EGG GELATINOUS MATRIX PROTECTS *AMBYSTOMA GRACILE* EMBRYOS FROM PROLONGED EXPOSURE TO AIR

ADOLFO MARCO[1] AND ANDREW R. BLAUSTEIN[2]

[1] Departamento de Biología Animal, Universidad de Salamanca, Salamanca 37071, Spain

[2] Department of Zoology, Oregon State University, Corvallis, OR 97331, USA

Desiccation during water recession at oviposition sites can be a major cause of embryo death in clutches of aquatic amphibians. However, aquatic eggs of the north-western salamander, *Ambystoma gracile,* can survive out of water for at least 30 days in the field. Females lay eggs surrounded by a firm jelly matrix which presumably protects embryos from desiccation. In a field study, embryos from clutches located either in or out of the water showed no differences in size or stage at hatching. In laboratory experiments, where genetic effects were controlled, air exposure had no effect on embryo survival rate, size or stage at hatching. The ability of *A. gracile* to survive prolonged droughts or water recession during embryonic development may be shared by other amphibians.

INTRODUCTION

Desiccation of aquatic habitats by drought, drainage, or abstraction for irrigation, could contribute to population declines of some amphibian species (Stebbins & Cohen, 1995). Specifically, decreasing hydroperiod at oviposition sites can be a major cause of death to developing embryos. Given that temperature accelerates embryonic and larval development (Brown, 1976; O'Hara, 1981), many species select warm oviposition sites, such as shallow water (Sype, 1975). However, clutches near the surface may have a higher risk of death during pond or stream desiccation.

The gelatinous egg envelopes of many amphibians can help protect the eggs against mechanical damage, predation, pathogens, and other damaging agents (Semlitsch, 1988; Waldman & Ryan, 1983; Ward & Sexton, 1981). Moreover, eggs in globular masses are better insulated and less likely to freeze than individual eggs or clumps (Zweifel, 1968). Arboreal egg masses of some tree frogs are encased in leaves which protect the eggs from desiccation. These clutches have egg-less capsules that provide water for the embryonated eggs (Pyburn, 1980). Some terrestrial and arboreal foam nests have dry outer surfaces that protect eggs from dehydration (Coe, 1974). In many terrestrial salamanders, females provide protection against dehydration of eggs (review in Jaeger & Forester, 1993). However, aquatic amphibian clutches are usually susceptible to dehydration when they become exposed to air. Forester & Lykens (1988) showed that communal egg deposition in *Rana sylvatica* can partially protect embryos from desiccation in aquatic clutches that became completely exposed to air. Nyman (1987) also found that aggregative egg deposition and the gelatinous matrix in *Ambystoma maculatum* can reduce desiccation in stranded egg masses. However, we have found no previous reports of significant survival of single amphibian clutches subjected to prolonged exposure to air. Our field observations suggest that egg masses of the Northwestern salamander (*Ambystoma gracile*) may be able to survive prolonged air exposure because its eggs are surrounded by a thick and firm extracellular jelly matrix. Eggs are deposited in globular masses 80 to 150 mm in diameter and ova are 1.5 to 2.5 mm in diameter. Clutch size varies from 40 to 270 eggs (Nussbaum *et al.,* 1983) and volume of jelly per egg is approximately 12.5 cm³.

Ambystoma gracile is an aquatic-breeder, and females attach clutches to branches in relatively shallow water, usually away from contact with other clutches (Nussbaum *et al.,* 1983; Stebbins, 1954). Breeding occurs from January to March in lowland areas, when the water temperature is relatively low. Embryos of *A. gracile* have a relatively long developmental time (30 to 60 days) and can tolerate relatively low water temperatures (Brown, 1976). Slow embryonic development may make *A. gracile* clutches susceptible to air exposure if hydroperiod decreases. To test the effect of air exposure on embryo survival rate, we conducted two laboratory experiments. These experiments were used in combination with field observations of clutches that became naturally exposed to air.

MATERIAL AND METHODS

FIELD OBSERVATIONS

We made field observations and conducted experiments from 20 February to 26 April 1996 at a pond near Fall Creek (Lincoln County, Coast Range, Oregon, elevation 200 m). Water depth receded more than 1 m from 20 February to 30 March. Between 16 and 19 March, 20 *A. gracile* clutches became completely exposed to air when embryos were at Harrison developmental stages 30-35 (Harrison, 1969). On 19 March, 10 of the 20 exposed clutches were selected at random and immersed in the pond water. We left the other 10 clutches out of water, either naturally stranded on land or suspended from branches. Water level in the pond never rose again to the level of the clutches located out of the water.

On 8 April (after at least 20 days of air exposure), seven clutches (1-7) located out of the water and seven clutches (8-14) located in the water were collected and

transported to the laboratory. We covered clutches with dechlorinated water in plastic containers (40 x 20 x 12 cm) until hatching, which finished on 16 April. On 19 April (after at least 31 days of exposure), the last three clutches that were out of the water and that remained in the field were transported to the laboratory. In the last three clutches that remained immersed in the pond, living embryos had already hatched when clutches were collected. Clutches that were out of the water for 31 days were also covered with dechlorinated water in plastic containers (40 x 20 x 12 cm) until hatching, which finished on 25 April. In the laboratory, clutches were maintained at a constant temperature of approximately 15°C. We recorded the number of dead embryos and hatchlings in each container. Because there were empty egg capsules in the outer portions of the jelly matrices when collected, we could not estimate embryo survival rates for whole clutches. Instead, we calculated the survival rate of embryos based on the number that occurred in the egg masses when they were collected and then compared among treatments. For all larvae, we recorded the day of hatching, total length (TL) and Harrison stage at hatching. To record total length and Harrison stage, we located hatchlings in small Petri dishes and observed them using a dissecting microscope. Total length was recorded with a calliper to the nearest of 0.1 mm.

LABORATORY EXPERIMENTS

We also conducted two experiments in the laboratory, to determine whether differences in embryonic development and survival existed between clutches located either in or out of water. We simulated the two situations that we observed in the field on clutches that became naturally exposed to air after water recession. We used 12 clutches of *A. gracile* from a pond that was adjacent to the original pond. Embryos were at Harrison stages 32-36 at the start of the experiments. Immediately before we began the experiments, clutches were cut in half and embryos with damaged perivitelline membranes were removed. Each half-clutch was placed in a plastic container (28 x 18 x 12 cm), and assigned to one of two treatments: air or water. In that way, we eliminated in the statistical analysis the variability due to differences among clutches. Stranded clutch halves in experiment 1 (clutches L 1-6) were placed on a solid plastic base 2 cm high. Suspended clutch halves in experiment 2 (clutches L 7-12) were placed on a metallic grille. The bottom of the grille was 5 cm above the bottom of the container and larvae could pass through the grille. In both experiments, containers with clutches assigned to the "water" treatment were filled to a depth of 10 cm with dechlorinated water and clutches were completely submerged. Containers with clutches assigned to the "air" treatment, were filled to a depth of 1 cm with dechlorinated water to permit hatchling survival after they dropped from the clutch to the bottom of the container. In the "air" treatment, clutches had no contact with water and were completely exposed to air. Water was replaced weekly and was not artificially aerated. Containers had no lids. Experiments were conducted at a constant laboratory temperature (15°C) and clutch temperature was recorded in the gelatinous surface of every egg mass.

We counted the number of living embryos in every egg mass at the beginning of the experiment. At the end of the experiment, we recorded the numbers of dead embryos and hatchlings 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 day of hatching, total length (TL), and Harrison stage at hatching. To record total length and Harrison stage, we located hatchlings in small Petri dishes and measured them under a dissecting microscope. Total length was recorded with a calliper to the nearest of 0.1 mm. After completion of the study, we released all larvae in the ponds from which they were collected.

DATA ANALYSIS

To compare day at hatching, hatchling stage, and size among treatments, we used average values for every clutch in field observations and half-clutch in the laboratory experiments. To analyse the overall effect of air exposure on embryos in the field (three treatments: 20 days in the water, 20 days out of water and 31 days out of water) we used One-way Multivariate Analysis of Variance (MANOVA). Dependent variables were: ratio of number of hatchlings to total number of embryos after collection, mean day at hatching, mean total length and mean Harrison stage at hatching. We also used One-way Univariate Analysis of Variance (ANOVA) to analyse the differences among treatments in the four variables. In laboratory experiments we used One-way MANOVA for the overall comparison (survival rate, mean day at hatching, mean total length and mean Harrison stage) and paired t-tests for univariate comparisons. To comply with normality assumptions, we arcsine-transformed values of the ratios of number of hatchlings to total number of embryos from the field observations and the survival rates from experiments 1 and 2.

RESULTS

CLUTCHES IN THE FIELD

There was no overall effect of air exposure on embryonic development (Table 1). On average (± 1SE), 83.1±0.6% of the hatchlings whose clutches were in water for 20 days hatched during the first day after collection, compared to 90.9±0.5% of hatchlings that were out of water for 20 days and 96.6±0.3% of hatchlings that were out of water for 31 days. There were no significant differences in the average day at hatching between these three groups of clutches (ANOVA, F=1.04, P=0.376), and consequently, laboratory conditions did not influence embryonic development of clutches exposed to the different treatments.

TABLE 1. Results of 3 MANOVAs that analyse the overall effect of exposure to air during embryonic development on *A. gracile* hatchlings (survival and day, size and Harrison stage at hatching). In the laboratory, clutches were stranded in experiment 1 and suspended in experiment 2.

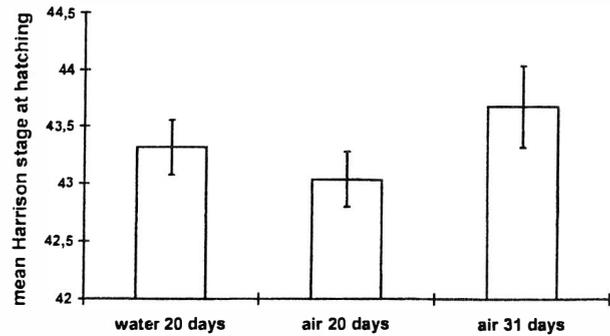|	Effect	df	F	P
Field obs.	Residual	4,11	37966.41	<0.001
	Treatment	8,22	1.00	0.461
Expt. 1	Residual	4,7	25702.13	<0.001
	Treatment	4,7	2.670	0.122
Expt. 2	Residual	4,7	65072.28	<0.001
	Treatment	4,7	2.51	0.136

FIG. 2. Mean ± 1 SE Harrison stage at hatching of *A. gracile* larvae from clutches located in water or in air, in the field. ANOVA: $F=2.564$, $P=0.112$, df=2, 14.
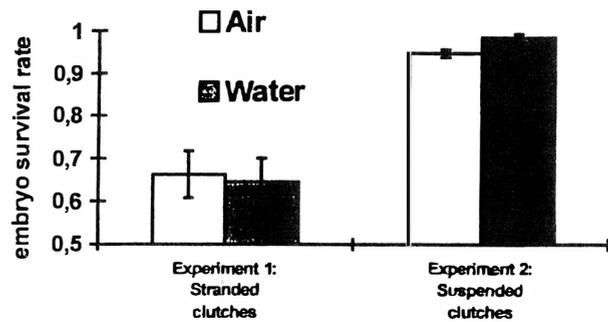

FIG. 1. Mean ± 1 SE total length of newly hatched *A. gracile* larvae from clutches in (water) and out (air) of water for at least 20 or 31 days in the field. ANOVA: $F=0.181$, $P=0.836$, df=2, 14.

FIG. 3. Mean ± 1 SE survival rate of *A. gracile* clutch halves located in (water) and out (air) of water from Harrison stages 32-36 in the laboratory. Left: stranded clutches (paired t-test: $t=0.145$, $P=0.891$, df=5). Right: suspended clutches (paired t-test: $t=2.52$, $P=0.053$, df=5). NS=$P>0.05$.

The average number of hatchlings, average number of dead embryos and mean ratio of the number of hatchlings to the total number of embryos after collection did not differ among the three groups of clutches (Table 2). There was no difference in average body size (Fig. 1) or in average stage at hatching (Fig. 2) among clutches located in and out of the water for 20 and 31 days.

LABORATORY EXPERIMENT 1: STRANDED CLUTCHES

The experiment began on 23 March and hatching occurred from 25 March until 13 April. In clutches exposed to air, larvae hatched on average after 7 days (range = 2-21 days) of exposure. There was no difference in clutch temperature between treatments (Student *t*-test: $t=0.703$, $P=0.498$). MANOVA showed no over-all significant difference among treatments (Table 1). Embryo survivorship (Fig.3), and mean day (paired *t*-test: $t=2.477$, $P=0.056$) and mean Harrison stage at hatching (paired *t*-test: $t=2.156$, $P=0.084$) were not affected by air exposure. Mean hatchling size was slightly smaller for exposed egg masses than for submerged egg masses (paired *t*-test: $t=2.647$, $P=0.046$).

LABORATORY EXPERIMENT 2: SUSPENDED CLUTCHES

This experiment began on 26 March and hatching occurred from 2 April until 17 April. Larvae hatched on average after 11.3 days (range = 5-22 days) of exposure to air. There was no difference in clutch temperature between treatments (Student *t*-test: $t=1.519$, $P=0.166$). MANOVA showed no overall significant difference among treatments (Table 1). Embryo survivorship (Fig.

TABLE 2. Average (± 1 SE) number of hatchlings and dead embryos, and average (± 1 SE) ratio of number of hatchlings to total number of embryos that remained in clutches of *A. gracile* located in or out of water in the field for at least 20 and 31 days. NS=$P>0.05$. 20d-IN = clutches located in water for 20 days; 20d-OUT = clutches located out of water for 20 days; 31d-OUT = clutches located out of water for 31 days.

	20d-IN	20d-OUT	31d-OUT	$F_{2,14}$
No. clutches	7	7	3	
No.hatchlings	45.6±6.62	46.7±6.75	28.3±10.11	1.67 NS
No.dead	17.1±4.69	8.6±4.39	2.7± 7.16	1.29 NS
Ratio	0.77±0.05	0.85±0.06	0.88± 0.07	0.38 NS

3), and mean day (paired t-test: $t=0.375$, $P=0.723$), mean size (paired t-test: $t=0.619$, $P=0.563$) and mean Harrison stage at hatching (paired t-test: $t=0.034$, $P=0.974$) were not affected by air exposure.

DISCUSSION

Several sources of amphibian embryo mortality have been reported, including freezing, low pH, UV-radiation, pollutants, pathogens and predation (Berrill et al., 1994; Blaustein et al., 1994, 1995; Harte & Hoffman, 1989; Kiesecker & Blaustein, 1995). Moreover, it seems obvious that desiccation at oviposition sites can be a major cause of embryo death in aquatic breeding amphibians. Air exposure in the field could expose clutches to increased risk of dehydration (air, sun, wind), freezing, mechanical damage and higher exposure to UV-B radiation. Forester & Lykens (1988) pointed out that communal egg-laying behaviour could protect some wood frog, Rana sylvatica, embryos from desiccation as ponds dry up. In laboratory experiments, they observed an exponential egg mortality rate for terrestrial exposure, but 11% of eggs were alive after 10 days and 4% survived for as long as 14 days. This relatively long survivorship was attributed to the protection of the viscous coating over the surface of the egg mass (Forester & Lykens, 1988). Nyman (1987) also observed that aggregative egg deposition in A. maculatum provided protection from desiccation to aquatic clutches exposed to the air. A. maculatum egg masses that were individually deposited masses or were in smaller aggregates did not survive an air exposure period of 35 days. However, A. maculatum eggs in the inner protected masses of the largest aggregates (40-80 masses) survived during that period (Nyman, 1987).

Our field observations of A. gracile indicated that embryos of individually deposited masses can survive inside the jelly matrix out of the water for at least 30 days. We observed no significant effects on size or stage at hatching due to terrestrial exposure of clutches in the field. In the laboratory, air exposure had no significant effect on embryo survivorship, day of hatching or developmental stage of single clutch halves. We only found a slight effect on stranded clutches that were exposed to air. In these clutches, hatchlings were smaller than those from clutches located in the water. A significant decrease of jelly-mass volume could have led to slight differences between treatments. The decrease of mass volume could have occurred as a result of the halving of clutches in the laboratory experiments. However, our field observations indicate that clutch volume reduction was not substantial in clutches exposed to air. Perhaps the outer jelly envelope provides most of the protection from desiccation. More than one A. gracile female may deposit a clutch on the same branch, but in general, clutches are not deposited communally. However, the relatively thick jelly matrix may allow A. gracile embryos to avoid water loss and survive drought or temporal water recession.

Clutches are usually attached to rigid branches or sticks and suspended in the water column (Nussbaum et al., 1983). When the water level decreases, clutches can remain suspended above the water or can become stranded on the soil. If larvae from suspended clutches hatch in the air, they could easily fall into the water and survive. Hatchlings from stranded clutches may not have access to water unless rainfall raises the water level before the embryos die.

It will be of interest to discover which other species have eggs resistant to desiccation. This capacity of clutches to survive desiccation is probably related, among other factors, to the structure and composition of the clutch jelly matrix. Many amphibains protect their eggs with gelatinous matrices, but the hardness and consistency of these matrices is very variable (Duellman & Trueb, 1994). Thus, many amphibian species having eggs surrounded by a jelly matrix may not survive prolonged exposure to air. For example, Rana aurora eggs laid in the same study pond at the same time, had 100% mortality during the first three days of exposure to air.

REFERENCES

Berrill, M., Bertram, S., McGillivray, L., Kolohon, M. & Pauli, B. (1994). Effects of low concentrations of forest-use pesticides on frog embryos and tadpoles. Environ. Toxicol. Chem. 13, 657-664.

Blaustein, A. R., Hoffman, P. D., Hokit, D. G., Kiesecker, J. M., Walls, S. C. & Hays, J. B. (1994). UV repair and resistance to solar UV-B in amphibian eggs: a link to population declines? Proc. Nat. Acad. Sci. 91, 1791-1795.

Blaustein, A. R., Edmond, B., Kiesecker, J. M., Beatty, J. J. & Hokit, D. G. (1995). Ambient ultraviolet radiation causes mortality in salamander eggs. Ecological Applications 5, 740-743.

Brown, H. A. (1976). The time-temperature relation of embryonic development in the northwestern salamander, Ambystoma gracile. Can J. Zool. 54, 552-558.

Coe, M. J. (1974). Observations on the ecology and breeding biology of the genus Chiromantis (Amphibia: Rhacophoridae). J. Zool., London 172, 13-34.

Duellman, W. E. & Trueb, L. (1994). *Biology of Amphibians*. Baltimore and London, The John Hopkins University Press.

Forester, D. C., & Lykens, D. V. (1988). The ability of wood frog eggs to withstand prolonged terrestrial stranding: an empirical study. *Can. J. Zool.* **66**, 1733-1735.

Harrison, R. G. (1969). Harrison stages and description of the normal development of the spotted salamander, *Ambystoma punctatum* (Linn.). In *Organization and Development of the Embryo*, 44-66. R. G. Harrison (ed.). Yale University Press, New Haven, Connecticut.

Harte, J., & Hoffman, E. (1989). Possible effects of acidic deposition on a Rocky Mountain population of the tiger salamander, *Ambystoma tigrinum. Conserv. Biol.* **3**, 149-158.

Jaeger, R. G. & Forester, D. C. (1993). Social behavior of plethodontid salamanders. *Herpetologica* **49**, 163-175.

Kiesecker, J. M. & Blaustein, A. R. (1995). Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. *Proc. Nat. Acad. Sci.* **92**, 11049-11052.

Nussbaum, R. A., Brodie Jr., E. D., & Storm, R. M. (1983). *Amphibians and Reptiles of the Pacific Northwest*. The University Press of Idaho, Moscow, Idaho.

Nyman, S. (1987). *Ambystoma maculatum* (Spotted salamander). Reproduction. *Herp. Review* **18**, 14-15.

O'Hara, R. (1981). *Habitat Selection Behavior in Three Species of Anuran Larvae: Environmental Cues, Ontogeny, and Adaptive Significance*. PhD thesis, Oregon State University, Corvallis, Oregon.

Pyburn, W. F. (1980). The function of eggless capsules and leaf in nests of the frog *Phyllomedusa hypochondrialis* (Anura: Hylidae). *Proc. Biol. Soc. Washington* **93**, 153-167.

Semlitsch, R. D. (1988). Allotopic distribution of two salamanders: Effects of fish predation and competitive interactions. *Copeia* **1988**, 290-298.

Stebbins, R. C. (1954). *Amphibians and Reptiles of Western North America*. McGraw-Hill, New York, New York.

Stebbins, R. C. & Cohen, N. W. (1995). *A Natural History of Amphibians*. Princeton University Press, Princeton, New Jersey.

Sype, W. E. (1975). *Breeding Habits, Embryonic Thermal Requirements and Embryonic and Larval Development of the Cascades Frog, Rana cascadae Slater*. Ph.D. Thesis. Oregon State University, Corvallis, Oregon.

Waldman, B. & Ryan, M. J. (1983). Thermal advantages of communal egg mass deposition in wood frogs (*Rana sylvatica*). *J. Herpetol.* **17**, 70-72.

Ward, D. & Sexton, O. J. (1981). Anti-predator role of salamander egg membranes. *Copeia* **1981**, 724-726.

Zweifel, R. G. (1968). Reproductive biology of anurans of the arid southwest, with emphasis on adaptation of embryos to temperature. *Bull. Amer. Mus. Nat. Hist.* **140**, 1-64.