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Persistence of an egg mass polymorphism in *Ambystoma maculatum*: differential performance under high and low nutrients

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Abstract. Polymorphisms play critical roles in allowing organisms to adapt to novel environments while enabling ecological speciation under divergent selection. *Ambystoma maculatum*, the spotted salamander, exhibits a unique polymorphism in the structure and appearance of its egg masses with two common morphs, white and clear. Amphibian egg jelly layers mediate interactions between embryos and the environment and are more responsive to ecological pressures of natural selection than other egg coat components. The *A. maculatum* egg mass polymorphism was hypothesized to be adaptive with regard to varying dissolved nutrient levels in ponds. We conducted two mesocosm experiments, collected field data, and constructed a population projection model to determine how dissolved nutrient levels affect embryonic and larval development and relate to the distribution of the morphs in natural ponds. We found that upon hatching there was an interaction between nutrient level and egg mass morph wherein individuals from white morphs were larger in low nutrient habitats. This interaction persisted throughout the larval stage, and along with the higher abundance of white morphs in ponds with low conductivity, we demonstrate that the white morph is advantageous in low nutrient environments. Our findings provide evidence for the role of environmental heterogeneity in enabling the persistence of a structural egg mass polymorphism, with maintenance occurring across multiple scales and persistence across its range. This indicates that polymorphisms can maximize performance in heterogeneous environments, while persisting over long timescales without leading to sympatric speciation.

Key words: Ambystoma maculatum; *egg mass; jelly layers; metapopulation; performance tradeoff; polymorphism; population model.*

Introduction

Selection for alternative phenotypes via polymorphisms, and dissimilarity between morphologies, can give rise to novel adaptations, effectively allowing a species to occupy multiple sympatric niches by buffering against local extinction (West-Eberhard 1986). The maintenance of a polymorphism presents a paradox, however, as a single morph should result both when selection favors one morph with fitness advantages, and when random drift acts to eliminate variation in a population when there are no fitness differences between morphs. Theoretical models have attempted to describe the evolution and long-term persistence of polymorphisms in populations (Hedrick 1986, Frank and Slatkin 1990, Leimar 2005), but if long-term persistence of polymorphisms were common, we would expect to observe the same polymorphism in closely related species (Forsman et al. 2008). Either recurrent mutation, a balance between divergent selection and dispersal between populations with different morphologies, or a form of balancing selection within a polymorphic population is required to maintain a stable polymorphism (Hartl and Clark 2007).

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Opposing selection in different environments is one of the strongest mechanisms for generating polymorphisms (Hedrick 1986). Divergence of morphs can be driven by adaptive tradeoffs between morphs in heterogeneous environments where each morph experiences higher fitness in the environment to which it is optimally matched (Skúlason and Smith 1995, Robinson 2000), and differential selective pressures can lead to further divergence by selecting for correlated characteristics (Nosil 2004). This process of divergence is reinforced if individuals actively choose habitats in which they have a fitness advantage (Fry 1996). Fitness differences among morphs of a polymorphic species in different environmental conditions can result in spatial mosaics of morphs matched to local conditions that are then maintained as a single species by dispersal and temporal variation in environmental conditions. Polymorphisms with environmental fitness differences can enhance species persistence during environmental change, as well as colonization of novel environments and range expansion (Forsman et al. 2008). However, polymorphisms can be short-lived on evolutionary timescales, representing intermediate stages during geographic or reproductive isolation or in the transition to a monomorphic population (Moran 1992, Gray and McKinnon 2007). Reproductive isolation can evolve via divergent selection on traits and lead to ecological speciation even in the presence of gene flow

(Maynard Smith 1966, Schluter 2001, Hollander et al. 2005). Work supporting the maintenance of polymorphisms has often focused on traits under sexual selection (Gamble et al. 2003, Gray and McKinnon 2007) or other biological interactions such as frequency-dependent selection (Punzalan et al. 2005, Vale et al. 2008), with little consideration of short-lived (at the individual level) structural differences in a species, such as egg structure (Hedrick 1986, 2006).

Amphibians produce jelly layers that comprise the outer layers of egg coats, surround ova, and exhibit remarkable variation in their structure, function, and production (Salthe 1963, Altig and McDiarmid 2007). Functions of egg jellies include attachment of eggs both to structures and to each other (forming egg masses) as well as enhancing the entry of conspecific sperm and preventing the entry of heterospecific sperm (Barbieri and del Pino 1975, Olson and Chandler 1999, Simmons et al. 2009). Egg jellies can also mediate the interactions between embryos and their external environment by protecting them from predators (Ward and Sexton 1981), desiccation (Marco and Blaustein 1998), contaminants (Marquis et al. 2006), pathogens (Gomez-Mestre et al. 2006), temperature (Beattie 1980), and ultraviolet light (Licht 2003), but also limit diffusion of dissolved oxygen (Seymour 1999).

Intraspecific variation in egg jelly properties provides a potential medium for natural selection, facilitating adaptation to environmental change and heterogeneity. Among all animals, layers of egg coats, including jelly layers, are an important source of maternal effects that have the potential to influence evolution on relatively short timescales (Carroll et al. 2007, Räsänen and Kruuk 2007). Egg coat proteins can evolve rapidly, but egg coat structure is relatively conserved across taxa in vertebrates, reflecting the need to maintain essential functions while under strong selective forces, particularly sperm-egg interactions and pathogens, respectively (Turner and Hoekstra 2008, Claw and Swanson 2012). Whereas egg coats play dual roles mediating both ecological and sperm-egg interactions, evolution of layers mediating organism-environment interactions can occur faster by natural selection than layers primarily regulating sperm-egg interactions due to the need to maintain essential reproductive functions associated with fertilization (Shu et al. 2015). Reproductive isolation can occur through adaptive divergence when there is strong, differential selection on egg coats in different environments (Nosil et al. 2005), potentially leading to ecological speciation (Rundle and Nosil 2005).

Ambystoma maculatum, the spotted salamander, exhibits a unique polymorphism in the structure and color of its egg masses, where the majority of egg masses produced have either a clear or white appearance (Appendix S1: Fig. S1) (Banta and Gortner 1914). A third intermediate (gray) morph can occur, but is either uncommon or completely absent in populations (Ruth et al. 1993). These morphs differ in that the outer jelly layer of white egg masses contains white crystals consisting of a hydrophobic protein that is produced along with jelly in the oviductal wall cells. The outer jelly layer of clear egg masses and the inner layer of all three morphs consist of a slightly smaller water soluble protein, while intermediate morphs contain both proteins in their outer layers (Hardy and Lucas 1991, Ruth et al. 1993). The total amount of protein, as determined from nitrogen analyses, does not differ between egg mass morphs. The morphs often occur sympatrically, but individual breeding sites and populations may contain predominately or solely one of the two common morphs (Hardy and Lucas 1991, Ruth et al. 1993, Brodman 1995, Glorioso et al. 2015). Individual females produce only one morph in their lifetime, as this egg mass polymorphism is due to a simple polymorphism of a single gene (Ruth et al. 1993).

Because the difference in egg mass structure in *A. maculatum* is due to the presence or absence of a protein, and not the relatively minor substitution of an amino acid within a protein, the structural difference would not likely be without function. However, this function has not definitively been determined. Larval size at hatching does not differ between the morphs in high/low light and high/low pH conditions, and oomycete infection resistance, and rates of hydration, desiccation, and freezing do not differ between the morphs (Ruth et al. 1993, Urban et al. 2015). Whereas Ruth et al. (1993) found that egg mass size and number of embryos did not differ between morphs, Brodman (1995) found that white egg masses had significantly more embryos, but morph did not affect hatching success or length of the incubation period. Although larval caddisflies (*Ptilostomis postica*) do not preferentially feed on the egg mass morphs or differentially affect embryonic growth in the egg masses (Rowe et al. 1994), spotted turtles (*Clemmys guttata*) have been observed feeding on clear egg masses but not white egg masses (Tyning 1990). Feeding by larval wood frogs (*Rana sylvatica*) on egg masses reduced survival of larvae and weight of egg masses significantly more in clear morphs than white morphs even though the number of *R. sylvatica* observed feeding did not differ between morphs (Petranka et al. 1998). Thus, Petranka et al. (1998) suggested that the crystals and resulting firmer consistency of white egg masses may present a physical barrier to grazing by larval anurans.

Ruth et al. (1993) found that the proportion of clear egg masses in natural ponds ranging from Louisiana to Pennsylvania was related to water chemistry, particularly the presence of potassium, sodium, calcium, and magnesium ions. Thus, they hypothesized that there may be differential fitness between the two morphs in environments with varying levels of these nutrients, although they did not experimentally test this hypothesis. If this polymorphism evolved in response to spatiotemporal variation in habitat quality, we would expect variation in performance differences between morphs in these different habitat types, particularly during the embryonic

stage. We conducted a series of experiments, collected field data, and constructed a population projection model to address the questions of whether the *A. maculatum* polymorphism results in performance differences between morphs, and if so, what allows for the persistence of this polymorphism? We specifically addressed this functionality question by asking: (1) Does hatchling size and time to hatching vary between egg mass morphs in high and low nutrient environments? (2) If so, do differences in larval size persist throughout the larval period? (3) Does the proportion of white egg masses in ponds correlate to pond conductivity, which can be an indicator of dissolved nutrient levels (Spencer and Blaustein 2001)? (4) How do performance differences between morphs influence population dynamics under different pond conditions?

Materials and Methods

Experimental design and surveys

Embryonic development.—*Ambystoma maculatum* egg masses were oviposited in fishless ponds at the University of Mississippi Field Station (UMFS) on the night of 8 February 2015. On 9 February we collected 12 clear egg masses, but were able to obtain only 11 white egg masses from this single night of breeding (N = 23). Eight wading pools (1.4 m diameter, 0.3 m depth, ~300 L) were established on 9 February in a field at UMFS, filled with unchlorinated well water, and had nutrient levels randomly assigned (high nutrient pools N = 4; low nutrient N = 4). Each wading pool contained either 1 kg of hardwood leaf litter (primarily *Fagus grandifolia* and *Quercus* spp.) (high nutrient pools) or no leaf litter (low nutrient pools). Treatments consisted of egg mass morph + nutrient level (N = 6 for all treatments except white egg mass/low nutrients where N = 5). Each egg mass was considered one replicate blocked by pool. Pools received three egg masses, except one pool that received two and an empty cage as a control. All egg masses were placed into individual cages (0.25 m diameter plastic cylinders with an open top and two 0.2 × 0.2 m side openings covered with window screening) to prevent physical interaction with the leaf litter and retain hatched larvae. As larvae hatched, they were collected daily, photographed, measured (total length) with ImageJ, and returned to a separate egg mass-specific cage in the same pool until all larvae hatched. Pool conductivity, temperature, and pH were measured with a YSI 63/25 FT meter and dissolved oxygen (DO) with a YSI 550 DO meter on 19 March, one day after the first individuals hatched.

Larval development.—We established wading pools (1.4 m diameter, 0.3 m depth, ~300 L, N = 28) in a field at UMFS on 30 January 2015 and filled them with well water. Treatments (nutrient level + egg mass morph) were randomly assigned to all pools. Hardwood leaf litter (primarily *F. grandifolia* and *Quercus* spp.) was placed in both high nutrient pools (2 kg) and low nutrient pools (0.25 kg) on 30 January. Leaf litter in the pools was allowed to leach and decompose until 8 April. To establish nutrient differences while retaining equivalent structural complexity, we removed all leaf litter and replaced it with 0.5 kg of new leaf litter. We matched morph/nutrient levels between the embryonic and larval development experiments and used the same set of individuals in both experiments. On 8 April we added seven larvae to each pool, with one larva from each egg mass plus randomly selected individuals to add a total of seven per pool. Each treatment (nutrient + morph) had seven replicates, except for the white egg mass/low nutrient treatment, which had six replicates due to a structural failure (draining) of one pool.

On 25 April we measured temperature, conductivity, pH, and DO and collected zooplankton samples from each pool. Zooplankton that colonized via passive dispersal were sampled by filtering four (from four separate locations) 400 mL water samples from each pool through an 80 μm mesh and preserving with Lugol's solution before being counted and identified to order. On 14 May, before larvae began to metamorphose, we collected all surviving larvae by exhaustively searching through the leaf litter and water. Larvae were massed, photographed, and measurements taken from the photos using ImageJ (head length, head width, snout-vent length (SVL), total length). We terminated the experiment on 14 May.

Pond surveys.—UMFS contains over 200 ponds, at least 60 of which are known to be fishless. Most of the fishless ponds are temporary and completely dry each year, but not all of them are used by ovipositing *A. maculatum*. We counted the number of white and clear eggs masses in 14 fishless ponds at UMFS on 29 January 2015 and again on 9 February following a second round of breeding. We surveyed 55 ponds at UMFS on 4 January 2016, counted egg mass morphs, and measured the pH and conductivity of each pond at the time of the survey.

Data analysis

We conducted a series of analyses to answer our questions regarding the functionality of the *A. maculatum* polymorphism. Blocking factors and covariates were excluded from analyses when $P > 0.25$. All analyses of variance used type III sums of squares, and all analyses used $\alpha = 0.05$ and were conducted in R v. 3.2.3 (R Core Team 2015).

Embryonic development.—We analyzed (1) mean hatching date using ANCOVA with clutch size (number of hatchlings per egg mass) as a covariate and (2) mean hatchling total length per clutch using ANCOVA with days to hatching as a covariate. We initially included clutch size as a covariate in the total length ANCOVA, but it was not significant ($P > 0.99$) and we rolled it into the error term. Analyses were conducted on log-transformed total lengths and square root transformed days and

clutch sizes. For both analyses block was not significant ($P > 0.8$) and included in the error term. Pool condition (3) (conductivity, DO, pH, temperature) differences between treatments were analyzed independently with ANOVAs on log transformed data (except pH).

Larval development.—In analyzing the effects of treatment on larvae in the larval development experiment we used four separate primary analyses plus constituent ANOVAs in multivariate analyses. In analyses (1–3) treatment consisted of morph plus nutrient level. We used (1) ANOVA on number of surviving larvae in each pool. To examine effect on larval body size (2), we used a MANCOVA on all five larval body measurements with nutrient level and egg mass morph as factors with survival as a covariate. However, survival was excluded from follow-up ANOVAs because it was not significant in any of them ($P > 0.44$). We (3) calculated and analyzed body condition (size independent mass) by mean-scaling mass to decouple variance from the measurement scale and means, regressing against SVL, and using the residuals in ANOVA (Berner 2011). Because of the strong correlation of SVL with total length ($r = 0.982$, $P < 0.0001$), mass ($r = 0.928$, $P < 0.0001$), and head width and head length, we used only SVL as a covariate in (4) MANCOVA and follow-up ANCOVAs on head length and head width to determine if head size varied independently of body size. Enlarged relative head size (particularly head width, which is a measure of gape size and feeding ability) has been linked to higher growth rates in salamanders (Kohmatsu et al. 2001). All body measurements were log transformed and count data square root transformed. Zooplankton abundance had no explanatory power, so it was not included in the size analyses. Zooplankton abundances (5) were independently analyzed with a MANOVA and ANOVAs on square root transformed counts of individuals from the orders cladocera, copepoda, ostracoda, and rotifera. Pool condition (6) (conductivity, DO, pH, temperature) differences between treatments were analyzed independently with ANOVAs on log transformed data (except pH), and pool temperature was included as a covariate in the DO analysis.

Pond surveys.—Kendall's rank correlation coefficient was used to determine if there was a relationship between the proportion of white egg masses found in ponds at UMFS and pond conductivity (log transformed) and pH.

Population modeling

We evaluated the potential importance of variable performance of the morphs in different conditions by constructing a stage-structured population projection model similar to that of Gibbs and Shriver (2005) using published parameter estimates of vital rates to estimate the number of adults ($N_{a,x,t}$) of each morph (x: c = clear; w = white) in a year (t) in populations within a metapopulation. Parameter estimates (Table 1) were based off of synopses of *A. maculatum* (Petranka 1998, Harper et al. 2008). For a single morph (clear in this example), this model is described by the equation:

$$N_{a,c,t} = N_{a,c,t-1} \times \sigma_a - E_{c,t} + I_{c,t} + R_{c,t}$$

where (σ_a) is the adult survival rate, (E) is emigration, (I) is immigration, and (R) is recruitment to the adult stage.

$$E_{c,t} = N_{a,c,t-1} \times e$$

$$I_{c,t} = (N_{a,c,t-1} + N_{a,w,t-1}) \times i \times p_c$$

where (e) is the emigration rate for the population wherein both morphs emigrate at equivalent rates and (i) is the

TABLE 1. Parameter estimates for the population projection model.

Parameter	Symbol	Estimate	
		Low nutrient	High nutrient
Adult survival	σ_a	0.7	0.7
Survival to metamorphosis	σ_m	0.06	0.06
Hatchling size	s_h		
Clear		0.92	1
White		1	0.97
Days to hatching	d_h		
Clear		1.04	1
White		1	0.83
Larval survival	σ_l		
Clear		0.76	1
White		1	1.32
Larval size	s_l		
Clear		0.87	1
White		1	0.83
Juvenile survival			
Winter 1	σ_{j1}		
Clear		0.59	0.61
White		0.61	0.59
Winter 2	σ_{j2}		
Clear		0.645	0.655
White		0.655	0.645
Winter 3	σ_{j3}		
Clear		0.68	0.68
White		0.68	0.68
Clutch size	ϕ	80	80
Number of clutches	N_ϕ	2	2
Probability of breeding	σ_b	0.38	0.38
Proportion female	ρ	0.33	0.33
Carrying capacity	K	200	200
Emigration rate	e	0.01	0.01
Immigration rate	i	0.01	0.01
Proportion in metapopulation	p		
Clear		0.714	0.714
White		0.286	0.286

immigration rate for the population. The proportion of immigrants is determined by the proportion of each morph (p) in the metapopulation, and this proportion was considered independent of the focal population. Metapopulation estimates were determined from the overall proportion of each morph during the 2015 and 2016 pond surveys at UMFS. Emigration and immigration rates were held constant relative to the focal population; there was no net morph-independent migration in this model. Site fidelity is very high among *A. maculatum*, so emigration and immigration rates were kept low (1%) (Shoop 1968, Phillips and Sexton 1989, Vasconcelos and Calhoun 2004).

Recruitment (R) is defined as:

$$R_{c,t} = \frac{N_{j1,c,t-1} \times \sigma_{j2,c}}{2} + N_{j2,c,t-1} \times \sigma_{j3,c}$$

where the number of young of the year juveniles (N_{j0}), number of one year old juveniles (N_{j1}), and number of two year old juveniles (N_{j2}) are defined as:

$$N_{j0,c,t} = N_{e,c,t} \times \sigma_{m,c} \times k_{adj,t}$$
$$N_{j1,c,t} = N_{j0,c,t-1} \times \sigma_{j1,c}$$
$$N_{j2,c,t} = \frac{N_{j1,c,t-1} \times \sigma_{j2,c}}{2}.$$

The number of eggs produced of a given morph ($N_{e,c}$) is a product of the number of adults of that morph in the population and the number of eggs produced per individual:

$$N_{e,c,t} = N_{a,c,t} \times \varphi.$$

The average number of eggs produced per individual (φ) is a product of the average clutch size (ϕ), number of clutches produced per female (N_{ϕ}), probability of breeding in a year (σ_b), and the proportion of individuals that are female within a population (ρ).

$$\varphi = \phi \times N_{\phi} \times \sigma_b \times \rho.$$

The morph-specific survival rate to metamorphosis is a product of the overall survival to metamorphosis and a habitat adjustment coefficient:

$$\sigma_{m,c} = \sigma_m \times h_c.$$

Rather than directly use our data to create parameter estimates of the embryonic and larval stages, we used our data to adjust the known survival rate to metamorphosis (σ_m) using a habitat coefficient (h). This was done because both embryonic (100%) and larval survival (69.3%) in our experiment were much higher than typical survival rates to metamorphosis (6%). The habitat coefficient was a weighted mean of four adjustment factors generated from our data: hatchling size (s_h) (calculated from total length), days to hatching (d_h), larval survival (σ_l), and larval size (s_l) (calculated from mass).

$$h_c = 0.3 \times s_{h,c} + 0.2 \times d_{h,c} + 0.25 \times \sigma_{l,c} + 0.25 \times s_{l,c}.$$

In the adjustments for hatchling size, larval survival, and larval size, the morph that was optimally matched to that habitat was assigned a value of 1 (clear in high nutrients, white in low nutrients), and the other morph received a value based on the ratio of its mean value from our data to that of the other morph. For time to hatching, we took the reciprocal of this ratio because smaller values (earlier time to hatching) could be more beneficial such that it could allow for feeding by larvae in suboptimal embryonic habitats. We placed the greatest weight on hatchling size because this is both an immediate effect of differential performance of the morphs and also because we would predict larger hatchlings to be better able to acquire prey and escape predation, imparting the greatest long-term effect of the four factors. Larval size and survival received intermediate and equivalent weights because they are both important factors that are related to each other but more temporally separated from effects imbued by the morphs during the embryonic stage than is hatchling size. The least weight was placed on days to hatching because it is an environmental condition-independent variable that we would expect to have a lower effect on survival to metamorphosis than the other variables.

Ambystoma populations are highly density-dependent and limited by the carrying capacity (K) of larval habitats, so we used an adjustment coefficient (k_{adj}) based on the proportion of larvae that would survive to metamorphosis (k_{calc}) (Vonesh and De la Cruz 2002).

$$k_{calc,t} = \frac{K}{N_{e,c,t} \times \sigma_{m,c} + N_{e,w,t} \times \sigma_{m,w}}$$

$$(k_{adj,t} = 1) \text{ if } (k_{calc,t} \geq 1) \text{ and } (k_{adj,t} = k_{calc,t}) \text{ if } (k_{adj,t} \leq 1).$$

In the southern portion of its range, most juvenile *A. maculatum* mature at an age of 2 or 3 yr (Walls and Altig 1986). We assumed that no 1-yr-old juveniles, 50% of 2-yr-old individuals, and all 3-yr-old individuals matured in a year. We predict that effects imparted by the morphs during the embryonic stage are primarily size- and survival-driven with differences in survival rates diminishing over time as individuals reach the adult stage. This is based on differential survival as larvae and equivalent body and egg mass sizes as adults (Ruth et al. 1993). However, whereas annual survival among juveniles is difficult to predict, using the assumption of equivalent adult survival rates we conservatively estimated differences between morphs based on published rates of 60% survival of juveniles during the first winter and 70% survival as adults.

In year 0, populations consisted of 50 individuals of each morph with no preexisting juveniles or larvae. For simplicity we assumed that morph is a maternally inherited characteristic, but this is not known. This is a reasonable assumption given intermediate morphs are uncommon or absent in populations and that morph is a maternally-produced trait. Although we would expect the carrying capacity of a low nutrient pond to be lower than that of a similarly sized high nutrient pond, we kept the two equivalent for comparison purposes; in our

results we emphasize relative, not absolute, performance differences between morphs/environments. We projected populations in both low and high nutrient ponds for 200 yr in our figures and determined the asymptote at which a pond's population stabilizes.

RESULTS

Embryonic development

There were (1) significant effects of both nutrients and egg mass morph on time to hatching, but no significant interaction: larvae took significantly longer to hatch in high nutrient pools and from white egg masses (Fig. 1a, b; Appendix S1: Table S1). There was (2) a significant interaction between nutrient level and egg mass morph and a significant positive effect of days to hatching on hatchling total length: hatchlings from white egg masses were larger than those from clear egg masses in low nutrient pools (Fig. 1c; Appendix S1: Table S1). We did not observe any dead unhatched embryos after all others had hatched and did not analyze survival to hatching. High nutrient pools (3) had significantly higher conductivity ($F_{1,6}$ = 16.49, P = 0.0066) and lower pH

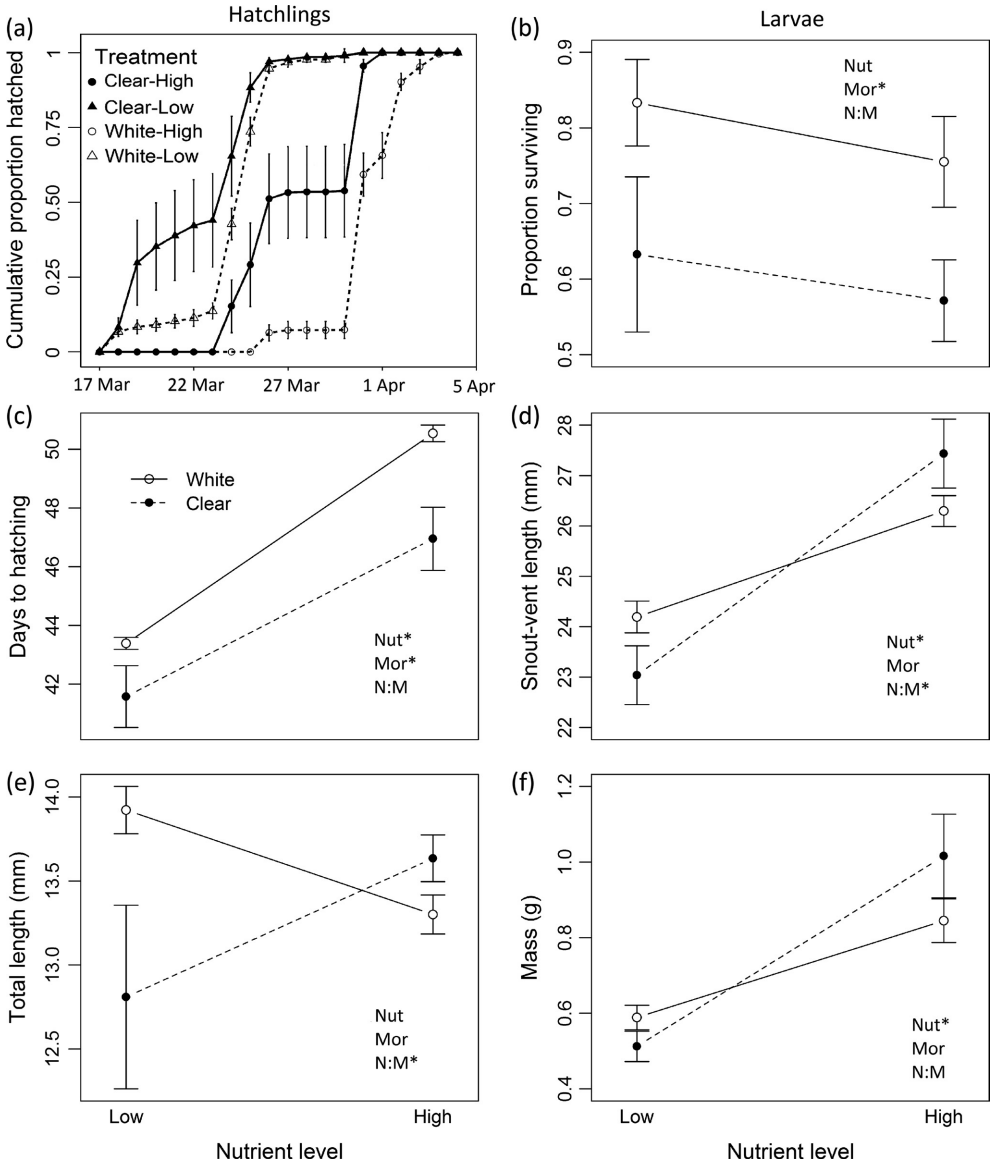

FIG. 1. (a) Cumulative proportion of individuals hatched by date in high/low nutrient pools and from white/clear morphs, (b) days to hatching, and (c) total length of hatchlings. (d) Proportion of larvae surviving, (e) larval SVL, and (f) larval body condition at the end of the larval development experiment. White morphs are indicated by white shapes and solid lines; clear morphs by black shapes and dashed lines (means ± 1 SE). * Indicates significance for the effects of nutrient (Nut), morph (Mor), or their interaction (N:M).

($F_{1,6}$ = 34.35, P = 0.0011) and DO ($F_{1,6}$ = 240.92, P < 0.0001). Temperature did not vary between pools (14.0°C) and was not analyzed.

Larval development

Larval survival (1) was significantly higher for white egg masses than for clear egg masses (Fig. 1d), and there was no interaction or effect of nutrients on survival (Appendix S1: Table S2). Larval size significantly covaried (2) with survival wherein larvae were smaller in pools with more surviving individuals (Appendix S1: Table S3, Fig. S2). Individual ANOVAs showed that all five body measurements, in addition to body condition (3), were significantly greater in high nutrient pools (Appendix S1: Table S4). The significant nutrient:morph interaction observed for size at hatching persisted for SVL (Fig. 1e), total length, and head length and width (Appendix S1: Fig. S3a–c), but not for body condition or mass (Fig. 1f). Larvae from white egg masses remained larger in low nutrient pools, while those from clear egg masses were larger in high nutrient pools. Larval head size (4) was significantly predicted by SVL, but did not vary with other factors (Appendix S1: Table S5, Fig. S3b, c). Zooplankton abundance did not significantly affect (P > 0.3) any larval size measures, so it was excluded from those analyses.

Zooplankton abundances (5) of all four orders (cladocera, copepoda, ostracoda, rotifera) were significantly higher in low nutrient pools than in high nutrient pools (Appendix S1: Table S6). While we did not sample them directly, in our zooplankton sampling we incidentally collected significantly more chironomid larvae from high nutrient pools (20.79 ± 12.49, mean ± SE) than low nutrient pools (2.31 ± 1.09; $F_{1,25}$ = 4.28, P = 0.0491). High nutrient pools (6) had significantly higher conductivity than low nutrient pools ($F_{1,25}$ = 41.00, P < 0.0001), but DO ($F_{1,24}$ = 1.32, P = 0.2616), temperature ($F_{1,25}$ = 0.56, P = 0.4594), and pH ($F_{1,25}$ = 1.36, P = 0.2537) were not significantly different. Temperature ($F_{1,24}$ = 3.44, P = 0.0758) was included as a covariate in the DO model. In a post hoc analysis, effect of pool position (row) was not significant (P > 0.25) for any pool condition measurements and was included in the error term.

Pond surveys

In 2015 we observed 114 clear, 51 white, and 0 intermediate egg masses in 14 ponds, and in 2016 we observed 193 clear, 72 white, and 0 intermediate egg masses in 26 ponds; 29 ponds had no egg masses. Among all ponds with egg masses, there was a significant negative correlation between pond conductivity (τ = −0.5398, P = 0.0002, Fig. 2a), but not pH (τ = 0.1364, P = 0.3476, Fig. 2b), and the proportion of white egg masses in those ponds: ponds with lower conductivity had higher proportions of white egg masses. We also did this same analysis only on ponds with five or more egg masses to eliminate potential biases from ponds with low number of egg masses, but the results were consistent for both conductivity (τ = −0.6498, P = 0.0005) and pH (τ = 0.3025, P = 0.1044).

Population modeling

In the simulated UMFS metapopulation, populations in low nutrient ponds stabilize with the dominant morph (white) accounting for 85.61% of the population (Fig. 3a), whereas populations in high nutrient ponds stabilize with the dominant morph (clear) accounting for 88.10% of the population (Fig. 3b). This stable point is reached faster in low nutrient ponds than in high nutrient ponds. In the long term, the proportion of each morph in a pond is strongly dependent on the proportion of the morphs in the metapopulation, but migration rates themselves did not affect the relative performance of each morph in a given habitat. Manipulation of the proportion of each morph in the metapopulation resulted in a wider range of the proportions of each morph in high nutrient ponds than in low nutrient ponds over 200 yr of simulations

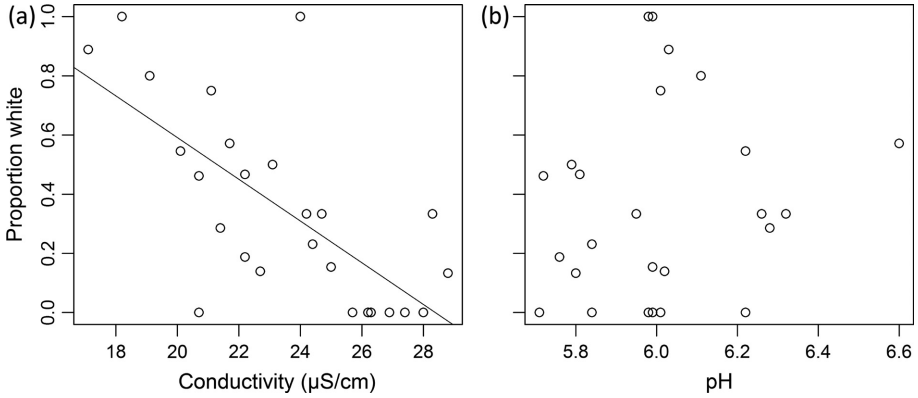

FIG. 2. The proportion of egg masses from the white morph in each pond during 2016 surveys vs. pond conductivity (a) and pH (b).

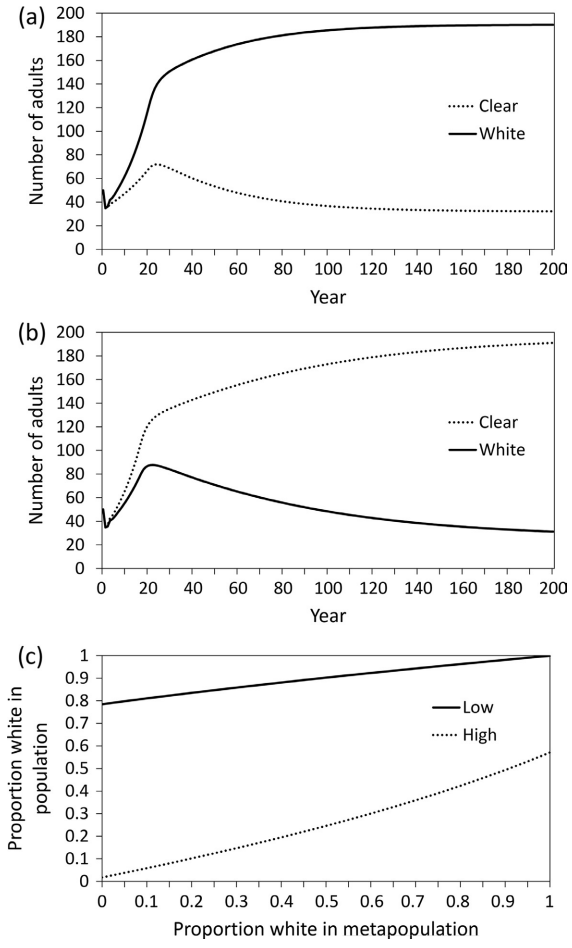

FIG. 3. Population projection models of the number of adults of clear and white morphs in (a) low nutrient pond and (b) high nutrient pond over 200 yr within the framework of the metapopulation at UMFS. (c) Variation in the proportion of each morph in a metapopulation combined with fitness differences of the two morphs in high and low nutrient ponds produce variation in the morph composition in individual populations after 200 yr of simulations.

(Fig. 3c). When the proportion of white morphs in the metapopulation approaches 0, white morphs accounted for 1.76% of individuals in high nutrient ponds and 78.46% in low nutrient ponds. When the proportion of white morphs in the metapopulation approaches 1, white morphs accounted for 57.11% of individuals in high nutrient ponds and 99.98% in low nutrient ponds (the relative role of site fidelity/dispersal is explored in Appendix S1: Fig. S4). These results reflect the greater degree to which white morphs outperform clear morphs in low nutrient ponds than do clear morphs outperform white morphs in high nutrient ponds.

DISCUSSION

The differential performance of the *A. maculatum* egg mass morphs in high and low nutrient environments, the persistence of these effects throughout the larval period, and the correlation of the proportion of white morphs to pond conductivity support the idea that this polymorphism is advantageous in, and maintained by, varying nutrient levels at breeding sites, as originally hypothesized by Ruth et al. (1993). Hatchlings from white egg masses were significantly larger in low nutrient pools, whereas those from clear egg masses were larger in high nutrient pools. The significant nutrient × egg mass morph interaction persisted throughout the larval period for more rigid measures of body size (total length, SVL, head width, head length), but not mass or body condition. This suggests that differences in larval body size between morphs within treatments was unaffected by availability or acquisition of food but rather dominated by the persistence of effects imparted by the egg jelly phenotypes during embryonic development. Because the difference between morphs is in the egg mass structure itself and not in the embryo, any effect of this polymorphism occurs during embryonic development (before hatching). This provides novel evidence of the role of environmental heterogeneity in the maintenance of a structural egg polymorphism.

Although not all females within a population breed every year (Phillips and Sexton 1989), the proportion of white egg masses in the UMFS metapopulation remained consistent between years (30.9% in 2015, 27.2% in 2016). This supports the findings of Ruth et al. (1993) that the proportion of morphs breeding in a population remains temporally consistent. Due to a limited number of ponds sampled both years (11) and low egg mass counts in several of these 11 ponds, our data are not sufficient to determine if the ratio of morphs within a pond is consistent between years. Because of the greater degree to which white morphs outperform clear morphs in low nutrient ponds, we would expect that the white egg morph would "fix" in low nutrient ponds, with both morphs persisting in higher nutrient ponds. This expectation is supported by our model, which shows that within a metapopulation framework, the proportion of white morphs in low nutrient ponds varies between 78.46% and 99.98% whereas in high nutrient ponds it varies between 1.76% and 57.11% (Fig. 3c). This assumes that females cannot assess either their own phenotype or the conditions of the pond. It would be interesting to determine whether, if given the choice, females preferentially oviposit in ponds that match their phenotype, with imperfect matching, or whether the correlation of white egg mass morph with conductivity is a function of differential, long term survival in populations, with the presence of both morphs perhaps maintained by temporal variation in nutrient inputs to ponds or lack of site fidelity. Regardless, it is interesting that this polymorphism is maintained at small spatial scales (between individual ponds) and persists across large spatial scales (entire range). The exact mechanisms underlying the maintenance of this polymorphism remain an interesting and unresolved question.

Fitness consequences for the morphs may result from long-term, size-related advantages, as larger individuals

typically have higher survival and fitness, and outcompete smaller individuals, which along with time to metamorphosis can have large effects on fitness, especially in temporary ponds (Semlitsch et al. 1988). When considering size at hatching, embryos from white morphs outperform those from clear morphs in low nutrient pools to a larger degree than clear morphs outperform white morphs in high nutrient pools (Fig. 1c). This differential performance is reflected in our model where, in a metapopulation consisting of equal frequencies of the two morphs, white morphs stabilize at a greater percent of the population in low nutrient ponds than do clear morphs in high nutrient ponds. However, larval size for both morphs was higher in high nutrient pools than in low nutrient pools, whereas survival did not differ with nutrient level. Therefore, in a habitat selection framework, we might expect breeding females of both morphs to preferentially oviposit in high nutrient ponds, but there is little evidence for oviposition site selection based on patch characteristics in *Ambystoma*. This indicates that the spatial distribution of the morphs is driven by differential environmentally-mediated fitness of the morphs (including site fidelity: Shoop 1968, Phillips and Sexton 1989, Vasconcelos and Calhoun 2004) rather than habitat selection by females.

In simulations of the UMFS metapopulation, white morph populations grow faster in low nutrient ponds than do clear morphs in high nutrient ponds (Fig. 3), and in neither case is the dominant morph able to relatively quickly eliminate the other morph from the population. Similarly, in simulations manipulating morph composition of metapopulations, as the proportion of white morphs in the metapopulation increases the clear morph represents a maximum of only approximately 50% of individuals in high nutrient ponds, compared to about 80% for white morphs in low nutrient ponds when the proportion of white morphs decreases in the metapopulation (Fig. 3c). Thus, we would expect under stable conditions these small environmentally-mediated fitness differences to result in a slow, asymmetrical process of fixation of first white morphs in low nutrient ponds, followed by clear morphs in high nutrient ponds. When we manipulated migration rates, we observed that even low rates of movement in a metapopulation were able to maintain both morphs in both habitats (Appendix S1: Fig. S4). Performance differences between the morphs were driven predominately by the habitat adjustment coefficient, and its effect was relatively robust against moderate differences (within about 20%) in its integrated components, well within the range of the small performance differences we observed in our experiments. These parameters indicate that this polymorphism can be maintained (1) over decades within individual populations due to relatively small performance differences between morphs, (2) spatially, as even low rates of dispersal in a metapopulation can support viable populations of each morph in its suboptimally matched habitat, and likely (3) temporally, as environmental perturbations cause changes that may favor one morph over the other.

In the few studies reporting the relative abundances of the morphs in natural populations, only Ruth et al. (1993) reported that the frequency of white morphs was greater at all sites, while our work and others report the clear morph is more common (Banta and Gortner 1914, Hardy and Lucas 1991, Brodman 1995, Glorioso et al. 2015). The reasons for these overall differences are unknown, but they could be due to founder effects, biases in study site selection, or generally poor documentation of egg mass morph composition of populations in many geographic areas. Selective forces can be only slight yet still create and maintain phenotypic differences over longer timescales (Fry 1996).

The *A. maculatum* egg mass polymorphism may be dually adaptive to both dissolved nutrient levels and feeding risk by *Rana* larvae or other predators (Cargo 1960, Seale 1980). Petranka et al. (1998) provided experimental and observational evidence that the white morph is subject to reduced feeding by larval *R. sylvatica*. *Ambystoma maculatum* egg masses are poor food sources for *Rana*, which are more likely to feed on egg masses when other food sources are lacking. This supports the idea that the white morph is dually adaptive, because we would expect low dissolved nutrient levels to correlate with both low abundances of periphyton, the preferred food for *Rana*, and overall lower ecosystem productivity (Van Buskirk and Relyea 1998).

Salamander larvae forage at all times of day, but typically only venture into the water column at night, spending daylight hours in the benthic zone (Branch and Altig 1981, Figiel and Semlitsch 1990). Zooplankton, along with aquatic insect larvae, make up a large portion of larval *Ambystoma* diets, especially at smaller size classes (Dodson and Dodson 1971, Petranka and Petranka 1980, Freda 1983). Larvae from both egg mass morphs were larger in our high nutrient pools even though zooplankton abundances were lower in these pools. We would expect larval size to be related to zooplankton abundance (Petranka 1989) and zooplankton to be more abundant in high nutrient pools (Leibold 1999). Our results could be due in part to temporal or cyclical variation in zooplankton populations (McCauley and Murdoch 1987), but we attribute greater larval size in high nutrient pools to greater abundance of the predominately benthic chironomid larvae, which are much larger and may be a more substantive food source than zooplankton.

Under strong selection, phenotypic divergence among populations has occurred on observable timescales, potentially indicating the early stages of speciation (Carroll et al. 2007). Although reproductive isolation often evolves in polymorphic species, the outer egg jelly layers likely play greater roles in regulating the interactions between the embryo and the environment and not sperm-egg interactions, thus allowing them to be more variable and responsive to the pressures of natural selection than inner layers (Shu et al. 2015). This is likely a contributing factor to why we see strong environmental performance differences between the *A. maculatum* egg

mass morphs, yet co-occurrence and likely interbreeding of the morphs (Ruth et al. 1993).

Contemporary observations of the origin of polymorphisms involve either local adaptation in a heterogeneous environment or colonization of a new environment (Reznick and Ghalambor 2001). Although the variable performance of *A. maculatum* egg mass morphs under different nutrient conditions occurs within a heterogeneous landscape of ponds, and the earliest record of the polymorphism dates to the early twentieth century (Banta and Gortner 1914), we doubt that this polymorphism is a contemporary adaptation to either a new environment or newly generated environmental heterogeneity. If the *A. maculatum* polymorphism is adaptive, it is to variable conditions (high/low nutrient levels) that are widely distributed across habitat patches on the landscape, often within the range of individual salamanders, likely contributing to the persistence of this polymorphism. Mitochondrial DNA analyses indicate that *A. maculatum* consists of two clades that diverged during isolation in separate glacial refugia (Zamudio and Savage 2003). These clades came into contact during post-glacial range expansion at several localities (including UMFS), but most populations still consist of only one of the clades' haplotypes. Given that this polymorphism occurs in individuals from both clades, it suggests that this is a very stable polymorphism that may have persisted for upwards of 100,000 yr, preceding the last glacial maximum. The high embryonic survival rate to hatching in our experiment and the co-occurrence of the *A. maculatum* egg mass morphs throughout its wide range may indicate it is not experiencing strong directional selection in different environments, thus limiting its potential as a force to drive local adaptation or ecological speciation. Studies of the lifetime reproductive success of individuals raised in different conditions, combined with long-term data on the proportion of morphs at individual sites and the overall prevalence of the intermediate morph, can help to provide an estimate of the strength of selection. In an adaptive framework, this polymorphism only allows individuals to maximize their fitness when egg mass phenotype is matched to local habitats, but the polymorphism also permits persistence under a variety of spatial and/or temporally variable conditions.

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