2019

Species Variation in a Pheromone Complex is Maintained at the Population Level in the Eastern Red-Backed Salamander

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Abstract.—Protein pheromones in salamanders of the genus *Plethodon* have experienced rapid and pervasive directional selection. Variation in mate recognition components, such as the sex-specific pheromones used by plethodontid salamanders, may influence sexual incompatibility and therefore provide a biochemical mechanism for the maintenance of discrete, isolated populations. Recent studies suggest that multiple, genetically distinct lineages of Eastern Red-Backed Salamanders (*Plethodon cinereus*) are present throughout their broad range. Representative populations from two of these lineages (the Ohio [OH] and Pennsylvania [PA] clades) span the southern shore of Lake Erie in northern Ohio. This distribution pattern creates a unique opportunity to study how phenotypic differences may reinforce population boundaries and possibly lead to speciation. The objectives of this study were to 1) characterize the pheromone profiles of male *P. cinereus* and 2) determine pheromone variation among populations and between the OH and PA clades. The composition of proteins associated with two known courtship pheromones, Plethodontid Modulating Factor (PMF) and Plethodontid Receptivity Factor (PRF), were compared among eight populations in northern Ohio. Analyses of Similarity (ANOSIM) suggest that both PMF and PRF profiles differ among populations but not between clades. These data suggest that the sex-specific pheromones of *P. cinereus* in the two clades are not yet different enough to allow reproductive isolation between the two genetic lineages. Although the relative roles of selection and genetic drift are unknown in our populations, specific pheromone isoforms and their effects on mate compatibility should be the focus of future studies aiming to determine mechanisms involved in maintaining population differences.

Studying pheromone divergence among populations that vary in degree of geographic isolation might help reveal the mechanisms responsible for reproductive isolation (reviewed in Smadja and Butlin, 2009). Geographic distance between populations can affect the ability of individuals to recognize conspecifics as potential mates. In *Desmognathus*, behavioral differences correlate with geographic distance among allopatric populations; populations that are farther apart geographically have potentially undergone longer divergence periods and may have more-disrupted gene flow (Tilley et al., 1990). In a classic example of a "ring species," *Ensatina* populations form a ring of adjacent, interbreeding populations around a central barrier but experience reproductive isolation where distributions reconnect (Kuchta and Wake, 2016). Pheromone chemistry in species such as plethodontid salamanders might be a driver in the sexual isolation of populations, and further studies should focus on differences in courtship pheromones via biochemical characterization (Arnold et al., 1993).

In most plethodontids and all *Plethodon*, each reproductive male develops a mental gland during the breeding season which functions exclusively to synthesize a nonvolatile courtship pheromone that directly affects female behavior (Woodley, 1994; Feldhoff et al., 1999). These pheromones, termed courtship pheromones, influence mate choice and reproductive success such that application increases female receptivity and decreases duration of courtship encounters (Houck and Reagan, 1990; Rollmann et al., 1999). Salamanders of the genus *Plethodon* exhibit a stereotyped courtship during which a reproductive male delivers courtship pheromones from his mental gland to the female by either a scratching or slapping mechanism (Arnold, 1972). Courtship behavior, gland morphology, and modes of pheromone delivery show relative evolutionary stasis in plethodontid salamanders whereas the pheromone protein complex appears to be undergoing rapid directional selection (Watts et al., 2004; Palmer et al., 2007, 2010). Two protein components, Plethodontid Modulating Factor (PMF) and Plethodontid Receptivity Factor (PRF), constitute the majority of the pheromone complex; each protein has multiple isoforms that males express in varying proportions (Feldhoff et al., 1999; Houck et al., 2007; Wilburn et al., 2012). Variation is present among and within populations (Rollmann et al., 2000; Chouinard et al., 2013). Although PMF is present in all plethodontid species, PRF can be found only in the four groups of eastern *Plethodon* (sensu Highton and Larson, 1979), most of which deliver pheromones using the derived mode of direct application to females' nares via slapping (Palmer et al., 2005; Wilburn et al., 2014). More basal clades of plethodontid salamanders also possess Sodefrin Precursor-Like Factor (SPF), and Palmer et al. (2007) hypothesized that PRF is replacing SPF in eastern *Plethodon*. Within one eastern group, the *Plethodon cinereus* complex of plethodontid salamanders, *P. cinereus* express PRF but retain the ancestral mode of pheromone delivery. In this mechanism males use hypertrophied premaxillary teeth to vaccinate the female, delivering pheromone directly into the bloodstream through dermal abrasions to the dorsum (Houck and Arnold, 2003). Because of this unique combination of the ancestral mode of pheromone delivery and the presence of the derived PRF protein complex, *P. cinereus* is a good candidate for studying the evolution of the pheromone complex as a whole.

Plethodon cinereus occupy a wide geographic range (Burton and Likens, 1975) and, across a continuous distribution, these dispersal-limited salamanders can show genetic differentiation when separated by as little as 200 m (Cabe et al., 2007). Consequently, there has been opportunity for isolation and evolutionary divergence. There are six genetically distinct lineages of this species in the eastern United States, two of which occur in northern Ohio: the Ohio (OH) and Pennsylvania (PA) clades. The close proximity of representative populations from both of these clades in northern Ohio allows for a unique and powerful opportunity to study possible mechanisms involved in the maintenance of distinct lineages.

TABLE 1. Locality information for populations used in this study. Reproductive adult *P. cinereus* were collected from eight populations in northern Ohio.

Clade	Locality name	ID	County	Latitude	Longitude
OH	Oak Openings Preserve	OAK	Lucas	41.54580	−83.84564
	West End Cemetery	WEC	Erie	41.31745	−82.49799
	Edison Woods Reserve	EDW	Erie	41.34042	−82.48331
	Findley State Park	FIN	Lorain	41.12204	−82.21156
PA	Brecksville Reservation	BRK	Cuyahoga	41.31430	−81.59882
	Manatoc Boy Scout Camp	MAN	Summit	41.22303	−81.52951
	The West Woods	WWD	Geauga	41.45626	−81.32829
	Idlewood Road	IDL	Ashtabula	41.75258	−80.91426

The goals of this study were to 1) characterize the pheromone profile of male *P. cinereus* and interpret findings in context of the evolution of pheromone delivery, and (2) compare pheromone profiles across populations and between the two distinct clades of *P. cinereus* located in northern Ohio. We hypothesized that populations from each of the two clades would differ in their pheromone composition (relative amount and presence of individual peaks) among both PMF and PRF; such differences would suggest that pheromones might act as a mechanism in the maintenance of distinct lineages. Therefore, we also hypothesized that differences between clades would be more pronounced than differences within clades, even if the geographic distance between clades is smaller than the distance between sites within a clade.

Materials and Methods

Collection and Housing.—We conducted fieldwork in the fall of 2015 (September–November); localities included eight populations across northern Ohio (see Table 1), and we visited each one in a randomized order to limit seasonal effects on pheromone composition. We collected 15 reproductive male *P. cinereus* by hand from under natural-cover objects at each locality. Because previous studies have reported differences in ecology and behavior between color morphs in this region (Anthony et al., 2008; Acord et al., 2013; Reiter et al., 2014; Paluh et al., 2015), we collected only striped animals for our study. We determined reproductive condition visually by the shape of the snout (Anthony et al., 2008), the presence of hypertrophied premaxillary teeth, and enlarged cloacal glands (Houck and Sever, 1994). In the laboratory, we housed each male in an individual glass 470-mL Pyrex dish with a moist, crumpled paper towel to create both a substrate and refugia. We housed animals at 16°C on a natural light-dark cycle. Once per week, we fed animals 25–30 wingless *Drosophila melanogaster*. We recorded snout–vent length (SVL) and mass (g) for each animal within 2 d of capture. Size of individuals varied in both SVL (32–50 mm) and mass (0.5–1.7 g).

Gland Removal.—We randomly placed reproductive males from each of the eight populations into three subsamples of five individuals. We surgically removed the mental gland of each male immediately after euthanasia (November 2015) following methods of pheromone extraction and analyses adapted from Rollmann et al. (1999). Using forceps, we lifted the skin of the mentum and cut using a #11 scalpel blade. We removed the section of ablated skin and any remaining glandular tissue and rinsed them in amphibian Ringer's solution. We pooled the glandular tissue of five individuals (a population subsample) together in 200 µL of 0.8 mM acetycholine chloride in amphibian Ringer's solution for 60 min with gentle mixing every 10 min to induce pheromone secretion from tissue. We then centrifuged the extract for 10 min at 10,000 × *g* and collected the supernatant. We then performed a second 10-min centrifugation and stored the final supernatant at −20°C until we conducted analyses at the University of Louisville (December 2015).

Pheromone Analysis.—We analyzed pheromone samples at the University of Louisville. We determined the protein content of each sample using a bicinchoninic acid assay (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, Massachusetts, USA). To determine relative differences in protein profiles among populations, we used 25 µg of each sample. We characterized samples using reverse phase–high-performance liquid chromatography (RP-HPLC) on a 2695 Alliance HPLC System with a 2487 dual wavelength absorbance detector set to 220 nm and with Empower Software (Waters Corporation, Milford, Massachusetts, USA). We eluted the column (C-18, 0.46 × 15 cm; Grace Davison Discovery Sciences, Chicago, Illinois, USA) with a gradient of 0–70% acetonitrile (ACN) at a rate of 1% ACN/min. We ran pheromone samples on the HPLC in three consecutive blocks. Each block included 10 runs on the machine: one 25-µg sample of *Plethodon shermani* whole extract and nine *P. cinereus* pheromone samples (25 µg each). *Plethodon shermani* whole extract has been previously characterized (Feldhoff et al., 1999; Wilburn et al., 2012) as a reference standard that allowed us to make comparisons of *P. cinereus* samples within and between blocks.

We performed two transformations of the raw data to calibrate the data set. First, we randomly selected a peak that was present in all samples to standardize the alignment of retention times. Then, we adjusted absorbance values to account for minor shifts that occur during sequential runs on the HPLC. Using pheromone profiles of the three *P. shermani* whole extract samples run at the beginning of each block, we created a linear regression between absorbance value and sample sequence number (out of 30 total samples) and applied it to the *P. cinereus* data. In all cases, standardizations resulted in minimal, if any, changes to data, and we performed them as a precaution. We analyzed peaks for retention times associated with isoforms of PMF and PRF components. We considered peaks within retention times of 28–37.98 min to be PMF-like and those with times of 49–59.98 min to be PRF-like (after Wilburn et al., 2014). We calculated the relative amount of protein in each individual peak (peak area) by approximating the area under the curve using multiple rectangles (Riemann sum) based on the raw output of the detector.

Statistical Analyses.—We performed two-way nested (populations within clades) analysis of similarity (ANOSIM) tests to detect differences in pheromone composition (both peak area and presence of individual peaks) across all subsamples. Similar to ANOVA, ANOSIM is a nonparametric test that instead uses dissimilarity matrices rather than raw data. Thus ANOSIM is

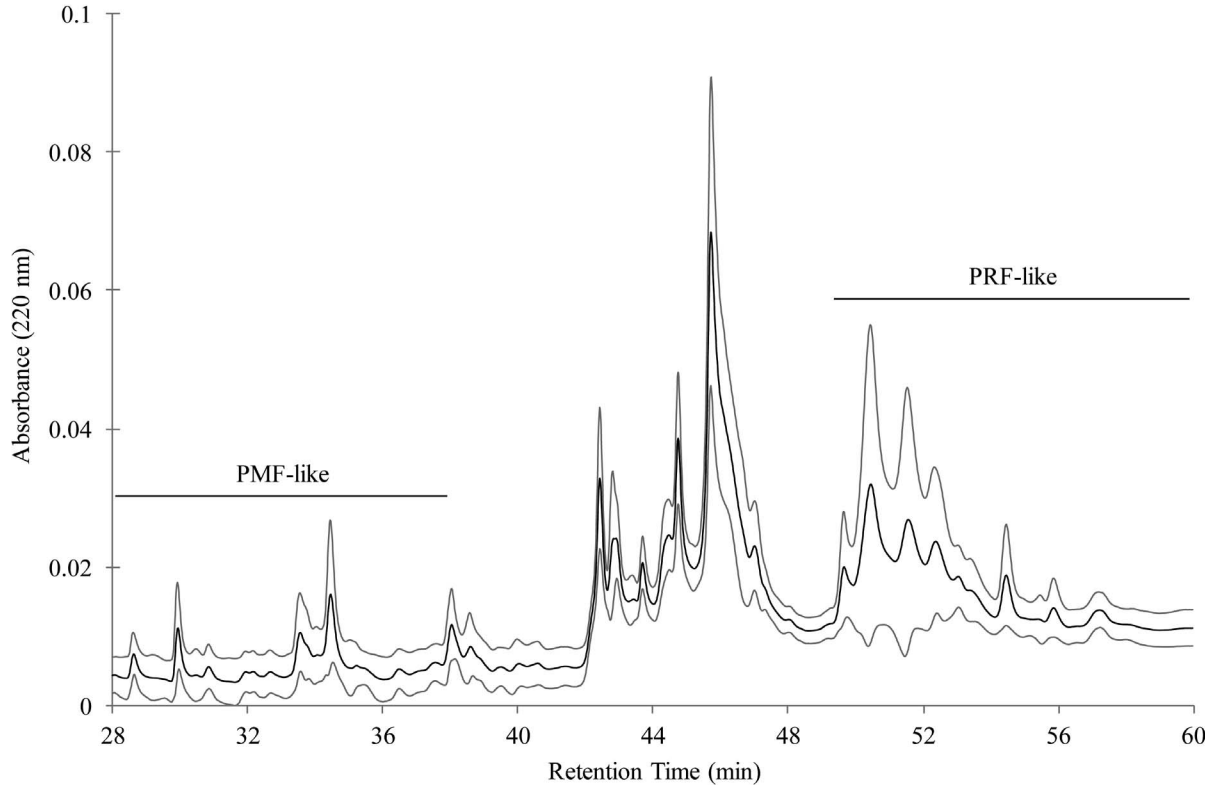

FIG. 1. Mean chromatogram of all samples ($n = 24$) in black with grey lines above and below showing ± standard deviation (SD) at each time point. Chromatogram created by plotting mean of sample absorbance at each retention time between 28–59.98 min. PMF-like proteins occur between 28–37.98 min; PRF-like proteins between 49–59.98 min. Intermediate peaks have yet to be characterized, but are thought to include some serum contaminants.

well-suited to testing for differences among defined groups such as species assemblages or, in our case, the pheromonal composition of different populations. We used nonmetric multidimensional scaling (nMDS) to visualize peak variation among populations and between clades for both PMF and PRF regions. We performed these analyses in PRIMER (v6) based on Bray-Curtis dissimilarity matrices (Beals, 1984).

We performed mantel tests in RStudio (v.0.99.893) using the package 'vegan' (vers. 2.3–4, R Foundation for Statistical Computing, Vienna, Austria, available from https://cran.r-project.org/web/packages/vegan/) to explore relationships between pheromone composition and both geographic distance and patristic (genetic) distance across populations. First, we compared matrices of pheromone composition and geographic distance of populations using the Pearson method. Then, we compared pheromone composition to patristic distance among populations (Radomski et al., unpubl. data). For each test, we identified the predictor variable as either geographic or patristic distance and the response variable as pheromone composition. We used dissimilarity matrices generated by the above ANOSIM tests to compare pheromone composition among populations (both PMF-like and PRF-like regions). We estimated geographic distance between sites with the distVincentyEllipsoid (default, WGS 1984) function in the 'geosphere' package in R (vers. 1.5–1, R Foundation for Statistical Computing, Vienna, Austria, available from https://CRAN.R-project.org/package=geosphere). We calculated patristic distance from tree branch length to describe the amount of genetic change or divergence within lineages (Stuessy and König, 2008).

RESULTS

We identified 33 peaks across all subsamples: 20 peaks with retention times between 28–37.98 min (PMF-like) and 13 peaks with retention times between 49–59.98 min (PRF-like). A measurement of variability showed that samples varied in their mean absorbance values across all retention times. This was especially evident within PRF-like peaks with retention times ranging from 49–51 min (Fig. 1).

Our first ANOSIM indicated that pheromone-like proteins significantly differed among populations in northern Ohio. Proteins showed statistically significant variation in overall pheromone composition (Fig. 2; Table 2). In the second ANOSIM, however, peak area did not differ between the OH and PA clades for either set of proteins, and clades did not differ in the presence of specific peaks within each pheromone-like component (Fig. 2; Table 2).

Geographic distance was not a significant predictor of pheromone differences among populations in either PMF-like or PRF-like proteins (Table 3). We detected no relationship between pheromone composition and genetic differences; there was no relationship between differences in composition and patristic distance among populations (Table 3).

DISCUSSION

Although Wilburn et al. (2014) examined pheromone composition of a pooled sample of male *P. cinereus* from a single population in Virginia, ours is the first study to examine differences in pheromone composition within and among populations of two distinct genetic clades. Our comparison among protein profile regions known to be associated with

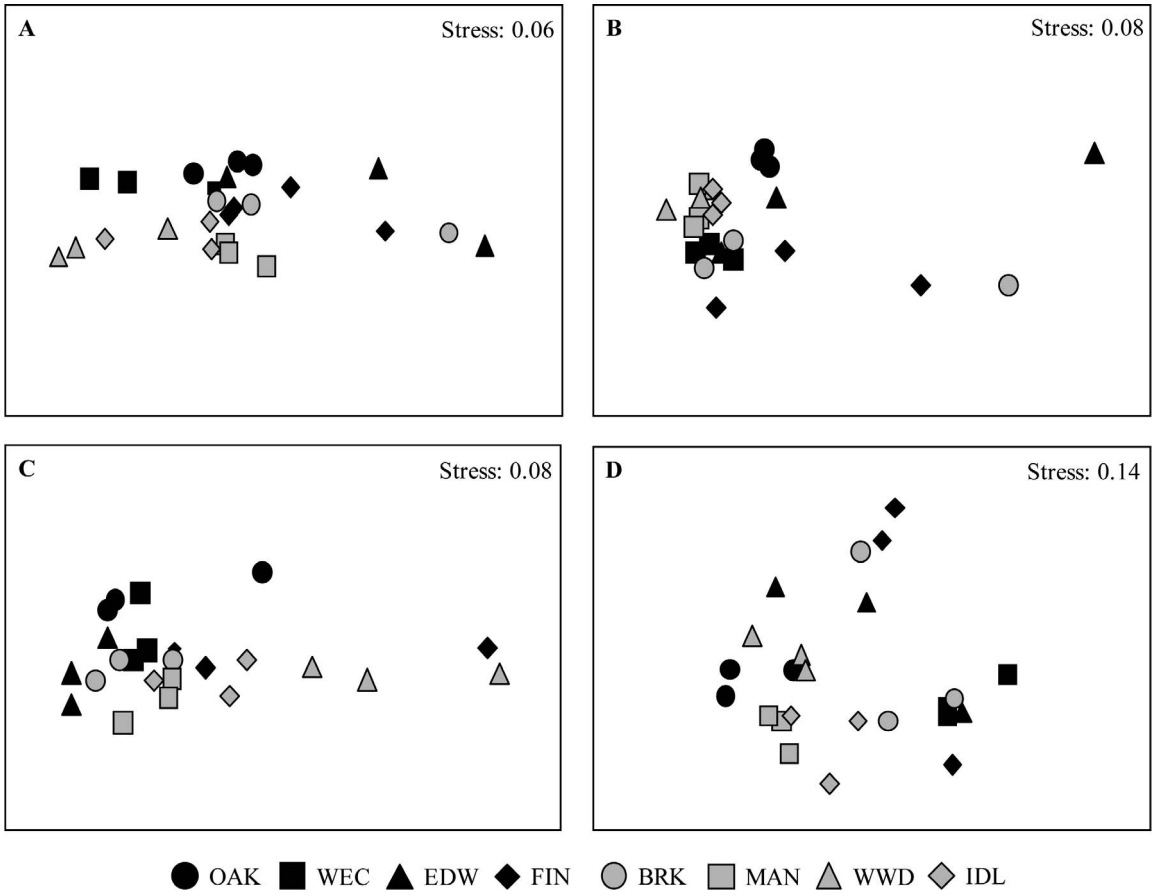

FIG. 2. Nonmetric multidimensional scaling (nMDS) plots depicting variation in male pheromone composition among pooled subsamples from eight populations and between two clades of *P. cinereus* in northern Ohio. Points that appear in close proximity represent subsamples that have similar pheromone composition. Stress values indicate how well the two-dimensional rendering reflects the multidimensional space. Stress values below 0.2 are considered very good. Populations are indicated by different symbols. Black symbols are populations within the OH clade and grey symbols represent populations in the PA clade. Top panels are PMF-like peaks (A) peak area and (B) presence. Bottom panels are PRF-like peaks (C) peak area and (D) presence.

plethodontid courtship pheromones (PMF and PRF) indicates that populations differ in peak area and presence of isoforms. Despite our expectations, clade membership was not a predictor of differences in pheromone composition in northern Ohio. Additionally, we found no evidence that geographic or genetic distance affected variation in pheromone composition.

Our study adds to a body of evidence suggesting that pheromone composition varies both within and between species. For example, species in the *Plethodon jordani*-complex (sensu Highton and Peabody, 2000; Weisrock and Larson, 2006) differ in their protein profiles as measured by the presence of peaks and relative amounts of PMF and PRF (Rollmann et al., 2000). Even within species, protein profiles differ. Similar to our study, Rollmann et al. (2000) found that the pheromone composition of male *Plethodon montanus* differed between two allopatric populations, and Chouinard et al. (2013) described variation among over 100 individual *P. shermani* from a single population. In both of these studies the total number of peaks differed among individuals (Chouinard et al., 2013) and populations (Rollmann et al., 2000). Another unique aspect of our study is that we compared multiple populations ($N = 8$)

TABLE 2. Two-way nested analysis of similarity (ANOSIM) tests were performed to detect variation in profile composition of PMF-like and PRF-like proteins among populations and between clades of male *P. cinereus*. Significant *P*-values are bolded.

Level of variation	Composition characteristic	Protein	Global R	*P*-value
Population	Peak Area	PMF	0.349	**0.002**
		PRF	0.458	**0.001**
	Presence	PMF	0.347	**0.001**
		PRF	0.448	**0.001**
Clade	Peak Area	PMF	0.042	0.343
		PRF	-0.125	0.829
	Presence	PMF	0.208	0.114
		PRF	0.083	0.371

TABLE 3. Mantel test results comparing variation in pheromone composition to both geographic and genetic distance among populations of male *P. cinereus* in northern Ohio.

Distance variable	Composition characteristic	Protein	*r*	*P*-value
Geographic	Peak Area	PMF	0.0361	0.426
		PRF	−0.0821	0.760
	Presence	PMF	−0.1072	0.793
		PRF	−0.0202	0.628
Genetic	Peak Area	PMF	−0.0679	0.845
		PRF	−0.0139	0.685
	Presence	PMF	−0.2593	0.998
		PRF	−0.2041	0.990

across geographic and genetic distance (two distinct clades). We found no effect of either genetic relatedness or geographic distance on pheromone composition in *P. cinereus*. In dispersal-limited species, genetic distance correlates with geographic distances (Slatkin and Maddison, 1990); however, genetic distance does not always correlate well with reproductive isolation. For example, Tilley et al. (1990) found that sexual isolation (via mate incompatibility) increased with geographic distance, but it was not correlated with genetic distance. Our inability to detect an effect of geographic distance on pheromone composition is surprising given the limited dispersal capability of *P. cinereus* (Cabe et al., 2007; Ousterhout and Liebgold, 2010). However, in estimating the distances between populations in our data analyses, we used the shortest distance between geographic coordinates and did not consider the permeability of the intervening landscape (Peterman et al., 2014; Cameron et al., unpubl. data). Geographic features such as streams and rivers (Marsh et al., 2007; Hantak et al., 2019) and fragmentation of habitat (Marsh et al., 2008; Cameron et al., unpubl. data) directly affect dispersal of *P. cinereus*. Therefore, the inaccuracy inherent in using straight line distances may have undermined our ability to detect a geographic effect.

Adaptive explanations for the patterns that we observed would necessarily invoke fitness differences in male pheromones. For example, previous studies suggest that female choice can affect the evolution of male pheromones via runaway selection (Palmer et al., 2010; Wilburn et al., 2015) and that variation within populations may be a consequence of specific pheromone isoforms differing in their efficacy in increasing female receptivity, as seen in studies of *P. shermani* (Wirsig-Wiechmann et al., 2006; Houck et al., 2008; Wilburn et al., 2015). Similarly, our observation that variation in PRF-like proteins exceeds that of PMF-like proteins might result from selection (via female choice) acting differentially on the PRF isoforms. Such processes could result in the pattern that we observed of apparently random differences among populations. One might be tempted to argue that differences may represent local adaptive peaks (sensu Wright, 1949); however, without further research into how pheromone variation influences reproductive success in our populations, the mechanisms behind our described patterns remain unidentified. Examining differences in the isoform presence and peak area of each pheromone component (PMF and PRF) would be a worthwhile avenue of future research, as such data could help to clarify the evolution of this pheromone complex.

Although adaptive explanations for our results seem feasible, we must consider that random patterns of isoform variation emerge through processes such as genetic drift. In other words, the patterns that we have described are simply random. This possibility raises interesting implications. For example, non-adaptive differences among populations may suggest that specific isoform types and quantities are less important to male fitness than is the production of any diverse complement of PRF-like proteins (Wirsig-Wiechmann et al., 2006; Wilburn et al., 2015). In other words, female salamanders may select males with diverse pheromone profiles, but the specific patterns of diversity may be unimportant. Additionally, if the evolution of isoform profiles is decoupled from geographic space and clade membership (either via random genetic drift or female choice), then reproductive isolation between populations becomes difficult to predict in a geographic and evolutionary context. One consequence may be that the likelihood of hybridization at contact zones between clades does not depend on pheromone variation but instead on time spent in isolation. For example, the extensive hybridization among species following rapid diversification in the *Plethodon glutinosus* group (Wiens et al., 2006), illustrates how short divergence times can undermine reproductive isolation in *Plethodon*.

In this study, we found that reproductive male *P. cinereus* vary in their pheromone-like protein profiles among populations in northern Ohio, but that suites of isoforms present in the males do not correlate to either genetic patterns between lineages or geographic differences among populations. Although components of mating systems might be expected to have an influence on reproductive isolation in groups of organisms that are geographically separated and genetically distinct, this pattern is not apparent in the species and populations studied. Because our results did not support our hypothesis, we are unable to rule out the possibility that differences among populations may simply be because of random variation and therefore do not extend to similar patterns of variation between clades. Future studies should focus on chemical pheromone variation as a potential driver in reproductive isolation. Examining differences in the isoform presence and peak area of each pheromone component (PMF and PRF), and performing behavioral trials to test the efficacy of, and female preference for, specific male pheromone isoforms, could help to clarify the roles of selection vs. random evolutionary processes in the evolution of this pheromone complex.

Acknowledgments.—We thank R. Saporito and J. Johansen for their comments on previous versions of this manuscript. We also thank D. Wilburn for his contribution to project design and analyses. P. Feldhoff and K. Bowen provided training and assistance to C. Kunkel on lab techniques. We thank S. Bolton, C. Kunkel, A. Cameron, B. Waldron, M. Ganzfried, and M. Hantak for field assistance. Funding for this project was provided by John Carroll University and a National Science Foundation collaborative grant to RCF: IOS-1146899. Collection of all specimens was conducted under an Ohio Department of Natural Resources collection permit (16-85) to CLK. This research was conducted with prior approval of the Institutional Animal Care and Use Committee at John Carroll University (IACUC protocol number JCU 1302).

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