Anthropogenic pressures on riparian communities: insights from genomic tools

by

Nina Röder

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Thesis examiners:

Prof. Dr. Klaus Schwenk, RPTU University of Kaiserslautern-Landau

Prof. Dr. Martin H. Entling, RPTU University of Kaiserslautern-Landau

Prof. Dr. Markus Pfenninger,

Senckenberg Biodiversity and Climate Research Centre

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I dedicate this thesis to all who pursue science with childlike curiosity. To all who choose not to take the easy path, drawn instead to the challenge. To all who continually question their own conclusions and never forget that every piece of knowledge carries with it a measure of uncertainty.

Content

Preamble

DNA is the universal code of life – a molecule that literally covers our planet. It can be found in the air, in the deepest parts of the ocean, hidden in the soil, or trapped in ice. Every organism carries it: millions of data points telling stories of inheritance, survival, and history. DNA is the manual of life, a manual we only recently discovered when Swiss chemist Friedrich Miescher first isolated it in 1869. Since then, our ability to read and understand these biological manuals has grown exponentially. The first complete genome was sequenced in 1976, marking the beginning of a new era where we started to “download” and interpret the full genetic blueprints of organisms. Yet, this was only the start of our journey into the vast complexity of life’s code.

We now stand at a point where we can read these biological manuals not just to understand single organisms, but to map the complex and dynamic webs they form in nature. In this thesis, I explore how humans impact riparian communities – the dynamic biocoenoses thriving where land and water meet. Instead of relying solely on traditional methods, I use the information carried within DNA to understand how community compositions, trophic interactions, and evolutionary processes shift under human influence. Through genomic tools, we gain new perspectives on how anthropogenic pressures reshape life at the water’s edge, often in ways invisible to the observer but recorded in the DNA that flows through every living being.

Summary

Riparian ecosystems, situated at the interface of aquatic and terrestrial environments, support exceptionally high biodiversity and provide critical ecosystem functions, including nutrient cycling, habitat provision, and cross-boundary energy transfer. Despite their ecological importance, these systems face increasing anthropogenic pressures such as pollution, hydrological alteration, and habitat degradation, which threaten their biodiversity and functioning. Understanding how riparian communities respond to such stressors requires an integrative perspective that considers three key dimensions: organismal composition, ecological interactions, and evolutionary processes.

This thesis combines genomic approaches with stress-ecology experiments to investigate how riparian invertebrate communities respond to human-induced stressors across these dimensions. In *Chapter I*, community metabarcoding was applied to assess how exposure to the mosquito control biocide *Bacillus thuringiensis israelensis* (Bti) alters chironomid emergence dynamics. A novel direct PCR metabarcoding workflow was developed, providing a cost-effective and reliable tool to capture community composition. Results indicated that altered emergence patterns were less driven by strong taxon-specific sensitivities than initially hypothesized.

In *Chapter II*, gut content metabarcoding of riparian spiders was used to evaluate how hydrological drought reshapes trophic interactions across aquatic-terrestrial boundaries. A bleach-wash protocol was validated to remove external DNA contamination, enabling reliable dietary analyses. Results uncovered cryptic diversity within *Tetragnatha* spiders and showed species-specific responses to drought, suggesting that shifts in ecological niches and competition, rather than changes in prey availability, structured riparian predator populations.

In *Chapter III*, an experimental evolution framework was applied to *Chironomus riparius* populations chronically exposed to Bti and copper. Population genomic analyses revealed pollutant-specific signatures of selection, linked to midgut cell repair in Bti-exposed populations and to detoxification pathways under copper exposure. These findings provide mechanistic insights into how genomic adaptation can occur within only a few generations, even before consistent phenotypic changes are detectable.

Taken together, the findings demonstrate that genomic tools can substantially refine our understanding of riparian communities by resolving hidden diversity, clarifying mechanisms underlying ecological interactions, and uncovering genetic pathways of adaptation. Beyond methodological advances, this thesis highlights the interconnectedness of ecological and evolutionary processes in shaping community resilience under anthropogenic stress. By integrating composition, interaction, and evolution, the work provides a framework for anticipating how riparian ecosystems, and other connected habitats, may persist, reorganize, or decline under ongoing global change.

Introduction

Riparian ecosystems represent ecologically rich and dynamic transition zones between terrestrial and aquatic environments, supporting disproportionately high levels of biodiversity relative to their spatial extent. These ecosystems play a pivotal role in maintaining ecosystem functions, including nutrient cycling, habitat provision, and the regulation of water quality (Singh et al., 2021). Despite their importance, riparian ecosystems have experienced significant degradation over the past century due to changes in river management, land use practices, and increasing anthropogenic pressures (Beechie et al., 2010). Given the diverse range of critical ecosystem services they provide, there is a critical need to understand and protect riparian ecosystems from further degradation (Gilvear et al., 2013). Studying the ecological processes that sustain these ecosystems is therefore essential for developing effective strategies to ensure their resilience in the face of environmental change.

Within riparian ecosystems, aquatic and terrestrial environments are interconnected not only through abiotic processes such as runoff, soil erosion, and sediment deposition during flooding events, but also through the activities of the organisms that inhabit these systems. Riparian communities play a critical role in biologically linking aquatic and terrestrial habitats, with many organisms actively transporting energy and nutrients between environments and fulfilling ecological roles in both habitats across their life stages (Polis et al., 1997). A prominent example of such organisms is the group of merolimnic insects, which develop as aquatic larvae before emerging as terrestrial adults. These insects play a vital role in supporting aquatic and terrestrial food webs, contributing significantly to the energy budgets of various predators, including fish, birds, bats, lizards, and spiders (Baxter et al., 2005). However, the ability of riparian communities to maintain these critical ecological linkages is increasingly challenged by a range of anthropogenic pressures, such as invasive species, hydromorphological changes, and anthropogenic pollutants (Schulz et al., 2024), that threaten the integrity and functioning of riparian ecosystems.

For this thesis, I define three dimensions that shape riparian and other ecological communities: (1) organismal composition, (2) ecological interactions between these organisms, and (3) evolutionary processes, dimensions that I use throughout this thesis to analyze how riparian communities respond to environmental change.

Anthropogenic pressures can directly affect any of these three dimensions, thereby threatening the integrity of riparian ecosystems (Figure 1). However, these dimensions do not respond independently to environmental conditions; rather, they are highly interconnected and influence one another. For example, prey community composition determines resource competition among predators (e.g., Garvey et al., 2022) and can trigger evolutionary processes such as niche partitioning under strong competition. Such feedbacks between ecological and evolutionary processes are characteristic of eco-evolutionary dynamics (Post & Palkovacs, 2009). Similarly, ecological interactions such as parasitism and mutualism can shape which species are present in a community (Hay et al., 2004; Skelton et al., 2016) and drive evolutionary changes through co-evolution. Likewise, evolutionary processes like local adaptation to flow velocities can give rise to traits that affect which species can establish in a community and how they interact with others.

A unique aspect of riparian communities is that anthropogenic stressors affecting any of these dimensions within aquatic habitats can induce responses across all dimensions in adjacent terrestrial habitats, and vice versa. For example, anthropogenic stress can reduce aquatic insect emergence, thereby decreasing prey availability for terrestrial predators and altering predator-prey dynamics with merolimnic insects (Jakob & Poulin, 2016; Kraus et al., 2014). As a consequence, terrestrial predators are often forced to switch to a more terrestrial diet (Gergs et al., 2014; Poulin et al., 2010; Trevelline et al., 2018). While earlier research has largely framed aquatic systems as receivers of terrestrial inputs, with limited attention to their reciprocal role as donors of resources to terrestrial ecosystems (Schulz et al., 2015), this thesis explicitly focuses on the reverse pathway – from water to land. By doing so, it addresses a critical but still underexplored aspect of cross-ecosystem connectivity (Schulz et al., 2024). The sensitivity of this complex connectivity makes studying the effects of anthropogenic pressures in riparian ecosystems particularly relevant and underscores the need to consider these dimensions in an integrated manner.

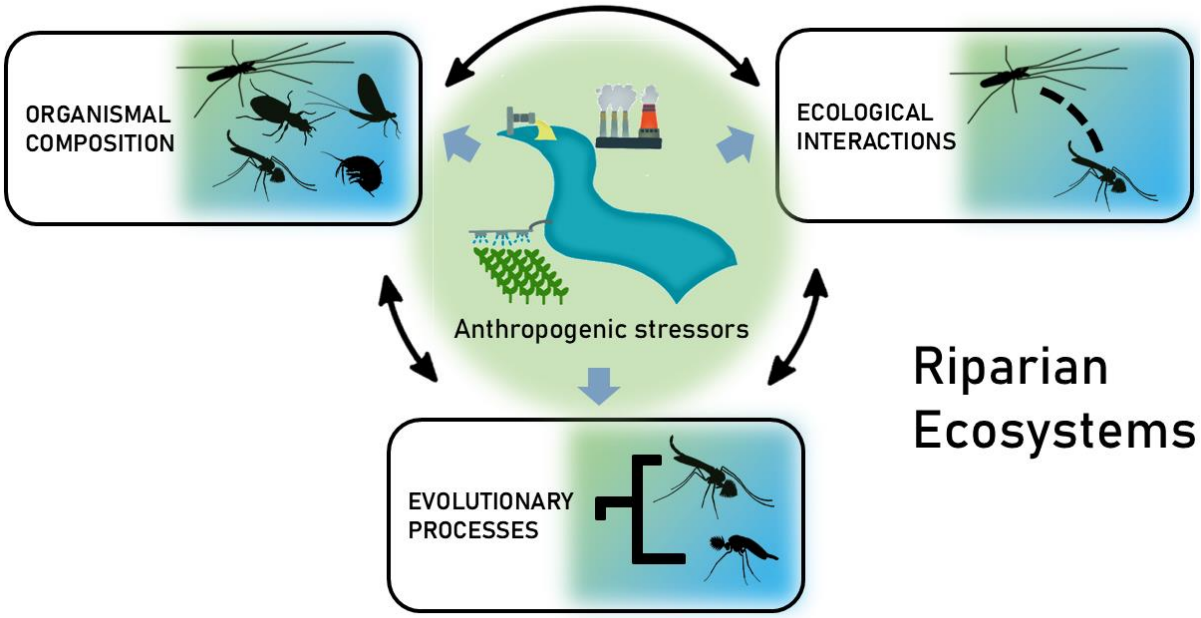

Figure 1 Conceptual framework illustrating three dimensions shaping riparian ecological communities: (1) organismal composition, (2) ecological interactions, and (3) evolutionary processes. Blue arrows indicate how anthropogenic pressures can directly impact each dimension, while black arrows indicate how changes in one dimension can influence the others. Together, these pressures and cross-dimensional feedbacks may compromise the integrity of riparian ecosystems.

Thesis outline

In this thesis, I focus on the three dimensions that collectively determine how riparian communities function and respond to anthropogenic pressures: organismal composition, ecological interactions, and evolutionary processes. Each chapter investigates one of these dimensions using an example system to elucidate mechanisms of riparian community responses to human-induced stressors (Figure 2). Given the interdisciplinary scope of this work and the high methodological diversity it entails – ranging from method development to micro- and mesocosm studies – I include a *Methodological approaches* chapter to outline the rationale and procedures underlying the studies presented in the thesis. *Chapter I* then examines how exposure to a model biocide for mosquito control alters the community composition of riparian midges, providing insights into the impacts of an example anthropogenic pollutant on riparian community structure. *Chapter II* focuses on trophic interactions between riparian predatory spiders and merolimnic insect prey under simulated hydrological drought conditions, illustrating how changes in water availability influence cross-ecosystem food webs. *Chapter III* analyses evolutionary responses in a riparian midge

species triggered by chronic exposure to two example anthropogenic pollutants, one biocide and one heavy metal, across multiple generations, highlighting the potential for rapid evolutionary change in response to environmental stressors. Collectively, these chapters address the following research questions:

I. *How does biocide application for mosquito control affect the community composition and diversity of riparian midges?*

II. *Does hydrological drought alter trophic interactions between riparian predatory spiders and emergent aquatic insects as their prey?*

III. *What are the genomic responses in a riparian midge population to chronic exposure to two pollutants, a biocide and a heavy metal, over several generations?*

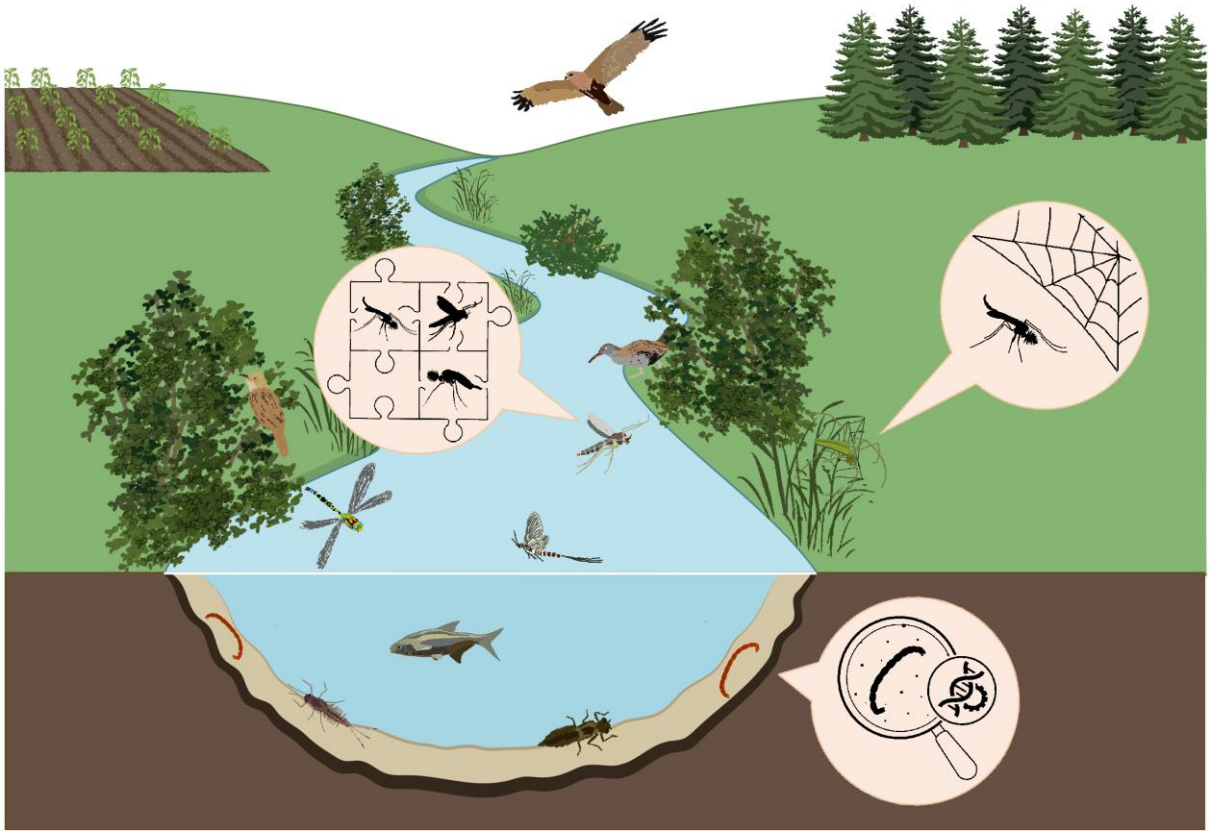

Figure 2 Schematic representation of a riparian ecosystem highlighting the three dimensions examined in this thesis: organismal composition, ecological interactions, and evolutionary processes. The diagram indicates how these are studied in example systems to reveal mechanisms of riparian community responses to anthropogenic pressures: community composition of emergent chironomids, trophic interactions between aquatic insects and riparian spiders, and evolutionary processes in chironomids triggered by pollutant exposure during the larval stage.

Methodological approaches

Study system: Merolimnic insects & riparian spiders

Riparian predatory spiders and merolimnic insect prey represent ecologically connected groups across aquatic and terrestrial boundaries, making them well-suited to investigate the effects of anthropogenic stressors on riparian communities. Merolimnic insects are often highly abundant and taxonomically diverse within freshwater systems, with their emergence patterns and community composition reflecting environmental conditions and stressor impacts (Nash et al., 2023; Schmidt et al., 2013). Among merolimnic insects, the family Chironomidae (Figure 3) is particularly ecologically important, as chironomids are frequently comprising a substantial portion of emergent biomass (Armitage et al., 1995). Their high species richness and functional diversity, alongside reported taxon-specific differences in susceptibility to environmental stressors (Armitage et al., 1995; King & Richardson, 2002; Lencioni et al., 2012; Serra et al., 2016), make chironomids an ideal target group for assessing how anthropogenic stressors can alter riparian communities.

Riparian spiders, such as long-jawed orb-weavers of the genus *Tetragnatha* (Figure 3), are often specialized to live and hunt in near-shore areas, where chironomids and other merolimnic insects typically constitute a high proportion of their diet (Bollinger et al., 2023; Krell et al., 2015). Environmental stress can lead to shifts in prey availability and, consequently, in the diets of riparian spiders (e.g., Gergs et al., 2014; Huszarik et al., 2024), making them useful indicators for assessing cross-boundary consequences of anthropogenic stressors in aquatic environments.

Figure 3 Merolimnic insects (left: *Chironomus riparius*) and riparian spiders (right: *Tetragnatha extensa)* are the study organisms in this thesis. Note that the two individuals are not shown at the same scale.

Chapter I – Community metabarcoding of aquatic insect emergence

Investigating the effects of anthropogenic stressors on natural chironomid communities presents significant challenges due to their high taxonomic diversity and dynamic responses to environmental fluctuations. Additionally, morphological identification of chironomids at lower taxonomic levels is time-consuming and requires (scarce) expert knowledge, as it relies on the examination of minute and often damaged or missing diagnostic traits (e.g., genitalia, antennae, setae patterns and wing venation) that are often difficult to interpret or to distinguish among cryptic species (cf. Chimeno et al., 2023; Ekrem et al., 2010; Gadawski et al., 2022). To overcome these limitations, we employed DNA metabarcoding, which enables high-resolution taxonomic identification without relying on morphological expertise. However, community metabarcoding can be costly and time-intensive, partially due to the DNA extraction step. Therefore, we first developed and tested a direct PCR metabarcoding approach, omitting the laborious extraction step by applying dissolved ground tissue powder directly to PCR.

In the second part of this chapter, we applied this approach to emerged chironomids collected during a previously conducted experiment at the Floodplain Pond Mesocosm (FPM; Manfrin et al., 2023) facility in spring and summer 2020. In this experiment, half of twelve artificial ponds were treated with our model biocide Bti which is regularly used for mosquito control (Brühl et al., 2020) and can have negative effects on non-target organisms such as other Diptera (e.g., Theissinger et al., 2019). In this experimental context, Kolbenschlag et al. (2023a) reported a time-dependent effect on chironomid emergence, with chironomids representing the dominant family (88% of collected individuals). As previous studies have shown taxon-specific differences in chironomid susceptibility to Bti (e.g., Allgeier et al., 2019; Liber et al., 1998), we expected differential responses between the genera, with varying levels of susceptibility likely linked to ecological traits such as feeding strategy. We applied an integrative approach, combining DNA metabarcoding with Hierarchical Modelling of Species Communities (HMSC), allowing us to simultaneously consider ecological traits and phylogenetic relationships while investigating chironomid responses to Bti exposure.

Chapter II – Gut content metabarcoding of riparian spiders

This chapter analyses consequences of hydrological drought on trophic interactions between riparian spiders and emergent aquatic insects. Spiders use extra-oral

digestion, secreting digestive enzymes onto or into their prey and ingesting liquefied tissues, which leaves no morphologically identifiable remains in their gut or excrement (Cohen, 1995). However, prey DNA can remain detectable in spider guts for days to weeks (Uiterwaal & DeLong, 2020). DNA metabarcoding is a highly sensitive method well-suited for analyzing degraded prey DNA, but its sensitivity also makes studies prone to contamination. Such contamination may arise during sample collection through accidental transfer of DNA (Liu et al., 2020) or from 'natural' external contamination, where spiders carry environmental DNA on their body surfaces after moving through their habitats. To reduce the risk of contamination introduced during sampling, careful hand sampling has been recommended (King et al., 2008). To also address natural surface contamination, the spider cuticle can be decontaminated to remove or destroy external DNA, for example using a bleach wash (cf. Greenstone et al., 2012). In the first part of this chapter, we therefore evaluated the effectiveness of spider surface decontamination using bleach and compared levels of external DNA contamination between spiders collected via wet pitfall traps and by hand.

Building on these results, the second part of this chapter explores the effects of hydrological drought on spider-insect trophic interactions using gut content metabarcoding. The study was conducted at the Riparian Stream Mesocosm (RSM; Manfrin et al., 2023) facility, where low-flow conditions were applied over more than five weeks in early summer 2021 to simulate hydrological drought as an anthropogenic stressor. As intended, the low-flow treatment induced hydromorphological changes, including a decrease in discharge and a reduction in stream width (Rovelli et al., 2024). Examining its effects on riparian arthropod communities, Ogbeide et al. (2025) reported that, while total emerging insect abundance and biomass remained largely unaffected, low-flow conditions caused a 2.9-fold reduction in the total abundance of emerging Ephemeroptera, Plecoptera, and Trichoptera (EPT) and a 2.6-fold reduction in the abundance of the web-building spider *Tetragnatha*. From these experimental mesocosms, *Tetragnatha* spiders were collected and decontaminated using a bleach-wash protocol to remove external DNA before gut content extraction, as described in the first part of this chapter. In this second part of the chapter, we analyzed the changes in riparian spider diets induced by hydrological drought. The gut content metabarcoding data presented here form part of a broader dataset that will be included in a forthcoming manuscript. In this we will evaluate the combined effects of hydrological drought on the spiders' diet composition, their pesticide body burdens and

their fatty acid profiles to assess physiological and ecological responses in riparian spiders.

Chapter III – Experimental evolution of C. riparius *populations*

Anthropogenic pollutants enter riparian ecosystems through various human activities, including agriculture, pest control, mining, and urban infrastructure, and can exert strong selective pressures on aquatic invertebrates. Investigating the evolutionary effects of anthropogenic pollutants on non-biting midges in riparian ecosystems is crucial for understanding population resilience under environmental stress. While ecological effects are typically well documented, evidence for their potential to drive adaptive genomic responses remains limited. To address this gap, we utilized the midge *Chironomus riparius*, an established model species in ecotoxicology and evolutionary ecology, to assess genomic responses to chronic exposure to two example pollutants, a biocide and a heavy metal, under controlled conditions. Building on a previous long-term exposure experiment (Kolbenschlag et al., 2024; Pietz et al., 2025), where populations were subjected to the microbial larvicide Bti or the heavy metal copper across approximately eight generations, we analyzed allele frequency changes using pool sequencing to identify genome-wide signatures of selection. By comparing these genomic patterns between treatments and controls, we disentangled pollutant-specific selective pressures from neutral processes and identified biological processes overrepresented among affected genes.

Chapter I: Effects of biocide use on the community composition of riparian midges

a) Röder N, Schwenk K: “Direct PCR meets high-throughput sequencing – metabarcoding of chironomid communities without DNA extraction”, published in *Metabarcoding and Metagenomics* (2023)

b) Röder N, Stoll VS, Jupke JF, Kolbenschlag S, Bundschuh M, Theißinger K, Schwenk K: “How non-target chironomid communities respond to mosquito control: Integrating DNA metabarcoding and joint species distribution modelling”, published in *Science of the Total Environment* (2024)

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Research Article

Direct PCR meets high-throughput sequencing – metabarcoding of chironomid communities without DNA extraction

Nina Röder[1], Klaus Schwenk[1,2]

1 RPTU Kaiserslautern-Landau, iES – Institute for Environmental Sciences, Landau, Germany
2 LOEWE Centre for Translational Biodiversity Genomics, Senckenberg Biodiversity and Climate Research Centre, Frankfurt am Main, Germany
Corresponding author: Nina Röder (nina-roeder@posteo.de)

Abstract

Aquatic emergent insect communities form an important link between aquatic and terrestrial ecosystems, yet studying them is costly and time-consuming as they are usually diverse and superabundant. Metabarcoding is a valuable tool to investigate arthropod community compositions, however high-throughput applications, such as for biomonitoring, require cost-effective and user-friendly procedures. To investigate if the time-consuming and labour-intensive DNA extraction step can be omitted in metabarcoding, we studied the difference in detection rates and individual read abundance using standard DNA extraction versus direct PCR protocols. Metabarcoding with and without DNA extraction was performed with artificially created communities of known composition as well as on natural communities both of the dipteran family Chironomidae to compare detection rates, individual read abundances and presence-absence community composition. We found that the novel approach of direct PCR metabarcoding presented here did not alter detection rates and had a minor effect on individual read abundances in artificially created communities. Furthermore, presence-absence community compositions of natural chironomid communities were highly comparable using both approaches. In conclusion, we showed that direct PCR protocols can be applied in chironomid metabarcoding approaches, with possible application for a wider range of arthropod taxa, enabling us to study communities more efficiently in the future.

Key words: cytochrome c oxidase I (COI or COX1), DNA isolation, metabarcoding, next-generation sequencing (NGS), presence-absence community composition, read abundance, size-sorting

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Introduction

A key global challenge in the 21st century is the attempt to reverse the ongoing global biodiversity decline and to mitigate its consequences. DNA metabarcoding for large-scale monitoring of species rich and abundant groups, such as insects, is labour- and cost-intensive, but has become more and more achievable since its development in the early 2000s (Gostel and Kress 2022; Guo et al. 2022). One typical processing step during the preparation of insect metabarcoding samples is the extraction of DNA from homogenized bulk samples (Yu et al. 2012; Creer et al. 2016). Although it depends on the choice of protocols

or commercial kits and the available capacities to automate processes, DNA extractions are often costly and time-consuming.

Typically, a laborious DNA extraction step prior to PCR is performed in order to purify and concentrate DNA from tissue samples. However, tissue from different invertebrate species has been successfully added to PCR reactions without prior DNA extraction in so-called direct PCR (dPCR) approaches (e.g., flies and starfish: Wong et al. 2014; mosquitoes: Werblow et al. 2016; spider mites: Sakamoto and Gotoh 2017; ants: Wang et al. 2018). Through the development of high-performance enzymes and buffers, PCR has become more robust against inhibitors, reducing the need to eliminate them via elaborate DNA extraction procedures. In addition, current high-throughput sequencing (HTS) technologies, such as sequencing by synthesis or nanopore sequencing, are very efficient in detecting even trace amounts of DNA. It has further been shown that fragments amplified by dPCR can be used in HTS approaches (e.g., in generating barcodes for individual chironomids: Baloğlu et al. 2018 and individual ants: Wang et al. 2018), although, to the best of our knowledge, this has neither been conducted for animal communities nor systematically tested and compared to conventional approaches before.

To investigate the suitability of direct PCR protocols in insect community metabarcoding we compared purified DNA with direct application of homogenized tissue as a substrate for PCR and subsequent metabarcoding. We chose communities of the family Chironomidae, since they are a superabundant and very species rich group of merolimnic insects. Although chironomids are important components of biomonitoring programmes worldwide, their morphological identification to species level is very difficult. Therefore, they are suitable target organisms for developing efficient metabarcoding techniques. We subjected artificial (known species composition) and natural communities (highly variable species composition) to standard vs. dPCR metabarcoding using previously established cytochrome c oxidase I (COI) markers (Elbrecht and Leese 2017). We compared the detection rates and individual read abundances of artificial communities generated using the standard and the dPCR metabarcoding approach. Based on the findings of Elbrecht and Leese (2015), we expected prior size-sorting to have an effect on both detection rates and read abundance, as our chironomid specimens varied in their individual mass up to a factor of more than 20. Therefore, we created artificial communities both with and without prior size-sorting. Furthermore, we compared the community composition of highly diverse natural chironomid communities (exposed to environmental stress) comparing both methods. We finally investigated the influence of using different amounts of purified or amplified DNA on read abundance, to assess whether (dPCR) metabarcoding can also be used for quantitative estimates.

Materials and methods

Chironomid origin

Chironomidae were retrieved from artificial ponds of the Eußerthal Ecosystem Research Station (EERES; 49°15'14"N, 7°57'42"E) near Landau, Germany, in 2019 and 2020. Adult specimens were collected from passive emergence

traps once to twice a week during spring and summer. In 2020, the artificial ponds were simultaneously used to study the effect of the mosquito control agent *Bacillus thuringiensis israelensis* (Bti) on merolimnic insect communities (Kolbenschlag et al. 2023).

Size-sorting and tissue preparation

Chironomid samples were sorted into four different size categories with known average weight per specimen (cf. Sabo et al. 2002; Suppl. material 1), to account for differences in individual mass that occur within and among chironomid species. They were subsequently stored in 70% ethanol. For tissue preparation, samples were dried at 60 °C for 16 to 24 hours and then finely ground by a TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for 3 × 1 min using two stainless steel beads (2–3 mm diameter). PCR-grade water (1 ml) was added to each sample and thoroughly vortexed. The tissue-water mixes were frozen at -20 °C until further analysis.

Sanger-sequencing and identification of individual specimens

Size-sorting of natural chironomid samples led to cases with only one specimen per size group and sample (due to low sample size). All these single specimens were individually Sanger sequenced. We used a direct PCR approach following Wong et al. (2014; see section 'Metabarcoding') with either the primer combination LCO1490 & HCO2198 (5'-GGTCAACAAATCATAAAGATATTGG-3' & 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'; Folmer et al. 1994) or, additionally, C1-J-1751 & C1 N2353 (5'-GGAGCTCCTGACATAGCATTCCC-3'; Simon et al. 1994 & 5'-GCTCGTGTATCAACGTCTATWCC-3'; Lewis et al. 2005) to target overlapping COI sequences of 658 bp and 557 bp length, respectively. The obtained Sanger sequences were aligned and taxonomically assigned using the BOLD Identification System (IDS) for COI (Ratnasingham and Hebert 2007; last accessed on 09/02/2023) by querying against the Public Record Barcode Database for animal identification (Suppl. material 2).

DNA extraction

We used 550 µl of the tissue-water mix to purify DNA from each sample with two technical replicates following an adapted high salt DNA extraction protocol after Aljanabi (1997). As the adapted protocol is designed for DNA extraction from dried tissue, smaller amounts of double concentrated buffer solutions were used in the beginning to account for the excessive water in the tissue-water mixes. Briefly, 450 µl of 2× SEB and 100 µl of 20% SDS buffer were added to 550 µl of tissue-water mix. After splitting into two technical replicates, 5 µl of Proteinase K (10 mg/ml) were added to each sample followed by 1 h of incubation at 60 °C. 350 µl of 5 M NaCl were added and – after centrifuging for 30 min – 600 µl of the supernatant were transferred to a fresh tube. An equal volume of isopropanol was added and the samples stored at -20 °C overnight. Samples were then centrifuged for 20 min at 4 °C. The resulting pellet was washed with 70% ethanol, dried and finally resuspended in 25 µl 1× TE buffer. Extraction blanks were included to ensure data reliability.

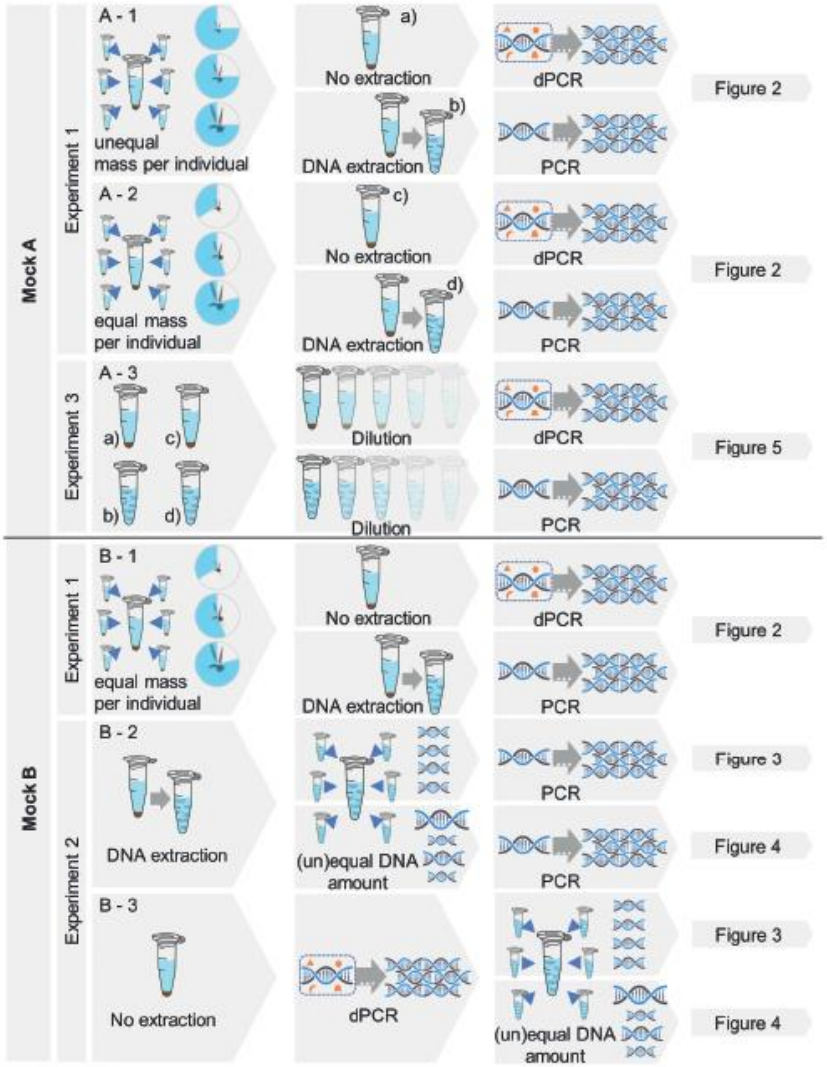

Figure 1. Schematic overview of the workflows during preparation of artificial chironomid communities. Two different sets of chironomid specimens (Mock A and Mock B) were analysed using different approaches of standard and direct PCR metabarcoding, to study the effect of direct PCR or DNA extraction protocols on detection rates and individual read abundances. Experimental setups included analysing the effects of variable mass per individual and different mock compositions (Experiment 1), variable or similar amounts of different input materials (Experiment 2), and variable concentrations of mock material to assess sensitivity (Experiment 3). Mocks were created by pipetting tissue-water mixes (Mocks A-1, A-2 and B-1), purified DNA extracts (Mock B-2) or PCR products (Mock B-3) of individual specimens. A subsample of Mocks A-1 (a, b) and A-2 (c, d) was used for sequential dilution (Mock A-3). Pie charts indicate if artificial communities were created under a size-sorting scenario (aiming for even masses per individual) or under a non-size-sorting scenario (where individual masses naturally vary). Purified DNA extracts were subjected to PCR, while non-purified tissue-water mixes were used in dPCR approaches. The labels 'Figure 2' to 'Figure 5' within the figure refer to the corresponding graphical representations of the results.

Artificial community composition

To assess the detection rates and specimen-specific read abundances under different metabarcoding approaches (Experiments 1–3), we created artificial communities with known composition of different chironomid species. Tissue-water mixes of individual chironomid specimens were selected based on the specimens' COI sequences. We aimed for taxonomically diverse artificial communities, while still being able to distinguish between specimens by their COI sequences targeted in the metabarcoding approach. We selected tissue-water mixes of 16 specimens from 2019 with at least 1.5% dissimilarity in the targeted region to create one artificial community ("Mock A", Suppl. material 2). In a second approach, we used tissue-water mixes of 13 specimens from the 2019 sampling campaign with at least 13% dissimilarity in the target region ("Mock B", Suppl. material 2) to investigate the effect of varying amounts of DNA input in detail. For a schematic overview of the preparation of mock communities see Fig. 1.

Experiment 1: Effect of size-sorting and tissue preparation on read abundance of specimens in artificial communities

Mock A was used to assess the effect of size-sorting (prior to sample preparations) and two different metabarcoding approaches i.e., with and without DNA extraction, on read-abundance per species. Each of the 5 replicates of Mock A were created by pipetting either the same amount of tissue-water mix (34 µl per specimen, concentrations varying between 0.0001 and 0.0014 mg tissue per 1 µl) simulating a "without size-sorting" scenario (Mock A-1, n = 5) or an adapted amount of tissue-water mix (4–116 µl) with approx. 0.006 mg tissue per each of the 16 specimen (Mock A-2: "with size-sorting" scenario, n = 5). For DNA extraction of the resulting tissue-water mixes, we followed the approach described in section 'DNA extraction' (but using only 495 µl tissue-water mix, the rest was needed for direct PCR applications). We further ran a size-sorting plus dPCR approach with Mock B (Mock B-1; n = 3, see Experiment 2 for details) to test if results are consistent when different specimens are used.

Experiment 2: Effect of DNA input variation on read abundance of specimens in artificial communities

With Mock B we assessed the effect of DNA input variation, i.e., varying or equal amount per specimen of either total DNA (Mock B-2) or target fragments (Mock B-3), on read abundance per specimen. DNA was extracted and purified from the 13 individual specimens and quantified using a Qubit fluorometer (Qubit dsDNA HS Assay Kit, Thermo Fisher Scientific). Both equal (5 ng) and varying (0.7–5 ng) amounts of DNA per specimen were then pooled into artificial communities (Mock B-2; n = 4), the latter using each 1.2 µl of the purified DNA extract per specimen. In addition, direct PCR was performed with each of the specimens individually and target fragments were quantified using a Tapestation 4200 (D1000 DNA Screentape Analysis Kit; Agilent Technologies, Santa Clara, CA, USA). Consequently, we pooled either the same (14, 31, 50 or 70 ng) or varying (14–80 ng) amounts of target fragments per specimen into artificial communities (Mock B-3; n = 4) and subjected them to the second PCR for tagging and adding of Illumina adapters.

Experiment 3: Effect of DNA input dilutions on detection rates of specimens in artificial communities

To assess sensitivity of the different methods, we determined specimen detection rates with different dilutions of the mock communities created for Experiment 1, but using only three of the five replicates per artificial mock (Mock A-3; n = 3). Subsamples of artificial communities were sequentially diluted 1:2 and PCR success was checked on a 1.5% TBE agarose gel. Due to limited sample capacity, five to six dilutions were chosen based on the quality of resulting bands to cover a representative range of dilutions that still yielded in PCR success. Further, only one technical replicate was used. We used sequential dilutions of up to 1:32 or 1:64 of the original mock for those resulting from direct PCR with or without prior size-sorting, respectively. For mocks based on purified DNA we chose dilutions between 1:8 and 1:512 (with prior size-sorting) and 1:16 up to 1: 1024 (without prior size-sorting) dilutions of the original mock.

Experiment 4: Natural communities under environmental stress

To assess the applicability of the dPCR approach compared to common metabarcoding protocols on natural chironomid communities, we used a subsample (eight out of 12 artificial ponds, five out of 26 sampling dates, N = 40) of an ongoing ecotoxicological study. Half of the eight artificial ponds had been treated with the mosquito control agent Bti (for details see Kolbenschlag et al. 2023), the five sampling dates corresponded to a time period of 17 days in June 2020. The adult specimens sampled were size-sorted and their tissue prepared (see section 'Size-sorting and tissue preparation'). Tissue-water mixes of different size-groups were pipetted to achieve even masses per specimen in each sample. All samples were then analysed using both metabarcoding approaches, i.e., with and without DNA extraction.

Metabarcoding

We followed a two-step PCR metabarcoding approach using the primers BF2 & BR2 (5'-GCHCCHGAYATRGCHTTYCC-3' & 5'-TCDGGRTGNCCRAARAAYCA-3''; Elbrecht and Leese 2017) to amplify a 421 bp COI fragment. PCRs included negative controls and two technical replicates per sample. When purified DNA was applied, each of the two purified DNA extracts per sample served as source for one of the technical replicates. For the initial PCR, we used per reaction either 1 µl of purified DNA extract or 5 µl of tissue-water mix, added 2 µl 10× buffer (TaKaRa, Shuzo, Japan), 1.2 µl dNTPs (2.5 mM), 1 µl of each primer (10 µM) and 0.15 µl of Ex Taq DNA Polymerase (TaKaRa, Shuzo, Japan), and filled up with PCR-grade water to achieve a final reaction volume of 20 µl (Wong et al. 2014). For the second PCR, the first-PCR product was 1:20 diluted (to reduce the amount of primers) and 1 µl was used as template. Primers were replaced by the corresponding fusion primers, including Illumina adapters for sequencing (P5 or P7) and inline barcodes of different length for individual tagging of samples (Elbrecht and Steinke 2018). PCRs had following cycling conditions: 94 °C for 5 min, 42 cycles (12 instead of 42 cycles in the second PCR) of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 1 min, ending with 72 °C for 10 min (adapted

from Wong et al. 2014). PCR success was checked on a 1.5% TBE agarose gel. The target fragment concentration was quantified using a Tapestation 4200 (D1000 DNA Screentape Analysis Kit; Agilent Technologies, Santa Clara, CA, USA). For each sample, the PCR product was pooled into a library proportionally to the number of specimens to ensure equal sequencing depth per specimen over all samples. Final libraries were purified and size-selected with magnetic beads (ratio: 0.65×, SPRIselect, Beckmann Coulter, Bread, CA, USA), retaining mainly fragments >300 bp. Libraries were sent to an external laboratory (CeGaT, Tübingen, Germany) for 2 × 250 bp (v2) paired-end sequencing on a MiSeq Illumina system. Samples were included in four different libraries: Sequencing of the library containing Mock A resulted in a total of 13.763.845 reads, sequencing of the library with Mock B resulted in 12.047.150 reads in total. Sequencing of the two libraries containing the natural community samples resulted in 5.928.530 and 9.336.106 reads, respectively. Each of the latter two libraries contained all samples from two treatment and two control ponds.

Bioinformatic analysis

Bioinformatic analysis was performed following the JAMP approach (https://github.com/VascoElbrecht/JAMP, package version 0.77). In short, raw data were demultiplexed and adapters were removed. Consequently, paired end-sequences were merged using usearch (https://drive5.com/usearch, version 11.0.667), then primers were removed using cutadapt (https://github.com/marcelm/cutadapt, version 3.5). After quality filtering, where sequences beyond the target length of 421 ± 10 bp (cutadapt) and more than 1 expected error (usearch) were discarded, operational taxonomic units (OTUs) were clustered with a 3% radius using usearch and vsearch (https://github.com/torognes/vsearch, version 2.21.1).

In the case of Mock A, specimens were too similar in the target COI region to be identified by the OTU clustering approach, i.e., two specimens could be clustered into one OTU. Therefore, we used the dada2 pipeline (Callahan et al. 2016) for these samples to analyse them after demultiplexing and adapter removal. Sequences were filtered and trimmed, then error rates were learned for sample inference. Finally, paired reads were merged and chimeras removed, resulting in the final amplicon sequence variant (ASV) table. The resulting 1184 ASVs were compared to nt database (last updated on 20.06.2022) of NCBI using BLAST+ 2.12.0 and 881 putatively non-dipteran ASVs were identified (i.e., 123 non-dipteran insects, 14 non-insect arthropods, 75 vertebrate, 3 non-arthropod invertebrates, 553 non-matching and 113 non-animals, mainly fungi, bacteria and plants) that accounted for 2.8% of the total reads in the samples. A small database consisting of only the Sanger sequences of the 16 specimens used for Mock A was generated in order to identify the ASVs with the assignTaxonomy function (bootstrap value 80). In this way, ASVs that resulted from minor variations in the sequences due to sequencing errors could be assigned to the corresponding specimen (OTU clustering). None of the putative non-dipteran ASVs were matched to one of the Sanger sequences. Of the putative dipteran ASVs, 254 ASVs were matched to one of the Sanger sequences, accounting for 99.96% of the reads, 49 ASVs were not matched and discarded. We compared the assigned ASVs with the respective Sanger sequence and discarded

additional 21 ASVs (representing 0.03% of reads), which differed more than 1% (genetic distance) from the respective Sanger sequence. In this way, 1–27 ASVs were clustered into one of the 16 OTUs, each representing a specimen in Mock A. Finally, the mean numbers of reads (based on two technical replicates) for the 16 OTUs were calculated.

Data preparation

Mock B

Each of the 13 most abundant OTUs over all Mock B samples matched with one of the Sanger sequences of the 13 individuals (100% identity). In each sample, 96.7 to 99.8% of the reads were assigned to the 13 OTUs, the rest of the reads were mainly associated with spurious non-chironomid DNA. Except for one sample, where one of the replicates showed a contamination by another OTU (9.2% of reads) and therefore only 89.4% of the reads were assigned to the 13 OTUs. However, the number of reads per OTU was in the same range as in the other technical replicate, thus the replicate was not discarded. We calculated the mean number of reads (based on two technical replicates) for the 13 relevant OTUs; all other reads/OTUs were deleted.

Natural communities

The 160 OTUs resulting from bioinformatic processing were compared to nt database (last updated on 20.06.2022) of NCBI using BLAST+ 2.12.0 and 41 putatively non-dipteran OTUs were discarded (i.e., 7 non-dipteran insects, 7 non-insect arthropods, 1 vertebrate, 1 parazoa, 13 non-matching and 12 non-animals, mainly fungi, bacteria and plants). Negative controls showed no sign of cross-contamination among samples as the sum of reads per negative control was always low (< 112 reads, around 10 reads on average; all non-control samples contained > 1500 reads, around 22.700 on average). However, we detected 209 low-read false positives in the negative control samples (95.7% of them with less than 10 reads) presumably derived from tag switching. Additionally, read abundances per OTU were generally in the same order of magnitude in each of the two technical replicates per sample (i.e., less than 1 order of magnitude apart in 97.8% of the comparisons). In 95.9% of cases where only one of the two technical replicates contained reads for an OTU, the read numbers were spurious (i.e., < 10). Therefore, for artificial communities, read abundances were calculated as the average of both technical replicates only when both technical replicates contained reads. To further prevent false-positives, that can be introduced in any step of the metabarcoding procedure (for example spurious contamination or tag-switching, cf. Drake et al. 2021), we defined minimum sequence copy thresholds utilizing two technical replicates per sample and a relatively high number of negative controls. We set this threshold by identifying the percentage of average read abundance per OTU which allowed us to keep as many putative target reads (i.e., those with reads in both technical replicates) as possible while removing reads in the controls (false-positives) as far as possible. The threshold was set at 1% of the average read abundance per OTU in samples with a read abundance larger than zero in both technical replicates.

Read abundances below this threshold, i.e., that were 100 times smaller than the average, were set to zero. In this way, 91.4% of reads in all negative controls were removed, while 97% of predominantly target reads (i.e., those with reads in both technical replicates) could be maintained. We assumed that OTUs with a read abundance larger than this specific threshold in both technical replicates indicated true presence of the species in a sample. Community compositions of natural communities were finally assessed based on presence-absence data and OTU sequences were taxonomically assigned to species level using the BOLD Identification System (IDS) for COI (Ratnasingham and Hebert 2007; last accessed on 14/02/2023) by querying against All Barcode Records on BOLD for animal identification. Five OTUs (out of 119) were not assigned to Chironomidae (3× Ceratopogonidae, 2× Chaoboridae) and therefore ignored in downstream analyses.

Statistical analysis

All statistical analyses were conducted in R version 4.2.0 (R Core Team 2019) using R Studio version 2022.07.1+554 (RStudio Team 2019). Plots were created using the packages dplyr version 1.1.0 (Wickham et al. 2021), ggplot2 version 3.4.1 (Wickham 2016), ggpmisc version 0.5.2 (Aphalo 2016) and ggpubr version 0.6.0 (Kassambara 2020). We used Hedges' *g* of the package effsize version 0.8.1 (Torchiano 2016) as a measure of the effect size for comparison between the read abundance variation in different taxa and the variation within taxa (derived from the two different methods) in Experiment 1. For analysing the difference in read abundance of specimens in artificial communities when using equimolar amounts of purified total DNA vs. target DNA fragments (Experiment 2), assumptions for parametric hypothesis testing were checked using the functions shapiro.test and leveneTest of the package car version 3.1-1 (Fox and Weisberg 2019). As assumptions were not met, the read abundances of the individual OTUs were statistically compared using Kruskal-Wallis rank sum test (kruskal.test), while the deviation from mean read abundances in both groups (purified total DNA vs. target DNA fragments) was statistically analysed using Wilcoxon rank sum test (wilcox.test). Further, we calculated the Pearson's correlation for linear regression analysis for analysing the effect on read abundance when using different amounts of purified total DNA and target DNA fragments (Experiment 2). For assessing the influence of the metabarcoding methods used for natural communities (Experiment 4), we conducted a Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) on the basis of Jaccard distance with 999 permutations using the function adonis2 and a correspondence analysis (CA) using the vegan package version 2.6-4 (Oksanen et al. 2019). To visualize the distribution of data, we utilized the ordiellipse function from the vegan package, which allowed us to draw ellipses based on the standard deviation of points.

Results

Bioinformatic analysis resulted on average in 22.299 (+/-4.068 SD) reads for Mock A samples (except one sample with 95.954 reads) and on average in 24.982 (+/-3.803 SD) reads for Mock B samples (except two samples with 54.942 and

38.809 reads, respectively). The natural community samples contained on average 163 (+/- 48 SD) reads per individual (except two samples with 6 and 31 reads per individual, resp., and one sample with 467 reads per individual).

Experiment 1: Effect of size-sorting and tissue preparation on read abundance of specimens in artificial communities

In total, 13 out of 16 specimens of Mock A and all specimens of Mock B were reliably detected in each replicate of every method. Three specimens of Mock A, corresponding to OTU_J, OTU_K and OTU_P, were detected in 97.5, 95 and 95% of the samples, respectively. For these three OTUs we found no reads in one of the two technical replicates in one or two samples. Variation in read abundance was higher between taxa (OTUs) within mock communities than within taxa comparing both metabarcoding approaches (Fig. 2B, D, F). The effect size Hedges' *g* indicates large differences between the two groups ($g > 0.8$) in each case. Pre-adjusting the mass per specimen did not level off this variation (compare "within mocks" read abundance variation in Fig. 2B, D). The variation

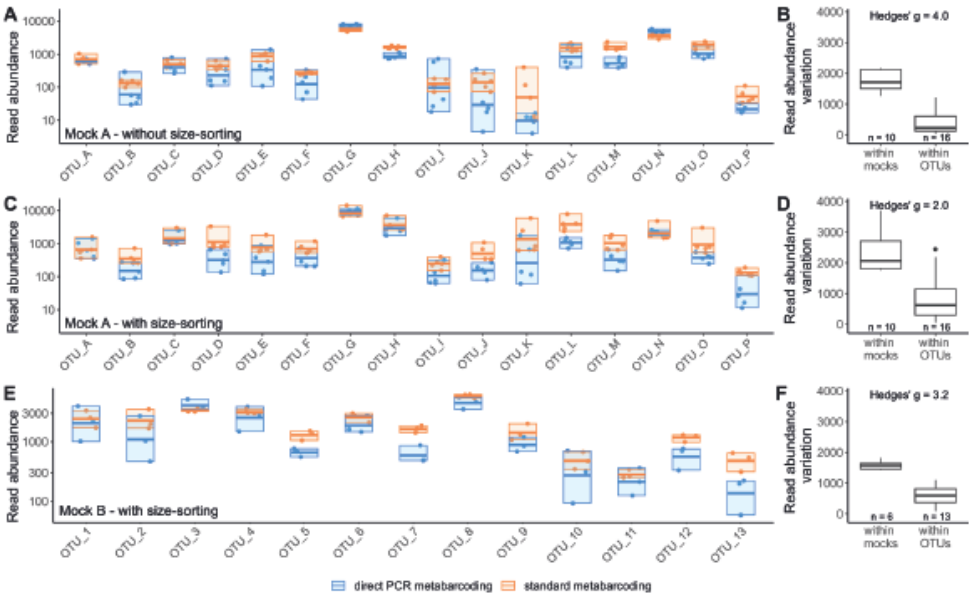

Figure 2. Effect of size-sorting and tissue preparation on individual read abundance. **A, C, E** show read abundances (logarithmic scale) per operational taxonomic unit (OTU) resulting from two different metabarcoding approaches (orange – purified DNA extracts, blue – direct PCR) with varying (**2A**) or equal (**2C, E**) mass per specimen from two different mock communities (Mock A, *n* = 5; Mock B, *n* = 3). Dots represent read abundances; means and ranges are indicated by vertical lines. **B, D, F** show boxplots illustrating variation in read abundance within mocks and within OTUs. Variation in read abundance is calculated as standard deviation of the compared values. The lower and upper hinges of boxplots correspond to the first and third quartiles. The upper whisker extends from the hinge to the largest value no further than 1.5 times the inter-quartile range from the hinge. The lower whisker extends from the hinge to the smallest value at most 1.5 times the inter-quartile range from the hinge. Data beyond the end of the whiskers, i.e., outliers, are plotted individually.

in individual read abundances exhibited substantial heterogeneity among the different taxa, which was observed in both analyses based on purified DNA and those based on dPCR (Fig. 2A, C, E).

Experiment 2: Effect of DNA input variation on read abundance of specimens in artificial communities

When using the same amount of purified total DNA per specimen, read abundance varied significantly between the different OTUs (Kruskal-Wallis rank sum test, $n = 4$, $p < 0.001$; Fig. 3, orange). Equalizing the amount of DNA fragments after the first PCR led to more even read abundances, with no significant differences between specimens (Kruskal-Wallis rank sum test, $n = 4$, $p = 0.42$; Fig. 3, grey). Deviations from mean read abundance were significantly higher when using the same amount of purified DNA per specimen as compared to when equalizing the amount of DNA fragments after the first PCR (Wilcoxon rank sum test, $n = 52$, $p < 0.001$). While there was no linear relationship between the amount of purified total DNA used in the PCR and read abundance in any of the four mock communities (Pearson's correlation, $R^2 = < 0.01$, DF = 11, $p = 0.83–0.93$; Fig. 4, orange), read abundance showed a positive linear correlation with the amount of target fragment used in the second PCR in all four replicates (Pearson's correlation, $R^2 = 0.74–0.94$, DF = 11, $p < 0.001$; Fig. 4, grey).

Experiment 3: Effect of DNA input dilutions on detection rates of specimens in artificial communities

When diluting the purified DNA extracts or tissue-water mixes prior to PCR, the reliability of OTU detection dropped in all approaches (Fig. 5). However, the de-

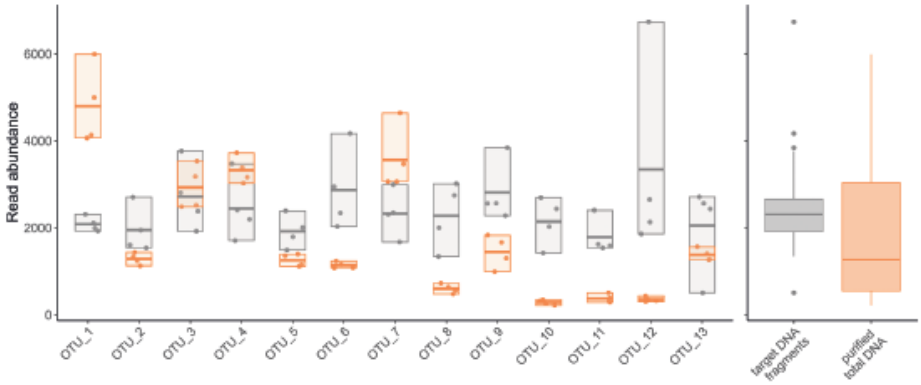

Figure 3. Individual read abundances resulting from equal amounts of purified total DNA or target DNA fragments. Read abundance of specimens in artificial communities ($n = 4$) after pooling equimolar amounts of purified total DNA (orange) or equal amounts of target DNA fragments (grey) per specimen. Dots represent read abundances, means and ranges are indicated by vertical lines. Overall read abundance per method is illustrated by boxplots on the right. The lower and upper hinges of boxplots correspond to the first and third quartiles. The upper whisker extends from the hinge to the largest value no further than 1.5 times the inter-quartile range from the hinge. The lower whisker extends from the hinge to the smallest value at most 1.5 times the inter-quartile range from the hinge. Data beyond the end of the whiskers, i.e., outliers, are plotted individually.

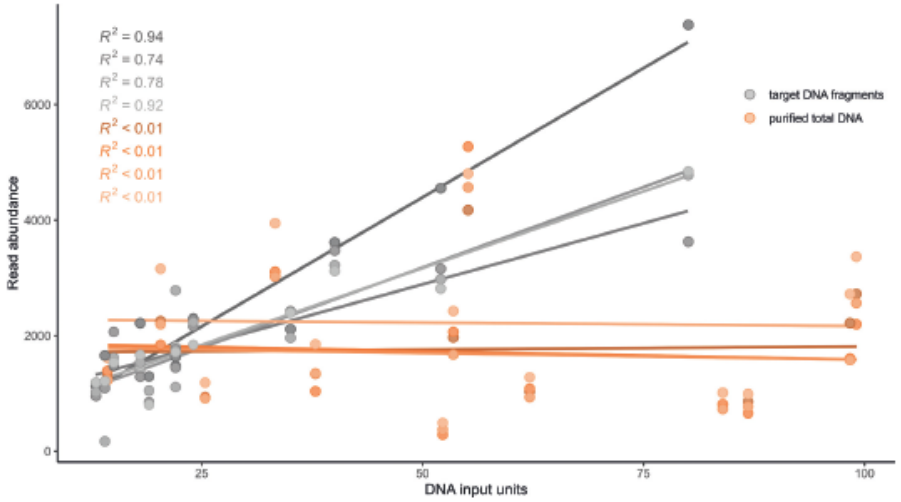

Figure 4. Individual read abundances resulting from varying amounts of purified total DNA or target DNA fragments. Read abundance of specimens in artificial communities when using different amounts of purified DNA (orange colours represent four mock communities) or different amounts of target DNA fragments (grey, four mock communities) per specimen for community pools. DNA input units correspond to 0.05 ng of purified total DNA and 1 ng of target DNA fragments. Shown are regression lines and multiple R^2 of the fitted linear regression models.

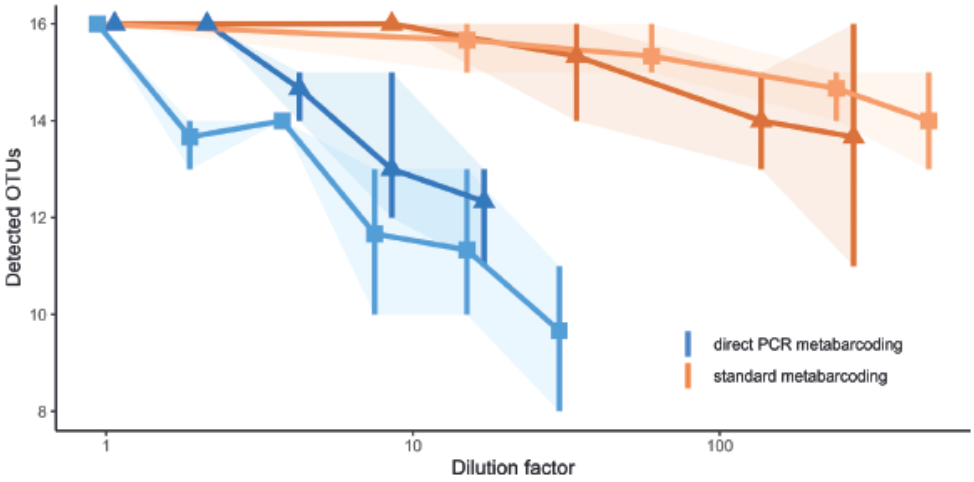

Figure 5. Effect of DNA input dilutions on detection rates in artificial communities. Mean number of detected OTUs (± range, n = 3) in a chironomid mock community with 16 individuals, using different metabarcoding methods and dilutions (logarithmic scale) of purified DNA extracts (orange) or tissue-water mixes (blue). We tested both the common and the direct PCR approach with (triangles, dark colours) and without (squares, light colours) prior size-sorting.

crease in OTU detection using purified DNA extracts is much lower than the decrease in detection rate based on tissue-water mixes, reflecting the faster deterioration of band intensity for dPCR samples visible during gel electrophoresis.

While the total read abundance kept stable in all dilutions (between 13.414 and 58.062 reads) due to equimolar pooling of PCR products during library preparation, read abundances of individual OTUs increased or decreased with progressing dilution. OTUs with low initial read abundance (< 50 reads) failed to be detected in at least one of the dilutions, while OTUs with high initial read abundance (e.g., > 700 reads) were almost constantly detected throughout the dilutions.

Experiment 4: Comparing standard versus dPCR metabarcoding approach for natural communities

Chironomid communities of eight different ponds in a mesocosm study were analysed using two different metabarcoding approaches, i.e., with and without DNA extraction. Ponds had been treated with Bti or left as a control and chironomid emergence was sampled on 5 sampling dates over a period of 17 days. In total, we detected 114 chironomid OTUs, corresponding to 36 different genera. Pairwise comparing each OTU in each sample, there were deviations between the two metabarcoding approaches in on average 8.3 (± 5.2 SD) percent of the OTUs per sample. In 6 out of 136 OTUs we saw a deviation between the two metabarcoding approaches in more than 4 samples (> 10%). These OTUs showed a relatively low mean read abundance (10 reads and less) compared to the overall mean read abundance (447 reads). Correspondence analysis showed high overlap in metabarcoding results from both approaches (Fig. 6). PERMANOVA indicated that a statistically significant effect on the community composition resulted from treatment (Bti vs. control) and sampling date, but not from the applied metabarcoding method (with or without DNA extraction; Table 1).

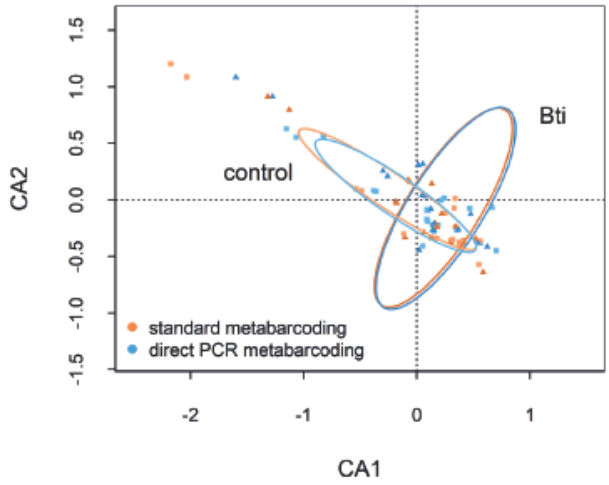

Figure 6. Correspondence analysis (CA) plot comparing standard versus dPCR metabarcoding approach for natural communities. Indicated are differences of chironomid communities from four Bti-treated (triangles, dark colours) and four control (squares, light colours) ponds of a mesocosm study over five sampling dates (N = 40). Two different metabarcoding approaches, i.e., with (orange) or without (blue) DNA extraction, were used for each community. Proportion explained per axis: CA1–7.8%, CA2–7.5%. Ellipsoids indicate standard deviations of points. Two very distant points are not visible in this figure.

Table 1. Results of the three-way PERMANOVA on natural communities. Assessed were the effects of Bti-treatment, sampling date, and DNA preparation method as well as their interactions on presence-absence chironomid community composition.

Source of variation	DF	sum of squares	*F* statistics	R^2	*p*
Bti-Treatment	1	0.870	3.730	0.046	0.001 ***
Sampling Date	4	2.534	2.715	0.135	0.001 ***
DNA Preparation Method	1	0.080	0.341	0.004	0.999
Bti-Treatment: Sampling Date	4	1.177	1.261	0.063	0.058
Bti-Treatment: DNA Preparation Method	1	0.004	0.018	< 0.001	1
Sampling Date: DNA Preparation Method	4	0.058	0.063	0.003	1
Bti-Treatment: Sampling Date: DNA Preparation Method	4	0.007	0.007	< 0.001	1
Residuals	60	14.001		0.747	
Total	79	18.731			

Discussion

In the current study, we showed for the first time that direct PCR protocols can be combined with insect community metabarcoding approaches. Metabarcoding of chironomid communities with and without DNA extraction, produced highly comparable results. Both methods led to similar detection rates in artificially created communities with known chironomid composition. Furthermore, samples of natural communities from an ongoing mesocosm study (Kolbenschlag et al. 2023), which had been processed with and without DNA extraction, showed to be highly similar in their species composition, allowing identical conclusions about the community response to an environmental stressor.

We observed high similarity between the presence-absence chironomid community composition detected by metabarcoding with and without DNA extraction. We explain the faster decrease of specimen detection rates in sequential dilutions of the dPCR approach as compared to the common approach with the stochastic effect that arises, when very small amounts of input material are pipetted (i.e., 1 µl of purified DNA extract or 5 µl of tissue-water mix). Stochasticity probably also explains the observed higher read abundance variation in the dPCR samples as compared to the common approach. As DNA is purified and concentrated during DNA extraction, the probability to represent the whole community in a small amount of purified DNA extract is much higher than in a similar amount of tissue-water mix. This lower representativeness could indeed raise concern about the reliability of direct PCR metabarcoding when studying natural communities that are typically composed of few abundant and many rare species (e.g., Fisher et al. 1943; Hubbell 2001). However, we found only minor differences between the dPCR and DNA extraction metabarcoding approach when studying natural chironomid communities (Fig. 6). In addition, the differences did not alter the conclusions about the outcome of the ecotoxicological case study. Therefore, we conclude that direct PCR metabarcoding is an equally valid tool to characterize chironomid community composition as compared to the common metabarcoding approach that includes DNA extraction.

While detection rates of the two approaches were largely similar, read abundance was influenced by the method used (Fig. 2). The individual read abundance was lower in direct PCR approaches for most of the specimens, while the variation between specimens was several factors higher. Elbrecht and Leese (2015) argued that the different body mass of specimens could have an effect on read abundance and recommended size-sorting of individuals prior to analysis. As our specimens varied in their individual mass up to a factor of more than 20, we expected an alignment of individual read abundances after equalizing the available tissue or DNA per specimen. Surprisingly, even though this treatment substantially improved the detection rates of very small specimens, individual read abundances still varied greatly between specimens (Fig. 2).

Investigating the reasons for differential individual read abundances, we found out that adjusting the availability of purified DNA prior to PCR did not eliminate variation. At the same time, varying the availably of DNA target fragments after PCR had a much larger influence on read abundance (Figs 3, 4). We therefore conclude that most of the variation arose during PCR probably due to primer bias, even though our specimens are closely related taxa and primers were highly degenerated. If and to what extent interspecific mitochondrial copy number variation (as described by Stefano et al. 2017) plays a role here is beyond the scope of this study and further studies are required to evaluate the potential impact of heteroplasmy and nuclear pseudogenes of mitochondrial origin on read abundance in insect metabarcoding studies (cf. D'Errico et al. 2004 and Guo et al. 2022). Given the weak relationship between individual tissue mass and read abundance, we agree with previous studies (e.g., Amend et al. 2010, Sun et al. 2015) that metabarcoding of arthropod communities should be evaluated based on presence-absence data, rather than assuming read abundance would reflect biomass proportions.

We tested the comparability of the two metabarcoding approaches using specimens from the family Chironomidae. However, dPCR protocols have been developed and successfully applied to several other invertebrate taxa, e.g., mosquitoes: Werblow et al. (2016), spider mites: Sakamoto and Gotoh (2017), ants: Wang et al. (2018). Wong et al. (2014) tested the suitability of dPCR protocols for a range of different invertebrates. While they were able to successfully optimize dPCR procedures for different flies and sea stars, the authors reported low success rates for beetles, copepods, ants and odonats. Wong et al. (2014) assumed that their dPCR protocol was less successful for heavily sclerotized taxa and/or those who carry a lot of PCR inhibitors (e.g., melanin, haemocyanin or secretions from exocrine glands). In contrast, the here presented dPCR approach, using finely ground tissue in water, produced COI sequences from specimens of the arthropod orders Araneae (*Pirata* and *Tetragnatha*), Coleoptera (*Dasytes* and *Neocrepidodera*), Diptera (*Bezzia* and *Helina*), Ephemeroptera (*Caenis* and *Cloeon*), Hemiptera (*Pyrrhocoris*), Hymenoptera (*Chelonus*, *Formica* and *Lasius*), Lepidoptera (*Eudonia*) and Odonata (*Coenagrion*). We assume that our thorough mechanical tissue-breakdown step (cf., Elbrecht and Leese 2015; Buchner et al. 2021), supported by the dilution of inhibitors when tissue is mixed with water, resulted in a higher PCR success for several difficult taxa. Additionally, longevity of samples could be easily improved by using buffer instead of water to dissolve ground tissue (see Anchordoquy and Molina 2007).

Our results are in accordance with Thongjued et al. (2019) who concluded that a thorough cell lysis, dilution of inhibitors and the use of an inhibitor tolerant DNA polymerase contribute to successful direct PCR even in challenging taxa. Although our initial positive results on Sanger sequencing of dPCR products of various arthropods suggest a successful extension to metabarcoding approaches, applicability of direct PCR metabarcoding to other arthropod groups needs to be verified. As shown for chironomids, representative mock communities need to be established to ensure reliable detection of relevant taxa in metabarcoding of mixed arthropod samples and to rule out dPCR-related biases between taxa. Further, upscaling the approach for very large sample sizes, such as from Malaise traps, could be tested. Given these initial tests are successful, dPCR metabarcoding might become available in mixed arthropod community samples which do not require laborious morphological sorting and therefore contribute to time and cost-efficient monitoring of natural, environmentally stressed or experimental communities.

In conclusion, we showed that DNA extraction can be omitted in chironomid community metabarcoding while preserving the informative value of the presence-absence community composition. As the adoption of our proposed direct PCR protocols is relatively easy and inexpensive, direct PCR metabarcoding has the potential to become a standard procedure in chironomid community analysis. With modern PCR reagents being more robust to contamination by inhibitors, we assume that direct PCR metabarcoding, where DNA extraction is replaced by solely a mechanical tissue-breakdown step, is applicable for a wide range of arthropod taxa and encourage further comparative studies. The opportunity to avoid DNA extraction steps might even aid the ongoing development to miniaturize the instrumental requirements for PCR and metabarcoding (e.g., for application in the field or for live on-site monitoring). In addition, laboratories implement more and more automation processes, in order to reduce the costs for high-throughput application of metabarcoding. Eliminating the DNA extraction step entirely might contribute a substantial improvement in this development. In the end, faster and cheaper metabarcoding procedures will boost our ability to monitor the diversity of arthropod communities and appropriately target conservation actions to combat the ongoing global biodiversity loss.

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Additional information

Conflict of interest

The authors declare they have no conflict of interest.

Ethical statement

No ethical statement was reported.

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Author contributions

NR and KS conceived the ideas and designed methodology; NR collected the data; NR and KS analysed the data; NR led the writing of the manuscript. Both authors contributed critically to the drafts and gave final approval for publication.

Author ORCIDs

Nina Röder https://orcid.org/0000-0002-9681-7538
Klaus Schwenk https://orcid.org/0000-0003-2427-4332

Data availability

Raw sequences were deposited in GenBank SRA archive and are available with the BioProject accession number PRJNA989176. Data and R scripts are available from Zenodo https://doi.org/10.5281/zenodo.8074454 (Röder and Schwenk 2023).

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Supplementary material 1

Overview of chironomid size classes

Authors: Nina Röder, Klaus Schwenk
Data type: additional methodological information
Explanation note: Chironomid individuals were sorted into four different size categories according to their body length (from the anterior margin of the head between the antennae to the end of the posterior abdominal segment) and shape (thin – usually males or thick – usually females). They were counted and average weight per specimen was determined.

Link: https://doi.org/10.3897/mbmg.7.102455.suppl1

Supplementary material 2

Composition of the two artificial chironomid communities

Authors: Nina Röder, Klaus Schwenk
Data type: additional methodological information
Explanation note: Sanger sequences of specimens were compared to BOLD database.

Link: https://doi.org/10.3897/mbmg.7.102455.suppl2

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How non-target chironomid communities respond to mosquito control: Integrating DNA metabarcoding and joint species distribution modelling

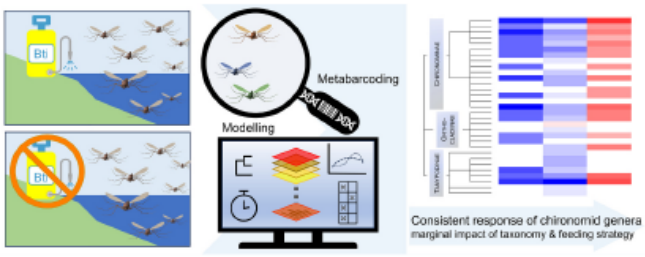
Nina Röder [a,*], V. Sophie Stoll [a], Jonathan F. Jupke [a], Sara Kolbenschlag [a], Mirco Bundschuh [a,b], Kathrin Theißinger [c], Klaus Schwenk [a,c]

[a] iES - Institute for Environmental Sciences, RPTU Kaiserslautern-Landau, Landau, Germany
[b] Department of Aquatic Science and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden
[c] LOEWE Centre for Translational Biodiversity Genomics, Senckenberg Biodiversity and Climate Research Centre, Frankfurt am Main, Germany

HIGHLIGHTS

- Mosquito control agent affects emergence dynamics of chironomid communities.
- DNA metabarcoding was combined with joint species distribution modelling.
- Consistent genus-specific responses to Bti treatment were observed.
- Phylogeny and larval feeding habits played a marginal role in explaining responses.
- Findings support understanding of ecological responses to an anthropogenic stressor.

GRAPHICAL ABSTRACT



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ABSTRACT

The conservation and management of riparian ecosystems rely on understanding the ecological consequences of anthropogenic stressors that impact natural communities. In this context, studies investigating the effects of anthropogenic stressors require reliable methods capable of mapping the relationships between taxa occurrence or abundance and environmental predictors within a spatio-temporal framework. Here, we present an integrative approach using DNA metabarcoding and Hierarchical Modelling of Species Communities (HMSC) to unravel the intricate dynamics and resilience of chironomid communities exposed to *Bacillus thuringiensis* var. *israelensis* (Bti). Chironomid emergence was sampled from a total of 12 floodplain pond mesocosms, half of which received Bti treatment, during a 16-week period spanning spring and summer of 2020. Subsequently, we determined the community compositions of chironomids and examined their genus-specific responses to the Bti treatment, considering their phylogenetic affiliations and ecological traits of the larvae. Additionally, we investigated the impact of the Bti treatment on the body size distribution of emerging chironomids. Our study revealed consistent responses to Bti among different chironomid genera, indicating that neither phylogenetic affiliations nor larval feeding strategies significantly contributed to the observed patterns. Both taxonomic and genetic diversity were positively correlated with the number of emerged individuals. Furthermore, our findings demonstrated Bti-related effects on chironomid body size distribution, which could have relevant implications for size-selective

* Corresponding author: RPTU Kaiserslautern-Landau, iES Landau - Institute for Environmental Sciences, Fortstraße 7, 76829 Landau, Palatinate, Germany.
E-mail address: nina.roeder@rptu.de (N. Röder).

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terrestrial predators. Hence, our study highlights the value of employing a combination of DNA metabarcoding and HMSC to unravel the complex dynamics of Bti-related non-target effects on chironomid communities. The insights gained from this integrated framework contribute to our understanding of the ecological consequences of anthropogenic stressors and provide a foundation for informed decision-making regarding the conservation and management of riparian ecosystems.

1. Introduction

Over the past century, riparian ecosystems have suffered degradation resulting from changes in river and land use practices (Beechie et al., 2010). Given the diverse range of ecosystem services they provide, including nutrient cycling and water quality protection, it is essential to safeguard these critical ecosystems from adverse anthropogenic influence (Gilvear et al., 2013). Merolimnic insects, i.e., insects with both aquatic and terrestrial life stages, play a vital role in riparian ecosystems. They support riparian and terrestrial food webs, contributing significantly to the energy budgets of various consumers, including birds, bats, lizards and spiders (Baxter et al., 2005). Among merolimnic insects, the family Chironomidae is a highly abundant, ecologically important and taxonomically diverse group. Chironomids frequently constitute a substantial portion of the merolimnic insects that emerge from aquatic environments (Armitage et al., 2012), making them an ideal target to study how anthropogenic stressors can alter riparian communities. However, investigating the effects of anthropogenic stressors on natural chironomid communities presents significant challenges due to their high diversity and dynamic responses to environmental fluctuations. Additionally, morphological identification of chironomids at lower taxonomic levels is time-consuming and requires (scarce) expert knowledge (cf. Chimeno et al., 2023; Ekrem et al., 2010; Gadawski et al., 2022). Consequently, many studies either analyzed chironomids at the (sub)family level (e.g., Robichaud et al., 2022) or have focused specifically on few dominant genera or morphospecies (e.g., Perrotta et al., 2023).

DNA metabarcoding, when combined with suitable reference databases, can help to overcome limitations in taxonomic expertise. In recent years, several studies have demonstrated the effectiveness of DNA metabarcoding for characterizing chironomid communities (e.g., Emilson et al., 2017; Theißinger et al., 2018; Beermann et al., 2018), as well as the non-target effects of the mosquito control agent *Bacillus thuringiensis* var. *israelensis* (Bti) on the emergence of chironomid communities (Theißinger et al., 2018, 2019). Dipterans, including the family Chironomidae, are known to be particularly susceptible to Bti. However, there is an ongoing debate regarding whether the practices used in Bti-based mosquito and black fly control pose a threat to non-target insects, such as the closely related chironomids. Numerous mesocosm and field studies, covering different countries and climatic regions, yielded variable and sometimes conflicting findings. For instance, Wolfram et al. (2018) found no significant effects on the abundance and species composition of chironomid larvae, while Theißinger et al. (2018) reported strong reductions in emergence and even weak effects on the community composition. Moreover, several studies have highlighted variations in the responses exhibited by different taxa within the Chironomidae family (e.g., Rodcharoen et al., 1991; Yiallouros et al., 1999; Stevens et al., 2013). Allgeier et al. (2019) and Liber et al. (1998) found no statistically significant Bti effects on the subfamily Tanypodinae as opposed to the subfamilies Orthocladiinae and Chironominae, attributing this to the prevalent predatory feeding strategy in Tanypodinae, which may substantially reduce Bti ingestion. In this study, we present an integrative approach, combining DNA metabarcoding with Hierarchical Modelling of Species Communities (HMSC), allowing us to consider factors such as the ecological traits and phylogenetic relationships of taxa simultaneously while investigating their responses to an environmental stressor. We applied this approach to unravel the impact of Bti on the emergence of non-target chironomid communities, in this way adding to a deeper understanding of the insights gained in a previously conducted extensive mesocosm study (Kolbenschlag et al., 2023).

Considering the findings of Kolbenschlag et al. (2023), which indicated an impact of Bti application on the abundance of emergent chironomids, we propose that the application of Bti also influences their community composition and diversity. Based on previous research (e.g., Stevens et al., 2013), we further hypothesize that Bti could affect diversity at both the genus and the subfamily level. We anticipate that closely related genera will exhibit more similar responses to the treatment, particularly those belonging to the subfamily Tanypodinae, which reportedly exhibit lower susceptibility to Bti. Building upon the findings of Liber et al. (1998) and Allgeier et al. (2019), we propose that chironomid genera with predatory larval stages will be less susceptible to Bti compared to those with non-predatory larvae, primarily due to lower uptake of the toxicant. Furthermore, taxonomic or trait-related differences in responses to Bti might lead to a shift in the body size distribution of emergent chironomids and thus alter terrestrial trophic interactions (via size-selective predation, cf. Turner, 1982). By addressing these research questions, we aim to extend existing approaches to unravel toxicant effects on natural non-target communities. Additionally, we seek to contribute to the understanding of the ecological implications of Bti treatment on chironomid communities, which represent valuable contributors to the overall functioning of riparian ecosystems.

2. Material and methods

2.1. Sample origin and experimental design

Chironomids were retrieved from a study investigating the effects of Bti on aquatic emergent insect dynamics (Kolbenschlag et al., 2023). The study was carried out in floodplain pond mesocosms at the Eußerthal Ecosystem Research Station (EERES) located in the Palatinate Forest near Landau, Germany (Manfrin et al., 2023; Stehle et al., 2022). The study site consisted of twelve floodplain ponds, each approximately 20 m × 5.2 m in size, arranged in parallel with approximately 3 m of distance between them. The ponds hosted a chironomid community that had developed over a span of more than two years through natural colonization. During the preceding summer months (July to September 2019) chironomids exhibited both taxonomic diversity and community similarity across the floodplain ponds (unpublished data). From mid of April to the end of May 2020, the water levels in the ponds were artificially raised from 30 to 50 cm on three occasions to simulate flooding. The mosquito control agent VectoBac WDG (Valent BioSciences, Illinois, USA), containing *Bacillus thuringiensis* var. *israelensis* (Bti), was applied at the highest recommended application rate (i.e., $2.88 * 10^9$ International Toxic Units [ITU]/ha) to every second pond on the third day of each flooding event (i.e., 14th April, 4th May, 25th May). One week after each application, excess water was released to restore the initial water levels of 30 cm. This treatment, conducted three times per season during flooding events, was designed based on mosquito control practices in the study region (Allgeier et al., 2019; Becker et al., 2018). Parallel biotests were performed to ensure the effectiveness of the Bti treatments and temperature, dissolved oxygen and pH were regularly measured to confirm uniform conditions in the floodplain ponds (see Kolbenschlag et al., 2023). To sample merolimnic insects, three floating emergent traps were deployed per pond, covering a total area of 3 m^2

(approx. 3 % of the surface area per artificial pond). These traps were equipped with collection bottles filled with ethylene glycol to capture and preserve the emerging insects. The collection bottles were emptied and replaced once to twice a week. Further information regarding the experimental design and sample collection can be found in Kolbenschlag et al. (2023).

2.2. Morphological sorting of chironomids and sample preparation

Aquatic insects were initially sorted at the family level. Within the family Chironomidae, additional visual sorting was performed to separate individuals into four distinct size classes (A. <3 mm and thin, B. <3 mm and bold or 3–5 mm and thin, C. 3–5 mm and bold or 5–8 mm and thin, D. 5–8 mm and bold), based on the body shape and the approximate body length from the anterior margin of the head between the antennae to the end of the posterior abdominal segment. The number of individuals in each sample was recorded. Following sorting, samples were dried at 60 °C for a minimum of 48 h until fully dry. Approximately 10 % of the samples were weighed after drying to estimate the mass per individual in each size class. Subsequently, all samples were finely ground three times for 1 min using a TissueLyser II (Qiagen) with two metal beads. The resulting tissue powder was mixed with 1 mL of PCR-grade water and vortexed thoroughly to create a homogenous tissue suspension. To ensure even masses per individual in each sample, tissue suspensions from different size classes were then combined by pipetting volumes ranging from 5 to 75 μL (cf. Sabo et al., 2002). The combined samples were finally stored at −20 °C until further analysis.

2.3. Direct PCR metabarcoding

We employed a two-step direct PCR metabarcoding approach (Röder and Schwenk, 2023) using the BF2 and BR2 primers (5′-GCHCCHGAYATRGCHTTYCC-3′ & 5′-TCDGGRTGNCCRAARAAYCA-3′; Elbrecht and Leese, 2017) to amplify a 421 bp cytochrome oxidase subunit I (COI) fragment. PCRs included negative controls and two technical replicates per sample. In the initial PCR, we used 5 μL of tissue suspension per reaction (cf. Röder and Schwenk, 2023), along with 2 μL 10× buffer (TaKaRa, Shuzo, Japan), 1.2 μL dNTPs, 1 μL of each primer and 0.15 μL of Ex Taq DNA Polymerase (TaKaRa, Shuzo, Japan). The reaction volume was adjusted to 20 μL with PCR-grade water. For the second PCR, the product from the first PCR was diluted 1:20, then 1 μL of the diluted product was used as the template. Fusion primers, including Illumina adapters for sequencing (P5 or P7) and inline barcodes of different lengths for individual tagging of samples (Elbrecht and Steinke, 2018), were used in the second PCR. The PCR cycling conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 42 cycles (12 cycles in the second PCR) of 94 °C for 30 s, 51 °C for 30 s and 72 °C for 1 min, with a final elongation step at 72 °C for 10 min (adapted from Wong et al., 2014). PCR success was verified using a 1.5 % TBE agarose gel. The concentration of the target fragment was determined using a Tapestation 4200 (D1000 DNA Screentape Analysis Kit; Agilent Technologies, Santa Clara, CA, USA). To ensure equal sequencing depth per individual across all samples, the PCR products from each sample were pooled into a library proportionally to the number of individuals. The final libraries were purified and size-selected using magnetic beads (ratio: 0.65×, SPRIselect, Beckmann Coulter, Bread, CA, USA), primarily retaining fragments larger than 300 bp. The libraries were sent to an external laboratory (CeGaT, Tübingen, Germany) for 2 × 250 bp paired-end sequencing on a MiSeq Illumina system. The samples were distributed among three different libraries, with each library separately sequenced and containing all sampling dates of two Bti-treated and two control ponds.

2.4. Bioinformatic processing and taxonomic assignment

We employed the "Demultiplexing_shifted" module of the JAMP bioinformatic pipeline (version 0.77; https://github.com/VascoElbrecht/JAMP) to segregate the raw data into individual files using their respective barcodes. Subsequently, the dada2 pipeline (Callahan et al., 2016) was utilized to generate an amplicon sequence variant (ASV) table by performing filtering, trimming, dereplication, sample inference, merging of paired reads and removal of chimeras. As our tagging approach involved parallel sequencing in forward and reverse direction (Elbrecht and Steinke, 2018), we finally identified and summed up pairs of identical ASVs in different directions, to obtain 3879 unique ASVs. Taxonomy was assigned using BOLDigger version 2.1.2 (Buchner and Leese, 2020) and NCBI-Blast+ version 2.13.0 (Camacho et al., 2009). Genus-level assignment was based on at least 95 % similarity and an e-value $<1.00 \times 10^{-3}$. In cases where more than one genus matched an ASV with at least 95 % in one or both databases, the more abundant genus in the best matches was chosen. This was typically the case when a genus occurred only once within the top results, likely due to taxonomic misidentification of individual database entries.

2.5. Taxon-specific trait assignment for chironomid genera

We utilized a trait database specific to European chironomids (Serra et al., 2016) to obtain information on larval traits, including 'feeding habits', 'food type' and 'maximal body size of the 4th instar', for 51 of the 52 identified chironomid genera. For the genus *Synendotendipes*, no information was available. To categorize larval feeding strategies, we assigned the genera into four groups ("facultative predators", "herbivores/detritivores", "diet ambiguous" and "diet unknown") using references from the database. For each genus, we summed up the number of references indicating a predatory feeding strategy, combining specific trait categories related to *predator* and *parasite* in the 'feeding habits' trait as well as *living microinvertebrates*, *living macroinvertebrates* and *living vertebrates* in the 'food type' trait. We compared the sum of references for predatory feeding strategy with the sum of references for non-predatory categories. Genera with a higher number of references indicating a predatory feeding strategy were categorized as "facultative predators" (total of 6 genera), while those with a lower count were classified as "herbivores/detritivores" (29 genera). None of the genera had references indicating an exclusively predatory feeding strategy. For nine genera, the number of references for predatory and non-predatory feeding strategies was equal, which we labelled as "ambiguous". Another eight genera had no related information available in the database ("unknown"). The body size of the 4th instar larvae was estimated by assigning sizes of 2.2 mm, 3.8 mm, 7.5 mm, 15 mm and 23 mm to the database's categories "SIZE1 (<2.5 mm)", "SIZE2 (>2.5–5 mm)", "SIZE3 (>5–10 mm)", "SIZE4 (>10–20 mm)" and "SIZE5 (>20–40 mm)", respectively. We then calculated the estimated average size for each genus, weighting it by the number of their references in each size category. Body size of 4th instar larvae was assumed to be representative for body size of adult chironomids (cf. Stanko-Mishic et al., 1999).

2.6. Categorisation of deviations from expected emergence

The effects of Bti on chironomid emergence exhibited significant variability, which was specific to individual ponds and overplayed by a gradual trend (Fig. 1). To capture this variability, alongside the conventional ecotoxicological analysis comparing control and treatment groups, we employed an additional approach. We assessed the communities in Bti-treated ponds by considering substantial deviations from the expected emergence patterns per week, which we referred to as 'emergence status'. Specifically, if the logarithm base 10 (log10) of the number of emerged chironomids per week in a Bti-treated pond was >0.05 higher than in the adjacent two control ponds (or the one adjacent pond in the case of Pond 12), we classified it as a 'Bti-induced increase' in chironomid emergence. Conversely, if the log10 value was >0.05 lower than in adjacent control ponds, it was considered a 'Bti-induced decrease' in emergence. All other cases in Bti-treated ponds were

	Pond 1 control	Pond 2 Bti	Pond 3 control	Pond 4 Bti	Pond 5 control	Pond 6 Bti	Pond 7 control	Pond 8 Bti	Pond 9 control	Pond 10 Bti	Pond 11 control	Pond 12 Bti
Week 16	28	47	15	66	39	29	27	105	42	32	47	116
Week 17	20	29	11	66	45	27	23	60	27	28	21	125
Week 18	44	112	54	104	83	94	65	107	41	39	36	138
Week 19	21	64	27	63	67	84	55	57	34	27	37	64
Week 20	161	224	150	225	277	284	168	339	120	236	173	293
Week 21	161	134	131	146	129	171	142	244	162	209	141	154
Week 22	198	124	91	184	268	176	186	412	186	86	141	97
Week 23	144	129	134	260	422	237	294	503	180	124	251	94
Week 24	236	246	253	305	531	503	450	482	500	153	479	163
Week 25	54	51	93	45	135	118	142	140	253	48	189	73
Week 26	182	193	271	236	525	328	419	381	501	108	554	167
Week 27	67	123	215	75	339	234	176	191	248	41	415	85
Week 28	112	97	173	72	212	176	83	153	231	59	352	64
Week 29	54	117	86	131	152	104	126	127	239	75	189	105
Week 30	42	160	89	195	101	162	129	124	330	70	173	88
Week 31	64	172	122	233	129	132	130	74	360	101	207	123

Fig. 1. Heatmap illustrating the number of emergent chironomids per week and pond. Cells are color-coded from red (indicating fewer emergent insects) to blue (indicating higher numbers). The color scheme is calibrated individually for each week, accentuating week-specific variations. Frames around specific cells and cell clusters highlight Bti-induced deviations from expected emergence, i.e., if the logarithm base 10 (log10) of the number of emerged chironomids per week in a Bti-treated pond was >0.05 higher or lower than that of the adjacent control ponds (red = decrease, blue = increase). Ponds with even numbers were treated with Bti, while the remaining ponds served as control.

categorized as 'emergence unaffected by Bti' (Fig. 1). We used a neighboring control pond-based approach to calculate expected emergence in order to account for local variation in chironomid emergence among ponds (i.e., emergence levels in ponds in close proximity were more similar). Alternatively, using the average emergence of all control ponds as a baseline would have led to the same classification of emergence status in 77 % of cases. However, this approach would have, for example, indicated a decrease in emergence in pond 2 from week 22 to 25 (51–246; average = 138), despite comparable numbers with pond 1 and 3 (54–253; average = 150), due to higher emergence levels in more northern control ponds (135–531; average = 288).

2.7. Data analysis

We carried out all analyses in R statistical program version 4.2.0 (R Core Team, 2022), using the program R Studio version 2023.03.0.386 (Posit team, 2023), and plotted results using the package GGPLOT2 version 3.4.1 (Wickham, 2016). To evaluate the similarity between technical replicates, the 'dist' function from the PROXY R package version 0.4-27 (Meyer and Buchta, 2022) was utilized. Approximately 3 % of the 311 samples exhibited a high Jaccard dissimilarity (>0.6), prompting individual examination. Consequently, for five samples only one technical replicate was considered in downstream analysis, as the other one either produced an insufficient number of reads or indicated contamination from a neighboring sample on the PCR plate. For all other samples, the technical replicates were combined and the table was transformed into presence-absence data. An ASV was marked as present in a sample only if both technical replicates had more than zero reads. We performed a comparative analysis of the number of individuals, genera, and ASVs in both treated and control ponds. A Pearson correlation test between the variables 'individuals' and 'genera' and 'individuals' and 'ASVs' was performed using the 'cor.test' function in R. Given the positive correlation observed between the number of individuals and both the number of genera and ASVs, we additionally calculated the normalized richness of 'genera' and 'ASVs' by dividing their respective counts by the total number of individuals in each sample. Linear mixed-effects models were employed using the 'lme' function from the NLME R package version 3.1-157 (Pinheiro et al., 2022) to investigate the relationship between the variables 'number of individuals', and 'genus richness'/'ASV richness' in relation to the treatment, time, and their interaction, as well as to the 'emergence status' (see Section 2.6). The random effects of pond and time were included in the treatment and emergence status models, respectively, to account for localized and seasonal effects. To assess the statistical significance of the treatment, time, and their interactions on the response variables, an analysis of variance (ANOVA) was conducted using the 'anova' function. In the emergence status models, a multiple comparison test was performed using the 'glht' function from the MULTCOMP R package version 1.4-23 (Hothorn et al., 2008). Tukey's tests were specifically employed to examine the differences among the various levels of the emergence status. Split violin plots were created using the R package VIOPLOT version 0.4.0 (Adler et al., 2022) to illustrate the distribution of chironomid genera and ASVs with different body sizes in Bti-treated vs. control ponds per week. Before configuring the HMSC model, preliminary insights into community structures were obtained using multivariate statistical approaches, specifically pairwise beta diversity analysis, PERMANOVA, and correspondence analysis for ordination (Supplementary material, Figs. S1 to S4).

2.8. Hierarchical modelling of species communities (HMSC)

We applied HMSC (Ovaskainen and Abrego, 2020; Ovaskainen et al., 2017), a Bayesian joint species distribution model (Warton et al., 2015) to analyze taxon-specific variation in responses to environmental covariates. The HMSC framework allows us to assess the contribution of environmental variables to the observed variation in taxon occurrences and abundances across various spatial or temporal scales. Our dataset consisted of ASV abundance information of different chironomid genera, with each metabarcoding sample serving as a sampling unit. ASV abundance, in this context, refers to the number of different ASVs within each genus, providing a measure of diversity associated with a specific genus. In total, we analyzed 311 metabarcoding samples collected from 26 sampling events across 12 artificial ponds, excluding one sample that lacked chironomid individuals.

To account for the zero-inflated nature of the data, we utilized a hurdle modelling approach, where we fitted two separate models: one

for the presence-absence component of the data and another for the abundance of ASVs conditional on their presence (hereafter referred to as the occurrence and the ASV abundance models). In the ASV abundance model, we excluded cases where the taxon was not recorded at all by specifying the response variables as NA, as described in Ovaskainen and Abrego (2020) for hurdle models within the HMSC framework. For the occurrence data, we employed probit regression to model the binary response variable, indicating the presence or absence of genera. In the ASV abundance models, which focused on the number of distinct ASVs (sequence variants) per genus given its presence, we utilized a log-normal Poisson model. To streamline the analysis, we excluded 'rare' genera that were encountered in five or fewer samples, following the approach outlined in Trivellone et al. (2017). As many of the genera had limited ASV abundance variation across samples, we considered only those in the ASV abundance models that exhibited a standard deviation of at least one when comparing ASV abundances across all samples.

Two distinct sets of models were employed to examine the influence of Bti on chironomid communities. The "treatment" models assessed the occurrences and ASV abundances of chironomids in response to the pond treatment (Bti or control), the sampling date, and their interaction. The 'emergence status' models analyzed how chironomid occurrences and ASV abundances were impacted by the community emergence response, which was categorized as "Bti-induced increase", "Bti-induced decrease" and "no Bti-induced effect" and compared to control conditions.

To investigate the factors influencing genus-specific responses to Bti, we incorporated information on larval feeding types and taxonomic affiliations for each genus. This allowed us to assess the extent to which the ecological characteristics and phylogenetic relationships of the genera contributed to their specific responses. To examine whether closely related taxa respond more similarly to Bti than expected based on their feeding type, we assumed that the residual variation in taxa responses is potentially phylogenetically structured (Ovaskainen et al., 2017). A positive estimate of the phylogenetic signal ρ ($0 < \rho < 1$) in the model implies that ecological traits, which are not included in the model but are relevant for the taxa responses, are phylogenetically structured. Conversely, if ρ is estimated to be 0, it indicates that the missing traits can be expected to be randomly distributed within the phylogeny or that all relevant traits are included in the model (Ovaskainen and Abrego, 2020). A taxonomic tree comprising three levels of taxonomic identifications (i.e., subfamily, tribe and genus) was used as a proxy of the phylogenetic relatedness. Equal branch lengths were assumed at each taxonomic level of the tree. Additionally, we included a spatially explicit random effect across the sample locations (artificial ponds) and treated the sampling date as a random effect in the "emergence status models" to address the repeated collection of chironomid samples.

The occurrence and the ASV abundance models were fitted using the HMSC R package version 3.0-13 (Tikhonov et al., 2020) with default prior distributions. In the occurrence models, we performed sampling from the posterior distribution using four Markov Chain Monte Carlo (MCMC) chains, each consisting of 187.500 iterations. The first 62.500 iterations were discarded as burn-in. For the ASV abundance models, we used four MCMC chains of 375.000 iterations, with a burn-in of 125.000 iterations. To reduce autocorrelation, the iterations were thinned by 500 for the occurrence models and 1000 for the ASV abundance models. This resulted in 250 posterior samples per chain, totaling 1000 posterior samples.

To assess chain convergence, we examined the potential scale reduction factors (psrf; Gelman and Rubin, 1992) for each model. A satisfactory MCMC convergence is indicated by a psrf value <1.05 for all β-parameters, which represent the responses of taxa to environmental covariates, (Tikhonov et al., 2020). The effect of a trait on the response is measured by γ-parameters (Ovaskainen and Abrego, 2020). Four genera were identified as "facultative predators" and included in our models as baseline γ-parameter estimates, namely *Ablabesmyia*, *Procladius*, *Parachironomus* and *Clinotanypus*, the latter being excluded from the ASVabu models. The explanatory power of the probit occurrence models was evaluated using genus-specific area under the receiver operating characteristic curve (AUC) and Tjur R^2 values. These metrics measure how well the occurrence probabilities discriminate sampling units as either occupied or empty. For the log-normal Poisson ASV abundance models, the explanatory power was assessed using the squared Spearman correlation between observed and predicted values, referred to as pseudo-R^2 (SR2). To calculate the explanatory power, model predictions were generated based on models fitted to the entire dataset. We partitioned explained variation among the fixed and random effects included in the model to quantify the effect of Bti on chironomid taxa.

3. Results

3.1. Sequencing statistics and taxonomic assignment

Sequencing the three libraries yielded a total of 12.047.150, 5.928.530 and 9.336.106 reads, respectively. On average, each sample had 229 (±131 SD) reads per individual. The analysis of negative controls revealed that 60 % of them had a total sum of reads equal to zero and the remaining had an average of 14 reads (with no more than 107 reads). Therefore, we considered a potential bias of our results due to cross-contamination among samples as neglectable.

Out of the 3879 ASVs, 2040 ASVs (representing 99.5 % of total reads) were successfully assigned to the family Chironomidae. Of those, 1843 ASVs were assigned to a total of 52 genera, which means that <10 % of the chironomid ASVs (representing 2.3 % of chironomid reads) could not be assigned to a genus. Genus-level assignments were generally consistent between NCBI and BOLD. Taxonomic assignment to the species level was not performed, however, preliminary analyses indicated that around 77 % of the genera were represented by one or two species. The remaining twelve genera contained an average of around six species, with the highest numbers being approximately ten species for *Chironomus* and 14 species for *Tanytarsus*.

After merging technical replicates, where ASVs were only counted as present when they occurred in both technical replicates, 625 ASVs were identified with no presence in any of the samples and were consequently excluded from further analysis. Those were considered negligible, as 99 % of them had occurred five times or less in the dataset before merging.

3.2. Community composition and diversity of emergent chironomids

A total of 1218 ASVs were considered in data analyses, corresponding to 52 chironomid genera. Among these genera, approximately 40 % were represented by only one or two ASVs (22 genera), and half ranged from 3 to 57 ASVs (with a median of 17 ASVs). The remaining four genera were particularly rich in sequence variants, with >100 ASVs each (up to 217 ASVs representing *Tanytarsus*). Around 60 % of the genera were present in both control and Bti-treated ponds regardless of the emergence status, i.e., the deviation from expected emergence. Of the remaining genera, 20 were considered 'rare', occurring in the dataset only up to five times, and *Limnophyes* was only missing in communities where Bti had induced a decrease in emergence. Overall, 44 genera were observed in control ponds, while 46 genera were found in Bti-treated ponds.

The number of genera and the number of ASVs, i.e., the genus and ASV richness, were positively correlated with the emergence of chironomids (Pearson correlation tests, both $p < 0.001$, Supplementary material Fig. S5). This is reflected by the similar patterns of the emergence (number of individuals), the genus richness and the ASV richness over time in the Bti-treated and control ponds (Supplementary material Fig. S6 and Fig. 2), where we observed an increasing trend over the study period, peaking around end of June. Furthermore, communities exhibited lower Sørensen dissimilarity when their sampling dates were closer together, regardless of whether they had received the same treatment (Bti or control) or different treatments (Bti vs. control;

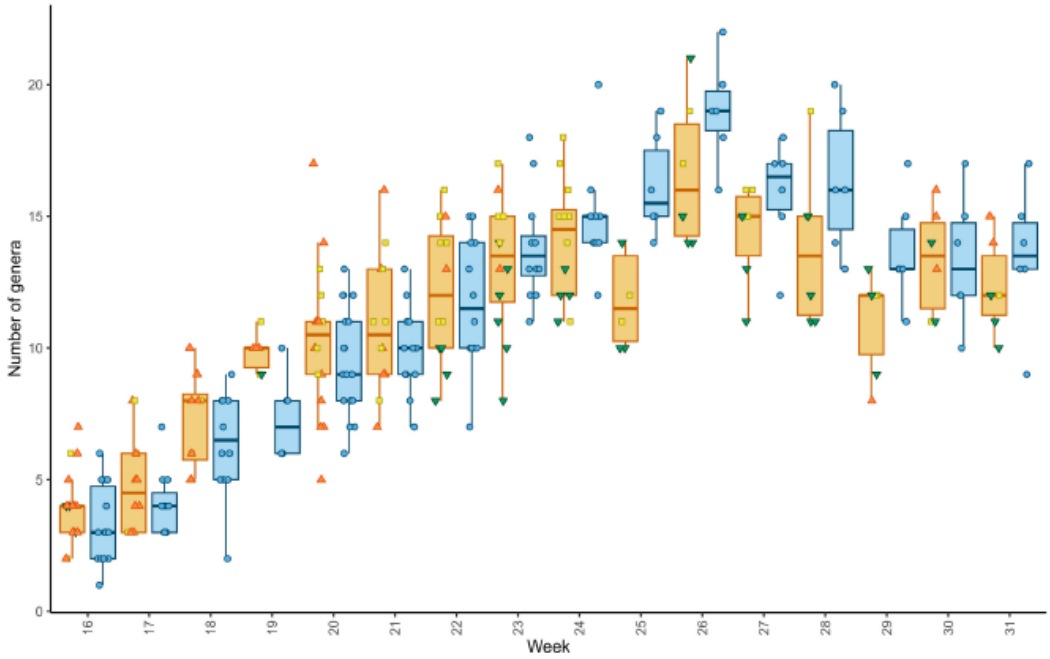

Fig. 2. Genus richness per week and treatment. Boxplots represent control (blue) and Bti-treated (orange) ponds (n = 6). Scattered symbols indicate individual ponds, with colors and shapes representing emergence status: orange triangle - increased emergence, yellow square - no deviation, green triangle - decreased emergence, blue circle - control.

Supplementary material, Fig. S1).

Bti treatment had no significant effect on the genus richness (ANOVA, F-statistics 0.1, p-value 0.74). However, both time and the interaction between time and treatment had a statistically significant effect on genus richness (time: F-statistics 447.1, p-value <0.001; treatment: time F-statistics 10.4, p-value 0.001). Similar patterns were observed for the number of individuals, the ASV richness and normalized ASV richness, and the normalized genus richness: time and the interaction between time and treatment consistently revealed statistical significance across these variables, while the effect of Bti treatment alone was not statistically significant. PERMANOVA indicated a significant influence of sampling date, Bti treatment, and their interaction on community composition, but sampling date accounted for the largest proportion of the explained variation (Supplementary material, Fig. S2). Ordinations revealed strong overlap between most communities with few more distant communities (Supplementary material, Figs. S3 to S4).

In cases of reduced emergence due to Bti, we observed a decrease in genus and ASV richness (Table 1). However, the normalized richness of genera and ASVs increased, despite the reduced number of individuals. Conversely, when Bti treatment led to an increase in chironomid emergence, the genus richness remained unaffected, but the ASV richness increased. Nonetheless, the normalized richness of both genera and ASVs decreased. In cases where Bti treatment did not impact chironomid emergence, no statistically significant effect was found on the (normalized and non-normalized) richness of genera or ASVs.

Out of the 311 samples analyzed, 39 samples contained a single 'rare' genus, which refers to a genus present in <2 % of all samples. Two samples contained two rare genera, while the remaining samples did not have any rare genera. Interestingly, the occurrence of rare genera was slightly more frequent in Bti-treated ponds compared to control ponds, with an average occurrence of 0.2 times per sample in Bti-treated ponds and 0.1 times per sample in control ponds. However, there was no significant correlation between the occurrences of rare genera and time (Pearson's correlation test, p = 0.8).

Table 1
Mean (±SD) number of individuals, genus/ASV richness and normalised genus/ASV richness in the control ponds and significant differences in the Bti ponds per emergence status (arrow up – significant increase, arrow down – significant decrease, equal sign – no significant deviation from the control).

	Number of individuals	Genus richness	ASV richness	Normalized genus richness	Normalized ASV richness
Mean (±SD)					
Control (n = 155)	106 (±108.0)	10.6 (±5.0)	28.7 (±18.9)	0.18 (±0.13)	0.38 (±0.17)
Linear mixed effect model outcome (Tukey, compared to control)					
Bti-induced decrease in emergence (n = 41)	↓	↓	↓	↑	↑
No effect on emergence (n = 54)	=	=	=	=	=
Bti-induced increase in emergence (n = 61)	↑	=	↑	↓	↓

3.3. Model convergence and fit

After exclusion of 'rare' genera, we retained a total of 31 genera for the occurrence (Occ) models. The selection criterion of a minimum ASV variability resulted in the inclusion of 13 genera in the ASV abundance (ASVabu) models. Psrfs were smaller than 1.05 for most β-parameters, indicating a good convergence of the Markov chains. The maximum psrfs observed were: $Occ_{treatment} = 1.01$, $Occ_{emerg.status} = 1.03$ (except *Dicrotendipes* Intercept 1.12), $ASVabu_{treatment} = 1.04$ and $ASVabu_{emerg.status} = 1.05$ (except *Paratanytarsus* Intercept 1.10, *Acricotopus* Intercept 1.17, 'Bti-induced decrease' 1.09, 'Bti-induced increase' 1.06). The explanatory power of the models varied widely among genera (Table 2).

3.4. Bti susceptibility with regard to taxonomic affiliations

The responses of the genera to environmental covariates exhibited a weak phylogenetic signal ρ in both the $Occ_{treatment}$ model ($Pr(\rho > 0) = 0.84$; $E(\rho) = 0.45$; 95 % confidence interval [CI] (ρ) = 0.00–0.94) and the $Occ_{emerg.status}$ model ($Pr(\rho > 0) = 0.61$; $E(\rho) = 0.27$; 95 % CI (ρ) = 0.00–0.89), implying that there was no clear evidence of a phylogenetic structure in chironomid genera occurrence distributions. However, the phylogenetic signal in the ASVabu models was stronger ($ASVabu_{treatment}$: $Pr(\rho > 0) = 0.91$; $E(\rho) = 0.70$; 95 % CI (ρ) = 0.00–1.00, $ASVabu_{emerg.status}$: $Pr(\rho > 0) = 0.95$; $E(\rho) = 0.76$; 95 % CI (ρ) = 0.00–1.00).

In the $Occ_{treatment}$ model (Fig. 3), a significant negative interaction between Bti treatment and time was strongly supported (i.e., 95 % of the values in the posterior distribution were below zero) for the majority of genera (18 out of 31). Among the remaining genera, 9 out of the 13 genera showed a positive association with time, following the general trend of an increasing diversity over the study period. Notably, *Metriocnemus* exhibited a strong negative association with time, being present only in the first half of the experiment. *Procladius* showed a positive association with Bti treatment and time, being present in 95 % of all samples, with the absence primarily observed in one control pond (pond 3). *Corynoneura* showed a positive association with Bti treatment, occurring more frequently in Bti-treated ponds (17 % of the samples) than in control ponds (10 % of samples), while *Cladopelma* showed no discernible association, occurring in only 3 % of all samples. In the $Occ_{emerg.status}$ model (Fig. 3), all responses to the 'decrease in emergence' status were negative (12 out of 31 genera), while associations with the 'increase in emergence' status were consistently positive (6 out of 31 genera). Three genera displayed a negative association and two genera a positive association with the 'no Bti-induced effect' status. Similarly, the $ASVabu_{treatment}$ models (Fig. 4) revealed significant support for a negative interaction between Bti treatment and time for seven out of 13 genera and strong support for a positive association with time in *Ablabesmyia* and *Acricotopus*, and with Bti treatment in *Cladotanytarsus*. The remaining three genera showed no discernible association. The $ASVabu_{emerg.status}$ model exhibited strong support for a positive association with the 'increase in emergence' status in two genera, while three genera showed a negative association with the 'decrease in emergence' status. Two genera displayed a negative association and *Chironomus* exhibited a positive association with the 'no Bti-induced effect on emergence' status.

3.5. Larval feeding strategy and Bti susceptibility

The response of chironomid genera to Bti treatment did not show a significant association with larval feeding strategy (i.e., <95 % posterior probability) in any of the models. The only exception was found in the feeding group 'diet unknown' which responded negatively in the 'Bti-induced decrease' status in the $Occ_{emerg.status}$ model. However, this response was specific to the genus *Acricotopus*, as it was the only representative of this feeding group included in the model. On the other hand, both $Occ_{treatment}$ and $ASVabu_{treatment}$ models clearly indicated a positive tendency of herbivores/detritivores under the Bti treatment (Fig. 5).

3.6. Bti effects on the body size distribution of chironomids

Fourth instar larval body size of the chironomid genera ranged from 3 mm (*Coryroneura*, *Thienemanniella*) to 1.7 cm (*Chironomus*). The size distributions were largely similar between communities from Bti-treated and control ponds (Fig. 6). However, some deviations were apparent. For example, during the first five weeks, the ASV richness in the very large size group (>16 mm; *Chironomus*) found in Bti-treated ponds was three to four times higher than in the control ponds, but two times lower in weeks 24 and 25. Likewise, in Bti-treated ponds, the ASV richness of chironomids with a size between 5 and 8 mm (representing 22 genera) was roughly twice as high in the first five weeks, but two to three times lower in weeks 24 to 27, compared to control ponds. Six genera in this body size group were mainly responsible for this pattern: *Acricotopus*, *Metriocnemus*, *Paratanytarsus*, *Stempellina*, *Tanytarsus* and *Zavrelimyia*. These are among the ten genera with the highest ASV richness in this body size group.

4. Discussion

Analyzing the non-target effect of Bti on natural chironomid communities presents a significant challenge due to their diverse nature and dynamic responses to environmental changes (Lundström et al., 2010). In our study, chironomid genera exhibited highly variable occurrence patterns: some were consistently present, while others occurred sporadically, showed no occurrence in certain ponds, or exclusively occurred in single ponds. Additionally, we observed a wide range of genetic variants (ASVs) in some genera, while many others were represented by only a single ASV. This complexity hinders the analysis of their response to an environmental stressor significantly. To tackle this challenge, we employed a novel approach combining DNA metabarcoding and HMSC in an attempt to unravel the underlying patterns. This integrated framework proved to be highly valuable to determine the contribution of trait variation and phylogenetic affiliations to taxon-specific responses within the chironomid community to Bti in the spatio-temporal context.

4.1. Bti effects on chironomid communities

In our study, we observed a substantial temporal influence on the impact of Bti on genus richness. In the initial phase of the experiment,

Table 2
Explained variance quantified by Tjur R^2 and AUC for occurrence models (Occ); R^2 for ASV abundance models (ASVabu) and partitioning of the explained variation among the fixed and random effects included in the model. Mean variance (%) explained by treatment, time, their interaction, emergence status and random effects are given.

				Fixed effects (%)				Random effects (%)	
Test	Model	Mean R^2 (range)	Mean AUC (range)	Treatment	Time	Treatment: Time	Emergence status	Time	Pond
Bti treatment	Occ	0.17 (0.01–0.60)	0.79 (0.60–0.98)	2.4	8.0	3.1			3.1
	ASV abu	0.21 (0.03–0.44)		4.1	4.5	4.4			8.3
Emergence status	Occ	0.25 (0.01–0.74)	0.84 (0.61–0.99)				4.4	15.8	4.6
	ASV abu	0.30 (0.06–0.55)					7.1	14.8	8.0

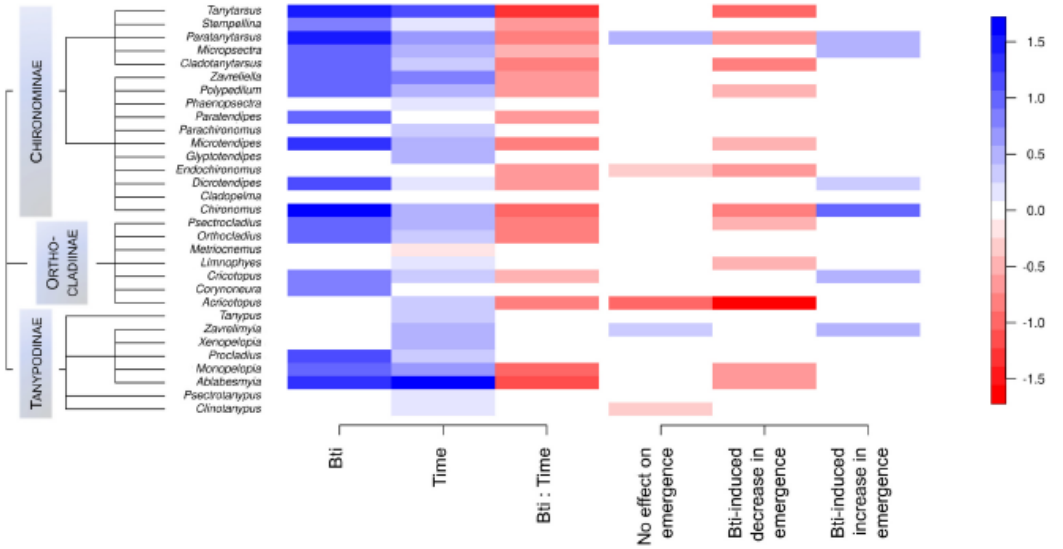

Fig. 3. Occurrence model results displaying genus-specific responses. Heatmap illustrating the estimated positive or negative β-coefficients across chironomid genera (y-axis) and environmental factors (x-axis). Positive associations with high (at least 95 % posterior probability) statistical support are shown in blue, while negative associations are displayed in red. Notably, for enhanced visibility, the β-coefficients for the environmental factors 'Time' and 'Bti: Time' were magnified by a factor of 20 and 100, respectively.

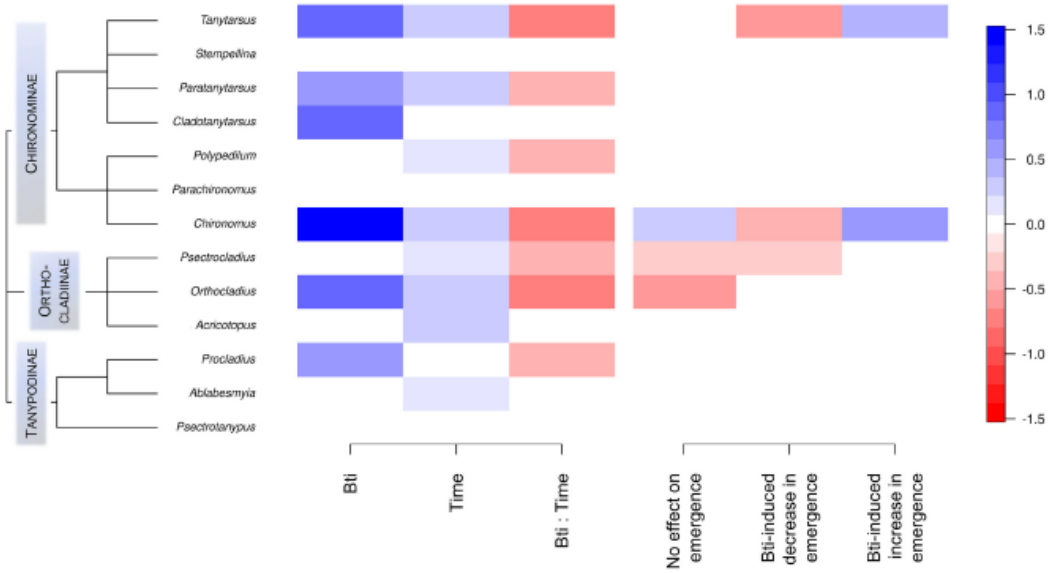

Fig. 4. ASV abundance model outcomes displaying genus-specific responses. The heatmap illustrates the estimated positive or negative β-coefficients across chironomid taxa (y-axis) and environmental factors (x-axis). Positive associations linked with high (at least 95 % posterior probability) statistical support are presented in blue, while negative associations are shown in red. Notably, for enhanced visibility, the β-coefficients for the environmental factors 'Time' and 'Bti: Time' were magnified by a factor of 20 and 100, respectively.

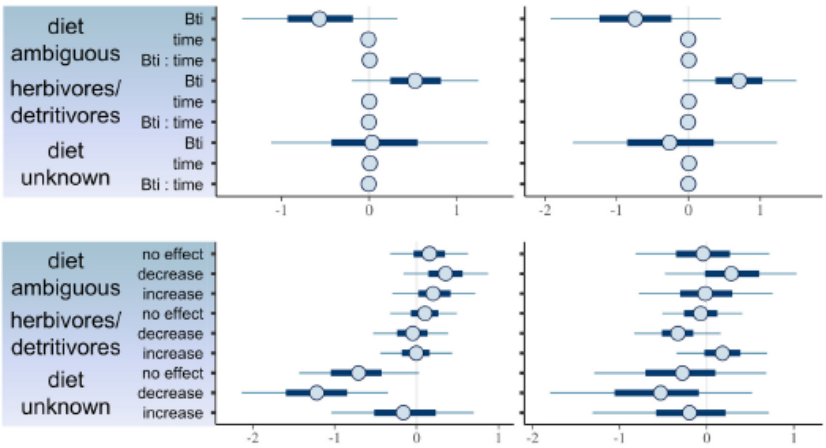

Fig. 5. Central posterior uncertainty intervals for the γ-parameters of the treatment models (top) and the emergence status models (bottom) with occurrence model outcomes diplayed on the left side and ASV abundance model outcomes on the right. Shown are medians (circles), 50 % probability intervals (bold lines) and 90 % probability intervals (thin lines).

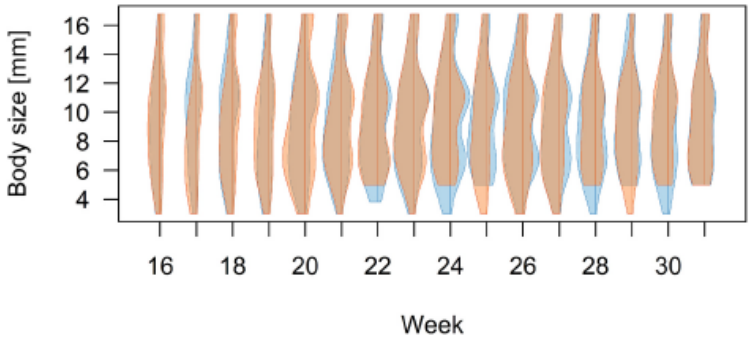

Fig. 6. Asymmetrical violin plots showing the body size of genera (left distributions) and ASVs (right distributions) that occurred in the respective week in Bti-treated (orange) or control (blue) ponds.

ponds treated with Bti exhibited a slight increase in genus richness compared to control ponds. However, this trend reversed as the weeks progressed. This differs from findings in other studies, where Bti effects on taxa diversity were either absent (Lundström et al., 2010, Theißinger et al., 2019; floodplain and forest wetlands) or distinctly negative (Theißinger et al., 2019; meadow wetlands). For instance, Lundström et al. (2010) found no significant Bti effect on chironomid species richness over six years, but their annual results were based on cumulative data from 19 sampling weeks, potentially overlooking short-term dynamics. Our study emphasizes the importance of monitoring emergence dynamics over an extended period of time following Bti application. Relying solely on cumulative data may average out temporal differences in the impact of Bti on chironomid diversity and/or community composition.

Despite the moderate impact of Bti on (normalized) genus- and ASV-level richness, our study revealed a remarkably regular pattern in the observed effects (Table 1), where (normalized) richness is (inversely) correlated with emergence. Specifically, a reduction in emergence correlated with decreased richness but increased normalized richness, and vice versa. When emergence levels were unaffected, (normalized and non-normalized) richness remained unchanged. This suggests a uniform impact of Bti on chironomid taxa, though the presence of slightly more rare taxa in Bti-treated ponds hints at potential effects on community composition. In the following, taxa-specific responses are discussed to elucidate the underlying dynamics of these findings, for example, taxonomic turnover.

4.2. Genus-specific responses of chironomids to Bti

In contrast to our expectation, we observed similar responses to Bti across various chironomid genera, corresponding to a weak phylogenetic signal (HMSC analysis). This pattern contradicts our initial hypothesis of taxon-specific responses, particularly in the Tanypodinae subfamily. Intriguingly, we observed a mix of positive and negative responses of different chironomid genera only when the overall chironomid emergence remained unaffected by Bti treatment (Figs. 3 and 4). This suggests the possibility of a taxonomic shift, where the reduced emergence of one or few taxa is compensated by the increased emergence of other taxa, as reported by Theißinger et al. (2019). Further research is necessary to gain a deeper understanding of the

circumstances under which reduced survival rates among some chironomid taxa can be compensated by taxon shifts.

The consistent response pattern of chironomid genera to Bti is in accordance with the minimal influence of Bti treatment on the community composition derived from community structure analyses (Supplementary material, Figs. S1 to S4). It supports the prevailing hypothesis that early-instar larvae generally exhibit greater susceptibility to Bti. Previous studies, focusing on representatives of the genus *Chironomus* (Treverrow, 1985; Ping et al., 2005; Kästel et al., 2017), have shown that chironomids exhibit a decreasing susceptibility to Bti with progressing larval developmental stages. If this observation extends to all chironomid taxa, this could explain the observed homogeneous response pattern. In our study, we observed an elevated emergence in all Bti-treated ponds during the initial phase, which lasted for two to seven weeks (Fig. 1). Notably, all observed responses displayed by chironomid genera in this phase were positive. It is likely that the Bti treatment exerted a direct negative impact primarily on the younger instar larvae, while those larvae that reached the stage of emergence within a short period after Bti application benefited from the enhanced availability of nutrients and reduced competition. However, the later phase, where we observed an emergence lower than expected in four of the treated ponds followed by a subsequent phase of noticeably higher emergence, could suggest a Bti-induced delay in larval development, as it has been observed for chironomids exposed to pesticides (e.g., Monteiro et al., 2019). On the other hand, the emergence in the two northernmost Bti-treated ponds consistently remained below the expected levels throughout the latter two-thirds of the experiment phase. These ponds consistently exhibited lower water temperatures than the other four Bti-treated ponds during April and May (70 to 90 % of the time), with temperature differences of up to 2 °C (data not shown), potentially leading to slower larval development (cf. Eggermont and Heiri, 2012) and consequently intensifying the impact of Bti application in spring as larvae were still in their vulnerable life stages. Additionally, our knowledge regarding the factors influencing the persistence of Bti in natural water bodies is limited, and it is plausible that the physico-chemical dynamics in the outer two ponds could have differed. Because of the similar community composition across all ponds, we do not assume that these ponds contained more Bti-sensitive taxa, although we cannot entirely dismiss this possibility since we did not identify the chironomids at the species level. The considerable variability in Bti effects on the emergence within our locally restricted replicated mesocosms underscores the role of contextual factors, particularly environmental influences, in affecting outcomes at the community level.

4.3. Larval feeding habits and Bti susceptibility

We hypothesized that predatory larvae would exhibit lower susceptibility to Bti compared to other feeding groups. However, our results revealed no substantial differences in the Bti effect across the different feeding strategies (Fig. 5). Our unexpected findings can be attributed to the challenges we encountered in identifying chironomid genera with strictly predatory larvae (cf. Theißinger et al., 2019). The chironomids we classified as "facultative predators" either belong to obligate predator genera (e.g., *Ablabesmyia*, Vallenduuk and Moller Pillot, 2013) or exhibit non-predatory behavior in their early larval stages (e.g., *Procladius*, Vallenduuk and Moller Pillot, 2013). Additionally, it is important to note that larval feeding strategies can vary between species of the same genus, therefore determining the precise feeding strategy for chironomid genera presents a potential additional level of variation. Unfortunately, information regarding the larval ecology at the species level is scarce (but see Serra et al., 2016). Our findings highlight the existing gaps in our understanding of the relationship between Bti susceptibility and chironomid larval feeding strategies. To advance this field, future research should focus on investigating chironomid feeding types at both the genus and species levels. Further research could involve molecular techniques, studying the trophic interactions within aquatic invertebrate communities, and conducting controlled laboratory experiments aimed at unravelling the underlying factors influencing Bti susceptibility in chironomid larvae with different feeding strategies. These future investigations might contribute to a more comprehensive understanding of chironomid ecology in general and aid in the development of effective protection strategies for non-target organisms subjected to mosquito control treatments.

4.4. Bti effect on chironomids of different size classes

The higher ASV richness in the first five weeks within the Bti-treated ponds, in comparison to the control ponds, is consistent with our observations during size-sorting, where we found a four-fold increase in the number of individuals from the largest size group. Also, the 1.5-fold higher abundance of individuals from the two smallest size groups in Bti-treated ponds during the initial phase, and the 1.5-fold lower abundance in the later weeks, is reflected by the observed ASV abundance patterns. These findings suggest a relation between the ASV abundance and the number of emerged chironomid individuals, although the number of emerged individuals cannot be directly inferred from ASV abundance (Iwaszkiewicz-Eggebrecht et al., 2023). Still, our observations indicate that the differences in ASV abundance can serve as valuable indicators of the potential impact of Bti treatment on the emergence of chironomid individuals across different size classes over time.

Moreover, our findings highlight the added value of evaluating taxon-specific trait information when studying the impacts of Bti treatments on chironomids. By examining the size distribution within the community, valuable insights can be gained that complement traditional metrics such as average individual weight or cumulative biomass. For example, Kolbenschlag et al. (2023) did not reveal disparities in the average individual weight of chironomids during the later experiment phase. However, the presence of a bimodal distribution of chironomid sizes (inferred from the distribution of ASV abundance across chironomid sizes; Fig. 6) likely obscured the effects. Changes in the size distribution of available prey can impact terrestrial predators exhibiting size-selective foraging behaviors within the size range considered in this study, such as bats (Divoll et al., 2022), adult dragonflies (Olberg et al., 2005) and birds (e.g., Turner, 1982). Consequently, it is crucial to conduct further research that specifically explores the interactions between size-selective predators and their prey in response to Bti treatments. This will help us understanding the potential impact of anthropogenic stressors on terrestrial predators that rely on emergent aquatic insects as an essential food source.

5. Conclusion and potential advancements of the model

Assessing the overall ecological impact of Bti on riparian ecosystems is challenging due to the exposure of larvae in the aquatic environment, while the interest lies in understanding its impact on the emergence and the consequences for higher trophic levels in the terrestrial environment. The variable developmental time of chironomid larvae, influenced by both species-specific characteristics (Jackson and Sweeney, 1995) and environmental conditions (e.g., Schütz et al., 2022), adds complexity to selecting the optimal sampling period for studying the effects of aquatic stressors on chironomid emergence. Our relatively simple models demonstrated an average explanatory power of 16.6 to 29.9 % for the chironomid community. Nevertheless, further advancements are possible. For instance, HMSC allows for the incorporation of a broader array of environmental variables. Various environmental factors have been identified to potentially influence the fate of Bti and resulting effects on chironomid communities. For example, studies have highlighted the role of flooding events (Lagadic et al., 2016), UV radiation (Poulin et al., 2022) and rainfall (Caquet et al., 2011) as significant drivers of Bti dynamics. Additionally, factors such as macrophyte coverage, temperature and water depth (Charbonneau et al., 1994) have also been implicated in shaping the fate and effects of Bti on chironomid

communities. The considerable variability in the effects of Bti on emergence, as observed in our replicated mesocosm study within a local context, underscores the significant influence of environmental factors on these outcomes. Our findings revealed, besides an overall interaction of time and treatment on the chironomid community composition, a consistent response pattern across various chironomid genera, a lack of phylogenetic constraints and minor differences in chironomid diversity and body size distributions in response to Bti treatment. They further indicated that considering the multifaceted nature of environmental influences is crucial for comprehending the fate and ecological consequences of anthropogenic stressors in aquatic habitats. The application of metabarcoding offers the potential for higher taxonomic resolution down to the genotypic (ASV) level, enabling more precise taxonomic assignments, as well as detailed phylogenetic and population genetic information. Including a number of taxon-specific traits could further contribute to our understanding of non-target effects of Bti. To summarize, the integration of metabarcoding and HMSC emerges as a powerful tool, enabling comprehensive analysis of aquatic and terrestrial communities subjected to anthropogenic stressors in complex mesocosm and field experiments.

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Declaration on the use of generative AI

During the preparation of this work the authors used Chat GPT, an AI language model developed by OpenAI, to enhance the manuscript's clarity and readability. After using this tool, the authors thoroughly reviewed and edited the content as necessary and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Nina Röder: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **V. Sophie Stoll:** Conceptualization, Investigation, Writing – review & editing. **Sara Kolbenschlag:** Conceptualization, Investigation, Writing – review & editing. **Mirco Bundschuh:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Kathrin Theißinger:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Klaus Schwenk:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Raw sequences were deposited in GenBank SRA archive and are available with the BioProject accession number PRJNA989176. Data (chironomid individual numbers and size sorting, chironomid mean individual mass) and R scripts (JAMP, dada2, HMSC and data analysis) are available from Zenodo doi:https://doi.org/10.5281/zenodo.10208032 (Röder et al., 2023).

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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.2023.169735.

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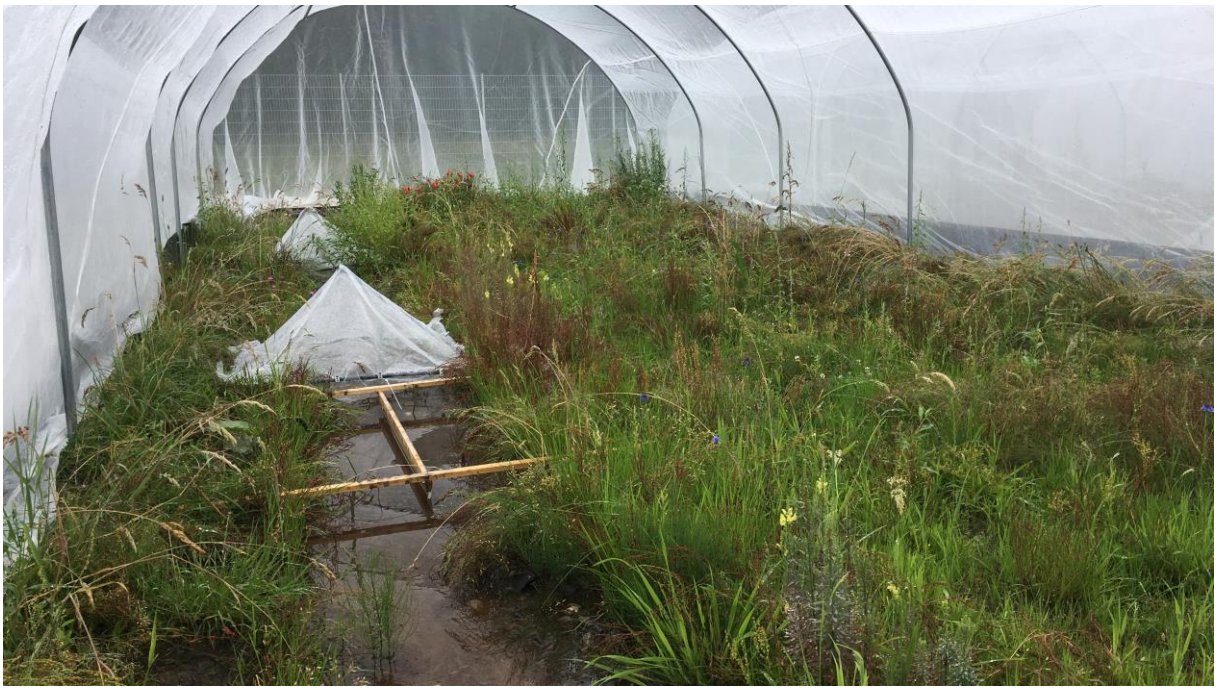
Chapter II: Does hydrological drought alter riparian spider diets?



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External DNA contamination and efficiency of bleach decontamination for arthropod diet analysis

Maike Huszarik[1] | Nina Röder[1] | Linda Eberhardt[1] | Susan Kennedy[2] | Henrik Krehenwinkel[2] | Klaus Schwenk[1] | Martin H. Entling[1]

[1]iES Landau, Institute for Environmental Sciences, RPTU Kaiserslautern-Landau, Landau in der Pfalz, Germany

[2]Department of Biogeography, University of Trier, Trier, Germany

Correspondence
Maike Huszarik, iES Landau, Institute for Environmental Sciences, RPTU Kaiserslautern-Landau, Fortstraße 7, 76829 Landau in der Pfalz, Germany.
Email: huszarik@uni-landau.de and maike.huszarik@rptu.de

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Abstract

DNA metabarcoding is increasingly used to analyze the diet of arthropods, including spiders. However, high sensitivity to DNA contamination makes it difficult to apply to organisms obtained from mass-sampling methods such as pitfall traps. An alternative is to hand-sample spiders, but it is unclear how effectively this prevents external contamination, especially with new knowledge showing the wide spread of eDNA in the environment. Protocols using bleach to remove external DNA have been tested on several invertebrates, though testing with both mass-sampling methods and spiders is lacking. Here, we used wolf spiders (Lycosidae) to assess the risk of external DNA contamination from pitfall trapping and hand sampling, and the efficacy of bleach decontamination. We first conducted a contamination experiment where we placed spiders in pitfall traps containing trapping medium and a nonprey insect species to simulate external DNA contamination. We also compared sampling methods by collecting spiders using pitfall traps and hand sampling. Spiders from the contamination experiment and sampling method comparison were either bleached or untreated, then metabarcoded using multiple primer pairs. The contamination experiment resulted in the contamination of almost all spiders from pitfall traps, which was successfully eliminated with bleaching. Interestingly, there was no difference in the number of amplicon sequence variants (ASVs) detected per spider between pitfall trapping and hand sampling but bleaching resulted in significantly fewer ASV detections for both methods. Additionally, bleaching, but not sampling method, affected the taxonomic diet composition for both hand-sampled and pitfall-trapped spiders, indicating similar levels of external contamination. Our results are the first to confirm that DNA metabarcoding can be used together with bleaching for spiders sampled from pitfall traps, and that hand sampling does not necessarily exclude external DNA contamination. Thus, diet studies using metabarcoding should address the risk of external contamination with field-sampled arthropods, regardless of sampling method.

KEYWORDS
Araneae, DNA metabarcoding, gut content, hand sampling, Lycosidae, pitfall trap

1 | INTRODUCTION

DNA metabarcoding of gut content has become an important tool for studying the diet of arthropod predators (Birkhofer et al., 2017; Valentini et al., 2009), and to describe trophic links within food webs (Nielsen et al., 2018; Pringle & Hutchinson, 2020; Roslin & Majaneva, 2016). It is especially useful for cryptic feeders such as spiders (Greenstone & Shufran, 2003), which externally digest their prey and leave no morphologically identifiable remains in their gut or excrement. Furthermore, spiders are good candidates for food web studies using DNA metabarcoding, as they play important predatory roles in a wide range of ecosystems (Nyffeler & Birkhofer, 2017) and retain a relatively long snapshot of their diet, with prey DNA remaining detectable in their guts for days to weeks (Harwood, 2008; Uiterwaal & DeLong, 2020).

DNA metabarcoding is a highly sensitive method that can detect small amounts of DNA, ideal for analyzing degraded prey DNA in the gut. However, this makes DNA contamination from sample collection an important consideration for studies using DNA metabarcoding (Liu et al., 2020). To avoid contamination of arthropods with external DNA, careful hand sampling has been recommended (King et al., 2008) and is often used to individually collect spiders for metabarcoding (Hambäck et al., 2016; Macías-Hernández et al., 2018). Hand sampling also limits the possible sampling effort for a study, however, as it is time- and labor-intensive (Chapman et al., 2010). Furthermore, recent surveys of insect environmental DNA (eDNA) have shown that insect eDNA is detectable within the terrestrial environment on surfaces such as leaves (Valentin et al., 2020), flowers (Thomsen & Sigsgaard, 2019) and even in air (Roger et al., 2022), so it is possible for spiders and other arthropods to be covered with nonprey DNA just from moving through their environment. Thus, if spiders are already externally covered with eDNA, hand sampling would not exclude external DNA contamination (Greenstone et al., 2011). This new knowledge calls for the testing and updating of sampling recommendations to assess and account for this risk.

Ideally, diet analysis and food web studies should use effective sampling methods, as they benefit from large sample sizes (Pringle & Hutchinson, 2020). Pitfall trapping is one common approach to passively collect high numbers of arthropods, especially ground-dwelling spiders such as wolf spiders (Lycosidae) (McCravy, 2018). These traps may be filled with preservative trapping medium to prevent in-trap predation, allow traps to be emptied less frequently (wet pitfall trapping; Weeks Jr & McIntyre, 1997) and preserve DNA (Nakamura et al., 2020). However, pitfall traps are not a recommended sampling method for molecular analysis, as there may be external DNA contamination of sampled individuals with nonprey organisms in the traps if DNA is transferred via trapping medium (Shokralla et al., 2010). Nevertheless, the risk for external DNA contamination of samples from wet pitfall traps has not yet been directly evaluated, although the benefits obtained by the use of diet analysis for pitfall trap samples are apparent.

One possibility for dealing with possible external DNA contamination of samples for metabarcoding studies is through the removal of external DNA. Dissecting the gut from the exoskeleton (Athey et al., 2017) is one approach which only includes internal DNA. However, this method is time-consuming and difficult to apply to spiders, which have complex gut structures (Macías-Hernández et al., 2018). Another strategy is the decontamination of the spider cuticle by removing or destroying any external DNA. Greenstone et al. (2012) developed a protocol for bleaching the exterior of beetles prior to molecular gut content analysis, which successfully removed external DNA contamination. Bleach is well-known for decontaminating lab surfaces (Champlot et al., 2010), and has also successfully been used to decontaminate other organisms including lepidopteran larvae (Hausmann et al., 2021), ticks (Binetruy et al., 2019), and rotifers (Oh et al., 2020) used for molecular analyses. Bleaching has also been tested for spiders from a field experiment (Miller-ter Kuile et al., 2021), but without explicit contamination to directly assess the removal of external DNA. Experimentally testing bleach and directly comparing its effects on spiders sampled with different methods would help define the need for and effectiveness of decontamination prior to molecular diet analysis of whole spiders. Indeed, a decontamination step using bleach would offer a way to ensure that external DNA contamination does not affect diet results.

Thus, we aimed to explicitly test surface decontamination of spiders using bleach, and to compare the external DNA contamination of spiders sampled by wet pitfall trapping and hand sampling. We first conducted a contamination experiment by adding wolf spiders to wet pitfall traps containing an exotic nonprey insect to assess whether wet pitfall trapping results in external DNA contamination of the spiders with the exotic insect DNA, and the ability of bleaching to remove contamination. We also compared the detected amplicon sequence variants (ASVs) and diet composition obtained from wolf spiders sampled by hand and by pitfall trap, as well as the effect of bleaching with both sampling methods. We used a multiplex DNA metabarcoding approach, which included three primer pairs specifically designed for spider diet analysis (Krehenwinkel et al., 2019), rather than one pair, to cover a broad range of possible arthropod prey taxa. Together, our results can contribute to informing sample collection and handling to avoid external DNA contamination of arthropods, while allowing DNA metabarcoding to be used for samples with assumed contamination (i.e., due to mass-sampling methods) and increasing the types of samples suitable for molecular diet analysis.

2 | MATERIALS AND METHODS

2.1 | Contamination experiment

Adult female wolf spiders, *Pardosa amentata* (Clerk, 1757) and *Pardosa agrestris* (Westring, 1861), were individually hand-sampled in a vineyard in Rhineland-Palatinate, Germany (Figure 1, Table S1). They were kept at room temperature in individual containers for 3 days and fed *Sinella curviseta* (Brook, 1882; Collembola) to obtain a known gut content. Simultaneously, four pitfall traps were prepared

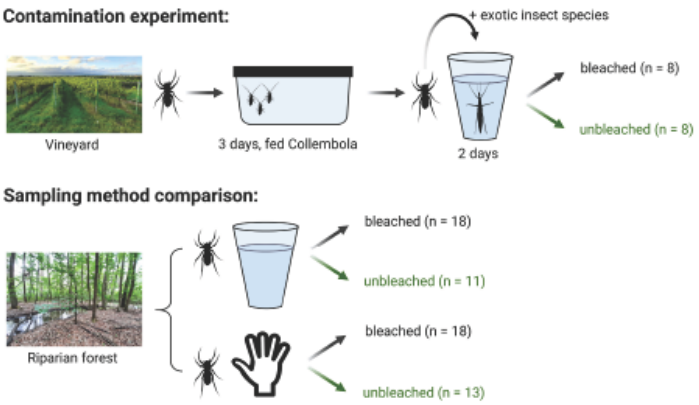

FIGURE 1 Overview of the collection and treatment of wolf spiders (Lycosidae) used for a contamination experiment and a comparison of two sampling methods for molecular diet analysis. Spiders for the contamination experiment (above) were collected by hand in a vineyard, then kept separated in the lab and fed *Sinella curviseta*, before being added to pitfall traps containing an exotic walking stick species, *Sungaya inexpectata*. For the sampling method comparison (below), spiders were either collected by hand or pitfall trap from riparian forests. Spiders from both parts of the study were then either bleached or left untreated, before being sequenced for DNA metabarcoding.

to simulate contamination occurring during trapping. We added multiple individuals of a non-native walking stick species, *Sungaya inexpectata* (Zompro 1996; Phasmatodea: Heteropterygidae), to unused plastic cups containing propylene glycol trapping medium (30% propylene glycol, 70% water, 1 mL/L dish soap and 10 mg/L denatonium benzoate – a deterrent for larger animals). As the spiders could not have previously fed on *S. inexpectata*, this species served as an indicator for DNA contamination caused by contact with nonprey organisms in the pitfall traps. After 3 days, the live spiders were added to the traps and immediately drowned, as would happen in the field. The traps were then set in forest ground (Table S1) to simulate field conditions, and covered to prevent additional material from entering. After 2 days, the spiders were removed, stored in 70% ethanol and frozen at −20°C. Finally, the ethanol was removed and the spiders were frozen individually in dry tubes at −20°C. Although the use of 70% ethanol and dry storage, common methods of storing arthropod samples to reduce brittleness, is not optimal for preservation of long-stranded DNA (Marquina et al., 2021), these methods are unlikely to have caused significant DNA degradation of our samples due to the short (<6 months) storage period (Stein et al., 2013), low temperatures (Vink et al., 2005), and short target fragment lengths.

2.2 | Sampling method comparison

We collected spiders by pitfall trap and hand sampling in the riparian zones of four forested streams in Rhineland-Palatinate, Germany (Figure 1, Table S1). Eight uncontaminated pitfall traps were set in the ground within 15 m of each stream bank. The pitfall traps were filled with 30% propylene glycol trapping liquid, as described for the contamination experiment, and emptied weekly over 5 weeks. Upon collection, the trap contents were immediately transferred to 70% ethanol and frozen at −20°C. The hand sampling of wolf spiders occurred on four occasions (Table S1), taking care to avoid external contamination. Each spider was frozen separately in a dry tube at −20°C. All female individuals of *Pardosa saltans* (Töpfer-Hofmann, 2000) and *Piratula hygrophila* (Thorell, 1872) from both hand and pitfall trap sampling were separated and individually stored in 96% ethanol at −20°C. Species identification of all spiders occurred on ice using Nentwig et al. (2019) and Roberts (1995).

2.3 | Bleaching

Approximately half of the spiders in each species and capture method group (pitfall trap, hand-sampled, experiment; Table 1, Figure 1) were bleached following Greenstone et al. (2012), to remove external DNA contamination. Bleaching was performed in the laboratory prior to DNA metabarcoding. Slightly more bleached spiders were included to ensure large enough sample sizes in case some individuals were damaged by the bleach treatment. Briefly, each spider was removed from its storage tube and added to a sterile tube containing 500 µL of 2.8% (w/w) commercial NaClO bleach (DANKlorix; Colgate-Palmolive GABA GmbH) for 40 min at 7.5°C, and gently shaken every 5 min. The bleach was then removed and the spiders were washed three times with distilled water and stored in 70% ethanol at −20°C.

2.4 | DNA extraction and PCR amplification

Ethanol was removed and spiders were dried in their tubes at 60°C for 1 h, then ground to a fine powder on a TissueLyser II (QIAGEN)

TABLE 1 The number of wolf spiders (Lycosidae) obtained with different sampling methods and treated with bleach for diet analysis with DNA metabarcoding.

	Pitfall trap		Hand-sampled		Experiment	
Spider species	Bleached	Unbleached	Bleached	Unbleached	Bleached	Unbleached
Pardosa saltans	8	5	8	5	–	–
Piratula hygrophila	10	6	10	8	–	–
Pardosa agrestris	–	–	–	–	2	2
Pardosa amentata	–	–	–	–	6	6
Total spiders (*n*)	18	11	18	13	8	8

using sterilized steel beads. DNA was extracted using a "high-salt" extraction method (Table S2; Aljanabi & Martinez, 1997), alongside seven negative control extractions. Next, prey DNA was selectively amplified in a multiplex PCR step using three primer pairs targeting variable regions of 18S (18S short and 18S long) and 28S rDNA, which are specifically designed to amplify a broad range of arthropods while suppressing spider amplification and providing taxonomic resolution comparable to COI at the order level (Krehenwinkel et al., 2019: 18SS, 18SL, and 28S; Table S3). For the multiplex PCR, 1.5 μL of the isolated DNA of each sample was mixed with 13.5 μL of the multiplex master mix (Table S4), then amplified (Table S5). Ten negative controls for DNA contamination containing only master mix were included. The success of the multiplex PCR was verified using gel electrophoresis. Then, all samples and negative controls were indexed and sequenced (Table 1).

The samples were indexed in a second PCR step using 96 unique combinations of 8 forward and 12 reverse indexing primers (Table S3). 0.5 μL of the multiplex PCR product was added to 8.5 μL master mix and 1 μL of the indexing primer pair (Table S6). This was then run with a shortened PCR program (Table S5), along with three negative controls containing only master mix. The success of the indexing PCR was verified with gel electrophoresis. The band strength on the gel was used to determine the amount of each sample to add to the final library for sequencing. The band strength was categorized as "weak," "middle," and "strong," and 4 μL, 2 μL, or 1 μL of sample was added, respectively, to include an approximately similar amount of DNA per sample in the library. The completed library was then shipped for the clean-up and sequencing steps. The library clean-up was performed at Trier University using 1X AMPure beads XP (Beckman Coulter). Finally, the library was sequenced with an Illumina MiSeq high-throughput sequencer using the MiSeq Reagent Nano Kit v2 (500-cycles; MS-103-1003; Illumina Inc.). Sequencing was performed at the Max Planck Institute for Evolutionary Biology in Plön, Germany.

2.5 | Sequence data processing and taxonomic assignment

The sequences were automatically demultiplexed by index barcode combination (bcl2fastq Conversion Software v1.8.4; Illumina Inc.). The adapter and primer sequences were removed using Cutadapt (Martin, 2011) at usegalaxy.eu (Afgan et al., 2018). The workflow was run once for each primer pair, and set to discard untrimmed reads (i.e. those belonging to the other primer pairs), with pair filtering set to both directions. The trimmed sequences were then processed with the DADA2 pipeline (v1.16; Callahan et al., 2016) in RStudio (R version 4.0.3; R Core Team, 2020). They were filtered (maxN = 0, maxEE = (2,2), truncQ = 2), and trimmed, with values for the "truncLen" variable based on the expected target sequence lengths of each primer pair and the quality plots produced with "plotQualityProfile" (18SL = 200, 160; 18SS = 150, 100; 28S = 200, 200). The sequences were then dereplicated, denoised, had chimeras removed and were merged to build the final amplicon sequence variant (ASV) table. In total, we identified 346,967 sequence reads and approximately 4565 reads per sample. The total number of reads and ASVs for each primer pair are presented in Table S7.

Next, taxonomic identities were assigned by matching the ASV sequences to entries in the GenBank nucleotide database (download date: 17 January 2022; Clark et al., 2016) using blastn MegaBLAST (BLAST+ version 2.12.0; Camacho et al., 2009), to obtain the first 100 matches, sorted by *e*-value. If the first 100 matches had the same *e*-value (i.e., no clear best sequences), more matches were obtained. Only matches with more than 85% identity, which has been tested as a threshold for assigning taxonomy at the order level for these primer pairs (Krehenwinkel et al., 2019), and at least 100 bp match length were included. However, the average match had over 97% identity. The NCBI BLAST name of the first 100 matches was used to assign each ASV to order level or higher, as this was sufficient to differentiate arthropods from nonprey groups. If the first 100 matches had the same BLAST name, then this taxonomic group was assigned to the ASV. If there were multiple matches, the name of the match with the best *e*-value and percent identity, query coverage and alignment length was selected. Specific ASVs were assessed and reblasted if conflicting orders were matched, and orders were assigned based on plausibility (i.e., considering unassigned uncultured environmental DNA matches, GenBank entries identified as contamination, mislabeled entries, and likely occurrence in study area). A second NCBI BLAST search (Johnson et al., 2008) was run only for spiders used in the contamination experiment, to identify Collembola ASV sequences with 100% identity matches to *S. curviseta*.

Amplicon sequence variants were then filtered to select only nonspider, noncrustacean arthropod orders, to avoid predator ASVs

from the spiders while keeping potential prey. In addition, ASVs only found in the negative DNA extraction and PCR controls were removed. After filtering and removing nontarget taxa, the diet analysis resulted in 77,117 target reads across all primer pairs (Table S7). To create an ASV presence-absence table, an ASV presence was defined as a read number greater than the minimum sequence copy of 0.001% of the maximum read number for that ASV (Drake et al., 2022). Singletons were excluded. As negative controls for DNA extraction and PCR did not show contamination of nonspider, noncrustacean arthropods, no adjustments for contamination were required.

2.6 | Statistical analysis

Statistical analyses were performed using R (R version 4.0.3; R Core Team, 2020). Data from each primer pair (18SS, 18SL, 28S) were analyzed separately, as it could not be confirmed that identical ASVs with identical taxonomic assignment from different genetic markers (i.e. different primer pairs) originated from the same organisms. First, to evaluate the effect of bleaching on the spiders from the contamination experiment, ASV counts for each spider were combined to form the three taxon groups "walking sticks" (the number of *S. inexpectata* ASVs), "known prey" (the number of ASVs with a 100% match to *S. curviseta*), and "other" (all other ASVs). The effect of bleaching on the average detections of each ASV group was evaluated using generalized linear models (GLMs) with a Poisson distribution, using the glm function in R.

Next, automated model selection was used to find the best model containing the variables affecting the number of ASVs detected per spider. First, a global GLM with a Poisson distribution was created, including all relevant predictor variables (spider species, sampling method and bleaching treatment) and their interactions, with the number of ASV detections as the response variable. All possible model subsets were computed and ranked by AICc (Burnham & Anderson, 2002; Hurvich & Tsai, 1989) using dredge (MuMIn; Barton, 2020). The best model (i.e. with lowest AICc) was chosen, except for 28S, where the second-best model was chosen as it included all three main predictor variables and had an AICc which was indistinguishable from the best model (delta AICc = 0.08; Burnham & Anderson, 2002). In addition, an initial GLM model had been conducted which included spider damage from bleach as a variable, to ensure that the exterior damage from bleaching observed in some spiders did not influence the diet analysis results. Damage was not significant for any primer pair 18SL: $Z(56,59) = 0.395$, $p = 0.495$; 18SS: $Z(55,59) = 1.135$, $p = 0.480$; 28S: $Z(57,59) = -2.265$, $p = 0.376$, so it was excluded in the final model selection. Finally, the effect of bleaching and sampling method on the spiders' recovered taxonomic diet composition was evaluated using a permuted MANOVA (adonis, vegan; Oksanen et al., 2019). The total number of ASV detections of each taxonomic group was calculated for each spider as the response variable. The predictor variables were spider species, bleaching treatment and capture method, as well as their interactions.

Generalized linear model assumptions and fit were verified for all models with check_model (performance, Lüdecke, Ben-Shachar, et al., 2021; see, Lüdecke, Patil, et al., 2021). To avoid problems with quasi-separation and over- and underdispersion, we derived the significance values for all GLMs from permutation tests (PermTest, pgirmess; Giraudoux, 2018). A significant result was considered $p < 0.05$. Figures were created using ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2020).

3 | RESULTS

3.1 | Contamination experiment

We detected walking stick ASVs in 7 of 8 unbleached spiders and none of the bleached spiders that had been placed in pitfall traps with walking sticks (*S. inexpectata*; Figure 2). Bleaching eliminated walking stick ASVs for all three primer pairs (18SL: $Z_{(14,15)} = 0.002$, $p < 0.001$; 18SS: $Z_{(14,15)} = 0.002$, $p < 0.001$; 28S: $Z_{(14,15)} = 0.002$, $p = 0.001$), but had no significant effect on the detections of the supplied prey *S. curviseta* or other taxa in any primer pair (all others $p > 0.41$; Figure 2).

3.2 | Sampling method comparison

The spider sampling method had no significant effect on the number of ASVs detected per spider in any primer pair (18SL: $Z_{(55,59)} = 2.450$, $p = 0.531$; 18SS: $Z_{(56,59)} = 0.000$, $p = 1.000$; 28S: $Z_{(56,59)} = 1.481$, $p = 0.417$; Figure 3). Furthermore, an interaction between the sampling method and bleaching was not included in the final models of 18SS nor 28S following model selection, and only showed a slight trend for 18SL ($Z_{(55,59)} = -2.390$, $p = 0.087$). Thus, sampling method also did not have an effect on the proportion of ASVs lost due to bleaching. Bleaching reduced ASV detections by 63.2% on average, namely 68.2% for hand-sampled and 58.3% for pitfall-trapped spiders. The negative effect of bleaching on insect DNA recovery was significant for all primer pairs (18SL: $Z_{(55,59)} = 4.221$, $p = 0.002$; 18SS: $Z_{(56,59)} = 2.027$, $p < 0.001$; 28S: $Z_{(57,59)} = 5.411$, $p = 0.002$; Figure 3). Finally, for both 18S primers, there was no effect of the spider species on the number of ASVs. However, 28S showed a significant difference in the number of ASVs found between *P. hygrophila* and *P. saltans* ($Z_{(57,59)} = 5.571$, $p < 0.001$), with *P. saltans* having approximately four times more ASVs detected per spider.

3.3 | Effect of bleaching and sampling method on detected diet composition of spiders

Springtails (Collembola), flies (Diptera), crickets (Orthoptera), and beetles (Coleoptera) composed most of the spider diet, with only minor differences in detected taxonomic diet composition between the three primer pairs (Figure 4). 18SS yielded the most

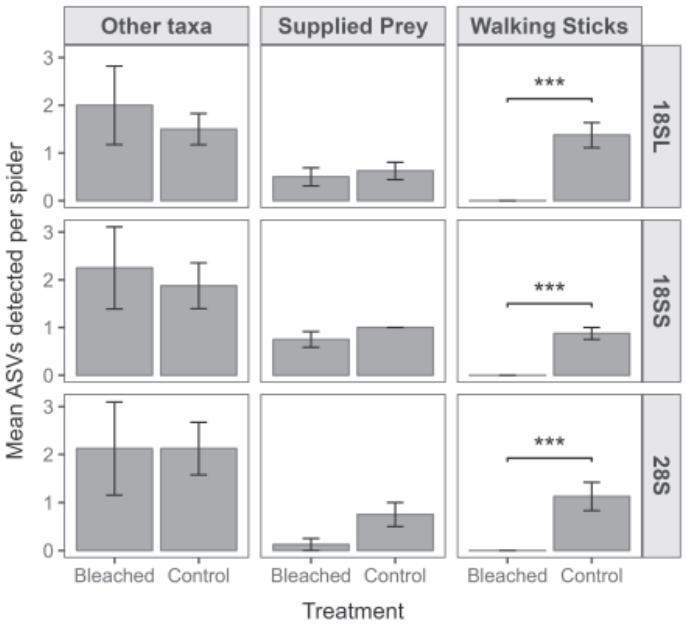

FIGURE 2 Effect of treatment with bleach on the average count of amplicon sequence variants (ASVs) obtained from wolf spiders (Lycosidae; $n = 8$ bleached, 8 control). "Walking sticks", *Sungaya inexpectata*, had been used to simulate external DNA contamination on the spiders from pitfall trap medium. The spiders had been fed the springtail species *Sinella curviseta* ("Supplied Prey") before being added to traps containing *S. inexpectata*. Spiders had either been treated with bleach to remove external DNA contamination (bleached) or not treated (control). The three primer pairs used for DNA metabarcoding are in separate panels on the vertical axis, and the taxonomic groupings of the ASVs on the horizontal axis. Significant differences are labeled with "***", indicating *p*-values of 0.001 or less, and standard errors are represented by black bars.

taxonomic groups, and 28S the fewest. Bleaching resulted in the recovery of a significantly different taxonomic diet composition in all three primer pairs, namely in fewer detections, rather than different taxa detected in the diet (18SL: $F_{(1,56)} = 4.31$, $p = 0.009$; 18SS: $F_{(1,56)} = 4.95$, $p = 0.003$; 28S $F_{(1,55)} = 6.38$, $p = 0.003$). Additionally, moths and millipedes were only detected in unbleached spiders. 18SL and 28S revealed a significant difference in the diet composition of *P. hygrophila* and *P. saltans* (18SL: $F_{(1,56)} = 3.17$, $p = 0.024$; 28S: $F_{(1,55)} = 10.64$, $p = 0.001$; Figure 4). 28S also showed an interaction between spider species and the bleaching treatment on diet composition ($F_{(1,55)} = 4.33$, $p = 0.012$). Furthermore, the sampling method was not associated with an effect on the diet composition of the spiders (18SL: $F_{(1,56)} = 0.203$, $p = 0.947$; 18SS: $F_{(1,56)} = 0.010$, $p = 0.652$; 28S $F_{(1,55)} = 0.009$, $p = 0.477$).

4 | DISCUSSION

4.1 | Contamination experiment

The results from our contamination experiment clearly confirm that wet pitfall trapping results in external DNA contamination of organisms used for molecular diet analysis. All unbleached spiders but one were contaminated with walking stick (*S. inexpectata*) DNA (Figure 2). Moreover, the fact that no bleached spiders had traces of *S. inexpectata* DNA indicates that the contamination in the pitfall traps was only external. Although this is the first study explicitly testing external DNA contamination from wet pitfall traps, our result is not unexpected. Propylene glycol can be used to store samples for molecular analysis (Nakamura et al., 2020), and can preserve DNA in aqueous solutions with concentrations as low as 20% (Ferro & Park, 2013). Our trapping medium contained 30% propylene glycol, which likely preserved the DNA of *S. inexpectata*, allowing it to come into contact with the spiders. Thus, such DNA contamination is also likely to occur in any other mass-sampling trap containing a DNA-preserving liquid.

There are several additional factors of trapping contamination that we did not explicitly evaluate with the experiment. For example, we did not consider the contribution of regurgitation to contamination within wet traps (King et al., 2008), nor the effect of time spent in trapping medium. However, when considering that insect eDNA can be widespread in the terrestrial habitat (Roger et al., 2022; Thomsen & Sigsgaard, 2019; Valentin et al., 2020), it is not surprising that mass-sampling traps are a source of contamination and are likely not the only concern when collecting arthropods. Our result calls for careful consideration when extracting DNA from whole organisms caught by mass-sampling traps, as any DNA on the target organism's cuticle can also be amplified by the primers targeting prey DNA from the gut.

4.2 | Bleaching decontamination

As expected, bleaching successfully removed the nontarget walking stick DNA from treated spiders in our contamination experiment, while not reducing the detection frequency of potential prey

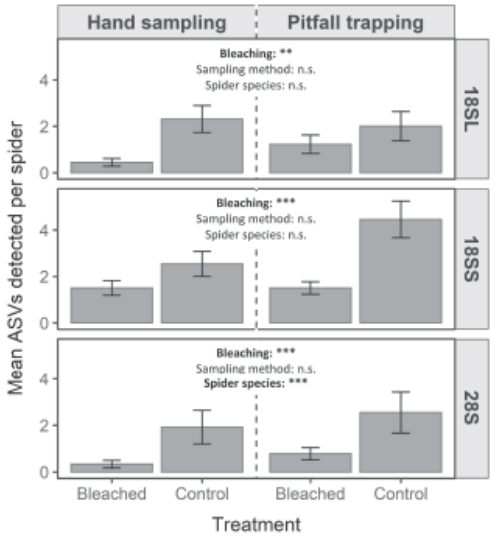

FIGURE 3 Effects of treatment with bleach on the average count of amplicon sequence variants (ASVs) found in wolf spiders (Lycosidae) sampled either by hand ($n = 18$ bleached, 13 control) or by pitfall trapping ($n = 18$ bleached, 11 control). The spider species included were *Pardosa saltans* and *Piratula hygrophila*. Spiders had either been treated with bleach to remove external DNA contamination (bleached) or not treated (control). The three primer pairs used for DNA metabarcoding are in separate panels on the vertical axis, and the spider sampling method on the horizontal axis. Significance is reported on the plots, with "n.s.", "**"and "***" representing p-values of >0.05, <0.01 and <0.001, respectively. Standard errors are represented by black bars.

(Figure 2). Bleached field-captured spiders also showed a strong reduction of arthropod ASV detections, with similar effects in both hand-sampled and pitfall-trapped spiders (Figure 3). However, a concern with bleaching is that it can destroy internal DNA if it enters the gut. The experiment did not show any effect of bleaching on the presence of ASVs matching with *S. curviseta*, which had been fed to the spiders to provide a known gut content. This indicates that the gut content was not significantly affected by the bleach. In fact, we detected other taxa which the spiders had probably consumed prior to being captured (Macías-Hernández et al., 2018), which were also not affected by bleaching. Additionally, a previous study testing the effectiveness of bleaching for surface decontamination of spiders also found that it did not significantly alter the measures of spider diet (Miller-ter Kuile et al., 2021). Thus, the results from our contamination experiment strongly suggest that bleach is a good treatment for removing external DNA, while keeping internal DNA intact.

Although there were no significant effects on gut content, our decontamination protocol could be further optimized. We exposed spiders to 2.8% NaClO for 40 min, based on Greenstone et al. (2012). However, Greenstone et al. (2012) used beetles in their study, which have a thicker cuticle than spiders and could likely withstand a stronger bleaching. While our results did not suggest that our protocol disrupted the DNA inside spiders, we could not directly confirm this. We did observe that the cuticle of a few spiders appeared slightly degraded following the bleaching treatment, but the initial statistical models confirmed that this visual damage did not significantly affect the number of ASVs detected. Other studies using bleach for surface decontamination have not found an effect on DNA in gut content (Binetruy et al., 2019; Hausmann et al., 2021; Miller-ter Kuile et al., 2021). However, their exposure times were shorter or at a lower concentration. Thus, we recommend exposing spiders and similar organisms with a thin cuticle to a shorter or less-concentrated bleach treatment to ensure external decontamination, while being certain to preserve internal DNA. Testing several concentrations and exposure times should be conducted to determine the ideal bleaching procedure for spiders and other arthropods.

In addition, bleaching may not be the only approach used for decontamination. Previous studies have already demonstrated that bleaching outperforms washing with ethanol for decontaminating invertebrate samples (Binetruy et al., 2019; Greenstone et al., 2012), as well as washing with distilled water and other decontaminants on surfaces (Champlot et al., 2010). However, bleaching may not be applicable to all samples, especially if they are particularly sensitive or if there are potential safety or environmental concerns. In this case UV exposure or washing with another nontoxic DNA-degrading liquid (as in Nilsson et al., 2022 or Champlot et al., 2010) could be tested. Nevertheless, the specific requirements for each study and organism should be considered and evaluated to decide on an appropriate protocol.

4.3 | Sampling method comparison

As carefully collecting spiders by hand has been suggested to avoid external contamination from mass-sampling techniques (King et al., 2008), we expected hand-sampled spiders to have fewer ASVs than those caught in pitfall traps in the sample method comparison. Surprisingly, our results did not reveal a difference in the number of ASVs detected between sampling methods (Figure 3). In fact, bleaching reduced the number of ASVs in both hand-sampled and pitfall-trapped spiders to a similar degree, which indicates that external DNA contamination was similar between hand-captured and pitfall-trapped spiders. The contamination of pitfall-trapped spiders can be explained by their contact with other arthropods in the traps via the trapping medium, as proven by the contamination experiment. However, the lack of difference between sampling methods indicates that most external DNA contamination is likely unrelated to pitfall traps, and cannot be avoided by hand sampling.

By combining the results of the method comparison and the contamination experiment, we can deduce the amount and source of external contamination of pitfall and hand-sampled spiders. Reductions through bleaching were similar across all arthropod orders in the sampling method comparison. Thus, there is no indication

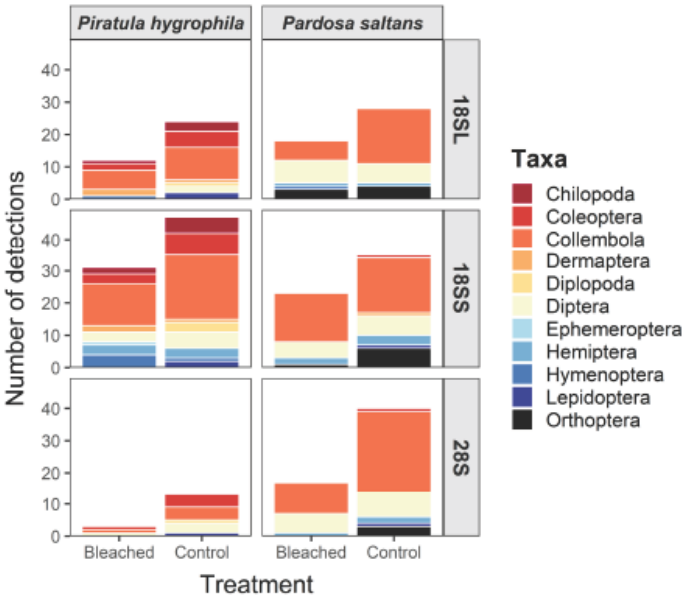

FIGURE 4 Taxonomic composition of gut content sequenced from *Piratula hygrophila* and *Pardosa saltans* using DNA metabarcoding. The spiders were collected by hand ($n = 18$ bleached, 13 control) and pitfall trap ($n = 18$ bleached, 11 control) from riparian forests in southwestern Germany. Spiders had either been treated with bleach to remove external DNA contamination (bleached) or not treated (control). The three primer pairs used for DNA metabarcoding are in separate panels on the vertical axis. Taxonomic composition was measured as the number of amplicon sequence variants (ASVs) detected per taxon per spider species. Note that there was no effect of sampling method on the diet composition and it is not shown in the figure.

that assumed prey, such as Diptera and Collembola, were less affected by bleaching than taxa that are less known to be consumed by wolf spiders, such as Chilopoda and Lepidoptera. Nonetheless, the contamination experiment suggests that bleach only acted externally, as bleaching only reduced *S. inexpectata* detections (Figure 2). Therefore, the strong, nonspecific effect of bleaching on DNA recovery from field-captured spiders combined with the poor effect on non *S. inexpectata* DNA in the contamination experiment suggest that both hand-captured and pitfall-trapped spiders had high levels of external contamination, albeit mainly with arthropods that are also part of their diet. This is not unexpected, because external contact of free-hunting spiders with arthropods will be most intense during prey attack. In addition, the generalist diet of the spiders in this study may mean that most of the organisms from the pitfall traps or local insect eDNA could also be potential prey. This contamination may not be considered severe if the DNA originates from prey or potential prey, but there still cannot be complete confidence in actual gut content versus external DNA if decontamination steps are not taken.

If wolf spiders are already externally contaminated in the field, we would expect that the bleached spiders from the contamination experiment would have shown a reduction of "other taxa" in addition to *S. inexpectata* detections. Although the spiders from our contamination experiment were hand sampled from the field prior to spending several days in the lab, they only showed *S. inexpectata* as external DNA contamination, and no difference in other taxa groups between bleached and control spiders. There is little information available about the fate of nonspider tissue and DNA on the spider cuticle. However, Valentin et al. (2021) found that insect eDNA rarely remained on surfaces for longer than a few days. Thus, we speculate that external DNA may have been lost from the spiders over time in the "clean" laboratory environment following hand capture. It could be interesting to investigate this further as another, specific, method of removing external DNA contamination in live samples.

Interestingly, our results indicate that hand sampling does not exclude external contamination of wolf spiders, and a decontamination step may be advisable regardless of sampling method. Greenstone et al. (2011) also tested whether hand sampling could eliminate contamination by releasing insects on plants where a different species had been incubated, and immediately recollecting them. They found that hand sampling did not necessarily eliminate contamination of their insect samples, indicating that there may still be a risk of external contamination due to the organisms coming into contact with eDNA in their environment, similar to our spiders. As discussed, much of the external DNA could originate from the handling of prey during attack and consumption. Furthermore, insect eDNA is found on plant surfaces and in air (Roger et al., 2022; Valentin et al., 2020), so spiders could have insect DNA on their cuticle simply from interacting with their environment. This is even more likely for free-hunting spiders which are much more mobile in their habitat than web-building species. If this is true, then spiders would also bring external DNA into pitfall traps, adding another source of contamination. Clearly, it is imperative to continue quantifying the risk of contamination of field-sampled organisms and whether it applies to different species and study designs.

4.4 | Effects of bleaching and sampling method on spider diet

In addition to the effect of bleaching and sampling method on the number of ASVs detected per spider, we also expected an effect of sampling method and bleaching on the recovered taxonomic diet composition of the spiders. However, we only observed that bleaching and spider species altered the taxonomic composition of recovered DNA, with no effect of sampling method (Figure 4). The difference in diet between the spider species is not surprising, given that *P. hygrophila* is more specialized on riparian areas whereas *P. saltans* is a forest specialist (Nentwig et al., 2019; Roberts, 1995). In addition, it makes sense that bleached spiders had a different DNA profile than control spiders if bleaching had removed external DNA contamination, which was likely slightly different than prey DNA in the gut. On the other hand, the fact that there was no difference between hand-sampled and pitfall-trapped spiders appears to indicate that both groups were exposed to similar taxa. This reinforces the idea that spiders from both sampling methods had external DNA contamination which was removed by bleaching, but also that hand sampling and pitfall trapping are equally suited to study the diet of wolf spiders.

4.5 | Future directions

Our results not only add to existing literature investigating bleaching as a decontamination method for invertebrates, but demonstrate that a decontamination step would enable food web studies to use mass-sampling techniques for collecting spiders, and other arthropods, for molecular diet analysis. This has the potential to greatly increase the sample size and reduce sampling effort required for such studies. For example, it would be easier to sample nocturnal ground-dwelling spiders or those in remote areas using pitfall traps, as the traps can be left unattended. In addition, different sampling methods are more efficient at collecting different species (McCravy, 2018), so increased sampling flexibility for molecular diet analysis would be a clear advantage. This means that DNA metabarcoding could more easily be used to fill knowledge gaps in the diets of a wider variety of species.

This study also highlights the need for updated sample-handling protocols which consider the risk of external contamination of field organisms with eDNA. We show that contaminating eDNA may originate from potential prey taxa of spiders, which could be mistaken for gut content. Thus, it is important for future studies to quantify the risk of external contamination of arthropod samples and to consider whether a decontamination step is necessary prior to performing molecular diet analysis. One strategy for this may be taking samples of storage ethanol (Shokralla et al., 2010) or the cuticle (Binetruy et al., 2019) to sequence as a control. However, this would not aid in distinguishing between gut and external DNA if both are potential prey taxa, as found in our study. In this case, external decontamination would ensure that sequenced DNA is only internal.

5 | CONCLUSION

In summary, our results confirm that there is a risk of external DNA contamination when using wet pitfall traps, but also that wolf spiders sampled by hand can be as contaminated as those from mass-sampling traps. Thus, external DNA contamination is a concern regardless of the sampling method. We also show that bleaching is effective as an additional decontamination step, enabling the use of DNA metabarcoding to analyze the diet of externally contaminated spiders. With additional testing and adjustment of the protocol, bleaching can also be applied to other mass-sampling techniques where external contamination is a concern, as well as for other arthropods. This would open the possibility of using metabarcoding for larger studies where samples cannot be individually collected, and help to provide important diet information to food web ecologists. Our results question the assumption that hand-sampled spiders are not contaminated with external DNA and call for a more careful approach to sample preparation in the context of DNA metabarcoding whole organisms.

AUTHOR CONTRIBUTIONS

ME, NR and LE conceptualized the study. ME, KS, SK, and HK supervised all study stages. NR and LE carried-out field sampling. MH, NR, SK and LE performed laboratory work. MH and NR conducted bioinformatics and MH performed data analysis. MH, ME, HK, SK, NR, and KS contributed to the interpretation of the data. MH wrote the manuscript with the assistance of all coauthors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw data for this study are available from Figshare (doi: 10.6084/m9.figshare.21197788).

ORCID

Maike Huszarik https://orcid.org/0000-0003-4470-2023

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Data analysis: Assessing the effect of hydrological drought on riparian spider diets

Nina Röder[1], Julia Gieser[1,2], Sebastian Pietz[1], Alexis Pieter Roodt[1], Collins Ogbeide[1], Mirco Bundschuh[1], Ralf Schulz[1,2], Martin Entling[1], Klaus Schwenk[1,3]

[1]iES Landau, Institute for Environmental Sciences, RPTU University Kaiserslautern-Landau, Fortstrasse 7, 76829 Landau, Germany

[2]Eußerthal Ecosystem Research Station, RPTU University Kaiserslautern-Landau, Birkenthalstraße 13, 76857 Eußerthal, Germany

[3]LOEWE Centre for Translational Biodiversity Genomics, Senckenberg Biodiversity and Climate Research Centre, Frankfurt am Main, Germany

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Introduction

Flowing freshwater ecosystems are increasingly threatened by anthropogenic pressures (Battin et al., 2023; Lemm et al., 2021). Escalating human water demand and climate change will likely lead to an increased frequency of hydrological droughts (Vörösmarty et al., 2000), characterized by reduced water availability and prolonged low-flow conditions in streams and rivers. Because many stream invertebrates are adapted to specific flow conditions (Kakouei et al., 2017), alterations in hydrology can substantially affect the composition and structure of benthic communities (Marino et al., 2024; Munasinghe et al., 2021; Wegscheider et al., 2023). Under low-flow scenarios, invertebrate richness often declines, largely due to reduced habitat diversity and the loss of microhabitats critical for different taxa (Dewson et al., 2007). These disruptions are particularly consequential because many benthic invertebrates are the aquatic larval stages of merolimnic insects, which emerge as winged adults and serve as important food resources for terrestrial predators such as birds, bats, and spiders (Baxter et al., 2005; Martin-Creuzburg et al., 2017). As a result, changes in aquatic community structure can weaken ecological linkages and lead to cascading effects across adjacent riparian ecosystems.

To examine how such hydrological changes propagate into riparian communities, we conducted a controlled experiment in riparian stream mesocosms. The experiment simulated reduced water flow conditions and measured multiple ecological responses, focusing on aquatic insect emergence and riparian predator dynamics. The current data chapter presents a targeted analysis of the effects of low-flow conditions on the prey composition of riparian

spiders of the genus *Tetragnatha*, a key group of riparian predators that rely heavily on aquatic subsidies (Iwata, 2007; Kato et al., 2003). This analysis is part of a broader investigation that includes fatty acid profiling and pesticide burden assessment of riparian spiders, aimed at understanding the multifaceted impacts of hydrological drought on terrestrial predator communities.

Based on field observations, we hypothesized the presence of two distinct *Tetragnatha* species in our experimental site, *Tetragnatha extensa* and *Tetragnatha montana*. Both species are aerial web builders adapted to riparian areas. Although both species prefer moist habitats, *T. montana* can also be found away from water (Nentwig et al., 2025; Pfister et al., 2015). Prior results from the same experiment reported a significant 2.6-fold reduction in the abundance of *Tetragnatha* spiders under low-flow conditions (Ogbeide et al., 2025). Additionally, total EPT (Ephemeroptera, Plecoptera, Trichoptera) abundance was significantly reduced in the low-flow treatment, showing a 2.9-fold reduction compared to the control. Dipterans of the family Chironomidae were the dominant taxa in the aquatic insect emergence. Furthermore, overall emergent insect abundance and biomass differed significantly between low-flow and control stream mesocosms in the final week of the five-week experiment (Ogbeide et al., 2025).

To assess how these changes in aquatic emergence influence spider diets, we used DNA gut content metabarcoding to analyze prey composition in individual spiders. Based on the observed reductions in aquatic insect emergence under low-flow conditions in the final experimental week, we expected corresponding shifts in spider feeding behavior. Specifically, we predicted that spiders from low-flow treatments would have consumed fewer prey items overall, reflecting reduced prey availability. Given the dominance of Chironomidae in the aquatic emergence, we expected this dipteran family to make up a major portion of the spiders' diets regardless of treatment. However, we also hypothesized that the consumption of EPT would decline as a consequence of reduced EPT emergence under low-flow conditions. In contrast, we predicted an increase in the proportion of terrestrial prey, as spiders might compensate for reduced aquatic inputs by relying more heavily on terrestrial food sources (Hunt et al., 2020). Finally, we expected a decline in overall prey diversity and a distinct shift in prey composition in response to altered hydrological conditions. Because *T. extensa* and *T. montana* were both likely present, we also considered that some of these dietary responses might vary between species.

Materials and Methods

Experimental design

The study site and experimental design are described in detail in Ogbeide et al. (2025). In brief, the experiment was conducted at the Riparian Stream Mesocosm (RSM) facility near Landau (Manfrin et al., 2023). We used 12 flow-through stream mesocosms (15 m x 1 m flumes with adjacent riparian zones), which were supplied with water from the river Queich, a stream influenced by agricultural and urban runoff.

Flumes were initially colonized passively by macroinvertebrates from the Queich and actively seeded with macroinvertebrates and organic material collected from the river. Riparian zones were seeded with a regional wetland plant mixture and allowed to develop under regular watering. Each unit was enclosed in a mesh tent to prevent the exchange of flying insects during the treatment phase.

At the start of the experiment, six flumes were randomly assigned as controls with a discharge rate of 1 L s^{-1}, while six flumes were subjected to a low-flow treatment, reducing discharge to 0.4 L s^{-1} to simulate hydrological drought. The experiment ran for five weeks and four days during summer 2021. Physical and chemical parameters, including flow velocity, water depth, temperature, dissolved oxygen, and conductivity, were regularly monitored throughout the experiment to ensure consistent conditions across treatments.

During the final four days of the 39-day experiment, we systematically hand-collected long-jawed orb weavers (*Tetragnatha* sp.) from riparian and overhanging vegetation within each mesocosm, carefully searching each area until no additional individuals were found. Spiders were transported to the laboratory on dry ice and immediately stored at –80°C.

Sample preparation

In total, 289 *Tetragnatha* sp. individuals were collected across all mesocosms. For gut content metabarcoding, we randomly selected 86 large, undamaged female individuals. Spiders were surface-sterilized prior to DNA extraction following the recommendations of Huszarik et al. (2023). Each spider was submerged in 1000 µl 2.8% (w/w) commercial sodium hypochlorite solution (DANKlorix; Colgate-Palmolive GABA GmbH) for one minute to remove external contaminants. The bleach was then carefully removed using a pipette, and 1000 µl of distilled water was added to each tube for 30 seconds to rinse residual bleach. The water was pipetted out, and the rinsing step was repeated once. After the final rinse, spiders were transferred to new, sterile tubes and freeze-dried for subsequent DNA extraction. The dried specimens were finely ground three times for 30 seconds using a TissueLyser II (Qiagen) with two metal beads. The resulting tissue powder was resuspended in 600 µl of PCR-grade water and vortexed thoroughly. The homogenous tissue suspension was then split and 300 µl were aliquoted and

stored for subsequent fatty acid analysis. For DNA extraction, we applied a high salt extraction protocol following Kaunisto et al. (2017). A detailed description of the processing steps is provided in the Supplementary Information (Tables S1 - S2).

DNA metabarcoding

We applied a two-step metabarcoding approach following Zizka et al. (2019). DNA extracts were diluted 1:10 prior to the first PCR ('amplicon PCR'), which was used to generate target amplicons for sequencing. The amplicon PCR employed primers carrying three length-varying inline tags and a universal tail as described in Leese et al. (2021). We used the primers BF2 (Elbrecht & Leese, 2017) and Spidprey_R (Melcher et al., 2024) to amplify an 85 bp COI fragment, with the reverse primer designed to reduce the amplification efficiency of spider DNA while targeting prey DNA. In the second PCR ('index PCR'), we used a 1:20 dilution of the amplicon PCR product as template, and Illumina adapters and unique dual indices were added using the indexing primers described in Buchner et al. (2021). For details on PCR reagents, thermal cycling conditions and primers, see Supplementary Information (Tables S3 – S5).

Index PCR products were pooled in approximately equimolar ratios (1 - 4 µl per sample) according to band intensity estimated on a 3% agarose gel, with 1 µl added for negative controls without visible bands. The final library pool included 40 negative controls, 8 positive controls, and, in two technical replicates each, samples from 86 individual spiders, 10 individual spider fecal samples, and 24 pooled spider fecal samples. All samples originated from the same sampling campaign and location, ensuring comparable expected prey communities across sample types. The pooled library was purified using 1.8x volume AmPure XP beads (Beckman Coulter, California, USA) and sent to CeGaT (Tübingen, Germany) for 2 × 250 bp paired-end sequencing on an Illumina MiSeq platform with a 5% PhiX spike-in.

Sequence data processing and taxonomic assignment

Sequencing of the library pool resulted in 11,929,383 reads. Raw data were initially demultiplexed using cutadapt (https://github.com/marcelm/cutadapt, version 3.5) and further demultiplexing by inline-tag was done with the python script "demultiplexer" (https://github.com/DominikBuchner/demultiplexer, version 1.2.0). Then primers were removed with cutadapt. Paired-end reads were further processed using DADA2 (version 1.34.0) following standard protocols (Callahan et al., 2016). Reads were first quality-filtered with filterAndTrim, allowing a maximum of 1 expected error for both forward and reverse reads. Error rates were learned separately for forward and reverse reads, and reads were dereplicated and denoised using the DADA2 algorithm to infer amplicon sequence variants (ASVs). Forward and reverse reads were merged, and chimeric sequences were removed. ASVs were length-filtered to retain only sequences between 84 - 86 bp, corresponding to the

target fragment. An ASV table containing 1,363 ASVs and 7,387,860 reads was generated for downstream analyses.

Amplicon sequence variants (ASVs) were taxonomically assigned using the NCBI BLAST+ pipeline (Camacho et al., 2009). We compared ASVs to the NCBI core nucleotide (core_nt) database (downloaded on 16 May 2025) using BLASTn version 2.16.0+, with an identity threshold of 80%, a word size of 11, and retaining up to 30 hits per query to capture taxonomic breadth. The output included relevant alignment metrics and NCBI taxonomic identifiers (taxids). For taxonomic lineage retrieval, we used TaxonKit (Shen & Ren, 2021; version 0.15.0), extracting lineage information based on taxids and reformatting outputs to standardized ranks (kingdom, phylum, class, order, family, genus, species). BLAST+ results paired with lineage information were further processed in R. We retained high-confidence hits per ASV by selecting the top 1% lowest e-values within each ASV group and excluding ASVs where all retained hits were non-arthropods. For each ASV, we determined the most frequent taxonomic assignment at each rank and its proportional representation among high-confidence hits. Subsequent filtering removed ASVs assigned to non-Arthropoda with ≥60% frequency, and for ASVs with ambiguous family-level assignments (low representation or missing values), manual curation based on inspection was performed to assign or exclude taxa as appropriate. Non-target aquatic arthropods (e.g., amphipods, crustaceans, sea spiders) were also excluded to focus on realistic arthropod prey. ASVs representing Acari (Mesostigmata, Sarcoptiformes, Trombidiformes) were ignored in downstream analysis, since they include parasitic and phoretic species and cannot be assigned as prey with confidence. However, only one third of spiders carried Acari DNA, with 1 (24.4%), 2 (8.1%) or 3 (2.3%) ASVs per spider.

A positive control consisting of a single exotic moth specimen (*Comadia redtenbacheri)* was included in the dataset. However, initial clustering yielded six ASVs for this specimen, indicating artificial splitting of true biological sequences due to sequencing errors not accounted for during denoising. To address this, we applied post-clustering curation using the LULU algorithm (Frøslev et al., 2017) to reduce redundancy and remove likely erroneous ASVs. The ASV table was processed alongside a BLAST-derived match list to identify potential parent-child relationships among ASVs. LULU was executed with a minimum match threshold of 96% (allowing up to three sequencing errors within the 85 bp fragment), a minimum abundance ratio of 1, and a relative co-occurrence threshold of 0.95. This curation reduced the dataset from 1,363 to 1,223 ASVs, discarding 140 ASVs. After LULU curation, only a single ASV corresponding to the positive control organism remained, confirming the successful removal of redundant ASVs.

Out of 40 negative controls (including PCR negatives and extraction blanks), only two prey ASVs were detected, each appearing in only a single spider sample. Given this extremely low

background occurrence, the dataset was considered highly reliable. We therefore applied no further post-bioinformatic filtering or minimum sequence copy thresholds (cf. Drake et al., 2022).

In 86 spiders, we identified 343 arthropod ASVs. Of those, 311 ASVs (90.7%) were classified as prey, with 212 ASVs (61.8%) identified as merolimnic, 1 ASVs (0.3%) as aquatic (Notonectidae) and 98 ASVs (28.6%) as terrestrial. Prey ASVs comprised 44 families. Notably, Chironomidae alone represented 50.8% of all prey ASVs, while all other prey families each comprised less than 7% of prey ASVs.

Due to a labeling error during laboratory processing, three spiders had to be excluded. For 67 out of resulting 83 *Tetragnatha* individuals, host identity was unambiguous, with >99% of host reads corresponding to a single species in both technical replicates. In six spiders, one of the two technical replicates lacked detectable host DNA while the other contained only low amounts, but consistently matching *T. extensa*. For seven spiders, reads from both *T. extensa* and *T. montana* were detected; however, *T. montana* was consistently present at lower proportions, with a maximum of 44% of reads in one replicate (mean across replicates: 14%). Consequently, those 13 spiders were assigned *T. extensa*. The host species could not be determined for three spiders due to either an absence of host DNA or only minimal, inconclusive traces of both species. These three spiders were therefore removed from the data set, resulting in a final dataset of 80 spiders, with 3 to 16 individuals per flume (median = 7).

The 160 samples (80 spiders, each two technical replicates) included in the analysis had an average of 24,246 total reads, with more than 96% of them between 6,000 and 60,000 reads.

Statistical analysis

Reads were rarefied to 6,000 reads per spider using the package VEGAN (Oksanen et al., 2001). Four samples were not rarefied as their total number of reads was too low (8, 774, 4,286 and 5,906, resp.). Reads were then transformed to presence-absence data, indicating presence when at least one technical replicate contained reads >0 of the respective ASV.

To analyze the effects of treatment and host on the number of prey ASVs, we used a generalized linear mixed model (GLMM) with a negative binomial distribution (nbinom2 parameterization) to account for overdispersion in count data. We specified treatment, host, and their interaction as fixed effects and included flume as a random intercept to account for non-independence within flumes. The model was fitted using the GLMMTMB package (McGillycuddy et al., 2025), and type III Wald chi-square tests were performed using the “Anova” function from the CAR package (Fox & Weisberg, 2019) to assess the significance of fixed effects and their interaction. We then computed estimated marginal means (EMMs) for treatment and host combinations using the EMMEANS package (Lenth, 2017).

To assess how treatment and host influenced the composition of prey types in spider diets, we modeled the relative abundance of key prey categories – Chironomidae, EPT and terrestrial arthropods – using separate generalized linear mixed models (GLMMs). For each category, we modeled the proportion of prey ASVs belonging to the focal group (e.g., Chironomidae ASVs / total ASVs) with treatment, host, and their interaction as fixed effects and flume as a random intercept. Models were fitted using the "glmer" function from the LME4 package (Bates et al., 2015) with a binomial error distribution. Residual diagnostics, including tests for overdispersion and zero inflation, were performed using the DHARMA package (Hartig, 2016). To evaluate significance of fixed effects, we used type III Wald chi-square tests from the CAR package. We computed EEMs on the response scale using the EMMEANS package.

We quantified the number of prey families detected per spider to assess the influence of treatment and host on spider diet diversity. Because spiders with more detected prey ASVs were expected to generally exhibit higher prey taxonomic richness, we first tested for positive relationships between the number of prey ASVs and prey family richness using Spearman's rank correlation with the function "cor.test". We then fitted linear models and compared slopes among treatments and host species using the linear model (lm) implemented in ggplot2. We further assessed dietary diversity at a finer taxonomic resolution for the dominant prey family, Chironomidae, by testing for associations between the number of chironomid ASVs and the number of chironomid genera per spider and comparing slopes from linear models across treatments and hosts.

To assess differences in spider diet composition between treatments and species, we calculated pairwise β-diversity of prey families using the Jaccard index with the function "beta.pair" in the BETAPART package (Baselga et al., 2025). This yielded matrices of total Jaccard dissimilarity, partitioned into prey turnover (replacement of prey families) and nestedness (subset patterns) components. We then calculated the mean and standard deviation of β-diversity across all individual spider pairs of different species within the same treatment or from different treatments within species. We further quantified the relative contributions of turnover and nestedness to overall dietary dissimilarity.

To further test whether prey composition differed between treatments and host species, we conducted a constrained ordination using canonical analysis of principal coordinates (CAP) with the "capscale" function from the VEGAN package. The analysis was based on a binary Jaccard dissimilarity matrix calculated from presence–absence data of prey ASVs per spider, with treatment, host, and their interaction specified as predictors. We assessed the overall model significance as well as the marginal (type III-like) effects of each predictor using the

“anova” function combined with permutation tests with 999 permutations. To further investigate variation in diet composition within the most important prey family, Chironomidae, we conducted a separate CAP analysis using genus-level data. For this, we calculated a Bray-Curtis dissimilarity matrix based on the abundance of ASVs assigned to each chironomid genus per spider. The constrained ordination included the same predictors – treatment, host species, and their interaction – and model significance was assessed using permutation tests with 999 iterations as described above.

All statistical analyses and visualizations were conducted in R version 4.5.0 (R Core Team, 2025), using the packages DPLYR (Wickham, François, et al., 2014), TIDYR (Wickham, Vaughan, & Girlich, 2014), TIDYVERSE (Wickham et al., 2019), GGPLOT2 (Wickham, 2016) and PATCHWORK (Pedersen, 2019) in R Studio version 2025.05.0+496 (Posit team, 2025).

Results and Discussion

We analyzed the gut contents of 80 *Tetragnatha* spiders using DNA metabarcoding to assess the impact of hydrological drought on riparian spider diets. Of those, 63 were *T. extensa* and 17 were *T. montana* (Figure S1). The number of *T. extensa* individuals in the control treatment was twice as high as in the low-flow treatment (n = 42, mean = 7 vs. n = 21, mean = 3.5). In contrast, *T. montana* occurred in similar numbers across both treatments (n = 7, mean = 1.2 in the control; n = 10, mean = 1.7 in the low-flow treatment). This is in accordance with Entling et al. (2007) who found that *T. montana* exhibits greater habitat flexibility and can also be found in more terrestrial, less hydrologically influenced environments. Although both species generally prefer moist habitats, *T. extensa* is considered a wetland specialist with a narrower ecological niche and an optimum in areas with higher moisture availability. The reduced number of *T. extensa* individuals in low-flow conditions likely reflects its stronger dependency on riparian microclimates, which are negatively impacted by hydrological drought. In contrast, the stable numbers of *T. montana* across treatments suggest a higher tolerance to habitat drying, possibly due to a broader physiological or behavioral adaptability that allows it to persist in suboptimal, drier conditions.

In total, we detected 1,407 prey occurrences across 43 arthropod families. Individual spiders had between 0 and 37 prey detections, with a median of 17. Comparable gut content metabarcoding studies on *Tetragnatha* spp. have reported averages between 1 and 11 prey OTUs or 8 prey species per spider (Huszarik et al., 2024; Kennedy et al., 2019; Sun et al., 2025). We assume that the higher prey detection rates in our study resulted from a combination of comparably high sequencing depths per individual, minimal host DNA amplification due to optimal primer choice, and the use of a relatively short target fragment (85 bp compared to the

200 - 400 bp range used in other studies), which enabled the detection of trace and highly degraded prey DNA.

Prey detections, defined as the number of prey amplicon sequence variants (ASVs) per spider, showed a significant interaction between spider species and low-flow treatment (GLMM, $\chi^2 = 10.96$, df = 1, $p = 0.001$). For *T. extensa*, the expected number of prey ASVs per individual was approximately 20 under control conditions and 17 under low-flow treatment. In contrast, *T. montana* consumed two times more prey ASVs in low-flow than in control condition (Figure 1A).

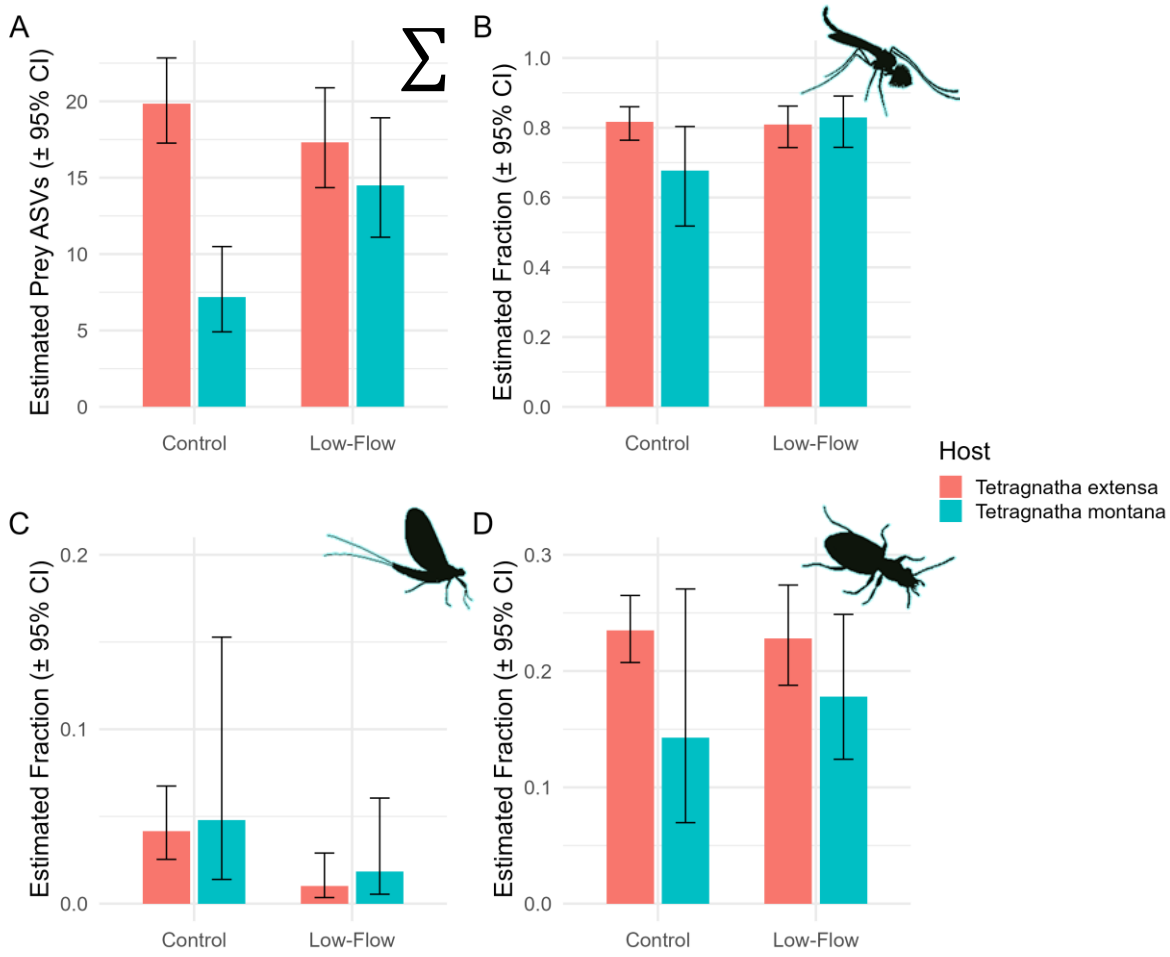

Figure 1 Effects of low-flow conditions on spider diet composition for the two species *T. extensa* and *T. montana*. (A) Estimated number of prey ASVs per spider. (B) Estimated fraction of chironomid prey ASVs. (C) Estimated fraction of EPT prey ASVs. (D) Estimated fraction of terrestrial prey ASVs. Bars show predicted means ± 95% confidence intervals from generalized linear mixed-effects models (GLMMs). *T. extensa*: n = 42 (control) and n = 21 (low-flow); *T. montana*: n = 7 for A and n = 6 for B–D (control), n = 10 (low-flow). One *T. montana* spider with no detected prey ASVs was excluded from the fraction calculations.

Chironomids represented approximately 80 % of all prey detections (Figure 1B), which is in accordance with Ogbeide et al. (2025), who identified dipterans of the family Chironomidae as

the dominant group in the aquatic emergence in our experimental units. However, there was a significant interaction between host species and treatment on the chironomid prey fraction, with a notably lower fraction observed in *T. montana* under control conditions (GLMM, $\chi^2 = 4.48$, df = 1, $p = 0.034$).

While the number of detected EPT ASVs per spider was generally low (ranging from 0 to 4), the treatment had a significant effect on the proportion of EPT prey (GLMM, $\chi^2 = 5.86$, df = 1, $p = 0.015$), with markedly lower fractions in low-flow treatments (Figure 1C). The lower fraction of EPT prey in low-flow treatments likely reflects reduced availability of these taxa, as shown by Ogbeide et al. (2025), who found significantly lower emergence rates of EPT under the low-flow conditions. Furthermore, terrestrial prey made up a relatively small portion of the diet, with a mean of 20.5% per spider. Neither host species nor treatment had a significant effect on the fraction of terrestrial prey, although *T. montana* tended to consume slightly less terrestrial prey (Figure 1D).

Sympatric occurrence of *Tetragnatha* species has been reported previously (Kennedy et al., 2019; Williams et al., 1995), involving differentiation in trophic niches and microhabitat preferences. In our study, *T. extensa* appears to be the more competitive species, as *T. montana* exhibited both lower prey consumption and consumed a smaller proportion of the nutritious and abundant chironomid prey under control conditions, where *T. extensa* was more abundant. This pattern suggests potential interspecific competition, with *T. montana* occupying a suboptimal niche when *T. extensa* is dominant. However, it is unlikely that *T. montana* shifted to more terrestrial areas within the riparian zone of the mesocosms, as the fraction of consumed terrestrial prey tended to be lower – not higher – for this species compared to *T. extensa,* especially under control conditions. The observed increase in prey consumption in *T. montana* under low-flow conditions may reflect reduced competitive pressure from *T. extensa*, which declined in abundance and prey consumption under these conditions.

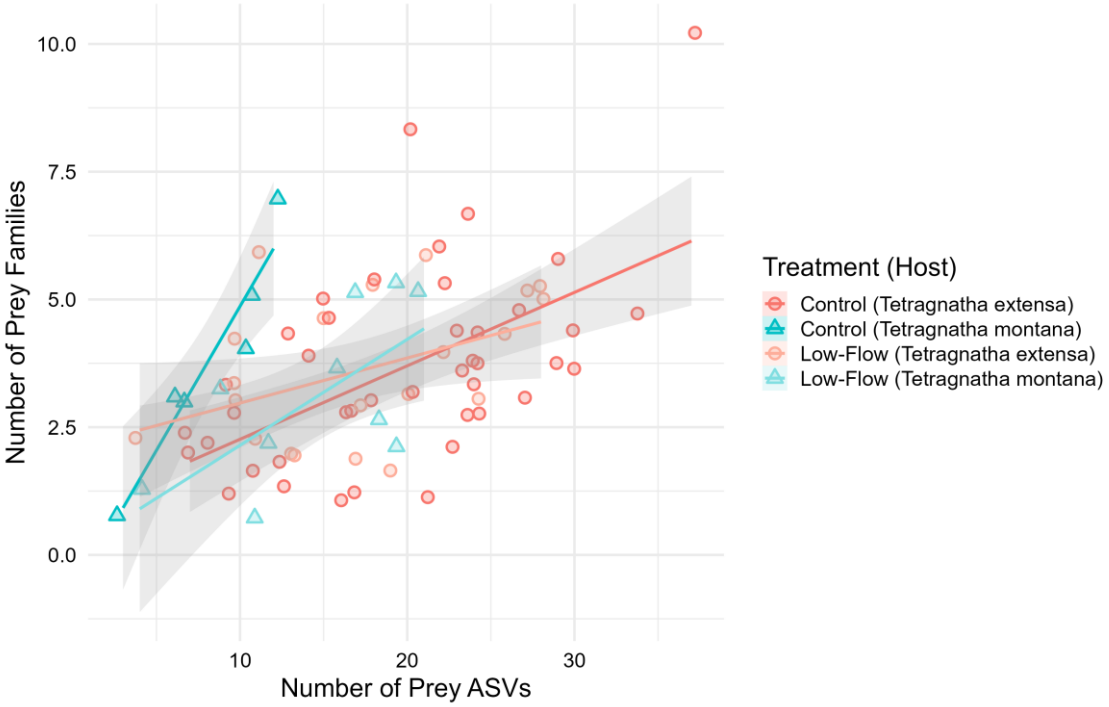

Figure 2 Relationship between the number of prey ASVs detected per spider and the number of prey families identified per spider. Each point represents an individual spider, with shape indicating host species and fill color indicating experimental group (treatment × host combination). Borders and regression lines are colored according to group identity. Linear regression lines (with shaded 95% confidence intervals) illustrate trends within each group. *T. extensa*: n = 42 (control) and n = 21 (low-flow); *T. montana*: n = 6 (control), n = 10 (low-flow).

We detected between 1 and 10 prey families per spider. As expected, there was a statistically significant moderate positive association between the number of prey families and the number of prey ASVs per spider (Spearman's rank correlation, $\rho = 0.48$, $p < 0.001$), indicating that spiders with more detected prey also showed greater taxonomic diversity in their diets. The steepest slope in the linear regression was observed for *T. montana* under control conditions, where *T. extensa* were dominant (Figure 2). This comparatively higher family richness, even in spiders with relatively few detected prey ASVs, suggests that *T. montana* may adopt a more opportunistic or divergent feeding strategy in response to competitive pressure from the dominant *T. extensa.* Such dietary broadening may reflect a behavioral or ecological flexibility that contributes to *T. montana's* persistence in competitive or resource-limited environments. For the dominant prey family Chironomidae, we detected between 2 and 15 chironomid genera per spider. The positive association between the number of chironomid genera and the number of chironomid ASVs per spider was even stronger (Spearman's rank correlation, $\rho = 0.93$, $p < 0.001$). Again, the steepest slope in the linear regression was observed for *T. montana* under control conditions (Figure S2). However, the maximum number of detected chironomid

ASVs per spider in this group was relatively low (7 compared to the overall maximum of 28), indicating that *T. montana* under control conditions generally consumed fewer chironomid prey.

Prey family composition differed strongly between spiders of the same species from different treatments and between spider species within treatments (mean Jaccard dissimilarities 0.69 - 0.73; Table S6). Partitioning showed that *T. extensa* mainly responded to drought by shifting to alternative prey (75% turnover), whereas *T. montana* exhibited a greater contribution of nestedness (44%). Between species, differences in diet were also marked (mean Jaccard dissimilarities ~ 0.7) and consistent across treatments, though slightly reduced under low-flow conditions. These results support that hydrological drought restructured prey use, with species-specific strategies: *T. extensa* compensated through prey switching, while *T. montana* seemed to shift from opportunistic feeding under high-competition conditions toward more selective foraging when competition is relaxed.

Constrained ordination (CAP) based on Jaccard dissimilarities revealed a significant interactive effect of host species and treatment on prey ASV composition ($F = 1.68$, $p = 0.001$). Despite this significance, the constrained model explained only around 6.3% of the total variation, which is expected in dietary studies of generalist predators due to the stochastic nature of feeding and fine-scale variability in prey availability (Alberdi et al., 2019; Mata et al., 2019). In the ordination space, *T. montana* individuals under control conditions formed a distinct cluster, while the three other groups (*T. extensa* under both treatments and *T. montana* under low-flow conditions) showed substantial overlap, indicating more similar prey compositions (Figure 3). This distinct clustering of *T. montana* under control conditions suggests a shift in dietary composition, potentially reflecting that *T. montana* may respond to increased competition by adding alternative prey types to their diet (cf. Svanbäck & Bolnick, 2007). In contrast, under low-flow conditions, when *T. extensa* was less abundant, prey composition in *T. montana* overlapped more strongly with that of *T. extensa*, suggesting shared prey preferences. These patterns align with the above mentioned observations of increased normalized family richness in *T. montana* under control conditions, supporting the idea of flexible foraging behavior in this species under competitive stress.

Focusing on the most relevant prey family, Chironomidae, CAP analysis based on Bray-Curtis dissimilarities of ASV abundances per genus also revealed a significant host-by-treatment interaction ($F = 3.26$, $p = 0.003$), with the constrained model explaining 10.1% of total variation. Similar to the overall prey patterns, *T. montana* under control conditions exhibited limited overlap with the other groups, which showed substantial overlap among themselves (Figure S3). This observation on a different taxonomic level reinforces the idea of dietary flexibility in *T. montana*, particularly under competitive stress.

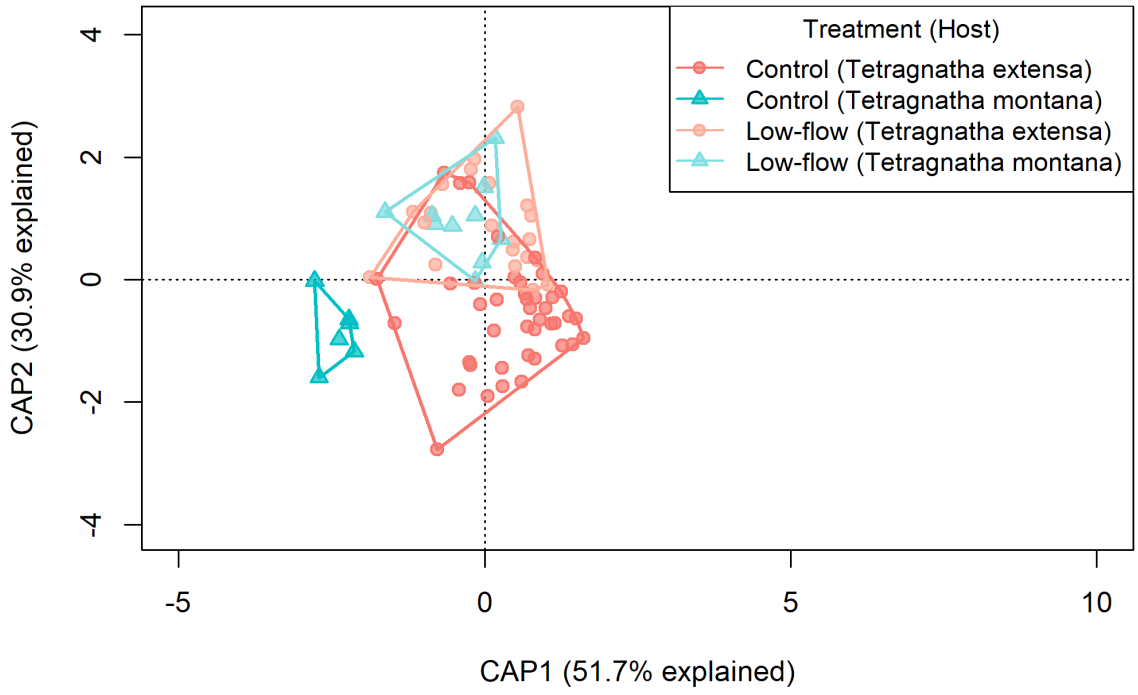

Figure 3 Constrained ordination (CAP) of prey composition in individual spider diets, based on Jaccard dissimilarities. Points represent individual spiders, colored by treatment and shaped by species. Convex hulls outline group dispersion for each treatment × species combination. The constrained axes explained 6.3% of the total variation in prey composition. The first two canonical axes (CAP1 and CAP2) captured 82.6% of this constrained variation, with CAP1 alone accounting for 51.7%.

To assess the importance of prey families in relation to host species and treatment, the relative frequency of each prey family across all spiders within a given treatment were calculated. Multiple occurrences of the same prey family within a single spider were counted only once to avoid overrepresentation (especially of Chironomidae). We found that the most frequently detected prey families largely overlapped across host species and treatments (Figure 4). Notable exceptions included the merolimnic Simuliidae and Culicidae, which were detected only in the control treatment, and the terrestrial Geometridae, which were detected exclusively under low-flow conditions.

Six prey families were consumed exclusively by *T. extensa*, with two of them – Ceratopogonidae and Ephydridae, both merolimnic taxa – gaining relevance in the low-flow treatment. Additionally, 12 families were detected only in *T. extensa* under control conditions; however, all occurred at low frequencies (< 3% relative frequency). Another six families were unique to *T. extensa* under low-flow conditions, again occurring only sporadically. This apparent exclusivity is likely a result of stochastic variation in prey detection, amplified by differences in sample size. As the number of analyzed *T. extensa* individuals was nearly four times higher than that of *T. montana*, rare prey items were more likely to be detected only in *T. extensa*, giving the impression of predator-specific dietary preferences, although they reflect

more likely random encounters with prey. Therefore, these observations should be interpreted with caution and are unlikely to indicate strong prey specialization.

On the contrary, the clearest pattern of dietary uniqueness was observed in *T. montana* under control conditions. This group was the only one to prey on Apionidae and Calliphoridae, while not preying on Drosophilidae and Miridae, which were otherwise more common. It was also the only group where Culicidae and Staphylinidae occurred in relevant frequencies (Figure 4). Although this group represented the smallest sample size, *T. montana* individuals under control conditions fed on several prey families not detected in *T. montana* under low-flow conditions or in *T. extensa* overall, while seemingly ignoring some commonly consumed taxa. This pattern may again result from the increased competition with *T. extensa* in the control treatment, potentially forcing *T. montana* to occupy less favorable microhabitats along the stream or within the three-dimensional riparian vegetation structure. Such shifts in web placement could limit access to certain prey groups while increasing encounters with others, helping to explain the distinct prey composition observed in this group. Similar microhabitat partitioning between sympatric *Tetragnatha* species has been observed in previous studies (Kennedy et al., 2019; Williams et al., 1995), and may also play a role in structuring diets under competitive conditions in our system.

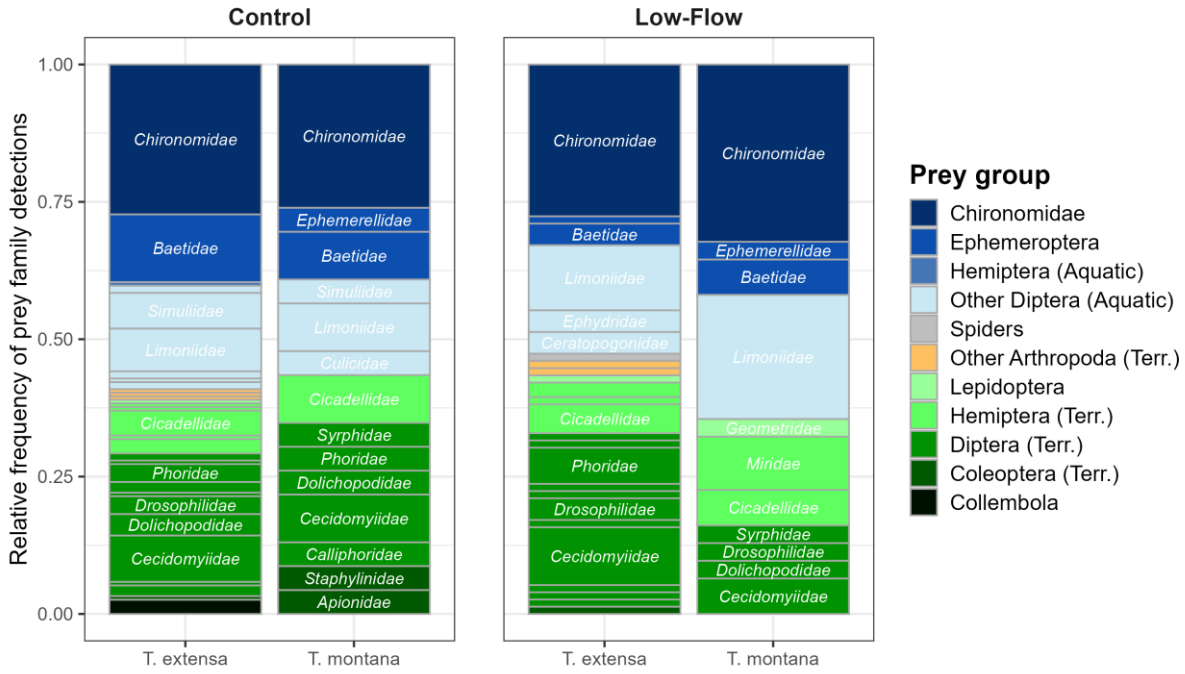

Figure 4 Relative frequency of prey family detections in *T. extensa* and *T. montana* under control and low-flow conditions. Relative frequency was calculated as the number of prey family detections per group divided by the total number of detections in that group. Multiple occurrences of the same prey family within a single spider were counted only once to avoid overrepresentation. Prey families exceeding 3% relative frequency are labeled.

Conclusion

Hydrological drought can profoundly impact stream and riparian ecosystems, influencing both habitat conditions and community interactions. In our study, while some prey families were exclusively consumed under either control or low-flow conditions, the direct effect of drought on spider diet composition was relatively subtle. Instead, the more pronounced impact of hydrological drought appeared to be indirect, mediated through changes in interspecific competition between two sympatric *Tetragnatha* species. Under control conditions, the wetland specialist *T. extensa* was more abundant and appeared to exert competitive pressure on the more generalist *T. montana*, resulting in niche differentiation and less dietary overlap. In contrast, under low-flow conditions, the decline in *T. extensa* abundance reduced this competition, allowing *T. montana* to shift its diet toward a more typical prey community, resulting in a greater overlap in realized dietary niches between the two species.

These findings suggest that hydrological drought can shift species interactions and resource use in riparian spider communities, potentially favoring more habitat-flexible species like *T. montana* at the expense of specialists such as *T. extensa*. If low-flow conditions become more frequent or prolonged due to escalating human water demand or climate change, there is a risk that generalist species with broader habitat tolerances could outcompete and displace specialist species, thereby altering community composition and ecosystem function in riparian zones. Whether these shifts represent temporary ecological fluctuations that reverse with the return of normal flow conditions, or more lasting environmental shifts leading to persistent changes in species composition and interactions, remains unclear. Long-term monitoring and experimental studies are needed to determine the resilience of riparian spider communities to recurring or prolonged hydrological droughts, especially given the critical role that dietary competition among sympatric species can play in shaping community dynamics and responses to environmental change.

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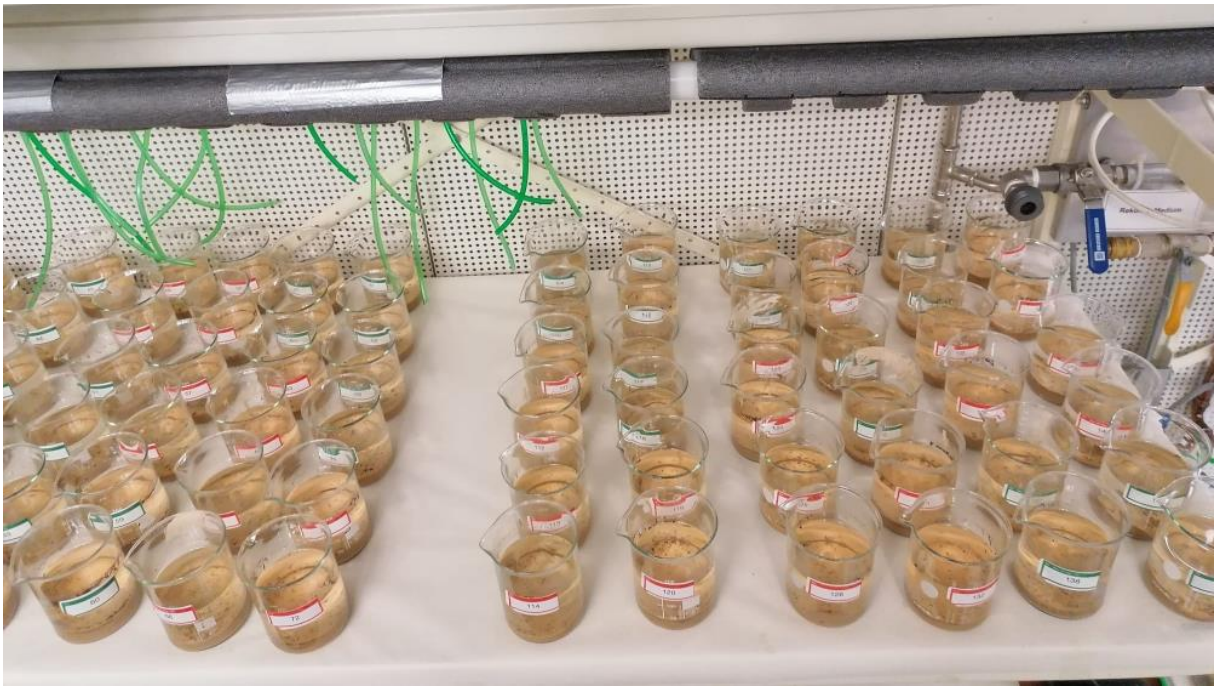
Chapter III: Pollutants shape evolutionary dynamics in a riparian midge



a) Röder N, Kolbenschlag S, Pietz S, Brennan RS, Bundschuh M, Pfenninger M, Schwenk K: “Pollution-Driven Selection in Riparian Ecosystems: Genome-Wide Responses to *Bacillus thuringiensis israelensis* and Copper in a Non-biting Midge”, manuscript under revision for *Molecular Ecology*

Title: Pollution-Driven Selection in Riparian Ecosystems: Genome-Wide Responses to *Bacillus thuringiensis israelensis* and Copper in a Non-biting Midge

Authors: Nina Röder[1], Sara Kolbenschlag[1], Sebastian Pietz[1], Reid S. Brennan[2], Mirco Bundschuh[1], Markus Pfenninger[3,4,5], Klaus Schwenk[1,3]

[1]iES Landau, Institute for Environmental Sciences, University of Kaiserslautern-Landau, Landau, Germany

[2]Marine Evolutionary Ecology, GEOMAR Helmholtz Centre for Ocean Research Kiel, Kiel, Germany

[3]LOEWE Centre for Translational Biodiversity Genomics, Senckenberg Biodiversity and Climate Research Centre, Frankfurt am Main, Germany

[4]Department of Molecular Ecology, Senckenberg Biodiversity and Climate Research Centre, Frankfurt am Main, Germany

[5]Institute of Organismic and Molecular Evolution, Johannes Gutenberg University, Mainz, Germany

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Abstract

Riparian ecosystems are vital interfaces between aquatic and terrestrial environments but are increasingly impacted by anthropogenic pollution. In these systems, merolimnic insects serve as crucial ecological links, occupying aquatic habitats as larvae and terrestrial environments as adults, thus being an essential food source in both. Consequently, pollutant exposure during the aquatic larval stage can have cascading effects across ecosystem boundaries. While the ecological consequences of such exposure are well documented, the evolutionary potential of merolimnic insects to adapt to chronic pollution remains poorly understood. To address this, we previously conducted a selection experiment exposing populations of the non-biting midge *Chironomus riparius* to the mosquito larvicide *Bacillus thuringiensis israelensis* (Bti) or heavy metal copper over approximately eight generations, which revealed only limited evidence of consistent phenotypic adaptation. Here we use whole-genome sequencing of these populations to assess their genomic responses to chronic pollutant exposure. Despite similar phenotypic sensitivity in pre-exposed and naïve populations, we detected distinct stressor-specific genomic responses. Copper exposure induced a significant genome-wide reduction in nucleotide diversity and evidence of selection-driven allele frequency changes, while Bti effects were dominated by heterogeneous, replicate-specific shifts, potentially reflecting drift or selection on multiple redundant pathways. Functional enrichment analyses indicated early-stage adaptation: immune- and apoptosis-related pathways were enriched under Bti, while metal detoxification and DNA repair pathways were enriched under copper, highlighting distinct adaptive mechanisms despite weak genome-wide signals of selection. Our findings demonstrate that Evolve and Resequencing approaches enable the detection of early genomic signals of adaptation even when phenotypic change is subtle or absent, offering a powerful framework for studying evolutionary responses to environmental pollution.

Introduction

Riparian ecosystems are ecologically rich and dynamic transition areas between land and water. They support high biodiversity and contribute to key ecosystem functions such as nutrient cycling, habitat provision, and the regulation of water quality (Singh et al., 2021). Many organisms in these systems link aquatic and terrestrial environments both physically – by moving between habitats, transporting energy and nutrients – and functionally, through their ecological roles in both habitats and across their life stages (Polis et al., 1997). A key example is the group of merolimnic insects, which develop as aquatic larvae and emerge as terrestrial adults. However, riparian ecosystems are increasingly affected by anthropogenic pressures (Feld et al., 2018; Lind et al., 2019). In addition to well-recognized stressors such as invasive species and hydromorphological changes, anthropogenic pollutants are now widely acknowledged as significant threats to riparian ecological integrity (Schulz et al., 2024).

Anthropogenic pollutants in riparian ecosystems originate from various human activities, including agriculture, pest control, mining, and urban infrastructure. These pollutants include both chemical compounds, such as heavy metals, and biological agents like microbial pesticides. *Bacillus thuringiensis israelensis* (Bti), a microbial larvicide widely applied to surface waters for mosquito control (Brühl et al., 2020), is intentionally introduced into aquatic ecosystems, raising concerns about its effects on other aquatic invertebrates like non-target insect larvae. In contrast, heavy metal copper enters freshwater systems unintentionally but consistently through surface runoff and soil erosion linked to agricultural and industrial activity (Comber et al., 2023; Pesce et al., 2025). It accumulates in sediments, where it remains bioavailable and toxic (Flemming & Trevors, 1989). This poses a significant risk to benthic organisms such as merolimnic insect larvae, which often inhabit or burrow into sediments.

Among the organisms affected by riparian pollutants are non-biting midges of the family Chironomidae, which are particularly important due to their high abundance and crucial roles in nutrient cycling and food web dynamics (Armitage et al., 1995). Chironomid larvae typically inhabit aquatic sediments in large numbers, where they serve as a major food source for aquatic predators (Serra et al., 2016). As they emerge from the water, chironomids often form large, synchronized swarms, becoming a predictable and energetically valuable resource for a variety of terrestrial predators, including birds, bats, and spiders (Baxter et al., 2005; Martin-Creuzburg et al., 2017). Consequently, disruptions to chironomid populations during their larval stage trigger cascading effects across both aquatic and terrestrial food webs in riparian ecosystems (Allgeier et al., 2019; Fukui et al., 2006; Kato et al., 2003; Kolbenschlag et al., 2023).

Bti-based larvicides are designed and applied to be relatively target-specific (Boisvert & Boisvert, 2000). Bti toxins act by binding to specific receptors in the gut epithelium of aquatic dipteran larvae, causing gut disruption and ultimately larval death (Bravo et al., 2007). Despite its specificity, a considerable

number of studies have reported adverse effects on chironomid larvae when exposed to Bti during mosquito control practices (Land et al., 2023). For example, mesocosm experiments have shown reductions in chironomid larvae abundance by up to 87% (Allgeier et al., 2019). Bti-induced chironomid reductions have been reported to propagate through aquatic and terrestrial food webs, affecting predators such as newts, dragonflies, damselflies, and insectivorous birds (Allgeier et al., 2019; Jakob & Poulin, 2016; Poulin et al., 2010). Thus, despite its targeted mode of action, Bti may disrupt riparian ecosystem interactions across aquatic and terrestrial boundaries.

In contrast to the relatively specific biological action of Bti toxins, sediment-bound copper exhibits a broad mode of toxicity, affecting multiple physiological processes across a wide range of benthic organisms (Roman et al., 2007). While copper is essential in trace amounts, elevated concentrations cause oxidative stress and perturb membrane integrity, protein function, and other vital cellular processes (Gaetke & Chow, 2003). In chironomids, copper exposure has been linked to reduced survival, delayed emergence, developmental deformities, and DNA damage (Bernabò et al., 2017; Marinković et al., 2011; Martinez et al., 2003). On the population level, copper has also been shown to impair reproductive timing, potentially affecting mating success (Servia et al., 2006). These findings underscore that sediment-associated copper can undermine chironomid life-history traits from molecular to population scales, posing a serious threat to benthic community stability.

While the ecological effects of Bti and copper on non-target chironomids are well documented, much less is known about the insects' capacity for adaptation to these pollutants. Resistance to individual Bti toxins has been demonstrated in some mosquito populations (Paris et al., 2011a; Tetreau et al., 2013), but there is currently no evidence for adaptation to commercial Bti formulations in either mosquitoes or chironomids (Becker et al., 2018; Brühl et al., 2020; Tetreau et al., 2013). In contrast, *C. riparius* has shown some potential for evolutionary responses to metal exposure. Early studies documented reduced sensitivity in historically contaminated (Groenendijk et al., 1999a; Postma et al., 1995a; Postma et al., 1995b), cross-bred adapted, (Groenendijk et al., 2002) or laboratory-selected lines (Marinković et al., 2012; Postma & Davids, 1995; Vogt et al., 2007), though these often lacked controls for maternal effects, phenotypic plasticity and/or unintended adaptation to laboratory conditions, making it difficult to attribute observed phenotypic changes strictly to genetic adaptation (Doria et al., 2022; Marinković et al., 2012). More recently, Im et al. (2019) provided physiological and epigenetic evidence of heritable adaptation to metal-contaminated sediments, and Doria et al. (2022) detected clear genomic signatures of cadmium tolerance in *C. riparius* after eight generations of laboratory exposure. However, robust evidence for copper-specific adaptation in *C. riparius* is currently lacking. Understanding the potential for genetic adaptation is essential for predicting population resilience and long-term ecological consequences of anthropogenic pollution in riparian ecosystems.

To shed light on this issue, we previously conducted a selection experiment with the non-biting midge *Chironomus riparius,* an established model species in ecotoxicology and evolutionary ecology (Foucault et al., 2019; Liu et al., 2025). We exposed populations to Bti or copper for approximately eight generations (Kolbenschlag et al., 2024; Pietz et al., submitted). Although some pre-exposure effects were observed – such as altered emergence timing or lipid content at certain Bti concentrations, and increased emergence success at one of the tested copper concentrations – evidence for consistent phenotypic adaptation was limited (Kolbenschlag et al., 2024; Pietz et al., submitted). Here, we build on this experiment by assessing genomic responses to chronic pollutant exposure in the same populations. Investigating allele frequency changes that may underlie adaptive processes allows us to explore potential genomic changes that may not have yet been reflected in the phenotype, for example due to the relatively short duration of the chronic exposure (cf. Doria et al., 2022). Specifically, this study addresses three key objectives: First, we aim to characterize genome-wide changes in *C. riparius* populations exposed to two distinct anthropogenic pollutants — a biologically targeted microbial pesticide vs. a broadly acting metal pollutant — and to compare these with untreated control populations. Second, we seek to determine the specific contribution of selective pressures imposed by these stressors to the observed genomic changes, distinguishing them from neutral processes such as genetic drift. Third, we investigate the functional basis of these genomic changes by identifying biological processes significantly overrepresented among affected genes, thereby uncovering the evolutionary responses triggered by each stressor. By linking pollutant-specific selective pressures to genomic signatures and associated biological functions, this study provides insights into how anthropogenic pollution may drive evolutionary change in riparian ecosystems.

Material and methods

Laboratory population establishment and culture conditions

To increase genetic diversity in the study population before chronic exposure, *C. riparius* in-house cultures were mixed with individuals of the same species from the laboratories of BASF SE (Ludwigshafen, Germany), ECT Oekotoxikologie GmbH (Flörsheim, Germany) and the LOEWE Centre for Translational Biodiversity Genomics (Frankfurt am Main, Germany) in the four months prior to the experiment. This approach was based on the assumption that long-established laboratory cultures may lack sufficient genetic variation to exhibit responses to selective pressures (Nowak et al., 2012). To minimize variability during the experiment due to newly introduced genotypes, no further individuals were added during the two months preceding the start of the exposure, allowing the laboratory population to stabilize. All cultures had been maintained for several months to years prior to their use in this experiment, following standard protocols for lab-based toxicity testing (e.g., OECD guidelines). Accordingly, food and sediment were provided for the aquatic larvae in water containers, which were

placed inside mesh cages tall enough to allow adult emergence and successful reproduction. Climate chambers were used to maintain stable temperature, humidity, and controlled day-night cycles. Although a new generation can appear in as little as two weeks (OECD, 2004), the exact number of generations that occurred within a longer time period is uncertain, as generation times can vary, with overlapping generations being likely (Foucault et al., 2019).

The experimental setup was conducted within three identical climate chambers, maintaining a temperature of 20±1 °C, 65% humidity, and a 16:8 day/night rhythm. Each experimental unit comprised a cage (50 x 35 x 50 cm, L x H x W, mesh size: 0.6 mm) housing two test vessels (32 x 7 x 22 cm). Within each vessel, there was 1.1 kg (wet weight) standardized sediment (composed of 75% sand, 20% clay, 5% peat; 40% water and 0.1% $CaCO_3$), and 2 L gently aerated SAM-5S medium (Borgmann, 1996), in accordance with the respective OECD guidelines for chironomid toxicity testing (OECD, 2010, 2023).

Egg collection, larval distribution, and chronic exposure

Between April 21 and 28, 2021, a total of 84 egg masses, each consisting of several hundred eggs, were collected from the culture over four days and stored in SAM-5S medium. Typically, *C. riparius* larvae begin to hatch 2 to 3 days after the eggs were laid at 20 °C (OECD, 2004). On days 4 and 6 after the end of the collection period, the freshly hatched larvae were split into 21 groups, with the aim of achieving an even number of individuals per replicate. Because each egg mass contains several hundred individuals, it is not feasible to individually count and allocate individuals manually to replicates, as the handling and the duration of the process might harm the larvae. Instead, we used a custom-built rotary dispensing device designed to ensure uniform distribution of larvae. This device consisted of a rotating basket with 21 glass tubes evenly distributed along the outer edge, paired with a pipette equipped with a funnel. By pouring the larval medium in a thin stream while rotating the basket, larvae were evenly dispensed into each tube. To estimate the numbers of larvae in each tube, three of the samples were individually counted under a stereomicroscope using pipettes modified with glass-tips. On day 4, these counts yielded an average of 350 larvae per replicate (range: 347–358), and on day 6, approximately 50 larvae per replicate (range: 45–60), resulting in a total of approximately 400 larvae per replicate. The remaining 18 replicates were assumed to contain similar numbers and were each allocated to one of 18 experimental units. Each group (day 4 and day 6) was introduced into one of the two vessels prepared for each experimental unit. The larvae used for counting were preserved in 70% ethanol at -20 °C to represent the genomic structure of the founding population, serving as a baseline for comparison with individuals sampled after chronic exposure.

The experimental conditions consisted of six replicates each of a control, a Bti treatment and a copper treatment. Treatment concentrations were selected based on preliminary tests (data not shown), with the goal of applying sufficient selection pressure without causing populations to collapse. In the Bti

treatment, each experimental unit was treated every two weeks with Bti using the commercial formulation VectoBac WDG (Valent BioSciences, Illinois, USA) at 33% of the recommended field rate, corresponding to 480 ITU/L. Repeated application was necessary to ensure comparable exposure of each chironomid generation, as the relatively high water temperatures, water turbidity and the absence of vegetation likely limited the duration of Bti toxicity (Brühl et al., 2020). Furthermore, it was assumed that the largest fraction of Bti toxins was lost during the biweekly test medium exchange (see below). The copper treatment involved sediment spiked with copper sulfate to achieve an environmentally relevant nominal concentration of 100 mg Cu/kg dry weight. These sediments were prepared and covered with medium two weeks prior to larval introduction to allow copper concentrations to equilibrate between sediment and porewater (OECD, 2004; Simpson et al., 2004). No additional copper was added during the chronic exposure phase, under the assumption that the majority remained bound to the sediment. The remaining six cultures served as untreated controls. To maintain stable water quality, the test medium was renewed every second week, within 48 hours prior to Bti application. Evaporation was compensated for by regularly refilling the vessels to maintain a constant water level. Larvae were fed ground TetraMin fish food (Tetra GmbH, Melle, Germany) twice a week at a rate of approximately 0.5 mg per larva per day.

After 26 weeks, about six months, of chronic exposure to copper and Bti (13 Bti applications), egg masses from each experimental unit were sampled on three consecutive days and stored in SAM-5S medium until hatching, resulting in a median of 10 egg masses per replicate (range: 4–22; SD = 5.6; see Supporting Information, Figure S1). Three days later, 100 larvae from each experimental unit were counted and stored in 70% ethanol at -20 °C for subsequent genomic analysis. Although generations likely overlapped during the chronic exposure period, we estimated that the sampled larvae represent approximately the eighth generation, based on an average generation time of three to four weeks.

DNA extraction and whole-genome sequencing

We used 100 larvae of each experimental unit (n = 18) and 400 larvae from the founding population, the latter of which was split into two technical replicates during DNA extraction. Following careful removal of ethanol from the samples, larvae were dried at 56 °C for approximately 4 hours. DNA extraction was carried out using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, involving a 2-hour proteinase K lysis step and elution in 25 µL elution buffer. Library preparation with a 450 bp insert size and subsequent sequencing of 150 bp paired-end reads were performed by Novogene Europe – UK on an Illumina NovaSeq platform, aiming for an expected coverage of 30x.

Single nucleotide polymorphism (SNP) identification

Reads were trimmed for quality and adapter contamination using trimmomatic v. 0.39 (Bolger et al., 2014) and then mapped on the latest *C. riparius* reference genome on NCBI (Bioproject number PRJEB47883) using BWA mem (Li & Durbin, 2009). Before trimming, the two technical replicates of the founding population were merged into one file. Variants were called using VarScan 2 (Koboldt et al., 2012) with a minimum variant frequency of 0.01, P value of 0.1, minimum alternate reads 2, and minimum coverage of 30x, resulting in 8,498,585 sites. Sites were then filtered for only biallelic sites with a coverage of >30x in all samples and a minimum minor allele frequency of 0.05 in at least six samples (i.e., one treatment). To control for mismapping, sites with depth per sample above the 97.5% quantile (951x for the founding population and 416x for all other populations) were excluded. The filtering resulted in a final set of 119,556 biallelic SNP sites.

Characterizing genomic variation

To assess the population genomic effects of the selection regimes, we estimated genome-wide levels of nucleotide diversity and linkage disequilibrium (LD) for all replicates. Nucleotide diversity was estimated as Tajima's pi (π) in the founding population and each evolved replicate using PoPoolation (Kofler et al., 2011a). We estimated π in 100-bp sliding windows with a 100-bp step size, resulting in 1,918,381 100-bp windows in 4 chromosomes and 10 scaffolds across the genome that were present across all samples. Each window required a minimum coverage of 25x, maximum coverage of 1,000x (to avoid mapping errors), and at least half of the window meeting these thresholds. To test for differences in π among treatments while accounting for replicate structure, we used a linear mixed-effects model (LMM) with treatment as a fixed effect and replicate as a random effect. The model was fitted using the *lmer* function from the `lme4` R package (Bates et al., 2015). To assess treatment differences, we performed pairwise comparisons of estimated marginal means using the `emmeans` package (Lenth, 2025), applying Holm correction for multiple testing. All statistics were performed in R (R Core Team, 2024). We summarized genome-wide variation in allele frequencies between all samples using principal component analysis (PCA) with the *prcomp* function, following an arcsine square root transformation of the allele frequency data to stabilize variances. LD was estimated using LDx, a Pool-Seq method that uses haplotype information from single read pairs to estimate linkage between pairs of SNPs over short distances (Feder et al., 2012). We estimate the decay of LD by regression of the log of physical distance with LD between base pairs; to estimate the slope and intercept of each treatment, we include replicate as a random effect with the R package `nlme` (Pinheiro et al., 2024).

Estimates of allele frequency change within and between treatments

We determined specific loci evolving due to selection by simulating the expected drift over eight generations using the `Pool-Seq` package in R (Taus et al., 2017) and following Barghi et al. (2020).

Using the starting allele frequencies at F0 (the founding generation), we mirrored our experimental design and simulated allele frequency trajectories for six replicates across eight generations with no selection under a Wright-Fisher model. We used the `Poolseq` R package to estimate the mean effective population size as 154 across all replicates, and simulated neutral allele frequency changes using this effective population size and a census size of 600. The census size is defined as the average number of reproducing individuals. Although individual counts were not tracked precisely, we estimated that each cage replicate supported approximately 600 reproducing individuals per generation based on observed densities and the assumption that each chironomid typically requires about 2 cm^2 of sediment surface to develop (OECD, 2004). We added the same variance as our sampling scheme by estimating allele frequencies from a sample size of 100 individuals and a simulated sequencing depth of 136x at F0 and 68x at F8 (the final generation), matching the observed depth. This simulation was repeated 500 times and, for each replicate, Fisher's exact tests in PoPoolation2 (Kofler et al., 2011b) were used to generate a null distribution of neutral allele frequency change. Empirical P values at the level of individual loci were calculated from this simulated distribution using *empPvals* in the `qvalue` R package (Storey et al., 2024), which estimate the proportion of Fisher's exact test statistics from the neutral simulations that are equal to, or more extreme than, those observed in the experimental data. This approach identifies loci where the observed allele frequency change is unlikely under genetic drift alone, thereby highlighting candidates for selection. Given that polygenic adaptation often involves genetic redundancy, where different replicate populations can reach similar phenotypic outcomes via distinct genetic routes (cf. Barghi et al., 2019), we did not require all replicates to show identical signals of selection. Candidate adaptive loci were sites with empirical P values less than 0.05 in at least four out of six replicates per treatment i.e., at least four replicates showed SNP variation exceeding expectations under genetic drift. Significance of overlap between sets of candidate SNPs between treatments was calculated using SuperExactTest in R (Wang et al., 2022).

Disentangling selection, drift, and laboratory adaptation in allele frequency changes

When selection acts on standing genetic variation in polygenic traits, the resulting allele frequency changes are often subtle and difficult to distinguish from genetic drift (Berg & Coop, 2014). To improve the detection of such signals, we applied a covariance-based approach that accounts for confounding effects like drift and laboratory adaptation (Buffalo & Coop, 2020), allowing us to quantify the contribution of selection to the variance in allele frequency change within and between treatments (cf. Brennan et al. 2022). This method partitions the variance in allele frequency change from F0 to F8 into components attributable to selection, drift, and lab adaptation, based on covariance across replicate populations within treatments. Covariance in allele frequency change was computed in 10,000-bp windows along the genome for each replicate. Uncertainty was assessed through bootstrap resampling

of windows. Temporal changes in allele frequencies that were consistent across at least four out of six replicates within a treatment were defined as the shared response for that treatment. To estimate the portion of this shared response attributable to selection, we subtracted the component attributable to laboratory adaptation. Since the control is expected to be selected only for culturing conditions, any covariance observed between the control and the treatment populations was interpreted as adaptation to the laboratory environment. When calculating shared responses based on subsets of four or five replicates, we conservatively identified the corresponding subset of control replicates (i.e., four or five out of six) that yielded the highest covariance with the treatment subset. We used this maximum covariance to quantify lab adaptation. Treatment-specific parallel changes were only attributed to selection if they exceeded this lab-adaptation signal.

Functional enrichment analysis

We used InterProScan (Jones et al., 2014) to assign Gene Ontology (GO) terms to predicted protein sequences. GO enrichment analysis was performed using topGO (Alexa & Rahnenfuhrer, 2024), applying the weight01 algorithm to identify significantly enriched biological processes. GO terms with fewer than five annotated genes were excluded by default during analysis. Prior to enrichment analysis, we filtered SNPs based on significance (false discovery rate < 0.05) in at least four, five, or all six replicates for each treatment. Then, topGO was run separately for each treatment group to assess treatment-specific GO term enrichment. A GO term was considered significant if at least one SNP associated with that GO term passed the threshold (Fisher statistic < 0.05). GO terms hierarchical information was retrieved from AmiGO2 (Carbon et al., 2009) and biological processes unique to treatments were identified. REVIGO (Supek et al., 2011) was used to generate a two-dimensional visualization of GO terms based on their semantic similarity, reflecting the degree of relatedness in their biological annotations.

Results

Genome-wide variation in response to experimental selection

To assess genome-wide responses to anthropogenic pollutants, we compared allele frequency changes in *C. riparius* populations exposed to chronic Bti or copper treatment, with unexposed controls and the founding population. We used pool-sequencing data from each replicate, yielding 119,556 SNPs with no missing data across all samples. The founding population possessed genome-wide genetic diversity on which selection could act (Tajima's π: 0.008 ± 0.010). Principal-component analysis (PCA; Figure 1) showed that the variance in genome-wide allele frequencies for all samples did not obviously cluster by treatment group, and not all F8 samples had notably diverged from the F0 founding population along PC1 (12.3% of the variation). There was no clear separation of the treatments from the control

along this axis. However, four replicates — two from copper-exposed populations and one each from the Bti-treated and control populations — were positioned slightly further apart from the rest along PC1. Along PC2 (8.4% of the variation), two of these replicates (one Bti- and one copper-treated population) also appeared further apart from the remaining samples, although this axis likewise did not group treatments into distinct clusters.

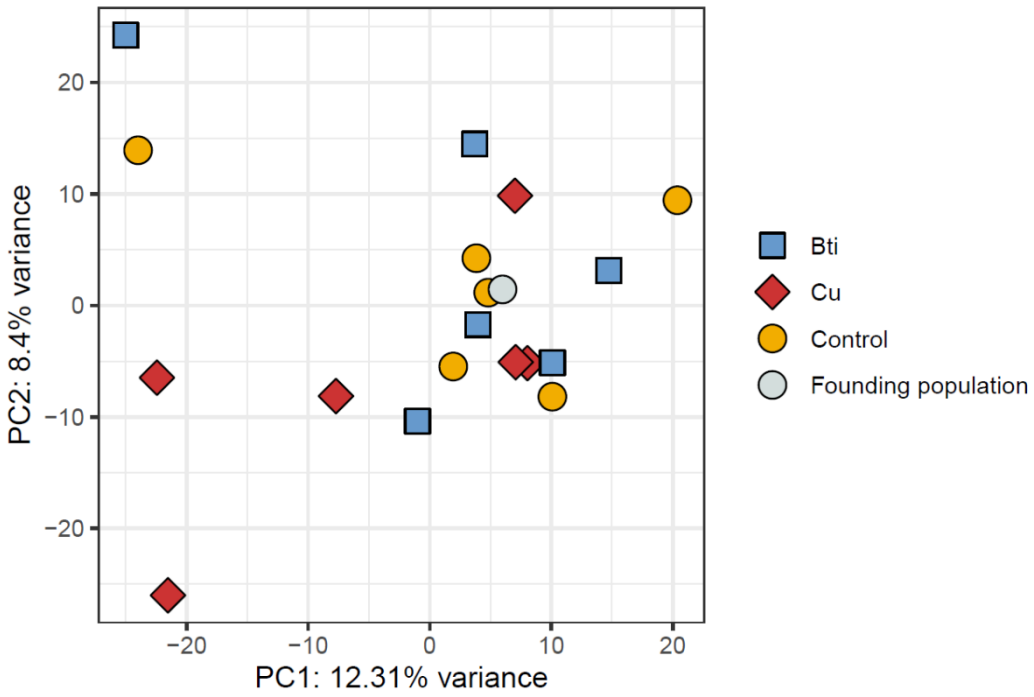


Figure 4 PCA of allele frequencies from 119,556 SNPs across the genome where color and shape distinguish treatment groups. The founding population is F0; all others are from F8.

We observed a slower rate of LD decay in the F8 populations compared to the founding population, indicating that all F8 populations underwent a bottleneck (Founding population: intercept: 0.3047 ± 0.0006; slope: -0.0447 ± 0.0003). The copper-exposed populations showed the largest increase in LD (intercept: 0.3096 ± 0.0036; slope: -0.0361 ± 0.0001), followed by Bti (intercept: 0.3101 ± 0.0039; slope: -0.0390 ± 0.0001) and control (intercept: 0.3113 ± 0.0022; slope: -0.0404 ± 0.0001). Because LDx estimates are sensitive to parameter settings (Feder et al., 2012), the LD values should be interpreted as relative differences between the treatments rather than exact measures of LD.

While adaptation to environmental stressors can promote population persistence, selection due to pollutant exposure typically reduces standing genetic variation. Consistent with this expectation, genome-wide nucleotide diversity (Tajima's π) decreased by between 4.8% and 8.4% across all treatment groups relative to the founding population. The greatest reduction was observed in the copper treatment (8.4% decrease; $\pi = 0.0076 \pm 0.0010$; LMM $p = 0.04$), followed by the Bti treatment (6.0% decrease; $\pi = 0.0078 \pm 0.0011$; $p = 0.20$), and the control populations (4.8% decrease; $\pi = 0.0079 \pm 0.0011$; $p = 0.20$).

Identifying potential selection targets

To pinpoint the genetic variation that may underlie adaptation to the different experimental selection regimes, we simulated the expected level of genetic drift over eight generations based on our experimental design (see *Material and methods*). We then employed Fisher's exact tests to identify loci that were evolving at a rate significantly higher than what would be expected due to drift alone in each replicate. Candidate adaptive loci were sites with empirical P values less than 0.05 in at least four out of six replicates per treatment i.e., at least four replicates showed SNP variation exceeding expectations under genetic drift. We found 1,932 (1.6% of all SNPs), 1,551 (1.3%), and 1,706 (1.4%) SNPs that were candidate targets of selection for Bti treated, copper-exposed, and control conditions, respectively (Figure 2). While most loci were unique within treatments, some were shared between treatments. All treatments showed a similarly high level of unique responses, with 1,718 SNPs (88.9% of significant loci) in the Bti-treated populations, 1,357 SNPs (87.5%) in the copper-exposed populations, and 1,495 SNPs (87.6%) in the control populations showing treatment-specific signals (Figure 2). However, the pairwise shared response between treatment groups was greater than by random chance (exact test, $P < 0.0001$) and similarly high (111 SNPs, 94 SNPs and 91 SNPs) for all pairs of treatments. There was a very small shared response between all treatments (9 SNPs), suggesting a weak signal of shared adaptation to laboratory conditions.

When stricter conditions were applied, i.e., requiring 5 out of 6 replicates per treatment to show significant SNPs, the number of treatment-specific SNPs decreased by a factor of approximately 7, with around 220 SNPs identified for each treatment instead of the ~1,500 SNPs under the initial condition (Supporting Information, Figure S2). The number of SNPs shared between two treatments also decreased to a range of 1 to 7 SNPs, compared to around 100 under the more lenient condition. Notably, no SNPs were shared across all three treatments. When even stricter conditions were applied, requiring all six replicates to show significant SNPs, the number of candidate SNPs further decreased to 20 for Bti-treated, 7 for copper-exposed, and 11 for non-treatment conditions, with no overlapping SNPs identified across any treatments (Supporting Information, Figure S2).

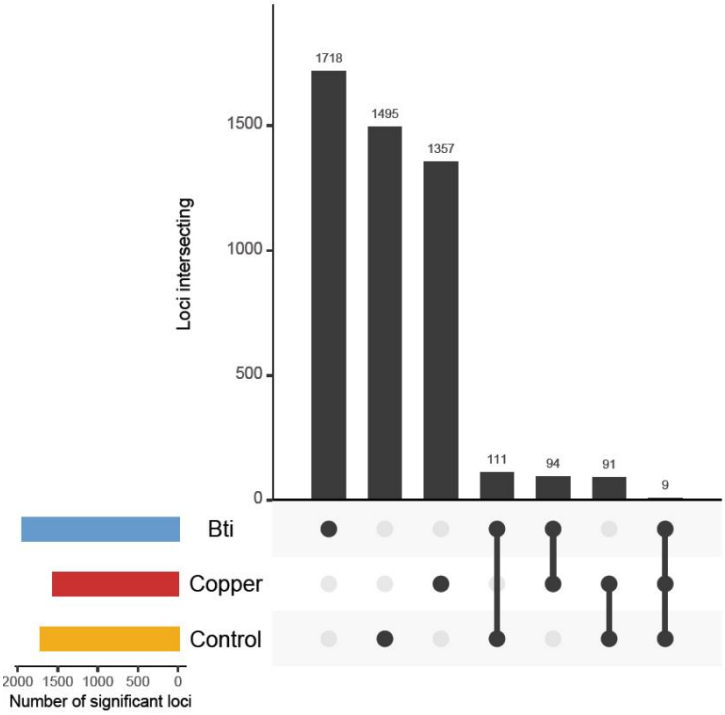


Figure 5 Candidate SNPs exceeding drift expectations identified from Wright–Fisher simulations in at least four out of six replicates. The horizontal bars indicate the total number of candidate loci in each group while the vertical bars show the number in each category. Black points show treatments in a category where multiple groups are indicated by connected lines. Note that the counts in each group are exclusive; for example, the number of loci in the control-alone category are those loci not shared with any other group.

Contribution to allele frequency change: selection, laboratory adaptation, drift and replicate-specific responses

By taking advantage of both the replicated and temporal aspects of our experimental design, we applied a temporal covariance-based method to detect even subtle signals of polygenic selection in genome-wide allele frequency changes. Unlike methods such as Cochran-Mantel-Haenszel (CMH), which focus on identifying strong, consistent selection at a limited number of loci, temporal covariance-based approaches can identify more subtle and weak responses to selection (Buffalo & Coop, 2020). In this way, we can assess the extent to which allele frequency changes across replicates are driven by shared selection pressures rather than random genetic drift or replicate-specific variation (Brennan et al., 2022). We calculated pairwise covariances in allele frequency changes from F0 to F8 within 10,000-bp windows across all replicate pairs. Using this, we derived the convergent correlation, a standardized measure of the similarity in allele frequency changes between replicates within or across treatments, reflecting a convergent selection response. A high convergent correlation indicates parallel allele frequency shifts driven by a shared selective pressure. In contrast, if changes were driven by genetic drift or replicate-specific selection, allele frequency shifts would be independent across replicates, resulting in a convergent correlation of zero. Additionally, if selection acts in divergent directions across populations, it generates negative convergent correlations. We observed comparably high convergent

correlations across all pairwise replicate comparisons, regardless of whether they were within the same selection regime or across different treatments (Figure 3).

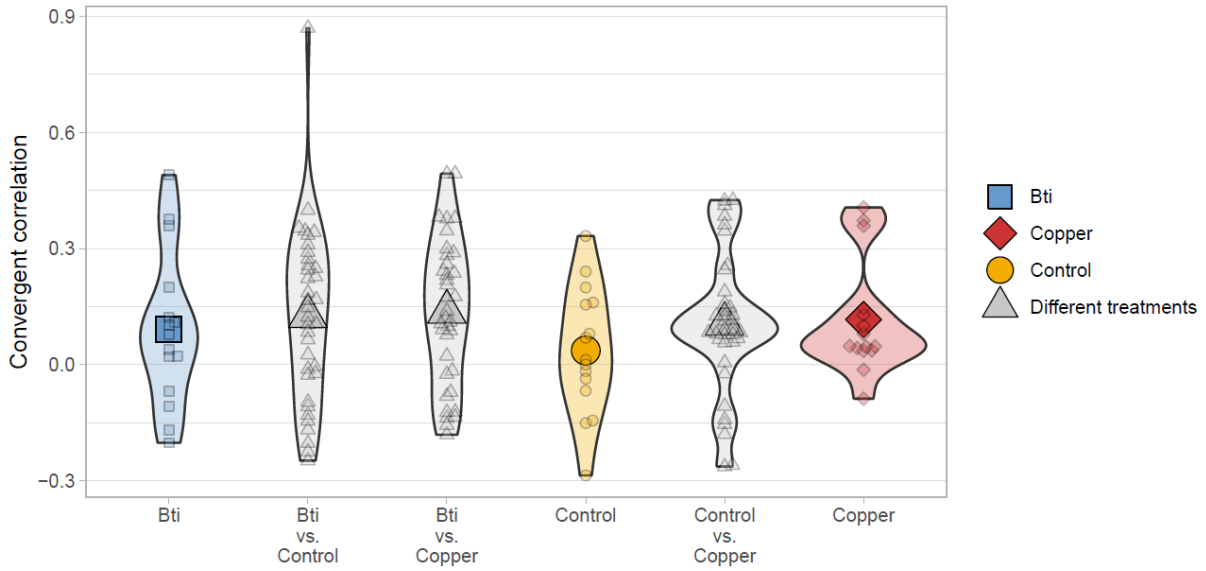


Figure 6 Convergent correlations of allele frequency change from F0 to F8. Higher values indicate a more similar change in allele frequency between two replicates. Small symbols represent convergent correlations from pairwise population comparisons; large symbols show the mean for each group comparison.

We partitioned the total variance in allele frequency change into components attributable to experimental selection, genetic drift, and laboratory adaptation (Figure 4). To conservatively identify signals of selection, we focused on sets of replicates within each treatment that showed a shared response with a positive lower confidence bound — indicating a statistically significant signal above zero. In both the Bti- and copper-treated populations, the strongest shared response was found in specific four-replicate subsets. Within these, the estimated contribution of laboratory adaptation to total variance was higher and more variable in Bti-treated (27%, 95% CI: [5-49%]) than in the copper-exposed populations (19%, 95% CI: [15-22%]). After accounting for lab adaptation, experimental selection explained 0% of the variance in Bti-treated populations (95% CI: [-44-20%]) and 6% in copper-exposed populations (95% CI: [2-11%]). In both treatments, most of the variance was due to drift or replicate-specific responses, with 85% of the total variance in Bti-treated (95% CI: [31-139%]) and 75% in copper-exposed populations (95% CI: [67-83%]; Figure 4).

Across all tested replicate groupings, only four produced statistically significant selection estimates (i.e., lower CI > 0): three four-replicate sets and the full six-replicate set from the copper-exposed populations. Among these, one four-replicate subset slightly outperformed the others, with a selection estimate of 7% (95% CI: [3-10%]). In contrast, no subset from the Bti treatment showed evidence of selection above zero, though the confidence intervals were notably wider than for the copper treatment, reflecting higher variability across genomic windows. Full results for all tested groupings,

including estimates of shared response and selection signal, are provided in Supporting Information, Figure S3 and Table S1.

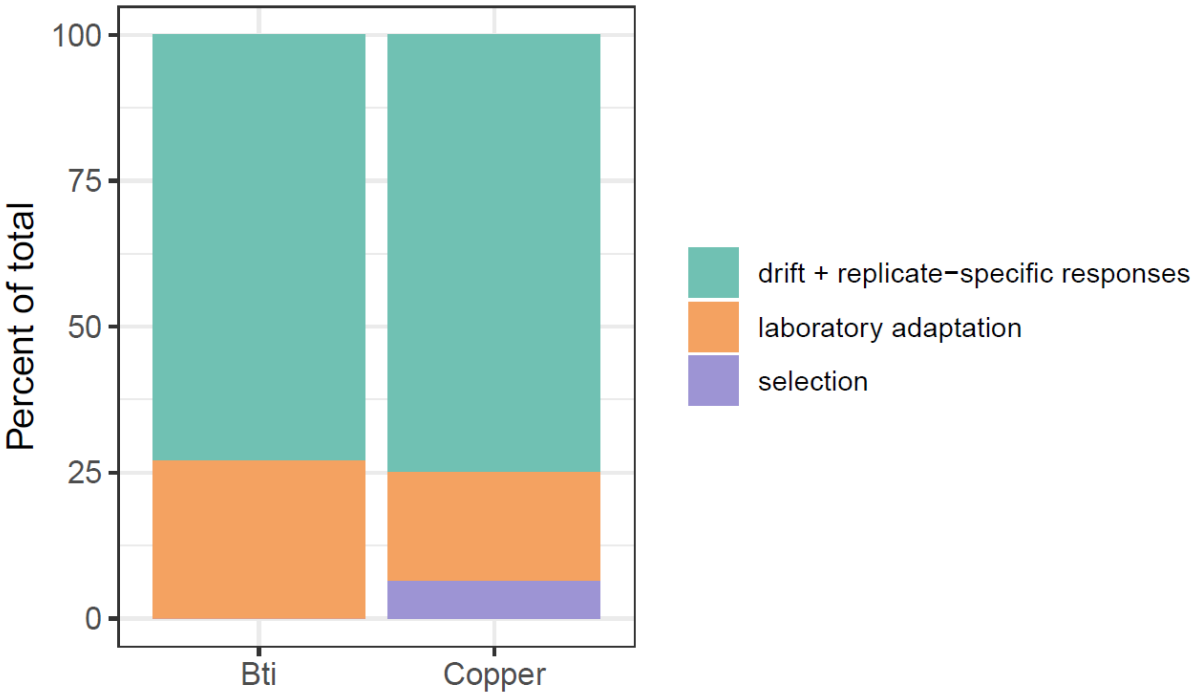


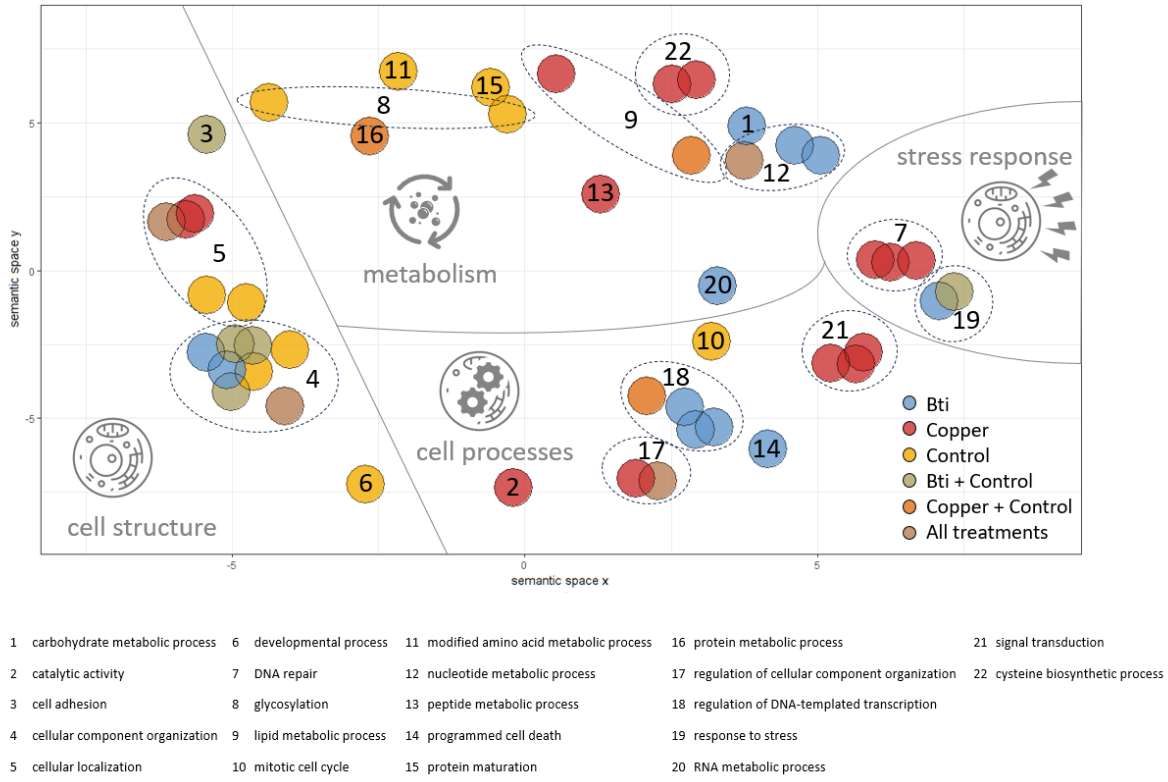
Figure 7 The contribution of laboratory adaptation, treatment selection, and drift plus replicate-specific responses to the total variance of allele frequency change from F0 to F8. The laboratory component was determined using the covariance of allele frequency change between the control and each of the two treatment groups while selection was identified as the covariance within a treatment group minus the laboratory adaptation. The remaining variance was attributed to drift and replicate-specific response to selection.

Gene ontology enrichment

Tests for gene ontology (GO) functional enrichment were used to gain insight into the mechanisms that may underlie adaptation to the different conditions. The GO enrichment analysis identified 45 biological processes associated with loci exhibiting significant allele frequency changes after chronic exposure (Supporting Information, Table S2). Among the treatments, we found enrichment for 11 GO terms in Bti-treated populations, 12 in copper-exposed populations, and 10 in control populations. Additionally, five GO terms were shared between Bti-treated and control populations, three between copper-exposed and control populations, and four were enriched across all treatments. All 45 enriched terms were predominantly related to biological regulation and cellular processes, including metabolic processes and organization or biogenesis of cellular components.

Enrichment of GO terms related to cell structure was primarily observed in control and/or Bti treatments, while those associated with cellular processes were more prominently enriched in the copper and Bti-treated populations. This trend was also evident in the enrichment of GO terms related to stress response, with no specific terms showing enrichment in the control groups. The number of

GO terms associated with metabolism was similar across all three treatments, although a distinct separation of clusters by treatment can be observed in the semantic space, as shown in Figure 5.



1	carbohydrate metabolic process	6	developmental process	11	modified amino acid metabolic process	16	protein metabolic process	21	signal transduction
2	catalytic activity	7	DNA repair	12	nucleotide metabolic process	17	regulation of cellular component organization	22	cysteine biosynthetic process
3	cell adhesion	8	glycosylation	13	peptide metabolic process	18	regulation of DNA-templated transcription		
4	cellular component organization	9	lipid metabolic process	14	programmed cell death	19	response to stress		
5	cellular localization	10	mitotic cell cycle	15	protein maturation	20	RNA metabolic process		

Figure 8 Scatterplot of 45 significantly enriched Gene Ontology (GO) terms, visualized in a two-dimensional semantic space using REVIGO (Supek et al., 2011). The x- and y-axes represent semantic space, where closer terms have higher functional similarity. Each colored circle represents a GO term, with colors indicating the treatment(s) in which the term was significantly enriched (see legend for color coding). Some functionally related GO terms are grouped within dashed-lined circles. Each colored circle or group is assigned a number, and below the figure, a corresponding list provides higher-level biological processes associated with the respective GO term(s). A full list of specific GO terms is available in the Supporting Information, Table S1.

Discussion

In this study, we investigated the genomic response of *C. riparius* to chronic exposure to two ecologically relevant anthropogenic pollutants, with the aim of identifying early genomic signals of selection even in the absence of unambiguous phenotypic adaptation. Linkage-disequilibrium (LD) patterns indicated a bottleneck in all three treatments, with the strongest signal in the copper populations, followed by Bti, and weakest in the controls. This pattern aligns with findings by Nieto-Blázquez et al. (2025), who reported that heavy metal exposure, such as cadmium, can suppress recombination. The observed gradient in LD strength was mirrored by corresponding reductions in genome-wide nucleotide diversity (Tajima's π), with the copper treatment exhibiting a statistically significant reduction in genome-wide nucleotide diversity. Together, these results suggest that

pollutant exposure intensified demographic effects, likely due to bottlenecks, compared to the controls, creating the necessary conditions for evolutionary responses to occur.

PCA and convergence correlation analyses revealed non-parallel genome-wide allele frequency changes across treatments (Figure 1). Both convergence and divergence were observed between replicate pairs (Figure 3), regardless of treatment, indicating the absence of consistent, parallel evolution. Following Barghi et al. (2019), genetic redundancy may explain why allele frequency changes occur only in subsets of replicates, even under similar selective pressures. To account for this, we identified candidate SNPs within subsets of replicated populations (Figure 2), i.e., candidate SNPs had to be significant only in at least four out of six replicated populations. The number of unique candidate SNPs was similar across replicates, while overlap among different treatments was limited, as expected under distinct selection regimes. Interestingly, copper treatment yielded the fewest candidate SNPs despite evidence of the strongest bottleneck and a significant selection signal via covariance analysis. Importantly, there was no indication that copper and Bti treatments shared a high number of general 'pollutant stress-response' SNPs, supporting the conclusion of stressor-specific genomic responses. However, the number of candidate SNPs may be an overestimation, as we tested each locus individually to identify candidates. Our calculations assumed independence among loci — a simplification, given that physical linkage likely extends across several hundred base pairs. As a result, some candidate SNPs may represent linked variants within the same selective sweep rather than independent signals of selection.

Covariance-based analysis revealed that non-parallel shifts—arising from genetic drift or selection responses occurring in fewer than four replicates—explained most of the genomic variation in both the copper- and Bti-treated populations (Figure 4). Taken together with the limited evidence for consistent phenotypic adaptation (Kolbenschlag et al., 2024; Pietz et al., submitted), this suggests that selective pressures were generally weak. Nevertheless, we found clear evidence that selection contributed to genome-wide allele frequency change in the copper treatment. In contrast, no such evidence was detected in the Bti treatment, where allele frequency changes appeared to be driven primarily by neutral or replicate-specific dynamics. This apparent lack of consistent selection signals may be explained by the nature of the Bti exposure regime. Unlike copper, which was continuously present in the sediment, Bti had to be applied every two weeks due to its presumed rapid degradation and loss during medium exchange. This intermittent exposure reflects common mosquito control practices, where Bti is applied multiple times per season (Becker et al., 2018). Given that generation times in our experiment likely exceeded two weeks (Hooper et al., 2003; OECD, 2004), it is reasonable to assume that all larvae experienced Bti exposure at least once during development. However, because older larvae are known to be less sensitive to Bti than younger ones (Kästel et al., 2017), the impact on

individual health likely varied with larval age at application date. This would lead to selection pressures that varied among individuals, rather than acting uniformly across the population. Nevertheless, an absence of genome-wide covariance of allele-frequency changes as seen in Bti treatments does not necessarily imply an absence of selection. Such signals can be masked by unselected sites and by opposing selection pressures at different loci (Lynch et al., 2024). Indeed, in the Bti treatment, we observed greater heterogeneity across genomic regions, as reflected in the broader confidence intervals across genomic windows. This pattern suggests that a few large-effect loci, rather than widespread polygenic adaptation, may have driven the observed changes (Supporting Information, Figure S4). This difference in the nature of the targets of selection may also explain the disagreement between number of candidate loci (Figure 2) and the covariance signals of selection for Bti and copper (Figure 4). For Bti, strong selective responses in few replicates could drive a significant Fisher's exact test result, but would result in a weak and variable covariance signal. In contrast, a diffuse polygenic signal may be missed by the Fisher's exact test but detected using covariance. Thus, the differences between the two results reveals the nature of the underlying genomic architecture of the response to selection for each treatment.

Although genome-wide signals of selection were heterogeneous, functional enrichment analyses revealed evolutionary responses across all treatments, supporting the conclusion that selective pressures acted in a stressor-specific manner. Stress-response and cellular processes were primarily enriched in the Bti- and copper-exposed populations, whereas GO terms related to cell structure were more prominent in controls (Figure 5). The distinct clustering of metabolically related GO terms provides further evidence that different adaptive pathways were followed in response to the specific selective environments. Notably, while Bti and copper treatments showed a similar number of enriched GO terms, there was no overlap between them, emphasizing the stressor-specific nature of these evolutionary responses to pollutant exposure.

Enrichment of the GO term "regulation of apoptotic process", as seen in all replicates of Bti-exposed populations, suggests a role of programmed cell death in maintaining gut integrity under Bti stress, consistent with midgut restoration mechanisms observed in *Drosophila* after *Bacillus thuringiensis* exposure (Loudhaief et al., 2017). Additionally, the Bti-related enrichment of the GO term "defense response" indicates activation of immune pathways aimed at limiting damage and promoting recovery. In copper-exposed populations, enriched cysteine biosynthesis pathways highlight the well-known role of cysteine metabolism in metal detoxification (Jeppe et al., 2014, 2017). Furthermore, GO terms related to DNA damage repair are consistent with the genotoxic effects of copper exposure documented in *C. riparius* (Michailova et al., 2006). Interestingly, phototransduction pathways were also enriched, which have previously been associated with cadmium exposure in *C. riparius* (Doria et

al., 2022), though the exact role of these pathways under metal stress remains speculative. In *C. riparius*, increased metal excretion and cuticle shedding have been described as responses to metal exposure and proposed as adaptive mechanisms to reduce metal burden (Postma et al., 1996; Groenendijk et al., 1999b). However, we found no enrichment of GO terms directly associated with these processes, suggesting either alternative detoxification strategies or that the observed mechanisms in natural populations reflect phenotypic plasticity rather than genetic adaptation.

In contrast, control populations showed enrichment of GO terms related to reproduction, abscission, cell cycle, and organelle functions, suggesting adaptive responses are driven by intraspecific competition and density-dependent selection in the absence of experimental stressors. This aligns with previous observations (Pfenninger & Foucault, 2020) that even under seemingly favorable laboratory conditions, selection on both life-history traits and the efficiency of basic cellular functions remains an ongoing evolutionary force.

Despite evidence for ongoing evolutionary processes at the genomic level, trait-based assessments of the evolved populations revealed limited and inconsistent phenotypic adaptation (Kolbenschlag et al., 2024; Pietz et al., submitted). A similar outcome was reported by Doria et al. (2022), who suggested that observable fitness-related phenotypic changes may require more than a few generations of selection to manifest. This idea is further supported by findings from Rigano et al. (2025), who conducted a multigeneration experiment in which *C. riparius* populations were exposed to polluted sediments and tracked across generations for both allele frequency changes and fitness traits. While shifts in allele frequencies were detectable from the first generation onward, phenotypic fitness (e.g., population growth rate) initially decreased in the first generations before showing signs of recovery—highlighting that genomic adaptation can precede detectable phenotypic responses.

Additionally, the choice of phenotypic endpoints can critically influence the detection of adaptive changes. For example, Ducrot et al. (2004) demonstrated that copper exposure in *C. riparius* reduced the proportion of emerging females capable of producing egg masses and increased egg-mass deformities. Similarly, Paris et al. (2011b) linked resistance to Bti toxins in the mosquito *Aedes aegypti* to reduced female fecundity (i.e., the number of eggs laid). Reproduction-related traits like these, reflecting actual reproductive success, were not assessed in our selection experiment. Thus, relevant adaptive phenotypes may have been overlooked. In the case of Bti, it is also possible that the adaptive processes occurring at the genetic level targeted specific toxins within the Bti mixture (cf. Paris et al., 2011a). However, the overall toxicity of the commercial Bti product, which contains multiple active components, potentially masked most measurable phenotypic effects (cf. Stalinski et al., 2014). Moreover, our trait-based assessments of the evolved populations may not have fully reflected the selective conditions during the chronic exposure, particularly since competition was substantially

reduced in test vessels containing only a limited number of organisms. Finally, biological variability likely limited the detection of subtle phenotypic changes; increased replication and more sensitive assays (e.g., transcriptomic profiling or enzymatic activity assays) may be needed to reveal early-stage phenotypic adaptations.

Conclusion

Taken together, our results suggest that, while non-parallel shifts dominated genome-wide allele frequency dynamics, selection has started to modestly shape treatment-specific adaptive responses. The observed patterns imply that adaptation to chronic pollutant exposure in *C. riparius* may initially proceed through subtle, pathway-specific modifications rather than through large-scale, parallel genomic shifts. Our study highlights the complexity of predicting adaptive responses to environmental pollutants. Secondary Evolve and Resequencing studies, where evolved populations are crossed back with the ancestral population and re-exposed to the same selective environment, could validate weak or replicate-specific selection signals (Burny et al., 2020). Additionally, computational simulations can aid in optimizing experimental designs, for instance by identifying the appropriate number of replicates, generations of selection, and starting haplotype diversity to robustly capture adaptive genomic responses (Vlachos & Kofler, 2018). Furthermore, integrating transcriptomic data (Doria et al., 2022) could provide functional insights by revealing gene expression changes associated with adaptive responses. Such complementary approaches may confirm candidate genes or uncover adaptive mechanisms that SNP-based analyses alone might overlook.

Understanding evolutionary responses is essential for predicting long-term ecological consequences, particularly in the face of persistent environmental pollution. Traditional ecotoxicological assessments predominantly focus on short-term toxicity (Padilla Suarez et al., 2023; Thoré et al., 2021) and acute effects on survival (Straub et al., 2020), often overlooking the potential for adaptive responses over multiple generations. Evolve and Resequencing approaches bridge this gap by integrating experimental evolution with genomics, providing direct insights into adaptive genetic change. This enables the detection of subtle or early-stage adaptations that may not yet manifest at the phenotypic level. In this way, by assessing population-level evolutionary responses to diverse pollutants, such as biocides and heavy metals, Evolve and Resequencing studies offer valuable empirical data on both the capacity and constraints of adaptation. However, translating laboratory-based findings to natural populations remains challenging (Phillips & Burke, 2021). Differences in generation times, effective population sizes, standing genetic variation, and environmental conditions — all of which can profoundly shape evolutionary trajectories — must be considered. Comparisons with natural populations are therefore essential to contextualize laboratory observations. However, despite its laboratory-based nature, this

study lays important groundwork for elucidating evolutionary processes driven by pollution in riparian systems.

Data availability

The sequence data generated in this study are available from the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA1282899. Code to run all analyses can be found on Zenodo: https://doi.org/10.5281/zenodo.15744395. Construction protocols for the chironomid-handling tools developed in this study are available on protocols.io: https://dx.doi.org/10.17504/protocols.io.3byl49qorgo5/v1.

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Author Contributions

NR, SK, SP, MB, and KS conceived and designed the study. KS, MB, and MP secured funding for the project. NR, SK, and SP conducted the experiments and collected the samples. NR processed the samples. Visualizations and code were based on previous work by RB. NR performed the bioinformatic and statistical analyses with support from RB and MP. NR wrote the first draft of the manuscript. All authors contributed to manuscript revisions and approved the final version.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Statement

This study did not involve work with vertebrate animals, endangered species, or human participants, and thus did not require formal ethical approval. All experimental procedures complied with local regulations and institutional guidelines.

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Synthesis and Outlook

This thesis demonstrates that anthropogenic stressors do not act on single components of riparian communities in isolation, but instead trigger complex, interconnected responses across species composition, ecological interactions, and evolutionary processes. Such cascading effects highlight riparian communities as dynamic systems, where aquatic and terrestrial linkages shape resilience in the face of environmental change. To advance understanding of these dynamics, this thesis develops and applies genomic approaches that reveal how riparian communities respond to anthropogenic pressures. In the following synthesis, I first discuss the methodological innovations developed in this work, then highlight how genomic tools were used to address ecological questions in coordination with accompanying studies, before turning to the broader ecological implications and future perspectives.

Advances in efficient metabarcoding for riparian communities

The first methodological advance was the demonstration that direct PCR metabarcoding without prior DNA extraction can reliably capture chironomid community composition (Röder & Schwenk, 2023). Compared to conventional extraction-based workflows, this approach reduces sample processing time, lowers costs, and minimizes opportunities for contamination, while maintaining high reproducibility. In a simultaneously published study, Stojan et al. (2023) showed that direct PCR produced results comparable to conventional extraction methods in metabarcoding of marine bacterial communities. Yet, aside from these studies, we are not aware of further applications of this approach, despite its promise for simplifying and accelerating metabarcoding workflows. This is particularly relevant because high-throughput sequencing remains largely unsuitable for direct field deployment, due to the need for specialized laboratory equipment, controlled conditions, and complex sample processing. To address this challenge, alternative strategies have been proposed, including ready-to-use microfluidic extraction systems (Fukuzawa et al., 2022), equipment-free cellulose paper-based extraction (Zou et al., 2017) or even PCR-free metabarcoding workflows using rapid cellulose column-based extraction coupled with recombinase polymerase amplification for library preparation (Plewnia et al., 2025). Additionally, portable sequencing platforms such as nanopore technology, which are small enough to be carried into the field, are likely to replace conventional high-throughput sequencing in future field applications of metabarcoding, and direct

PCR metabarcoding of bacterial communities has already been successfully combined with nanopore sequencing (Kai et al., 2019). Direct PCR thus represents a promising yet underexplored tool for streamlining metabarcoding workflows.

In addition to workflow efficiency, we tackled contamination issues by showing that bleach decontamination effectively removes external DNA, enabling reliable spider diet metabarcoding even from pitfall-trapped specimens (Huszarik et al., 2023). This provides a practical solution to both reduce costs and minimize contamination risks in field-based metabarcoding. Our decontamination protocol was successfully tested and has since been adopted in related contexts, including spider (Pekár et al., 2025) and ant (Lin et al., 2025) diet analyses. While we used 2.8% (w/w) bleach, subsequent work has advanced towards establishing standardized concentrations to reduce inconsistencies across studies and enhance the reliability of DNA-based dietary analyses. For example, Silva Wijeyeratne & Gweon (2025) suggested that bleach concentrations as low as 0.5% (w/w) may be sufficient. Moving towards standardized decontamination protocols represents an important step in addressing the broader challenge of discriminating actual trophic interactions from artefacts caused by surface contamination, a methodological issue that is only beginning to receive attention as evidence accumulates that DNA from the environment adheres to living organisms (Huszarik et al., 2023).

Together, these methodological advances – simplified bulk metabarcoding (*Chapter I*) and effective decontamination protocols (*Chapter II*) – enable more efficient, reliable, and ecologically meaningful characterization of community composition and trophic interactions. By reducing biases and enhancing reliability, these approaches open new possibilities for studying biodiversity and food-web dynamics in riparian systems and other ecosystems, and provide methodological developments that can be adopted more broadly in genomic ecology research.

Genomic insights into community and evolutionary responses

In this thesis, genomic analyses were applied to riparian invertebrate samples from previously conducted experiments, thereby complementing parallel stress-ecology studies and adding more detailed insights into community responses (Table 1). By integrating molecular data with traditional methods such as abundance counts and physiological assays, we were able to detect hidden diversity, clarify species-specific responses, and uncover the genetic mechanisms underlying adaptation to human-

induced environmental pressures. This finer-scale perspective highlights how genomic tools complement ecological approaches in revealing the complex ways riparian communities respond to anthropogenic stressors.

For example, Kolbenschlag et al. (2023a) reported that the emergence dynamics of chironomids differed between Bti-treated and control artificial ponds, with an approximately 10-day shift leading to an earlier and reduced peak. In Bti-treated ponds, emergence was higher during April and May but lower during June and July compared to controls. The authors suggested that these changes could reflect shifts in community composition, as local chironomid assemblages comprised species with diverse breeding cycles and feeding strategies, which were potentially affected differently by Bti exposure. A complementary study on stable isotope ratios in the aquatic emergence and riparian spiders from the same experiment found that Chironomidae from Bti-treated mesocosms had elevated nitrogen isotope levels, which the authors interpreted as a shift in community composition toward taxa with higher trophic positions, i.e. more predatory species (Kolbenschlag et al., 2023b).

By applying community metabarcoding with HMSC, our work provided additional resolution to the community composition of aquatic chironomid emergence that refined these interpretations (*Chapter I*). We observed broadly similar responses to Bti across various chironomid genera, indicating only a weak phylogenetic signal in sensitivity, which contradicted the initial hypothesis of taxon-specific responses. Furthermore, our results showed no substantial differences in the Bti sensitivity among chironomids with different feeding strategies. These insights were only possible because genomic data allowed genus-level resolution, revealing patterns that could not be captured by emergence counts or isotopic signatures alone. The additional results make it more plausible that the altered emergence patterns and elevated nitrogen isotope values were likely driven by opportunistic feeding that many chironomid larvae exhibit (Serra et al., 2016; Vallenduuk & Moller Pillot, 2013). Specifically, after Bti exposure, older larvae may have consumed dead or weakened younger larvae, providing a temporary boost in available resources and leading to a short-term increase in emergence. The later decline in emergence occurred because the susceptible larvae were no longer present in the population and thus could not contribute to the expected emergence peak. Thus, the elevated nitrogen isotope values may not necessarily reflect a shift toward inherently predatory chironomid species, but rather the temporary increase in

intraguild predation among larvae following Bti exposure. In this way, genomic tools helped revealing the mechanisms driving chironomid community composition and emergence dynamics under Bti exposure, providing a more detailed understanding of genus-level responses to anthropogenic stressors.

A similar approach was applied to riparian spiders, revealing species-specific responses under low-flow conditions. Ogbeide et al. (2025) reported a statistically significant 2.6-fold reduction in the abundance of *Tetragnatha* sp. under low-flow conditions compared to the control in riparian stream mesocosms. The authors hypothesized that this decline was driven by changes in habitat structure and by reduced availability of EPT taxa. Using gut content metabarcoding, we not only characterized the spiders' diet but also used host DNA to show that the *Tetragnatha* sp. considered in the study actually comprised two sympatric species (*Chapter II*). Of these, one species was strongly negatively affected by the low-flow treatment, while the other appeared to benefit from reduced competitive pressure. This revealed that low-flow conditions directly altered riparian spider populations in addition to reshaping aquatic prey communities. Although consumption of EPT taxa was reduced under low-flow conditions – directly reflecting reduced EPT emergence – these prey items contributed only minimally to spider diets and were therefore unlikely to drive changes in spider abundance. Instead, our results indicate that differences in ecological niches and competitive dynamics played a stronger role. Several recent studies have similarly applied DNA-based and 'genes-to-ecosystems' approaches to investigate riparian and aquatic-terrestrial interactions (Becker et al., 2018; Crutsinger et al., 2014; Huszarik et al., 2024; Perrotta et al., 2025). In this context, our findings further demonstrate the added value of genomic approaches in revealing cryptic diversity and clarifying ecological mechanisms that link aquatic and terrestrial communities under stress.

While the previous studies focused on short-term community responses, the next experiment used an experimental evolution approach to examine longer-term adaptation in replicated *C. riparius* populations exposed to two anthropogenic pollutants, Bti and copper. For Bti, Kolbenschlag et al. (2024) reported that after six months of culturing, some tested exposure scenarios resulted in higher protein or lipid contents in pre-exposed *C. riparius* populations compared to naïve populations, suggesting a limited level of phenotypic adaptation to Bti. The authors proposed that this observation could be caused by a more efficient use of available energy reserves

in adapted populations. In the case of copper, Pietz et al. (2025) found higher emergence in pre-exposed compared to naïve populations, but only under one of the many tested exposure scenarios that varied in copper concentration and food. The authors proposed a potential trade-off between individual fitness and metal adaptation as an explanation. They further hypothesized that adaptive capacity may depend on the initial presence of traits required for increased tolerance, with experimental duration possibly insufficient to promote their development or establishment.

By applying population genomics, we provided a mechanistic understanding of these observed physiological and life-history patterns (*Chapter III*). For Bti, adaptation appeared to be linked to more effective mechanisms for the loss and restoration of affected midgut cells, which could explain the elevated protein and lipid contents through improved digestion and nutrient uptake. For copper, genomic analyses revealed selection primarily on genes associated with DNA repair and detoxification mechanisms, which may have enabled higher emergence in one exposure scenario, but possibly at the cost of individual fitness, particularly reproductive capacity. These results demonstrate how molecular approaches can uncover the genetic basis of adaptive responses, linking physiological changes and life-history traits to underlying evolutionary processes.

Table 1 Overview of accompanying studies, summarizing the stressors, reported effects, and proposed explanations. Additional insights provided by genomic analyses and the corresponding thesis chapter are indicated.

Reference	**Stressor**	**Reported effect**	**Proposed explanation**	**Added insight / evidence**	**Thesis chapter**
Kolbenschlag et al. (2023a)	Bti	Earlier and lower chironomid emergence peak	Taxa responding differently caused altered chironomid communities	Bti responses were largely consistent across chironomid genera	I b)
Ogbeide et al. (2025)	Hydrological drought	Reduced abundance of riparian *Tetragnatha* spiders	Reduction caused by habitat changes and EPT decline	Only one of two sympatric spider species declined, EPT played minor role in dietary composition	II b)
i) Kolbenschlag et al. (2024) ii) Pietz et al. (2025)	i) Bti ii) Copper	phenotypic adaptation in some exposure scenarios	i) More efficient energy use after adaptative phase ii) Tolerance traits might not have been developed or established	i) Immune- and apoptosis-related pathways were enriched ii) Metal detoxification and DNA repair pathways were enriched	III a)

From genomic resolution to ecological understanding

Taken together, the findings of this thesis demonstrate how genomic approaches add ecological relevance by resolving organismal composition, clarifying ecological interactions, and uncovering evolutionary processes that shape riparian communities under anthropogenic stress.

Metabarcoding approaches substantially improved the resolution of chironomid and spider assemblages (*Chapter I and Chapter II*), revealing hidden diversity and species-specific responses that traditional emergence counts or morphological surveys would have overlooked without considerable taxonomic expertise and time investment. The family Chironomidae and the genus *Tetragnatha* provide prominent examples of riparian taxa that are often treated as undifferentiated taxonomic groups rather than analyzed at species level (e.g., Byeon & Kim, 2025; Fierro et al., 2021; Graf et al., 2020; Miller et al., 2020; Popescu et al., 2021; Todd et al., 2024). Yet, as also shown in this thesis, feeding strategies, ecological niches, and stressor tolerances can vary markedly among species within these groups, and overlooking this diversity risks masking important ecological dynamics.

At the same time, while genomic tools facilitate species-level identification, they do not automatically provide information on ecological traits or functional roles. Riparian communities are shaped by more than taxonomic composition or genetic diversity. They are also structured by functional diversity (e.g., redundancy that stabilizes ecosystems), phenological dynamics (life-cycle timing, emergence, reproduction), and key organismal traits such as body size, health, mobility, and behavior. These attributes strongly influence community responses to stressors but remain largely invisible to DNA-based approaches. Future research should therefore combine genomic data with trait-based or functional analyses, as well as imaging or morphological surveys, to more directly link taxonomic resolution to ecological function.

Gut-content metabarcoding provided detailed insights into trophic linkages, showing how prey choice can directly and indirectly be influenced by anthropogenic stressors (*Chapter II*). The high taxonomic resolution of this method makes it particularly valuable for cryptic feeders such as spiders (Greenstone & Shufran, 2003), which externally digest their prey and leave no morphologically identifiable remains in the gut or excrement. In comparison, stable isotope analyses are highly effective for quantifying the relative contribution of aquatic versus terrestrial prey sources in riparian predators

(Doucett et al., 2007), but they provide no resolution within these broad groups, for example distinguishing Baetidae from Chironomidae (cf. Kolbenschlag et al., 2023b). By contrast, metabarcoding can identify prey to finer taxonomic levels, thereby refining our understanding of predator-prey interactions.

However, gut-content metabarcoding only reveals which prey taxa terrestrial spiders have consumed recently, without providing information on the quantity ingested or the nutritional value obtained. Yet stressors may alter not only the taxonomic composition of prey but also their physiological condition or nutrient profiles (Kolbenschlag et al., 2024; Pietz et al., 2023; Scherer et al., 2020), thereby reshaping energy transfer across aquatic-terrestrial boundaries. To capture these dynamics, genomic dietary data should be integrated with complementary approaches such as stable isotope analysis and physiological fitness measures (e.g., fatty acid composition). This combination would provide both taxonomic resolution and functional insights into how anthropogenic stressors affect trophic interactions and cross-ecosystem energy flow.

Population genomic analyses revealed candidate mechanisms of adaptation to anthropogenic pollutants, linking molecular signatures of selection to physiological changes (*Chapter III*). These results demonstrate the potential of experimental evolution frameworks to anticipate the long-term resilience of riparian species. Remarkably, within only a few months, *C. riparius* populations showed genomic signals of adaptation to two anthropogenic model stressors, even before consistent phenotypic responses were detectable. Similar studies have further corroborated the value of this approach by integrating genomic with transcriptomic data, thereby linking genetic variation (the potential for adaptation) with gene expression patterns (the traits actively expressed under stress) to capture early adaptive changes (e.g., Doria et al., 2022).

At the same time, laboratory experiments highlight that adaptation is rarely a fixed outcome, but instead an ongoing and dynamic process, even under seemingly stable conditions (Pfenninger & Foucault, 2020). Extrapolating these findings to natural populations remains challenging, as adaptation in the wild is shaped by many more fluctuating and interacting ecological factors. Future work should therefore combine experimental evolution with field-based genomic surveys to assess how adaptive potential observed under controlled conditions translates into natural riparian ecosystems.

Together, these strengths and limitations highlight the need for integrative approaches that combine genomic analyses with ecological, physiological, and functional data. Such frameworks can better illuminate not only the immediate ecological consequences of anthropogenic pressures but also the evolutionary potential that may buffer – or fail to buffer – riparian ecosystems against future change.

Concluding remarks and future directions

Taken together, the findings of this thesis highlight how genomic, ecological, and evolutionary approaches can be integrated to understand riparian communities as interconnected systems under anthropogenic stress.

Understanding the feedbacks in riparian systems provides a model for other connected ecosystems, such as estuaries, coastal wetlands, and mangrove forests, where cross-boundary interactions and rapid environmental changes create similar dynamics. To fully capture these complex processes, future research should combine genomic data with functional trait measurements and ecological monitoring under natural field conditions. Long-term studies are needed to determine whether early genomic signals of adaptation indeed translate into population-level resilience. Experimental manipulations, coupled with genomic and ecological data, could further disentangle causality within eco-evolutionary dynamics, revealing which mechanisms drive observed patterns.

Ultimately, this thesis demonstrates that integrating genomic, ecological, and evolutionary perspectives provides both methodological innovation and ecological insight. This dual approach advances the toolbox for biodiversity and food-web research while simultaneously improving our understanding of how riparian ecosystems respond and adapt to anthropogenic stressors. Such integrative frameworks illuminate both the immediate ecological consequences of human pressures and the longer-term capacity of riparian communities to persist, transform, or collapse under global change. These insights provide a foundation for designing monitoring, management, and conservation strategies that can detect, anticipate, and support resilient riparian ecosystems across connected aquatic-terrestrial landscapes.

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I would also like to thank my mother **Lisa Röder**, who raised me to become an independent mind, capable of critical reflection, and always mindful of how our actions as humans affect the environment we depend on. Her contribution to this achievement cannot be overstated.

Finally, to my **family**, who were never tired of asking me when I would “finally be finished with my studies”:

Dear family, I will never truly be finished – but here, at last, is my completed thesis.

Appendix

Curriculum Vitae

Status and author contributions of publications included in this dissertation

Declarations

Supplementary information

Curriculum Vitae

Personal Information

Nina Röder

nina.roeder@rptu.de

Education

Since 10/2019

PhD Student in Environmental Sciences

RPTU - University of Kaiserslautern-Landau (Landau, Germany)

- Working title: "Transfer of environmental stress across ecosystems - community and species response to aquatic pesticide load" (Supervisor: Prof. Dr. Klaus Schwenk)

10/2014 – 09/2017

Master Programme in Environmental Sciences

University of Koblenz-Landau (Landau, Germany)

- Master thesis: „Does fungicide exposure alter interspecific relationships of aquatic leaf-decomposing fungi? - A case study using modern biochemical tools" (Supervisors: Prof. Dr. Ralf Schulz, Dr. Jochen Zubrod)

02/2015 – 06/2015

Study Abroad Experience

University of Queensland (Brisbane, Australia)

- Marine Sciences
- Australia's Terrestrial Environment
- Sustainable Development
- Environmental Communication
- Environmental Management

10/2010 – 09/2014

Bachelor Programme in Environmental Sciences

University of Koblenz-Landau (Landau, Germany)

- Bachelor thesis on "The molecular genetic identification of *Pseudocalanus acuspes* in the framework of the BIOACID II project" (Supervisors: Prof. Dr. Klaus Schwenk, Dr. Anne Thielsch)

Research Experience

Since 05/2023	**Scientific Laboratory Manager** Responsible for lab operations in molecular ecology labs and laboratory safety compliance. ▪ Supported researchers with safety and regulatory documents ▪ Coordinated lab safety and documentation
12/2024	**Practical Experience with Coral Stress Testing** Participated in the "Coral Bleaching Automated Stress System (CBASS)" Workshop, organized by MaRHE Center (Magoodhoo, Maldives) ▪ Generated standardized thermal tolerance data for three different Maldivian coral taxa ▪ Collaboratively contributed to drafting a manuscript summarizing the findings
09/2021	**Practical Experience with Artificial Reef Structures** Participated in the workshop "Living Shorelines: Green Engineering Methods for Coral Reef Habilitation", organized by rrreefs and Coreles de Paz (San Andres, Colombia) ▪ Assisted in pilot project to build an artificial reef structure using 3D-printed clay bricks
01/2020	**European Scientific Diver Certification** Certified by the Italian Association of Scientific Divers (AIOSS) after practical and theoretical training (Elba, Italy)
10/2017 – 09/2019	**Scientific Associate** University of Koblenz-Landau ▪ Investigated the effects of the mosquito control agent *Bacillus thuringiensis israelensis* (Bti) on chironomid communities using metabarcoding
2014 – 2017	**Research Assistant** Assisted in organizing the annual International Summer Academy on Spatial Ecotoxicology and Ecotoxicological Risk Assessment ▪ Support for international participants
09/2016 – 10/2016	**Guest Researcher** University of Eastern Finland, Department of Environmental and Biological Sciences (Kuopio, Finland) ▪ Molecular genetic analysis of *Aphanomyces astaci* ▪ Assisted in sediment sampling of Finnish lakes
05/2016	**Internship in Reef Ecology** New Heaven Reef Conservation Program (Koh Tao, Thailand) ▪ Coral taxonomy and nursery ▪ Management and maintenance of (artificial) coral reefs
02/2014 – 04/2014	**Volunteering in Marine Conservation** Red Sea Environmental Centre (Dahab, Egypt) ▪ Monitoring of corals, invertebrates, and fish

Teaching Experience

Since 03/2023	**Metabarcoding-Club** Initiated biweekly meetings and currently mentor young scientists from different working groups dealing with metabarcoding analyses
2024 – 2025	**Lecturer for Bachelor and Master Courses** Teaching of *Genetics and Evolution* and *Molecular Ecology I*
2018 – 2025	**Phylogenetic and Population Genetic Analysis Seminars** Responsible for the *Phylogenetic and Population Genetic Analysis* seminars for master students
2014 – 2017	**Instructor for R Statistics Tutorials** Initiated and conducted *R Statistics* tutorials, funded by the student college "docendo discimus" from 2016
2014 – 2017	**Scientific Assistant during Practical Courses** Assisted in the *Microscopic-Biological Introductory Practicum*

Peer-Reviewed Publications

1. **Röder N,** Kolbenschlag S, Pietz S, Brennan RS, Bundschuh M, Pfenninger M, Schwenk K (accepted): "Pollution-Driven Selection in Riparian Ecosystems: Genome-Wide Responses to *Bacillus thuringiensis israelensis* and Copper in a Non-biting Midge", *Molecular Ecology*.

2. Pietz S, **Röder N**, Kolbenschlag S, Schöndorfer A, Schwenk K, Bundschuh M (2025): "Effects of copper, food quality and exposure history on aquatic insect emergence: Insights from a multigeneration study", *Ecotoxicology and Environmental Safety*. DOI: 10.1016/j.ecoenv.2025.118893.

3. Ogbeide C, Manfrin, A, Burgazzi G, Burgis F, Knäbel A, Pietz S, **Röder N**, Roodt AP, Schreiner VC, Schwenk K, Bundschuh M, Schulz R (2025): "Flow Reduction in a Pesticide-Exposed Stream Mesocosm Affects Emerging Aquatic Insects and Alters Riparian Spider Communities", *Archives of environmental contamination and toxicology*. DOI: 10.1007/s00244-025-01146-5.

4. Stoll VS, **Röder N**, Gerstle V, Manfrin A, Schwenk K (2025): "Effects of Bti on the diversity and community composition of three Chironomidae subfamilies across different micro-habitats", *Environmental Pollution.* DOI: 10.1016/j.envpol.2024.125490.

5. Kolbenschlag S, Pietz S, **Röder N**, Schwenk K, Bundschuh M (2024): "Phenotypic adaptation of *Chironomus riparius* to chronic Bti exposure: effects on emergence time and nutrient content", *Aquatic Toxicology.* DOI: 10.1016/j.aquatox.2024.107013.

6. **Röder N**, Stoll VS, Jupke JF, 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*. DOI: 10.1016/j.scitotenv.2023.169735.

7. **Röder N**, Schwenk K (2023): "Direct PCR meets high-throughput sequencing – metabarcoding of chironomid communities without DNA extraction", *Metabarcoding and Metagenomics*. DOI: 10.3897/mbmg.7.102455.

8. Huszarik M, **Röder N**, Eberhardt L, Kennedy S, Krehenwinkel H, Schwenk K, Entling MH (2023): "External DNA contamination and efficiency of bleach decontamination for arthropod diet analysis", *Environmental DNA*. DOI: 10.1002/edn3.410.

9. Schneeweiss A, Schreiner VC, Liess M, **Röder N**, Schwenk K, Schäfer RB (2023): "Population structure and insecticide response of *Gammarus spp*. in agricultural and upstream forested sites of small streams", *Environmental Sciences Europe*. DOI: 10.1186/s12302-023-00747-y.

10. Pietz S, Kolbenschlag S, **Röder N**, Roodt AP, Steinmetz Z, Manfrin A, Schwenk K, Schulz R, Schäfer RB, Zubrod JP, Bundschuh M (2023): "Subsidy Quality Affects Common Riparian Web-Building Spiders: Consequences of Aquatic Contamination and Food Resource", *Environmental Toxicology and Chemistry*. DOI: 10.1002/etc.5614

11. Manfrin A, Schirmel J, Mendoza-Lera C, Ahmed A, Bohde R, Brunn M, Brühl CA, Buchmann C, Bundschuh M, Burgis F, Diehl D, Entling MH, Ganglo C, Geissler S, Gerstle V, Girardi JP, Graf T, Huszarik M, Jamin J, Joschko TJ, Jungkunst HF, Knäbel A, Kolbenschlag S, Lorke A, Muñoz K, Ogbeide C, Osakpolor SE, Pietz S, Riess K, Roodt AP, Rovelli L, **Röder N**, Rösch V, Schaumann GE, Schäfer RB, Schmitt T, Schmitz D, Schützenmeister K, Schwenk K, Stehle S, Schulz R (2023): "SystemLink: Moving beyond Aquatic-Terrestrial Interactions to Incorporate Food Web Studies", *Limnology and Oceanography Bulletin*. DOI: 10.1002/lob.10557.

12. Roodt AP, **Röder N**, Pietz S, Kolbenschlag S, Manfrin A, Schwenk K, Bundschuh M, Schulz R (2022): "Emerging Midges Transport Pesticides from Aquatic to Terrestrial Ecosystems: Importance of Compound- and Organism-Specific Parameters", *Environmental Science & Technology*. DOI: 10.1021/acs.est.1c08079.

13. Konschak M, Zubrod JP, Duque Acosta TS, Bouchez A, Kroll A, Feckler A, **Röder N**, Baudy-Groh P, Schulz R, Bundschuh M (2021): "Herbicide-Induced Shifts in the Periphyton Community Composition Indirectly Affect Feeding Activity and Physiology of the Gastropod Grazer *Physella acuta"*, *Environmental Science & Technology*. DOI: 10.1021/acs.est.1c01819.

14. Baudy-Groh P, Zubrod JP, Konschak M, **Röder N**, Nguyen TH, Schreiner VC, Baschien C, Schulz R, Bundschuh M (2021): "Environmentally relevant fungicide levels modify fungal community composition and interactions but not functioning", *Environmental Pollution*. DOI: 10.1016/j.envpol.2021.117234.

15. Konschak M, Zubrod JP, Baudy-Groh P, Fink P, Kenngott KGJ, Englert D, **Röder N**, Ogbeide C, Schulz R, Bundschuh M (2021): "Chronic effects of the strobilurin fungicide azoxystrobin in the leaf shredder *Gammarus fossarum* (Crustacea; Amphipoda) via two effect pathways", *Ecotoxicology and Environmental Safety*. DOI: 10.1016/j.ecoenv.2020.111848.

16. Baudy-Groh P, Zubrod JP, Konschak M, **Röder N**, Baschien C, Feckler A, Schulz R, Bundschuh M (2019): “A glance into the black box: Novel species-specific quantitative real-time PCR assays to disentangle aquatic hyphomycete community composition”, *Fungal Ecology*. DOI: 10.1016/j.funeco.2019.08.002.

17. Theissinger K, **Röder N**, Allgeier S, Beermann AJ, Brühl CA, Friedrich A, Michiels S, Schwenk K (2019): “Mosquito control actions affect chironomid diversity in temporary wetlands of the Upper Rhine Valley”, *Molecular Ecology*. DOI: 10.1111/mec.15214.

18. Wenisch B, **Röder N**, Link M, Gergs R, Entling MH, Schäfer RB (2015): “Aquatic prey subsidies to riparian spiders in a stream with different land use types”, *Limnologica*. DOI: 10.1016/j.limno.2014.10.001.

Submitted Publications

1. Kripp A, Eitzinger B, Hatamli K, Huszarik M, Melcher A, **Röder N**, Entling MH (submitted): “Separation of the trophic niches in a community of riparian generalist predators”

Publications included in this thesis are underlined.

Status and author contributions of publications included in this dissertation

Chapter I

Title: Direct PCR meets high-throughput sequencing – metabarcoding of chironomid communities without DNA extraction

Authors: Röder N, Schwenk K

Status: published 2023 in Metabarcoding and Metagenomics (DOI: 10.3897/mbmg.7.102455).

Contribution: I contributed to the conception of the ideas and the design of the methodology, collected and analyzed the data, and led the writing of the manuscript.

Title: How non-target chironomid communities respond to mosquito control: Integrating DNA metabarcoding and joint species distribution modelling

Authors: Röder N, Stoll VS, Jupke JF, Kolbenschlag S, Bundschuh M, Theißinger K, Schwenk K

Status: published 2024 in Science of the Total Environment (DOI: 10.1016/j.scitotenv.2023.169735).

Contribution: I conducted the formal analysis and led the writing of the manuscript. Additionally, I contributed to the conceptualization, investigation, methodology and visualization.

Chapter II

Title: External DNA contamination and efficiency of bleach decontamination for arthropod diet analysis

Authors: Huszarik M, Röder N, Eberhardt L, Kennedy S, Krehenwinkel H, Schwenk K, Entling MH

Status: published 2023 in Environmental DNA (DOI: 10.1002/edn3.410).

Contribution: I contributed to the conceptualization of the study and carried out field sampling and laboratory work with assistance. I conducted bioinformatic analyses, contributed to the interpretation of the data and assisted in writing the manuscript.

Chapter III

Title: Pollution-Driven Selection in Riparian Ecosystems: Genome-Wide Responses to *Bacillus thuringiensis israelensis* and Copper in a Non-biting Midge

Authors: Röder N, Kolbenschlag S, Pietz S, Brennan RS, Bundschuh M, Pfenninger M, Schwenk K

Status: Manuscript accepted by Molecular Ecology

Contribution: I contributed to the conception and design of the study, conducted experiments, and was responsible for sample collection and processing. I performed the bioinformatic and statistical analyses and led the writing of the manuscript.

Declarations

Declaration according to §8 of the Doctoral Degree Regulations of the Faculty of Natural and Environmental Sciences of the University of Kaiserslautern-Landau, dated 14 June 2013, as amended on 19 August 2014

I, the author of this work, hereby declare that I have independently conducted the research presented in this thesis entitled “Anthropogenic pressures on riparian communities: insights from genomic tools”. All sources and aids used have been fully acknowledged, and the contributions of any collaborators or co-authors are clearly indicated within the respective publications.
I further declare that I have not used any paid services from commercial counseling or mediation agencies (‘Promotionsberater’ or similar) in preparing this thesis.
I confirm that I have not previously submitted this dissertation, in the same or similar form, for examination as part of a state or academic degree at any university or scientific institution, either in Germany or abroad, and that I have not submitted the same or a different dissertation to another faculty or university, nor has any such submission taken place elsewhere.
I am aware that any violation of the above statements may result in the withdrawal of the doctoral degree and may lead to further legal consequences.

Declaration on the Use of Generative AI

During the preparation of this dissertation and two related manuscripts (Röder et al., 2024; Röder et al., accepted), I used ChatGPT, an AI language model developed by OpenAI, to enhance clarity, readability, and linguistic quality. Additionally, I used ChatGPT to generate and adapt code for bioinformatic and statistical analyses during these projects.
After using this tool, I thoroughly reviewed, verified, and edited all AI-assisted outputs, including written content and code, and take full responsibility for the content, interpretations, and analyses presented in this dissertation.

Supplementary information

Supplemental Information for:

Direct PCR meets high-throughput sequencing – metabarcoding of chironomid communities without DNA extraction

Nina Röder, Klaus Schwenk

Table S1

Table S1 Overview of chironomid size classes. Chironomid individuals were sorted into four different size categories according to their body length (from the anterior margin of the head between the antennae to the end of the posterior abdominal segment) and shape (thin - usually males or thick - usually females). They were counted and average weight per specimen was determined.

Size group	Body length	Body shape	Mean dry weight [mg] per specimen	Number of weighed samples	Number of specimens in the weighed samples
Very large	> 5 mm	thick	1.44	6	between 10 and 50
Large	> 5 mm	thin	0.54	7	between 3 and 40
	3-5 mm	thick			
Medium	3-5 mm	thin	0.18	12	between 16 and 110
	< 3mm	thick			
Small	< 3mm	thin	0.05	7	between 21 and 44

Supplemental Information for:

Direct PCR meets high-throughput sequencing – metabarcoding of chironomid communities without DNA extraction

Nina Röder, Klaus Schwenk

Table S2

Table S2 Composition of the two artificial chironomid communities. Sanger sequences of specimens were compared to BOLD database.

Artificial community	Specimen ID	Size class	Length Sanger Sequence	BOLD best match BIN ID	BOLD best match species	BOLD best match similarity (%)	Corresponding metabarcoding OTU
Mock A	IS33	LARGE	655 bp	ACD8095	*Endochironomus tendens*	99.3	OTU_A
Mock A	IS36	MEDIUM	601 bp	AAU2481	*Phaenopsectra flavipes*	99.8	OTU_B
Mock A	IS52	SMALL	647 bp	ACO6911	*Monopelopia tenuicalcar*	100.0	OTU_C
Mock A	IS61	SMALL	571 bp	AAR9373	*Cladotanytarsus pallidus*	99.6	OTU_D
Mock A	IS63	MEDIUM	630 bp	ABY9333	*Ablabesmyia monilis*	99.6	OTU_E
Mock A	IS78	VERY LARGE	621 bp	AAW4677	*Kiefferulus tendipediformis*	99.5	OTU_F
Mock A	IS107	VERY LARGE	657 bp	AEB2387	*Endochironomus albipennis*	99.2	OTU_G
Mock A	IS108	VERY LARGE	630 bp	AAJ4282	*Chironomus luridus*	99.0	OTU_H
Mock A	IS118	MEDIUM	632 bp	ACW5385	*Procladius culiciformis*	100.0	OTU_I
Mock A	IS146	MEDIUM	622 bp	ACY8325	*Procladius crassinervis*	100.0	OTU_J
Mock A	IS153	MEDIUM	612 bp	ACN4387	*Tanytarsus occultus*	99.7	OTU_K
Mock A	IS154	MEDIUM	617 bp	AAU6327	*Psectrocladius schlienzi*	100.0	OTU_L
Mock A	IS157	LARGE	647 bp	ABY3509	*Microtendipes pedellus*	99.7	OTU_M
Mock A	IS159	MEDIUM	658 bp	AAD8015	*Zavrelimyia melanura*	99.8	OTU_N
Mock A	IS173	LARGE	653 bp	ACD8095	*Endochironomus albipennis*	100.0	OTU_O
Mock A	IS186	MEDIUM	658 bp	AAF3633	*Ablabesmyia monilis*	99.2	OTU_P
Mock B	LB102	VERY LARGE	838 bp	ACD4470	*Glyptotendipes pallens*	99.9	OTU_4
Mock B	LE1	MEDIUM	834 bp	ACK3818†	*Ablabesmyia sp. 2ES*	99.5	OTU_1
Mock B	LE12	LARGE	838 bp	ACD8095	*Endochironomus albipennis*	99.9	OTU_13
Mock B	LE26	LARGE	658 bp	ADC7811†	*Clinotanypus nervosus*	99.7	OTU_12

† "No match" in Public Record Barcode Database, results based on All Barcode Records on BOLD.

Supporting Material to: How non-target chironomid communities respond to mosquito control: Integrating DNA metabarcoding and joint species distribution modelling

Nina Röder[1], V. Sophie Stoll[1], Jonathan F. Jupke[1], Sara Kolbenschlag[1], Mirco Bundschuh[1,2], Kathrin Theißinger[3], Klaus Schwenk[1,3]

[1] iES - Institute for Environmental Sciences, RPTU Kaiserslautern-Landau, Landau, Germany

[2] Department of Aquatic Science and Assessment, Swedish University of Agricultural Sciences, Uppsala, Sweden

[3] LOEWE Centre for Translational Biodiversity Genomics, Senckenberg Biodiversity and Climate Research Centre, Frankfurt am Main, Germany

AUTHOR INFORMATION

Corresponding Author

Nina Röder

RPTU Kaiserslautern-Landau

iES Landau - Institute for Environmental Sciences

Fortstraße 7

76829 Landau/Palatinate

Germany

Email: nina-roeder@posteo.de

A) Sørensen beta-diversity

The Sørensen dissimilarity was computed pairwise for all 311 samples in R, utilizing the beta.pair function from the betapart package (Baselga et al., 2023). The plot shows Sørensen dissimilarity for chironomid communities in samples with the same treatment (both Bti or both control) or with different treatments (Bti vs. control).

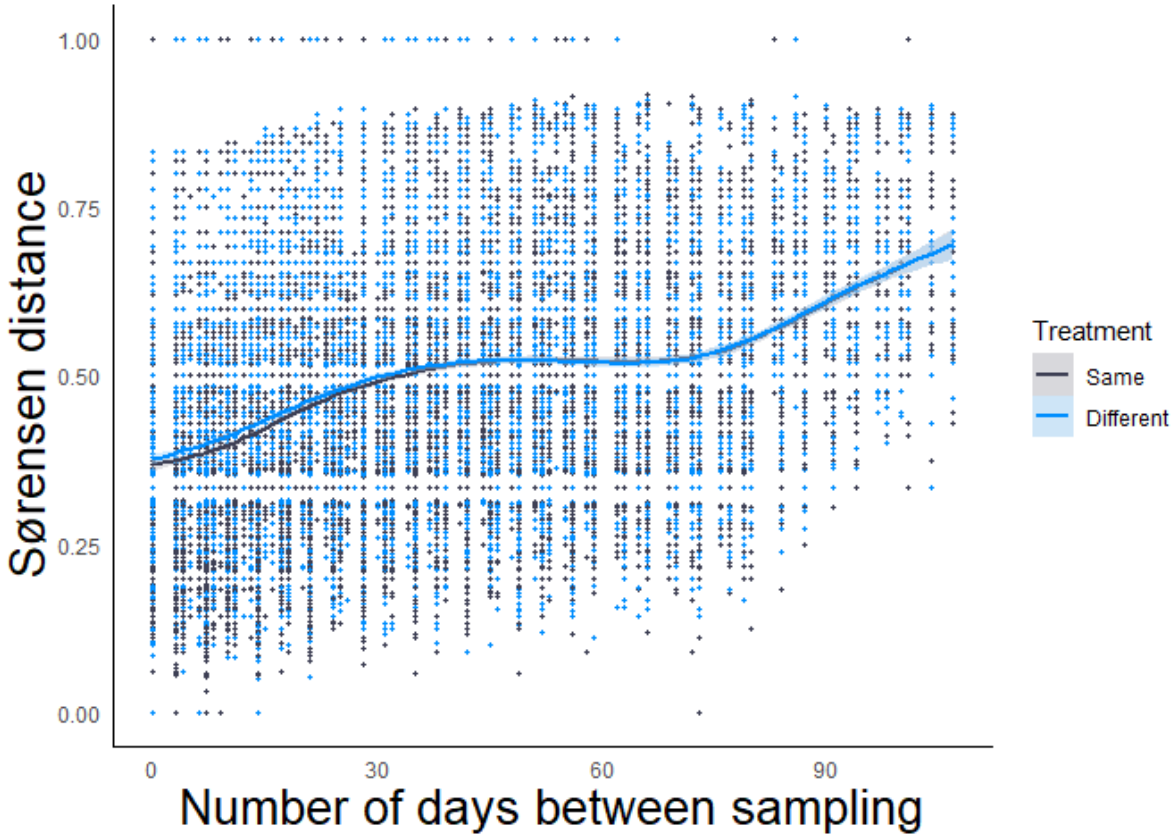

Figure S9 Pairwise beta-diversity of 311 samples. Compared were samples with the same treatment (both Bti or both control) or with different treatments (Bti vs. control). Mean Sørensen dissimilarity was higher when sampling dates were further apart.

B) PERMANOVA

To assess the influence of Bti treatment and sampling date (day of year = DOY) on the chironomid communities, we performed a PERMANOVA using the function adonis2 of the vegan R package (Oksanen et al. 2022). We applied Jaccard distance to our presence-absence data and used 999 permutations.

```
Permutation test for adonis under reduced model
Terms added sequentially (first to last)
Permutation: free
Number of permutations: 999

adonis2(formula = Dat_OTUs ~ Treatment * DOY, data = data, permutations = 999,
method = "jaccard")
               Df SumOfSqs      R2       F Pr(>F)
Treatment       1    0.656 0.01006  3.6302  0.004 **
DOY             1    8.639 0.13234 47.7779  0.001 ***
Treatment:DOY   1    0.474 0.00726  2.6218  0.004 **
Residual      307   55.512 0.85035
Total         310   65.282 1.00000
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure S10 Outcome of the PERMANOVA; the effect of Bti treatment, sampling date and their interaction on the community composition was assessed. All three parameters show a significant effect, however sampling date explains the largest share of the explained variation.

C) Ordination (Correspondence Analysis)

We performed a correspondence analysis using the cca function of the vegan R package (Oksanen et al. 2022) for ordination. To reduce variation, we summarized the communities in each pond per month, resulting in four communities per pond over the experiment phase.

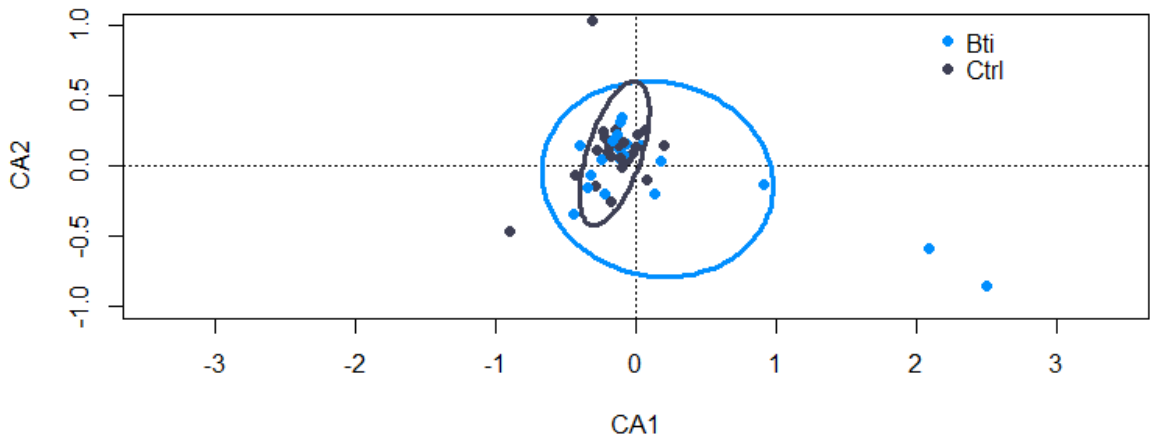

Figure S11 Correspondence analysis plot showing communities from Bti-treated and control ponds. Ellipsoids indicate standard deviations of points. The first and second axis explain 9.2 % and 8.6 %, respectively. Two distant data points are excluded from the plots.

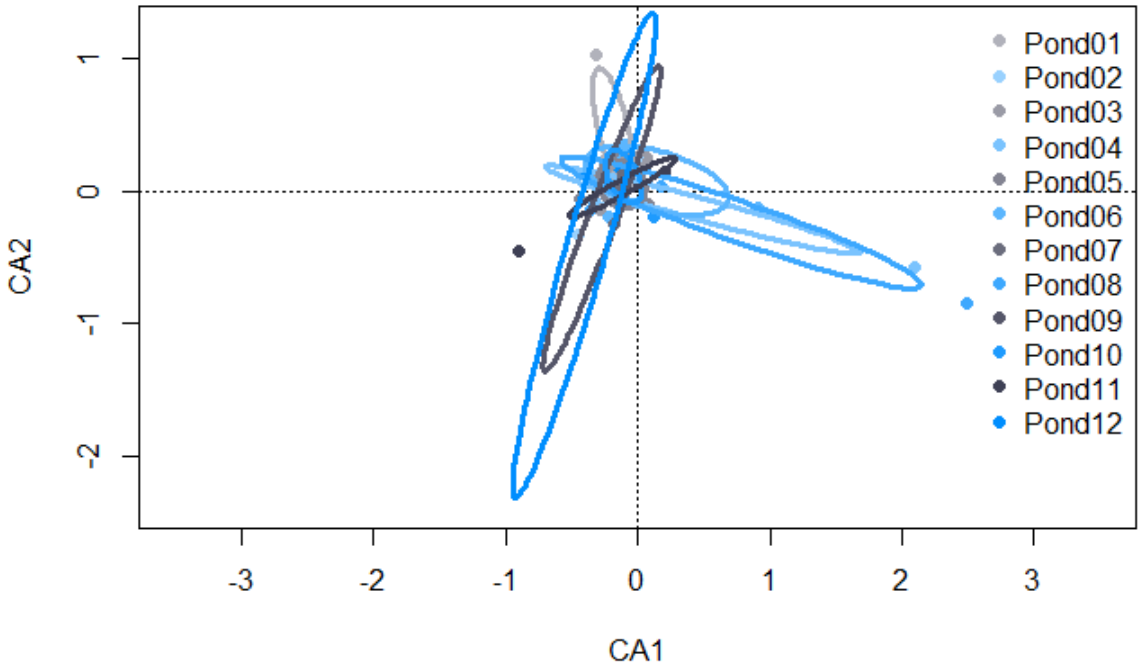

Figure S12 Correspondence analysis plot showing communities from the twelve different ponds. Ponds with even numbers where Bti-treated. Ellipsoids indicate standard deviations of points. The first and second axis explain 9.2 % and 8.6 %, respectively. Two distant data points are excluded from the plots.

D) Emergence Dynamics

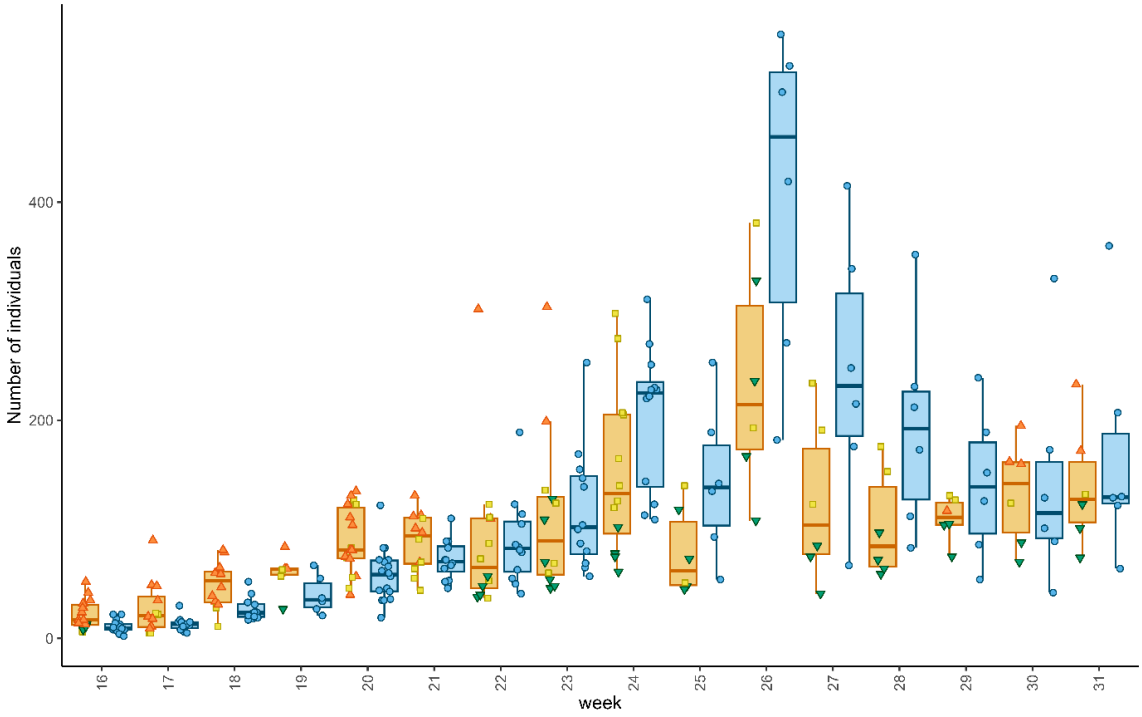

Figure S5 Number of individuals per week and treatment. Boxplots represent control (blue) and Bti-treated (orange) ponds (n=6). Scattered symbols indicate individual ponds, with colors and shapes representing emergence status: orange triangle - increased emergence, yellow square - no deviation, green triangle - decreased emergence, blue circle - control.

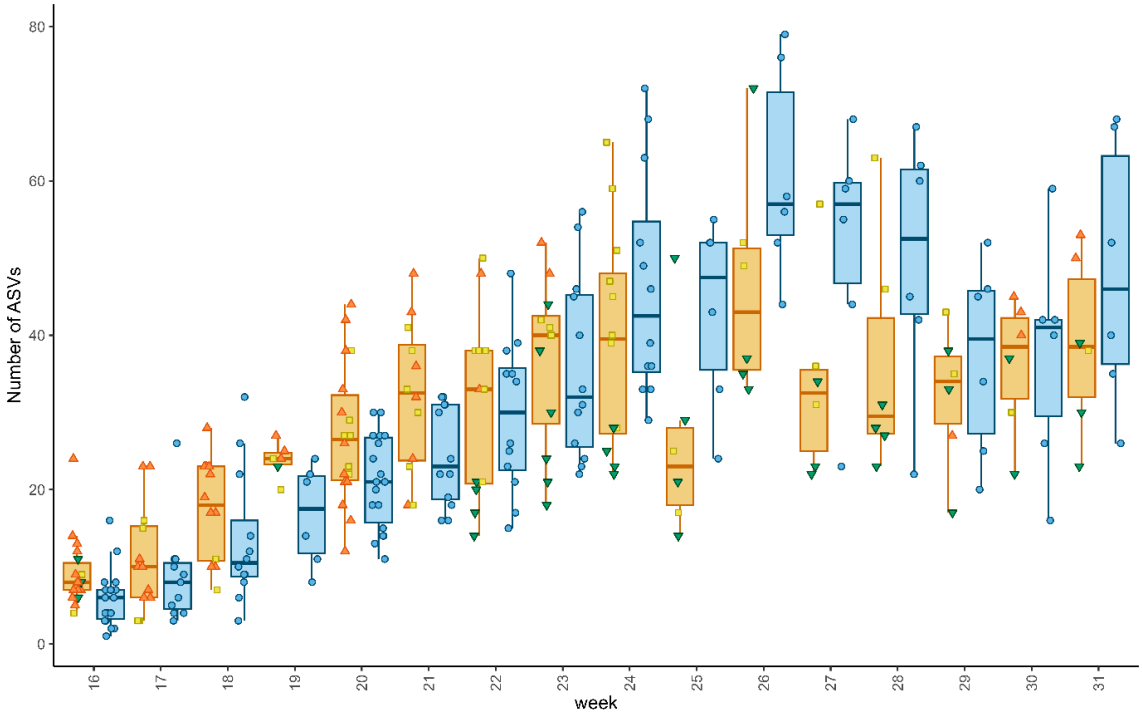

Figure S6 ASV richness per week and treatment. Boxplots represent control (blue) and Bti-treated (orange) ponds (n=6). Scattered symbols indicate individual ponds, with colors and shapes representing emergence status: orange triangle - increased emergence, yellow square - no deviation, green triangle - decreased emergence, blue circle - control.

E) References

Baselga A, Orme D, Villeger S, De Bortoli J, Leprieur F, Logez M, Martinez-Santalla S, Martin-Devasa R, Gomez-Rodriguez C, Crujeiras R (2023). _betapart: Partitioning Beta Diversity into Turnover and Nestedness Components_. R package version 1.6, <https://CRAN.R-project.org/package=betapart>.

Oksanen J, Simpson G, Blanchet F, Kindt R, Legendre P, Minchin P, O'Hara R, Solymos P, Stevens M, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, De Caceres M, Durand S, Evangelista H, FitzJohn R, Friendly M, Furneaux B, Hannigan G, Hill M, Lahti L, McGlinn D, Ouellette M, Ribeiro Cunha E, Smith T, Stier A, Ter Braak C, Weedon J (2022). _vegan: Community Ecology Package_. R package version 2.6-4, <https://CRAN.R-project.org/package=vegan>.

Supporting Information:

"External DNA contamination and efficiency of bleach decontamination for arthropod diet analysis"

Table S1: Coordinates of spider sampling sites and pitfall traps and sampling dates

Event	Stream (If applicable)	Location	Date
Vineyard for hand sampling		49.2038456, 8.0950493	August 19th, 2019
Forest for simulated pitfall trap		approx. 49.2046261, 8.1060383	
Riparian forests for sampling	Michelsbach	49.1848960, 8.3514450	May-July 2019 (Hand sampling June 2019)
	Neuer Tiefer Graben	49.2191616, 8.3205017	
	Queich	49.2197106, 8.3233606	
	Otterbach	49.0935382, 8.2609278	

Table S2: High salt extraction protocol used for DNA extraction from whole spiders, which had been dried and ground into a powder.

Step	Proceedure
1.	Add 450 µL SEB and 100 µL SDS to dried and ground samples
2.	Add5 µL Proteinase K and vortex
3.	Incubate 1 hr at 60 °C in a 400-rpm shaker
4.	Add 350 µL NaCl (5M) and vortex
5.	Centrifuge at 16200 x g for 30 minutes
6.	Transfer 600 µL supernatant to new tube and add 600 µL ice-cold isopropanol. Mix briefly
7.	Freeze at -80 °C for 20 minutes
8.	Centrifuge at 4 °C for 20 minutes (16200 x g)
9.	Discard supernatant, careful not to disturb pellet. Add 200 µL ice-cold 70% ethanol
10.	Centrifuge at 4 °C for 10 minutes (16200 x g)
11.	Discard supernatant, careful not to disturb pellet. Dry tube with pellet in heat block at 60 °C
12.	Elute in 25 µL 1x TE buffer

Table S3: Pairs of forward and reverse primers used for selective amplification of prey DNA in wolf spider gut content with a multiplex PCR step, as used by *Krehenwinkel et al. (2019)*. The barcode portion of the indexing primers is shown in bold red.

Primer Type	Primer Name	Sequence (5' to 3')
Multiplex forward 1 (18SS)	18SrDNA F1046	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTCTGGTTRATTCCGRTAACGAA
Multiplex forward 2 (18SL)	18SrDNA F985	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTCTTTCTYGATTCRGTGGGT
Multiplex reverse 1&2	18SrDNA R1238	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCACAGACCTGTTATTGCTCAA
Multiplex forward 3 (28S)	28SrDNA F1020	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCGTCTTGAAACACGGACCA
Multiplex reverse 3	28SrDNA R1333	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGWCCTCCATCAGGGTTTCCC
Index forward	F_01	AATGATACGGCGACCACCGAGATCTACAC**TAGATCGC**ACACTCTTTCCCTACACGA
Index forward	F_02	AATGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**ACACTCTTTCCCTACACGA
Index forward	F_03	AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**ACACTCTTTCCCTACACGA
Index forward	F_04	AATGATACGGCGACCACCGAGATCTACAC**AGAGTAGA**ACACTCTTTCCCTACACGA
Index forward	F_05	AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**ACACTCTTTCCCTACACGA
Index forward	F_06	AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**ACACTCTTTCCCTACACGA
Index forward	F_07	AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**ACACTCTTTCCCTACACGA
Index forward	F_08	AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**ACACTCTTTCCCTACACGA
Index reverse	R_01	CAAGCAGAAGACGGCATACGAGAT**ATTGGTCA**GTGACTGGAGTTCAGACGTG
Index reverse	R_02	CAAGCAGAAGACGGCATACGAGAT**TAAAAATG**GTGACTGGAGTTCAGACGTG
Index reverse	R_03	CAAGCAGAAGACGGCATACGAGAT**ATCACTGT**GTGACTGGAGTTCAGACGTG
Index reverse	R_04	CAAGCAGAAGACGGCATACGAGAT**TATTTCAC**GTGACTGGAGTTCAGACGTG
Index reverse	R_05	CAAGCAGAAGACGGCATACGAGAT**ATATTGGC**GTGACTGGAGTTCAGACGTG
Index reverse	R_06	CAAGCAGAAGACGGCATACGAGAT**TATACAAG**GTGACTGGAGTTCAGACGTG
Index reverse	R_07	CAAGCAGAAGACGGCATACGAGAT**ATGATCTG**GTGACTGGAGTTCAGACGTG
Index reverse	R_08	CAAGCAGAAGACGGCATACGAGAT**TACTCTAC**GTGACTGGAGTTCAGACGTG
Index reverse	R_09	CAAGCAGAAGACGGCATACGAGAT**ATAAGCTA**GTGACTGGAGTTCAGACGTG
Index reverse	R_10	CAAGCAGAAGACGGCATACGAGAT**TAGTATAG**GTGACTGGAGTTCAGACGTG
Index reverse	R_11	CAAGCAGAAGACGGCATACGAGAT**ATTACAAG**GTGACTGGAGTTCAGACGTG
Index reverse	R_12	CAAGCAGAAGACGGCATACGAGAT**TAATTGGC**GTGACTGGAGTTCAGACGTG

Table S4: Components of the multiplex PCR master mix to selectively amplify prey DNA from wolf spider samples

Ingredient	Volume (µL)
18S_F1046	0.42
18S_F985	0.54
18S_R1238	0.96
28S_F1020	0.75
28S_R1333	0.75
Multiplex PCR Master Mix (QIAGEN)	7.5
H_20	2.58
Master Mix total	***13.5***
Isolated DNA	***1.5***

Table S5: Timings and temperatures for multiplex and index PCR steps to extract prey DNA from wolf spider samples

	Temperature (°C)	Time (mm:ss)
Step 1	95	15:00
Step 2	94	0:30
Step 3	55	1:30
Step 4	72	1:30
Step 5	*Repeat steps 2-4, 34 times (multiplex) or 5 times (index)*	
Step 6	72	10:00
Step 7	12	infinite hold

Table S6: Components of the indexing PCR master mix to selectively amplify prey DNA from wolf spider samples

Ingredient	Volume (µL)
Multiplex PCR Master Mix (QIAGEN)	5
H_20	3.5
Indexing Master Mix total	***8.5***
Multiplex PCR product	***0.5***
Indexing primer (forward + reverse)	***0.5 + 0.5***

Table S7: Count of sequence reads obtained from spider diet analysis using three primers. Target reads were non-spider non-crustacean arthropods.

Primer	18SL	18SS	28S
Number ASVs total	274	593	327
Number target ASVs	82	125	82
Number reads total	52,080	157,628	137,259
Average reads bleached	567	1604	1961
Average reads control	753	2473	1586
Number target reads	14,926	50,963	11,228
Average reads bleached	134	395	53
Average reads control	282	1049	279
Spiders with no reads	3 (all bleached)	0	0
Spiders with no target reads	26 (4 control)	10 (2 control)	33 (6 control)

Data analysis: Assessing the effect of hydrological drought on riparian spider diets

– Supplementary Information –

Table S1 Components of the SEB buffer used in DNA extraction.

Ingredient	Volume (µL)
NaCl (5M)	2548
1M Tris-HCl (pH 8)	319
0.5M EDTA (pH 8)	127.5
H_2O	618
Master Mix total	***3612.5***
Buffer per sample	***42.5***

Table S1 High salt extraction protocol following Kaunisto et al. (2017) used for DNA extraction from whole spiders, which had been dried, ground into a powder and dissolved in PCR-grade water.

Step	Procedure
1.	Add 42.5 µL SEB and 7.5 µL SDS (10%) to tissue suspension
2.	Add 50 µL Proteinase K (10 mg/ml) and vortex
3.	Incubate 3 hours at 60 °C in a 350-rpm shaker
4.	Add 300 µL NaCl (5M) and vortex
5.	Centrifuge at 11,000 x g for 20 minutes
6.	Transfer 600 µL supernatant to new tube and add 600 µL ice-cold isopropanol. Mix briefly
7.	Freeze at -20 °C overnight
8.	Centrifuge at 4 °C for 20 minutes (14,000 x g)
9.	Discard supernatant, careful not to disturb pellet. Add 1,000 µL ice-cold 70% ethanol
10.	Centrifuge at 4 °C for 10 minutes (14,000 x g)
11.	Discard supernatant, careful not to disturb pellet. Dry tube with pellet in heat block at 56 °C
12.	Elute in 20 µL 1x TE buffer

Table S3 Timings and temperatures for amplicon and index PCR steps to amplify prey DNA from spider samples

	Temperature (°C)	Time (mm:ss)
Step 1	95	5:00
Step 2	95	0:30
Step 3	54 (amplicon) / 61 (index)	1:30
Step 4	72	0:30
Step 5	*Repeat steps 2-4, 35 times (amplicon) or 12 times (index)*	
Step 6	68	10:00

Table S4 Components of the PCR master mix to selectively amplify prey DNA from spider samples.

Ingredient	Volume (µL)
Multiplex PCR Master Mix (QIAGEN)	5
H_20	2
Dye (QIAGEN)	1
Master Mix total	***8***
DNA extract / PCR product	***1***
Primer (forward + reverse)	***0.5 + 0.5***

Table S5 Pairs of forward and reverse primers used for selective amplification of prey DNA in spider gut content. The barcode portion of the indexing primers is shown in bold red.

Forward Universal Tail	Tag	Forward Primer Sequence	Forward Primer Name
ACACTCTTTCCCTACACGACGCTCTTCCGATCT	**CTGT**	GCHCCHGAYATRGCHTTYCC	BF2
ACACTCTTTCCCTACACGACGCTCTTCCGATCT	**TCTCA**	GCHCCHGAYATRGCHTTYCC	BF2
ACACTCTTTCCCTACACGACGCTCTTCCGATCT	**GTCCTA**	GCHCCHGAYATRGCHTTYCC	BF2
Reverse Universal Tail	**Tag**	**Reverse Primer Sequence**	**Reverse Primer Name**
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	**GTCCTA**	RGGRTANACNGTTCAWCCWGT	Spidprey_R
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	**GAACA**	RGGRTANACNGTTCAWCCWGT	Spidprey_R
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	**CTGT**	RGGRTANACNGTTCAWCCWGT	Spidprey_R

Table S6 Pairwise partitioning of diet dissimilarity (Jaccard β-diversity) between riparian spiders. Values represent mean ± SD for total β-diversity, and its turnover and nestedness components. Turnover fraction indicates the proportion of dissimilarity due to replacement of prey families, while nestedness fraction reflects differences attributable to subset patterns. Comparisons are shown between species (*T. extensa* vs. *T. montana*) under the same treatment, and between treatments (Control vs. Low-Flow) within species.

Group A	Group B	Beta diversity	Turnover	Nested-ness	Turnover fraction [%]	Nestedness fraction [%]	Comparison type
Low-Flow *Tetragnatha extensa*	Low-Flow *Tetragnatha montana*	0.69 ± 0.15	0.44 ± 0.32	0.24 ± 0.27	64.3	35.7	between Species
Control *Tetragnatha extensa*	Control *Tetragnatha montana*	0.72 ± 0.17	0.46 ± 0.35	0.26 ± 0.30	63.3	36.7	between Species
Control *Tetragnatha extensa*	Low-Flow *Tetragnatha extensa*	0.73 ± 0.14	0.54 ± 0.30	0.19 ± 0.23	74.5	25.5	between Treatments
Control *Tetragnatha montana*	Low-Flow *Tetragnatha montana*	0.70 ± 0.17	0.39 ± 0.35	0.31 ± 0.31	56.0	44.0	between Treatments

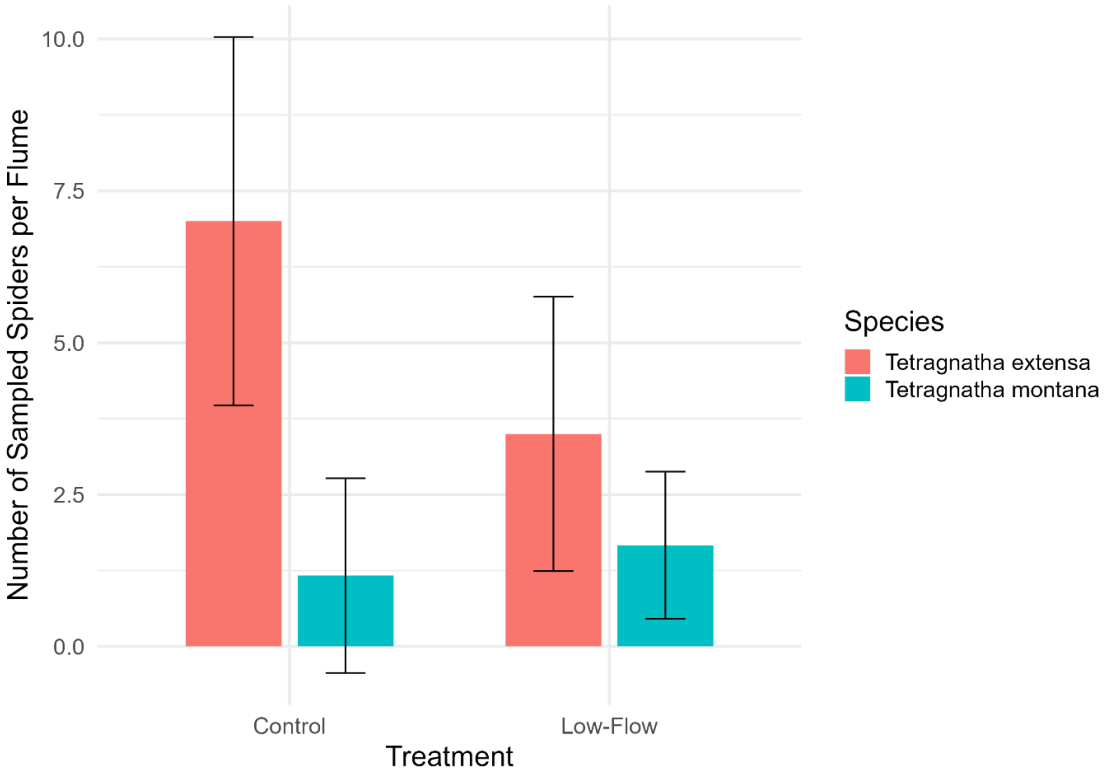

Figure S1 Average number of spiders sampled per flume for each spider species across treatments. Error bars represent ±1 standard deviation from the mean.

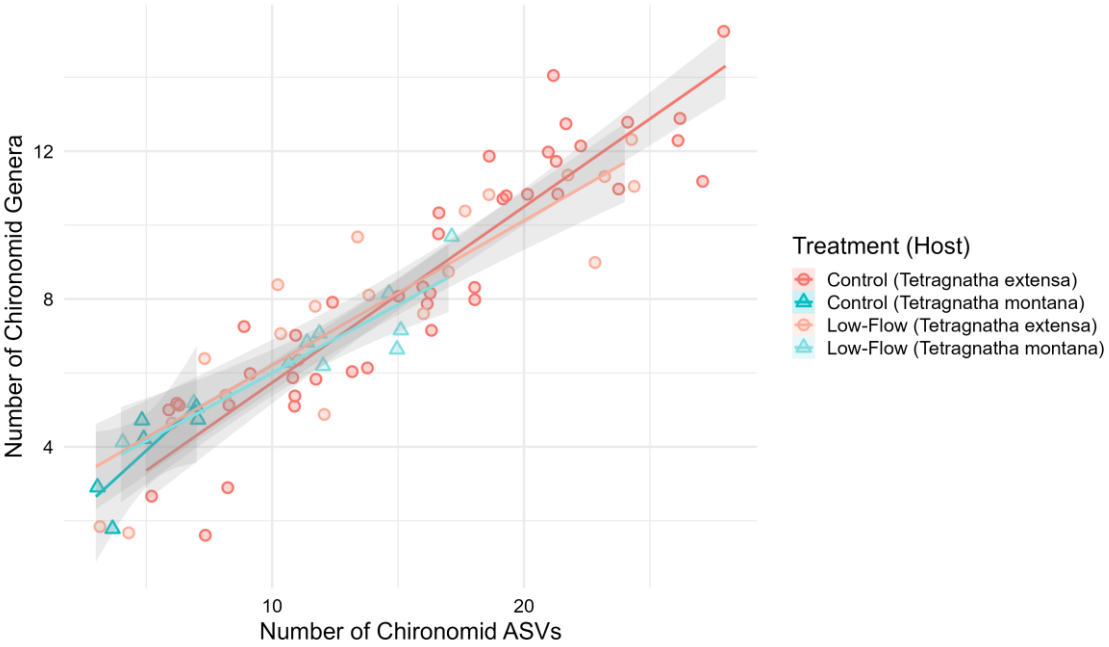

Figure S2 Relationship between the number of chironomid prey ASVs detected per spider and the number of chironomid genera identified per spider. Each point represents an individual spider, with shape indicating host species and fill color indicating experimental group (treatment × host combination). Borders and regression lines are colored according to group identity. Linear regression lines (with shaded 95% confidence intervals) illustrate trends within each group. *T. extensa*: n = 42 (control) and n = 21 (low-flow); *T. montana*: n = 6 (control), n = 10 (low-flow).

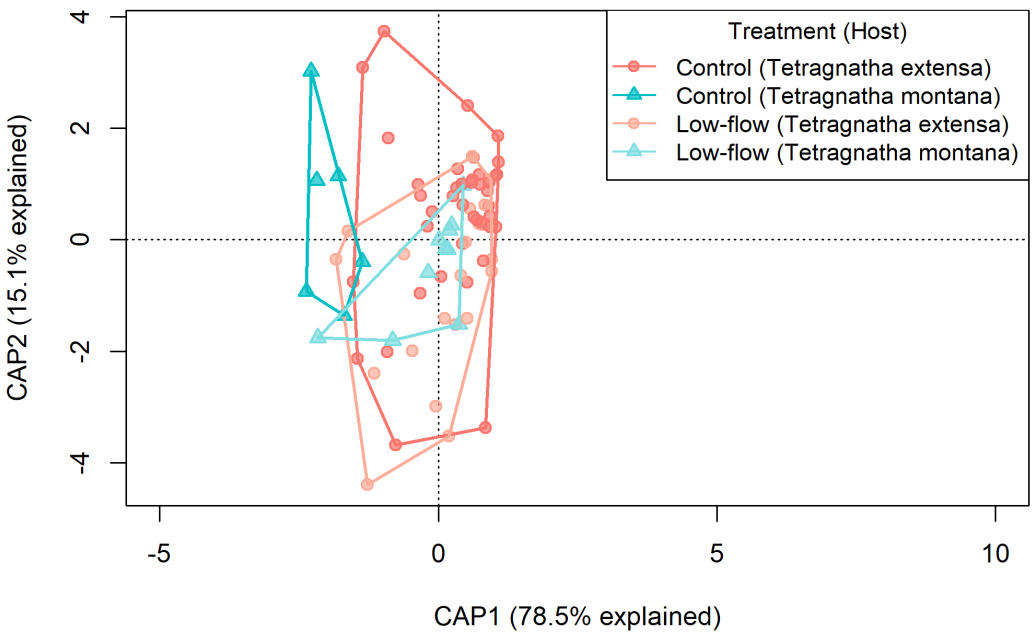

Figure S3 Constrained ordination (CAP) of chironomid prey composition in individual spider diets, based on Bray-Curtis dissimilarities of ASV abundances per genus. Points represent individual spiders, colored by treatment and shaped by species. Convex hulls outline group dispersion for each treatment × species combination. The constrained axes explained 10.1% of the total variation in prey composition. The first two canonical axes (CAP1 and CAP2) captured 93.6% of this constrained variation, with CAP1 alone accounting for 78.5%.

Supporting Information to

"Pollution-Driven Selection in Riparian Ecosystems: Genome-Wide Responses to *Bacillus thuringiensis israelensis* and Copper in a Non-biting Midge"

List of contents

Figure S1

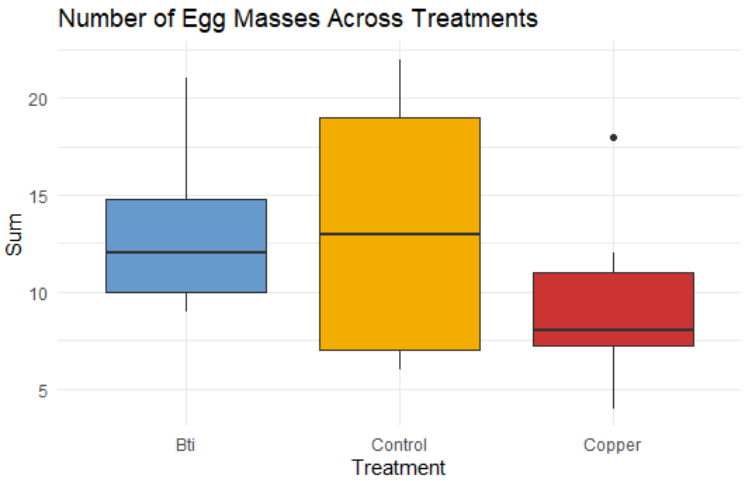

Figure S13 Number of egg masses sampled per treatment (n = 6) following 26 weeks of chronic exposure. Egg masses were collected over three consecutive days and stored in SAM-5S medium until hatching. Larvae hatching from these egg masses were used for population genomic analyses. Boxplots indicate the interquartile range (IQR), horizontal lines denote the median, whiskers extend to 1.5× IQR, and points beyond this range are shown as outliers.

Figure S2

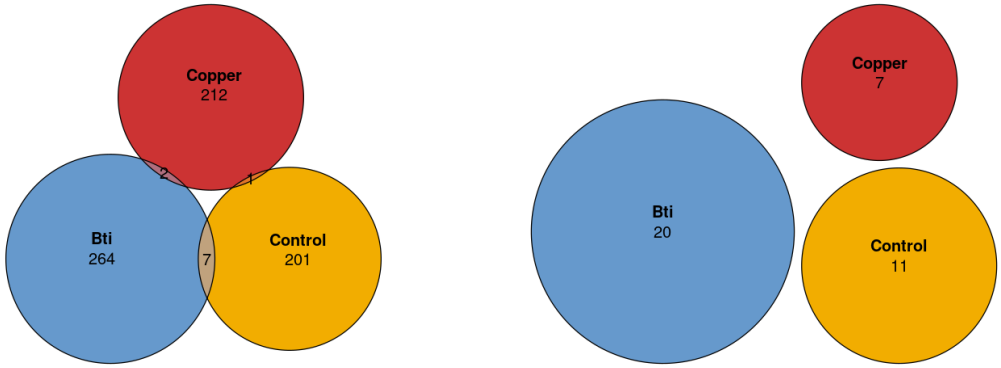

Figure S14 Venn diagrams showing the number of candidate SNPs under stricter significance thresholds: (left) ≥5 of 6 replicates, and (right) all 6 replicates per treatment. Stricter criteria greatly reduced both treatment-specific and shared SNPs.

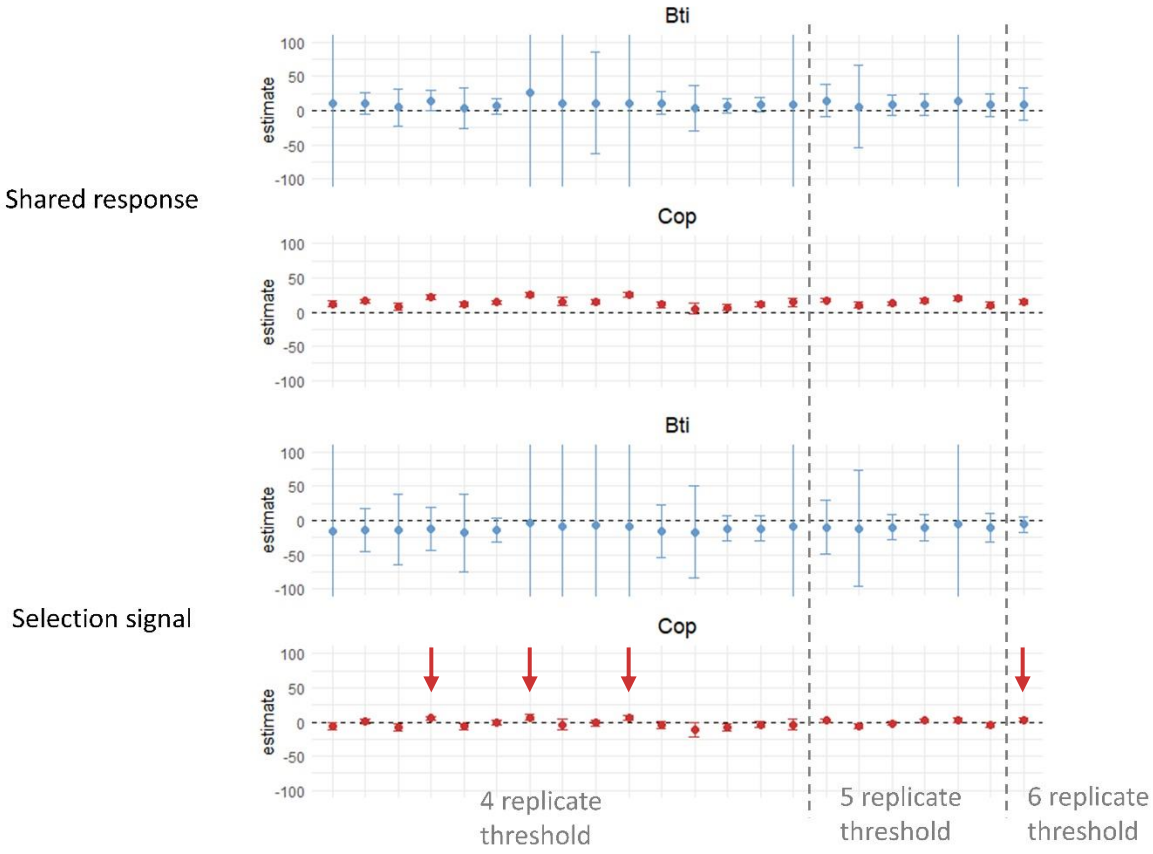

Figure S15 Partitioning of allele frequency change variance into shared and selection components across replicate combinations for Bti- and copper-treated populations. Upper panels show the percentage of total variance attributed to shared allele frequency change (i.e., parallel change), and lower panels show the variance attributed to selection after accounting for laboratory adaptation. Statistically significant positive selection estimates are highlighted with arrows. Each point represents a replicate combination (15 four-replicate, 6 five-replicate, and 1 six-replicate combination per treatment), with 95% confidence intervals indicated by error bars. Variance components are expressed as percentages of total variance in allele frequency change from F0 to F8. Error bars extending beyond the plotting range (±100%) are truncated visually.

Figure S4

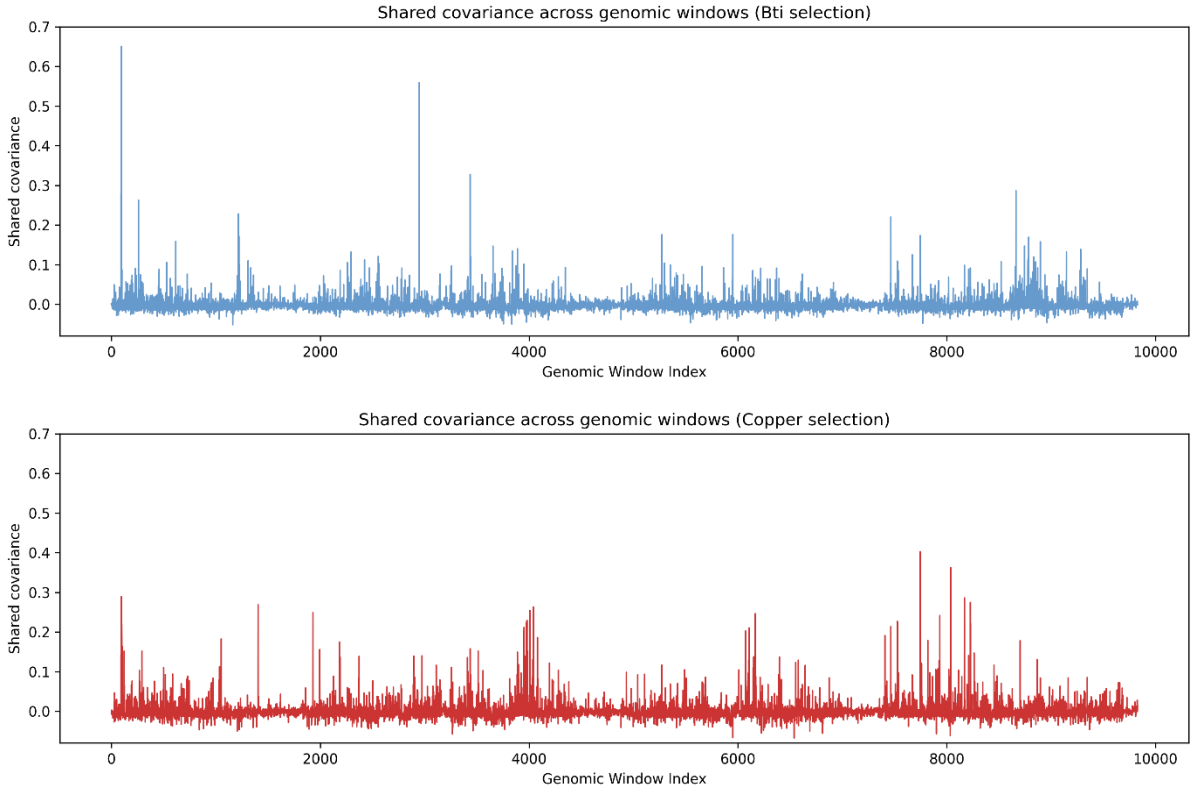

Figure S16 Genome-wide distribution of shared covariance across genomic windows in six-out-of-six Chironomus riparius *populations exposed to Bti (top) and copper (bottom). For each genomic window, the proportion of total genetic variance attributable to shared allele frequency changes across replicates is shown.*

Table S1

Table S2 Summary statistics of variance partitioning results for all tested replicate combinations across treatments. Each row reports the estimate and confidence interval (lower and upper error bounds) for one of four variance components—total variance in allele frequency change, the shared response, the portion attributable to laboratory adaptation, and the inferred contribution of experimental selection—for a specific replicate combination in either Bti-treated or copper-exposed populations. The combination number refers to a specific set of replicates, and the number of replicates used in each is indicated. The range column reflects the span between the upper and lower error bounds. See Supplementary Figure S2 for graphical visualization of the shared and selection variance components.

Sort	treatment	variable	combination number	lower_err	estimate	upper_err	range	based on .. replicates
1	Bti	total	1	0.0169	0.0178	0.0188	0.0019	4
2	Bti	shared	1	-618.215	10.951	640.118	1258.333	4
3	Bti	lab	1	-1147.781	26.757	1201.296	2349.077	4
4	Bti	selection	1	-1818.411	-15.806	1786.799	3605.21	4
5	Bti	total	2	0.0198	0.0209	0.022	0.0022	4
6	Bti	shared	2	-4.19	11.295	26.78	30.97	4
7	Bti	lab	2	4.008	24.414	44.82	40.812	4
8	Bti	selection	2	-44.733	-13.119	18.496	63.229	4
9	Bti	total	3	0.0196	0.0206	0.0216	0.002	4
10	Bti	shared	3	-21.682	4.925	31.531	53.213	4
11	Bti	lab	3	-10.192	18.075	46.341	56.533	4
12	Bti	selection	3	-64.265	-13.15	37.965	102.23	4
13	Bti	total	4	0.0196	0.0207	0.0217	0.0021	4

14	Bti	shared	4	0.519	15.037	29.555	29.036	4
15	Bti	lab	4	4.995	27.112	49.229	44.234	4
16	Bti	selection	4	-44.032	-12.074	19.883	63.915	4
17	Bti	total	5	0.0194	0.0204	0.0214	0.002	4
18	Bti	shared	5	-26.713	3.348	33.409	60.122	4
19	Bti	lab	5	-9.36	20.738	50.836	60.196	4
20	Bti	selection	5	-73.975	-17.39	39.195	113.17	4
21	Bti	total	6	0.0224	0.0235	0.0246	0.0022	4
22	Bti	shared	6	-5.543	6.489	18.521	24.064	4
23	Bti	lab	6	9.459	19.443	29.426	19.967	4
24	Bti	selection	6	-30.425	-12.953	4.518	34.943	4
25	Bti	total	7	0.014	0.015	0.0159	0.0019	4
26	Bti	shared	7	-6455.978	26.603	6509.184	12965.162	4
27	Bti	lab	7	-206971.779	29.687	207031.152	414002.931	4
28	Bti	selection	7	-203467.4	-3.084	203461.232	406928.632	4
29	Bti	total	8	0.0138	0.0147	0.0155	0.0017	4
30	Bti	shared	8	-4093.184	10.619	4114.422	8207.606	4
31	Bti	lab	8	-12646.692	18.289	12683.27	25329.962	4
32	Bti	selection	8	-16774.891	-7.671	16759.55	33534.441	4
33	Bti	total	9	0.0168	0.0178	0.0187	0.0019	4
34	Bti	shared	9	-63.186	11.572	86.33	149.516	4
35	Bti	lab	9	-163.396	18.1	199.597	362.993	4
36	Bti	selection	9	-258.171	-6.528	245.115	503.286	4
37	Bti	total	10	0.0166	0.0175	0.0185	0.0019	4
38	Bti	shared	10	-150.599	10.841	172.281	322.88	4
39	Bti	lab	10	-283.03	19.377	321.785	604.815	4
40	Bti	selection	10	-469.503	-8.537	452.43	921.933	4
41	Bti	total	11	0.019	0.02	0.021	0.002	4
42	Bti	shared	11	-4.597	11.452	27.5	32.097	4
43	Bti	lab	11	0.442	26.251	52.059	51.617	4
44	Bti	selection	11	-53.086	-14.799	23.488	76.574	4
45	Bti	total	12	0.0187	0.0197	0.0207	0.002	4
46	Bti	shared	12	-29.799	3.26	36.32	66.119	4
47	Bti	lab	12	-18.091	19.644	57.379	75.47	4
48	Bti	selection	12	-84.125	-16.384	51.357	135.482	4
49	Bti	total	13	0.0217	0.0228	0.0239	0.0022	4
50	Bti	shared	13	-3.959	7.181	18.321	22.28	4
51	Bti	lab	13	7.722	18.458	29.194	21.472	4
52	Bti	selection	13	-29.486	-11.277	6.933	36.419	4
53	Bti	total	14	0.0215	0.0226	0.0236	0.0021	4
54	Bti	shared	14	-0.998	9.581	20.16	21.158	4
55	Bti	lab	14	9.454	20.868	32.283	22.829	4
56	Bti	selection	14	-29.321	-11.288	6.745	36.066	4
57	Bti	total	15	0.0159	0.0169	0.0178	0.0019	4
58	Bti	shared	15	-502.029	9.807	521.643	1023.672	4

59	Bti	lab	15	-1207.532	18.043	1243.618	2451.15	4
60	Bti	selection	15	-1741.909	-8.236	1725.438	3467.347	4
61	Cop	total	1	0.0275	0.0288	0.0301	0.0026	4
62	Cop	shared	1	7.306	11.841	16.377	9.071	4
63	Cop	lab	1	13.779	17.589	21.398	7.619	4
64	Cop	selection	1	-10.937	-5.747	-0.557	10.38	4
65	Cop	total	2	0.032	0.0333	0.0347	0.0027	4
66	Cop	shared	2	12.548	15.866	19.184	6.636	4
67	Cop	lab	2	12.577	15.375	18.173	5.596	4
68	Cop	selection	2	-2.73	0.492	3.713	6.443	4
69	Cop	total	3	0.0261	0.0273	0.0285	0.0024	4
70	Cop	shared	3	2.629	7.509	12.388	9.759	4
71	Cop	lab	3	12.339	16.06	19.781	7.442	4
72	Cop	selection	3	-13.67	-8.551	-3.432	10.238	4
73	Cop	total	4	0.0339	0.0353	0.0367	0.0028	4
74	Cop	shared	4	18.714	21.813	24.913	6.199	4
75	Cop	lab	4	14.077	16.484	18.892	4.815	4
76	Cop	selection	4	2.214	5.329	8.444	6.23	4
77	Cop	total	5	0.0281	0.0293	0.0305	0.0024	4
78	Cop	shared	5	7.036	11.032	15.028	7.992	4
79	Cop	lab	5	14.15	17.245	20.34	6.19	4
80	Cop	selection	5	-10.655	-6.213	-1.772	8.883	4
81	Cop	total	6	0.0325	0.0338	0.0352	0.0027	4
82	Cop	shared	6	11.077	14.155	17.232	6.155	4
83	Cop	lab	6	12.678	14.953	17.228	4.55	4
84	Cop	selection	6	-3.749	-0.798	2.153	5.902	4
85	Cop	total	7	0.0299	0.0312	0.0325	0.0026	4
86	Cop	shared	7	21.421	25.238	29.055	7.634	4
87	Cop	lab	7	15.456	18.931	22.405	6.949	4
88	Cop	selection	7	1.969	6.307	10.645	8.676	4
89	Cop	total	8	0.0241	0.0252	0.0264	0.0023	4
90	Cop	shared	8	10.192	15.658	21.123	10.931	4
91	Cop	lab	8	14.168	19.357	24.546	10.378	4
92	Cop	selection	8	-11.233	-3.699	3.835	15.068	4
93	Cop	total	9	0.0285	0.0297	0.031	0.0025	4
94	Cop	shared	9	11.639	15.39	19.142	7.503	4
95	Cop	lab	9	13.417	16.608	19.799	6.382	4
96	Cop	selection	9	-5.41	-1.217	2.975	8.385	4
97	Cop	total	10	0.0304	0.0317	0.033	0.0026	4
98	Cop	shared	10	21.447	24.86	28.274	6.827	4
99	Cop	lab	10	15.332	18.15	20.968	5.636	4
100	Cop	selection	10	3.133	6.711	10.288	7.155	4
101	Cop	total	11	0.0281	0.0293	0.0306	0.0025	4
102	Cop	shared	11	6.433	10.954	15.475	9.042	4
103	Cop	lab	11	12.436	16.116	19.796	7.36	4

104	Cop	selection	11	-10.335	-5.162	0.011	10.346	4
105	Cop	total	12	0.0223	0.0234	0.0244	0.0021	4
106	Cop	shared	12	-3.099	4.864	12.827	15.926	4
107	Cop	lab	12	9.757	15.854	21.95	12.193	4
108	Cop	selection	12	-21.689	-10.99	-0.291	21.398	4
109	Cop	total	13	0.0267	0.0279	0.0291	0.0024	4
110	Cop	shared	13	2.21	6.801	11.392	9.182	4
111	Cop	lab	13	10.492	13.943	17.395	6.903	4
112	Cop	selection	13	-12.264	-7.142	-2.021	10.243	4
113	Cop	total	14	0.0286	0.0299	0.0311	0.0025	4
114	Cop	shared	14	7.247	11.285	15.323	8.076	4
115	Cop	lab	14	12.608	15.539	18.471	5.863	4
116	Cop	selection	14	-8.636	-4.254	0.128	8.764	4
117	Cop	total	15	0.0246	0.0258	0.0269	0.0023	4
118	Cop	shared	15	8.409	14.136	19.862	11.453	4
119	Cop	lab	15	13.106	18.133	23.16	10.054	4
120	Cop	selection	15	-11.576	-3.997	3.582	15.158	4
121	Bti	total	1	0.0179	0.0189	0.0198	0.0019	5
122	Bti	shared	1	-8.773	14.51	37.794	46.567	5
123	Bti	lab	1	3.593	24.236	44.879	41.286	5
124	Bti	selection	1	-48.684	-9.726	29.233	77.917	5
125	Bti	total	2	0.0177	0.0186	0.0196	0.0019	5
126	Bti	shared	2	-53.939	6.276	66.491	120.43	5
127	Bti	lab	2	-11.454	17.763	46.98	58.434	5
128	Bti	selection	2	-95.967	-11.487	72.993	168.96	5
129	Bti	total	3	0.0201	0.0211	0.0221	0.002	5
130	Bti	shared	3	-7.507	8.14	23.786	31.293	5
131	Bti	lab	3	9.2	17.686	26.171	16.971	5
132	Bti	selection	3	-28.138	-9.546	9.047	37.185	5
133	Bti	total	4	0.02	0.0209	0.0219	0.0019	5
134	Bti	shared	4	-7.027	8.961	24.95	31.977	5
135	Bti	lab	4	10.241	18.768	27.295	17.054	5
136	Bti	selection	4	-28.699	-9.807	9.084	37.783	5
137	Bti	total	5	0.0155	0.0164	0.0172	0.0017	5
138	Bti	shared	5	-15437.727	13.629	15464.984	30902.711	5
139	Bti	lab	5	-17149.623	19.281	17188.185	34337.808	5
140	Bti	selection	5	-32608.194	-5.652	32596.889	65205.083	5
141	Bti	total	6	0.0194	0.0204	0.0214	0.002	5
142	Bti	shared	6	-8.315	8.226	24.767	33.082	5
143	Bti	lab	6	8.889	17.913	26.937	18.048	5
144	Bti	selection	6	-30.387	-9.687	11.012	41.399	5
145	Cop	total	1	0.0303	0.0316	0.0329	0.0026	5
146	Cop	shared	1	14.18	17.401	20.621	6.441	5
147	Cop	lab	1	12.669	15.178	17.688	5.019	5
148	Cop	selection	1	-0.637	2.222	5.081	5.718	5

149	Cop	total	2	0.0257	0.0268	0.028	0.0023	5
150	Cop	shared	2	5.851	10.283	14.715	8.864	5
151	Cop	lab	2	12.692	15.836	18.98	6.288	5
152	Cop	selection	2	-9.348	-5.553	-1.758	7.59	5
153	Cop	total	3	0.0292	0.0304	0.0316	0.0024	5
154	Cop	shared	3	9.01	12.229	15.448	6.438	5
155	Cop	lab	3	11.647	14.136	16.625	4.978	5
156	Cop	selection	3	-4.534	-1.907	0.721	5.255	5
157	Cop	total	4	0.0308	0.032	0.0332	0.0024	5
158	Cop	shared	4	13.873	16.859	19.845	5.972	5
159	Cop	lab	4	12.608	14.72	16.832	4.224	5
160	Cop	selection	4	-0.393	2.139	4.671	5.064	5
161	Cop	total	5	0.0276	0.0287	0.0299	0.0023	5
162	Cop	shared	5	15.823	19.443	23.063	7.24	5
163	Cop	lab	5	13.728	16.48	19.232	5.504	5
164	Cop	selection	5	-0.383	2.963	6.309	6.692	5
165	Cop	total	6	0.0261	0.0272	0.0284	0.0023	5
166	Cop	shared	6	5.388	9.734	14.08	8.692	5
167	Cop	lab	6	11.53	14.594	17.658	6.128	5
168	Cop	selection	6	-8.62	-4.86	-1.1	7.52	5
169	Bti	total	1	0.0185	0.0194	0.0203	0.0018	6
170	Bti	shared	1	-14.464	9.809	34.082	48.546	6
171	Bti	lab	1	-1.771	15.415	32.6	34.371	6
172	Bti	selection	1	-16.954	-5.606	5.743	22.697	6
173	Cop	total	1	0.0283	0.0295	0.0306	0.0023	6
174	Cop	shared	1	11.283	14.484	17.686	6.403	6
175	Cop	lab	1	8.186	11.153	14.119	5.933	6
176	Cop	selection	1	0.835	3.332	5.829	4.994	6

Table S2

Table S3 Results of Gene Ontology (GO) enrichment analysis for loci showing significant allele frequency changes after chronic exposure across treatments. The table lists the 45 enriched biological process GO terms identified in Bti-treated, copper-exposed, and control populations, including those shared across treatments. Terms were detected across replicate combinations showing statistically significant shared responses, with the corresponding number of replicates indicated.

Sort	GO ID	GO term	treatment	based on … replicates
1	GO:0016358	dendrite development	all	6
2	GO:0031114	regulation of microtubule depolymerization	all	5
3	GO:0007097	nuclear migration	all	4
4	GO:0006196	AMP catabolic process	all	4
5	GO:0042981	regulation of apoptotic process	Bti	6
6	GO:0006952	defense response	Bti	5
7	GO:0000122	negative regulation of transcription by RNA polymerase II	Bti	5
8	GO:0045893	positive regulation of DNA-templated transcription	Bti	4
9	GO:0006338	chromatin remodeling	Bti	4
10	GO:0006741	NADP biosynthetic process	Bti	4

11	GO:0019674	NAD metabolic process	Bti	4
12	GO:0006997	nucleus organization	Bti	4
13	GO:0006357	regulation of transcription by RNA polymerase II	Bti	4
14	GO:0033499	galactose catabolic process via UDP-galactose	Bti	4
15	GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	Bti	4
16	GO:0007602	phototransduction	Copper	5
17	GO:0046513	ceramide biosynthetic process	Copper	5
18	GO:0006606	protein import into nucleus	Copper	5
19	GO:0043171	peptide catabolic process	Copper	5
20	GO:0000012	single strand break repair	Copper	4
21	GO:0006535	cysteine biosynthetic process from serine	Copper	4
22	GO:1902275	regulation of chromatin organization	Copper	4
23	GO:0008543	fibroblast growth factor receptor signaling pathway	Copper	4
24	GO:0007190	activation of adenylate cyclase activity	Copper	4
25	GO:0019343	cysteine biosynthetic process via cystathionine	Copper	4
26	GO:0006303	double-strand break repair via nonhomologous end joining	Copper	4
27	GO:0061512	protein localization to cilium	Copper	4
28	GO:0051295	establishment of meiotic spindle localization	Control	5
29	GO:0061077	chaperone-mediated protein folding	Control	5
30	GO:0006487	protein N-linked glycosylation	Control	5
31	GO:0007051	spindle organization	Control	5
32	GO:0044878	mitotic cytokinesis checkpoint signaling	Control	4
33	GO:0046901	tetrahydrofolylpolyglutamate biosynthetic process	Control	4
34	GO:0009838	abscission	Control	4
35	GO:0032979	protein insertion into mitochondrial inner membrane from matrix	Control	4
36	GO:0070085	glycosylation	Control	4
37	GO:0006325	chromatin organization	Control	4
38	GO:0007156	homophilic cell adhesion via plasma membrane adhesion molecules	Control-Bti	5
39	GO:0045104	intermediate filament cytoskeleton organization	Control-Bti	4
40	GO:0042060	wound healing	Control-Bti	4
41	GO:0031122	cytoplasmic microtubule organization	Control-Bti	4
42	GO:0000492	box C/D snoRNP assembly	Control-Bti	4
43	GO:0034244	negative regulation of transcription elongation by RNA polymerase II	Control-Copper	5
44	GO:0006685	sphingomyelin catabolic process	Control-Copper	5
45	GO:0019919	peptidyl-arginine methylation, to asymmetrical-dimethyl arginine	Control-Copper	4