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Does chronic exposure to ammonium during the pre-metamorphic stages promote hindlimb abnormality in anuran metamorphs? A comparison between natural-habitat and agrosystem frogs

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Abstract. Despite their detrimental effects on locomotion, prevalence of hindlimb abnormalities is increasing in anuran populations worldwide. Among others, agrochemical pollution during the larval stage is a potential cause. However, populations exposed to such a strong selective pressure could evolve resistance. In this work, we examine the potential effects of chronic exposure to ammonium during the pre-metamorphic stages of *Pelophylax perezi* frogs on metamorph hindlimb abnormality prevalence, as compared with unpolluted-water-reared conspecifics. We conducted the experiment on tadpoles either from natural-habitat or from agrosystem parents. We detected no effect of chronic exposure to ammonium on hindlimb abnormality prevalence in frogs from either habitat, which suggests that the lack of effect detected is not related to resistance evolved in agrosystem frogs.

Keywords. Amphibian, anuran, anomaly, malformation, *Pelophylax perezi*.

Functioning appendages are fundamental for whole-organism performance of most anurans (Johansson et al., 2010; Zamora-Camacho et al., 2019). Hind limb morphology is directly responsible for locomotion of metamorph (Zamora-Camacho and Aragón, 2019a) and adult anurans (Zamora-Camacho, 2018), either from terrestrial (Gomes et al., 2009) or aquatic environments (Herrel et al., 2012), regardless of their locomotion mode (Enriquez-Urzelai et al., 2015). Therefore, hindlimb abnormality in this group is likely eradicated by natural selection due to its severe negative effects on locomotion (Zamora-Camacho and Aragón, 2019b). Consistently, prevalence of anuran appendage abnormality appears generally below 5% (Ouellet, 2000; Mester et al., 2015).

Nonetheless, limb abnormality rates are increasing in anurans worldwide (Johnson and Bowerman, 2010; Laurentino et al., 2016). These include diverse malformations, such as lacking and extra limbs and digits, as well as fused or misshaped limbs (Johnson and Bowerman, 2010; Reeves et al., 2013). Limb abnormalities are particularly common in metamorphs (Kiesecker, 2002; Piha et al., 2006), seemingly because reduced locomotor performance (Zamora-Camacho and Aragón, 2019b) might cause their death shortly after metamorphosis.

Besides a genetic origin (Droin and Fischberg, 1980), hindlimb abnormalities in anurans have been related to biotic interactions such as predatory pressure (Johnson and Bowerman, 2010) or parasite infections (Roberts and Dickinson, 2012), as well as abiotic factors such as ultra-

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violet-B radiation (Pahkala et al., 2001). However, human perturbance frequently provokes these malformations (Blaustein and Johnson, 2003), which are more common next to roads (Reeves et al., 2008) or in agrosystems (Ouellet et al., 1997; Spolyarich et al., 2011). Agrochemicals such as fungicides (Bernabo et al., 2016), pesticides (Jayawardena et al., 2010), and fertilizers (Xu and Oldham, 1997) increase limb abnormality prevalence in anurans. The aetiology of these malformations is often multiple (Meteyer et al., 2000): trematode infections (Haas et al., 2018) and predator attacks (Reeves et al., 2010) boost the effects of agrochemicals. Albeit, greater selective pressures could also drive the appearance of resistance to the environmental stressors (Miaud and Merilä, 2001), which could eventually reduce the prevalence of abnormalities in agrosystem populations.

Metamorph morphology is often related to tadpole growth history (Tejedo et al., 2000). In this work, we compare the prevalence of hindlimb abnormality in metamorphs of *Pelophylax perezi* (López Seoane, 1885) frogs resulting from tadpoles chronically exposed to ammonium contamination with unpolluted-water-reared conspecifics. Ammonium is among the most common compounds derived from agricultural fertilizers, with several negative effects on amphibian populations (Ortiz et al., 2004). We checked any possible resistance to contamination evolved in agrosystems by applying this treatment to tadpoles from natural habitats and from agrosystems. We expected higher prevalence of hindlimb abnormalities in metamorphs from the ammonium treatment. However, if agrosystem populations have evolved resistance, this effect would be greater in natural-habitat tadpoles.

Pelophylax perezi is a Ranid that occurs naturally throughout the Iberian Peninsula and southern France (Egea-Serrano, 2014), in a wide variety of habitats, but always in or not far from waterbodies, either pristine or polluted (Egea-Serrano, 2014). Indeed, it often inhabits human-altered habitats, such as urban or agricultural environments (Egea-Serrano, 2014).

Fieldwork was conducted in pristine Pinares de Cartaya *Pinus pinea* grove and surrounding agrosystems (SW Spain, 37º20’ N, 7º09’ W). Agrosystems are about 6 km away from pine grove, and consist of a traditional extensive vegetable crop area that has lately transitioned into intensive plantations regularly added fertilizers and at owners’ discretion.

In April 2018, 10 adult males and 10 adult females were randomly caught from each habitat. Capture was manual, and males were recognized for their greyish forelimb nuptial pads and their vocal sacs in the mouth commissures (Egea-Serrano, 2014). Frogs were pooled separately according to their provenance in two adjacent outdoor semi-natural enclosures with ponds (Fig. S1 in Supplementary Material). Ponds were daily checked for the presence of egg masses, which we transferred to the laboratory within 12 hours after they had been laid.

In the laboratory, we immediately separated eggs randomly in groups of 15. Each group was placed in an aquarium (Length×Width×Height: 38×27×19 cm) with 6 L of untreated spring water. In half of the aquariums, randomly chosen for each egg mass, we added 178.87 mg of 99.7% pure NH_4Cl, so we obtained a concentration of 10 mg NH_4^+/L. In a previous study on this species, a concentration of 13.5 mg NH_4^+/L caused circa 70% mortality rate in a mid-term experiment on larvae of this species from natural habitats (Egea-Serrano et al., 2009). We chose a concentration slightly lower in order to avoid such mortality rates, while triggering sublethal effects. The other aquaria contained untreated spring water, as a control. Thus, we had 15 aquaria with eggs from frogs from each habitat and treatment, totalling 60 aquaria, in a 2 × 2 factorial experimental design.

Aquaria were kept in shelves in the laboratory until larvae finished metamorphosis. Water was completely replaced twice a week, and each time we maintained the treatment and randomly changed the position of each aquaria within the shelves. A window let natural daylight in, permitting adjustment of circadian rhythms. Because tadpole diet can affect limb abnormality rates in this species (Martínez et al., 1992), all specimens were standardly fed boiled spinach *ad libitum*. In Gosner stage 42, preceding tail resorption (Gosner, 1960), tadpoles were transferred to tilted aquaria to allow them to exit the water as metamorphosis ended.

Some metamorphs presented an abnormality in one of their hindlimbs (Fig. 1). Abnormal limbs were aberrantly inserted in the pelvis with an approximate angle of 270º with respect to the body axis (Fig. 1). Moreover, the knee-joints were unable to fold normally in resting position (Fig. 1). In all cases, only one hindlimb was abnormal in each individual affected, either the right or the left appendage. We calculated the proportion (number of abnormal metamorphs divided by number of total surviving metamorphs) of abnormal-limbed metamorphs from each aquarium.

Data met the criteria of homoscedasticity and residual normality (Quinn and Keough, 2002), so we conducted a two-way ANOVA to test the effects of habitat, treatment, and their interaction on the proportion of abnormal-limbed metamorphs, using the software Statistica 8.0.

The total numbers and proportions of abnormal-limbed metamorphs from each habitat and treatment are in Table S1 in Supplementary Material. The effects of habitat ($F_{1, 56} = 0.026$; $P = 0.874$; Fig. 2), treatment ($F_{1, 56}$

Fig. 1. *Pelophylax perezi* metamorph affected by the hindlimb abnormality described, with a measuring tape in cm.

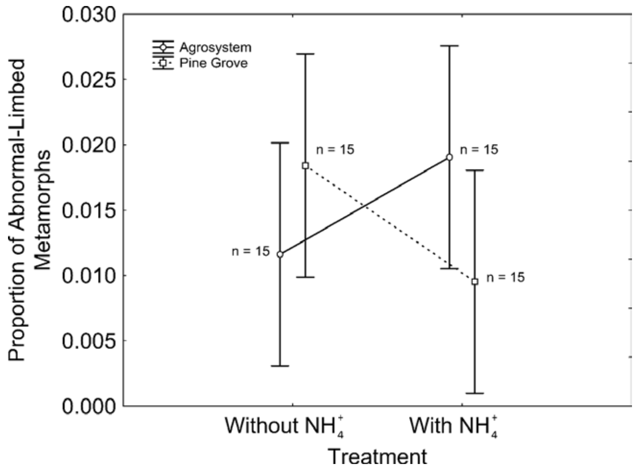

Fig. 2. Effects (mean ± 1SE) of treatment and habitat on the proportion of abnormal-limbed metamorphs obtained from each aquarium, calculated as the number of abnormal metamorphs divided by the total number of surviving metamorphs. Sample sizes indicated represent the number of aquaria in each treatment.

= 0.007; P = 0.932: Fig. 2), and their interaction ($F_{1,\,56}$ = 0.914; P = 0.343; Fig. 2) on the proportion of abnormal-limbed metamorphs obtained in each aquarium were non-significant.

At the concentration used, chronic exposure to ammonium during the larval stage does not increase the prevalence of hindlimb abnormality in these frogs. However, a subchronic exposure to even lower concentrations of this compound reduces survivorship (Egea-Serrano et al., 2009) and affects behaviour of *P. perezi* larvae (Egea-Serrano et al., 2011). Prevalence of limb abnormality in *Bufo bufo* toad metamorphs were higher following an acute exposure to 100 mg/L ammonium nitrate during the larval stage than following a subchronic exposure to 50 and 100 mg/L ammonium nitrate (Xu and Oldham, 1997). Those results could be a consequence of greater mortality of larvae in the subchronic exposure treatment (Xu and Oldham, 1997), which could mask potential limb abnormalities if future bearers die. Chronic exposure to other pollutants, such as mercury in *Rana sphenocephala* frogs (Unrine et al., 2004), and nickel, cobalt, or cadmium chlorides in *Xenopus laevis* frogs (Plowman et al., 1994), causes malformations in metamorphs. Also, subchronic exposure to carbamate and organophosphate pesticides causes malformations in *P. perezi* (Alvarez et al., 1995).

Juvenile *P. perezi* from agrosystems are smaller, and show increased limb fluctuant asymmetry, than conspecifics from natural habitats (Burghelea et al., 2013). However, we detected no effect of habitat on hindlimb abnormality prevalence on either treatment. Aligned with our results, prevalence of limb abnormality was not greater in *Rana temporaria* frogs from agrosystems than from natural habitats (Piha et al., 2006). Nevertheless, these findings contrast with others that detected increased prevalence of limb abnormality close to agrosystems in several anurans (Kiesecker, 2002; Guerra and Aráoz, 2016). Our results do not support the hypothesis of resistance in agrosystem frogs. We obtained an overall prevalence of hindlimb abnormality notably below the 5% detected in other wild amphibian populations (Ouellet, 2000; Mester et al., 2015). Low prevalence in both habitats could be a consequence of the capability of this species to thrive in polluted waters (Egea-Serrano et al., 2008).

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at < http://www.unipv.it/webshi/appendix > Manuscript number 10016.

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