

THE DYNAMICS OF AQUATIC SUBSIDIES: HOW DISTURBANCE CAN PROPAGATE FROM
AQUATIC INSECTS TO RIPARIAN SPIDERS,
EXEMPLIFIED BY *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS*

by

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Mischief managed

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DECLARATION

I hereby declare that I independently conducted the work presented in this thesis entitled “The dynamics of aquatic subsidies: how disturbance can propagate from aquatic insects to riparian spiders, exemplified by *Bacillus thuringiensis* var. *israelensis*”. All used assistances are mentioned and involved contributors are either co-authors of or are acknowledged in the respective publication.

This thesis has never been submitted elsewhere for an examination, as a thesis or for evaluation in a similar context to any department of this university or any scientific institution. I am aware that a violation of the aforementioned conditions can have legal consequences.

Place, date

Signature

This thesis is a cumulative dissertation based on the following peer-reviewed publications:

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C. A., & Bundschuh, M. (2023). A temporal perspective on aquatic subsidy: Bti affects emergence of Chironomidae. *Ecotoxicology and Environmental Safety*, 250, 114503. <https://doi.org/10.1016/j.ecoenv.2023.114503> [**Appendix A.1**]

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Kolbenschlag, S., Pietz, S., Röder, N., Schulz, R., & Bundschuh, M. (Under review). Long-term exposure to Bti alters the sensitivity of *Chironomus riparius* populations. Submitted to a peer-reviewed journal [**Appendix A.3**]

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ABSTRACT

Aquatic habitats are closely linked to the adjacent riparian area. Fluxes of nutrients, energy and matter through emerging aquatic insects are a key component of the aquatic subsidy to terrestrial systems. In fact, adult insects serve as high-quality prey for riparian predators. Stressors impacting the aquatic subsidy can thus translate to consequences for the receiving terrestrial food web, while mechanistic knowledge is extremely limited.

Against this background, this thesis aimed at (i) assessing the impact of a model stressor specifically targeting insect emergence, that is the mosquito control agent *Bacillus thuringiensis* var. *israelensis*, on quantity, temporal dynamics and (ii) quality of emerging aquatic insects. For this purpose, outdoor floodplain pond mesocosms (n = 6) were employed. Since emergence is, in most cases, no point event but occurs over a longer period emergence was monitored over 3.5 months. The model stressor, i.e., Bti applied three times during spring at 2.88×10^9 ITU/ha, shifted the emergence time of aquatic insects, especially of non-biting midges (Diptera: Chironomidae), by ten days with a 26% reduced peak, while the nutrient content was not altered. On this basis, (ii) the propagation of the effects in aquatic subsidy emergence to riparian predators was investigated. Stable isotope analyses were used to assess the diet of a model predator, that is the web-building riparian spider *Tetragnatha extensa*. Results suggested changes in the composition of the spider's diet to replace missing Chironomidae by other aquatic and terrestrial prey organisms pointing to further negative consequences. Finally, the thesis aimed at (iii) the understanding of processes underlying an altered emergence of aquatic subsidy mainly consisting of chironomids. Using a laboratory-based test design, populations of *Chironomus riparius* (n = 6) were assessed for their sensitivity towards Bti under different food qualities (high and low nutritious) before and after a long-term (six months) Bti exposure. Signs of phenotypic adaptation were observed in emergence time and nutrient content over multiple generations, resulting in changes in chironomids' quantity and quality as food source.

Overall, it can be concluded that direct and indirect effects of an aquatic stressor, as well as the adaptive response to it, can alter ecosystems at different levels, including individual, population and community level. Furthermore, this thesis highlights the importance of a temporal perspective when investigating the impact of aquatic stressors beyond ecosystem boundaries. It illustrates potential bottom-up effects on riparian predators through altered emergence of aquatic insects, feeding our understanding of meta-ecosystems and how stressors and their effects are transferred across systems. These insights will support efforts to protect and conserve natural ecosystems.

1. INTRODUCTION

1.1 Ecological relevance of aquatic subsidies

Aquatic and adjacent riparian ecosystems are closely linked forming an aquatic-terrestrial meta-ecosystem (Soininen et al., 2015). They reciprocally subsidize each other by cross-boundary fluxes of matter, nutrients and energy from donor to recipient system, supporting the productivity of the recipient (Baxter et al., 2005; Polis et al., 1997). While the subsidy of aquatic ecosystems by terrestrial resources, for example through leaf litter, has been intensively studied for decades, aquatic subsidies for riparian food webs have been lacking behind (Schulz et al., 2015). This is particularly true when aiming at studies targeting the consequences of disturbances in aquatic ecosystems and the linked riparian food webs. This lack of knowledge may be triggered by the complexity of the topic calling for research at different scales (Polis et al., 2004). In the present thesis, this gap was addressed by using outdoor pond mesocosms (Appendix A.1 & A.2) and a laboratory approach (Appendix A.3).

The inputs of riparian leaf litter and terrestrial invertebrates are, as indicated above, examples for the flow of terrestrial resources into aquatic habitats (Fisher & Likens, 1973). Conversely, a major compound of aquatic-to-terrestrial subsidies are emerging aquatic insects when mastering metamorphosis (Paetzold et al., 2005). In most cases, aquatic subsidy consists of adult individuals of Diptera, Ephemeroptera, Plecoptera, Trichoptera, and Odonata of which Diptera are with up to 99% the most abundant ones (Baxter et al., 2005; Kolbensschlag et al., 2023; Leeper & Taylor, 1998). Aquatic insects function as prey for a variety of terrestrial consumers including spiders, birds, bats, or lizards, and can account for substantial proportions of these predators' diet (Arlettaz et al., 2001; Fukui et al., 2006; Gray, 1993; Henschel et al., 2001; Kato et al., 2003; Nakano & Murakami, 2001; Paetzold et al., 2005; Recalde et al., 2021; Sabo & Power, 2002). While the magnitude of terrestrial subsidy to aquatic ecosystems is often superior over the other direction than vice versa, aquatic subsidy is considered to be of higher quality (Bartels et al., 2012). Nutrient and lipid contents of aquatic insects tend to be greater compared to terrestrial prey (Armitage et al., 1995; Schindler & Smits, 2017). In addition, aquatic systems produce essential highly unsaturated fatty acids, which are by definition of high nutritious value (Twining et al., 2016). Thus, aquatic subsidy is not only another source of energy but provides important nutrients that can be hardly acquired by terrestrial prey (Hixson et al., 2015). In consequence, aquatic subsidies induce high densities and great

diversity of consumers in riparian zones (Fukui et al., 2006; Gray, 1993; Nakano & Murakami, 2001).

As a result, development and life stages of riparian consumers depend on the magnitude but also temporal availability of aquatic subsidy. The timing of emergence is highly variable but can be influenced by different factors such as nutrient availability, water temperature or climate (Armitage et al., 1995; Goedkoop et al., 2007; Schindler & Smits, 2017). In temperate regions, the peak of aquatic emergence occurs between spring and early summer followed by low fluxes until early spring (Nakano & Murakami, 2001; Sabo & Power, 2002). In contrast, the in situ biomass of terrestrial habitats in the same climatic zone peaks in late summer and is low during winter and spring (Nakano & Murakami, 2001). Hence, aquatic subsidy buffers for low abundances of terrestrial prey in spring due to the seasonal asynchrony (Wesner, 2010). The high density of prey items during peak emergence times extends up to several hundred meters (Muehlbauer et al., 2014; Schindler & Smits, 2017) and usually attracts consumers such as migrating birds or bats (Fukui et al., 2006; Gray, 1993; Recalde et al., 2021). In addition, also residential consumers can be temporarily dependant on aquatic subsidy since peak emergence occurs in parallel to periods of high energy demand such as the end of hibernation and during reproduction (Altringham, 1996; Bryant & Westerterp, 1983; Fukui et al., 2006; Vaughan, 1997; Zahn et al., 2007).

Besides vertebrate predators, terrestrial arthropods living in the riparian zone can be substantially dependent on aquatic subsidy. While ground-dwelling and free-hunting predators show proportions of up to 70% aquatic diet (O'Callaghan et al., 2013; Schulz et al., 2015), consumption of aquatic prey can be even higher in riparian web-building spiders due to their web-based foraging strategy (Hawley et al., 2014; Jensen et al., 2011). Indeed, particularly the density of horizontal orb-weavers (Tetragnathidae) follows the emergence dynamics of aquatic insects likely due to aquatic prey contributions of up to 90% (Kato et al., 2003; Krell et al., 2015; Wiczorek et al., 2015). One species strongly featuring this relation is *Tetragnatha extensa* (Akamatsu et al., 2004; Graf et al., 2020; Wiczorek et al., 2015) which was thus chosen as model riparian predator in this thesis.

1.2 Effects of anthropogenic stressors on aquatic subsidies

Predators consuming aquatic insects respond to variations in prey availability. This dependence is explained by their physiological and behavioural adaptation to cycles of emergence and hence the timing of energy fluxes (Armstrong et al., 2016; Marczak &

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Richardson, 2008). As detailed above, consumers might temporarily rely on the aquatic nutrient and energy supply, thus disturbance of the emergence dynamics of aquatic insects can transfer through the aquatic-terrestrial food web (Paetzold et al., 2011; Stepanian et al., 2020). Besides natural factors such as seasonal variation, anthropogenic modifications can result in quantitative as well as qualitative changes of aquatic subsidies (Kraus et al., 2020; Paetzold et al., 2005; Schulz et al., 2015). Those anthropogenic modifications comprise hydromorphological alterations, invasive species and water pollution (Gergs et al., 2014; Greig et al., 2012; Schulz et al., 2015). Pollutants enter aquatic ecosystems, among others, through run-off or spray-drift inducing lethal and sublethal (e.g., physiological stress, behavioral alteration and reduced reproduction) effects on aquatic species (Schulz, 2004). Given that species differ in their sensitivity towards toxicants (Newman et al., 2000), this often results in shifts in species composition (Liess & Ohe, 2005), with consequences on intra- and interspecific interactions (Liess, 2002; Preston, 2002). Such indirect effects can be of equal or even higher importance than direct effects and may propagate within and across the boundaries of aquatic systems (Fleeger et al., 2003; Schulz et al., 2015). These effects are not solely quantity-driven (e.g., biomass) but may also mediate through changes in the emergence time and nutritional quality of aquatic subsidy (Bundschuh et al., 2019; Dewey, 1986; Kotalik & Clements, 2019; Langer-Jaesrich et al., 2010; Pietz et al., 2023; Tassou & Schulz, 2013). Given the high dependence of riparian spiders on aquatic subsidy, indirect effects on these spiders have repeatedly been shown in response to pollution of aquatic ecosystem (Kato et al., 2003; Kraus et al., 2014; Krell et al., 2015; Paetzold et al., 2011). These effects can, for example, comprise the dietary exposure towards inorganic and organic pollutants but also less persistent compounds may reduce species richness and density of riparian spiders (Graf et al., 2019; Paetzold et al., 2011; Schulz & Bundschuh, 2020).

A stressor with a selective effect in one group of emerging insects (e.g., nematoceros Diptera) is the bacterial mosquito control agent *Bacillus thuringiensis* var. *israelensis* (Bti; Després et al., 2011). Bti was chosen as model stressor to mechanistically assess the propagation of disturbed aquatic subsidy to terrestrial predators. While most other stressors are unintentionally introduced into the environment, Bti is deliberately applied to freshwater bodies to control mainly larvae of mosquitoes and blackflies (Becker, 1997; Boisvert & Boisvert, 2000; Després et al., 2011). Applications in the Upper Rhine Valley are usually done between April and September, ranging from one to eleven times per year with an average of five applications (Becker et al., 2018; Brühl et al., 2020).

The larvicidal activity of Bti is described by δ -endotoxins that become activated in the alkaline milieu of the larval midgut. The binding to specific receptors results in membrane-perforation and ultimately mortality within hours (Ben-Dov, 2014; Dyló et al., 2014; Vachon et al., 2012). Although this mode of action is described as taxa-specific it includes also larvae of other nematoceros dipterans, which can be explained by their phylogenetically close relation (Woodley et al., 2009). While several studies on Bti demonstrated adverse effects on non-target organisms (reviewed in Boisvert & Boisvert, 2000; Brühl et al., 2020) reported direct consequences on non-biting midges (Diptera: Chironomidae) are especially relevant in the context of disturbed aquatic subsidy (Allgeier et al., 2019; Charbonneau et al., 1994; Kästel et al., 2017; Liber et al., 1998). Chironomidae often dominate aquatic subsidy, in some cases with up to 90% (Armitage et al., 1995; Leeper & Taylor, 1998), and are considered high-quality prey for a wide range of riparian predators (Bergeron et al., 1988; de la Noüe & Choubert, 1985). Thus, potential Bti-induced alterations of their emergence could affect subsidy for higher trophic levels in the terrestrial ecosystem which might cascade through the terrestrial food web (Henschel et al., 2001; Jakob & Poulin, 2016; Poulin et al., 2010). However, knowledge on the impact of Bti on aquatic-terrestrial meta-ecosystems is scarce.

2. THESIS OUTLINE AND OBJECTIVES

The main objective of this thesis was to obtain a temporal perspective on effects of an anthropogenic stressor (i.e., Bti) on aquatic insects and the propagation of these effects to riparian spiders. Therefore, Bti was applied to floodplain pond mesocosms (FPMs) employing a field relevant scenario. The first research objective (RO 1) was to investigate changes in the community of emerging aquatic insects and their temporal emergence pattern over 3.5 months as consequence of Bti [Appendix A.1]. In order to link potential effects in aquatic subsidy to the subsidized terrestrial food web, the second objective (RO 2) targeted the investigation of the diet of spiders living in the riparian vegetation of the FPMs [Appendix A.2]. Relevant factors to assess were the prey quality (nutrient content), the spiders' diet composition as well as the spiders' trophic position using stable isotope analyses. To expand the understanding of temporal effects in the field study (RO 3), a long-term laboratory experiment on populations of chironomids was performed [Appendix A.3] targeting adaptation processes towards chronic Bti exposure using a model species for aquatic subsidy, i.e., *Chironomus riparius*.

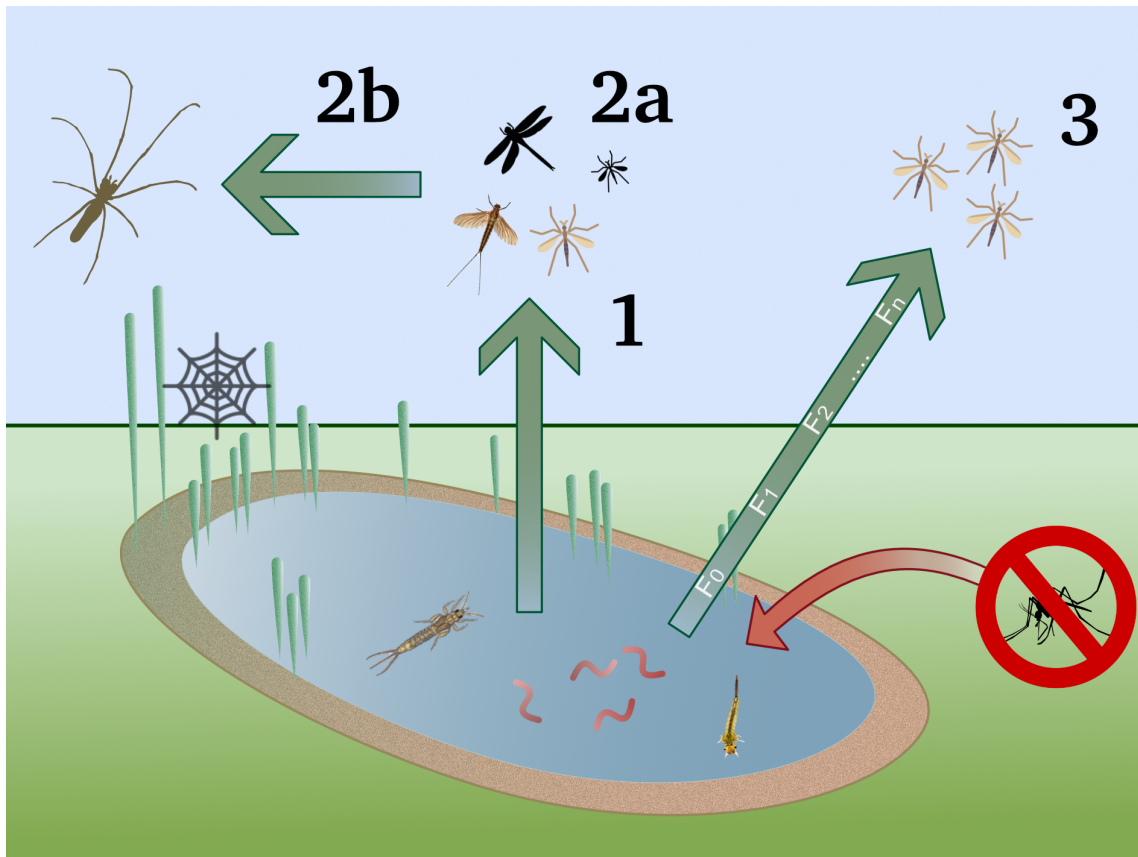


Fig. 1: Overview of this thesis providing the research objectives and their implementation in the following publications and chapters (Appendix A.1 – A.3).

RO 1: Assessment of Bti-induced alterations in emergence quantity, dynamics and biomass of aquatic insects [Appendix A.1]

RO 2: Evaluation of the impact of Bti on riparian spiders through changes in prey quality (a) and spider diet (b) [Appendix A.2]

RO 3: Consequences of long-term Bti exposure on the sensitivity of chironomids [Appendix A.3]

3. METHODS

3.1 Experimental design: field study

Study site

The field study, which formed the basis for two publications investigating RO 1 and 2, was conducted from April to July 2020 in twelve artificial floodplain pond mesocosms (FPMs; area $\approx 104 \text{ m}^2$; Fig. 2) of the Eußerthal Ecosystem Research Station (EERES), Germany. The FPMs were constructed in 2017 and were allowed to develop naturally (Stehle et al., 2022) with the aim to study the impact of stressors on aquatic subsidy in an environmentally realistic and at the same time replicated manner. The water level of each FPM could be regulated via adjustable water in- and outflows connected to a small natural stream. The vegetation of the FPMs comprised coontails, waterweeds, green algae, rushes and bulrushes. The pH of the FPMs was measured on a regular basis, while temperature and dissolved oxygen were logged continuously to validate similar conditions among treatments.



Fig. 2: Top view of the FPM system with color-indicated allocation to control (cyan) or Bti (orange) treatment.

Bti application

Applications of Bti in floodplain areas such as in the Upper Rhine Valley are usually linked to flooding events inducing hatching of larvae of target species (Becker et al., 2018). To recreate a realistic application scenario in flooded wetlands, water levels of all FPMs were raised from 30 to 50 cm three times. On the third day of each flood (i.e., 14th April, 5th May and 25th May) Bti was applied in the maximum field rate, i.e., 2.88×10^9 ITU/ha, which is used for water bodies deeper than 10 cm or larval infestation of high density or developmental stages (BAuA, 2018). A suspension of the Bti-containing mosquito control agent Vectobac WDG (Valent BioSciences, Illinois, USA) was evenly applied to six out of

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twelve FPMs (Fig. 2) using a conventional knapsack sprayer (prima 5, GLORIA, Germany). One week after each application, water levels were reset to 30 cm. A detailed time schedule can be found in the Supplementary Material of Appendix A.1.

Since no method was available to determine the concentration of Bti toxins analytically, a biotest was conducted in parallel to each application. The toxicity of Bti was assessed by the comparison of survival rates of mosquito larvae (i.e., *Culex sp.*) in control and Bti-treated FPMs. Successful applications were verified by observing at least 90% mortality compared to control mosquitoes which matches mortality rates usually achieved by Bti (Becker, 1997, 2003).

Arthropod sampling

To collect emerging aquatic insects, six floating emergence traps, each covering an area of 0.33 m² (Cadmus et al., 2016), were installed on every FPM (Fig. 3). Three of them were equipped with collection bottles filled with ethylene glycol which were emptied once or twice a week. In parallel, organisms caught in the remaining three traps were collected by vacuuming the living individuals from the inside. In addition, terrestrial arthropods living in the floodplain vegetation were collected during the first two weeks of June using a suction sampler (modified Stihl SH 86; Stihl, Waiblingen, Germany). On June 15, five female *T. extensa* were gathered from the riparian vegetation of each FPM. All arthropods collected alive were cryo-conserved and stored at -80 °C. Aquatic and terrestrial arthropods were identified either to family or to order level (Klausnitzer, 2011; Köhler et al., 2015), depending on their further use.



Fig. 3: Top view of one FPM (104 m²) with the floodplain area and water inflow pipe on the left. White squares are floating emergence traps (0.33 m²) for emerging insect collection.

3.2 Experimental design: laboratory study

Test setup

The laboratory study was conducted under climate-controlled conditions of 20 ± 1 °C, 65% humidity and a 16:8 day/night-rhythm at the Institute for Environmental Sciences of the RPTU Kaiserslautern-Landau, Germany, from April to December 2021. At test initiation (Sensitivity test 1, ST 1), the sensitivity of *C. riparius* larvae (in terms of quantitative and qualitative aspects) was investigated using a factorial design with different treatments of Bti crossed with food quality ($n = 6$; Fig. 4). In parallel, cultures of *C. riparius* larvae were set up for long-term culturing with (pre-exposed) and without (naïve) exposure to Bti ($n = 6$) to assess for potential adaptations of chironomid populations. After six months (i.e., November 2021), the sensitivity of the pre-exposed and the naïve populations was re-assessed in a second test (Sensitivity test 2, ST 2) following the procedure employed under ST 1 (Fig. 4). An overview of all treatments can be found in the Supplementary Material of Appendix A.3.

Both sensitivity tests were conducted using 250-mL beakers with 150 mL aerated SAM-5S medium (Borgmann, 1996) and 80 g standardized sediment (OECD, 2004, 2010) which were covered with mesh tents. Each beaker contained 20 vivid first-instar larvae whose emergence was documented on a daily basis. The larvae were fed with two food sources, either Spirulina or TetraMin, to test the influence of food quality.

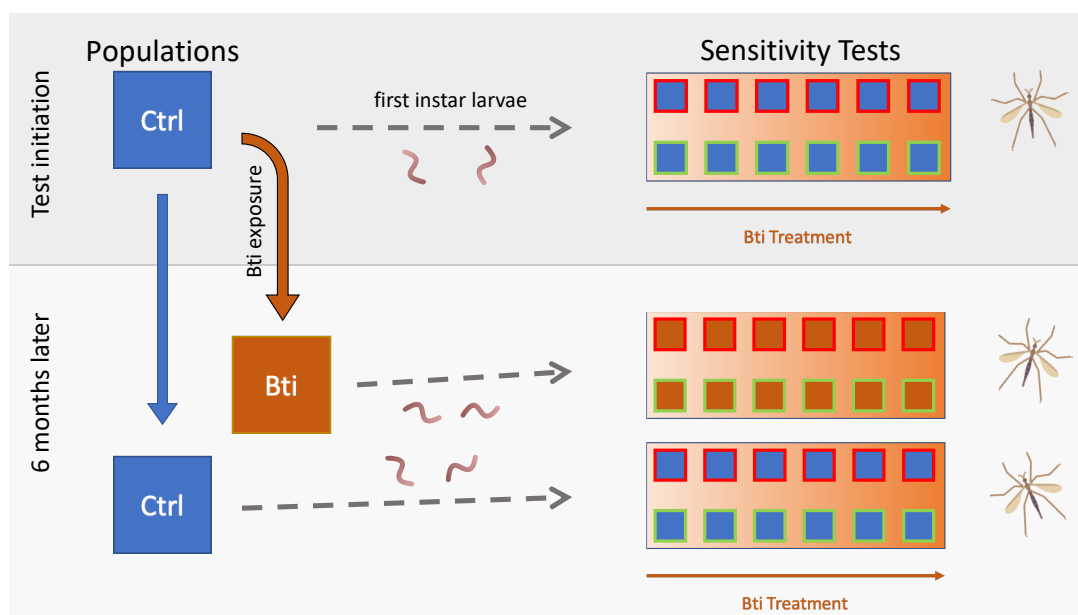


Fig. 4: Schematic illustration of the multi-generation laboratory study on *C. riparius*. Unexposed control populations (naïve) and the respective larval origin are represented in blue, populations chronically exposed to Bti (pre-exposed) and the respective larval origin in brown. Sensitivity tests comprised two food sources, i.e., Spirulina (green outlines) and TetraMin (red outlines). Different Bti treatments were tested in the sensitivity tests depicted by a gradient from bright to dark brown.

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Bti application

To apply Bti, the formulation VectoBac WDG (Valent BioSciences, Illinois, USA) 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 to induce some selective pressure but without the risk of population extinction. The Bti treatments tested in the sensitivity tests were a combination of application day, i.e., day 0 and/or day 10, and dose, i.e., 33% FR (i.e., 480 ITU/L), 100% FR (i.e., 1440 ITU/L), 200% FR (i.e., 2880 ITU/L). Those were selected to cover realistic doses as regularly applied to German floodplain areas. The application times were chosen on the basis of varying susceptibility of different instars to target sensitive early-stage as well as more robust late-stage larvae (Kästel et al., 2017).

3.3 Investigated endpoints

Research objective 1

From the data of collected and determined insects, different endpoints were calculated and investigated for Bti-related differences. These include the total number of emerged individuals (for each family), temporal course of fluxes in individuals per day and m² (for all insects, Chironomidae, and Baetidae), temporal course of the diversity of communities (Shannon diversity index H' , taxa richness S and evenness E), and community composition based on Bray-Curtis dissimilarities.

Three families of aquatic insects, i.e., Chironomidae, Baetidae, and Coenagrionidae, were further investigated for the weight of individuals due to their dominance in aquatic subsidy either by size or numbers. Besides the average individual weight, the temporal course of the individual weight was compared between treatments [Appendix A.1].

Research objective 2

Chironomids collected from Bti-treated and control FPMs were analysed for their nutrient content in eight time periods of about two weeks. Thus, the contents of proteins, carbohydrates, lipids, and glycogen in these samples were determined following the procedure of Foray et al. (2012), adapted for chironomids. A detailed description of the method can be found in the Supplementary Material of Appendix A.2. Nutrient composition and glycogen content over the study duration were compared between treatments [Appendix A.2].

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In order to investigate the food webs and the diet of the collected spiders, stable isotope ratios of C and N of spider opisthosomas as well as of all relevant aquatic (i.e., Chironomidae, Baetidae, Coenagrionidae) and terrestrial prey organisms (i.e., Auchenorrhyncha, Sternorrhyncha, Lepidoptera, Nabidae, Linyphiidae, Collembola) were determined. Opisthosomas were selected due to their fast turnover rate (i.e., 8 days) which leads to an appropriate reflection of the resources sampled 1-2 weeks before the spiders (Belivanov & Hambäck, 2015). Proportions of aquatic and terrestrial prey in spiders' diet were estimated with Bayesian mixing models. The niche sizes were assessed as Bayesian standard ellipse area (SEA) normalized to the SEA of resources for comparability between treatments. The trophic position of the spiders was estimated with a two-baseline model with Baetidae and Aphidina as baseline organisms for the aquatic and terrestrial habitat, respectively [Appendix A.2].

Research objective 3

As quantitative measures of sensitivity, emergence success, emergence time and reproductive potential of the populations were examined for each treatment. As a representation of emergence success, the mean number of emerged chironomids was calculated. To assess the mean emergence time of the different treatments, EmT50s (time to 50% emergence) were computed using dose-response modelling. Following Charles et al. (2004), the reproductive potential was assessed as the maximum number of fertilized females using the respective dynamics of the emergence success. As qualitative endpoints, dry weight per individual, protein and lipid content were measured. Protein and lipid contents were determined using the same approach as established in RO 2.

The influence of the tested factors, i.e., food source, Bti dose, application day, and exposure history, on the different endpoints was tested using either generalized linear models or linear mixed effect models followed by ANOVA. In addition, significant influences of exposure history on the response to Bti, as a sign of adaptation, were investigated by performing two-factor ANOVAs on each Bti × food source scenario of each endpoint [Appendix A.3].

4. RESULTS AND DISCUSSION

The results gained during the experimental part of this thesis are detailed in two peer-reviewed publications [Appendix A.1 & A.2] and one manuscript submitted to a peer-reviewed journal [Appendix A.3].

4.1 RO 1: Bti affects emergence dynamics of Chironomidae

These results are discussed in detail in the peer-reviewed publication in Appendix A.1.

The aquatic subsidy collected from the FPMs consisted mostly of Chironomidae (Diptera; 87.8%) followed by Baetidae (Ephemeroptera; 9.4%; Fig. 5). The total abundance of insects and of relevant insect families differed non-significantly between Bti-treated and control ponds (all insects: -11.7%). However, diversity and community composition changed significantly over the study duration under the influence of Bti. These changes were mainly related to the emergence dynamics of Chironomidae as key component of the aquatic subsidy, which were significantly affected by Bti. In summary, more chironomids emerged from Bti-treated than from control FPMs (average factor of 1.7) during the first two months (Fig. 6). Those individuals were also on average 21% heavier than their control counterparts. Consequently, the peak emergence in treated FPMs was shifted forward by about 10 days and was reduced by 26%. During the following 1.5 months, less chironomids emerged under Bti exposure compared to the controls (average factor of 1.3). In comparison, no such Bti-related differences in the emergence dynamics could be observed for Baetidae (Fig. 6).

Those results showed that although the total number of emerged aquatic insects was not significantly changed by Bti, the exposed systems were adversely affected which was only visible by including the temporal perspective. The detected variations in the emergence dynamics of Chironomidae were the first of its kind to be described for Bti. However, other aquatic contaminants were previously observed to affect the temporal emergence dynamics of aquatic subsidy such as nanoparticles, pyrethroids, triazines or metals (Bundschuh et al., 2019; Dewey, 1986; Gruessner & Watzin, 1996; McCahon & Pascoe, 1991; Schulz & Liess, 2000). Explanations were mostly contaminant-specific or unclear, but included also resource-related theories which was also suggested to partly explain the results of this study.

Different factors are known to influence the susceptibility of chironomid larvae to Bti including species-specific properties, developmental stage, which is partly related to

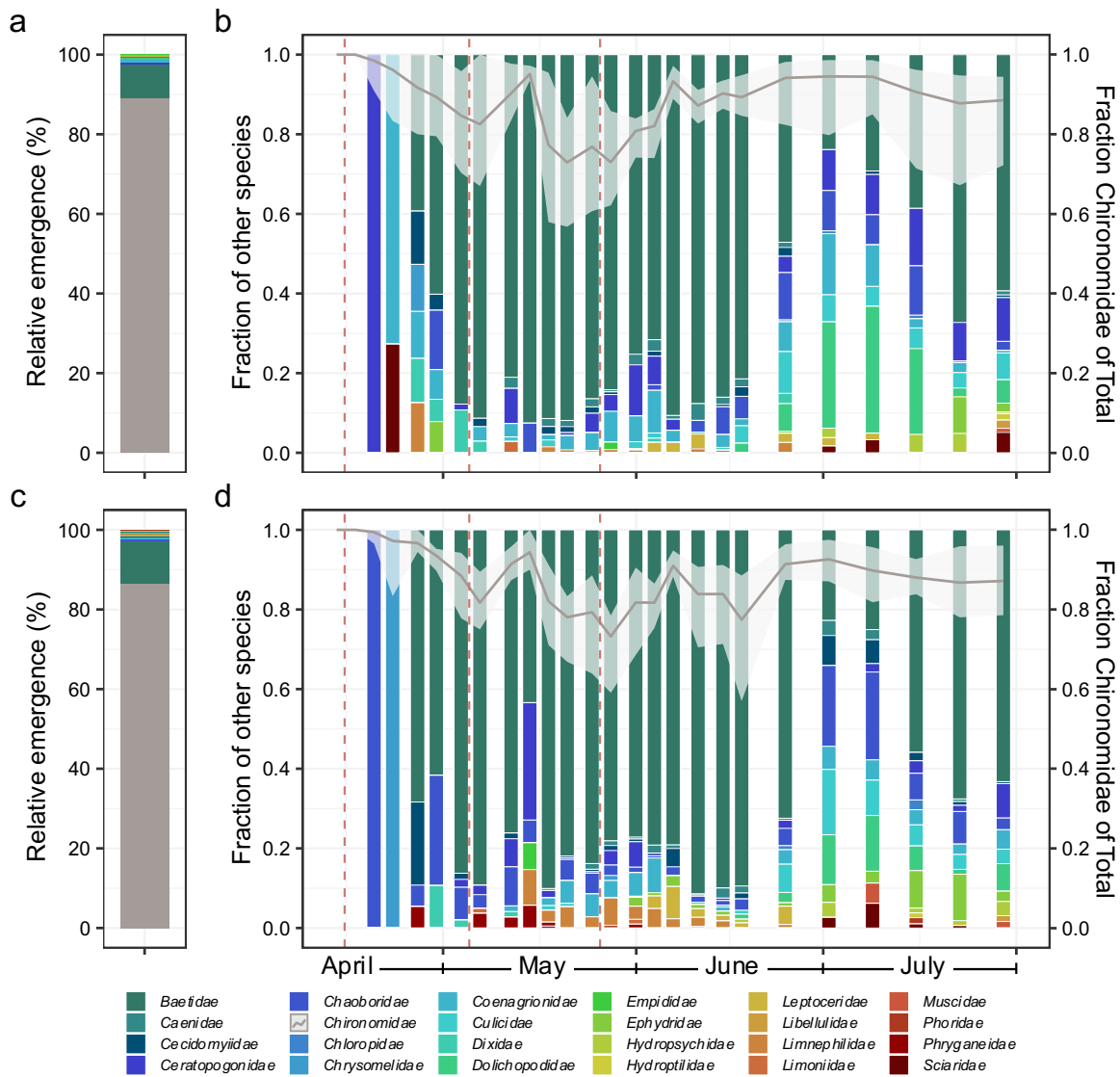


Fig. 5: Left: Composition of total collected insects from a) control FPMs ($n = 6$) and c) FPMs treated with Bti ($n = 6$). Right: Composition of the collected insects for every sampling time point from b) control FPMs ($n = 6$) and d) FPMs treated with Bti ($n = 6$) three times (dashed red lines). Fractions of Chironomidae are displayed on the right and are represented by the grey line. Fractions of other families on the remain are displayed on the left and shown as bars. Figure taken from Appendix A.1.

the number of breeding cycles (voltinism), or feeding strategy (e.g., filter-feeding or predatory), which all vary between taxa (Ali et al., 1981; Armitage et al., 1995; Kondo et al., 1995; Liber et al., 1998). The resulting variations in the susceptibility to Bti of chironomid species may translate to an altered emergence dynamic of the whole family as observed here. Additionally, the decreasing susceptibility of chironomid larvae towards Bti with progressing development (Kästel et al., 2017; Ping et al., 2005) suggests that larvae of early developmental stage have been strongly negatively affected by Bti explaining the reduced emergence during the last 1.5 months. This is further supported by a simultaneous investigation of the benthic communities in the FPMs which found Bti-

induced reductions in larval chironomid abundances (on average 41%; Gerstle et al., 2023). A reduced density of chironomid larvae due to high sensitivity towards Bti of some species or developmental stages lowers the pressure of resource competition within the treated FPMs. Hence, less sensitive larvae may have been able to acquire energy and nutrients more efficiently leading to an accelerated development, thus earlier emergence, and also to higher individual weight of adults in the first two months (Oliver, 1971; Péry et al., 2002). Indeed, larval growth, development and adult weight can be positively influenced by higher energy supply (Hooper et al., 2003; Ristola et al., 1999). Hence, potential energy-demanding stress responses induced by Bti (Kooijman, 2000; Saraiva et al., 2020) might have been masked in the complex benthic communities of the FPMs.

The missing effects on Baetidae can be explained by the phylogenetic distance between Ephemeroptera and Diptera, the main target group of Bti. Missing physiological prerequisites in the gut prevent Bti from acting (Boisvert & Boisvert, 2000). Consequently, the present study provides further evidence for the prevailing assumption that especially Chironomidae are among the non-target species most likely directly affected by Bti under natural conditions. Nevertheless, the complex interrelations in aquatic insect communities also hold the potential for indirect effects on other taxa (Giller, 2012).

Furthermore, differences in chironomid larvae reflected in changed emergence pattern also mean variations in the availability of high-quality prey for riparian predators (Polis et al., 1997; Schindler & Smits, 2017). This can be especially relevant during times of low terrestrial prey biomass (Nakano & Murakami, 2001) or during periods of increased energy demand such as reproductions phases or after hibernation (Fukui et al., 2006; Zahn et al., 2007). Consequential dietary shifts or variations in the nutrition of the predators (Polis et al., 1997; Shipley et al., 2022; Toft, 2013) can affect the survival as well as the reproductive success of temporal-dependent predators leading to changes in abundances and population densities (Kato et al., 2003; Marczak & Richardson, 2007; Poulin et al., 2010). As most terrestrial predators themselves function as consumers but also as prey within the terrestrial ecosystem, their diet and abundance can affect higher as well as lower trophic levels (Hershey et al., 1998; Recalde et al., 2020, 2021). Consequently, changes in the temporal emergence dynamics of aquatic subsidy could alter the whole food web of linked terrestrial ecosystems.

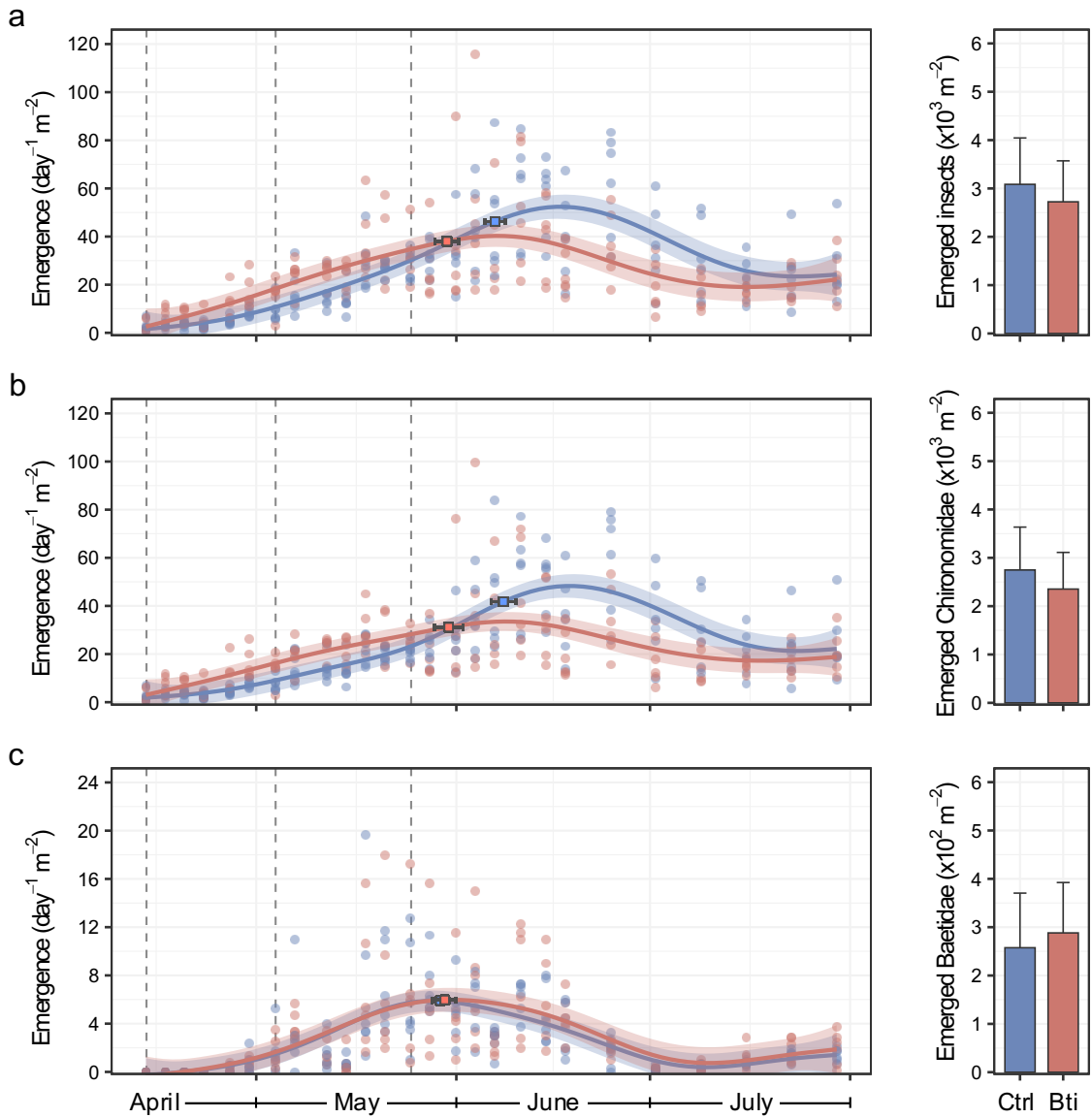


Fig. 6: Number of emerged individuals per day and m² over 16 weeks (left) and mean (with 95% CI) total emergence (right) of a) all insects, b) the most abundant insect family: Chironomidae and c) the second most abundant insect family: Baetidae. Six out of twelve FPMs were treated with Bti (red line; control: blue line) three times (dashed lines). The time points of 50% emerged specimens are indicated with squares (with 99% CI). Figure taken from Appendix A.1.

4.2 RO 2: Disturbed aquatic subsidy can propagate to riparian spiders

The respective data is discussed to a greater extent in the peer-reviewed publication in Appendix A.2.

Quality of aquatic subsidy

Neither the macronutrient composition (i.e., protein, lipids, carbohydrates) nor the proportion of glycogen in chironomids were significantly affected by Bti. Although both variables changed dynamically over time, no distinct difference between the treatments could be observed (Fig. 7). These results contradicted the hypothesis that the previously observed shift in the emergence pattern of Chironomidae from Bti-treated FPMs [Appendix A.1] would be reflected in their nutritional value. Since the observed earlier emergence was mainly explained by a reduced larval density in treated replicates (Gerstle et al., 2023) and increased availability of resources for the surviving less-sensitive

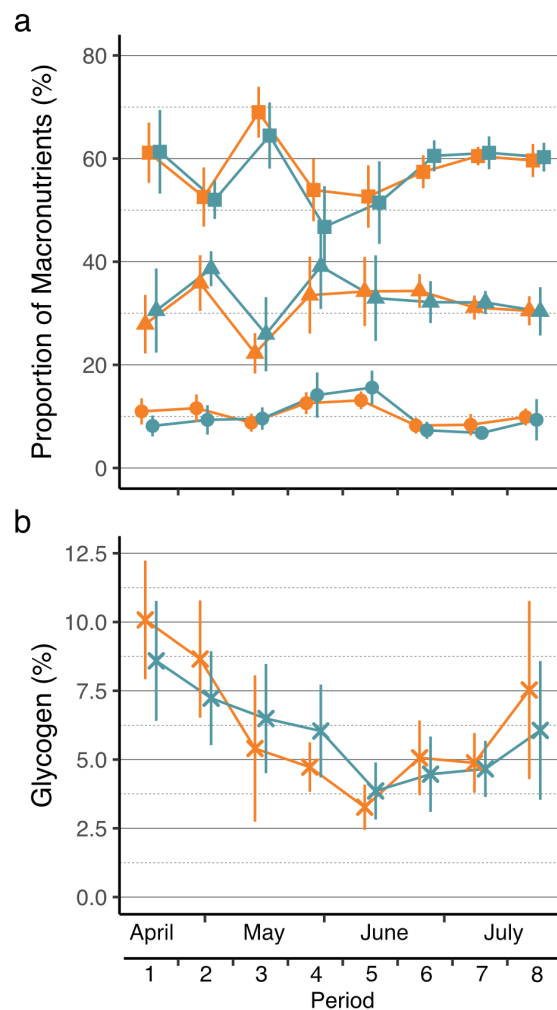


Fig. 7: a) Mean (\pm 95% CI) composition of macronutrients (\bullet carbohydrates, \blacktriangle lipids, \blacksquare proteins) and b) mean (\pm 95% CI) proportion of glycogen in Chironomidae from control (cyan; $n = 6$) and Bti-treated (orange; $n = 6$) FPMs between April and July, measured in eight time periods. Bti was applied three times during April and May. Figure taken from Appendix A.2.

chironomid larvae, higher nutrient content was expected (Goedkoop et al., 2007; Péry et al., 2002). The deviating results were attributed to the fact that larvae have to fulfil different size checkpoints to enter metamorphosis (Nijhout, 1975). The associated complex sequence of processes necessary to reach the adult life stage hold potential for temporal alterations and, at the same time, independent changes in quality (Mirth & Riddiford, 2007). However, studies on the nutrient content of adult Diptera showed a negative correlation to larval density (Morimoto et al., 2019; Nguyen et al., 2019; Takken et al., 2013) thus underlying mechanisms require further investigation.

In general, temporal variations in the nutritional value of aquatic prey can transfer aquatic disturbance to riparian spiders (S. Wilder, 2011; S. M. Wilder et al., 2010). In contrast to herbi- or omnivores, and also other carnivores, web-building spiders, such as *T. extensa*, show limited ability to regulate the intake of nutrients through their prey (Hawley et al., 2014; Mayntz et al., 2005; Persson et al., 2010). Their web-based foraging strategy as well as regular limitations in prey quality and quantity lead to an opportunistic and less selective feeding behaviour compared to free-hunting carnivores (Hawley et al., 2014; Jensen et al., 2011). Consequently, spiders' performance and fitness could be negatively affected if available prey does not match their nutritional demands and vice versa (Mayntz & Toft, 2001; S. Wilder, 2011). Thus, temporal fluctuations in macronutrient and glycogen levels of aquatic insects and their relevance for terrestrial predators should be subject of future studies.

Spider diet

In accordance with literature and our expectation, aquatic prey contributed with ~50% substantially to the diet of *T. extensa* (Fig. 8; Akamatsu et al., 2004; Krell et al., 2015). Contrary to the hypothesis, the aquatic proportion differed only marginally between treatments (Bti = 52.9% (43.6-62.4), Ctrl = FPMs (55% (43.9-66.3)). Based on several studies (Brühl et al., 2020) and the data of RO 1 [Appendix A.1] showing a temporal reduction in Chironomid emergence by Bti, increased consumption of terrestrial prey was assumed to replace lower abundance of aquatic prey. However, it needs to be acknowledged that chironomid emergence in Bti-treated FPMs was reduced by only 10% during the respective period, which allows the conclusion of no change in spiders' diet.

However, a small difference in the dietary niche of *T. extensa* (i.e., relative niche size; Bti = 0.42 (0.28-0.63) %⁰², Ctrl = 0.47 (0.31-0.70) %⁰²) suggested an altered food intake in Bti-treated FPMs compared to controls. This was further supported by a lower

trophic level estimate of treated *T. extensa* (3.42 (3.23-3.61)) compared to controls (3.62 (3.45-3.80)). Both changes were most likely related to an increased consumption of prey with $\delta^{15}\text{N}$ values below chironomids, which applies to primary consumers, i.e., herbivores and detritivores, from both aquatic and terrestrial habitats (Fig. 8). The respective aquatic and terrestrial prey were well separated in their $\delta^{13}\text{C}$ values and deviated from *T. extensa* to different extents (i.e., $\Delta^{13}\text{C}_{\text{Baetidae-spiders}} = -3.4\text{‰}$; $\Delta^{13}\text{C}_{\text{Terr. prey-spiders}} = 1.7\text{‰}$). Based on this, it is assumed that increased intake of alternative prey of aquatic origin (i.e., Baetidae) may have masked higher consumption of terrestrial prey items compared to control. Thus, the terrestrial proportion of the diet of spiders from Bti-treated FPMs could be underestimated explaining the insignificant difference in aquatic proportion between treatments. This shortcoming of mixing models could be counteracted by the inclusion of additional tracers (e.g., fatty acids; [Twining et al., 2020](#)) or DNA-based analyses of the spiders' diet ([Piñol et al., 2014](#)) to increase the understanding of ecological interactions in complex food webs ([Pacioglu et al., 2019](#); [Stock et al., 2018](#)).

Although dietary changes may have been partially masked in this study, even small differences in spiders' diet composition, induced by anthropogenic stressors, can result in relevant ecological consequences. Variations in the nutrient content of different insect taxa ([Gerber et al., 2022](#)) could lead to changes in the nutrition of spiders with effects on performance and reproductive success ([Blamires et al., 2009](#); [Salomon et al., 2008](#); [Toft, 2013](#)). Resulting consequences in spider populations might propagate effects further on to higher and lower trophic levels due to their dual role in terrestrial food webs as both prey and consumers ([Kato et al., 2003](#); [Marczak & Richardson, 2007](#); [Paetzold et al., 2011](#); [Recalde et al., 2021](#)).

RESULTS AND DISCUSSION

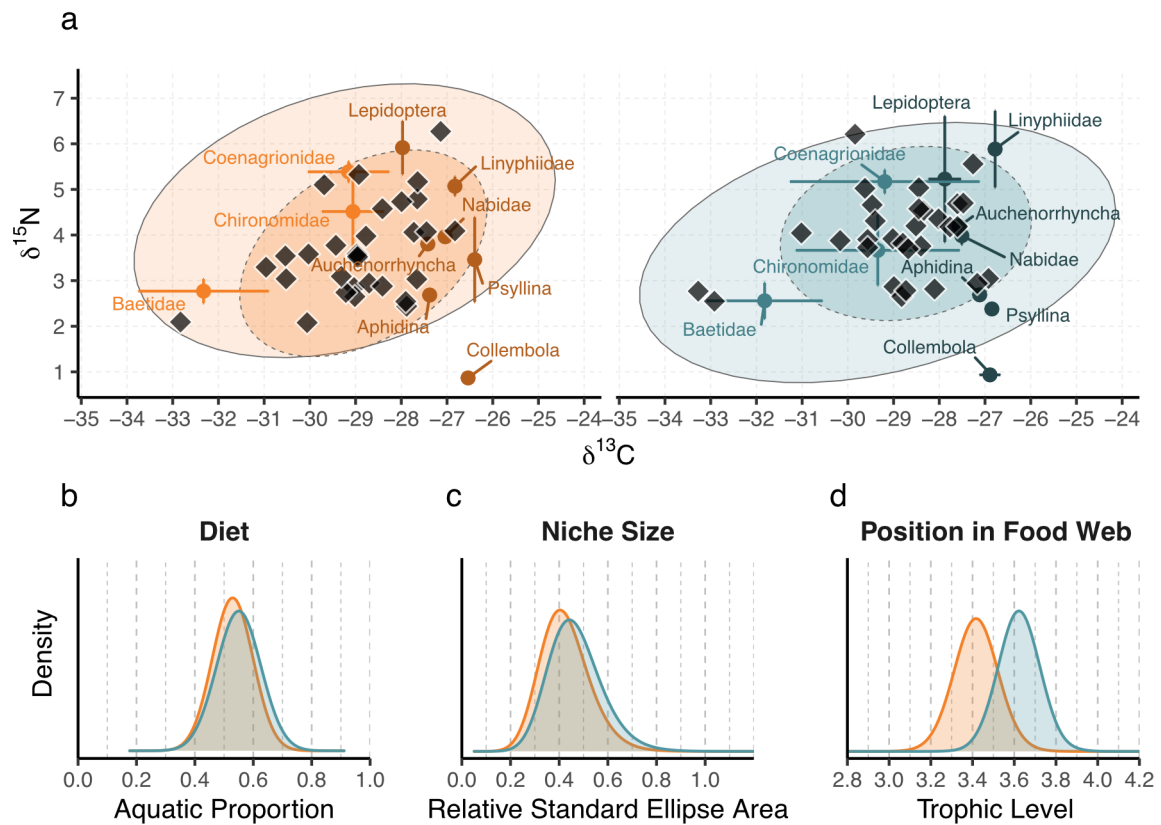


Fig. 8: a) 95%-confidence ellipse of *T. extensa* (dark shade) determined from the stable isotope ratios of C and N (black diamonds) and 95%-confidence ellipse (bright shade) of resources adjusted for trophic enrichment. Means \pm SD of aquatic and terrestrial prey organisms ($n = 5$) are plotted as bright and dark dots, respectively. b) aquatic proportion in diet of *T. extensa*, c) niche size of *T. extensa* determined from relative SEA, and d) the trophic position of *T. extensa*, in Bti-treated (orange) and control (cyan) FPMs ($n = 6$). b), c), and d) show probability densities which are unitless. Figure taken from Appendix A.2.

4.3 RO 3: Chronic Bti exposure alters sensitivity of *C. riparius* populations

These results are further elaborated in the submitted manuscript in Appendix A.3.

Quantitative perspective

In line with previous studies, the initial sensitivity test (ST 1) confirmed lethal effects of Bti on non-target chironomids (Fig. 9; reviewed in [Brühl et al., 2020](#)) as well as decreasing susceptibility of larvae with advancing development (Kästel et al., 2017). Moreover, accelerated emergence of surviving individuals in replicates with increased mortality (due to Bti applied on day 0) may have pointed out the connection between lower larval densities, lower competition for food and faster development (Goedkoop et al., 2007) as observed in RO 1 [Appendix A.1]. Alternatively, Bti may have triggered the emergence process as a mechanism to escape harmful environmental conditions. Besides emergence time, also the reproductive potential was negatively affected by Bti, probably due to the above-mentioned mortality.

The low-quality food source did not affect the emergence success but the emergence time of the initial populations (ST 1). In comparison to the high-quality food source, larvae could acquire energy less efficiently, hence less energy can be allocated to

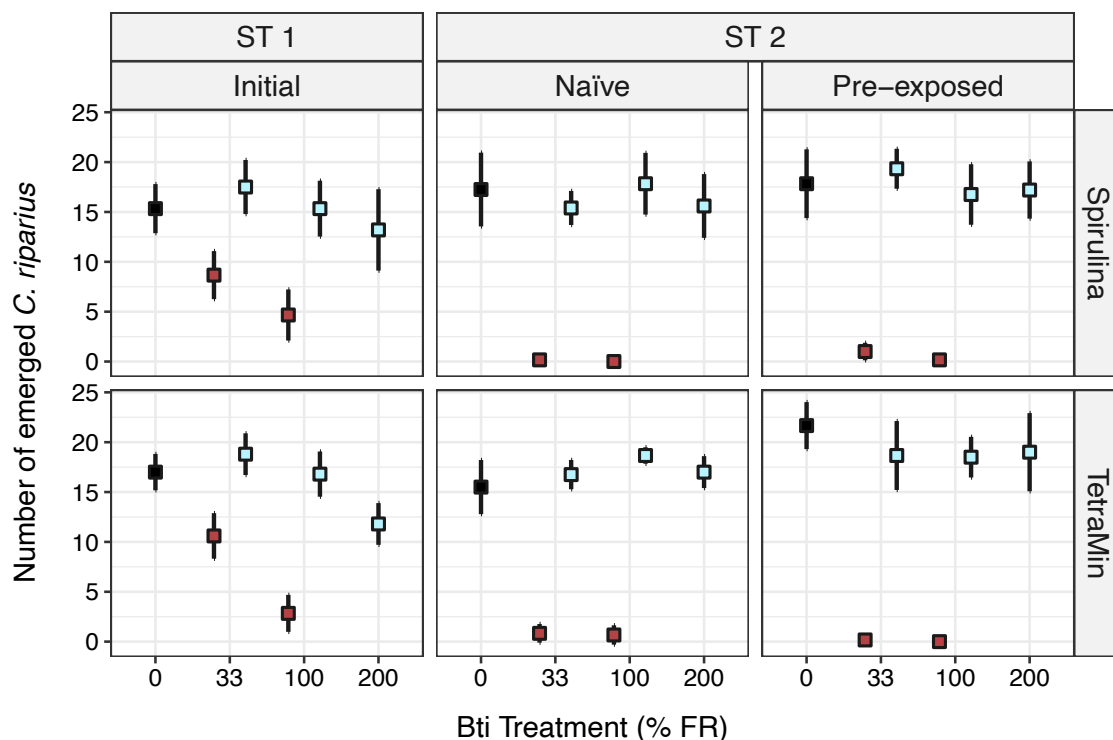


Fig. 9: Mean number of emerged *C. riparius* per treatment with 95% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four different doses expressed as % field rate (FR) at two time points, i.e., Day 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). Figure taken from Appendix A.3.

development leading to a delayed emergence under low-quality food (cf. dynamic energy budget theory; [Kooijman, 2000](#)). Due to the increased energy demand during their development, female chironomids emerge later than males (Armitage et al., 1995; Frouz et al., 2002). Logically, female emergence was further delayed when low-quality food was provided.

The long-term culturing over six months had a strong effect independent of the 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. This observation might be explained by genetic impoverishment of the isolated populations over time (attributed to low initial genetic variation and genetic drift) resulting in lower fitness and high susceptibility towards stress (Nowak et al., 2007). Also contrary to the expectations, pre-exposed populations did not show lower sensitivity to Bti than their naïve counterparts. Due to the selective pressure of Bti, it was suggested that more tolerant individuals prevail, leading to less sensitive populations (Vogt et al., 2007). Potential explanations could be: a) the nature of Bti operating as a mixture of toxins with synergistic interactions resulting in a lower potential for tolerance development (Ben-Dov, 2014; Georghiou & Wirth, 1997; Paris et al., 2011); b) a lack of the required traits in the assessed populations; c) high costs associated with developed mechanisms for an elevated tolerance of Bti leading to lower individual fitness (Férard & Blaise, 2013); d) low selective pressure induced by the chronic exposure to Bti which was potentially superimposed by an age-dependent selection based on the varying susceptibility of different instars (Kästel et al., 2017). However, one out of 18 tested factor combinations (3 Bti doses x 2 food sources x 3 endpoints) showed signs for phenotypic adaptation: emergence time was earlier when chironomids were fed with TetraMin and exposed to 33% FR. As mentioned above, leaving an unfavourable environment early could be beneficial.

Furthermore, the response to Bti of long-term cultured populations differed between the food sources. Populations fed with low-quality food showed later emergence when exposed to lower Bti doses. This might be attributed to a higher need for food intake to cover increased energy requirements of stress responses or repair mechanisms (Goedkoop et al., 2007; Sokolova et al., 2012). When offering high-quality food, lower doses of Bti induced earlier emergence. The unfavourable environment may have triggered the larvae to allocate more of the available energy to their development while retaining the ability to combat the effects of Bti (Kooijman, 2000).

RESULTS AND DISCUSSION

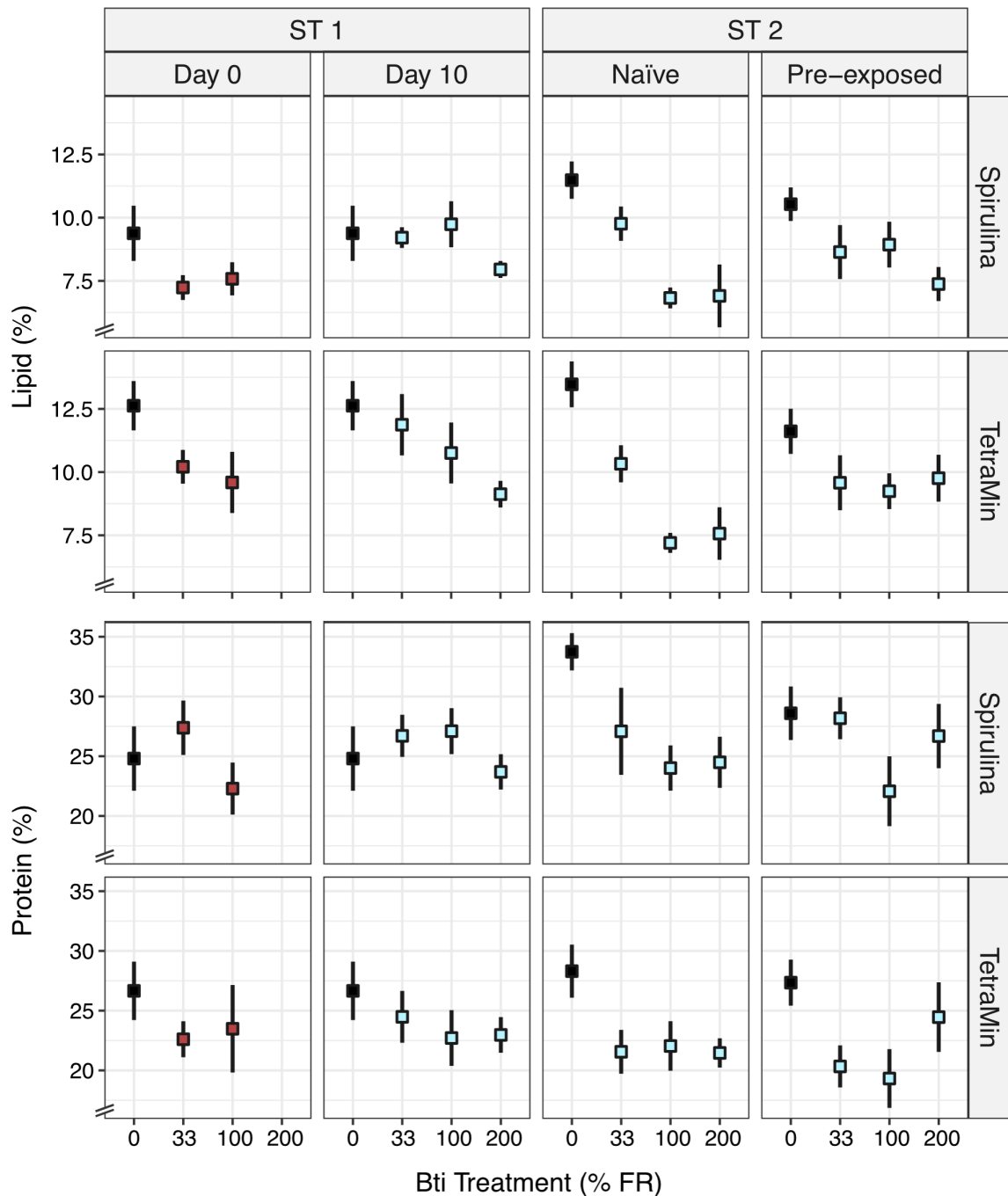


Fig. 10: (Top) Mean proportion of lipid (%) and (Bottom) mean proportion of protein (%) in emerged *C. riparius* per treatment with 95% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four different 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 values could be determined for application day 0 in ST 2 due to high mortality. Figures taken from Appendix A.3.

Quantitative perspective

Increased dry weight of chironomids fed with high-quality food might be explained by the higher carbohydrate content of TetraMin. Protein and lipid content of chironomids were most likely affected by differences in the nutritional quality of the food sources, including protein content, lipid content and also fatty acid composition. Such differences could explain deviations in the effects of Bti treatments on chironomids' nutrient contents.

The protein and lipid contents of the long-term cultured populations were negatively affected by Bti independent of the exposure history. However, one out of six tested scenarios showed higher protein content in pre-exposed chironomids suggesting adaptation to Bti. Similarly, lipid contents of pre-exposed populations were less reduced by Bti than of naïve populations. Pre-exposure to Bti had a significant effect in three out of six tested scenarios indicating adaptation. These results could imply a more efficient use of available energy reserves for combating Bti (Kooijman, 2000; Sokolova et al., 2012) as consequence of chronic Bti exposure. Since chironomids are an important food source for many riparian predators, changes in their nutritional quality could potentially affect the fitness and reproduction of the next trophic levels of consumers.

5. CONCLUSION AND OUTLOOK

The ecological consequences of the aquatic model stressor Bti are still under debate (reviewed in Brühl et al., 2020). This thesis provides further evidence that this stressor has an impact on larvae of aquatic non-target insects, mainly Chironomidae, translating to altered aquatic emergence dynamics and potential long-term impacts.

Observations from the mesocosm-scale experiment were likely attributed to direct (i.e., mortality of highly sensitive larvae) and indirect (i.e., altered resource competition) effects on the benthic community. These insights demonstrate the complexity of disturbance in aquatic food webs. Given that the total abundance of emerging aquatic insects was not meaningfully affected by the stressor, the altered temporal pattern of their emergence is a highly-relevant perspective. This is of particular importance when considering the seasonal dependence of some riparian predators on prey of aquatic origin. Investigated on the riparian spider *T. extensa* in mesocosms, differences in the temporal availability of aquatic prey translated to a shift in their diet to alternative prey of both aquatic and terrestrial origin. This could be documented using stable isotope analyses of C and N which, however, implied advantage of combining the approach with fatty acids or molecular gut content analyses to enable more solid conclusions. The altered diet of *T. extensa* indicate potential for consequences of anthropogenic stressors across the boundaries of aquatic habitats. Thus, this thesis adds knowledge on propagating effects from the aquatic to the terrestrial ecosystem through food webs (Schulz et al., 2015). Unexpectedly, quality parameter (i.e., macronutrients) assessed in adult chironomids from mesocosms did not reflect the altered emergence dynamics, calling for studies mechanistically addressing the impact and interaction between relevant factors such as larval density, food availability, timing of emergence and quality of adults. Accordingly, the results of the laboratory approach substantiated the relation between larval density, food availability, and emergence time. However, no direct link could be drawn between these factors and the quality of adults in terms of nutrient content. Hence, it highlights the complexity of the impact of stressors on the insect metabolism especially during development.

Assessing effects of long-term exposure to Bti yielded small changes in quantitative (emergence time) and qualitative (nutrient content) aspects of chironomid populations, represented by *C. riparius*. The selective pressure of a stressor is one of the driving forces behind adaptation that could lead to long-term alterations of chironomid populations, and

CONCLUSION AND OUTLOOK

hence of aquatic subsidy. Additionally, mesocosm data reported changes in the community composition of aquatic subsidy, most likely towards more tolerant taxa (considering the concept of pollution-induced community tolerance; Nikinmaa, 2014). Alterations at population and community level of aquatic subsidy in terms of quantity, quality, and timing of emergence as consequences to anthropogenic stressors constitute further potential for impact on terrestrial consumers in the long-term.

As continuation, the conducted studies suggest the necessity of considering also effects on physiological endpoints of riparian predators as a consequence of alternative prey and altered prey quality and quantity. Fatty acid composition, macronutrient content, but also reproductive success and lifespan could enhance our understanding of potential impacts of aquatic stressors on higher trophic levels of subsidised terrestrial food webs.

Lastly, the gained insights are due to the properties of Bti (i.e., low persistence and high specificity) almost exclusively linked to food web-mediated responses. As a consequence, the findings of this thesis may be applicable beyond the used model stressor, thus relevant for the development and validation of ecological models helping to mechanistically predict effects in the aquatic-terrestrial meta-ecosystem.

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APPENDICES

Appendix A.1

A temporal perspective on aquatic subsidy: Bti affects emergence of Chironomidae

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Abstract

Emerging aquatic insects serve as one link between aquatic and adjacent riparian food webs via the flux of energy and nutrients. These insects provide high-quality subsidy to terrestrial predators. Thus, any disturbance of emergence processes may cascade to higher trophic levels and lead to effects across ecosystem boundaries. One stressor with potential impact on non-target aquatic insects, especially on non-biting midges (Diptera: Chironomidae), is the widely used mosquito control agent *Bacillus thuringiensis* var. *israelensis* (Bti). In a field experiment, we investigated emerging insect communities from Bti-treated (three applications, maximum field rate) and control floodplain pond mesocosms (FPMs) over 3.5 months for changes in their composition, diversity as well as the emergence dynamics and the individual weight of emerged aquatic insects over time. Bti treatments altered community compositions over the entire study duration – an effect mainly attributed to an earlier (~10 days) and reduced (~26%) peak in the emergence of Chironomidae, the dominant family (88% of collected individuals). The most reasonable explanation for this significant alteration is less resource competition caused by a decrease in chironomid larval density due to lethal effects of Bti. This is supported by the higher individual weight of Chironomidae emerging from treated FPMs (~21%) during Bti application (April – May). A temporal shift in the emergence dynamics can cause changes in the availability of prey in linked terrestrial ecosystems. Consequently, terrestrial predators may be affected by a lack of appropriate prey leading to bottom-up and top-down effects in terrestrial food webs. This study indicates the importance of a responsible and elaborated use of Bti and additionally, highlights the need to include a temporal perspective in evaluations of stressors in aquatic-terrestrial meta-ecosystems.

Keywords: aquatic insects, emergence dynamics, riparian food web, aquatic-terrestrial linkage, mosquito control, floodplain mesocosms

Introduction

Aquatic and terrestrial ecosystems are linked through fluxes of nutrients, matter and energy (Soininen et al., 2015). These donor-controlled fluxes subsidize the receiving ecosystem and thereby increase productivity (Polis et al., 1997). While the input of leaf litter and invertebrates from terrestrial ecosystems supports freshwater food webs (Cole et al., 2006; Fisher and Likens, 1973), emerging aquatic insects subsidize terrestrial predators (Baxter et al., 2005; Henschel et al., 2001). In fact, emerging insects of aquatic origin are considered high-quality prey for several riparian predators such as bats, birds, arthropods (e.g., spiders) and lizards (Bartels et al., 2012; Gratton and Vander Zanden, 2009; Henschel et al., 2001; Nakano and Murakami, 2001; Paetzold et al., 2005; Salvarina et al., 2018). Thus, alterations in quantity, nutritious quality and timing of aquatic subsidy may induce changes in recipient terrestrial ecosystems either through bottom-up or top-down cascades (reviewed in Schulz et al., 2015).

Changes in quantitative and qualitative parameters of subsidy are induced by several factors of which seasonal variation is dominant (Baxter et al., 2005; Paetzold et al., 2005). In addition to natural factors, anthropogenic modifications in aquatic ecosystems (e.g., hydromorphological and hydraulic changes, invasive species and pollution) impact subsidy (Gergs et al., 2014; Greig et al., 2012; Schulz et al., 2015). Emergence of aquatic insects has generally been identified as a sensitive indicator of anthropogenic influences, e.g., chemical stress (Schmidt et al., 2015; Schulz and Liess, 2001). However, many studies only investigated alterations of the total emergence by anthropogenic stressors, while few examples focused on the temporal emergence dynamics (Kotalik, 2020). Indeed, a temporal decoupling between the emergence of aquatic insects and the periods of increased energy demands for terrestrial consumers, e.g., riparian spiders, breeding birds or nursing bats, may have far-reaching ecological consequences (Baxter et al., 2005; Kurta et al., 1989; Twining et al., 2018; Zahn et al., 2007).

One stressor of increasing concern is the mosquito control agent *Bacillus thuringiensis* var. *israelensis* (Bti; Belousova et al., 2021; Brühl et al., 2020). Bti carries parasporal toxins, activated in the alkaline milieu of the larval gut, bind to specific receptors and perforate the midgut epithelium causing substantial damage (Boisvert and Boisvert, 2000). Due to its distinct mode of action, Bti is assumed to act taxa-specifically on several nematoceros dipterans (e.g., mosquito and blackfly larvae). It is, moreover, considered biodegradable with a short half-life (Boisvert and Boisvert, 2000; Federici et

al., 2012). These beneficial properties stimulated the global use of Bti as an environmentally friendly biocide at breeding sites of target dipterans, mostly wetlands, to reduce nuisance and transmission of diseases.

Although applied to act specifically on target dipterans, Bti has been found to cause adverse effects on non-target dipterans, especially on non-biting midges (Chironomidae), in a number of laboratory and (semi-)field studies (Allgeier et al., 2019; Charbonneau et al., 1994; Kästel et al., 2017; Liber et al., 1998). Other studies found no substantial impact on non-target dipterans (Lagadic et al., 2016; Lundström et al., 2010; Timmermann and Becker, 2017; Wolfram et al., 2018), however, in some of these studies the efficacy of the Bti treatment was not reported. The indication for non-target effects on Chironomidae, which are phylogenetically closely related to the mosquito target species, is of particular concern, especially against the background of aquatic subsidy of terrestrial food webs. Chironomidae contribute substantially to emergent subsidy, in some cases up to 90% (Armitage et al., 1995; Leeper and Taylor, 1998). At the same time, they are considered easily digestible high-protein prey for a variety of terrestrial predators (Bergeron et al., 1988; de la Noüe and Choubert, 1985). Accordingly, the potential of Bti to impact Chironomidae and their emergence may affect the subsidy for terrestrial predators which might cascade bottom-up or top-down through the terrestrial food web (Henschel et al., 2001; Jakob and Poulin, 2016; Poulin et al., 2010).

We designed a replicated field study using twelve artificial floodplain pond mesocosms (FPMs) harbouring natural communities (Stehle et al., 2022). In contrast to previous (semi-)field studies using enclosures (e.g., Allgeier et al., 2019; Duchet et al., 2015) or individual ponds/wetlands at different locations (e.g., Charbonneau et al., 1994; Lundström et al., 2010), this design allowed comparable and realistic conditions among all FPMs (Stehle et al., 2022), and a field-relevant as well as controlled Bti application (Becker et al., 2018). Half of the FPMs received the maximum field rate of Bti three times during spring while the remaining six FPMs served as unexposed control. We determined the emergence at each FPM in terms of abundance, diversity and weight with a particular emphasis on their dynamics between April and July 2020 (3.5 months). We hypothesized that (1) Bti reduces the emergence of insects, mainly those of the family Chironomidae, with consequences on the composition of the emerging insect communities. Driven by potential sublethal implications in the larval stage of this family we additionally hypothesized (2) a temporal delay of emergence with (3) lower individual weight of successfully emerged insects. The latter hypotheses are informed by the dynamic energy

budget theory suggesting the allocation of energy to maintenance protecting against toxic stress which in turn reduces the energy available for development and growth (Kooijman, 2000; Péry et al., 2002).

Material and methods

Study sites

The study was conducted from April to July 2020 in twelve artificial floodplain pond mesocosms (FPMs; Fig. 1) at the Eußerthal Ecosystem Research Station (EERES) of the University of Kaiserslautern-Landau, Germany, located in the Palatinate Forest. The FPM system was constructed in 2017 and the ecological development as well as the natural colonization of the FPMs, were monitored for more than two years as described in detail by Stehle et al. (2022). The initial insect communities of the FPMs consisted mainly (< 99%) of Chironomidae, Baetidae, and Coenagrionidae (Stehle et al., 2022). To regulate the water level, every FPM is equipped with an adjustable water supply from a small stream as well as a closable outlet. The structural and ecological quality of the supplying stream is classified as high with negligible anthropogenic influences (Stehle et al., 2022). Since one shore is very shallow, the FPM surface area changes with increasing water level. At application time (see *Bti application*), every FPM covered a surface area of approx. 104 m² (~20 m x 5.2 m). The vegetation in the FPMs included waterweeds and coontails, green algae as well as emergent plants such as bulrushes and rushes. Over the whole study duration, temperature and dissolved oxygen were monitored with data loggers, and pH was measured manually (Fig. S1).

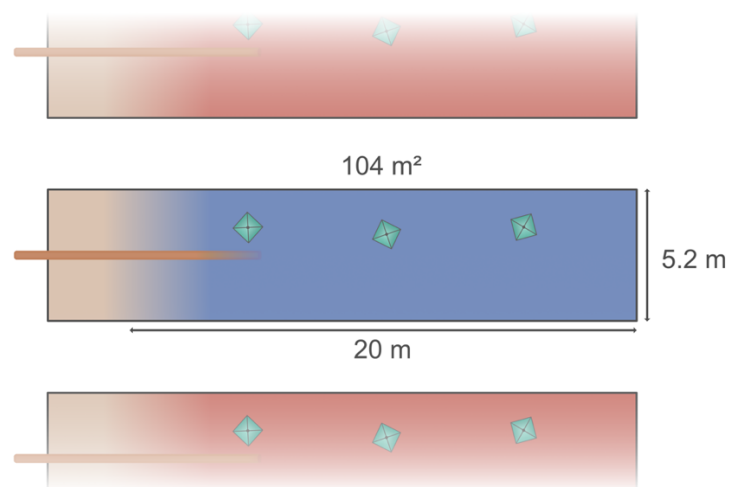


Figure 1: Schematic figure of a control FPM (blue) and the neighbouring Bti-treated FPMs (red) with dimensions. Light blue squares represent the emergence traps (each 0.33 m²). The floodplain area at the left shore is implied by a fawn gradient.

Bti application

Between mid of April and the end of May 2020, the water levels of the FPMs were raised from 30 cm to 50 cm three times to mimic flooding. Floods usually trigger the hatching of target mosquito larvae, mainly the floodwater mosquito *Aedes vexans*, and thus the application of Bti in the study region, the Upper Rhine Valley (Becker et al., 2018). Here, the application frequencies can vary between one and eleven times per year with an average of five applications, usually between April and September (Becker et al., 2018; Brühl et al., 2020). On the third day of each flooding (i.e., 14th April, 4th May, 25th May), the mosquito control agent VectoBac WDG (Valent BioSciences, Illinois, USA), containing *Bacillus thuringiensis* var. *israelensis* (Bti), was applied to six FPMs. In Germany, the application rate and method depend on the water depth (i.e., below or above 10 cm) as well as the density and developmental stage of mosquito larvae (BAuA, 2018). To treat the FPMs with the highest recommended application rate (i.e., $2.88 \cdot 10^9$ ITU/ha), a suspension of VectoBac WDG was evenly applied to the FPM surface using a conventional knapsack sprayer (prima 5, GLORIA, Germany). To prevent cross-contamination of control ponds, the spraying was exclusively done on calm days and when no gusts appeared. One week after each application, excess water was released to obtain initial water levels (i.e., 30 cm water height) as a precondition for the next application. A detailed time schedule is provided in the supplementary material (Tab. S1).

To verify the efficiency of Bti treatments, a biotest was conducted in parallel to every application since no analytical method was available to determine the concentration of Bti toxins. Thus, twelve buckets with filtered FPM water (mesh filter, pore size: 55 μ m) and a known number of local mosquito larvae (*Culex* sp.) were prepared in the morning of each application day. One open bucket was placed in every treatment and control FPM over the course of each application leading to the exact same treatment of FPMs and buckets. Subsequently, the number of dead and emerged mosquitoes in every bucket was ascertained daily for seven days. All treatments were considered successful since the mortality of mosquitoes in treated buckets reached at least 90% compared to control mosquitoes which is consistent with official mortality rates achieved by Bti (Becker, 2003, 1997).

Emergent insect collection and identification

Three floating pyramidal emergence traps (Cadmus et al., 2016) covering a total area of 1 m² were installed at each FPM. The traps were equipped with collection bottles filled with

ethylene glycol to catch and preserve emerging insects. From mid-April until mid-June, all bottles were emptied and replaced twice a week being continued with a reduced frequency (i.e., once per week) until the end of July (Tab. S1). Every insect was determined to family level using a stereomicroscope (SZX 9, Olympus) with a transmitted brightfield illumination base (SZX-ILLB200, Olympus) and taxonomical keys (Klausnitzer, 2011; Köhler et al., 2015). Subsequently, all insects were stored in 70% ethanol for biomass quantification. Organisms without an aquatic life stage and those with a total abundance of only one were considered bycatch and excluded from further analyses.

Biomass determination of emergent insects

The weight of individuals of three families, namely Chironomidae, Baetidae and Coenagrionidae, was determined after drying at 60°C for at least 48 h and used to calculate the biomass of the respective family. Those families have been selected as they dominated the community composition either by numbers or size. Baetidae and Coenagrionidae were weighed to the nearest 0.01 mg, while Chironomidae were instead sorted into four size classes based on length and body shape resulting in an average weight per specimen as prerequisite for further analyses.

Calculations and statistics

The abundances of all collected and determined families were cumulated to obtain the total number of emerged insects. Using the total number of emerged insects as well as subsets for Chironomidae and Baetidae, fluxes as emerged individuals per day and m² were calculated. EmT50 values (Time until 50% of the individuals emerged) were calculated from cumulative abundance data by dose-response modelling using the package “drc” (Ritz et al., 2015). To investigate differences in the diversity of communities, Shannon diversity index H' as well as taxa richness S and evenness E were calculated for every time point using the package “vegan” (Oksanen et al., 2020). The average dry weight (dw) of Chironomidae at each time point was calculated from the following equation

$$dw = \frac{1}{n_{total}} \sum_{i=1}^4 n_i \cdot dw_i \quad (1)$$

where n_{total} is the sum of individuals, i is the size class (1 to 4), n_i is the number of individuals of size class i and dw_i is the average dry weight per individual of size class i .

To assess the impact of Bti treatments over the whole study on total abundances, average individual weight, and diversity endpoints, pairwise comparisons between Bti-treated and control FPMs were performed via t-tests after checking for normality and homoscedasticity. To investigate the influence of Bti treatments on the emergence of Chironomidae and Baetidae as well as on the diversity of communities and the weight of emerged individuals over time, autoregressive mixed effect models (LME) were used with treatment, time and their interaction as fixed effects and FPM as a random effect to account for repeated measurements and autocorrelation. For these analyses, fluxes and weight were log₁₀-transformed to fulfil assumptions (i.e., normality and homoscedasticity). Statistical significance of factors in these models was tested using an analysis of variance (ANOVA) with an alpha level of 0.05. Multiple comparisons among factor combinations were done using a Tukey-adjusted post-hoc test based on least-squared means (SAS Institute Inc., 2012).

To further investigate Bti-induced changes in community composition, permutational multivariate analysis of variance (PERMANOVA) in 9999 permutations was performed on Bray-Curtis dissimilarities with treatment and time as predictors. To account for repeated measurements, FPM ID was set as strata. Homogeneity of group dispersion was confirmed using the “betadisper” function (Oksanen et al., 2020). To reduce the influence of dominant species, data were square-root transformed and standardized using the “decostand” function (method: “total”; Oksanen et al., 2020). Additionally, community composition during Bti application (April – May) and afterwards (June – July) were compared. Non-metric multidimensional scaling (NMDS) ordination plots were used for visualization.

All calculations, statistics and data visualizations were conducted with R (4.1.2, R Core Team, 2021) using the packages “dplyr” (Wickham et al., 2021), “plyr” (Wickham, 2011), “tidyr” (Wickham, 2021), “tidyverse” (Wickham et al., 2019), “vegan” (Oksanen et al., 2020), “nlme” (Pinheiro et al., 2021), “emmeans” (Lenth, 2021), “ggpubr” (Kassambara, 2020), “scales” (Wickham and Seidel, 2020), “patchwork” (Pedersen, 2020) and “ggplot2” (Wickham, 2016).

Results

Collected insects

Between mid of April and the end of July, we collected a total of 34,855 emerged insects of aquatic origin belonging to 24 families within five orders: 31,243 flies (Diptera), 3,333

mayflies (Ephemeroptera), 163 dragon- and damselflies (Odonata), 112 caddisflies (Trichoptera) and four beetles (Coleoptera). The most abundant families were Chironomidae (Diptera) and Baetidae (Ephemeroptera) which accounted for 87.8% and 9.4% of the collected insect specimens, respectively. Every other family constituted less than 0.5% (Tab. S2).

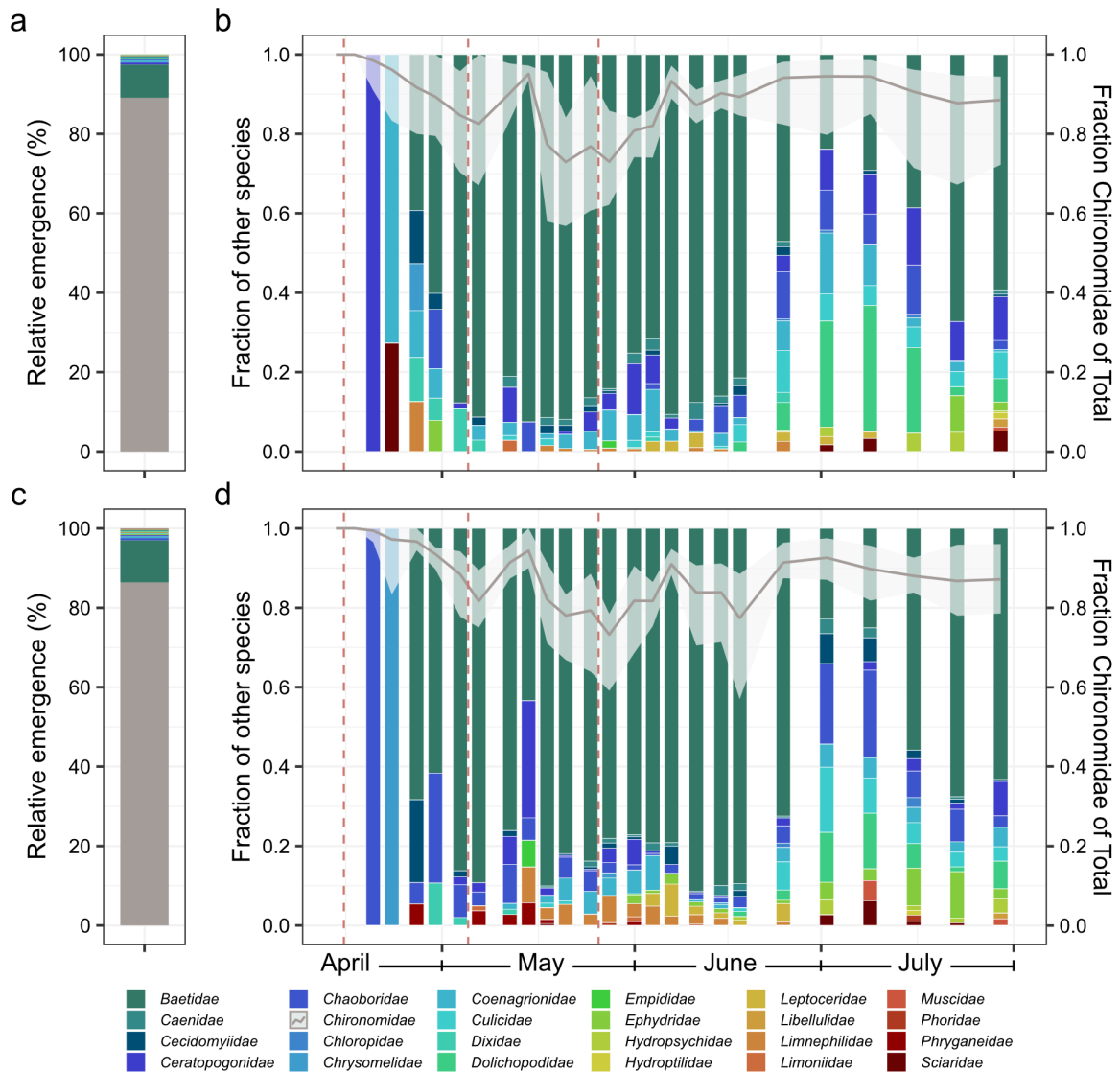


Figure 2: Left: Composition of total collected insects from a) control FPMs (n = 6) and c) FPMs treated with Bti (n = 6). Right: Composition of the collected insects for every sampling time point from b) control FPMs (n = 6) and d) FPMs treated with Bti (n = 6) three times (dashed red lines). Fractions of Chironomidae are displayed on the right and are represented by the grey line. Fractions of other families on the remain are displayed on the left and shown as bars.

Effects of Bti on community structure

Total abundance of emerged insects from FPMs treated with Bti was reduced by 11.7% relative to the control. Proportions of all insect families were in equal ranges with small

differences between treatments (< 3%; Fig. 2a, c). Consequently, we did not find statistically significant effects of Bti on the total number of collected insects (t.test, $df = 9.850$, $p = 0.485$) nor of collected Chironomidae (t.test, $df = 9.764$, $p = 0.405$) or Baetidae (t.test, $df = 9.936$, $p = 0.619$). Quantities of mosquitoes (Culicidae) were also in equal ranges (Tab. S2) as they did not occur in the FPMs during the months of Bti application but afterwards, i.e., June and July. Since their developmental time during summer is usually less than two weeks (Clements, 1992), the present individuals were not affected by Bti. Analyses of insect families with a proportion of at least 0.1% revealed a statistically significant difference only for one family of caddisflies, that is Limnephilidae (t.test, $df = 7.965$, $p = 0.022$). However, Bti-related differences, positive as well as negative, were also observed in other families (Tab. S2) but due to low abundances and high variability in the data those differences are difficult to interpret and did not turn out statistically significant. Thus, we refrain from a detailed discussion due to substantial uncertainty.

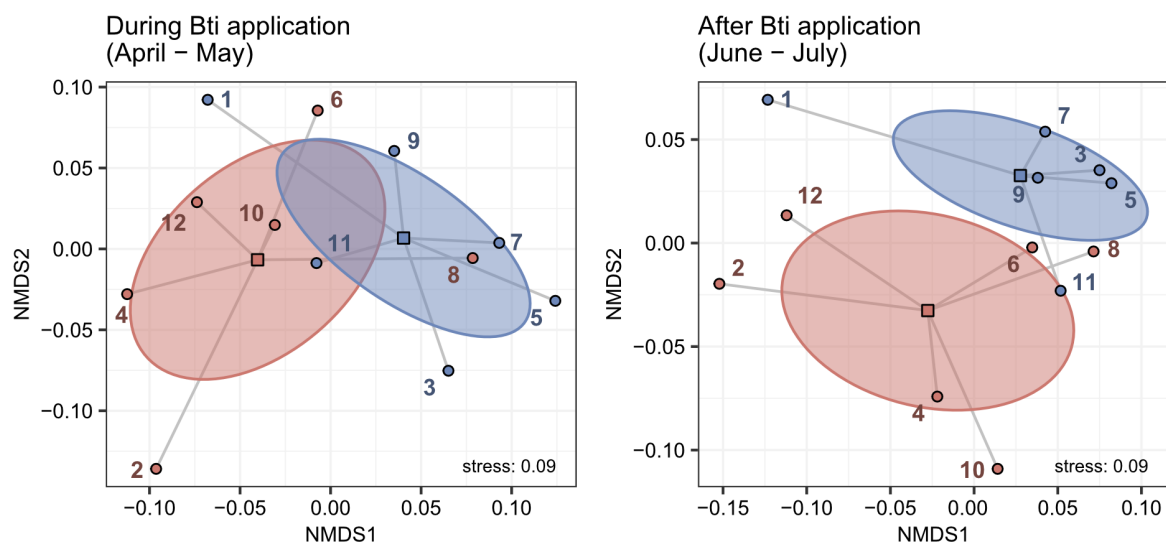


Figure 3: Non-metric multidimensional scaling (NMDS) ordination for the composition of insect communities from Bti-treated (red; $n = 6$) and control FPMs (blue; $n = 6$) during Bti application (left) and afterwards (right). The stress values are stated as a “goodness-of-fit” measure, indicating a good ordination for values below 0.1 (Clarke, 1993).

Investigating the community composition (Fig. 2b, d), we found a significant Bti effect over the whole study duration (PERMANOVA, $F_{1,308} = 2.719$, $p = 0.0001$). We further compared the communities during (April – May; Fig. 3a) and following Bti application (June – July; Fig. 3b; PERMANOVA, $F_{1,308} = 2.875$, $p = 0.034$) and found communities after Bti application were more dissimilar than before. Additionally, we detected differences in the temporal dynamics of total emerging insects between treatments (Fig. 4a, ANOVA,

$F_{25,249} = 1.751$, $p = 0.0174$). Both results were related to Chironomidae constituting almost 90% of the emerged organisms, thus their dynamics (Fig. 4b; ANOVA, $F_{25,249} = 1.817$, $p = 0.012$) dominated changes on community level (SIMPER, Chironomidae: 79.9%, Baetidae: 14.6%) and also the emergence dynamics of all insects (Fig. 4a, b).

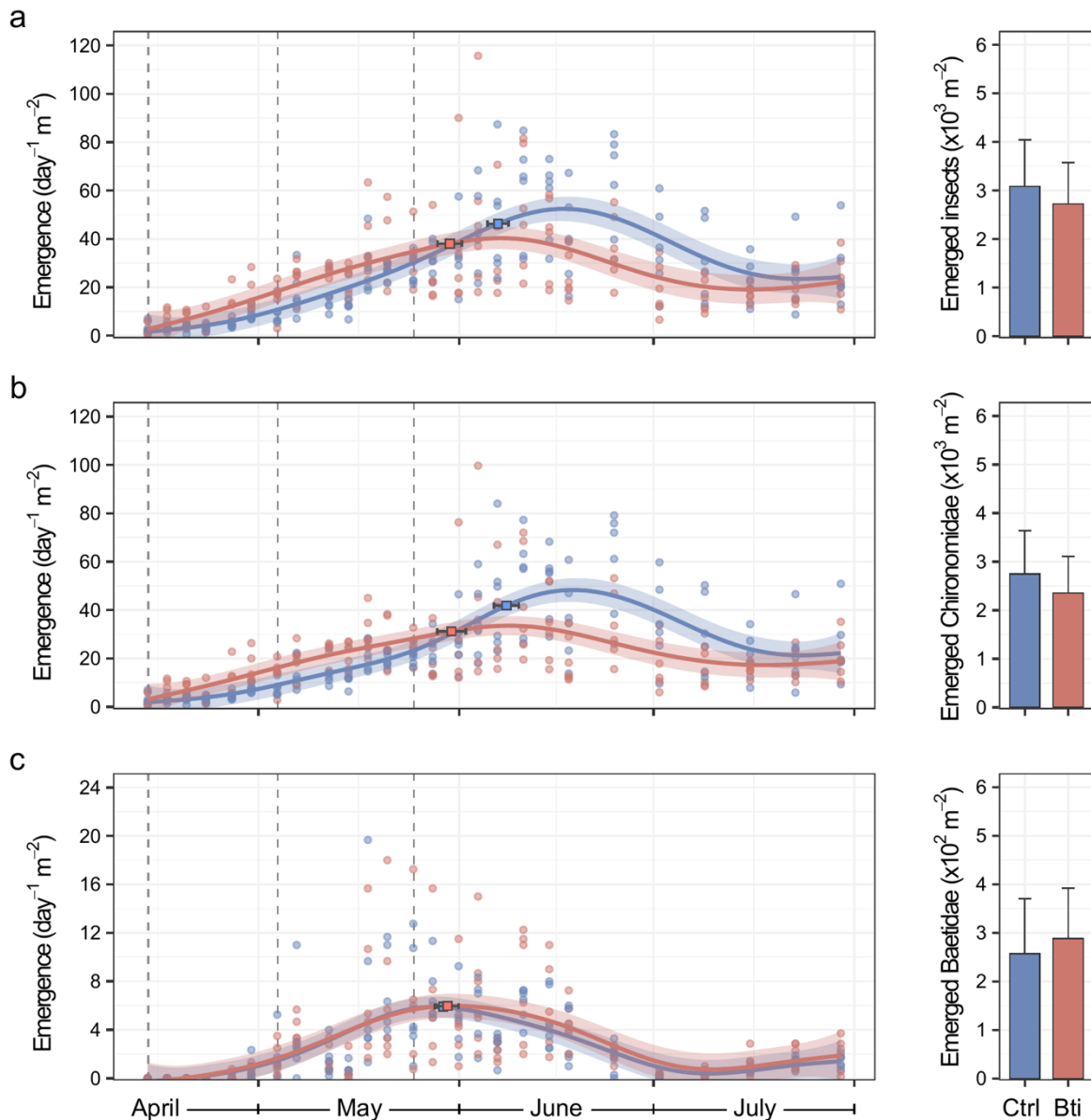


Figure 4: Number of emerged individuals per day and m² over 16 weeks (left) and mean (with 95% CI) total emergence (right) of a) all insects, b) the most abundant insect family: Chironomidae and c) the second most abundant insect family: Baetidae. Six out of twelve FPMs were treated with Bti (red line; control: blue line) three times (dashed lines). The time points of 50% emerged specimens are indicated with squares (with 99% CI).

During the period of Bti application, the emergence of Chironomidae in Bti-treated FPMs was on average 1.7 times higher relative to their untreated counterparts (least-square means, $p = 0.016$). Moreover, the peak of Chironomidae emergence was about 10

days earlier under Bti exposure (i.e., beginning of June) with an approximately 26% lower abundance compared to the peak of the control (i.e., mid of June). Consequently, Chironomidae emergence from treated FPMs reached 50% 8.5 days earlier than from control FPMs ($EmT50_{Bti} = 46.9$ d, $EmT50_{Control} = 55.4$ d). In the period following Bti applications, numbers of emerging Chironomidae from treated FPMs were on average a factor of 1.3 below the control (least-square means, $p = 0.037$). We could not confirm a similar pattern or any other Bti-related difference between treatments for Baetidae (Fig. 4c; ANOVA, $F_{21,191} = 0.250$, $p = 0.999$).

Effects of Bti on diversity

To analyse the diversity of communities, we compared Shannon diversity indices of Bti-treated FPMs ($H' = 0.54 \pm 0.07$, mean \pm 95% CI) and control FPMs ($H' = 0.47 \pm 0.08$;

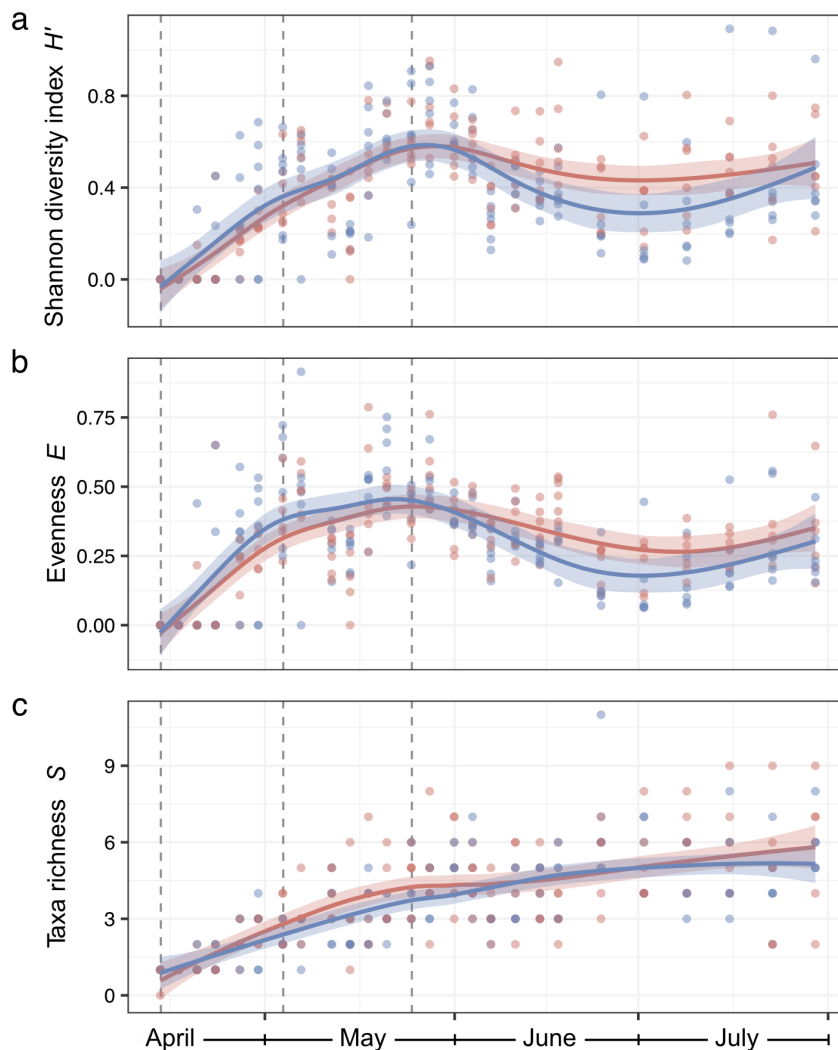


Figure 5: Diversity endpoints for insect communities from control FPMs (blue; $n = 6$) and FPMs treated with Bti (red; $n = 6$) three times (dashed lines). All indices are based on family level.

ANOVA, $F_{1,10} = 0.139$, $p = 0.717$) and their dynamics (ANOVA, $F_{23,205} = 1.031$, $p = 0.429$). Although Bti had no statistically significant effect on those indices, diversity in Bti-treated FPMs was by tendency higher than in control FPMs in the period following Bti application (June – July, Fig. 5a). Investigating the evenness of taxa ($E_{Bti} = 0.19 \pm 0.02$, $E_{Control} = 0.18 \pm 0.03$; Fig. 5b) revealed no significant changes over time (ANOVA, $F_{23,205} = 1.249$, $p = 0.207$). However, evenness was higher in control FPMs during Bti application followed by a higher evenness in treated FPMs, which is an inversion of the emergence pattern of Chironomidae (cf. Fig. 4 & 5). We further compared the average number of collected taxa (richness S) between treatments. In total, we collected slightly more taxa in Bti-treated FPMs ($S = 16.67 \pm 1.20$) than in control FPMs ($S = 14.17 \pm 1.71$) which seemed to be confined to the period of Bti application (Fig. 5c). On average, the number of collected taxa per FPM was 7% higher in Bti-treated FPMs than in control FPMs (ANOVA, $F_{1,10} = 1.033$, $p = 0.333$) and changed equally over time (ANOVA, $F_{25,249} = 0.517$, $p = 0.974$; Fig. 5c). Compared to the dominance of Chironomidae, the additional taxa have less influence on the diversity index.

Effects of Bti on the weight of individuals

Chironomidae from Bti-treated FPMs were 11% heavier than control individuals (weight_{Bti} = 0.23 mg, weight_{Control} = 0.21 mg) though not statistically significant over the entire study duration (ANOVA, $F_{1,10} = 1.902$, $p = 0.198$) or between time points (ANOVA, $F_{25,247} = 0.978$, $p = 0.497$). During the period of Bti application, the average dry weight of Chironomidae in both Bti-treated and control FPMs decreased from ~0.45 mg to ~0.2 mg, with individuals from the Bti treatment being on average 21% heavier (Fig. 6). Due to the method of size sorting, these observations can be attributed to differences in the body condition but also to changes in the dominant species. Following the Bti application period, the average weight remained stable and comparable among both treatments.

Similarly, the average dry weight of Baetidae was not impacted by Bti treatment (t.test, $df = 7.482$, $p = 0.705$). Nonetheless, we observed a general decrease in the individual dry weight of Baetidae over the course of the season (Fig. 6). It was not possible to analyse the dynamics of the weight of Coenagrionidae since numbers were too low. However, dry weight of Coenagrionidae emerging from Bti-treated and control FPMs differed on average by only 0.2% (t.test, $df = 7.512$, $p = 0.976$). Overall, weight changes were mainly related to seasons instead of Bti, thus the total biomass of the assessed families collected over 3.5 months did not significantly differ between treatments (Fig. 6,

right; $t.test_{Chironomidae}$, $df = 8.684$, $p = 0.723$; $t.test_{Baetidae}$, $df = 9.599$, $p = 0.675$; $t.test_{Coenagrionidae}$, $df = 7.892$, $p = 0.373$). Consequently, the dynamics of emerged biomass of Chironomidae as well as Baetidae showed nearly the same pattern as the emerged individuals (Fig. S2).

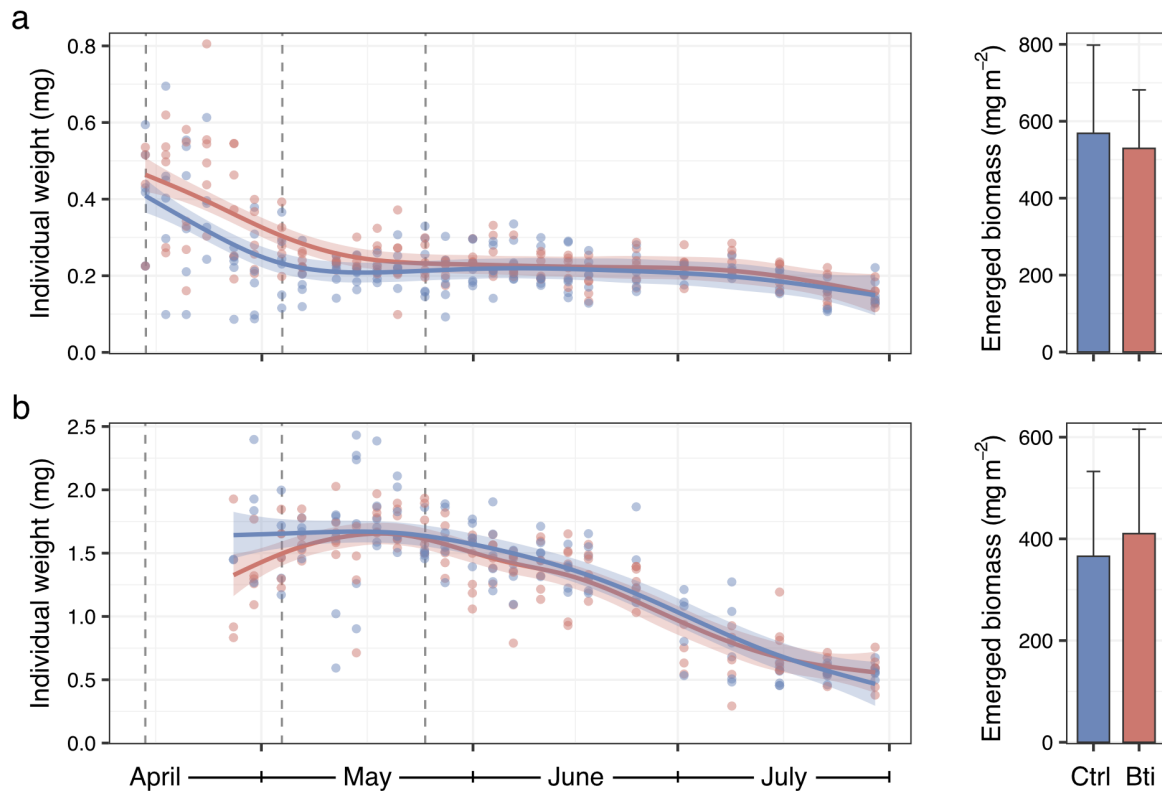


Figure 6: Mean individual weight in mg over 16 weeks (left) and mean (with 95% CI) total biomass in mg per m² (right) of a) the most abundant insect family: Chironomidae and b) the second most abundant insect family: Baetidae in control FPMs (blue; $n = 6$) and FPMs treated with Bti (red; $n = 6$) three times (dashed lines).

Discussion

Effects on emerging insects

By including a temporal perspective, we could show that even with no significant effects of Bti treatments on the total number of emerging aquatic insects – which was also observed by other studies (reviewed in Brühl et al., 2020) – Bti affected the community composition as well as the number of emerging insects over the course of the study. This points towards alterations in the temporal emergence dynamics of exposed systems. Similarly, diversity indices indicate consequences of Bti mainly for the post-application period (i.e., June – July). As most of the identified taxa contributed less than 0.5% to the emergence, these shifts in diversity and fluxes are mostly driven by implications on the

most abundant taxa, Chironomidae and Baetidae, justifying a focus on these in the following.

Emergence dynamics of Chironomidae from Bti-treated and control FPMs differed by a main point: the emergence was shifted by about 10 days leading to an earlier and reduced peak associated with higher emergence during April and May and fewer during June and July. This highly dynamic pattern is, to the best of our knowledge, not yet described as an impact caused by Bti. However, changes in the temporal emergence dynamics of aquatic subsidy were previously observed for other aquatic contaminants, e.g., nanoparticles, pyrethroids or metals, in terms of a delay or a shortening of the emergence peak (Bundschuh et al., 2019; McCahon and Pascoe, 1991; Schulz and Liess, 2000) but also described earlier emergence (Dewey, 1986; Gruessner and Watzin, 1996). Explanations for the observed pattern were mostly contaminant-specific but reasons for earlier emerging insects remained mainly unclear. Our observations may be explained by local chironomid communities assembled from various species comprising a wide range of breeding cycles (voltinism) and feeding strategies (Armitage et al., 1995). Chironomidae species are known to be varyingly susceptible to Bti which can be partly attributed to the feeding strategy (Ali et al., 1981; Kondo et al., 1995, 1992; Theissinger et al., 2019). Collecting-gathering, grazing or filter-feeding chironomids, for instance, are more likely to directly ingest Bti and thus might be compromised to a higher extent than predatory representatives (Liber et al., 1998; Pillot, 2014a, 2014b). The variations in the susceptibility to Bti of Chironomidae species with different breeding cycles may translate to an altered emergence dynamic of the whole family as observed here. A shift towards more resistant species of larger size could further explain the increased individual weight of emerged Chironomidae from treated FPMs during April and May. Analysing the community composition of the Chironomidae collected in the present study on a lower taxonomic level by using metabarcoding and considering ecological traits such as feeding mode, could give a deeper insight into the underlying mechanisms of taxa-specific Bti susceptibility. It would also provide information on the number and abundance and thus the importance of multivoltine chironomid species (species with more than one generation per year; Pinder, 1986) equally ovipositing in FPMs of both treatments which could have mitigated the observed effects of Bti. Additionally, larvae from the same species can be at different developmental stages in an FPM and thus vary in their sensitivity to Bti (Armitage et al., 1995). In fact, the sensitivity of larvae to Bti decreases with increasing developmental stage (Kästel et al., 2017; Ping et al., 2005). Hence, it may

be well feasible that individuals at initial developmental stage have been strongly negatively affected by Bti during its application, which may have translated to a reduced emergence of Chironomidae during the post-application phase (i.e., June and July). This is further supported by a simultaneous investigation of benthic communities in the FPMs during June which found Bti-induced reductions in larval chironomid abundances by on average 41% (Gerstle et al., 2022). Since the average developmental time of Chironomidae larvae is between two and four months (Pillot, 2014a, 2014b), an extended study duration could have revealed further consequences of Bti on the dynamics of Chironomidae emergence.

A lower abundance of early-stage larvae or highly susceptible species reduces the pressure of resource competition for further developed and less sensitive specimens. Consequently, these organisms may have been able to acquire nutrients and energy more efficiently leading to a quicker development and thus earlier emergence as indicated by the emergence pattern of Chironomidae (Fig. 4; Oliver, 1971; Péry et al., 2002). Also, other species or families of emerging insects could have benefitted from lower densities of chironomid larvae since they could expand their trophic niches leading to higher portions in Bti-treated FPMs (i.e., Limnephilidae; Fig. 2). An increased availability of energy resources is further underlined by heavier Chironomidae emerging from Bti-treated FPMs during the application period. We initially hypothesized lighter individuals due to stress-induced energy allocation from storage to increased maintenance costs and repair mechanisms (Kooijman, 2000; Saraiva et al., 2020; Sokolova et al., 2012). In fact, several studies documented higher energetic costs as a consequence of immunological or biochemical stress responses leading to retarded growth and delayed emergence of individual species (Bordalo et al., 2020; Saraiva et al., 2017; Silvestre Pereira Dornelas et al., 2020). Thus, increased resource availability in a much more complex benthic and in particular chironomid community within our FPMs could have concealed potential energy-demanding effects of Bti. Beyond that, a higher energy supply can accelerate larval growth and development and, in some species, influence adult weight (Hooper et al., 2003; Ristola et al., 1999) explaining the unexpected results in our study. A closer look into underlying processes, namely the energetic budget of the emerged organisms, might help to further explain our observations, especially concerning reported higher protein content as a consequence of Bti exposure (Bordalo et al., 2020). Additionally, it could improve the assessment of qualitative changes in aquatic subsidy, i.e., in which form energy is transferred, for the nutrition of riparian predators. Given the known differences

between sexes in adult weight and emergence time (“Protandry”; Armitage et al., 1995), investigating sex-specific emergence patterns could further deepen the understanding of complex communities.

No impact of Bti on the emergence dynamics or the weight of Baetidae was observed, which is in line with our expectations and informed by the following considerations: The phylogenetic distance between Baetidae (Ephemeroptera) and the Bti target organisms mosquitoes (Culicidae, Diptera) leads to missing prerequisites in their gut for Bti to act (Boisvert and Boisvert, 2000). Thus, the possibility of negative effects is limited which is confirmed by studies reporting no Bti-induced effects on Baetidae (cf. Boisvert and Boisvert, 2000). Consequently, the present study provides further evidence for the prevailing assumption that especially Chironomidae are among the non-target species most likely directly affected by Bti under natural conditions. Nevertheless, the complex interrelations in aquatic insect communities also hold the potential for indirect effects on other taxa. As mentioned above, lower competitive pressure can positively affect population development of other consumers. Furthermore, the decrease of one taxon, especially of a highly abundant one such as Chironomidae, can induce a restructuring of the ecological niches between its competitors but also predators leading to both negative and positive indirect effects on their abundance (Gerstle et al., 2022; Giller, 2012). In fact, differences in the abundance data of a few taxa could further point to an alteration in the species interactions within the assessed communities, even though not statistically significant.

Consequences of altered dynamics of aquatic subsidy

The shift in the emergence dynamics of Chironomidae, which are the main constituent (e.g., 88%) of the assessed and other natural communities and drive aquatic subsidy, can lead to temporal (un)availability of prey (Allgeier et al., 2019; Hershey et al., 1998). This can be as limiting to consumers as lower overall abundances since predators may be behaviourally and physiologically adapted to cycles of emergence and thus timing of energy fluxes (Armstrong et al., 2016). Furthermore, fluxes of prey subsidy from aquatic to terrestrial food webs and vice versa can be seasonally asynchronous depending on the productivity of the donor habitat (Nakano and Murakami, 2001). In fact, the *in situ* prey biomass in some terrestrial habitats (e.g., forests in continental climate) is lowest during winter and spring, and peaks in late summer. Reversely, the flux of aquatic prey to terrestrial systems is highest between spring and early summer, as observed in our

systems, buffering for the low availability of prey of terrestrial origin (Nakano and Murakami, 2001). Additionally, insects of aquatic and terrestrial origin feature different nutrient compositions regarding fatty acids. Aquatic insects contain up to 34 times higher amounts of omega-3 long-chain polyunsaturated fatty acids which are essential for several terrestrial consumers since they are unable to synthesize them themselves leading to even higher importance of aquatic subsidy (Shiple et al., 2022). Consequently, the observed shifts in aquatic subsidy may have implications for temporarily dependent terrestrial predators (Polis et al., 1997; Schindler and Smits, 2017). Indeed, the density of terrestrial spiders, particularly horizontal orb-weavers (Tetragnathidae), follows the dynamics of aquatic emergence driven by the high degree of dependency on aquatic subsidy (Kato et al., 2003; Krell et al., 2015; Marczak and Richardson, 2007). Moreover, subsidy-induced changes in spider abundances can cause top-down effects on terrestrial prey as observed by Henschel et al. (2001). Besides spiders, vertebrate consumers such as insectivorous bats and birds show seasonal dependences on aquatic subsidy mainly due to limited terrestrial prey during spring (Altringham, 1996; Nakano and Murakami, 2001; Vaughan, 1997; Zahn et al., 2007). During this season energy demands of both bats and birds are elevated due to reproduction (Arlettaz et al., 2001; Bryant and Westerterp, 1983; Fukui et al., 2006; Zahn et al., 2007). Additionally, the end of hibernation of bats as well as the return of migrant birds during spring are further examples pointing to the importance of well-timed aquatic prey (Gray, 1993; Zahn et al., 2007). Since the application of Bti reduces mosquitoes intentionally by up to 90% (Becker, 1997), additional changes in the temporal availability of other aquatic subsidy, as indicated by our results, could lead to a lack of appropriate nutrition during sensitive time periods (e.g., reproduction phases). In fact, a field study by Poulin et al. (2010) supports this assumption showing negative effects on chick survival of breeding house martins which the authors linked to a Bti-induced reduction in aquatic subsidy. Hence, side effects of Bti on non-target Chironomidae in terms of shifted emergence dynamics may affect the survival as well as the reproductive success of temporal-dependent predators and lead to consequences in other parts of the linked terrestrial systems. Therefore, we suggest to reassess the use of Bti considering alternative mosquito control measures (e.g., traps or natural predators; Acquah-Lampsey and Brandl, 2018; Poulin et al., 2017) in sensitive natural areas and ecologically valuable wetlands (such as protected areas for nature conservation; BMUV, 2022) or during vulnerable seasons.

Conclusion

Our results show that Bti has the potential to change the emergence dynamics of insect families that are closely related to target mosquitoes. If those insects are an important subsidy for adjacent terrestrial food webs, such as Chironomidae, a time-shifted emergence can translate to effects at higher trophic levels. Seasonal dynamics in the availability of *in situ*, as well as allochthonous prey, cause a temporal dependence of terrestrial predators on aquatic subsidy to cover energy demands. Thus, altered emergence dynamics could lead to a lack of appropriate prey inducing a shift in the diet – if other prey organisms are available – which holds the potential for further indirect effects. As terrestrial predators themselves function as consumers but also as prey within the terrestrial ecosystem, their diet and abundance can affect higher as well as lower trophic levels. Consequently, changes in the temporal emergence dynamics of aquatic subsidy could alter the whole food web of linked terrestrial ecosystems.

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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.

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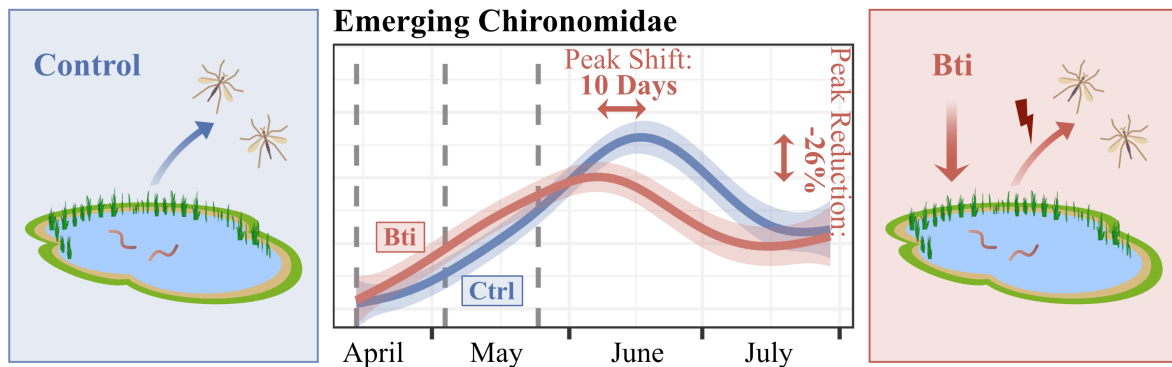
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Graphical Abstract



Supplementary Material – Appendix A.1

Table S1: Time schedule of treatments in FPMs and insect samplings

Date	FPM treatment	Sampling
11 April 2020	Flooding, 30 > 40 cm	
12 April 2020	Flooding, 40 > 50 cm	
14 April 2020	1 st Bti application	X (before application)
17 April 2020		X
20 April 2020		X
21 April 2020	Release water, 50 > 40 cm	
22 April 2020	Release water, 40 > 30 cm	
23 April 2020		X
27 April 2020		X
30 April 2020		X
02 May 2020	Flooding, 30 > 40 cm	
03 May 2020	Flooding, 40 > 50 cm	
04 May 2020	2 nd Bti application	X (before application)
07 May 2020		X
12 May 2020	Release water, 50 > 40 cm	X
13 May 2020	Release water, 40 > 30 cm	
15 May 2020		X
18 May 2020		X
21 May 2020		X
23 May 2020	Flooding, 30 > 40 cm	
24 May 2020	Flooding, 40 > 50 cm	
25 May 2020	3 rd Bti application	X (before application)
28 May 2020		X
01 June 2020		X
02 June 2020	Release water, 50 > 40 cm	
03 June 2020	Release water, 40 > 30 cm	
04 June 2020		X
07 June 2020		X
11 June 2020		X
15 June 2020		X
18 June 2020		X
25 June 2020		X
02 July 2020		X
09 July 2020		X
16 July 2020		X
23 July 2020		X
30 July 2020		X

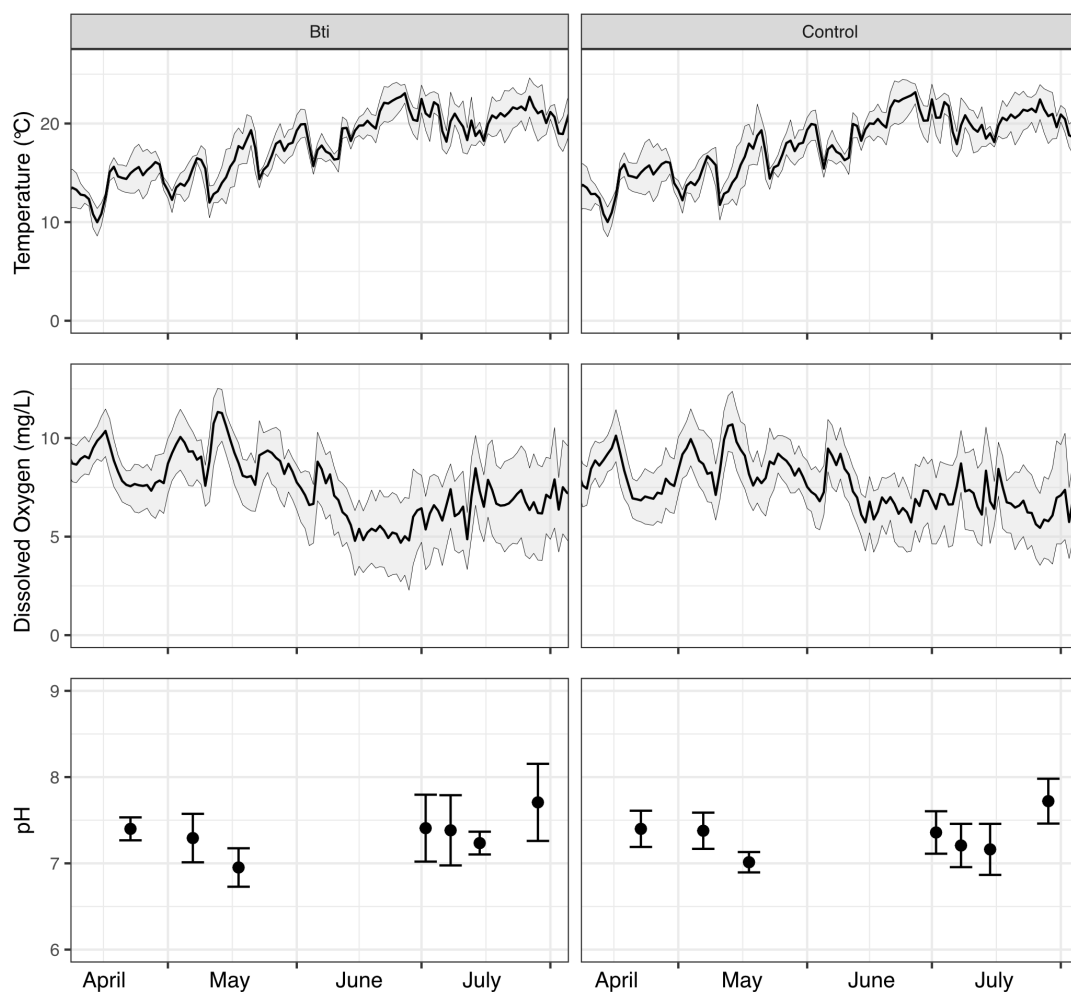


Figure S1: Temperature in °C (mean with 50% quantile), dissolved oxygen in mg L⁻¹ (mean with 50% quantile) and pH (mean with 95% confidence interval) in treated FPMs (Bti; n = 6) and control FPMs (n = 6) during the study duration (3.5 months).

Table S2: Overview of aquatic insects collected with emergence traps (total area: 1 m²) over 3.5 months

Order	Species	Control	Bti	Total
<i>Coleoptera</i>	Chrysomelidae	1	3	4
<i>Diptera</i>	Cecidomyiidae	21	21	42
	Ceratopogonidae	109	53	162
	Chaoboridae	51	102	153
	Chironomidae	16492	14122	30614
	Chloropidae	4	3	7
	Culicidae	45	39	84
	Dixidae	11	4	15
	Dolichopodidae	48	36	84
	Empididae	2	2	4
	Ephydriidae	9	41	50
	Limoniidae	1	3	4
	Muscidae	1	5	6
	Phoridae	0	3	3
	Sciaridae	8	7	15
<i>Ephemeroptera</i>	Baetidae	1545	1729	3274
	Caenidae	33	26	59
<i>Odonata</i>	Coenagrionidae	91	67	158
	Libellulidae	2	3	5
<i>Trichoptera</i>	Hydropsychidae	8	10	18
	Hydroptilidae	1	1	2
	Leptoceridae	17	18	35
	Limnephilidae	11	38	49
	Phryganeidae	0	8	8

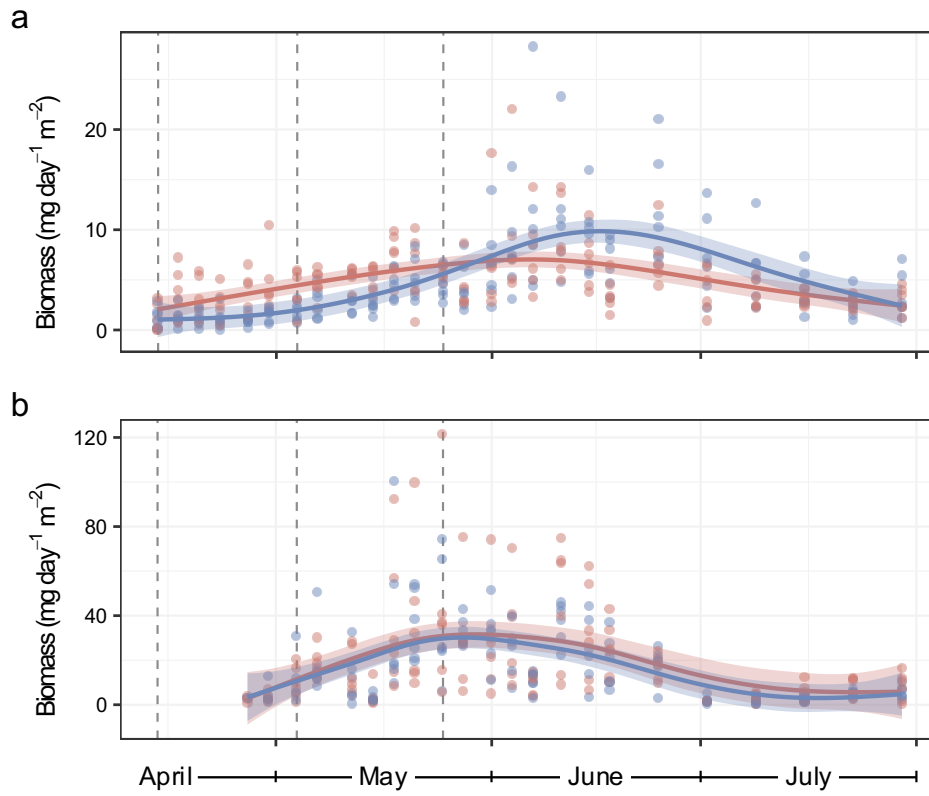


Figure S2: Mean emerged biomass in mg per day and m² over 16 weeks of a) the most abundant insect family: Chironomidae and b) the second most abundant insect family: Baetidae in control FPMs (blue; n = 6) and FPMs treated with Bti (red; n = 6) three times (dashed lines).

Appendix A.2

Impact across ecosystem boundaries – Does Bti application change quality and composition of the diet of riparian spiders?

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Abstract

Emerging aquatic insects link aquatic and adjacent terrestrial food webs by subsidizing terrestrial predators with high-quality prey. One of the main constituents of aquatic subsidy, the non-biting midges (Chironomidae), showed altered emergence dynamics in response to the mosquito control agent *Bacillus thuringiensis* var. *israelensis* (Bti). As riparian spiders depend on aquatic subsidy, they may be affected by such changes in prey availability. Thus, we conducted a field study in twelve floodplain pond mesocosms (FPMs), six were treated with Bti (2.88×10^9 ITU/ha, VectoBac WDG) three times, to investigate if the Bti-induced shift in chironomid emergence dynamics is reflected in their nutritional value and in the diet of riparian spiders. We measured the content of proteins, lipids, glycogen, and carbohydrates in emerged Chironomidae, and determined the stable isotope ratios of female *Tetragnatha extensa*, a web-building spider living in the riparian vegetation of the FPMs. We analysed the proportion of aquatic prey in spiders' diet, niche size, and trophic position. While the content of nutrients and thus the prey quality was not significantly altered by Bti, effects on the spiders' diet were observed. The trophic position of *T. extensa* from Bti-treated FPMs was lower compared to the control while the aquatic proportion was only minimally reduced. We assume that spiders fed more on terrestrial prey but also on other aquatic organisms such as Baetidae, whose emergence was unaffected by Bti. In contrast to the partly predaceous Chironomidae, consumption of aquatic and terrestrial primary consumers potentially explains the observed lower trophic position of spiders from Bti-treated FPMs. As prey organisms vary in their quality the suggested dietary shift could transfer previously observed effects of Bti to riparian spiders conceivably affecting their populations. Our results further support that anthropogenic stressors in aquatic ecosystems may translate to terrestrial predators through aquatic subsidy.

Keywords: Chironomidae, *Tetragnatha extensa*, stable isotope analysis, nutrient content, *Bacillus thuringiensis* var. *israelensis*, aquatic-terrestrial linkage

Introduction

The reciprocal flow of resources between aquatic and terrestrial habitats, as it naturally occurs in riparian zones, links food webs and increases their respective productivity (Polis et al., 1997). While the input of terrestrial organic matter is well known to support aquatic food webs, flying insects of aquatic origin are especially relevant for riparian predators, such as spiders (Baxter et al., 2005; Marczak and Richardson, 2007; Richardson et al., 2010). Emergence of adult aquatic insects (e.g., midges or mayflies) increases the prey availability in terrestrial habitats and supports riparian predators. At the same time, these insects of aquatic origin serve as high-quality prey due to their significant nutritional value for these spiders (Akamatsu et al., 2004; Kato et al., 2003).

However, the linkage through aquatic subsidy may transfer disturbances from aquatic to terrestrial systems through changes in timing, nutritional value, or abundance of adult life stages of aquatic insects (Schulz et al., 2015; Stepanian et al., 2020). Indeed, various forms of human activity, spanning from hydromorphological modifications and invasive species to pollution, can modify the subsidy of terrestrial food webs by aquatic resources (Gergs et al., 2014; Greig et al., 2012; Kraus et al., 2020). Among those disturbances, the mosquito control agent *Bacillus thuringiensis* var. *israelensis* (Bti) directly targets parts of the aquatic subsidy, mainly mosquitoes and blackflies, when deliberately applied to freshwater bodies (Boisvert and Boisvert, 2000). As biological alternative to chemical insecticides with an assumed taxa-specific mode of action against the larvae of several nematoceros dipterans, Bti is considered relatively environmentally friendly and safe for humans explaining its worldwide application (Després et al., 2011). The larvicidal activity is based on δ -endotoxins which are solubilized and activated in the larval midgut after ingestion. Binding to specific receptors impairs the midgut epithelium resulting in damage of the gut walls and ultimately mortality within hours (Dylo et al., 2014; Vachon et al., 2012).

Despite the presumed high specificity of Bti against target dipterans, several studies demonstrated negative responses of phylogenetically closely related non-target organisms (Boisvert and Boisvert, 2000; Brühl et al., 2020; Jakob and Poulin, 2016). Especially non-biting midges (Diptera: Chironomidae) were identified as a sensitive member of aquatic macroinvertebrate communities (Allgeier et al., 2019; Bordalo et al., 2021, 2020; Gerstle et al., 2023; Kästel et al., 2017). Unintended negative impacts on Chironomidae, which often dominate aquatic subsidy with shares of up to 90 % to total emergence, hold the potential for aquatic-to-terrestrial propagation as suggested by

Kolbensschlag et al. (2023). In this predecessor publication, the authors report a significant alteration of the emergence dynamics over 3.5 months as a consequence of Bti applications (Kolbensschlag et al., 2023). In contrast to chironomids, the second most abundant taxon (Ephemeroptera: Baetidae) did not show any response to Bti. In fact, the emergence of Chironomidae from treated floodplain pond mesocosms (FPMs) peaked 10 days earlier at an approximately 26 % reduced abundance. At the same time, the overall number of emerged chironomids (i.e., over the entire study duration) was slightly reduced by 14 % (Kolbensschlag et al., 2023) reflecting the lower larval densities in Bti-treated FPMs observed in parallel (Gerstle et al., 2023). Although the study by Kolbensschlag and colleagues (2023) points towards Bti-induced shifts in the insect emergence and thus temporal changes in the quantity of aquatic subsidy, it lacks the consideration of the quality, i.e., the nutritional value of adult chironomids for terrestrial predators. Reduced larval densities could relax resource competition enabling surviving individuals to build up larger reserves of energy and nutrients (Arrese and Soulages, 2010). Furthermore, potential alterations on the linked terrestrial food web are not yet assessed but are of substantial ecological relevance.

To close this gap of knowledge, we expanded on published studies by analysing energy reserves (i.e., macronutrients and glycogen) of emerged chironomids from control and Bti-treated FPMs. In addition to prey quality, we investigated the consequences of Bti treatments on subsidized riparian spiders. We focused on the web-building *Tetragnatha extensa*, as it is known to strongly rely on aquatic prey (Kato et al., 2003; Wieczorek et al., 2015) and thus responds to changes in its availability (Graf et al., 2020). Adults are usually abundant between May and August, and reproduce in late summer (Bellmann, 2016). Their diet as opportunistic feeders includes small flying insects caught in their web such as diptera, aphids and mayflies but also e.g., damselflies (Nyffeler and Benz, 1981; Wieczorek et al., 2015). By measuring stable isotope ratios, we compared the spiders' diet, trophic level, and niche size between control and Bti-treated FPMs. We hypothesised that the Bti-induced changes in the emergence dynamics are reflected (1) in higher levels of nutrients in adult chironomids from Bti-treated FPMs due to lower resource competition in the aquatic habitat. Furthermore, the changed emergence dynamics caused an intermittently lower availability of aquatic subsidy. Given that spiders' diet often follows the relative availability of food sources (Ryabov et al., 2015; Wilder, 2011) and the mentioned dependence of *T. extensa* on flying aquatic insects, we hypothesised (2) a shift

to higher proportions of terrestrial prey for *T. extensa* in Bti-treated FPMs relative to controls detectable via their stable isotope ratios.

Materials and methods

Study setup

This study was conducted between April and July 2020 in twelve artificial floodplain pond mesocosms (FPMs) located at the Eußerthal Research Station (EERES), Germany, in parallel to other studies (Gerstle et al., 2023; Kolbensschlag et al., 2023). These FPMs were designed to assess implications of stressors on aquatic subsidy in an environmentally realistic and at the same time replicated manner. Therefore, natural colonization and succession were allowed to occur in these mesocosms since their construction in 2017 (Stehle et al., 2022). The water level of each FPM can be regulated via adjustable in- and outflows directly connected to an adjacent stream of high ecological quality. One shore in each FPM represents a floodplain area with a flat slope (3°), leading to differences in the surface area with changes in water level. When the water level reached 50 cm, which is the maximum used in the present study, the water surface of each FPM measured about 104 m² (~20 m × 5.2 m). All FPMs were vegetated mostly by waterweeds, coontails, green algae, bulrushes, and rushes. During the study, the pH was regularly measured while dissolved oxygen and temperature were continuously logged. Data suggest similar conditions between the treatments as detailed in the Supplementary Material (Fig. S1).

Bti application

The hatching of the target mosquito larvae in the Upper Rhine Valley, mainly *Aedes vexans*, is initiated by flooding and hence the application of Bti is temporally linked to such events (Becker et al., 2018). To simulate flooding, the water height of every FPM was increased by 20 cm (from 30 to 50 cm) three times in an interval of three weeks which corresponds to a realistic frequency of Bti applications in the Upper Rhine Valley (Becker et al., 2018). Three days after the onset of each flood (i.e., 14th April, 4th May, 25th May), the maximum field rate (i.e., 2.88×10^9 ITU/ha) of the mosquito control agent *Bacillus thuringiensis* var. *israelensis* (Bti) was applied to six out of twelve FPMs (Fig. S2). For this, the formulation VectoBac WDG (Valent BioSciences, Illinois, USA) was suspended in pond water and homogeneously applied to the water surface using a conventional knapsack sprayer (prima 5, GLORIA, Germany). All Bti products applied in Germany need to be sterilized by gamma radiation to annihilate viable spores or cells which lowers long-term persistence

of Bti (Becker et al., 2018; Poulin et al., 2022). Applications of Bti were exclusively done on windless days in order to prevent any unintended contamination of control (unexposed) FPMs. The water levels were reset to 30 cm one week after each application (see for details Kolbensschlag et al., 2023).

Since the successful application of Bti could not be verified analytically, a bioassay, which uses the toxicity of Bti towards mosquito larvae (i.e., *Culex sp.*) as response variable, was employed as an alternative method. In parallel, the rate of larval survival in control FPMs was used to rule out any cross-contamination. Following this procedure, we indirectly verified a successful Bti application by observing at least 90 % mortality of *Culex sp.* compared to the controls.

Arthropod sampling and identification

Over the entire study period, emerging aquatic insects were caught using three floating emergence traps per FPM, each covering an area of 0.33 m² (Cadmus et al., 2016). These traps were emptied once or twice a week by vacuuming all living insects from the inside with a self-constructed hand-operated suction sampler (Fig. S3). For stable isotope analyses, terrestrial arthropods were collected in the first two weeks of June twice a week from the floodplain vegetation of control and treated FPMs using a suction sampler (modified Stihl SH 86; Stihl, Waiblingen, Germany) for about 2 min per FPM. Additionally, five *T. extensa* females were gathered from the riparian vegetation of each FPM on June 15, 2020. All arthropods were conserved in liquid nitrogen and stored at -80 °C until further analyses. Aquatic insects and terrestrial arthropods were identified to family or order level (Klausnitzer, 2011; Köhler et al., 2015), respectively, using a stereomicroscope (SZX 9, Olympus) with a transmitted brightfield illumination base (SZX-ILLB200, Olympus).

Nutrient composition

Chironomids caught in the emergence traps were pooled in eight periods of approximately two weeks, representing the emergence of the respective sampling dates (Table S5). This pooling was done for each replicate (i.e., FPM) separately. Chironomid samples were freeze-dried for at least 48 h and ground in a mixer mill by adding a stainless-steel ball. From the pulverised biomass, 1.9 mg (\pm 0.1 mg) were weight into 2-mL Eppendorf tubes to analyse proteins, lipids, glycogen, and carbohydrates largely following Foray et al. (2012) but adapted to Chironomidae (see Supplementary Material

for further details). Briefly, proteins were solubilized in a phosphate buffer and their content was measured using the Bradford assay. In a second step, chloroform-methanol and sodium sulphate were added to the remaining sample allowing the quantification of glycogen and carbohydrates using an anthrone-based assay while the lipid content was determined utilizing phospho-vanillin reactions. To account for recovery rates of each endpoint, larvae of darkling beetles, i.e., *Zophobas morio* and *Tenebrio molitor*, were measured and compared to their known nutrient contents.

Stable isotope analysis

The opisthosomas of *T. extensa* were separated from the body and analysed for their stable isotope signature. Spiders' opisthosoma and prosoma show significant differences in their isotopic signatures (Wieczorek et al., 2015) due to differences in their elemental turnover rate. The focus on opisthosomas is justified by the relatively fast turnover rate (i.e., 8 days) compared to other body parts which reflects the resources sampled 1-2 weeks before the spiders (Belivanov and Hambäck, 2015). The resources were selected based on abundance and relevance for *T. extensa* as a web-building spider. Thus, Chironomidae, Baetidae, and Coenagrionidae were selected as aquatic prey. As terrestrial prey, Auchenorrhyncha, Sternorrhyncha (Aphidina, Psyllina), and Lepidoptera as herbivores, Nabidae, and Linyphiidae as carnivores, and Collembola as detritivores were chosen due to their size and their ability of jumping, flying or ballooning – all traits that allow the species to be caught by *T. extensa*. All samples, except *T. extensa* and Coenagrionidae, consisted of several individuals due to the low body weight of individuals.

All samples were dried for at least 48 h at 60 °C and ground to fine powder as detailed above for the energy reserves. For each sample and replicate, a mass of 0.6 mg (\pm 0.1 mg) was packed in tin capsules (5 mm \times 9 mm, IVA, Meerbusch, Germany) using an ultrafine balance (Sartorius, Goettingen, Germany) with an accuracy of 0.1 μ g. Elemental contents and isotope ratios of C and N were measured by an elemental analyzer (EA, Flash 2000 HT, Thermo Scientific, Bremen, Germany) coupled to an isotope ratio mass spectrometer (IRMS, Delta V Advantage, Thermo Scientific, Bremen, Germany). Values are reported in the delta notation:

$$\delta X = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \cdot 1000\text{‰} \quad \text{Eq. 1}$$

where δX is either $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$, and R_{sample} and R_{standard} are the abundance ratios of the heavy to the light isotope of the sample and the international standard, respectively (Vienna Pee Dee Belemnite for C, atmospheric air for N). A working standard (i.e., casein) was measured in duplicate every ten samples with a precision of $<0.06\text{ ‰}$.

Calculations and statistics

The contents of each of the four nutrients were calculated from the respective calibration curves. To investigate the influence of Bti on the different nutrients and their dynamics, autoregressive mixed effect models (LME) were generated with FPM as random effects to account for repeated measurements and autocorrelation. Treatment, time period and their interaction were included as explanatory variables. In order to identify statistically significant differences among the factor levels, analyses of variance (ANOVAs) were used with an alpha level set at 0.05. All variables were checked for normality and homoscedasticity.

Prior to further use in stable isotope mixing models, terrestrial sources were grouped by k-means clustering with the optimal number of clusters being determined based on within sum of squares. This step was done to reduce the number of sources included in the mixing models and thus lower their underdetermination (Phillips et al., 2014). The proportions of aquatic and terrestrial resources in the diet of *T. extensa* were estimated with Bayesian mixing models for each FPM applying a generalist prior (i.e., $\alpha = 1$) using the R package “MixSIAR” (chainLength = 300,000, burn = 200,000, thin = 100, chains = 3, resid_err = TRUE, process_err = TRUE, version 3.1.12, Stock et al., 2018). Since stable isotope signatures are systematically increasing through the food web (i.e., trophic enrichment), resources were adjusted by $0.5 \pm 0.19\text{ ‰}$ for $\delta^{13}\text{C}$ and $2.3 \pm 0.24\text{ ‰}$ for $\delta^{15}\text{N}$ (McCutchan Jr et al., 2003). Niche sizes were assessed as Bayesian standard ellipse area (SEA) using the R package “SIBER” (Version 2.1.6, Jackson et al., 2011) and normalized to the SEA of resources for comparability between treatments. The trophic position of the spiders was estimated with a two-baseline model using the R package “tRophicPosition” (Version 0.8.0, Quezada-Romegialli et al., 2022) with Baetidae and Aphidina as baseline organisms for the aquatic and terrestrial habitat, respectively, and the same trophic enrichment factors as specified above. All calculations, statistics, and data visualizations were conducted with R (4.2.1, R Core Team, 2022) using the packages “ggplot2” (Wickham, 2016), “ggpubr” (Kassambara, 2020), and “nlme” (Pinheiro et al., 2021).

Results & discussion

Quality of aquatic subsidy: Nutrient composition

The composition of macronutrients (i.e., protein, lipids, carbohydrates) and the proportion of glycogen were not affected by the Bti treatment relative to the control (Table 1, Fig. S6). These parameters changed over the course of the study, however with no statistically significant interaction with Bti (Table 1, Fig. 1). While the variations in the macronutrients did not follow a consistent pattern over time (Fig. 1a), average glycogen content tended to decrease when progressing from spring to summer (Fig. 1b). This decrease in the glycogen content might be linked to increasing temperatures as this energy reserve is known to be mobilized under temperature stress to produce cell-protecting disaccharides (Watanabe et al., 2002). Nevertheless, the temporal patterns showed no obvious link to the shift in the emergence dynamics of Chironomidae from Bti-treated FPMs, that is an earlier and reduced emergence (Kolbensschlag et al., 2023). This is in contrast to our hypothesis, which was built on the assumption that Bti reduced the number of sensitive chironomid larvae leading to increased resource availability for surviving chironomids. Ultimately, accelerated larval development could explain the documented earlier emergence (Kolbensschlag et al., 2023) which should also be reflected in the nutrient composition of adults. Recolonization of the FPMs via ovipositing multivoltine species during the study could have partly mitigated any Bti-induced effect. To further understand the discrepancy between the observation and our hypothesis, we need to recognize that insect larvae have to meet size checkpoints to enter metamorphosis (Nijhout, 1975). More specifically, reaching the *minimal viable weight* assures sufficient nutrient storage in larvae to survive metamorphosis while the *critical weight* influences the adult size and can be affected by environmental conditions (Davidowitz et al., 2003; Mirth and Riddiford, 2007). Against this background, we conclude that the presumed increased resource availability in Bti-treated FPMs indeed promoted an earlier accomplishment of metamorphosis checkpoints supported by the earlier emergence (Kolbensschlag et al., 2023). However, this might not necessarily change the critical weight and thus does not mean that substantially more macronutrients and glycogen are accumulated during the larval development as reflected in our data. Alternatively, metamorphosis is an energetically costly process (Arrese and Soulages, 2010), which might have masked any energetic advantage chironomids might have acquired during their aquatic life stage in the Bti-treated systems. Considering that the few studies on the nutritional value of adult dipterans suggest lower contents with

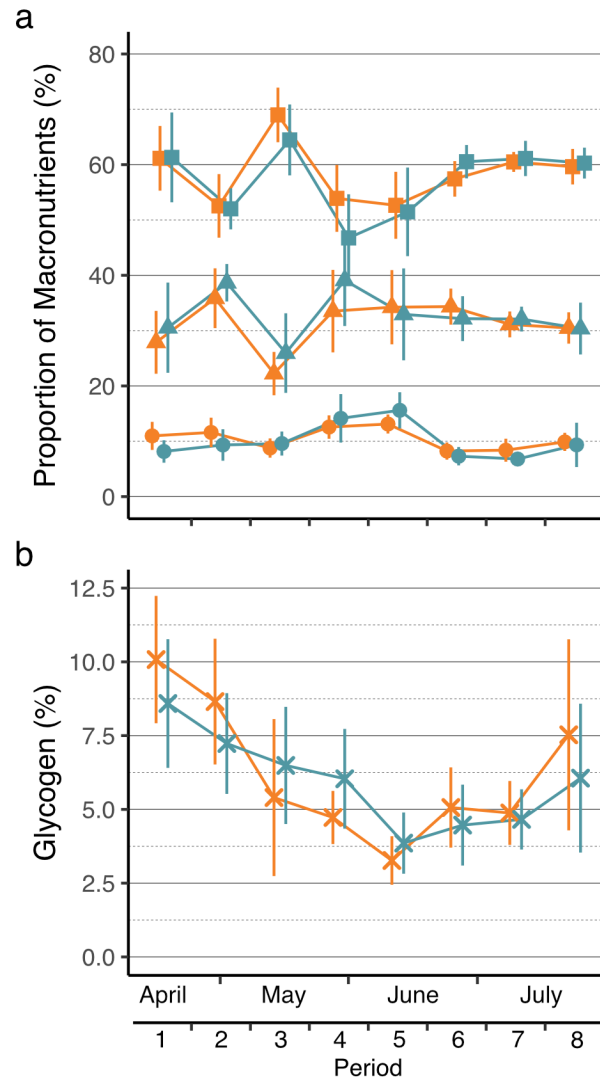


Fig. 1: a) Mean (\pm 95 % CI) composition of macronutrients (\bullet carbohydrates, \blacktriangle lipids, \blacksquare proteins) and b) mean (\pm 95 % CI) proportion of glycogen in Chironomidae from control (cyan; $n = 6$) and Bti-treated (orange; $n = 6$) FPMs between April and July, measured in eight time periods. Bti was applied three times during April and May. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increasing larval density (Morimoto et al., 2019; Nguyen et al., 2019; Takken et al., 2013), we propose targeted studies on the nutrient content of larval and adult Chironomidae reared at different larval densities to elucidate the mechanism behind our observations. Additionally, analysing the nutrient content of the collected chironomids grouped for their feeding habits (i.e., filter-feeding or predatory) could help to develop a more sophisticated understanding of the Bti-induced changes in this ecologically highly relevant family.

Though not obviously linked to the Bti treatment, fluctuations in macronutrient and glycogen levels over time could be relevant for arachnid consumers (Wilder, 2011; Wilder et al., 2010). While herbivores and omnivores possess a selective nutrient intake

to maintain nutritional homoeostasis (Persson et al., 2010), carnivores (e.g., spiders) seem to be more heterogeneous in their ability to respond to variations in the nutrient composition (Mayntz et al., 2005). Wandering spiders such as wolf spiders or jumping spiders could behaviourally and physiologically adapt to varying nutrient content in prey (Jensen et al., 2011; Koemel et al., 2019; Toft et al., 2010). In contrast, web-building spiders, to which *T. extensa* belongs, showed limited regulation of their nutrient intake based on their prey (Hawley et al., 2014). This may be related to regular limitations in prey amount and quality over the year. Due to their web-based foraging, *T. extensa* fully depend on insects caught in their webs presumably leading to an opportunistic and less selective feeding behaviour compared to free-hunting spiders (Hawley et al., 2014; Jensen et al., 2011). Consequently, if the consumed prey does not match spiders' nutritional demands, their fitness and performance may be negatively affected (Mayntz and Toft, 2001; Wilder, 2011). Vice versa, high-nutrient prey is expected to increase survival, reproductive success as well as the tolerance towards toxic substances and to accelerate juvenile growth (Mayntz and Toft, 2001). Thus, the observed temporal fluctuations in macronutrient and glycogen levels of aquatic insects and their relevance for terrestrial predators should be addressed further – particularly in situations in which disturbances in aquatic systems are affecting besides the quantity also the quality of this subsidy (Pietz et al., 2023).

Table 1: Treatment, time, and interaction effects on contents of glycogen, lipid, protein, and carbohydrates analysing the variance (ANOVA) of the respective mixed effect models. Significant p-values are printed in bold.

Component	Factor	numDF	denDF	F-value	p-value
Glycogen	Treatment	1	10	0.1764	0.6834
	Time	7	69	12.2092	<0.0001
	Treatment:Time	7	69	1.0871	0.3811
Lipid	Treatment	1	10	0.0459	0.8347
	Time	7	70	6.9741	<0.0001
	Treatment:Time	7	70	0.7090	0.6645
Protein	Treatment	1	10	2.4393	0.1494
	Time	7	70	40.5383	<0.0001
	Treatment:Time	7	70	0.9324	0.4873
Carbohydrates	Treatment	1	10	3.2538	0.1014
	Time	7	70	6.3603	<0.0001
	Treatment:Time	7	70	1.3311	0.2488

3.2 Spider diet

The structures of the analysed food webs were comparable between Bti-treated and control FPMs in terms of raw stable isotope signatures of C and N, and the size of the resource polygons (Fig. 2a; Bayesian $SEA_{Bti} = 9.07$ (7.58-10.94) ‰², Bayesian $SEA_{Ctrl} = 9.04$ (7.52-10.98) ‰², median with 95 % equal-tail interval). Aquatic and terrestrial prey were well separated with higher $\delta^{13}C$ values in the latter allowing for a reasonable distinction between resources of different origins (Fig. 2a). In accordance with literature (Akamatsu et al., 2004; Krell et al., 2015), aquatic insects were an important resource for *T. extensa*, contributing approximately 50 % to their diet (Fig. 2b). Based on several studies reporting a negative impact of Bti on Chironomidae (reviewed in Brühl et al., 2020), which is with around 90 % the largest contributor to aquatic subsidy in the FPMs, a lower abundance of Chironomidae was expected, resulting in increased consumption of

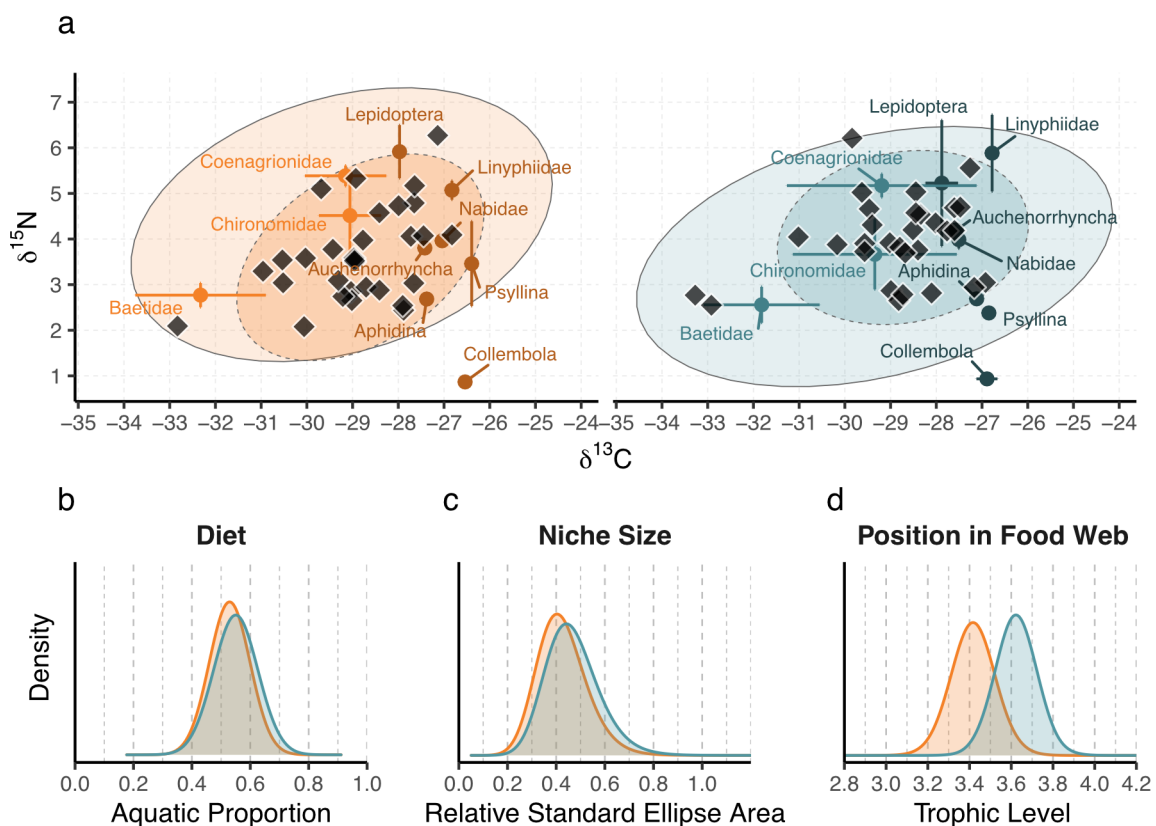


Fig. 2: a) 95 %-confidence ellipse of *T. extensa* (dark shade) determined from the stable isotope ratios of C and N (black diamonds) and 95 %-confidence ellipse (bright shade) of resources adjusted for trophic enrichment, for Bti-treated (orange) and control (cyan) FPMs ($n = 6$). Means \pm SD of aquatic and terrestrial prey organisms ($n = 5$) are plotted as bright and dark dots, respectively. b) aquatic proportion in diet of *T. extensa*, c) niche size of *T. extensa* determined from relative SEA, and d) the trophic position of *T. extensa*. b), c), and d) show probability densities which are unitless. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

terrestrial prey by spiders. Contrary to this hypothesis, Bti reduced the contribution of aquatic prey to *T. extensa*'s diet by only 2 % (52.9 % (43.6-62.4)) compared to control FPMs (55 % (43.9-66.3)) suggesting only marginal changes in the spiders' diet (Fig. 2b). This relatively low shift may be driven by the varying effect of Bti on the emergence dynamics of aquatic insects (Kolbensschlag et al., 2023). In fact, during the period which is reflected in the spiders' stable isotope signature, chironomid emergence was reduced by only roughly 10 % in the Bti-treated FPMs. Consequently, sampling spiders at a later stage of the experiment, during which the number of emerging chironomids was decreased by up to 50 %, might have yielded a more prominent change in the diet.

Despite the only marginal reduction of aquatic prey in spiders' diet (Fig. 2b), the dietary niche of *T. extensa* (i.e., relative niche size) differed slightly between treatments (Bti = 0.42 (0.28-0.63) ‰², Ctrl = 0.47 (0.31-0.70) ‰²; Fig. 2c) indicating an altered food intake. This was mainly attributed to an expansion of the niche of spiders from Bti-treated FPMs towards lower $\delta^{15}\text{N}$ values compared to controls ($\Delta^{15}\text{N}_{\text{Bti-Ctrl}} = -0.34$ ‰), which strongly suggests a higher consumption of prey lower in $\delta^{15}\text{N}$ than Chironomidae. Such values were observed in most terrestrial prey but also in Baetidae, the second most abundant taxon in aquatic emergence. As ^{15}N accumulates with higher trophic level the stable isotope ratio of N can be used as an indication of food chain length (McCutchan Jr et al., 2003). Chironomidae vary strongly in their feeding habits including also predaceous species (Allan et al., 2021; Armitage et al., 1995) leading to high $\delta^{15}\text{N}$ (Fig. 2a). In contrast, larvae of Baetidae consume algae and organic detritus and are thus lower in $\delta^{15}\text{N}$. The same applies to terrestrial herbivores and detritivores as primary consumers. Consequently, the lower trophic level estimate of *T. extensa* (Fig. 2d) in Bti-treated FPMs (3.42 (3.23-3.61)) compared to controls (3.62 (3.45-3.80)), supports the suggested dietary shift towards an increased feeding on both Baetidae and terrestrial prey (herbivorous or detritivorous). In addition, we measured 0.9 ‰ higher $\delta^{15}\text{N}$ values in Chironomidae from Bti-treated FPMs (Fig. 2a) indicating alterations in the species composition of this family towards more predaceous species. Thus, even in a situation of equal chironomid consumption by spiders of both treatments, the higher $\delta^{15}\text{N}$ in chironomids from Bti-treated FPMs would increase the spiders' trophic level estimate. As we observed the opposite (Fig. 2d), this is additional evidence for the assumption of a dietary replacement of chironomids by Baetidae and terrestrial prey.

Beyond this, we acknowledge that an increased contribution of Baetidae to the diet of spiders from Bti-treated FPMs could cause an underestimation of the proportion of

terrestrial prey and thus explain the only marginal difference in aquatic proportion between treatments (Fig. 2b). More specifically, a higher dietary intake of Baetidae, which were low in $\delta^{13}\text{C}$ (-32.6‰ , $\Delta^{13}\text{C}_{\text{Baetidae-spiders}} = -3.4\text{‰}$), would shift spiders residing at Bti-treated FPMs towards lower $\delta^{13}\text{C}$ values (i.e., to the “aquatic side”; Fig. 2a) compared to control spiders. Conversely, an equally increased consumption of terrestrial prey would rise spiders’ $\delta^{13}\text{C}$ values (i.e., to the “terrestrial side”) to a much lesser extent owing to only slightly higher $\delta^{13}\text{C}$ than *T. extensa* ($\Delta^{13}\text{C}_{\text{Terr. prey-spiders}} = 1.7\text{‰}$). Since $\delta^{13}\text{C}$ values of spiders did not differ between treatments, a higher consumption of terrestrial prey by spiders from Bti-treated FPMs might be masked by the increased contribution of Baetidae leading to artificially obscured treatment effects on the dietary proportions of prey origins. This could also be partly attributed to a slightly increased relative density of Baetidae in aquatic subsidy (Factor of 1.13) since they did, in contrast to Chironomidae, not experience Bti-induced reductions. As mentioned above, prey availability mainly determines the diet of web-building spiders as opportunistic feeders (Kato et al., 2003; Nakano and Murakami, 2001).

Since we could only partially detect the mentioned potential changes in the diet using mixing models, the inclusion of additional tracers (e.g., fatty acids; Twining et al., 2020) or DNA-based analyses of the spiders’ diet (Piñol et al., 2014) could shed light on the underlying processes. By providing more details or a better separation of the resources in complex food webs the above-discussed potential underestimation of dietary shifts could be resolved and our understanding of the ecological interactions might be increased (Pacioglu et al., 2019; Stock et al., 2018).

Despite that dietary shifts may have been masked in mixing models, even small reductions in the aquatic proportion of spiders’ diet induced by anthropogenic disturbances hold the potential for relevant ecological consequences. A shift in consumed prey can also involve a change in the available nutrients for the predator (Toft, 2013). In fact, the nutrient content varies depending on the insect order, the mating system and the dispersal strategy (Gerber et al., 2022), which may affect spider performance including the web quality (Blamires et al., 2009) or the reproductive success (Salomon et al., 2008), parameters especially related to the availability of proteins and lipids, respectively. Such an impact on the individual level may, in the long run, have consequences for the development of spider populations: densities of web-building riparian spiders usually decrease with altered or reduced aquatic subsidy (Kato et al., 2003; Marczak and

Richardson, 2007; Paetzold et al., 2011) which could lead to propagating effects on consumers feeding on spiders (Recalde et al., 2020).

Conclusion

The present study points towards limited changes in the nutrient transfer from aquatic to terrestrial ecosystems via adult chironomids as a consequence of Bti application. However, extending the analyses by, partly essential, amino and fatty acids could be a valuable amendment to increase the understanding of changes in the aquatic-terrestrial meta-ecosystem. Despite no detectable effects of Bti on the nutrient content of aquatic subsidy, consequences of Bti for riparian spiders are suggested in terms of an altered composition of their diet. While the observed change in the proportion of aquatic prey was rather low due to a potential underestimation, the analyses of the trophic position indicated shifts to alternative prey which suggest ecologically relevant consequences. Altered performance and density of spider populations could propagate to higher as well as lower trophic levels due to their linking role in terrestrial food webs functioning as both consumers and prey.

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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.

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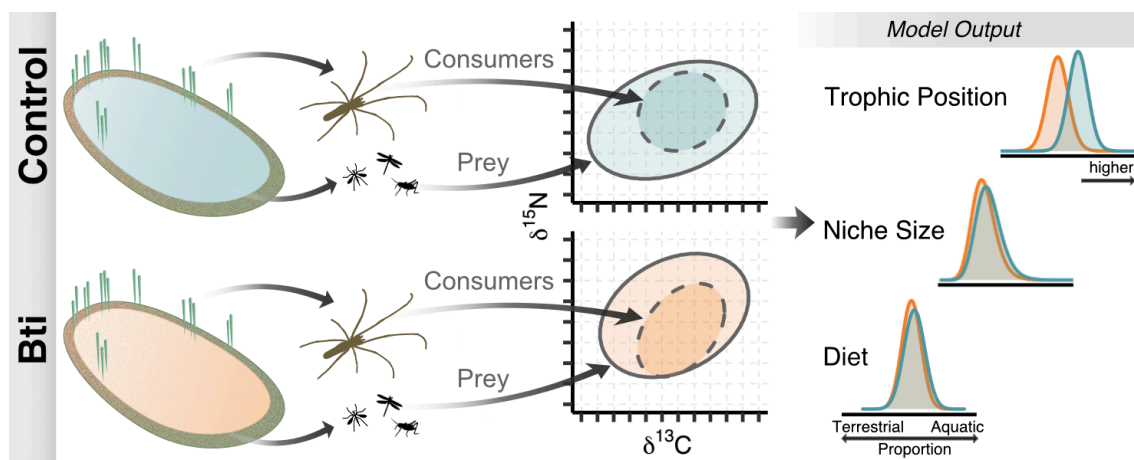
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Graphical Abstract



Supplementary Material – Appendix A.2

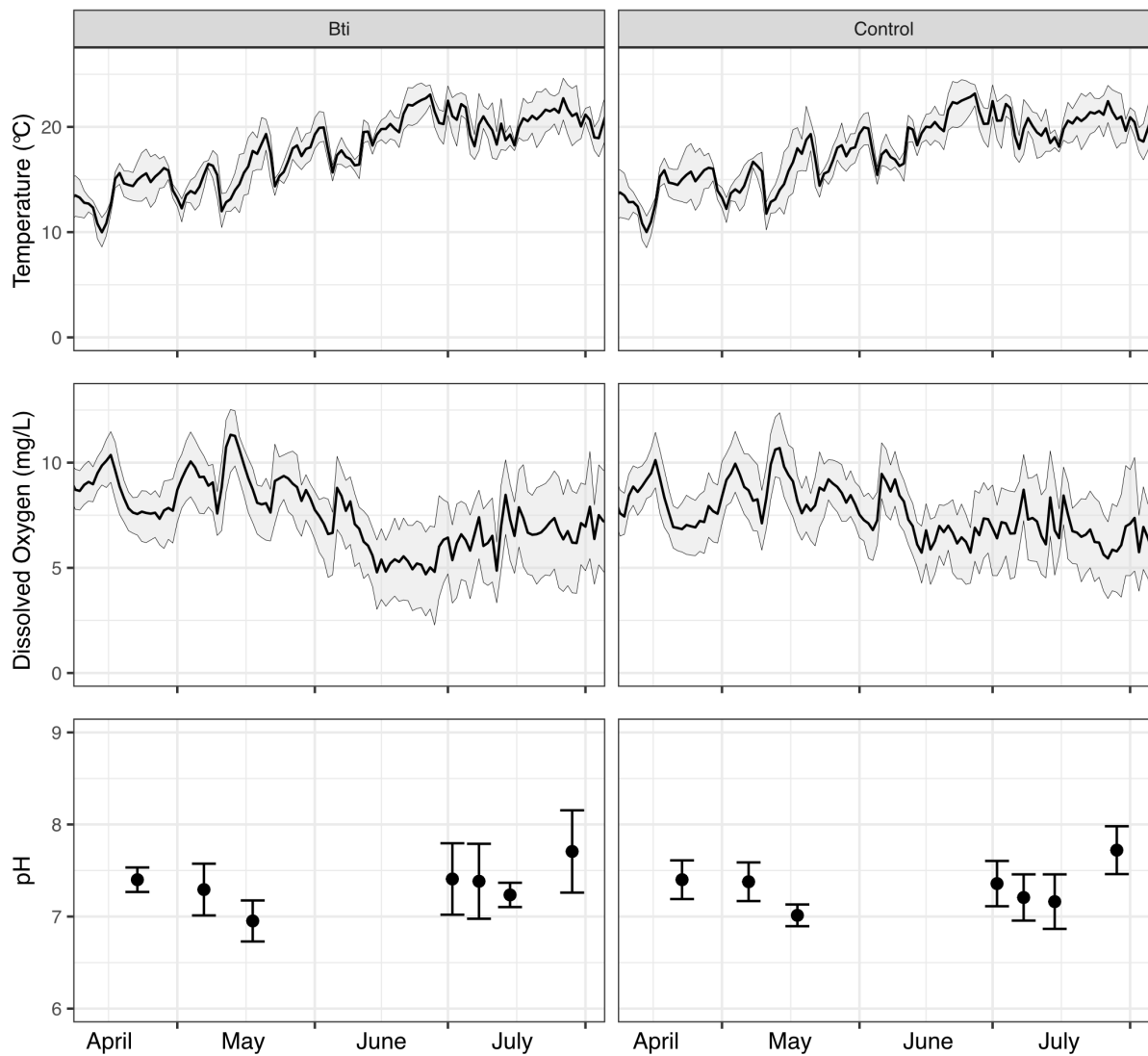


Fig. S1: Environmental parameters measured from April to July in all FPMs. (Figure adapted from Kolbenschlager et al. (2023) as both studies were conducted in parallel.)



Fig. S2: Picture of the FPM system with color-indicated allocation to control (cyan) or Bti (orange) treatment.

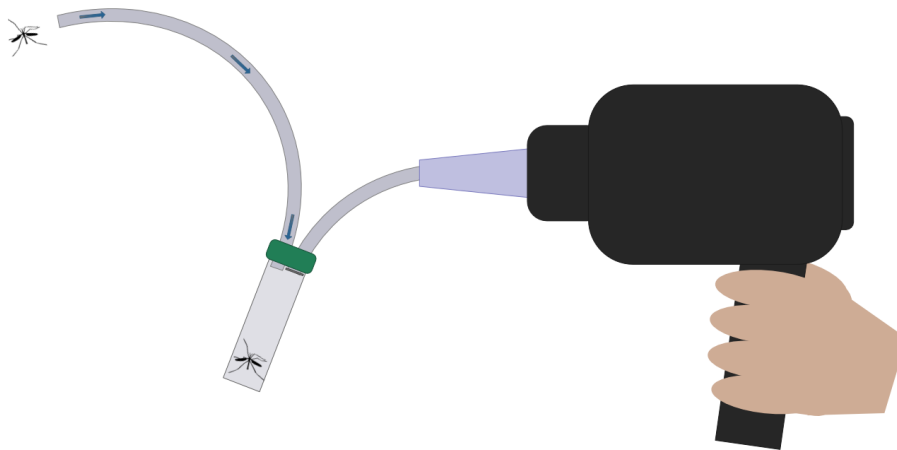


Fig. S3: Self-constructed hand-held suction sampler built from a portable vacuum cleaner, a 10-mL pipette tip, plastic tubes and a 50-mL falcon tube.

Adapted method for nutrient analyses following (Foray et al., 2012)

Preparation and protein content

Every sample of insect powder was homogenized with 180 μL lysis buffer and one stainless-steel ball in a mixer mill (MM 301, Retsch) for 30 seconds and afterwards centrifuged for 15 min at 4 $^{\circ}\text{C}$ and 400 g. Two times 2.5 μL of supernatant were transferred into a 96-well microplate and 250 μL of Bradford reagent (B6916, Sigma-Aldrich) were added to each well. After 25 min of incubation at room temperature, the plate was shaken for 3 sec at 10 Hz. Absorption was determined at 595 nm using a microplate reader (Infinite 200, Tecan).

The removed volume of supernatant was replaced by 5 μL buffer. After adding 20 μL 20% sodium sulphate solution and 1000 μL chloroform-methanol (1:2, v:v) each sample was vortexed and centrifuged as detailed above. The entire supernatant was transferred into a new 2-mL Eppendorf tube for the subsequent analyses of carbohydrates and lipids. The pellet was washed with 400 μL 80% methanol by vortexing and centrifuging for 5 min at 16,000 g and 4 $^{\circ}\text{C}$. After discarding the supernatant, the pellet was stored with another 400 μL 80% methanol at -20 $^{\circ}\text{C}$ until analyses of glycogen.

Carbohydrates

Two times 150 μL of supernatant were transferred into a 96-well quartz plate (730.009-QG, Hellma Analytics). The plate was placed in an oven at 40 $^{\circ}\text{C}$ for 1.5 h to evaporate the sample in a water bath. To each well, 240 μL anthrone reagent (1.42 g/L in 70% sulphuric acid) were added. After 15 min of incubation at room temperature, the plate was covered with a sealing film and placed in a water bath at 90 $^{\circ}\text{C}$ for 15 min. The reaction was then

stopped on ice and the absorbance was measured at 625 nm (Infinite 200, Tecan).

Lipids

Two times 100 μ L of supernatant were transferred into a 96-well quartz plate (730.009-QG, Hellma Analytics). The plate was placed in an oven at 90 °C for 30 min to evaporate the entire sample. Subsequently, 10 μ L 98% sulphuric acid were added to each well and the plate was incubated in a water bath at 90 °C for 2 min. After cooling down on ice, 190 μ L vanillin reagent (1.2 g/L in 68% ortho-phosphoric acid) were added to each well, the plate was shaken for 30 sec and incubated for 20 min at room temperature. After an additional shaking of 30 sec, absorbance was measured at 525 nm (Infinite 200, Tecan).

Glycogen

The pellets, stored in 80% methanol, were vortexed and centrifuged for 5 min at 4 °C and 16,000 g. After discarding the supernatant, 1.5 mL anthrone reagent were added to each sample, vortexed and incubated for 15 min in a water bath at 90 °C. The reaction was stopped on ice and the samples were filtered through low-protein binding membranes (Chromafil Xtra PVDF-45/13, Macherey-Nagel). To dilute the samples 1:2, 350 μ L were transferred in a new tube and 350 μ L anthrone reagent were added. The samples were again incubated for 15 min at 90 °C in a water bath and cooled down on ice. After transferring two times 250 μ L into a 96-well microplate, the absorbance was measured at 625 nm (Infinite 200, Tecan).

Standards

For each of the four energy reserves, a standard series as indicated in Table S4 was prepared and measured in duplicates with the samples of each plate.

Table S4: Standards for the calibration curves given in μ g mL⁻¹ for each endpoint with the R² in %.

Endpoint	Standard	Solvent	Concentrations in μg mL⁻¹	R² in %
Protein	Bovine serum albumin	Buffer	2500, 1500, 900, 540, 320, 200, 0	99.0 – 99.1
Carbohydrates	Glucose	Chloroform-methanol (1:2)	60, 45, 33.75, 25.31, 18.98, 14.24, 0	97.3 – 97.8
Lipids	Triolein	Chloroform-methanol (1:2)	400, 200, 100, 50, 25, 12.5, 0	90.3 – 97.3
Glycogen	Glucose	25% ethanol	480, 240, 120, 60, 30, 15, 0	99.5 – 99.7

Table S5: Information about the time periods according to which Chironomidae were pooled for analyses of nutrients.

Time interval	Period	Days	Sampling dates
1	April 14 – April 25	12	April 18, April 25
2	April 26 – May 7	12	April 30, May 4, May 7
3	May 8 – May 21	14	May 12, May 15, May 18, May 21
4	May 22 – June 4	14	May 25, May 28, June 1, June 4
5	June 5 – June 18	14	June 7, June 11, June 15, June 18
6	June 19 – July 2	14	June 25, July 2
7	July 3 – July 16	14	July 9, July 16
8	July 17 – July 30	14	July 23, July 30

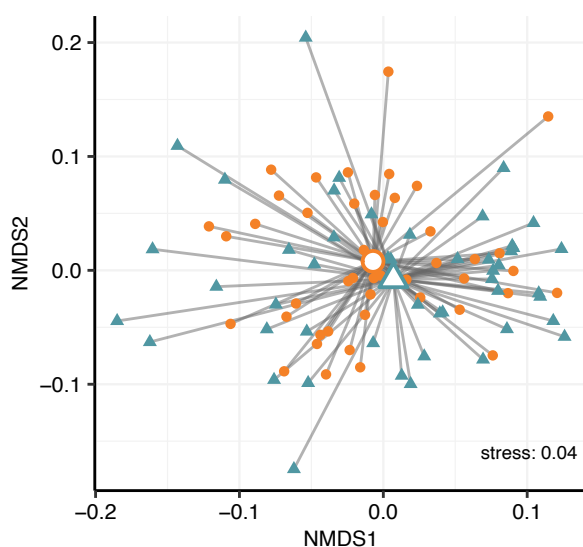


Fig. S6: Non-metric multidimensional scaling (NMDS) ordination for the composition of macronutrients and glycogen in Chironomidae from control (cyan triangles; $n = 6$) and Bti-treated FPMs (orange dots; $n = 6$) during the study duration from April to July. The stress values are stated as a “goodness-of-fit” measure, indicating a good ordination for values below 0.1 (Clarke, 1993).

Supplementary Material – References

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Appendix A.3

Long-term exposure to Bti alters the sensitivity of *Chironomus riparius* populations

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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, *Chironomus riparius*, a species of Chironomidae (Diptera), was selected as model organism. This selection is motivated by its high abundance and its role as high-quality 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. Ultimately, the results suggest small adaptation on population level. However, adaptation might also happen at community level with more tolerant species prevailing. (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.

Keywords:

Chironomidae, *Bacillus thuringiensis* var. *israelensis*, emergence, adaptation, energy reserves

Abbreviations:

- Bti *Bacillus thuringiensis* var. *israelensis*
- ST sensitivity test

Introduction

Freshwater systems are closely coupled with adjacent riparian zones (Bartels et al., 2012; Soinen 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: 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). However, already during their larval life stages (Armitage et al., 1995) chironomids may be subjected to aquatic disturbances affecting their development (Kraus et al., 2020).

These disturbances can be of anthropogenic origin such as the mosquito control agent *Bacillus thuringiensis* var. *israelensis* (Bti). 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 quantity, dynamics and quality (Allgeier et al., 2019; Boisvert and Boisvert, 2000; Bordalo et al., 2020; Brühl et al., 2020; Kolbensschlag 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). Even if applied sterilized (Becker, 2002) 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 aquatic organisms were mostly conducted under field conditions or by employing mesocosms (Allgeier et al., 2019; Kolbensschlag et al., 2023; Lundström et al., 2010; Theissing et al., 2019; Vinnersten et al., 2010). However, studies specifically assessing alterations in population tolerance towards Bti, particularly in terms of adaptation (Bundschuh et al., 2023), 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 nutrient (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 consequence to (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 presumable higher than that of chronically exposed and potentially adapted populations.

Materials and methods

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, individuals from other laboratories (i.e., BASF SE (Ludwigshafen, Germany), ECT Oekotoxicology GmbH (Flörsheim, Germany) and SBiK-F/LOEWE-TBG (Frankfurt a.M., Germany)) were introduced to 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 (≤ 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.

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 x 35 x 50 cm, L x H x W, mesh size: 0.6 mm) with two test vessels (32 x 7 x 22 cm). Each vessel contained 1.1 kg (wet weight) standardized sediment (dry weight: 75% sand, 20% clay, 5% peat; 40% water, 0.1% CaCO₃) 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 first instar larvae were introduced into the vessels of each 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).

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

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).

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, 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).

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 protein and lipid contents were calculated from the respective calibration curves. 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 done 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, an ANOVA was performed on each Bti × food source scenario of each endpoint 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).

Results

Quantitative perspective

Emergence success

In ST 1, increasing Bti doses significantly decreased the number of emerged chironomids (ANOVA, $p < 0.001$; Fig. 1, 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.1, 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).

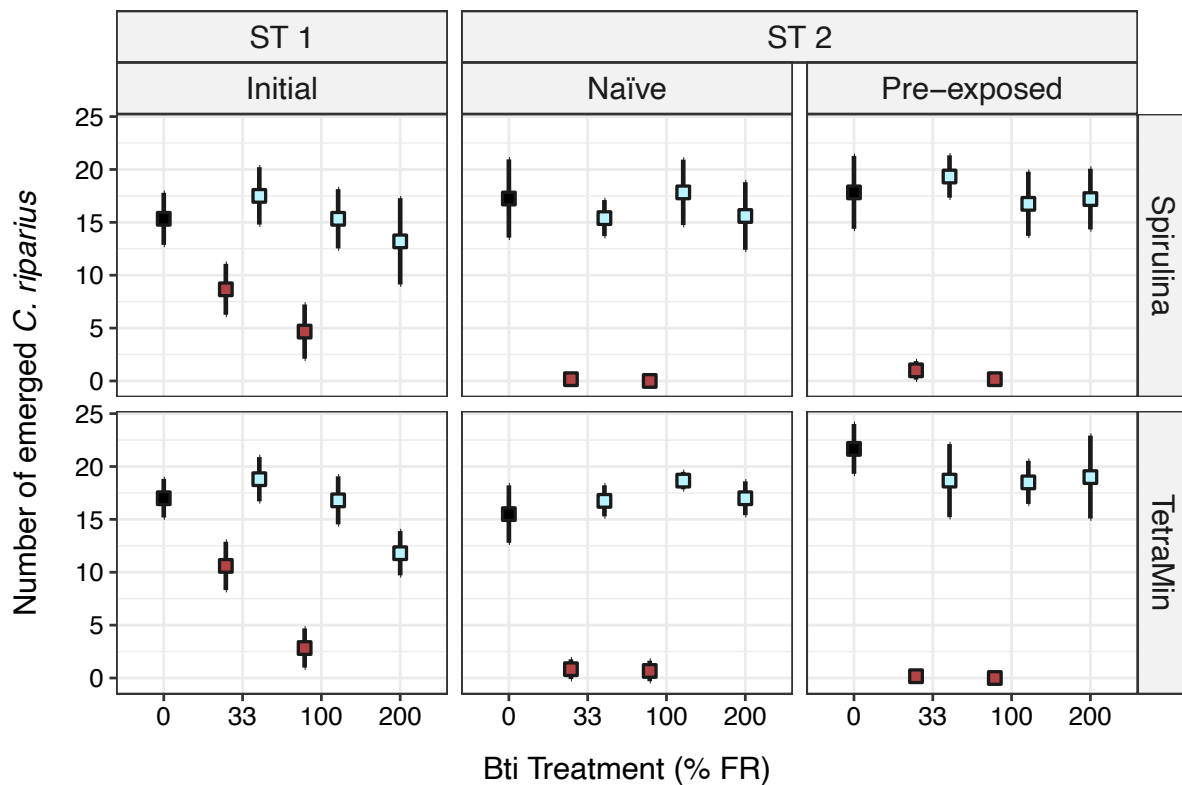


Fig. 1: Mean number of emerged *C. riparius* per treatment with 95% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four doses expressed as % field rate (FR) at two time points, i.e., Day 0 (red) 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).

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. 2, 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. 2, 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. 2, bottom). When Spirulina was provided as food (Fig. 2, 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 by 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).

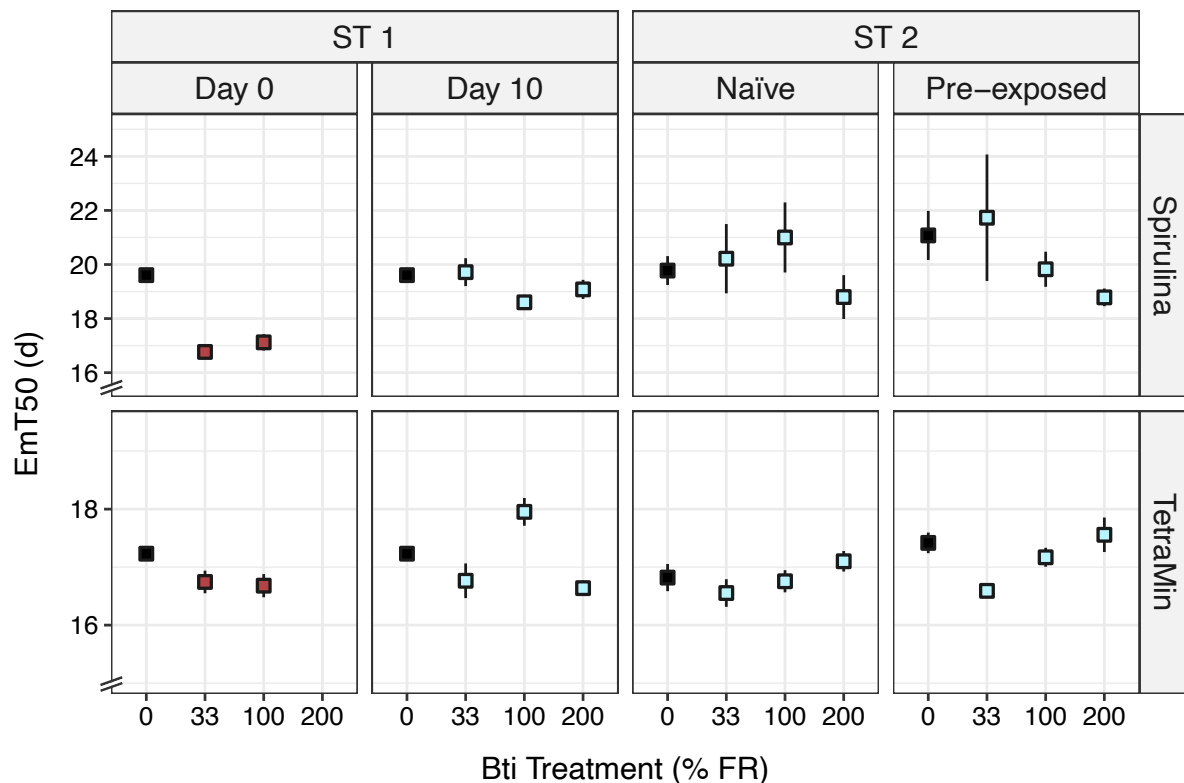


Fig. 2: Mean emergence time (EmT50 in days) of emerged *C. riparius* per treatment with 99% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four doses expressed as % field rate (FR) at two time points, 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 population 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 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) \times 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

Reproductive potential

Over all treatments, females emerged later than males with the difference ranging from 1 to 6 days (EmT50; Fig. S3). 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. 3). In ST 1, the application day of Bti had a significant influence on the reproduction rate (ANOVA, $p = 0.022$) independent of the food source. Bti applications on day 0 reduced the reproduction rate by 63%, while applications on day 10 led to 14.7% lower rates. In ST 2, effects of Bti application were not significantly affected by exposure history (ANOVA, $p = 0.945$) nor food source (ANOVA, $p = 0.674$).

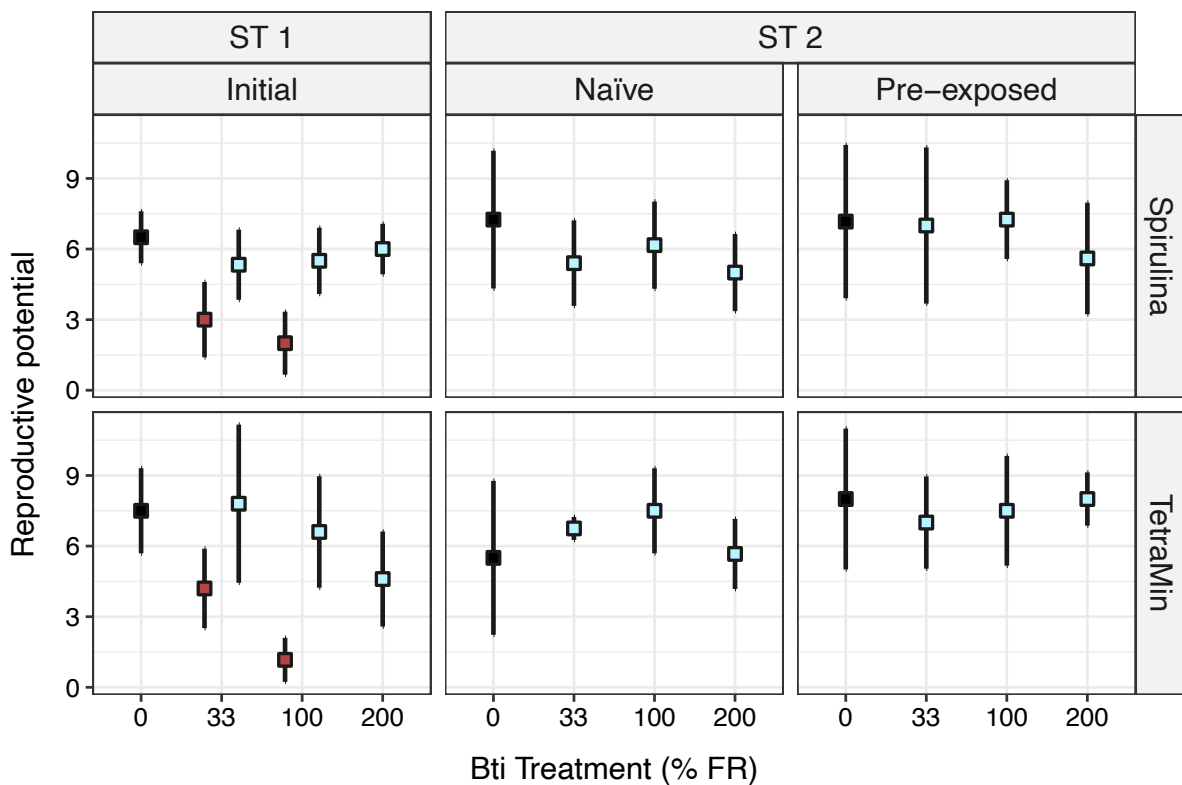


Fig. 3: Mean reproductive potential (maximum possible number of fertilized females) with 95% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four doses expressed as % field rate (FR) at two time 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.

Qualitative perspective

Dry weight of emerged individuals

The dry weight of chironomids that emerged from replicates fed with Spirulina, i.e., 0.9 mg \pm 0.06 mg, was significantly lower (-22%; ANOVA, $p < 0.001$) than the dry weight of TetraMin-fed individuals, i.e., 1.14 mg \pm 0.08 mg (Fig. S4). Females were approximately twice as heavy, i.e., 1.4 mg (\pm 0.05 mg), compared to males, i.e., 0.66 mg (\pm 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.

Protein content

In ST 1, the effect of Bti treatments differed significantly between the two food sources (ANOVA, $p = 0.01$; Fig. 4, left). When fed with TetraMin, Bti treatments caused 13.1% (\pm 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% (\pm 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. 4, 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% (\pm 4.6%) and 23.3% (\pm 3.5%), respectively. In contrast, pre-exposed chironomids showed the strongest reduction in the medium Bti treatment (i.e., 100% FR) with 22.7% (\pm 10.2% Spirulina) and 29.3% (\pm 9%, TetraMin) lower protein contents and a less pronounced reduction in the highest treatment (i.e., 200% FR), i.e., -6.6% (\pm 9.4%, Spirulina) and -10.3% (\pm 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 ($p_{\text{adj}} = 0.043$; Table 1).

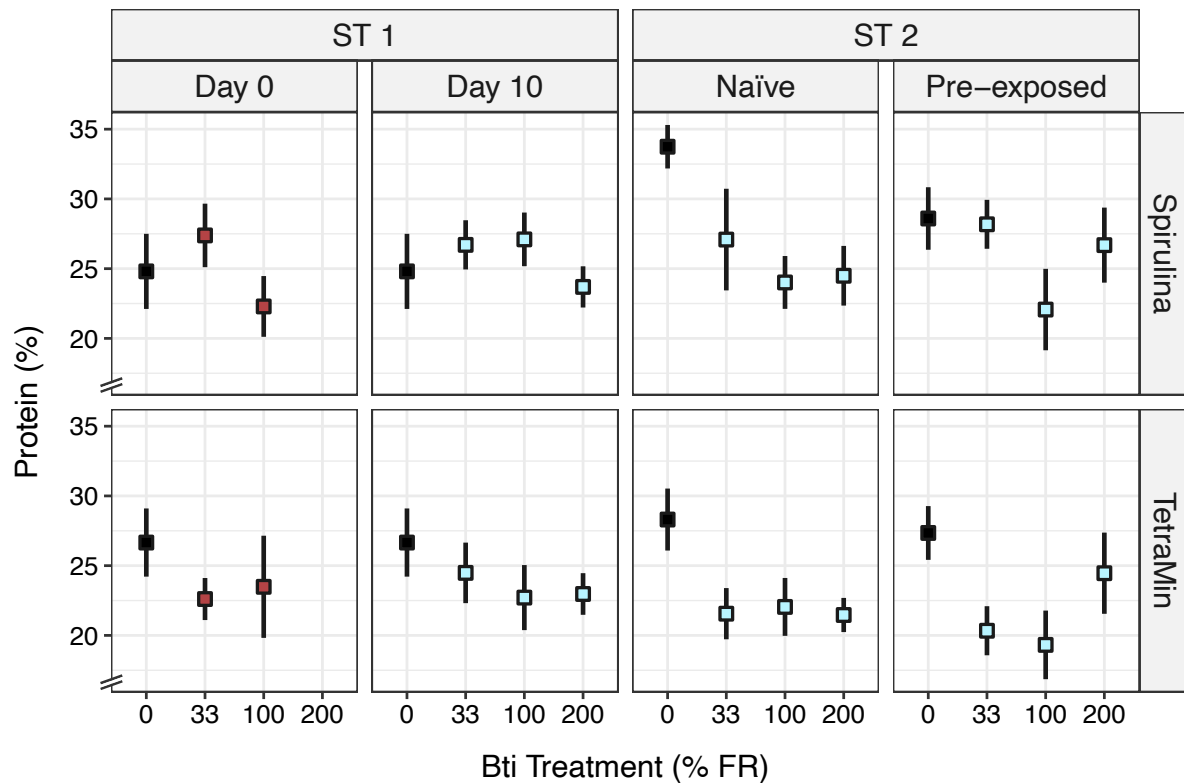


Fig. 4: Mean proportion of protein (%) in emerged *C. riparius* per treatment with 95% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four doses expressed as % field rate (FR) at two time points, 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.

Lipid content

Overall, the lipid content was significantly higher in individuals fed with TetraMin, i.e., 10.2% lipid ($\pm 0.3\%$), than with Spirulina, i.e., 8.7% lipid ($\pm 0.3\%$; Fig. 5; 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 ($-21.2\% \pm 4.2\%$) and TetraMin-fed chironomids ($-21.1\% \pm 5.1\%$), 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 = $-40.3\% \pm 5.2\%$ and TetraMin = $-45.2\% \pm 4.2\%$). In

chironomids of pre-exposed origin, the lipid content was equally reduced in all Bti treatments (Spirulina = $-20.6\% \pm 5.1\%$, TetraMin = $-17.6\% \pm 4.6\%$). 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 ($p_{\text{adj}} = 0.002$; Table 1). With TetraMin as food source, the interaction of exposure history and Bti was significant for 100% FR ($p_{\text{adj}} < 0.001$) and 200% FR ($p_{\text{adj}} = 0.006$).

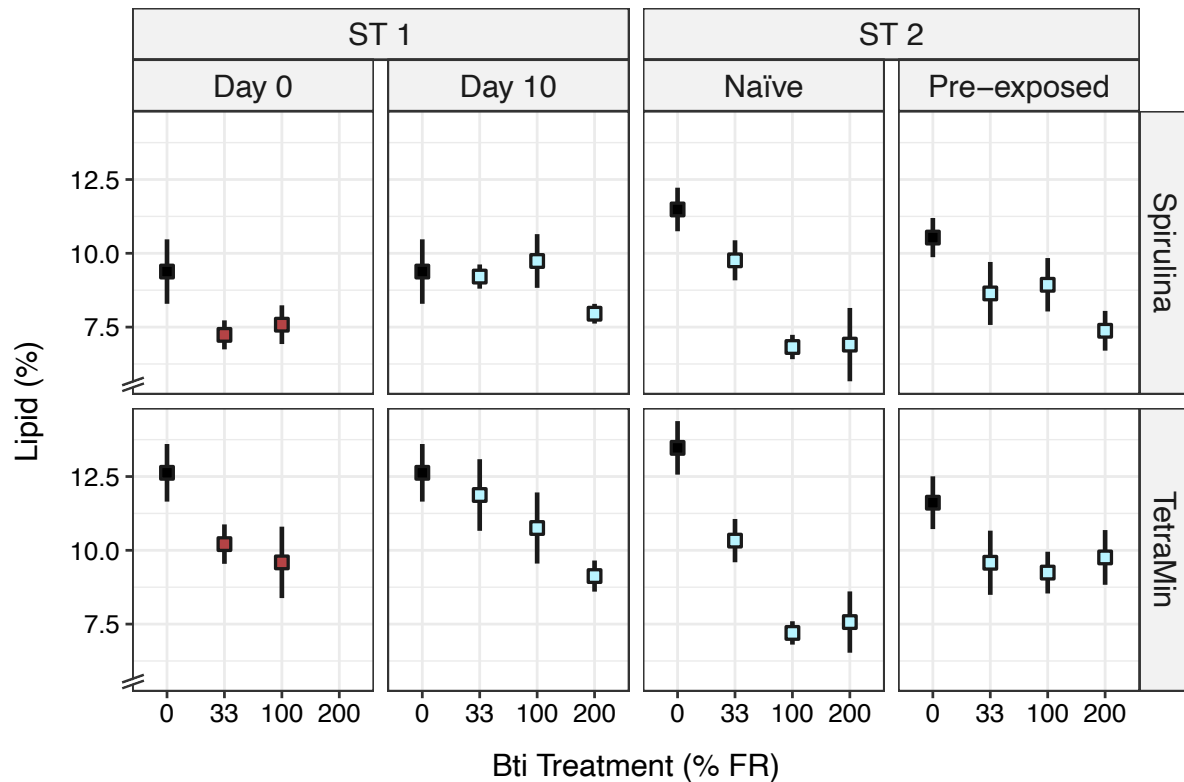


Fig. 5: Mean proportion of lipid (%) in emerged *C. riparius* per treatment with 95% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four doses expressed as % field rate (FR) at two time points, 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.

Discussion

Quantitative perspective – Population

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), the less energy can be acquired from a food source, the 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. 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 dipterans, 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 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. 1; 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).

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 x 2 food sources x 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 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, further analyses of the genetic information of pre-exposed and naïve populations could help to understand the underlying processes.

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 supplier 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 profile is 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, adaptation processes as a consequence of repeated Bti treatments that lead to chironomid populations with less affected nutrient contents, could be beneficial 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 (Kolbensschlag 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).

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.

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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.

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Supplementary Material – Appendix A.3

Table S1: Bti treatments (dose in % field rate (FR) and ITU/L combined with different application times), food resource and exposure history (origin) tested in sensitivity test (ST) 1 and 2. Each factor combination was tested in six replicates.

Included in	Origin	Food	Dose (% FR)	Dose (ITU/L)	Day of application
ST1, ST2	Naïve	TetraMin	0	0	-
			33	480	Day 0
			100	1440	Day 0
			33	480	Day 10
			100	1440	Day 10
			200	2880	Day 10
ST1, ST2	Naïve	Spirulina	0	0	-
			33	480	Day 0
			100	1440	Day 0
			33	480	Day 10
			100	1440	Day 10
			200	2880	Day 10
ST2	Pre-exposed	TetraMin	0	0	-
			33	480	Day 0
			100	1440	Day 0
			33	480	Day 10
			100	1440	Day 10
			200	2880	Day 10
ST2	Pre-exposed	Spirulina	0	0	-
			33	480	Day 0
			100	1440	Day 0
			33	480	Day 10
			100	1440	Day 10
			200	2880	Day 10

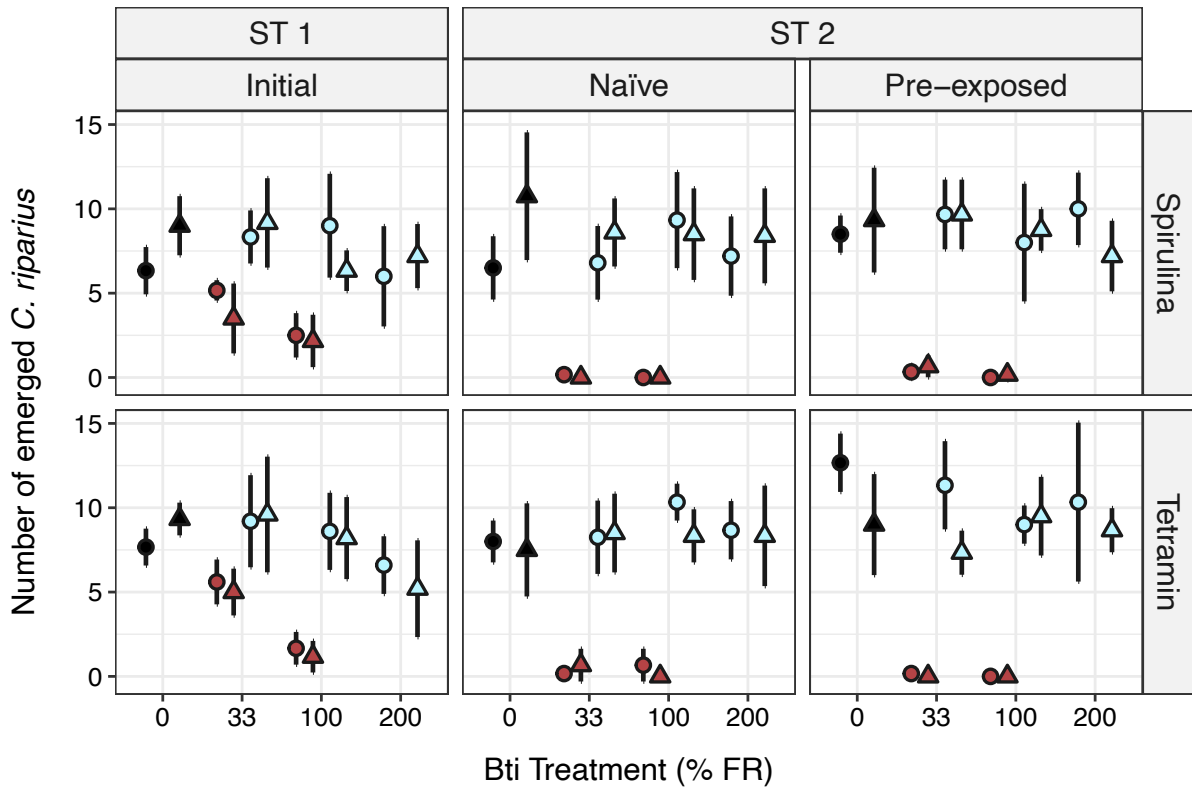


Fig. S2: Mean number of emerged male (circles) and female (triangles) *C. riparius* per treatment with 95% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four different doses expressed as % field 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).

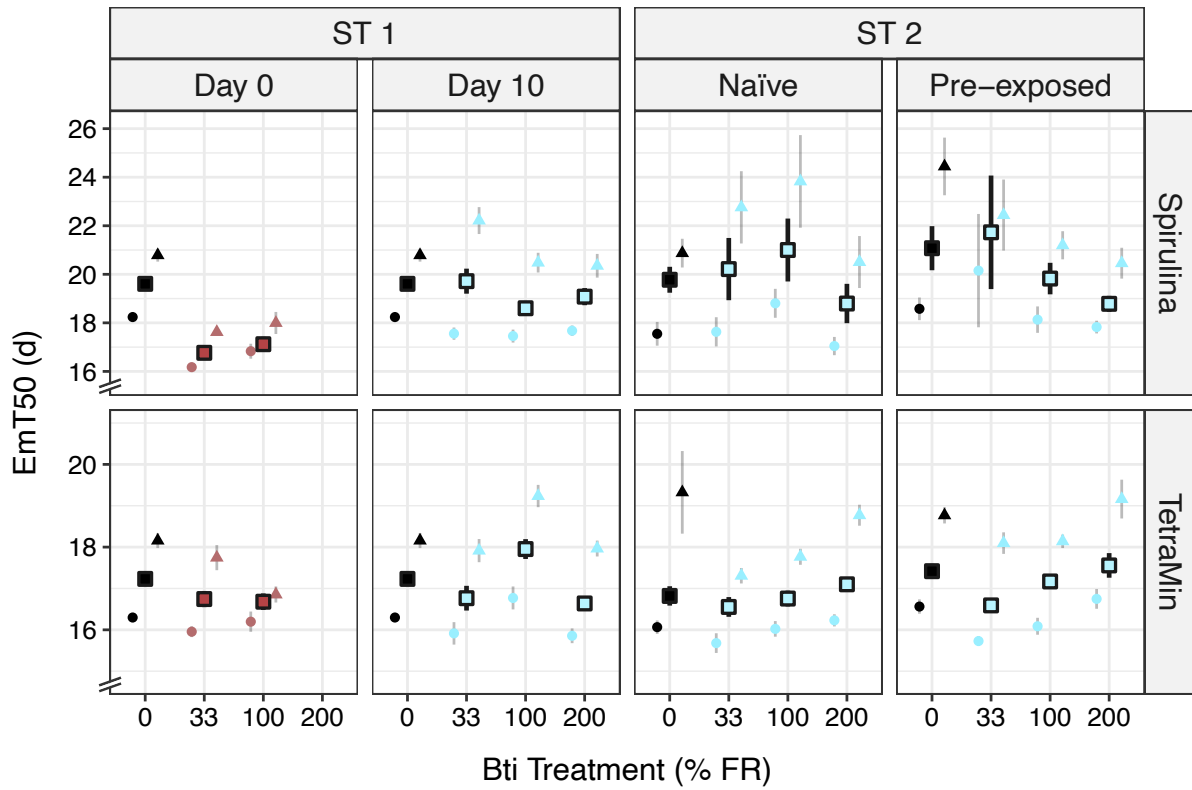


Fig. S3: Mean emergence time (EmT50) of all emerged *C. riparius* (squares), of males (circles) and of females (triangles) per treatment with 99% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four 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. y-axes of Spirulina and TetraMin differ.

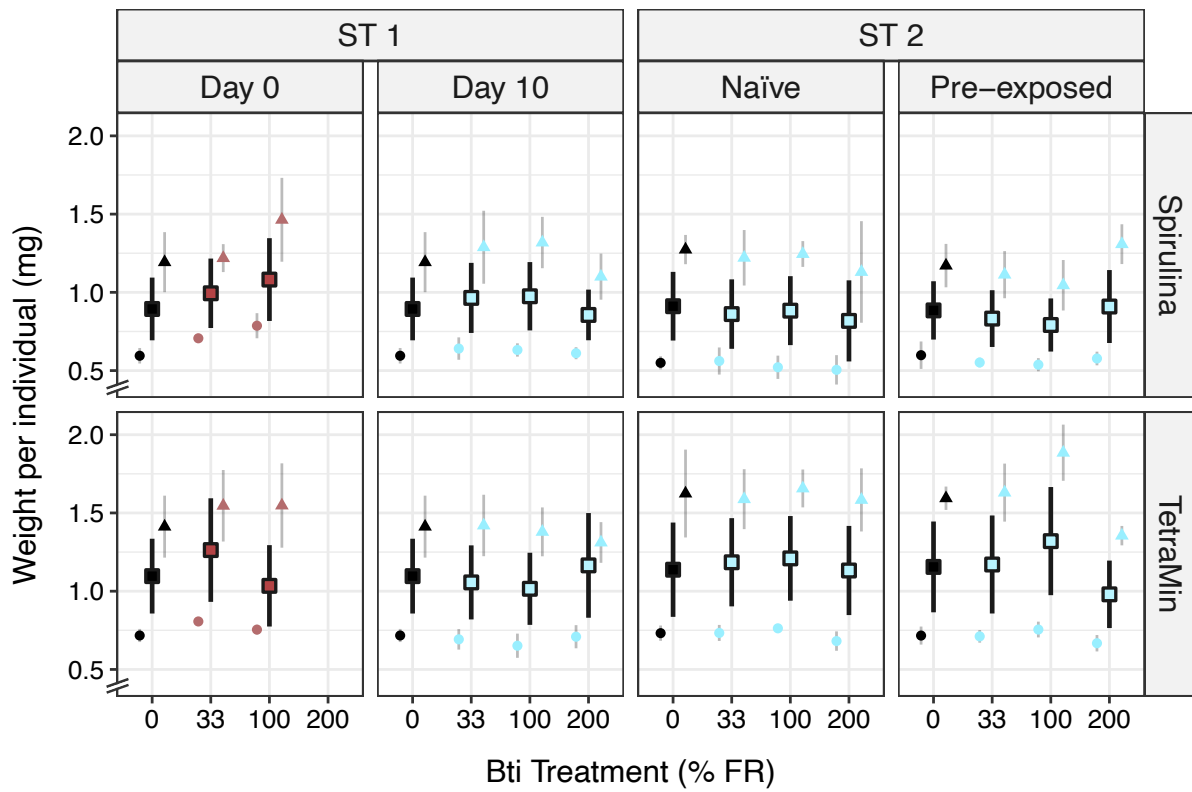


Fig. S4: Mean dry weight of all emerged *C. riparius* (squares), of males (circles) and of females (triangles) per treatment with 95% confidence interval (CI) from sensitivity test (ST) 1 and 2. Chironomids were fed with two food sources, either Spirulina or TetraMin. Bti was applied at four different 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 dry weight could be determined for application day 0 in ST 2 due to high mortality.

CURRICULUM VITAE

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Education

Since 10/2019	PhD candidate, Environmental Sciences RPTU Kaiserslautern-Landau – DFG GRK Systemlink Thesis topic: Subsidy dynamics
10/2016 – 09/2019	Master of Science, Ecotoxicology University of Koblenz-Landau Thesis title: “Do juvenile common toads (<i>Bufo bufo</i>) behaviourally avoid pesticide-contaminated areas?”
10/2012 – 03/2016	Bachelor of Science, Biosciences Technische Universität Kaiserslautern Thesis title: “Effects of mutations in the respiratory chain on petit- and ρ^0 -cells in <i>Saccharomyces cerevisiae</i> ”
2003 – 03/2012	Higher education entrance qualification Werner-Heisenberg-Gymnasium, Bad Dürkheim

Experience

10/2018 – 08/2019	Student assistant International knowledge transfer University of Koblenz-Landau
10/2017 – 03/2018	Tutor for tutorial “Advanced Environmental Chemistry” University of Koblenz-Landau
06/2017 – 08/2017	Internship , 3 months Experimental Toxicology and Ecology, BASF SE, Ludwigshafen
02/2016 – 08/2016	Research assistant Cellular Biochemistry, TU Kaiserslautern
09/2015 – 10/2015	Internship , 2 months Institut für Pathologie, Ludwigshafen
07/2012	Internship , 2 weeks Pollichia-Pfalzmuseum für Naturkunde, Bad Dürkheim

PUBLICATION LIST*Peer-reviewed articles*2024

Kolbenschlag, S., Pietz, S., Röder, N., Schwenk, K., Bundschuh, M. (Under review). Long-term exposure to Bti alters the sensitivity of *Chironomus riparius* populations. Submitted to a peer-reviewed journal.

Röder, N., Stoll, V. S., Jupke, J. F., **Kolbenschlag, S.**, Bundschuh, M., Theißinger, K., & Schwenk, K. (2024). How non-target chironomid communities respond to mosquito control: Integrating DNA metabarcoding and joint species distribution modelling. *Science of The Total Environment*, 913, 169735. <https://doi.org/10.1016/j.scitotenv.2023.169735>.

2023

Kolbenschlag, S., Bollinger, E., Gerstle, V., Brühl, C. A., Entling, M. H., Schulz, R., & Bundschuh, M. (2023). Impact across ecosystem boundaries – Does Bti application change quality and composition of the diet of riparian spiders? *Science of The Total Environment*, 873, 162351. <https://doi.org/10.1016/j.scitotenv.2023.162351>

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Pietz, S., **Kolbenschlag, S.**, Röder, N., Roodt, A. P., Steinmetz, Z., Manfrin, A., Schwenk, K., Schulz, R., Schäfer, R. B., Zubrod, J. P. & Bundschuh, M. (2023). Subsidy Quality Affects Common Riparian Web-Building Spiders: Consequences of Aquatic Contamination and Food Resource. *Environmental Toxicology and Chemistry*, 42: 1346-1358. <https://doi.org/10.1002/etc.5614>

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Ganglo, C., Mendoza-Lera, C., Manfrin, A., Bolpagni, R., Gerstle, V., **Kolbenschlag, S.**, Bollinger, E., Schulz, R., & Lorke, A. (2023). Does biocide treatment for mosquito control alter carbon dynamics in floodplain ponds? *Science of The Total Environment*, 872, 161978. <https://doi.org/10.1016/j.scitotenv.2023.161978>

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Salinas, E. R., Bozich, J. S., **Kolbenschlag, S.**, Kary-Heinrich, M., Hopp, P. W., Lukas, R., Zok, S., & Hidding, B. (2020). Aquatic testing guidelines insufficiently control the influence of dilution water toc and hardness on cationic polymer toxicity – A proposal to improve standardized test procedures. *Chemosphere*, 259, 127473. <https://doi.org/10.1016/j.chemosphere.2020.127473>

CONFERENCE PRESENTATIONS

Only first author contributions

Oral presentations

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C.A., Bundschuh, M. (2022) Bti-induced changes in aquatic subsidy propagate to linked terrestrial food webs.

SFE2, GfÖ & EEF Joint Meeting, International Conference on Ecological Sciences, Metz, 21-25 November 2022.

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C.A., Bundschuh, M. (2022) Mosquito control agent *Bacillus thuringiensis* var. *israelensis* alters emergence dynamics of insects from freshwater ponds.

36th Congress of the Society of Limnology, Berlin, 7-10 August 2022.

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C.A., Bundschuh, M. (2022) Mosquito control agent *Bacillus thuringiensis* var. *israelensis* alters emergence dynamics of insects from freshwater ponds.

SETAC Europe, 32nd annual meeting, Copenhagen, 15-19 May 2022.

Poster presentations

Kolbenschlag, S., Bollinger, E., Gerstle, V., Brühl, C.A., Schulz, R., Bundschuh, M. (2023) Bti-induced changes in aquatic subsidy dynamics transfer to linked terrestrial food webs. SETAC Europe, 33rd annual meeting, Dublin, 30 April - 4 May 2023.

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C.A., Bundschuh, M. (2022) Effects of a mosquito control agent on emerging insect communities in natural pond mesocosms.

YRS 2022, Kaiserslautern, 22 July 2022.

1. Place, Award for Best Poster

Kolbenschlag, S., Gerstle, V., Röder, N., Eberhardt, J., Bosch, L., Brühl, C.A., Bundschuh, M. (2021) Effects of the mosquito control agent *Bacillus thuringiensis* var. *israelensis* on natural aquatic insect communities - a semi-field floodplain study.

SETAC Europe, 31st annual meeting, Online, 3-6 May 2021.

Kolbenschlag, S., Kary-Heinrich, M., Salinas, E.R. (2017) Bewertung des Einflusses von Organischem Kohlenstoff und Wasserhärte auf die Toxizität von Kationischen Polymeren auf das Wachstum von Algen.

SETAC GLB, 22. Jahrestagung, Neustadt a.d. Weinstraße, 12-14 November 2017.

Kolbenschlag, S., Zimmermann, J., Morgan, B. (2016) Investigating the role of Nde1 and Nde2 in cellular redox homeostasis.

GBM study group redox biology, 3rd Meeting, Düsseldorf, 3-5 July 2016.

AUTHOR CONTRIBUTION

The authors' contribution to the peer-reviewed articles contained in this cumulative dissertation was as follows:

Publication 1:

Kolbenschlag, S., Gerstle, V., Eberhardt, J., Bollinger, E., Schulz, R., Brühl, C. A., Bundschuh, M. (2023). A temporal perspective on aquatic subsidy: Bti affects emergence of Chironomidae. *Ecotoxicology and Environmental Safety*, 250, 114503. <https://doi.org/10.1016/j.ecoenv.2023.114503>

Sara Kolbenschlag: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing.

Verena Gerstle: Methodology, Writing – review & editing.

Julian Eberhardt: Investigation, Writing – review & editing.

Eric Bollinger: Formal analysis, Visualization, Writing – review & editing.

Ralf Schulz: Conceptualization, Writing – review & editing.

Carsten A. Brühl: Methodology, Writing – review & editing.

Mirco Bundschuh: Conceptualization, Methodology, Supervision, Writing – review & editing.

Publication 2:

Kolbenschlag, S., Bollinger, E., Gerstle, V., Brühl, C. A., Entling, M. H., Schulz, R., Bundschuh, M. (2023). Impact across ecosystem boundaries – Does Bti application change quality and composition of the diet of riparian spiders? *Science of the Total Environment*, 873, 162351. <https://doi.org/10.1016/j.scitotenv.2023.162351>

Sara Kolbenschlag: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

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Carsten A. Brühl: Methodology, Writing – review & editing.

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Ralf Schulz: Conceptualization, Resources, Writing – review & editing.

Mirco Bundschuh: Conceptualization, Resources, Supervision, Writing – review & editing.

Publication 3 (Under review):

Kolbenschlag, S., Pietz, S., Röder, N., Schwenk, K., Bundschuh, M. Long-term exposure to Bti alters the sensitivity of *Chironomus riparius* populations. Submitted to a peer-reviewed journal.

Sara Kolbenschlag: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

Sebastian Pietz: Conceptualization, Methodology, Investigation, Writing – review & editing

Nina Röder: Conceptualization, Methodology, Investigation, Writing – review & editing

Klaus Schwenk: Conceptualization, Methodology, Writing – review & editing, Funding acquisition

Mirco Bundschuh: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition