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CORRECTION

Intracapsular algae provide fixed carbon to developing embryos of the salamander *Ambystoma maculatum*

Erin R. Graham, Scott A. Fay, Adam Davey and Robert W. Sanders

There was an error published in *J. Exp. Biol.* **216**, 452-459. The calculations of carbon fixation and translocation rates, which are presented in supplementary material Table S2, were incorrect. The error was not in the measured data (disintegrations per minute of radioactive samples), but a mathematical error that occurred when calculating the amount of carbon in the sample incubations. Thus, the error does not affect the percent carbon translocation values, only the calculated rates of carbon fixation and translocation per hour.

In Results, Carbon translocation (p. 455, lines 8–10), the text should read:

> The average hourly rate of carbon fixation was 589.1±56.7 ng carbon per sample, or 294.5 ng carbon per egg. An estimated average 15.4 ng fixed carbon was translocated to each egg per hour.

The correct version of Table S2 appears below:

Table S2. Data from carbon translocation experiments using [^{14}C]bicarbonate

Sample	Net DPM whole egg[a]	Net DPM embryo[b]	Percent carbon translocation egg^{-1} [c]	Rate of carbon fixation (ng C h^{-1} egg^{-1})[d]	Rate of carbon translocation (ng C h^{-1} egg^{-1})[e]
1	9634.3	877.1	9.1	235.7	21.5
2	12485.4	1644.5	13.2	305.4	40.2
3f	4199.4	1247.3	29.7	102.7	30.5
4	20278.7	96.2	0.5	496.0	2.4
5	13234.3	992.6	7.5	323.7	24.3
6	5920.2	445.7	7.5	144.8	10.9
7	10335.4	577.1	5.6	252.8	14.1
8	28224.7	654.1	2.3	690.4	16.0
9	5783.7	643.4	11.1	141.5	15.7
10	10515.7	100.1	1.0	257.2	2.5
11	4000.6	247.9	6.2	97.9	6.1
Mean	12041.3	627.9	6.4%	294.5	15.4

Samples consist of two eggs combined in one vial. DPM = disintegrations per minute.
[a]DPM of egg (including embryo) incubated in light minus average DPM of egg samples (*n*=5) incubated in darkness.
[b]DPM of embryo only incubated in light minus average DPM of embryos (*n*=5) incubated in darkness.
[c]Net DPM of embryo/net DPM of whole egg × 100.
[d]Carbon fixation per hour = dissolved inorganic carbon (DIC) in sample × (DPM total egg/DPM of ^{14}C added/hours incubation). DIC was estimated according to Wetzel and Likens (2000).
[e]Carbon fixation (or translocation) per hour = DIC in sample × [DPM total egg (or embryo fraction)/DPM of ^{14}C added/hours incubation]. DIC was estimated according to Wetzel and Likens (2000).
[f]Sample not included in mean calculations.

The authors sincerely apologise for the errors in the original manuscript, but assure readers that they do not affect any of the major findings or conclusions of the work.

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RESEARCH ARTICLE

Intracapsular algae provide fixed carbon to developing embryos of the salamander *Ambystoma maculatum*

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SUMMARY

Each spring, North American spotted salamander (*Ambystoma maculatum*) females each lay hundreds of eggs in shallow pools of water. Eggs are surrounded by jelly layers and are deposited as large gelatinous masses. Following deposition, masses are penetrated by a mutualistic green alga, *Oophila amblystomatis*, which enters individual egg capsules, proliferates and aggregates near the salamander embryo, providing oxygen that enhances development. We examined the effects of population density of intracapsular *O. amblystomatis* on *A. maculatum* embryos and show that larger algal populations promote faster embryonic growth and development. Also, we show that carbon fixed by *O. amblystomatis* is transferred to the embryos, providing the first evidence of direct translocation of photosynthate from a symbiont to a vertebrate host.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/3/452/DC1

Key words: *Oophila amblystomatis*, *Ambystoma maculatum*, algae, salamander, carbon translocation, symbiosis.

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INTRODUCTION

Over 120 years ago, it was discovered that a mutualistic, flagellated green alga, *Oophila amblystomatis* (Lambert), inhabits the eggs of a few species of ambystomatid salamanders and ranid frogs (Orr, 1888). The spotted salamander, *Ambystoma maculatum* (Shaw 1802), is common throughout North America and has served as a model organism for studying this unique alga–vertebrate symbiosis. *A. maculatum* females deposit hundreds of eggs in shallow vernal pools each spring. The eggs are surrounded by multiple jelly layers and are deposited as masses enclosed in a thick jelly matrix that protects the embryos from pathogens, predators and contaminants (Altig and McDiarmid, 2007). Egg masses remain below the surface of the water and are often attached to vegetation. Within hours of deposition, the masses are penetrated by benthic *O. amblystomatis* that swim to the egg masses (Gilbert, 1944); however, the mechanism by which *O. amblystomatis* locates *A. maculatum* eggs remains unknown. Once inside, *O. amblystomatis* multiplies and traverses the middle membranes of the eggs, ultimately aggregating as nonmotile spherical or ovoid cells on the inner membrane of individual eggs, adjacent to the developing salamander embryo (Gilbert, 1942). A recent discovery revealed that *O. amblystomatis* is also endosymbiotic; a portion of algal cells penetrates the embryo near the blastopore and settles within tissues and cells of the embryonic salamander (Kerney et al., 2011).

O. amblystomatis benefits *A. maculatum* embryos, presumably by increasing oxygen within the egg capsules (Valls and Mills, 2007; Pinder and Friet, 1994), which contributes to faster embryonic growth and development, increased survivorship and more synchronous hatching (Tattersall and Spiegelaar, 2008; Gilbert, 1944; Gilbert, 1942). In turn, *O. amblystomatis* receives embryonic nitrogenous waste (Goff and Stein, 1978) and possibly CO_2 (Hammen, 1962). Another possible benefit for the salamander embryo is contribution of photosynthetically fixed carbon from *O. amblystomatis* (reviewed by Kerney, 2011), which could provide additional nutrition during development. Translocation of sugars from symbiont to host is common among alga–invertebrate symbioses, such as in tropical cnidaria–zooxanthellae symbioses, but remains unverified in an alga–vertebrate mutualism.

The recent work of Kerney et al. (Kerney et al., 2011) has increased our understanding of the *O. amblystomatis–A. maculatum* mutualism, yet several questions regarding this relationship remain unanswered. First, is there a direct relationship between *O. amblystomatis* abundance and *A. maculatum* growth and development? Second, aside from increasing oxygen in egg capsules, does *O. amblystomatis* provide any additional benefits to salamander embryos, such as photosynthetic products? To address these questions, we tested the effects of *O. amblystomatis* abundance on growth and development of *A. maculatum* embryos by comparing egg masses maintained in light, dark and with a photosynthesis inhibitor. We hypothesized that algal population density would be positively correlated with embryonic development and growth, irrespective of light. Although *O. amblystomatis* increases intracapsular oxygen during photosynthesis, eggs inhabited by algae have been shown to experience periods of anoxia during the night when the combination of algal and salamander respiration is not countered by oxygen production from photosynthesis (Pinder and Friet, 1994). Therefore, we hypothesized that oxygen may not be the primary benefit for the embryos and that photosynthetically fixed carbon may accelerate growth and development. We used [^{14}C]bicarbonate to test the hypothesis that *O. amblystomatis* provides *A. maculatum* embryos with fixed carbon from photosynthesis. Our results reveal that fixed carbon is translocated from *O. amblystomatis* to salamander embryos and that there is

a direct relationship between *O. amblystomatis* abundance and *A. maculatum* embryonic growth and development. Our results also suggest that algae protect embryos from harmful microorganisms, possibly by inhibiting bacterial growth. This study provides the first evidence of direct translocation of carbon from a photosynthetic symbiont to a vertebrate host and demonstrates that *O. amblystomatis* contributes multiple benefits to the salamander in this mutualism.

MATERIALS AND METHODS

Ambystoma maculatum egg masses were collected from a vernal pool in Bucks County, PA, USA on 28 March 2011 and 15 March 2012. Clutches were attached to underwater roots and brush ~10 cm below the water surface. On the dates of collection, the water temperature was 4°C and 10°C, respectively. Clutches were transferred to the laboratory, where they were rinsed in dechlorinated tapwater and staged using an axolotl embryo staging series (Ambystoma Genetic Stock Center, University of Kentucky, USA; viewed at www.ambystoma.org/education/embryo-staging-series; see Table 1). To test the effects of algal population density on embryonic growth and development, 10 egg masses were separated into three portions each and placed into 1 liter of dechlorinated tapwater assigned to one of three treatments: (1) control, (2) photosynthesis inhibitor [80 µl of 100 mmol l^{-1} 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), created by dissolving 200 mg DCMU in 8.6 ml 95% ethanol] or (3) dark. All control and dark containers also received 80 µl of 95% ethanol to account for the ethanol solvent used in the DCMU solution. To approximate natural conditions, all eggs were maintained at ~1.5×10^{15} quanta s^{-1} cm^{-2} at 8°C on a 12 h:12 h light:dark cycle for 30 days. Dark treatment eggs were housed in a light-tight box in the same incubator with other treatments. Water was changed every 5 days. In addition, five uncut egg masses were maintained in pond water in the incubator under the same light and temperature conditions. Eggs from these masses were used to determine (1) algal density in the inner and outer egg membranes as well as in embryonic tissue and (2) carbon translocation (described below).

Algal and embryo measurements

Eggs were collected from treatment and control mass portions on days 10, 20 and 30. Each egg and embryo was only used for one measurement and then discarded. Algal population abundance was determined by rinsing individual eggs with phosphate-buffered saline (PBS), rupturing the eggs and removing the embryos, which were rinsed with PBS and placed in 10% formalin in PBS in individual microcentrifuge tubes. The egg fluid and membranes were transferred to a separate microcentrifuge tube, and 1150 µl deionized water and 50 µl 70% ethanol (to emulsify large globules) were added. The egg fluid mixture was vortexed, then centrifuged at 2000 ***g*** for 10 min. After centrifugation, the supernatant was removed and the algal pellet resuspended in 1200 µl deionized water. Samples were vortexed, loaded into a Phycotech settling chamber, and algal cells were counted with a Zeiss Axiovert inverted microscope at 1000×. Cell population density was determined by averaging cell counts of 50 grids. Embryos were staged and measured using a Wild M8 dissecting microscope at 12×. To compare the outer and inner egg membranes, the outer membrane only was ruptured first and collected and then the vitelline membrane was ruptured and collected. To examine algae contained inside embryo tissues, fixed embryos were rinsed with PBS and homogenized in 500 µl PBS using a glass homogenizing tube and Teflon pestle. The homogenate was centrifuged at 2000 ***g*** for 8 min, then the supernatant was discarded and the pellet resuspended in dechlorinated water, vortexed and loaded into a settling chamber. Algal cells were counted as described above.

DNA extraction and amplification

We extracted DNA from algae using a cetyltrimethylammonium bromide (CTAB)/hot detergent extraction protocol (Gast et al., 2004; Kuske et al., 1998). Algal DNA was PCR amplified using eukaryotic 18S rRNA gene primers EukA (5′-AACCT-GGTTGATCCTGCCAGT-3′) and EukB (5′-TGATCCTT-CTGCAGGTTCACCTAC-3′) (Medlin et al., 1988). Amplified DNA was directly sequenced (Genewiz, New Jersey, USA), and the resulting 1689 bp consensus sequence was deposited into GenBank (accession no. JQ779881).

Carbon translocation

Carbon translocation from *O. amblystomatis* to *A. maculatum* embryos was measured using methods modified from previous studies on sea anemones (Engebretson and Muller-Parker, 1999). Salamander eggs (stages 37–41) were placed in replicate scintillation vials (two eggs per vial) containing 5 ml dechlorinated water with 3 µl of sodium [^{14}C]bicarbonate for a final specific activity of 111 kBq per sample (55.5 kBq per egg). Eggs were incubated in the light (1.5×10^{15} quanta s^{-1} cm^{-2}) at 8°C for 1.75 h. Control samples were incubated in darkness for the same duration. Following incubation, eggs were transferred to non-labeled dechlorinated water and placed in the dark for a dark chase period (2 h). Embryos were removed from their egg capsules, rinsed twice in dechlorinated water and homogenized in 3 ml clean dechlorinated water. Subsamples of homogenized embryos (0.5 ml) were transferred to clean vials. The remaining embryo homogenate was combined with its respective egg capsule material and mixed. Subsamples (0.5 ml) of the total egg homogenate (embryo, algae and capsule) were taken. All samples were acidified with 6 mol l^{-1} HCl in a fume hood overnight to remove unincorporated ^{14}C, then neutralized with 6 mol l^{-1} NaOH before adding scintillation fluid. Radioactivity was measured using a scintillation counter (Beckman LS-3801); average counts per minute were converted to disintegrations per minute (d.p.m.) using a quench correction curve. Average d.p.m. of dark controls for embryo alone and total egg were subtracted from the light incubations of embryo alone and total egg, respectively, to determine carbon fixation. After correction with dark controls, the percent translocation was calculated from the ratios of radioactivity of embryo only to that of the total egg for each sample. Actual rates of carbon fixation and translocation were calculated using the equation of Engebretson and Muller-Parker (Engebretson and Muller-Parker, 1999): carbon fixation (or translocation) per hour = dissolved inorganic carbon (DIC) in sample × d.p.m. total egg (or embryo fraction) / d.p.m. of ^{14}C added / hours incubation. DIC was estimated according to Wetzel and Likens (Wetzel and Likens, 2000).

To test for ^{14}C assimilation by endosymbiotic algae, we incubated newly hatched embryos in sodium [^{14}C]bicarbonate under the conditions described above, with one salamander larvae per vial. Seven salamanders were incubated in the light and six in the dark. After incubation and dark chase period, we homogenized salamanders and collected 0.5 ml samples for analysis. We counted algal cells in the remaining homogenate using a settling chamber at 400× as previously described.

Statistical analysis

To examine the effects of algal population density and treatment on embryonic growth and development, random effects regression

models ($y_{it}=\alpha+x_{it}+\nu_i+\varepsilon_{it}$; Stata 12, Stata Corp., College Station, TX, USA) were estimated with robust standard errors to allow for potential heteroskedasticity. These models were compared to corresponding fixed-effects models using Hausman specification tests, and no evidence of unobserved heterogeneity was detected. Algal densities were transformed by natural logarithms [ln(x+1)] prior to analysis. Due to the death of all embryos in the DCMU treatment, two sets of analyses were performed: one set consisted of all treatments (control, DCMU and darkness) at day 10 only, and the second set compared control and darkness treatments only from days 0–30. For the first analysis, models were fitted independently to determine the effects of treatment and clutch on algal population density and to determine the effects of treatment, clutch and algal population density on embryonic growth and development. In the second set of analyses, we examined algal population density using time and treatment as main effects as well as the time × treatment interaction, and clutch membership. Models predicting length and development over 30 days in control and darkness were estimated using time, treatment (and the time × treatment interaction), clutch membership, and log-transformed algal density as variables.

Algal densities in the inner and outer egg envelopes and carbon translocation data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Location and morphology of *O. amblystomatis*

Algal DNA sequences from *A. maculatum* eggs were 99.8% similar (1685/1689 base pairs) to GenBank HM590634, confirming affinity to *O. amblystomatis* (Kerney et al., 2011). During the tailbud period of embryonic development (Table 1), *O. amblystomatis* was abundant throughout the outer and middle egg envelopes. As embryonic development progressed, *O. amblystomatis* was significantly more abundant in the inner envelope and vitelline membrane surrounding the embryo (Fig. 1A). We discovered *O. amblystomatis* cysts in eggs of prehatched embryos (Fig. 1B). In addition, *O. amblystomatis* transferred to DYIV culture media (Sanders et al., 2001) became cysts that have remained in the cyst form in culture for over one year.

Oophila amblystomatis was observed within intact salamander embryos using dissecting and compound fluorescence microscopy; algal masses were located near the developing heart. Internal examination of pond-water embryos in developmental stages ranging from early tailbud (stage 25) to hatchling (stage 44) revealed that 25 of 28 (89%) embryos contained endosymbiotic *O. amblystomatis*. The population density of internal *O. amblystomatis* cells ranged from 17,560 to 526,822 cells ml^{-1} of homogenate; however, there was no correlation between developmental stage and algal cell abundance (data not shown). The three embryos that did not contain endosymbiotic algae were in different developmental stages (41, 28, 35) and originated from different egg masses.

O. amblystomatis population density and embryonic growth and development

Darkness and photosynthesis inhibitor (DCMU) significantly reduced *O. amblystomatis* abundance in *A. maculatum* eggs. After 10 days, eggs reared in darkness and DCMU contained less than one-third and one-tenth, respectively, of the amount of algal cells in control eggs reared in light without DCMU. This result was primarily due to treatment conditions (R^2=0.54, P<0.001; Fig. 2A), although egg masses accounted for ~16% of the variance. Algal density was significantly lower in the DCMU (121,650±13,293 cells ml^{-1}, mean ± s.e.m.; P<0.001) and darkness (341,715±46,709 cells ml^{-1}; P=0.023) at day 10 compared to eggs reared in the light (1,261,344±304,413 cells ml^{-1}) (Fig. 2A; supplementary material Table S1).

In addition to differences in algal population densities, there were significant differences in both the length and developmental stage of embryos reared in DCMU and darkness compared with control eggs. Throughout the experiment, embryo length was strongly correlated with developmental stage (r=0.89, P<0.001) in control eggs. Fifty-eight percent of the variance in salamander length was due to treatment condition (P<0.001), and 27% of the variance was associated with egg mass. There was no significant difference between length of embryos reared in control *versus* DCMU conditions at 10 days of development; however, embryos reared in darkness were an average 2.08 mm shorter (P<0.001; Fig. 2B; supplementary material Table S1). Apart from treatment condition, algal density alone did not add significantly to predicted length (supplementary material Table S1). Similar to our model predicting embryo length, a parallel model predicting embryo developmental stage assigned 68% of the variance to differential treatment conditions (P<0.001). Embryos reared in darkness were an average of 7.6 developmental stages behind those reared under control (light) conditions (Fig. 2C; supplementary material Table S1). Clutch membership accounted for 25% of the variability in embryonic development. Unlike our length model, however, there was a significant effect of algal population density on embryo development, above and beyond treatment condition, such that higher algal populations in the eggs corresponded to more developed embryos (P=0.008; supplementary material Table S1).

At day 20, no live embryos remained in the DCMU treatment. For this reason, the statistical analysis that evaluates day 0 to day 30 includes control and darkness treatments only. From day 10–30, algal population density was affected primarily by treatment condition (R^2=0.69, P<0.001) and, to a lesser extent, by egg mass (accounted for 10% variance). Over the 30 days, darkness was a significant predictor of embryo length (R^2=0.89, P<0.001) and development (R^2=0.90, P<0.001); embryos reared in the dark averaged 1.94 mm shorter and were an average 8.02 developmental stages behind those in control conditions (P<0.001) (supplementary material Table S1). Above and beyond treatment condition (darkness), algal population density had a significant positive effect on embryo length (P=0.019) and development (P=0.001) from day 10 to day 30. Egg mass still contributed 13% of the variance in both the models predicting length and development. There were also significant effects of time on both length and development, as well as time × treatment interactions in both models (supplementary material Table S1).

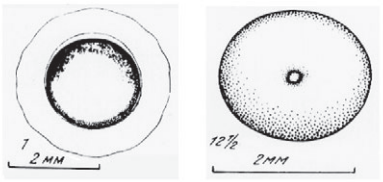
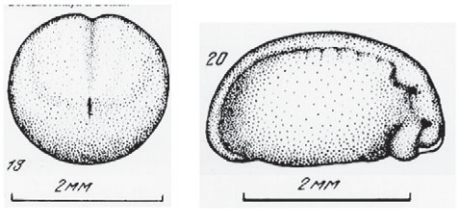
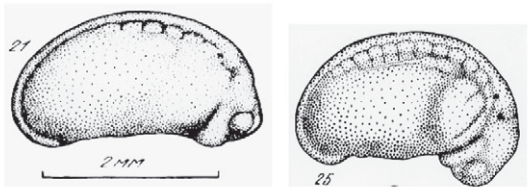
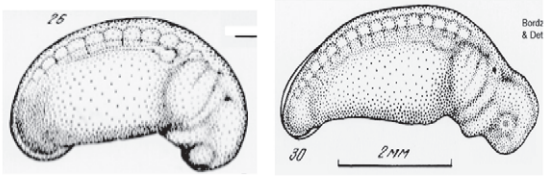
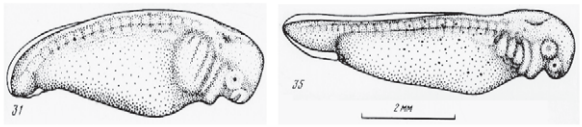
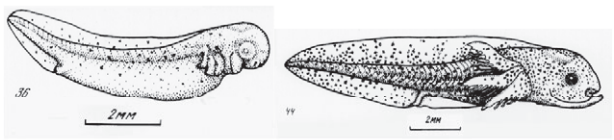
Bacterial colonization of DCMU eggs

On day 20, all of the embryos in each of the 10 masses in the DCMU treatment were completely disintegrated, appearing as dark streaks inside the egg capsules. Microscopical analysis of these egg capsules (N=12) revealed large numbers of bacteria throughout all of the capsules. *O. amblystomatis* population density was extremely low (mean 41,770 cells ml^{-1}), and algal cells remaining in the eggs were degraded and/or lysed. All samples in DCMU were discarded before the 30-day analysis due to bacterial colonization and deterioration of the egg masses.

Carbon translocation

Salamander eggs incubated in the light (N=10) had higher radioactivity than those maintained in the dark (N=5; Mann–Whitney, P<0.001). The corresponding embryos extracted

Table 1. Major time points during *A. maculatum* development

Period of development	Images of the beginning and end of each period	Stages	Time* (h)
Fertilization through gastrulation		1–12.5	49–51
Neurula		13–20	70.5
Early tailbud		21–25	83
Middle tailbud		26–30	102
Late tailbud		31–35	122
Pre-hatched through hatched		36–44	342

Embryonic and larval illustrations, as well as stage and time data, are from Bordzilovskaya et al. (Bordzilovskaya et al., 1989), reproduced with permission from Oxford University Press. *Time indicates hours elapsed post-fertilization for the latest stage shown.

from eggs incubated in the light (N=10) also had significantly higher activity than embryos from eggs maintained in the dark (N=5; Mann–Whitney, P=0.018). In other words, all embryos in the light received carbon from the surrounding egg capsule or from endosymbionts. Translocation percentages (embryo d.p.m./total egg d.p.m.) ranged from 0.5% to 13.2% (N=10), with an average of 6.4±1.3% (mean ± s.e.m.) (see supplementary material Table S2). The estimated average hourly rate of carbon fixation was 1.5±0.3 ng carbon per sample, or 0.8 ng carbon per egg. An estimated average 0.04 ng fixed carbon was translocated to each egg per hour.

Endosymbiotic algae were observed in all salamander larvae examined (N=13). The average population density of algae inside embryonic tissue was 14,314±2500 cells ml^{-1} (~715 cells per larvae) and ranged from 2954 cells ml^{-1} to 25,021 cells ml^{-1}. Despite the presence of endosymbiotic *O. amblystomatis* in all of the salamander larvae, there was no significant difference in radioactivity between salamander larvae incubated in the dark *versus* light (Mann–Whitney, P=0.554).

DISCUSSION

O. amblystomatis morphologies and locations

Algae in the present study were genetically identified as *O. amblystomatis*, the same species reported from *A. maculatum* egg masses collected in Nova Scotia, Canada (Kerney et al., 2011). This supports the previous suggestions that the mutualism is exclusive between *A. maculatum* and *O. amblystomatis*. Similar to others (Kerney et al., 2011; Gilbert, 1942), we observed several heterotrophic protist species, including a tetrahymenid ciliate and a heterolobosean amoeba, within *A. maculatum* eggs. However, their presence or absence did not appear to affect salamander embryos or *O. amblystomatis*.

Three morphologies of *O. amblystomatis* have been described previously: spherical and ovoid cells 6–30 µm in diameter, and an elongated biflagellate form 15–25 µm long (Gilbert, 1942). We observed these forms in addition to a cyst form that was present in eggs in the final stages of development. The cysts observed in this study are morphologically distinct from encased *O. amblystomatis*

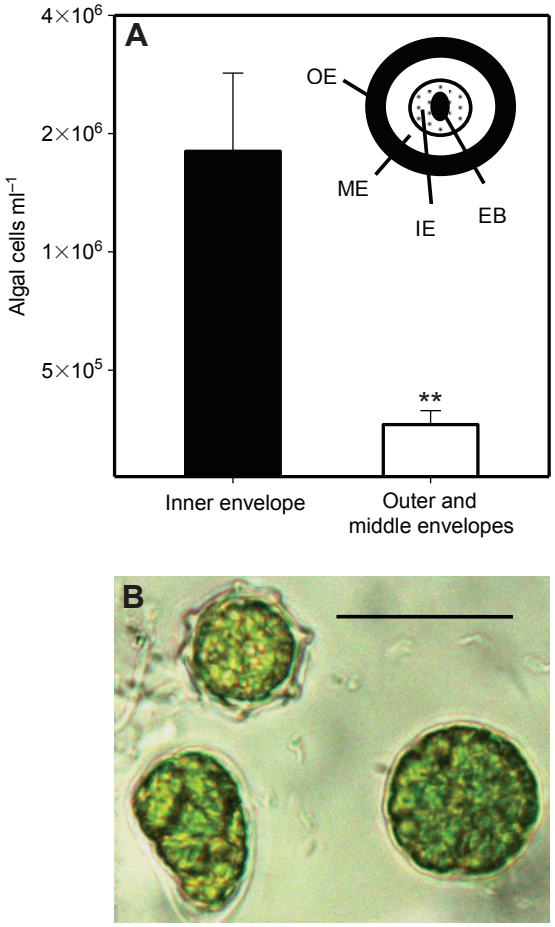


Fig. 1. Characteristics of *O. amblystomatis* in *A. maculatum* eggs of embryos in hatchling stages (36–44). (A) Algal cells were significantly more abundant in the inner envelope and vitelline membrane (Mann–Whitney, $^{**}P$=0.01), although some remain in the middle and outer egg envelopes. Values are means + s.e.m. (N=17). Inset shows eggs layers: OE, outer envelope; ME, middle envelope; IE, inner envelope; EB, embryo surrounded by vitelline membrane. (B) Algal cells observed in the inner envelope were predominantly spherical (lower right), but included a few cyst-like (upper left) and ovoid cells (lower left). Scale bar, 10 μm.

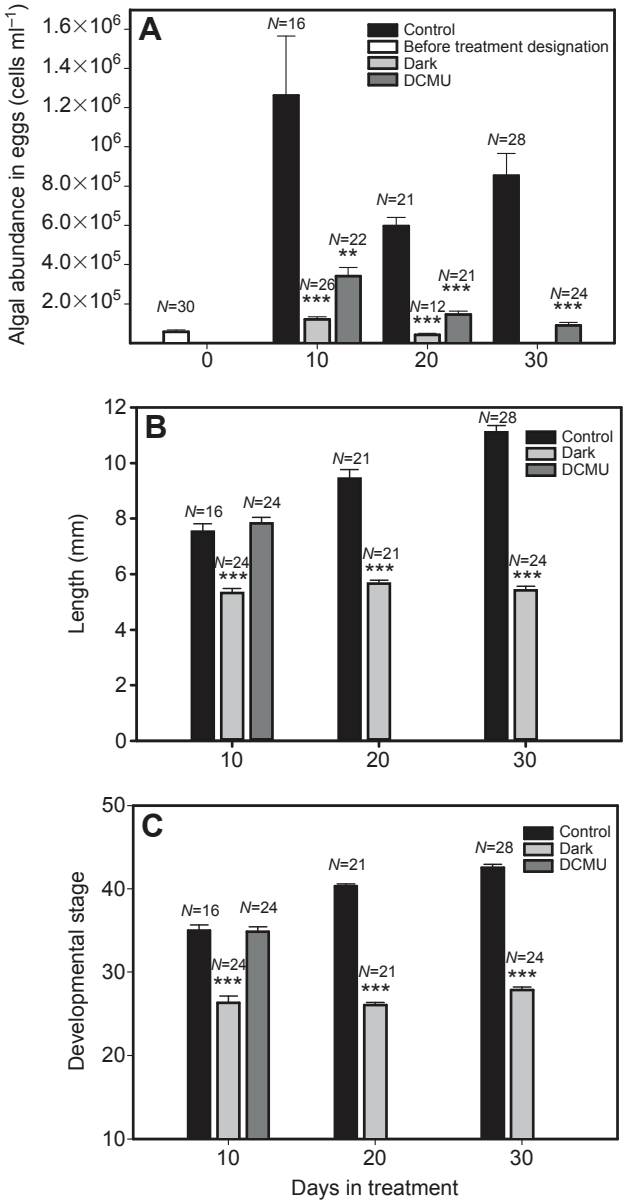


Fig. 2. The effects of darkness and algaecide on (A) *O. amblystomatis* abundance in *A. maculatum* eggs, (B) growth (length) and (C) development of *A. maculatum* embryos within eggs inhabited by *O. amblystomatis*. Time zero represents eggs from three egg masses prior to separation into treatments. Bars represent means + s.e.m. Statistical significance was evaluated by ANOVA ($^{***}P$=0.001, $^{**}P$=0.01).

previously observed on the inner egg membranes of early-stage embryos and intracellular algae with envelopes (Kerney et al., 2011). It is possible that the cysts observed in our study may represent the cyst morphology of free-living *O. amblystomatis* that presumably inhabits soils until vernal pools form in the spring and new egg masses are deposited. The *O. amblystomatis* cysts identified in the present study (Fig. 1B) resemble those of other flagellated green algae including *Chlamydomonas* and *Basichlamys* (Nozaki, 2003).

During neurula and early tailbud (stages 13–25), eggs in all treatments contained primarily tear-drop (ovoid) shaped, motile algae, which were replaced by spherical non-motile algae as development progressed. Some eggs maintained in the dark contained elongated algae. Algal population densities were significantly higher in the inner envelope and vitelline membrane compared with the outer egg membranes. This supports the hypothesis that *O. amblystomatis* enters the egg, travels through the outer and middle egg envelopes and attaches to the inner envelope as embryonic development progresses (Gilbert, 1942).

Similar to Kerney et al. (Kerney et al., 2011), we observed endosymbiotic algae in embryos throughout their development. We initially observed clumps of algal cells near the blastopore of embryos in late neurula and early tailbud (stages 17–25). In stage 25–44 embryos, algal population density was approximately 6–20-fold lower in embryonic tissue compared with the egg capsule; however, 89% of the embryos examined contained algae, suggesting that intraembryonic invasion is common. This invasion does not appear to affect growth, development or hatching. We observed no correlation between abundance of intraembryonic algal cells and developmental stage of the embryo. Endosymbiotic algae were observed in 13 hatched salamander larvae examined in this study.

Kerney et al. also observed intracellular algae in liver and cartilage of salamander larvae, although intracellular algae were much less abundant in larvae compared with stage 35–44 embryos (Kerney et al., 2011).

Alga–salamander interactions

Several studies have concluded that *O. amblystomatis* promotes faster embryonic growth and development and more synchronous hatching of eggs (Tattersall and Spiegelaar, 2008; Gilbert, 1944; Gilbert, 1942). In addition, Marco and Blaustein reported that algal population density was positively correlated with reduced mortality and larger, more-developed embryos in *Ambystoma gracile* (Marco and Blaustein, 2000). Our study also found a significant positive relationship between algal population density and embryonic growth and development. Although treatment condition was the primary predictor of embryo length and development throughout the experiment, increased *O. amblystomatis* abundance alone also had a significant positive effect on both length and development over 30 days. These positive effects are likely due to increased oxygen from photosynthesis (Valls and Mills, 2007; Pinder and Friet, 1994) in addition to receiving fixed carbon (discussed below).

Eggs maintained in the dark had lower algal populations, and embryos were smaller and less developed. Several studies have reported that *A. maculatum* embryos reared in darkness averaged 1–4 developmental stages behind those reared in the light (Goff and Stein, 1978; Gilbert, 1944; Gilbert, 1942) and were an average 1.3 mm shorter in length (Gilbert, 1944). Embryos in the present study averaged 7 stages behind and 1.94 mm shorter than eggs reared in light; this increased effect may be due to the lower incubation temperatures that we used, which are typical of local early spring. Similar to Gilbert (Gilbert, 1944), the present study shows that delays in growth and development in embryos maintained in the dark are not simply due to the absence of light but are effects caused by *O. amblystomatis* being maintained in darkness. In the absence of photosynthesis, *O. amblystomatis* respiration contributes to anoxia in egg capsules (Pinder and Friet, 1994); therefore, eggs with *O. amblystomatis* maintained in the dark not only lack photosynthetic oxygen but also become more susceptible to anoxia when *O. amblystomatis* respires. Similar results were reported by Gilbert (Gilbert, 1944), who created algae-free egg masses by breeding adult salamanders in the lab and preventing their exposure to *O. amblystomatis*. In his study, embryos from algae-free egg masses maintained in both light and darkness had similar growth and development to control (*O. amblystomatis*-inhabited) eggs in the light; however, embryos from *O. amblystomatis*-inhabited eggs in the dark were significantly smaller and less developed than both control eggs in the light and algae-free eggs (Gilbert, 1944), indicating that *O. amblystomatis* benefits salamander embryos only as long as photosynthesis is possible.

During our experiments, embryos maintained in DCMU suffered mass mortality within 20 days. It is unlikely that DCMU directly killed the salamander embryos. The effects of DCMU have been studied in several species of amphibian tadpoles and eggs, and the lowest reported LC_{50} (dose resulting in 50% mortality) was 5.4 mg l^{-1} in *Xenopus laevis* tadpoles maintained in DCMU for 14 days; this is nearly threefold more than our dose of 1.9 mg l^{-1} (Schuytema and Nebeker, 1998). Schuytema and Nebeker also found that the lowest observed adverse effect level (LOAEL) values were above 14.5 mg l^{-1} for all amphibians maintained at the dose concentration of DCMU for 10–21 days (embryos of *X. laevis* and *Pseudocriss regilla* and tadpoles of *X. laevis*, *P. regilla*, *Rana catesbeiana* and *Rana aurora*) (Schuytema and Nebeker, 1998). In the present study, *A. maculatum* embryos in the DCMU treatment appeared to have died following bacterial colonization in the eggs. Brodman suggested that *O. amblystomatis* protects *A. maculatum* eggs from pathogenic microorganisms (Brodman, 1995), and our study strongly supports the hypothesis that *O. amblystomatis* inhibit bacterial colonization. Alternatively, tissue from dead embryos could enhance bacterial growth. However, embryos placed in DCMU continued to grow and develop at the same rate as control embryos until there were no observable healthy *O. amblystomatis* cells remaining. At this time (approaching day 10), bacteria began colonizing the egg capsules, and by day 20, all of the embryos were dead and bacteria were abundant throughout the egg capsules. Bacterial colonization following the death of *O. amblystomatis* could have caused embryonic mortality by depleting oxygen or by excreting toxins. In either case, the absence of healthy algal symbionts appears to have facilitated the colonization. This hypothesis is further supported by the fact that bacteria did not appear in eggs maintained in the dark, despite low numbers of *O. amblystomatis* cells, because the remaining algal cells were not lysed and appeared healthy apart from some being reduced in size. We propose that the presence of a small population of healthy *O. amblystomatis* inhibited bacterial colonization in the dark treatment, compared to the DCMU treatment, where all *O. amblystomatis* cells were deteriorated. We did not test for the mechanism by which *O. amblystomatis* inhibits bacteria; however, some eukaryotic algae, including the closely related genus *Chlamydomonas*, can exude antibiotics (Proctor, 1957). Additional studies are needed to confirm whether *O. amblystomatis* also releases antibiotics in egg capsules.

Translocation of carbon

Fixed carbon was translocated from *O. amblystomatis* to salamander embryos in all of the samples radiolabeled with ^{14}C. Our translocation values likely underestimate the amount of fixed carbon translocated from *O. amblystomatis* to *A. maculatum* embryos in nature because we were not able to create higher light intensities representative of those in the field *in vitro* without affecting the incubator temperature. However, light intensity was sufficient enough to encourage algal growth when eggs were placed in the incubator (Fig. 2A). *O. amblystomatis–A. maculatum* translocation (maximum 13%, mean 6.4%) was similar to percentages reported for the freshwater sponge *Ephydatia fluviatilis*, which receives 9–17% of photosynthetically fixed carbon from its facultative algal symbiont (Wilkinson, 1980), and the freshwater hydra, *Chlorohydra viridissima*, which acquires 10–20% of its symbiont's photosynthate (Muscatine and Lenhoff, 1965). Our results were, however, lower than amounts reported for other photosynthetic symbionts using ^{14}C methods (see Table 2). For example, dinoflagellates (zooxanthellae) in temperate sea anemones translocate as much as 50% of their fixed carbon (Muller-Parker and Davy, 2001), and tropical zooxanthellae give 50–97% of their photosynthate to invertebrate hosts (Fitt and Cook, 2001; Steen and Muscatine, 1984; Muscatine et al., 1984). However, these latter symbioses are often obligate and intracellular, suggesting a long evolutionary relationship, and may involve a 'host factor' that stimulates release of algal photosynthate (Gates et al., 1995). The amount of carbon translocated from *O. amblystomatis* to salamander embryos is expected to vary because translocation can be affected by several factors, including temperature and irradiance. Moreover, length of the dark-chase period can alter translocation estimates (Engebretson and Muller-Parker, 1999), and using ^{14}C to quantify translocation can alone lead to underestimation (reviewed by Muller-Parker and Davy, 2001). Hatched salamander larvae did not incorporate ^{14}C on their own, nor did their

Table 2. Percentages of fixed carbon translocated from photosynthetic symbionts to hosts based on ^{14}C measurements

Species	Photosynthetic symbiont	Translocation %	Reference
Sea anemones	Zooxanthellae *Symbiodinium*		
Aulactinia stelloides		21–29%	Smith, 1986
Aiptasia pulchella		12–22%	Muller-Parker and Davy, 2001
Anemonia viridis		50%	
Coral	Zooxanthellae *Symbiodinium*		
Plesiastrea versipra		10%	Hinde, 1987
Stylophora pistillata		>95%	Muscatine et al., 1984
Heteroxenia fuscescens (octocoral)		17%	Schlichter et al., 1983
Zoanthids	Zooxanthellae *Symbiodinium*		
Zoanthus robustus		12–42%	Hinde, 1987
Palythoa variabilis		89%	Steen and Muscatine, 1984
Zoanthus sociatus		95%	
Molluscs	Zooxanthellae		
Tridacna maxima		39–45%	Trench et al., 1981
Other marine invertebrates	Zooxanthellae *Symbiodinium*		
Pteraeolidia ianthina		25–50%	Hinde, 1987
Cassiopea andromeda		5–10%	Hofmann and Kremer, 1981
Myrionema amboinense		52%	Fitt and Cook, 2001
Freshwater invertebrates			
Ephydatia fluviatilis	Green algae	9–17%	Wilkinson, 1980
Chlorohydra viridissima	Green algae	10–20%	Muscatine and Lenhoff, 1965
Vertebrates			
Ambystoma maculatum	*Oophila*	0.5–13.2%	Present study

endosymbiotic algae. If any ^{14}C was assimilated by algae within the salamanders, the quantity was too small to identify using standard methodology.

Fixed carbon can be translocated to hosts as several compounds. The most common products translocated in alga–invertebrate symbioses include glycerol and glucose, although C4 acids, succinate/fumerate, maltose, lipids, and amino acids can also be transferred (Yellowlees et al., 2008; Whitehead and Douglas, 2003). Products released by algae can also change in response to environmental conditions; therefore, *O. amblystomatis* may translocate a number of photosynthetic compounds depending on the environmental conditions surrounding the egg mass. In addition to acquiring nutrition from algal symbionts, studies on algae in eggs of other organisms revealed that embryos actually consumed algae in the egg capsule. Embryos of the marine polychaete *Axiothella mucosa* have a fully developed digestive system within their first five days, which allows them to graze on diatoms in their eggs during the rest of development (Peyton et al., 2004). Although *O. amblystomatis* likely provide nutrition to hatched salamanders during their initial foraging, it is unlikely that algae are consumed by embryos. The digestive system of *A. maculatum* is not functional until stage 44–45 and does not complete development until after the salamander hatches (Harris, 1967).

Amphibian eggs deposited in aquatic environments usually have a lower amount of yolk than those deposited terrestrially, and the yolk is incorporated into the gut at an early stage. The additional nutrition provided by photosynthate would ensure that adequate nutrition was available to embryos until hatching. Yet, is carbon translocation necessary for the development and survival of *A. maculatum* embryos? Although rare, algal-free egg masses can be found in nature (Gilbert, 1942), indicating that embryos do survive without photosynthetically derived carbon. But the positive relationship between *O. amblystomatis* abundance and *A. maculatum* embryo survival and development argues for the overall importance of the relationship for *A. maculatum*. A recent study suggests that associations between egg masses and photoautotrophs may be more prevalent than previously thought, and more heterotrophic organisms may rely on products from photoautotrophs during development. Woods and Podolsky examined aquatic egg masses spanning a variety of invertebrate species and found that eggs were often either deposited on substrates covered by macrophytes or the masses themselves contained microalgae (Woods and Podolsky, 2007). No egg masses in that study were associated with both macrophytes and microalgae; however, in either case, oxygen in the egg masses increased from association with a phototroph. The apparent facultative nature of the *O. amblystomatis–A. maculatum* mutualism may be influenced by the presence of macroalgae or macrophytes in the water surrounding the egg masses. It is possible that masses lacking *O. amblystomatis* (e.g. Gilbert, 1942) were deposited on macrophyte-covered surfaces and, despite the absence of an intracapsular alga, embryos would still benefit from the associated photoautotroph. Overall, however, the importance of *O. amblystomatis* to development in *A. maculatum* is emphasized by the rarity of algal-free egg masses in nature and the high mortality rate of those eggs (Gilbert, 1944; Gilbert, 1942).

Conclusions

The present study shows a direct positive relationship between *O. amblystomatis* abundance and the growth and development of salamander embryos and suggests that *O. amblystomatis* inhibits bacteria from invading *A. maculatum* eggs, or at least inhibits bacterial growth. In addition, we have identified a cyst form of *O. amblystomatis* that is the likely form in which *O. amblystomatis* survives outside of eggs in vernal pools. *O. amblystomatis* benefits *A. maculatum* embryos by increasing oxygen content and probably by removing nitrogenous waste in the egg capsule. Furthermore, as shown in the present study, *O. amblystomatis* provides supplemental nutrition from photosynthetic products. Although additional experiments are required to identify what compounds are exchanged between *O. amblystomatis* and *A. maculatum*, it is clear that salamander embryos do incorporate fixed carbon from algal photosynthesis. To our knowledge, this is the first study to identify carbon translocation in an alga–vertebrate symbiosis. Other salamander and frog species have similar alga–egg symbioses, and

it is likely that translocation occurs in those associations as well. Indeed, there may be alga–egg relationships that have not yet been identified, and phenomena similar to those known for *O. amblystomatis* and *A. maculatum* may be more common than suspected from current reports.

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