

Differential fitness maintains polymorphism in *Ambystoma maculatum*

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Abstract

Genetically based polymorphisms are difficult to explain because differential fitness should lead to the extinction of one morph, but selection on alternative morphs may be maintained if relative fitness varies spatially or temporally or habitat-dependent selection. Egg masses of *Ambystoma maculatum* exhibit a polymorphism in coloration, appearing as either opaque or clear. *Ambystoma maculatum*, egg mass morph is genetically determined and both morphs co-occur throughout much of the species' range. White masses have been shown to have an advantage over the clear masses with regards to decreased predation by *Lithobates sylvaticus* tadpoles, but there is no known condition that favors the clear masses. The focus of this study is to better understand advantages of the clear morph, which we believe would vary with water depth because of its correlations with light availability, dissolved oxygen, and temperature. We hypothesize that microhabitat variability contributes to the maintenance of the polymorphism in *Ambystoma maculatum* egg mass coloration, leading to white and morph egg masses having differential success based on oviposition. Egg masses of both morphs were contained in mesh and anchored at water surface and at the pond substrate. Temperature and dissolved oxygen were measured weekly and masses were inspected regularly for hatchlings. Masses with hatchlings were returned to the lab to collect developmental and survivorship data. Egg membranes from each mass were harvested and preserved. We determined that egg masses at the substrate took a longer time to hatch, regardless of morph. Survivorship of white morphs was unaffected by depth, but the clear masses had significantly higher survivorship at the surface of the water. Clear masses receiving surface treatment had the highest survivorship out of any other morph and depth treatment combinations. These results support our hypothesis that microhabitat fluctuations result in differential fitness of opaque and clear *Ambystoma maculatum* egg mass morphs.

Keywords: Polymorphism, *Ambystoma maculatum*, *Oophilia amblystomatis*, amphibian, development, symbiosis

1. Introduction

Genetically based polymorphisms are difficult to explain because differential fitness should lead to the extinction of one morph, but selection on alternative morphs may be maintained if relative fitness varies spatially or temporally¹. Egg mass color is a heritable polymorphism in the salamander *Ambystoma maculatum* that corresponds with the presence or absence of a crystalline protein². Presence of the crystal results in opaque color; absence results in clear appearance. This crystalline protein may relate to fertilization, cleavage, or early development². Although the genetic and molecular mechanisms responsible for this polymorphism are well understood, it is not yet clear how this polymorphism is maintained.

Ambystoma maculatum egg masses are typically attached to submerged vegetation. The two egg mass color morphs co-occur across the species range. Previous studies sampling *Ambystoma maculatum* populations in Louisiana, Texas,

and Arkansas found that ratio of clear to opaque masses varied among populations, with a range of 0 to 1. In addition to this microgeographic^{2,3} variation, it has also been found that the ratio of clear to opaque egg masses correlated with predator (*Lithobates sylvaticus*) density and nutrient availability⁴. It has been proposed that egg predation plays a significant role in maintaining high frequency of the opaque morphs in western North Carolina⁴. Previous research studying natural populations in western North Carolina found that when the population was exposed to predation by *Lithobates sylvaticus* tadpoles, there was a significantly higher proportion of opaque egg masses⁴. A microcosm study determined that white masses were less likely than clear masses to be predated upon by *Lithobates sylvaticus* tadpoles, but this advantage varied with the level of supplemental nutrients available to the predators⁴. This may be owing to a selective advantage for opaque masses, either at the embryonic or larval stage, but functional advantage is difficult to determine due to subtle selective forces⁵, especially if the advantage varies with environmental conditions like nutrient availability. If opaque morphs do have an advantage in the presence of *Lithobates sylvaticus* predators, then the question remains, why are clear morphs maintained?

This study seeks to investigate the advantages of the clear morph in order to better understand how selection has resulted in maintaining this polymorphism. One possible advantage is that the clear morphs better facilitate the colonization of *Oophilia amblystomatis*, which could result in a selective advantage with regards to survivorship, growth, and development. *Oophilia amblystomatis* is a benthic, flagellated photoautotrophic protist that penetrates the egg mass soon after it is laid and colonizes embryonic membranes⁶, it has also been determined that the alga will penetrate the developing embryo near the blastopore and colonize as an endosymbiont⁷. The *O. amblystomatis* colonies receive nitrogenous waste from the embryos, while the embryos receive photosynthetic oxygen and translocated carbon from the algae⁸. Previous research has supported a direct correlation between algal abundance (photosynthetic production) and embryonic growth rate⁸. The colonization of *O. amblystomatis* has been shown to benefit the *A. maculatum* embryos by increasing survivorship and rate of development⁸. When population growth of *O. amblystomatis* is hindered with either absence of light or photosynthetic inhibitor (DCMU), *A. maculatum* embryos experience increased mortality as well as delayed growth and development⁶.

Consumption of O₂ by *O. amblystomatis* at night leads to periodic oxygen deprivation of the *A. maculatum* embryos with which they have a symbiotic relationship. Chronic hypoxia negatively affects embryonic development, resulting in delayed development or premature hatching⁹. Because the embryos are positioned closely together within an impermeable mass, O₂ cannot surround individual eggs to be used for respiration, making them susceptible to hypoxia. In *A. maculatum* egg masses, convection is absent, unlike some other amphibian egg masses, and diffusion cannot provide all O₂ necessary, especially to embryos close to center. Depending on the relative location of embryos with regards to center of mass, varying degrees of hypoxia may occur. In aquatic anamniotes, extended or chronic hypoxia has been shown to delay development^{10,11}, reduce survivorship^{12,13} and, in some cases, result in premature hatching and developmental abnormalities¹⁴. Dissolved oxygen content in ponds typically fluctuates throughout a given 24-hour period¹⁵, but in some cases the symbiotic algae drastically influences fluctuations in oxygen content within the egg mass. Dissolved oxygen content fluctuations within the egg mass can have varying effects on the developing embryos depending on respiratory demands of the mass (number of embryos) and dissolved oxygen content of the aquatic habitat.

Because the clear egg masses have a translucent gelatinous matrix and allow for transmittance of light, we believe they may better facilitate colonization by *O. amblystomatis*. If the opaque morph egg masses do not allow for sufficient penetration of light, algal population density may be lower, resulting in less oxygen production. This would mean that the quality of the symbiosis would vary depending on morph of the egg mass. However, any advantage of the clear morph may vary with light availability due to water depth. As water depth increases, light availability, dissolved oxygen content, and temperature decrease. At greater depths, where there is lower light availability, colonization by *O. amblystomatis* may be hindered. If colonization is not hindered, it may still differentially affect embryos based on the dissolved oxygen content and temperature of their microhabitat. Embryos from egg masses that are colonized to the same extent, but are subject to different microhabitat conditions may experience differential fitness. This means that clear egg masses at greater depth may not experience the advantage to algal cell colonization that would occur at higher light availability. Lower water temperatures and dissolved oxygen content may offset the advantage due to algal cell colonization, particularly because the alga consumes oxygen at night, which can lead to periods of hypoxia⁹.

2. Methods

On March 14 and March 15, 2015, approximately 100 opaque and clear *Ambystoma maculatum* egg masses were collected from Sandy Bottom Preserve, Buncombe County, NC. A total of twenty PVC pipes, with holes drilled for points of attachment, were staked into the pond floor to serve as anchors for egg masses. The first ten poles were arranged 2-3 meters apart in a straight line along the deepest of the pond. The remaining ten poles were randomly scattered in a nearby open area with emergent vegetation. Zip-ties and carabiners were used to attach four egg masses contained in cloth, mesh bags to each pipe. Opaque and clear egg masses of similar age and size were paired and randomly attached to poles at either the surface or substrate treatment. Egg masses that received surface treatment were attached at the surface of the water, the substrate treatment egg masses were attached to the pole 30 cm lower. For six weeks following setup the site was visited once weekly. During site visits, each egg mass was inspected for hatchlings and aquatic environmental conditions were measured. Each week dissolved oxygen content and water temperature were measured using YSI-75 meter (Yellow Spring Instruments, Yellow Springs, Ohio, USA) at surface and substrate treatments of each replicate. Water depth was also measured.

After the initial hatching event, site visits occurred more frequently so that masses could be inspected for hatchlings. Masses containing hatchlings were transported back to the laboratory where age (days) of hatchlings was calculated and median developmental stage of hatchlings was identified according to Harrison's stages¹⁶. The probability of surviving was also calculated for each egg mass. This value was defined as the number of hatchlings and live embryos out of the total number of hatchlings and embryos. Any dead hatchlings were included in the count of living individuals because they survived to hatch. For each mass, ten embryonic membranes were preserved in Shandon™Glyo-Fixx™ (Richard Allan Scientific, Kalamazoo, MI, USA).

The effects of egg mass morph, depth treatment, and their interaction on age and stage at hatching were analyzed using a generalized linear mixed effect model, in which pole identity was a random effect. We used the lme function of the nlme package¹⁷ of the programming language R (R Development Core Team, 2010), in which progressively simpler models for the data are compared using analysis of variance and the test statistics for the comparison are F statistics. Egg mass data for probability of surviving were analyzed as a binomial response variable using generalized linear models with a logit link function and binomial error distribution using the glm function of the nlme package of R. Models contained continuous and discrete fixed effects only and their fit was compared using analysis of deviance.

3. Results

Trends in dissolved oxygen content did not differ between surface and substrate treatments (Figure. 1), nor did temperature (Figure. 2).

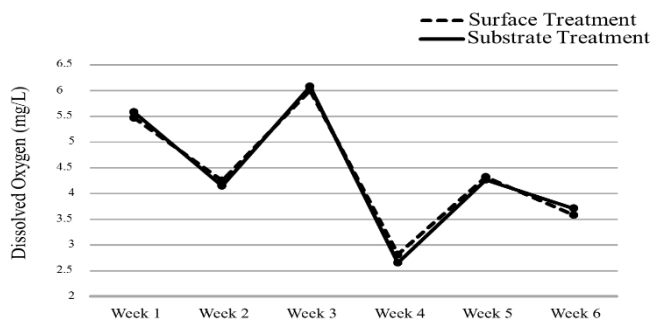


Figure 1. Six week trends in average dissolved oxygen content of water at surface and substrate treatments.

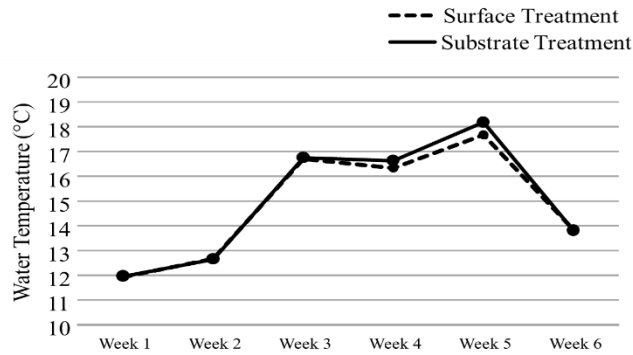


Figure 2. Six week trends in average water temperature at surface and substrate treatments.

Despite the consistency in measured environmental conditions across treatment groups, survivorship for an egg mass was significantly affected by its depth treatment ($D_1 = -55.0$, $p < 0.0001$), morph ($D_1 = -87.2$, $p < 0.0001$), and the interaction of morph and depth ($D_1 = -60.6$, $p < 0.0001$). At the surface treatment, embryos from opaque masses were less likely to survive to hatching ($\text{Pr}(\text{hatching})=0.61$) compared to embryos from clear masses ($\text{Pr}(\text{hatching})=0.84$). Both opaque and clear morphs responded similarly to substrate treatments (Figure. 1). Depth treatment did not have an effect on probability of surviving for embryos from opaque masses, but embryos from clear masses were significantly more likely to hatch at the water surface. In addition, probability of surviving increased with initial dissolved oxygen concentration, as measured during the first week of the experiment ($D_1 = -310.7$, $p < 0.0001$). Embryos from egg masses that experienced higher initial dissolved oxygen concentration were significantly more likely to survive to hatching. Dissolved oxygen concentration did not fluctuate significantly over the course of the 6-week field experiment.

Age at hatching was affected significantly by depth treatment ($F_{1,118} = 127.683$, $p < 0.0001$), but not by egg mass morph ($F_{1,118} = 1.569$, $p = 0.2128$) or the interaction of morph and depth ($F_{1,118} = 1.914$, $p = 0.1692$). Regardless of color morph, the egg masses that were fixed at the substrate took 4.9 days longer to hatch than masses fixed at the surface of the water (Figure. 2).

Developmental stage at hatching was also significantly influenced by depth ($F_{1,114} = 7.06$, $p < 0.0001$), but was not significantly influenced by morph ($F_{1,114} = 0.18$, $p = 0.67$) or the interaction between morph and depth ($F_{1,114} = 2.66$, $p = 0.11$). Clear and opaque egg masses were influenced similarly by depth; masses subjected to the substrate depth treatment hatched at .215 of a stage earlier than surface treatment masses (Figure. 3).

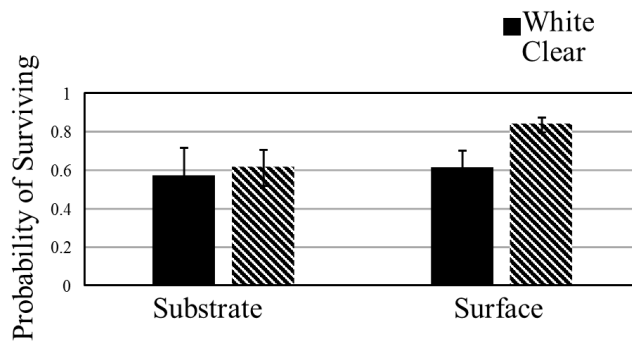


Figure 3. Survivorship data. Probability of hatching \pm SE for opaque and clear egg masses from surface and substrate treatments.

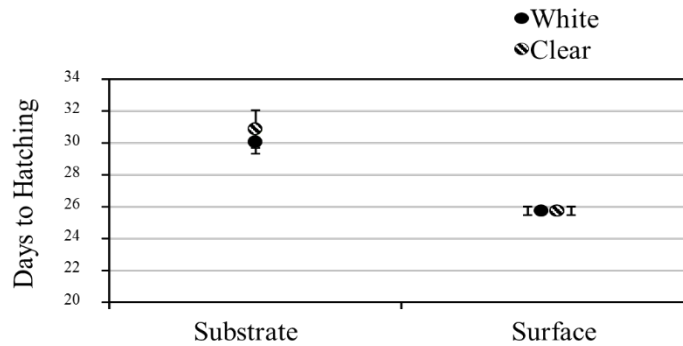


Figure 4. Average age in days at first hatching event for opaque and clear egg masses at surface and substrate treatments. Note that data points and error bars for surface treatment have been separated for clarity

4. Discussion

The differential survivorship and rate of development between clear morph egg masses at the surface and substrate treatments support our proposed circumstantial advantage of clear morph egg masses. Clear masses receiving surface treatment experienced a drastically higher hatching success compared to any other morph and treatment combination, so it is logical to conclude that environmental conditions closer to the water surface favor clear masses. It is not likely that egg masses that were anchored at the substrate had lower survivorship and delayed hatching because of lower dissolved oxygen or water temperature because those environmental conditions did not vary between treatment groups. We did not measure light availability, but it is still possible that depth in combination with greater transmittance of light by the clear gelatinous matrix was advantageous for embryos from clear egg masses.

A greater transmittance of light through the gelatinous matrix of the egg mass would better facilitate colonization of *Oophilia amblystomatis*, resulting in higher production of O₂. Algal cell densities need to be determined before this explanation can be confirmed. Future research should seek to determine differences in colonization by *O. amblystomatis* between opaque and clear morph egg masses of *A. maculatum*. We would expect membranes originating from clear masses that received surface treatment to have greater algal cell densities or larger algal cells than clear masses that were subjected to substrate treatment and opaque masses that received either depth treatment.

Depth was found to have a significant effect on age and stage at hatching, but it is important to note that the effect on stage at hatching is too miniscule to have realistic implications. According to our findings, masses that are attached to twigs or other vegetation closer to the water surface hatch significantly earlier but emerge at roughly the same stage, regardless of morph. At substrate treatments, both morphs took longer to hatch but still emerged at roughly the same stage as egg masses that received the surface treatment.

5. Acknowledgements

The author wishes to express gratitude to Rebecca Hale and Caroline Kennedy for their invaluable wisdom, guidance, and mentorship. I am also extremely grateful to Graham Reynolds for his feedback and inspiration. Thanks to my incredible network of family, friends and UNC-Asheville faculty who have supported me along this journey.

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