Bull. Environ. Contam. Toxicol. (2004) 72:983–990

DOI: 10.1007/s00128-004-0340-1

Bulletin of Environmental Contamination and Toxicology

Arsenic Uptake by Reptile Flexible-Shelled Eggs from Contaminated Nest Substrates and Toxic Effect on Embryos

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Received: 7 March 2003/Accepted: 5 March 2004

In many oviparous species, factors affecting embryo survival may represent a very important cause of population declines (Overall 1994). Among other perturbations, environmental contaminants can severely affect embryonic development and can potentially contribute to embryo mortality. Reptile and bird eggs have been considered well protected from the external environment (Rose et al.1999, Sparling et al. 2000) and the presence of environmental contaminants on eggs of these vertebrates has been assumed to respond to the maternal transference during egg formation (Kleinow et al. 1999). Maternal transference is a logical source of contaminants during vitellogenesis and oviductal egg retention. However, eggs are exposed to many environmental contaminants in the nest environment during incubation. Some contaminants could enter into the eggs affecting embryos. Recently, Canas and Anderson (2002) have demostrated uptake of organochlorine pesticides by snake eggs incubated in contaminated nest material. However, there is little evidence of the potential risk of contaminated soils to terrestrial vertebrate eggs (Di Giulio and Tillitt 1999, Sparling et al. 2000).

We have tested whether arsenic present in the nest material may cross through the flexible eggshell of reptiles affecting embryos. Reptile eggs usually develop in soil without parental care and the soil environment decisively affects embryonic development. Flexible-shelled eggs are especially sensitive to their hydric environment and can take large amounts of water from the soil increasing their weight up to three or four times from egg laying (Thompson 1987, Packard and Packard 1988). Reptile eggshells are also permeable to some gases such as oxygen and carbon dioxide (Ackerman 1980). To test whether soil contaminants can cross through the reptilian eggshell, we exposed eggs of the Iberian rock lizard (*Lacerta monticola cyrenni*) to an artificial substrate watered with a solution with four concentrations of arsenic (50, 100, 250 and 500 ppb of As) and a control. We also analyzed the effects of the detected levels of arsenic on eggs. Arsenic is a naturally occurring metalloid found in soils and is neurotoxic to a variety of organisms (Chang 1996).

MATERIALS AND METHODS

We collected 13 gravid Iberian rock lizard females (*Lacerta monticola cyrenni*)

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from Gredos Mountains (Avila, Spain), during the last week of June 2001. Females were collected in areas where they were abundant and there were no close sources of pollution. All animals were captured and cared for throughout the experiment in accordance with the principles and guidelines of the Spanish laws regarding the use of wild animals for scientific research. They were housed individually in 30 L plastic containers in the laboratory at approximately 26 °C under natural daylight allowing for some exposure to UV radiation. Dechlorinated drinking tap water treated for human consume was provided ad libitum. The floor of the containers was filled with 8 cm of wet washed sand that was watered regularly with drinking tap water. Arsenic concentrations in drinking tap water and washed sand were considered negligible. Females were fed with living *Tenebrio* sp larvae ad libitum, previously dusted with multivitamin powder. Egg laying took place after a maximum of 15 days of captivity. All females laid an average of 7 eggs per clutch.

Immediately after egg-laying, the eggs were extracted from the sand, cleaned with a soft brush, weighed using a digital scale (± 0.01 g) and measured with a digital caliper (± 0.1 mm). Before the beginning of the experimental treatment, the eggs were incubated at 26 °C in washed sand watered with distilled water. After egg laying, females were released to the same locations in which they were collected.

The permeability of eggshells to arsenic and its bioaccumulation and effects in embryos were tested incubating single eggs in wet vermiculite with As water concentrations ranging from 0 to 500 ppb. One fertile egg of each clutch was

Table 1. Concentration of Cu, Cd, Zn, Pb and As on lizard eggshells and in embryos of *Lacerta monticola cyrenni* incubated on vermiculite with different concentrations of arsenic (ng As/g).

Tissue	Substrate [As]	As (ng/g)	Cu (µg/g)	Cd (ng/g)	Zn (µg/g)	Pb (ng/g)
Eggshell	0	14.7±0.9	1.08±0.01	3.48±0.00	9.19±0.05	142.6±0.0
	50	29.6±1.3	1.19±0.01	4.14±0.22	7.59±0.01	59.3±2.2
	100	57.4±0.3	1.66±0.02	7.10±0.08	15.52±0.05	81.9±4.4
	250	64.4±1.9	0.74±0.03	2.52±0.06	11.22±0.03	48.2±2.3
	500	88.5±0.0	0.79±0.01	2.85±0.14	9.30±0.09	42.6±0.4
Embryo	0	nd	0.79±0.01	3.00±0.18	14.03±0.06	31.7±1.1
	50	16.0±1.0	0.73±0.00	3.00±0.19	13.26±0.07	61.2±1.1
	100	12.9±0.6	0.49±0.00	3.19±0.18	13.82±0.10	55.5±0.2
	250	25.9±1.3	0.71±0.01	2.73±0.12	11.26±0.09	140.9±3.5
	500	38.2±1.4	0.54±0.01	2.83±0.17	13.26±0.03	96.6±0.2

nd = non detected. N=4 for both tissues and all As concentrations

randomly assigned to each of five experimental treatments (0, 50, 100, 250 and 500 ppb As). The ninety eggs were individually introduced inside plastic containers (110 ml) filled with 100 ml of sterile As-free vermiculite. The substrate completely covered the eggs. Selected As concentrations were obtained from a concentrated arsenic standard solution of As pentoxide (arsenic acid in nitric acid 0.5 mol/l)(Merck, Germany) in distilled water. The same amount of each As concentration solution or distilled water (for controls) was added to the vermiculite of each container until the acquisition of a water potential of –150 KPa following methodology proposed by Packard (1991). The substrate pH was recorded in each container before the addition of the egg and was not influenced by the As solution. Previous studies on lizard eggs indicate that the impact of the nitrate added with the As is negligible (Marco et al., unpubl. data). The containers were closed with lids to minimize evaporation. The experiment was conducted in July and August 2001 in an incubation chamber at 26 °C (Sanyo Incubator MIR 52, Japan). The selected values for temperature and water potential fall within the ranges of values measured for natural nests. In order to reduce the risk of fungal infection and substantial changes on substrate water potential, substrates were replaced every 14 days. During the substrate replacement, eggs were treated carefully and relocated in the new substrate conserving the original egg position. At the beginning of the exposure to As and when the substrate was replaced, we recorded the survival and the external shape and aspect of eggs and weighed them using a portable digital scale to the nearest of 0.01 g. The increased rate of egg mass was calculated by dividing the increase of egg mass during the experimental incubation (a period of 18 days) by the egg mass at the beginning of the experiment. From 5 to 7 days before hatching and when embryos were in the last developmental stages, the incubation of four eggs from each treatment was ended in order to analyse them for As and other metals. The four eggs analyzed from each treatment correspond to the same 4 clutches, thus reducing the influence of genetic or maternal effects. These analyzed eggs had been exposed to As from 24 to 26 days. The rest of the eggs were incubated in their As treatments until hatching in order to analyse the impact of As on hatching success and hatchling size and fitness.

As, Cd, Cu, Pb, and Zn concentrations were measured separately on embryos and eggshells. For element determination, 0.5 g of fresh tissue was placed in hermetic Teflon digester containers. Digestion was performed in an acid medium with 0.5 ml HNO_3 and H_2O_2 (4 drops), with the sample in the furnace at 100 °C for 4 hours. Analyses of Zn were performed using a flame Atomic Absorption Spectrometer (AAS) (Spectra A-100, Varian, Palo Alto, California, U.S.A.). Cu, Cd, Pb and As were measured using a Perkin-Elmer (PE) longitudinal AC Zeeman (AAnalyst 600) AAS equipped with a Transversely Heated Graphite Atomizer (Perkin-Elmer Hispania, S.A., Madrid, Spain). The instrumental detection limits (LOD) were: 0.2 ng/ml for As; 0.008 ng/ml for Cd; 0.1 ng/ml for Cu; 0.06 ng/ml for Pb and 10 ng/ml for Zn. All specimens were analysed in batches, with method blanks, known standards, and reference material, DORM-2 (dogfish (*Squalus acanthias*) liver), and TORT-2 (lobster hepatopancreas), from U.S. National Research Center (Washington D.C., U.S.A.). Accepted recoveries of reference material ranged from 88 to 110%. Relative standard deviation (RSD) in replicates and

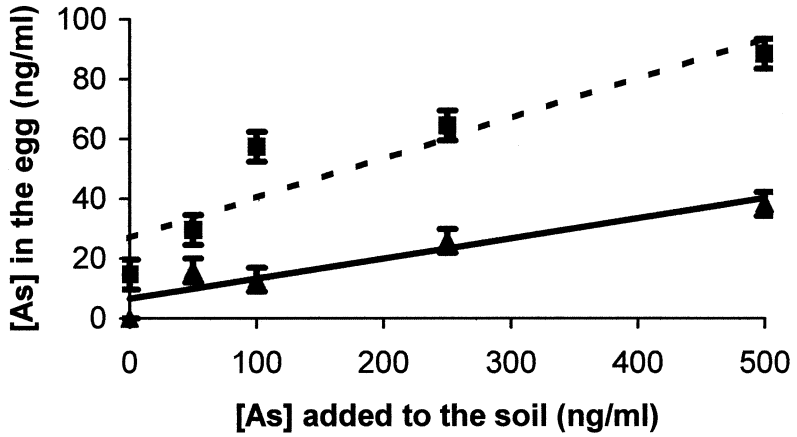

Figure 1. Relationship between As concentration (± SE) of water substrate and lizard eggshells and embryos after an exposure of eggs to different As concentrations during incubation. Squares and the dashed line correspond to eggshells and triangles and the continuous line correspond to embryos.

reference material was always below 10%. The bioconcentration factor (BCF) was calculated as the ratio of the concentration of the chemical in the eggshell or embryo to the concentration in the substrate water.

The remaining eggs hatched after an average of 36.3 days of incubation and 31.4 days of exposure to As. Immediately after hatching we measured hatchling snout-vent length (SVL) and body mass. We also examined hatchlings for external morphological abnormalities and behavioral alterations. Hatchlings were housed in 30 L plastic containers in the laboratory at approximately 26 °C under natural light and were fed with crickets that were dusted with multivitamin powder before being presented. To determine whether substrate As concentration during incubation had an effect on hatchling locomotor ability, we measured the running speed of each individual within the first 24 h after hatching. All hatchlings had the tail intact. Hatchlings were forced to run a distance of 1 m by chasing them by hand. The track was constructed of cardboard 120 cm long with vertical walls 30 cm high positioned 20 cm apart and the floor was lined with sand. To calculate running speed we considered the time hatchlings took to run 100 cm, excluding the first and the last 10 cm of the track. Running times were recorded with a stopwatch to the nearest of 0.1 s. We tested each hatchling once. At the end of the experiment, hatchlings were released in the area where females had been collected.

RESULTS AND DISCUSSION

At the end of the experiment, As in control eggs was very low on eggshells and undetectable in embryos (Table 1). For that reason, and given that the eggs from all treatments were produced simultaneously by the same females, we believed

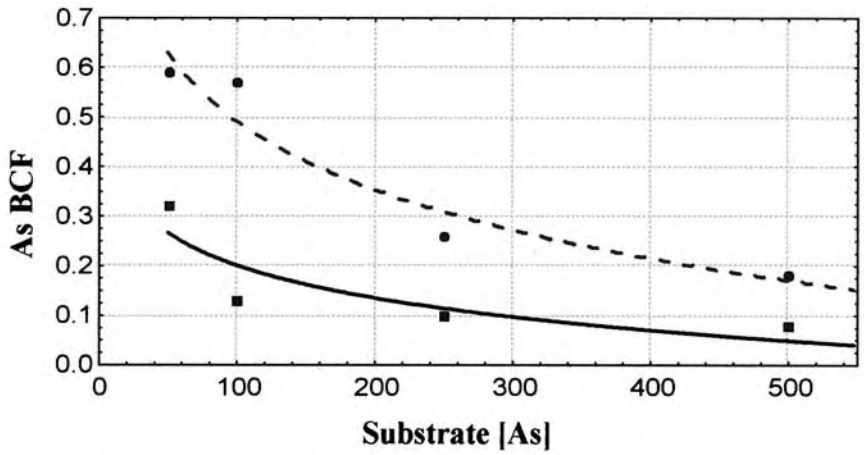

Figure 2. Variation in arsenic bioconcentration factor with exposure concentration of eggshells (dashed line) and embryos (full line) of Iberian rock lizard eggs incubated in As-contaminated substrates.

that at the beginning of the experiment, As concentration was also very low in all of the incubated eggs. As a result, we believe that maternal transfer of As to the incubated eggs was very low. However, in As-contaminated substrates, eggs absorbed and embryos accumulated during incubation significant amounts of As. We found a positive and highly significant relationship between the amount of As in the substrate water and As concentration both in eggshells ($r=0.923$, $F_{1,3}=17.32$, $P=0.025$) and in embryos ($r= 0.943$, $F_{1,3}=24.3$, $P=0.016$)(Fig. 1). There was no relationship found between As concentration in the substrate water and concentration of Cd, Zn, Cu or Pb either on eggshells or in embryos ($P>0.1$ in all cases).

As concentration was significantly higher in eggshells than in embryos (paired t-test: $t=4.222$, $P=0.013$) indicating that the eggshell partially blocked the passing of As into the eggs. However, there was no difference between embryo and eggshell concentration for the rest of trace elements (paired t-test: Cu - $t=2.269$, $P=0.086$; Cd - $t=1.427$, $P=0.227$; Zn - $t=1.783$, $P=0.149$; Pb - $t=0.065$, $P=0.951$). The bioconcentration factor of As in eggs was negatively related to exposure concentration (Fig. 2). BCFs in eggshells ranged from 0.629 at 50 ng As /ml in water to 0.171 at 500 ng/ml. BCFs in embryos ranged from 0.265 at 50 ng/ml As in water to 0.048 at 500 ng/ml. Embryonic As varied from 8 to 32 % of As in substrate water. These results indicate that eggshells do not fully protect reptilian embryos from soil contaminants. As in soils may easily exceed concentrations that we used in our experiment and an excess of As in soils could be harmful to flexible-shelled reptile eggs. Further studies are necessary to understand the extent of the problem of permeability of reptile eggs to soil pollutants during incubation.

Permeability of eggs to soil contaminants should be considered before using eggs collected in their nests for the biological monitoring of toxicants on adults. Soil

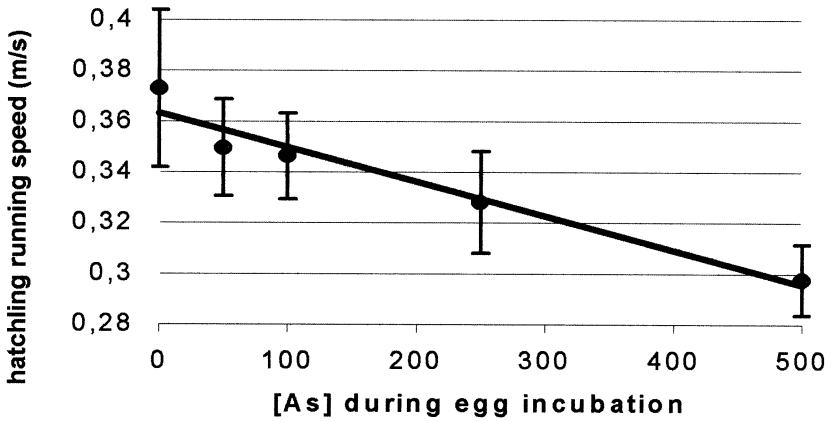

Figure 3. Relationship between average hatchling running speed (± SE) and exposure to arsenic in the substrate water during lizard embryonic development.

contamination should be considered as an important factor in environmental risk assessment for reptile species with flexible-shelled eggs.

One embryo died during the experiment. We did not find any external developmental abnormalities in eggs or hatchlings. The tested levels of As had no effect on embryo survival, incubation duration and hatchling size. There was a strong negative relationship between the average hatchling running speed for each treatment and the As in the substrate water (Pearson correlation: $r = -0.975$, $F_{1,3} = 57.03$, $P = 0.005$)(Fig. 3) and this variability was independent of hatchling SVL. Substrate As containation hampered hatchlings in running and hence reduced escape and foraging efficiency. Escape using fast movements is an important defensive behavior for many lizard species and may be considered as an indicator of fitness (Bauwens and 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 the egg incubation on contaminated substrates may influence survival or success of juveniles.

Reptilian species are declining on a global scale and environmental contaminants have been suggested as one of the main causes (Gibbons et al. 2000). Reptiles are exposed to numerous environmental contaminants and many studies have documented their bioaccumulation in different tissues and eggs (Sparling et al. 2000). Most of these studies have been conducted in turtles and crocodilians (Meyers-Schöne and Walton 1994, Crain and Guillette 1998), and very few studies have been conducted on snakes or lizards (Campbell and Campbell 2002, Talent et al. 2002). Moreover, the bioaccumulation of toxicants or any resulting toxic effect on eggs has been assumed to have its only origin in maternal transference during egg formation (Kleinow et al. 1999, Russell et al. 1999). Very few experiments document the effects and the dose-effect relationship of contaminants to reptiles

(Sparling et al. 2000). However, this study provides evidence of an uptake of a contaminant from the nest environment and its bioaccumulation and toxic effect on embryos. We suspect that soil contamination may be contributing to the decline of reptiles with flexible-shelled eggs.

Acknowledgments. We thank M.J. González Carlos and M. Fernández for their help with chemical analysis conducted in the IQOG (CSIC, Madrid, Spain). We also thank C. Díaz Paniagua, N. Sillero, A. Perera, N. Perez-Santigosa, J. Hidalgo y M. González. This study was funded by the Spanish Ministry of Science and Technology and the regional Government of Castilla and Leon.

REFERENCES

Ackerman RA (1980) Physiological and ecological aspects of gas exchange by sea turtle eggs. Amer Zool 20:575-583.

Avery RA, Mueller CF, Smith JA, Bond DJ (1987) The movement patterns of lacertid lizards: speed, gait and pauses in *Lacerta vivipara*. J Zool 211: 47-63.

Bauwens D, Thoen C (1981) Escape tactics and vulnerability to predation associated with reproduction in the lizard *Lacerta vivipara*. J Anim Ecol 50: 733-743.

Campbell KR, Campbell TS (2002) A logical starting point for developing priorities for lizard and snake ecotoxicology: a review of available data. Environ Toxicol Chem 21: 894-898.

Canas JE, Anderson TA (2002) Organochlorine contaminants in eggs: the influence of contaminated nest material. Chemosphere 47: 585-589.

Chang LW (1996) Toxicology of metals. Lewis Publ, Boca Raton, FL, USA.

Crain DA, Guillette LJ Jr (1998) Reptiles as models of contaminant-induced endocrine disruption. Anim Reprod Sci 53: 77-86.

Di Giulio RT, Tillitt DE, eds (1999) Reproductive and developmental effects of contaminants in oviparous vertebrates. SETAC Press, Pensacola, FL, USA.

Gibbons JW, Scott DE, Ryan TJ, Buhlmann KA, Tuberville TD, Metts BS, Greene JL, Mills T, Leiden Y, Poppy S, Winne CT (2000) The global decline of reptiles, déjà vu amphibians. Bioscience 50: 653-666.

Kleinow K, Baker J, Nichols J, Gobas F, Parkerton T, Muir D, Monteverdi G, Mastrodone P (1999) Exposure, uptake, and disposition of chemicals in reproductive and developmental stages of selected oviparous vertebrates. In: Di Giulio RT, Tillitt DE, (eds) Reproductive and developmental effects of contaminants in oviparous vertebrates. SETAC Press, Pensacola FL, p 9-112.

Linder G, Grillitsch B (2000) Ecotoxicology of metals. In: Sparling DW, Linder G, Bishop CA (eds) Ecotoxicology of amphibians and reptiles. SETAC Press, Pensacola, FL, p 325-460.

Meyers-Schöne L, Walton BT (1994) Turtles as monitors of chemical contaminants in the environment. Rev Environ Contam Toxicol 135: 93-153.

Overall KL (1994) Lizard egg environments. In: Vitt LJ, Pianka ER (eds) Lizard ecology: historical and experimental perspectives. Princeton University Press, NJ, USA, p 51-72.

Packard GC (1991) Physiological and ecological importance of water to embryos

of oviparous reptiles. In: Deeming DE, Ferguson MWJ (eds) Egg incubation: its effects on embryonic development in birds and reptiles. Cambridge University Press, Cambridge, UK, p 213-228.
Packard GC, Packard MJ (1988) The physiological ecology of reptilian eggs and embryos. In: Gans C, Huey RB (eds) Biology of the Reptilia,Vol. 16 - Ecology B, Defense and life history. Alan R Liss Inc, New York, NY, USA, p 523-606.
Rose KA, Brewer LW, Barnthouse LW, Fox GA, Gard NW, Mendonca M, Munkittrick KR, Vitt LJ (1999) Ecological responses of oviparous vertebrates to contaminant effects on reproduction and development. In: Di Giulio RT, Tillitt DE (eds) Reproductive and developmental effects of contaminants in oviparous vertebrates. SETAC Press, Pensacola, FL, p 225-282.
Russell RW, Gobas FPC, Haffner GD (1999) Maternal transfer and in ovo exposure of organochlorines in oviparous organisms: a model and field verification. Environ Sci Technol 33: 416-420.
Sparling DW, Linder G, Bishop CA, eds (2000) Ecotoxicology of amphibians and reptiles. SETAC Press, Pensacola, FL, USA.
Talent LG, Dumont JN, Bantle JA, Janz DM, Talent SG (2002) Evaluation of western fence lizards (*Sceloporus occidentalis*) and eastern fence lizards (*S. undulatus*) as laboratory reptile models for toxicological investigations. Environ Toxicol Chem 21: 899–905.
Thompson MB (1987) Water exchange in reptilian eggs. Physiol Zool 60:1-8.