

2023

**MATE RECOGNITION IN GENETICALLY DISTINCT POPULATIONS
OF THE EASTERN RED-BACKED SALAMANDER (PLETHODON
CINEREUS)**

Courtney Thomas

Follow this and additional works at: <https://collected.jcu.edu/masterstheses>



Part of the [Biology Commons](#)

MATE RECOGNITION IN GENETICALLY DISTINCT POPULATIONS OF THE EASTERN
RED-BACKED SALAMANDER (*PLETHODON CINEREUS*)

A Thesis Submitted to the
Graduate School of
John Carroll University
In Partial Fulfillment of the Requirements
For the Degree of
Master of Science

By
Courtney A. Thomas
2023

Table of Contents

Abstract.....4

Introduction.....5

Methods.....11

 Site Selection.....11

 Salamander housing and experimental set up12

 Experiment 1: Female behavioral response to male chemical cues.....13

 Experiment 2: Female association with male chemical cues.....15

 Statistical Design.....16

Results.....17

Discussion.....18

Acknowledgements.....27

Literature Cited.....28

Tables and Figures.....43

 Table 1.....43

Table 2.....	44
Table 3.....	45
Table 4.....	46
Table 5.....	47
Figure Legends.....	48
Figure 1.....	49
Figure 2.....	50
Figure 3.....	51

Mate recognition in genetically distinct populations of the Eastern Red-backed Salamander
(*Plethodon cinereus*)

ABSTRACT

Lineages that have undergone genetic divergence and subsequently experience secondary contact present fascinating situations related to the potential for reproductive isolation. Within species hybrid zones provide unique opportunities to test the strength of reproductive barriers through behavioral experiments when genetic relatedness between groups is established. Among the complex interactions that operate to maintain species boundaries, mate recognition and mate choice are two behaviors that serve to promote evolutionary independence across diverse taxonomic lineages. I explored mate recognition in genetically distinct populations of the Eastern Red-backed Salamander, *Plethodon cinereus*, a small, abundant, terrestrial salamander with a widespread distribution throughout northeastern United States and in southeastern Canada. Earlier studies identified distinct mitochondrial and nuclear clades in northern Ohio, despite some populations of different clades existing in close geographic proximity. To investigate whether female *P. cinereus* showed a preference for male odors based on clade membership, individuals of two clades were collected and mate recognition trials were performed in the laboratory. One experiment tested the level of interest of females of both clades by quantifying the number of nose taps directed at male odors and a second experiment assessed association by females with male odor via time in proximity to odors. Females did not discriminate between male odors of either cluster based on number of nose taps and the results of the association experiment yielded mixed results. The results of these behavioral experiments corroborate recent studies suggesting that the sex-specific pheromones of *P. cinereus* in the two clades studied are not yet different enough to result in reproductive isolation between the two genetic lineages.

Other explanations for female response to male odors include avoidance behavior and asymmetric mate recognition, among other possibilities. I compare my results with similar studies on mate recognition and discuss future research directions concerning this ecologically valuable salamander species.

INTRODUCTION

Recognizing mechanisms that drive the origin of novel species represents a central goal of evolutionary biology. The variety of ways in which divergence arises in populations through natural selection is reflected by the diversity of processes through which populations can become reproductively isolated. Niche specialization, some forms of sexual selection, and the ecological and behavioral interactions of populations can drive divergent selection and promote ecological speciation (West-Eberhard, 1983; Rundle and Nosil, 2005). Such forces may act in tandem rather than as solitary agents of selection, as specific environments present unique conditions to which species respond. When organisms living in different environments experience divergent selective regimes, reproductive isolation (RI) and ecological speciation may occur (Schluter, 2000) and can do so in populations that are allopatric or sympatric, resulting from either prezygotic or postzygotic barriers, or a combination of each (Turelli et al., 2001). Reproductive isolation can arise from the evolution of varying morphological characters that are advantageous in different environments, along with the accumulation of genetic differences that influence physiological and ecological traits between populations (Dobzhansky, 1946; Mayr, 1954). Reproductive barriers and gaps in geographic distribution between species may also lead to differences in behavior, which can reveal important information used to identify species boundaries (Hillis, 2019). Among the array of observed behavioral phenomena in animals, mate recognition and

mate choice are two that serve to maintain species boundaries, and ultimately biodiversity (Bickford et al., 2007; Maan and Seehausen, 2011; Servedio and Boughman, 2017).

Mate recognition is defined as the process of detecting, assessing, and deciding whether to accept or reject a potential mate (Pfennig, 1998). The ability of an individual to identify a genetically compatible mate has obvious implications on the success of reproduction. Mate choice may also impart direct and indirect fitness benefits on offspring or the selected individual (Deutsch and Reynolds, 1973). For example, male Gray Tree Frogs (*Hyla versicolor*) that advertise long calls produce offspring with increased fitness during larval stages compared to offspring of males that advertise short calls, suggesting a mechanism for an honest indicator of genetic quality (Welch et al., 1998). Similarly, sexually dimorphic female Mottled Sculpin (*Cottus bairdi*) preferentially mate with large males, which results in increased reproductive success because egg mortality is lower at egg deposition sites occupied by larger males than at those occupied by smaller males (Downhower et al., 1983). Mate recognition and mate choice vary greatly across taxa and may involve a suite of strategic choices during the phases of gathering and processing information (Real, 1990). In natural populations, individuals often prefer certain trait values over others when selecting their mates including vocal signals, behavioral repertoires, morphological compatibility, and pheromones (Schwander et al., 2013). Taxonomic lineages that have undergone extensive adaptive radiation such as salamanders within Plethodontidae are ideal systems to study patterns of ecological divergence because of the inherent diversity they represent, including traits associated with mate recognition and mate choice.

The Eastern Red-backed Salamander, *Plethodon cinereus*, (Green, 1818) is a small, fully terrestrial salamander that inhabits temperate forests throughout New England, southward to

western and northeastern North Carolina, and northwestward to western Minnesota (Petranka, 1998). Despite its broad geographic distribution, genetic analyses and mark-recapture studies suggest that dispersal is low, especially in areas where abundance is high (Mathis, 1991; Gillette, 2003; Cabe et al., 2007). Indeed, prior work on *P. cinereus* has shown that geographical features that may appear minor, such as first order streams and forest roads, can limit movement and potentially restrict gene flow (Marsh et al., 2005, 2007). Individuals do not undergo migrations (but see Woolbright et al., 2014); they occupy small home ranges in which they exhibit site fidelity and territoriality, a characteristic likely related to their low rates of dispersal (Kleeberger and Werner, 1982; Jaeger, 1981; Gergits and Jaeger, 1990). In Ohio, reproduction is biennial (Anthony and Pfungsten, 2013), and females rely on moist microhabitats either underground or below cover objects to deposit eggs (Jaeger, 1971). Lungless salamanders like *P. cinereus* are thought to be important indicators of ecosystem integrity due to their unique restrictions concerning leaf litter, soil moisture, and temperature (Welsh and Droege, 2001) particularly with regard to fine-scale, soil-level processes. Careful experiments have revealed that this species plays an important role in trophic systems by exerting strong regulation over guild members as well as top-down, predator-mediated regulation of ecosystem functions (Hickerson et al., 2012; Hickerson et al., 2017; Walker et al., 2018). Empirical evidence for intra- and interspecific competition has been shown in several field and laboratory studies (Jaeger, 1984; Mathis, 1990a; reviewed in Jaeger et al., 2016). Additionally, *P. cinereus* exhibits extensive phenotypic variation in the form of color polymorphism, with two genetically based color morphs that occur most commonly throughout its widespread geographic range: striped and unstriped (Moore and Ouellet, 2014). The striped morph is characterized by the presence of a red stripe on the dorsum, whereas this feature is absent in the unstriped morph. The composition and frequency of this

polymorphism varies throughout the range of this species (Anthony and Pfingsten, 2013) and may be influenced by geographical features such as waterways (Highton, 1999; Hantak et al., 2019). In a population occurring in Cuyahoga Valley National Park, assortative pairing based on color morph was observed in the field (Anthony et al., 2008; Acord et al., 2013), and females were more likely to be associated with striped males compared to unstriped males in the laboratory (Acord et al., 2013). These attributes combined with the wealth of research on the ecology and behavior of *P. cinereus* make this species of particular interest when addressing questions related to the potential of ecological divergence and associated behaviors such as mate recognition and mate choice.

Most plethodontid salamanders rely on olfaction to navigate and interpret the environment, including the recognition and selection of potential mates (Jaeger and Gergits, 1979; Uzendoski and Verrell, 1993; reviewed in Houck and Verrell, 1993). Behavioral responses of plethodontid salamanders to pheromonal signals presented from conspecific members of the opposite sex have been well documented in the laboratory (Ovaska, 1989; Walls et al., 1989; Karuzas et al., 2004) and are often accompanied by the investigation of chemosensory cues. Male and female *P. cinereus* produce pheromones that convey a repertoire of chemical signals involved in social communication (Jaeger and Wise, 1991; Jaeger and Forester, 1993). Like other plethodontid salamanders, *P. cinereus* assess chemosensory cues through a behavior termed nose tapping (NT), in which the animal physically taps the tip of the upper lip to a substrate to identify non-volatile markings (Brown, 1968; reviewed in Mathis et al., 1995). Nose tapping is a discrete behavior directly associated with chemosensory investigation by the animal, is easily recorded by observation, and can be quantified in the field or lab (Schubert et al., 2008; Jaworski et al., 2018). Chemosensory cues are conveyed from the substrate by way of NT into the vomeronasal

organ via the nasolabial groove, which runs from the tip of the upper lip into the nasal cavity (Dawley and Bass, 1988, 1989). Chemical signals are then interpreted by the animal, which can contain important information involved in a variety of social interactions (reviewed in Jaeger and Forester, 1993). In the laboratory, plethodontid salamanders have been shown to recognize individual (Dawley, 1984; Kohn and Jaeger, 2009), conspecific (McGavin, 1978), and sex of the signal-sender (Jaeger and Gergits, 1979), as well as the presence of an intruder in their territory (Martin et al., 2005) by substrate cues alone, representing a key requirement of complex mating systems (Gillette et al., 2000). Additionally, Mathis (1990b) showed that red-backed salamanders can communicate information about the relative size of conspecifics based on chemosensory cues and Jaeger (1981) showed that such cues can function in ‘dear enemy’ recognition in the absence of visual or physical contact via NT. When presented with fecal pellets derived from males fed a high-quality diet (termites) compared to males fed a low-quality diet (ants), gravid females spent more time visually inspecting and associating with male fecal pellets of the high-quality diet (Walls et al., 1989). Their observations suggest the potential of an honest signal obtained via olfactory sampling, which may increase female fitness by selecting males whose diets and territory quality are reliably conveyed by fecal pellets. Thus, nose-tap frequency can be used as an indicator of a salamander's level of interest in a substrate and time spent preferentially associating with substrates marked by specific individuals can inform hypotheses regarding mate recognition and preference (Tristram, 1977; Dawley, 1984; Herring and Verrell, 1996; Schubert et al., 2008; Evans et al., 2010).

Integrating biogeography and behavioral ecology is a valuable approach that may elucidate how historical events have shaped current species distributions. Glaciation has dramatically influenced biogeographic patterns in *P. cinereus*: more than 75% of its current

geographic range consists of regions that until recently, were glaciated and uninhabitable (Highton and Webster, 1976). In contrast to most other members of *Plethodon*, this species has successfully reinvaded previously glaciated areas of eastern North America since the last glacial maximum (LGM) which occurred approximately 22,000-18,000 years ago (Dyke, 2004). Radomski et al. (2020) performed a phylogeographic investigation using mitochondrial loci and identified several distinct genetic clades in *P. cinereus*. In addition, they found that three of the clades (Ohio, Pennsylvania and Northern clades) had colonized regions north of the glacial boundary after the LGM, with their molecular clock placing the ancestor of *P. cinereus* appx 1.5 myr ago within the Appalachian physiographic provinces of North America (Fig. 1). Hantak et al. (2019) used microsatellite markers to examine landscape genetics in *P. cinereus* and identified three distinct genetic clusters (Eastern, Central, and Western) in northern Ohio (Fig. 2) populations. Whereas microsatellite and mtDNA data disagree on the exact location of contact zones among clades, this information presents a unique opportunity to study differences between known genetic populations across a potential contact zone.

I explored the possibility of reproductive isolation via divergence in mate recognition between two genetically distinct populations of *P. cinereus* using a behavioral approach. To test the hypothesis that females show an increased interest in males from the same genetic cluster compared to males from a different genetic cluster, I conducted a laboratory study to observe female behavioral response to male chemical cues. Because these populations appear to have not been in contact since before the LGM (Radomski et al., 2020), I hypothesized that females would show stronger recognition toward males of the same genetic cluster compared to males of a different genetic cluster when presented with male chemical cues. Understanding mate recognition as a mechanism that may drive reproductive isolation among genetically distinct

populations is an important first step in understanding the potential for divergence within this species.

METHODS

Site selection

Salamanders were collected in April 2019 from four distinct populations in northern Ohio: two populations from the Central Cluster including Rocky River (41.42073, -81.85917) and West Creek (41.39006, -81.69121) reservations, and two from the Eastern Cluster including Tinker's Creek (41.37557, -81.57355) reservation and Doan Brook watershed (41.49361, -81.593533; Fig. 1; Table 1). These sites correspond to known genetic clusters identified by Hantak et al. (2019) and were selected due to their relatively large F_{ST} values (ranging from F_{ST} (0.29-0.37) between Eastern and Central study populations). Variation in the ecology and behavior between striped and unstriped color morphs of *P. cinereus* has been shown (Anthony et al., 2008; Anthony et al., 2017; Reiter et al., 2014) including weak assortative mating based on color morph (Acord et al., 2013), so to reduce differences resulting from differences in morph frequency among populations, only sites exhibiting proportions between 0.6-0.8 striped individuals were visited, and no unstriped individuals were collected. Some variation in habitat quality was observed among sites but each consisted of at least secondary deciduous forest with canopies dominated by beech (*Fagus grandifolia*) and other mixed deciduous species (*Quercus* spp.; *Acer* spp. and *Carya* spp.). To mitigate the impact of geographic variability among salamander populations, the distance between the most western site in the Central Cluster and the most eastern site in the Eastern Cluster was minimized (approximately 25 km). Within the two genetic clusters, 20 males and 20 females were collected by hand from each of the four sites within the two genetic clusters, for a combined total of 160 individuals. Female *P. cinereus* in

Ohio are considered adults when SVL >34 mm, and males when SVL >32 mm (Anthony and Pfingsten, 2013); only adults were collected for experiments. In the field, male and female salamanders were distinguished primarily based on snout morphology: in spring when in reproductive condition, adult males display a large snout relative to females, which exhibit a blunt shape compared to a male (Anthony et al., 2008). The presence of cirri in reproductively active males also serves as an important sexually dimorphic feature. The largest average home range reported for this species is 5m^2 (Kleeberger and Werner, 1982) and familiarity has been shown to influence interactions between salamanders with regard to courting behavior (Guffey et al., 1998). For instance, male and female *P. cinereus* can remember their natural partners after a five-day separation period in the lab (Jaeger et al., 2002); therefore, to avoid collecting potential pairs, individuals encountered within 5m^2 were not collected. Salamanders were carefully transported in clean, individually labeled Falcon 50mL conical centrifuge tubes containing dampened paper towel substrate to John Carroll University, University Heights, OH, USA.

Salamander housing and experimental set up

All salamanders were housed in individual glass 474 mL Pyrex containers containing leaf litter collected in the field that was regularly moistened until experiments began. This species has been shown to detect differences in diet quality through chemical cues alone (Walls et al., 1989). All individuals were placed on a strict feeding schedule upon housing of approximately 25-30 *Drosophila melanogaster* once per week to standardize the non-pheromonal composition of their fecal pellets, which was maintained for the duration of the experiment. Laboratory conditions were kept at a temperature of $15 \pm 2\text{ }^\circ\text{C}$ under a natural light-dark cycle. One week prior to experiments trials, salamanders were transferred from their Pyrex dishes into clean, individual 15 x 1.5 cm polystyrene petri dishes containing filter paper substrate regularly moistened with

natural spring water. Salamanders were allowed to establish a territory in their petri dishes for six days prior to each trial; a period of five days in the laboratory is sufficient for individuals to establish territorial ownership via substrate marking (Jaeger, 1981; Nunes and Jaeger, 1989). Animals were size matched to SVL within ± 2 mm to remove any potentially confounding effects of size because this species can recognize relative size of conspecifics derived from chemical cues (Mathis, 1990b). Finally, because *P. cinereus* is most active at night (Heatwole, 1962) all trials were conducted under red light during the dark photoperiod in the same room in which the animals were housed. Between experiments 1 and 2, the salamander care schedule as earlier stated was maintained.

Experiment 1: Female behavioral response to male chemical cues

This experiment tested the hypothesis that females would show increased investigation of male chemical cues in their own genetic cluster compared to those of males in the adjacent genetic cluster. Experimental trials were conducted over the course of nine weeks (22 May - 16 July 2019) between 20:00 and 04:00 (320 ten-minute trials). Salamanders were housed in the lab for approximately six weeks to acclimate to laboratory conditions until behavioral trials began. Each female (Central Cluster: n=41; Eastern Cluster: n=40) was exposed to four different treatments: exposure to 1) a male donor chemical cue from a member of the same genetic cluster (i.e. female from Central Cluster exposed to male donor chemical cue from Central Cluster); 2) a male donor chemical cue from a member of a different genetic cluster (i.e. female from Central Cluster exposed to male donor chemical cue from Eastern Cluster); 3) the female's own chemical cue; and 4) a control which was a blank, moistened filter paper. Individual female salamanders only experienced each treatment once (four treatments total) and underwent only one trial per week (four trials total). The order in which females were exposed to treatments was randomized such

that treatments were interspersed among all replicates (Hurlbert, 1984) and to reduce observer-expectancy bias, all individuals were assigned random alphanumeric codes such that the observer was blind to clade membership and treatment (Balph and Balph, 1983; Kardish et al., 2015). Using clean gloves, male salamanders were carefully removed from their territorial chambers and placed into clean petri dishes, then female salamanders were carefully placed into the center of the treatment chamber containing one of four chemosensory stimuli. After the female made her first movement, the number of NT was recorded for 10 min using TrueBasic Event-PC 3.0 data collection software (TrueBASIC, Inc.). The number of NT is proportional to the degree of *investigation* of chemical signals (Chouinard, 2012); therefore, NT was treated as a proxy for the degree of interest in the chemical cue presented. After the trial was over, each salamander was placed into a clean petri dish with fresh, moistened filter paper (same as initial set up) to prepare for trials the following week.

A generalized linear mixed effects regression model (glmer) with a Poisson error distribution (R package: lme4, function: glmer) was used to test the effect of genetic cluster on behavioral response when females were exposed to male chemosensory cues. This is an appropriate test for data that are not normally distributed and right skewed. Clade membership was included as a fixed effect and because each female experienced four different trials, female ID was included as a random effect to account for any variation due to repeated measures. To quantify deviation of alpha diversity measures between treatments, estimated marginal means (least squared means) of pairwise differences were calculated for each genetic cluster independently. The R package “emmeans” was used to estimate pairwise comparisons, a 95% confidence interval (95% CI) and p-values were adjusted using the Tukey method for comparing a family of multiple estimates. The Kenward-Roger method for approximating degrees of

freedom was selected because it is a more conservative estimation of this statistical parameter (Kenward and Roger, 1997; Schaalje et al., 2002). The Poisson glmer model provided an estimated relationship between female nose tap counts representing level of investigation across each treatment.

Experiment 2: Female association with male chemical cue

This experiment tested the hypothesis that female *P. cinereus* would associate preferentially with chemical cues of male donors in their own genetic cluster compared to chemical cues of male donors in a different genetic cluster. Experimental trials were conducted over the course of six weeks (07 October - 16 November 2019) between 21:00 and 05:00 h for a total of 80 eight-hour trials. As in Experiment 1, each female (Eastern Cluster: n=39; Central Cluster n=41) experienced four different treatments: exposure to 1) a male donor chemical cue from a member of the same genetic cluster; 2) a male donor chemical cue from a member of a different genetic cluster; 3) the female's own chemical cue; and 4) a blank, moistened filter paper which was used as a control. Individual salamanders were only exposed to each treatment once and underwent only one trial per week. In contrast to Experiment 1, females were presented with all four treatments simultaneously. In this experiment, each treatment was a ¼ pie section of the filter paper from an established territory of a male from each genetic cluster, the female's own territory, and a control. An experimental arena (245 x 245 x 20 mm polystyrene bioassay dish) was lined with clean, moistened (spring water) paper towels. To determine the location of the odor stimuli within the experimental chamber, a random number generator was used to select each of the four corners so that treatments were placed randomly. Cameras (Raspberry Pi NoIR Camera V2) were secured above each experimental chamber and a single frame was exposed once every two minutes for eight hours (21:00 - 05:00). To determine the amount of time spent

associating with each odor, the salamander's position in relation to the chemosensory cue was recorded from each photo (240 photos per individual = 19,200 photos total). "Time in proximity" was used to indicate female spatial *association* with each male odor and was defined as the proportion of time spent with at least half of her body in physical contact with the filter paper containing the odor (Chouinard, 2012). After each trial, salamanders were carefully returned to their respective Pyrex dishes as discussed in salamander housing.

To determine if females preferentially associate with odors of male donors in their own genetic cluster compared to odors from an adjacent genetic cluster, I used a generalized linear model (glm) with a Poisson error distribution (package: lme4, function: glm). The glm was performed separately for Central and Eastern clusters and is an appropriate statistical test for data that are not normally distributed with categorical predictor variables (clade membership) and continuous response variables (time spent in proximity to odor). The interaction between treatment and clade was also evaluated by combining both genetic clusters and comparing predictor variables using the glm function. As in Experiment 1, estimated marginal means of pairwise differences were calculated for each genetic cluster independently, R package "emmeans" was used to estimate pairwise comparisons and a 95% confidence interval (95% CI), and p-values were adjusted using the Tukey method for comparing a family of multiple estimates and the Kenward-Roger method for approximating degrees of freedom was used. The Poisson glm model provided an estimated relationship between female time spent associating with each treatment presented. All statistical analyses were performed using R Statistical Software Version 1.1.456 (R Core Team, 2018).

RESULTS

Experiment 1

Females did not differ in the number of nose taps directed towards male odors of members of the same and different genetic clusters in either the Central or Eastern Cluster but all other pairwise comparisons differed significantly (Table 3). Females nose tapped a similar number of times towards males of their own cluster compared to males of a different cluster: Central Cluster females nose tapped Central Cluster male odors an average of 14.3 times and Eastern Cluster males 15.73 times while Eastern Cluster females nose tapped Eastern Cluster male odors an average of 14.15 times and Central Cluster males an average of 14.30 times (Central Cluster: z value = 1.719; p = 0.314; Eastern Cluster: z value = 0.179; p = 0.998; Table 2). Females of both clusters nose tapped same-cluster males significantly more than they did their own odor (Central Cluster: z value = -10.043; p < .0001; Eastern Cluster: z value = -9.495; p < .0001) or water control (Central Cluster: z value = 12.800; p < .0001; Eastern Cluster: z value = 13.969; p < .0001). Females of both clusters also nose tapped different-cluster males significantly more than they did their own odor (Central Cluster: z value = 11.577; p < .0001; Eastern Cluster: z value = 9.658; p < .0001) or water control (Central Cluster: z value = 14.195; p < .0001; Eastern Cluster: z value = 14.106; p < .0001). Additionally, females from both clusters nose tapped their own odors significantly more than they did the water control (Central Cluster: z value = 3.334; p = 0.0048; Eastern Cluster: z value = 5.565; p < .0001).

Experiment 2

Eastern Cluster females did not differ significantly in time in proximity to any treatment presented except for own odor compared to water control, with females spending significantly more time associating with their own odor than the water control (z value = -2.936; p = 0.01;

Table 5). All other pairwise comparisons were not different, with females spending a similar amount of time in proximity to each treatment presented (Table 4). For Central Cluster females, all pairwise comparisons were significant except time in proximity to own odor and Eastern Cluster male odor (z value -0.362; $p = 0.9838$). Central Cluster females spent significantly more time with Eastern Cluster male odors than with Central Cluster male odors (z-value = 9.218; $p < .0001$). Central Cluster females also spent significantly more time in proximity to Eastern Cluster male odors than to the water control (z value = -4.921; $p < .0001$). Central Cluster females also spent significantly more time with the water control than Central Cluster male odors (z value = 4.431; $p = 0.0001$). Finally, Central Cluster females spent significantly more time in proximity to their own odor (21.7) than to Central Cluster male odors (z value = 9.561; $p < .0001$) and the water control (z value = 4.431; $p < .0001$). The interaction between treatment and clade was significant with the Central Cluster exhibiting a differential response to the control treatment compared to the Eastern Cluster ($p < .0001$) and driving the overall significant interactive effect between treatment and clade.

DISCUSSION

Although signal evolution in the form of pheromones is thought to drive diversification in plethodontid salamanders (Herrboldt et al., 2021) and can operate in the context of mate recognition and mate choice (Watts et al., 2004), there is much to learn about the complex processes involved in maintaining species boundaries in *Plethodon cinereus*. In contrast to my hypothesis, clade membership was not a predictor of differences observed in the behavior of female salamanders toward male odors, despite strong genetic differentiation between the two study groups. This was indicated in both experiments: Central and Eastern Cluster females performed a similar number of nose taps toward odors belonging to males of their own clade

compared to the adjacent clade in Experiment 1 and did not spend significantly more time in proximity to odors belonging to males of their own genetic cluster compared to the adjacent cluster in Experiment 2. Taken together, these behavioral experiments corroborate recent studies suggesting that the sex-specific pheromones of *P. cinereus* in the two clades are not differentiated enough to allow reproductive isolation between the two genetic lineages studied. Kunkel et al. (2019) investigated mental gland pheromone profiles of male *P. cinereus* from the Pennsylvania clade (Eastern Cluster) and the Ohio clade (Central Cluster) using liquid chromatography to characterize samples and did not detect variation between the two phylogenetic clades. However, they found significant differences in pheromonal profiles between populations suggesting that pheromone composition varies both within and between clades. Female preferences for odors presented in Experiment 1 are straightforward, but results of trials for time spent in proximity to odors in Experiment 2 yielded mixed results.

Experiment 1: Female behavioral response to male chemical cues - Analyses from Experiment 1 revealed a strong similarity between the two genetic clusters. Overall, females from the Central Cluster displayed a pattern of increasing interest from the water control < own odor < male odors but did not differentiate between odors belonging to males of the Central Cluster compared to males of the Eastern Cluster (Fig. 2b, Table 3). This pattern was mirrored for the Eastern Cluster, with females showing increasing interest from the water control < own odor < male odors, regardless of the genetic cluster to which the donor belonged (Fig. 2a, Table 3). The tendency of females to exhibit increased nose tapping behavior in the presence of male odors when compared to the water control or the female's own odor demonstrates the ability of *P. cinereus* to detect important differences among odor sources under an effective experimental design. While chemical-based signaling is considered the most ancient and widespread form of

communication and has been shown in at least 15 orders of organisms representing taxa ranging from bacteria to mammals (El-Sayed, 2006), much of the research on mate recognition and mate choice in amphibians has historically focused on visual and acoustic cues (Johansson and Jones, 2007). For plethodontid salamanders, chemical communication is the dominant sensory modality (Dawley, 1986b) and recent research on the morphology and physiology of salamander pheromonal systems reveal increasing evidence of variation and complexity in terms of behavioral effects across species (Woodley and Staub, 2021). Although much attention has been given to *P. cinereus* in the literature, identifying experimental designs that directly test hypotheses related to mate assessment via chemical cues could elucidate patterns associated with reproductive isolation and speciation. Additionally, exploring *P. cinereus* populations that are more deeply divergent, either phylogenetically or ecologically, and testing mate recognition and mate choice hypotheses in the context of pheromonal evolution may help to reveal the relationship between pheromonal divergence and population divergence.

Experiment 2: Female association with male chemical cue - On average, Eastern Cluster females spent more time associating with Central Cluster male odors than with Eastern Cluster male odors, but results of the glm were not significant (Fig. 3a, Table 5). Additionally, Eastern Cluster females spent more time associating with their own odor than any other treatment but only differences in time in proximity to own odor compared to water control were significant (Fig. 3a, Table 5). The interaction model indicates that the Central Cluster is driving the overall significant effect between treatment and clade, suggesting that Central Cluster females were more sensitive to the odor cues than were Eastern Cluster females. Of the four treatments presented, Central Cluster females spent the least amount of time associating with Central Cluster male odors, increased time in proximity to water control, and a similar time in

proximity to Eastern Cluster male odors and own odors (Fig. 3b, Table 5). Why females avoided male substrates in Experiment 2 is not yet clear, but one explanation may involve behaviors associated with familiarity observed in this species. For example, Guffey et al. (1998) found that male *P. cinereus* exhibited greater aggression toward unfamiliar females compared to those with which they had previously interacted in the laboratory, suggesting that males likely show aggression toward both male and female intruders in natural populations. As a result, females may tend to avoid territories of aggressive, unfamiliar males. In a laboratory experiment by Peterson et al. (2000) gravid females were more attracted to pheromones of unfamiliar females than to those of unfamiliar males, which may cause a female to avoid such territories (or markings) in response to male aggression.

Laboratory studies on chemical communication in *P. cinereus* exploring a broad range of research questions reveal behaviors like those observed in Experiment 2 of the present study. Jaeger and Gergits (1979) performed two-choice tests and found that female *P. cinereus* preferred to associate with their own previously marked substrates compared to substrates marked by conspecific males. They hypothesize that these movements are determined by a withdrawal from the appropriate conspecific pheromone rather than an approach to the individual's own pheromone (Jaeger and Gergits, 1979). Similarly, Simon and Madison (1984) used a series of choice tests to investigate the source of chemical cues that function in conspecific identification and found that salamanders preferred their own odors to those of conspecific males, which may influence the spatial behavior of *P. cinereus*. Jaeger et al. (1986) developed an artificial burrow chamber to test the hypothesis that male *P. cinereus* use pheromones contained in fecal pellets to identify territories marked by their own pheromones and male-marked territories of conspecifics. They found that salamanders spent more time in

burrows marked with their own fecal pellets than in those marked with either conspecific or surrogate (control) pellets and spent more time in surrogate-marked burrows than in those marked with another individual's pellets, supporting the idea that this species uses pheromones in territorial identification. When presented with chemical cues, female *P. cinereus* have been shown to behave similarly to males. Horne and Jaeger (1988) focused on female salamanders to test the hypothesis that, like males, female *P. cinereus* deposit pheromones associated with fecal pellets. They found that salamanders spent more time in their own burrow when presented with a pellet of a conspecific female, supporting their hypothesis and demonstrating that males and females share more behavioral traits than previously known.

Collectively, the studies discussed previously may help to provide insight into the results of Experiment 2 in terms of avoidance behavior. An important distinction between Experiment 2 in the present study and previous studies is the number of choices presented: two versus four. This discrepancy may help to explain a caveat in the interpretation of these results related to the ability of *P. cinereus* to detect volatile odors (Chouinard, 2012). Specifically, it is possible that one or more of the signals are dampened when the female was simultaneously introduced to multiple stimuli, or they may be associating with one stimulus but responding to another because of difficulty differentiating volatile odors in the test chamber.

Alternatively, differences in time spent associating with male odors in the Central Cluster may be related to asymmetric mate recognition, such that Central Cluster females may be less selective or more attracted to the unfamiliar Eastern Cluster male odor compared to the familiar Central Cluster male odors. Although this situation may not represent the norm in animal mating systems, asymmetric mate recognition has been documented in a variety of taxa such as insects (Hochkirch and Lemke, 2011), fish (Ryan and Wagner, 1987; Egger et al., 2010), amphibians

(Osikowski, 2012), reptiles including birds (Rohwer et al., 2001; Hardwick et al., 2013; Sardell, 2016; Semenov et al., 2017), and mammals (Shurtliff et al., 2013). Asymmetric hybridization mediated by female mate choice occurs in Spadefoot Toads (*Spea multiplicata* and *Spea bombifrons*) under conditions in which the traits of hybrid offspring are favored. In shallow ponds, which dry sooner, hybridization may be advantageous for females because of faster development in their hybrid offspring, allowing hybrids to be more likely than pure *S. bombifrons* offspring to reach metamorphosis before pond drying. In contrast, deep ponds provide ample time to complete metamorphosis and so *S. bombifrons* females have higher fitness by mating with conspecifics (Pfennig 2007). Such variation in female mate choice may suggest how some hybrids are maintained in sympatric populations and influence the process of sexual selection in profound ways.

In other taxa, asymmetrical mate preference is influenced by color morph, as observed in locally adapted and ecologically distinct populations of the Eastern Fence Lizard, *Sceloporus undulatus*, from the Chihuahuan Desert. Blanched, melanic, and brown color morphs are found in gypsum-characterized White Sands substrate, black basalt Carrizozo lava flows, and dark brown Chihuahuan scrubland, respectively. When paired by ecomorph in the laboratory during mate choice trials, only White Sands males preferred local females, whereas black lava and dark soil males showed no preference for local females, indicating that color may be playing an important role in both adaptation and RI, and providing behavioral evidence that White Sands lizards are undergoing incipient ecological speciation (Hardwick et al., 2013). Evidence that odor, like color, may act as a means of asymmetric isolation has also been found in other taxa (Rafferty and Boughman, 2006; Smadja and Butlin, 2009). While divergence in *S. undulatus*

across the White Sands ecotone has occurred in parapatry, populations that experience allopatric divergence and subsequent recontact also provide evidence for speciation.

Secondary contact zones, where populations that were once isolated later reestablish gene flow, represent ideal natural systems to examine processes by which lineage identity is defined and maintained, both intra- and interspecifically. Studies that consider the genetic structure of contact zones offer valuable insight to the complex nature of hybridization processes (Pedall et al., 2011). Divergence in mate recognition within sympatric populations may arise if selection against reproduction between distantly related populations compared to closely related populations is at play (Higgie et al., 2000) or when different traits such as those involved in communication experience reproductive character displacement across populations within a contact zone (Howard, 1993; Lemmon, 2009). In the Spotted Salamander, *Ambystoma maculatum*, secondary contact following population expansion has resulted in a hybrid zone with pronounced introgression of nuclear loci across the contact boundary of two diverging lineages (Johnson et al., 2015). Like *P. cinereus*, *A. maculatum* shows discordance between mitochondrial and nuclear DNA, such that the hybrid zone is characterized by a narrower mtDNA cline that is noncoincident with the broader nuclear cline. Female mate choice across the contact zone demonstrates potential for the first asymmetric stages of prezygotic isolation in this species and may have contributed to the genetic discordance previously described, potentially due to inequalities in mate quality between the two lineages resulting in asymmetric introgression between the two groups (Johnson et al., 2015).

Waldron et al. (2019) investigated the nature of a *P. cinereus* contact zone in northern Ohio and found a similar pattern of mitonuclear discordance. They detected a clinal transition in mtDNA but little differentiation in nuclear loci, and infer that lineage sorting, male-biased

dispersal, or historical introgression of mtDNA may have contributed to the disagreement in genetic markers or, alternatively, a nuclear cline outside of their sampling region may exist (Waldron et al., 2019). In the two genetic lineages of *P. cinereus* examined in the present study, differences in female behavior toward male odors based on genetic cluster may follow a similar pattern of asymmetry in mate choice, with females of the Central Cluster being less choosy than those of the Eastern Cluster. The Cuyahoga River acts as a barrier to gene flow between the Eastern and Central Clusters (Fig. 1), with genetic drift playing an important role in divergence (Hantak et al., 2019). If Central Cluster females do prefer Eastern Cluster males or are less selective than Eastern Cluster females based on clade membership, it may be related to genetic admixture in the Central Cluster. Population 17 (Fig. 1) appears to have low but detectable admixture consisting of Central Cluster and Eastern Cluster individuals (Hantak et al., 2019), suggesting that when Central Cluster females are presented with male odors of both clusters, they are not choosy. In contrast, no admixture was detected for Eastern Cluster individuals, which may help to explain why no significant difference was detected in time in proximity of male odors in Eastern Cluster females in Experiment 2. In an intralinear hybrid zone, females must contend with conspecific recognition in addition to mate quality recognition, representing a potential conflict when traits used for each form of recognition differ (Pfennig, 2000). If *P. cinereus* incur any costs in interlineage reproduction, females would be expected to choose intralinear males in such a contact zone and as a result, the few males that successfully migrate across the contact zone are unlikely to experience reproductive success unless selection acts to favor males from the opposite lineage (Johnson et al., 2015).

Future studies, that consider how the processes of species and mate quality recognition interact, may improve our understanding of the underlying genetic mechanisms by which

recognition evolves within and between species. Although *P. cinereus* is among the most well-studied salamanders, the link between genetic divergence of populations and reproductive isolation is not well understood. Evidence that chemical cues are important for species recognition and the maintenance of lineage identity between sympatric salamander species is widespread in the literature (Dawley, 1986a; Verrell, 1989; reviewed in Arnold et al., 1993), but for speciation to proceed past the initial stages, multiple forms of reproductive isolation and possibly the action of both natural selection and sexual selection may be required (Ritchie, 2007; van Doorn et al., 2009). For example, ecological, physiological, and/or morphological adaptation to novel environments, accompanied by biogeographic processes that promote differentiation or limit gene flow, may also contribute to reproductive isolation, but identifying these potential influences can be challenging in recently diverged or rapidly diverging lineages, or in lineages that contain cryptic diversity (Worsham et al., 2017), such as *P. cinereus*. Thus, research efforts that analyze both genetic and ecological interactions between lineages in secondary contact may offer insight to historical processes that maintain evolutionary independence as well as provide valuable evidence to help fill major gaps in our understanding of speciation. Although this species is classified as least concern (IUCN, 2020), there appears to be considerable geographic variability in conservation status throughout its range, with some populations undergoing expansion (Page et al., 2020) and others showing precipitous declines (Mitchell, 2015). Nearly all *Plethodon* species appear to be in decline throughout eastern North America, though the extent of decline varies among populations (Highton, 2005). Growing evidence that *P. cinereus* is sensitive to disturbances such as forest loss and fragmentation (deMaynaider and Hunter, 1995), invasive species (Maerz et al., 2009; Ziemba et al., 2015), and climate change (Caruso et al., 2014; Muñoz et al., 2016), combined with its rich genetic and ecological diversity, make it an

important species to study when considering species boundaries to which biodiversity is intimately tied.

ACKNOWLEDGEMENTS

I would like to extend my sincerest gratitude to my advisors, C. Anthony and C. Hickerson, for their unyielding support and imparting special knowledge on *P. cinereus* ecology and behavior; my lab companions, T. Evers, K. Garner, and J. Ryan for their meaningful collaboration; M. Hantak for providing genetic details and sampling locality data. Olivia Brooks, A. Eucker, D. Frost, J. Galinis, M. Green, S. Kale, S. Kocheff, C. Lindstrom, E. Palmer, H. Parson, H. Roberts, and A. Veselka assisted with salamander collection in the field. T. Evers, K. Garner, C. Lindstrom, H. Roberts, and J. Ryan maintained *Drosophila* colonies and carefully tended to live animals in the lab for the study duration. Jennifer Murphy, A. Perez, and J. Watling advised appropriate statistical analyses. I thank R. Drenovsky for her valuable comments and ideas to improve the manuscript. Special thanks to J. Thomas, L. Thomas, R. Thomas, and C. Venard for their enduring patience and support. Finally, I wish to acknowledge the rich, scientific community of staff and students alike in the Department of Biology, as their camaraderie and zeal for science deeply enriched my experience as a graduate student at John Carroll University. The Ohio Department of Natural Resources granted scientific collection permit #ODNR 22-018, allowing us to obtain animals for research purposes and the Institutional Animal Care and Use Committee of John Carroll University (Protocol no. 1800) approved ethical work with live animals in the laboratory.

LITERATURE CITED

- Acord, M.A., Anthony C.D., and C.M. Hickerson. 2013. Assortative mating in a polymorphic salamander. *Copeia*, 2013: 676-683.
- Anthony, C.D., and R.A. Pfungsten. 2013. Eastern Red-backed Salamander, *Plethodon cinereus* (Green 1818). Pp. 335–360 in Amphibians of Ohio, Ohio Biological Survey Bulletin New Series, Volume 17, Number 1 (R.A. Pfungsten, J.G. Davis, T.O. Matson, G. Lipps, Jr., D. Wynn, and B.J. Armitage, eds.). Ohio Biological Survey, USA.
- Anthony, C.D., Venesky, M.D. and C.M. Hickerson. 2008. Ecological separation in a polymorphic terrestrial salamander. *Journal of Animal Ecology*, 77: 646-653.
- Anthony, C.D., Hickerson, C.M., Jaworski, K., and M. Messner. 2017. Differences in prey availability within the territories of striped and unstriped eastern red-backed salamanders (*Plethodon cinereus*). *Herpetological Review*, 48: 509–514.
- Arnold, S.J., Reagan, N.L., and P.A. Verrell. 1993. Reproductive isolation and speciation in plethodontid salamanders. *Herpetologica*, 49: 216–228.
- Balph, D.F. and M.H. Balph. 1983. On the psychology of watching birds: the problem of observer-expectancy bias. *The American Ornithologists' Union*, 100(3): 755-757.
- Bickford, D., Lohman D.J., Sodhi, N.S., Ng, P.K., Meier, R., Winker, K., Ingram, K.K. and I. Das. 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution*, 22: 148-155.
- Brown, C.W. 1968. Additional observations on the function of the nasolabial grooves of plethodontid salamanders. *Copeia*, 1968:728-731.

- Cabe, P.R., Page, R.B., Hanlon, T.J., Aldrich, M.E., Connors, L., and D.M. Marsh. 2007. Fine-scale population differentiation and gene flow in a terrestrial salamander (*Plethodon cinereus*) living in continuous habitat. *Heredity*, 98: 53–60.
- Caruso, N.M., Sears, M.W., Adams, D.C., & K.R. Lips. 2014. Widespread rapid reductions in body size of adult salamanders in response to climate change. *Global Change Biology*, 20(6): 1751-1759.
- Chouinard, A.J. 2012. Rapid onset of mate quality assessment via chemical signals in a woodland salamander (*Plethodon cinereus*). *Behavioral Ecology and Sociobiology*, 66: 765-775.
- Dawley, E.M. and A.H. Bass. 1988. Organization of the vomeronasal organ in a plethodontid salamander. *Morphology*, 198: 243-255.
- Dawley, E.M., and A.H. Bass. 1989. Chemical access to the vomeronasal organs of a plethodontid salamander. *Journal of Morphology*, 200:163-174.
- Dawley, E.M. 1984. Recognition of individual, sex and species odours by salamanders of the *Plethodon glutinosus*-*P. Jordani* complex. *Animal Behaviour*, 32(2): 353–361.
- Dawley, E.M. 1986a. Behavioral isolation mechanisms in sympatric terrestrial salamanders. *Herpetologica*, 42(1986): 156-164.
- Dawley, E.M. 1986b. Evolution of chemical signals as a premating isolating mechanism in a complex of terrestrial salamanders. In *Chemical Signals in Vertebrates 4* (pp. 221-224). Springer, Boston, MA.
- DeMaynadier, P.G., and M.L. Hunter Jr. 1995. The relationship between forest management and amphibian ecology: a review of the North American literature. *Environmental Reviews*, 3(3-4): 230-261.

- Deutsch, J.C., and J.D. Reynolds. 1973. Design and sexual selection: the evolution of sex differences in mate choice. *Perspectives in ethology: volume 11 behavioral design*. Plenum Press, New York, and London.
- Dobzhansky, T. 1946. Complete reproductive isolation between two morphologically similar species of *Drosophila*. *Ecology*, 27: 205–211.
- Downhower, J.F., Brown, L., Pederson, R., and G. Staples. 1983. Sexual selection and sexual dimorphism in mottled sculpins. *Evolution*, 37(1): 96-103.
- Dyke, A.S. 2004. An outline of North American deglaciation with emphasis on central and northern Canada. *Developments in Quaternary Sciences*, 2: 373-424.
- El-Sayed, A.M. 2006. The Pherobase: Database of insect pheromones and semiochemicals. <http://www.pherobase.com>
- Egger, B., Mattersdorfer, K., and K.M. Sefc. 2010. Variable discrimination and asymmetric preferences in laboratory tests of reproductive isolation between cichlid colour morphs. *Journal of Evolutionary Biology*, 23(2): 433-439.
- Evans, A.L., Forester, D.C., and B.S. Masters. 2010. Recognition by population and genetic similarity in the Mountain Dusky Salamander, *Desmognathus ochrophaeus* (Amphibia: Plethodontidae). *Ethology*, 103(10): 865-875.
- Gergits, W.F., and R.G. Jaeger. 1990. Site attachment by the red-backed salamander, *Plethodon cinereus*. *Journal of Herpetology*, 24: 91-93.
- Gillette, J.R. 2003. Population ecology, social behavior, and intersexual differences in a natural population of red-backed salamanders: A long-term field study. Ph.D. Dissertation, University of Louisiana at Lafayette, Lafayette, Louisiana, U.S.A.

- Gillette J.R., Kolb, S.E., Smith, J.A., and R.G. Jaeger. 2000. Pheromonal attractions to particular males by female redback salamanders (*Plethodon cinereus*). In *The Biology of Plethodontid Salamanders* (pp. 431-440). Springer, Boston, MA.
- Guffey, C., Makinster, J.G., and R.G. Jaeger. 1998. Familiarity affects interactions between potentially courting territorial salamanders. *Copeia*, 1998(1): 205– 208.
- Hantak, M., Page, R., Converse, P., Anthony, C.D., Hickerson, C.M., and S. Kuchta. 2019. Do genetic structure and landscape heterogeneity impact color morph frequency in a polymorphic salamander? *Ecography*, 42(8): 1383-1394.
- Hardwick, K.M., Robertson, J.M., and E.B. Rosenblum. 2013. Asymmetrical mate preference in recently adapted White Sands and black lava populations of *Sceloporus undulatus*. *Current Zoology*, 59(1): 20-30.
- Hartwell, H., Welsh, J.R., and S. Droege 2001. A case for using plethodontid salamanders for monitoring biodiversity and ecosystem integrity of North American forests, *Conservation Biology* 15(3): 558–569.
- Heatwole, H. 1962. Environmental factors influencing local distribution and activity of the salamander, *Plethodon cinereus*. *Ecology*, 43(3): 460–472.
- Herrboldt, M.A., Steffen, M.A., McGouran, C.N., and R.M. Bonett. 2021. Pheromone gene diversification and the evolution of courtship glands in plethodontid salamanders. *Journal of Molecular Evolution*, 89(8): 576-587.
- Herring, K., and P. Verrell. 1996. Sexual incompatibility and geographical variation in mate recognition systems: tests in the salamander *Desmognathus ochrophaeus*. *Animal Behavior*, 52: 279-287.

- Hickerson, C.M., Anthony, C.D., and B.M. Walton. 2012. Interactions among forest floor guild members in structurally simple microhabitats. *American Midland Naturalist*, 168:30–42.
- Hickerson, C.M., Anthony, C.D., and B.M. Walton. 2017. Eastern Red-backed Salamanders regulate top-down effects in a temperate forest-floor community. *Herpetologica*, 73: 180-189.
- Higgie, M., Chenoweth, S., and M.W. Blows. 2000. Natural selection and the reinforcement of mate recognition. *Science*, 290(5491): 519-521.
- Highton, R. 1999. Geographic protein variation and speciation in the salamanders of the *Plethodon cinereus* group with the description of two new species. *Herpetologica*, 55(1): 43-90.
- Highton, R. 2005. Declines of Eastern North American woodland salamanders (*Plethodon*). Pages 34-46 in M. Lanoo, editor. *Amphibian Declines: Conservation Status of United States Species*. University of California Press, Berkeley.
- Highton, R., and T.P. Webster. 1976. Geographic protein variation and divergence in populations of the salamander *Plethodon cinereus*. *Evolution*, 30: 33-45.
- Hillis, D.M. 2019. Species delimitation in herpetology. *Journal of Herpetology*, 53(1), 3-12.
- Hochkirch, A., and I. Lemke. 2011. Asymmetric mate choice, hybridization, and hybrid fitness in two sympatric grasshopper species. *Behavioral Ecology and Sociobiology*, 65(8): 1637-1645.
- Horne, E.A., and R.G. Jaeger. 1988. Territorial pheromones of female red-backed salamanders. *Ethology*, 78: 143-152.

- Houck, L.D., and D.M. Sever. 1994. Role of the skin in reproduction and behaviour, p. 351–381.
In: Amphibian biology, vol. 1. H. Heatwole and G. T. Barthalmus (eds.). Surrey Beatty and Sons, Chipping Norton, New South Wales, Australia.
- Houck, L.D., and P.A. Verrell. 1993. Studies of courtship behavior in plethodontid salamanders: a review. *Herpetologica*, 49: 175-184.
- Howard, D.J. 1993. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. Pp. 46–69 in R. G. Harrison, ed. Hybrid zones and the evolutionary process. Oxford Univ. Press, Oxford
- Hurlbert, S.H. 1984. Pseudoreplication and the design of ecological field experiments. *Ecological Monographs*, 54(2): 187-211.
- IUCN. 2020. The IUCN red list of threatened species. Version 2020–1.
<https://www.iucnredlist.org>
- Jaeger, R.G. 1971. Moisture as a factor influencing the distributions of two species of terrestrial salamanders. *Oecologia*, 6: 191-207.
- Jaeger, R.G. 1981. Dear enemy recognition and the costs of aggression between salamanders. *The American Naturalist*, 117: 962–974.
- Jaeger, R.G. 1984. Agonistic behavior of the red-backed salamander. *Copeia*, 1984: 309-314.
- Jaeger, R.G., and D.C. Forester. 1993. Social behavior of plethodontid salamanders. *Herpetologica*, 49(2): 163-175.
- Jaeger, R.G., and W.F. Gergits. 1979. Intra- and interspecific communication in salamanders through chemical signals on the substrate. *Animal Behavior*, 27: 150-156.
- Jaeger, R.G., and S.E. Wise. 1991. A reexamination of the male salamander 'sexy faeces hypothesis'. *Herpetology*, 25: 370-373.

- Jaeger, R.G., Goy, J.M., Tarver, M., and C.E. Márquez. 1986. Salamander territoriality: pheromonal markers as advertisement by males. *Animal Behavior* 34: 860-864.
- Jaeger, R.G., Gillette, J.R., and R.C. Cooper. 2002. Sexual coercion in a territorial salamander: males punish socially polyandrous female partners. *Animal Behaviour*, 63: 871-877.
- Jaeger, R.G., Gollmann, B., Anthony, C.D., Gabor, C.R., and N.R. Kohn. 2016. Behavioral Ecology of the Eastern Red-Backed Salamander, Oxford University Press, New York, NY, 248 p.
- Jaworski, K.E., Lattanzio, M.S., Hickerson, C.M., and C.D. Anthony. 2018. Male mate preference as an agent of fecundity selection in a polymorphic salamander. *Ecology and Evolution*, 8(17): 8750-8760.
- Johansson, B.G., and T.M. Jones. 2007. The role of chemical communication in mate choice. *Biological Reviews*, 82(2): 265–289.
- Johnson, B.B., White, T.A., Phillips, C.A., and K.R. Zamudio. 2015. Asymmetric introgression in a spotted salamander hybrid zone. *Journal of Heredity*, 106(5): 608-617.
- Kardish, M.R., Mueller, U.G., Amador-Vargas, S., Dietrich, E.I., Ma, R., Barrett, B., and C.C. Fang. 2015. Blind trust in unblinded observation in ecology, evolution, and behavior. *Frontiers in Ecology and Evolution*, 3, 51.
- Karuzas, J.M., Maerz, J.C., and D.M. Madison. 2004. An alternative hypothesis for the primary function of a proposed mate assessment behaviour in red-backed salamanders. *Animal Behaviour*, 68(3): 489–494.
- Kenward, M.G., and J.H. Roger. 1997. Small sample inference for fixed effects from restricted maximum likelihood. *Biometrics*, 983-997.

- Kleeberger, S.R., and J.K. Werner. 1982. Home range and homing behavior of *Plethodon cinereus* Northern Michigan. *Copeia*, 1982: 409–415.
- Kohn, N.R., and R.G. Jaeger. 2009. Male salamanders remember individuals based on chemical or visual cues. *Behaviour*, 146(11): 1485-1498.
- Kunkel, C.L., Anthony, C.D., Hickerson, C.M., and R.C. Feldhoff. 2019. Species variation in a pheromone complex is maintained at the population level in the Eastern Red-backed Salamander. *Journal of Herpetology*, 53(3): 173-178.
- Lemmon, E.M. 2009. Diversification of conspecific signals in sympatry: geographic overlap drives multidimensional reproductive character displacement in frogs. *Evolution: International Journal of Organic Evolution*, 63(5): 1155-1170.
- Maan, M.E., and O. Seehausen. 2011 Ecology, sexual selection and speciation. *Ecology Letters*, 14: 591–602.
- Maerz, J.C., Nuzzo, V.A., and B. Blossey. 2009. Declines in woodland salamander abundance associated with non-native earthworm and plant invasions, *Conservation Biology*, 23(4): 975–981.
- Marsh, D.M., Milam, G.S., Gorham, N.P., and N.G. Beckman. 2005. Forest roads as partial barriers to terrestrial salamander movement. *Conservation Biology*, 19(6): 2004-2008.
- Marsh, D.M., Page, R.B., Hanlon, T.J., Bareke, H., Corritone, R., Jetter, N., Beckman, N.G., Gardner, K., Seifert, D.E., and P.R. Cabe. 2007. Ecological and genetic evidence that low-order streams inhibit dispersal by red-backed salamanders (*Plethodon cinereus*). *Canadian Journal of Zoology*, 85(3), 319-327.
- Martin, S.B., Jaeger, R.G., and E.D. Prosen. 2005. Territorial red-backed salamanders can detect volatile pheromones from intruders. *Herpetologica*, 61(1): 29-35.

- Mathis, A. 1990a. Territoriality in a terrestrial salamander: the influence of resource quality and body size. *Behaviour*, 112: 162–175.
- Mathis, A. 1990b. Territorial salamanders assess sexual and competitive information using chemical signals. *Animal Behaviour*, 40(5): 953-962.
- Mathis, A. 1991. Territories of male and female terrestrial salamanders: costs, benefits, and intersexual spatial associations *Oecologia*, 86: 433-440.
- Mathis, A., Jaeger, R.G., Keen, W.H., Ducey, P.K., and B.W. Buchanan. 1995. Aggression and territoriality by salamanders and a comparison with the territorial behavior of frogs, p. 633-676. In: *Amphibian Biology. Vol 2, Social Behavior*. H. Heatwole and B.K. Sullivan (eds.). Surrey Beatty and Sons, Chipping Norton, NSW, Australia.
- Mayr, E. 1954. Change of genetic environment and evolution. In *Evolution as a process* (ed. J. Huxley, A.C. Hardy, and E.B. Ford), pp. 157-80. Allen and Unwin, London, UK.
- McGavin, M. 1978. Recognition of conspecific odors by the salamander *Plethodon cinereus*. *Copeia*, 1978(2): 356-358.
- Mitchell, J.C. 2015. Amphibian and small mammal assemblages in a northern Virginia forest before and after defoliation by gypsy moths (*Lymantria dispar*). *Virginia Journal of Science*, 66: 403–411.
- Moore, J.D., and M. Ouellet. 2014. A review of colour phenotypes of the eastern red-backed salamander, *Plethodon cinereus*, in North America. *Canadian Field-Naturalist*, 128: 250–259.
- Muñoz, D.J., Hesed, K.M., Grant, E.H.C., and D.A. Miller. 2016. Evaluating within-population variability in behavior and demography for the adaptive potential of a dispersal-limited species to climate change. *Ecology and Evolution*, 6(24): 8740-8755.

- Nunes, V.S., and R.G. Jaeger. 1989. Salamander aggressiveness increases with length of territorial ownership. *Copeia*, 1989: 712-718.
- Osikowski, A. 2012. Asymmetric female preferences for courtship pheromones in two closely-related newt species, the smooth newt (*Lissotriton vulgaris*) and the Carpathian newt (*L. montandoni*) (Salamandridae). *Zoological Science*, 29(6): 390-395.
- Ovaska K. 1989. Pheromonal divergence between populations of the salamander *Plethodon vehiculum* in British Columbia. *Copeia*, 1989(3): 770-775.
- Page, R. B., Conarro, C., Quintanilla, D., Palomo, A., Solis, J., Aguilar, A., Bezold, K., Sackman, A.M., and D.M. Marsh. 2020. Genetic variation in *Plethodon cinereus* and *Plethodon hubrichti* from in and around a contact zone. *Ecology and Evolution*, 10(18): 9948-9967.
- Pedall, I., Fritz, U., Stuckas, H., Valdeón, A., and M. Wink. 2011. Gene flow across secondary contact zones of the *Emys orbicularis* complex in the Western Mediterranean and evidence for extinction and re-introduction of pond turtles on Corsica and Sardinia (Testudines: Emydidae). *Journal of Zoological Systematics and Evolutionary Research*, 49(1): 44-57.
- Peterson, M.G. 2000. Alternative life styles in a terrestrial salamander: Do females preferentially associate with each other? p. 417–429. In: *The Biology of Plethodontid Salamanders*. R. C. Bruce, R. G. Jaeger, and L. D. Houck (eds.). Kluwer Academic/Plenum, New York.
- Petranka, J.W. 1998. *Salamanders of the United States and Canada*. Smithsonian Institution Press, Washington, D.C.

- Pfennig, K.S. 1998. The evolution of mate choice and the potential for conflict between species and mate-quality recognition. *Proceedings of the Royal Society B: Biological Sciences*, 265(1407): 1743–1748.
- Pfennig, K.S. 2000. Female spadefoot toads compromise on mate quality to ensure conspecific matings. *Behavioral Ecology*, 11(2): 220-227.
- Pfennig K.S. 2007. Facultative mate choice drives adaptive hybridization. *Science*, 318 (5852): 965-967.
- R Core Team. 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.
- Radomski, T., Hantak, M.M., Brown, A.D., and S.R. Kuchta. 2020. Multilocus phylogeography of eastern red-backed salamanders (*Plethodon cinereus*): cryptic Appalachian diversity and postglacial range expansion. *Herpetologica*, 76(1): 61-73.
- Rafferty, N.E., and J.W. Boughman. 2006. Olfactory mate recognition in a sympatric species pair of three-spined sticklebacks. *Behavioral Ecology*, 17(6): 965-970.
- Real L. 1990. Search theory and mate choice. I. Models of single-sex discrimination. *The American Naturalist*, 136: 376-405.
- Reiter, M.K., Anthony, C.D., and C.A.M. Hickerson. 2014. Territorial behavior and ecological divergence in a polymorphic salamander. *Copeia*, 2014(3): 481-488.
- Ritchie, M.G. 2007. Sexual selection and speciation. *Annual Review of Ecology, Evolution, and Systematics*, 79-102.
- Robertson, J.M., Nava, R., Vega, A., and K. Kaiser. 2018. Uniformity in premating reproductive isolation along an intraspecific cline. *Current Zoology*, 64(5): 641-652.

- Rohwer, S., Bermingham, E., and C. Wood C. 2001. Plumage and mitochondrial DNA haplotype variation across a moving hybrid zone. *Evolution*, 55: 405–422.
- Rundle, H.D., and P. Nosil. 2005. Ecological speciation. *Ecology Letters*, 8: 336–352.
- Ryan, M.J., and W.E. Wagner Jr. 1987. Asymmetries in mating preferences between species: female swordtails prefer heterospecific males. *Science*, 236(4801): 595-597.
- Sardell, J.M., and J.A.C. Uy. 2016. Hybridization following recent secondary contact results in asymmetric genotypic and phenotypic introgression between island species of *Myzomela* honeyeaters. *Evolution*, 70(2): 257-269.
- Sayler, A. 1966. The reproductive ecology of the red-backed salamander, *Plethodon cinereus*, in Maryland. *Copeia* 1966(2): 183-193.
- Schaalje, G.B., McBride, J.B., and G.W. Fellingham. 2002. Adequacy of approximations to distributions of test statistics in complex mixed linear models. *Journal of Agricultural, Biological, and Environmental Statistics*, 7(4), 512-524.
- Schluter, D. 2000. The ecology of adaptive radiation. Oxford University Press, Oxford.
- Schubert S.N., Houck L.D., Feldhoff, P.W., Feldhoff, R.C., and S.K. Woodley. 2008. The effects of sex on chemosensory communication in a terrestrial salamander (*Plethodon shermani*). *Hormones and Behavior*, 54: 270-277.
- Shurtliff, Q.R., Murphy, P.J., Yeiter, J.D., and M.D. Matocq. 2013. Experimental evidence for asymmetric mate preference and aggression: behavioral interactions in a woodrat (*Neotoma*) hybrid zone. *BMC Evolutionary Biology*, 13.1(220): 1-13.
- Schwander, T., Arbuthnott, D., Gries, R., Gries, G., Nosil, P., and B.J. Crespi. 2013. Hydrocarbon divergence and reproductive isolation in *Timema* stick insects. *BMC Evolutionary Biology*, 13(1): 1-14.

- Semenov, G.A., Scordato, E.S.C., Khaydarov, D.R., Smith, C.C.R., Kane, N.C., and R.J. Safran. 2017. Effects of assortative mate choice on the genomic and morphological structure of a hybrid zone between two bird subspecies. *Molecular Ecology*, 26(22): 6430-6444.
- Servedio, M.R., and J.W. Boughman. 2017. The role of sexual selection in local adaptation and speciation. *Annual Review of Ecology, Evolution, and Systematics*, 48: 85–109.
- Simon, G.S., and D.M. Madison. 1984. Individual recognition in salamanders: cloacal odours. *Animal Behaviour*, 32(4): 1017-1020.
- Smadja, C., and R.K. Butlin. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. *Heredity*, 102(1): 77-97.
- Tristram, D.A. 1977. Intraspecific olfactory communication in the terrestrial salamander *Plethodon cinereus*. *Copeia*, 1977(3): 597–600.
- Turelli, M., Barton, N.H., and J.A. Coyne. 2001. Theory and speciation. *Trends in Ecology & Evolution*, 16(7): 330-343.
- Uzendoski K., and P. Verrell. 1993. Sexual incompatibility and mate-recognition systems: a study of two species of sympatric salamanders (Plethodontidae). *Animal Behaviour*, 46: 267-278.
- van Doorn, G.S., Edelaar, P., and F.J. Weissing. 2009. On the origin of species by natural and sexual selection. *Science*, 326, 1704–1707.
- Verrell, P.A. 1989. An experimental study of the behavioral basis of sexual isolation between two sympatric plethodontid salamanders. *Desmognathus imitator* and *D. ochrophaeus*. *Ethology*, 80(1989): 274-282.

- Waldron, B.P., Kuchta, S.R., Hantak, M.M., Hickerson, C.A.M., and C.D. Anthony. 2019. Genetic analysis of a cryptic contact zone between mitochondrial clades of the Eastern Red-Backed Salamander, *Plethodon cinereus*. *Journal of Herpetology*, 53(2): 144-153.
- Walker, D.M., Murray, C.M., Talbert, D., Tinker, P., Graham, S.P., and T.W. Crowther. 2018. A salamander's top down effect on fungal communities in a detritivore ecosystem. *FEMS Microbiology Ecology*, 94(12): fty168.
- Walls, S.C., Mathis, A., Jaeger, R.G., and W.F. Gergits. 1989. Male salamanders with high-quality diets have faeces attractive to females. *Animal Behaviour*, 38(3): 546-548.
- Watts, R. A. 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.
- Welch, A.M., Semlitsch, R.D., and H.C. Gerhardt. 1998. Call duration as an indicator of genetic quality in male gray tree frogs. *Science*, 280(5371): 1928-1930.
- West-Eberhard, M.J. 1983. Sexual selection, social competition, and speciation. *The Quarterly Review of Biology*, 58(2): 155-183.
- Woodley, S.K., and N.L. Staub. 2021. Pheromonal communication in urodelan amphibians. *Cell and Tissue Research*, 383: 327–345.
- Woolbright, L.L., and Martin, C.P. 2014. Seasonal migration by red-backed salamanders, *Plethodon cinereus*. *Journal of Herpetology*, 48(4): 546-551.
- Worsham, M.L., Julius, E.P., Nice, C.C., Diaz, P.H., and D.G. Huffman. 2017. Geographic isolation facilitates the evolution of reproductive isolation and morphological divergence. *Ecology and Evolution*, 7(23): 10278-10288.

Ziemba, J.L., Hickerson, C.A.M. and Anthony, C.D. 2016. Invasive Asian earthworms negatively impact keystone terrestrial salamanders. *PloS one*, 11(5): e0151591.

TABLE 1 - Salamander collection localities and F_{st} values displaying genetic differentiation and geographical distance measured in kilometers between paired study populations. Populations corresponding to known genetic clusters (Hantak et al., 2019) were used to explore mate recognition trials – two from the Central Cluster (17 and 18) and two populations from the Eastern Cluster (22 and 23). Population numbers are from Hantak et al., 2019.

Central Cluster	Eastern Cluster	F_{st}	Distance (km)
17	22	0.29	15.07
17	23	0.29	23.50
18	22	0.37	9.99
18	23	0.36	14.07

TABLE 2 - *Experiment 1*. Simple statistics showing the mean number of nose taps (NT), standard error (SE), degrees of freedom (df), and standard deviation (SD) for females of the two genetic clusters (GC), Eastern Cluster (E) and Central Cluster (C), when exposed to treatments (Trt) including male donor odors of either genetic cluster (C or E), her own odor (Own), and a water control (HO). Eastern Cluster (n=40) Central Cluster (n=41).

GC	Trt	NT	SE	df	SD	GC	Trt	NT	SE	df	SD
E	HO	4.15	0.72	3	4.53	C	HO	5.12	0.61	3	3.88
E	Own	7.13	0.99	3	6.27	C	Own	6.93	1.30	3	8.34
E	E	14.2	1.60	3	10.10	C	C	14.27	1.86	3	11.91
E	C	14.3	1.67	3	10.571	C	E	15.73	1.98	3	12.65

TABLE 3 - *Experiment 1*. Multiple comparisons of means using Tukey contrasts for number of nose taps across each treatment in the genetic clusters (GC) studied, the Eastern Cluster (E) and the Central Cluster (C) Eastern Cluster (n=40); Central Cluster (n=41). Treatments (Trt) showing the combination between genetic cluster and stimulus presented are shown with associated Z-values (z) and p-values (p).

G C	Trt	SE	D f	z	p	G C	Trt	SE	D f	z	p
E	C-E	0.05	39	0.18	0.9980	C	E-C	0.057	40	1.72	0.3138
E	C-Own	0.07	39	9.66	<.0001	C	E-Own	0.07	40	11.57	<.0001
E	C-HO	0.09	39	14.11	<.0001	C	E-HO	0.08	40	14.20	<.0001
E	E-Own	0.07	39	-9.50	<.0001	C	C-Own	0.07	40	-10.04	<.0001
E	E-HO	0.09	39	13.97	<.0001	C	C-HO	0.08	40	12.80	<.0001
E	Own-HO	0.097	39	5.57	<.0001	C	Own-HO	0.09	40	3.33	0.0048

TABLE 4 - *Experiment 2*. Simple statistics showing the mean time in proximity (TIP) to each treatment, standard error (SE), degrees of freedom (df), and standard deviation (SD) for females of both genetic clusters (GC) when simultaneously exposed to treatments (Trt) including odors of males of either genetic cluster (C or E), the female's own odor (Own), and a water control (HO). Eastern Cluster (n=39) Central Cluster (n=41)

GC	Trt	TIP	SE	df	SD	GC	Trt	TIP	SE	df	SD
E	E	17.9	2.14	3	13.53	C	C	12.7	1.34	3	8.35
E	C	18.6	2.71	3	17.17	C	E	21.3	3.13	3	19.76
E	Own	20.1	3.07	3	19.41	C	Own	21.7	3.96	3	25.16
E	HO	17.2	2.54	3	16.07	C	HO	16.5	2.88	3	18.26

TABLE 5 - Experiment 2. Multiple comparisons of means using Tukey contrasts for time spent in proximity to each treatment (Trt) in the genetic clusters (GC) studied, the Eastern Cluster (E) and the Central Cluster (C). Eastern Cluster (n=39) and Central Cluster (n=41).

GC	Trt	SE	df	z-value	p-value	GC	Trt	SE	df	z-value	p-value
E	C-E	0.05	3	0.69	0.90	C	E-C	0.06	3	9.22	<.0001
E	C-Own	0.05	3	-1.44	0.47	C	E-Own	0.05	3	-0.36	0.98
E	C-HO	0.05	3	-1.49	0.43	C	E-HO	0.05	3	-4.92	<.0001
E	E-Own	0.05	3	2.129	0.14	C	C-Own	0.06	3	9.561	<.0001
E	E-HO	0.05	3	-0.81	0.85	C	C-HO	0.06	3	4.431	0.0001
E	Own-HO	0.05	3	-2.94	0.02	C	Own-HO	0.05	3	-5.28	<.0001

FIGURE 1 - Adapted from Hantak et al. (2019). Map of Northern Ohio showing select sampling localities of *Plethodon cinereus*. Numbered circles represent salamander populations and numbers between paired populations represent F_{st} values. Pie colors represent the proportion of striped (white) to unstriped (black) salamanders at each locality. Two genetic clusters: C = 'Central Cluster' and E = 'Eastern Cluster' were identified using microsatellite loci. The dashed line represents a break between the two microsatellite clusters and the solid black line separates mtDNA clades (Radomski 2020). Populations examined in this study include 17, 18, 22, and 23.

FIGURE 2 - *Experiment 1*. Differences in the number of nose taps in response to treatments presented to females in the A) Eastern Cluster (n=41) and B) Central Cluster (n=40). Data are presented as boxplots with horizontal lines representing medians, the boxes indicating interquartile range, the \diamond symbol denoting means, and the solid circles representing outliers.

FIGURE 3 - *Experiment 2*. Differences in time spent in proximity to treatments measured by number of photos in which individual females were observed associating with each treatment in A) Eastern Cluster females (n=39) and B) Central Cluster females (n=41). Data are presented as boxplots with horizontal lines representing medians, the boxes indicating interquartile range, the \diamond symbol denoting means, and the solid circles representing outliers.

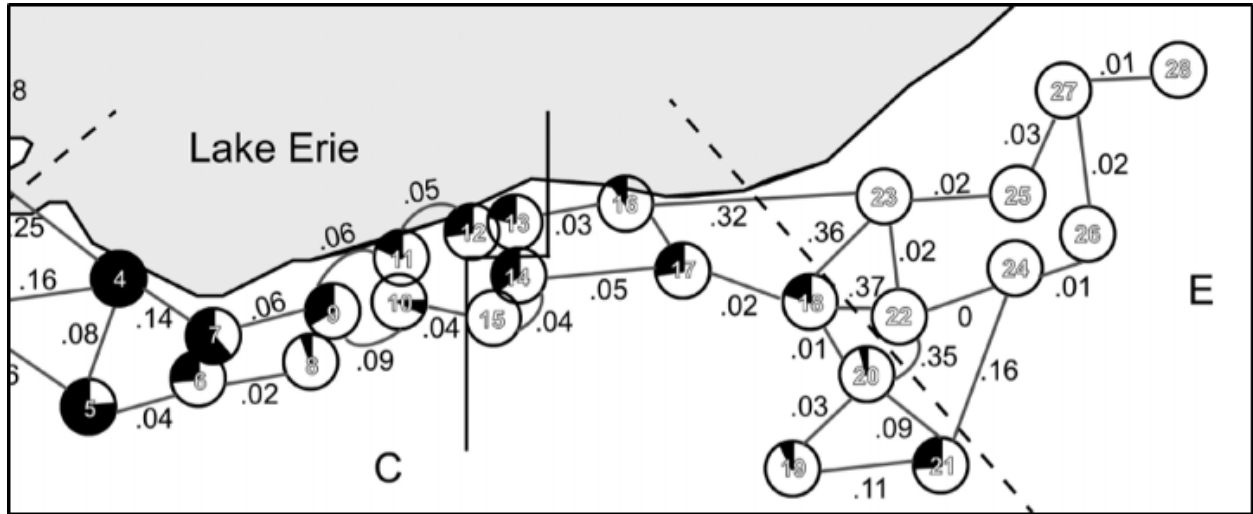
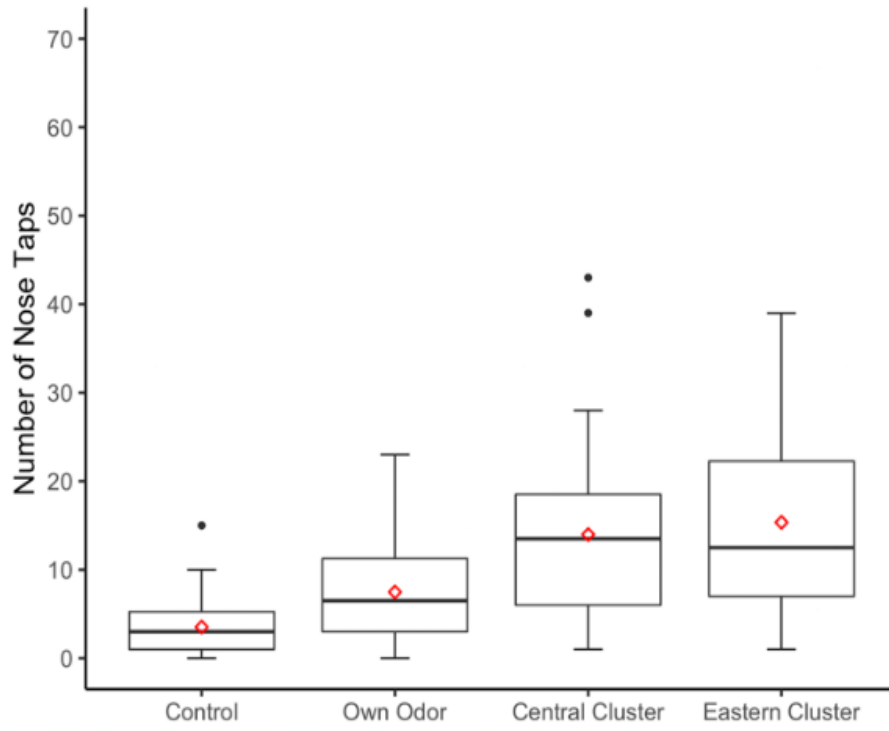


FIG. 1.

A.



B.

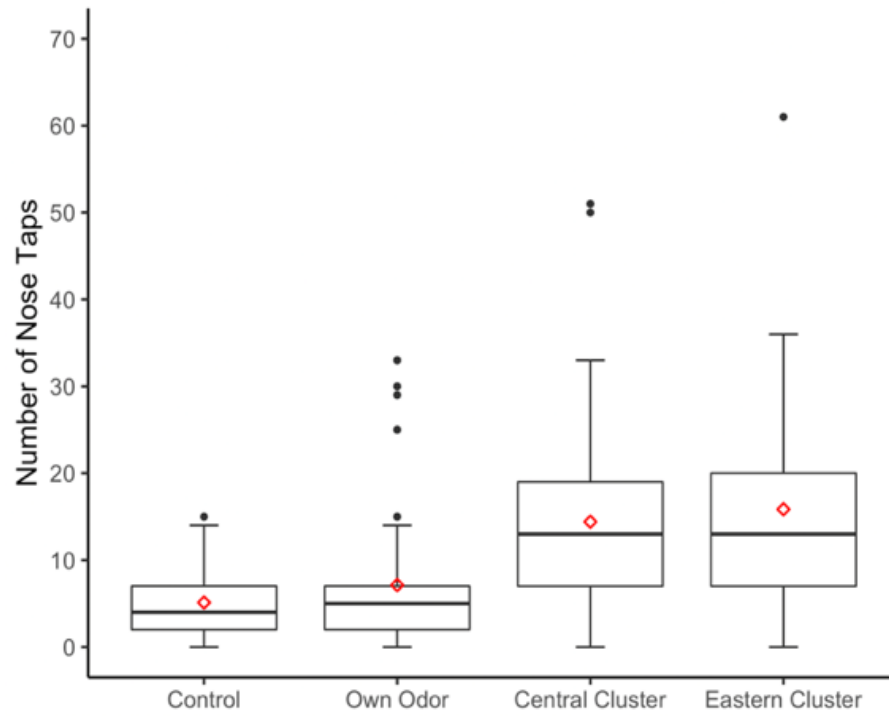
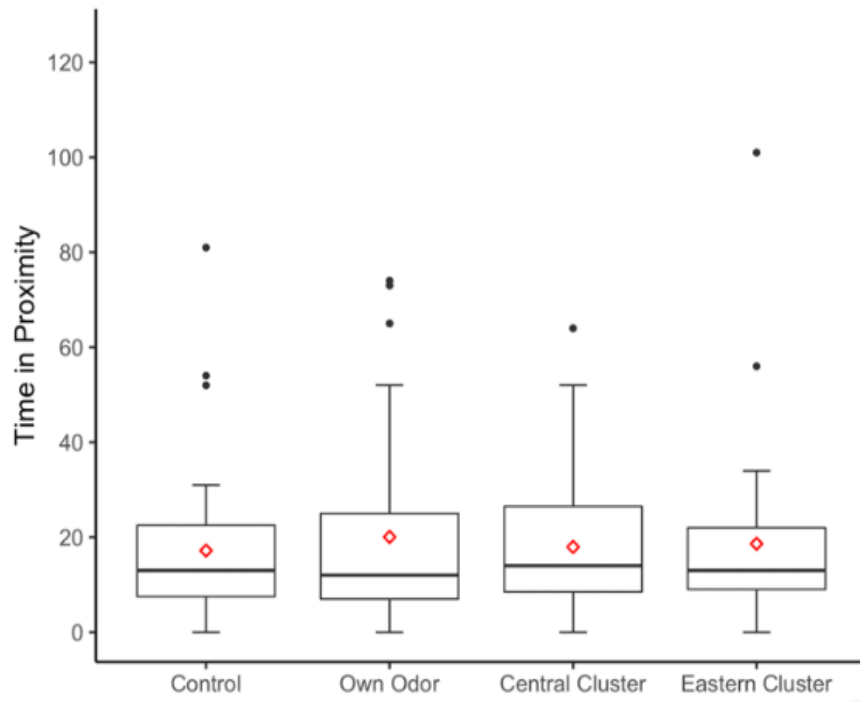


FIG. 2.

A.



B.

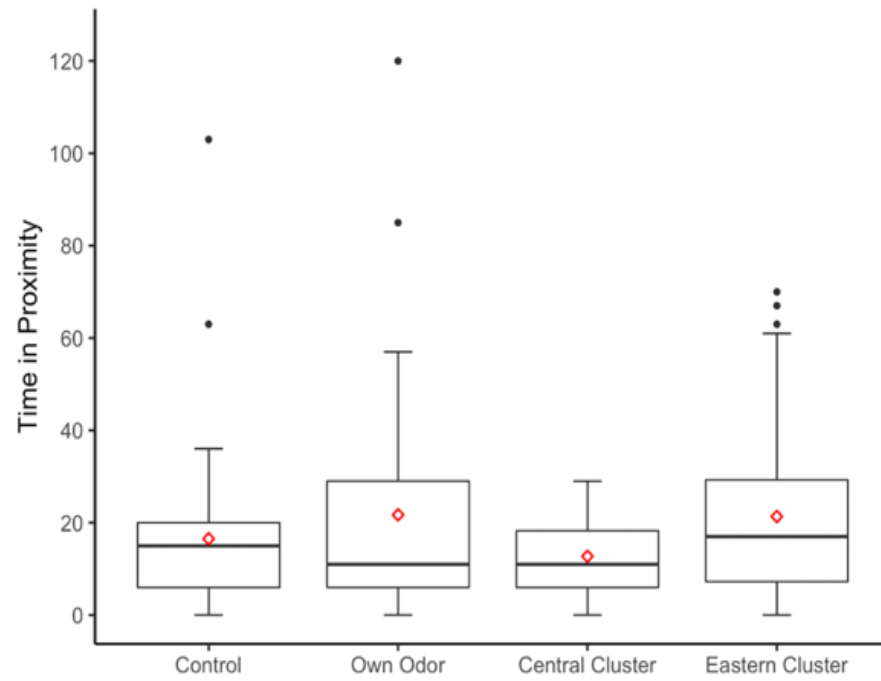


FIG. 3.