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Phenotypic adaptation of *Chironomus riparius* to chronic Bti exposure: effects on emergence time and nutrient content

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ABSTRACT

Insects with aquatic larval and terrestrial adult life stages are a key component of coupled aquatic-terrestrial ecosystems. Thus, stressors applied to water bodies adversely affecting those larvae have the potential to influence the riparian zone through altered emergence, with differences in prey availability, timing, or nutrition. In this study, the common model organism *Chironomus riparius*, a species of Chironomidae (Diptera), was used. This selection was further motivated by its wide distribution in European freshwaters and its importance as prey for terrestrial predators. A stressor of high importance in this context is the globally used mosquito control agent *Bacillus thuringiensis* var. *israelensis* (Bti) which has been shown to affect Chironomidae. Here, we investigated the ability of chironomid populations to adapt to a regularly applied stressor, leading to a reduced impact of Bti. Therefore, the initial sensitivity of laboratory populations of *C. riparius* was investigated under the influence of field-relevant Bti treatments (three doses × two application days) and different food sources (high-quality TetraMin vs. low-quality Spirulina). Following a chronic exposure to Bti over six months, the sensitivity of pre-exposed and naïve populations was re-evaluated. Food quality had a strong impact on emergence timing and nutrient content. In addition, alterations in emergence time as well as protein and lipid contents of chronically exposed populations indicated a selection for individuals of advantageous energetics, potentially leading to a more efficient development while combating Bti. Signs of adaptation could be confirmed in five out of 36 tested scenarios suggesting adaptation to Bti at the population level. Adaptive responses of one or several species could theoretically (via eco-evolutionary dynamics) result in a community shift, favouring the prevalence of Bti-tolerant species. (In)direct effects of Bti and the adaptive responses at both population and community levels could affect higher trophic levels and may determine the fate of meta-ecosystems.

1. Introduction

Freshwater ecosystems are closely coupled with adjacent riparian zones (Bartels et al., 2012; Soininen et al., 2015). These terrestrial ecosystems are, among others, subsidized through fluxes of energy and nutrients via emerging aquatic insects (Baxter et al., 2005; Polis et al., 1997). In this context, the adult life stage of aquatic non-biting midges (Diptera: Nematocera: Chironomidae) plays an important role serving as high-quality, widely abundant prey for terrestrial predators such as spiders, bats or birds (Nakano and Murakami, 2001; Paetzold et al., 2005; Richardson et al., 2010). Within the aquatic ecosystems, chironomid larvae are a valuable food source due to their abundance and high protein content (Armitage et al., 1995; de la Noüe and Choubert, 1985). During their larval life stages, chironomids may be subjected to aquatic disturbances which can affect their development (Armitage et al., 1995; Kraus et al., 2020).

These disturbances can be of anthropogenic origin such as the larvicide *Bacillus thuringiensis* var. *israelensis* (Bti). Bti is predominantly used for the regulation of mosquito or blackfly larvae, although it can be utilized against other Nematocera (Diptera) species as well (Boisvert and Boisvert, 2000; Vaughan et al., 2008). When used as mosquito control agent, Bti affects co-occurring non-target insects in treated freshwater bodies such as chironomid larvae (Brühl et al., 2020). Especially first-instar larvae show high sensitivity towards Bti that decreases with further development (Kästel et al., 2017). Ultimately, Bti changes the aquatic-terrestrial linkage in terms of altered chironomid emergence

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Abbreviations: Bti, *Bacillus thuringiensis* var. *israelensis*; ST, sensitivity test.
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quantity, dynamics and quality (Allgeier et al., 2019; Boisvert and Boisvert, 2000; Bordalo et al., 2020; Brühl et al., 2020; Kolbenschlag et al., 2023). Depending on the local regulations, Bti can be applied in a viable and sporulating state leading to prolonged persistence (Poulin et al., 2022). Some countries, including Germany, only allow the application of the sterilized form of Bti which degrades faster, especially when affected by environmental factors, such as UV radiation or temperature (Becker, 2002; Becker et al., 1992). Even if applied sterilized, the repeated application during the growing season (Becker et al., 2018) could lead to pseudo-persistence of Bti, thereby subjecting chironomid populations to chronic stress. Such chronic exposure to a stressor can drive selection processes for (beneficial) attributes leading to adapted, more tolerant populations (Newman and Clements, 2008). The underlying processes of natural selection might be particularly fast in species with short generation times, such as some multivoltine members of the family Chironomidae (Pinder, 1986). This suggests that chironomid populations may be capable of rapidly developing tolerance towards Bti.

Previous assessments of chronic Bti exposure on non-target aquatic organisms were mostly conducted under field conditions or by employing mesocosms (Allgeier et al., 2019; Kolbenschlag et al., 2023; Lundström et al., 2010; Theissinger et al., 2019; Vinnersten et al., 2010). However, studies specifically assessing alterations in population tolerance towards Bti, particularly in terms of adaptation, are lacking. To target this gap, we conducted a laboratory experiment over approximately eight months using *Chironomus riparius* and Bti as model species and stressor, respectively. At test initiation, we investigated the sensitivity of *C. riparius* populations towards Bti crossed with two food qualities (high, low). The assessed Bti treatments included increasing doses and targeted two developmental stages of the chironomid populations with presumably different Bti sensitivity (Kästel et al., 2017). After a chronic exposure of *C. riparius* populations towards Bti over approximately six months, the sensitivity of the populations was re-assessed.

This approach allowed us to investigate the effects of the chronic Bti exposure on chironomids from two perspectives: first, a quantitative perspective of population development by assessing the emergence success (number of emerged chironomids), the emergence time (time until 50 % emerged) and the reproductive potential (maximum fertilized females). Second, a quality-based food web perspective by measuring the weight of emerged chironomids and their protein and lipid content. We hypothesised that (A), in accordance with the dynamic energy budget theory (Kooijman, 2000), a low-quality food source leads to increased sensitivity towards a stressor due to limited availability of energy and nutrients (Goedkoop et al., 2007; Sokolova et al., 2012). This might cause less and later emergence with lower reproductive potential as well as lower weight, protein and lipid contents. Furthermore, we suggested that (B) the chronic exposure to Bti leads to more tolerant populations, relative to the unexposed populations (Nikinmaa, 2014). Since adaptation processes can result in, e.g., more efficient stress responses such as detoxification processes (Férard and Blaise, 2013), chronically exposed populations might be less sensitive and feature higher weights and contents of protein and lipid, as well as more and earlier emergence when treated with Bti, compared to Bti-naïve populations. As a consequence of (A) and (B), we hypothesised that (C) the low-quality food source could have a larger impact on the Bti-sensitivity of naïve populations since their energy demand for combating Bti is presumably higher than that of chronically exposed and potentially adapted populations.

2. Materials and methods

2.1. Test organism and general test design

Larvae of *C. riparius* were obtained from an in-house culture (RPTU Kaiserslautern-Landau, Landau, Germany). Four to one month prior to this study, adult individuals from other laboratories (i.e., BASF SE (Ludwigshafen, Germany), ECT Oekotoxikologie GmbH (Flörsheim, Germany) and SBiK-F/LOEWE-TBG (Frankfurt a.M., Germany)) were introduced into this culture with the intention to increase the genetic diversity. It was assumed that this approach leads to more robust populations with an increased potential for adaptation.

Over several days, freshly laid egg ropes ($\leq$ 24 h) were collected and stored in SAM-5S medium (Borgmann, 1996). Larvae hatching over the next 96 h were used for the first sensitivity test (ST 1) in April 2021. In parallel, the same procedure was employed to start twelve long-term populations as described in *Chronic populations*. After approx. six months, freshly laid egg ropes were collected from those populations to set up the second sensitivity test (ST 2) in November 2021 following the procedure of ST 1. This schedule was designed to cover the duration of an application season of Bti in the local floodplain areas, which is usually about six months (Becker et al., 2018). A schematic overview of the test design is given in Fig. 1.

2.2. Chronic populations

Twelve populations were set up in climate chambers with temperature set at 20±1 °C, 65 % humidity and a 16:8 day/night-rhythm. Each population consisted of a cage (50×35×50 cm, L×H×W, mesh size: 0.6 mm) with two test vessels (32×7×22 cm). Each vessel contained 1.1 kg (wet weight) standardized sediment (dry weight: 75 % sand, 20 % clay, 5 % peat; 40 % water, 0.1 % $CaCO_3$) and 2 L gently aerated SAM-5S medium (Borgmann, 1996), in accordance with the respective OECD guidelines (OECD, 2010, 2004). At test start, approx. 400 randomly selected first instar larvae, from a total of 84 egg ropes, were introduced into the vessels to found each experimental population. Every two weeks, half of the populations received a Bti application (see *Bti application*) simulating a chronic exposure (pre-exposed), while the remaining six populations served as naïve controls. To maintain a stable water quality, the test medium of the vessels was exchanged every second week, within 48 h prior to Bti application. Additionally, the water level was kept constant by regular refilling to counteract evaporation. Larvae were fed with the ground fish food TetraMin (Tetra GmbH, Melle, Germany) two times a week (0.5 mg/larva/day).

2.3. Sensitivity tests

Both sensitivity tests were conducted under the same climatic conditions as detailed above. ST 1 comprised 72 test units. Every unit consisted of a 250-mL beaker with 80 g standardized sediment and 150 mL gently aerated SAM-5S medium (see *Chronic populations*; OECD, 2010, 2004). Using a stereomicroscope and converted pipettes, 20 first instar larvae were selected and introduced into each beaker. Twelve beakers served as untreated control while the remaining 60 beakers received five Bti treatments which were a combination of dose and application time (Table S1, see *Bti application*). Every two days, half of the units of each treatment ($n = 6$) were fed with a highly nutritious food (i.e., TetraMin) at a dose of 0.5 mg/larva/day (OECD, 2004). The remaining replicates ($n = 6$) received Spirulina (BioNutra, BTG Berlin Trade GmbH, Germany), a low-nutritional food consisting of cyanobacteria of the genus *Arthrospira*, at the same dose (Table S1). To counteract constant evaporation, medium was adjusted to a volume of 150 mL every second day. To be able to associate emerged individuals to each replicate, all beakers were individually covered with mesh tents, which were emptied daily. After recording emerged females and males per unit, collected individuals were stored at −80 °C until protein and lipid analyses.

ST 2 was conducted approximately half a year after ST 1 using larvae from the naïve populations and from the pre-exposed populations (i.e., chronically exposed to Bti, see *Chronic populations*) which allows to assess for potential adaptations of chironomid populations. While the number of test units doubled relative to ST 1, the experimental procedure was kept constant allowing for a direct comparison of the results.

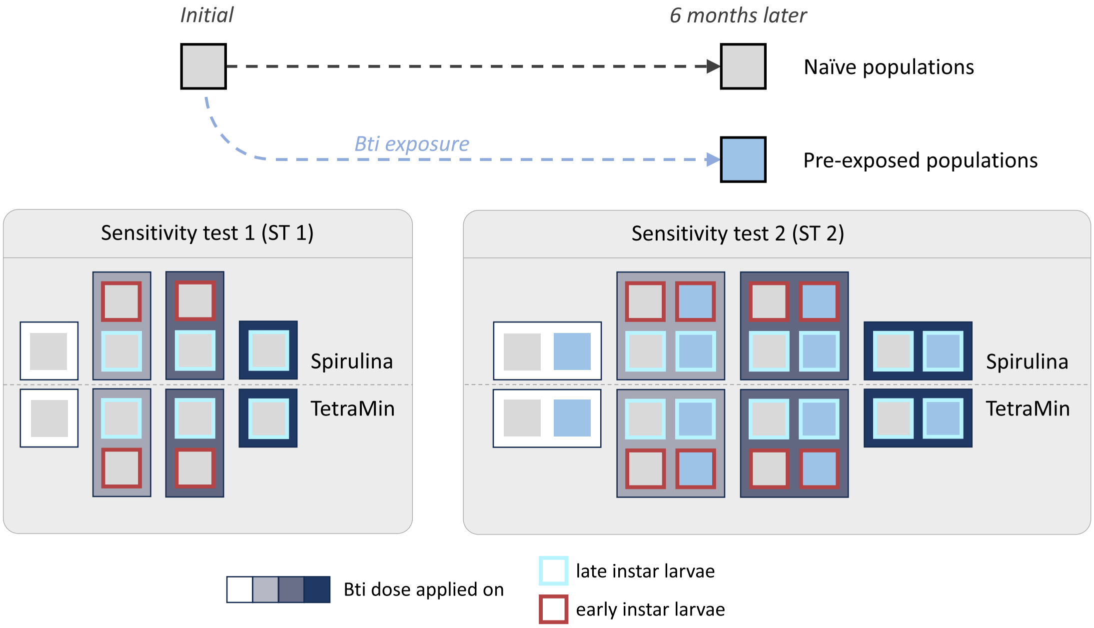

Fig. 1. Schematic overview of the test design using *C. riparius*. Unexposed control populations (naïve) and the respective larval origin are represented as grey squares, populations chronically exposed to Bti (pre-exposed) and the respective larval origin as blue squares. Sensitivity tests (ST 1 & 2) comprised two food sources, i.e., Spirulina (top) and TetraMin (bottom). Bti doses tested in the sensitivity tests, depicted by increasing shades of blue, were 0 % FR (field rate), 33 % FR, 100 % FR, and 200 % FR. Bti was applied on two developmental stages of larvae, i.e., early instar (red outlines) and late instar larvae (bright blue outlines).

All treatments of the sensitivity tests are summarized in Fig. 1 and Table S1.

2.4. Bti application

To apply Bti, the formulation VectoBac WDG (Valent BioSciences, Illinois, USA) containing *Bacillus thuringiensis* var. *israelensis* was suspended in bacteria-free, distilled water. The chronic exposure of the populations was realised by applying 33 % field rate (FR), i.e., 480 ITU/L, every two weeks. This dose was chosen on the basis of preliminary tests to induce some effect but without the risk of population extinction. The Bti doses tested in the sensitivity tests, i.e., 33 % FR (i.e., 480 ITU/L), 100 % FR (i.e., 1440 ITU/L), and 200 % FR (i.e., 2880 ITU/L), were selected to cover realistic doses as regularly applied to German floodplain areas to reduce abundance of mosquito larvae. In this context, the double FR (200 % FR) is recommended for areas with e.g., high larval densities or predominance of late developmental stages (BAuA, 2018). Since chironomid larvae are less susceptible to Bti as their developmental stage increases (Kästel et al., 2017), Bti was applied at one of two time points, i.e., day 0 or day 10, to target sensitive early-stage as well as more tolerant late-stage larvae (Table S1).

2.5. Nutrient content

The adult chironomids collected during the sensitivity tests were analysed for their protein and lipid content following the procedure of Foray et al. (2012). Therefore, two samples, i.e., one for each sex, were generated from each replicate. To achieve the biomass needed for a reliable quantification of both parameters, randomly selected individuals were pooled to reach a dry weight between 2 and 3 mg. After freeze-drying, samples were crushed in lysis buffer using a stainless-steel ball and a mixer mill (MM301, Retsch, Germany). Low-spin centrifuging allowed for the use of the supernatant in a Bradford-assay to determine the protein content spectrophotometrically at 595 nm (Infinite 200, Tecan). Bovine serum albumin in buffer was used as standard in a series of 0, 0.2, 0.32, 0.54, 0.9, 1.5, and 2.5 mg/mL.

After adding sodium sulphate solution and chloroform-methanol, samples were low-spin centrifuged. Determination of the lipid content in the supernatant was done according to the vanillin assay procedure described in Foray et al. (2012). Absorbance was measured spectrophotometrically at 525 nm (Infinite 200, Tecan) with triolein in chloroform -methanol serving as standard (0, 12.5, 25, 50, 100, 200, 400 µg/mL).

2.6. Calculations and statistics

The emergence success was calculated as total number of emerged chironomids. Replicates outside the 95 % CI ($n = 17$) or a total number of emerged individuals above 125 % ($n = 8$) were considered as outliers and eliminated from further statistical assessments. The emergence time was investigated as EmT50 (time to 50 % emergence) for all individuals and in addition for males and females separately. The EmT50s were calculated from cumulative emergence data using the package “drc” for dose-response modelling (Ritz et al., 2015). The reproductive potential was calculated following Charles et al. (2004) as the maximum possible number of fertilized females. Females can be fertilized once during the four days after emergence. Males can fertilize females for three days after emergence with decreasing ability: 1.5 females on the first day, 1 female on the second day and 0.5 females on the third day (Downe, 1973). Using the dynamics of the emergence success, the maximum number of fertilized females could be calculated for every replicate. To receive the dry weight per individual, the weight of the male and female samples used for the nutrient analyses were divided by the number of individuals. The amounts of protein and lipid were determined from the respective calibration curves and the proportion of the content calculated as follows:

$$\text{Content } (\%) = \frac{\text{P/L (mg)}}{\text{S (mg)}} \times 100 \tag{1}$$

with P/L being the total amount of protein or lipid per sample in mg, and S being the total weight of organism per sample in mg. The mean values of mg nutrient (P/L) per mg organism (S) are given in Table S4. Replicates with measured values outside the 95 % CI were excluded from the statistical analysis as outliers ($n_{\text{protein}} = 16$; $n_{\text{lipid}} = 8$).

To investigate the influence of the different factors, i.e., food source, Bti dose, application day, and exposure history on emergence success, generalized linear models (GLMs) with a Poisson distribution were generated for ST 1 and ST 2. In case of zero-inflated data (in ST 2), an additional model was done using a binomial distribution. The impact of the tested factors on the reproductive potential was assessed using a GLM with a Poisson distribution for each sensitivity test. For the endpoints weight, protein, and lipid content, linear mixed effect models were used with food source, Bti dose, application day, and exposure history as fixed effects and sex as random effect to account for the different magnitudes of values between sexes. Models for ST 1 included food source, Bti dose and application day, while models for ST 2 included food source, Bti dose and exposure history. To test for significant differences between factor levels, analyses of variance (ANOVAs) were performed with an alpha level of 0.05. EmT50 values were compared by confidence interval testing using the "drc" package.

To evaluate whether exposure history (comparison of naïve and pre-exposed populations) determined the sensitivity of chironomid populations to Bti, additional statistical analyses were conducted for each endpoint: an ANOVA was performed for each Bti × food source scenario on a linear model with exposure history (naïve vs. pre-exposed) and Bti dose (treatment vs. control) as independent variables. *P*-values were adjusted using the Holm method.

Visualisations, calculations and statistics were conducted with R (4.3.2, R Core Team, 2023) and the packages "lme4" (Bates et al., 2015), "lmerTest" (Kuznetsova et al., 2017), "ggh4x" (Brand, 2024), "ggpubr" (Kassambara, 2020) and "tidyverse" (Wickham et al., 2019).

3. Results

3.1. Quantitative perspective

3.1.1. Emergence success

In ST 1, increasing Bti doses significantly decreased the number of emerged chironomids (ANOVA, $p < 0.001$; Fig. 2, left). This effect was strongly influenced by the day of application (ANOVA, $p < 0.001$). Specifically, emergence success of chironomids that were exposed to Bti on day 0 was reduced by 41 % (33 % FR) or 77 % (100 % FR). However, if Bti was applied on day 10, numbers of emerged chironomids did only decrease at the highest dose, i.e., 200 % FR (−23 %).

During ST 2, the response to Bti differed in comparison to ST 1 but independent of the exposure history (ANOVA, $p = 0.449$; Fig. 2, right). While the applications of Bti on day 0 reduced the emergence success in all treatments to almost zero (ANOVA, $p < 0.001$), no significant influence of Bti applications on day 10 could be observed (ANOVA, $p = 0.905$). In both sensitivity tests, emergence success was not affected by the food source. Over all treatments, no sex-related pattern was observed (Fig. S2).

3.1.2. Time to 50 % emergence

In ST 1, Bti applied on day 0 led to significantly shorter emergence times relative to the controls (CI overlap testing) which was more pronounced when Spirulina served as food ($\Delta_{\text{Spirulina}} = 2.7$ d, $\Delta_{\text{TetraMin}} = 0.5$ d; Fig. 3, left). In ST 2, no emergence time could be calculated for Bti applications on day 0 due to very high mortality. Effects of Bti applications on day 10 differed significantly between food sources (ANOVA, $p < 0.001$) but were not affected by the exposure history (ANOVA, $p = 0.054$; Fig. 3, right). Low and medium doses of Bti (i.e., 33 % FR and 100 % FR) applied on day 10, caused slightly earlier emergence when fed with TetraMin (up to −0.8 d; Fig. 3, bottom). When Spirulina was provided as food (Fig. 3, top), only the highest dose, i.e., 200 % FR, resulted in shorter emergence time compared to the controls (0 % FR; −1.7 d). To some extent, low and medium doses (i.e., 33 % FR and 100 % FR) increased the emergence time (up to 1.2 d). When testing all treatments separately for effects of exposure history on the response to Bti, the interaction was significant only for 33 % FR when fed with TetraMin ($p_{\text{adj}} = 0.028$; Table 1). Additionally, emergence times of pre-exposed populations were slightly longer compared to naïve populations (+ 0.45 d). Overall, emergence times of Spirulina-fed chironomids were longer, i.e., 19.4 d (± 0.73 d), compared to TetraMin, i.e., 17 d (± 0.22 d).

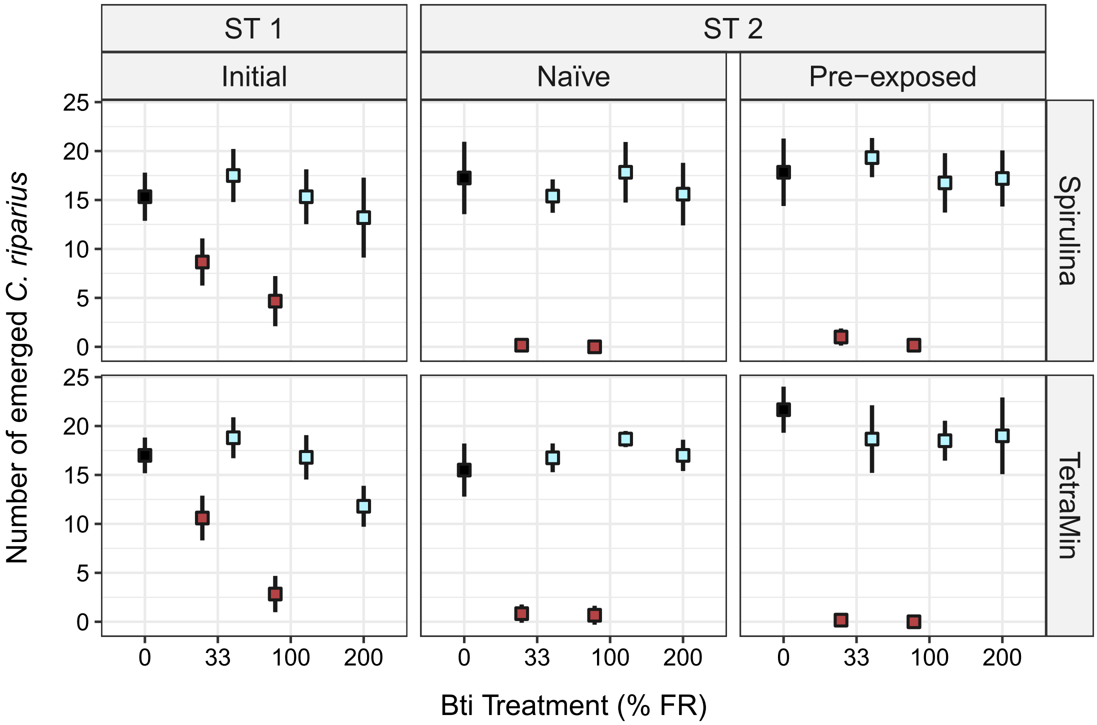

Fig. 2. Mean number of emerged *C. riparius* per treatment with 95 % confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four doses expressed as % field rate (FR) at two time points, i.e., Day 0 (red) or Day 10 (bright blue). The untreated controls are depicted in black. Chironomids used in ST 2 originated from either populations without exposure (naïve) or populations chronically exposed to Bti (pre-exposed).

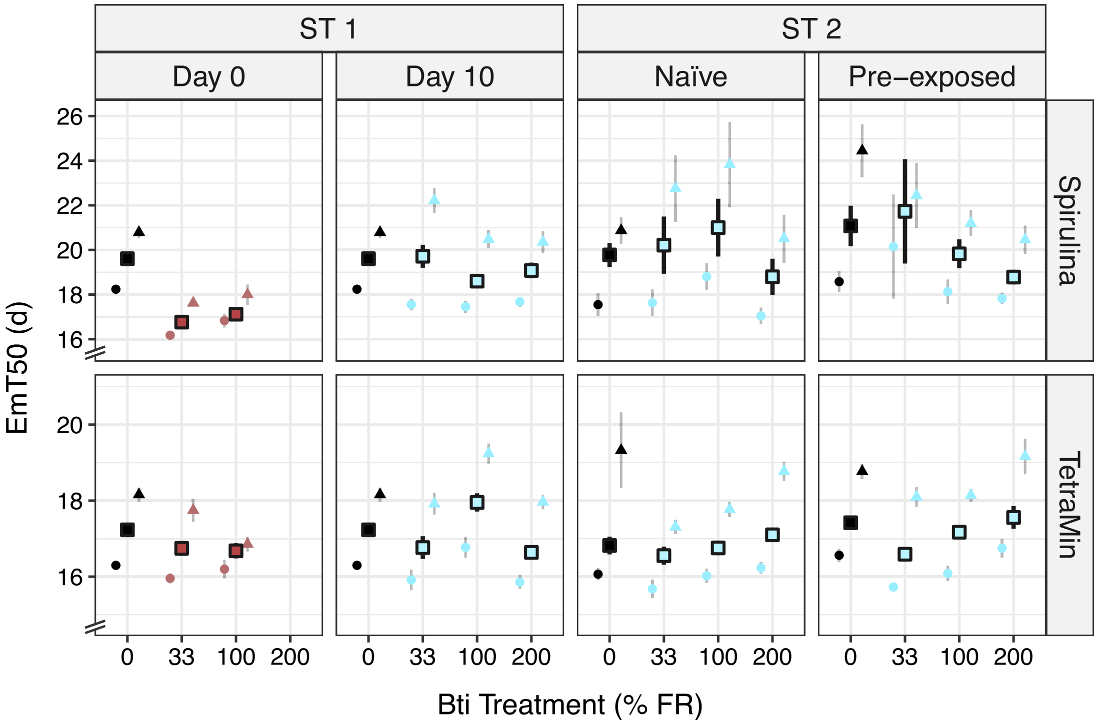

Fig. 3. Mean emergence time (EmT50 in days) of all emerged *C. riparius* (squares), of males (circles) and of females (triangles) per treatment with 99 % confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four different doses expressed as % field rate (FR) at two time points, i.e., Day 0 (red) or Day 10 (bright blue). The untreated controls are depicted in black. Chironomids used in ST 2 originated from either populations without exposure (naïve) or populations chronically exposed to Bti (pre-exposed). No EmT50 could be calculated for application day 0 in ST 2 due to high mortality. Please note that y-axes of Spirulina and TetraMin differ.

Table 1
Outcome of ANOVA analyses on each factor combination of food source (TetraMin and Spirulina) and Bti dose (33 %, 100 % and 200 % FR): *p*-values, adjusted using the Holm method, of the interaction of Bti (dose vs. control) × exposure history (naïve vs. pre-exposed). Bold numbers represent significant *p*-values (i.e., < 0.05).

Endpoint	*p*-value (adjusted): TetraMin / Spirulina		
	33 % FR	100 % FR	200 % FR
Emergence success	0.35 / 0.77	0.35 / 0.77	0.35 / 0.77
Emergence time	***0.03*** / 0.48	0.65 / 0.05	0.65 / 0.18
Reproductive potential	0.59 / 0.73	0.94 / 0.73	0.59 / 0.73
Weight	0.93 / 0.70	0.47 / 0.58	0.49 / 0.39
Protein content	0.92 / 0.09	0.81 / 0.28	0.51 / ***0.04***
Lipid content	0.23 / 0.75	***< 0.001*** / ***0.002***	***0.006*** / 0.16

3.1.3. Reproductive potential

Over all treatments, females emerged later than males with the difference ranging from 1 to 6 days (EmT50; Fig. 3). This difference was by tendency larger when chironomids were fed with Spirulina, i.e., 3.3 d (± 0.73 d), instead of TetraMin, i.e., 2.1 d (± 0.31 d). Based on the data on male and female emergence times, the reproductive potential (maximum possible number of fertilized females) was calculated for all treatments (Fig. 4). In ST 1, the application day of Bti had a significant influence on the reproductive potential (ANOVA, $p = 0.022$) independent of the food source. Bti applications on day 0 reduced the reproductive potential by 63 %, while applications on day 10 led to 14.7 % lower rates. In ST 2, effects of Bti application on reproductive potential were not significantly affected by exposure history (ANOVA, $p = 0.945$) nor food source (ANOVA, $p = 0.674$).

3.2. Qualitative perspective

3.2.1. Dry weight of emerged individuals

The dry weight of chironomids that emerged from replicates fed with Spirulina, i.e., 0.90 mg ± 0.06 mg, was significantly lower (−22 %; ANOVA, $p < 0.001$) than the dry weight of TetraMin-fed individuals, i.e., 1.14 mg ± 0.08 mg (Fig. S3). Females were approximately twice as heavy, i.e., 1.40 mg (± 0.05 mg), compared to males, i.e., 0.66 mg (± 0.02 mg), independent of the food source. No effect of Bti treatment or exposure history was observed, and neither a difference between ST 1 and 2.

3.2.2. Protein content

In ST 1, the effect of Bti treatments on protein content differed significantly between the two food sources (ANOVA, $p = 0.01$; Fig. 5, left). When fed with TetraMin, Bti treatments caused 13.1 % (± 3.7 %) lower protein contents compared to the controls (0 % FR). When Spirulina served as food source, the lower treatments (i.e., 33 % FR and 100 % FR) increased the protein content by on average 9.3 % (± 4.6 %).

The different exposure histories tested in ST 2 led to significantly different effects of Bti on the protein contents (ANOVA, $p = 0.039$; Fig. 5, right) independent of the food source. Without previous exposure to Bti, all treatments similarly reduced the protein contents in Spirulina-fed as well as TetraMin-fed chironomids by 25.2 % (± 4.6 %) and 23.3 % (± 3.5 %), respectively. In contrast, pre-exposed chironomids showed the strongest reduction in the medium Bti treatment (i.e., 100 % FR) with 22.7 % (± 10.2 % Spirulina) and 29.3 % (± 9 %, TetraMin) lower protein contents and a less pronounced reduction in the highest treatment (i.e., 200 % FR), i.e., −6.6 % (± 9.4 %, Spirulina) and −10.3 % (± 10.6 %, TetraMin). When testing all treatments separately for effects of exposure history on the response to Bti, the interaction was significant for 200 % FR when fed with Spirulina ($p_{adj} = 0.043$; Table 1).

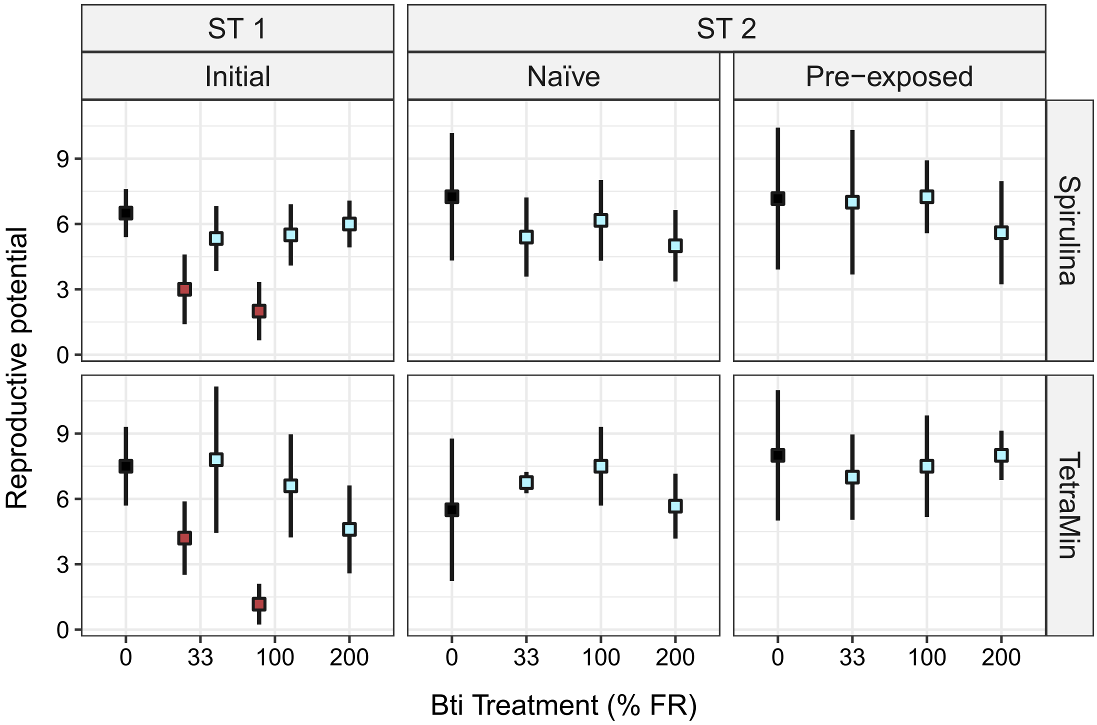

Fig. 4. Mean reproductive potential (maximum possible number of fertilized females) with 95 % confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four doses expressed as % field rate (FR) at two time points, i.e., Day 0 (red) or Day 10 (bright blue). The untreated controls are depicted in black. Chironomids used in ST 2 originated from either populations without exposure (naïve) or populations chronically exposed to Bti (pre-exposed). No reproductive potential could be calculated for application day 0 in ST 2 due to high mortality.

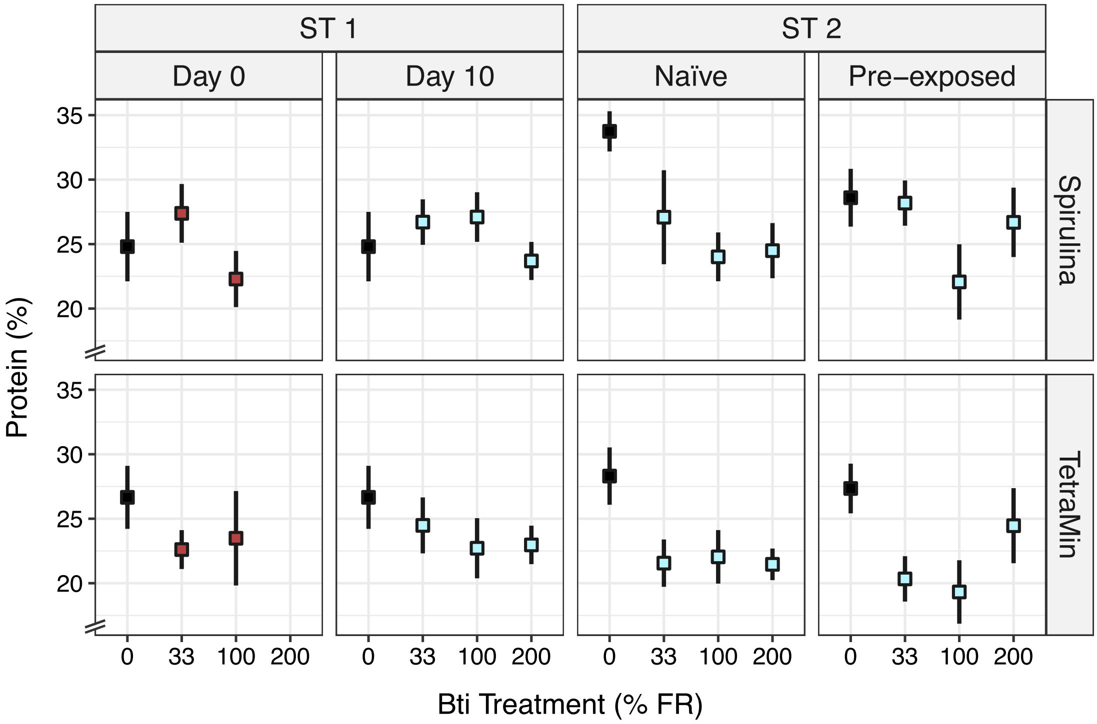

Fig. 5. Mean proportion of protein (%) in emerged *C. riparius* per treatment with 95 % confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four doses expressed as % field rate (FR) at two time points, i.e., Day 0 (red) or Day 10 (bright blue). The untreated controls are depicted in black. Chironomids used in ST 2 originated from either populations without exposure (naïve) or populations chronically exposed to Bti (pre-exposed). No values could be determined for application day 0 in ST 2 due to high mortality.

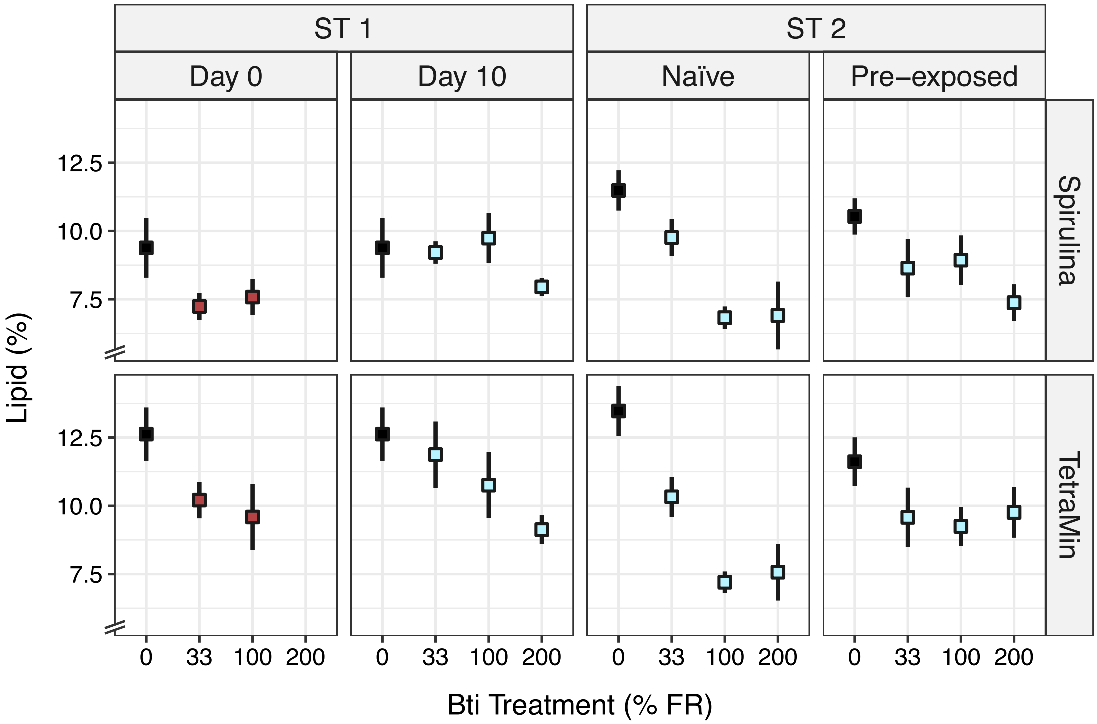

Fig. 6. Mean proportion of lipid (%) in emerged *C. riparius* per treatment with 95 % confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four doses expressed as % field rate (FR) at two time points, i.e., Day 0 (red) or Day 10 (bright blue). The untreated controls are depicted in black. Chironomids used in ST 2 originated from either populations without exposure (naïve) or populations chronically exposed to Bti (pre-exposed). No values could be determined for application day 0 in ST 2 due to high mortality.

3.2.3. Lipid content

Overall, the lipid content was significantly higher in individuals fed with TetraMin, i.e., 10.2 % lipid (± 0.3 %), than with Spirulina, i.e., 8.7 % lipid (± 0.3 %; Fig. 6; ANOVA, $p < 0.001$). In addition, significantly lower lipid content was measured in Bti treatments than in untreated controls (0 % FR; ANOVA, $p < 0.001$). In ST 1, the effects of Bti differed between the two food sources. While all Bti doses applied on day 0 led to lower lipid contents in both Spirulina (−21.2 % ± 4.2 %) and TetraMin-fed chironomids (−21.1 % ± 5.1 %), Bti doses applied on day 10 resulted in decreasing lipid content in TetraMin-fed (−5.6 % to −27.5 %) but not in Spirulina-fed chironomids.

In ST 2, the exposure history caused a significantly different effect of Bti (ANOVA, $p < 0.001$) independent of the food source. In the naïve populations, the lowest Bti treatment (33 % FR) led to moderately less lipid in chironomids (Spirulina = −15.1 %, TetraMin = −23.7 %), while the higher Bti treatments caused an equally higher reduction of lipid content (Spirulina = −40.3 % ± 5.2 % and TetraMin = −45.2 % ± 4.2 %). In chironomids of pre-exposed origin, the lipid content was equally reduced in all Bti treatments (Spirulina = −20.6 % ± 5.1 %, TetraMin = −17.6 % ± 4.6 %). When testing all treatments separately for effects of exposure history on the response to Bti, the interaction was significant for 100 % FR when fed with Spirulina ($p_{adj} = 0.002$; Table 1). With TetraMin as food source, the interaction of exposure history and Bti was significant for 100 % FR ($p_{adj} < 0.001$) and 200 % FR ($p_{adj} = 0.006$).

4. Discussion

4.1. Quantitative perspective – populations

In line with previous studies, the initial sensitivity test (ST 1) showed that increased Bti doses led to decreased emergence success highlighting the lethal effect of Bti towards non-target chironomids (reviewed in Brühl et al., 2020; Land et al., 2023). Furthermore, our observations of higher mortality when Bti was applied on day 0 relative to day 10 (indicated by lower emergence success) confirm increased susceptibility to Bti in early-stage larvae (Kästel et al., 2017). Besides increased mortality, Bti application on day 0 induced accelerated emergence of those surviving. Earlier emergence may be explained by lower larval densities as consequence of Bti, resulting in more resources being available for surviving larvae (Postma et al., 1994; Vos et al., 2000). With lower competition for food, larvae could reach metamorphosis checkpoints during their development earlier (Mirth and Riddiford, 2007) leading to an earlier emergence (Goedkoop et al., 2007; Hooper et al., 2003). Alternatively, Bti may have triggered the emergence process as a mechanism to escape unfavourable environmental conditions (as shown for drought stress by Drummond et al., 2015).

Only partially in line with our hypothesis (Hypothesis A), the low-quality food source had no impact on the emergence success of the initial populations (ST 1), but increased emergence times. Elaborated in the dynamic energy budget theory (Kooijman, 2000), when less energy can be acquired from a food source, less energy can be allocated to developmental processes, resulting in a delayed emergence (Goedkoop et al., 2007). Since female chironomids require in general high energy reserves for reproduction (e.g., egg production), they show an increased energy demand during their development and thus emerge later than males (Armitage et al., 1995; Frouz et al., 2002). Consequently, the low-quality food source (i.e., Spirulina) increased the time lag between male and female emergence due to the lower energy supply. However, the factor food source did not affect the reproductive potential of the populations. The respective calculations are partially based on the fecundity of males and females, which was not directly evaluated in this study. This introduces some uncertainties regarding the influence of food quality on the reproductive potential. Nonetheless, differences in the nutritional quality of TetraMin and Spirulina could indeed affect the adult life span and the period of fecundity (Souza et al., 2019) resulting in lower reproductive success. The quality of food supply under natural conditions might be in between the two tested food scenarios as they represent a balanced (i.e., TetraMin) and a one-sided diet (i.e., Spirulina). Freshwater bodies usually comprise different sources of food although various (environmental) factors influence their availability and quality. In contrast to the food source, Bti treatments decreased (when compared to the control) the reproductive potential which might be mainly attributed to the above-mentioned mortality in Bti-treated populations.

Contrary to our expectations (Hypothesis B), the strongest effect of long-term culturing on larval sensitivity was independent of the Bti exposure history: sensitivity of early instar larvae (ST 2) increased substantially, demonstrated by nearly complete mortality in all treatments that received Bti on day 0. A potential explanation could be genetic impoverishment of our populations due to the limited genetic variation of the initial populations ("founder effect") combined with a lack of gene flow (Nowak et al., 2007). The continuous loss of alleles (genetic drift) over six months (i.e., approximately eight generations) might have resulted in lower fitness of the populations and high susceptibility to experimental conditions and Bti (Nowak et al., 2007). Besides this general observation, chironomids from pre-exposed populations emerged slightly later than their naïve counterparts. This suggests increased sensitivity towards Bti whereas we hypothesised a reduced sensitivity due to selection for more tolerant individuals (Vogt et al., 2007). The higher sensitivity of chironomids chronically exposed to Bti may be based on the fact that the toxicity of Bti is induced by a mixture of δ-endotoxins (Cry and Cyt) causing membrane-perforation (Ben-Dov, 2014; Schnepf et al., 1998). While resistance towards individual toxins have indeed been observed in mosquitoes, the mixture of these toxins shows lower potential for tolerance development (Becker et al., 2018; Georghiou and Wirth, 1997; Paris et al., 2011; Tetreau et al., 2013). This common observation might be attributed to a synergistic interaction of the toxins, their different modes of action, and their activation and interaction with specific enzymes or (membrane) receptors (Ben-Dov, 2014; Poncet et al., 1995). Consequently, tolerance may be due to modifications to those receptors or altered activity of enzymes, such as proteases (Morin et al., 2003; Oppert et al., 1997). While resistance to the complete Bti mixture has not previously been observed in chironomids, observations on mosquito populations are not consistent, reporting either an absence of resistance or resistance to a certain degree (Becker et al., 2018; Georghiou and Wirth, 1997; Paris et al., 2011; Su, 2016; Tetreau et al., 2013).

While we expected tolerant individuals to prevail, it is possible that the required traits were just not present in the assessed populations. Alternatively, mechanisms for an increased tolerance of Bti could be associated to high costs leading to a decreased individual fitness (Férard and Blaise, 2013) hampering tolerance establishment. In addition, the selective pressure induced by the chronic exposure to Bti (i.e., 33 % FR) might have been too weak and was potentially superimposed by an age-dependent selection. The latter refers to the less strong effect in late instar larvae relative to younger individuals (Fig. 2; Kästel et al., 2017), a process, which indeed selects for age and not genetically manifested tolerance. The resulting decrease in genetic variation (*natural selection*) on top of the above suggested genetic impoverishment of the laboratory populations (*genetic drift*) could have ultimately reduced the biological fitness of the populations leading to an even higher susceptibility to Bti (Armbruster and Reed, 2005; Nowak et al., 2007).

Decreased genetic diversity in our long-term cultured populations might also be a reason for divergent influence of the food sources on the emergence time during ST 2 (Hypothesis C). Chironomids fed with low-quality food (i.e., Spirulina) emerged later when exposed to lower doses of Bti (i.e., 33 % FR & 100 % FR, day 10). To cover their elevated energy requirements as consequence of Bti exposure, larvae may have had to extend the time of food ingestion and were thus able to increase their energy uptake to successfully complete emergence (Goedkoop et al., 2007). In contrast, when offering high-quality food (i.e., TetraMin), lower doses of Bti induced earlier emergence of chironomids. Potentially triggered by the unfavourable environment due to the presence of Bti, larvae fed with high-quality food may, according to the dynamic energy budget theory (Kooijman, 2000), be able to allocate more energy to their development while still combating the impact of Bti. This observation was not confirmed at the highest Bti dose (i.e., 200 % FR) probably due to even higher energy requirements for biochemical stress responses or repair mechanisms depleting available resources on the expense of development (Kooijman, 2000; Sokolova et al., 2012). Moreover, chironomids fed with Spirulina had to cope with a switch of food source since long-term populations were fed with TetraMin. This alteration in the feeding conditions could have affected the response to Bti in addition to the lower quality of the food source Spirulina.

As described above, the general response to Bti (over all doses and food sources) was not significantly affected by the exposure history for emergence success, emergence time and reproductive potential. Despite this, one specific factor combination (out of 18 factor combinations: 3 Bti doses × 2 food sources × 3 endpoints) did show signs for phenotypic adaptation: when TetraMin served as food source and chironomids were exposed to Bti at 33 % FR, the emergence time of *C. riparius* was significantly reduced (compared to the control) due to pre-exposure. Such response might be explained by the fact that larval development is a sensitive part of insects' life cycle and adaptive responses may first be seen in their emergence time. In fact, earlier emergence could be a helpful feature to escape unfavourable environmental conditions, an aspect we discussed in further detail above. While the data suggests the possibility to adapt to Bti exposures over several generations, ongoing analyses of the genomic responses in pre-exposed and naïve populations will help to understand the underlying processes (unpublished data, Röder et al.).

4.2. *Qualitative perspective – food web*

The high-quality food source (i.e., TetraMin) resulted in higher chironomid dry weight compared to Spirulina which matches our hypothesis and is in line with the results of similar experiments (Souza et al., 2019; Strandberg et al., 2020). TetraMin contains higher amounts of carbohydrates which are easy to respire and mainly used as an energy source (Souza et al., 2019; Vos et al., 2000). In addition, both food sources are good protein suppliers, with contents between 45 % and 70 % (manufacturers' information: BTG Berlin Trade GmbH, 2024; Tetra GmbH, 2024). Higher protein contents in Spirulina could explain to some extent the observed pattern in chironomid protein content in the first sensitivity test (ST 1). Lipid contents are low in both resources, but differences in their nutritional quality can also be ascribed to their fatty acid composition (Pietz et al., 2023; Strandberg et al., 2020). However, fatty acid profiles are beyond the scope of this publication. Contrary to our expectations, Bti affected protein and lipid contents of adults but not their dry weight. Permanent body structures such as the exoskeleton or wings seemed to make up for the largest part of chironomid dry weight, overriding small variations in nutrient contents.

After six months of long-term culturing, protein and lipid contents of chironomids were negatively affected by Bti in both pre-exposed and naïve populations (ST 2). Although the effects of Bti on protein content varied only slightly across exposure histories, one of the six scenarios tested (i.e., 200 % FR × Spirulina) showed a significantly higher protein content in pre-exposed chironomids compared to naïve chironomids, suggesting an adaptation to Bti. Similarly, lipid contents of pre-exposed populations were more robust and less reduced by Bti than of naïve populations. In three out of six tested scenarios (i.e., 100 % FR × Spirulina, 100 % FR × TetraMin, 200 % FR × TetraMin) significant interaction terms were observed, indicating adaptation to Bti. These findings could suggest a more efficient use of available energy reserves for combating Bti (Kooijman, 2000; Sokolova et al., 2012) as a result of chronic Bti exposure. Since Bti in general reduced the content of both lipids and proteins, adaptive changes to those effects could have further consequences for the linked food web. This is particularly relevant as chironomids are an important food source for numerous predators in the riparian zone. This is not only driven by their quantity with up to 90 % of the emerging insects (Kolbenschlag et al., 2023), but also by their quality (Pietz et al., 2023; Schindler and Smits, 2017). Thus, observed changes in the nutritional quality of emerged chironomids can potentially affect predators such as spiders, lizards, bats or birds through the food chain (Fukui et al., 2006; Gray, 1993; Kato et al., 2003; Sabo and Power, 2002). In that way, the impact of a stressor on chironomids may extend to the next trophic level of consumers, potentially affecting their fitness and reproduction. This, in turn, could have cascading consequences on their population development through indirect effect pathways (Bartels et al., 2012; Schulz et al., 2015).

5. Conclusion

Populations of *Chironomus riparius* showed only limited levels of adaptation to Bti exposure under laboratory conditions. No effects of the Bti pre-exposure were observed on emergence success and reproductive potential. However, in one out of six tested scenarios emergence time showed phenotypic adaptation with earlier emergence in pre-exposed compared to naïve populations. Moreover, alterations in chironomids' nutrient content (mostly increases in pre-exposed chironomids) suggest potential adaptation in *C. riparius* populations to Bti which could be confirmed for four out of twelve tested scenarios. Since chironomids play a key role in riparian food webs, stress-caused changes in chironomids' quantity (emergence time) or quality as food source (nutrient content) might have consequences for higher trophic consumers and predators. In addition, direct and indirect effects of a biocide such as Bti as well as the adaptive response to it could not only alter individuals and populations but also communities. Thus, the observed effects could be scaled up and linked to species-rich chironomid communities (e.g., Kolbenschlag et al., 2023; Röder et al., 2024; Theissinger et al., 2019) which needs to be addressed in future studies. Enhancing the understanding of the eco-evolutionary dynamics of long-term exposed ecosystems can help to improve the assessment and regulation of biocide applications.

Besides for mosquito control, Bti is also used to combat infestations by Chironomidae as they are considered a nuisance or health threat (e.g., in the USA, the U.K., Australia or China; Ali et al., 1981; Ping et al., 2005; Stevens et al., 2013; Vaughan et al., 2008). However, Bti doses applied in this context are usually at least ten times higher than those relevant to the use case assessed in the present study. Understanding the potential for adaptation under those conditions might also be relevant from a human health perspective but remain to be explored.

CRediT authorship contribution statement

Sara Kolbenschlag: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Sebastian Pietz:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Nina Röder:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Klaus Schwenk:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Mirco Bundschuh:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data of this article is available through zenodo: Kolbenschlag, S., Pietz, S., Röder, N., Schwenk, K., & Bundschuh, M. (2024). Emergence data from multi-generation study on Chironomus riparius [Data set]. Zenodo. https://doi.org/10.5281/zenodo.11321653.

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Supplementary materials

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