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Sensitivity and Behavior of the Iberian Newt, *Triturus boscai,* Under Terrestrial Exposure to Ammonium Nitrate

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During the last sixty years an increase in the addition of nitrogen to the environment has been observed, mainly as part of chemical fertilizers (Vitousek et al. 1997). This nitrogen excess is known to be potentially dangerous for amphibians and has been proposed as a possible cause of the global decline of populations (Blaustein et al. 1994). One of the most widely used fertilizers around the world is ammonium nitrate (FMA, MAFF & SOAFD 1993). This substance is applied on crop fields as granules and, because of its high solubility, a great percentage of the amount applied is washed by runoff to the nearest water bodies where amphibians breed (Crews and Peoples 2004). The negative effects of the nitrogen excess on amphibian embryos and larvae have already been demonstrated (e.g. Ortiz et al. 2004). However, when fertilizer is still in the soil, it may also affect terrestrial stages of amphibians. Actually, several isolated cases of adult amphibian mortality in the field have been related to the application of nitrogenous fertilizers (Schneeweiss and Schneeweiss 1997). Terrestrial stages of amphibians might detect and, thus, avoid polluted areas. However, both the selection ability and the potential effects of terrestrial habitat pollution on amphibians have hardly been analyzed. In spite of the apparently higher sensitivity of aquatic stages of amphibians, the effects of pollutants on terrestrial stages could affect populations at a higher level by altering breeding migrations or reproductive habitat selection.

The aim of this work is to analyze the sensitivity of adult Iberian newt (*Triturus boscai*) to the application of ammonium nitrate on substrate by using filter papers as experimental substrates, which allow the control of fertilizer doses. On the other hand, we assess if newts are able to detect ammonium nitrate on substrate by conducting an experiment of substrate selection. *T. boscai* is an endemic species from the western Iberian Peninsula. Adult individuals of the species present a terrestrial phase which usually coincides with the driest periods (Caetano and Leclair 1999). Before the reproductive season, adults migrate towards their breeding points. Although habitat pollution has been proposed as one of the main threatening factors for the species, the effects of pollutants on the species, both in terrestrial and aquatic environments, have not been evaluated.

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MATERIALS AND METHODS

We conducted two experiments. In the first, we assessed the sensitivity of newts by exposing them to seven different doses of ammonium nitrate. Then, we selected, for the second experiment, four fertilizer concentrations which were combined in order to assess if newts were able to select substrates with non-dangerous concentrations. We collected forty-four adult *T. boscai* (22 males and 22 females) in Vilvestre, Salamanca (Western Spain) in October 2002, coinciding with migrations of the species towards the breeding ponds, when newts are fully exposed to the substrate pollution. Newts were collected with the permission of the regional authorities. Newts were taken to the laboratory where they were individually placed in aquaria containing 2 L dechlorinated tap water at 20°C and with the natural sunlight photoperiod. Each individual was randomly assigned to one of the two experiments (28 to the first experiment and 16 to the second), in such a way that the same number of males and females was assigned to each experiment. Experiments were conducted between October 23rd and 25th 2002.

In the first experiment, we used terrariums of 15.3 x 15.3 cm of base on which we placed filter papers with different doses of ammonium nitrate. Each terrarium was assigned to one of the following treatments: 0, 10, 25, 40, 55, 70 and 85 Kg N / ha, which implied the addition of 0, 0.067, 0.167, 0.268, 0.368, 0.468 and 0.568 g of ammonium nitrate respectively. These levels are below those that are usually applied on crop fields (60-260 kg N / ha, Kraft and Stites 2003). Each treatment was replicated four times. Ammonium nitrate was applied as granulated salt (99% maximum purity. Merck KGaA®, Darmstad, Germany). After application, 5 ml dechlorinated tap water was sprayed on each terrarium in order to give the newts a wet substrate and to dissolve the ammonium nitrate. Fifteen minutes after application, one newt was placed in the center of each terrarium. Assignation of newts to the different terrariums was random, and the four replicates of every treatment were constituted by two males and two females. Each newt was exposed until some anomalous effect was observed; in the cases in which effects were not observed, individuals were kept in terrariums a maximum of five minutes. The time of appearance of effects was compared among treatments with a Kruskal – Wallis test. The kind of observed effect in each newt was recorded. We distinguished between two kinds of effects: strong effects, such as irregular and convulsive movements or respiratory difficulties, that presumably would have led to death if the newts had not been removed from terrariums in time, and slight effects, such as the appearance of slow and clumsy movements accompanied with body curving. All the individuals were washed with clean water after their exposure. We used probit regressions to calculate the medium doses (ED_{50}) of appearance of any kind of effects (strong or slight), as well as medium doses of appearance of strong and slight effects separately.

In the second experiment, we exposed sixteen newts individually to four ammonium nitrate doses as represented in Fig. 1. Each paper inside a terrarium was randomly assigned to one of the four ammonium nitrate doses (0, 10, 25 and 40 kg N / ha, which implied the addition of 0, 0.084, 0.210 and 0.336 g of

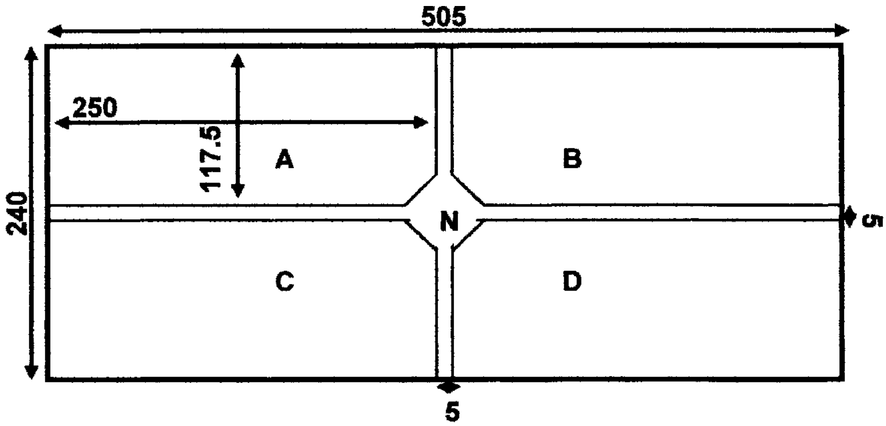

Figure 1. Experimental design of the terrarium in the second experiment. Numbers indicate measures (in mm). Letters A – D indicate the four filter papers on which ammonium nitrate was applied at different doses. "N" indicates the point where newt was placed at the beginning of the trial.

ammonium nitrate, respectively). These four doses were selected from the results of newt sensitivity to substrate pollution obtained in the previous experiment. After fertilizer application, we sprayed every paper with 5 ml of dechlorinated tap water. One newt was placed in the center of every terrarium 15 minutes after fertilizer application. Two of the sixteen newts began to run quickly just after being introduced in their terrariums, which was considered as an anomalous response; these two replicates were removed from the analyses. Newts were kept in the terrariums until they definitely stayed motionless on one of the four sectors. Sectors which animals initially moved towards, as well as sectors that the newts selected to definitely stay were recorded. To analyze if both initial and definitive selection depended on the treatment we used chi-square tests. In some cases, newts initially moved towards a sector in which they definitely stayed. In the rest of the cases, newts moved from one sector to another before their definitive selection. The time that newts stayed in every treatment during these movements was measured. To assess if the behavior of the newts (stay on the first sector or change) depended on the initially selected treatment, we made a contingency table which was analyzed with a chi-square test, with the initially selected treatment and the response of the animals as the entrances of the table. Finally, for newts that moved, the time of stay on each treatment was analyzed with a Kruskal-Wallis test.

RESULTS AND DISCUSSION

None of the newts exposed to non-contaminated papers or to the lowest ammonium nitrate dose (10 kg N / ha) showed any effect after the maximum exposure time. Time of appearance of effects significantly decreased with increased ammonium nitrate doses (Fig. 2) ($\chi^2 = 23.204$; 6 *df*; $P = 0.001$). All the individuals exposed to doses over 25 kg N / ha were affected by the pollutant. The

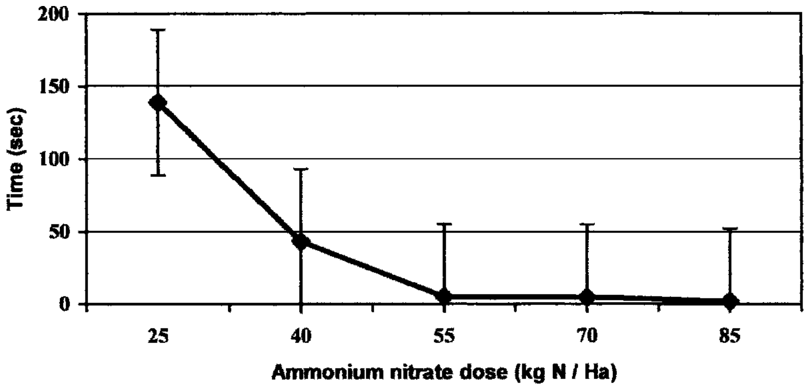

Figure 2. Overall time (±SE) of appearance of anomalous effects (generally body curving and respiratory difficulties) in adult *T. boscai* exposed to different doses of ammonium nitrate applied on substrate. The two lowest doses are not shown because they did not produce any effect.

ED_{50} based on the appearance of slight effects was 25.00 (±2.61) kg N / ha, and the ED_{50} based on the appearance of strong effects was 43.14 (±1.41) kg N / ha. Finally, the global ED_{50} if we consider both kinds of effects altogether was 22.94 (±2.14) kg N / ha.

The two pieces of filter paper with the non-dangerous ammonium nitrate doses (0 and 10 kg N / ha) were selected initially by 71.4% of individuals, and definitely by 100% of individuals (Fig. 3). No significant differences were detected among the three treatments (0, 10 and 40 kg N / ha) that were initially selected (χ^2 = 1.857; 2 *df*; $P = 0.395$). Among the two lower treatments, which were the only definitely selected, newts preferred the control (χ^2 = 4.571; 1 *df*; $P < 0.033$). Ten of the fourteen newts moved over their terrariums before selecting a definitive sector. The four individuals that did not change their initial selection were on controls, while none of the newts that initially selected a sector different from the control stayed at that sector. Although this result was not statistically significant (χ^2 = 5.600; 2 *df*; $P = 0.066$), this *P*-value is low enough to consider the influence of initial treatment on behavior of newts. The time spent on each sector by individuals that moved tended to be lower with the increased ammonium nitrate doses (Fig. 4). However, differences among treatments in time of stay were not significant (χ^2 = 4.448; 3 *df*; $P = 0.217$).

Our results show that doses of ammonium nitrate on substrate over 25 kg N / ha affected adult *T. boscai*. With doses over 55 kg N / ha, effects were strong and potentially lethal. These levels are below the minimum recommended for fertilizer application on crop fields (Kraft and Stites 2003). To our knowledge, there is only one study that has analyzed the effects of ammonium nitrate on amphibians in the terrestrial environment. Oldham et al. (1997) demonstrated that the fertilizer produced toxic effects on adult *Rana temporaria* in acute exposures.

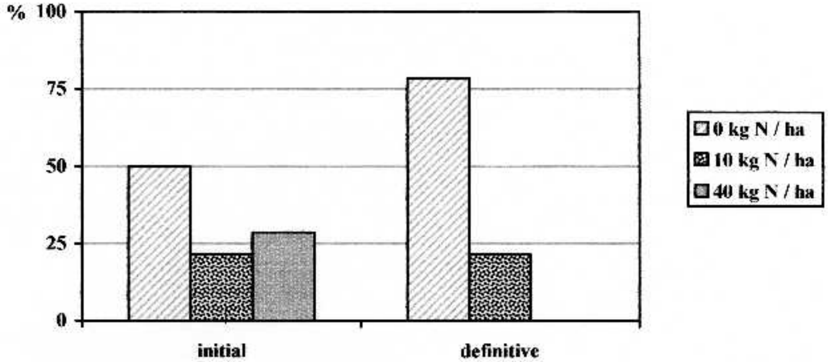

Figure 3 Percentages of initial and definitive selection of ammonium nitrate doses showed by adult *T. boscai*. Results from 25 kg N / ha are not shown because this treatment was not selected by any newt.

When filter papers were used as substrates for ammonium nitrate application, the effects appeared at doses of 3.1 g NO_3NH_4 / m^2 (10.85 kg N / ha) after 180 minutes of exposure. This dose did not produce any effect in our experiment, although the great difference in exposure times between studies did not allow the comparison of results. The ED_{50} for *R. temporaria* based on alteration of respiratory frequency was 6.9 g NO_3NH_4 / m^2 (24.15 kg N / ha) (Oldham et al. 1997), which is very similar to the value obtained from our results. However, time of effect was again very different, because while the effects on *R. temporaria* appeared after 5-330 minutes of exposure, only between 2 and 139 seconds were needed for the negative effects to be observed in *T. boscai*.

Individuals exposed to the highest ammonium nitrate doses showed abnormalities when breathing. In line with our results, Oldham et al. (1997) observed how the respiratory rate in *R. temporaria* was affected by the exposure to the fertilizer. Furthermore, during the experiments conducted by these authors, two frogs died in the post-exposure period as a consequence of the ammonium nitrate effects on the respiratory system. Species with a higher degree of cutaneous respiration present more permeable skin, and thus are more vulnerable to pollutant diffusion than species with bucopharyngeal or pulmonary respiration (Shoemaker et al. 1992). Marco et al. (2001) observed that the rough skin salamander, *Taricha granulosa*, was very tolerant to urea exposure in the terrestrial environment. This is a very terrestrial species whose skin is relatively thick, so it would be well protected against chemical diffusion. The other two species tested in that work, *Plethodon vehiculum* and *Rhyacotriton variegatus*, were more sensitive to urea (Marco et al. 2001). These two species present a high degree of cutaneous respiration as well as the absence or significant reduction of the lungs, which implies that their skins are rather thinner than that of *T. granulosa*. Species of the genus *Triturus*, because of their aquatic habits, have poorly little developed lungs, and the efficiency of their pulmonary respiration is very low, which induces them to use cutaneous respiration more actively (Duellman and Trueb 1994). Thus, *T. boscai* have very thin, permeable skin, which might explain the high sensitivity to

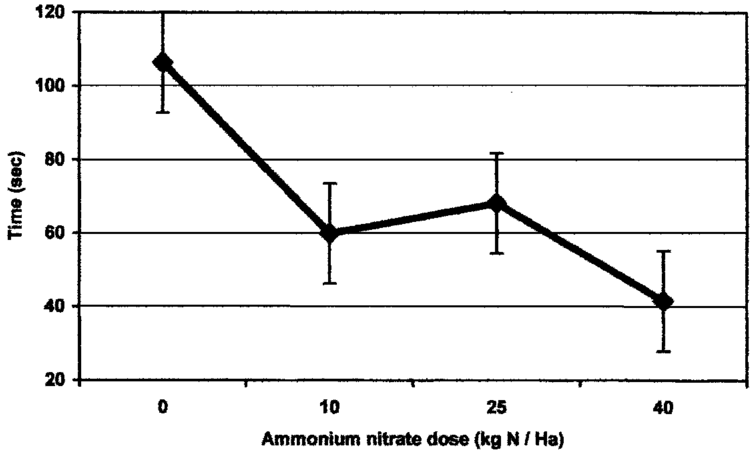

Figure 4. Overall time of stay (±SE) of adult *T. boscai* in each one of the four treatments during their movements over the terrariums.

ammonium nitrate observed in our study.

Although we have observed a high sensitivity of *T. boscai*, even at levels below those recommended for fertilizer application, we must consider several points in the interpretation of our results. We have used papers as substrates, where fertilizer properties may be different than those observed when it is applied on soil. Oldham et al. (1997) demonstrated that ammonium nitrate doses that affected *R. temporaria* when fertilizer was applied on paper were lower than those which caused the same effects when applied on soil. We must also consider the persistence of the fertilizer in the environment. Ammonium nitrate has a high solubility in water, which implies that it is quickly washed from the soil. Oldham et al. (1997) observed how, in the field, the fertilizer was dissolved completely in two hours after application, and its effects on *R. temporaria* disappeared after 110 minutes. Since the field application of fertilizer is usually accomplished during the daytime and most amphibians are active during the night, the low persistence of ammonium nitrate would decrease the probability of individuals becoming exposed to the highest environmental doses of fertilizer.

In our second experiment, newts preferred the harmless treatments. However, there was not an initial selection of these harmless treatments and newts' behavior did not depend on the initially selected substrate. This suggests that newts are not able to identify the appropriate substrates if they do not taste them before. This hypothesis would be supported by the fact that the time spent on each sector was not related to the ammonium nitrate dose. Pollution-related avoidance behavior has been analyzed in amphibians by a few works. Marco et al. (2001) observed how adult *P. vehiculum*, *T. granulosa* and *R. variegatus* exposed in terrariums in which half of the floor had been sprayed with urea, avoided exposure to the fertilizer by using mostly the clean half. Furthermore, our results partially coincide with those obtained by Hatch et al. (2001), who observed that newly metamorphic individuals of *Bufo boreas* and *Rana cascadae* avoided doses of 100 kg urea / ha when papers were used as substrates, while when fertilizer was applied on soil, the individuals were not able to discriminate polluted from control zones.

Amphibian behavior during their breeding migrations would depend on several factors. Chemical cues seem to be implied in the terrestrial habitat selection (Spieler and Linsenmair 1997). Ammonium nitrate could be detected by individuals and inform them about the unsuitability of a habitat, or, on the other hand, interact with the chemical cues used by amphibians and thus affect their ability to correctly select the habitat. Unfortunately, nothing is known about the effects of pollution on habitat selection by amphibians.

The application of chemical fertilizers in temperate regions of the northern hemisphere is mainly accomplished during spring (Chalmers et al. 1990), so it coincides with the reproductive season of many amphibian species. Between 10 and 60 % of fertilizer applied on crop fields is never used by plants (MAFF 1993). This produces a nitrogen excess both in soil and, when fertilizer is washed, in water bodies (Cameira et al. 2003). In spite of the factors that do not allow us to extrapolate our results to what is happening in the environment, we believe our study demonstrates that the direct contact of adult *T. boscai* with low and habitual doses of ammonium nitrate may produce strong effects in a very short time. An initial measure to avoid risk for individuals would be the improvement of fertilization efficiency. For example, Meyer-Aurich et al. (1998) demonstrated that a variation in the timing of fertilization increased the efficiency of fertilizers by decreasing the required excess of nutrients that plants have during the coldest months. Furthermore, this change in the time of fertilization minimized the risk of exposure of amphibians during breeding migrations. In most cases, a previous evaluation of soil characteristics and the synchronization of the fertilization with nitrogen demand would allow a better adjustment of the required amount of fertilizer in order to improve its efficiency (Crews and Peoples 2004), and thus decrease the potential risk for wildlife.

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