. 1998). I also recorded the number of the salamander's nose taps for each side of the testing chamber.

For the analysis of my data I utilized JMP software and a one-sample t test to determine if the male preference for local females was significantly greater than the chance expectation of 50%. This was done to both the data from side preference and nose tap data.

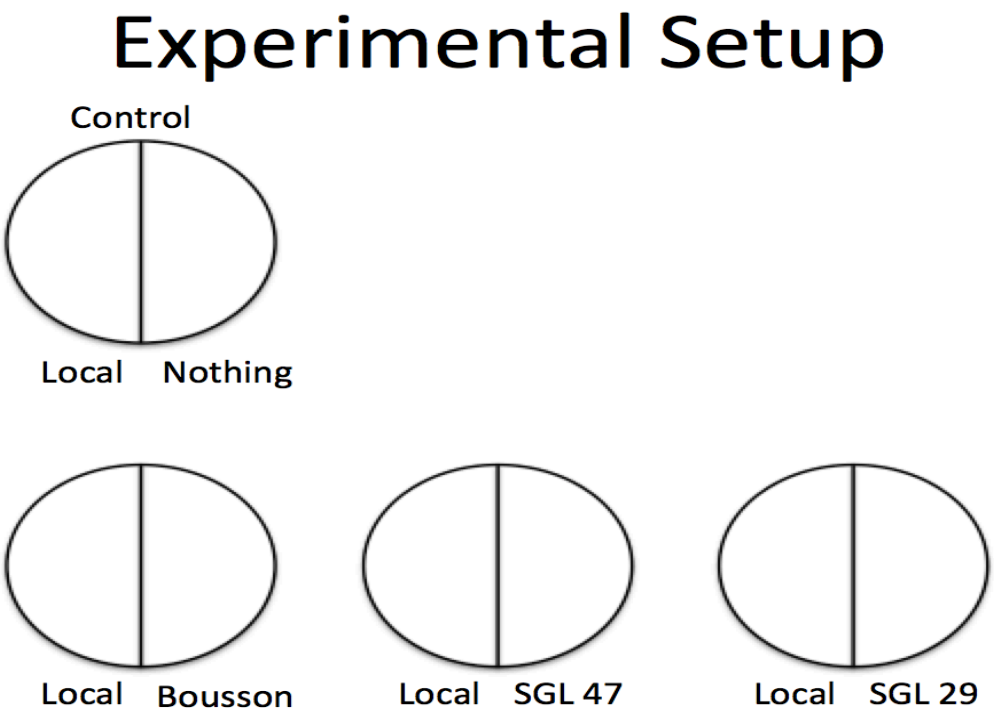


Figure 1. A visual representation of the set-up of the experiment. Each circle represents a confined circular plastic container.

Results

Location Preference

Males showed no significant preference for local females in both the Local-Bousson and Local-Control treatments (Figure 2; p=0.71 and p=0.50, respectively). However, males spent significantly more time on the paper towels of local females in both the Local-State Game Lands 47 treatment (p=0.014) and the Local-State Game Lands 29 treatment (p=0.002; Figure 2).

Nose-Tap Preference

Males showed no significant preference for local females in only the Local-Bousson treatment (p=0.14; Figure 2). Males gave significantly more nose-taps to the paper towels of local females in the Local-State Game Lands 47 treatment (p=0.003), the Local-State Game Lands 29 treatment (p=0.007), and the control treatment (p=0.001; Figure 2).

Individual Variation

The male salamanders varied in the amount of local nose taps they provided in total for the experiment (Figure 3). Some salamanders showed a lot of variation between the four trials, like salamander 7. Others, like salamander 4, were relatively consistent in the amount of taps they gave per trial (Figure 4).

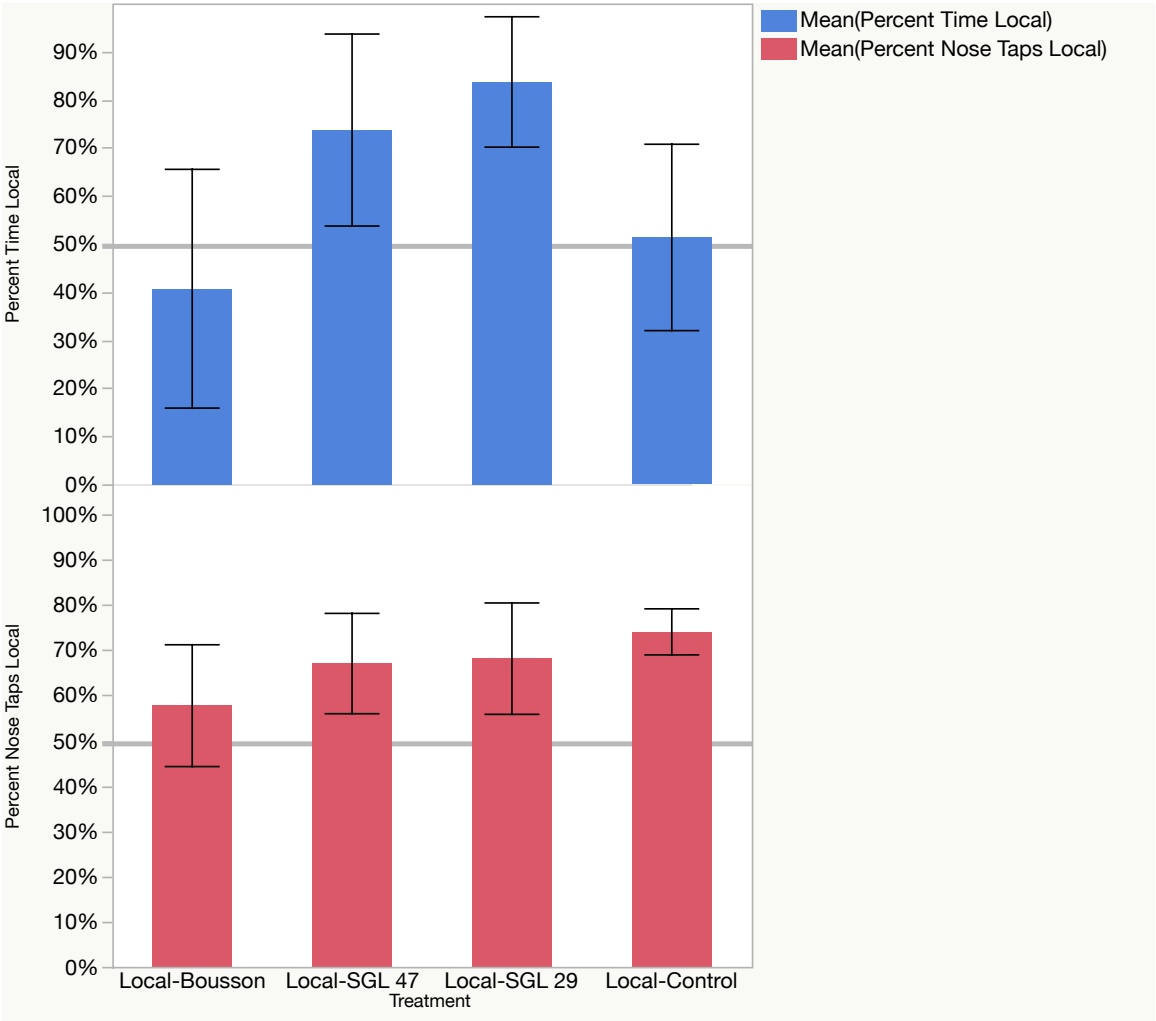


Figure 2. The mean percent nose taps local and the mean percent time local versus each treatment. Error bars represent the 95% confidence interval; treatments where the error bars do not encompass the 50% reflect a significant male preference for local females.

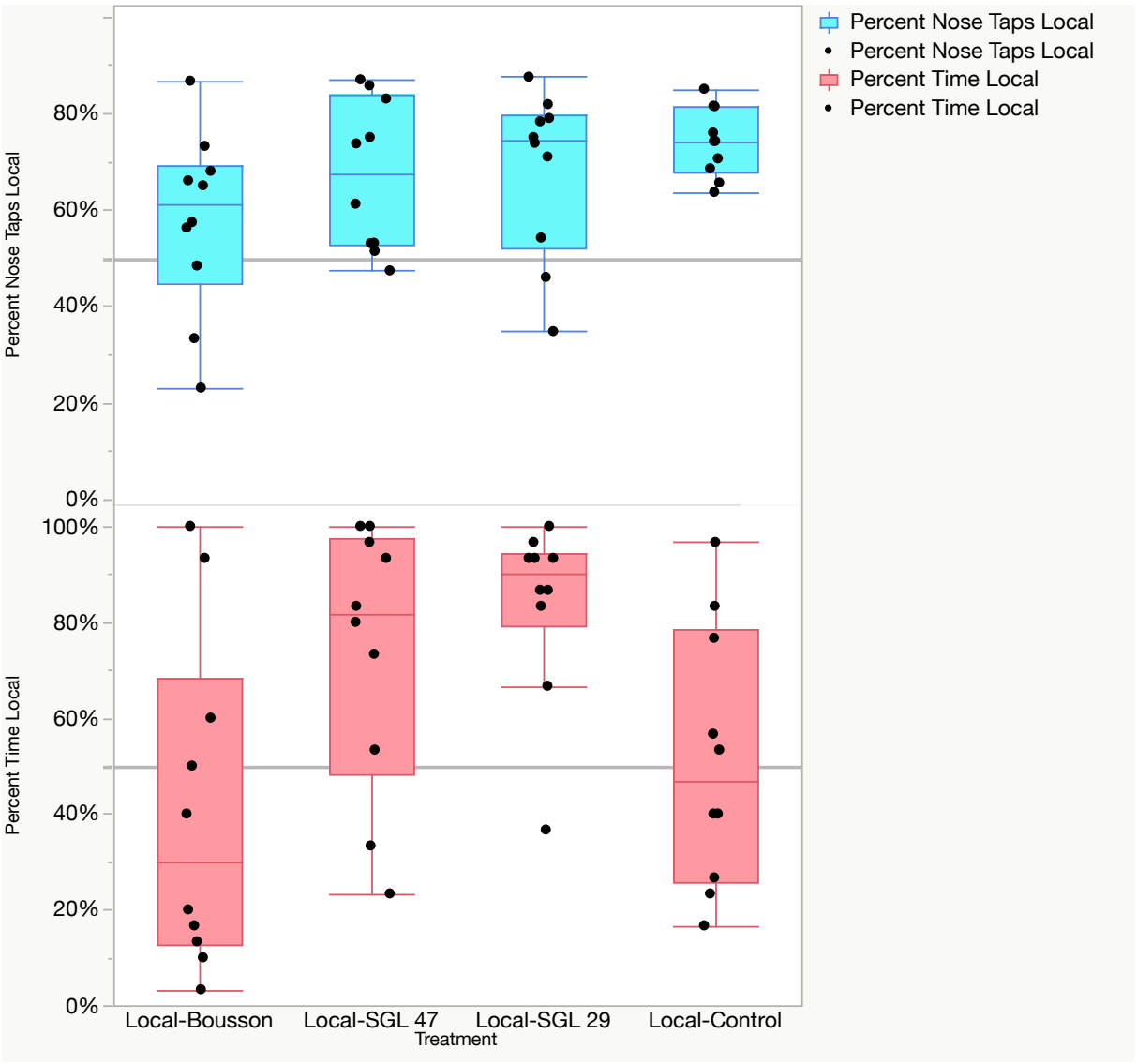


Figure 3. The percent nose taps local and the percent time local versus each treatment. Points above 50% indicate a preference for local females.

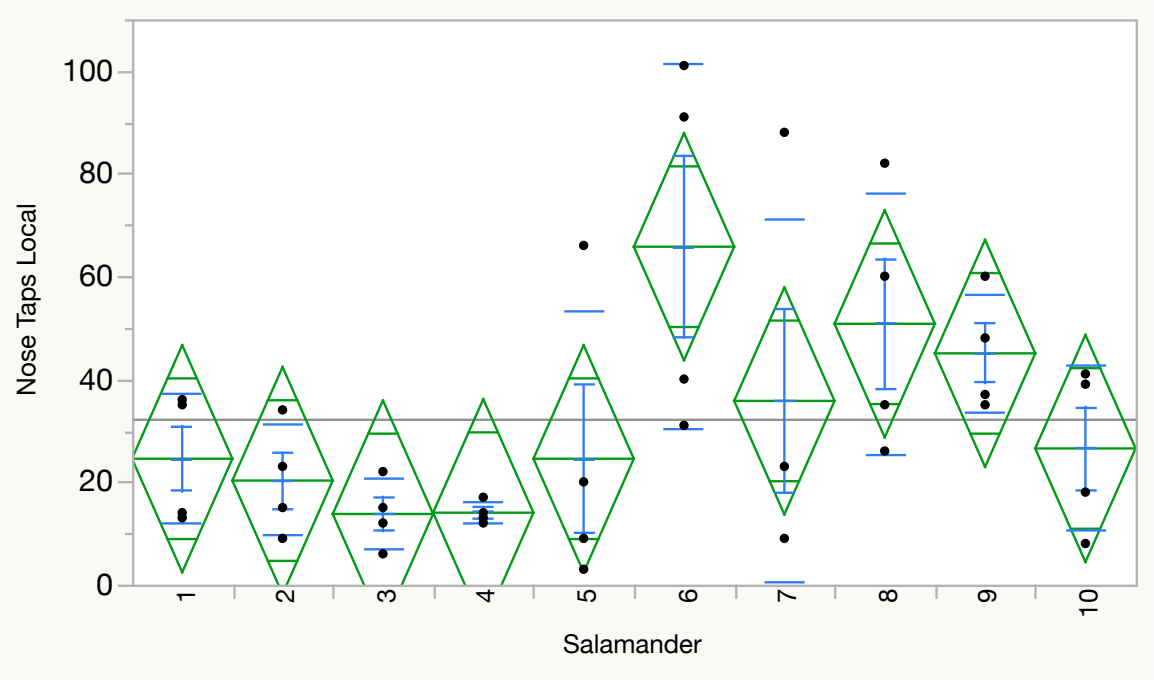


Figure 4. Local nose taps for each salamander, shows visible variation between and within salamanders.

Discussion

The objective of my research was to determine if red-backed salamanders show a preference for the chemical cues of potential mates from local salamander populations relative to those produced by salamanders from more distant populations. This would help determine the extent of phylogenetic difference in mate-attraction signals over set distances of salamanders. My hypothesis was that the salamander *Plethodon cinereus* would show a preference to the chemical cues of potential mates from a local salamander population rather than cues of potential mates from more distant populations.

Rollmann et al. (2000) examined salamander populations that have been geographically isolated from each other. Salamanders from the four different locations were found to have significantly different chemical make-ups of a pheromone PRF (Rollmann et al. 2000). Although my experimental choices are different I still think this study is useful due to the same genus and my separation of populations by distance. Nöel et al. (2007) studied eastern red-backed salamanders from isolated urban areas and areas in continuous habitats. Allelic richness and heterozygosity were found to be lower in the urban populations. This led me to believe that the salamanders would show a significant preference to salamanders from their own specific location due to the population differentiation and phylogenetic differences.

The results, for the most part, supported the hypothesis. Local males from Robertson spent significantly more time on the side of local females when presented with chemical cues from females of the farthest locations (Figure 2). Along with that,

they also did not show a significant preference for local females in comparison to Bousson females, which was the closest alternative distance. The one questionable result was the control trial. Males only spent around half of their time on the local female side of the testing chamber when presented with a moist paper towel on the other side containing no chemical cue. It was predicted that they would spend a majority of time on the local side with the only chemical cues provided on that trial. These results may have been obtained because on days the salamanders were fed they also got their paper towels changed. So, the salamanders may associate that fresh and clean paper towel with getting fed and that may be why they spent so much time on that side.

All of the treatments, except for Local-Bousson, showed a significant preference for nose tapping on the local female's side of the testing chamber. This could indicate a strong preference for the chemical cues of local Robertson females. Because the local females and Bousson females are close in distance, it is plausible that their chemical cues do not have very different makeups and that could cause Robertson males to show less of a preference in that condition.

In order to get more conclusive results, this experiment should be repeated again. More salamanders and more trials could help to support or refute the results found here. A more widespread collection distance would also be beneficial. Due to the nature of the experiment obtaining samples from a very large distance was not possible. Along with the extended range, more intermediate sample distances would also be a beneficial addition because then the salamanders' preferences could become more visible between more specific distances.

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