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Carry-over effects of ultraviolet-B radiation on larval fitness in *Rana temporaria*

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A number of studies have failed to find evidence for negative effects of ultraviolet-B radiation (UVBR) on amphibian early-embryonic performance, leading to the conclusions, first, that the embryonic stages of many species are tolerant to UVBR, and second, that the increased amount of UVBR reaching the Earth's surface is not likely to have any direct negative effects on many amphibian populations. However, possible carry-over effects of exposure to UVBR in the embryonic stages to the larval stages have received less attention. We studied the effects of UVBR experienced during the embryonic stages (age less than 11 days) on the later performance (age 11–75 days) of common frog, *Rana temporaria*, larvae. In a factorial laboratory experiment, newly fertilized embryos were divided into three different UVBR treatments (no UVBR (control), 1.25 kJ m^{-2} (normal) and 1.58 kJ m^{-2} (26% enhanced)), after which the individual larvae were raised until metamorphosis in the absence of UVBR. No effects of UVBR on embryonic survival rates, frequency of developmental anomalies or hatchling size were found, corroborating the earlier results indicating that *R. temporaria* embryos are tolerant to UVBR. However, analyses of larval performance revealed that larvae exposed to enhanced levels of UVBR as embryos suffered from an increased frequency of developmental anomalies and metamorphosed later and at a smaller size than larvae that had been protected from UVBR as embryos. These results suggest, in contrast to the earlier studies, that UVBR has direct negative effects on *R. temporaria* embryos, but these effects are expressed mostly or only during the later life stages. To this end, our results support the contention that carry-over effects from one life stage to another may be an important source of phenotypic variation in fitness.

Keywords: amphibians; anomalies; environmental stress; metamorphosis; ultraviolet-B radiation

1. INTRODUCTION

A global decline in amphibian populations (Alford & Richards 1999; Houlahan *et al.* 2000) has prompted intensive research into environmental factors that could explain the decline of populations not only in anthropogenically influenced areas but also in undisturbed areas (e.g. Wake 1998; Alford & Richards 1999; Corn 2000). One possible global agent is the increased solar ultraviolet-B radiation (UVBR) reaching the Earth's surface as a consequence of stratospheric ozone depletion (Kerr & McElroy 1993; Madronich *et al.* 1998; McKenzie *et al.* 1999). UVBR alone, or in combination with other stresses, has now been shown to cause mortality or developmental anomalies in the embryonic stages of many amphibian species (Worrest & Kimeldorf 1975, 1976; Grant & Licht 1995; Kiesecker & Blaustein 1995; Long *et al.* 1995; Nagl & Hofer 1997; Ankley *et al.* 1998, 2000; Hatch & Burton 1998; Walker *et al.* 1998; Zaga *et al.* 1998; Monson *et al.* 1999; Belden *et al.* 2000; Hatch & Blaustein 2000; Pahkala *et al.* 2000; Smith *et al.* 2000; see Blaustein *et al.* 1998 for a review of earlier literature). However, the results are not entirely consistent across different investigations and species, and many studies have not found any negative effects of UVBR on amphibian embryos (e.g. Blaustein *et al.* 1995, 1999; Anzalone *et al.* 1997; Corn 1998; Cummins *et al.* 1999; Langhelle *et al.* 1999; Starnes *et al.* 2000). The common frog, *Rana temporaria*, is a case in point: all published studies to date indicate that the embryos of this species are highly tolerant of normal and enhanced levels of UVBR (Cummins *et al.* 1999; Langhelle *et al.* 1999; Merilä *et al.* 2000*a*; Pahkala *et al.* 2000).

Most of the studies of amphibian UVBR tolerance have used a protocol where the early embryonic stages (or both early embryonic and larval stages) have been exposed to UVBR, and the experiments have usually been terminated when the embryos have hatched (Blaustein *et al.* 1998; but see Grant & Licht 1995; Nagl & Hofer 1997; Ovaska *et al.* 1997; Ankley *et al.* 1998, 2000; Crump *et al.* 1999; Langhelle *et al.* 1999; Broomhall *et al.* 2000; Smith *et al.* 2000). Although this study design is ecologically relevant in the sense that the free-swimming larvae are usually able to seek cover from UVBR (but see Nagl & Hofer 1997), it leaves open the question of possible delayed lethal or sub-lethal effects of UVBR, which may become expressed only at the later developmental stages. That this issue may be of some concern is also suggested by the increasing number of studies showing that environmental stresses experienced during early life (or even in earlier generations; Rossiter 1996; Desai & Hales 1997) may have permanent negative effects on later life fitness (Pechenik *et al.* 1998; Lindström 1999). Hence, before concluding that UVBR does not have negative effects on individual fitness, we have to ascertain that the direct effects of exposure to UVBR are not expressed, with a time-lag, in later developmental stages.

The aim of this study was to test whether exposure to UVBR during early life (from fertilization until hatching, *ca.* 11 days) might have negative effects on the later life

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(age 11–75 days) fitness of *R. temporaria* tadpoles. If such a delayed negative effect of UVBR was detected, this would mean that the previous studies focusing on effects at embryonic stages might have underestimated the potential consequences of increased UVBR on amphibian fitness and, consequently, perhaps also on population dynamics (cf. Lindström 1999).

2. MATERIAL AND METHODS

(a) *The study species and populations*

R. temporaria breeds in a wide range of freshwater habitats across central and northern Europe (Gasc *et al.* 1997). In southern Sweden breeding occurs in late March and early April, and normally lasts for less than 3 weeks (A. Laurila and J. Merilä, unpublished data). Eggs are laid in shallow water so that the uppermost eggs typically reach the water surface (A. Laurila and J. Merilä, unpublished data). Thus, eggs of this species are exposed to direct UVBR.

Effects of UVBR on common-frog embryos and larvae were studied in a laboratory experiment in Uppsala, Sweden, between April and June 2000. Five adult males and five adult females from southern Sweden (Tvedöra, 55°42′N, 13°26′E) were brought to the laboratory in Uppsala, where each male was artificially mated (see Berger *et al.* 1994) with a single female, resulting in five full-sib families (eggs produced by a single pair). Artificial mating ensured that the eggs had no prior exposure to UVBR. Any damaged eggs were discarded prior to experimentation. After fertilization, the eggs (less than 2 h old) were divided into batches of about 30 (the eggs are individually within a gelatin matrix, and this was not removed when a cluster of eggs was separated from the main clump) and placed into polypropane vessels (0.25 l, 5 cm high and 5.5 cm in diameter) about 4.5 cm below the water surface.

(b) *Experimental design and procedures*

To study the effects of UVBR on embryonic and larval performance, two experiments were performed. Both experiments were conducted in a constant-temperature room (17°C) using reconstituted soft water (APHA 1985) consisting of $NaHCO_3$ (48 mg l^{-1}), $CaSO_4{\cdot}2H_2O$ (30 mg l^{-1}), $MgSO_4{\cdot}7H_2O$ (61.37 mg l^{-1}) and KCl (2 mg l^{-1}).

(i) *First part of the experiment*

The first part of the experiment aimed to test the effects of UVBR on embryonic traits; it was conducted in three aquaria systems, each of which consisted of two experimental aquaria (120 cm × 120 cm × 25 cm, *ca.* 320 l) situated on top of each other, with a reservoir tank (90 cm × 90 cm × 35 cm, *ca.* 280 l) below them. Each aquarium system was filled with reconstituted soft water, which was continuously circulated (flow rate, 3 l min^{-1}) to reduce temperature fluctuations. To maintain water temperatures at the desired level (see below), each aquarium system was equipped with a water-cooling unit.

The experiment consisted of three UVBR treatments (see below), which were applied to four replicates from each of the five families. The replicates were randomly placed in the aquarium so that the total number of vessels per treatment combination within each aquarium was the same. The placement of the vessel within the aquarium was changed randomly each day to control for possible variations of irradiance in the aquarium.

The experimental vessels were placed on plastic netting situated 5 cm below the water surface. The bottom of each vessel was replaced with fine mesh, which allowed water to circulate into the vessels. As a direct consequence of the radiation from the greenhouse lamps (see below) there were regular daily temperature variations (13.8–18.6 °C) in the aquaria. The mean (±s.e.m.) temperatures in the different UVBR treatments differed slightly (15.7 ± 0.24 °C, 16.4 ± 0.24 °C and 16.6 ± 0.24 °C for the control, normal and enhanced UVBR treatments, respectively) but significantly ($F_{2,75} = 3.77$, $p = 0.028$). Therefore, the mean temperature experienced by each experimental unit was included in the analyses as a covariate in order to control for possible temperature effects.

The UVBR treatments were divided into six blocks (two for each UVBR treatment) over the three aquaria systems, each system thus containing two blocks. The daily photoperiod was 17 L:7 D and the UVBR exposure periods were centred around noon (11.00–14.00), mimicking the situation in nature (Josefsson & Karlsson 1997). A computer model (Björn & Murphy 1985; Björn & Teramura 1993) was used to calculate the daily irradiance of UVBR in Uppsala on April 24 (the normal breeding time of *R. temporaria*) as well as the daily increase in UVBR that would follow from 15% ozone depletion under clear-sky conditions, resulting in a 26% enhancement of UVBR above normal levels. The 15% decrease in ozone is within the observed daily variation in central Sweden (Josefsson 2000). The calculation was based on spectrally weighting the radiation (lamps irradiated 0.39 W m^{-2}) with Caldwell's plant action spectrum as parameterized by Thimijan *et al.* (1978). However, to facilitate comparisons with other experiments on frogs, we have expressed the radiation in DNA-weighted units. The DNA-weighted daily UVBR exposures corresponded to 1.25 kJ m^{-2} and 1.58 kJ m^{-2} for the normal- and enhanced-UVBR treatments, respectively. The levels of UVBR were adjusted by regulating daily irradiation regimes in the following ways: for the normal-UVBR treatment the irradiation time was 2 h 17 min day^{-1}, for the enhanced-UVBR treatment the irradiation time was 2 h 53 min day^{-1} and for the control treatment the irradiation time was 2 h 17 min day^{-1} but ultraviolet B and ultraviolet C were blocked with a Mylar filter (0.10 mm; Erik S. Ekman, Stockholm, Sweden). UVBR for each aquarium was provided using four fluorescent tubes (120 cm, 40 W; Q-PANEL, UV-B 313, Cleveland, OH) pre-burned for 100 h to give a stable output. In each aquarium, the four fluorescent tubes were placed 50 cm above the water level, uniformly parallel (40 cm between each lamp) to each other. The mid-sections (*ca.* 40 cm) of the two central tubes were covered with aluminium foil to obtain an even radiation distribution in the aquarium. For the normal- and enhanced-UVBR treatments the radiation was passed through a cellulose diacetate filter (0.13 mm; Courtaulds, Derby, UK) to cut out ultraviolet-C (< 280 nm) radiation. (For details of filter properties see Pahkala *et al.* (2000).) Filters were placed about 25 cm above the water level to allow air circulation beneath them.

To ensure sufficient background light for normal functioning of light-dependent DNA damage-repair mechanisms (Zhao & Mu 1998), two 400 W greenhouse lamps (Powerstar HQI-BT 400 W/D, OSRAM Malmö, Sweden) were fitted over each of the six aquaria. The amount of white light was measured using a Li-Cor Light Meter (Li-Cor, Lincoln, NE) with a quantum sensor, giving an irradiance of 320 μmol m^{-2} s^{-1}.

The response variables measured at the end of the experiment (when the majority of the larvae had reached Gosner (1960) stage 25) were first, survival (proportion of eggs surviving from the beginning to the end of the experiment),

second, the proportion of developmentally anomalous larvae (see below) and third, hatchling size. Hatchling size was determined from five alcohol (70%) preserved larvae from each replicate, as the total length (from nose to tail tip) of the larvae under a stereomicroscope (to the nearest 0.1 mm). Individuals were classified as anomalous if they had flexure of the tail or oedema.

(ii) *Second part of the experiment*

When the majority of the embryos in the first part of the experiment had reached development stage 25 (Gosner 1960), one or two randomly selected (though seemingly healthy) tadpoles from each experimental unit (90 in total, 30 per UVBR treatment) were placed into opaque plastic vessels (0.9 l) and reared individually until metamorphosis, in the absence of UVBR. The vessels were allocated randomly into two blocks (each consisting of two shelves in a constant-temperature (17 °C) room, daily photoperiod 17 L:7 D). The tadpoles were fed with lightly boiled spinach *ad libitum* every seventh day. Tadpoles were raised in reconstituted soft water (aerated and aged for at least 24 h before use) and the water was changed every seventh day in conjunction with feeding.

From the time the first individuals approached metamorphosis, the vessels were checked daily and metamorphosed individuals (defined as the emergence of the first foreleg, stage 42 (Gosner 1960)) were removed from the containers, weighed with an electronic balance (to the nearest 0.1 mg) and measured for total length (to the nearest 0.01 mm). Age at metamorphosis was defined as the number of days between fertilization and metamorphosis. At this point, the number of anomalies was also recorded. Examples of these anomalies are shown in the form of photographs in electronic Appendix A.

(c) *Statistical analyses*

The effects of the UVBR treatments on survival, frequency of developmental anomalies and total length (the first part of the experiment), and age and size at metamorphosis (the second part of the experiment), were analysed with analyses of variance (ANOVAs) as implemented in PROC GLM of SAS (Littell *et al.* 1996). In the second part of the experiment the effects of UVBR treatment on survival and the frequency of developmental anomalies were investigated using generalized linear model as implemented in PROC GENMOD of SAS (SAS Institute Inc. 1999). Logit link function, binomial error structure and type III sums-of-squares were used (Littell *et al.* 1996). In all models, the UVBR treatments and the block (in the second part of the experiment) effects were treated as fixed effects. Since block and block × treatment interaction were never significant in the analyses of metamorphic traits ($p > 0.16$ in all cases), the block effects were pooled to error term. In the first part of the experiment the random term 'family' was included in the models to control for variation due to maternal phenotypes. Temperature was included as a covariate to control for temperature variation (in the first part of the experiment). For analyses of hatchling size (in the first part of the experiment) the mean size of the hatchlings in each replicate was used. Before statistical testing (in the first part of the experiment) both survival and anomaly estimates were arcsine–square-root transformed to normalize their distributions. All measurements were taken blind with respect to the experimental treatment. In the second part of the experiment we also analysed the body condition of the metamorphs by introducing total length into the model when analysing total weight. We reasoned that metamorphs that are heavier for a given total length have a higher body condition.

3. RESULTS

(a) *First part of the experiment*

(i) *Survival, developmental anomalies and hatchling size*

There was no UVBR-treatment effect on survival rate at the point of hatching (figure 1*a*; $F_{2,52} = 0.35$, $p = 0.70$). Likewise, no UVBR-treatment effect on the frequency of developmental anomalies was detected (figure 1*b*; $F_{2,55} = 0.15$, $p = 0.86$). Different families differed considerably in their mean survival rates ($F_{4,52} = 8.21$, $p < 0.001$, mean ± survival of family 1 = 98.9 ± 0.47%, mean ± survival of family 2 = 99.2 ± 0.43%, mean ± survival of family 3 = 96.4 ± 0.98%, mean ± survival of family 4 = 85.1 ± 4.54% and mean ± survival of family 5 = 98.1 ± 0.74%) but the frequency of developmental anomalies was independent of family ($F_{4,51} = 0.37$, $p = 0.82$). The UVBR treatment did not have a significant effect on hatchling size (figure 1*c*; $F_{2,51} = 1.08$, $p = 0.35$), and hatchling size was also independent of family ($F_{4,51} = 2.03$, $p = 0.10$).

(b) *Second part of the experiment*

(i) *Survival and frequency of anomalies*

Survival rates until metamorphosis were highest in the control treatment and lower in the normal- and enhanced-UVBR treatments (figure 2*a*) but these differences were not significant (table 1). However, the effect of UVBR treatment on the frequency of developmental anomalies was significant (table 1). Anomaly frequencies were significantly higher in the enhanced-UVBR treatment (33% of all metamorphosed tadpoles) than in the control (10%) and normal-UVBR (7%) treatments (figure 2*b* and table 1). Anomalies consisted of either tail (flexure, 7.7% of anomalies) or hind-limb (ectromelia, 15.4% of anomalies; or ectrodactyly, 69.2% of anomalies) malformations similar to those observed in UVBR-treated individuals of *Rana pipiens* (Ankley *et al.* 1998, 2000). Out of the animals having ectrodactyly, 62.5% also had seriously bent knees.

(ii) *Age and size at metamorphosis*

Both age and size at metamorphosis were significantly affected by UVBR treatment (table 1). Tadpoles from the enhanced-UVBR treatment metamorphosed, on average, 4 days later than those from the control and normal-UVBR treatments (table 1 and figure 2*c*). Likewise, size at metamorphosis, whether measured as total length or mass, was largest in the control group, intermediate in the normal-UVBR treatment and smallest in the enhanced-UVBR treatment (figure 3*a*,*b*). However, in the case of total length only the difference between the control group and the enhanced-UVBR treatment was significant (Tukey, $p < 0.05$), whereas in the case of mass both the differences between the control group and the enhanced-UVBR treatment and between the normal- and enhanced-UVBR treatments were significant (Tukey, $p < 0.05$). Furthermore, individuals from the control group and the normal-UVBR treatment were heavier for a given total length than individuals from the enhanced-UVBR treatment (table 1 and figure 3*c*).

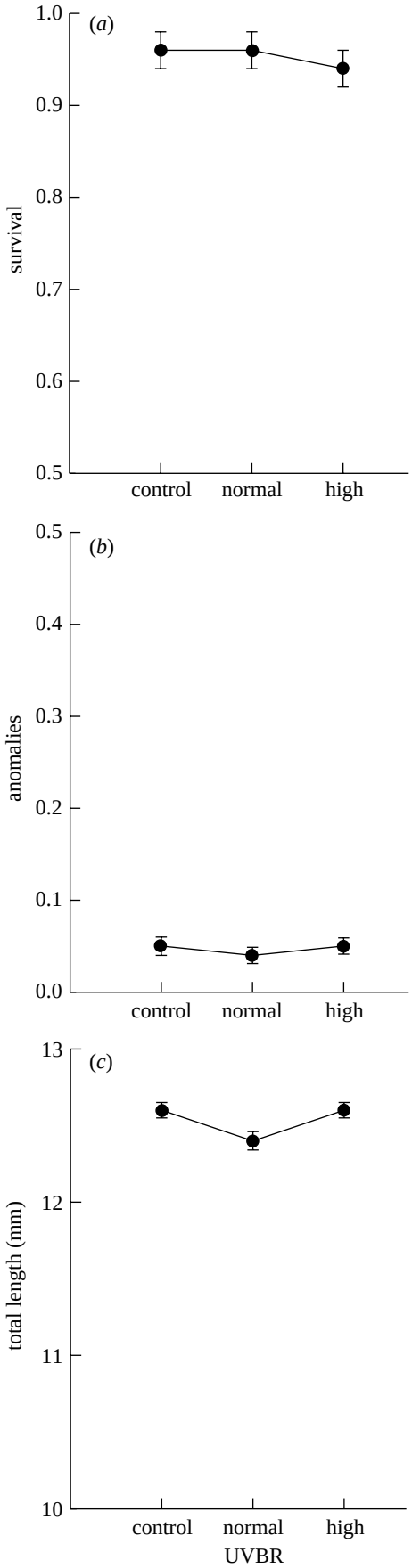

Figure 1. Effects of ultraviolet-B radiation treatment on (*a*) survivor, (*b*) incidence of anomalous development and (*c*) hatchling size in *Rana temporaria* at stage 25 (Gosner 1960) (first part of the experiment). All values are means ± s.e.m.

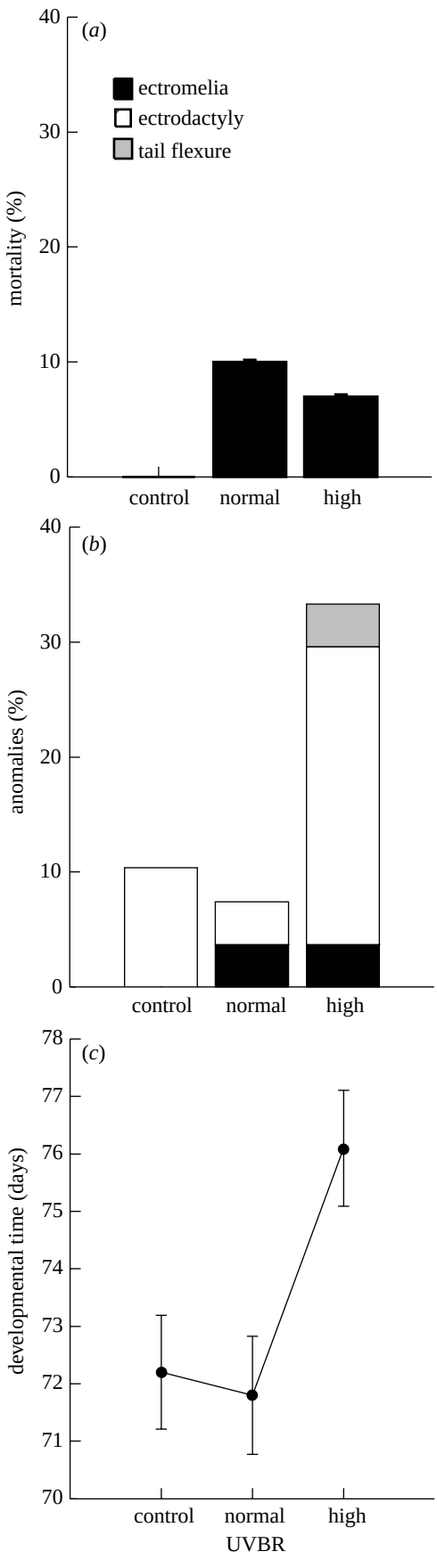

Figure 2. Effects of ultraviolet-B radiation treatment on (*a*) mortality rates, (*b*) incidence of anomalous development and (*c*) developmental time in *Rana temporaria* in the second part of the experiment. Values for age at metamorphosis are least-square means ± s.e.m.

Table 1. Effects of ultraviolet-B radiation treatments on metamorphic traits of *Rana temporaria*.

treatment effect	d.f.	χ^2	p
mortality	2	4.33	0.1148
anomalies	2	7.41	0.0246
age	2	4.96	0.0093
mass	2	8.64	0.0004
total length	2	5.44	0.0061
condition	2	6.48	0.0025

4. DISCUSSION

Our results show that embryonic exposure to UVBR can have negative effects on individual fitness but that these negative effects are expressed mainly in the later stages of development. More specifically, when exposing *R. temporaria* eggs to UVBR until hatching and then rearing the larvae in the absence of UVBR until metamorphosis, we found that the early exposure to UVBR increased the frequency of developmental anomalies among the metamorphs, delayed metamorphosis and reduced size and body condition at metamorphosis, compared with eggs in the control group. There were no effects of UVBR on embryonic survival rates, frequency of developmental anomalies or hatchling size, corroborating earlier studies with this species (Cummins *et al.* 1999; Langhelle *et al.* 1999; Merilä *et al.* 2000*a*). However, in these earlier studies the experiments were terminated when the embryos hatched. By inference, our results suggest that these earlier studies may have underestimated the potential consequences of increased UVBR on amphibian fitness and, consequently, perhaps also on population dynamics.

There is only one study that has looked at the carry-over effects of UVBR on larval development. In a recent work, Smith *et al.* (2000) found that *Rana blairi* tadpoles exposed to high levels of UVBR as embryos had slower growth and development rates than tadpoles from a low-UVBR treatment, but they did not continue the experiment until metamorphosis and did not report hind-limb malformations. However, Ankley *et al.* (1998, 2000) found that when exposing larvae of *R. pipiens* to UVBR (from stage 25–26 to forelimb emergence), individuals exhibited dose-dependent elevations in the frequency of hind-limb malformations, similar to those detected in our study.

The fact that UVBR had negative, and apparently dose-dependent, effects on the timing of and size at metamorphosis underlines the contention that negative effects of UVBR on amphibian development are not always conspicuously lethal but also that more subtle (sub-lethal) effects are possible (Worrest & Kimeldorf 1975, 1976; Grant & Licht 1995; Nagl & Hofer 1996; Zaga *et al.* 1998; Belden *et al.* 2000; Blaustein *et al.* 2000; Kats *et al.* 2000; Pahkala *et al.* 2000). These findings are significant because effects on timing of and size at metamorphosis can have cascading effects on individuals' later-life fitness. For example, delayed metamorphosis can increase the risks associated with habitat drying or predation (Newman 1992), and small size at metamorphosis can expose individuals to a greater risk of desiccation or starvation (Goater 1994; Newman & Dunham 1994) as well as increased vulnerability to terrestrial predators (John-Alder & Morin 1990). Also, smaller size at metamorphosis can lead to delayed maturation, reduced fecundity and/or lower

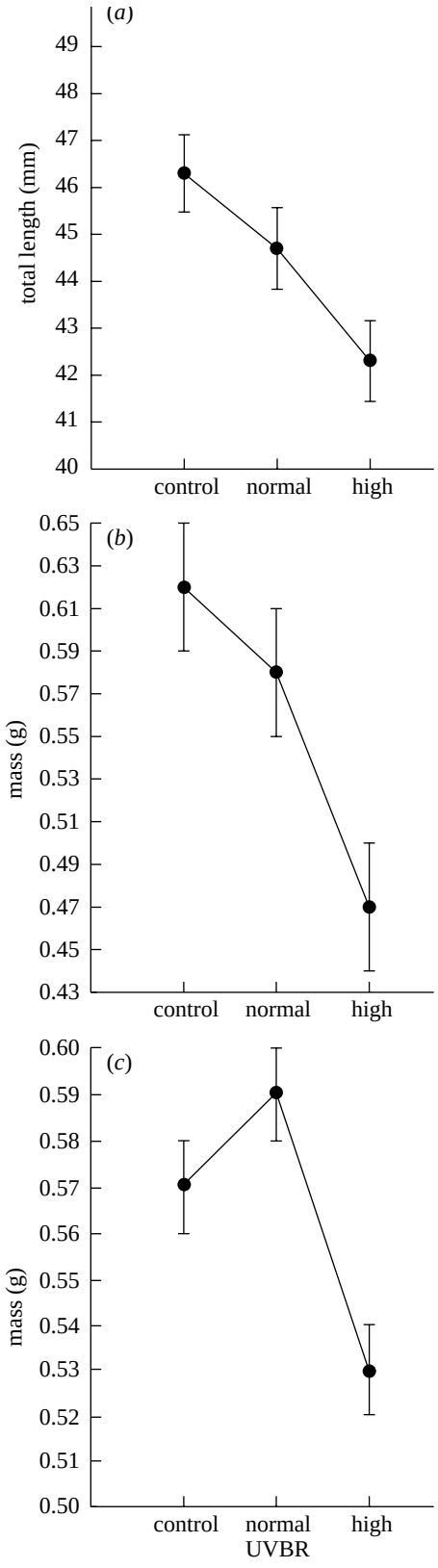

Figure 3. Effects of ultraviolet-B radiation treatment on size ((*a*) total length and (*b*) weight) and (*c*) relative weight of *Rana temporaria* metamorphs in the second part of the experiment. All values are least-square means ± s.e.m.

mating success (Kaplan & Salthe 1979; Howard 1980; Smith 1987; Semlitsch *et al.* 1988; Berven 1990). Consequently, the negative effects of embryonic exposure to UVBR can have far-reaching consequences from an individual's, and perhaps also from a population dynamic, point of view (Lindström 1999).

As to the proximate mechanisms causing the observed effects, we can only raise the obvious point that trade-offs between different functions could be involved. For example, as suggested earlier (Pahkala *et al.* 2000), a trade-off between the energy allocated to development and growth and that allocated to cellular-DNA damage repair could be involved. Hence, if the mechanisms responsible for UVBR damage repair are costly and use up part of the energy that could otherwise be allocated to faster growth and development, then delayed development and smaller size at metamorphosis would be expected. However, it is also entirely possible that no trade-offs are involved, but that UVBR-induced photoproducts interfere directly with early growth and development and have a permanent influence on later life performance. These types of persistent effects of early environmental conditions on individuals' subsequent performances are widespread both in frogs (e.g. Travis 1984; Semlitsch *et al.* 1988; Audo *et al.* 1995; Kaplan 1998; Merilä *et al.* 2000*b*) and in other organisms (Rossiter 1996; Desai & Hales 1997; Merilä & Svensson 1997; O'Steen 1998).

The amount of UVBR that the embryos were exposed to was based on clear-sky conditions. It is clear that in many years clear-sky conditions do not prevail throughout the embryonic period. However, in some years and areas clear-sky conditions can prevail during the whole embryonic period (A. Laurila, unpublished data). Another factor that diminishes the UVBR levels in nature is the amount of dissolved organic carbon (e.g. Nagl & Hofer 1996; Schindler *et al.* 1996). However, *R. temporaria* eggs are laid in shallow water, so that the uppermost eggs usually reach the water surface. Hence, we consider our normal-UVBR levels to be realistic, and note that the radiation levels in our enhanced-UVBR treatment are within the range of natural variation in the amount of UVBR in Scandinavia (Josefsson 2000).

In conclusion, our results demonstrate that exposure of *R. temporaria* embryos to enhanced levels of UVBR can induce the expression of developmental anomalies in later life stages as well as delay and reduce the size at metamorphosis. To this end, our results contradict the earlier conclusions (e.g. Cummins *et al.* 1999; Langhelle *et al.* 1999) that *R. temporaria* embryos are highly tolerant to UVBR, and caution against conclusions drawn from short-term experiments where no negative effects of UVBR treatment have been detected. Further studies are required to understand whether the effects uncovered by this study are applicable to other species implicated to be highly tolerant of high doses of UVBR.

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