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Accepted: 20 July 2000.

*Journal of Herpetology*, Vol. 34, No. 4, pp. 617–621, 2000


## Symbiosis with Green Algae Affects Survival and Growth of Northwestern Salamander Embryos

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Ova of many amphibian species are surrounded by transparent gelatinous envelopes that protect the eggs in aquatic environments (Duellman and Trueb, 1994; Stebbins and Cohen, 1995). These gelatinous envelopes can help protect the eggs against mechanical damage, desiccation, some kinds of predation and penetration by pathogens, and other damaging agents (Ward and Sexton, 1981; Semlitsch, 1988). Moreover, eggs in globular masses are better insulated and less likely to freeze than single masses (Zweifel, 1968). If hydroperiod recess at oviposition sites and clutches become exposed to air, jelly matrix may also protect embryos from desiccation (Marco and Blaustein, 1998).

The transparent envelopes of some amphibian species such as some ambystomatid salamanders and ranid frogs often have significant growth of green algae (*Chlamydomonas* sp.) in the gelatinous matrix and inside the perivitelline membrane. Flagellate algae penetrate the gelatinous matrix after eggs are laid and usually are more abundant in the inner envelopes. When flagellate algal cells penetrate in the perivitelline fluid, they attach to the inner surface of the vitelline membrane and transform to nonflagellate morphs. Nonflagellate algae are spherical and have a characteristic size range (Goff and Stein, 1978).

Early studies concerning the algal-amphibian egg relationship suggested a symbiosis (Orr, 1884; Gilbert, 1942). In this relationship, algae obtain carbon dioxide and nitrogenous waste from the embryo, and the embryo gets oxygen and lower concentration of ammonia in the vitellin fluid (Hutchinson and Hammen, 1958; Hammen and Hutchinson, 1962; Gatz, 1973; Goff and Stein, 1978; Bachmann et al., 1986; Pinder and Friet, 1994). Apparently, this relationship favors the survival and development of *Ambystoma maculatum* embryos (Gilbert, 1944). When Gilbert (1944) experimentally reared embryos in the presence of green algae, embryos showed lower mortality and higher growth rates compared with embryos that were reared in the dark, in the absence of algae. However, in nature, ambystomatid embryos are exposed to solar radiation and permanent darkness is not a natural condition of development. In addition, Anderson (1971) found a positive correlation between algal growth and embryo mortality in *Ambystoma tigrinum*, suggesting algae are detrimental.

We tested the effect of green algae on embryonic development in *Ambystoma gracile* clutches from three different populations in the laboratory. Aquatic-breeding female *A. gracile* attach clutches to branches in relatively shallow water, usually away from contact with other clutches (Stebbins, 1954; Nussbaum et al, 1983). Embryos of *A. gracile* have a relatively long developmental time (30 to 60 d) and can tolerate relatively low water temperatures (5–6 C) compared with other ambystomatids (Anderson, 1972; Brown, 1976; Marco and Blaustein, 1998). Slow embryonic development of egg masses makes *A. gracile* clutches susceptible to significant algal growth and perhaps to substantial effects of algae on embryonic development.

We conducted four experiments in the laboratory to determine whether density of algae inside the perivitelline membrane influences embryonic development and embryo survival on *A. gracile*. We collected *A. gracile* eggs masses from three different locations and at different stages of embryonic development (Harrison, 1969) in areas where they were abundant. For experiments 1 and 2, we used clutches from Trout Lake, Klickitat County, Washington, USA (altitude: 600 m)(Site 1). Eggs were collected on 12 April. Eggs for experiment 1 were collected from 18 clutches at Harrison stages 1–9. This experiment was conducted in April–June 1996. Eggs from ten clutches were collected at Harrison stages 10–13 for experiment 2 which was conducted in April–May 1996. For experiment 3, we used eggs from 16 clutches from one pond in Lincoln County, Oregon, USA (altitude: 200 m)(Site 2). This experiment was conducted in February–April 1997 with eggs that were collected on 17 February at Harrison stages 10–13. For experiment 4, we used eggs from 24 clutches from one pond in Linn County, Oregon, USA (altitude: 900 m)(Site 3). This experiment was conducted in April–June 1997 with eggs that were collected on 22 April at Harrison stages 14–18.

In the laboratory and for each experiment, we cut one portion of each collected clutch using a scalpel. The rest of the clutch was not used in the experiment. Embryos with damaged perivitelline membranes were removed. The number of live embryos in every egg mass was counted at the beginning of the experiment. All portions had similar spherical shape and the number of eggs per portion varied from 15 to 38. Each clutch piece was placed into separate 4 L containers in the laboratory and eggs were allowed to hatch. Containers were filled with 3 L of dechlorinated water and the gelatinous matrix was completely submerged. Water was replaced weekly. The experiments were conducted at 15 C under a 14h:10h light:dark

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TABLE 1. Results of the one-way MANOVAs that analyze the overall difference in embryonic development among egg masses within the four experiments and one-way ANOVAs that analyze differences among egg masses for each variable (day at hatching, hatchling length and Harrison stage at hatching). We indicate the $F$ values and the significance level. NS: $P > 0.05$; *: $P < 0.05$; **: $P < 0.001$; ***: $P < 0.001$.

| | One-way MANOVAs | | One-way ANOVAs | | | |
|---|---|---|---|---|---|---|
| | d.f. | Overall | d.f. | Day | Length | Harrison stage |
| Experiment 1 | 51, 1007 | 5.179*** | 17, 340 | 8.001*** | 3.588*** | 2.008* |
| Experiment 2 | 27, 882 | 14.989*** | 9, 304 | 12.030*** | 28.185*** | 7.009*** |
| Experiment 3 | 45, 710 | 4.523*** | 15, 241 | 4.702*** | 6.084*** | 1.664 NS |
| Experiment 4 | 69, 1378 | 5.629*** | 23, 463 | 7.559*** | 3.697*** | 2.169** |

photoperiod. All clutches were maintained under similar conditions of artificial light, indirect sunlight, and temperature.

At the end of the experiment, the numbers of dead embryos and hatchlings were recorded in each container. Survival rate was calculated for each container as the ratio of the number of hatchlings to the initial number of living embryos. For all larvae, we recorded the day of hatching and total length (TL) and Harrison stage at hatching (Harrison, 1969). Total length was measured with a caliper to the nearest 0.1 mm. At the completion of the study all larvae were released into the ponds from which they had been collected. Given that algae cells grew during incubation, the density of green algae cells of each egg was estimated immediately after hatching. We considered that the algal density at the end of the incubation is a good indicator of the algal density during the embryonic development. We counted the number of algal cells attached to the perivitelline membranes inside of rectangles of 0.15 $mm^2$ (1.5 × 0.1 mm). For each egg mass, we measured the algal density in 10 different eggs, and in five different areas of the perivitelline membrane of each egg. Both eggs and areas of the membrane were selected randomly. The selected perivitelline membranes were extracted from the jelly mass and the algal cells were counted under a dissecting microscope. The diameter, shape, and color intensity of the algal cells were homogeneous among eggs and egg masses, but their densities varied among egg masses. We classified the clutch portions in accordance with the level of algal density in three categories: low density when algal density was lower than 25 cells/$mm^2$; medium for algal densities varying from 25 to 50 cells/$mm^2$; and high when algal density was higher than 50 cells/$mm^2$.

We used one-way Multivariate Analysis of Variance (MANOVA) to analyze the overall difference in embryonic development (day, length, and Harrison stage at hatching) among egg masses within an experiment. Then, we used two-way Multivariate Analysis of Variance (MANOVA) to analyze the overall difference in embryonic development (mortality rate and average values of day, length, and Harrison stage at hatching) among experiments and among levels of algal density. Then we applied two way ANOVA and Scheffé multiple contrast test to analyze differences for each variable. To comply with the assumption of normality, values of the mortality rates were Arcsin-transformed. We used the Bartlett Chi-square test to compare the homogeneity of variances of algal density among experiments and the Kruskal-Wallis test (heteroscedasticity for that variable) to compare the median values of algal density among experiments. To analyze the effect of algal density on embryonic development within each experiment, we used Pearson product moment correlation, considering average density of algal cells as the independent variable, and average day at hatching, total length, and Harrison stage at hatching as dependent variables. To analyze the effect of algal density on embryo survival we used a Spearman correlation. We used average values of every egg mass for density of algal cells, day at hatching, and hatching size and stage in the two-way MANOVA and the correlation analysis.

One-way MANOVA showed an overall significant difference among egg masses within the four experiments (Table 1). One-way ANOVAs indicated that there were significant differences among egg masses in size and day at hatching within the four experiments and in stage at hatching within the experiments 1, 2, and 4 (Table 1). Number of eggs per egg mass was not correlated with algal density, survival rate, hatchling size, day at hatching, or Harrison stage at hatching in any of the four experiments with the exception of a positive correlation between number of eggs per egg mass and Harrison stage in experiment 1 (Pearson product-moment correlation: r = 0.854, $P < 0.001$).

There were significant differences among experiments in variance (Bartlett Chi-square = 53.25, $P < 0.001$) and median values of algal density (Kruskal-Wallis test: Chi-square = 32.26, $P < 0.001$). Two-way MANOVA showed an overall significant difference on embryonic survival and development among experiments ($F_{12,140} = 12.70$, $P < 0.001$) and among levels of algal density ($F_{8,106} = 6.19$, $P < 0.001$). However, there was no interaction between experiments and algal density (Table 2). There were significant differences among experiments in embryo mortality, hatchling size, and Harrison stage and day at hatching (Table 2). There were significant differences among levels of algal density in size and stage at hatching (Table 2).

In eggs collected in site 1 at stages 1–9, algal density was lower and embryo mortality was higher than in the rest of the experiments (Fig. 1, Table 3). There was a negative correlation between algal density and embryo mortality (Spearman correlation: $r_s = -0.59$, $P = 0.032$). There was no correlation between algal density and day at hatching (Pearson correlation: r = 0.238, $P = 0.341$), stage at hatching (Pearson correlation: r =

TABLE 2. Results of the two-way ANOVAs that analyze the overall difference in embryonic development among experiments and levels of algal density. Two-way ANOVA and Scheffé multiple contrast tests were applied to analyze differences for each variable. We indicate the F values and the significance level. NS: $P > 0.05$; ** : $P < 0.001$; *** : $P < 0.001$.

| | d.f. | Survival rate | Day at hatching | Hatchling length | Harrison stage |
|---|---|---|---|---|---|
| Effect | | | | | |
| Algal density | 2, 56 | 1.619 NS | 1.793 NS | 5.302** | 12.986*** |
| Experiment | 3, 56 | 1.069 NS | 13.420*** | 41.282*** | 4.698** |
| Interaction | 6, 56 | 1.054 NS | 1.529 NS | 1.752 NS | 2.148 NS |

0.279, $P = 0.261$) or size at hatching (Pearson correlation: r = 0.070, $P = 0.781$).

In eggs collected in sites 1 and 2 at stages 10–13, algal density in eggs was variable when comparing different egg masses (Fig. 1). Embryo mortality was relatively low (Table 3) and was not correlated with algal density (Exp. 2: Spearman correlation: $r_s = -0.22$, $P = 0.501$; Exp. 3: Spearman correlation: $r_s = 0.18$, $P = 0.495$). Algal density was positively correlated with hatchling size (Exp. 2: Pearson correlation: r = 0.885, $P < 0.001$; Exp. 3: Pearson correlation: r = 0.667, $P < 0.01$), and hatchling Harrison stage (Exp. 2: Pearson correlation: r = 0.888, $P < 0.001$; Exp. 3: Pearson correlation: r = 0.504, $P < 0.05$). Day at hatching was not correlated with algae density in any of both experiments (Pearson correlation in experiment 2: r = 0.319, $P = 0.369$; Pearson correlation in experiment 3: r = 0.087, $P = 0.750$).

In eggs collected at stages 14–18 in site 3, algal density, hatchling size and hatchling Harrison stage were less variable among egg masses than in experiments 2 and 3 (Fig. 1). Embryo mortality was very low; embryos developed slower and hatched at larger sizes comparing with the other experiments (Table 3). Embryo mortality was not correlated with algal density (Spearman correlation: $r_s = 0.22$, $P = 0.533$). Algal density was not correlated with hatchling size (Pearson correlation: r = 0.19, $P = 0.357$), with hatchling Harrison stage (Pearson correlation: r = 0.04, $P = 0.866$) or with day at hatching (Pearson correlation: r = 0.06, $P = 0.782$).

We demonstrated that under light conditions, the alga-egg relationship benefits the embryonic development of *Ambystoma gracile*. Eggs with fewer algal cells had higher embryonic mortality or hatched at earlier Harrison stages and smaller sizes than eggs in clutches that were extensively colonized by algae. Environmental factors that affect algal colonization and growth in the field may also affect *A. gracile* embryo success.

Embryonic survival and growth varied among experiments. In clutches collected during the first hours after egg laying and with relatively low rates of algal colonization, there was a negative correlation between algal density inside the perivitelline membrane and embryo mortality. In experiments with high variability in algal density among egg masses, there was a positive correlation between algal density, hatchling size, and developmental stage. In egg masses with low variability and relatively high levels of algal density, we did not find any effect of algal density on embryonic development. Factors affecting the presence of algae in breeding habitats could affect the reproductive success of *A. gracile*. Intraspecific variability in embry-

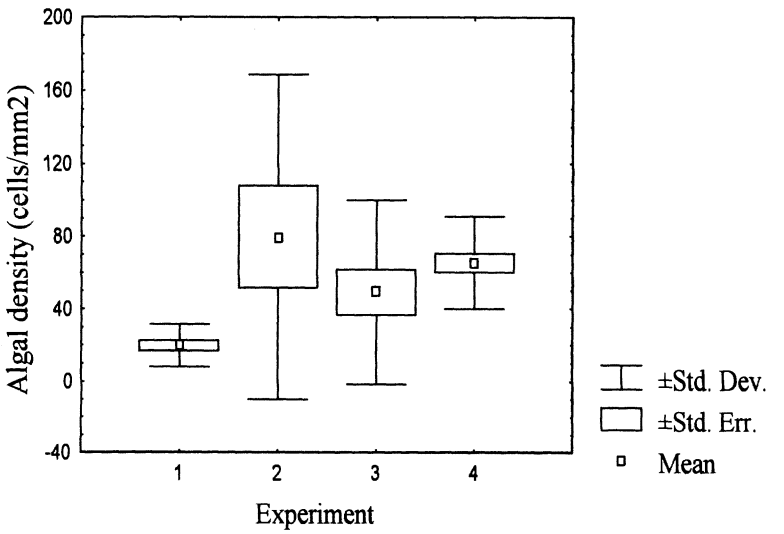


FIG. 1. Average values and variability (SD and SE) of algal density (cells/mm²) in the perivitelline membrane of *A. gracile* eggs from the four experiments.

TABLE 3. Average (range) of density of algal cells (cells/mm²) in perivitelline membranes, and embryo survival rate (%), hatchling size (mm) and hatchling stage at hatching in egg masses of *A. gracile* in four experiments. NM indicates the number of egg masses and NE the number of eggs of each experiment. Different letters near average values indicate significant statistical differences in post-hoc analyses (Scheffé multiple contrast test) of the significant ANOVAs that compare each variable among experiments.

| | NM | NE | Algal density | Mortality | Hatchling length | Harrison stage | Day at hatching |
|---|---|---|---|---|---|---|---|
| Exp. 1 | 18 | 417 | 19.7 (6–55) | 12.6$^{c}$ (0–46) | 13.1$^{b}$ (12.4–13.9) | 41.1$^{a}$ (40.2–42.1) | 12.71$^{b}$ (8.1–16.3) |
| Exp. 2 | 10 | 335 | 79.5 (11–292) | 5.8$^{b}$ (2.6–15) | 12.4$^{a}$ (11.5–13.9) | 41.5$^{a}$ (40.8–42.8) | 8.79$^{a}$ (3.2–15.4) |
| Exp. 3 | 16 | 275 | 49.3 (8–190) | 6.1$^{b}$ (0–18) | 12.2$^{a}$ (11.4–13.6) | 41.3$^{a}$ (40.7–42.2) | 9.71$^{a}$ (4.5–15.4) |
| Exp. 4 | 24 | 487 | 65.4 (17–130) | 0.9$^{a}$ (0–7) | 14.7$^{c}$ (13.8–15.2) | 42.0$^{b}$ (41.7–42.5) | 15.56$^{c}$ (12.7–21) |

onic development may be partially due to the different origin of the embryos. Anderson (1972) found intraspecific variation in embryonic development of some species of the genus *Ambystoma* studying eggs from different populations, in the laboratory and under standard conditions. We also found differences among experiments in the algae-embryo relationship. However, certain density of algae had similar effects in each experiment. We suggest that variability in algal colonization may explain part of the variability among experiments. Immediately after egg deposition, egg masses are usually small and eggs are in very close proximity to one another. During the first days after egg deposition, the gelatinous matrix swells as water is absorbed (Duellman and Trueb, 1994). Algae probably penetrate into the eggs during this process (Gatz, 1973). When we collected egg masses in the field at Harrison stages greater than 13 (experiment 4), and introduced them in algae-free environments, algal growth was great in all masses and similar to that of clutches raised in their natural habitat. However, when we collected egg masses at earlier Harrison stages (experiments 1–3), algal growth in water without algae was variable compared among different masses. Some egg masses were densely colonized by algae, whereas others were not. Egg masses collected during the first 24 hours after egg deposition (Harrison stages lower than 10; experiment 1) showed the lowest level of algal growth and, thus, algal colonization in the field had not been optimal when they were collected.

Increasing depth is correlated with lower temperatures and lower light intensity. Low temperatures and light intensities at oviposition sites could affect embryo survival directly by increasing development time (Brown, 1976; Brodman, 1995) and also indirectly by decreasing the presence and growth of symbiotic algae. On the other hand, eggs laying near the water surface could favor algal growth because of increased light and temperature. At shallow water, eggs may also found oxygen rich environments. However, clutches would be more prone to desiccation (air, sun, wind) if eggs become exposed as the water level recedes. Moreover, exposure to air could induce freezing, mechanical damage, and higher exposure to ultraviolet radiation (Ward and Sexton, 1981; Semlitsch, 1988; Blaustein et al., 1994, 1995; Marco and Blaustein, 1998). There may be a trade-off in how salamanders lay eggs with regard to depth. Females may avoid the extremes of this trade-off by attaching clutches to vegetation in relatively shallow water at 10 to 50 cm (Stebbins, 1954; Nussbaum et al., 1983; pers. obs.).

Algal growth in salamander clutches may help protect embryos from UV-B radiation that can harm salamander embryos. Ambient UV-B radiation causes mortality and abnormalities in *A. gracile* eggs (Blaustein et al., 1995, 1997). Gelatinous matrix and egg envelopes are transparent in clutches of many ambystomatids and in previous experiments (Blaustein et al., 1995, 1997) did not protect embryos from the negative effect of UV-B radiation. However, algae create a green filter around embryos that may impede UV-B radiation. Phytoplankton and dissolved organic carbon attenuate the penetration of UV-B radiation in water (Morris et al., 1995; Scully et al., 1997). Thus, embryos surrounded by green algae may be exposed to lower and perhaps sublethal levels of UV-B radiation. Moreover, the UV-B blocking effect of algae could permit salamander clutches to grow in environments with higher levels of UV-B radiation, such as shallow water. In this case, both algae and eggs could develop quickly, increasing the reciprocal benefits of a possible symbiotic interaction. However, algae may also be affected by UV-B radiation. The protection afforded to salamander eggs by algae may diminish if algal cells cannot take hold under increased UV-B radiation.

*Acknowledgments.*—We thank Carmen Díaz-Paniagua, Doug P. Chivers, Joseph M. Kiesecker, Erica L. Wildy, Jill Devito, Miguel Lizana, Valentín Pérez-Mellado, Bill Masen, Christine Durrant, Karen Goodwin and Tom Goodwin for their help. This study was partially supported by the Spanish Ministry of Education and Science (Grant E95-16796691), Oregon State University, Department of Zoology Research Founds, and National Science Foundation of USA (Grant DEB 9423333).

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Accepted: 22 July 2000.

*Journal of Herpetology,* Vol. 34, No. 4, pp. 621–624, 2000


## Absence of Polarity Perception by Rattlesnakes of Envenomated Prey Trails

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Snakes use chemicals in reproductive and in predatory contexts (Gillingham et al., 1990). Sex pheromones permit snakes to make sex and species recognition, as well as stimulate courtship behaviors (Noble, 1937; Devine, 1977; Ford, 1986). Further, some pheromones are presented in such a way as to permit detection of the polarity of the chemical trail, its directionality from early to later deposition, and thereby determine the direction of deposition of the chemical trail (Ford and Low, 1984).

Rattlesnakes strike and usually release rodent prey (Gans, 1966; Radcliffe et al., 1980; Kardong, 1986; Chiszar et al., 1992). The envenomated prey, which dashes off until the onset of immobilization and death, must then be relocated. During this poststrike relocation, the rattlesnake possesses extraordinary chemosensory acuity (Halpern, 1992), being able to discriminate the scent trail of the envenomated mouse from even that of littermates (Robinson and Kardong, 1991; Lavín-Murcio et al., 1993). However, it is not known if the rattlesnake can detect the directionality of a scent trail deposited by an envenomated prey. Therefore, our goal was to see if rattlesnakes could, in addition to recognizing the scent trail of an enven-

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