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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

Table 1
Application rates of the three formulations (Ice, Liquid, Sand) based on the nominal (1×), doubled (2×) and tenfold (10×) field rate (FR) used in the German mosquito control strategy (KABS e.V., 2016). Aquarium surface area = 0.046 m^2; Volume = 1.7/L.

	Ice				Liquid				Sand			
	FR		Application rate		FR		Application rate		FR		Application rate	
	[kg/ha]	[10^9 ITU/ha]	[mg/L]	[ITU/L]	[L/ha]	[10^9 ITU/ha]	[µl/L]	[ITU/L]	[kg/ha]	[10^9 ITU/ha]	[mg/L]	[ITU/L]
1× FR	15	1.44	40.56	3900	2	1.92	5.41	6494	25	1.2	67.61	3247
2× FR	30	2.88	81.13	7800	4	3.84	10.82	12988	50	2.4	135.21	6494
10× FR	150	14	405.65	39000	20	19.2	54.12	64940	250	12	676.06	32470

ITU = International Toxic Unit.

population, water depth and temperature. In wetlands with deep-water areas and older mosquito larvae (2nd, 3rd stages) the nominal field rate is routinely doubled in order to reach a sufficient treatment success (Becker, 1998, 2003). The tenfold field rate was employed both to assess dose-dependent effects and to include a worst-case exposure. In mosquito control programs, Bti is applied repeatedly whenever mosquito populations start to develop in monitored wetlands. A mean application frequency of three was chosen related to typical Bti application numbers in wetlands in the Upper Rhine Valley within the last ten years between the months March and September. Formulations were directly applied on the water surface without further mixing to recreate realistic exposure conditions.

2.4. Experimental design

2.4.1. Multiple exposure experiment

When tadpoles developed gill buds at GS 19, they were transferred into 100 separate plastic aquaria (7 × 16 × 22 cm) in which the experiments took place. Each aquarium contained five *R. temporaria* individuals in 1.7 L of filtered (0.2 µm Supor, Pall Corporation, Port Washington) and aerated tap water. Overall, Bti was applied three times, referred to as application frequency, at three time points in a fully crossed design: three formulations × three field rates. Each treatment and one Bti-free control was replicated ten times (n = 10). The applied volumes of the respective formulation were calculated based on the surface area of the aquaria (Table 1).

The first Bti application took place 3 days after test start when tadpoles reached GS 21–23 and larvae start to feed autonomously (Fig. 1). Embryos were not included in the experiment since the most likely way of tadpole exposure towards Bti is orally via food intake (Mokany and Shine, 2003). The second application was conducted after additional 6 days when the external gills receded at GS 24–28. Finally, a third application took place after a further 32 days, before the forelimbs started to become visible at GS 36–40. The determination of GS took place on a randomized basis prior to applications. Tadpoles used for biomarker analyses were assigned to GS 23, 25 and 39 and sampled 48 h after each application (n = 10). Euthanasia took place using 0.1% MS-222, after which individuals were shock-frozen in liquid nitrogen and individually stored at −80 °C until further analyses. After the third application (GS 39), five randomly chosen individuals were sampled for biomarker analysis at 2× and 10× FR, since remaining individuals were used for establishing the biomarker assay.

Larval mortality was evaluated every second day and additionally 24 h after each application. During the experiment, tadpoles were fed ad libitum with rearing food for aquarium animals (Sera Micron, Sera GmbH, Heinsberg), while half of the water was replaced every second day. As soon as the forelimbs of one froglet became visible, the aquaria were placed in an inclined position in order to offer dry areas to avoid drowning after completion of metamorphosis. On completion (GS 47), frog metamorphs were sampled and euthanized in 0.1% MS-222 and time to metamorphosis was calculated individually. Body length was evaluated at the end of metamorphosis using the software AxioVision® (Carl Zeiss; Oberkochen, Germany) and a digital photograph (Finepix F500EXR, Fujifilm) of the metamorph. Associated wet body mass was recorded by weighing the dabbed dry metamorph to the nearest 100 mg (Mettler PM6000, Columbus, USA). Body length and mass were used to compute body condition of individuals, using the scaled mass index ($\hat{M}_i$) (Peig and Green, 2009).

2.4.2. Single exposure experiment

To account for the impact of repeated Bti applications on biochemical responses of *R. temporaria* tadpoles in early developmental stages, a single exposure experiment was performed. In contrast to the multiple exposure experiment, tadpoles were exposed to Bti (Ice,

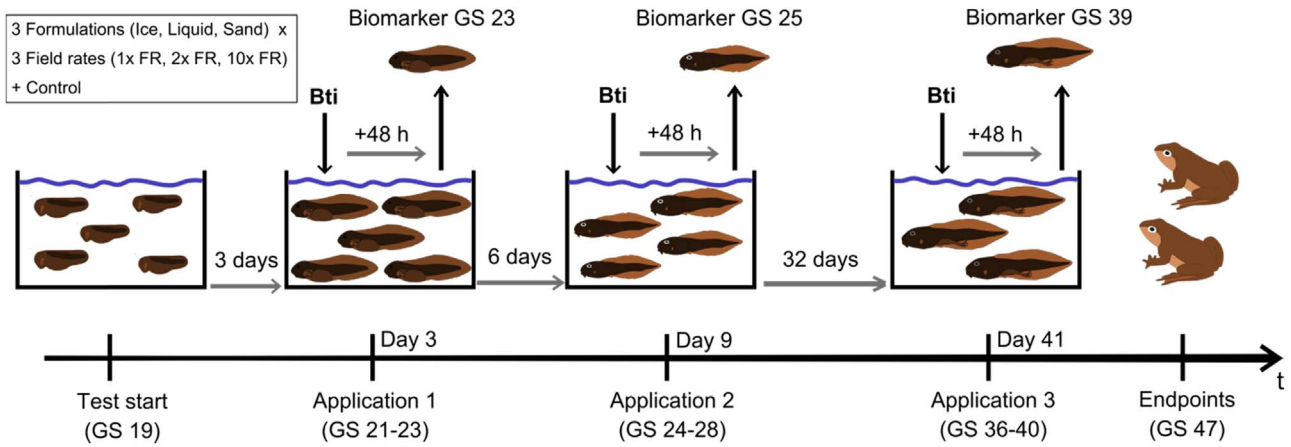

Fig. 1. Schematic overview of the experimental design using *R. temporaria* tadpoles. Three formulations (Ice, Liquid, Sand) at three field rates (FR) (1×, 2×, 10×) were applied three times (Application 1–3) at three consecutive time points (Day 3, 9, 41) after the test start at Gosner stage (GS) 19 until recording of endpoints (mortality, physiological parameters) at GS 47 (n = 10). Tadpoles for biomarker analysis were sampled 48 h after each application having GS 23, 25 and 39 (n = 10).

Liquid, Sand) the first time between GS 24 and 28. 48 h after the application tadpoles assigned to GS 25 (n = 10) were euthanized using 0.1% MS-222, shock-frozen in liquid nitrogen and individually stored at −80 °C for further biomarker analyses. To reduce the number of animals due to animal protection regulations, the experiment consisted of a control and all formulations in one field rate (2× FR). If not stated otherwise the experiment was conducted under the same conditions mentioned in sections 2.1 and 2.4.1.

2.5. Biomarker assays

Since Lajmanovich et al. (2015) found signs of oxidative stress in larval amphibians after Bti exposure, we investigated the enzymatic biomarkers GST and GR. GST is a phase II detoxifying enzyme while GR serves as an antioxidant enzyme (Steinberg, 2012). Both are widely used to assess pesticide effects on amphibians (Venturino and D'Angelo, 2005). Additionally, AChE was examined since it plays an important role in the function of nerve impulse transmission which makes it an important and well-studied biomarker for the detection of neurotoxic properties in aquatic organisms (Venturino and D'Angelo, 2005). The specific activities of the selected enzymes were determined spectrophotometrically using a multi plate reader (Synergy HT-I, BioTek, Winooski, USA) at 25°C in duplicates. All biomarker assays were conducted according to the protocol described in Mingo et al. (2016) modified for tissue, using a 1:10 dilution of tadpole homogenate. Protein concentrations, needed to calculate enzyme specific activities, were determined according to the Bradford method, using bovine serum albumin (BSA) as a standard (Bradford, 1976). GST activity was measured following Habig et al. (1974). GR activity was assayed according to Carlberg and Mannervik (1985). AChE activity was measured following the Ellman method (Ellman et al., 1961).

2.6. Calculations and statistics

2.6.1. Calculations

The scaled mass index ($\hat{M}_i$) as described by Peig and Green (2009) was chosen as a body condition index (CI) to assess test animals' fitness based on length and mass data. Compared to other conventional body condition indices, it proved to successfully account for the detection of body size changes (Peig and Green, 2010). It is calculated according to the following equation:

$$\hat{M}_i = M_i \left[L_0/L_i\right]^{b_{SMA}}$$

To compute this index, a standardized major axis (SMA) regression was performed on ln-transformed data of all body mass versus length measurements. The index adjusts the body composition of each individual to the arithmetic mean of all individual length measurements L_0. When two individuals reached metamorphosis, the mean values of body mass, length, time to metamorphosis and $\hat{M}_i$ were calculated for each replicate.

2.6.2. Statistical analyses

To test for statistically significant differences in enzymatic responses, body condition parameters, time to metamorphosis and mortality rates in the Bti treatments compared to the respective control, analysis of variances (ANOVA) was used whenever the assumptions of normality and homoscedasticity were met. Normality of data was examined using the Shapiro–Wilk test, as well as visual inspection. Homoscedasticity was tested with the Levene test. ANOVA was followed by Dunnett's post-hoc test. If assumptions of normality and homoscedasticity were not met or the number of observations was unequal, a Kruskal–Wallis test was used followed by Dunn's test for post-hoc comparisons of control and treatments (Zar, 2010).

To test whether the exposure to Bti influenced the activity of the enzymatic biomarkers, a generalized linear model (GLM) was implemented. Time (from experiment start), FR, formulation and the interaction between formulation and time and between dose and time were implemented in the model as explanatory variables. Time after experiment start was included as a continuous variable to account for proceeding tadpole development under the increasing number of applications. The best model was chosen using F-test-based backward model selection. For GST, a gamma probability distribution and an identity link function was used. Since GR and AChE activities showed a high number of zero-values, a GLM following a tweedie distribution (var.power = 1.7, link.power = 0) was conducted. The tweedie distribution was chosen since it reflected the data properly, adjusting a point mass at zero before following a regular exponential curve (Dunn and Smyth, 2005). All calculations and statistical analyses were performed in R (R developmental Core Team, Vienna, R version 3.3.2).

3. Results

3.1. Multiple exposure experiment

3.1.1. Mortality, body condition parameters and time to metamorphosis

Common frog survival was neither significantly affected by the different Bti substances nor the application rate (chi-square = 3.35, p = 0.95), although mortalities of up to 10% occurred with the nominal and the doubled field rate treatments (Table 2). The number of days tadpoles needed to finish metamorphosis ranged from the highest value of 59.75 (± 4.12) in the control to the shortest time of 51.90 (± 2.90; ± 3.09) in Ice and Sand applications (1× FR) where frogs left the water around 8 days prior to the control, but without statistically significant difference (chi-square = 11.7, p = 0.23). Body length ranged from 13.80 mm (± 1.27) in the control to 15.10 mm (± 1.25) in Ice (1× FR) without statistically significant differences (chi-square = 9.08, p = 0.85). Tadpoles showed no significant difference in weight (chi-square = 7.18, p = 0.62), although the lowest weight of 0.17 g (± 0.02) was measured in the control and in Ice (2× FR), while the highest weight (0.21 g ± 0.04) was recorded in Ice (1× FR) and Liquid (2× FR). $\hat{M}$ was comparable throughout all treatments and ranged between

Table 2
Mortality, time to metamorphosis (TTM) and physiological parameters of *R. temporaria* metamorphs at Gosner stage 47 when metamorphosis was completed in different treatments (mean values ± 95% Confidence Interval, $\hat{M}$ – scaled mass index, FR – field rate, n = 10).

Treatment (Application rate)	Formulation	Mortality [%]	TTM [d]	Body length [mm]	Body mass [g]	$\hat{M}$
Control	–	0	59.75 ± 4.12	13.80 ± 1.27	0.17 ± 0.02	0.19 ± 0.02
1× FR	Ice	10 ± 13.07	51.90 ± 2.90	15.10 ± 1.25	0.21 ± 0.04	0.18 ± 0.02
	Liquid	10 ± 19.60	56.94 ± 5.96	13.94 ± 1.36	0.19 ± 0.05	0.20 ± 0.02
	Sand	5 ± 9.80	51.90 ± 3.09	14.34 ± 1.65	0.19 ± 0.02	0.19 ± 0.02
2× FR	Ice	0	53.44 ± 3.43	14.02 ± 1.10	0.17 ± 0.02	0.20 ± 0.03
	Liquid	10 ± 13.07	57.05 ± 5.42	14.94 ± 1.30	0.21 ± 0.04	0.20 ± 0.02
	Sand	5 ± 9.80	54.80 ± 3.70	14.29 ± 1.24	0.17 ± 0.03	0.18 ± 0.03
10× FR	Ice	5 ± 9.80	54.89 ± 3.59	14.07 ± 0.77	0.19 ± 0.02	0.20 ± 0.01
	Liquid	0	52.70 ± 2.17	14.73 ± 1.08	0.18 ± 0.02	0.18 ± 0.02
	Sand	5 ± 9.80	54.30 ± 2.89	14.94 ± 0.63	0.20 ± 0.03	0.19 ± 0.03

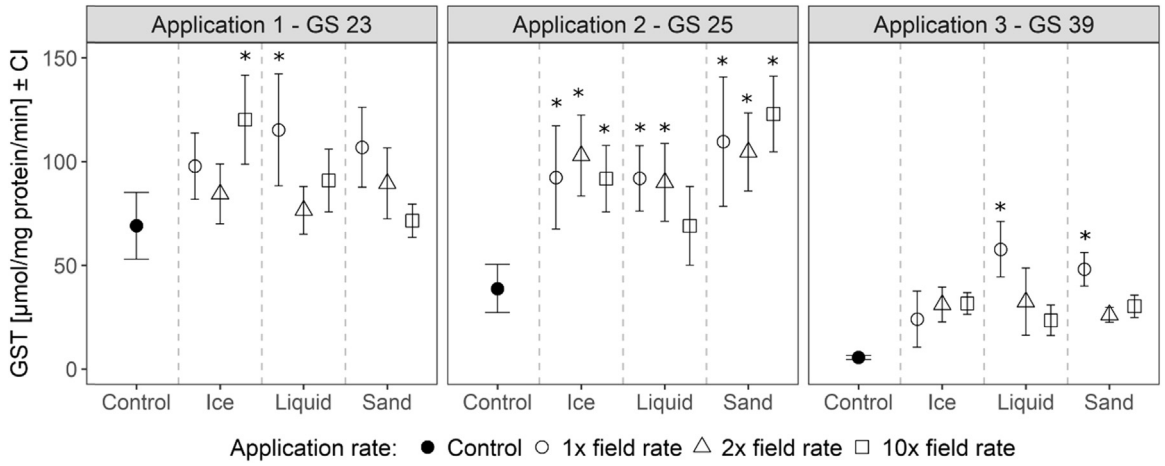

Fig. 2. Mean GST activity rates (± 95% CI) of *R. temporaria* larvae in control and Bti treatments (Ice, Liquid, Sand) at different application rates (1×, 2×, 10× field rate) for three Gosner stages (GS) (23, 25, 39) (n = 10, except n = 5 in 2× and 10× FR at Gosner 39). Asterisks indicate statistically significant differences to the respective control $p < 0.05$.

0.18 and 0.20 ($F = 0.52$, $p = 0.85$).

3.1.2. Enzymatic activities

3.1.2.1. GST activity. Generally, mean GST activity levels in tadpoles decreased significantly over the course of the experiment and proceeding tadpole development ($t = -9.98$, $p < 0.001$) (Fig. 2). However, GST activity levels of all treatments increased statistically significant compared to the respective control activities after Bti applications in all three formulations (Ice: $t = 6.26$, $p < 0.001$; Liquid: $t = 4.70$, $p < 0.001$; Sand: $t = 6.26$, $p < 0.001$). The mean increase in activity was about 37% after Application 1, 150% after Application 2 and 550% after Application 3 irrespective of the applied formulation. The application of higher field rates tended to lead to lower GST activities ($t = -1.90$, $p = 0.06$) primarily apparent with Liquid and Sand. Proceeding time of the experiment interacted significantly negative with the application of the formulations Ice ($t = -3.11$, $p < 0.01$) and Sand ($t = -2.03$, $p < 0.05$), leading to higher treatment effects at the early phases of the experiment.

3.1.2.2. GR activity. GR showed decreasing activity rates with proceeding experimental time ($t = -11.87$, $p < 0.001$), but less distinct than in GST responses (Fig. 3). Mean GR activities increased after the Bti applications compared to control levels, showing the highest increase of 140% after the second application at GS 25 (Application 1: 5%, Application 3: 24%). While some treatments showed differences after the first two applications, there was no statistically significant difference of any treatment combination after the third application (Fig. 3). Increases were not driven by the different Bti formulations (Ice, Liquid, Sand: $p > 0.05$) or any interactions with time but rather by field rate. The application of higher field rates significantly increased GR activities ($t = 4.13$, $p < 0.001$) which is in contrast to the GST results.

3.1.2.3. AChE activity. The activity pattern of AChE was similar to the GST and GR responses and showed decreasing activity rates with proceeding experimental treatment and tadpole development (Time: $t = -30.21$, $p < 0.001$) (Fig. 4). After the first two applications, AChE levels of all treatments increased by an average of 38% (Application 1) and 137% (Application 2) while the third Bti application showed no increases compared to control levels. AChE increases occurred independently from the applied formulation (Ice: $t = 4.20$, $p < 0.001$; Liquid: $t = 4.02$, $p < 0.001$; Sand: $t = 3.10$, $p < 0.01$). As with GR activities, field rate generated a significant positive input to the AChE responses ($t = 0.02$, $p < 0.01$). Detailed results on the model output of all biomarker GLMs can be found in the Supplementary material B.

3.2. Single exposure experiment

After the first Bti application at GS 25, mean GST activity levels increased of about 48% compared to control levels, showing statistical

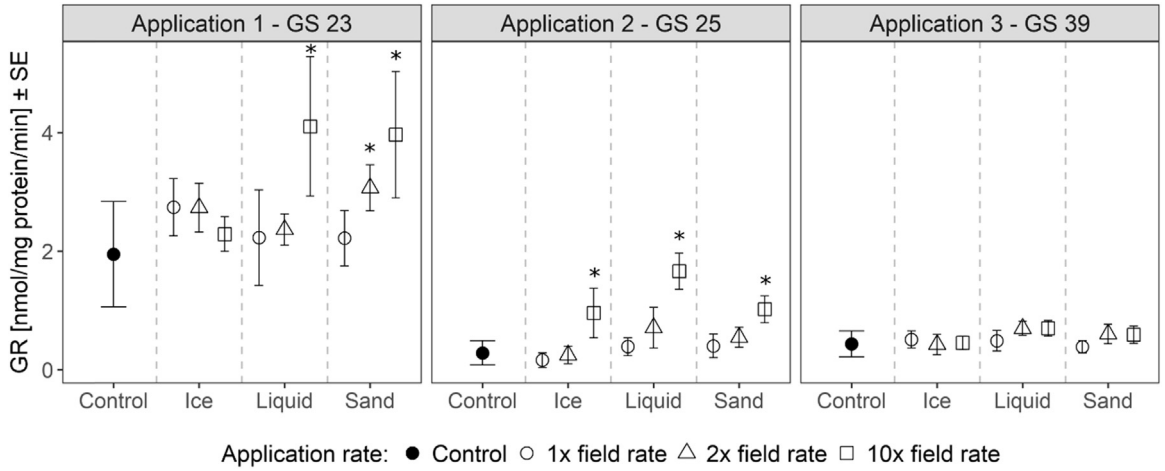

Fig. 3. Mean GR activity rates (± 95% CI) of *R. temporaria* larvae in control and Bti treatments (Ice, Liquid, Sand) at different application rates (1×, 2×, 10× field rate) for three Gosner stages (GS) (23, 25, 39) (n = 10, except n = 5 in 2× and 10× FR at Gosner 39). Asterisks indicate statistically significant differences to the respective control $p < 0.05$.

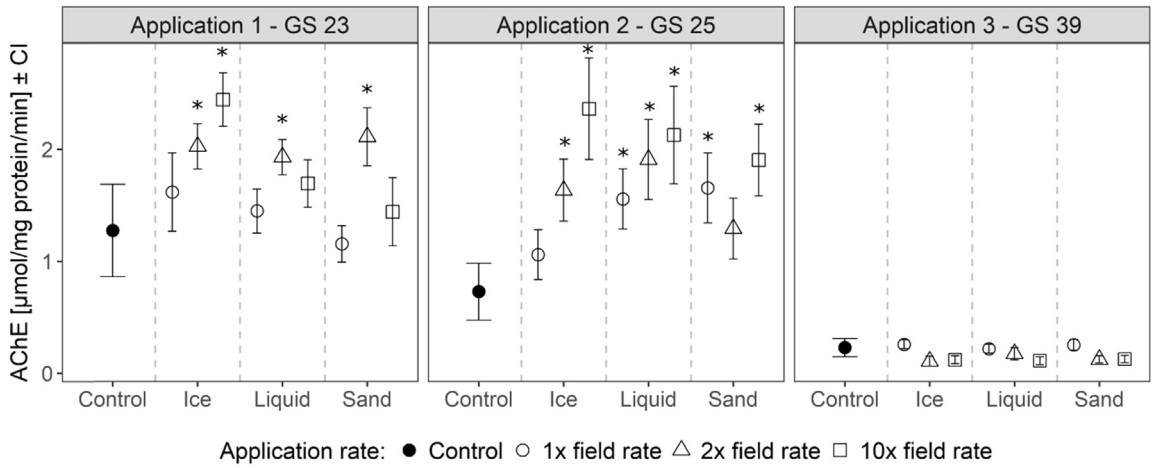

Fig. 4. Mean AChE activity rates (± 95% CI) of *R. temporaria* larvae in control and Bti treatments (Ice, Liquid, Sand) at different application rates (1×, 2×, 10× field rate) for three Gosner stages (GS) (23, 25, 39) (n = 10, except n = 5 in 2× and 10× FR at Gosner 39). Asterisks indicate statistically significant differences to the respective control $p < 0.05$.

significance in Liquid ($p < 0.05$) and Sand ($p < 0.01$). GR activity increased significantly after the exposure to all formulations (Ice: $t = 2.61$, $p < 0.05$; Liquid: $t = 2.5$, $p < 0.05$; Sand: $t = 4.65$, $p < 0.001$), resulting in a mean increase of 88%. AChE showed similar activity levels to the control, except for a statistically significant decrease in Ice ($p < 0.05$).

4. Discussion

In our experimental setup, common frog larvae experienced exposure conditions similar to realistic mosquito control in the Upper Rhine Valley. Regardless of the formulation, delivery form or application rate, tadpole survival rates and time to metamorphosis tended to be reduced after repeated Bti exposures. All exposed individuals, once again irrespective of application rate or formulation, revealed statistically significant deviations in the level of antioxidant enzyme activity when compared to control individuals. Our study indicates that field-relevant Bti applications induce metabolic processes of detoxification, antioxidant defenses and alter neuronal activity in tadpoles.

Contrary to our expectations, neither of the applied Bti formulations (VectoBac®12AS and VectoBac®WG), in any of the different delivery forms (Liquid, Ice, Sand) induced acute mortality to *R. temporaria* tadpoles. Nevertheless, we found a slight, but not significant, increase in the cumulative mortality after three consecutive Bti applications (Table 2). However, high survival rates made it possible for this study to examine adverse effects on a sublethal level. While our study is in line with older studies that show no Bti induced mortality in larval frogs (inter alia *R. temporaria*), newts, salamanders or toads (Becker and Margalit, 1993; Boisvert and Boisvert, 2000; WHO, 1999), it contradicted the findings of a recent study on the south American common frog *Leptodactylus latrans*. Lajmanovich et al. (2015) found toxic effects of the liquid Bti formulation Introban®, causing 100% mortality at a concentration of 22.45 mg/L which corresponds to 48,000 ITU/L. ITU is referring to the quantity of toxicity driving endotoxins, responsible for toxic effects in targeted insects (Skovmand and Becker, 2000). In terms of ITU, the concentration of the liquid VectoBac®12AS in our study was even higher (64,940 ITU/L), but did not significantly affect survival rates. Lethal effects towards amphibians that are based on the same very specific toxic mode of action of Bti towards insects are rather unlikely, due to the absence of suitable receptor sites in the neutral intestine of amphibians (Broderick et al., 2006; McDiarmid and Altig, 1999). Besides endotoxins, formulations also contain additives, which do not need a public declaration but constitute a major part of the formulation, varying from 62.6% (VectoBac®WG) to 98.8% (Introban®). These additives may be as responsible for the divergent results on mortality as the potential difference in the sensitivity of the tested amphibian species. In fact, many studies concerning the risks of pesticide applications for human health and wildlife have already highlighted the importance of additives and surfactants as drivers for toxicity (Cox and Surgan, 2006; Puglis and Boone, 2011; Wagner et al., 2013).

Surprisingly, we found a trend towards a faster larval development in the Bti treated tadpoles compared to control animals (Table 2). This trend was consistent throughout all Bti treatments. Amphibian larvae are able to change their behaviour, morphology or physiology in order to adapt to different environmental conditions, collectively termed as phenotypic plasticity (Newman, 1992). In this case, tadpoles could have used Bti proteins as an additional food source, which would enable them to capitalize from better aquatic growth opportunities, gain body mass and allow for a faster growing. However, this could not be supported by the data on body condition at the time of metamorphosis, since body mass, as well as body condition, did not differ between control and treated individuals (Table 2). Alternatively, amphibian larvae are also known to escape unfavorable conditions in their larval environment as soon after they reach a certain threshold body mass (Morey and Reznick, 2000). Such a reaction was already detected under the influence of pesticides (Cauble and Wagner, 2005). In addition to that, all the examined biochemical markers showed significant changes in activity levels after contact to Bti throughout the course of the experiment (see Section 3.2). These alterations indicate that tadpoles possibly underwent enhanced stress conditions during their stay in Bti treated water, determined by increases in the detoxification process (GST), the antioxidant defence (GR) and in AChE levels. Moreover, higher activities of the antioxidant enzymes GPx and GR have already been found in amphibian larvae experiencing development acceleration (Burraco et al., 2017).

In fact, Bti induced higher activity of GST and GR after each application in our study (Figs. 2 and 3). As detoxification enzyme, GST can be involved in the detoxification of Bti which was already suggested in association with resistance against Bti in mosquitoes (Boyer et al., 2007). However, given the size of the toxin and the extracellular location (the mode of action requires binding to a receptor in the midgut epithelium) it is unlikely that detoxification occurs before the interaction with the receptors. To our knowledge there is no biochemical evidence how Bti toxins could be detoxified by GST which is why Boyer et al. (2012) suggested that the increase in GST could also be a response to stress caused by either Cry toxins or associated additives.

GR is one of the key antioxidant enzymes that protects cells in stress conditions by re-establishing or maintaining redox homeostasis after an excessive increase of reactive oxygen species (ROS). Therefore, high antioxidant activities could be seen as an adaptive response in order to

avoid oxidative stress. However, if ROS is not sufficiently balanced by an upregulation of the antioxidant defenses, it may lead to oxidative stress (Monaghan et al., 2009). There are two conceivable ways for ROS to actively be created: first off, within phagocytic cells as immune response to fight the bacteria or other harmful particles contained in the Bti formulation (Steinberg, 2012). Secondly, ROS may also be induced when ingested Bti is detoxified in the biotransformation system. In the phase I metabolism, xenobiotic compounds get functionalized by cytochrome P450 enzymes (Steinberg, 2012). As a result, ROS can be generated although this step is necessary for the provision of reactive sites needed in the conjugating reaction (phase II) involving GST (Steinberg, 2012).

Increasing GST activities have also been found in tadpoles exposed to sublethal concentrations of another Bti formulation (Introban®) (Lajmanovich et al., 2015). A notable difference is, however, that the increases in GST activities after VectoBac® treatments are dose-independent and led to rather constant or even slightly decreasing activities. A potential explanation might be that the detoxification potential of GST is limited and the threshold is already reached after the exposure to nominal field rates. Subsequently, this leads to free ROS that could not be degraded by GST anymore in higher field rates (Steinberg, 2012). An oversaturation of the complete glutathione related uptake pathway at the nominal field rate could also be a feasible alternative, when GR responses would show a similar pattern to GST. However, considering that the activity pattern of GR displays an activity increase (indicating the emergence of ROS) with increasing application rates, the first assumption seems more plausible. Presuming the limitation of the GST detoxification capacity, increased field rates may nevertheless lead to higher stress levels which needs to be clarified with more biomarker assays within the phase II metabolism or ROS measurements. If the increased antioxidant responses already indicate the presence of oxidative stress cannot be finally stated by means of the analysed biomarkers in this study. Building on this, biomarker of oxidative damage in the targeted key molecules, notably DNA, proteins or lipids would need to be evaluated further (Monaghan et al., 2009).

Moreover, common frog tadpoles responded to the first two Bti applications with significant increases in AChE activity, which were up to 137% higher than control levels. As a biomarker for neurotoxic effects, it is widely accepted that a change of more than 25% in AChE activity indicates harmful effects after pesticide exposures (Beyers and Sikoski, 1994; Stansley, 1993; Sturm et al., 2007). However, deleterious neurotoxic effects are known from pesticides like organophosphates, carbamates or organochlorines and are linked to AChE inhibitions, rather than excitation (Venturino and D'Angelo, 2005). Regarding amphibians, AChE increases have also been observed in *Rhinella arenarum* and *Rana clamitans* tadpoles being exposed to organophosphorus pesticides (Rosenbaum et al., 2012; Sparling et al., 1997). As an explanation for AChE increases, Sparling et al. (1997) assumed that suffering under prolonged exposure stress could have stimulated the nervous system in tadpoles leading to the production of more ACh and consequently AChE. Besides, increased AChE levels have been found in bees after sublethal concentrations (Badiou et al., 2008). Thus, AChE increases may also be a response in order to avoid neurotoxicity to a certain degree. However, mechanisms behind AChE increases after Bti exposures have not been described so far, but should be studied further since our results indicate some unknown alterations in the neural transmission.

Subcellular responses were induced independently of formulation and delivery form, suggesting that either certain proteins, chitinases, spore-associated factors (Benz and Perron, 1967; Sampson and Gooday, 1998), a common additive or some Bti specific virulence factors such as beta-exotoxins or vegetative insecticidal may trigger biochemical alterations. However, as the surfactants of the formulations remain unknown, definitive assertions regarding the mode of action cannot be made. Furthermore, all enzymatic activities decreased with proceeding time of the experiment. Since the decrease can be seen in the control as well, changes in activity levels are probably associated with metabolic changes during the larval development (Ferrari et al., 2008). The extent of the Bti effect after individual applications varied, showing lowest activity increases after the first application followed by increases about 140% when Bti is applied a second time at a later developmental stage. Additionally, treatment effects on GST were higher in the early stages of the experiment according to the significant interaction term (Section 3.1.2.1). Due to the linkage of application frequency and larval development in the experimental design, it can hardly be distinguished if either GS 25 is a very sensitive larval stage or short-term consecutive applications intensify observed effects. In fact, the latter can be supported by the enzymatic activities gained from the single exposure experiment where Bti was applied at GS 25 for the first and only time (Section 3.2). While the increase in GST (37%) after an application at GS 23 is comparable to the increase (48%) at GS 25, a second consecutive application at the same developmental stage enhanced GST activities twice as much (Fig. C.1a). At the same time, GR activities increased as well, about 15% (Fig. C.1b). Thus, our results suggest that consecutive Bti applications in a short period of time may increase the risk for the induction of detoxification and antioxidant responses. At the time of the third exposure (32 days following the second) a recovery of the cellular responses seems most plausible (Mingo et al., 2016) and is supported by GR and AChE activities. However, latent effects from former exposures cannot be completely excluded since GST activities showed further increases.

Our results are of particular importance for mosquito control strategies in seasonal wetlands, because Bti can be applied up to 12 times a year (Becker, 1997) in intervals less than one week (KABS e.V., unpublished) depending on the incidence of flooding events that induce massive mosquito hatchings. Therefore, an application frequency of three times, chosen for this study, is a rather conservative approach in assessing Bti induced effects. While early Bti applications against snowmelt mosquitoes in marshy woodlands are implemented in March or early April, treatments against floodwater mosquitoes in temporary flooded ponds along streams are applied during summer months. Hence, various native amphibian species can come into contact with Bti, ranging from early spawning anurans that reach their spawning habitats in early spring (mid of March) such as *R. temporaria* or *R. dalmatina*, to the *Phelophylax* frogs which spawn later during May and June (Günther, 1996). Anuran tadpoles often spend their larval time in very shallow waters where the assumed water phase of 7.5 cm in height is indeed low but still realistic. For example, standardized calculations on Bti concentrations in ponds are mostly based on an assumed mean water height of 10 cm (Schnetter et al., 1981). Besides, application rates in mosquito control programs are calculated depending on the surface area of waterbodies which can vary greatly in depth or structure. Additionally, according to our results, effects on the subcellular level do not show strong dose-effect relationships.

Generally, investing in cellular responses to xenobiotics is an energy demanding process for animals (Steinberg, 2012). Tadpoles will take trade-offs due to increased costs for maintenance, which, if not resulting in direct mortality, may lead to the impairment of other life-history components such as behaviour, reproduction or life-span that, again, affect their fitness (Lushchak, 2011; Monaghan et al., 2009). Furthermore, experiences in early larval development result in latent effects that may be first exhibited in juveniles or adults (Pechenik, 2006). Such effects yet cannot be assessed by this study but are likely to determine amphibian health in later life. Amphibians inhabiting any kind of wetlands largely contribute to habitat interconnectivity, thus, potential effects on life-history or reproduction would adversely influence the transfer of an appreciable portion of energy and biomass across ecosystem boundaries (Gibbons et al., 2006).

5. Conclusion

In the light of global climate change, proceeding globalization and

the ongoing spreading of tropical mosquito species in Europe, in future, mosquito control will gain in importance with regard to human health. Consequently, the application of products based on Bti will probably rise alike worldwide due to its propagated environmental compatibility in various aquatic habitats. In view of the above, the present work indicates that the decision for such an expanded use should not be taken lightly. This is due to the induced subcellular biochemical alterations in young amphibian larvae after consecutive exposures with Bti. Subsequently, this may adversely affect amphibian health. The mode of action behind these alterations is probably different to the toxic mechanisms involved in insects. Hence integrative approaches that combine several different enzymatic biomarkers and endpoints related to the reproductive potential, need to be incorporated in further research in order to fully understand the extent of effects of Bti on non-target amphibians. Unfortunately, the current environmental risk assessment for Bti strain AM65-52 bases the risk for all non-target aquatic organisms, like basically for all insecticides, on toxicity data of fish and daphnids (European Comission, 2011). In the context of worldwide amphibian declines (Stuart et al., 2004), the implementation of a threshold on application numbers as well as a minimum interval between individual Bti treatments may help to reduce the potential risk for adverse effects on amphibians. Such precautions should be especially considered for wetlands located in designated nature conservation areas further apart from human residential areas. These areas, by definition, focus on the protection of nature and environmental health over human convenience, sometimes even in a legally binding matter. Adopting existing management accordingly would be an important first step towards environmentally safe mosquito control programs.

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Declaration of interest

The authors declare that they have no competing interests.

Authors' contribution

Study design and supervision: SA, CB; Experimental work: BF, SA; Data analysis: SA; Biomarker analysis: VM; Manuscript writing: SA

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Ethical approval

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 Rhineland-Palatinate, Germany, NTP-ID: 00008349-1-2).

Available at: https://www.animaltestinfo.de/dsp_show_ntp.cfm?ntpID=8349&showPage=qry_param_showPage&CFID=6064570&CFTOKEN=d072edf1052c8142-83F45ED1-0616-BD4B-7918E447721E422D.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2018.01.010.

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