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Model toxin level does not directly influence the evolution of mimicry in the salamander *Plethodon cinereus*

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Abstract The resemblance between palatable mimics and unpalatable models in Batesian mimicry systems is tempered by many factors, including the toxicity of the model species. Model toxicity is thought to influence both the occurrence of mimicry and the evolution of mimetic phenotypes, such that mimicry is most likely to persist when models are particularly toxic. Additionally, model toxicity may influence the evolution of mimetic phenotype by allowing inaccurate mimicry to evolve through a mechanism termed 'relaxed selection'. We tested these hypotheses in a salamander mimicry system between the model *Notophthalmus viridescens* and the mimic *Plethodon cinereus*, in which *N. viridescens* toxicity takes the form of tetrodotoxin. Surprisingly, though we discovered geographic variation in model toxin level, we found no support for the hypotheses that model toxicity directly influences either the occurrence of mimicry or the evolution of mimic phenotype. Instead, a link between *N. viridescens* size and toxicity may indirectly lead to relaxed selection in this mimicry system. Additionally, limitations of predator perception or variation in the rate of phenotypic evolution of models and mimics may account for the evolution of imperfect mimicry in this salamander species. Finally, variation in predator communities among localities or modern changes in environmental conditions may contribute to the patchy occurrence of mimicry in *P. cinereus*.

Keywords Batesian mimicry · *Notophthalmus viridescens* · *Plethodon cinereus* · Relaxed selection · Tetrodotoxin

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Introduction

Batesian mimicry, in which a palatable mimic species resembles an unpalatable model species (Bates 1862), has long stimulated questions on the origins of interspecific resemblance. For example, how does model unpalatability influence mimic phenotype? Experimental studies have found that model unpalatability can promote the evolution and maintenance of mimicry (Goodale and Sneddon 1977; Lindström et al. 1997), though the relationship between unpalatability and mimicry remains understudied (Darst and Cummings 2006). In many Batesian mimicry systems, unpalatability is the consequence of toxic chemicals produced by the model (Ruxton et al. 2004). The toxicity of models may influence both the occurrence of mimicry (Endler 1991; Lindström et al. 1997) and the evolution of mimic phenotype (Duncan and Sheppard 1965; Goodale and Sneddon 1977; Lindström et al. 1997). For example, Endler (1991) and Lindström et al. (1997) have hypothesized that mimicry evolves and is maintained where models are most toxic. Once established in a community, mimic phenotype may evolve in one of two ways. One might expect that in the presence of highly toxic models, mimics will evolve toward an ever more perfect resemblance of model phenotype.

However, mimics are frequently observed that closely, but not perfectly, resemble their models (Ruxton et al. 2004). In such cases, model toxicity is thought to play a role by deterring predators from attacking individuals carrying the model phenotype, including imperfect mimics. Under the relaxed selection hypothesis, predators will increasingly avoid mimics as the penalty for mistakenly attacking models increases, particularly through increased toxicity (Schmidt 1958; Duncan and Sheppard 1965; Sherratt 2002; Penney et al. 2012; Kikuchi and Pfennig 2013). Thus, selection against imperfect mimics will weaken as models become more toxic (Duncan and Sheppard 1965; Darst et al. 2006). Support for the relaxed selection hypothesis would consequently appear as a negative relationship between model toxicity and mimetic perfection, with imperfect and variable mimics occurring with highly toxic models (Goodale and Sneddon 1977; Lindström et al. 1997). Finally, high intrapopulation variability in model toxicity may be more aversive to predators than low variability (Barnett et al. 2014), and thus may be positively associated with mimic presence and imperfect mimicry.

The salamanders *Notophthalmus viridescens* (model) and *Plethodon cinereus* (mimic) are an ideal system to study the relationship between model toxicity and mimicry. Juvenile *N. viridescens* are bright orange-red and confer toxicity through tetrodotoxin (TTX; Yamashita and Mebs 2001). TTX is a potent neurotoxin that blocks the pore region of voltage-gated sodium channels (Narahashi et al. 1967) and potential predators find TTX noxious (Brodie 1968). After tasting and rejecting an *N. viridescens* individual, predators tend to avoid any prey that phenotypically resemble *N. viridescens* (Brodie 1968). As such, predators have generalized this rejection to other species of orange-red salamanders (Howard and Brodie 1973), including the erythristic color morph of *P. cinereus* (Brodie and Brodie 1980; Tilley et al. 1982). Predators do not avoid non-mimic *P. cinereus*, which indicates that *P. cinereus* are not toxic to predators (Brodie and Brodie 1980; Tilley et al. 1982). Thus, erythristic *P. cinereus* are hypothesized to be Batesian mimics of *N. viridescens* (Lotter and Scott 1977).

In this study, we tested for a relationship between model toxin level and mimicry by sampling terrestrial salamander communities from 32 localities across western Massachusetts. From each community we collected erythristic *P. cinereus* and *N. viridescens* for color quantification, and from the *N. viridescens* individuals we quantified dermal

concentrations of TTX to estimate toxin level. If model toxicity influences the distribution of mimicry, we predict that *N. viridescens* toxin level will be positively associated with erythristic *P. cinereus* presence and abundance. Additionally, variation in toxin level within localities will be positively associated with erythristic *P. cinereus* presence and abundance. Finally, if model toxicity influences the evolution of mimic phenotype, we predict that imperfect mimicry (as measured by color variation or difference between mimics and models) will be positively associated with *N. viridescens* toxin level and toxin variability.

Materials and methods

Study system

We examined the relationship between model toxicity and Batesian mimicry with two salamander species: the model *Notophthalmus viridescens* (Rafinesque 1820) and its Batesian mimic, the erythristic color morph of *Plethodon cinereus* (Green 1818). These species are widely distributed in northeastern North America and overlap across much of their respective ranges. *N. viridescens* has a triphasic life cycle that includes a secondary, juvenile (eft) stage, in which efts are terrestrial and strikingly red–orange in coloration (Petranka 1998). Eft skin contains TTX (Mebs et al. 2010), which is a potent neurotoxin that makes them highly unpalatable to predators (Brodie 1968). The orange coloration of efts is interpreted as a warning signal of toxicity to most natural predators (Brodie 1968). The terrestrial salamander *Plethodon cinereus* exhibits several discrete color morphs, including striped, unstriped, and erythristic. Only the erythristic form of *P. cinereus* is qualitatively similar in coloration to efts (Lotter and Scott 1977). *P. cinereus* are unlike *N. viridescens* in that they lack TTX and are palatable to predators (Brodie and Brodie 1980; Tilley et al. 1982). Of several potential predator classes, birds are considered the predators driving the evolution of mimicry in this system (Lotter and Scott 1977; Brodie and Brodie 1980; Tilley et al. 1982; Kraemer and Adams 2014). Interestingly, while both *N. viridescens* and *P. cinereus* are commonly encountered at many localities in western Massachusetts, the erythristic color morph of *P. cinereus* is uncommon and typically occurs only in a subset of localities (Tilley et al. 1982; Kraemer and Adams 2014).

Salamander collection

In May and June 2011, we collected a total of 123 erythristic *P. cinereus* and 318 eft-stage *N. viridescens* salamanders from 32 localities in western Massachusetts, USA. We visited each locality on three separate occasions, intensely searching under cover objects and in leaf litter for 1 h per occasion. *P. cinereus* are territorial and non-migratory (Petranka 1998). Visual searching for this species is therefore the most effective method for detection (Smith and Petranka 2000). Furthermore, visual searches were unlikely to introduce novel patterns in the data, as an identical search format was used at each locality. *N. viridescens* were found at 32 localities, and erythristic *P. cinereus* were found at 14 localities (*see* Supporting Information Table S1). All individuals were first anesthetized using tricaine methanesulfonate (MS-222: *P. cinereus*) or by applying benzocaine to the head (*N. viridescens*). There is the potential for differences in anesthetization to affect estimates of coloration between salamander groups. However, such physiologically-based color changes have only been observed in members of an unrelated salamander lineage (Garcia et al. 2003), and we did not observe such changes in our salamanders.

Color quantification

After anesthetization in the field, we measured salamander spectral reflectance from a single point in the mid-dorsal region of each erythristic *P. cinereus* salamander using a portable JAZ-PX spectrometer (OceanOptics, Dunedin, FL) fitted with a 100 μm entrance slit, a pulsed xenon lamp, and a QR400-7-UV-BX reflectance probe. This probe was equipped with a tip that standardized the measured patch to a 2 mm diameter circle at a distance of 20 mm between probe and measured patch while excluding ambient light. We held the probe perpendicular to measured patches and used a Spectralon white reflectance standard between each animal to correct for drift in lamp intensity (see e.g., Kraemer et al. 2012). Collecting reflectance measurements at a perpendicular angle can introduce undesirable spectral glare, in particular under the circumstances in which coloration is influenced by structural elements in the target animal (Endler 1990). However, the dorsal coloration of these salamanders is composed of pigments with limited structural elements (Bagnara and Taylor 1970), which reduced the potential for problems associated with spectral glare in this study. We measured each spectrum at 1 nm intervals from 300 to 700 nm.

TTX quantification

After collection, we field-preserved *N. viridescens* individuals in liquid nitrogen to be transported to Iowa State University (Ames, IA) for TTX quantification. From each individual, we removed a 5 mm diameter punch of skin (0.015 g) from the dorsal surface between the pelvic and pectoral girdle. Toxin extractions from each punch were prepared sensu Hanifin et al. (2002). Briefly, each sample was finely ground at room temperature with 600 μl of 0.1 M aqueous acetic acid and placed in a boiling water bath for 5 min, then centrifuged at 13,000 RPM for 20 min. All resulting supernatant was transferred to a Durapore PVDF 0.22 μm centrifugal filter tube (Millipore) and spun for 20 additional minutes at 13,000 RPM. This extraction procedure is highly repeatable ($r = 0.95$; Hanifin et al. 1999), and thus does not introduce a significant degree of variation in toxin level estimates. We then quantified the concentration of TTX in each sample using Liquid Chromatography-Mass Spectrometry (LC–MS). To each sample, we added caffeine at a final concentration of 40 ng/μl and applied either 0.2 μl of sample to an LC-C18 column with 90 % MeOH and 10 % H_2O at a flow rate of 0.8 ml/min, or 1 μl of sample to an LC-C18 column with 100 % MeOH at a flow rate of 0.8 ml/min. After separation, samples were sent to an Agilent QTOF 6540 mass spectrometer set to positive ion mode for detection and quantification. Concentration curves used to estimate sample TTX concentration were calculated from the known concentration of caffeine present in each sample (40 ng/μl) and TTX standards prepared from commercial TTX (Abcam). After estimating the concentration of TTX in each sample, we calculated the amount of TTX in each gram of *N. viridescens* skin for analyses.

Model/mimic matching

We modified a visual model developed by Vorobyev et al. (2001) to characterize salamander coloration (Kraemer and Adams 2014) and to estimate the degree of model-mimic matching at each locality. This analytical model estimates discriminability of visual signals from the signal to noise ratio of predator photoreceptors (Vorobyev et al. 1998). The model

yields estimates of visual contrast between targets (model and mimic salamanders), and thus can be used to estimate similarity between mimics and models from the perspective of relevant predators. Because erythristic *P. cinereus* are thought to mimic *N. viridescens* on the basis of color, and not brightness (Kraemer and Adams 2014), we used the visual model to calculate contrasts between models and mimics for the chromatic visual channel (ΔS), which describes the aspects of visual stimuli pertaining to coloration (i.e. chroma and hue). Large contrast scores indicate large and potentially discriminable differences between targets and backgrounds from the predator's perspective, while smaller contrasts indicate close mimicry that is potentially indistinguishable. Specifically, contrasts greater than 1.0 are considered discriminable and potentially apparent to potential predators (Vorobyev et al. 1998).

The visual model requires reflectance measures of targets (i.e. models and mimics), background habitat irradiance (i.e. light environment), and photoreceptor sensitivities of the predator. We used a forest shade irradiance measure reported elsewhere (sensu Kraemer and Adams 2014). Although there are several likely predators of salamanders in this system (Petranka 1998), prior work indicated that the evolution of mimicry in *P. cinereus* is likely the result of predation from tetrachromatic birds (Brodie and Brodie 1980; Tilley et al. 1982; Kraemer and Adams 2014), which we approximated by using the spectral sensitivities of the blue tit (Hart et al. 2000). A full description of visual model calculations is found in Kraemer and Adams (2014). All analyses were conducted in R 3.0.2 (R Development Core Team 2013).

Statistical analyses

We first calculated variation in erythristic *P. cinereus* coloration using brightness-standardized reflectance spectra. We then tested for significant variation in *N. viridescens* toxin level across localities. We tested for a relationship between model toxin level and mimicry by comparing the presence and abundance of mimics at each locality to the mean and variance of *N. viridescens* toxin level. Finally, we tested for a relationship between model toxin level and imperfect mimicry by comparing several measure of mimicry at each locality (the degree of erythristic *P. cinereus–N. viridescens* color match and the degree of multivariate dispersion of erythristic *P. cinereus* coloration) to the mean and variance of *N. viridescens* toxin level.

Color variation

We estimated disparity in erythristic *P. cinereus* coloration among localities by first brightness-standardizing the reflectance spectra from each salamander and then performing a principal components analysis on the standardized reflectance data (sensu Leal and Fleishman 2004). In this representation, all variation remaining after standardization represent aspects of chroma and hue (Endler 1990; Endler and Thèry 1996; Grill and Rush 2000) and thus the color that each spectrum represented. From these data we calculated the multivariate dispersion of salamander coloration at each locality (using 'betadisper' in the vegan library in R).

TTX variation

We performed an analysis of variance (ANOVA) with *N. viridescens* toxin level as estimated from LC–MS as the dependent variable and locality as the group factor to identify if

there is significant variation in *N. viridescens* toxin level across localities. We then tested for spatial dependency of variation in toxin level using the Moran's I statistic as calculated in the 'ape' package of R (Paradis et al. 2004).

Toxicity and mimicry

To identify if mimics occurred where models were most toxic, we performed a logistic regression with the presence of erythristic *P. cinereus* as the response variable and mean *N. viridescens* toxin level as the predictor variable. We then performed a logistic regression with the presence of erythristic *P. cinereus* as the response variable and variance of *N. viridescens* toxin level as the predictor variable. We conducted a series of linear regressions between the mean *N. viridescens* toxin level and erythristic *P. cinereus* abundance (as calculated by total number of mimics observed, the ratio of mimics to models, and the ratio of mimics to all *P. cinereus* observed at the locality). We also conducted a series of linear regressions between the variance of *N. viridescens* toxin level and erythristic *P. cinereus* abundance to identify if any measure of mimic abundance co-varied with model toxin level.

Toxicity and imperfect mimicry

To test for a relationship between imperfect mimicry and model toxicity we performed two sets of linear regressions, (1) between *N. viridescens* toxin level and the degree of color match between *N. viridescens* and erythristic *P. cinereus* from the perspective of bird predators, and (2) between *N. viridescens* toxin level and the multivariate dispersion of erythristic *P. cinereus* coloration among localities. Each linear regression was performed using both mean *N. viridescens* toxin level and the variance in *N. viridescens* toxin level calculated at each locality. TTX dose–response curves are unknown for bird predators, so it is possible that even low levels of *N. viridescens* toxins may deter predators. To account for this we log-transformed TTX concentrations and ran a parallel set of analyses to those described above. Since our results are qualitatively the same, we report only the analyses that use untransformed data. Additionally, dose–response curves and other aspects of predator physiology are required to directly estimate the 'toxicity' of an animal (e.g. Brodie et al. 2002). Consequently, we used toxin levels to estimate toxicity for this study.

Results

TTX variation

We found significant variation in *N. viridescens* toxin levels across localities (Fig. 1; adjusted $R^2 = 0.19$; $F_{31} = 3.24$; $P < 0.001$), with individuals at some localities possessing no detectable TTX and individuals at other localities possessing over 1.0 mg TTX/g of *N. viridescens* skin. This level of variation is comparable to most populations of *Taricha* newts in the Pacific Northwest, with the exception of the most toxic populations of that species (Hanifin et al. 1999). One specimen outlier, possessing over 5 mg TTX/g skin, was removed from analysis. When the spatial proximity of populations was considered, we found no significant geographical autocorrelation of toxin level across localities (Moran's $I = -0.01$; $P = 0.55$).

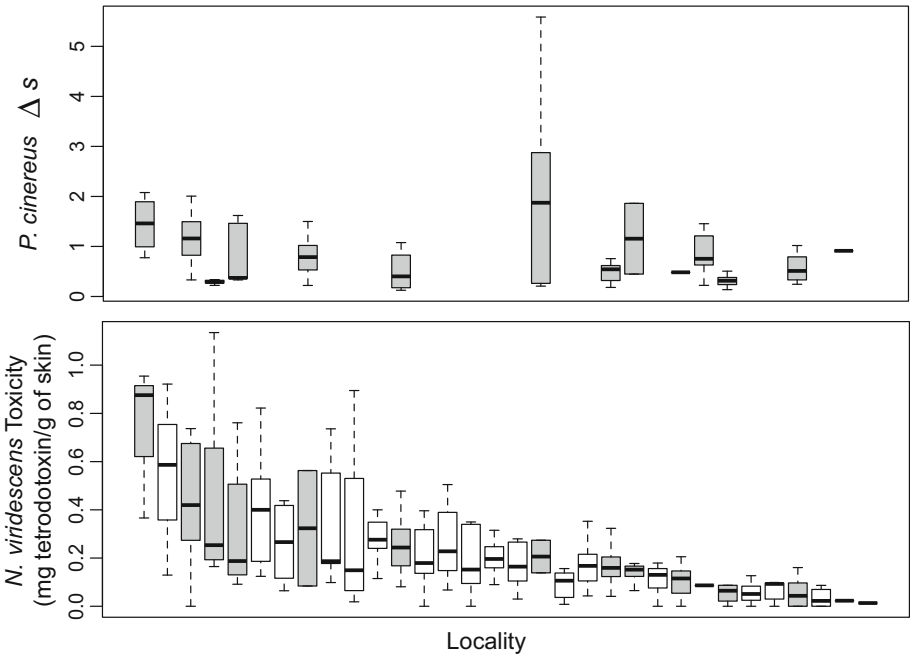

Fig. 1 Variation in mimicry and model toxin level among localities. *Top panel* variation in model-mimic matching as calculated by ΔS between erythristic *P. cinereus* and the average *N. viridescens* found at each locality. *Bottom panel* the concentration of tetrodotoxin of *N. viridescens* individuals among localities. Note that localities are arranged in the same order between panels. *Grey boxes* denote localities with erythristic *P. cinereus*

Toxicity and mimicry

We observed no relationship between *N. viridescens* toxin level and erythristic *P. cinereus* presence (Table 1). We also found no relationship between *N. viridescens* toxin level and erythristic *P. cinereus* abundance as calculated by number of erythristic *P. cinereus* individuals observed at a locality, the ratio of observed erythristic *P. cinereus* to *N. viridescens*, or the ratio of erythristic *P. cinereus* to total salamanders observed at a locality (Table 1).

Toxicity and imperfect mimicry

We found no relationship between *N. viridescens* toxin level and degree of color match between *N. viridescens* and erythristic *P. cinereus*, nor between *N. viridescens* toxin level and multivariate dispersion of erythristic *P. cinereus* coloration (Table 1). Thus, these results do not identify any of the patterns predicted from the hypothesized link between *N. viridescens* toxicity and mimicry in *P. cinereus.*

Discussion

Theoretical (Pilecki and O'Donald 1971) and experimental (Duncan and Sheppard 1965; Goodale and Sneddon 1977; Lindström et al. 1997) studies have indicated that model unpalatability may influence the evolution and maintenance of Batesian mimics. The aim

Table 1 The relationship between *N. viridescens* (model) toxin level and mimicry

	Mean toxin level		Variance in toxin level	
	Z value	*P*	Z value	*P*
(A)				
Erythristic *P. cinereus* presence	0.435	0.663	0.564	0.573

	Mean toxin level			Variance in toxin level		
	Sum sq	F value	*P*	Sum sq	F value	*P*
(B)						
Erythristic *P. cinereus* abundance	20.200	0.384	0.547	37.700	0.697	0.423
Erythristic *P. cinereus*: *N. viridescens*	1.580	0.193	0.669	1.580	0.193	0.669
Erythristic *P. cinereus*: total *P. cinereus*	0.002	0.214	0.652	0.003	0.405	0.539
(C)						
Model-mimic matching	0.220	1.225	0.290	0.019	0.080	0.783
Mimic multivariate dispersion	1.552×10^{-4}	1.630	0.226	3.710×10^{-6}	0.345	0.570

Analyses on the left summarize comparisons between mean model toxin level at each locality and mimicry, while analyses on the right correspond to comparisons between the variance of model toxin level at each locality and mimicry. Note no relationship between (A) mimic presence and model toxin level, (B) any measure of mimic abundance and model toxin level, or (C) between either measure of imperfect mimicry and model toxin level

of this study was to observe the microevolutionary patterns that such a relationship predicts in a natural mimicry system of two salamander species. We found significant variation in model (*Nothophthalmus viridescens*) toxin level among localities, but no spatial dependency in this variation. Toxin level was not directly linked to the presence or abundance of mimics (erythristic *Plethodon cinereus* salamanders), nor was there a relationship between model toxin level and two measures of imperfect mimicry. Furthermore, we found no support for the hypotheses that model toxicity directly influences either the maintenance of mimicry or the evolution of the mimetic phenotype.

Erythristic *P. cinereus* are rare mimics of *N. viridescens* and never exceed $\sim$25 % of the *P. cinereus* population at any locality in western Massachusetts (Tilley et al. 1982; Kraemer and Adams 2014). Kraemer and Adams (2014) previously identified a relationship between mimicry and model presence in this system, such that mimics are only found with models, while models are frequently encountered without mimics. This observation suggests that the presence of unpalatable models at a locality is necessary, but not sufficient, to maintain the presence of mimicry. Kraemer and Adams (2014) also found no relationship between model and mimic abundance where they are syntopic, further indicating that other factors influence the distribution of mimicry in this system. For variation in *N. viridescens* unpalatability to play a role in driving mimicry evolution in western Massachusetts, we expected significant variation in model toxin level among localities (Fig. 1). Despite the variation we found, there was no relationship between model toxin level and the distribution of mimicry. The findings of this study indicate that variation in unpalatability does not directly influence the maintenance of mimicry in this system. While

the breakdown of mimicry in the absence of models is well established, such examples of mimicry breakdown typically involve larger spatial scales than those examined here (e.g. Platt and Brower 1968; Greene and McDiarmid 1981; Pfennig et al. 2001; Prudic and Oliver 2008). Little research has attempted to address the absence of mimics at localities within the geographic ranges of both models and mimics. Those studies that do examine such fine-scale variation (such as Edmunds and Reader 2014) often find some support for basic predictions of Batesian mimicry, but also evidence that other mechanisms affect mimic distributions. Similarly, additional factors (such as selection from mammal predators; Kraemer et al. *in review*), may limit the distribution of mimics to a subset of localities where *N. viridescens* are present.

Examples of Batesian mimicry are often used to illustrate the power of natural selection to drive phenotypic convergence (Ruxton et al. 2004; Forbes 2009), where selection for mimicry is predicted to result in mimics well matched to their model species (Ruxton et al. 2004). Despite this expectation, many species imperfectly resemble their models, such as hoverflies (Penney et al. 2012), snakes (Savage and Slowinski 1992), and spiders (Edmunds 2000). Imperfect mimicry may evolve under several scenarios (Kikuchi and Pfennig 2013), including relaxed selection on mimics (Schmidt 1958; Duncan and Sheppard 1965; Sherratt 2002; Penney et al. 2012). Relaxed selection states that as the cost:benefit ratio of attacking models and mimics increases, a smaller proportion of mimics will be attacked. Thus, as models become increasingly unpalatable, selection on mimics will relax (see Figure 2 from Kikuchi and Pfennig 2013), resulting in imperfect mimics that are as fit as perfect mimics. Consequently, where models are the most toxic we predicted that mimic phenotype would be the most 'imperfect' and most variable. At the 14 localities where both erythristic *P. cinereus* and *N. viridescens* were present, we found no relationship between model toxin level and our two measures of mimetic imperfection. We also found no relationship between imperfect mimicry and either strict model abundance or relative abundance of mimics and models (Table 1). However, we did find that size and toxin level covaried in *N. viridescens* (Supporting Information Figure S2A,B). Furthermore, where *N. viridescens* were the largest, mimic coloration was more variable and less similar to model coloration (Supporting Information Figure S2C,D). These findings suggest that relaxed selection from predators may be responsible for imperfect mimicry in this system not directly because of variation in toxicity among localities, but perhaps through variation in model size. Large *N. viridescens* individuals may be more effective at deterring predators from attacking orange-red salamanders simply because larger *N. viridescens* individuals carry more effective warning signals. Alternatively, the link between *N. viridescens* size and toxin level might result in a more generalized avoidance of orange-red salamanders by predators at localities where *N. viridescens* are larger, even when *N. viridescens* are not especially toxic. More research that focuses on this relationship is needed, particularly because predator perception of model profitability may be directly responsible for a link between toxin level and size (Speed and Ruxton 2014). Variation in model size among localities may influence the cost:benefit ratio of attacking models and mimics, thereby resulting in relaxed selection on mimics when models are particularly large, although this has not been tested. Thus, the imperfect mimicry we observed may be in part the result of relaxed selection on mimics, though not directly through variation in model toxicity.

Kikuchi and Pfennig (2013) summarize 11 non-exclusive mechanisms that may contribute to the evolution of imperfect mimicry, including relaxed selection. Of these, five mechanisms may play a role in the evolution of mimicry in *P. cinereus* in addition to relaxed selection. First, most color variation in erythristic *P. cinereus* appears to be below the level of detection for relevant bird predators (Kraemer and Adams 2014), which may

prevent predators from distinguishing between models and 'imperfect' mimics (the 'eye-of-the-beholder' hypothesis; Cuthill and Bennett 1993; Dittrich et al. 1993). However, although we find a close match between mimics and models at many localities (Fig. 1, top panel, localities with ΔS values below 1.0), several localities appear to contain imperfect mimics that would be apparent to predators (Fig. 1, top panel, localities with ΔS values above 1.0), which suggests that other factors may be responsible for the presence of imperfect mimicry in this system. For example, differences in brightness (Kraemer and Adams 2014), body shape, or behavior between erythristic *P. cinereus* and *N. viridescens* may allow for satyric mimicry in this system, which requires imperfect mimics to possess sensory cues that confuse potential predators, resulting in greater latency between detection and attack (Howse and Allen 1994). Kraemer et al. (2015) found that *N. viridescens* are most conspicuous at localities with erythristic *P. cinereus*, and among localities where both are found, erythristic *P. cinereus* and *N. viridescens* conspicuousness covaries. These findings suggest that chase-away selection, in which models evolve away from mimics and a time-lag prevents the immediate evolutionary response of mimics (Nur 1970; McGuire et al. 2006; Franks et al. 2009), may be responsible for imperfect mimicry (Kraemer et al. 2015). Finally, multiple predators may select for imperfect mimicry in this system, with some predators selecting for mimicry while other predators impose opposing selective pressures on mimic phenotype. This possibility is particularly interesting, as birds are considered the only predators that select for mimicry in *P. cinereus* (Lotter and Scott 1977; Brodie and Brodie 1980; Tilley et al. 1982). Other predators, such as mammals, likely predate on *P. cinereus* (Petranka 1998), but may not perceive erythristic *P. cinereus* as mimics of *N. viridescens* (Kraemer and Adams 2014). Instead, mammals likely select for inconspicuousness and novel phenotypes in *P. cinereus* (Kraemer et al. *in review*). Additional predators may also influence the links between models and mimics in other ways. For example, the perceived toxicity of models is influenced by the method of handling. Predators that sample salamanders before ingesting, like birds (Tilley et al. 1982), will be more sensitive to the concentration of TTX in newt skin than the total amount of TTX carried by the entire animal. Alternatively, snakes that swallow salamanders whole (Hanifin et al. 2004) will be affected by total-animal TTX. Thus, the influence of TTX on mimicry depends on the way toxicity is calculated. To account for predators affected by total-animal TTX, we tested for a relationship between mimicry and toxicity after accounting for *N. viridescens* size (results not shown). Our results from this set of analyses were concordant with the results reported above, indicating that *N. viridescens* toxicity does not influence the maintenance or evolution of mimicry by predators that ingest whole animals without sampling. Based upon our results, it is unclear how these five mechanisms may interact to contribute to imperfect mimicry in *P. cinereus*. Thus, a better understanding of the predators involved in this system and the variation in predation pressure among localities may help to disentangle the influence of these mechanisms.

In conclusion, we find no direct influence of model toxicity on mimic presence, abundance, or degree of imperfection among localities. The absence of mimics where models are present is an interesting problem that has been given little attention. We find that in *P. cinereus*, the absence of the mimic morph at 18 localities where models occur cannot be attributable to variation in model toxin levels. Instead, other locality-specific factors, such as variation in the predator community (Pekár et al. 2011) or availability of alternative prey (Carpenter and Ford 1953), may influence the distribution of the mimic morph. Additionally, changes in land use, climate, and predator communities among the sampled localities may contribute to the current distribution of erythristic *P. cinereus*. Work that examines the historical and current distribution of *P. cinereus* color morphs in

light of such changes may be able to elucidate the impact of environmental change on the distribution of mimicry in this system. Our findings also allow us to reject the hypothesis that imperfect mimicry in *P. cinereus* is due to relaxed selection from predators because of variation in model toxicity. Instead, imperfect mimicry in *P. cinereus* may be due to variation in model size, limitations of predator vision, the ability of mimics to confuse predators, a time lag between model and mimic evolution, or the existence of multiple predators, each of which will need to be explicitly tested to better understand the distribution and evolution of mimicry in this salamander species.

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