# RESPONSES TO SEX- AND SPECIES-SPECIFIC CHEMICAL SIGNALS IN ALLOPATRIC AND SYMPATRIC SALAMANDER SPECIES

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## 1. INTRODUCTION

Sexually reproducing organisms depend on a close association between one sex's ability to produce and transmit a signal in a given environment and the ability of the opposite sex to decipher and respond to that signal. While continuously confronted with an assortment of visual, acoustic and chemical stimuli from their surroundings, many species readily distinguish signals produced by prospective mating partners. This tightly linked signaler/receiver system enables these animals to quickly locate or attract mates, assess mate quality, and avoid the reproductive costs that may be associated with hybridization. In many organisms, reproductively isolated populations exhibit differences in their mating signals, and these signals act as barriers to gene exchange (Ryan, 1990; Coyne and Oyama, 1995; McLennan and Ryan, 1997, 1999). It is well accepted that reproductive isolation can evolve gradually in geographically separated populations as the populations diverge in response to adaptations to different environments or to other selective pressures. More controversial, however, is the idea that natural selection may rapidly increase divergence in mate recognition by way of selection against hybridization following secondary contact. The reinforcement of mate recognition by this process will result in the pattern of reproductive character displacement, whereby the signals that reduce mating between populations diverge more dramatically between sympatric populations than they do between allopatric populations (Dobzhansky, 1937; Dawley, 1987; McKinnon and Liley, 1986; Reagan, 1992).

Sexual isolation has arisen among numerous species of geographically isolated populations of salamanders (review by Arnold et al., 1993). Behavioral experiments indicate that the degree of reproductive isolation is stronger between sympatric species of large *Plethodon* than it is between allopatric populations of these species (Reagan, 1992). Sexual isolation may be due to species-specific differences in the chemosensory systems

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of these terrestrial vertebrates (Arnold, 1976; Dawley, 1986; Verrell, 1989). Plethodontid salamanders rely on chemical cues for territorial advertisement, sex identification, mate assessment and, in some cases, species recognition (review by Mathis et al., 1995; Marco et al., 1998). Surprisingly, very little research has been carried out to determine if substrate-borne chemical signals are used to maintain reproductive barriers between these salamander species (but see Verrell, 1989; Verrell, 2003). The goal of the present study is two-fold. First, we aim to determine whether closely related large Eastern *Plethodon* species are capable of accurately assessing sex- and species-specific substrate-borne chemical signals. Secondly, we ask if variation in the chemical signal alone is enough to explain the existing patterns of isolation, wherein sexual isolation is stronger between sympatric species than it is between allopatric species.

## 2. CHEMICAL COMMUNICATION IN TERRESTRIAL SALAMANDERS

Salamanders from the genus *Plethodon* are a morphologically conservative group of nocturnal, terrestrial animals that are incapable of vocalizing. Visual and auditory signals therefore play minor roles in their ability to find, attract or sexually persuade mates. During the breeding season, male plethodontid salamanders detect and precisely track pheromone trails left by passing females by tapping their nasolabial grooves on the substrate (Gergits and Jaeger, 1990). Nasolabial grooves are furrows on the snout, extending from the edge of each naris down to the upper lip. When an animal taps its snout to the substrate (or the skin of another salamander), moisture containing chemosensory information is passed up the grooves and into the nares through capillary action (Brown, 1968; Dawley and Bass, 1989). The chemicals are then directed laterally to chemoreceptors in the highly specialized vomeronasal epithelium (Dawley and Bass, 1989). The vomeronasal receptor neurons project to the accessory olfactory bulb (Schmidt, 1988), and presumably the information is then transmitted via the amygdala to a region in the hypothalamus known to be involved in reproductive behavior (cf Halpern, 1987). Thus, these salamanders can easily acquire information via substrate-borne chemical signaling and these signals may play a fundamental role both in promoting conspecific interactions and in maintaining reproductive barriers between species.

This study involves three closely related species of large, Eastern salamanders within the genus *Plethodon*: *P. shermani*, *P. montanus* and *P. teyahalee*. Two of these species, *P. montanus* and *P. shermani*, are geographically separated from one another and sexual isolation has evolved in allopatry (Stalker index = 0.342, where 0 = random mating and 1 = complete isolation; Reagan, 1992). Where *P. teyahalee* and *P. montanus* are found in sympatry, morphological, molecular and behavioral evidence indicate that hybridization does not occur (Stalker index = 1.0, Reagan, 1992; Highton and Peabody, 2000). In contrast, *P. teyahalee* and *P. shermani* hybridize extensively in many areas of contact and, in one region, the parental species have been replaced entirely by hybrids (Stalker index = 0.017 - 0.586; Reagan, 1992; Highton and Peabody, 2000). There are no obvious differences in the courtship behaviors of these species (Arnold, 1976; Reagan, 1992), nor is there a correlation between genetic divergence and levels of reproductive isolation (Reagan, 1992).

Dawley (1984, 1986, 1987) demonstrated that some species of large Eastern *Plethodon* are capable of detecting and responding to sex- and species-specific air-borne chemical stimuli. When presented with substrate-borne odors, however, these *Plethodon*

species were unable to distinguish sex- or species-specific odors (Dawley, 1984). This lack of detection is surprising for three reasons: (1) our understanding of the structure and function of nasolabial grooves, as well as the associated vomeronasal organ, illustrates a mechanism by which substrate-borne chemical cues can be readily acquired and processed, (2) *Plethodon* salamanders inhabit moist terrestrial environments where transport of these signals is easily facilitated and where non-volatile molecules can persist for longer periods than volatile ones, and (3) small Eastern *Plethodon* species have been shown to detect and respond to substrate-borne territorial signals (review by Mathis et al., 1995). For these reasons, it is generally well accepted that terrestrial salamanders rely on substrate-borne chemical cues when selecting reproductive partners, despite a limited amount of experimental evidence. The animals used in Dawley's (1984) experiments, however, were not in reproductive condition, and sensitivity to odors is known to increase during the mating season (Toyoda and Kikuyama, 2000). Furthermore, amphibian sex pheromones show considerable seasonal variation and are usually only produced during the breeding season (Rollmann et al., 2000; Wabnitz et al., 1999). If the chemical signals used as sex and/or species identifiers are produced and broadcast during the mating season, we should be able to detect individual preferences at that time.

## 3. METHODS AND RESULTS

### 3.1. Collection and Maintenance of Salamanders

We collected 20 adult males and 20 adult females for each of three species, *P. teyahalee, P. montanus* and *P. shermani,* between 2-16 August 2001. The *P. shermani* were captured from Macon County, North Carolina (lat 35°10'48", long 83°33'38"), and *P. teyahalee* and *P. montanus* were collected from a single locality in Madison County, North Carolina (lat 35°48'50", long 82°56'58"). We obtained females with enlarged ova and males with visible mental glands to ensure that the animals used in the experiment were in reproductive condition. The animals were shipped to Oregon State University where they were maintained at 13-15°C on a natural (Corvallis, OR) photoperiod. The salamanders were housed individually in plastic shoeboxes (31 X 16.5 X 9 cm) with damp paper towel substrates and fed two waxworms (*Galleria mellonella*) each week.

### 3.2. Test Protocol

The odor preference experiments were conducted in the laboratory from 27 August to 18 October 2001, a time period corresponding with the breeding season for each of these species. Odor sources were obtained by lining the bottom of a small plastic box (17 X 12 X 6 cm) with a piece of single-ply filter paper moistened with dechlorinated water. A scent-marking animal was placed on top of the filter paper and allowed to move about the box for a period of 24 hr. A blank scent was produced by moistening a piece of filter paper with dechlorinated water and storing it in a covered enclosure for 24 hr.

Fifteen salamanders of each sex and of each species were tested in a set of eight odor preference trials (Figure 1; 120 tests per species). The order in which an animal performed a particular trial was assigned randomly. For each trial, we used a clear, rectangular box (31 X 16.5 X 9 cm) as the experimental chamber. Each side of the box contained a piece of marked filter paper (or a blank) with a space of 3.5 cm between the

two substrates to prevent diffusion of chemical cues between sides.  The side of the box in which the odors were placed was randomized.  At 1800 hr, the experimental animal was introduced to the center of the experimental box and the animal's behavior was observed under dim red illumination.  Data were collected every two minutes for a period of 2 hrs (60 observations / animal).  We recorded:  (1) the side of the box where the animal's head was positioned, (2) whether the animal was tapping the substrate (i.e. nasolabial grooves in repeated contact with the substrate, Arnold 1976), and (3) if the animal was located on the wall of the experimental chamber (indicating lack of contact with chemical signals).  Nose tapping behavior was recorded to assess the general activity level of the animals and is not considered an independent measure from which to infer odor preferences.  We simultaneously tested 55 animals (on average) each trial night, and provided salamanders with at least one day of rest between trial nights.

### 3.3. Data Analysis

Individuals were tested for side preferences (right versus left) by comparing the number of times an individual animal was found on either side of the experimental chamber during each of its eight trials.  Animals displaying a preference for one side of the box over the other, regardless of odor choices, were excluded from the analyses.  In addition, if an animal spent more than half of the testing period on the wall of the experimental chamber it was excluded from the analysis.  For each scent preference trial, we tallied the number of observations out of 60 that the animal spent on each side of the experimental arena.  Data were subjected to a Wilcoxon matched-pairs signed ranks test to test the null hypothesis that a salamander would spend an equal amount of time (30 observations) on both sides of the experimental chamber if it had no odor preference.

### 3.4. Female Odor Discrimination

For each of the three *Plethodon* species, a single female exhibited a statistically significant preference for one side of the box over the other throughout her eight trials.  These three females were excluded from the analysis.  In addition, individuals from 22 *P. teyahalee* tests, 25 *P. shermani* tests and 19 *P. montanus* tests spent more than half of the time on the wall of the experimental chamber and so were not included in the statistical analyses.  Females did not exhibit a preference for one substrate odor over the other in 22 of 24 trials (Table 1).  However, when given a choice between a blank and the odor of a *P. shermani* male, *P. montanus* females spent the majority of their time on the substrate with no odor (Table 1, P = 0.01).  Similarly, *P. shermani* females spent most of their time on the substrate with no odor when the other option was a substrate marked by a *P. teyahalee* male (Table 1, P = 0.03).

### 3.5. Male Odor Discrimination

Two *P. shermani* males and one *P. montanus* male demonstrated a preference for one side of the box over the other throughout the eight trials and were excluded from the study.  In individual tests, 16 *P. teyahalee* tests, 11 *P. shermani* tests and 13 *P. montanus* tests had males that remained on the wall of the experimental chamber for over half of the experimental duration and were not included in the analyses.  Males did not exhibit a preference during blank versus conspecific male odor trials, blank versus either

heterospecific female odor trials, or trials in which two heterospecific female odors were present (Table 1). In most trials, males did not display a preference for conspecific female versus heterospecific female odors. The one exception is the *P. montanus* trial in which males exhibited a preference for conspecific female odors over *P. teyahalee* female odors (Table 1, P = 0.03). Males of all three species preferred the odor of conspecific females to that of a blank (Table 1, *P. teyahalee*: P = 0.03; *P. shermani*: P = 0.03; and *P. montanus*: P = 0.001). Males of all species also displayed a preference for conspecific female odors over conspecific male odors (*P. teyahalee*: P = 0.04; *P. shermani*: P = 0.01; and *P. montanus*: P = 0.001). In all trials, males were significantly more active (more nose taps) than females (P = 0.001).

**Table 1.** Preferences of three species of *Plethodon* in trials with two substrate-borne odor choices. In each trial, the female or male was given a choice of two substrates marked by another salamander. Response to substrate was measured every two minutes for two hours (for a total of 60 observations). T is the value of the Wilcoxon matched-pairs signed-ranks test, p is the two-sided probability with significance of * α <= 0.05 and ** α <= 0.01. C = conspecific, H = heterospecific. For *P. teyahalee*, H1 = *P. shermani*, H2 = *P. montanus*; for *P. shermani*, H1 = *P. montanus*, H2 = *P. teyahalee*; and for *P. montanus*, H1 = *P. shermani*, H2 = *P. teyahalee*

| | FEMALES: | | | | | MALES: | | | |
|---|---|---|---|---|---|---|---|---|---|
| Species | N | Mean response to substrate | | T | p | Species | N | Mean response to substrate | | T | p |
| **Trial 1** | | Blank vs. C♂ | | | | **Trial 1** | | Blank vs. C♂ | | | |
| *P. teyahalee* | 14 | 34.2 | 25.8 | 39.5 | 0.41 | *P. teyahalee* | 13 | 38.5 | 21.5 | 25.0 | 0.27 |
| *P. shermani* | 8 | 17.1 | 42.9 | 8.0 | 0.19 | *P. shermani* | 11 | 34.9 | 25.1 | 21.5 | 0.31 |
| *P. montanus* | 10 | 22.3 | 37.7 | 14.5 | 0.16 | *P. montanus* | 11 | 28.3 | 31.7 | 29.0 | 0.72 |
| **Trial 2** | | Blank vs. C♀ | | | | **Trial 2** | | Blank vs. C♀ | | | |
| *P. teyahalee* | 11 | 28.7 | 31.3 | 30.0 | 0.79 | *P. teyahalee* | 12 | 16.7 | 43.3 | 9.5 | 0.03* |
| *P. shermani* | 9 | 29.6 | 30.4 | 22.0 | 0.95 | *P. shermani* | 11 | 18.8 | 41.2 | 1.0 | 0.03* |
| *P. montanus* | 11 | 36.5 | 23.5 | 24.0 | 0.42 | *P. montanus* | 14 | 10.8 | 49.2 | 7.0 | 0.00** |
| **Trial 3** | | C♂ vs. C♀ | | | | **Trial 3** | | C♂ vs. C♀ | | | |
| *P. teyahalee* | 11 | 31.8 | 28.2 | 30.0 | 0.79 | *P. teyahalee* | 11 | 11.0 | 49.0 | 10.0 | 0.04* |
| *P. shermani* | 12 | 28.5 | 31.5 | 28.5 | 0.69 | *P. shermani* | 13 | 16.8 | 43.2 | 5.0 | 0.01* |
| *P. montanus* | 14 | 27.2 | 32.8 | 52.0 | 0.98 | *P. montanus* | 12 | 17.4 | 42.6 | 2.0 | 0.00** |
| **Trial 4** | | Blank vs. H1♂ | | | | **Trial 4** | | Blank vs. H1♀ | | | |
| *P. teyahalee* | 12 | 32.2 | 27.7 | 31.0 | 0.53 | *P. teyahalee* | 12 | 18.2 | 41.8 | 23.0 | 0.21 |
| *P. shermani* | 13 | 35.2 | 24.8 | 36.0 | 0.51 | *P. shermani* | 12 | 30.7 | 29.3 | 38.0 | 0.94 |
| *P. montanus* | 9 | 52.0 | 8.0 | 0.0 | 0.01* | *P. montanus* | 12 | 26.8 | 33.2 | 20.0 | 0.45 |
| **Trial 5** | | Blank vs. H2♂ | | | | **Trial 5** | | Blank vs. H2♀ | | | |
| *P. teyahalee* | 12 | 28.9 | 31.1 | 38.5 | 0.97 | *P. teyahalee* | 13 | 33.3 | 26.7 | 33.0 | 0.64 |
| *P. shermani* | 10 | 47.3 | 12.7 | 6.0 | 0.03* | *P. shermani* | 13 | 29.4 | 30.6 | 43.0 | 0.86 |
| *P. montanus* | 10 | 29.4 | 30.6 | 26.5 | 0.92 | *P. montanus* | 10 | 41.4 | 18.6 | 14.5 | 0.19 |
| **Trial 6** | | C♂ vs. H1♂ | | | | **Trial 6** | | C♀ vs. H1♀ | | | |
| *P. teyahalee* | 8 | 26.6 | 33.4 | 16.5 | 0.83 | *P. teyahalee* | 14 | 36.0 | 24.0 | 37.5 | 0.94 |
| *P. shermani* | 13 | 33.5 | 26.5 | 35.5 | 0.49 | *P. shermani* | 11 | 34.3 | 25.7 | 16.5 | 0.26 |
| *P. montanus* | 12 | 30.6 | 29.4 | 32.0 | 0.58 | *P. montanus* | 14 | 27.1 | 32.9 | 44.5 | 0.62 |
| **Trial 7** | | C♂ vs. H2♂ | | | | **Trial 7** | | C♀ vs. H2♀ | | | |
| *P. teyahalee* | 11 | 28.5 | 31.5 | 30.0 | 0.79 | *P. teyahalee* | 14 | 41.8 | 18.2 | 30.0 | 0.16 |
| *P. shermani* | 13 | 27.1 | 32.9 | 36.0 | 0.51 | *P. shermani* | 12 | 34.1 | 25.9 | 25.0 | 0.27 |
| *P. montanus* | 14 | 25.9 | 34.1 | 39.0 | 0.40 | *P. montanus* | 13 | 42.2 | 17.8 | 15.0 | 0.03* |
| **Trial 8** | | H1♂ vs. H2♂ | | | | **Trial 8** | | H1♀ vs. H2♀ | | | |
| *P. teyahalee* | 11 | 22.6 | 37.4 | 24.0 | 0.42 | *P. teyahalee* | 15 | 25.5 | 34.5 | 46.0 | 0.43 |
| *P. shermani* | 9 | 20.9 | 39.1 | 11.0 | 0.17 | *P. shermani* | 10 | 30.7 | 29.3 | 27.5 | 0.99 |
| *P. montanus* | 13 | 27.9 | 32.1 | 39.5 | 0.68 | *P. montanus* | 13 | 31.5 | 28.5 | 39.5 | 0.68 |

## 4. DISCUSSION

This study indicates that mating interactions for large Eastern *Plethodon* species depend largely on the male's ability to perceive, distinguish and respond to substrate-borne chemical signals produced by the female. Although females of these species produce a chemical signal that attracts males, females did not use substrate-borne odors to locate mating partners during our laboratory experiments. These females may, however, assess male chemical signals during courtship interactions as the male makes physical contact and applies courtship pheromones directly onto the female's nares.

Males of all three species displayed a strong preference for substrates previously occupied by a conspecific female when the alternative was a conspecific male odor or a substrate with no salamander odor. These males showed no interest in heterospecific female odors under the same testing conditions, suggesting that these chemical signals have diverged in allopatry. When males were given a choice between a substrate previously occupied by a conspecific female odor and a substrate containing a heterospecific female odor, however, the attraction for the conspecific was no longer apparent. Thus, the strength of the conspecific signal is dampened when the male is simultaneously introduced to a heterospecific female's scent. In one case, however, male *P. montanus* displayed a strong preference for the female conspecific when the alternate odor was that of a heterospecific *P. teyahalee* female. In this case, *P. montanus* and *P. teyahalee* exist in sympatry without hybridizing, indicating that the divergence of chemical cues is greater between sympatric species.

### 4.1. Discussion of Female Behavior

Female *P. shermani, P. montanus* and *P. teyahalee* did not respond preferentially to sex-specific or species-specific substrate-borne chemical cues in our laboratory experiments. The lack of a distinct preference and reduced activity that we observed could accurately reflect the natural behavior of these species during the mating season. In most signal/receiver systems involved in mate attraction, one sex produces a signal while the other sex detects and pursues that signal. While there have been observations of male *P. shermani* rapidly following the trails of passing females (Gergits and Jaeger, 1990; Reagan, 1992), similar behaviors have not been reported for females. In our study, male salamanders tapped the substrate much more frequently than did females, indicating that the males more actively sample the environment, presumably in search of a mate. This difference in male and female search behavior may correlate with a sexual dimorphism of the vomeronasal organ (VNO). In VNO studies of a related plethodontid salamander, *P. cinereus,* the male has a significantly larger VNO than the female during the breeding season (Dawley, 1992). Furthermore, observations from staged courtship encounters in the laboratory indicate that males pursue and initiate courtship, whereas the females are initially passive (CP, personal observations). In our tests, *P. shermani* and *P. montanus* females spent approximately twice as much time on substrates marked by conspecific males than on substrates with no salamander odor (a trend that was not statistically significant). Similarly, in four out of six trials, females visited the substrate with no salamander odor more frequently than the substrate bearing a heterospecific male odor, but in only two of these four trials was this difference statistically different.

A similar odor-choice experiment was conducted by Dawley (1986, 1987), who examined female responses to air-borne chemical signals in large Eastern *Plethodon*

species. Dawley (1986) concluded that females always showed indifference to hetero-specific male odors, but were attracted, repulsed or unresponsive to conspecific males. Results from two of our trials also suggest that females are repulsed by substrates bearing heterospecific male odors. The *P. shermani* females avoided *P. teyahalee*, and *P. montanus* females avoided *P. shermani* male odors, both spending significantly more time on the substrate with no salamander odor. When these females were exposed to substrates marked by the same heterospecific males in two other trials, however, the avoidance response was no longer apparent (Table 1). For this reason, evidence for a genuine avoidance response seems lacking. In fact, based on the data from all eight trials, we conclude that females of the *Plethodon* species used in this study do not use substrate-borne chemical cues to appraise and/or locate potential mating partners.

Our conclusion that females do not use substrate-borne odors to actively locate mates does not imply that these salamanders are incapable of detecting and assessing male chemical signals. Pheromones delivered during initial contact between mates, as well as during courtship interactions, may play a significant role in female mate choice. Chemical signals can be conveyed to the female directly from the surface of the male's body as the pair comes into physical contact (Arnold, 1976). Furthermore, pheromones are delivered directly to the female's nares when the male 'slaps' his mental gland onto her snout during courtship (Organ, 1958; Arnold, 1976). Experimental studies show that the application of this proteinaceous courtship pheromone results in a significant increase in female receptivity, indicating that pheromone delivery improves courtship success (Houck et al., 1998; Rollmann et al., 1999). Courtship pheromone delivery does not guarantee insemination, however, and the female may leave the courting male following pheromone delivery (Reagan, 1992). Thus, females may use chemical signals during courtship to identify and assess appropriate mating partners and these courtship pheromones may play a role in sexual isolation.

### 4.2. Discussion of Male Behavior

Male *P. shermani, P. montanus* and *P. teyahalee* are fully capable of discriminating between sex-specific odors and show a strong preference for female chemical cues. When the males were given a choice between a conspecific female odor and a substrate containing no odor, all three species showed a significant preference for the female odor. In addition, males preferred substrates marked by conspecific females to those marked by conspecific males. In a third type of trial, males presented with a conspecific male odor and a substrate with no salamander odor did not discriminate between substrates. These results provide compelling evidence that males are displaying a sex-specific response and are not merely attracted to the scent of any other salamander and/or avoiding substrates that have been marked by conspecific males.

Large eastern *Plethodon* species emerge from their underground burrows at nightfall to forage and to mate. Because these animals occupy and defend their own burrows, they do not have immediate access to a mating partner on a given night, nor do they rely on acoustic or visual signals to attract or discern an appropriate mate. Instead, the results of our laboratory tests suggest that a strong sex-specific chemical signal is produced by the female and this signal can easily be detected by a conspecific male. When the female deposits this pheromone on a moist, terrestrial substrate in a natural setting, the male should be able to locate the female relatively quickly. In Dawley's (1984) experiments, males did not respond to female substrate-borne odors outside of the breeding season.

We infer that immature and non-gravid females produce a signal that is distinct from the odor advertised by gravid females or that this sex-specific signal is absent altogether in non-breeding females.

Male preferences for the odor of a conspecific female are straightforward, but results of tests using heterospecific female odors yield mixed results. When *P. shermani*, *P. teyahalee* and *P. montanus* males were allowed to choose between a substrate marked by a heterospecific female and a second substrate with no odor, the males did not display a preference. Behavioral observations in the laboratory reveal that male terrestrial salamanders frequently fail to initiate courtships with heterotypic females (review by Arnold et al., 1993). Our results indicate that the odors of the heterospecific females are either not recognized by the male or that these chemical signals fail to carry reproductive significance and are simply ignored. Although *P. teyahalee* males spent more than twice as much time on substrates bearing *P. shermani* female odors than on the substrate with no salamander odor, the results were not statistically significant. However, *P. teyahalee* males may indeed be attracted to *P. shermani* female odors given that these two species hybridize in many areas where they come into contact with each other (Highton and Peabody, 2000).

Both *P. shermani* and *P. teyahalee* males failed to respond to a conspecific female odor when a heterospecific female odor was presented simultaneously. This result is similar to that observed for female swordtail fish, wherein the response to a conspecific odor was stronger when the alternative choice was water rather than a heterospecific odor (Crapon de Caprona and Ryan, 1990). This pattern of discrimination suggests that although large Eastern *Plethodon* males prefer the odor produced by their own females, males are capable of perceiving heterospecific female signals. The fact that males are capable of perceiving differences between these odors supports the notion that mate-recognition systems can evolve in allopatry (Crapon de Caprona and Ryan, 1990).

The *P. montanus* males, on the other hand, maintained a strong preference for conspecific female odors when the second odor was that of a *P. teyahalee* female. Similarly, *P. teyahalee* males spent more than twice as much time on conspecific female odors than on substrates marked by *P. montanus* females, but this result was not statistically significant. The asymmetry in male response to conspecific and heterospecific female odors (*P. teyahalee* showed no strong preference, *P. montanus* preferred conspecific female odors) is unexplained, as these two species are sympatric and reproductive isolation is complete (Reagan, 1992). Male *P. montanus* spent more than twice the amount of time on the substrate without a salamander scent than on the substrate containing *P. teyahalee* female odors (results were not statistically significant). Thus, there is strong evidence that female signals attract conspecific mates, and there is some suggestion that these signals may also function to repel sympatric, heterospecific males. In any event, the chemical cue produced by the female salamander provides species-specific information that may play a large role in maintaining this reproductive barrier. The mate-recognition system of large Eastern *Plethodon* species has evolved in allopatry, but the signaling system is more specialized in areas where the species co-exist.

## 5. SUMMARY AND PROPOSAL FOR FUTURE WORK

In this terrestrial salamander system, chemical signals are broadcast in the environment by reproductively active females and are detected by the males as they tap

their nares to the substrate. These signals function in mate recognition and have evolved in allopatry. There is evidence that these chemical cues have diverged further in areas where closely related salamander species are sympatric, presumably to prevent hybridization. Whether the pattern of signal divergence disclosed by this study reflects true character displacement as defined by Dobzhansky (1937) has yet to be established. To date, research on hybrid viability in this system has not been conducted, presumably because of the difficulties that are associated with mass-rearing of terrestrial salamander eggs in the laboratory. A focus on post-mating isolation is essential, for pre-mating isolation may evolve in response to post-mating consequences.

Furthermore, the chemical signals involved in sexual isolation and their site of production have yet to be identified. Proteins are good candidate molecules as sex-attractants in terrestrial salamanders and warrant investigation. To date, all of the pheromones that have been characterized for amphibian mating systems have been proteins (Rollmann et al., 1999; Wabnitz et al., 1999; Toyoda and Kikuyama, 2000). In addition, a protein signal may have greater stability in terrestrial environments than other molecules. For example, rodents release proteins in their urine and these proteins encase a bound volatile molecule. In this arrangement, the volatiles are transformed into stable signals as they are time-released into the environment during the relatively slow process of protein degradation (Hurst et al., 1998). If the signals used in salamander mate-recognition are indeed proteins, the sequences for the genes encoding the proteins can be determined. Furthermore, the evolution of these sequences can be analyzed and models of the selective pressures acting on the gene (i.e., neutral, diversifying or stabilizing selection) can be tested (Yang, 2000). From this analysis, we can gain a better understanding of what processes caused the signal to change. Ultimately, this system has the potential to help us understand the selective processes involved in the evolution of reproductive isolation.

## 6. ACKNOWLEDGEMENTS

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