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A Comparative Look At Embryonic Development In Two Fall Breeding Ambystomatid Salamanders

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Abstract

Embryonic development is highly variable in different salamander species, even within the same genus. Also variable are the breeding and egg laying patterns of those different species. Previous studies have found variation in aspects of embryonic development, but could not isolate whether these differences likely were due to differences in breeding season, nesting habitat, or egg attendance. Both species in my study were known to be late summer and early fall breeders. All embryos observed were collected in Arkansas, where the species breed in similar ponds. *Ambystoma opacum* is a terrestrial egg laying species that practices parental care by covering and staying near the eggs after they are laid. *Ambystoma annulatum* is an aquatic egg laying species that lacks the practice of parental care entirely. I observed the embryos of each species over time in a lab setting to assess variation in development. Survivorship, time to hatching, stage at hatching, and mass at hatching were all recorded for analysis. It was predicted that some significant differences would be found in the development, eliminating the possibility that breeding season is the primary cause for variation in embryonic development. The results of the study confirmed this with *A. opacum* having a higher age, stage, and mass at hatching. This confirmation allows us to examine more closely the relationship of terrestrial breeding and parental care to these traits in embryonic development, as well as the differing effects of aquatic breeding and the lack of parental.

1. Introduction

Embryonic development in animals can be highly variable, both within species and between closely related species[1,2]. For example, within *Adalaria proxima* molluscs, much variation is found in clutch size and egg size in different locations across Europe[3]. In the oviparous rough-skinned newt, *Taricha granulosa*, significant variation is found in degree of embryonic development and age at hatching between clutches from different females[4]. Particularly, the developmental patterns in amphibians are so highly variable, that zoology uses them as the model organism to study developmental biology[5]. Differences in embryonic development have an effect on fitness and therefore are considered to be the results of selective pressure on an adaptive trait. Rate of development impacts yolk consumption and mass at hatching. Time of hatching is seen as the first life-history switch point for an organism and is closely related to risk of predation.

All of these traits are interdependent and are either due to adaptive plasticity or evolve slowly because despite costs, they do have some advantages. Due to the variable nature of embryonic development, it is precarious to suggest the causation of selective pressures on specific differences. To understand why hatch timing and rate of development vary in closely related species of the salamander genus *Ambystoma*, variance in breeding and egg laying patterns can be examined. Breeding season, egg laying environment, and presence of parental care are potential selective pressures to be considered for differences in embryonic development.

1.1. Season

One factor that appears to influence developmental traits is breeding season, due to differences in availability of water, predation, and temperatures. For example, temperature in different breeding seasons can impact hatch time in many amphibians. In *T. granulosa,* there is less variance in hatch time at high temperatures[4]. In *Ambystoma gracile*, hatch time is shorter at higher temperatures, reaching as few as 12.7 days at 20 degrees celsius[6]. In Ambystomatid salamanders, development time is extended in the colder temperatures found in early winter; winter-breeding *A. maculatum* take 3-4 weeks to reach hatching in nature, compared to 1-2 weeks in the fall-breeding *A. annulatum*[7,8].

Water availability in different seasons also should impact development, but at the evolutionary level. Many pond-breeding amphibians breed in winter or early spring, and their larvae must reach metamorphosis before ponds dry in later summer. Research has shown that manipulating the drying time of an environment will directly impact the rate at which *Ambystoma talpoideum* juveniles metamorphosize[9]. Days to metamorphosis increases with days to drying in this species[9]. Fall breeding has evolved in three species of *Ambystoma*, *A. opacum*, *A. annulatum*, and *A. cingulatum*[10]. By breeding in the fall, these species' larvae have more time to grow and reach metamorphosis before ponds dry. This early breeding also gives *A. opacum* hatchlings a size, competitive, and predatory advantage over the hatchlings of late breeders such as *A. talpoideum*[11].

1.2. Habitat

Developmental traits might also be influenced by egg laying environment. Most *Ambystoma* species are aquatic breeders and come out of hiding to visit breeding ponds where they will deposit their eggs in gelatinous clutches. In aquatic species, the time of embryo metamorphosis is influenced by the drying time of whatever water they are in. In contrast, *A. opacum* and *A. cingulatum* are both terrestrial breeders. Both species lay eggs in the fall by depositing them in terrestrial environments that will later naturally fill with water, such as ditches and dry shallow ponds or swamps[12,13]. Both of these terrestrial breeders also lay their eggs in hidden locations under logs or leaf litter[13]. For both aquatic and terrestrial breeders, embryonic development rate and survivorship should be impacted by time to pond filling. However, the terrestrial egg-laying species have the added pressure of remaining as embryos until water inundates the nest. In *A. opacum*, embryos enter a developmental stasis at late embryonic stages and do not hatch until they are submerged in water[14]. Selection for this developmental stasis may actually favor slower development. In *A. opacum*, this terrestrial breeding is accompanied by parental care.

1.3. Parental care

A final feature that might influence embryonic development is parental care. Some amphibians display this trait while others do not. Parental care is defined by the male or female parent staying to guard the embryos[15]. This period of guarding typically lasts from the time of deposition till embryos hatch and begin to feed[16]. Many amphibians leave their embryos immediately. Parental care is directly associated with hatching plasticity. In the glassfrog, *Hyalinobatrachium fleischmanni*, when the parent leaves the eggs, hatching occurs sooner as a direct defense response[17]. In salamanders, parental care is strongly connected to large egg size, long embryonic period, large embryos, and hidden nest sites[16]. This is seen clearly in lotic breeders due to the need for protection during the longer development period, as this usually means higher mortality rate. Terrestrial breeders were derived from lotic breeders, so it is clear that terrestrial breeding is not the origin for the development of parental care[15]. In the past, the "Safe Harbor" theory suggested that large egg size and parental care had a causal relationship, but it has become clear that these traits are both a result of paternal investment[15]. Extensive research has been done to understand the relationship between parental care and embryonic development in Ambystomatid salamanders, but there is much more to learn.

1.4. Previous study

In a previous study done to compare the embryonic development of *A. maculatum* and *A. opacum*, Hale et al. (2016) examined the impact that different aspects of breeding ecology may have on differences in development. It was suggested that these two species evolved to breed in different seasons to rear more competitive embryos by different means[10]. The breeding patterns of these two species are vastly different, even beyond season. *A. maculatum* is a late, aquatic breeder that does not practice parental care, and *A. opacum* is the opposite in all aspects. *A. opacum* displayed

a higher age and stage at hatching, but a smaller mass. It is possible that *A. opacum* embryos have a longer development time and hatch at a higher stage because earlier breeding improves survivorship by allowing embryos to develop further before facing competitors and eliminates the pressure of habitat drying[9,10]. The embryos were likely smaller due to preserving yolk for an extended period[18]. However, it is also possible that some of these differences preceded the evolution towards different breeding seasons. Moving forward, I aimed to eliminate a variable in order to examine an even smaller system of breeding ecology.

1.5. Present study

For my study, two Ambystomatid species that breed in the fall were chosen for comparison to eliminate the variable of breeding season. *A. opacum* is the same terrestrial breeding marbled salamander species from the previous study and *A. annulatum* is the ringed salamander species that breeds aquatically during that same season. *A. opacum* exhibits parental care and *A. annulatum* does not. By looking at the embryonic development between these two species, I can examine the ecological and evolutionary selective pressures of egg laying environment and parental care on embryonic development, while controlling for their breeding season. If no difference is found between these two species, then the developmental differences observed by Hale et al. (2016) between *A. opacum* and *A. maculatum* likely were due to differences in the species' breeding season. However, if I find differences between *A. opacum* and *A. annulatum* that are similar to those observed between *A. opacum* and *A. maculatum*, a deeper look can be taken into the impacts of parental care and egg laying environment on hatch age, hatch stage, and hatch mass. I began this study expecting to still see some differences in development consistent with the previous study, but assumed that they may be less significant.

2. Methods

2.1. Collection

Ambystoma annulatum embryos were collected from various sites in Arkansas in September 2019. Twenty-four clutches were collected from two sites in Ozark National Forest (Franklin County, Washington County), 30 clutches from four sites in McIlroy Madison Wildlife Management Area (Madison County), and 5 clutches from one site in Baxter County. In some cases, gravid females were placed in 0.6 m diameter Rubbermaid bins to lay their eggs to ensure collection during the trip. Bins had holes in the bottom to prevent water retention, were covered with netting attached by a bungee cord, and contained 10-15 cm depth of leaf litter. *A. opacum* embryos were collected from similar sites in Arkansas in October 2019; however, far fewer were found. One clutch was collected from St. Francis National Forest (Phillips County), 4 from Murray Park (Pulaski County), and 1 clutch from a site in Pope County. Clutches were collected directly from the environment or the same Rubbermaid bin setup. In all cases, clutches were kept separate in semi-permeable bags for transport back to the lab in Asheville, NC.

2.2. Treatment Set Up

In the lab, embryos were separated, counted, and selected by survivorship to reach our final sample sizes of embryos for *A. annulatum* (n=1280 in 32 clutches) and *A. opacum* (n=240 in 6 clutches). Each clutch was divided into two groups of 20 embryos that were placed into two different treatments. I used 150 ml specimen jars for the treatments. One jar was an air treatment and the other was a water treatment. The air treatments included 20 embryos fully exposed to air and lids with holes slightly ajar. Drops of water were added as needed to prevent the embryos from drying out. The water treatment included 20 embryos and enough dechlorinated water to completely submerge the embryos. The same lids were placed on these jars with tubing through the whole that held an air stone for gently circulating air from a pump outside of the growth chamber. All jars were placed at random into a Conviron CMP6050 growth chamber at 20 degrees Celsius, with a light cycle of 12 hours on and 12 hours off. The light period was from 7am to 7pm.

2.3. Staging Embryos and Measuring Larvae

All jars were taken out of the growth chamber and observed one by one under a dissecting microscope and development was recorded using the Harrison developmental stage chart[19]. For each jar I observed each embryo and

recorded a range of the Harrison stages observed. I also recorded the number of dead embryos and removed them from the jar. When the embryos began hatching, they were euthanized and the first five were preserved with Shandon's Glyo-fixx (Thermo Scientific) in 0.5 ml centrifuge tubes when applicable. The Harrison stages of the preserved hatchlings were also recorded. After all embryos died or hatched, the preserved hatchlings were dried. I used a pipette to remove excess Glyo-fixx and covered each tube with parafilm with a small hole in the top. Tubes were placed into a vacuum drier for at least 48 hours until completely dry. Then each preserved embryo was weighed to the nearest XX mg with a scale and the mass was recorded. When entering data, the median stage was used when there was not a single value.

2.4. Data Analysis

Survivorship of embryos of the two species placed in the air and water treatments was analyzed in a generalized linear mixed effect model that included species, treatment, and species x treatment interaction as fixed effects and egg mass as a random effect. Inclusion of the random effect allowed us to use data collected from individual hatchlings while accounting for embryos being collected from the same egg mass and, thus, sharing at least one parent. Analysis was conducted in R (R Core Team) using the glmer function of the lme4 package and specifying binomial error[20].
Treatment and species effects on Harrison stage, age, and mass at hatching were evaluated using linear mixed effect models using the lmer function of the lme4 package of R. Models included species, treatment, and species x treatment interaction as fixed effects and egg mass as a random effect. Marginal means were estimated using the emmeans package of R[21]. For these analyses, only embryos that survived to hatching were included.

3. Results

Considerable variation was found within both species, with interesting differences between species and in some analyses, an interaction between species and treatment.

3.1. Survivorship

Survivorship to hatching differed significantly between air and water treatments (Wald $\chi^2_1 = 6.13$, $p = 0.013$), but did not differ between species (Wald $\chi^2_1 = 0.41$, $p = 0.52$). Survivorship was higher in water (mean ± SD: 17.1 ± 3.5 embryos) than in air (14.1 ± 5.2 embryos; Figure 1), but this effect may have been driven by *A. annulatum*, of which there were considerably more replicates.

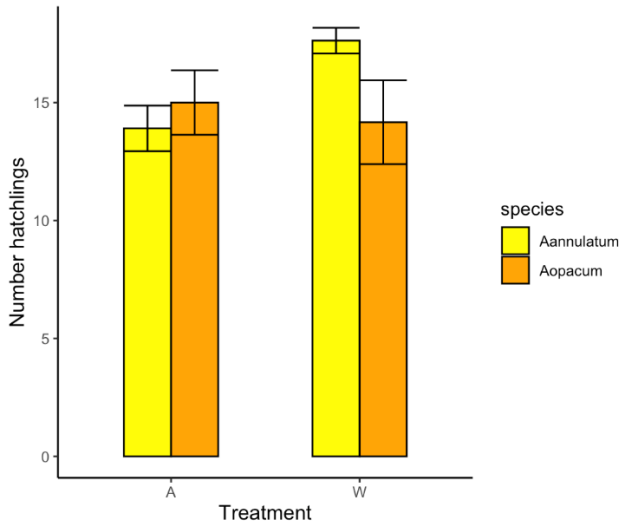

Figure 1. Mean (± SE) number of embryos surviving to hatching per replicate, out of 20 embryos placed in each replicate.

3.2. Hatch Stage

Stage at hatching was affected by an interaction between species and treatment (species x treatment: Wald $\chi^2_1 = 134.88$, $p < 0.0001$); stage at hatching was affected by treatment in *A. opacum*, but not in *A. annulatum*. In air, *A. opacum* hatched approximately 3.5 Harrison stages further developed than *A. annulatum* (mean (95% CI): 44.3 (43.2,45.4) versus 40.9 (40.5,41.4)), but there was no difference between species in stage at hatching when reared in water (40.5 (39.4,41.6) versus 40.1 (39.7,40.6); Figure 2).

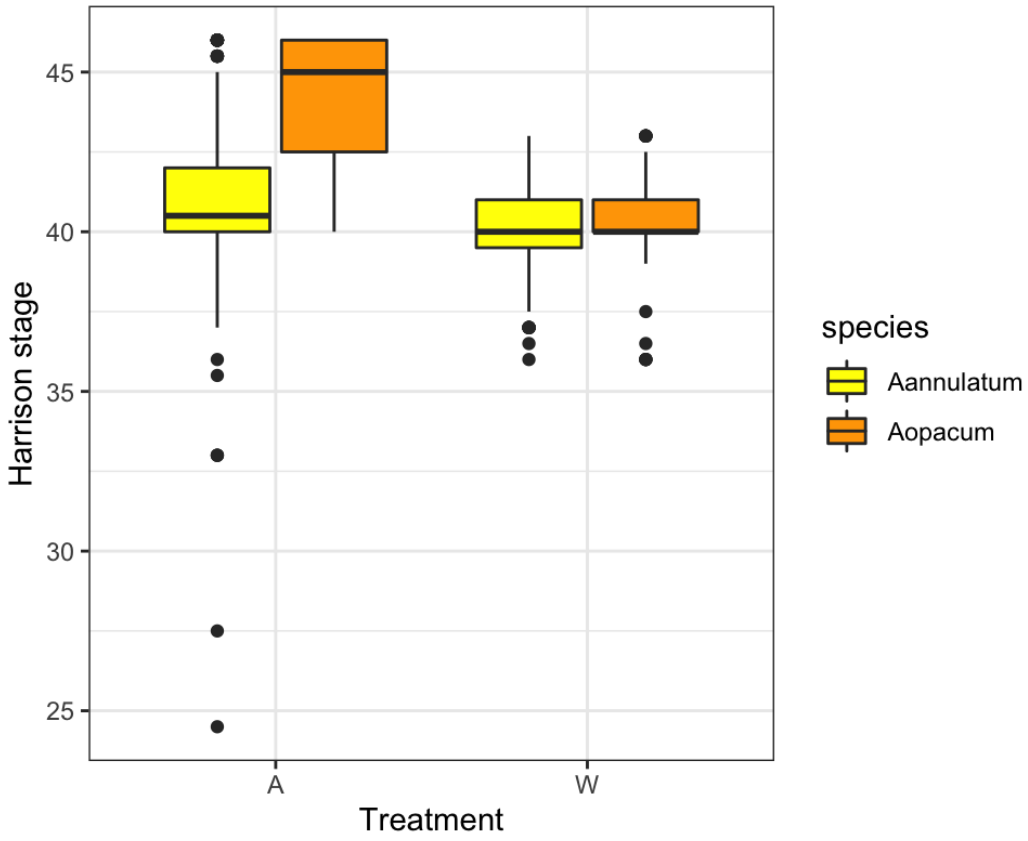

Figure 2. Harrison stage at hatching for *A. annulatum* and *A. opacum* embryos reared in either moist air or water. Boxplots show medians, first, and third quartiles. Dots represent outliers.

3.3. Age

Age at hatching also was affected by an interaction between species and treatment (species x treatment: Wald $\chi^2_1 = 135.0$, $p < 0.0001$); treatment affected age at hatching in both species, but had a stronger effect in *A. opacum*. In air, *A. opacum* hatched at nearly twice the age as *A. annulatum* on average (mean (95% CI): 26.75 (23.97,29.5) versus 13.46 (12.25,14.7)). In water, *A. opacum* hatched slightly over 7 days after *A. annulatum* on average (16.94 (14.15,19.7) versus 9.86 (8.66,11.1); Figure 3).

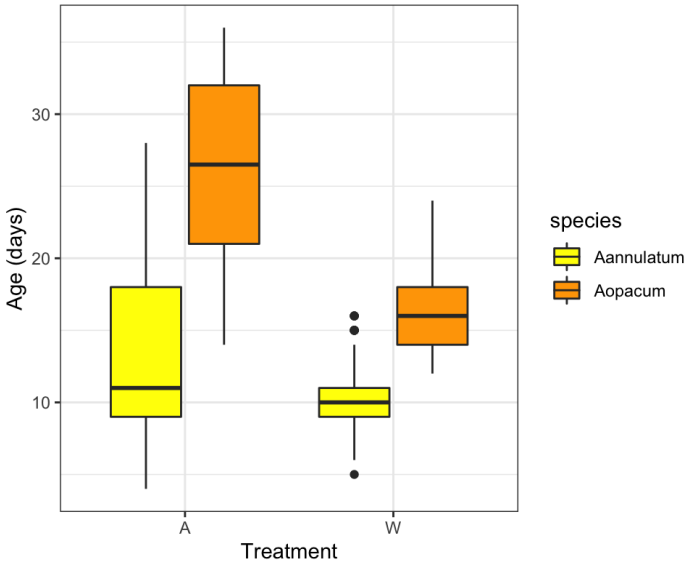

Figure 3. Age at hatching for *A. annulatum* and *A. opacum* embryos reared in either moist air or water. Boxplots show medians, first, and third quartiles. Dots represent outliers.

3.4. Mass

In contrast, species and treatment both had significant effects on mass at hatching (species: Wald χ^2_1 = 12.92, p = 0.000; treatment: Wald χ^2_1 = 20.10, p < 0.0001), but the species x treatment interaction did not have an effect. In air, *A. opacum* hatchlings had an average mass of approximately 1 mg greater than *A. annulatum* (mean (95% CI): 5.79 (5.03,6.56) versus 4.63 (4.29,4.97)) and likewise in water, *A. opacum* hatchlings had an average mass of approximately 1(mg) greater than *A. annulatum* (6.18 (5.41,6.94) versus 5.08 (4.75,5.41); Figure 4).

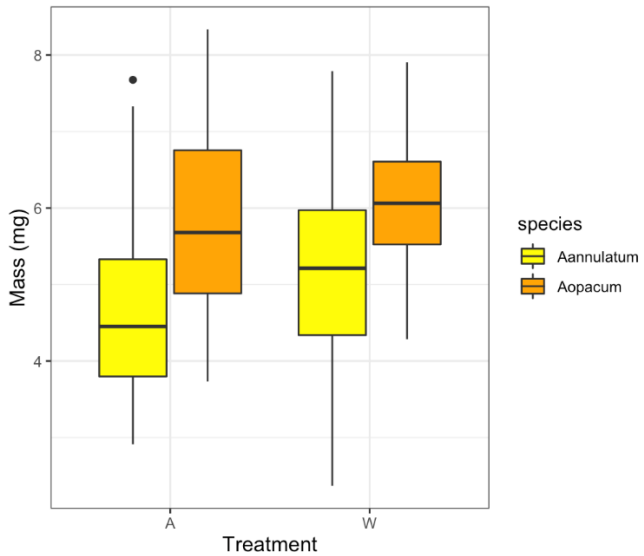

Figure 4. Mass at hatching for *A. annulatum* and *A. opacum* embryos reared in either moist air or water. Boxplots show medians, first, and third quartiles. Dots represent outliers.

4. Discussion

The results show that in air *A. opacum* takes longer to hatch and hatches at a higher stage than *A. annulatum*. In water, *A. opacum* takes longer to hatch, but both species hatch around the same stage with the median being Harrison stage 40 for both. *A. opacum* hatched at a greater mass in both treatments. When considering what could impact these dependent variables, differences in breeding ecology must be examined. In this case, those differences include egg laying habitat and the presence or absence of parental care. These results could be due *A. opacum* being a terrestrial breeder and *A. annulatum* being an aquatic breeder, or it could be to *A. opacum* practicing parental care, while *A. annulatum* does not.

In a previous study, Hale et al. (2016) similarly found *A. opacum* to take longer to hatch and hatch at a higher stage than *A. maculatum*. In contrast, their results showed *A. opacum* hatchlings to have a smaller mass. The study presented the variable of breeding season as having the potential to be a primary cause for the differences found in embryonic development. *A. opacum* is a fall breeder, while *A. maculatum* is a winter breeder. However, these two species also differ in both egg laying habitat and patterns of parental care. In fact, the two species in this study differ in those variables identically to the two species in my study. In light of the results from my study, I suggest that the differences in embryonic development found were due to either nesting habitat or patterns of parental care, but not breeding season.

Terrestrial and aquatic breeders have typically been associated with differing ages at hatching. In regards to terrestrial breeders, extended time to hatching in *A. opacum* has been observed as the eggs wait for pond filling to occur before hatching[14]. More developed hatchlings have increased survivorship and an advantage in the predator, prey, and competitor dynamic[11]. The interaction between species and treatment in age at hatching supports evidence that this response variable is a combination of adaptation and plasticity. There was a significant difference in age at hatching between the two treatments, most noticeably in *A. opacum*. While it is known that time to hatching is selected for due to the advantages, hatch time has also shown plasticity in amphibians. Environmental triggers such as pressure, pathogens, water, and gas have all been observed to directly delay or trigger hatching[14,22]. The significant difference between A. opacum's time to hatching in the two treatments, could be due to this combination of evolution and plasticity.

In regards to the practice of parental care, there is much evidence to suggest that the presence or absence of parental care has an impact on many of the response variables within embryonic development. Shine's safe harbor hypothesis from 1978 starts with parental care and asserts that as the cornerstone for a longer embryonic development period, larger egg size, and larger hatchling. This hypothesis states that although embryonic development is usually a high-risk life stage, parental care makes it a low risk stage and therefore natural selection inevitably favors extending this safe development stage while shortening the juvenile stage to increase population survivorship[23]. The data from *A. opacum* certainly agree with this hypothesis. It has also been argued that the advantages of a larger egg and propagule size put the selective pressure on parental care instead of being a result of it[16]. Either way, my study confirms that the presence of parental care is correlated with an increase in egg size, age, stage, and mass at hatching.

Another option to consider is that maybe all differences in response variables were driven by differences in egg size. One explanation for the smaller hatching size in *A. annulatum* hatchlings may be that, anecdotally, the eggs appear to be smaller. Smaller egg size would have implications for development, age, and mass, as smaller eggs produce smaller larvae[16]. I did not measure egg size or record propagule size in any case. Perhaps that would be another variable to consider or control for in future studies. However, this wouldn't explain the effect of air versus water, only the differences found between the two species. Continuing this research comparing a variety of *Ambystoma* species with more breeding ecology patterns in common, particularly egg laying habitat, would be the obvious next step. If it could be narrowed to only one trait difference across two species in several similar studies, each trait could be studied separately without being blurred by trait interactions.

5. References

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