Bull. Environ. Contam. Toxicol. (2004) 73:125–131

DOI: 10.1007/s00128-004-0403-3

Bulletin of Environmental Contamination and Toxicology

Toxic Effects of Ammonium Nitrate Fertilizer on Flexible-Shelled Lizard Eggs

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Received: 15 April 2003/Accepted: 20 April 2004

The use of chemical fertilizers is a very common practice all over the world and very often there is an excess of nitrate and ammonium in agricultural areas (Tamm 1991). Several studies document the impact of nitrogen saturation of soils on crops, grasslands and surrounding areas or the impact in aquatic ecosystems of nitrogen loadings (Vitousek 1997; Aber 1992). However, few studies analyze the effect of an excess of nitrate or ammonium on soil fauna (Tamm 1991). Many oviparous species deposit their eggs in subterranean nests. During embryonic development, eggs of these species maintain physical contact with the surrounding soil. Moreover, many of these terrestrial species lay eggs with permeable shells that during incubation exchange gasses and water with the soil (Packard and Packard 1988). For these reasons, these type of eggs are exposed to perturbations of soil quality and specifically to an excess of chemical fertilizers.

Unionized ammonia can be neurotoxic and rapidly lethal. Nitrate can reduce to nitrite and this ion can cause methemoglobinemia where the blood can no longer bind oxygen (Lewis and Morris 1986). Newborn humans are particularly susceptible to nitrate poisoning because fetal hemoglobin is more readily oxidized to methemoglobin (Kross et al. 1992). Embryos and larvae of some aquatic species such as fishes and amphibians are also very sensitive to high levels of chemical fertilizers in the water (Williams and Eddy 1989; Marco et al. 1999). We hypothesized that an excess of ammonium nitrate in the soil could have detrimental effects for the embryonic development of oviparous species. Fertilizers could directly affect the embryos or may modify the egg environment, thus influencing water or gas exchange between the egg and the surrounding soil.

We have tested whether nitrogen pollution affects embryonic development of two small lizard species, *Podarcis hispanica* and *Podarcis carbonelli.* We have also tested the influence of vegetation on the egg-fertilizer interaction. Plant roots absorb nutrients such as nitrate and ammonium from the soil. Thus, the presence of roots near the eggs could reduced the impact of fertilizers on embryos.

MATERIALS AND METHODS

We collected 12 gravid Carbonell wall lizard females (*Podarcis carbonelli*) and 7 gravid Spanish wall lizard females (*Podarcis hispanica*) from mountain areas in

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Sierra de Francia (Salamanca, Spain), during the last week of May 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. Dechlorinated tap water was always available. The bottom of each container was filled with 8 cm of wet sand that was watered regularly. Females were fed living *Tenebrio* sp larvae ad libitum. Egg laying took place within 15 days of captivity. Females laid an average of 2.14 eggs per clutch for *P. hispanica* and 2.58 eggs per clutch for *P. carbonelli*. Immediately after oviposition, eggs were extracted from the sand, cleaned with a soft brush, individually marked with a graphite pencil on the eggshell, weighed (± 0.01 g) and measured (± 0.1 mm). Approximately 1 to 13 days before the beginning of the experiment eggs were incubated at 26 °C in wet sand. After egg laying, females were released at their respective collection sites.

The effects of ammonium nitrate on lizard egg incubation were tested incubating single eggs in wet vermiculite. One fertile egg of each clutch was randomly assigned to one of four treatments: 1) plants and fertilizer; 2) plants and control; 3) no plants and fertilizer; 4) no plants and control). For *Podarcis hispanica* we incubated 4 eggs at treatment 1, 4 eggs at treatment 2, 3 eggs at treatment 3, and 4 eggs at treatment 4. For *Podarcis carbonelli* we incubated 8 eggs at treatment 1, 8 eggs at treatment 2, 8 eggs at treatment 3, and 7 eggs at treatment 4. The experiment was conducted in June and July (2001) in open-air enclosures at an average temperature of 26 °C. The experiment was conducted until egg hatching.

At the beginning of the experiment we recorded egg weight to the nearest of 0.01 g. The forty six eggs were individually introduced inside plastic containers (110 ml) filled with 100 ml of sterile vermiculite that completely covered the eggs. In containers corresponding to treatments 1 and 2, we deposited 20 selected seeds of *Festuca rubra* (BASF S.A, Barcelona) at a depth of 5 mm. Then, in containers corresponding to treatments 1 and 3 we added to the vermiculite 40 ml of a concentrated solution of ammonium nitrate (250 mg NH_4NO_3 / L) to obtain a concentration in the substrate of 100 mg/L. This concentration is high but realistic in the upper layers of fertilized soils after applications of ammonium nitrate of 80-120 Kg N/ha (Tamm 1991). Then, the substrate of each container was moistened with distilled water until the acquisition of a water potential of –150 kPa following methodology proposed by Packard et al. (1987). Substrates with this water potential are wet and permit correct water absorption by eggs and successful embryonic development of most of reptiles (Packard and Packard et al., 1988). In controls we only added distilled water until the acquisition of the same water potential. At this point each container was weighed to the nearest 0.01 g. During the experiment we periodically weighed each container to control for water losses and distilled water was added to the containers until the initial weight was obtained. Water loss never exceeded 20 % of initial weight. Incubation lasted on average 63 days and eggs were exposed to treatments for an average of 50 days. Immediately after hatching, we measured hatchling total length and weight (individuals with intact tail). We also checked for abnormalities or behavioral alterations. Vegetation (leafs and roots) was cleaned and dried at 80 °C until

constant weight and then, was weighed to the nearest 0.01 g. Hatchlings were housed in 30 L plastic containers in the laboratory at approximately 26 °C under natural light and were fed crickets.

To determine whether the exposure to chemical fertilizer during incubation had an effect on hatchling locomotor's abilities, 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 continuously 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 positioned 20 cm apart; the floor was lined with filter paper. To calculate the running speed we considered the time that hatchlings took to travel 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 twice and considered the mean running speed for each individual. Between each trial, hatchlings were allowed to rest for 2 min. Air temperature during the tests was approximately 26 °C. At the end of the experiment hatchlings were released in the area where females had been collected.

To determine the effect of chemical fertilizers on embryo survival we used the Chi-square test. To determine whether fertilizers or plants had an overall effect on egg incubation, we used four separate 3 way-ANCOVAs, one for each dependent variable. We considered as dependent variables: time to hatching, hatchling weight and length, and running speed. The covariate for the 3 first dependent variables was the initial weight of eggs and for the running speed was the hatchling length. The factors were the species, the presence of fertilizer and the presence of plants. We used Tukey honest significance differences for post-hoc comparisons of ANCOVAs. All the assumptions of these analysis were previously verified.

RESULTS AND DISCUSSION

Mortality was only detected in treatment with fertilizers and without plants, and was significantly different from expected considering the four treatments (Chi-square test = 9.546, df=3, $P<0.023$). 27 % of eggs exposed to fertilizers and without plants died but there was no mortality in the rest of treatments. There were differences between species on incubation duration and hatchling weight (Table 1). Fertilizers had a significant negative effect on hatchling weight and length (Table 1, Fig. 1). We did not find any external developmental abnormalities on eggs and hatchlings. However, hatchlings from eggs incubated with fertilizers were significantly smaller on body length (Table 2) and weight (10 %) than controls (Fig. 1). Vegetation did not evidence any significant effect on hatchling features. Only in *P. carbonelli* a trend to heavier hatchlings were observed when eggs were incubated without plants. (Table 1 and Fig. 1). There was no effect of fertilizers on hatchling running speed (3-way ANCOVA, effect: fertilizer, $F=0.018$, $P=0.894$; effect: fertilizer x species, $F=302$, $P=0.587$; effect: fertilizer x vegetation, $F=0.868$, $P=0.359$; effect: fertilizer x vegetation x species, $P=0.079$, $P=0.780$).

Table 1. Results of ANCOVAs that analyze the effect of ammonium nitrate and vegetation on embryonic development of two Iberian lizard species.

Variable	Incubation duration		Hatchling mass		Hatchling length	
Effect	F	P	F	P	F	P
Initial egg mass*	33.77	< 0.001	9.05	0.005	19.12	< 0.001
Fertilizer	0.87	0.358	4.14	0.049	4.26	0.047
Vegetation	0.52	0.478	0.14	0.714	0.16	0.693
Species	22.93	< 0.00	13.72	< 0.001	2.13	0.154
Fertilizer x Vegetation	0.01	0.93	0.26	0.613	3.38	0.075
Fertilizer x Species	0.53	0.471	1.83	0.185	0.02	0.878
Vegetation x Species	0.73	0.399	0.69	0.411	0.09	0.770
Fertil x Vegetat x Species	0.03	0.856	0.16	0.690	0.30	0.587

*Covariate

Chemical fertilizers, sewage, and livestock residues are very common pollutants that are contributing to the alteration of the global soil nitrogen cycle (Vitousek et al. 1997; Aber 1992). Nitrogen is one of the most important limiting nutrients of terrestrial, freshwater, and marine ecosystems and the impacts of elevated levels of a major limiting nutrient are well documented. The results of this study demonstrate that an excess of chemical fertilizers into the soil can also affect the embryonic development of terrestrial oviparous species with flexible-shelled eggs.

Reptilian eggs and embryos are affected profoundly by the availability of water in their environment (Ackerman 1991; Packard 1999). Many factors affect the exchange of water between a reptilian egg and its environment (Packard 1999). For example, the soil water potential in the nest may influence hatching success and hatchling size (Packard and Packard 1988). In our experiment, we incubated all the eggs in wet substrates and enough water was present near the eggs to guarantee an optimal embryonic development. However, an excess on ammonium nitrate can decrease soil water potential and eggs could have lower availability of water during incubation (Killham 1994). Further studies are necessary to understand the physiological mechanisms that explain the impact of ammonium nitrate on embryonic development of flexible-shelled reptiles.

Hatchlings from eggs of the two lizard species studied, incubated in fertilizer-polluted substrates had lower body mass and size than controls. This is a similar effect as the one described for eggs incubated in dry versus wet environments. Large or well-hydrated hatchlings usually survive better than small during the neonatal period (Packard 1999; Ferguson and Fox 1984). For example, large hatchlings may be better than small emerging from the dry nests, avoiding

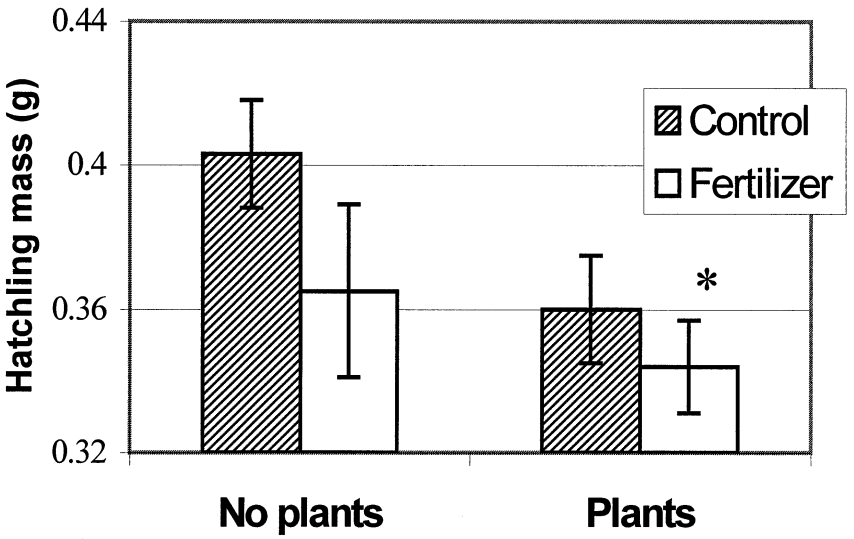

Figure 1. Effect of incubation substrate pollution by ammonium nitrate (100 mg N/L) and presence of vegetation on hatchling weight (mean ± SE) of Carbonell wall lizard. * Significant differences from the control with no plants (Tukey test, $P<0.05$).

predators or capturing larger preys (Packard 1999). Vegetation was expected to reduce the effect of fertilizers on embryonic development. It partially protected embryos from mortality, which was only detected in treatments with no plants, but did not reduce the negative effects that caused smaller hatchling size.

Many reptiles lay in underground nests eggs with flexible and permeable shells (Packard and Packard 1988). Eggs can absorb water from the soil increasing their weight and volume up to three or four times from egg laying. Water absorption and gas exchange are necessary to successfully complete embryonic development (Packard and Packard 1988). Some studies have analyzed the maternal transference of pollutants and their bioaccumulation on reptile eggs (for example: Russell et al. 1995) and estimated the impact of pollutant concentration inside the eggs on embryonic survival and development. However, there are no studies that analyze the impact of soil pollution on reptile eggs (reviews of reptile ecotoxicology in: Sparling et al. 2000, Di Giulio and Tillitt 1999, Campbell and Campbell 2002).

Reptiles are exposed to numerous environmental contaminants in the field and can accumulate them in different tissues (Sparling et al. 2000). During egg formation females can transfer pollutants to eggs and thus, affecting embryos (Russell et al. 1999). However, until now there was no evidence of a direct effect of soil pollution on reptile eggs that are developing in underground nests. This is the first study that demonstrate the impact of a widespread soil pollutant on reptile eggs.

Table 2. Influence of the experimental exposure to ammonium nitrate (100 mg N/L) on *Podarcis hispanica* and *Podarcis carbonelli* egg incubation and hatchlings. Data correspond to average (SD) values of the incubation duration, hatchling size and running speed.

	Podarcis hispanica		*Podarcis carbonelli*	
	Fertilizer	Control	Fertilizer	Control
Incubation duration (d)	63.87 (1.079)	66.00 (0.881)	61.75 (0.673	61.00 (0.645)
Hatchling weight (g)	0.30 (0.018)	0.33 (0.016)	0.35 (0.012)	0.38 (0.012)
Hatchling length (mm)	60.31 (1.236)	61.88 (1.089)	60.47 (0.770)	63.28 (0.770)
Running speed (m/s)	0.178 (0.041)	0.160 (0.026)	0.155 (0.020)	0.130 (0.020)

Reptile species are declining on a global scale and environmental pollution has been suggested as one of the main causes (Gibbons et al. 2000). Soil pollution could be an important source of reptile egg mortality and may be contributing to the decline of reptiles with flexible-shelled eggs.

Acknowledgments. The Spanish Ministry of Science and Technology funded this study (PB 97-1162). We thank Valentín Pérez-Mellado, Ana Andreu y Matati Pérez-Santigosa for their help.

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