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The Role Of *Oophila amblystomatis* In The Maintenance Of Egg Mass Color Dimorphism In *Ambystoma maculatum*: Effects On Larval Morphology, Performance, And Survival

Mischa D'Errico
Department of Biology
The University of North Carolina Asheville
2500 University Heights
Asheville, North Carolina 28804 USA

Faculty Advisors: Caroline Kennedy, Rebecca Hale

Abstract

The spotted salamander, *Ambystoma maculatum*, exhibits two color morphs of egg mass, white and clear. Factors that contribute to the maintenance of this dimorphism have been explored, but no consensus has been reached. White egg masses are preyed upon less than clear egg masses, suggesting an advantage to white masses. Previous work has shown that larvae from clear egg masses have greater mass than those from white; however, the mechanisms and consequences of this are unknown. *A. maculatum* embryos of both color morphs have a mutualistic relationship with a green alga, *Oophila amblystomatis*, that grows in the egg capsules. The mutualism may benefit clear egg masses, as the translucent jelly may allow for greater algal density than in the white egg masses. I asked whether larvae from clear and white masses differ in survivorship or performance and, if so, whether this difference is mediated by differences in algal density. I paired clear and white masses in a pond during the embryonic period and then determined egg capsule algal densities. Larvae from these eggs were subject to swim trials and were subsequently measured. I also conducted a mesocosm predation study to determine if there were differences in larval survival between color morphs. There was no significant difference in egg membrane algal density between clear and white masses. Subsequently, there were no differences in larval performance trials and morphometric measurements between morph types or any significant differences in larval survival between morphs.

1. Introduction

The spotted salamander, *Ambystoma maculatum*, has a wide range across much of eastern North America. They are most commonly found in forests and swamps, near water, of the piedmont and mountain regions. *A. maculatum*, like most mole salamanders, bury under tree roots, fallen logs, and large boulders. Adult salamanders leave their retreat-sites for ephemeral ponds in the late winter to early spring exhibiting explosive breeding patterns when conditions are suitable. Females excrete large jelly egg masses containing up to a few hundred embryos each encapsulated within their own membrane[12]. Many populations exhibit an egg mass polymorphism in which clear and white egg masses are produced[6]. The two egg masses are visually different in color because the white egg mass jelly contains a crystalline glycoprotein, while the clear egg mass jelly protein is water soluble[19]. The production of the crystalline glycoprotein by the mother is determined by a single nucleotide polymorphism (SNP)[19]. Although the physiological and chemical differences between morphs are now understood, the advantages of each morph are still under investigation. This genetic polymorphism results in co-occurring and contrasting phenotypes; however, evolutionary theory predicts that only one phenotype should persist over the other due to any increased level of fitness of one morph, decreasing the allelic frequency of the other. The persistence and prevalence of polymorphisms with no local extinction is most likely due to a variation in selective forces that favor each morph. Hale et al.[5] suggested that variation in selection over time or among microhabitats could explain the persistence of this polymorphism. Research has shown an advantage to white egg masses in ponds with wood frog tadpoles (*Lithobates sylvaticus*) and eastern newts (*Notophthalmus*

viridescens)[7,13]. Wood frog tadpoles predate on white egg masses more than clear egg masses, suggesting an advantage to white egg masses in habitats of high predator density. The difference in predation rate is hypothesized to be due to (1) the crystalline glycoprotein within the white jelly being particularly distasteful to predators or (2) the white masses being more cryptic than clear egg masses.

An advantage of clear egg masses has yet to be confirmed, but may be related to the embryos' mutualistic relationship with green algae (*Oophila amblystomatis*). Only hours after egg masses are deposited, the benthic *O. amblystomatis* penetrate the masses and cover the outer membrane of individual embryo egg capsules[4]. As the algae photosynthesize, they supply oxygen to embryos, which may be of vital importance during times of high metabolic rates in warmer temperatures[5]. Preliminary data from Jacobsen[7] noted higher algal cell counts in clear egg masses than in white egg masses, which could help explain the maintenance of the clear morph. Embryos with algae present in egg capsules during development tend to have faster growth and larger size than embryos in capsules with no algae[3]. Similarly, Hale et al.[5] found larvae from clear egg masses were larger than those of white egg masses. These differences in size may be due to differences in oxygen supply during development, as amphibian embryonic development and hatchling morphology is known to vary with biotic and abiotic conditions including oxygen[9,11,14]. An increase of *O. amblystomatis* colonization on embryo capsules should result in more oxygen for growth and development, which has been shown to influence hatching morphology[11]. Furthermore, increased oxygen during development leads to higher fitness of juveniles in other species[20].

Locomotor performance and survival of larvae from clear and white masses can provide valuable information on the maintenance of the dimorphism. Environmental interactions through competition and predation are indicators of important evolutionary history in amphibians[10]. Previous studies have concluded a direct relationship between morphology and predator avoidance tactics[2,10]. Landberg and Azizi[10] consider salamander morphometrics to be directly related to burst swimming rather than distance swimming. If salamander larvae are supplied more oxygen during development via algal growth, it is expected that they will be larger and, consequently, stronger and faster in predator-prey interactions or intraspecies interactions.

The goal of this study was to explore further the interaction between *O. amblystomatis* and spotted salamander embryos to determine whether there are algal density differences between egg morphs, whether algal density and/or morph type is associated with differences in larval morphology and performance, and whether such differences directly translate to predator avoidance and survival. To establish a link between larval morphology and performance, which are typically measured in the lab, a biologically relevant measure of fitness, I quantified survival under semi-natural conditions by introducing larvae to predators in mesocosms. I predicted that larvae from clear egg masses will have higher algal density, exhibit morphology associated with faster burst speed, swim faster, and exhibit higher survivorship when exposed to predators, than larvae from white masses. I also predicted that, independent of morph type, algal density will correlate with larval shape, size, and swim speed.

2. Methods

2.1 Site Description

All field components of this research were done from mid-January to April at Sandy Bottom Nature Preserve adjacent to the middle French Broad River near Asheville, NC. Sandy Bottom is composed of about 35-acres of wetland and upland forest containing an ephemeral pond in which *Ambystoma maculatum* and other amphibians breed, owned by the University of North Carolina Asheville and Long Branch Environmental Education Center and partners with NC Wildlife Resource Commission (Sandy Bottom Conservation and Management Plan). Lab experiments were completed from February to May of 2018 in the Biology Department of Zeis Hall.

2.2 Field Study

Based off previous observation and research, I began making trips to Sandy Bottom in mid-January 2018 in search of egg masses. On February 16, 2018, 40 egg masses were obtained: 20 white and 20 clear. Each egg mass was bagged individually in tulle fabric to prevent predation in the field, then a clear and white mass were paired together and given either a green or red identification tag of the same number on a carabiner. A green tag was used for the identity of white egg masses and a red tag was used for the identity of clear egg masses. Each pair was assigned and attached to a PVC pipe at the same depth in the water; however, 10 of the poles were contained in a narrow straight of the pond, while the other 10 poles were held in a larger open space about eight feet away. Beginning on February 20, 2018 I

took the following measurements twice a week for five weeks: air temperature (°F), dissolved oxygen (mg/L), water temperature (°C), and PPFD light measurements.

2.3 Algal Cell Density

On March 23, 2018 the first hatching larvae were identified in two masses. The masses were taken back to the lab for survival counts and algal cell photographs. Masses were carefully separated into smaller chunks to identify individual embryo counts, dead or alive. Five embryos still contained in their individual membrane from each egg mass, were transported to individual labeled containers with part deionized water and part Sandy Bottom water. At the time of their hatching, individual membranes were extracted from their container, then manipulated on a slide with a grid cover slip. Then they were carefully photographed at one layer of algal cells, using Motic Images Plus3.0 and Motic Images Device. The larvae were kept alive for two weeks until performance testing. For the next month, the rest of the egg masses were brought to the lab in the same fashion and photographed.

Photos were analyzed using ImageJ. Five algal photos from each of the 39 egg masses, totaling 195 photos, were imported individually into ImageJ for initial cropping. Each photo had to be cropped to set a consistent scale of size for cell counts. Once that was completed, the photos were counted for total algal density. In ImageJ parameters of color threshold: black and threshold method: minimum, were set to change the color threshold of the photo. Particles were then analyzed and automatically counted using another set of parameters, size= 10 – infinity and circularity = 0.20 – 1. For the individual algal cells in each photo that were not automatically counted using ImageJ, they were manually counted and added to the total count using a clicker.

2.4 Larval Performance And Morphology

After two weeks since individual larvae hatched and algal membrane were photographed (during which they were fed brine shrimp and organic matter from fresh Sandy Bottom water), three out of the five larvae from each egg mass were randomly chosen for performance trials. Salamander larvae were put through three trials of swimming speed in a clear plastic footlong gutter-like contraption filled with deionized water. Two measuring tapes were placed underneath the gutter, with two lamps overhead for optimal lighting reducing shadows with the camera stationed directly above the gutter for recording. After two minutes of acclimation to the water, larvae were prodded perpendicularly to the base of the body to induce swimming[2]. When the larvae stopped, they were given a minute of rest then prodded two more times with a minute of rest in between. All trials were filmed with a Canon Vixia HD20 video recorder (Canon USA, New York). One trial with each larvae was analyzed in Tracker 5.0.6 (© Douglas Brown, Open Source Physics) to quantify the total distance traveled (cm), maximum velocity (cm/s), and time to travel three body lengths (s). After filming, larvae were euthanized with an overdose of MS-222 and photographed for head width, total length, tail length, and tail depth using ImageJ 2.0.0[10,18].

2.5 Larval Survivorship

Larval survival of the two morphs was quantified in a mesocosm study. Before running the experiment, 100g of leaves were collected and distributed into each of 28 “kiddie pools” (~25 gallons) of water. Surviving larvae from masses collected in the first five days of hatching, that were not involved in algal counts or performance, were used. About 500 larvae of each egg mass morph placed in two kiddie pools based on their morph type at UNCA’s semi-natural field site. Twenty eight adult newts were held in a third kiddie pool, used as the predator species for treatments. One week later, 22 larvae from the clear and white masses were placed into four treatments of white mass individuals with and without predators, and clear mass individuals with and without predators and replicated seven times for a total of 28 kiddie pools. Two adult newts were used as the predator species for the predator treatments, as they commonly consume spotted salamander larvae (Petranka, pers. comm.). Survivors were counted over two days after allowing the study to run for 12 days.

2.6 Statistical Analyses

All analyses were performed in R and the specific packages are indicated, below[17]. Algal cell density was compared between morphs using a linear mixed effect model in which egg capsule was nested within clutch using the nlme package[16]. Total distance traveled, maximum velocity, and time to travel three body lengths were not normally

distributed, so the performance data were compared between larvae from clear and white morphs with Wilcoxon Signed-Rank tests using the stats package[17]. The four morphological measurements were analyzed using principal components analysis using the vegan package[8]. The effect of algal cell density on performance and morphology were evaluated with Spearman Rank Correlations using the stats package[17]. Finally, survivorship of hatchlings in mesocosms was evaluated with logistic regression in a generalized linear model using the nlme package[16].

3. Results

3.1 Algal Cell Density

Algal cell density ranged between 661 and 2063 cells / mm^2 for clear masses and 224 and 1987 cells/mm^2 for white masses. There was no difference in algal cell density between egg capsules from clear and white egg masses ($F_{1,147}$ = 0.03, p = 0.86; Fig. 1, 2).

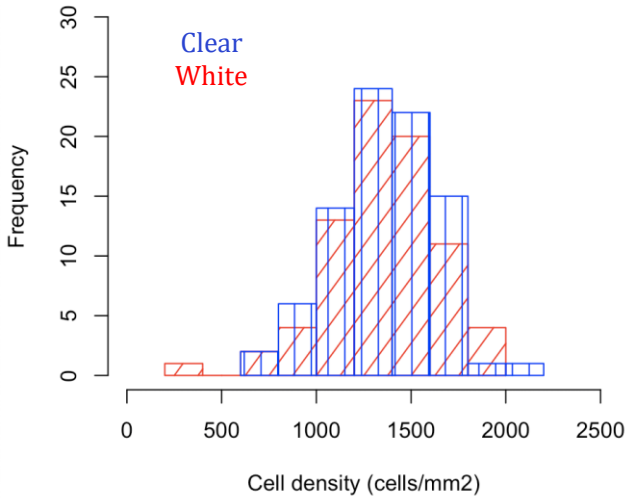

Figure 1. Frequency distribution of algal density for egg capsules from clear (blue bars) and white (red bars) egg masses.

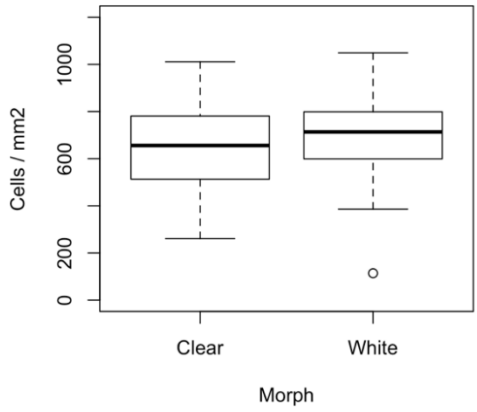

Figure 2. Box plot of algal cell density (cells/mm^2) for spotted salamander egg mass embryos from morph types: clear and white.

3.2 Larvae morphology and performance

Total distance traveled was normally distributed, but maximum velocity and time to travel three body lengths were not, so all performance variables were compared between clear and white morphs using Wilcoxon [BH1] signed rank tests. Larvae from clear and white egg masses did not differ in total distance traveled (W=1304, N = 102, p=0.92; Fig. 3), maximum velocity (W=1335, N=102, p=0.75; Fig. 3), or time to travel three body lengths (W=702.5, N = 73, p=0.69; Fig. 3). Sample size was lower for time to travel three body lengths because total length measurements were not available for all larvae.

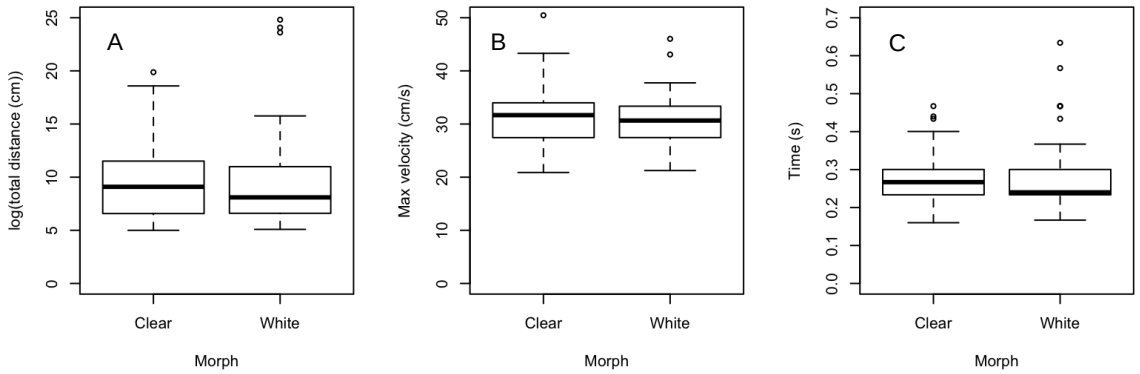

Figure 3. Larval performance measurements total distance (cm), maximum velocity (cm/s), and time to travel three body lengths (s) between salamander egg mass morphs, clear and white.

Principal components analysis of larval morphology are summarized in Table 1; principal component 1 (PC 1) explained 62% of the variance in morphology and principal component 2 (PC 2) explained 18%. All measurements loaded positively on PC 1, indicating that PC 1 captured variance in overall body size. Head width and tail depth loaded positively on PC 2, whereas total length and tail length loaded negatively on PC 2, indicating that PC 2 captured variance in shape (Fig. 4). Neither body size (PC 1; W=623, N = 67, p=0.545) or shape (PC 2; W=533, N = 67, p=0.74) differed between larvae from clear and white masses. Although neither performance nor morphology differed between larvae from clear and white masses, this did not preclude the possibility that algae could influence performance or morphology. Therefore, we analyzed whether algal density was related to performance variables or morphology using Spearman rank correlations in the stats package of R, when egg mass morphs were pooled. Algal density was not correlated with total distance traveled (S = 107050, N = 102, p = 0.82, = 0.024; Fig. 5), maximum velocity (S = 120590, N = 102, p = 0.3612, = -0.099; Fig. 5), or time to travel three body lengths (S = 40656, N = 73, p = 0.59, = 0.025; Fig. 5). Further, algal density was not correlated with PC 1 (S = 36924, N = 67, p = 0.551, = -0.079; Fig. 6) or PC 2 (S = 31996, N = 67, p = 0.624, = 0.065; Fig. 6) scores.

Table 1. Summary of principal components analysis for four measurements of *A. maculatum* larval morphology, where PC1 describes body size and PC2 describes body shape. Measurements taken after performance trials at day 14.

Measurement	PC1	PC2	PC3
Head Width	0.436	0.655	0.570
Total Length	-0.561	-0.365	0.280
Tail Length	0.546	-0.499	-0.121
Tail Height	0.444	0.434	-0.763
Proportion variance explained	62.1%	18.4%	15.8%

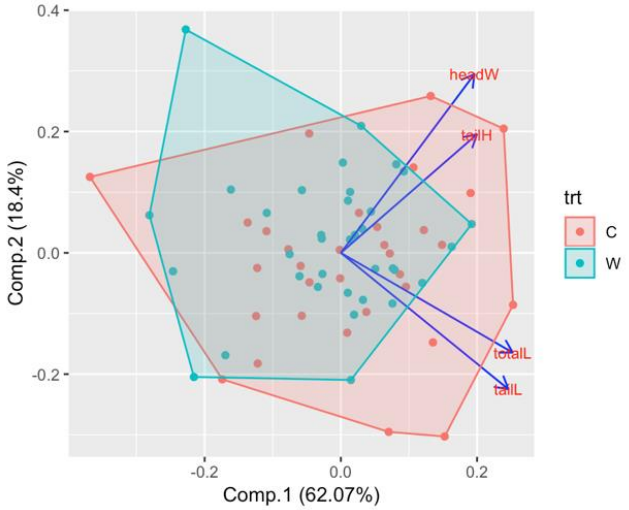

Figure 4. Principle components analysis biplot of individual larvae morphometrics (head width, tail height, total length, and tail length) between clear (red points) and white (green points) egg masses.

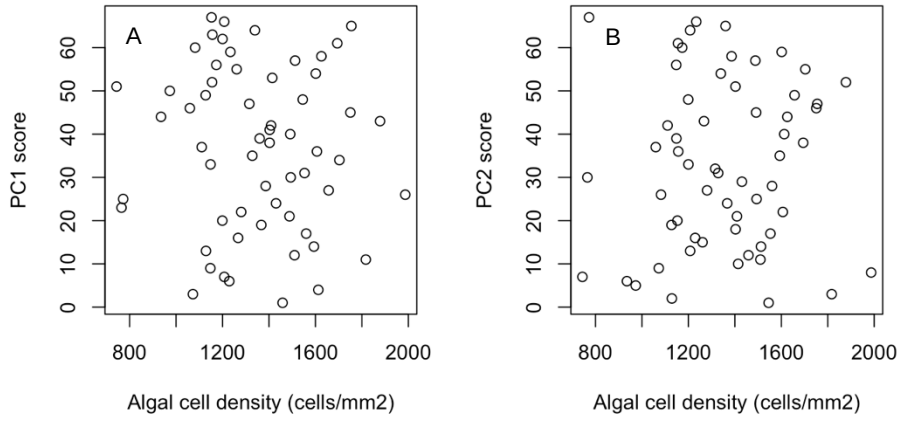

Figure 5. Relationship between algal density and (A) principal component 1 and (B) principal component 2 scores.

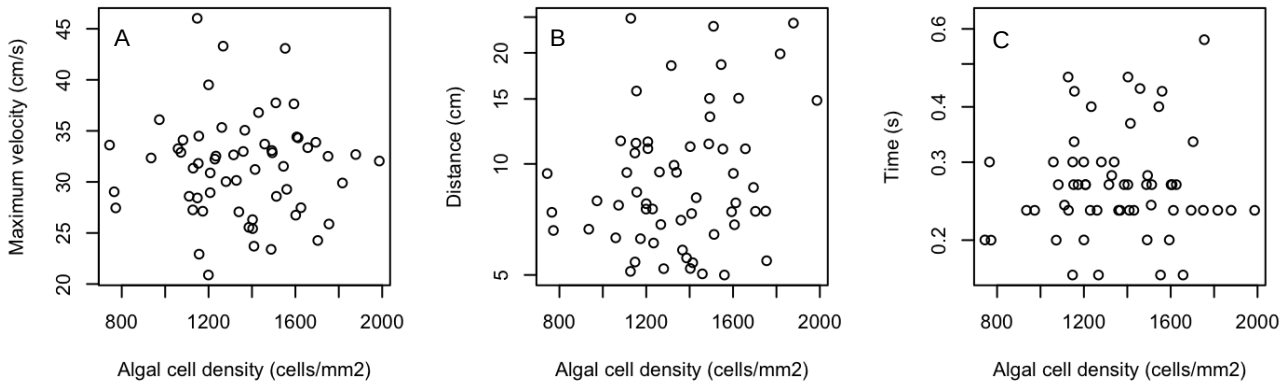

Figure 6. Relationship between algal density and performance metrics: (A) maximum velocity observed during the movement away from the stimulus, (B) total distance traveled during the movement, and (C) time to travel three body lengths.

3.3 Mesocosm

Survival rates in the presence of eastern newt predators did not differ significantly between egg mass color morphs (Morph: D_{res}=-0.06, df=26, p=0.8, Predator: D_{res} = -7.23, df=26, p=0.007, interaction: D_{res}=-0.006, df=24, p=0.94; Fig. 7).

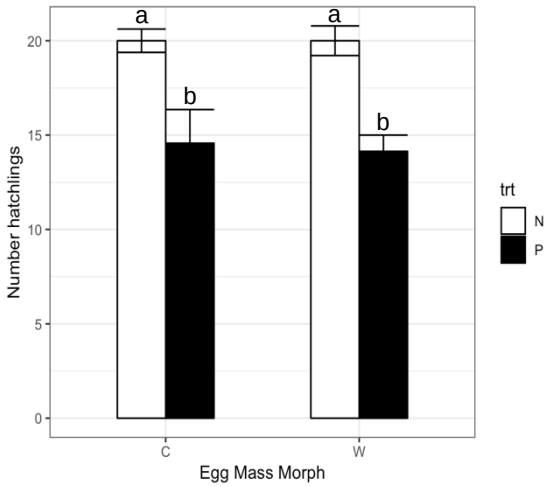

Figure 7. Mean ± SE number of larvae survived in mesocosm predation study between spotted salamander egg mass morphs: clear (C) and white (W) of two treatments no predator (N; white) or predator (P; black). Means with the same letter were not significantly different.

4. Discussion

My results indicate there is no difference in algal density of spotted salamander egg membranes between clear and white egg masses (Fig. 1, 2). In addition, there were no significant differences in morphological measures of body size and shape of larvae between clear and white egg masses (Fig. 4). Furthermore, performance measures of total distance swam (cm), maximum velocity (cm/s), and time to swim three body lengths (cm) were not significantly different between egg mass morphs (Fig. 3). Since there were no discrepancies in body size and shape of larval

morphometrics it is not surprising that performance trials did not yield noteworthy differences either. I further investigated possible algal density effects on each performance variable and principal components analysis of morphometrics, independent of morph type. Algal density did not have an effect on larval morphometrics (Fig. 5) or performance measures (Fig. 6).

Previous research on algal cell density in *A. maculatum* egg masses has provided an array of conflicting data. Algal density has been noted to have both a positive and negative correlation with growth and development of embryos and larvae. For example, Gilbert[3] showed that hatchlings reared from masses with algae were larger than those reared in the absence of algae, whereas Hale et al.[5] and Tattersall and Spiegleaar[21] found no effect of algae on hatchling size. Conflicting evidence for the role in *O. amblystomatis* on spotted salamander embryonic development and subsequent larval size and shape may be explained by varying spatial and temporal selection that makes each type of egg mass morph more advantageous in different environmental conditions of predation, sunlight, and pond nutrients[6,715,19].

I predicted that algae would occur in clear egg mass membranes at a higher density, resulting in larger size and better performance; however, I found no differences in larval morphology and performance between morphs. Allowing larvae to develop for two weeks in a controlled environment, individually could have impacted results of performance and morphometrics. Larvae were possibly able to equalize with no interactions with other salamander larvae or predators, disrupting the natural evolutionary conditions in which they are born into. There has been a difference in larval size immediately following hatching and when embryos were removed from their jelly prior to hatching[5]. In this experiment, embryos remained in their jelly until hatching and not measured until 10-14 days following. Any possible differences between larvae equalized, or differences do not occur when reared in jelly, which is more consistent with Hale et al.[5] previous data. Body and developmental stage, known to change with ontogeny, are not often used in evaluating ecological interactions due to the unpredictability[10]. Swimming performance and survival in mesocosm study could have been affected by the absence of predators from larvae to two-week old time period because mortality and predation are density dependent and decrease with increasing body size so selection for escape performance/burst speed decreases through the larval stage[10]. I did not use normalized tail area that was the best predictor for swim performance in Landberg and Azizi[10]. Buskirk and McCollum[2] found a deeper tail fin had a positive correlation with swimming performance which was no indicated by this studies results.

Future research should continue to test the fitness differences of larvae from clear and white egg masses in context to algal colonization. Algal density counts should be repeated, and perhaps counted on egg membranes throughout embryonic development, as algae may colonize embryos at different rates. Work should focus on similar performance trials of individual larvae but with the introduction to chemical cues for predation and competition to simulate natural selective pressures immediately following hatching in the lab. Since predation pressures are so high on small salamander larvae immediately after hatching, conducting controlled lab experiments under as natural conditions as possible will hopefully produce natural results from competitive larvae in morphometrics and swimming performance.

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