

Embryonic motility and hatching success of *Ambystoma maculatum* are influenced by a symbiotic alga

Glenn J. Tattersall and Nicole Spiegelaar

Abstract: To augment O₂ supply through the jelly mass and egg capsule, embryonic yellow-spotted salamanders (*Ambystoma maculatum* (Shaw, 1802)) take advantage of a unicellular alga, *Oophila ambystomatis*. Convective currents from surface cilia, however, may also enhance O₂ transport, whereas muscular contractions could either enhance delivery or contribute to O₂ consumption. Embryonic motion is, therefore, potentially vital to salamander development. We examined embryonic motility across multiple developmental stages, survivorship, and hatching timing in response to different algal levels by rearing salamander egg masses under three different diel light cycles: 24 h dark, 12 h light, and 24 h light per day. Embryos raised in continuous light hatched synchronously and at slightly earlier developmental stages than embryos raised in the dark or in 12 h light per day. We removed eggs at multiple stages to examine embryonic rotation and muscular contraction rates under 180 min periods of both light and dark. Rotational movements occurred more frequently in alga-free than in algae-inhabited eggs, and more frequently in algae-inhabited eggs in the dark than in light. At later developmental stages, muscular contractions were more frequent in embryos from algae-inhabited egg masses in light than those in the dark; thus embryos with less O₂ reduced muscular activity, thereby reducing energy consumption when O₂ availability was compromised.

Résumé : Afin d'augmenter l'apport d'O₂ à travers la masse gélatineuse et la capsule de l'œuf, les embryons de la salamandre maculée (*Ambystoma maculatum* (Shaw, 1802)) tirent avantage de la présence de l'algue unicellulaire *Oophila ambystomatis*. Cependant, les courants de convection des cils superficiels peuvent aussi favoriser le transport d'O₂, alors que les contractions musculaires peuvent ou bien améliorer le transport ou alors contribuer à la consommation d'O₂. Les mouvements embryonnaires sont donc potentiellement essentiels au développement des salamandres. Nous avons examiné la motilité embryonnaire dans des conditions diverses de stade de développement, de survie et de période d'éclosion en réaction à des densités différentes d'algues en élevant des masses d'œufs de salamandres sous trois cycles photopériodiques journaliers, soit 24 h d'obscurité, 12 h de lumière et 24 h de lumière par jour. Les embryons élevés en lumière continue éclosent en synchronie et à un stade de développement relativement plus précoce que les embryons gardés à l'obscurité ou à 12 h d'éclaircissement par jour. Nous avons retiré des œufs à différentes étapes de leur développement pour examiner la rotation de l'embryon et les taux de contraction musculaire pour des périodes de 180 min, tant à la lumière qu'à l'obscurité. Les mouvements de rotation s'observent plus fréquemment chez les œufs sans algues que chez ceux qui portent des algues et plus fréquemment chez les œufs porteurs d'algues dans l'obscurité que dans la lumière. Aux stades plus avancés du développement, les contractions musculaires sont plus fréquentes chez les masses d'œufs porteurs d'algues à la lumière qu'à l'obscurité; ainsi, les embryons qui ont moins d'O₂ réduisent leur activité musculaire, diminuant par conséquent leur consommation d'énergie lorsque la disponibilité d'O₂ est réduite.

[Traduit par la Rédaction]

Introduction

The yellow-spotted salamander (*Ambystoma maculatum* (Shaw, 1802)) spends its adult life underground in woodland habitats, migrating to small, freshwater ponds to breed in the spring (Findlay 1985; Brodman 2002), generally preferring shallow ponds with persistent vegetation to which their egg masses can be attached below the surface of the water (Findlay 1985; Brodman 2002; Egan and Paton 2004). These egg masses vary in size from 10 to 20 cm in diameter, and from 50 to 100 eggs (Brodman 2002; Egan and Paton 2004).

Within masses, each embryo is protected by perivitelline fluid enclosed by a vitelline membrane and gelatinous envelopes varying in thickness throughout development (Gilbert 1942; Mills et al. 2001). These envelopes are held together by a firm collective jelly (Pinder and Friet 1994), which acts as the interface between the eggs and the environment. The dense composition of the gelatinous mass and the egg capsule, however, prevents convective O₂ transport, leaving diffusion to supply O₂ to the embryos from the surrounding water (Mills et al. 2001).

The low diffusion constant and solubility of O₂ in water

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constrain the O₂ supply for aquatic eggs (Lee and Strathmann 1998). In addition, many *Ambystoma* lay eggs in shallow waters with abundant decaying organic matter that reduces the local O₂ availability (Bachmann et al. 1986; Mills et al. 2001). In gelatinous egg masses of frogs, salamanders, and gastropods, P_{O₂} typically decreases from the surface of the mass towards the centre where it approaches and often exceeds the lower tolerance limit of embryos (Seymour and Bradford 1987; Pinder and Friet 1994; Seymour 1995; Cohen and Strathmann 1996). Furthermore, a progressive increase in metabolic rate with development will exacerbate egg mass hypoxia (Seymour 1995; Territo and Burggren 1998). Mortality, deformation, slowed development, and premature hatching are all potential consequences of hypoxia (Adolph 1979; Mills et al. 2001; Valls and Mills 2007). Certain strategies, however, could improve embryonic O₂ balance: (i) an association with photosynthetic algae; (ii) convective mixing of the in ovo environment via cilia or whole body movements; or (iii) cessation of motility entirely, thereby reducing energetic expenditure and O₂ consumption.

Egg capsules of *A. maculatum* are usually colonized by a green alga (first described as *Oophila ambystomatis* by Lambert in 1910; Gilbert 1942). This unicellular, flagellated alga penetrates the envelopes from the surrounding water and multiplies rapidly, becoming most abundant in the perivitelline space around well-developed embryos (Gilbert 1942). This relationship is often considered a symbiosis (Gilbert 1942, 1944; Bachmann et al. 1986; Pinder and Friet 1994), although equivocal data exist (Anderson 1971; Marco and Blaustein 2000). Embryos from algae-inhabited egg masses are longer at hatching and have greater hatching success than those from masses without algae (Gilbert 1944; Marco and Blaustein 2000). The alga has been suggested to protect embryos from harmful UV-B radiation, allowing eggs to develop in shallow waters (Marco and Blaustein 2000). However, more common explanations for their success are the beneficial effects of local O₂ provision (Bachmann et al. 1986; Pinder and Friet 1994), CO₂ elimination, or nitrogenous waste elimination (Goff and Stein 1978). When light is available, the algae supply O₂, which may be particularly valuable for embryos centrally located in the egg mass (Pinder and Friet 1994); however, algae also consume O₂ under dark conditions (Hutchison and Hammen 1958; Cohen and Strathmann 1996; Woods and Podolsky 2007).

Embryos themselves may also behaviourally alter their O₂ supply and demand. Both ciliary action and muscular contraction induce intra ovum convective mixing (Warkentin 2007; Goldberg et al. 2008). Epidermal cilia serve many roles before and after hatching, including the movement of mucus, microorganisms, and debris, rotations before hatching and gliding movements after hatching, and water-quality assessment, as well as disruption of boundary layers to improve gas exchange (Burggren 1985; Doran et al. 2004; Nokhbatolfoghahai et al. 2005) (Nokhbatolfoghahai et al. 2005, 2006). In fact, the number of surface cilia increases with development until the embryo approaches hatching (Kessel et al. 1974). The convection generated by these cilia should also work to enhance gas transport across the egg capsule, and ultimately throughout the entire egg mass

(Burggren 1985), alleviating diffusion limitations. Indeed, Hunter and Vogel (1986) observed higher diffusion rates in egg masses with rotating embryos than those with nonrotating embryos. Coordinated muscle contraction does not occur until later in development (Harrison 1969); it could aid convective mixing at that point, but it would also increase O₂ demand.

In this study, we assessed the importance of photosynthetic algae and the behavioral responses of embryos to variation in O₂ availability in *A. maculatum*. Our hypotheses were (i) that algae accelerate salamander development and improve hatching success, (ii) that embryonic rotation in algae-inhabited eggs increases under dark (hypoxic) conditions to enhance O₂ delivery, and (iii) that O₂-demanding muscular activity is reduced in algae-inhabited eggs in dark (hypoxic) conditions. We assessed these hypotheses by manipulating algal content and photosynthesis. We reared eggs under three diel light cycles (24 h darkness, 12 h light : 12 h darkness, and 24 h light) to generate different algal levels. We then quantified embryonic motion under short-term (180 min) light and dark exposures, altering O₂ in algae-inhabited eggs. Since algal density and embryonic ciliary abundance and muscular activity all change developmentally, we examined embryonic motility at multiple developmental stages.

Materials and methods

Egg collection

We collected 22 egg masses of *A. maculatum* that were laid on 24 April 2007 (day 0) from Bat Lake in Algonquin Park, Ontario, Canada (45.5850°N, 78.5181°W), on 25–26 April. These were the first egg masses laid in 2007, based on daily inspections for 4 days prior. Given the size of the lake (~2.6 ha), the total number of egg masses laid (typically 2000–4000 egg masses are laid at this location; Findlay 1985), and the dispersed collection sites (>30 m apart), each egg mass was likely from a unique pairing.

Oviposition depth and algal colonization in Bat Lake

Egg masses were found attached to submerged vegetation (leatherleaf, *Chamaedaphne calyculata* (L.) Moench). To assess oviposition sites, we measured the depth (top of mass to water surface) for 330 egg masses accessed by row boat. We sampled all egg masses visible within a 1 m radius at random distances from 1 to 20 m apart along the 670 m perimeter of the lake. After the egg masses hatched (~8 June), 18 evacuated egg masses (containing only jelly and algae) were collected haphazardly from a range of depths (0–80 cm) and photographed to estimate relative algal content. Empty egg masses were placed into containers (height controlled to ensure similar jelly density), arranged together on a uniform background, and photographed (single image) with a digital camera (model DMC-FZ10, Lumix Panasonic). This image was analysed using Adobe Photoshop version 6.0. Equal-sized regions of interest were selected from each jelly mass and the mean luminosity (L^*) determined. The L^* channel is a linear scale (0–100) depicting the relative brightness of an image (Drimbarean and Whelan 2001). Dark green jelly masses had low L^* values, whereas clear, alga-free egg masses had higher L^* values.

Egg mass light:dark incubation protocol

The egg masses collected on 25–26 April were randomly assigned to 12 containers, 4 for each of the 3 long-term light incubation treatments, with 1–2 masses per container (depending on size) so that each treatment had similar numbers of eggs (~350–450 eggs in total per treatment). All containers were housed within a common incubator for temperature control. The eggs were kept at 5 °C in complete darkness for 1 week to ensure developmental stasis while equipment was prepared. Within the incubator, different light incubation treatments were then established in three separate, light-proof regions. On 5 May, the temperature was raised to 10 °C and the long-term light incubations began; one group of egg masses was kept in complete darkness (DD), the second group was subjected to a 12 h light : 12 h dark cycle (LD), and the third group was exposed to continuous light (LL). Temperatures within all light treatment regions were determined to be similar (± 0.2 °C). Each container was aerated continuously. Field O₂ measurements at natural oviposition sites during egg development were 8–12 mg·L⁻¹ (19–21 kPa; early morning measurements), similar to values obtained by aeration in the laboratory.

Behaviour recordings under light and dark conditions

Behavioural observations on embryos from all three incubation treatments (DD, LD, and LL) were made using video recordings at eight intervals throughout development. At day 12 after oviposition the embryos were still spherical (stage 11–13; Harrison 1969), thus rotation could not readily be detected. Behavior was recorded every 2–3 days from day 20 (stage 20–22) to day 45, on individual eggs removed from their masses and exposed to 180 min of light or dark.

For each recording, nine eggs were selected from the four containers (container chosen randomly) within each incubation group (DD, LD, LL), and staged according to Harrison (1969). Eggs were removed from masses with no bias for centre or peripheral location, extracting them gently with a spoon. Each egg was placed in a small observation compartment and all 27 eggs placed in a small incubator at 10 °C, where they were recorded simultaneously with a video camera (model DCR-HC90 Sony Handycam). This procedure was conducted twice per observation day, once under fluorescent light within the incubator and once in visible darkness using infrared illumination from the camera. Separate embryos were used for each light condition ($N = 54$ eggs per recording day) and treatment order randomized. Eggs were first incubated 150 min under light or dark conditions to allow any changes in O₂ levels from algal photosynthesis and algal and salamander respirations to manifest (Bachmann et al. 1986). They were then recorded for 30 min. After video recording, eggs were placed into a separate container in the incubator under their original light:dark cycle until hatching. No eggs were reused; all behavioural observations were made on newly extracted eggs that had developed within the egg mass.

From the 30 min video recordings we quantified two behaviours for each embryo. (1) We viewed tapes at high speed (16×) to count the number of embryonic rotations, subsequently expressed as rotation rate per minute. Rotation was unidirectional, continuous, and generally slow. (2) We viewed tapes at slower speeds (2× to 4×) to count the

number of contractions (brief, rapid, contractions along the body axis, typically lasting <1 s), subsequently expressed as contraction rate per minute.

Hatching and survivorship

From day 45, after behaviour recordings were complete, no further eggs were extracted from masses. We then checked all containers with egg masses daily for hatched salamanders until no live eggs were left (day 80). Hatchlings were staged under a microscope and any deformities noted (e.g., abnormal growths, twisted spines, missing appendages). We also recorded and removed dead embryos daily. Survival, hatching, and deformity data were recorded separately for each container, but stage data were recorded only for treatment groups. Hatchlings from separated eggs were returned to Bat Lake, but not included in survival or hatching analyses.

Statistical analysis

Cumulative live hatching (% of total eggs) was analysed using a repeated-measures ANOVA, with time (day 48, 52, 56, 60, 64, and 68) as the within-subjects effect, long-term incubation (LL, LD, DD) as the between-subjects effect, and containers as replicates. Since hatching stage data were pooled across containers within treatments, the effect of incubation treatment was tested with χ^2 tests of independence, and standardized residuals were compared using z scores (Grafen and Hails 2006). Deformity data were analysed with a one-way ANOVA, using the containers as replicates.

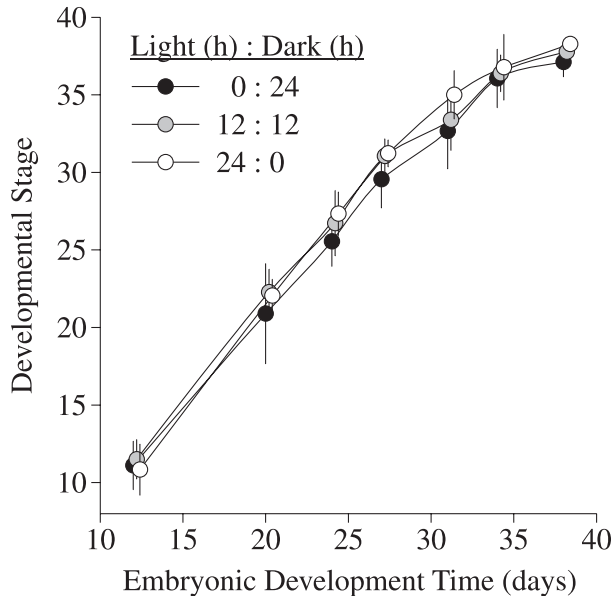
Development data from extracted eggs violated normality and homoscedasticity assumptions of a two-way ANOVA. We therefore first regressed stage on age with an ordinary least squares general linear model, then tested the residuals from this relationship for effects of incubation treatment with a one-way ANOVA. Stages above 38 were not included in the model because algae obscured developmental markers. Embryonic rotation data were analysed with a three-way ANOVA, with long-term incubation (DD, LD, LL), experimental incubation (light, dark), and age (days 20, 24, 27, and 31) as factors, after arcsine square root transformation to meet assumptions. Non-normality and heteroscedasticity precluded the three-way ANOVA of muscular contractions. We therefore analyzed each day (31, 34, and 38) separately for effects of long-term and experimental incubation treatments with two-way ANOVAs on log-transformed or ranked data. Analyses of contractions and rotation used subsets of development (3 and 4 ages each, respectively) to exclude stages with numerous zero values for these behaviours. When ANOVAs revealed significant effects, post hoc comparisons were performed using the Holm–Šidák method, with $\alpha = 0.05$. Results are presented as means (\pm SD) unless otherwise indicated.

Results

Oviposition depths and algal colonization

Oviposition site depth averaged 33.8 cm (SD = 16.8 cm, $N = 330$, range 5–80 cm). Algal content (L^*) was negatively correlated with depth in a subset ($N = 18$) of evacuated jelly

Fig. 1. Development of embryonic *Ambystoma maculatum* reared under three diel light cycles to manipulate algal colonization and photosynthesis. Staging follows Harrison (1969). Dark-reared embryos lacking algae are, on average, less developed for their age than are alga-colonized eggs. Data are means \pm SD; time points are displaced horizontally to allow error bar visualization.



masses photographed following hatching in the field ($P = 0.036$, $r^2 = 0.24$).

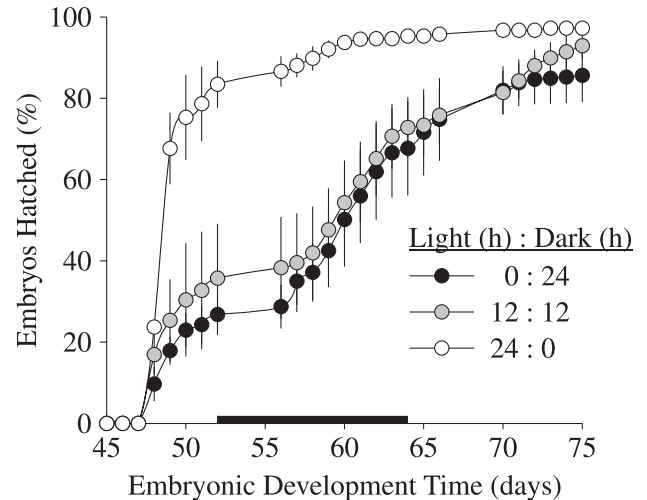
Development and hatching patterns

Developmental stage increased linearly over time ($F_{[5,480]} = 1103$, $P < 0.0001$, $r^2 = 0.91$; Fig. 1). Long-term incubation conditions (i.e., algal colonization) significantly, but only slightly, affected development ($F_{[2,483]} = 6.77$, $P = 0.0013$, $r^2 = 0.027$). Post hoc analysis showed that DD embryos were, on average, slightly less developed for their age than LD and LL embryos (DD vs. LD: $t_{[161]} = 2.69$, $P = 0.007$; DD vs. LL: $t_{[161]} = 3.52$, $P = 0.0005$). There was no discernible difference between the LL and the LD groups.

Survival to hatching was generally high (85%–97%; Fig. 2) but was nevertheless affected by algal colonization ($F_{[2,9]} = 7.3$, $P = 0.013$). A time \times incubation treatment interaction ($F_{[10,45]} = 4.36$, $P = 0.002$) revealed that the algae affected the temporal pattern of hatching. The LL group began hatching rapidly after the 48th day, with most (95%) eggs hatching within 10 days, whereas the LD and DD groups began hatching much more gradually. The mean times to hatching were affected by algal presence ($F_{[2,9]} = 5.7$, $P = 0.025$), with the LL group requiring less time (50.6 ± 1.5 days) than the LD group (58.1 ± 5.8 days) or the DD group (58.7 ± 2.5 days).

Deformities occurred in $\sim 1\%$ of embryos and were not significantly affected by algal colonization ($F_{[2,9]} = 0.215$, $P = 0.811$). Although larvae hatched in stages 41–43, hatching stage varied with incubation treatment ($\chi^2_{[4]} = 277$, $P < 0.00001$; Table 1). The comparison of all three treatments was heavily influenced by the early, rapid hatching of the LL group ($z = 9.0$, $P = 1 \times 10^{-18}$); however, a second χ^2 test of independence comparing DD vs. LD also revealed

Fig. 2. Cumulative hatching patterns of embryonic *Ambystoma maculatum* housed in three diel light cycles: DD, LD, and LL. Data are mean (\pm SE) percent hatched from four groups of eggs per treatment. Embryos raised in continuous light had a rapid onset of hatching, whereas the other incubation groups hatched gradually. The bar indicates days at which cumulative hatching was higher ($P < 0.05$) in the LL group compared with the LD and DD groups.



a treatment effect on stage at hatching ($\chi^2_{[2]} = 14.5$, $P = 0.001$). The DD group had fewer than expected stage 43 larvae ($z = -2.5$, $P = 0.01$), thus the relative order of hatching stage was LL $<$ DD $<$ LD (Table 1).

Embryonic rotation

Embryonic rotation was affected by a three-way interaction between long-term incubation treatment, short-term light exposure, and developmental stage ($P = 0.0333$; Fig. 3A, Table 2), as well as by a stage \times light exposure interaction ($P = 0.000691$). There was, however, no effect of the long-term incubation \times light exposure interaction ($P = 0.116$). There was a significant main effect of algal colonization ($P = 0.00104$), borne out by an overall elevated embryonic rotation rate in the DD group (DD vs. LL: $t_{[159]} = 3.75$, unadjusted $P = 0.0003$; DD vs. LD: $t_{[159]} = 2.40$, unadjusted $P = 0.018$) (Fig. 3A). Embryonic rotation began between days 12 and 20 and ceased in late development (Figs. 3A, 4). Within the age range of active rotation, a significant three-way interaction indicates that the effect of age (developmental stage) on rotation rate depends on both incubation treatment (algae) and illumination (Table 2). Indeed, illumination initially increases rotation rate in all long-term incubation groups (day 20: $t_{[80]} = 3.1$, unadjusted $P = 0.003$). Later in development, illumination mostly does not influence rotation rates. However, in the LL group at day 27, rotation rate was higher in the dark ($t_{[17]} = 3.64$, unadjusted $P = 0.002$; Fig. 3A).

Muscular contractions

Muscle contractions began between days 26 and 31, peaked at day 34 or 38, and then declined (Figs. 3B, 4). Significant incubation \times short-term illumination interactions occurred at days 31 and 34 (Table 3). In algae-inhabited eggs (LD and LL), illumination dramatically increased con-

Table 1. Percentage of larval *Ambystoma maculatum* that hatched at each particular developmental stage.

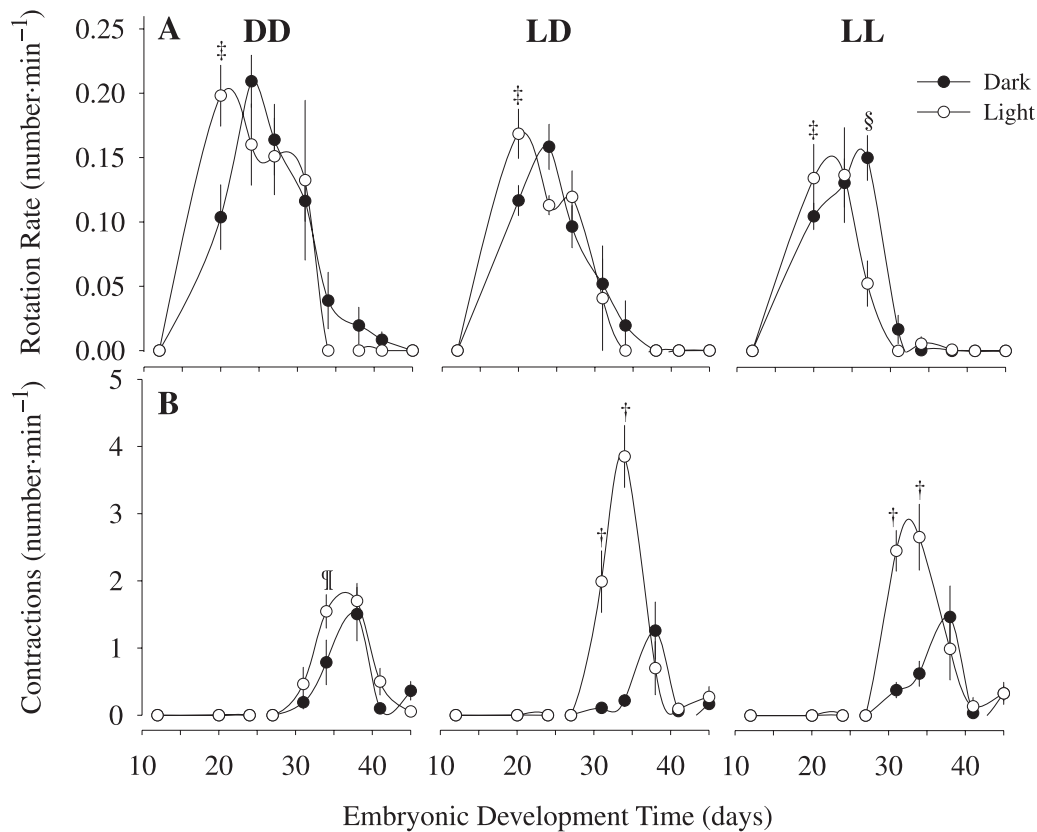
Long-term incubation (light (h) : dark (h))	Stage			P	P
	41	42	43		
DD (0 : 24)	38.2	52.1	9.7		
LD (12 : 12)	34.3	46.2	19.5	<0.000001*	0.001†
LL (24 : 0)	85.6	11.8	2.5		

Note: Values were compared using χ^2 contingency tables on the actual frequency of occurrences.

*A significant overall effect of incubation, $\chi^2_{[4]} = 277, N = 1176$.

†A significant difference between DD and LD, $\chi^2_{[2]} = 14.5, N = 779$.

Fig. 3. Rotation rate (A) and spontaneous muscular contractions (B) of embryonic *Ambystoma maculatum* reared under three diel light cycles (LL, LD, DD as indicated) during 30 min observations under dark and light conditions. Incubating embryos in complete darkness (DD) to prevent algal colonization increases embryonic rotation regardless of illumination. Illumination initially increases rotation (‡, day 20, $P = 0.003$). Later in development, it decreases rotation in LL eggs only (§, day 27, $P = 0.002$). Illumination affected muscle contractions, especially in alga-colonized eggs (LD, LL) at days 31 and 34 (†, $P < 0.001$) but also in DD embryos at day 31 (¶, $P = 0.02$). For visual clarity, error bars are not shown.



traction rates on day 31 (LD: $t_{[17]} = 4.58$, unadjusted $P = 3 \times 10^{-5}$; LL: $t_{[17]} = 4.04$, unadjusted $P = 0.00018$) and day 34 (LD: $t_{[17]} = 6.28$, unadjusted $P = 1 \times 10^{-8}$; LL: $t_{[17]} = 3.17$, unadjusted $P = 0.003$). Embryos raised in complete darkness (DD, lacking algae) generally exhibited no changes in muscle contractions with short-term changes in light, except for a small stimulatory effect of light on muscle contractions at day 34 ($t_{[17]} = 2.57$, unadjusted $P = 0.02$).

On day 31, the DD group exhibited fewer contractions under light exposure than both LL and LD groups ($t_{[17]} = 5.33$ and 3.99 , unadjusted $P = 0.0000552$ and 0.000948 , re-

spectively). A similar trend existed on day 34 ($t_{[17]} = 2.64$ and 4.02 , unadjusted $P = 0.0172$ and 0.000888 , respectively). Contractions under dark illumination on days 31 and 34 were not different among the long-term incubation groups. The influence of incubation, short-term illumination, and the incubation \times short-term illumination interaction at day 38 were all not significant (Table 3).

Discussion

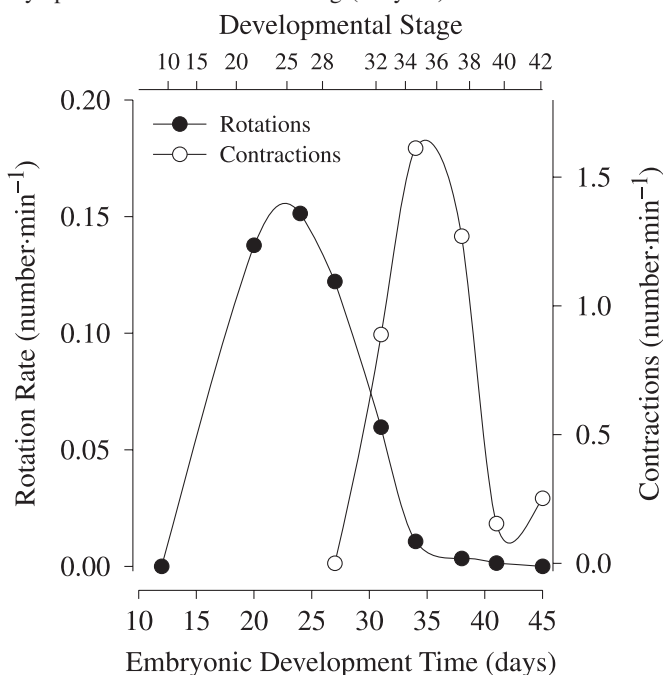
The presence of unicellular algae living within the egg

Table 2. ANOVA summary table for the analysis of rotation rates in embryonic *Ambystoma maculatum* under different periods of long-term light incubation and short-term light illumination.

Effect	df	MS	F	P
Long-term incubation (LTI)	2	0.0745	7.21	0.00104
Short-term incubation (STI)	1	0.00079	0.0770	0.782
Stage	2	0.0240	2.33	0.101
LTI × STI	2	0.0226	2.19	0.116
Stage × LTI	4	0.00870	0.842	0.501
Stage × STI	2	0.0791	7.66	0.000691
LTI × STI × Stage	4	0.0278	2.69	0.0333
Error	144	0.0126		

Note: This analysis is restricted to a subset of stages when rotation occurred in all treatments, thus the analysis does not capture larger developmental changes in presence or absence of rotation.

Fig. 4. Summary of the onset and ontogeny of embryonic rotation and muscular contraction in embryonic *Ambystoma maculatum*. Data are means of all three long-term incubation conditions measured under dark conditions only. As embryonic rotation rates decrease, muscular contractions rise to a climax falling only a few days prior to the onset of hatching (~day 48).



capsule of developing salamanders has long been speculated to serve a symbiotic role. The current study lends support to that idea with some caveats. Salamander eggs raised under continuous light hatched more synchronously and at earlier stages in development compared with eggs under lower light levels, suggesting that continuously photosynthesizing algae accelerate hatching. The presence of algae, however, diminishes embryonic rotation, suggesting alterations in the expression or control of epidermal cilia. Since embryos with algae reduce contractions when placed in the dark, it also appears that muscular contractions are positively dependent on the level of O_2 and that reducing metabolic expenditure is the impetus behind changing muscular activity in hypoxia.

Nature of the symbiosis

Gilbert (1942) observed that *Ambystoma* eggs raised in the light were colonized by algae and hatched over a shorter period of time than alga-free eggs reared in the dark group. Our results support this, particularly with respect to hatching success and synchrony. The presence of algae result in an increase in O_2 consumption in embryonic *A. maculatum* by 45.4% in earlier stages (12–13) and 74.8% in later stages (32–34) relative to alga-free egg capsules (Hutchison and Hammen 1958). Thus, the algae play an increasingly important role in enhancing embryonic respiration rates through ontogeny, allowing for higher rates of metabolism (Pinder and Friet 1994) and promoting synchronous hatching at an earlier developmental stage (Table 1, Fig. 2). Since much of the O_2 from the surrounding environment may be consumed before it reaches the centre of the egg mass, O_2 production within the capsule would be most vital to successful development and hatching of the innermost embryos. Gilbert (1944) also suggested that this relationship is symbiotic because algae grew more vigorously when associated with embryos than without embryos. By inhabiting egg capsules, *O. ambystomatis* likely utilize CO_2 and nitrogenous wastes excreted by the developing embryo, allowing for rapid algal growth (Gilbert 1944; Hutchison and Hammen 1958). Whether O_2 is the primary factor, or whether other factors (e.g., nutrition, waste management, buoyancy; cf. Peyton et al. 2004) also assist embryonic development, remains to be demonstrated.

Ontogeny of embryonic motility

An intriguing result of the present study is the overall developmental pattern of embryonic motility. Rotation initially rises in early development, decreasing with the onset of muscular contractions (Fig. 4). It is unlikely that rotation is limited by elongation of the embryo in the constrained space of the egg capsule, since straightening and elongation of the embryo occurs at stages 33–35 (Harrison 1969), while rotation rates decrease earlier following stage 26 (Fig. 4). Furthermore, the capsular chamber is capable of swelling under hypoxic conditions (Mills et al. 2001; Valls and Mills 2007). During amphibian development, epidermal cilia initially increase in density, and subsequently decrease as ciliated cells disappear (Nokhbatolfoghahai et al. 2005, 2006). The abundance patterns of surface cilia in larval sala-

Table 3. ANOVA summary table for the analysis of muscular contraction rates in embryonic *Ambystoma maculatum* under different periods of long-term light incubation and short-term light illumination.

Effect	df	MS	F	P
Day 31				
Long-term incubation (LTI)	2	1300	10.9	0.000125
Short-term incubation (STI)	1	2920	24.6	9.24×10 ⁻⁶
LTI × STI	2	749	6.32	0.00366
Error	48	118		
Day 34				
LTI	2	0.132	0.821	0.446
STI	1	7.50	46.8	1.30×10 ⁻⁶
LTI × STI	2	0.675	4.22	0.0205
Error	48	0.160		
Day 38				
LTI	2	1.69	2.84	0.0682
STI	1	0.654	1.10	0.300
LTI × STI	2	0.901	1.52	0.230
Error	48	0.595		

Note: This analysis is restricted to a subset of stages when muscular contractions occurred in all treatments, thus the analysis does not capture larger developmental changes in presence or absence of contractions.

manders match the changes in rotation rates in the present study (Billett and Courtenay 1973; Kessel et al. 1974; Landstrom 1977), suggesting a role of ciliary rotation in embryonic development and respiration (Burggren 1985).

In terms of the proximate mechanisms leading to changes in embryonic motility, it is important to consider whether the short changes in light levels indirectly influence photosynthetic O₂ supply to those embryos with algae, or if light itself triggers ciliary beating. The present study shows minor influences of short-term changes in light on embryonic rotation (Fig. 3), although during the early stages of development (day 20, stages 23–25), embryonic rotation appears to be positively influenced by light. This suggests that early in development, ciliary beating is linked to photoreceptor input, particularly because this stimulating effect of light exists in the alga-free embryos, which would not be expected to exhibit hypoxia in the dark. Later in development (~stage 30) in the LL group (which should possess the highest algal density), there is a stimulatory effect of dark incubation on rotation, suggesting that toward the time of peak ciliary activity, low O₂ will stimulate ciliary activity. The fact, however, that dark incubation does not stimulate rotation in the LD group or at other developmental stages may be related to a low threshold P_{O₂} threshold of stimulation. Larval pond snails require P_{O₂} values of <30 mm Hg before noticeable changes in rotation occur (Kuang et al. 2002). If the combined algal and embryonic respiration does not drive P_{O₂} levels low enough, rotation may not be stimulated.

Consistent with Harrison (1969), we noted that muscular movements commence around stage 33. These early contractions are electrogenic, not resulting from nervous input (Lewis and Hughes 1960; Muntz 1975). A change in cellular O₂ levels (by exposing an alga-inhabited egg to darkness) may simply shut down muscle contractions rather than stimulate them as might be expected in a neurally coordinated system where the embryo could attempt intra ovum mixing

or escape related behaviours (at hatching-competent stages). As development proceeds and neuromuscular junctions reinforce neural control, there is an overall suppression of these spontaneous muscle contractions, as well as a diminishment of the influence of the dark (i.e., hypoxia). One caveat, however, is that muscle contractions did not differ among all of the incubation groups during dark exposure, raising the possibility that elevated O₂ from light exposure in the alga-inhabited eggs stimulates muscular contractions, rather than hypoxia suppressing activity. A suppression of muscular activity, however, has been observed in larval rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) exposed to hypoxia (Ciuhandu et al. 2007), highlighting a common strategy for energy conservation in larval fish and amphibians. Nevertheless, it is unlikely that muscular contractions occur to facilitate convective mixing during hypoxia.

Developmental consequences of varying O₂

Given the differential influence of algae on embryonic motility and hatching, it is prudent to consider the consequences of O₂ to salamander development. Chronic hypoxia can be detrimental to survival in developing salamanders; development is slowed or arrested, hatching is either delayed or forced prematurely (Detwiler and Copenhaver 1940). These effects are most severe in later developmental stages; later stage embryos succumb to anoxia much sooner than younger embryos, although those that survive show deformities and slowed development, but are capable of elongating, acquiring cilia, and flexing the body after being reoxygenated (Adolph 1979).

Oxygen partial pressure in the egg is influenced not only by photosynthesis of the associated algae, but by respiration from algal, embryonic, and microbial sources (Bachmann et al. 1986). Thus, the presence of algae in the egg creates diurnal P_{O₂} cycles where photosynthesis in the day may produce hyperoxic conditions within the egg, while dark

respiration leads to a net decline of O₂ at night (Bachmann et al. 1986; Pinder and Friet 1994). The periodic anoxia that may result from dark respiration may become increasingly challenging for the developing embryo as the embryo metabolism and the algal density increase. In particular, ocaation within the egg mass produces challenges for the developing embryos in the core, as steep O₂ gradients are known to prevail in algae-inhabited egg masses exposed to low light (Pinder and Friet 1994).

In embryonic *A. maculatum* exposed to alternating oxygenation protocols, Valls and Mills (2007) observed slower development and delayed and developmentally premature hatching. Premature hatching may act to eliminate respiratory barriers of the egg capsule (Valls and Mills 2007), and conforms to the notion that hypoxia may be a trigger for hatching in certain amphibian embryos, particularly terrestrial developers (Petranka et al. 1982). Interestingly, in the current study, early hatching did not occur in salamanders raised in the dark or under the 12 h light : 12 h dark regime, consistent with previous work demonstrating that hypoxic *Ambystoma* egg masses take longer to hatch than ones raised at elevated O₂ tensions (Mills and Barnhart 1999). In the present study, the most ecologically meaningful treatment (the LD group) exhibited a slower rate of hatching than the LL group. Translating these results to the field would require measurements of algal density and the O₂ conditions surrounding the egg mass. All of the egg masses in the current study, however, received constant aeration from external air stones, and although convective currents do not play a role in O₂ delivery to the egg (Pinder and Friet 1994), a continuously elevated external O₂ environment would have alleviated overall egg mass hypoxia. The LL group, however, did exhibit rapid, synchronous hatching, suggesting that elevated O₂ stimulates hatching. It is possible that the enhanced muscle activity during light exposure assists in freeing the embryo from the egg. Furthermore, photosynthesizing algae should raise O₂ levels throughout the egg mass, allowing those embryos in the diffusion-limited centre to develop at similar rates to those in the periphery, resulting in more synchronous hatching. The hatching patterns in the LD and DD groups (Fig. 2) suggest that “waves” of hatching occurred, with embryos hatching from the outside of the egg mass first, and subsequent hatching occurring in the central core as diffusion barriers became reduced with jelly and capsule degradations. One way to establish the importance of O₂-stimulated hatching would be to monitor the diurnal patterns of hatching in egg masses held under different light regimes to verify if higher daytime activity facilitates hatching.

Conclusions

Our results show that the presence of continuously photosynthesizing algae induces rapid, coordinated hatching of salamander larvae, decreasing the overall time to hatch. Since algal density varies in egg masses in nature (Marco and Blaustein 2000), a potential benefit of coordinated hatching could result from the “predator dilution” effect, where simultaneous hatching swamps potential generalist predators, thereby reducing individual mortality (Ims 1990; Tucker et al. 2008). Developmentally, algae may mitigate the embryo’s reliance on ciliary beating, since embryonic

rotation is lower in eggs containing algae. Algal respiration, however, contributes to generating hypoxic conditions in the dark. This appears to influence rotation later in development, enhancing rotation in the dark in the embryos containing the potentially highest density of algae, suggesting some role for ciliary-induced convective mixing of the perivitelline fluid. The most profound effect of algal colonization, however, is seen by their influence on embryonic muscle activity, where embryos with algae reduce their muscle contractions in the dark, suggesting that hypoxia limits muscular activity. If muscle contractions are virtually absent, this may influence their ability to hatch in the dark, forcing them, in the wild, to leave the egg mass under conditions of greater risk to visual predators.

Since oviposition sites in the field are often in shallow water, this raises the question of whether depth selection occurs by female when ovipositing eggs to submergent vegetation, particularly since egg masses at greater depths develop fewer algae. Future exploration of the effects of depth, water quality, and turbidity on the alga–salamander symbiosis would shed light on the potential costs (e.g., predation, desiccation, UV-B exposure) and benefits (e.g., algal growth, oxygenation, metabolism) of oviposition site selection to the success of larval salamanders.

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