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Consequence of terrestrial egg laying in amphibians: a comparison of embryonic oxygen sensitivity in two Ambystomatid salamanders

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Abstract

Many amphibian species lay their eggs in ephemeral ponds where competition for resources is high and there is pressure to develop quickly and grow to large metamorphic size. The adaptive nature of rapid growth and metamorphosis is essential for overcoming the intense resource competition and fluctuating oxygen availability observed in ephemeral pools. Low concentrations of dissolved oxygen can influence the duration of developmental phases in aquatic salamander eggs, resulting in extended durations of poorly adapted phases of their lifecycle, or by forcing rapid metamorphosis to smaller sized adults. This study focused on two species of closely related salamanders, *Ambystoma maculatum*, and *Ambystoma opacum*, differing by aquatic and terrestrial egg laying preferences respectively. Embryonic development of these two species was studied under laboratory conditions, comparing growth, development, and hatching success in a moist terrestrial environment and in an aquatic environment, under a range of dissolved oxygen concentrations. Because *A. maculatum* prefers development in aquatic environments with lower dissolved oxygen than the terrestrial habitats of *A. opacum*, observation of *A. maculatum* embryos should indicate faster development and/or be larger at metamorphosis, particularly in lower oxygen environments. The study concluded that while *A. maculatum* developed faster in all treatments, the difference between species was greatest in higher oxygen treatments, instead of the predicted lower treatments. The low oxygen treatment of *A. maculatum* showed the only significance difference in larval mass among treatments, with a more anaerobic environment producing larger larvae.

1. Introduction

Many amphibians lay their eggs aquatically, but some have evolved to lay their eggs terrestrially in moist soil. Indeed, terrestrial laying has evolved independently in many amphibian taxa, including plethadontids. In ambystomatid salamanders aquatic egg laying is thought to be ancestral but there are two ambystomatid salamanders (*Ambystoma cingulatum* and *Ambystoma* opacum) that have evolved terrestrial breeding reproductive behaviors. The selective evolutionary pressure attributed to terrestrial egg laying is to prevent predation of highly vulnerable aquatic eggs[1] by predators such as wood frog tadpoles (*Rana sylvatica)*[2], leeches, (*Macrobdella decora)*[3], and caddisflies, (*Banksiola dossuaria)*[4]. Though, the terrestrial environment comes with its share of hazards to eggs, including desiccation[24] and fungal infection. Predators also differ for terrestrially laid eggs, consisting of invertebrates along with other amphibian species[5] and even birds[6]. In many amphibians that lay terrestrially have also developed forms of parental care[7-10], which can increase embryonic survival by keeping eggs moist, decreasing instance of fungal infection, and ward off predatory insects and conspecifics[11]. However, the higher oxygen present in terrestrial environments may greatly benefit developing embryos, allowing them to develop faster, larger, or to a later stage before hatching. This could make terrestrial laying beneficial since it allows time for embryos to develop before

rains fill ephemeral ponds then hatch already able to feed and defend themselves to an extent when the rains do come.

Small ovum size is generally associated with faster embryo development, earlier hatching, and lower oxygen requirements[12]. *Ambystoma maculatum* (spotted salamander) eggs are smaller than those of the terrestrially laid *A. opacum.* This is most likely because the aquatically laid eggs of *A. maculatum* must rapidly develop in order to survive in the highly competitive environments of filled ephemeral pools while *A. opacum*, which is laid while pools are dry, has already developed and can hatch to begin feeding and competing as soon as pools fill. *Ambystoma maculatum* eggs have a gelatinous casing which is a deterrent to predation but can also be a hindrance to oxygen transfusion for the embryos, especially those in the center of the mass[13]. To combat this, *A. maculatum* has developed a symbiotic relationship with *Oophila amblystomatis*, commonly referred to as salamander algae, which helps facilitate oxygen diffusion inside the egg[14], through photosynthesis[15-17]. Because *A. maculatum* are not laid until after ephemeral ponds have filled, they are under significant pressure to develop quickly and to a large size in order to compete with much larger *A. opacum* offspring, other competitors, and the possible sudden drying of their habitat after hatching[18-20].

Without the added suspension of water, oxygen diffusion in terrestrial eggs is more restricted due to gravity and surface tension[12]. Oxygen is key to the hatching success of many terrestrial egg-layers. To ensure survival of newly hatched aquatic dependent larvae, hatching must be delayed until spring rains have filled the ephemeral ponds. Therefore, selection has resulted in the ability for terrestrially laid egg species to delay hatching. When the eggs are submerged in water the PO2 outside the eggs is reduced, therefore degrading oxygen consumption and triggering hatching in the process[21].

The main hypotheses of this study are that *A. maculatum* must retain an advantage to developing in lower dissolved oxygen environments. Thus, if placed in a low dissolved oxygen environment *A. opacum* embryos would not develop as quickly nor reach the same masses as *A. maculatum.*

2. Methods

2.1. Embryo collection

Ambystoma opacum eggs were collected in September of 2012 and 2014. Eggs collected in 2013 were too far along in development at collection to use in the study. Collections were made at ephemeral ponds at Sandy Bottom Preserve (Buncombe County, NC), in early September after days of rain. Adults were found by combing through the leaf litter and then placed into, 0.6 m diameter Rubbermaid bins with holes drilled in the bottom to prevent water retention. These bins were filled with 10-15 cm leaf litter and left at Sandy Bottom. They provided breeding and nesting habitat for the marbled adults and were used to increase probability of collecting eggs at the earliest stages of development. For every female in a bin, two to three males were also placed in the bin. Bins were covered with fiberglass window screening secured by bungee cords to deter predation and allow airflow, while preventing escape of adult *A. opacum.* Bins were checked daily for new egg masses.

We collected *A. maculatum* egg masses in February 2013 and 2014, when ephemeral ponds filled. Clear rather than opaque egg masses were chosen[22] to make developmental staging easier. Individual eggs were removed from the jelly mass and counted. Each of the eggs was assessed using the Harrison staging chart[23], a chart comprising numbered illustrations of morphological differences during salamander development, to determine the average stage of each jar. *Ambystoma opacum* egg masses collected in September 2012 and 2014 were rinsed with dechlorinated tap water to remove dirt and debris. The number of eggs and median Harrison stage[23] was identified for each mass.

2.2. Oxygen manipulation

In the laboratory, embryos were divided into groups of 10-25 and placed in a 150 ml specimen jar; embryos from different clutches were not mixed in order to control for any factors specific to clutches that could either aide or deter developmental progress. Groups of embryos were then randomly assigned to an oxygen treatment (ambient air, saturated dissolved oxygen, medium dissolved oxygen, low dissolved oxygen), resulting in a randomized block design in which egg mass identity was the block. Each season included five to ten replicate blocks, depending on egg mass availability. Jars were placed in a growth chamber set to a constant 80% relative humidity, 15˚C, and a 12h:12h light:dark cycle. During the 2013 trials with *A. maculatum*, *Oophila amblystomatis*[14] was discovered growing within the eggs. *Oophila amblystomatis* is well-documented to grow within the egg membranes of *A.*

macultum and thought to have a symbiotic relationship with the salamander. To exclude possible effects of this alga on embryonic development in *A. maculatum*, perhaps providing extra oxygen to growing larvae under oxygen stress, 2014 trials with both species were run in complete darkness.

Dissolved oxygen treatments contained about 150 mL of dechlorinated water, enough to fill jars without overflowing. Air treatments did not contain water, except for a small amount to prevent dehydration of embryos. Dissolved oxygen treatments were maintained by bubbling 10% O_2 : 90% N_2 mixed gas (low treatment), 15% O_2 : 85% N_2 mixed gas (medium treatment), or air (saturated treatment) through an air stone into the water. Air treatments received air through an air stone from an air pump set inside the growth chamber to maintain high humidity within the jar. Mixed gas was supplied from gas cylinders to distribution manifolds through tubing fed through a hole in the wall of the chamber. Smaller tubes distributed gas from the manifold through the lid of each specimen jar to an air stone. Dissolved oxygen levels were monitored with a FOXY-OR125 oxygen probe attached to a Neofox light source (Ocean Optics Inc., Dunedin, FL, USA), and were recorded daily in 2012-2013 and periodically in 2014 because we noticed very little variation in years previous.

Staging of embryos was conducted daily, using the Harrison developmental stage chart[12]. The number of live and dead embryos was recorded while staging. Embryos were considered dead and discarded if the membrane surrounding them was completely clouded gray or embryo had compacted to one side. The median Harrison stage per jar was recorded and any hatchlings were individually staged before being removed from the jars. The first ten hatchlings in every jar were euthanized with MS-222, and then preserved in 5mL centrifuge tubes with Shandon™ Glyo-Fixx™ solution (Thermo Scientific). Any other hatchlings were moved to hatchery tanks and kept alive until the end of the experiment when they were returned to Sandy Bottom. After all embryos hatched, those preserved were then vacuum dried and weighed using a Mettler XS3DU microbalance (Mettler-Toledo).

The goal was to determine whether the effect of oxygen treatment differed between species. We evaluated the effect of species, treatment, and their interaction on age, stage, and mass at hatching with generalized linear mixed models using the lme function of the programming language R (R Core Development Team) and comparing fit of progressively simpler models using likelihood ratio tests. The two sampling years were combined for these analyses. Significant interactions between species and treatment made it difficult to interpret the effect of treatment within species. Therefore, each species-year combination was analyzed for treatment effects separately using ANOVA for a total of 12 separate analyses (2 species x 2 years x 3 response variables).

3. Results

There were significant effects of species ($L = 28.46$, $p < 0.0001$), treatment ($L = 122.51$, $p < 0.0001$), and their interaction ($L = 86.57$, $p < 0.0001$) on age at hatching (Table 1). Inspection of the coefficients associated with these effects indicated that *A. maculatum* embryos consistently hatched at a significantly earlier age than *A. opacum* embryos. Similarly, there were significant effects of species ($L = 68.02$, $p < 0.0001$), treatment ($L = 24.20$, $p < 0.0001$), and their interaction ($L = 75.66$, $p < 0.0001$) on stage at hatching (Table 2). Inspection of the coefficients of these effects indicate that *A. opacum* consistently hatched at a later stage, but that the effects of treatment differed between species.

There was no effect of treatment on mass at hatching, but *A. maculatum* hatched at greater mass than *A. opacum*, when years were combined ($L = 4.75$, $p = 0.03$) (Table 3). This difference appears driven by the larger mass of *A. maculatum* embryos in 2014, when experiments were run in the dark to prevent growth of symbiotic algae.

Overall, *A. maculatum* was affected very little by dissolved oxygen treatment, as indicated by no significant differences in stage at hatching between low, medium, and saturated dissolved oxygen treatments in either year. Dissolved oxygen did affect age at hatching in *A. maculatum* in 2013 (Table 1), hatched quicker in oxygen saturated conditions.

The effect of developing in air was quite different between species. *A. maculatum* hatched sooner (Table 1) and at an earlier stage (Table 2) in air than in the water treatments. In contrast, *A. opacum* embryos took longer to hatch and hatched at a later stage in air than in any water treatment in 2014. The data from *A. opacum* in 2012 were difficult to interpret; we had very low hatching success in *A. opacum* reared in air due to desiccation, and those that hatched did so early.

Table 1. Effect of oxygen treatment on age at hatching. Results of individual ANOVA analyses testing the effect of treatment, separately for each species and year combination. Treatment differences were determined with a Tukey-Kramer *post hoc* test.

Species	Year	F	df	P	Treatment Differences
A. opacum	2012	5.794	3, 18	0.0078	sat > med, low
A. opacum	2014	33.184	3, 46	<0.00001	air > sat, med, low
A. maculatum	2013	6.768	3, 27	0.0018	low > air, sat
A. maculatum	2014	18.345	3, 29	<0.00001	sat, med, low > air

Table 2. Effect of oxygen treatment on stage at hatching. Results of individual ANOVA analyses testing the effect of treatment, separately for each species and year combination. Treatment differences were determined with a Tukey-Kramer *post hoc* test.

Species	Year	F	df	P	Treatment Differences
A. opacum	2012	14.52	3, 18	0.00010	sat > med, low; air > low
A. opacum	2014	21.828	3, 46	<0.00001	air > sat > med, low
A. maculatum	2013	6.193	3, 27	0.0029	sat > air
A. maculatum	2014	6.932	3, 29	<0.00084	sat, med, low > air

Table 3. Effect of oxygen treatment on mass. Results of individual ANOVA analyses testing the effect of treatment, separately for each species and year combination. Treatment differences were determined with a Tukey-Kramer *post hoc* test.

Species	Year	F	df	P	Treatment Differences
A. opacum	2012	0.895	3, 17	0.47	none
A. opacum	2014	2.059	3, 46	0.12	none
A. maculatum	2013	5.265	3, 26	0.0065	low > air, sat
A. maculatum	2014	0.709	3, 39	0.55	none

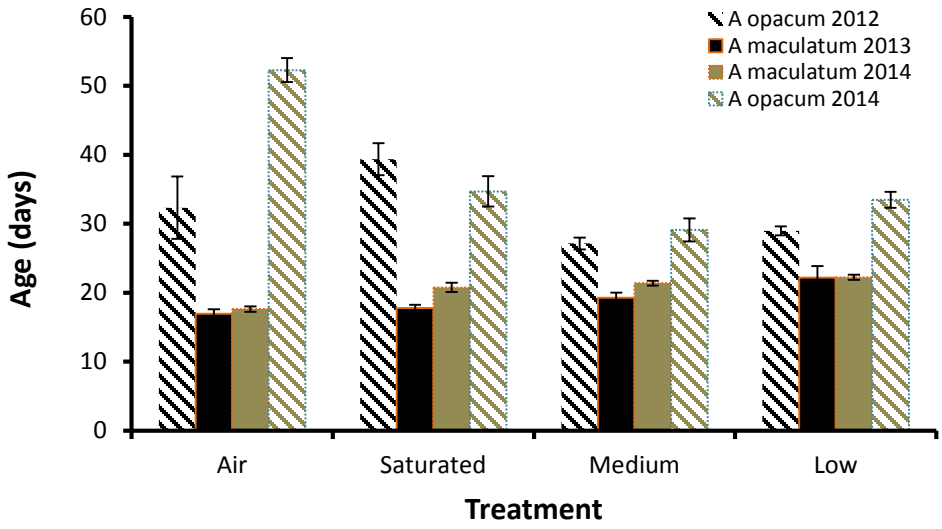

Figure 1. Age (days; ± SE) at hatching for *A. opacum* 2012 (dark striped), *A. opacum* 2014 (light striped), *A. maculatum* 2013 (dark solid), and *A. maculatum* 2014 (light solid) reared in four dissolved oxygen treatments. T-tests indicated that *A. opacum* took longer to hatch in all treatments.

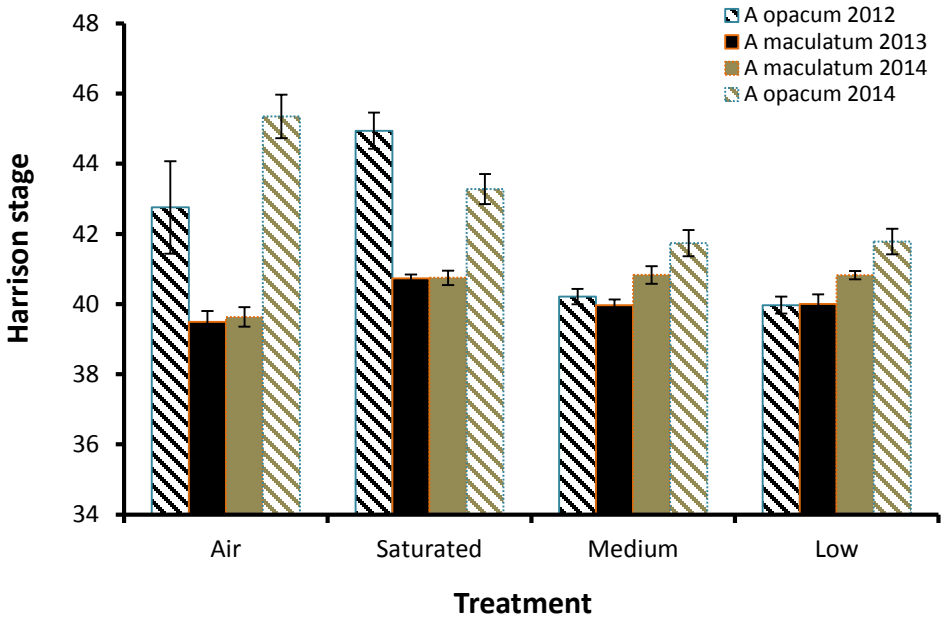

Figure 2. Mean Harrison stage (± SE) for *A. opacum* 2012 (dark striped), *A. opacum* 2014 (light striped), *A. maculatum* 2013 (dark solid), and *A. maculatum* 2014 (light solid) reared in four dissolved oxygen treatments. T-tests indicate that *A. opacum* hatched at a later stage than *A. maculatum* in air and oxygen-saturated water treatments.

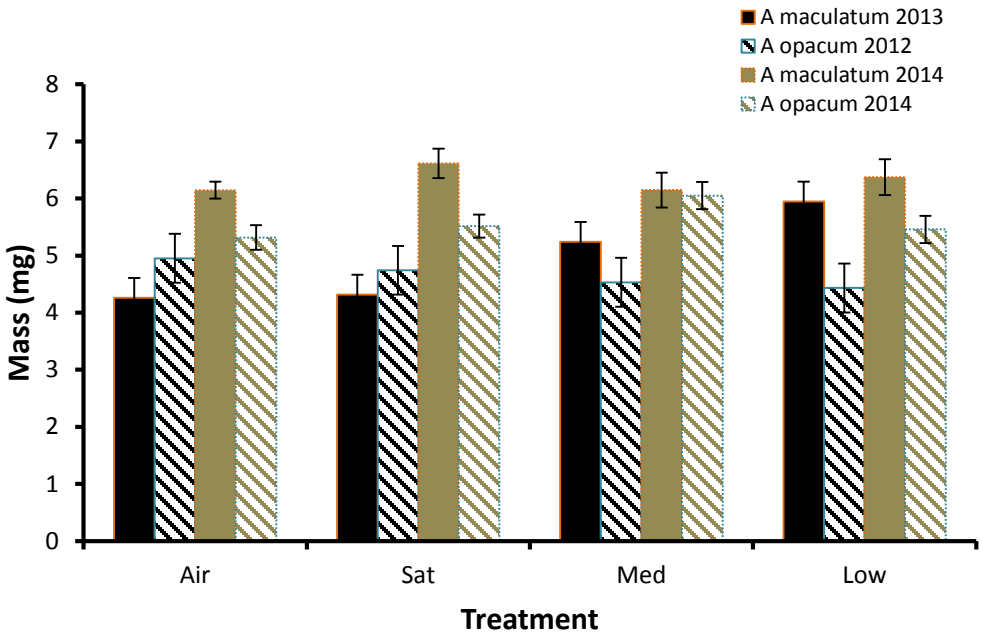

Figure 3. Mean mass (mg; ± SE) at hatching for *A. opacum* 2012 (dark striped), *A. opacum* 2014 (light striped), *A. maculatum* 2013 (dark solid), and *A. maculatum* 2014 (light solid) reared in four dissolved oxygen treatments.

4. Discussion

We expected *A. maculatum*, to develop faster and to a later stage before hatching than *A. opacum*, particularly in lower oxygen environments. However, we found that the greatest difference in development between the species occurred in oxygen-saturated water and air treatments.

This suggests that the two species are similarly constrained by low dissolved oxygen during development, but that they respond to high oxygen differently. Their disparate responses may be a consequence of their distinct breeding ecologies. Because *A. opacum* breeds early and has time to develop fully before ponds fill in the spring, they do not necessarily benefit from fast development. Instead, they can delay hatching a few days, reach a later stage, and still enter the pond before competitor species. In contrast, *A. maculatum* breeds after ponds have filled and may experience strong selection to develop quickly and hatch as early as they are viable. Indeed, delaying hatching even a few days may be detrimental to larvae.

Over the course of experimentation, alga was discovered growing within the eggs of *A. maculatum*. To determine whether this alga played a significant role in embryonic development, perhaps providing extra oxygen to growing larvae, light was removed from growth chambers of both species in 2014, even though *A. opacum* showed no alga growth under lighted conditions, in the 2012. Though it appears light, and thus alga, played no significant role in aiding embryo development stage or age at hatching, it is interesting to note that the masses of both species appeared to increase in 2014. It is unclear whether this increase in mass can be contributed to the omission of light or other variables such as variance among gathered clutches, environmental changes from differing years of collection, etc. More conclusive data could be retrieved from replication of the experiment wherein clutches from the same season are divided so half are reared in light and the other half reared without light, to control for any undetermined variables.

In summary, there are clear differences between the species in developmental progress and these differences are consistent with species divergence in breeding ecology, but not with the prediction that they have evolved diverse physiological responses to low dissolved oxygen. In future studies, the effect of light and alga growth could play on mass and development of embryos will be explored in more depth, controlling for outside variables.

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