Egg-wrapping behaviour protects newt embryos from UV radiation

ADOLFO MARCO*, MIGUEL LIZANA†, ALBERTO ALVAREZ† & ANDREW R. BLAUSTEIN‡

*Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas

†Departamento de Biología Animal, Universidad de Salamanca

‡Department of Zoology, Oregon State University

Oviparous species that do not guard their eggs during development may suffer significant embryonic mortality. However, the way females lay eggs may help prevent this. For example, females of several newt species carefully wrap single eggs into leaves of aquatic vegetation in shallow water. Wrapping behaviour may protect eggs from predators and from mechanical damage. We hypothesized that egg-wrapping behaviour also protects newt embryos from the negative effects of ultraviolet (UV) radiation. In field experiments, we tested the effects of ambient levels of UV radiation on marbled newt, *Triturus marmoratus,* embryos exposed to sunlight. Of these embryos 95–100% died during the first 14 days of exposure. The eggs showed loss of their round shape, skin damage, oedemas and fungal infection. In contrast, during the same period, mortality of embryos protected by UV-blocking filters was only 20%. In laboratory experiments, we exposed marbled newt embryos to an artificially elevated intensity of UV-B radiation and tested the protective effect of leaves. The mortality of eggs wrapped in leaves and exposed to UV-B radiation was low and similar to that of unwrapped eggs that were shielded from UV-B radiation with a UV-blocking filter, whereas 119 of 120 unwrapped eggs exposed to UV radiation died within 5 days.

Oviparous species that do not guard their eggs during incubation may suffer high embryonic mortality (Trivers 1972; Stebbins & Cohen 1995). Specifically, eggs of the majority of amphibian species are exposed to many environmental risks. However, females that do not care for their eggs directly may still influence their survival (Arnold 1994; Bernardo 1996). For example, they may limit the eggs' exposure to harmful biotic and abiotic agents. During egg laying, females of some species of the genus *Triturus* carefully wrap their eggs in leaves of aquatic plants in shallow water (Díaz-Paniagua 1989; Duellman & Trueb 1994; Griffiths 1995; Miaud 1995) by manipulating a single egg and the leaf with their back legs. This process takes several minutes as the female holds its breath and remains motionless above the plant (Díaz-Paniagua 1989; Griffiths 1995). This behaviour may protect eggs from predation or mechanical damage, and

Correspondence: A. Marco, Estación Biológica de Doñana, CSIC, Apartado 1056, Sevilla 41080, Spain (email: amarco@cica.es). M. Lizana and A. Alvarez are at the Departamento de Biología Animal, Universidad de Salamanca, Campus Miguel de Unamuno, Salamanca 37071, Spain. A. R. Blaustein is at the Department of Zoology, Oregon State University, Cordley Hall 3029, Corvallis, Oregon 97331-2914, U.S.A.

may prevent the eggs from sinking into mud that could inhibit respiration (Winpenny 1951; Miaud 1993; Griffiths 1995). Miaud (1994) found that unwrapped eggs of three *Triturus* species were quickly eaten in the field while wrapped eggs suffered a much lower predation rate. We hypothesized that the egg-wrapping behaviour also protects newt eggs from the negative effects of UV radiation which kills embryos in several amphibian species (Blaustein et al. 1994a, 1997; Anzalone et al. 1998; Lizana & Pedraza 1998; Langhelle et al. 1999; Broomhall et al., in press).

Amphibian eggs are usually surrounded by transparent gelatinous envelopes that protect them in aquatic environments (Duellman & Trueb 1994; Stebbins & Cohen 1995), but UV radiation can pass through the envelopes. Blaustein et al. (1994a) predicted that the effects of UV radiation on eggs will vary according to their natural exposure to sunlight. Increasing depth, cloudy water or an opaque surface above the eggs can attenuate exposure to sunlight (Blaustein et al. 1998). However, *Triturus* females usually select clear and shallow water in which to lay their eggs and egg frequency decreases as a function of depth (Miaud 1995). We investigated how egg-wrapping behaviour in the marbled newt, *Triturus marmoratus,*

protects developing embryos in shallow water from UV radiation.

METHODS

We collected marbled newt embryos in April 1999 from an artificial pond in Salamanca, Spain (800 m elevation). In the field, all collected eggs were wrapped in leaves of *Mentha pulegium* at a depth of 5–20 cm. Eggs were collected within 48 h of laying, with the surrounding gelatinous matrix intact. The pond water where eggs were collected was clear throughout the breeding period. We collected 520 eggs from one pond and selected 432 of them for use in the two experiments.

Field Experiment

In the field we tested the sensitivity of exposed eggs to ambient UV radiation. Field experiments were carried out in April 1999 in an artificial pond at Salamanca, Spain. We selected 192 eggs and randomly distributed them into 12 fibre-glass mesh enclosures (40 × 40 cm and 12 cm high; 16 eggs per enclosure) that allowed the circulation of water and air. The portion of the leaf in contact with the upper part of the egg was carefully pinned back from all the eggs so that they could be exposed to solar radiation. In previous experiments we observed that the unwrapping procedure, when conducted carefully, had no effect on embryonic development (personal observations). The enclosures were lined with Styrofoam film 2 cm thick. Because eggs attached to the leaf floated, each piece of leaf containing a single egg was attached to the Styrofoam with two sewing pins. Pins never made physical contact with the egg and cast no shade over it.

We used filters to expose eggs to one of three levels of UV radiation. Four enclosures were covered with UV-blocking Llumar filter film (Llumar Window Film UVCL SR HPR, Courtaulds Performance Films, Martinsville, VA, U.S.A.) and four with a UV-transmitting polyester film. Both Llumar and polyester filters were placed 10 cm above the enclosure to permit air circulation. The remaining four enclosures were not covered and were fully exposed to UV radiation. The filter characteristics of the Llumar and polyester films were tested before and after the experiments with a UV-Visible Spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) showing that the Llumar filter completely blocked rays below 380 nm. The polyester filter permitted the passage of light over 280 nm, blocking only ca. 10–15% of the UV radiation. The properties of both filters remained unchanged after the experiment.

The 12 enclosures were placed in a concrete pond (ca. 4 × 8 m and 1.6 m deep) filled with rain water. The pond contained aquatic vegetation, invertebrates and tree frogs, *Hyla arborea*. The enclosures were partially submerged to a depth of 10 cm and kept afloat with polystyrene floats. Every 2 days, in each enclosure we recorded egg mortality, embryonic developmental stage and water temperature. The experiment was completed in 14 days.

Laboratory Experiment

In a laboratory experiment, we tested whether eggs covered by leaves were protected from UV radiation. We also recorded the presence and frequency of abnormalities in UV-exposed and nonexposed embryos. We selected 240 marbled newt eggs covered by a leaf of *M. pulegium* and with the gelatinous matrix intact. Before the experiment eggs naturally covered by leaves were maintained in 40-litre tanks with water from the pond where they were collected. We exposed eggs to four conditions: (1) eggs not covered by a leaf and protected with a UV-B blocking filter; (2) eggs covered by a leaf and exposed to UV-B radiation for 5 days; (3) eggs not covered by a leaf and exposed to UV-B radiation for 3 days; and (4) eggs not covered by a leaf and exposed to UV-B radiation for 5 days. There were three replicates for each treatment. Eggs were placed in plastic tanks (30 × 15 cm and 8 cm high) that were filled with 5 cm of dechlorinated tap water. The tank was lined with Styrofoam 2 cm thick. Because eggs attached to leaves floated, each piece of leaf containing a single egg was attached to the Styrofoam with two sewing pins. Pins never made physical contact with the egg and cast no shade over it. Eggs were placed 1 cm apart.

All tanks were exposed for 5 days to daily doses of UV-B radiation of 14 000 J/m^2 (1 h of daily exposure to an intensity of 3.8 W/m^2). UV-B radiation during April can reach maximum intensities near 2 W/m^2 and daily doses of 45 000 J/m^2 at Salamanca's latitude (data from ELDONET: European Light Dosimeter Network, European Union). The source of UV-B radiation was fluorescent tubes (Philips UVB-TL 20W/12) with a peak of spectral emission at 310 nm. The fluorescent tubes were installed 10 cm above the water surface and adequately aged to stabilize the emission. We measured UV radiation in the centre of the container and at the water surface with a broad-band UV radiometer. In treatment 1, tanks were always covered with a Llumar filter that totally blocked the transmission of UV-B radiation. In treatment 3, tanks were covered with a Llumar filter during the last 2 days of exposure to UV-B radiation. Treatments 2 and 4 were never covered by filters. In treatment 2, eggs were totally covered by a leaf as in nature, while in the rest of the treatments the portion of the leaf in contact with the upper part of the egg was carefully pinned back to permit exposure to UV-B radiation. The experiment was completed in 7 days after 2 final days with no experimental exposure to UV.

For each tank, we recorded the incidence of fungal infection and abnormalities and embryo mortality every 24 h, except for the tanks in treatment 2, which were checked only at the end of the experiment. During the experiment, we used a microscope to observe embryos inside their tanks. We considered several types of abnormalities following the criteria of the Atlas of abnormalities of FETAX (Bantle et al. 1991): necrosis, yolk leaking,

roundness contour, oedema, skin damage with loss of dermal tissue and axial malformation.

Data Analysis

To compare the mortality rate and the frequency of fungal infection and abnormalities among treatments, we used ANOVA, after checking for normality and homogeneity of variances. Values of mortality rate were arcsine square root transformed.

Ethical Note

The study was conducted with the permission of the Consejería de Medio Ambiente, Junta de Castilla y León, Spain. We tried to minimize the impact on the individuals and the population of origin. We also consider that the environmental impact of the study was low. We collected 520 eggs from one pond and selected 432 of them for use in the two experiments. The majority of the remaining eggs (excluding a few that died during the selection process or while being held) were returned to the same pond. Immediately (within 24 h) after the experiments, the surviving experimental eggs were also returned to their original pond.

Marbled newts are abundant in Spain, including agricultural areas, and they inhabit the majority of ponds in Salamanca. Clutch size varies between 250 and 500 eggs. In the pond where we collected the eggs, we observed a minimum of 22 breeding females, but the number was probably higher because we did not systematically sample the pond to count the females. If we consider a mean clutch size of 300 eggs per female (most females are small), we collected only ca. 8% of the fertile eggs in the pond. Several other ponds with marbled newts were also present locally. In the field, newt egg mortality from predation, fungal infection and desiccation is usually high and egg survival in our experiments, might have been higher than in the field. We did not capture or disturb any female during egg collection. For these reasons, we believe that our impact on the population was low.

The effects of UV radiation on amphibians are poorly known and before we started our study we did not know how long it would take for UV damage to occur. We knew of no preliminary sign of damage at which we could stop the experiment and prevent mortality. When embryos became affected by UV radiation they usually quickly showed an abnormality or infection or stopped development and died. The recovery rate of affected eggs was very low. We used a small sample size (20 and 16 per enclosure), especially considering that mortality from a chromosomal syndrome was relatively high in all tanks. We conducted the experiments at environmental levels of natural and artificial UV radiation and did not overexpose the eggs. In the field, some of the 14 days of the experiment were cloudy or wet and the eggs were thus exposed to relatively few hours of solar radiation. In the laboratory, eggs were exposed to a maximum of 5 days of radiation. The first effects were observed after day 11 in the field and day 4 in the laboratory.
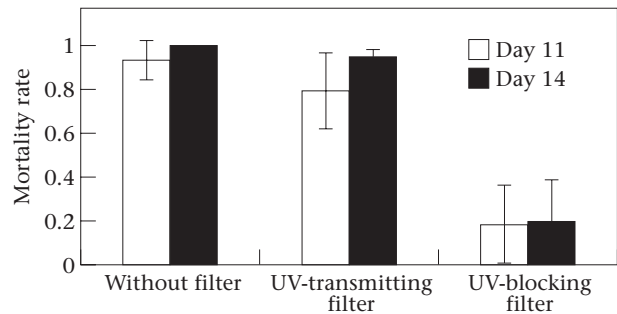
Figure 1. Mean±SD embryonic mortality rate of *Triturus marmoratus* embryos exposed to three regimes of UV radiation in the field. There were four enclosures per treatment and 16 eggs per enclosure.

RESULTS

Field Experiment

An ANOVA (nested design) indicated that there were no block effects among the enclosures in the field experiment where the top surface of the leaf in which the egg was wrapped was pinned back in all conditions. We found highly significant differences in the survival of the embryos between the three treatments (one way ANOVA: $F_{2,9}=43.090$, $P<0.001$). The average mortality of the embryos was 20.3% in the four enclosures with UV-blocking Llumar filters, 95.3% with UV-transmitting polyester filters and 100% without filters (Fig. 1). A post hoc Tukey test showed differences between the treatment with UV-blocking filters and the other two treatments (Tukey test: in both cases $P<0.001$). Mortality was not related to the embryo developmental stage. There were no differences between treatments in water temperature (one way ANOVA: $F_{2,9}=0.147$, $P=0.865$).

Laboratory Experiment

The mortality of eggs covered by leaves and exposed to artificial UV-B radiation for 5 days was similar to that of eggs not covered by leaves and not exposed to UV-B radiation; however, mortality of eggs directly exposed to UV-B radiation was significantly higher (ANOVA: $F_{3,8}=53.97$, $P<0.001$; Fig. 2). Embryos exposed to UV-B radiation suffered a high incidence of abnormalities. Those at early developmental stages displayed yolk leaking, necrosis or loss of their round shape. At medium or late developmental stages, we observed oedema and skin damage with loss of dermal tissues (Table 1). The majority of the abnormal individuals died within a few hours of the appearance of the abnormality. Frequency of loss of shape, oedema, skin damage and loss of dermal tissue was significantly higher in eggs exposed to UV-B radiation (ANOVA: $F_{3,8}=24.582$, $P<0.003$). Eggs exposed to UV-B radiation for 5 days showed a higher incidence of oedema than eggs exposed for 3 days. Infection by water mould (*Saprolegnia* sp.) also varied between treatments (ANOVA: $F_{3,8}=11.76$, $P<0.003$). The number of infected embryos was significantly higher in the treatment with unwrapped eggs exposed for only 3 days to UV-B radiation (Table 1), but there were no differences in rate of fungal infection
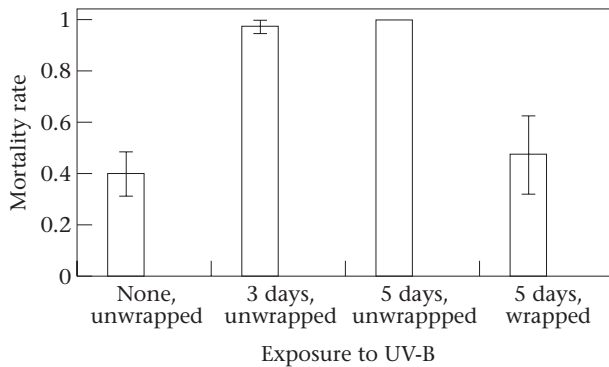
Figure 2. Mean percentage±SD of surviving *Triturus marmoratus* embryos for the four treatments in a laboratory experiment. Wrapped eggs were naturally enveloped in a leaf. In unwrapped eggs, the upper part of the leaf was carefully pinned back to permit the direct incidence of solar radiation. There were three enclosures per treatment and 20 eggs per enclosure.

between the other three treatments. We found no relationship between the embryo developmental stage and the appearance of abnormalities and embryo death.

DISCUSSION

Marbled newt embryos are extremely sensitive to ambient UV radiation. In the field, all the embryos died after 14 days of direct exposure to ambient solar radiation. In the laboratory, when exposed to artificial UV-B radiation, they suffered severe abnormalities and a high incidence of water mould infections. Relatively high levels of UV-B radiation killed all the embryos in less than 7 days. Embryos not exposed to UV-B radiation still suffered a relatively high mortality rate, which may be related to the developmental arrest syndrome observed in some species of large newts such as *T. cristatus* and *T. marmoratus* (Macgregor & Horner 1980; Horner & Macgregor 1985). Embryo arrest is a consequence of homomorphism of chromosome 1, which leads to the death of half of all the embryos. Normal development requires heteromorphism for chromosome 1 (Horner & Macgregor 1985). Although this intrinsic embryo mortality (ca. 50%) was probably a significant part of the mortality in treatments exposed to UV, the differences in abnormality frequencies, fungal infection and mortality rates between treatments indicate that *T. marmoratus* embryos are very sensitive to UV radiation. The relatively high survival found in some tanks (higher than 50%) in the field experiment could be related to the low water temperature: the experiment finished at approximately the middle of the embryonic development which was slow. At that time, the arrest syndrome affected only the more developed embryos and some with this syndrome were still alive.

The gelatinous matrix and egg envelopes of some salamandrids and ambystomatids may not always protect embryos from the negative effect of UV-B radiation (Blaustein et al. 1997; Anzalone et al. 1998; Langhelle et al. 1999). Some of these species may protect their eggs from UV radiation by laying them in deep or cloudy water or under opaque surfaces (Blaustein et al. 1998) and, in clutches of some ambystomatids, the growth of symbiotic green algae may create a green filter around embryos that may impede UV radiation (Marco & Blaustein 1998). For species that lay in clear and shallow water, we suggest that wrapping behaviour allows eggs to develop by limiting exposure to UV-B radiation and its negative effects. In the laboratory, with high levels of UV-B radiation, wrapped eggs did not suffer a higher mortality rate than embryos protected by UV-blocking filters. Thus, it is likely that wrapped eggs in the field are well protected from ambient levels of UV-B.

The larvae of other newt species are also sensitive to UV-B radiation. *Triturus cristatus* larvae show reduced survival when exposed to UV radiation (Langhelle et al. 1999). UV radiation also causes severe skin damage in *T. alpestris* larvae with lysis of epithelial cells and death (Nagl & Hofer 1997). Some aquatic habitats, especially in mountainous areas, lack aquatic vegetation. However,

Table 1. Effect of enhanced UV-B radiation on marbled newt embryos in the laboratory that were either wrapped in a leaf or not wrapped

	No UV-B unwrapped	3 days of UV-B unwrapped	5 days of UV-B unwrapped	5 days of UV-B wrapped	ANOVA
Number of eggs†	60	60	60	60	
Yolk leaking	2	8	11	4	NS
Axial malformation	0	2	0	0	NS
Necrosis	1	5	2	0	NS
Roundness contour	4	9	10	2	*
Oedema	1	1	8	0	**
Fungal infection	22	44	28	20	**
Skin damage	7	30	35	5	***
Death at day 7	24	59	60	29	***

†Number of marbled newt embryos with different types of abnormalities or infected by water mould in the three replicates of four different regimes of exposure to artificial UV-B radiation. Abnormalities and fungal infection were recorded when they occurred except for the wrapped eggs where they were recorded at the end of the experiment.
*$P<0.05$; **$P<0.01$; ***$P<0.001$.

newt females must select ponds with aquatic vegetation so that they can wrap their eggs. Selecting these types of habitats for oviposition may also provide refuges from UV radiation for sensitive larvae.

Díaz-Paniagua (1986) observed active selection by marbled newts for specific aquatic plants to envelop their eggs. For example, leaves of selected plants (mainly *Mentha* spp.) are flexible and wide, which may facilitate the wrapping process (Díaz-Paniagua 1986). Miaud (1995) found similar female preferences for the oviposition site in three species of European newts. Females may also select leaves that fully envelop the eggs, eliminating any direct exposure to sunlight of embryos.

Amphibian eggs often become infected by aquatic Saprolegniaceae fungi (Blaustein et al. 1994b; Beebee 1996). Eggs laid communally are more prone to fungal infection than those laid singly (Kiesecker & Blaustein 1997). Green (1999) suggested that laying eggs singly reduces infection by water moulds in newts. He found that eggs placed 1 cm apart were significantly less cross-infected than eggs in physical contact with each other. In our experiments, in all treatments, eggs were placed 1 cm apart reducing the possibility of infection. Because marbled newt females lay single eggs, the incidence of fungal infection may be decreased. Wrapping behaviour may also prevent eggs from becoming infected by fungal spores in the field. Nevertheless, in our experiments, wrapped eggs had a similar infection rate as unwrapped eggs that had not been exposed to UV-B radiation. Thus, with our data, we cannot conclude that leaves protect embryos from fungal infection.

Eggs exposed to UV-B radiation for 5 days showed a lower fungal infection rate than eggs exposed for 3 days. UV radiation may negatively affect the immune system of amphibians, increasing the likelihood of infection (Tevini 1993; Blaustein et al. 1994b; Kiesecker & Blaustein 1995), but longer exposure to UV-B radiation may also reduce fungal growth. Both fungal infection and UV radiation may contribute to embryonic mortality in marbled newts. In our laboratory experiments, eggs exposed to UV-B radiation suffered severe skin damage, a characteristic of trauma caused by UV radiation (Worrest & Kimeldorf 1976).

A low and variable percentage of newt eggs are observed unwrapped in the field (personal observation). This situation may be caused by disturbances during egg laying that induce females to leave before wrapping the eggs. Moreover, eggs may become unwrapped during embryonic development because of mechanical disturbance, leaf manipulation or leaf consumption by small herbivores, disintegration or death of the leaf or disintegration or denaturation of the gelatinous mass that attaches the leaf to the egg. Our results suggest that unwrapped eggs will quickly die if they become exposed to sunlight.

The harmful effects of UV radiation may have been an important selective pressure in the evolution of egg-wrapping behaviour in marbled newts. As our study shows, unwrapped, developing newt embryos are very sensitive to UV radiation. Presumably, over evolutionary time, those females that did not wrap their eggs or those that wrapped them inefficiently would leave fewer offspring than females that protected their eggs from UV radiation by wrapping leaves around them. Moreover, although UV penetration is greatest in shallow water, egg-wrapping behaviour has allowed newts to lay eggs in this habitat.

Acknowledgments

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