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Ontogenetic Effects of Hatching Plasticity in the Spotted Salamander (Ambystoma maculatum) due to Egg and Larval Predators

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Abstract

The ability to respond plastically to the environment has allowed amphibians to evolve a response to spatial and temporal variation in predation threat (Benard 2004). Embroys exposed to egg predation are expected to hatch out earlier than their conspecifics. Larval predation can induce a suite of phenotypic changes including growing a larger tail area. When presented with cues from both egg and larval predators, embryos are expected to respond to the egg predator by hatching out earlier because the egg predator presents an immediate threat. However, hatching early may be costly in the larval environment in terms of development, morphology, and/or behavior. We created a laboratory experiment in which we exposed clutches of spotted salamander (*Ambystoma maculatum*) eggs to both egg (caddisfly larvae) and larval (*A. opacum*) predators to test this hypothesis. We recorded hatching time and stage and took developmental and morphological data of the animals a week after hatching. Larvae were entered into lethal predation trials with a larval predatory sunfish (*Lepomis* sp.) in order to study behavior. We found that animals exposed to the egg predator cues hatched out earlier and at earlier developmental stages than conspecifics regardless of whether there was a larval predator present. Animals exposed to larval predator cues grew relatively larger tails and survived longer in the lethal predation trials. However the group exposed to both predators showed a cost of early hatching in terms of lower tail area and shorter survival time in predation trials. The morphological and developmental effects measured of hatching plasticity were transient as there were no developmental or morphological differences between the treatment groups at metamorphosis. Hatching plasticity may be transient but it is important to the development and survival of many amphibians.

Introduction

Amphibians have evolved the ability to plastically respond to the environment around them. Transitions from egg to larva or from larva to adult are instances during which amphibians can alter development or morphology in order to adaptively contend with the environment around them (Rose 2005). The ability to accelerate metamorphosis and speed up development allows animals to leave the aquatic larval environment to avoid desiccation or predation. Since predation may be patchy in time and space, altering hatching time lets animals adjust to the environment around them. Egg predators may induce early hatching to avoid being eaten while they are immobile in their egg capsules. However, with any plasticity there may be costs. These costs can be in terms of development, morphological differences, or the ability to escape from predation and will be incurred and measured in future life stages.

Studies have found that embryos will often hatch out early when faced with an egg predator. Gomez-Mestre *et al.* (2006) found that spotted salamanders (*Ambystoma maculuatm*), wood frogs (*Rana sylvatica*), and American toads (*Bufo americanus*) hatched out more quickly, at smaller size and earlier developmental stage when exposed to a deadly water mold. Work with the Red-Eyed Tree Frog (*Agalychnis callidryas*) found that embryos exposed to egg predation would hatch out earlier than conspecifics that were undisturbed (Warkentin 1995, 1999, 2000). Vonesh (2005) found that the African reed frog (*Hyperolius spinigularis*) responded in the same way with early hatching in response to egg predation. Chivers *et al.* (2001) discovered that Pacific Tree Frogs (*Pseudacris regilla*) and Cascades frogs (*Rana cascadae*) hatch early due to contact from both predatory leeches and non-predatory earthworms. They also found that *P.*

regilla also hatches earlier when exposed to chemical cues of egg predators and injured eggs.

Larval predators do not reveal consistent effects across species. Sih and Moore (1993) examined the effects of larval predator flatworms on hatching time of the Stream-side salamander (*Ambystoma barbouri*). They found that both the presence of flatworms and the presence of flatworm cues caused the embryos to delay hatching compared to animals in either the control freshwater or non-predatory isopod treatments. Anderson and Petranka (2003) however found no change in hatching time in response to larval predation. They exposed *R. sylvatica* and *A. maculatum* embryos to fed and unfed dragonfly larvae (*Anax junius*), conspecifics or control freshwater. Neither *R. sylvatica* nor *A. maculatum* responded to any of the treatments in terms of hatching time. In general, embryos seem to remain in protective eggs for as long as possible in order to grow and develop away from the larval environment unless an environmental change or predator forces them out. While some embryos delay hatching in the presence of a larval predator, embryos seem to respond more strongly to the more immediate threat of an egg predator.

The adaptive value of early hatching is clearly escaping from the egg predator. What, however, are the costs of such plasticity? These may take multiple forms. For example, both size and shape at hatching can be affected by plasticity. Early hatching embryos will tend to leave the egg at a smaller size and earlier developmental stage. This difference in size between early and later hatching animals is a cost to altering hatching time. Anderson and Petranka (2003) found no difference in size of larvae between treatments but they measured only total length. When faced with predation animals may

not grow longer but may grow a larger tail to enhance escape ability (Van Buskirk and Relyea 1998; McIntyre *et al*. 2004). Vonesh *et al*. (2000) also found that surviving Cinnamon-bellied Reed Frog (*Hyperolius cinnamomeoventris*) that had been exposed to fly predation as embryos were significantly smaller at hatching. Being smaller at hatching may have a cost in a reduced ability to compete for food or escape from predation (Beckerman *et al*. 2007).

In the aquatic environment tadpoles and salamander larvae use their tail fins for propulsion: a larger tail area will create more force and thus more speed (Wassersug & Hoff 1985; Aziz & Landberg 2002; McCollum *et al*. 1997). A larger tail may also be a lure for a predator (Johnson *et al*. 2008; Blair & Wassersug 2000; Van Buskirk *et al*. 2000a) because if the predator bites off a piece of the tail the animal is still alive and safe, whereas a smaller tail may not attract a predator and may not be large enough to ensure the body of the animals does not get bitten. Larvae raised with predators are expected to have larger tails in order to escape from larval predation (Van Buskirk *et al*. 2000b; Steiner 2007; Relyea & Werner 2000). Van Buskirk and Relyea (1998) found that *R. sylvatica* reared in ponds with predators had deep tail fins, and dragonflies preferentially killed tadpoles that were smaller with shorter tail fins. Relyea (2004) found increased predation of *R. sylvatica* resulted in deeper tails. Relyea and Hoverman (2003) found that gray tree frog tadpoles (*Hyla versicolor*) grew a deeper tail fin with a shorter body when exposed to *Anax* larvae. However, there may be a cost to this tail plasticity.

When animals are exposed to multiple life stage predators there needs to be a mechanism by which they respond to multiple threats (Relyea 2001b; Van Buskirk 2001; Van Buskirk 2002a). The animals will be responding to either the most immediate or the

most deadly threat. Relyea (2001a) found that animals have predator specific responses. Ireland *et al.* (2007) exposed embryos of green frogs (*R. clamitans*) to an egg predator, a larval predator, a combination of both predators, and a control with no predators. Following the trend in other studies, they found that the egg predators alone induced early hatching at an earlier developmental stage and a smaller size than the control. The larval predator however caused a delay in hatching with a later developmental stage and a larger size. However, there were no significant differences in hatching time in the treatment with both predators when compared with either of the single predator treatments. The lack of response to the combination of predators led the authors to speculate that the combination of predators dampened the effect of either threat.

Relyea (2003a) exposed *R. sylvatica* tadpoles to four different predators that varied in the life stage that they threatened. Animals were raised in single predator treatments as well as combined predator treatments. Animals appear to have responded to the predator that was the greatest threat at a given time. Unlike in Ireland *et al.*, these animals responded to one of the combination of predators. Vonesh *et al*. 2003 found that the combined effects of egg and larval predators on *H. spinigularis* were non-additive. The difference between these results and those of Ireland *et al.* may have been due to the different species of *Rana* or the predators used.

We designed an experiment to test whether there are phenotypic costs of hatching plasticity in spotted salamanders (*Ambystoma maculatum*). We had two main objectives: to examine effects of egg predators on hatching time, and to study the effects that early hatching may have on the ability to respond to larval predators. To do this we used four treatments: egg predators, larval predators, both predators and no predators. We

hypothesized that embryos that are exposed to an egg predator will respond by hatching earlier and at younger developmental stages than animals in the larval predator treatment or control. We expected that regardless of whether there is a larval predator present or not, the embryos will respond to the immediate threat of egg predation, not the future threat of larval predation. However, there may be a cost to hatching out early. Larvae that remain in the egg longer should have the time and resources to put toward a larger tail area, which could be useful for escape behavior. Animals exposed to larval cues should also be more adept at escaping predation. Finally, animals that hatch out early may end up metamorphosing later than their conspecifics that hatched out on a regular schedule.

Materials & Methods

Experimental Design

Six spotted salamander (*Ambystoma maculatum*) clutches were collected from the Fenton River in Storrs, CT (on 4 May 2007), and placed in forty-eight thirty-eight-liter aquaria, arranged in two spatial blocks. The animals ranged in stage from 37 – 39 (Harrison 1969). Each clutch was separated into eight equal parts for two replicates each of four treatments: control (no predator), egg predators (caddisfly larvae), larval predators (*Ambystoma opacum*), and both egg and larval predators. Tanks were randomly assigned to a treatment. Egg and larval predators were collected from the Fenton River in April 2007 to create an environment as close to reality as possible (Miner *et al*. 2005).

Animal husbandry

Air temperature was maintained at 15ºC, with a 12 hr light: 12 hour dark cycle. Tanks were divided with a fiberglass screen (2 mm pore size) in a Plexiglas™ frame (Figure 1A). Each tank was then half filled with distilled water and adjusted with R/O Right™ salt to approximate physiological osmolarity. Each tank had a constant supply of air being bubbled from a pump. Experimental animals were fed aquatic invertebrates (~90% bloodworms) collected from a local fish hatchery. Water was changed weekly, removing and refilling about 15 liters.

About a month into the experiment there were full tank deaths most likely due to a bacterial infection. The animals had curved tails, exploded yolk sacs, and had lost their color. All animals died in tanks 5, 12, 15, 17, 18, 19, 20, 21, 23, 24, 25, 26, 28, 29, 30, 38, 40, 42. Following tank deaths the tanks were emptied and cleaned with a mix of Bleach, soap and water. Animals were redistributed on June 6, 2007 keeping animals in

the same treatment and clutch together. After redistribution there were: clutch 1 – 7 tanks, clutch 2 – 9 tanks, clutch 3 – 10 tanks, clutch 4 – 6 tanks, clutch 5 – 8 tanks, clutch 6 – 8 tanks. For treatments: egg predator – 10 tanks, egg/larval predator – 14 tanks, control – 12 tanks, and larval predator – 12 tanks.

Predator treatments

Predator cues differed between treatments. In the egg predator treatment, caddisfly larvae were placed in the side of the tank with the egg clutch. They were observed walking around on the egg masses and manipulating them, but were not observed eating them. In the larval predator treatment, *A. opacum* were placed on the other side of the divider from the clutch of experimental animals. These were fed with other *A. maculatum* larvae every 2-3 days. This treatment allowed the chemical cues from the larval predator and their conspecifics to reach the egg masses without allowing predation on the experimental animals. Treatments were terminated June 23rd, by removing all predators when *A. opacum* began to metamorphose (26 days after final hatching). *A. maculatum* were allowed to continue developing until August 30th(week 18) when animals in the first block were euthanized, photographed, tagged, and preserved in ethanol; animals in the second block were treated identically on September 6th(week 19).

Developmental staging

Upon entering the experiment, all animals were staged in the egg. Daily records were kept for the number of hatchlings per tank and the stage of hatched animals. Newly hatched animals were staged following the Harrison staging table (Harrison, 1969). All animals had hatched by day 20. The Harrison staging table, which stops at stage 46 (animals with two front limb toes, one front limb toe bud and a hind limb bud), was

extended from stage 47 through 55 using similar criteria to the Harrison Staging table (Table 1).

Table 1 – Continuation of the Harrison (1969) staging table for *Ambystoma maculatum*.

Stage	Front Limb Toe Bud[1]	Front Limb Toe	Hind Limb Bud[2]	Hind Limb Toe Bud	Hind Limb Toe
45	0	2	Yes	0	0
46	1	2	Yes	0	0
47	0	3	Yes	0	0
48	1	3	Yes	0	0
49	1	3		2	0
50	1	3		1	2
51	0	4		0	3
52	0	4		1	3
53	0	4		0	4
54	0	4		1	4
55	0	4		0	5

[1]A toe was differentiated from a bud by being longer than wide.

[2]The hind limb was a bud until toes started to form.

We recorded developmental stages again at the end of the experiment. We created a metamorphic staging table based on three characteristics that are known to change during metamorphosis: gill size, skin color pattern, and tail fin development (Figure 2). The salamander gills are large and robust (0) during the larval period and then become reduced (1); subsequently the filaments are resorbed leaving a bare rachis (2) until the

rachis is resorbed by the end of metamorphosis (3). Skin coloration changes from a drab, solid color (0) during the larval stage to mottled with yellow pigment (1), through indistinct spots with ragged edges (2) to fully and distinctly spotted (3). The tail fin of larvae extends far up the back to the head (0) during the larval period, gradually recedes posteriorly (1) and is ultimately completely resorbed leaving the adult's muscular tail (2). Each animal was scored for all three traits and received a total score from 0 (fully larval) to 8 (fully metamorphosed).

Morphology

After hatching, photographs were taken weekly of each experimental animal. Each animal was photographed in a 10.2 x 10.2 x 7.6 cm Plexiglas™ box that had a scale bar for calibration. Morphometric traits were measured from lateral view photos analyzed with Image J software (freeware available at: http://rsb.info.nih.gov/ij/). Measurements were taken of total length, snout-vent length, maximum tail height, and tail area (including the fin above the back anterior to the pelvis) according to Azizi & Landberg, 2002.

Predation Trials

Predation trials were run on May 29th, May 30th, May 31st and June 1st, running half a block, twelve tanks, per day. Originally the predation trials were designed using the same larval predator the marbled salamander (*Ambystoma opacum*) which was providing cues in the experimental tanks. Before beginning predation trials with the experimental animals we ran predation trials with another population of *A. maculatum*. While *A. opacum* would eat salamanders in the experimental tank, the predators would

only consume one hatchling per predation trial and most would not eat at all. Due to the fact that we needed voracious predators we switched to sunfish (*Lepomis* sp.) and the fish trained very quickly. By the time our experimental animals entered predation trials the fish had run enough trials that there was no learning curve on what they were eating.

Five animals from each tank were randomly sampled, staged and photographed. These five animals became a predation trial replicate and were assigned to one of four predators (green or bluegill sunfish (*Lepomis cyanellus* and *L. macrochirus* respectively). Fish ranged in size from 9.6 cm to 10.2 cm. Since the primary interest was in the relationship between tail morphology and escape performance, the predation tank was an open arena; there were no hiding places. This design maximizes reliance on escape swimming performance rather than refuge seeking behavior. The hatchlings were placed into a clear cylindrical Plexiglas™ predation trial tank (51 cm diameter, 23 cm deep) that had been filled with 30 liters of water (Figure 1B). A fish had previously been placed in the water so that all treatment groups started with the same predator cues. Hatchlings were allowed to acclimate to the water for one minute before the fish was added. Each trial was allowed to run for 10 minutes. Investigators left the room and filmed trials from above at 30 frames/sec (Panasonic PV-GS59 digital camcorder and Sony mini digital video cassettes). Each fish participated in three trials per day so fatigue or satiation was not an issue (i.e., all predators ate all the salamanders in every trial).

Analysis of the predation trials was done from the videos uploaded to a Macintosh computer into iMovie HD. First, the number of hatchling moves during the "pre-trial" was recorded in order to determine a baseline for movement. Movement rate in the presence of fish (moves/animal/minute), the time at which each predation event occurred,

and the number and time of each escape response was recorded. An escape response was defined as a high curvature movement (also known as a startle or escape response or c-start; Azizi & Landberg, 2002) that the hatchling made while being targeted by the fish, which may or may not have ended in predation. The end of the trial was the time at which the fish ate the fifth hatchling.

Metamorphosis

In order to determine if hatching plasticity affected the animals at later life stages, we looked at their development and morphology at metamorphosis. We preserved animals from each block on a single day in order to get a cross-sectional look at their developmental morphology. Animals in block two developed more slowly than block one and were given an additional week to develop (week 18 and 19 respectively). Total length and snout-vent length were measured from lateral view photographs, and developmental stages were calculated (criteria in Figure 2).

Statistical Analysis

Tanks are the unit of replication, so tank means are the data points. All analyses were conducted with a linear model ANOVA and significant treatment differences were diagnosed with Tukey's post-hoc test using JMP 5.0. All statistical tests included treatment, clutch and block as fixed factors.

For hatching, the proportion hatched each day and developmental stage of the hatched animals were the variables of interest. These data were analyzed for days 5-15 when most of the hatching occurred. Day and the interaction of treatment*day were included in the ANCOVA model.

Total length was analyzed with ANOVA with block, clutch and treatment as fixed factors. Tail area from weeks 4 and 14 was analyzed with ANCOVA with total length as the covariate. The interaction between total length and treatment was not significant and thus was excluded from the model. The block*treatment interaction showed that three of the treatments (control, egg/larval, and larval) had larger tails in block 2 than in block 1. This did not affect our interpretation of the data in any way because the comparison between treatments in blocks was the same and therefore was also excluded from the model.

The ANCOVA for the predation trials had predation trial duration as the variable of interest with developmental stage and total length as covariates and predator ID (which fish was used) as fixed factor. To see if escape or movement rates differed among treatment groups we ran ANCOVA with escape and movement rates as the variables of interest, with stage and total length as covariates. Finally, to see if movement rate or escape rate affected survival time, we ran an ANCOVA with predation trial duration as the response, and escape rate, movement rate, stage and total length as covariates.

Rate of metamorphosis was analyzed with an ANCOVA with metamorphic stage as the variable of interest and included the interaction between treatment and total length. Since the interaction was not significant, it was excluded. Total length was also a variable of interest and we analyzed it with an ANOVA.

Results

Hatching

The animals in the egg predator and egg and larval predator treatment groups hatched significantly earlier than those in the control and larval treatment groups (Tukey post-hoc test; Figure 3). Hatching (number hatched/total animals alive in tank) was analyzed using ANCOVA and the overall model was highly significant ($p<0.0001$) as were all of the factors and covariates (Table 2)

The animals in the egg predator and egg/larval predator treatment groups hatched at significantly earlier developmental stages than the animals in the control and larval treatment groups (Tukey post-hoc test; Figure 4). Stages were analyzed using ANCOVA and the overall model was highly significant ($p<0.0001$) as were most of the factors and covariates (Table 3).

Hatchling Morphology

We ran an ANCOVA on hatching morphology data to see if any of the morphological traits were different between the treatment groups. There was no significant differences in the ANCOVA for total length ($p>0.05$).

The ANCOVA for tail area in week 4 was highly significant ($p<0.0001$) and explained 91% of the variation. Tail area was greatest for the larval predator treatment and least for the egg predator treatment group (significant Tukey differences; Figure 5A). Animals in the egg and larval and control were intermediate and not statistically different from other groups. All of the covariates and factors that were put into the ANCOVA were significant (Table 4).

Tail area in week 14 did not differ across treatment groups (Figure 5B). Thus, the treatment effects observed in week 4 did not persist throughout the larval period. (Table 5)

Predation Trial Results

Survival time in the lethal predation trials was significantly affected by predator treatment and developmental stage (Figs 6 & 7). The ANCOVA model for survival time was statistically significant ($p=0.0088$) as were some of the factors and covariates (Table 6). Animals in the larval predator treatment group survived longer than those in the egg predator treatment group with the other groups intermediate. Developmental stage negatively affected survival time (Figure 6). Neither clutch nor predator ID had a detectable effect on the survival times.

Metamorphosis

Time to metamorphosis (as measured by developmental stage in weeks 18 & 19 for blocks 1 & 2 respectively) was not affected by predator treatment (Figure 8A). The ANCOVA model with metamorphic stage as the response variable was not significant ($p=0.3169$). There was however one variable (total length; $p=0.0249$) that was significant indicating that larger animals were at a later developmental stages. The rest of the variables were not significant (Table 7).

Body size (total length in week 18/19) was not affected by predator treatment (Figure 8B). The ANOVA model was not significant (Table 8).

Discussion

Early exposure to egg predator cues appears to significantly affect hatching in the spotted salamander (*Ambystoma maculatum*). The embryos in the two treatments with egg predators (egg only and egg/larval) hatched out earlier than in treatments lacking egg predators (fig. 3), supporting work in other studies (Warkentin 1995, 1999, 2000; Vonesh 2005; Chivers 2001). The immediate threat of potential egg predation appears to outweigh the future threat posed by larval predators. The larval predator did not appear to have an effect on hatching time because the larval predator treatment group and the control were not significantly different from each other in terms of hatching time.

As found in other studies (Warkentin 1995, 1999, 2000; Vonesh 2005; Chivers 2001) the animals in the egg predator and egg/larval predator treatment groups hatched at significantly earlier stages (fig 4) than the animals in the larval and control treatment groups. This means they were having less time to develop in their eggs and doing more development in the larval environment. Again, there was no evidence that the presence of the larval predator affected hatching stage because the larval and control groups were not statistically different from each other showing that the later life threat will not cause them to hatch at even later life stages.

These data confirms previous results of Anderson and Petranka (2003) who found that *A. maculatum* would not alter hatching time, hatching synchrony or developmental stage in the presence of an odonate larval predator. This is most likely due to the animals remaining inside the relative safety of their egg for as long as possible unless something forces them out. Our experiment, however, included the egg predator cues as well, which was the threat to cause the animals to hatch out of the egg, and in the treatment group

with both egg and larval predation cues the animals were confronted with threats in multiple life stages. These animals still responded to the threat of egg predator cues and this effect was not dampened by the threat of the larval predator. This proves that in the presence of both an egg and larval predator, the animals still respond to the immediate egg predator threat over the future life stage larval predator.

Pictures of all the animals were taken weekly during the experiment so that trends in morphology and development could be measured. While total length, snout-vent length and maximum tail height did not differ significantly between the treatment groups at any stage, the animals in the larval predator only treatment group had significantly larger tail areas for their body size than the animals in the egg predator only treatment group one week after hatching (fig 5A). These data are consistent other work in which amphibians exposed to predator cues had larger tail areas. A larger tail may help to increase thrust during locomotion to propel hatchlings faster in the water (Van Buskirk *et al.* 2000b; Van Buskirk and Relyea 1998; Wilson *et al*. 2005) as well as create a lure to draw larval predators away from the body (Johnson *et al.* 2008; Van Buskirk *et al.* 2003).

The cost of early hatching on hatchling morphology can be seen in the egg and larval predator treatment group. These animals hatched out early to avoid predation by the egg predator but at an apparent cost in their tail morphology. They also received larval predator cues but their tail morphology was intermediate and not significantly different from either the egg predator treatment or larval predator treatment. This is consistent with the idea that in order to deal with both of the potential threats, the larvae hatched out early, but were not able to devote as many resources to building the larval tail fin.

Anderson and Petranka (2003) also looked at morphology to see if they could find morphological differences between their treatment groups. They measured total length, tail length, tail depth and muscle depth in the tail and found no significant differences between any of the treatment groups. Similarly we found no difference in total length, snout-vent length, or maximum tail height, but we did find a difference in tail area. We believe that if they had also measured tail area or population differences they may have had a different result. Tail area is expected to be more closely correlated with escape performance because it measures the full surface area that the animal has to create thrust to propel itself through the water or lure a predator.

The lethal predation trials were run at week 4, about one week after the animals finished hatching. At this point the animals also had significant tail area differences. We designed the trials to measure the behavioral differences of the treatment groups. The first, somewhat surprising discovery was that the animals that were at later developmental stages were surviving for shorter times in the trials (fig. 6). This seems counterintuitive because generally one might think that animals that are more developed are better able to escape predators. Development in this case reduced survival time; however, neither escape rate nor movement rate contributed significantly. This means that the animals at younger stages were not escaping more often from the predators in order to survive longer and the animals at higher developmental stages were not moving significantly more to attract the predators. Since the animals at higher developmental stages were slightly larger they may have been bigger targets. This trend is consistent with another data set showing similar results (unpublished data: Artrip, Brown-Wilusz, Landberg). In that study more developed animals were also eaten at a higher rate, but

with more trials we found a negative correlation between movement rate and survival time. However, there could also be chemical, visual or behavioral cues that were not measured in this experiment.

In addition to development, predator treatment also affected survival time. The larval predator treatment group survived significantly longer in the predation trials than in the egg predator treatment group (fig. 7). The control and egg/larval predator treatment groups were intermediate. This result mirrors the tail area data. We do not know the mechanism underlying the difference in this fitness metric but it could be something chemical, behavioral, or visual used to avoid predation. Since the animals in the larval predator treatment group were already exposed to larval predator they may have already known to behave to avoid detection and thus predation.

The differences in tail area seen shortly after hatching did not persist throughout larval development. One explanation for this could be that all the predators were removed from the experimental tanks 8 weeks into the experiment when the larval predators began to metamorphose. If the larval predators had remained in the tanks for the duration of the experiment, tail area differences may have been maintained. Inducible defenses have been show to be reversible in other systems (Relyea 2003b; Van Buskirk 2002b; Schoeppner 2008).

Another reason for this could have been the mortality that occurred during the 4th and 5th week of the experiment. After redistribution some of the clutches were represented more than others and there were fewer animals in the experiment. Infection may have caused the animals that were left to grow smaller, or there could have been a reduction in statistical power.

Metamorphosis is the other major history transition in amphibian development. Therefore, we wanted to find out if hatching out early would mean that the animals would metamorphose at a later date. Metamorphosing later is typically interpreted as a cost in amphibian development. We examined a cross section of the metamorphic stages and staged all of the animals on the same day. This way we could determine which animals were farther along in development on a particular day.

Animals that were larger were farther along in development. This result makes sense because since metamorphosis is costly the animals that are bigger would have more resources to be able to devote to metamorphosis. This pattern of larger animals being at later developmental stages was a pattern that was found across treatment groups.

Unlike other studies with larval predators (Vonesh and Warkentin 2006) there were no effects of predator treatment on either size or developmental stage at metamorphosis. Although the egg predator induced early hatching, animals did not develop at different rates to metamorphosis. This means the animals in the egg and egg/larval treatment groups were able to keep up with their counterparts in larval and control treatment groups.

Future research on hatching plasticity would need to include work to determine what exactly the cue is that the embryos are receiving. The animals exposed to the egg predator may be receiving mechanical, chemical or visual cues that cause them to hatch out early. In order to determine which cue it is, there would need to be treatments that just had chemical cues of caddisfly larvae, treatments that had non-lethal manipulation of the eggs, and the visual cue of the caddisfly without allowing the animals to receive chemical or mechanical cues. Warkentin (2005) found that Red-Eyed Tree Frog

(*Agalychnis callidryas*) embryos responded to disturbance by a snake egg predator by hatching out early. However, when she exposed the embryos to non-specific vibrations the animals did not hatch out. This means that the animals can detect the differences in vibration and will only hatch out early when they are actually threatened. As for the larval predator cues, the embryos did not have direct contact with the larval predators so the cue must be visual, chemical or a combination of both. In order to determine which cue was contributing embryos would need to be exposed to only chemical cues, or the visual cue of the predator without chemical cues. There is literature however to support the idea that it is generally a chemical cue that the embryos respond to (Moore *et al.* 1996; Chivers *et al.* 2001) but more work does need to be done.

Conclusion

Hatching plasticity is a unique evolutionary strategy that allows animals to respond to environmental or predator cues around them. In an attempt to understand this ability, scientists have performed experiments with varying levels of predation, competition, and environmental changes. To fully understand salamander hatching plasticity animals need to be exposed to multiple life stage predators. Animals will rarely be in an environment with just one type of predator cue and the response to the interaction between the multiple stage predators is very compelling. Two studies on *Ambystoma maculatum* have shown that these animals do not delay hatching when exposed to larval predator cues, but our study shows that they will change their body morphology by growing a larger tail area. They will respond to egg predator cues by hatching out earlier, but this comes at a cost. Even if hatching plasticity is the main goal of the study, the effects on later life stages must also be taken into consideration. The cost to hatching out early was seen in later life as the animals had smaller tail areas, and survived for shorter amounts of time in lethal predation trials. By studying multiple predator effects on multiple life stages, the fascinating phenomenon of hatching plasticity may finally be understood.

Tables

Table 2: ANCOVA for Hatching Proportions by Tank (R^2 = 63%)				
Sources	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Day	1	14.32	467.11	<.0001
Treatment	3	1.03	11.22	<.0001
Block	1	0.25	8.14	0.005
Clutch	5	11.41	74.45	<.0001
Treatment*Day	3	0.29	3.18	0.024

Table 3: ANCOVA for Hatching by Developmental Stage (R^2 = 62%)				
Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Day	1	148.02	447.27	<.0001
Treatment	3	11.99	12.08	<.0001
Block	1	0.04	0.12	0.73
Clutch	5	5.3	3.21	0.008
Treatment*Day	3	22.02	22.18	<.0001

Table 4: ANCOVA for Tail Area Week 4 (R^2 = 92%)				
Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Treatment	3	0.0012	4.36	0.0053
Block	1	0.0005	5.27	0.0227
Clutch	5	0.0061	12.95	<.0001
Total Length	1	0.145	1560.12	<.0001
Block*Treatment	3	0.001	3.62	0.0139

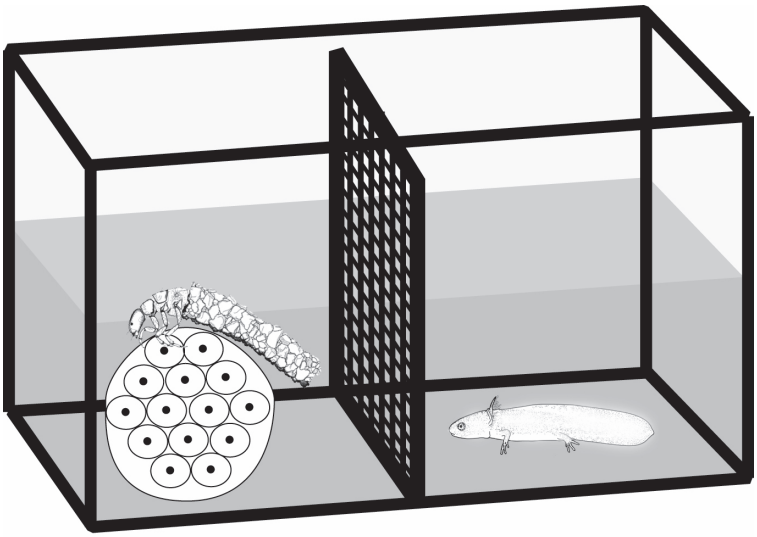
Table 5: ANCOVA for Week 14 Tail Morphology (R^2 = 93%)				
Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Treatment	3	0.023	0.69	0.56
Block	2	0.104	4.68	0.011
Clutch	5	0.16	2.91	0.016
Total Length	1	16.61	1492	<.0001

Table 6: ANCOVA for Predation Trial Data (R^2 = 41%)				
Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Treatment	3	49893.61	3.24	0.03
Clutch	5	37466.22	1.46	0.22
Developmental Stage	1	59610.18	11.6	0.0013
Total Length	1	16372.7	3.19	0.081
Predator ID	3	35897.5	2.33	0.086

Table 7: ANCOVA for Metamorphic Stage (R^2 = 8%)				
Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Treatment	3	9.93	1.41	0.24
Block	1	5.36	2.28	0.13
Clutch	5	6.27	0.53	0.75
Total Length	1	12.12	5.15	0.02

Table 8: ANOVA for Total Length at Metamorphosis (R^2 = 10%)				
Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Treatment	3	1.71	1.95	0.13
Block	1	0.71	2.44	0.12
Clutch	5	1.46	1.003	0.42

A



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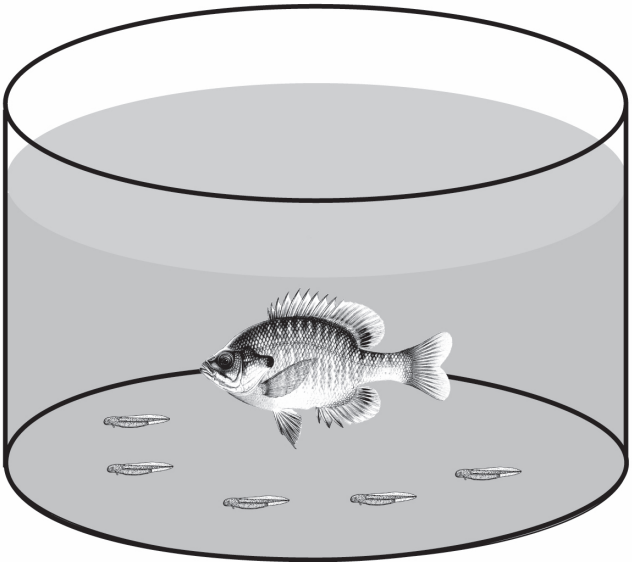

Figure 1. Experimental animals were kept in (A) 48 38-liter tanks with four combinations of egg and larval predators. Treatment groups were egg predator only, larval predator only, both predators, and no predators. Egg predators were kept on the same side as the experimental animals and larval predators were separated from experimental animals by a screen. Once all the animals were hatched they were put into (B) lethal predation trials. Five animals were randomly sampled from each tank for each predation trial. The animals were put into the 51 cm diameter predation tank and allowed to acclimate for one minute. A fish predator (*Lepomis* sp.) was put into the tank and allowed ten minutes in which to eat the salamanders. Trials were filmed from above. Trial length, time to predation events, escape rate, and movement rate were calculated from the video recordings. (Drawings by T. Landberg)

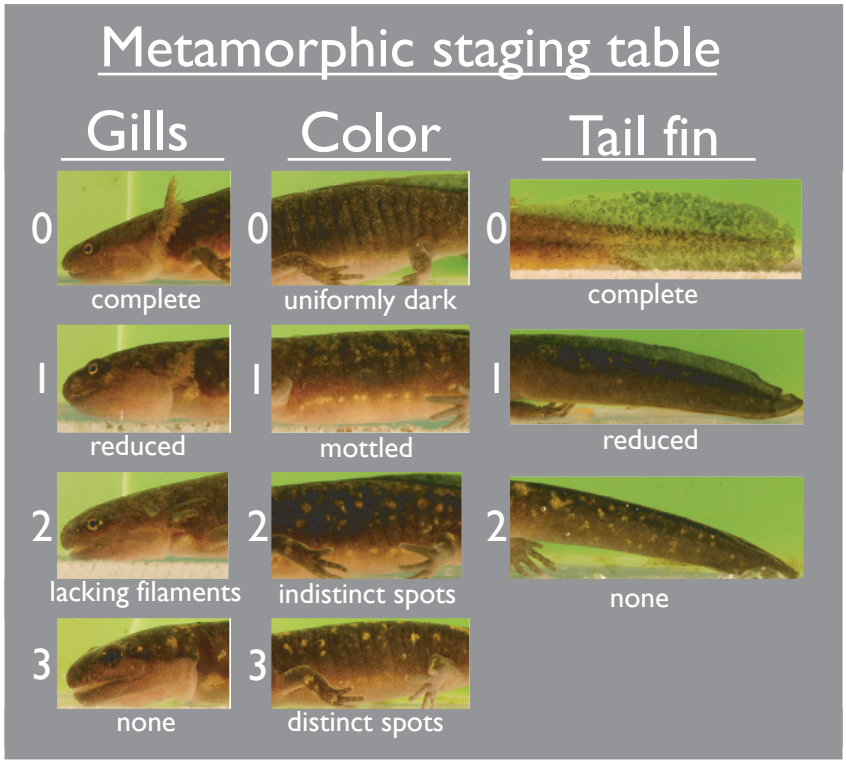

Figure 2. At the end of the experiment the metamorphic stage (0-8) was determined by the sum of three traits for each animal according to the staging table we created. During metamorphosis gills go from complete (0), to reduced (1), then lose filaments leaving a bare rachis (2) and are finally totally resorbed (3). The animals go from a uniformly dark larval color (0), to dark with yellow mottling (1), to indistinct spots with ragged edges (2), to distinct spots (3). The tail fin goes from extending up to the back of the head (0), to reduced (1), to completely resorbed leaving the animal with the muscular adult tail (2).

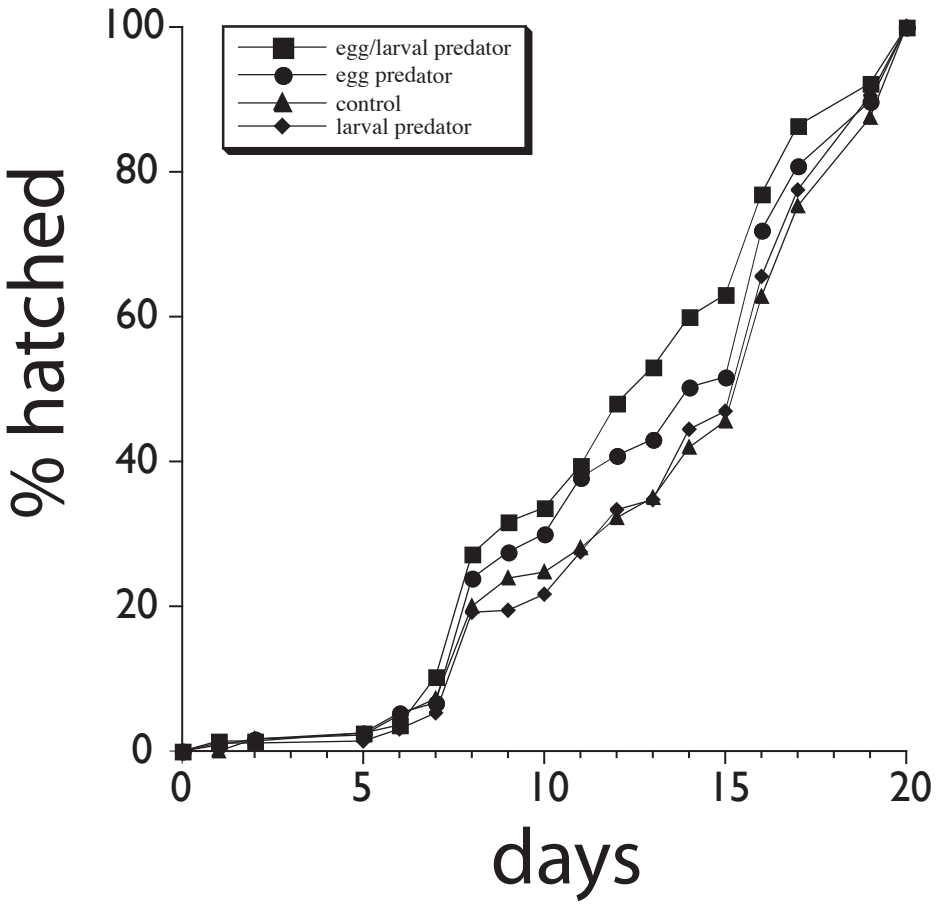

Figure 3. Hatching percentage as a function of treatment. Individual points on the graphs are the treatment means of tank proportions on a given day. The animals in the egg predator and egg & larval predator treatment groups hatched significantly earlier than the animals in the control and larval predator only treatment groups (Tukey test).

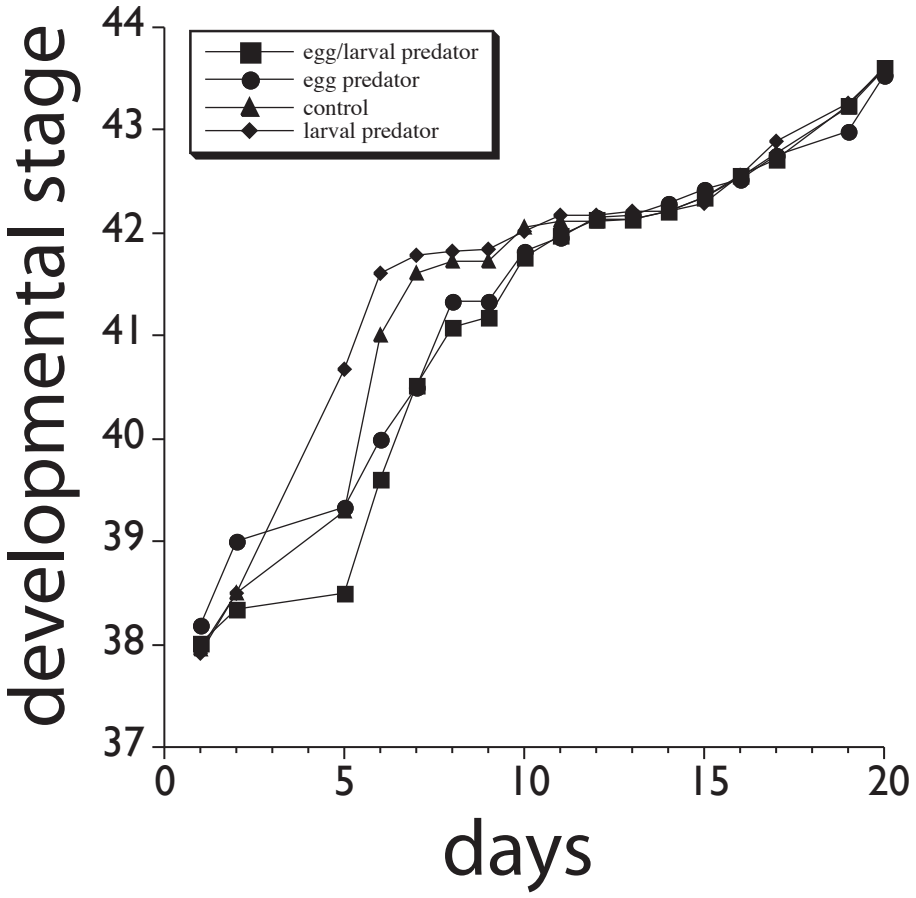

Figure 4. Developmental stage at hatching as a function of treatment. Animals in the egg and egg/larval predator treatment groups hatched at earlier developmental stages than the animals in the control and larval predator treatment groups (Tukey test).

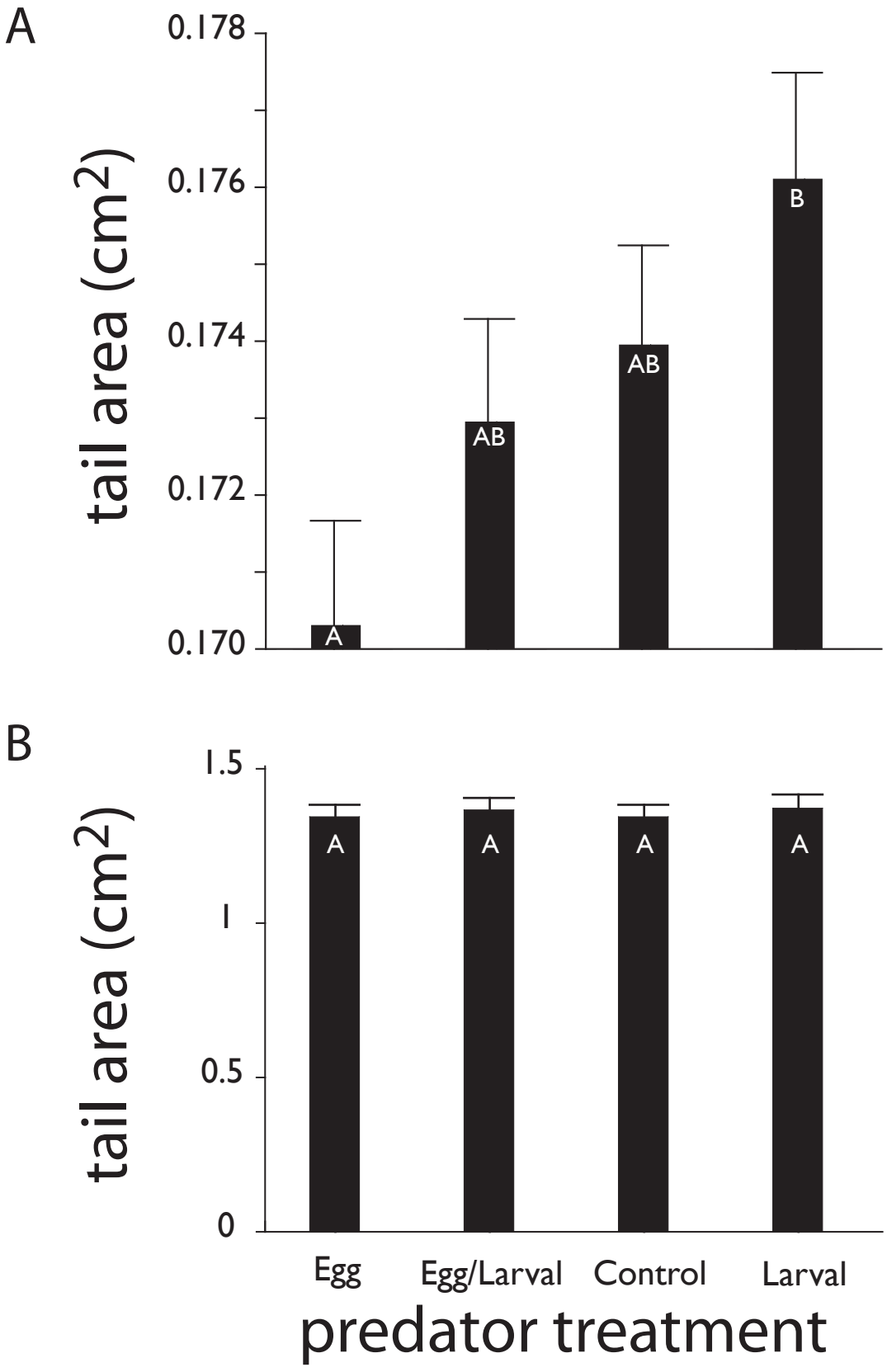

Figure 5. Relative tail area in week 4 (A) and week 14 (B) as a function of predator treatment. During week 4 animals in the larval predator treatment group had significantly larger tail areas than the animals in the egg treatment group; control and egg/larval treatment groups were intermediate (Tukey test). Different letters represent treatments that are statistically different from each other. During week 14 treatment effects were not significant (p=0.56).

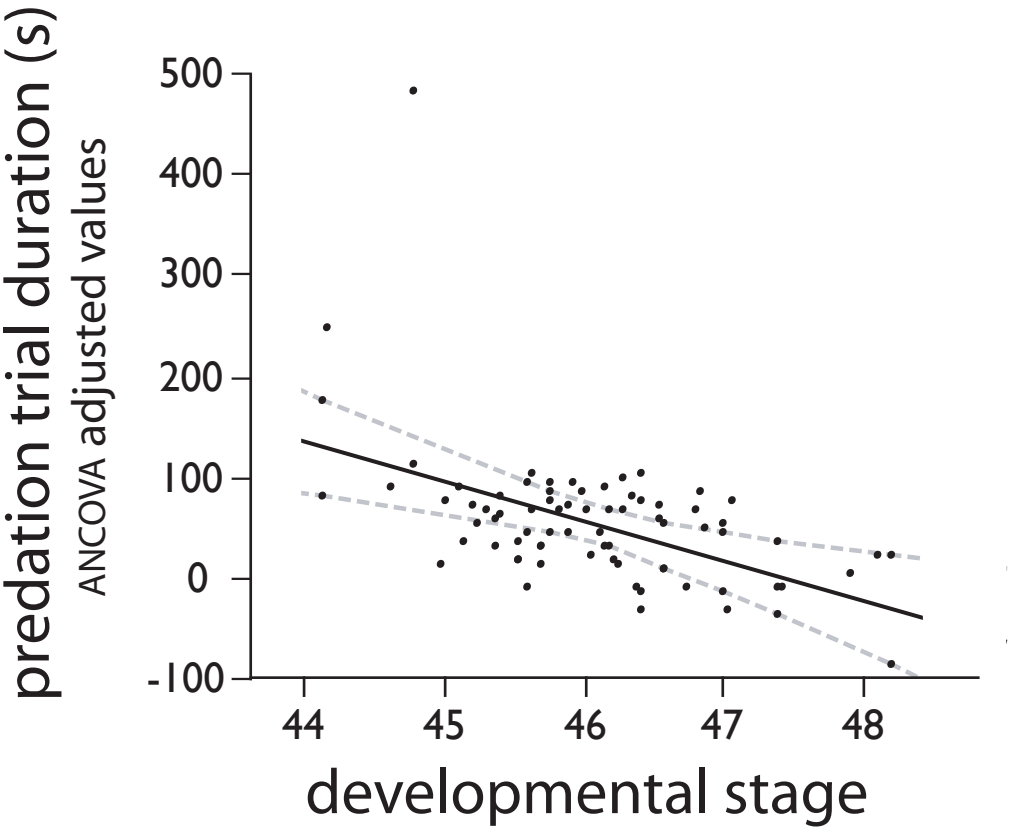

Figure 6. Survival time in lethal predation trials by developmental stage. Animals at later developmental stages survived for shorter times in predation trials (ANCOVA; p=0.0013). The overall ANCOVA model for survival time was statistically significant (p=0.009) and explained 41% of the variation. Some values are negative because the durations (s) were adjusted for treatment, total length, and fish predator ID to allow for comparison of the trials.

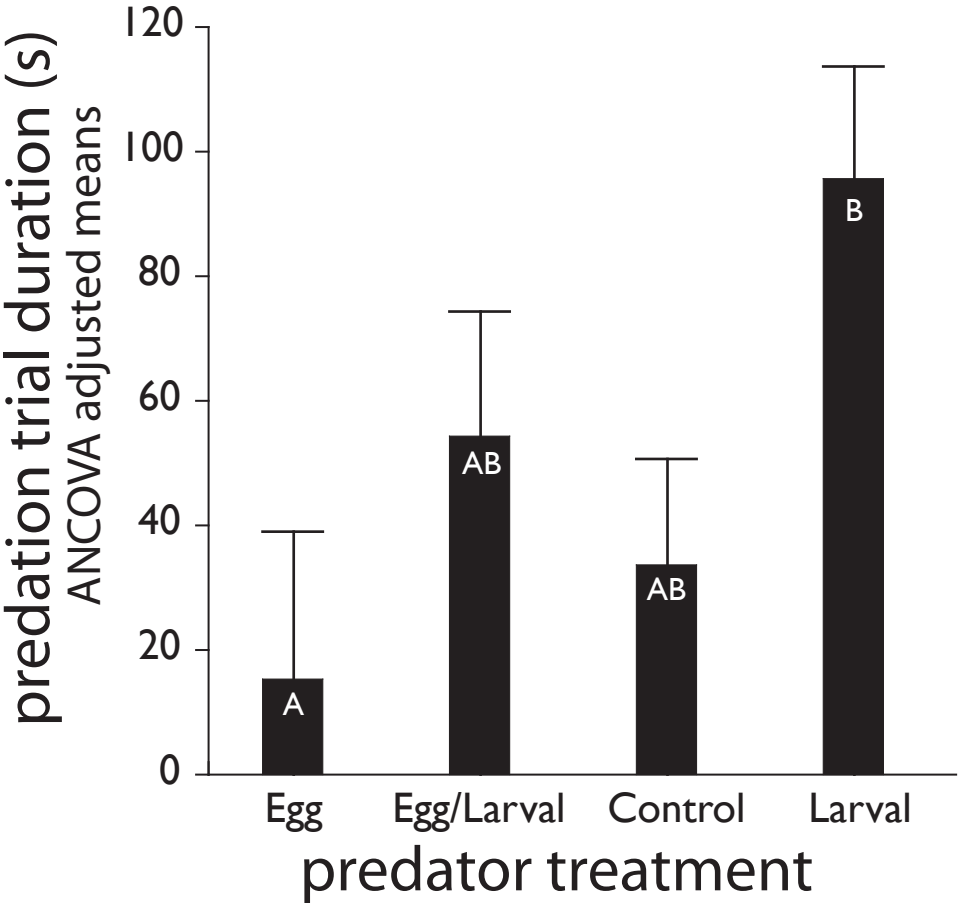

Figure 7. Survival time by treatment. Previous exposure to predators had a statistically significant effect on survival time (p=0.030). The animals in the larval predator treatment group had significantly longer survival time than the animals in the egg predator treatment group. The animals from the egg/larval and control treatment groups were intermediate. (Different letters represent treatments that are statistically different than each other.) The predation trial duration (s) were adjusted for developmental stage, total length, clutch and fish predator ID.

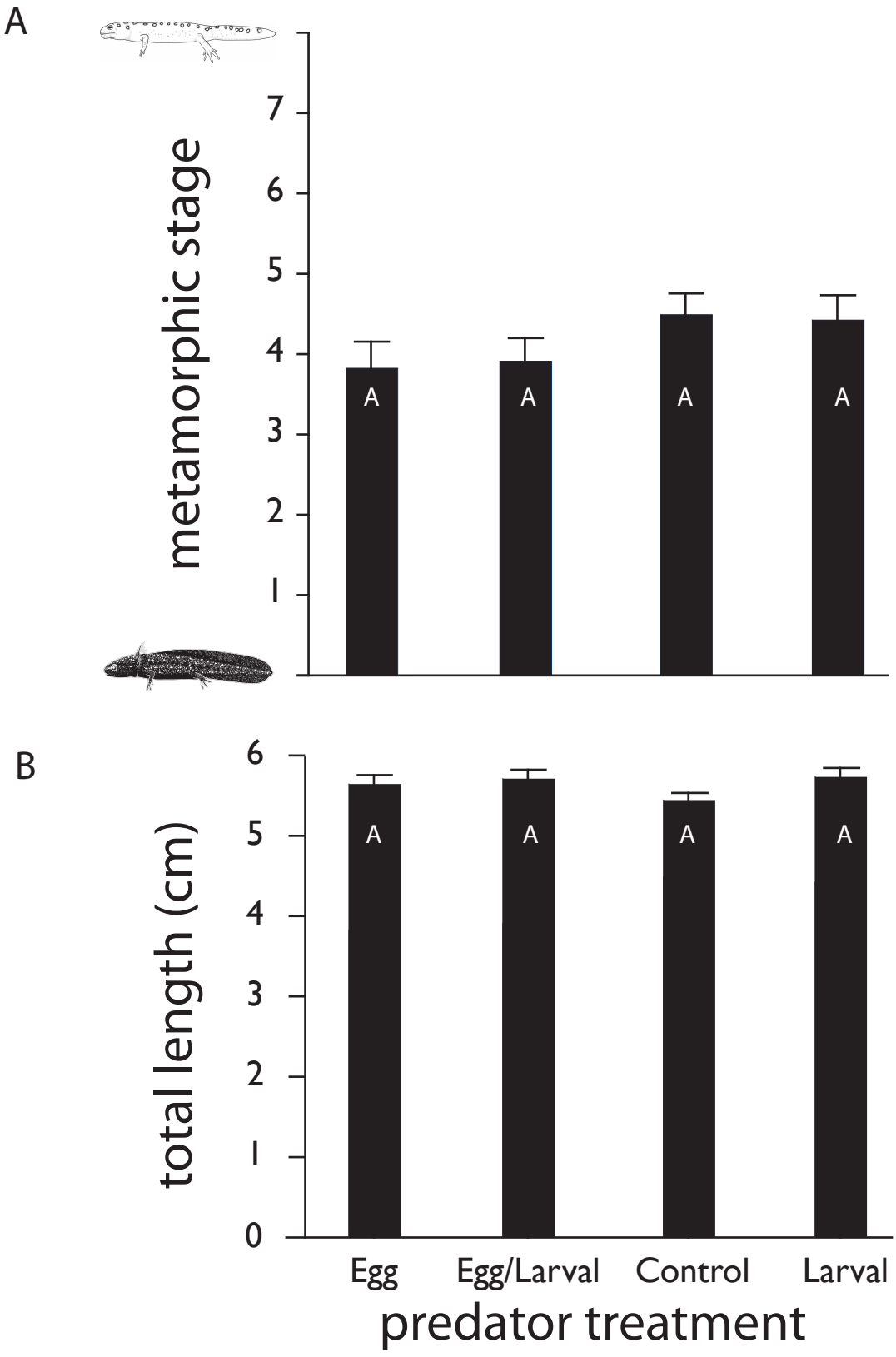

Figure 8. Metamorphic stage (A) and total length (B) in weeks 18 (block 1) and 19 (block 2) respectively. Neither developmental stage nor total length was affected by the predator treatment (ANCOVA $p>0.05$). There was a positive effect of total length on development but no treatment * total length interaction.

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