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Mosquito control based on *Bacillus thuringiensis israelensis* (Bti) interrupts artificial wetland food chains

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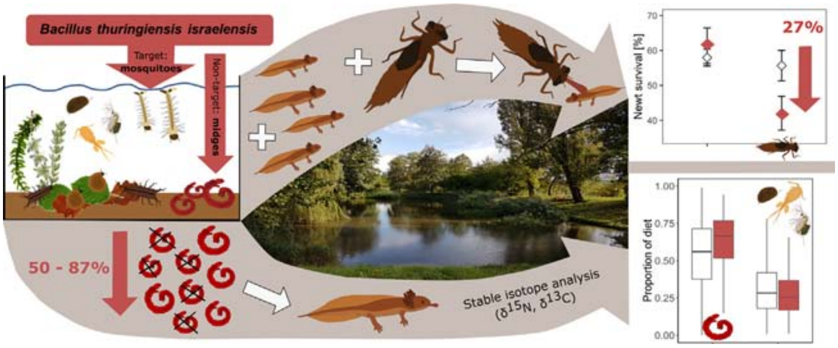
HIGHLIGHTS

- Chironomidae are the non-target aquatic invertebrates most affected by Bti.
- Development of newt larvae was indirectly affected in Bti treated food chains.
- The dragonfly *Aeshna cyanea* decreased newt survival by 27% when Bti was applied.
- Larval chironomids are a key component in the diet of aquatic predators.

GRAPHICAL ABSTRACT



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ABSTRACT

The biocide *Bacillus thuringiensis israelensis* (Bti) has become the most commonly used larvicide to control mosquitoes in seasonal wetlands. Although Bti is considered non-toxic to most aquatic organisms, the non-biting chironomids show high susceptibilities towards Bti. As chironomids are a key element in wetland food webs, major declines in their abundance could lead to indirect effects that may be passed through aquatic and terrestrial food chains. We conducted two mesocosm experiments to address this hypothesis by assessing direct and indirect effects of Bti-modified availability of macroinvertebrate and zooplankton food resources on the predatory larvae of palmate and smooth newts (Urodelans: *Lissotriton helveticus*, *Lissotriton vulgaris*). We examined newt survival rates and dietary composition by means of stable isotope (δ^{15}N and δ^{13}C) analysis in the presence of Bti treatment and a predator (Odonata: *Aeshna cyanea*). We assessed palmate newts' body size at and time to metamorphosis while developing in Bti treated mesocosms. Chironomid larvae were the most severely affected aquatic invertebrates in all Bti treated food chains and experienced abundance reductions by 50 to 87%. Moreover, stable isotope analysis revealed that chironomids were preferred over other invertebrates and comprised the major part in newts' diet (56%) regardless of their availability. The dragonfly *A. cyanea* decreased survival of newt larvae by 27% in Bti treated mesocosms showing affected chironomid abundances. Increasing intraguild predation is most likely favored by the Bti-induced reduction of alternative prey such as chironomid larvae. The decreased food availability after Bti treatment led to slightly smaller *L. helveticus* metamorphs while their developmental time was not affected. Our findings highlight the crucial role of chironomids in the food webs of freshwater ecosystems. We are also emphasizing the importance of reconsidering human-induced indirect effects of mosquito control on valuable wetland ecosystems particularly in the context of worldwide amphibian and insect declines.

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1. Introduction

The biocidal usage of the bacterium *Bacillus thuringiensis israelensis* (Bti) is considered one of the most environmental friendly methods to control mosquito populations emerging from stagnant water (Lawler, 2017). The treated water bodies range from temporary flooded wetlands to small ditches that are inhabited by various freshwater organisms (Becker et al., 2010). In principle, Bti is believed to be relatively safe for aquatic non-target organisms such as freshwater molluscs, crustaceans, vertebrates and most insects due to its' specific toxic mode of action (Boisvert and Boisvert, 2000; Lacey and Merritt, 2003; Lagadic et al., 2016, 2014). The larvicidal activity of Bti is based on δ-endotoxins produced during sporulation (Ben-Dov, 2014). After ingestion, they bind to specific receptor sites in the midgut epithelium of the targeted species, mosquitoes (Culicidae) and black flies (Simuliidae), which leads to perforation and subsequently to a rapid death of their larvae (Becker and Margalit, 1993; Ben-Dov, 2014).

Next to mosquitoes and black flies, non-biting midges of the family Chironomidae also show a high susceptibility towards Bti (Kästel et al., 2017; Stevens et al., 2005). Therefore, Bti is even applied against chironomids to reduce nuisance associated with their ability to form huge swarms upon emergence (Ali et al., 2008; Vaughan et al., 2008). However, chironomids are usually non-target organisms with regard to Bti applications in wetland mosquito control. Nonetheless, several field studies showed that Bti reduced abundances of chironomid larvae and adults by 50% and more in wetlands that were subject to regular mosquito control activities (Allgeier et al., 2019; Hershey et al., 1998; Jakob and Poulin, 2016) while other non-target invertebrates were not affected (Duchet et al., 2008; Lagadic et al., 2016, 2014). In contrast, other studies did not find negative effects of Bti on chironomids (Duchet et al., 2015; Lagadic et al., 2016; Lundström et al., 2010b; Wolfram et al., 2018) which is why the susceptibility of chironomids in the field is still a matter of debate in the current literature.

Nevertheless, a Bti-induced reduction in chironomid availability is of great concern since chironomids form a major component of the macroinvertebrates in most ecosystems as they are characterized by high abundances and species richness (Armitage et al., 1995). Besides their ubiquitous occurrence, their high protein content and digestibility makes them a quality food resource for aquatic and terrestrial organisms such as predatory insects, fish, amphibians, bats and birds (Armitage et al., 1995; Arnold et al., 2000; De La Noüe and Choubert, 1985; Jakob and Poulin, 2016). Some field studies on routine Bti treatments linked reduced availability of chironomid adults to lower breeding success in birds (Poulin et al., 2010; Poulin, 2012) or declining numbers and richness of adult dragonflies (Jakob and Poulin, 2016). In contrast, other studies did not find any indirect effects on birds that are related to Bti applications (Hanowski et al., 1997; Niemi et al., 1999; Timmermann and Becker, 2017).

Compared to insectivorous birds which are relatively mobile terrestrial predators, aquatic predators cannot escape from Bti treated ponds where they are exposed to Bti and have to subsist on available prey. The predatory performance of backswimmers on the mosquito *Aedes aegypti* was enhanced after Bti exposure (Gutiérrez et al., 2017) whereas Bti-based control of floodwater mosquitoes increased the abundance of medium-sized diving beetles (Vinnersten et al., 2009). However, there have been no studies on aquatic predators investigating indirect effects of Bti-induced changes in wetland food resources aside from mosquitoes.

Seasonal ponds and flood areas of small creeks offer optimal breeding grounds for mosquito larvae but also amphibians by providing an aquatic environment with standing water free of predatory fish (Batzer and Wissinger, 1996; Rubbo et al., 2011). Larval development of most European amphibian species coincides with recurrent mosquito control activities in spring and summer months (Allgeier et al., 2019; Becker et al., 2010; Günther, 1996). In these ecosystems, carnivorous newts (Urodelans) are among the top-predators and can have a major impact on freshwater invertebrate communities as they are opportunistic feeders that select their prey on availability and size (Mettouris and Giokas, 2017). The quality and quantity of food accessible during their larval stage has implications for time to emergence and body size at metamorphosis. Decreased food levels lead to smaller anuran metamorphs (Leips and Travis, 1994) whereas the length of the larval period largely depends on growth rates (Newman, 1992; Tejedo and Reques, 1994). Body size at metamorphosis is closely associated with individual fitness further affecting fecundity, reproductive success or survival as adults (Leips and Travis, 1994; Semlitsch et al., 1988).

Although Bti is supposed to have no direct lethal effect on amphibians, field application rates can evoke stress in amphibian larvae that is manifested in alterations of oxidative stress enzymes and changes in swimming behavior (Allgeier et al., 2018; Junges et al., 2017; Lajmanovich et al., 2015). Other than human-induced contaminants, amphibian larvae face multiple biotic and abiotic stressors in wetland ecosystems such as pond drying, varying temperatures, food limitation and the presence of natural predators (Wilbur, 1997). The combination of several sublethal stressors can have adverse interactive effects on amphibians (Boone et al., 2007; Rohr et al., 2004). For example, pesticides can become more lethal to amphibians in the presence of stress induced by predator cues (Relyea, 2003; Relyea and Mills, 2001). Conversely, predator-prey interactions may be affected by pesticide-induced changes in antipredator traits (Janssens and Stoks, 2012; Relyea and Edwards, 2010).

Amphibians are engaged in predator-prey interactions with dragonfly nymphs that prey on anurans and newts (Blois, 1985; Van Buskirk and Schmidt, 2000). Furthermore, predatory newts and dragonfly nymphs are also competitors for shared food resources. This mixture between competition and predation of species with comparable trophic niches is defined as intraguild predation (Holt and Polis, 1997). However, the coexistence of different species in the same trophic guild can be facilitated by high abundances of alternative prey, like chironomids, being present in wetland ecosystems (Preston et al., 2017).

This study addressed direct and indirect effects of Bti-based mosquito control on experimental mesocosm food webs with focus on larval newts (genus: *Lissotriton*). Due to the essential role of chironomids in wetland food webs (Leeper and Taylor, 1998; Williams, 2006) and their controversially discussed vulnerability in higher tier studies, chironomids were in the center of this research. Our aim was to examine how Bti treatments affect resource use and development of newt larvae and the trophic dynamics in a more complex trophic food web including a predator, the dragonfly nymph *Aeshna cyanea*. We used stable carbon and nitrogen isotopes (δ^{15}N and δ^{13}C) analysis to examine dietary composition and isotopic niche widths of the predators. The latter provides quantitative information on the isotopic niche space occupied by newts and dragonflies and thus insights to their adapted ecological niches and trophic diversity (Newsome et al., 2007). First, we hypothesized that (i) the dietary composition of newts feeding in Bti-treated aquatic communities would change towards the consumption of less chironomid larvae as a result of their reduced availability. On this basis, we assumed that (ii) a reduced availability of nutritional chironomids would affect the development of individual newt larvae leading to smaller metamorphs in terms of body condition. Moreover, we expected that the Bti-induced modification of basic food resources (iii) increased intraguild predation between dragonfly nymphs and newts due to reduced availability of alternative prey.

2. Materials and methods

2.1. Newt sampling and breeding

To obtain larval newts, we collected fertilized *Lissotriton helveticus* and *Lissotriton vulgaris* females in a pristine pond in the Bienwald forest, Rhineland-Palatinate, Germany (49°01′ N, 8°10′ E) and brought them to the laboratory for oviposition. Two females at a time were placed in 30 L glass tanks (50 × 30 × 20 cm) containing filtered tap water (0.2 μm Supor, Pall Corporation, Port Washington) and blades of couch grass (*Elymus* sp.) for egg deposition. Newt females were fed ad libitum with larvae of mosquitoes and chironomids obtained from forest ponds. Grass blades were checked for eggs every other day and replaced if necessary, along with the renewal of water. Eggs were placed into aerated plastic aquaria (7 × 16 × 22 cm) containing pond water. After hatching, larvae were fed with small *Artemia* and *Daphnia* from in-house cultures. Female newts were released back in the pond of origin after 21 days if enough eggs had been obtained for the experiments. All experimental procedures were evaluated and approved by the Institutional Animal Care and Use Committee at the University Koblenz-Landau and performed in accordance with Directive 2010/63/EU.

2.2. Study system

The present study comprised two mesocosm experiments conducted at the Landau Stream Mesocosm Facility at the University of Koblenz-Landau (Germany). The experiments were implemented in two subsequent years and followed the same study system, set-up, inoculation with invertebrates and sampling procedure as explained in more detail in Allgeier et al. (2019).

We established 48 (Experiment 1) and 16 (Experiment 2) aquatic model ecosystems in artificial ponds (70 × 40 × 30 cm, 90 L, polyethylene mortar buckets, JOPA, Ahlen, Germany). Mesocosms were placed into the water flow-through stream channels and covered by a shading net (30% reduction) (Fig. S.1). All mesocosms were filled with 40 L tap water, inoculated with 30 L pond water and supplied with quartz sand, a black alder/oak leaf mixture typically found in swamp forests and two aquatic plants (*Elodea canadensis*, *Myriophyllum spicatum*). To establish suitable prey for predators, we obtained aquatic invertebrates from local wetlands (*Asellus aquaticus*, snails, zooplankton, chironomids, mosquitoes) and in-house cultures (*Culex pipiens*, *Chironomus riparius*, *Daphnia magna*). Aquatic invertebrates were added to each mesocosm continuously in the same abundance or volume starting six weeks before the experimental phases in order to obtain standardized invertebrate communities. In total, 14 snails (Physidae, Planorbidae, Lymnidae) and 24 *Asellus aquaticus* were initially added to each mesocosm. A zooplankton mixture (Cladocera, Copepoda, Ostracoda) obtained from local wetlands (60 μm net) was homogenized with *Daphnia magna* and allocated into 48 (Experiment 1) and 16 (Experiment 2) equal volumes prior to their addition to the mesocosms. Chironomid larvae (pet-shop, 90 mL; in-house culture) and eggs (in-house culture; local wetlands) were portioned into aliquots of the same volume and added to the mesocosms once a week to ensure a diverse age structure and species mixture. Mosquitoes were treated equally to chironomids and added three and one week prior to the experiment start.

Each mesocosm was covered with an emergence trap (70 × 40 × 85 cm) to prevent colonization by external organisms and to catch emerging insects in collecting bottles positioned on top of the traps. Collecting bottles were filled with glycol and a detergent and activated once a week for a period of three consecutive days. Emerged insects were preserved in 70% ethanol and determined to order level and the order Diptera to family level using a Leica M80 binocular with a 10× magnification. Insect emergence within the entire sampling period expressed the number of successfully emerged insects that were not used as prey during their larval stage. Additionally, sampling emergence enables the detection of effects on 1st or 2nd instar chironomids which are difficult to count in natural samples due to their small sizes.

Aquatic invertebrates were sampled once prior to the Bti application and otherwise once a week during the course of the experiments using a tube sampler (Fig. S2, method described in (Allgeier et al., 2019). After gently mixing the mesocosm water, the tube (Ø 12 cm) was lowered quickly through the water column into the sand of each mesocosm and closed with a Perspex lid. The invertebrates contained in the water/sediment sample of the tube (volume: 1.5 L) were immediately identified and counted on site to class (Ostracoda, Hirudinea), order (Copepoda, Cladocera), genus (*Hydra*, *Tubifex*), family (Physidae, Planorbidae, Chironomidae, Culicidae, Chaoboridae) or species (*Asellus aquaticus*). Samples were poured back into the respective mesocosm after determination.

2.3. Experiment 1: community experiment

2.3.1. Experimental design

The first experiment examined the effects of a regular Bti application and predation on the dietary intake and survival of a natural newt larvae assemblage comprising *L. helveticus* and *L. vulgaris*. Both species are common in Central Europe and among the smallest newts in seasonal wetlands. They breed in early spring and their aquatic larval period coincides spatially and temporally with the application of Bti for mosquito control (Allgeier et al., 2019; Günther, 1996). Both species further share a similar feeding behavior and have a high food niche overlap which is why they are considered as a single group within food analyses (Griffiths, 1986). We used a randomized 3 × 2 factorial design manipulating two factors in the aquatic communities to assess the impacts on the food chain: three levels of predators [*no predators* (*predation control* (*PC*))/*newt larvae* (*N*)/*newts and dragonfly larvae* (*ND*)] and two levels of Bti treatment [*control*/*Bti treated*]. *Aeshna cyanea* (Odonata: Anisoptera) nymphs were inserted as a generalist predator that is efficient in consuming a wide range of prey in the wild including zooplankton, amphipods and amphibian larvae (Blois, 1985; Van Buskirk and Schmidt, 2000). All treatment combinations were replicated eight times and randomly assigned to 48 previously established pond mesocosms. Results on invertebrates in PC were recently published in Allgeier et al. (2019) and are used again here for the assessment of the impact of predators on the aquatic communities in N and ND.

The community experiment lasted for nine weeks from May 8 to July 12, 2015. On May 10, we applied 34.8 mg VectoBac®WG granules (3000 International Toxic Units (ITU)/mg, Valent BioSciences Corporation, Illinois, USA) directly onto the water surface (0.29 m^2) of the treated mesocosms. The application rate equaled a high field rate (2.88 × 10^9 ITU/ha) which is regularly applied in the German mosquito control program at water levels >10 cm and when mosquito larvae are larger than 2nd larval stage (Allgeier et al., 2019; Becker, 2003). We performed six samplings of aquatic invertebrates at −2, 2, 9, 18, 32, and 47 days after application (DAA) and emergence at DAA 0, 5, 12, 18, 32, 45. Dissolved oxygen, pH and water temperature were measured mid-morning of each sampling day in a randomized design using a WTW Multi 340i (WTW GmbH, Weilheim, Germany) (Supplementary material).

Late-instar *A. cyanea* nymphs were collected by dip-netting from a Bti-untreated pond near Landau (49°15′N, 7°57′E). One nymph (mass: 568.75 mg ± 47.6, length: 36.69 mm ± 1.4) was randomly added to each ND mesocosm on May 6. Initially, six newt larvae (length: 10 mm ± 1) were inserted in N and ND mesocosms on DAA 3. We added another five newt larvae (length: 15 mm ± 1) into each mesocosm three weeks later because of high mortality rates at the beginning of the experiment. As a reaction to progressing hatching rates of dragonfly nymphs we measured body length (ruler) and mass (Mettler PM6000, Columbus, USA) on site at DAA33 (38 days after addition). Newt assessment was performed twice per week as soon as the first newt larvae showed signs of metamorphosis such as the resorption of the external gills and the tail. Shortly before metamorphosis, individual newts were transferred into sealed aquaria to finish metamorphosis without escaping the mesocosm tanks. We recorded newt survival as the proportion of newts that finished metamorphosis in each mesocosm and kept 54 individuals for stable isotope analyses.

2.3.2. Stable isotope analysis

Sampling of aquatic food web components took place between July 9 and July 12 after the last newts left the mesocosms because tanks needed to be emptied entirely to reach a sufficient sample amount of resources (Cladocera, Copepoda, Ostracoda, Chironomidae, *Asselus* *aquaticus*, Planorbidae, Physidae, Lymnaeidae). For the evaluation of newts' dietary composition we selected newt individuals of N-control and N-Bti that finished metamorphosis between July 8 and July 20 (n = 19). Moreover, we analyzed the trophic niche spaces occupied by dragonflies and newts that finished metamorphosis between June 29 and July 5 when metamorphosis rates peaked in all treatment combinations (n(dragonfly) = 13, n(newts) = 35).

Newt metamorphs and dragonflies were euthanized by immersion in 0.1% MS-222 and individually stored at −20 °C until processing for stable isotope analyses. MS-222 is commonly used for euthanizing amphibians and no effects on stable isotope measurements have been reported (Caut et al., 2013). We dissected tail muscle samples from newt metamorphs and thoracic muscle tissue from dragonflies for bulk stable isotope analyses. All samples were kept separate in 1.5 mL safe-lock tubes (Eppendorf, Hamburg, Germany), oven-dried at 60 °C for at least 48 h and grounded to a fine and homogenous powder using stirring spatulas (120 mm, 30 × 3 mm, Ø 3 mm, PS, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). 0.4 to 1 mg of ground sample was weighed into tin capsules (8 × 5 mm, ThermoFisher Scientific) and stored at 60 °C until analysis of carbon and nitrogen isotopic values (δ^{13}C and δ^{15}N). Bulk stable isotope analysis was performed at the Landau Stable Isotope Facility using a flow ratio mass spectrometer (Delta V Advantage) interfaced to a high-performance Flash 2000 HT elemental analyzer via a ConFlo IV (Thermo Finnigan, Bremen, Germany). Stable isotope ratios of carbon and nitrogen are expressed in the standard δ-notation, which is defined as the difference between the stable isotope value of the sample and the international reference standards relative to Vienna Pee Dee Belemnite and atmospheric N2 in per mille (‰). The accuracy of repeated analysis of the international standard Casein was ≤0.057‰ and 0.097‰ for carbon and nitrogen, respectively.

2.4. Experiment 2: newt development experiment

The second experiment examined the individual development of *L. helveticus* larvae under regular Bti treatments by assessing time to metamorphosis (TTM) and body size at metamorphosis. Therefore, we established eight controls and eight Bti treatments following the general study system and set-up. The experimental phase lasted for nine weeks between May 25 and July 22, 2016. Mesocosms were treated with Bti twice (May 26 and June 8, 2016) using ice pellets (IcyPearls) from the stock material of the German Mosquito Control Association. IcyPearls were manufactured with a VectoBac®WG suspension that is converted to 4 mm grain sized granules with the help of liquid nitrogen (Becker, 2003). Again, we applied the high field rate (2.88×10^9 ITU/ha) which equals the application of 870 mg IcyPearls on the water surface of each mesocosm. At DAA11, two days prior to the second Bti application, we added additional mosquito larvae to record the efficiency of the second Bti treatment. We sampled aquatic invertebrates at DAA -1, 6, 13, 21, 26, 35 and 40 and emergence at DAA 6, 13, 19, 26, 33 and 42. Temperature and pH were measured three times while dissolved oxygen and conductivity were measured two times during the sampling period (Supplementary material).

One larval *L. helveticus* individual (11.44 ± 1 mm) was added to each mesocosm the day after the initial Bti application. To prevent newt metamorphs from escaping the mesocosms, we transferred the nearly finished metamorphs into closed plastic aquaria for a maximum of four days until they finally finished metamorphosis. Time to metamorphosis displays the time between experiment initiation and date of complete metamorphosis. Body length and snout to vent length (SVL) were evaluated at the beginning of the experiment and at the end of metamorphosis using the software AxioVision® (Carl Zeiss; Oberkochen, Germany) on a digital photograph of the individuals. Associated body mass was recorded by weighing the dabbed dry metamorphs (Mettler Toledo XA105 DualRange; Giessen, Germany). Metamorphs were released next to their pond of origin after final body size was assessed.

2.5. Data analysis

All analyses were performed using the statistical software R, version 3.5.1 (R Core Team, 2014). Significant differences were denoted by asterisk in graphical representations of the data.

2.5.1. Food web composition

Differences in taxa abundances due to a certain treatment referred to cumulative reductions over the entire sampling period. Taxa contributing >0.1% to the overall invertebrate abundances were included in multivariate analyses on invertebrate communities. We performed a permutational multivariate analysis of variance (PERMANOVA) to reveal the influence of time, Bti, predator and their interactions on invertebrate communities in 999 permutations. Pairwise comparisons (post-hoc) were performed using Wilks test statistic, 999 permutations and Bonferroni corrections (function: pairwise.perm.manova). Furthermore, Bti effects on the invertebrate composition in the newt development experiment were analyzed using the principal response curve (PRC) method (Van den Brink and Braak, 1999). We ln(2x 1) transformed the abundance data beforehand to avoid false discrepancies between zero abundances and low abundances (Van den Brink et al., 2000). Differences at single sampling dates were assessed by redundancy analysis (RDA) followed by Monte Carlo permutation tests.

A generalized linear mixed-effect model (GLMM) with negative binomial errors was implemented to assess whether the treatments influenced taxa abundances. We specified taxa abundance as response variables and included DAA and Bti, predator and Bti ∗ predator (community experiment) or DAA and Bti (development experiment) as predictor variables. Mesocosms were included as random effect to account for repeated measures. The interaction term was removed when non-significant. Differences at each sampling day were analyzed using Student's *t*-test with Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). We applied Kruskal-Wallis test when data were not normally distributed (tested with Shapiro-Wilk test) and Welch's t-test with unequal variances (tested with Levene's test). Differences in abiotic parameters were tested with a generalized linear effect model using a Poisson distribution and displayed in the Supplementary material. We used the R packages “vegan” (Oksanen et al., 2018) and “glmmADMB” (Skaug et al., 2013) for the analyses.

2.5.2. Predator performances

We estimated chironomid prey intake by predators as difference between the amount of emerged chironomids in the predator-free controls and both predator scenarios during the entire sampling period. To assess individuals fitness we calculated a body condition index (BCI) for newts and dragonfly nymphs according to the scaled mass index described by Peig and Green (2009). For the index, ln-transformed data of individual mass and length measurements were used to perform a standardized major axis (SMA) regression. Furthermore, the slope was used to adjust the body composition of each individual to the mean length of all newt or dragonfly individuals. Newt survival was analyzed with a two-way analysis of variance (ANOVA with Type III SS without sequentially fitting of terms) of fitted linear mixed effect models with treatment, predator and their interaction as fixed and mesocosm as random effect. Residuals were tested for normality using a Shapiro-Wilk's test. We calculated the mean increases in mass, length and the respective BCI of dragonfly nymphs and analyzed differences in body parameters using a Student's t-test. Whether Bti treatment had an effect on individual newt development (mass, SVL, length, length increase, BCI) was assessed by an ANCOVA in which TTM was included as covariate to account for possible time-dependent effects. Influences on TTM were analyzed using a two-way ANOVA. The analysis of mass related parameters is based on n(Bti) = 7 and n(control) = 8 newt individuals since one body mass value was noted incorrectly.

Table 1
Results of PERMANOVA of invertebrate community compositions in relation to sampling time, Bti treatment and predator groups. *P* values in bold indicate statistical significance.

Factors	F	r^2	*P*
Main effects			
Time	91.87	0.261	**0.001**
Bti	18.95	0.054	**0.001**
Predators	2.77	0.015	**0.008**
Interactions			
Bti × Time	2.06	0.006	0.081
Predators × Time	0.63	0.004	0.764
Bti × Predators	1.36	0.008	0.200

2.5.3. Stable isotope analysis

Differences of raw δ^{13}C and δ^{15}N values of analyzed organisms in control and Bti treatment were evaluated using Student's t-test. Effects of treatment or predator on δ^{13}C and δ^{15}N values of newts at the metamorphosis peak were analyzed with a two-way ANOVA. We used "MixSIAR", a Bayesian framework for conducting stable isotope analysis in R (Stock et al., 2018), to estimate newts' relative consumption of prey. We applied trophic discrimination factors (TEFs) specified for the analysis of muscle tissue without any treatment for lipid removal as recommended by McCutchan et al. (2003) (1.1 ± 0.35 for δ^{13}C; 2.8 ± 0.4 for δ^{15}N). To reduce the number of sources in the model, we summarized isotopic ratios of Cladocera, Copepods and Ostracods as "zooplankton" and Planorbidae, Physidae and Lymnidae as "snails" a posteriori resulting in four systematically distinguished prey types: chironomids, zooplankton, snails and *Asellus aquaticus*. We used an informative prior for the mixing model based on abundance data to account for the Bti effect on prey abundances. Cumulative abundance data for control and Bti treatment were scaled so that the sum of α for each prey type is 4 which is the number of sources (Table S1). The mixing model was fitted on three Markov chain Monte Carlo (MCMC) chains and convergence was assessed with the Gelman-Rubin diagnostic (Gelman et al., 2013).

We explored their isotopic niche widths to analyze dietary similarity and potential niche shifts between predators in control and Bti mesocosms. Although the isotopic niche of a consumer is derived from abiotic and biotic processes within a biological system, it highly correlates with the trophic niche when it is primarily driven by consumer-resource interactions (Bearhop et al., 2004). For its calculation, we used Bayesian methods for populations with unequal and small sample sizes included in the R package 'SIBER' (Jackson et al., 2011). The analysis generates a standard ellipse area (SEA) of the isotopic niches which is a measure of the mean core population isotopic niche not affected by sample size. Moreover, we calculated a corrected SEA_C value (minimizes bias due to small sample sizes) and a Bayesian SEA_B value to compare niche width statistically (based on 10,000 posterior draws). Differences in SEA_C were estimated via Bayesian inference according to Jackson et al. (2011). Dietary niche overlap is given as a proportion of the non-overlapping area of two isotopic niches.

3. Results

3.1. Experiment 1: community experiment

3.1.1. Food web composition

Invertebrate community structure differed significantly as a result of proceeding time, the Bti treatment and the presence of predators (PERMANOVA, Table 1). Both predator treatments influenced the invertebrate community composition when compared to PC (pairwise

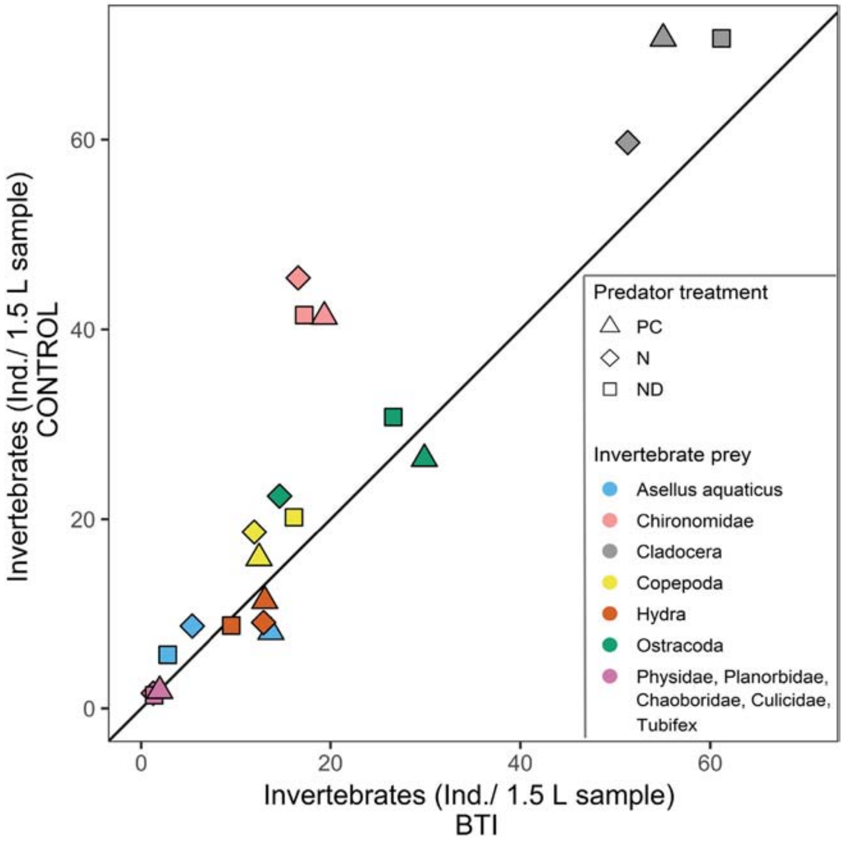

Fig. 1. Mean abundances of aquatic invertebrate prey (Individuals in 1.5 L sample) over all sampling days (N = 5) in Bti relative to control treatments. Abundances are displayed in predator treatments (predation control: PC, newt larvae: N, newts dragonfly larvae: ND). Taxa with mean abundances <4 are summarized in one group (purple). Taxa above the solid line indicate that taxa are more abundant under control conditions while taxa under the line are more abundant in Bti treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

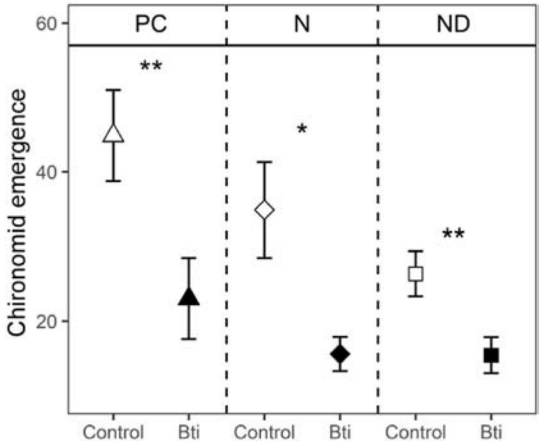

Fig. 2. Mean chironomid emergence rates (±SE) over the entire sampling period (7 weeks) in the different predator groups (predation control: PC, newt larvae: N, newts dragonfly larvae: ND) and control (white) and Bti (black) treatment (n = 8 mesocosms for each treatment).

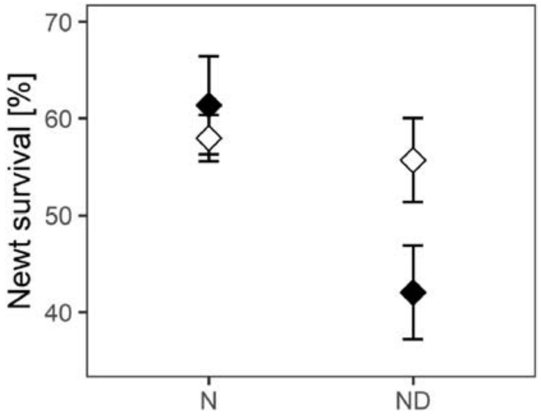

Fig. 3. Mean proportion (±SE) of surviving newts (*L. helveticus*, *L. vulgaris*) in the absence (N) and presence (ND) of one *A. cyanea* nymph under control (white) and Bti treatment (black) (initially introduced newt larvae: n = 11).

PERMANOVA, N: $P = 0.003$, ND: $P = 0.03$). However, there were no differences in the invertebrate composition between N and ND (pairwise PERMANOVA).

Total invertebrate density decreased as a result of Bti treatment (GLMM: $z = -5.73$, $P < 0.001$) whereas differences were larger in the presence of predators (GLMM, N: $z = -2.88$, $P = 0.004$; ND: $z = -1.69$, $P = 0.09$). Available invertebrate prey occurred in varying abundances (Fig. 1). Chironomid larvae and small-bodied zooplankton (Cladocera, Ostracoda, Copepoda) were highly abundant in all treatments. Chironomid larvae were evenly spread over the predator treatments, however, Bti reduced their abundances by 53 to 64% (GLMM: $z = -10.47$, $P < 0.001$). Within the zooplankton, relatively less Cladocera and Copepoda occurred with Bti (GLMM: Cladocera: $z = -1.89$, $P = 0.059$, Copepoda: $z = -3.38$, $P < 0.001$) while the presence of newts reduced the abundances of Cladocera and Ostracoda in N (GLMM: Cladocera: $z = -3.09$, $P = 0.002$; Ostracoda: $z = -2.54$, $P = 0.01$; Fig. 1). Other less abundant organisms like snails, annelids and other dipteran larvae appeared too infrequently to evaluate a pattern (mean abundance < 4). The most abundant organisms were included in the subsequent analysis of stable isotopes except Hydra which was no longer found in the final sampling.

3.1.2. Predator performance

The presence of the different predators influenced the overall number of emerging chironomids (GLMM: N: $z = -1.92$, $P = 0.055$; ND: $z = -2.81$, $P = 0.005$; Fig. 2) that comprised the majority (>90%) of emerging insects. Predators reduced the number of chironomid adults by 22% in N-control (mean: 34.90 ± 6.4) and 41% in ND-control (mean: 26.38 ± 3.0) when compared to PC-control (mean: 44.88 ± 6.1) (Fig. 2; Table S2). The presence of predators in Bti treated mesocosms equally reduced chironomid emergence by 34% in N-Bti (mean: 15.60 ± 2.3) and ND-Bti (mean: 15.4) (Fig. 2, Table S2). In Bti treated mesocosms, newts consumed 22% less chironomids than in the control while 57% less chironomids were consumed by newts and the dragonfly nymph. Chironomid adults exhibited Bti-induced abundance reductions by 43 to 56% (GLMM: $z = -5.05$, $P < 0.001$) comparably to the reductions in their larval state. Mosquitoes emergence (<2% of all emerged insects) were reduced by 45% due to Bti (GLMM: $z = -2.46$, $P = 0.014$) but were not influenced by predators.

Proportional newt survival was affected by *A. cyanea* ($X^2 = 10.09$, $P = 0.001$) but not by the Bti treatment (Fig. 3). However, Bti caused a significant decrease in survival rates by 27% when applied in mesocosms with dragonfly predator ($X^2 = 3.93$, $P = 0.047$; Fig. 3).

Dragonfly body length, mass and the respective increases did not significantly differ between Bti and control at DAA33 (Fig. 4). However, whereas dragonflies had an equal initial BCI in both treatments at the beginning of the experiment (mean: 599.12 ± 36.2; $t = 1.448$, $P = 0.17$), nymphs in the Bti treatment showed a significantly lower BCI after 38 days (Fig. 4C).

3.1.3. Stable isotope analysis

Newts' $\delta^{15}N$ values were enriched relative to their prey (Table 2). No significant shifts in stable isotope values of either predator or prey occurred between control and Bti, except for slightly higher $\delta^{13}C$ in chironomids in the Bti treatment (Table 2, dual isotope spaces: Fig. S3). Isotopic values of newts at the metamorphosis peak did not differ significantly as a result of Bti, predator presence or their interaction (two-way ANOVA, $P > 0.2$). The dragonfly nymph showed highest $\delta^{13}C$ values of all taxa and 1.4‰ lower $\delta^{15}N$ values than newts (Table 2) which is why we could not perform a stable isotope mixing model to assess dragonflies' dietary composition. Thus, the mixing model analyses were focused on the dietary composition of newts.

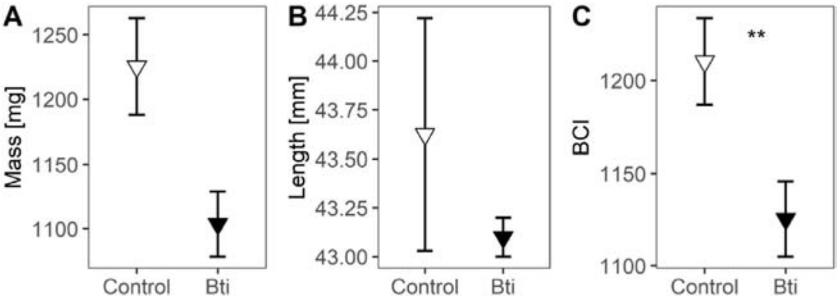

Fig. 4. Mean (±SE) (A) body mass, (B) length and (C) body condition index (BCI) of *A. cyanea* nymphs in control (n = 8, white) and Bti treatment (n = 5, black) at DAA33.

Table 2
Mean ± SD $\delta^{13}C$ and $\delta^{15}N$ values from bulk stable isotope analysis of predators and prey in control and Bti treated mesocosms (uncorrected for isotopic discrimination) used in the analyses of dietary composition of *Lissotriton* newts (N) and for isotopic niche widths of newts and *A. cyanea* at the metamorphosis peak (N/ND). n = number of individuals (*Lissotriton*, *A. cyanea*) or prey samples analyzed. Values written in bold indicate statistically significant difference from control.

	Control			Bti		
	n	$\delta^{13}C$ ± SD (‰)	$\delta^{15}N$ ± SD (‰)	n	$\delta^{13}C$ ± SD (‰)	$\delta^{15}N$ ± SD (‰)
Dietary composition (N)						
Lissotriton	10	−24.21 ± 0.38	5.73 ± 0.47	9	−24.32 ± 0.36	5.73 ± 0.26
Chironomids	9	−25.17 ± 0.51	3.01 ± 0.9	9	**−25.84 ± 0.46**	2.71 ± 0.64
Asselus aquaticus	5	−24.00 ± 0.57	2.04 ± 0.73	5	−24.32 ± 0.38	1.69 ± 0.27
Snails	15	−26.03 ± 1.92	1.94 ± 0.75	13	−26.71 ± 2.15	2.18 ± 0.79
Zooplankton	15	−23.91 ± 3.29	3.37 ± 1.34	15	−23.14 ± 3.68	3.04 ± 1.38
Isotopic niche width (N/ND)						
Lissotriton (N)	8	−24.78 ± 0.49	5.76 ± 0.27	10	−24.19 ± 0.83	6.00 ± 0.38
Lissotriton (ND)	10	−24.39 ± 0.90	5.86 ± 0.43	7	−24.32 ± 0.71	5.97 ± 0.54
A. cyanea (ND)	7	−29.11 ± 0.43	4.39 ± 0.26	6	−28.96 ± 1.29	4.49 ± 0.43

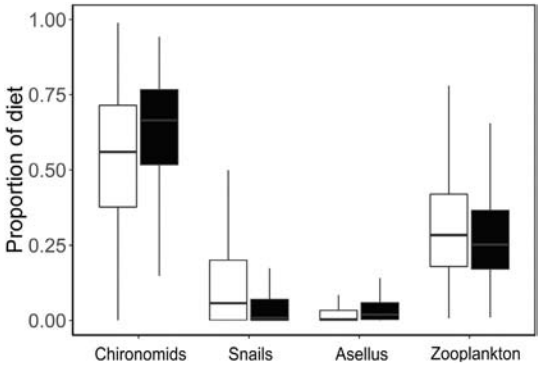

Fig. 5. Proportional contributions (lines = median, box = 50% CI, error bars = 95% CI) of each prey item to the diet of *Lissotriton* newts calculated with the abundance data (abundance prior) in control (white) and Bti (black) mesocosms.

The diet of newts was dominated by a combination of mostly chironomids (>56%) and zooplankton (>25%, Fig. 5) according to the mixing model estimates including the abundance priors. Moreover, chironomids comprised a similar proportion of newts' diet in control and Bti treatment despite their different abundances.

Isotopic niche widths reflect populations' food resource diversity and varied in-between treatments indicated by increasing SEA_B and SEA_C values (Fig. 6). Newts without any treatment (N-control) had the smallest isotopic niche width (SEA_C, Fig. 6A). Treatment with Bti resulted in 1.3 (N) and 1.7 times (ND) enhanced niche widths (SEA_C) of newts compared to their respective controls. Accordingly, the presence of *A. cyanea* increased newt niche widths by 2.3 times compared between controls and 1.7 times when comparing Bti treatments (Fig. 6A). Lowest niche overlap (33%) was found between newts in N-control and ND-Bti whereas highest niche overlap (74%) occurred in newts of N-Bti and ND-control (Fig. S4). Isotopic niche widths of *A. cyanea* individuals were five times wider when feeding in Bti treated mesocosms than in Bti-free controls (Fig. 6B). Moreover, dragonflies in Bti and control conditions showed just a small niche overlap of 20% (Fig. S5).

3.2. Experiment 2: newt development experiment

3.2.1. Food web composition

The aquatic invertebrate communities were dominated by zooplankton (Cladocera: 71% of individuals, Ostracoda: 5%, Copepoda: 5%) and midge larvae (Chironomidae: 9%, Culicidae: 4%). Mean invertebrate densities were higher than in the community experiment (control: 230.25 ± 10; Bti: 206.61 ± 9.9) but also reduced by the Bti treatment (GLMM: $z = -1.96$, $P = 0.05$). Control and Bti treated mesocosms differed in their invertebrate community (Bti: F = 25.00, $P = 0.001$) according to the PRC analysis (Fig. S6). Differences were statistically significant at all sampling dates except the initial sampling prior to the first Bti treatment (DAA -1). Monte Carlo permutation tests indicated that 76% of the total variance was captured by the first PRC axis. Overall, 41% of the variance could be attributed to time whereas Bti treatments explained 16%. Analysis of taxa weights (b_k) showed that chironomids were the most affected taxa ($b_k = 3.28$) followed by mosquitoes (b_k

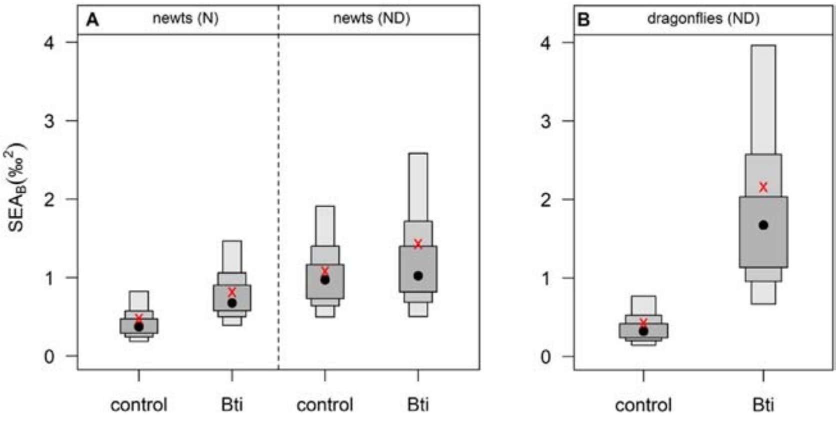

Fig. 6. Density plots of standard ellipse area (SEA) displaying the isotopic niche width for (A) newts and (B) *A. cyanea* in predator (N/ND) and Bti treatments (control/Bti). Boxed areas represent SEA_B value (black dot) with Bayesian 50, 70 and 95% credible intervals (gray bars). The red cross indicates the sample size corrected SEA_C value. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

= 1.24). Mosquito larvae were reduced by at least 99% within the first six to eight days after both Bti applications (DAA6: $X^2 = 12.39$, $P < 0.001$; DAA21: $X^2 = 8.45$, $P = 0.003$) (Fig. S7). Moreover, Bti affected chironomid larvae from six days after the initial Bti application and cumulatively reduced their abundances by 87% over the entire sampling period (GLMM: $z = -12.10$, $P < 0.001$) (Fig. S7). While Cladocera decreased over time (GLMM: $z = -6.36$, $P < 0.001$) other aquatic invertebrates showed increasing abundances. However, none of them was affected by the Bti treatment.

3.2.2. Newt development

Body sizes of newt metamorphs ranged from 167.62 to 203.81 mg (33.08–38.25 mm) in the Bti treatment and from 185.27 to 219.10 mg (34.66–37.94 mm) in the controls. Newt metamorphs appeared to be slightly smaller in Bti treated mesocosms, while TTM showed no differences between control and Bti treatment (Fig. 7, Table S4). The smaller sizes were reflected in a trend towards a 7% lower body weight of *L. helveticus* newts at metamorphosis ($F = 4.07$, $P = 0.067$).

4. Discussion

This is the first study that showed adverse effects of Bti-based mosquito control in environmentally relevant application rates on aquatic predators such as newt larvae. Our experiments revealed that Bti increased vulnerability of newt larvae to intraguild predation by a dragonfly nymph, while both predators co-existed in Bti-free environments. As chironomids are the most severely affected non-target food resource in the Bti treated food chains (Allgeier et al., 2019), this study highlights the crucial role of chironomids as food resource in confined aquatic systems.

4.1. Bti effects on food web composition

Bti treatment changed the invertebrate community structure and affected the density of available food resources by significantly reducing abundances of mosquitoes but also non-target chironomids (Fig. 1). Availability of chironomids was reduced by more than half (53 to 87%) which was confirmed repeatedly in both our mesocosm experiments. In accordance with previous studies, other aquatic invertebrates were not directly affected due to the Diptera-specificity of Bti (Boisvert and Boisvert, 2000; Lacey and Merritt, 2003). Chironomids usually constitute a major proportion of invertebrate biomass and secondary production in temporary ponds and other wetlands in terms of abundances (Allgeier et al., 2019; Leeper and Taylor, 1998; Lundström et al., 2010b; Williams, 2006) and species diversity (Lundström et al., 2010a; Theissinger et al., 2018; Wolfram et al., 2018). On this account, chironomids as the only adversely affected non-target invertebrates are central for our further considerations, especially since previous results on the existence and extent of negative effects on chironomids have been inconsistent (Allgeier et al., 2019; Lagadic et al., 2014; Lundström et al., 2010b).

Chironomids showed similar effect sizes (55 to 75% reduction) in another pond mesocosm study where the formulation VectoBac®G was used in application rates up to 9×10^9 ITU/ha (Liber et al., 1998). Moreover, the effect on chironomids in our mesocosm was verified by several field studies that analyzed chironomid communities and found reduced abundances (Allgeier et al., 2019; Hershey et al., 1998; Jakob and Poulin, 2016; Theissinger et al., 2018). Nevertheless, there are other semi-field and field approaches in which chironomid abundances or species diversities were unaffected (Charbonneau et al., 1994; Lagadic et al., 2014; Lundström et al., 2010b, 2010a; Pont et al., 1999; Wolfram et al., 2018).

There may be several reasons for the discrepancy of the results in field studies. First, the detection of Bti effects on chironomid

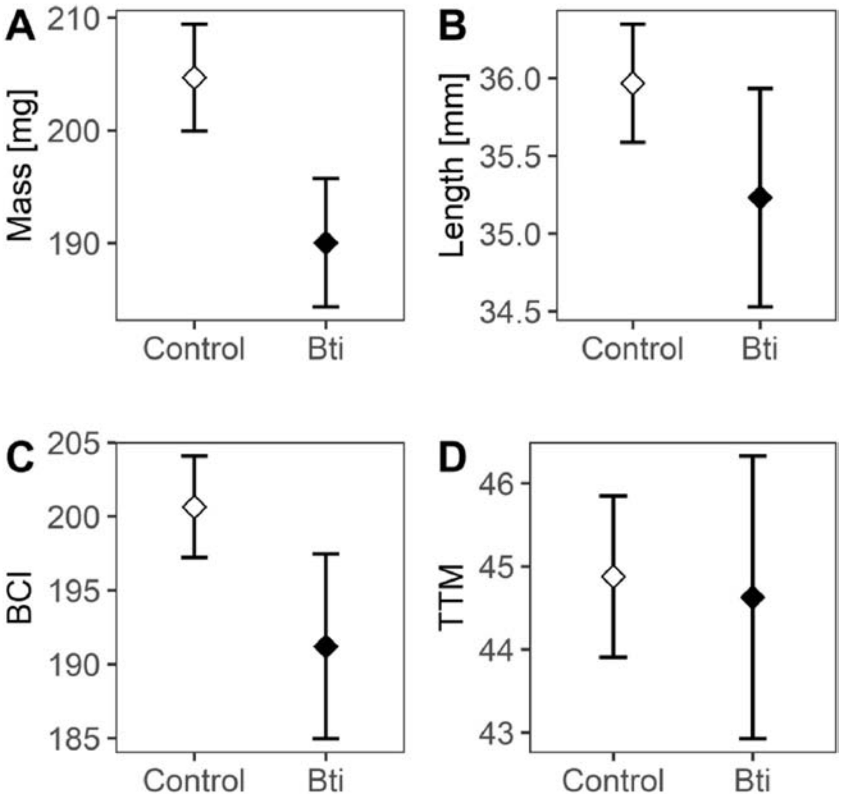

Fig. 7. Mean (±SE) (A) body mass, (B) length, (C) body condition index (BCI) and (D) time to metamorphosis (TTM) of *L. helveticus* metamorphs in control (n = 8, white) and Bti treatment (n = 7, black).

communities in the field may be facilitated in sampling periods longer than a few days after the Bti application were effects on chironomids could recently not be detected (Wolfram et al., 2018). Furthermore, not all of the above mentioned field studies on non-target effects explicitly assessed abundances of mosquitoes (Allgeier et al., 2019). However, significant effects on the target organism should be used as a proxy for Bti treatment efficiency that allows estimating non-target effect sizes on other organisms. Besides, several environmental factors (e.g. biotic and abiotic wetland characteristics, desiccation and flooding events, species community structure, timing, rate and formulation of Bti applications) might modify or superimpose Bti-induced effects in natural wetlands. Even if these factors generally influence chironomid abundances, a Bti-induced reduction of this magnitude would additionally limit prey available for several aquatic predators such as newts, insects or fish.

4.2. Diet of larval newts

According to the stable isotope mixing model, chironomids constituted the major part of newts' diet when compared to more abundant zooplankton or Cladocera in particular (Fig. 5). A diet comprised of chironomids and zooplankton is in accordance with stomach content observations of *L. helveticus* larvae and the syntopic species *Ichthyosaura alpestris* and *Triturus cristatus* (Braz and Joly, 1994). However, stomach contents in Romanian *L. vulgaris* contained mainly Cladocera in quasi-permanent ponds (Covaciu-Marcov et al., 2010) and Asselidae in the Carpathian Mountains (Roşca et al., 2013). Mosquitoes were considered less important in the diet of crested and smooth newt populations (Bogdan et al., 2013) due to their peak occurrences after flooding events and the fast larval development (<three weeks) at temperatures above 15 °C (Becker et al., 2010). Chironomids are independent of flooding events and show continued availabilities with developmental times ranging from several months to more than one year (Armitage et al., 1995). Hence, chironomids depict an important and reliable insect food resource for predators in wetlands.

In contrast to our assumption, newt larvae seemed to prey on chironomids preferentially instead of feeding opportunistically as a result of higher availability of other prey. This is probably due to their high conspicuousness to optical predators associated with the benthic zone (Dolmen and Koksvik, 1983). *Lissotriton* newts effectively suppressed chironomid emergence rates but consumed 22% less chironomids in Bti-treated mesocosms. Thus, instead of switching to alternative prey in the Bti treatment, newts might have eaten less in terms of actual biomass. This is surprising as at least *L. vulgaris* adults are considered generalists with high trophic prey diversities (Covaciu-Marcov et al., 2010; Griffiths, 1986; Roşca et al., 2013). Larvae of the genus *Lissotriton* change their feeing behavior during ontogeny ranging from small crustaceans to larger insects (predominantly chironomids) (Kuzmin, 1991) which can both be accounted for with the analysis of stable isotopes.

Newt larvae neither exposed to Bti nor predator had the most specialized diet while these factors caused increasing niche widths (Fig. 6). A trophic niche expansion can indicate a higher diversity of used resources for instance within the group of zooplankton that have different feeding strategies ranging from predatory copepods to filter-feeding daphnids. Wider niches might also reflect suboptimal environmental conditions due to increased stress triggered by the presence of a contaminant (Bayona et al., 2014), food limitation or inadequate food quality (Karlson et al., 2018).

4.3. Development of larval newts

The development of *L. helveticus* individuals indicated a trend towards slightly smaller metamorphs in Bti treatments, albeit the difference was not significant at a 0.05 significance level (Fig. 7). Amphibian body size at metamorphosis is determined by food intake experienced during the later developmental stages (Alford and Harris, 1988; Leips and Travis, 1994) which is why reduced chironomid prey can be reflected in smaller newts. However, sublethal Bti concentrations induced early signs of detoxification and oxidative stress in tadpoles causing additional stress for amphibians (Allgeier et al., 2018; Lajmanovich et al., 2015). Thus, reduced growth can be a result of the prey limitation but also of a higher energetic investment in detoxification mechanisms (Wright and Wright, 1996).

Freshly metamorphosed *L. helveticus* were recorded to reach mean body weights of 100 mg (Schlüpmann et al., 1996). As this is less than observed in our mesocosm (190 mg–205 mg) we cannot exclude that a potential food oversupply in the development experiment led to an underestimation of effects. However, natural wetland conditions, such as higher habitat complexity, lower invertebrate densities and the presence of competitors, might increase the adverse effects on newt development in the field. Reduced sizes during the aquatic phase show several disadvantages such as lower survival probabilities during their juvenile period and higher vulnerability towards predation by fish, beetles or dragonflies (Formanowicz, 1986; Smith, 1987).

4.4. Trophic interactions in Bti-treated food webs

Depending on wetland community structures, dragonflies and newts are engaged in intraguild predation that can be intensified when dragonflies as intraguild predators utilize resources of newts as the intraguild prey (Holt and Polis, 1997; Yurewicz, 2004). In our experiment, the dragonfly *A. cyanea* was 27% more lethal to newt larvae when they were exposed to Bti. Interestingly, *A. cyanea* had a minor effect on newt survival in control mesocosms (Fig. 3) even though dragonflies are one of the most efficient predators in seasonal wetlands (Van Buskirk and Schmidt, 2000; Wilbur and Fauth, 1990). Amphibian tadpoles recognize predators mainly through chemical cues (Relyea and Mills, 2001). To escape predation risk they develop physiological modifications designed to move faster (deep tails, small bodies) or adapt a anti-predator behavior by decreasing their activity (Relyea, 2001; Van Buskirk and Schmidt, 2000). Larval *L. helveticus* showed increasing refuge use (resulting in lower activity rates) to reduce detection and encounters by predatory brown trouts (Orizaola and Braña, 2003).

While the exposure to predator cues resulted in adaptive behavior or physiology (Reeves et al., 2011; Van Buskirk and Schmidt, 2000) the presence of actual predators reduced amphibian survival significantly (Relyea, 2018; Van Buskirk and Schmidt, 2000). Nymphs of *Anax junius* reduced tadpoles by 30 and 92% irrespectively of their potential anti-predator behavior (Relyea, 2018). The increased amphibian mortality when dragonflies interfere with Bti is in line with a mesocosm study by Pauley et al. (2015) where Gray treefrog (*Hyla versicolor*) tadpoles experienced even higher mortalities of >80%. However, dragonflies reduced their survival by around half even when not exposed to any insecticide (Pauley et al., 2015). The mechanism behind this synergistic effect was not explicitly studied but did not involve intraguild predation as herbivores treefrogs are no competitors to dragonflies.

High chironomid prey abundances in the controls may have facilitated the coexistence of newts and dragonflies by suppressing intraguild predation due to the increased availability of valuable alternative prey. Both predators reduced overall chironomid emergence suggesting that chironomid larvae were not only eaten preferably by newts but also by dragonfly nymphs. Additionally, the increased growth rate of dragonfly nymphs in the control (Fig. 4) indicated that they were well-fed which facilitates the coexistence with newt larvae. Bti-affected body sizes were already found in *Erythemis simplicicollis* nymphs which were smaller after repeated Bti applications (Painter et al., 1996). Furthermore, the correlation between the presence of more preferable alternative prey and decreased predation rates was recently shown for amphibian larvae and a mosquitofish predator in outdoor mesocosms (Preston et al., 2017).

The much higher intra-population variability in the isotopic niche of Bti-treated *A. cyanea* reflects a high diet diversification and trophic diversity that may be the result of the predation on larval newts. The estimation of dragonflies' diet proportions was not possible because their isotopic composition was lower than the one of their potential prey (Table 2). In terms of isotopic composition a high trophic level is displayed in enriched δ^{15}N values relative to the respective prey, while δ^{13}C values depend on the source of dissolved carbon in the water and change little among the food chain (Peterson and Fry, 1987). Late-instar dragonfly nymphs presumably did not assimilate enough tissue during the experiment and thus reflect the signature of putative prey in the permanent pond of their origin. Moreover, Seifert and Scheu (2012) proposed that large-bodied Odonata larvae integrate temporal variations in prey δ^{13}C signatures including periods with depleted prey signatures, especially in autumn and winter. Hence, dragonfly nymphs in the late-instar would need more time to incorporate isotopic signals prevailing in our mesocosms.

4.5. Direct effect of Bti on amphibians

The exposition to Bti as the only stressor did not lead to acute toxic effects on newts which is consistent with the majority of studies on vertebrates (Boisvert and Boisvert, 2000; Lacey and Merritt, 2003). Nevertheless, Lajmanovich et al. (2015) recently detected increased mortality of a South American frog after applying a liquid Bti formulation (Introban®). On the contrary, ten times the field rate of VectoBac®WG did not reveal mortality in tadpoles of the common frog *Rana temporaria* (Allgeier et al., 2018). However, Bti exposure induced behavioral changes in tadpoles that resulted in decreased swimming activity (Junges et al., 2017) which has already been detected with other insecticides (Relyea and Edwards, 2010). While activity reduction is a common behavioral response to avoid short-term predation (Relyea, 2001), a reduced activity induced by contaminants has no clear benefits for tadpoles but rather long-term implications such as less foraging time, smaller sizes and longer time to metamorphosis (Relyea, 2007). Moreover, sublethal effects induced by contaminants often increase susceptibility to predation in amphibians for instance by inhibiting the development of an antipredator behavior (Hayden et al., 2015; Polo-Cavia et al., 2016; Reeves et al., 2011). Changes in in larval newts' behavior induced by Bti would be responsible for the reduced dietary intake and the higher vulnerability to predation.

4.6. Importance for wetlands

Natural wetlands can be packed with additional stressors for amphibian larvae not predicted from our experiment such as several predators, competition or various chemical contaminants that often have interactive effects (Boone et al., 2007; Rohr et al., 2004). Thus, the observed effects on newt survival can even be more pronounced when Bti further decreases essential chironomid food resources as they often represent the most dominant taxa in wetlands (Allgeier et al., 2019; Hershey et al., 1998; Leeper and Taylor, 1998). Adult and larval amphibians largely contribute to habitat interconnectivity due to their biphasic life cycle and to several ecosystem services including the biological control of mosquitoes, pests and related diseases (Hocking and Babbitt, 2014). Hence, less amphibian metamorphs leaving Bti treated ponds will have implications on entire ecosystems for instance by reducing the transfer of biomass and energy across ecosystem boundaries (Gibbons et al., 2006).

Our results are of particular concern because large parts of Bti treated areas all across Europe are situated in national and international nature conservation sites (RAMSAR, Natura 2000) (Allgeier et al., 2019; Lagadic et al., 2014; Land and Miljand, 2014). These areas intrinsically focus on the preservation of biodiversity and the conservation of species particularly worthy of protection such as amphibians. Moreover, wetlands are valuable parts of our landscape as they largely contribute to ecosystem services and freshwater biodiversity (Biggs et al., 2017; Mitsch et al., 2015) and should be managed in a sustainable manner especially in light of the upcoming biodiversity extinction crisis (Ceballos et al., 2015).

5. Conclusion

Despite the increasing use of Bti in mosquito control (van den Berg et al., 2012), adverse effects on wetland ecosystems are still a matter of debate especially when it comes to long-term and indirect effects on the non-target biodiversity (Land and Miljand, 2014; McKie and Goedkopp, 2010). This study highlights the crucial role of non-target chironomids in the food webs of seasonal ponds where Bti increased vulnerability of newt larvae to predation. Besides from using Bti in mosquito control, the bacterium Bt is also extensively applied over forests to eradicate other pests such as phytophagous caterpillars (UBA and BfN, 2018) resulting in a ubiquitous occurrence of Bt in many amphibian habitats. Taking into account that amphibians suffer from worldwide population declines (Stuart et al., 2004), Bt-based pest control may pose an additional threat to amphibian populations already faced with multiple stressors in their habitats.

In future, the control of vector mosquitoes will most definitely gain in importance with regard to human health aspects. Since most vectors are container-breeding species their control requires small-scale strategies rather than large-scale Bti treatments of wetlands often integrated in nature protection areas. Thus, the application of Bti in habitats relevant for amphibian and nature conservation such as the Camargue (France) and the Upper Rhine Valley (Germany) should be reconsidered.

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Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

CRediT authorship contribution statement

Stefanie Allgeier: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft. **Anna Friedrich:** Conceptualization, Methodology, Investigation. **Carsten A. Brühl:** Conceptualization, Writing - review & editing, Supervision.

CRediT authorship contribution statement

Stefanie Allgeier: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft. **Anna Friedrich:** Conceptualization, Methodology, Investigation. **Carsten A. Brühl:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.05.358.

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