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Effects of an environmentally relevant temporal application scheme of low herbicide concentrations on larvae of two anuran species

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HIGHLIGHTS

- Short time exposure to low herbicide concentrations can induce mortality at later development.
- Time point of exposure is relevant for mortality.
- The two test species were differently affected.
- Time point of exposure should be considered in risk assessments of pesticides.

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ABSTRACT

Cultivation of herbicide-tolerant crops involves repeated applications of the complementary herbicide throughout the growing season, while in conventional corn production, herbicide application is restricted to the beginning of cultivation. Repeated application of herbicides increases both the likelihood an organism will be exposed to the herbicide and the concentration it may be exposed to. We examined effects of short and pulsed exposure of the cycloxydim-based herbicide formulation Focus® Ultra at doses close to the calculated LC5 (0.01 and 0.5 mg a.i. L^{-1}) and LC10 values (0.05 and 1.0 mg a.i. L^{-1}) on early premetamorphic and prometamorphice larvae of two anuran model organisms, *Xenopus laevis* and *Discoglossus scovazzi*. In addition, larvae were repeatedly exposed, i.e. at all considered developmental stages. The herbicide did not induce effects on body size at and time to metamorphosis or increase deformation rates in both species. Exposure to calculated LC5 values did not increase mortality or cause clinical signs in both species. At calculated LC10 values, narcotic effects were seen in all developmental stages. There was no clear evidence of developmental-specific mortality. Metamorphic success was independent of time point and duration of application in *X. laevis*. Only repeated exposure significantly increased mortality at metamorphosis in *D. scovazzi*. Narcosis may result in increased mortality under field conditions due to rise of predation risk. Different sensitivity of the test species to the compound was attributed to their physiological properties. Different filtering rates were understood as an accompanying factor influencing exposition.

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1. Introduction

Amphibian populations are dramatically declining worldwide, with more than one third of the ca. 7400 known species threatened with extinction (Alford and Richards, 1999; Houlahan et al., 2000; Stuart et al., 2008). Various factors, partly interacting, are at work and environmental pollution is one (Collins and Storfer, 2003). In general, pesticide use is strongly affecting farmland biodiversity (Geiger et al., 2010). Actually, it is suggested that this also applies to amphibians in cultivated landscapes (Mann et al., 2009), in particular when utilizing agricultural water bodies for reproduction (Knutson et al., 2004). Causal relationships between pesticide use and amphibian population declines are still poorly understood (Schmidt, 2004; Wagner et al., 2013). Nevertheless, in several field studies negative effects at the individual level have been found, including the increased development of abnormal gonads (McCoy et al., 2008), decreased body size and mass at metamorphosis (Attademo et al., 2014; Wagner et al., 2014a). In addition, stress due to alterations in enzymatic activity was reported (Attademo et al., 2007, 2011, 2014; Lajmanovich et al., 2010, 2011). Stress may furthermore lead to increased infection risk of pathogens (Attademo et al., 2011). In addition, numerous laboratory and mesocosm studies have shown deleterious effects of pesticides at

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environmentally relevant concentrations on amphibians, mainly on aquatic life stages of anurans (Howe et al., 2004; Relyea, 2009; Bernabò et al., 2013). Beside acute toxic (Relyea, 2005; Belden et al., 2010; Brühl et al., 2013), chronic and delayed effects (Bridges, 2000; Howe et al., 2004; Cauble and Wagner, 2005; Jones et al., 2009; Williams and Semlitsch, 2010) after exposure to sublethal concentrations of pesticides have been found in anurans. Prolonged and shortened metamorphosis as well as reduced body indices were seen (Bridges, 2000; Howe et al., 2004; Cauble and Wagner, 2005; Williams and Semlitsch, 2010). Indirect effects of pesticide use included avoidance of contaminated breeding sites (Takahashi, 2007; Vonesh and Buck, 2007; but see Wagner and Lötters, 2013). Exposure risks and adverse effects of pesticides are specific in three ways. They were found to be formulation-specific and species-specific (Mann and Bidwell, 1999; Wagner et al., 2013, 2014b). Additionally effects were reported whose nature was understood as developmental-specific (Greulich and Pflugmacher, 2003; Biga and Blaustein, 2013). After steps of embryonic development organogenesis starts and continues in the early larvae (early premetamorphosis). A late phase of dramatic developmental changes is prometamorphosis with destruction and rebuilding of organs (Gilbert, 2010). For these critical phases increased sensitivity to pesticides has to be suggested (see Wilson and Warkany, 1965).

Animal studies on toxic effects of insecticides are more common than of herbicides (Weir et al., 2012). In the Americas and Europe, corn cultivation has already increased in the last few decades – especially for fodder crop production – but currently, corn cultivation is remarkably expanding for biofuel production (Landis et al., 2008). While in the Americas and other parts of the world, corn cultivation is mainly based on genetically modified crops with a resistance against the herbicide glyphosate (Duke and Powles, 2008), in most European countries, genetically modified crop cultivation is rather marginal because most such crops are still to be approved (Böll et al., 2013). Herbicide-resistant corn cultivation is mainly based on cycloxydim-resistant corn hybrids (Vancetovic et al., 2009) with some exceptions such as Spain where nearly one-third of corn cultivation is based on genetically modified plants (www.isaaa.org/). Herbicide-resistant crop cultivation enables repeated applications of the complementary herbicide (not only at beginning of cultivation like in conventional crops), such as the cycloxydim-based herbicide formulation Focus® Ultra. We here investigated the effects of two low, environmentally relevant concentrations of Focus® Ultra on larvae of the African clawed frog (*Xenopus laevis*, family Pipidae) and the Moroccan painted frog (*Discoglossus scovazzi*, family Alytidae) when exposed repeatedly and at different developmental stages. The two species drastically differ in their phylogeny, morphology and life history (Duellman and Trueb, 1986). Orton (1953) described four different anuran larval types by means of outer morphology. *X. laevis* develops type I larvae which are understood as derived. The ancestral larval types are types III and IV (Sokol, 1975; Haas, 2003; Roelants et al., 2011), in this study represented by *D. scovazzi* (larval type III). Different sensitivity to pesticides based on physiological properties and different exposure to the test item due to different feeding behaviour including the volume of water pumped through their buccopharynx (Viertel, 1990, 1992) are suggested.

In accordance with studies that chronically exposed anuran larvae to herbicides (Howe et al., 2004; Cauble and Wagner, 2005; Williams and Semlitsch, 2010), we hypothesized (i) that only repeated exposure to low pesticide concentrations – that are described as "sublethal" based on acute toxicity tests – would affect mortality at metamorphosis, clinical signs, time to and body size at metamorphosis and that, (ii) effects were species-specific because the tested anuran species represent different phylogenetic lineages. Furthermore, we suggested that, (iii) early larvae would be more sensitive concerning acute toxic effects (i.e. clinical signs after 1 and 2 d and mortality after 1 week) than later stages (Jones et al., 2010; Biga and Blaustein, 2013).

2. Material and methods

2.1. Test substance

The tested formulation is a selective herbicide containing 100 g L^{-1} (=10.8%) of cycloxydim (CAS 101205-02-1) as active ingredient (a.i.) and 50% of Solvent Naphtha (CAS 64742-94-5) and 4% of sodium dioctylsulphosuccinate (CAS 577-11-7) as added compounds. It is also used for foliar spraying against perennial grasses in conventional rape, sugar beet, potato, green bean and field bean cultivations (EFSA, 2010). Its a.i. cycloxydim is a cyclohexene oxime herbicide, i.e. it inhibits acetyl-CoA carboxylase in grasses while dicotyle plants and cycloxdyim-resistant corn hybrids remain unaffected (Burton et al., 1989).

2.2. Test organisms

The African clawed frog (*X. laevis*) originates from the southern part of Africa (Nieuwkoop and Faber, 1956). Developmental biology of *X. laevis* is well known, which was the reason to include the species into the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX: ASTM Standard E1439, 1998). Reproduction was initiated by injection of human chorionic gonadotropin into the dorsal lymph sac of both genders. Embryos and larvae are easy to rise.

Eggs of *D. scovazzi* are available all over the year. The spontaneous egg deposition and the relatively fast development of aquatic life-stages (1–2 months until metamorphosis) are beneficial to laboratory work. *D. scovazzi* is distributed in the Mediterranean region of Morocco and the Spanish exclaves Ceuta and Melilla (Salvador, 1996; Fromhage et al., 2004).

2.3. Experimental procedure

Experiments were designed to demonstrate effects of an environmentally relevant temporal scheme of herbicide applications on different developmental stages of both species. Endpoints were clinical signs (mild and full narcosis of larvae), mortality, body size at metamorphosis, time to metamorphosis and morphological changes. Based on data from a dose range-finding study, a 96-h LC50 value of 0.1 ± 0.01 mg a.i. L^{-1} (=1 mg formulation L^{-1}) for early *X. laevis* larvae was found. The 96-h LC50 value for early *D. scovazzi* larvae was 1.45 ± 0.01 mg a.i. L^{-1} (=14.5 mg formulation L^{-1}). From these values, we calculated the LC 5 and LC10, which corresponds to rounded 0.01 and 0.05 mg a.i. L^{-1} for *X. laevis*, and 0.5 and 1.0 mg a.i. L^{-1} for *D. scovazzi*. The experiments were conducted in a climate chamber at Trier University at 23 ± 1 °C. The experimental design included triplicates of controls and four different time points of exposure, which correspond to different application time points in the field. Tests solutions were freshly prepared using FETAX solution (ASTM guideline E1439, 1998). Water was changed weekly, dead animals were removed daily, and relevant parameters (oxygen, ammonium, nitrate, pH, hardness and conductivity) were measured before and after water changes. Larvae were fed with 2.5 mg Sera Micron®/animal/day and food rations were doubled after *X. laevis* larvae reached NF stage 51 (Nieuwkoop and Faber, 1956) or *D. scovazzi* larvae Gosner stage 30 (Gosner, 1960). After 24 h and 48 h of exposure to Focus® Ultra, all individuals were tapped on the tail with a blunt glass rod to assess narcosis by eliciting the escape response. In accordance with Mann and Bidwell (2001), "mild narcosis" was noted if an individual failed to swim or in an uncoordinated manner, and "full narcosis" if the animal did not

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