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European common frog *Rana temporaria* (Anura: Ranidae) larvae show subcellular responses under field-relevant *Bacillus thuringiensis* var. *israelensis* (Bti) exposure levels

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ABSTRACT

Bacillus thuringiensis var. *israelensis* (Bti) is presumed to be an environmental friendly agent for the use in either health-related mosquito control or the reduction of nuisance associated with mosquitoes from seasonal wetlands. Amphibians inhabiting these valuable wetlands may be exposed to Bti products several times during their breeding season. Up until now, information regarding effects on the non-targeted group of amphibians has to be considered rather inconsistent. On this account, we evaluated how three repeated exposures to frequently used Bti formulations (VectoBac®12AS, VectoBac®WG) in field-relevant rates affect European common frog (*Rana temporaria*) larvae. In a laboratory approach, we assessed potential effects with regard to enzymatic biomarkers (glutathione-S-transferase (GST), glutathione reductase (GR), acetylcholine esterase (AChE)), development, body condition and survival until the end of metamorphosis. Although survival and time to metamorphosis were not significantly affected, larval development tended to be shortened in the Bti treated water phase. Furthermore, exposure to Bti induced significant increases of GST (37–550%), GR (5–140%) and AChE (38–137%) irrespectively of the applied formulation, indicating detoxification, antioxidant responses as well as an alteration of neuronal activity. GST activity increased twice as much after two repeatedly executed Bti applications within a time period of 6 days. The examination of several biochemical markers is needed to fully evaluate the ecotoxicological risk of Bti for amphibian populations, especially in the context of worldwide amphibian declines. Nevertheless, following the precautionary principle, it may be advisable to implement certain thresholds for application numbers and intervals in order to ensure environmentally friendly mosquito control programs, especially in areas designated for nature conservation.

1. Introduction

In mosquito control, the widespread use of synthetic insecticides like organophosphates and pyrethroids had several downsides such as the development of insect resistances or adverse effects on environment and human health (Hemingway and Ranson, 2000). Consequently, the usage of more specifically acting bio-pesticides increased substantially over the last decades. Above all, commercial formulations containing the active ingredient *Bacillus thuringiensis* serotype *israelensis* (Bti) represent one of the main bacterial insecticides for the control of larval mosquitoes, blackflies and chironomids (Becker, 2006; Lacey and Merritt, 2003) with global application amounts of 70–300 t of formulated product per year (van den Berg et al., 2012). Comparatively, the output quantity of organophosphates amounts to 163 t per year (van den Berg et al., 2012).

On a global scale, Bti is largely applied for human health issues by controlling vector-borne diseases in subtropical and tropical urban breeding sites (van den Berg et al., 2012). However, temperate regions such as the Upper Rhine Valley in Germany look back on more than 40 years of Bti treatments in river floodplains with the objective of reducing nuisance for the local population (Becker, 2006). To this end, more than 30.000 ha wetlands along the river Rhine are periodically treated against floodwater and snowmelt mosquitoes (KABS e.V., 2016). Noteworthy, the majority of treated wetlands is protected by the EU's Natura 2000 network (KABS e.V., unpublished; Swedish Chemicals Agency, 2015). Bti is generally considered environmentally safe in regard to non-target aquatic organisms due to its specific mode of action (Boisvert and Boisvert, 2000). The driver of toxicity are endotoxins (Cry-toxins) that get activated after ingestion and bind to specific receptor sites in the midgut epithelium of the target organism. The

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preceding activation depends on several factors such as the alkaline condition and the number of receptors in the midgut (Bravo et al., 2007). Nevertheless, during the last years, some studies revealed uncertainties about the environmental compatibility of ordinary Bti applications. Contrasting results can be found especially when it comes to adverse effects on chironomids (Kästel et al., 2017; Lagadic et al., 2016) that may be propagated upward in wetland food chains (Jakob and Poulin, 2016; Poulin et al., 2010) affecting environmental health.

In addition to the mass occurrence of larval mosquitoes, temporary flooded wetlands also offer suitable breeding sites for many other aquatic organisms, including amphibians (Blaustein and Margalit, 1996). The latter are currently considered the most globally threatened group of vertebrates and their populations are declining worldwide at alarming rates (Stuart et al., 2004). One of the reasons held responsible for the dramatic population decline is the growing rate of human-induced environmental contamination, most notably the influence of pesticides (Sparling et al., 2001). In contrast to chemical pesticides that largely reach the water body indirectly through spray drift, run-off or atmospheric transport (Mackay et al., 2014; Schulz, 2001), Bti reaches amphibian habitats by a direct application to the water surface (Becker, 2006) during the spawning season of many amphibian species. Consequently, amphibian larvae may be exposed to Bti during multiple instances during their development.

Despite of the potential exposure risk for amphibians, (eco-) toxicological research on Bti and amphibians has been quite scarce, especially compared to studies on agricultural pesticides. Basic knowledge was gained through direct toxicity studies conducted in the 1980s and '90s that indicated mortalities in anurans at high dosages of several Bti products or self-produced bacterial laboratory cultures (Channing, 1998; Morawcsik, 1983; Paulov, 1985). A recent study found intestine damage and increasing glutathione-S-transferase and catalase activity levels after the exposure of a tropical frog species to sublethal Bti concentrations of a commercial Bti formulation (Introban®) (Lajmanovich et al., 2015). Induced enzyme activities were directly linked to lethal effects at high Bti concentrations, resulting in a LC_{50} at 22.45 mg Bti/L (Lajmanovich et al., 2015) which is comparable to actual application rates in the Upper Rhine Valley, particularly when older mosquito larvae are present (Becker, 1998). However, toxic effects might not be caused entirely or at all by the active ingredient Bti: for pesticides, it has been shown that additives in commercial formulations can potentiate amphibian toxicity (Puglis and Boone, 2011; Relyea and Jones, 2009) or, in other cases, have been shown to be the main cause of toxicity (Cox and Surgan, 2006; Wagner et al., 2013). The effects caused by Bti formulation additives were so far not considered in scientific studies or the environmental risk assessment. In addition, the German Mosquito Control Association (GMCA, Speyer, Germany) even applies different delivery forms of commercially available Bti VectoBac® formulations, depending on application type, habitat accessibility and wetland size (KABS e.V., 2016) which may also change the toxic properties of the final product. Considering that amphibians are key components for energy transfers between aquatic and terrestrial habitats (Gibbons et al., 2006), highlights the need of ecotoxicological data in order to ensure environmental health of wetland ecosystems.

The goal of the present study was to examine the effects of three Bti delivery forms on the common frog *Rana temporaria* which is widely distributed throughout Europe. Its spawning habitats range from stagnant shallow to temporary ponds (Schlüpmann and Günther, 2004) co-occurring with mosquito larvae. We simulated the current practice of mosquito control using environmentally relevant rates (Table 1) and frequencies of Bti adapted to the control program in the Upper Rhine Valley, where smallest temporary wetlands are treated several times a year in short intervals. By doing so, tadpoles were exposed to three consecutive Bti applications with three common Bti delivery forms at three different stages during their larval development, in a fully crossed design. Based on the findings by Lajmanovich et al. (2015) and the scanty toxicity information on VectoBac® formulations in the pesticide risk assessment (European Food Safety Authority, 2013), we hypothesized that consecutive applications of Bti formulations would affect survival, physiological fitness (judged by growth) and developmental time of tadpoles depending on the application rate. Moreover, we selected three well studied biomarkers of effect in anuran larvae to examine sublethal effects: glutathione-S-transferase (GST), glutathione reductase (GR) and acetylcholine esterase (AChE) (Venturino and D'Angelo, 2005). We expected subcellular alterations in the enzymatic activity rates of GST and the antioxidant enzyme GR after each Bti application at sublethal concentrations similarly to the effects of Introban®. Furthermore, we assumed the absence of any neurotoxic effects (AChE) due to the specific mode of action of Bti.

2. Material and methods

2.1. Tadpole collection and animal husbandry

To ensure the lack of previous exposure and a high genetic variability of tested individuals, six freshly laid (up to 3 days old) *R. temporaria* egg clutches were collected from a pristine pond in the Bienwald forest, Rhineland-Palatinate, Germany (49°01'19.2'' N, 8°10'46.1'' E) in March 2016. Egg clutches were randomly assigned to aerated 30 L glass tanks (50 × 30 × 20 cm) filled with filtered tap water (0.2 µm Supor, Pall Corporation, Port Washington) until embryos developed to freely swimming tadpoles which were used in the following experiment. Tadpoles were fed with commercially available rearing food for aquarium animals (Sera Micron, Sera GmbH, Heinsberg) three times a week during the renewal of water. Housing, rearing and all experimental procedures took place at 18–24 °C and a 16/8-h light/dark cycle. All experimental procedures in our study were evaluated and approved by the Institutional Animal Care and Use Committee at the University Koblenz-Landau and the federal investigation office (Landesuntersuchungsamt – LUA, NTP-ID: 00008349-1-2). All animals not used in the experiment were euthanized using 0.1% MS-222. Tadpole development stages (GS) were determined using a binocular (Leica KL300 LED, Wetzlar, Germany) according to Gosner (1960).

2.2. Bti formulations

Bti formulations were chosen according to the application practice in the German mosquito control program. Two commercially available formulations containing the active ingredient Bti (strain AM 65-52) are used in Germany: VectoBac®WG and VectoBac®12AS. VectoBac®WG is a water dispersible granule formulation (37.4% a.i. 3000 ITU/mg) whereas VectoBac®12AS is an aqueous suspension (11.6% a.i. 1200 ITU/mg) (Valent BioSciences Corporation, Illinois, USA). No further information on other ingredients is provided by the manufacturer. The GMCA uses VectoBac®WG and VectoBac®12AS as a basis for the preparation of three different delivery forms: ice-pellets, sand-granule and liquid. Ice-pellets are manufactured with a suspension of VectoBac®WG that is converted to 4 mm grain sized granules with the help of liquid nitrogen (Becker, 2003). In case of Bti sand-granule, the respective amount of VectoBac®WG granules is bound to coarse sand as a mineral carrier with the use of vegetable oil. The liquid formulation is prepared as a 1:10 solution of VectoBac®12AS and tap water. All formulations were obtained from stock material of the GMCA. In the following, ice-pellets are referred to as formulation "Ice", sand-granule as formulation "Sand" and the liquid formulation as "Liquid".

2.3. Exposure conditions

To adequately simulate realistic exposure conditions, three concentrations according to the actual field rates were used (KABS e.V., 2016): the nominal field rate (1×), twice (2×) and tenfold (10×) the nominal field rate (FR). According to the control strategy of the GMCA, field rates depend on the age structure of the mosquito larvae