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Aquatic Toxicology

Low levels of chemical anthropogenic pollution may threaten amphibians by impairing predator recognition

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ABSTRACT

Recent studies suggest that direct mortality and physiological effects caused by pollutants are major contributing factors to global amphibian decline. However, even sublethal concentrations of pollutants could be harmful if they combined with other factors to cause high mortality in amphibians. Here we show that sublethal concentrations of pollutants can disrupt the ability of amphibian larvae to recognize predators, hence increasing their risk of predation. This effect is indeed much more important since very low amounts of pollutants are ubiquitous, and environmental efforts are mostly directed towards preventing lethal spills. We analyzed the effects of two common contaminants (humic acid and ammonium nitrate) on the ability of tadpoles of the western spadefoot toad (*Pelobates cultripes*) to recognize chemical cues from a common predator, nymphs of the dragonfly *Anax imperator*. We compared the swimming activity of tadpoles in the presence and absence of water-borne chemical cues from dragonflies at different concentrations of humic acid and ammonium nitrate. Tadpoles reduced swimming activity in response to predator cues in the absence of pollutants, whereas they remained unresponsive to these cues when either humic acid or ammonium nitrate was added to the water, even at low concentrations. Moreover, changes in tadpole activity associated with the pollutants themselves were non-significant, indicating no toxic effect. Alteration of the natural chemical environment of aquatic systems by pollutants may be an important contributing cause for declines in amphibian populations, even at sublethal concentrations.

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1. Introduction

Chemical pollutants have been pointed out as one of the major drivers of human-induced environmental change (Millennium Ecosystem Assessment, 2005; Sih et al., 2011; Diamond et al., 2015). Pollutants are indeed considered among the five ultimate factors causing global decline of amphibians, the most vulnerable group of vertebrates (approximately 41% of the species endangered; Hoffmann et al., 2010). Most studies to date describe wastewater contaminants as a major threat to amphibian species, based on their lethal physiological and toxicological effects (see Egea-Serrano et al., 2012). However, sublethal levels of pollution can act indirectly causing physiological stress, reduced growth rates, or developmental and reproductive failures in amphibian populations (Boone and Bridges, 2006; Hayes et al., 2010; Baker et al., 2013). Pollution may also critically affect amphibians by disrupting chemoreception and altering individual behavior (Lürling and Scheffer, 2007; Lürling, 2012).

Chemical communication is of primary importance, particularly in aquatic systems that have poor lighting conditions, which reduce the effectiveness of visual signals, and where many organisms rely primarily on semiochemicals for mate recognition and predator detection (Brönmark and Hansson, 2000; Johansson and Jones, 2007). Accurate detection and recognition of chemical information released by predators, or by prey under attack, allow aquatic prey to respond plastically through inducible defenses (Chivers and Smith, 1998; Kats and Dill, 1998). Prey can often alter their morphology, life history, and/or behavior upon detection of predator kairomones, i.e., a predator's signature odor (Sih and Moore, 1993; Tollrian and Harvell, 1999; Lass et al., 2005; Chivers et al., 2008; see Ferrari et al., 2010 for an extensive review). Such plastic antipredator responses are generally fine-tuned to the level of risk experienced (Van Buskirk and Arioli, 2002; McCoy et al., 2012) and are the result of joint evolutionary history between predator and prey (Ferrari et al., 2010).

However, various anthropogenic activities have resulted in the contamination of freshwater ecosystems with a wide array

of harmful contaminants (i.e., pesticides, herbicides, fertilizers, or metals) (Kolpin et al., 2002; Carpenter et al., 2011; Lürling, 2012; Rasmussen et al., 2015). In addition to their direct toxic effects, chemical pollutants released in freshwater habitats alter the chemical environment, causing interferences in the cue recognition systems of aquatic organisms and impairing chemoreception (Seehausen et al., 1997; Wolf and Moore, 2002; Fabian et al., 2007). Disruption of chemical communication may have drastic ecological and evolutionary consequences, affecting foraging, navigation, mate choice, species recognition, and possibly predator detection or evasion (Brönmark and Hansson, 2000; Wolf and Moore, 2002; Wyatt, 2003; Fisher et al., 2006; Ward et al., 2008; Gill and Raine, 2014).

In water, some contaminants may bind to pheromones and block or damage chemoreceptors (Hubbard et al., 2002; Yang et al., 2002; Mesquita et al., 2003). For instance, elevated levels of humic acid are known to reduce the olfactory response of goldfish (*Carassius auratus*) and other teleosts to sexual pheromones (Hubbard et al., 2002). Humic acid also promotes hybridization of poeciliid fish (*Xiphophorus* sp.) due to the loss of mating preferences of females for conspecific males (Fisher et al., 2006), and has been demonstrated to interfere with species recognition in zebrafish (*Danio rerio*, Fabian et al., 2007). Likewise, chemical pollutants could affect predator–prey recognition in aquatic organisms by impairing chemical detection of predators (Dodson et al., 1995; Preston et al., 1999a,b; Schulz and Dabrowski, 2001).

Chemical pollution can affect many aquatic animals, but impacts on amphibian species are of special concern due to global declines of amphibian populations in the last decades (Hoffmann et al., 2010). Amphibian declines are correlated with proximity to agricultural lands (Bishop et al., 1999; Joly et al., 2001; Davidson, 2004), likely as a result of runoff of pesticides and fertilizers (Relyea et al., 2005; Relyea and Hoverman, 2006; Miaud et al., 2011). Ecologists have made a great effort to understand direct toxicological impacts of pesticides on larval amphibians (Diana et al., 2000; Boone and Semlitsch, 2001, 2002; Boone et al., 2001; Boone and Bridges, 2003; Boone and James, 2003; Sparling et al., 2010; Egea-Serrano et al., 2012), and studies have also addressed the synergistic effects of pesticides and predator cues on tadpole activity (Bridges, 1999; Rohr and Crumrine, 2005; Relyea and Edwards, 2010), growth (Kerby et al., 2011) and survival rates (Relyea and Mills, 2001; Relyea, 2003a, 2005). Nevertheless, the complex indirect effects of chemical pollutants that arise from disrupted sensory systems of tadpoles remain unclear (but see Mandrillon and Saglio, 2007). Many amphibian larvae respond to water-borne predator cues by strongly reducing their activity levels, thus avoiding detection in predator encounters (Stauffer and Semlitsch, 1993; Wilson and Lefcort, 1993; Holomutzki, 1995; Kiesecker et al., 1996; reviewed in Kats and Dill, 1998; Chivers and Smith, 1998). However, anthropogenic chemicals directly or indirectly spilled on freshwater ecosystems might interfere with tadpoles' ability to recognize predator cues, hence seriously compromising their odds of survival.

Here we examine the effects of anthropogenic alteration of the chemical environment on the ability of tadpoles of the western spadefoot toad (*Pelobates cultripes*) to accurately respond to chemical cues from a common predator, nymphs of the dragonfly *Anax imperator*. We compared swimming activity of tadpoles in the presence and absence of water-borne chemical cues from dragonflies, at different concentrations of two contaminants that are commonly present in freshwater habitats as a consequence of human activities: humic acid (HA) and ammonium nitrate (AN). HA is a naturally occurring substance in aquatic environments resulting from biodegradation of dead organic matter, but, associated with agricultural manure, it may highly increase due to eutrophication (Thomas, 1997; Atiyeh et al., 2002; Kappler and Haderlein, 2003). HA is known to alter natural behaviors of aquatic species by disturb-

ing their sensory environment (Hubbard et al., 2002; Fisher et al., 2006; Fabian et al., 2007). On the other hand, nitrogen pollution resulting from agricultural runoff, including AN, negatively affects growth and development of embryos, and reduces larval survival in several amphibian species (Marco et al., 1999; Jofre and Karasov, 1999; Laposata and Dunson, 2000; De Solla et al., 2002; Ortiz et al., 2004; Griffis-Kyle, 2005; Ortiz-Santaliestra et al., 2007). In addition, nitrates may influence tadpole behavior and affect the outcome of competition and predation in the amphibian larval guild (Smith et al., 2006). We hypothesize that the presence of either HA or AN in water could interfere the ability of tadpoles to discriminate and adaptively respond to chemical cues from their natural predators, disrupting their behavioral defenses (i.e., reduced activity in the presence of predators) and increasing prey detectability.

2. Materials and methods

2.1. Animal care and housing

We collected 50 eggs (Gosner stages 10–12, Gosner 1960) from each of three clutches of western spadefoot toad (*P. cultripes*) at a temporary peridune pond (36°59′N, −6°30′W) within the Biological Reserve of Doñana (southwestern Spain) and transported them to walk-in climatic chambers (16 °C, 12:12 L:D) at the Doñana Biological Station in Seville. In the laboratory, eggs from the three clutches were pooled together and placed on two 5 L plastic trays with carbon-filtered dechlorinated tap water and aerators until hatching. Experimental tadpoles were, hence, not in contact with predator cues prior to the experiments. When tadpoles reached the free-feeding stage (Gosner stage 25), tadpoles were haphazardly allocated to 3 L plastic buckets filled with carbon-filtered dechlorinated tap water, where they were raised individually at 20 °C and under a natural photoperiod (12:12 L:D). We renewed water twice weekly and tadpoles were fed with ground rabbit chow ad libitum.

We also captured dragonfly nymphs (*A. imperator*) at several ponds within the park, to be used as predator cue donors. Dragonflies (3–5 cm length) were also housed individually in 1 L plastic buckets in a climatic chamber (20 °C, 12:12 L:D) separated from that of tadpoles, to avoid chemical or visual contact with the predators prior to the experiments. To provide necessary perches to the nymphs, we placed plastic plants in all aquaria, thoroughly rinsed to eliminate any artificial cues.

2.2. Predator cue preparation

To prepare predator chemical cues, we filled each donor dragonfly container with 0.5 L of dechlorinated tap water, to be pervaded with predator cues. Dragonflies were fed *P. cultripes* tadpoles, once per day. This way predator cues contained not only dragonfly kairomones, but also alarm cues and post-digestion cues from conspecific tadpoles, better reflecting natural conditions. Since predator cues last approximately 2–4 days in water (Peacor et al., 2006), we extracted and mixed the water from three dragonfly aquaria every two days, and froze it in 10 mL aliquots at −20 °C until use (Woody and Mathis, 1998; Gonzalo et al., 2007; Polo-Cavia et al., 2010; Polo-Cavia and Gomez-Mestre, 2014). Control water was prepared following the same procedure but without placing dragonflies in the aquaria.

2.3. Experimental procedure

Experimental tadpoles (n = 75; Gosner stages 30–35) were randomly assigned to five treatments (n = 15 each) consisting of water containing HA (Sigma–Aldrich, 53680) or AN (Merck, 101188) at different concentrations ('HA-20 mg/L', 'HA-200 mg/L', 'AN-20 mg/L'

or 'AN-80 mg/L') and a "control" treatment consisting of carbon-filtered dechlorinated tap water. For each pollution treatment, we compared swimming activity of each individual tadpole across two predator cue treatments ('presence' vs. 'absence' of water-borne chemical cues from dragonflies), in random order. Concentrations of pollutants were selected based on previous studies to avoid lethal effects on tadpoles and according to ecologically realistic levels (Ortiz et al., 2004; Fisher et al., 2006; Fabian et al., 2007; Relyea and Edwards, 2010). For example, concentrations of HA in freshwater ecosystems can fluctuate from trace levels to over 200 mg/L, being highly variable in both space and time (Thomas, 1997; Steinberg, 2003).

Experiments took place in grey, U-shaped gutters (101 × 11.4 × 6.4 cm) sealed at both ends with plastic caps and marked inside to visually divide them into five equal parts. Gutters were filled either with 3 L of carbon-filtered dechlorinated tap water, or with 3 L of dechlorinated tap water supplemented with HA or AN depending on the pollution treatment. Then, we added 10 mL of control water or predator cue water to each end of each gutter (two frozen aliquots per gutter) to favor cue diffusion. We waited 5 min for the aliquots to thaw entirely, and then, we placed a single tadpole in the middle of each gutter, waiting another 5 min before the trials to allow tadpoles to acclimate. For the trials, we recorded activity of each individual tadpole for 30 min, registering the quadrant that each tadpole occupied in the gutter every 1 min (30 scans per tadpole in total). We then calculated swimming activity of tadpoles from the number of marks crossed by each tadpole in each trial. This scan sampling method is widely used to measure activity rates and reasonably approximates continuous sampling (Rohr and Madison, 2001; Gonzalo et al., 2007; Polo-Cavia et al., 2010; Polo-Cavia and Gomez-Mestre, 2014). Gutters were thoroughly cleaned after each trial to eliminate residual pollutants or predator chemical cues. All behavioral observations in the study were carried out blindly so that the observer was unaware of the type of cue placed in each gutter in each trial.

2.4. Data analysis

To compare swimming activity of tadpoles (i.e., number of marks crossed by tadpoles over the total observation time) in the presence or absence of predator cues, and at different concentrations of contaminants in water, we used a two-way repeated measures analysis of variance with predator cue treatment (two levels: 'presence' vs. 'absence' of dragonfly cues) as a within-subjects factor, and with pollution treatment (five levels: clean water, HA-20 mg/L, HA-200 mg/L, N-20 mg/L, AN-80 mg/L) as a between-subjects factor. We checked data normality using Kolmogorov–Smirnov test ($p \geq 0.2$ for all variables) and verified homogeneity of variances with Levene's test ($p \geq 0.37$ for all variables). Post hoc pairwise comparisons among treatments were made using Tukey's honestly significant difference tests (Sokal and Rohlf, 1995). Analyses were performed using Statistica 8.0 software.

3. Results

We found a significant interaction between predator cues and presence of contaminants affecting swimming activity of tadpoles (two-way repeated measures ANOVA, predator cue treatment × pollution treatment: $F_{4,70} = 3.24$, $p = 0.017$). Thus, the response to predator cues depended on the pollution treatment. In the absence of pollutants, tadpoles effectively responded to predator cues by reducing their swimming activity by an average of 44% with respect to predator absence (Tukey's test, $p = 0.02$; Fig. 1). In
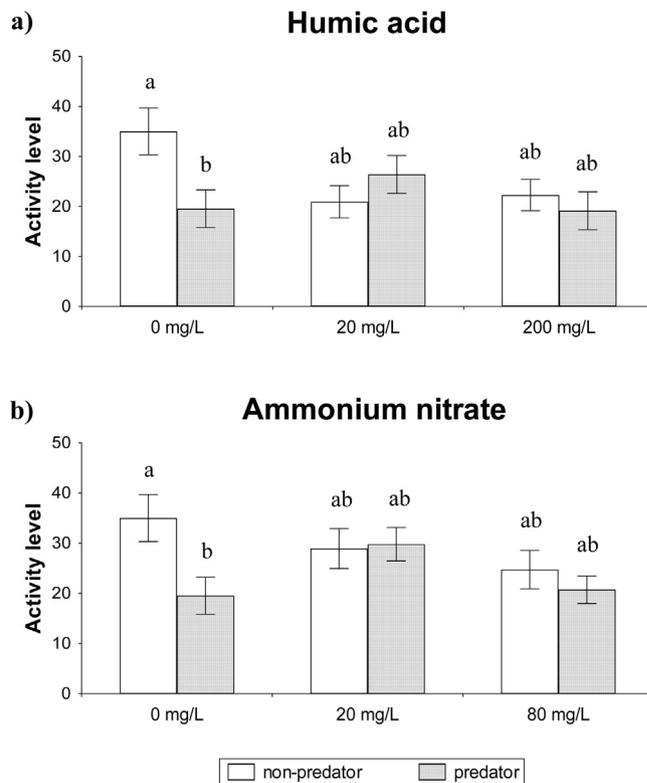

Fig. 1. Activity levels (mean ± SE number of marks crossed during 30 min) of *Pelobates cultripes* tadpoles in water with different concentrations of (a) humic acid and (b) ammonium nitrate, in presence and absence of chemical cues from a common predator (*Anax imperator*). Bars labelled with different letters differed significantly from one another.

contrast, in presence of either HA or AN, tadpoles maintained high activity levels when exposed to predator cues ($p \geq 0.96$ in all cases; Fig. 1). Post-hoc tests also indicated that tadpole activity remained unchanged when either HA or AN were added to the water, whether predators were present ($p \geq 0.61$ in all cases) or not ($p \geq 0.17$ in all cases) (Fig. 1), which suggested a lack of direct toxic effects on tadpole activity. Main effects were not significant: swimming activity of tadpoles did not vary significantly in presence and absence of dragonfly cues when considered across all pollutant levels together (predator cue treatment, $F_{1,70} = 2.82$, $p = 0.1$), and tadpoles did not alter their activity as a consequence of pollutants either (pollution treatment, $F_{4,70} = 1.42$, $p = 0.24$).

4. Discussion

Our findings demonstrate that chemical pollutants in aquatic systems interfere with the innate behavioral response of amphibian prey to cues from a native odonate predator. Spadefoot toad tadpoles responded to the presence of dragonfly cues by steeply reducing their swimming activity (44% reduction on average), but only in the absence of pollutants (Fig. 1). In contrast, when HA or AN were present in the water, tadpoles remained unresponsive to predator cues, suggesting that sublethal levels of chemical pollution impaired cue recognition of the tadpoles, hence compromising their ability to detect and respond to common predators.

High activity levels increase prey detectability and the probability of encountering a predator (Taylor, 1984; Anholt and Werner, 1995), thus predicting a decrease in tadpole survival when exposed to HA and AN at concentrations within the range of those used in this study. Therefore, a higher impact of predation would be expected in amphibian populations inhabiting water bodies affected by agricultural runoff carrying these pollutants, even if at

low concentrations. This prediction needs to be tested in future experiments considering also the effects of pollutants on predator behavior (Ujszegi et al., 2015), by assessing tadpole survival against predators when both predator and prey are exposed to chemical contaminants.

Previous studies on amphibians have examined the effects of pesticides on predator–prey interactions, suggesting that sublethal concentrations of pollutants in combination with exposure to predator cues may act synergistically to decrease survival (Relyea and Mills, 2001; Relyea, 2003a, 2005). Other studies have found that pesticides can increase predation rates, but the underlying mechanisms responsible for such effects are unknown (Broomhall, 2002, 2004; Mandrillon and Saglio, 2007; Ortiz-Santaliestra et al., 2010). Some studies to date have investigated the effect of chemically altered environments on predator recognition (reviewed in Lürling, 2012). Leduc et al., 2004, 2006, 2007 and Brown et al. (2012) reported that response to alarm cues and learned recognition of novel predators by salmonids can be impaired under reduced pH conditions. Also, elevated CO_2 causes fish to fail to respond to predation cues (Dixson et al., 2010; Ferrari et al., 2011). In amphibians, Mandrillon and Saglio (2007) found that the presence of sublethal concentrations of an herbicide impaired the acquired recognition of a non-native predator by *Bufo bufo* tadpoles. More recently, Troyer and Turner (2015) have shown that water quality (i.e., pH and conductivity) affects the magnitude of antipredator responses by tadpoles of *Hyla versicolor*. To our knowledge, our study is the first to demonstrate interference of innate defensive behavior against local predators by amphibian larvae due to exposure to organic chemical pollutants. A review of the literature further suggests that typical pesticides, herbicides and heavy metals present in natural waters can similarly act as disruptors of inducible defenses in larval amphibians (Lefcort et al., 1998; Raimondo et al., 1998; Lürling, 2012). Within this frame, our findings provide a potential mechanism explaining why chemical exposure can increase predation rates in amphibian populations.

With regard to toxic effects of pollutants, we found no evidence for alteration of tadpole swimming activity in the non-predator treatment associated with the presence of HA or AN (Fig. 1). Activity rates of tadpoles have been observed to decrease dose-dependently in presence of pesticides (Relyea and Edwards, 2010). Nevertheless, other studies using much lower concentrations have found no effect of pesticides on tadpole activity (Hanlon and Relyea, 2013), suggesting that the effect is dependent upon the type of pollutant and its concentration. The concentrations of HA and AN in our study seem to have been too low to cause a rapid toxic effect, based on tadpole behavior, although concentrations were high enough to impair the response of tadpoles to predator cues.

Anthropogenic chemicals such as HA or AN are pervasive in freshwater ecosystems worldwide, greatly affecting amphibian habitats (Harris et al., 1998; Relyea, 2003b; Relyea and Hoverman, 2006). Sublethal concentrations of these pollutants may be hardly detectable in the short-term by conservation programs since they cause no immediate or visible impacts on wildlife populations, but have the potential for causing important damage by severely disturbing the chemical environment. The capacity of aquatic prey to reliably recognize water-borne chemical cues from predators is critical to survival because it allows the activation of adaptive inducible defenses (i.e., plastic changes in life history, morphology or behavior), and consequently reduces the risk of predation (Gomez-Mestre and Diaz-Paniagua, 2011; Polo-Cavia and Gomez-Mestre, 2014). Hence, even slight concentrations of pollutants may pose a threat to tadpole survival against predators, threatening the long-term persistence of amphibian populations.

While most toxicological studies have pointed out the physiological effects of pesticides as a factor responsible for amphibian declines (e.g., Perkins et al., 2000; Fordham et al., 2001; Sparling

et al., 2001, 2010; Ortiz-Santaliestra et al., 2011; Egea-Serrano et al., 2012), indirect impacts of sublethal concentrations of pollutants disrupting tadpoles' ability to detect natural predators might be a key contributing factor explaining the local extinction of many amphibian populations in the last decades. Given that aquatic organisms are likely exposed to several chemicals combined (Hayes et al., 2006; Relyea, 2009; Rasmussen et al., 2015), our study may underestimate the indirect effects of pollutants and their interactions on survival of aquatic prey mediated through impacts on their chemically-based predator recognition systems. Further studies exploring the actual effects of these chemical mixtures on the sensory systems of aquatic prey, along with their effects on the predators themselves, will ultimately elucidate the relative role of sublethal levels of water pollution on prey population viability.

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