SOIL ACIDIFICATION NEGATIVELY AFFECTS EMBRYONIC DEVELOPMENT OF FLEXIBLE-SHELLED LIZARD EGGS

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Many reptile species dig underground nests where they deposit eggs with flexible and permeable shells that have physical contact with the soil and are highly permeable to soil water and gases. Iberian rock lizard eggs (*Lacerta monticola cyrenni*) incubated in acidic substrates suffered significant impairment to their development. Therefore, soil pollution could be affecting embryonic development. Low pH had a negative effect on egg water exchange, hatchling size and locomotor performance. In all cases, pH had no effect on incubation duration and embryo survival. At substrate pH of 4 and 10, eggs absorbed less water and final egg size and weight was lower than at neutral pH. Hatchlings from eggs incubated in acidic substrates had lower mass (up to 28 % of weight), SVL, and tail length than controls. Running speed – a good indicator of lizard fitness – was also affected by substrate pH. Embryos incubated at pH 4 ran slower than controls. The alteration of the water absorption process that low substrate pH had on eggs during incubation partially explained the observed effects on hatchling characteristics. These sublethal effects may influence the survival or success of juveniles during or after their first wintering.

Key words: ecotoxicology, incubation, lacertids, soil pH

INTRODUCTION

Soil pollution, and more specifically the alteration of soil pH, may influence embryonic development and hatching success of these species. They usually inhabit areas that are susceptible to acidification such as industrialized areas, conifer forests, heathlands, moorlands or mountain grasslands. This impact could be particularly significant on species that have flexible and permeable shelled eggs, such as terrestrial egg-layer amphibians and many reptiles (Packard & Packard, 1988). During incubation, flexible-shelled eggs exchange gases and water with the nest environment. Eggs can absorb water from the soil increasing their weight and volume up to three or four times. Water absorption and gas exchange are necessary to successfully complete embryonic development (Thompson, 1987; Overall, 1994).

We were interested in the study of the impact of soil quality on flexible-shelled reptile eggs and, more specifically, the effect of soil pH on reptile embryonic development. Extreme soil pHs could have detrimental effects on the eggshell or egg content. Soil pH could also modify the egg environment, thus influencing water or gas exchanges between the egg and the surrounding soil, or it could directly modify egg content pH, and therefore affect embryonic development. To test the sensitivity of flexible shelled reptile eggs to soil pH, we incubated Iberian rock lizard eggs from mountain areas in substrates with pH levels ranging from 4 to 10.

The Iberian rock lizard, *Lacerta monticola cyrenni*, (the species selected as a model), is a medium-sized lizard inhabiting mountain areas of Central Spain which is susceptible to the affects of acid deposition. During the early summer, females deposit 4 to 10 eggs in nests burrowed in the soil in rocky or grassy areas. It is a vulnerable species with a reduced and fragmented distribution and is susceptible to a number of environmental stresses (Pérez-Mellado, 1997; Barbadillo *et al.*, 1999).

MATERIALS AND METHODS

EGG COLLECTION

We collected 10 gravid Iberian rock lizard females (*Lacerta monticola cyrenni*) from the Gredos Mountains (Avila, Spain), during the last week of June 2001. Females were collected in areas where they were abundant. They were housed individually in 30 L plastic containers in the laboratory at approximately 26 °C and were exposed to daylight, allowing some exposure to UV radiation. The bottom of the containers was filled with 8 cm of sand that was watered regularly. Dechlorinated tap water was always available. Females were fed with living *Tenebrio* larvae *ad libitum*, previously dusted with multivitamin powder. Egg laying took place after a maximum of 15 days in captivity. All females laid an average of 6.2 eggs per clutch. Immediately after egg-laying, the eggs were extracted from the sand, cleaned with a soft brush, marked individually by a number on the eggshell with a graphite pencil, weighed on a digital scale (± 0.01 g) and measured with a caliper (± 0.1 mm). Before the beginning of the experiment, the eggs were incubated at 26 °C in wet sand. After egg laying, females were released to the same places where they had been collected.

EXPERIMENTAL PROCEDURES

The effects of substrate pH on lizard egg incubation were tested by incubating single eggs in wet vermiculite

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at the selected substrate pHs ranging from 4 to 10. We used vermiculite as substrate to reduce pH variation. One fertile egg from each clutch was randomly assigned to each of five pH treatments (4, 5, 7, 9 and 10) in a block design that controlled the effect of the intra-clutch variability. All eggs began experimental incubation on the same day. The experiment was conducted in July and August (2001) in an incubation chamber at 26 °C (Sanyo Incubator MIR 52). The selected values for temperature and water potential fall within the ranges of values measured for natural nests at the collection site. The experiment was conducted until the eggs hatched.

The fifty eggs were individually incubated inside plastic containers (110 ml) filled with 100 ml of sterile vermiculite. Selected substrate pHs were obtained and adjusted by adding concentrated solutions or HCl or NaOH to the vermiculite. These acid solutions were made by adding concentrated HCl or NaOH to 50 ml of distilled water. Then, the substrate of each container was watered with distilled water until a water potential of 150 KPa was reached (following the methodology proposed by Packard & Packard (1988)). Eggs were added to the containers after selected pH and water potential were obtained. The substrate covered the eggs completely. The containers were covered with lids to minimize evaporation. To control the substrate pH, we introduced into the incubator an additional container for each pH treatment with the wet vermiculite at the corresponding pH. The pH of the wet vermiculite before being placed in the experimental containers, and of the wet vermiculite in the enclosures without eggs, was measured periodically using a portable pH meter (Handylab 1 BNC, Schott-Geräte GmbH, Germany). In order to avoid substantial alterations of the selected pH values, substrates were replaced every 8 to 10 days with new substrates at the selected pH.

At the beginning of the exposure to the pH treatments and when the substrate was replaced, we recorded the survival rate and the external shape and aspect of eggs. We also weighed them on a portable digital scale to the nearest 0.01g. Immediately after hatching, we measured hatchling snout-vent length (SVL) and mass. We also checked for morphological or behavioural alterations. Hatchlings were housed in 30 L plastic containers in the laboratory at approximately 26 °C under natural light and were fed with crickets previously dusted with vitamin powder.

To determine whether substrate pH during incubation had an effect on hatchling locomotor abilities, we measured the running speed of each individual within the first 24 hr of hatching. All hatchlings had the tail intact. Hatchlings were forced to run a distance of 1m by chasing them by hand, following a simplified version of the methodology proposed by Huey *et al.* (1981). The track was constructed of cardboard and was 120 cm long with vertical walls 30 cm high which were positioned 20 cm apart; the floor was lined with filter paper. To calculate the running speed, we considered the time that hatchlings took to displace 100 cm, excluding the first and the last 10 cm of the track. Running times were recorded with a stopwatch to the nearest 0.1s. We tested each hatchling twice and considered the mean running speed for each individual. Between each trial, hatchlings were permitted to rest for 2 min. At the end of the experiment, hatchlings were released in the area where females had been collected.

Analysis of Data

To determine whether substrate pH has an overall effect on egg incubation, we used analysis of covariance (ANCOVA), considering as dependent variables: final egg mass, time to hatching, hatchling mass, SVL, tail length and running speed. The covariate was the initial egg mass and the factor was the substrate pH. To determine the effect of substrate pH on embryo survival, we used the Chi-square test. We also used ANCOVA to determine the effect of pH on tail length and running speed considering hatchling SVL as covariate. We used the Tukey honest significant difference for post-hoc comparisons of means of ANCOVAs. All the assumptions of these analyses were previously verified.

RESULTS

ANCOVA indicated an effect of substrate pH on lizard egg incubation (Rao's R=5.261; df=24,102; P<0.001). Nine eggs died during the experiment. Eight of them corresponded to two clutches. We did not find any external developmental abnormality on eggs or hatchlings. The tested levels of substrate pH had no effect on embryo survival (χ^2=1.508; df=4; P=0.825) and incubation duration (Table 1). On average, incubation lasted 35.6 days and hatching started after a mean time of exposure to pH treatments of 21 days. However, the substrate pH affected egg (Fig. 1) and hatchling mass (Fig. 2), tail length (Table 2) and running speed (Fig. 3). Post-hoc Tukey tests indicated that eggs incubated at a substrate pH of 4 and 10 absorbed less water and pre-hatching egg size and weight were lower than those at neutral pH (Table 1; Fig. 1). Post-hoc Tukey tests also indicated that hatchlings from eggs incubated at pH 4 were smaller (28%) than those incubated at pH 7 (Fig. 2). Similar results were obtained for SVL and tail length (Table 2). When we included hatchling SVL as a covariate in the ANCOVA analysis, the effect of

TABLE 1. Results of ANOVA that analyze the effect of substrate pH on initial egg mass and of ANCOVAs that analyze the effect of substrate pH on other parameters of Iberian rock lizard embryonic development using as the initial egg mass as a covariate.

Variable	df	*F*	*P*
Initial egg mass	4,41	0.02	0.999
Final egg mass	4,41	4.85	0.003
Incubation duration	4,36	1.27	0.266
Hatchling mass	4,36	17.34	<0.001
Hatchling SVL	4,36	2.32	0.075
Hatchling tail length	4,36	5.14	0.002
Running speed	4,36	2.96	0.032

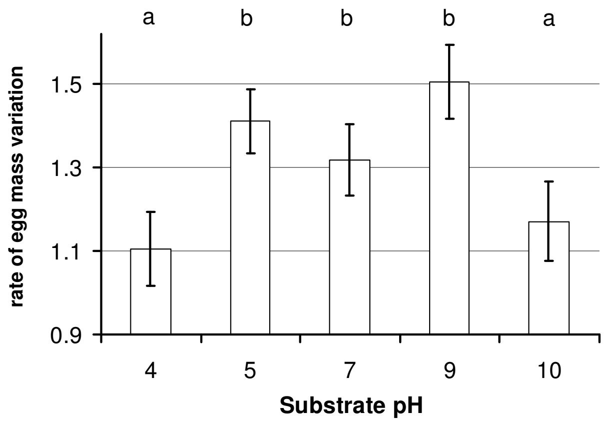

FIG. 1. Average rate of egg mass variation (± SE) of Iberian rock lizard eggs incubated at different substrate pHs. Letters over the bars indicate mean separation in post-hoc Tukey tests.

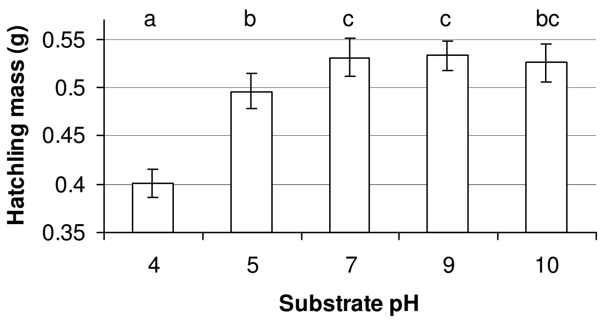

FIG. 2. Average mass (± SE) of Iberian rock lizard hatchlings from eggs incubated at different substrate pHs. Letters over the bars indicate mean separation in post-hoc Tukey tests.

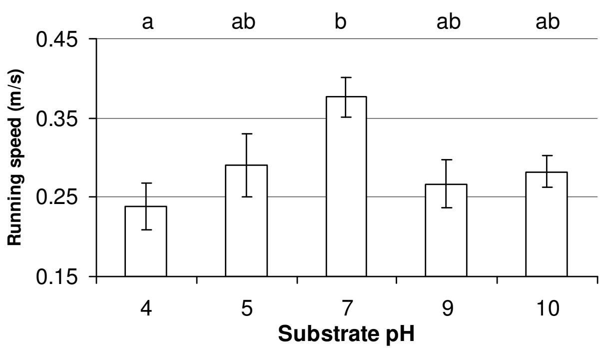

FIG. 3. Effect of substrate pH during flexible-shelled eggs' incubation on the running speed (± SE) of Iberian rock lizard hatchlings. Letters over the bars indicate mean separation in post-hoc Tukey tests.

substrate pH on hatchling mass was still significant ($F_{4, 36}$=7.195, P< 0.001). Running speed varied significantly among treatments (Table 1) and was positively correlated with hatchling mass (Pearson correlation: r=0.467, F=19.92, P<0.001). Embryos incubated at pH 4 and 5 generated hatchlings that ran significantly slower than controls (Fig. 3). The effect of soil pH on running speed was also significant when we included hatchling SVL as a covariate in the ANCOVA ($F_{4, 36}$=3.181, P=0.025).

DISCUSSION

The results of this study demonstrate that flexible-shelled reptile eggs are sensitive to the pH of their nest environment. Low pH has a significant effect on egg water exchange, and also on hatchling size and running speed. These sublethal effects may influence the survival or success rates of juveniles. In all cases, pH had no effect on incubation duration and embryo survival. Basic pHs only slightly affected the egg mass variation during incubation. They had no effect on hatchling morphology. Eggs were exposed to the pH treatments during the last 60 % of their embryonic development. Exposing eggs to extreme pHs during the full incubation period could increase the negative impacts on embryonic development.

Many factors affect the exchange of water between a reptilian egg and its environment (Packard & Packard, 1988; Ackerman, 1991). For example, the size of the egg, the proportion of the egg in contact with the substrate or the physical characteristics of the eggshell such as the porosity and rigidity can all determine how much water the egg can absorb or retain. Environmental factors such as the soil water potential or the temperature also influenced water exchange. We have found that the substrate pH may also affect the water absorption process that flexible-shelled reptile eggs experience during incubation. Acid and basic soil pHs alter the permeability of the eggshells or modify the availability of the soil water to the eggs in the nest environment.

Reptilian eggs and embryos are affected profoundly by the availability of water in their environment (Packard, 1991). Soil water potential in the nest may influence hatching success (Thompson, 1987; Packard, 1991). Moreover, embryos of flexible-shelled eggs incubated on wet substrates consume more oxygen, have a higher metabolic rate and grow more than those incubated in dry environments (reviewed in Packard, 1991).

TABLE 2. Mean (± SD) values of some characteristics of *Lacerta monticola* eggs and hatchlings exposed to different substrate pHs during incubation.

Substrate	4	5	7	9	10
Initial egg mass	0.435 (0.01)	0.435 (0.01)	0.43 (0.01)	0.432 (0.02)	0.432 (0.01)
Final egg mass	0.889 (0.04)	1.043 (0.03)	0.990 (0.03)	1.076 (0.04)	0.963 (0.04)
Incubation Duration	34.4 (0.54)	35.8 (0.57)	36.4 (0.57)	35.4 (0.65)	34.9 (0.60)
Dead eggs	1	3	1	2	2
Hatchling SVL	25.6 (0.41)	26.4 (0.43)	26.8 (0.43)	27.1 (0.49)	27.0 (0.46)
Hatchling tail length	39.9 (1.13)	42.8 (1.20)	46.1 (1.20)	45.6 (1.36)	43.6 (1.27)

Hydric conditions during incubation can also influence the hatchling locomotor performance of some reptiles (Miller *et al.*, 1987). In our experiment, we incubated all the eggs in wet substrates (–150 KPa) and enough water was available to guarantee optimal embryonic development. We demonstrate that substrate acidification causes similar effects on egg size, hatchling size and running speed as those of dry nest environments. Embryos in moist environments tend to remain in the egg longer before hatching than do embryos incubating in drier conditions (Packard, 1991). In moist environments, embryos grow for a longer period and hatch with larger sizes. However in our study, a decrease in the incubation period did not cause the observed effects related to substrate pH, because there were no differences in incubation duration among treatments.

Hatchlings from eggs incubated in acid substrates had lower body mass than controls and part of this difference was independent of hatchling SVL. One explanation for these results is the soil pH influence on the level of hydration of newly-hatched hatchlings. Other studies have shown that embryos incubated in dry substrates hatch less hydrated than those incubated on wet substrates (Packard, 1999). The level of hydration of hatchlings can explain the differences found on body mass and at the same time the absence of variability on body length among treatments. The state of desiccation has a strong influence on hatchling survival, especially in arid areas where hatchlings have difficulty accessing water sources. Large or well hydrated hatchlings usually survive better than smaller hatchlings during the neonatal period (Ferguson & Fox, 1984; Vleck, 1991). For example, large hatchlings may be better than smaller ones at emerging from dry nests, avoiding predators or capturing large prey (Packard, 1999).

Substrate pH had an effect on hatchling running speed. Hatchlings incubated at neutral pH were larger and ran faster than those incubated at acid pH and this difference was partially independent from hatchling SVL. The level of hydration may have an influence on locomotor performance. Alternatively, hatchlings incubated at acid pH may have developed less muscle mass. Lizards with a larger body size tend to have greater absolute stamina (Garland & Losos, 1994). Tail length could also influence hatchling locomotor performance. Short tails reduce running speed in some lizards (Ballinger *et al.*, 1979). Substrate acidification hampered hatchlings' capacity for running and hence reduced escape and foraging efficiency. Escape using fast movements is an important defensive behaviour for many lizard species and may be considered as an indicator of fitness (Bauwens & Thoen, 1981). Moreover, most reptiles have an intensive foraging strategy and they travel considerable distances, often at a very high running speed (Avery *et al.*, 1987). Decreased locomotor ability and increased energetic costs of locomotion due to egg incubation on acid substrates may influence the survival or success rates of juveniles.

Reptile species are declining on a global scale and environmental pollution has been suggested as one of the main causes (Gibbons *et al.*, 2000). Reptiles are exposed to numerous environmental contaminants and many studies document their bioaccumulation in different tissues and eggs (Sparling *et al.*, 2000). Most studies on reptile ecotoxicology have been conducted on turtles and crocodilians, but there are very few studies conducted on snakes or lizards. On the other hand, very few experiments document the effects and the dose-effect relationship of pollutants on reptiles (Kleinow *et al.*, 1999; Sparling *et al.*, 2000; Campbell & Campbell, 2002). Finally, it is assumed that the accumulation of toxicants or any other toxic effect on eggs originates from maternal transference during egg formation. There is very little evidence indicating that environmental pollution can have a direct impact on egg incubation. For example, oil applied to the external surface of bird eggshells produced high embryo mortality, but partial sealing of the shell and reduced gas exchange could be the cause of the death (Jessup & Leighton, 1996). Another study documents that organochlorine contaminants may pass through the eggshell of snake eggs incubated in contaminated nests (Cañas & Anderson, 2002). Our study documents the impact of soil pH on embryonic development of reptiles with flexible-shelled eggs. Acid rain, dry deposition or other sources of soil acidification could be contributing to the decline of reptiles with flexible-shelled eggs. Though there has been a significant reduction in anthropogenic acid deposition from the ‘60s, many aquatic and terrestrial ecosystems are still showing a very slow recovery or no recovery at all (Likens *et al.*, 1996; Alewell *et al.*, 2000).

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