



Developmental temperature influences color polymorphism but not hatchling size in a woodland salamander

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Abstract

Phenotypic plasticity can be an important adaptive response to climate change, particularly for dispersal-limited species. Temperature frequently alters developmental and phenotypic traits including morphology, behavior, and reproductive cycles. We often lack crucial information about if and how thermal conditions during development will interact with genetic responses and facilitate persistence or adaptation under climate change. Polymorphic species offer an ideal test for this, as alternative morphs often confer differential adaptive advantages. However, few studies have examined the effects of incubation temperature on color expression or development in polymorphic taxa. Here we test if developmental temperature mediates morph frequency in the polymorphic salamander *Plethodon cinereus*. Although previous research suggests geographic variation in morph proportions results from differential climate adaptation, it remains unknown if plasticity also contributes to this variation. We used a split-clutch common garden experiment to determine the effects of developmental temperature on the color and development of *P. cinereus*. Our results indicate developmental temperature affects coloration in *P. cinereus*, either via plasticity or differential mortality, with eggs incubated at warmer temperatures yielding a higher proportion of unstriped individuals than those from cooler temperatures. This temperature response may contribute to the spatial variation in morph frequencies in natural populations. Surprisingly, we found neither temperature nor egg size affected hatchling size. Our study provides important insights into the potential for climate-induced responses to preserve diversity in dispersal-limited species, like *P. cinereus*, and enable time for adaptive evolution.

Keywords Plasticity · Adaptation · Plethodontidae · Amphibians · Spatial variation

Introduction

Climate is projected to change rapidly in the coming decades and will likely alter the structure and functioning of biological communities (Petchey et al. 1999; Gilman et al. 2010). Populations may vary in their resilience to climate change either due to underlying evolutionary potential or phenotypic plasticity (Charmantier et al. 2008). Genetic variation

in traits such as phenology, physiology, and behavior has been demonstrated to alter species responses to climate change (Merilä and Hendry 2014). Yet plasticity might be as important as genetic variation for responding to climate change by facilitating rapid shifts in ecologically important traits (Charmantier et al. 2008; Merilä and Hendry 2014). Changes in environmental factors, such as temperature, induce variation in many phenotypic and developmental traits, including morphology (Gomez-Mestre et al. 2010; Tejedro et al. 2010), coloration (Uhlenhuth 1919; Harkey and Semlitsch 1988; Garcia et al. 2003), sex (Valenzuela and Lance 2004), behavior (Brodie III and Russell 1999; Ballen et al. 2015), and reproductive cycles (reviewed in Gotthard and Nylin 1995; Urban et al. 2014).

Polymorphisms are useful for understanding responses to climate change because the long-term persistence of alternative morphs suggests differential adaptive advantages (Moran 1992; Roulin 2004; Gray and McKinnon 2007). Color polymorphisms are by definition under

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genetic control (Fogleman et al. 1980; O'Neill and Beard 2010), although alternative color forms can also occur, at least in part, by phenotypic plasticity (Davison 1964; Harkey and Semlitsch 1988; Garcia et al. 2003; Leimar 2009). Here we use a classic climate-linked polymorphic species, the eastern red-backed salamander (*Plethodon cinereus*), to evaluate the importance of developmental temperature in mediating morph proportions. This species has two main color morphs, striped and unstriped, which also differ in physiology (Moreno 1989; Petruzzi et al. 2006), diet (Anthony et al. 2008; Stuczka et al. 2016), behavioral responses to predators (Moreno 1989; Venesky and Anthony 2007; Reiter et al. 2014), and disease susceptibility (Venesky et al. 2015).

The striped and unstriped color morphs of *P. cinereus* vary in proportion across the species' geographic range (Gibbs and Karraker 2006; Cosentino et al. 2017), and multiple hypotheses have been proposed to explain the observed distribution patterns. In particular, the temperature-dependence hypothesis suggests unstriped individuals tolerate warmer and drier conditions than striped individuals. This hypothesis is supported by observations of greater proportions of unstriped individuals in warmer regions (Lotter and Scott 1977; Gibbs and Karraker 2006; Anthony et al. 2008; Evans et al. 2018; but see Moore and Ouellet 2014) and lower energetic costs for unstriped individuals during warm periods due to lower metabolic rates (Moreno 1989; but see Petruzzi et al. 2006). Early studies suggested that one or a few loci of major effect likely underlie this polymorphism (Highton 1959, 1975), but these studies measured genetic effects in a single environment. Highton (1959) indicated the need for controlled experiments to rule out possible effects of environmental factors. However, no one has tested this possibility, and yet studies continue to assume that the color forms are completely genetically determined (Test 1952; Williams et al. 1968; Lotter and Scott 1977; Pfungsten and Walker 1978; Venesky and Anthony 2007; Fisher-Reid et al. 2013). If this assumption is wrong, then predictions about the genetic and plastic responses of populations to future temperatures also might be inaccurate.

We used a split-clutch common garden experiment to determine how temperature affects the coloration, growth, and development rate of *P. cinereus* embryos. We predicted that eggs incubated at warmer temperatures would yield a higher proportion of unstriped individuals than those incubated at cooler temperatures, due to the temperature-dependence hypothesis. We also tested the effects of temperature on the development and growth rates of *P. cinereus* from multiple populations. We predicted that regardless of color morph or source population, eggs incubated at warmer temperatures (20 °C) would yield smaller hatchlings than those at colder temperatures (15 °C) due to higher metabolic costs experienced by ectotherms at warmer temperatures. This

prediction aligns with the temperature-size rule predicted for ectotherms (Atkinson 1994; Zuo et al. 2012).

Given that life history characteristics can also determine evolutionary responses of populations to climate change, we also compared the reproductive output of the two color morphs. In *P. cinereus*, it has been suggested that unstriped individuals delay reproductive maturity to achieve larger body sizes, whereas striped individuals may reach reproductive maturity earlier and then produce more egg clutches with fewer eggs per clutch across their lifetime (Lotter 1978). Consequently, we predicted that, regardless of source population, color morphs of *P. cinereus* would differ in reproductive output, with unstriped females producing larger clutch sizes, thereby matching the lifetime reproductive output of striped individuals (Lotter 1978).

Methods

We used a split-clutch common garden experiment to examine the effects of incubation temperature on embryo development and growth. We collected 369 wild gravid female salamanders from six polymorphic populations, three in Ohio and three in Connecticut (Online Resource 1). Females were collected between May and June over 3 years (2016–2018), shortly before natural oviposition occurs (Fraser 1980). For all gravid females collected, we recorded morph (striped or unstriped). We also photographed each gravid female collected and measured snout–vent length (SVL, mm) and tail length (TL, mm) from photographs using ImageJ, version 1.48. For practical reasons, we measured SVL from the tip of the snout to the posterior edge of the rear legs, rather than the posterior edge of the vent. This measure is highly correlated with the more typical SVL measure (Mott et al. 2010). Tail length was measured from the posterior edge of the hind legs to the tail tip. To maximize egg sample sizes, we only collected gravid females carrying a minimum of four eggs (eggs are visible through the female's body wall), and where possible, we attempted to collect equal numbers of striped and unstriped individuals even if this meant not collecting females of a certain morph with more than four eggs. During our 2018 sampling, we also recorded the number of eggs yolked by all gravid females (both those collected and not collected from the field), and we photographed gravid females we did not collect before release. The collected gravid females were brought into the laboratory where they were housed in individual plastic containers lined with damp paper towels and fed fruit flies (*Drosophila melanogaster*) ad libitum. The animals were kept at 20 °C and under 24-h darkness to mimic their behavior of retreating inside cover objects or beneath the ground prior to oviposition. Salamanders were given a minimum of 24 h to acclimate to laboratory conditions before oviposition was induced.

To induce oviposition, we injected each salamander intra-abdominally with 0.1 ml of 0.05 mg/ml of mammalian luteinizing hormone-releasing hormone (LHRH, Sigma-Aldrich #L4513), a standard procedure for obtaining eggs in plethodontid salamanders (Jockusch 1996). Eggs obtained via hormonal injections develop normally (Collazo and Marks 1994) and, therefore, this procedure is unlikely to affect the study results. For females that did not oviposit or deposited an incomplete clutch, this injection procedure was repeated up to two more times, with at least 1 week between subsequent injections. Salamanders were chilled on ice for 20–30 min before being injected to reduce stress. We also applied a topical anesthetic Bactine spray to the injection site immediately prior to injections to minimize pain and any potential for infection. For each resulting clutch, we recorded the date(s) of oviposition and number of eggs. We photographed each egg clutch and measured initial egg width and length from photographs using ImageJ, version 1.48. These measurements were used to determine initial egg size, which we calculated as the volume of an ellipsoid ($V = \frac{4}{3}\pi(r_1 \times r_2^2)$), where r_1 is the longer radius and r_2 is the shorter radius of the two initial egg measurements (Furtula et al. 2008). Half of each clutch was assigned to the 15 °C treatment and half to the 20 °C treatment (Homyack et al. 2010). We selected these two experimental temperatures as they reflect temperatures of the forest floor during summer months (Taub 1961; Heatwole 1962) when wild *P. cinereus* are incubating egg clutches.

Within each treatment, each egg was randomly assigned to a well of a six-well perforated histology cassette, and each cassette was placed within a plastic container lined with damp filter paper (Houck et al. 1985). To control for a potential block effect, no eggs within a single cassette ('block') originated from the same clutch, and block position within the incubator was rotated daily. To reduce mortality due to fungal pathogens, we washed eggs daily in 0.5% hydrogen peroxide solution followed by a rinse in distilled water and 0.1 × Marc's Modified Ringer solution (Online Resource 2). Eggs with fungal infection or decay were removed. Upon hatching, we recorded the date, total size (snout–tail length, from photographs measured using ImageJ) and color morph (striped or unstriped) of each hatchling.

Statistical analyses

To determine the effects of incubation conditions on coloration and development and growth of *P. cinereus* embryos, we used a Markov Chain Monte Carlo (MCMC) approach to fit Bayesian generalized linear mixed models in the MCMCglmm package ver. 2.2.2 (Hadfield 2010). To test for temperature effects on hatchling coloration, we used the phenotype (striped or unstriped) of hatchling salamanders as our response variable and assumed binomial errors. For

all coloration plasticity models, we included incubation temperature (15 °C or 20 °C) and mother's phenotype (striped or unstriped) as fixed factors, and year, population, and family as random factors. We used two data sets: (1) a paired dataset that only included salamander clutches with hatchlings surviving at both incubation temperatures (paired sibling families), and (2) data from all surviving hatchlings. Due to high egg mortality, we did not include either Dunhamtown Forest (hatchlings = 0) or Hoffman Forest Metropark (hatchlings = 6, only 2 related) in plasticity analyses. We used a χ^2 distribution with one degree of freedom as our prior distribution on the family variance components (de Villemereuil et al. 2013) and fixed the residual variance at one (Hadfield 2010). Model parameters (iterations, burn-in length and thinning interval) are listed in Online Resource 3. For these, and all subsequent models, we checked for suitable model convergence and mixing using Geweke (1991) and Gelman and Rubin (1992) diagnostics as well as visual inspection of trace plots and posterior distribution plots.

To test the effects of incubation temperature on development and growth of *P. cinereus* embryos, we fit Bayesian linear mixed models with a Gaussian family where the response variable was either development time (number of days between oviposition and hatching) or offspring size at hatching (total length, mm). Hatchling size was transformed using a square root transformation to improve normality. We included temperature, color morph of mother, and color morph of hatchling as fixed factors, and family, population and experimental block as random factors (Gomez-Mestre and Tejedo 2003). We fitted the family variance components using a prior with a χ^2 distribution and the residual variance using an inverse Wishart prior with $V = 1$ and $nu = 0.002$, where V is an estimate of variance and nu is the parameter for the degree of belief in V (Hadfield 2010). To improve model fit, we removed one hatchling outlier with a developmental deformity that failed to hatch on its own, shown in diagnostic tests to have a significant influence on the models.

To test for differences in reproductive output between *P. cinereus* color morphs, we fitted Bayesian generalized linear mixed models using a Poisson distribution, where the response variable was either clutch size (number of eggs laid), or number of yolked eggs (number of eggs visible through the female body wall prior to laying). The analysis of number of eggs yolked was restricted to females recorded during the 2018 surveys (including females both collected and not collected in the wild), as data on wild gravid females encountered but not collected were not recorded for 2016–2017 samples. For all reproductive output models, we included female morph (striped or unstriped), SVL and TL as fixed effects, and source population as a random factor. We used Bayesian linear regression to test for differences in mean initial egg volume (egg size) and mean clutch volume (product of mean egg volume and clutch size) (Milanovich

et al. 2006). Mean egg volume and mean clutch volume were both square root transformed to improve normality. We also included clutch size as a predictor variable when modeling mean initial egg volume. Model parameters (iterations, burn-in length, and thinning interval) varied between our general development models and reproductive models (see Online Resource 3). We determined which parameters were significant by evaluating whether the 95% highest posterior density intervals (HPDI) around the posterior mean overlapped zero. We also estimated the pMCMC statistic, which is calculated as two times the smallest MCMC estimate of the probability that a parameter value is either greater than or less than zero. For each random variable, we assessed heterogeneity, which we calculated as the proportion of the total model variance (i.e., the sum of all variance components in the model) associated with that factor (Prokop et al. 2012). Finally, we used Spearman's rank correlation test to determine whether a relationship existed between initial egg volume and hatchling snout–tail length. All analyses were run in R (version 3.2.3, R Core Team 2013). Data generated or analysed during this study are available in the Online Resources associated with this article.

Results

Over 3 years, we captured 369 gravid females, of which 270 (73%) successfully laid eggs following LHRH injections (Online Resources 1 and 4). Most females began oviposition 3–4 days following LHRH injections, and very few females (1.6%) laid eggs following a third LHRH injection. We successfully raised 100 hatchlings from 51 families across four out of six original populations (Table 1) despite substantial, but normal (Highton 1960; Jockusch 1996), laboratory

mortality from egg to hatchling stage. We raised fewer hatchlings ($n=38$) at 15 °C than at 20 °C ($n=62$).

Phenotypic plasticity Temperature had a marginally significant effect [posterior mean: 0.360, 95% highest posterior density interval (HPDI): $-0.023, 0.768$, Fig. 1a, Online Resource 5] on the morph of hatchling salamanders when using our full hatchling dataset (100 individuals), with 13% more unstriped individuals observed at warmer temperatures compared to cooler temperatures, as predicted (Fig. 2a, Table 1). Likewise, female phenotype in the full hatchling dataset also significantly affected hatchling phenotype (posterior mean: -2.697 , 95% HPDI: $-5.125, -0.243$, Fig. 1a, Online Resource 5) as expected by genetics, with striped females producing 31% more striped hatchlings than unstriped females. However, neither temperature (posterior mean: 0.296, 95% HPDI: $-0.215, 0.785$, Fig. 1a, Online Resource 5) nor female coloration (posterior mean: -3.145 , 95% HPDI: $-7.292, 0.977$, Fig. 1a, Online Resource 5) was significant for the smaller (51 individuals, 16 families) paired sibling dataset (Table 1, Fig. 2b). Including Hoffman Forest Metropark, which had low sample size, in the full plasticity analysis did not change conclusions (Online Resource 6).

We found no differences in the development time between hatchlings of different color morphs (posterior mean: 1.617, 95% HPDI: $-1.925, 5.238$, Fig. 1b, Online Resources 5 and 7), and we also found that hatchling size did not significantly differ between morphs (posterior mean: -0.305 , 95% HPDI: $-0.161, 1.027$) or developmental temperatures (posterior mean: 0.010, 95% HPDI: $-0.180, 0.199$, Fig. 1c, Online Resources 5 and 8). As expected, individuals developed significantly faster at 20 °C with a mean development time of 58 days, compared to 82 days in the 15 °C treatment (posterior mean: -5.197 , 95% HPDI: $-5.727, -4.675$, Online

Table 1 The number of salamander families and hatchlings of each color morph that successfully hatched in each developmental temperature treatment for each population included in our plasticity models

Population	Temperature	Full hatchling dataset		Paired hatchling dataset	
		Families	Hatchlings (striped, unstriped)	Families	Hatchlings (striped, unstriped)
Edison Woods (OH)	15	7	7 (6, 1)	5	6 (5, 1)
	20	13	17 (11, 6)		6 (4, 2)
Hinckley Reservation (OH)	15	8	11 (11, 0)	3	3 (3, 0)
	20	9	16 (15, 1)		5 (4, 1)
Shenipsit State Forest (CT)	15	9	13 (11, 2)	5	8 (7, 1)
	20	10	15 (11, 4)		10 (8, 2)
Moss Tract (CT)	15	4	7 (7, 0)	3	6 (6, 0)
	20	7	14 (12, 2)		7 (7, 0)
Global	15	28	38 (35, 3)	16	23 (21, 2)
	20	39	62 (49, 13)		28 (23, 5)

The full hatchling dataset includes all hatchling salamanders, regardless of whether related hatchlings were present in both of the temperature treatments and the paired dataset only includes salamander families with related hatchlings in both temperature treatments

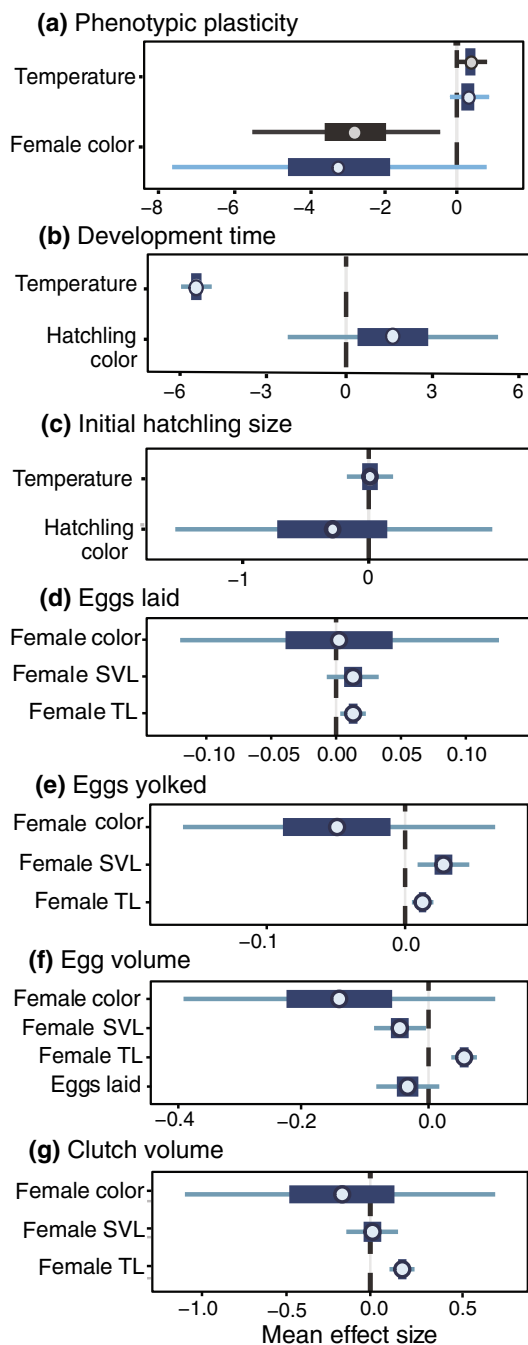


Fig. 1 Mean effect sizes for **a** full hatchling coloration plasticity model (in black, top) and the paired hatchling coloration plasticity model (in blue, bottom), **b** hatchling development time, **c** initial hatchling size, and four reproductive output models for the salamander *Plethodon cinereus*, **d** number of eggs laid, **e** number of eggs yolked, **f** egg volume, and **g** clutch volume. Circles represent the posterior means for each parameter and the horizontal lines represent the 50% (thick lines) and 95% (thin lines) credible intervals. SVL indicates ‘snout–vent length’ and TL indicates ‘tail length’, both measured in mm. Additional information is available from Online Resource 5 (for panels a–d) and Online Resource 10 (for panels e–g)

Resources 5 and 7). Source population, salamander family, and experimental block explained 14.7%, 14.3%, and 1.1% (respectively) of the total variance (heterogeneity) in development time and 16.5%, 10.4%, and 5.5% (respectively) of the total variance in hatchling size (Online Resource 9).

Reproductive output Data on clutch size, egg volume, and clutch volume were collected from 267 egg clutches (3 clutches laid were eaten by the female before data were collected). Color morphs showed no significant differences in any of the four reproductive output measures we recorded after accounting for female size (Fig. 1d–g, Online Resources 10 and 11). For both striped and unstriped females, all four reproductive output measures were significantly positively related to female tail length (Fig. 1d–g, Online Resource 10), meaning that females with longer tails not only yolked and laid more eggs but that egg and clutch volumes were also greater. On average, for every additional 1 mm of tail length, the number of eggs yolked and laid by females increased by 10.1% while average egg and clutch volumes increased by 10.6% and 12.0% respectively. We found no significant differences in tail length between color morphs ($t = 0.360$, $p = 0.719$) but morphs differed significantly in SVL ($t = 2.023$, $p = 0.045$), with unstriped individuals being on average 0.80 mm longer than striped individuals. Only two reproductive output measures were significantly influenced by female SVL—the number of eggs yolked and mean initial egg volume (Fig. 1e, f, Online Resource 10)—where females with larger bodies yolked a larger number of smaller eggs. On average, for every 1 mm of body length, females yolked an additional 1.6 eggs but the average volume of each egg laid decreased by 0.04 mm^3 . Finally, we found that population differences explained 11% of the variance in mean egg volume and 12% of the variance in mean clutch volume. In contrast, population differences explained 92% of the variance in both our models of clutch size and number of eggs yolked (Online Resource 9).

Discussion

Coloration plasticity

Our study suggests that we cannot assume, as many earlier studies have done, that alternative phenotypes in polymorphic species are completely genetically determined. Our work provides the first evidence that color morph may be influenced by temperature during development in plethodontid salamanders. Thus, thermal developmental conditions likely explain some of the variations in relative frequency of color morphs observed in natural populations that has, to this point, remained enigmatic (Harkey and Semlitsch 1988). Recent studies have shown that incorporating measures of genetic variation and evolution into species–climate

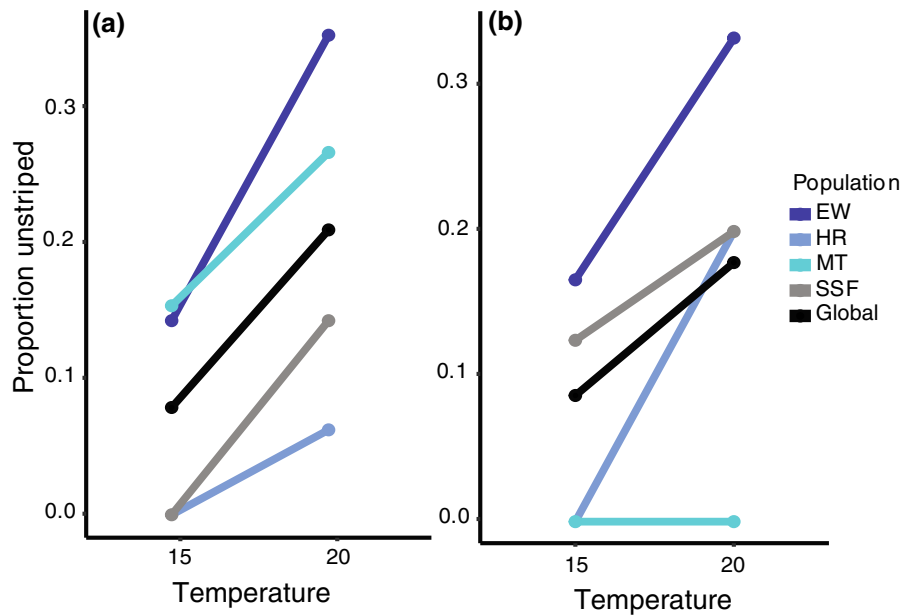


Fig. 2 Population reaction norms of the proportion of unstriped individuals of *Plethodon cinereus* hatching at 15 °C and 20 °C developmental temperatures. Plot **a** shows the reaction norms for the full hatchling dataset regardless of whether related hatchlings were present in both of the temperature treatments; plot **b** shows the reaction norms using the paired dataset (only including salamander

families with related hatchlings in both temperature treatments). The black line shows the global plasticity pattern across all populations, obtained by pooling all hatchling data within each of the developmental temperatures. See Table 1 for sample sizes. Population abbreviations are EW (Edison Woods, OH), HR (Hinckley Reservation, OH), MT (Moss Tract, CT), and SSF (Shenipsit State Forest, CT)

predictive models can substantially alter model predictions (Kearney et al. 2009; Pearman et al. 2010; Chevin et al. 2010; Benito Garzón et al. 2019). Plasticity is taxonomically widespread and can vary within and between populations (Scheiner 1993). Therefore, incorporating measures of plasticity into mechanistic models may resolve some of the ongoing challenges associated with accurately predicting species responses to climate change (Urban et al. 2016; Riddell et al. 2018).

In most polymorphic species, once established, color morph is fixed throughout the life of an individual, yet surprisingly little is known about the role of temperature-induced coloration, particularly in amphibians. To date, previous work is restricted to just four studies on two anurans [ornate chorus frog *Pseudacris ornata* (Harkey and Semlitsch 1988), leopard frog *Rana pipiens* (Davison 1964)] and three salamander species in the genus *Ambystoma* [*Ambystoma barbouri*, *A. texanum* (Garcia et al. 2003), and *A. tigrinum* (Uhlenhuth 1919)]. Our results combined with these previous studies suggest that developmental plasticity could contribute to the maintenance and spatial variation of color polymorphisms in amphibians. Alternative color morphs often differ in ecologically important traits (Forsman et al. 2008), and heterogeneous environments typically impose differential selection on organism traits (DeWitt and Scheiner 2004). Therefore, climate-induced plasticity in ecologically relevant traits may help buffer populations (Canale

and Henry 2010), particularly those that are dispersal-limited such as *P. cinereus*, from rapid climate change.

The expression of the striped phenotype at warmer temperatures as well as cold is likely related to the genetic mechanism(s) that also influences coloration in this species (Highton 1959). Although the results from our full hatchling dataset suggested that developmental temperature may influence coloration in *P. cinereus*, this effect was not significant in the paired sibling dataset, which had a wider confidence intervals, likely the result of small sample sizes in the paired dataset. The temperature effect shown in our results could also be driven by differential mortality during the incubation stage rather than phenotypic plasticity (Burger and Zappalorti 1988; Viets et al. 1994; Reichling and Gutzke 1996). It is difficult to assess the effects of differential mortality as death often occurs before phenotypic traits of interest are detectable (Burger and Zappalorti 1988). Because the genetic architecture underlying the color polymorphism in *P. cinereus* remains uncertain (Highton 1959, 1975), and most egg mortality events occurred prior to the formation of the neural crest (where chromatophores are produced), we could not directly test whether differential mortality altered the morph proportions of surviving hatchlings in our study. We sought to test if selective mortality contributed to our finding that developmental temperature affected the frequency of color phenotypes by testing if greater embryonic mortality occurred in families expected to produce the morph selected

against in each thermal environment. However, this was not the case. Post hoc analyses showed no evidence for differences in egg mortality as a function of female morph or population at either developmental temperature tested in our study (Online Resource 12). Despite this evidence, we cannot completely reject the hypothesis of differential mortality given the limitations of our system. An additional constraint we faced is that sperm storage (Sever 1997) and multiple paternity within clutches are common in this species (Liebgold et al. 2006). It is, therefore, possible that some of our temperature–coloration results could be genetic if eggs from one father made it into one treatment and another father into another treatment. However, because eggs were randomly assigned to temperatures, this possibility should not significantly affect the overall results other than to increase the variance.

Variation in development and reproductive output

Life history characteristics and reproductive output are also expected to be important for determining the ecological and evolutionary responses of populations to climate change (Bradshaw and Holzapfel 2008; Reed et al. 2011). Phenological shifts are among the most common climate change responses (Root et al. 2003; Thackeray et al. 2016), and are often the result of plasticity (Urban et al. 2014). In ectotherms, temperature-induced plasticity in developmental traits such as growth rate, morphology, and time to hatching are frequently observed (Atkinson 1994; Du and Shine 2015), with cooler developmental temperatures typically inducing slower growth and development rates, larger initial hatchling sizes, and an increase in developmental abnormalities (Voss 1993; Kaplan et al. 2006; Ringia and Lips 2007). Our study suggests that *P. cinereus* might benefit from faster developmental rates under projected climate changes without having to compromise on initial hatching size. This species is, therefore, an exception to the ectotherm developmental temperature–size rule (Atkinson 1994; Zuo et al. 2012) as temperature did not significantly affect hatchling size. The reasons for this pattern remain unknown, although they may relate to the physiology and efficiency of yolk absorption (Montague 1987).

Our finding that hatchling size is not correlated with initial egg size contrasts with another general reproductive trend often observed in ectotherms (Salthe 1969; Salthe and Mecham 1974; Kaplan 1980; Duellman and Trueb 1994; Wells 2007). For the trade-off between clutch size and egg size to persist, larger eggs must experience some meaningful benefit over smaller eggs, and in our experiment, post hoc tests revealed that larger eggs developed more rapidly than smaller eggs (posterior mean: -0.199 , 95% HPDI: -0.318 , -0.073 , Online Resource 13), which might reduce the risk of fungal infections (Warkentin et al. 2001) and enable

offspring to forage longer and grow larger before having to retreat to winter refuges (Fraser 1980).

Our work suggests that female body condition (particularly tail length) is responsible for much of the variation observed in reproductive success of this species. Tail size in plethodontids affects many ecological traits ranging from the ability to defend a high-quality territory (Wise et al. 2004), to escape from predators via autotomy (Jamison and Harris 1992), and time to maturity (Maiorana 1977). In most plethodontids, clutch size for the upcoming breeding season is determined during the year preceding oviposition by factors such as female size (Nagel 1977; Fraser 1980; Herbeck and Semlitsch 2000) or environmental conditions like precipitation (Milanovich et al. 2006). Once established, clutch size cannot be altered during the year of oviposition (Fraser 1980). In contrast, final ovum volumes (at maturation) are determined after clutch size has been fixed and, therefore, are more easily altered based on the addition or loss of energy reserves in the tail (Fraser 1980).

Most studies on clutch size relationships in salamanders examine the number of eggs yolked (ovarian eggs) rather than number of eggs laid as the measure of reproductive success (Blanchard 1928; Nagel 1977; Lotter 1978; Fraser 1980; Hom 1987; Herbeck and Semlitsch 2000). However, detectable differences between these two clutch size measures and post hoc tests suggest that the number of eggs yolked (rather than eggs laid) significantly affected average egg volume (posterior mean: -0.037 , 95% HPDI: -0.075 , 0.000 , Online Resource 12). This discrepancy suggests the importance of distinguishing the different effects of these two measures of reproductive potential when considering the viability and life history traits of wild populations. For example, while we did not detect significant differences in reproductive success between *P. cinereus* color morphs after correcting for female size, our results do suggest that unstriped individuals have greater reproductive potential due to their overall larger SVL and the positive correlation between SVL and number of eggs yolked (but not number of eggs laid). The frequency and benefits of egg reabsorption in the wild are uncertain (Ng and Wilbur 1995), but if natural egg reabsorption occurs less frequently than in the laboratory, this higher reproductive potential in unstriped individuals may ultimately translate into higher reproductive output (increased clutch size), although at a cost to initial egg size.

Conclusion

For dispersal-limited, environmentally sensitive species, plasticity can provide a crucial and potentially favorable response to climate change. Despite decades of research, little work has directly tested if and how phenotypic

plasticity affects the development and reproductive success of many polymorphic species. Here, we provide the first evidence that developmental temperature influences coloration in the salamander *P. cinereus*, suggesting that temperature likely influences the morph frequency distribution in this species, although the relative effects of plasticity compared to other mechanisms like differential mortality remain uncertain. This result suggests the need to test for plasticity in coloration in other polymorphic species. We also unexpectedly found that temperature did not affect the initial size of individuals at hatching, which contrasts with observations in other taxa. Given that *P. cinereus* embryos can benefit from faster developmental rates without having to compromise on initial hatching size, this may allow *P. cinereus* populations to derive reproductive benefits from projected climatic changes. Finally, our work highlights the previously overlooked importance of female tail condition (lipid storage) for reproductive output. Knowing how environmental conditions may affect the fitness of individuals and populations will improve our ability to predict how populations will respond to future climate change. Our study provides important insights into the potential for climate-induced responses like plasticity to preserve diversity within and among populations of polymorphic species, like *P. cinereus*, thus potentially enabling time for adaptive evolution.

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Author contribution statement AE designed the project, collected and analyzed the data, and wrote and revised the manuscript; MU and EJ contributed to project design and manuscript revisions.

Compliance with ethical standards

Research involving human participants and/or animals All applicable institutional and/or national guidelines for the care and use of animals were followed. This research was conducted under approvals from the University of Connecticut IACUC (protocol numbers: A15-023, A18-025), Federal Fish and Wildlife permit (MA93731B-0), Ohio permits (numbers: 17-271, 18-184, and 19-151), and Connecticut permits (numbers: 0915008 and 0920008).

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