Science of the Total Environment

Pharmaceuticals and endocrine disrupting compounds modulate adverse effects of climate change on resource quality in freshwater food webs

--Manuscript Draft--

Manuscript Number:	STOTEN-D-23-17284R3
Article Type:	Research Paper
Keywords:	climate change; Pollution; caddisflies; sex specific stress response; aquatic-terrestrial subsidies; ecosystem subsidies
Corresponding Author:	Ana Previsic, Ph.D. University of Zagreb Faculty of Science Zagreb, CROATIA
First Author:	Iva Kokotovic
Order of Authors:	Iva Kokotovic
	Marina Veseli
	Filip Lozek
	Zrinka Karacic
	Marko Rozman
	Ana Previsic, Ph.D.
Abstract:	Freshwater biodiversity, ecosystem functions and services are changing at an unprecedented rate due to the impacts of vast number of stressors overlapping in time and space. Our study aimed at characterizing individual and combined impacts of pollution with pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) and increased water temperature (as a proxy for climate change) on primary producers and first level consumers in freshwaters. We conducted a microcosm experiment with a simplified freshwater food web containing moss (Bryophyta) and shredding caddisfly larvae of Micropterna nycterobia (Trichoptera). The experiment was conducted with four treatments; control (C), increased water temperature +4 °C (T2), emerging contaminants' mix (EC = 15 PhACs & 5 EDCs), and multiple stressor treatment (MS = EC + T2). Moss exhibited an overall mild response to selected stressors and their combination. Higher water temperature negatively affected development of M. nycterobia through causing earlier emergence of adults and changes in their lipidome profiles. Pollution with PhACs and EDCs had higher impact on metabolism of all life stages of M. nycterobia than warming. Multiple stressor effect was recorded in M. nycterobia adults in metabolic response, lipidome profiles and as a decrease in total lipid content. Sex specific response to stressor effects was observed in adults, with impacts on metabolome generally more pronounced in females, and on lipidome in males. Thus, our study highlights the variability of both single and multiple stressor impacts on different traits, different life stages and sexes of a single insect species. Furthermore, our research suggests that the combined impacts of warming, linked to climate change, and contamination with PhACs and EDCs could have adverse consequences on the population dynamics of aquatic insects. Additionally, these findings point to a potential decrease in the quality of resources available for both aquatic and potentially terrestrial food webs.
Response to Reviewers:	

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Revision
MS ref. STOTEN-D-23-17284

Title: Pharmaceuticals and endocrine disrupting compounds modulate adverse effects of climate change on resource quality in freshwater food webs

Authors: Iva Kokotović, Marina Veseli, Filip Ložek, Zrinka Karačić, Marko Rožman, Ana Previšić
Science of the Total Environment

Dear Prof. Sabater,

Thank you for the constructive comments that help to improve the manuscript. I would also like to thank the reviewer for time and effort invested in the second round, we really appreciate the constructive comments. We have integrated all the comments and suggestions and corrected the manuscript accordingly. More specifically, we have reanalysed parts of the data as suggested and subsequently rewritten parts of the manuscript.

We appreciate yours as well as the reviewer's feedback, which prompted us to revise and clarify our sampling strategy description. Hence, we have included a paragraph explaining the background of our sampling approach into the Methods section.

As instructed, we have added detailed clarifications to all comments, thus, please find attached the file with a point-by-point response on the queries provided to our submission.

Thank you for giving us the opportunity to revise our work and I am looking forward to receiving your reply.

Kind regards,

Ana Previšić on behalf of the authors

Response letter

MS ref. STOTEN-D-23-17284R1

Title: Pharmaceuticals and endocrine disrupting compounds modulate adverse effects of climate change on resource quality in freshwater food webs

Authors: Iva Kokotović, Marina Veseli, Filip Ložek, Zrinka Karačić, Marko Rožman, Ana Previšić

Following the suggestions of the reviewer, we have corrected the manuscript to improve the quality. All changes we made are listed and explained in detail following the reviewers' comments below (**reply in green colour**). Page and line numbers correspond to the revised (clean) manuscript, hence they may differ in the file with track changes (due to changes in the document).

Reviewer #1:

General comments:

The authors have made substantial modifications, according to the reviewers' comments, which greatly improve the manuscript's quality.

However, I have a significant concern regarding the differentiation between technical and biological replicates in the methodology employed in this study. To detect the effects of the treatments on the caddisfly population accurately, biological replicates are essential. While I understand that in some cases, pooling individuals may be necessary to obtain sufficient tissue for analysis, it is crucial to emphasize that biological replicates are vital for extrapolating observed effects on the samples to the entire population. Technical replicates, on the other hand, primarily assess the reliability of the analytical method. Pooling individuals to reduce variability, as mentioned in the paper, is an unconventional approach. Individual variation within biological replicates is essential for making meaningful extrapolations to the broader population. Unless the authors can provide robust justification for this method, with references to established practices or prior studies, it is challenging to validate such an approach.

Upon investigation, I found that in Previšić, et al. 2020, the authors employed 'at least two replicate samples' per treatment, which aligns with common biological replicate practices. However, in Previšić, et al. 2021, a departure from this approach is evident as samples were pooled also to reduce variability. This discrepancy raises concerns about the consistency and reliability of the methodology employed in this study.

To maintain the rigor and validity of the research, it is advisable to reconsider the approach to replicates and justify its use, especiall when it deviates from widely accepted scientific practices. For further clarification on this topic, I recommend reviewing the following sources:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3321166/

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4825082/#:~:text=Broadly%20speaking%2C%20biological%20replicates%20are,the%20measuring%20equipment%20and%20protocols.

https://onlinelibrary.wiley.com/doi/pdf/10.1002/ejlt.201200260

Reply: We are grateful to the reviewer for highlighting areas in need of improvement. The initial description of our sampling strategy was acknowledged as inappropriate, and we have rephrased it in response to the reviewer's valuable feedback. The phrase "pooling biological replicates" has been reworked, recognizing its intuitive illogicality. We aimed to avoid overemphasizing potential extreme effects of individual microcosms. Thus, we have added a paragraph explaining the background of our approach (P7-8; L195-207) and two relevant references (Kraufvelin, 1998 and Sanderson 2002).

In the context of working with micro and mesocosms, the conventional use of biological replicates for generalization, widely accepted in ecology, faces challenges. As pointed out by (Kraufvelin, 1998), the high variability in these

systems often makes it difficult or impossible to find statistically significant deviations from the controls, even with rigorous replication and large effect sizes. A review on replicability in pesticide studies found that 88% of test biological variables showed no statistical significance, despite a theoretically adequate number of replicates (Sanderson, 2002). Similar trends were observed across different systems and taxa groups (e.g. Knauer et al., 2005; Pandey et al., 2017), leading to the recognition of these challenges in guidance on aquatic ecotoxicology assessment (European Food Safety Authority, 2013). Moreover, the increasing complexity and duration of such test systems reveal problems like "aquarium individuality," where initially identical replicates diverge from one another due to random factors or chance events, even when no experimental errors are made (Kraufvelin, 1998).

In our current experiment, as well as in the experiment presented in Previšić et al. 2021 (ES&T), we incorporated simplified food webs, exposing parts of natural systems to treatments over 2 months and spanning different life stages. Despite efforts to achieve uniformity among individual microcosms, random effects leading to differences in the final stages cannot be fully excluded. However, we ensured replication by employing three replicates for each treatment, although we opted to pool tissue samples to address potential extreme effects. Additionally, the weight reduction of adult aquatic insects during metamorphosis posed a challenge, resulting in high tissue demand. The applied approach aims to strike a balance between providing ecologically meaningful results with non-model organisms and minimizing methodological constraints.

European Food Safety Authority, 2013. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA J. 11. https://doi.org/10.2903/j.efsa.2013.3290

Knauer, K., Maise, S., Thoma, G., Hommen, U., Gonzalez-Valero, J., 2005. Long-term variability of zooplankton populations in aquatic mesocosms. Environ. Toxicol. Chem. 24, 1182–1189. https://doi.org/10.1897/04-010R.1

Kraufvelin, P., 1998. Model ecosystem replicability challenged by the “soft” reality of a hard bottom mesocosm. J. Exp. Mar. Bio. Ecol. 222, 247–267. https://doi.org/10.1016/S0022-0981(97)00143-3

Pandey, L.K., Bergey, E.A., Lyu, J., Park, J., Choi, S., Lee, H., Depuydt, S., Oh, Y.T., Lee, S.M., Han, T., 2017. The use of diatoms in ecotoxicology and bioassessment: Insights, advances and challenges. Water Res. 118, 39–58. https://doi.org/10.1016/j.watres.2017.01.062

Sanderson, H., 2002. Pesticide studies: Replicability of micro/mesocosms. Environ. Sci. Pollut. Res. 9, 429–435. https://doi.org/10.1007/BF02987597

Other comments:

In reference to my previous comments on the use of PCA and PRC, I would like to clarify that I did not object to the application of PRC to your data. Instead, my concern was the absence of the y-axis in the PRC representation.

Reply: We thank the reviewer for these remarks. As we did not focus on a few particular metabolites/lipids the y axis would be filled with a lot of values representing m/z values as there are hundreds of metabolites/lipids extracted from the data and that would not give any additional information to the reader (as an example on appearance we added a raw figure with the y axis; Fig.1). Moreover, we were only able to identify some metabolites/lipids and those were putatively annotated. However, putative annotation does not provide definitive identification of a metabolite/lipid, and further verification is needed to confirm its identity using methods such as targeted metabolomics/lipidomics which is beyond the scope of our study. Thus, we have chosen to omit the y axis when presenting the current results.

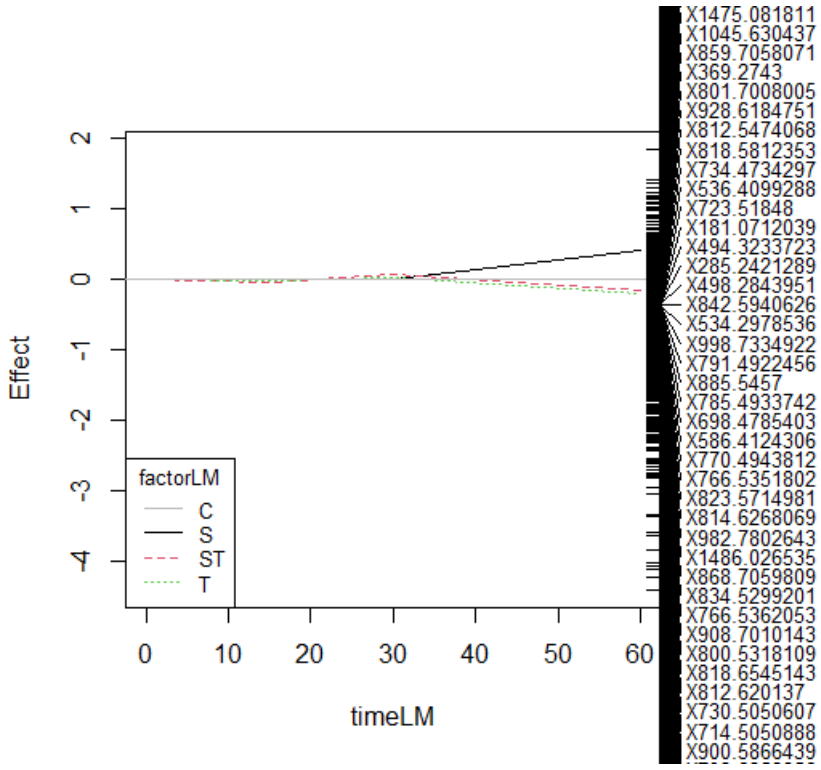


Fig.1. Lipidome profiles of the caddisfly *Micropterna nycterobia;* PRC of changes in the lipidome profiles of caddisfly life stages and female adults exposed to treatments in relation to control (please note that the figure is generated in R without any editing so color of lines and treatment abbreviations differ from those in the manuscript).

Additionally, I suggested to consider running RDA as an alternative to PCA for two reasons: 1) PCA is primarily a visual tool and not a formal statistical analysis; 2) given the notable time effect in your data, RDA would enable you to include time as a covariate, enhancing the analysis.

We agree with the reviewer that PCA is primarily a visualization tool and we used it primarily as a method of visualisation, moreover, formal statistical analysis was provided by the PRC (tables with statistics provided in supporting information). However, due to the missing data for metabolome and lipidome of moss at day 30 (treatment with ECs, as explained in the section 2.4.2. at P12) we did not run the PRC for that dataset. Therefore, in order to compensate for this oversight and include a formal statistical analysis for this dataset, we added the RDA analysis for moss metabolome and lipidome changes. Accordingly, parts of the text in materials and methods (P12 paragraph 2.4.2. Lines 295-299) as well as results (P13 Lines 328-331) and discussion (section 4.1. P21-22) were rewritten. In addition, new figures were created and integrated into the supporting information (Fig. S2).

However, as we wanted to emphasize the effect of time i.e. plant development and growth rather than the effects of treatment in moss, as the latter was not evident in HSP response or total lipid content changes, we decided to keep the previous PCA figures in the paper.

Introduction:

Line 112: a full stop is missing.
Line 117: shouldn't you say global warming then?
Line 127: aquatic organisms (plural)

Reply: Corrected as suggested.

Line 136: How do you want to address that? Or do you mean assess instead of address?

Reply: The sentence was rewritten as suggested (P4-5, Lines 115-118).

In your hypothesis, it is not explicitly stated that you also intend to test the response across the different stages. This omission was the basis of my earlier comment that comparing data between different dates and life stages seems incongruent with the study's objectives. It would be beneficial to explicitly include this in your hypothesis.

Reply: We thank the reviewer for pointing this out. We have added the information that changes in non-model organisms were evaluated across their live cycles into the hypothesis section (P6, Lines 146-149).

Materials and methods:

Please provide clarification in the description of the Generalized Linear Models (GzLM) regarding which data you analyzed using a normal distribution and which ones you analyzed using a gamma distribution.

Reply: Added as suggested, P11, Lines 277-279: “Normal distribution linear scale response was used for all data except for body weight of adults where gamma scale response with log link was used as the data did not achieve normal distribution.”

Discussion:

The discussion is clear and very well-written.

Pharmaceuticals and endocrine disrupting compounds modulate adverse effects of climate change on resource quality in freshwater food webs

Iva, Kokotović[1]; Marina, Veseli[1]; Filip, Ložek[1,2]; Zrinka, Karačić[3]; Marko, Rožman[3]; Ana, Previšić[1]

[1] Department of Biology, Zoology, Faculty of Science, University of Zagreb, Zagreb, Croatia

[2] South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic

[3] Ruđer Bošković Institute, Zagreb, Croatia

Iva Kokotović: iva.kokotovic@biol.pmf.hr

Marina Veseli: marina.veseli@biol.pmf.hr

Filip Ložek: lozekf@frov.jcu.cz

Zrinka Karačić: zrinka.karacic@irb.hr

Marko Rožman: marko.rozman@irb.hr

Ana Previšić: ana.previsic@biol.pmf.hr

*Corresponding author:

Ana Previšić

Department of Biology, Zoology, Faculty of Science, University of Zagreb

Horvatovac 102a, 10000 Zagreb, Croatia

ana.previsic@biol.pmf.hr

These two authors contributed equally: Ana Previšić and Marko Rožman

Abstract

Freshwater biodiversity, ecosystem functions and services are changing at an unprecedented rate due to the impacts of vast number of stressors overlapping in time and space. Our study aimed at characterizing individual and combined impacts of pollution with pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) and increased water temperature (as a proxy for climate change) on primary producers and first level consumers in freshwaters. We conducted a microcosm experiment with a simplified freshwater food web containing moss (Bryophyta) and shredding caddisfly larvae of *Micropterna nycterobia* (Trichoptera). The experiment was conducted with four treatments; control (C), increased water temperature +4 °C (T2), emerging contaminants' mix (EC = 15 PhACs & 5 EDCs), and multiple stressor treatment (MS = EC + T2). Moss exhibited an overall mild response to selected stressors and their combination. Higher water temperature negatively affected development of *M. nycterobia* through causing earlier emergence of adults and changes in their lipidome profiles. Pollution with PhACs and EDCs had higher impact on metabolism of all life stages of *M. nycterobia* than warming. Multiple stressor effect was recorded in *M. nycterobia* adults in metabolic response, lipidome profiles and as a decrease in total lipid content. Sex specific response to stressor effects was observed in adults, with impacts on metabolome generally more pronounced in females, and on lipidome in males. Thus, our study highlights the variability of both single and multiple stressor impacts on different traits, different life stages and sexes of a single insect species. Furthermore, our research suggests that the combined impacts of warming, linked to climate change, and contamination with PhACs and EDCs could have adverse consequences on the population dynamics of aquatic insects. Additionally, these findings point to a potential decrease in the quality of resources available for both aquatic and potentially terrestrial food webs.

Keywords:

climate change, pollution, caddisflies, sex specific stress response, aquatic-terrestrial subsidies, ecosystem subsidies

Highlights:

- warming was a dominant stressor for development-related traits of the caddisfly
- negative effects of warming on lipids were increased by presence of PhACs&EDCs
- pollution with PhACs & EDCs has higher impact on caddisfly metabolism than warming
- trait-, sex- and life stage-specific responses to multiple stressors were observed
- impacts of warming and PhACs & EDCs cross the aquatic-terrestrial interface

1. INTRODUCTION

Freshwater ecosystems are susceptible to various anthropogenic stressors (e.g. chemical pollution, climate change, habitat loss, invasive species) which mostly coincide. Pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) are a diverse group of pollutants designed to be biologically active at low doses, targeting specific metabolic and molecular pathways in humans and animals, thus posing risk for aquatic organisms even at low environmental concentrations (Ebele et al., 2017; Tijani et al., 2013; Wilkinson et al., 2017). Moreover, EDCs encompass a wide range of chemicals (e.g. personal care products, cleaning products, food preservatives, etc.) that interfere with normal function(s) of the endocrine system (Ebele et al., 2017). PhACs and EDCs were shown to affect aquatic insects in many ways, causing changes in growth and development (Jarvis et al., 2014), biomass (López-Doval et al., 2012), enzymatic activity (Pestana et al., 2014), metabolome composition (Grgić et al., 2023; Previšić et al., 2020; Späth et al., 2022), behavior (Jarvis et al., 2014; Späth et al., 2022) and survival rate (López-Doval et al., 2012; Maenpaa and Kukkonen, 2006). Furthermore, PhACs and EDCs have the potential to bioaccumulate in aquatic insects (Grabicova et al., 2015; Lagesson et al., 2016; Previšić et al., 2021; Veseli et al., 2022) and to cross ecosystem boundaries through emerging aquatic insects and thus contaminate terrestrial habitats (Previšić et al., 2021; Veseli et al., 2022). Moreover, in freshwater ecosystems these compounds rarely occur one at a time but rather in complex mixtures of several different emerging contaminants, thus making it harder to investigate their effects (Wilkinson et al., 2017).

Climate change, with its long-term shifts in global weather patterns, poses significant challenges to freshwater ecosystems. Within freshwater ecosystems, aquatic insect groups, such as caddisflies, mayflies, and stoneflies, play a crucial role as inter-habitat linkages between aquatic and terrestrial ecosystems, facilitating the flow of energy and nutrients (Huryn and Wallace, 2000). Additionally, they serve as valuable bioindicators for assessing the health of freshwater environments (Water Framework Directive (WFD) 2000/60/EC). Their vulnerability to climate change is influenced by their specific biological traits and ecological preferences, with a considerable proportion of them belonging to cold-adapted taxa (Conti et al., 2014; Hershkovitz

et al., 2015). The impact of climate change, characterized by rising temperatures and changes in precipitation levels (Webb et al., 2008), is particularly profound on these cold-adapted taxa in higher altitudes, making them highly vulnerable to warming (Krajick, 2004; Macadam et al., 2022). As a consequence of climate change, temperature increase affects various aspects of aquatic insects' lives. It directly impacts their growth, development, and body size (Cogo et al., 2020), as well as their emergence patterns (Finn et al., 2022). The anticipated rise in the frequency and extent of intermittent rivers and streams, which periodically cease flow or even completely dry, is a direct consequence of global climate change and the increasing human demand for freshwater resources (Blackman et al., 2021). This trend holds significant implications for aquatic insect communities, as considerably altered environmental conditions not only impact their geographic distribution (Cogo et al., 2020 and references therein), but also their population dynamics (Nukazawa et al., 2018), and community structure (Dorić et al., 2023).

Global warming~~Climate change~~ is likely to exacerbate impact of other anthropogenic stressors (Wrona et al., 2006). For instance, as temperature rises, the solubility and mobility of PhACs and EDCs in water can increase, leading to higher concentrations and potentially greater toxicity (Kazmi et al., 2022; Noyes et al., 2009). Temperature change may also alter degradation rates of some chemical contaminants, with increasing temperature usually shortening their half-life and reducing the overall risk (Bhangare et al., 2022; Noyes et al., 2009). While accelerated decomposition reduces the concentration of the parent compound, it increases the concentration of the degradation products, which in some cases could be even more toxic for aquatic organisms (Noyes et al., 2009). Warmer water usually increases the metabolic rate of aquatic organisms, thus leading to potentially increased uptake of these chemicals (Kazmi et al., 2022). In addition to increasing uptake, warming can also affect behaviour and physiology of aquatic organisms making them more susceptible to stressors (Polazzo et al., 2022). Toxicity of chemicals in water can depend on variety of abiotic (e.g. photolysis, hydrolysis, etc.) and biotic processes (e.g. biotransformation, biodegradation, etc.) (von Schiller et al., 2017). Moreover, the impact of warming on the toxicity of PhACs and EDCs in water is complex and can depend on a variety of factors, including the type of the chemical (Serra-Compte et al., 2018), aquatic species (Duchet et al., 2023, preprint), and life-stage tested (DeCourten and Brander, 2017). Due to the

increase in temperature caused by climate change, the temperature-dependent toxicity of PhACs and EDCs in water will become a matter of growing significance that warrants assessment~~an increasingly important issue that needs to be addressed~~.

It is generally very challenging to gain empirical understanding of the effects of climate change in comparison with other stressors (Halsch et al., 2021), as well as their combined effects (Dinh et al., 2022). Consequently, there have been only a handful of studies investigating single and combined effects of increased water temperature and PhACs and EDCs in aquatic invertebrates (e.g. Barbosa et al., 2017; Cruzeiro et al., 2019; Heye et al., 2019). More specifically, carbamazepine and higher temperatures increased *Chironomus riparius* mortality (Heye et al., 2019); fluoxetine combined with higher temperature reduced reproductive success and population growth in *Daphnia magna* (Barbosa et al., 2017), whereas levonorgestrel and increased temperature did not cause DNA damage in *Gammarus locusta* cells (Cruzeiro et al., 2019). To address this knowledge gap, we conducted a study aimed at characterizing effects of increased water temperature and exposure to PhACs and EDCs on primary producers (non-vascular macrophytes; moss) and first level consumers (shredding aquatic insects) in freshwaters. Considering the importance of aquatic insects as fundamental links between aquatic and terrestrial food webs and reliable bioindicators to pollution (Erasmus et al., 2021; Muñoz et al., 2015) we chose caddisflies as our model organisms. Caddisflies are a species-rich and ecologically diverse insect order, that is well-suited to reflect effects of various stressors on aquatic ecosystems (Hering et al., 2009). Despite of the high ecological plasticity and thermal tolerance of intermittent rivers specialists (Stubbington et al., 2017) we hypothesized that the individual and combined stressor effects of both, increased water temperature and PhACs and EDCs, will trigger a stress response in an intermittent river caddisfly, *Micropterna nycterobia* (McLachlan, 1875). Chronic *in situ* exposure to pollution with ECs results in measurable changes of metabolite levels in relatively pollution-tolerant caddisfly larvae (Previšić et al., 2020), whereas increased temperature results in depletion of lipids and reduces developmental period of mayfly larvae (Chou et al., 2018). Moreover, variable impacts on emergence and survival were observed in dragonflies and an aquatic heteropteran exposed to increased water temperature, PhACs and their combination (Duchet et al., 2023, preprint). Hence, we conducted a 78-day microcosm

experiment composed of a simplified freshwater food web exposed to a mixture of PhACs and EDCs and increased water temperature as a proxy for climate change using the randomized factorial design. We analyzed the physiological changes in caddisflies and moss via non-targeted metabolomics and lipidomics, i.e. by evaluating alterations in the metabolite and lipid profiles of non-model organisms across their life cycles, as well as changes in emergence patterns, body weight and total lipid content.

2. MATERIALS AND METHODS

2.1 Microcosm Experiment: Experimental Design and Sample Collection

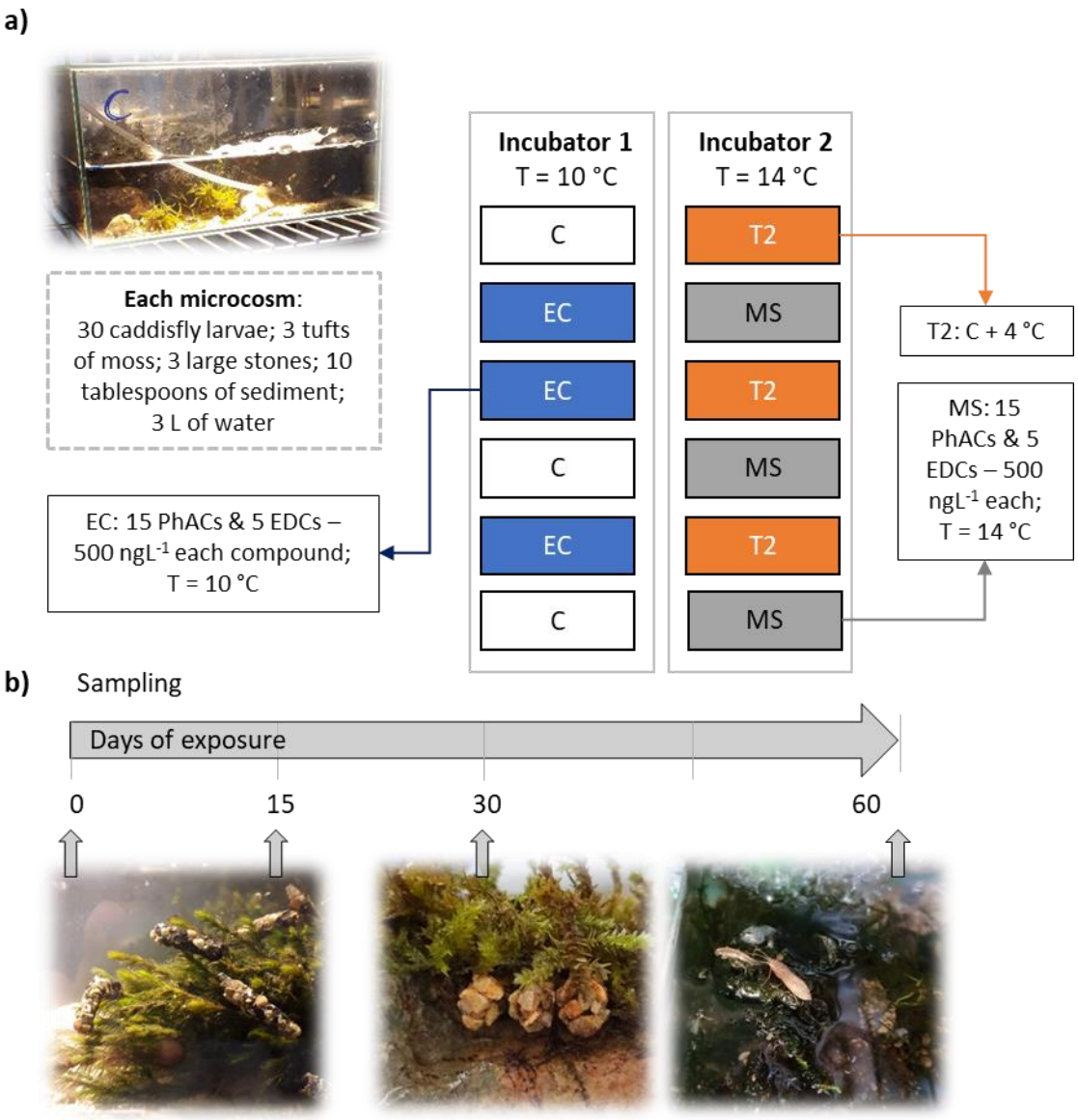
We conducted the microcosm experiment with a simplified freshwater food web containing nonvascular macrophytes (Bryophyta), hereafter moss and caddisfly larvae *Micropterna nycterobia* (McLachlan, 1875) (Limnephilidae, Trichoptera) feeding mainly as shredders. Trichoptera larvae, moss (*Cinclidotus aquaticus* (Hedw.) Bruch & Schimp and *Rhynchostegium riparioides* (Hedw.) Cardot), water, sand and stones collected from the pristine Krčić River (N44.027321 E16.318936), minimally impacted by anthropogenic activity, were used for the experiment. Microcosm setup followed Previšić et al., 2021 with modifications in number of microcosms, temperature regime and mixture of moss species. Upon collection, 12 microcosms (aquaria 30 × 20 × 15 cm) were installed with 3 L of water,10 tablespoons of sand, 3 large stones (> 10 cm),10 small stones (2–5 cm), 3 tufts of moss (6–8 cm in diameter and plants up to 15 cm in length) and 5th instar *M. nycterobia* larvae (ca. 30 larvae per aquarium). Constant oxygen levels were kept using aquaria air pumps, and to minimize evaporation, each microcosm was covered with a glass cover. Natural light and day-night regime was supplemented with the artificial light (with sunlight's spectrum) in the regime of 12 hours of light followed by 12 hours without light. All microcosms were acclimatized for 10 days at 10 °C under aforementioned conditions preceding the start of the experiment. The experiment was conducted in the randomized factorial design, with treatments as follows: Control (C), Increased temperature (T2), ECs mix (EC), multiple stressor treatment = ECs mix + increased temperature (EC + T2 = MS), with three replicates of each. Subsequently, 6 of them were exposed to a mixture of 20 ECs over a 78-days

period. The composition of the ECs mixture was selected based on occurrence of particular compounds in freshwaters in Europe (Mandaric et al., 2015), and included 15 PhACs; azaperol, acetaminophen, thiabendazole, levamisol hydrochloride, dexamethasone, ketoprofen, naproxen, ranitidine hydrochloride, soltalol hydrochloride, valsartan, diphenhydramine, clopidogrel hydrogen sulfate, hydrochlorothiazide, sertraline hydrochloride, cimetidine and 5 EDCs; benylparaben, ethylparaben, propylparaben, estriol, estradiol-β. The volume of water was kept constant by adding fresh dechlorinated tap water (ca. 100 mL every week), and the concentration of each compound was kept at a pseudo-constant concentration of 500 ng L^{-1}. Taking into consideration knowledge gained from our previous experiments (Cetinić et al., 2022; Grgić et al., 2023; Previšić et al., 2021), three stock solutions were prepared and 100 µL of the solution was added each day, every three days or once a month, depending on the compound. In this way, abiotic attenuation (sorption and/or (photo)degradation) was taken in consideration and the nominal concentration of each compound was maintained, more details provided in Supporting information (SI 1). As different temperature regimes needed to be followed, aquaria were placed in two different incubators (POL-EKO APARATURA, Poland). "Natural" temperature regime (T1) mimicked the regime of the Krčić spring reach before the drying phase (10 °C) and temperature was successively increased 0.5 °C every 15 days. Increased temperature regime (T2) followed the same pattern but with temperature increased by 4 °C, in accordance with patterns of reduced flow observed in the selected river coupled with projections of temperature increase due to climate change in the Mediterranean montane regions (Bravo et al., 2008).

Biota sampling included initial (after the acclimatization period - day 0 (D0)) and several consecutive collections (ca. day 15 (D15), day 30 (D30) and day 60 (D60) of exposure) including different life stages (larvae, pupae and adult stage), in accordance with the life cycle of holometabolous caddisflies. At each sampling date, we collected replicate samples from each microcosm, consisting of 2 g of moss and 3–5 Trichoptera larvae/pupae. However, these samples were pooled per treatment to mitigate the potential extreme effects of individual microcosms (Kraufvelin, 1998). The complexity and extended duration of micro and mesocosm test systems exacerbate issues like "aquarium individuality," where initially identical replicates diverge due to random effects, introducing significant variability and complicating the identification of

statistically significant deviations (Kraufvelin, 1998; Sanderson, 2002). Additionally, the weight reduction of adult aquatic insects during metamorphosis (Huryn and Wallace, 2000) poses a challenge, leading to high tissue demand. Consequently, analytical replicates for each treatment per sampling date were created after homogenizing the pooled samples (details under 2.2, Biota Sample Processing). The chosen approach aims to strike a balance between providing ecologically meaningful results with non-model organisms and minimizing methodological constraints. Trichoptera larvae were kept in clean aquaria for 24 h to allow for gut clearance prior to collection (Van Geest et al., 2010). Additionally, emerging adult *M. nycterobia* were collected as they emerged (daily), and sex of each individual was determined. These samples were pooled depending on sex for subsequent analyses due to tissue requirements and to match the overall sampling scheme, according to temperature treatments. Emergence following the „natural" temperature regime (T1) started ca. 2 weeks later than in T2, thus samples were pooled for dates 45-60 and 60-75 days of exposure. Samples in elevated temperature regime (T2) were pooled for dates: 30-37 and 38-47 days of exposure. We combined adult *M. nycterobia* from 45-60 days of exposure in T1 and 38-47 days of exposure in T2 to one time point (day 60 of exposure) in order to have approximately the same time period of exposure to selected stressors. All samples were freeze-dried upon collection and stored at −80 °C until further processing.

Oxygen concentration (mg L^{-1}), oxygen saturation (%), pH and electrical conductivity (mS cm^{-1}) were measured in every microcosm at the beginning of the experiment (D0) and subsequently every two weeks (D15, D30, D45 and D60) using Hach HQ40D portable multi-parameter probe (Hach, Germany).



223

224 Figure 1. A) Experiment design with the following treatments: C - Control (T1 = 10 °C), T2 -
225 Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (EC + T2) and b)
226 sampling scheme with caddisfly larvae, pupae and adults sampled at various days of exposure.

227 2.2. Biota sample processing: extractions of metabolites and lipids

228 Within this study, the term "metabolome" refers to the complete set of low molecular weight
229 molecules or metabolites present within an organism, while "lipidome" is used to describe the
230 complete lipid profile within an organism (i.e. all lipids).

231 Biota samples processing, metabolite and protein extraction and metabolite profiling analyses
232 followed (Grgić et al., 2023). In order to detect Hsp70 proteins, we performed Western blot
233 analysis of protein samples, the details are provided in Supporting Information (SI 2). As there
234 was no detection of the aforementioned proteins no results are shown.

235 The Folch lipid extraction method (Folch et al., 1957) was used and performed following the
236 protocol of (Sarafian et al., 2014) with modifications. Briefly, for lipidome profiling and
237 determination of the total lipid content (TLC), each sample (30 mg) was dissolved in 600 µL $CHCl_3$
238 : MeOH (2:1 v:v). Samples were vortexed at medium speed (IKA® Vortex Genius 3, Germany) for
239 5 min. After 10 min of incubation at room temperature, samples were cooled at -20 °C for 10 min
240 and additional lysis was done via ultrasonic probe (Sonoplus HD4050, Bandelin electronic GmbH,
241 Germany) for 1 min at 50% of intensity. Samples were stored overnight at −20 °C to improve
242 protein precipitation and then centrifuged at 14 000g for 20 min (Tehtnica-Centric 200R,
243 Slovenia). The supernatant was collected (600 µL) in a previously weighed tube, filtered through
244 a PVDF filer (MILLEX® - GV Syringe Filter 0.22 µm Hydrophilic PVDF, 13 mm, Sterile) and
245 evaporated to dryness. The tube was weighed to determine the TLC.TLC was determined using
246 the following equation; TLC = weight of "full" tube – empty tube. Samples were dissolved in 200µL
247 IPA : ACN : H2O (2:1:1, v:v:v) for subsequent LCMSMS non-target analysis.

248 For both metabolome and lipidome analysis, set of quality control samples was prepared by
249 taking small aliquot of the each sample solution from the entire set and pooling them together.
250 Subaliquots of this pooled sample are regarded as set of quality control samples.

251

252 2.3 Non-target metabolome and lipidome analysis

253 Non-target analyses of the metabolom and lipidome samples were performed using a high-
254 resolution mass spectrometry system; LTQ-Orbitrap VelosTM (Thermo Fisher Scientific, USA)
255 coupled with an ultra-performance liquid chromatography (UPLC) system (Ultimate 3000
256 RSLCnano system, Dionex, Amsterdam, Netherlands). Instrument parameters and UPLC gradients
257 are provided in Supporting Information (SI). Data extraction, chromatographic deconvolution and
258 final alignment were done using the MZmine program (Katajamaa et al., 2006). Steps and settings

used in the MZmine program are provided in Supporting information (SI 3). The exported .csv files were further filtered and sorted using modified parts of Bqunat script written in Mathematica (Wolfram Research Inc.,Campaign, IL, USA) (Rožman et al., 2018). Data were cleaned by removing of all blank-related features. Feature was considered as blank related if an intensity ratio sample:blank was < 10. Quality acceptance criteria for each metabolite were: detection rate > 70%, relative standard deviation < 30% and dispersion ratio < 40%. Based on the exact mass match, metabolite and lipid identification was performed in http://ceumass.eps.uspceu.es/ and by searching Metlin, Kegg, LipidMaps, PubChem, and HMDB databases. It is worth noting that the metabolites and lipids reported here are only the metabolites that were putatively annotated.

2.4 Statistical Analysis

2.4.1 Body weight changes & total lipid content of *Micropterna nycterobia* and non-vascular macrophytes, and physico-chemical water parameters

The effects of experimental treatments on body weight (evaluated individually in *M. nycterobia* specimens; N = 12 per treatment per collecting date) and total lipid content (TLC; evaluated in composite samples of *M. nycterobia* pooled per life stage and treatment; N = 3 per treatment per collection date) of different life stages of *M. nycterobia* were analysed using Generalized Linear Models (GZLMs) constructed in IBM SPSS Statistics 27.0 (IBM Corporation). Additionally, for adults the differences between the sexes were determined. Normal distribution linear scale response was used for all data except for body weight of adults where gamma scale response with log link was used as the data did not achieve normal distribution. Maximum likelihood estimate was used for parameter estimation. Pairwise contrasts of estimated means were performed using Wald's statistics.

The changes in total lipid content in moss, as well as changes in the physico-chemical water properties, between different treatments over time were tested using repeated measures ANOVA using the IBM SPSS Statistics 27.0 (IBM Corporation). Pairwise comparisons were

conducted with Bonferroni adjustment for multiple comparisons. Obtained data were analyzed and visualized using Principal Component Analysis (PCA) in Primer 7.

2.4.2. Data analysis of non-target metabolomic and lipidomic profiles of *Micropterna nycterobia* and non-vascular macrophytes

Obtained metabolomic and lipidomic data matrices were forth root transformed and~~was~~ analyzed using Principal Component Analysis (PCA) and Principal Response Curves (PRC) in package vegan (version 2.5-7) RStudio version 4.1.2 (Oksanen et al., 2020). ~~PRC analysis was performed using forth root transformed data.~~ Additionally, significance of the results was tested using the Monte Carlo test in the 'permute' package, with 99 permutations, more specifically significance of the 1st canonical axis of the PRC and significance of sampling date/insect stage was tested. Due to the missing moss sample D30 EC, Redundancy Analysis (RDA) was performed instead of PRC for moss metabolome and lipidome data using CANOCCO software (version 5.11,ter Braak and Šmilauer, 2012). We used treatment as the categorical explanatory variable, metabolites and lipids as response, respectively and time as a covariate. The RDA significances were tested using Monte Carlo test with 999 permutations. Values of MZ masses of the metabolome dataset were normalized and processed by Principal Component Analysis (PCA) in Rstudio. The PCA was visualized in Primer 7 (Clarke and Gorley, 2015). The first 100 variables (MZ masses) that contribute most to separation along certain PCs were selected for identification of metabolites.

3. RESULTS

3.1. Physico-chemical properties of water during microcosm experiment

Oxygen concentration (mg L-1) and oxygen saturation (%) throughout the experiment did not differ significantly among treatments, they were impacted only by the day of measurement (repeated measures ANOVA, Table S1, S2; and PCA analysis, the total variance accounted by the two components shown was ~69 %; variance from [PC1, PC2]≈[46.4 %, 22.6 %]; Fig. S1). Day of measurement and interactive effects; temperature x ECs, temperature x time and ECs x time

caused changes in pH levels (repeated measures ANOVA, Table S3). Specifically, pH was higher on D15 and D60 in treatments with ECs mixture (EC and MS). Additionally, on D30 and D45 in treatments with elevated water temperature (T2 and MS) pH was higher and lower, respectively (repeated measures ANOVA, Table S3). Electrical conductivity differed between treatments, i.e. treatments with increased water temperature (T2 and MS) had higher conductivity compared to the "natural" temperature regime (C and ECs) (repeated measures ANOVA, Table S4). Moreover, electrical conductivity was generally rising throughout the whole experiment in all treatments, thus it was also impacted by the day of exposure (repeated measures ANOVA, Table S4).

3.2. Total lipid content, metabolome and lipidome profiles of non-vascular macrophytes

Principal component analysis (PCA) based on non-target metabolic profiles and lipidome profile of moss revealed clustering primarily based on the duration of the experiment rather than treatment (Fig. 2A&B). In the analysis of the metabolome and lipidome, the total variance accounted by the first two components was ~45.5 % (variance from [PC1, PC2]≈[31.9 %, 13.6 %] and ~41,5 % (variance from [PC1, PC2]≈[28.8 %, 12.7 %]), respectively. In both analyses, the first principal component (PC) axis separated the profiles between D0 and D15 from D60, with D30 lacking a consistent grouping patterns (Fig. 2A&B; note however, that D30 EC samples are missing, as they were lost during the processing). In addition, RDA analyses shows that metabolome and lipidome profiles differed significantly among treatments (RDA: pseudo-F = 3.0, p = 0.002, explained variability = 12.1 %; Fig. S2A and RDA: pseudo-F = 2.2, p = 0.004, explained variability = 7.7 %; Fig. S2B, respectively). Majority of metabolite groups (17 in total) showing the most significant changes in abundance to stressor treatments were terpenoids, terpenes class (50%) followed by lipids and fatty acids (29%), amino acids, peptides and proteins (11%), carbonyl compounds (5%), organic acids, carboxylic acids and monocarboxylic acids (5%) (Fig. S3, Table S5). Within the lipidome, most significant changes in abundance of 51 lipids to stressor treatments were noticed in glycerophospholipids (51%) followed by glycerolipids (20%), sterol lipids (12%), sphingolipids (8%), prenol lipids (8%) and fatty acyls (2%) (Fig. S4, Table S6). The lowest total lipid content in all treatments was measured on D 15, however, no significant

changes in TLC was observed related to days of exposure or treatments (Fig. S5, repeated measures ANOVA, Table S7).

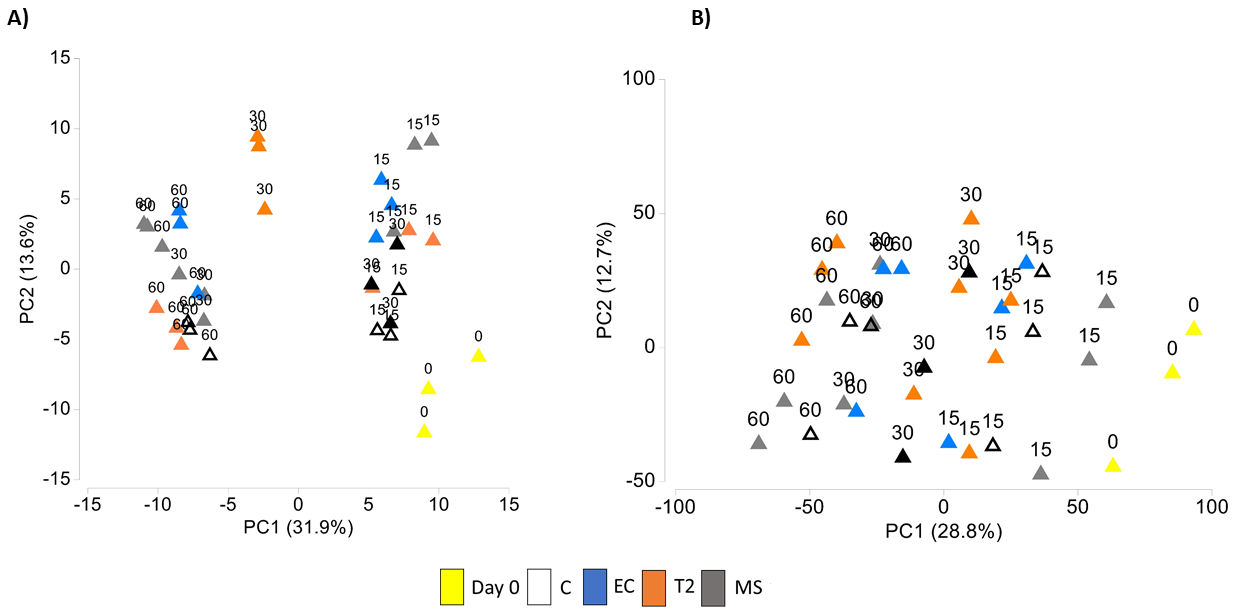


Figure 2. PCA plot showing separation based on A) metabolome and B) lipidome of moss in different treatments. Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC); Day 0 – pre-exposure sample.

3.3. Phenology (emergence patterns) of the caddisfly *Micropterna nycterobia*

Increased water temperature caused earlier emergence of both males and females, resulting in approximately three-week shift in peak emergence between the normal (C and EC) and elevated temperature (T2 and MS) treatments (Fig. 3). Overall, more males emerged throughout the experiment, however, the majority of pupae that did not emerge by the end of experiment were females. Only minor differences between emergence patterns of males and females were observed within treatments (Fig. 3).

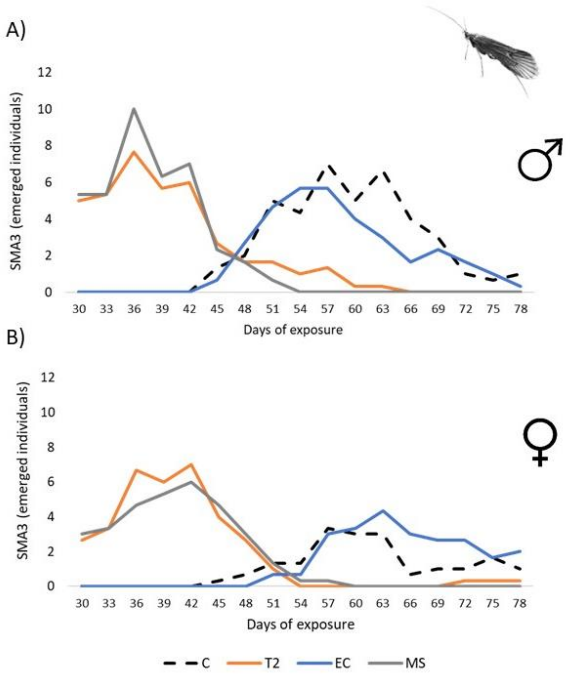
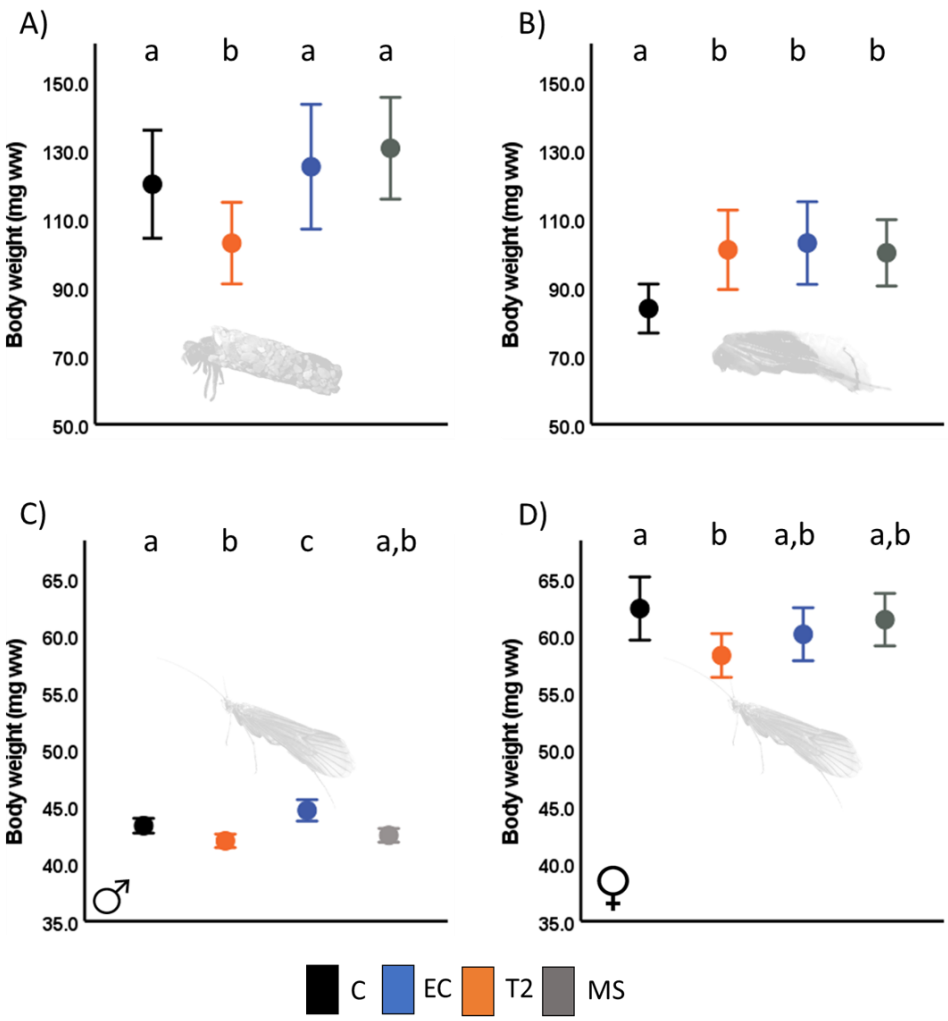


Figure 3. Three-day simple moving average (SMA3) of emerged A) male and B) female adults of *Micropterna nycterobia* across treatments. Abbreviations of treatments are as follows: C - Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC).

3.4. Body weight and total lipid content changes of the caddisfly *Micropterna nycterobia*

Overall, a loss in body mass was recorded in *M. nycterobia* throughout the life cycle, with larvae having the highest body weight and adults the lowest (Fig. 4A-D). A statistically significant drop in body weight was observed in larvae in T2 compared to control (4A, Table S8). All experimental treatments increased the body weight of pupae compared to control (Fig. 4B, Table S9). Adult females had significantly higher body weight compared to males (Fig. 4C & 4D, Table S10), however, in adults of both sexes a statistically significant drop in body weight was observed in T2 compared to control (Fig. 4C & 4D, Table S11 and S12).



372

373 Figure 4. Model predictions illustrating the effect of treatment on body weight of A) larvae B)
374 pupae C) adult males and D) adult females of *M.* nycterobia. Mean values of twelve replicates are
375 presented with 95% confidence intervals. Different letters indicate significant differences among
376 treatments ($p < 0.05$). Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 -
377 Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC).

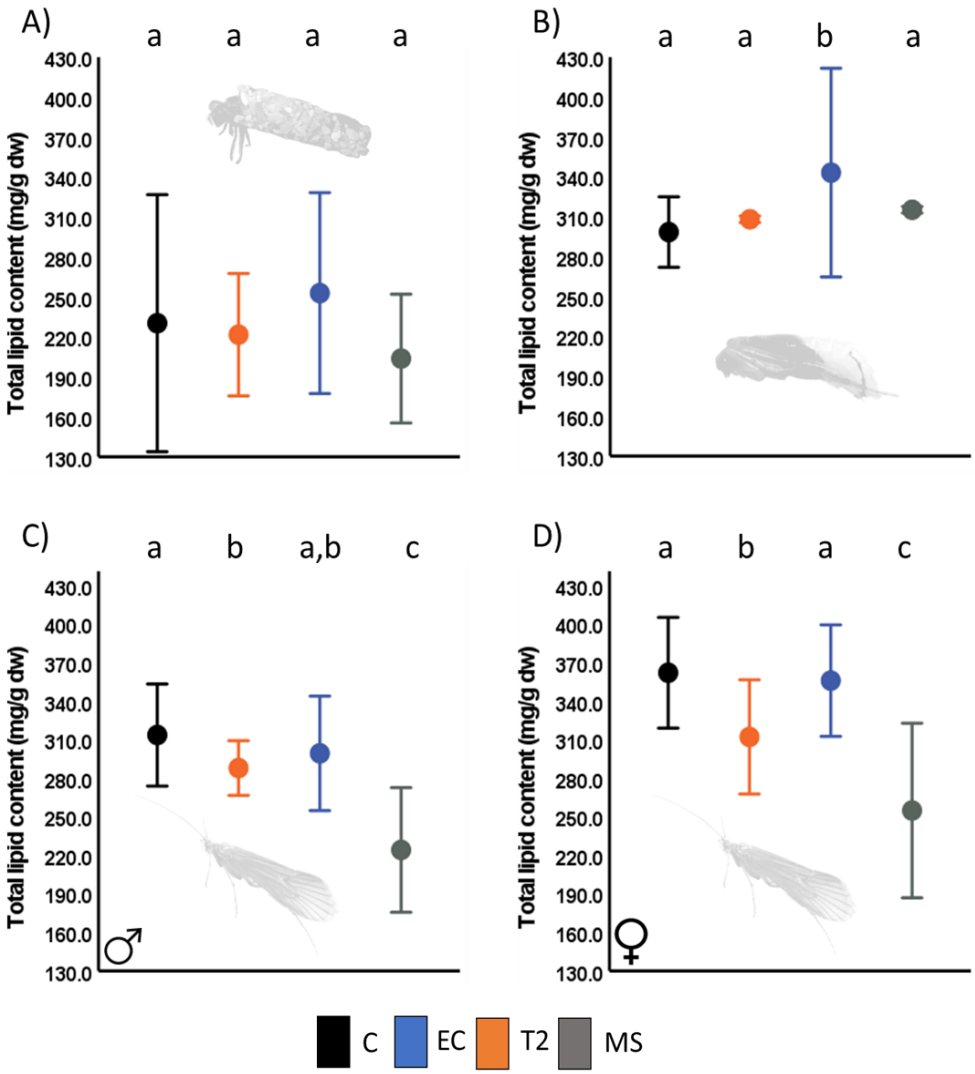


Figure 5. Model predictions illustrating the effect of treatment on total lipid content of A) larvae B) pupae C) adult males and D) adult females of *M. nycterobia*. Mean values of three replicates are presented with 95% confidence intervals. Different letters indicate significant differences among treatments ($p < 0.05$). Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC).

Changes in the TLC were more pronounced post-metamorphoses (Fig. 5A-D). Experimental treatments had no effect on TLC on larvae (Fig. 5A, Table S13), while pupae exhibited an increase in the EC treatment compared to control (Fig. 5B, Table S14). Total lipid content of adults was

lower compared to control in treatments with increased temperature (T2 and MS) (Fig. 5C & 5D, Table S15 & S16). Moreover, the greatest drop in TLC of adults was observed in the MS treatment ((Fig. 5C & 5D, Table S15 & S16), corresponding with the negative synergistic effect according to (Piggott et al., 2015). Females had significantly higher total lipid content compared to males (Figure 5C & 5D, Table S17).

3.5. Metabolome profiles of the caddisfly *Micropterna nycterobia*

Principal Response Curves (PRC) of non-target metabolome profile of larvae, pupae and adult caddisflies show that the metabolome of all caddisfly life stages was mostly affected by the ECs mixture (EC and MS), as evident by the highest deviation from the control (Fig. 5A & B). Deviations were observed in larvae at D15 already, and further increased in pupae and adults. The MS treatment showed highest impact on metabolome of both male and female adult caddisflies (Fig. 5A & B). Additionally, 62.6 % of total variance in males and 59.5 % in females could be attributed to time, whereas 22.1 % in males and 25.8 % in females could be attributed to the treatment (including its interaction with time, Table S18). The first PRC axis was significant (males – F (1, 32) = 18.714 $p < 0.05$, females – F (1, 32) = 28.172, $p < 0.05$) and based on the Monte Carlo tests per sampling date, the treatment regime had a significant influence on all sampling dates.

Principal component (PCA) analysis based on non-target metabolic profiles of all life stages of *M. nycterobia* revealed clear separation of pre-metamorphosis larvae and post-metamorphosis pupae and adult imagines (Fig. S6, PC1). However, when particular life stages were analyzed separately, the PCA also revealed separation into distinct groups based on treatments. In larvae, for instance, PC1 axis separating larvae in ECs and MS treatments from D0, C and T2 treatments accounted for 37.7 % of variability (Fig. 4C; variance from [PC1, PC2]≈[37.7 %, 15 %]).

Majority of metabolite groups (18 in total) showing the most significant changes in abundance to stressor treatments in *M. nycterobia* were lipids and fatty acids (39%) followed by amino acids (22%), quinones (6%), acylcholine (6%), adrenergic agents (6%), lactones (6%), alcohols (5%), pyridines (5%), alkaloids (5%) (Fig. S7, Table S19.

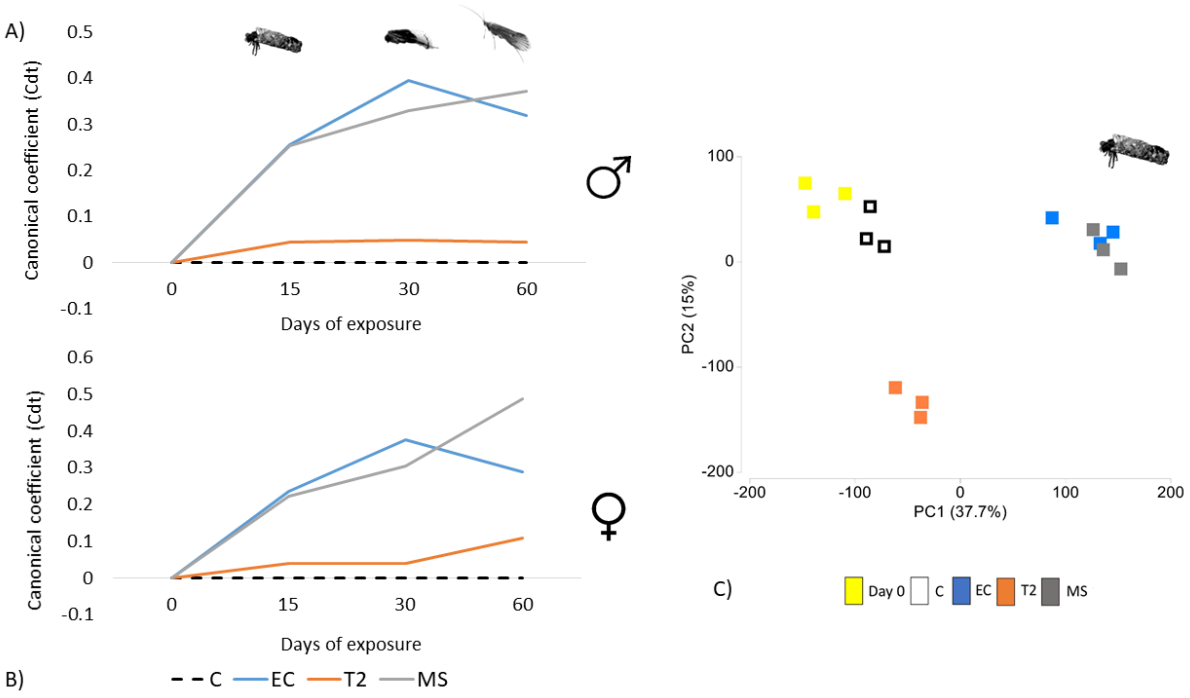


Figure 5. Metabolome profiles of the caddisfly *Micropterna nycterobia;* PRC of changes in the metabolic profiles along the caddisfly life cycle in A) male and B) female adults exposed to treatments in relation to control. Numbers on x-axis denote days of exposure. C) PCA plot showing separation based on metabolome of *M. nycterobia* larvae in different treatments at 15 days of exposure. Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC); Day 0 – pre-exposure sample.

3.5. Lipidome profiles of the caddisfly *Micropterna nycterobia*

Principal Response Curves (PRC) of non-target lipidome profile of larvae, pupae and adult caddisflies shows the highest deviation from the control in male and female adults, with an opposing individual stressor effect (EC vs T2; Fig. 6A & B). In MS treatments, increased temperature was the dominant stressor for both sexes, however, there were differences between sexes, as male caddisflies lipidome was more affected in T2 and MS treatments, whereas female caddisflies lipidome was more affected in the EC (Fig. 6A & 5B). Moreover, table S20 shows that 79.8 % of total variance in male and 68.4 % in female caddisflies can be attributed

to time, whereas 13.3 % in male and 17.3% in female can be attributed to the treatment (including its interaction with time). The first PRC axis was significant (males - F (1, 32) = 17.475 $p < 0.05$, females – F (1, 32) = 14.583, $p < 0.05$) and based on the Monte Carlo tests per sampling date, the treatment regime had a significant influence on all sampling dates.

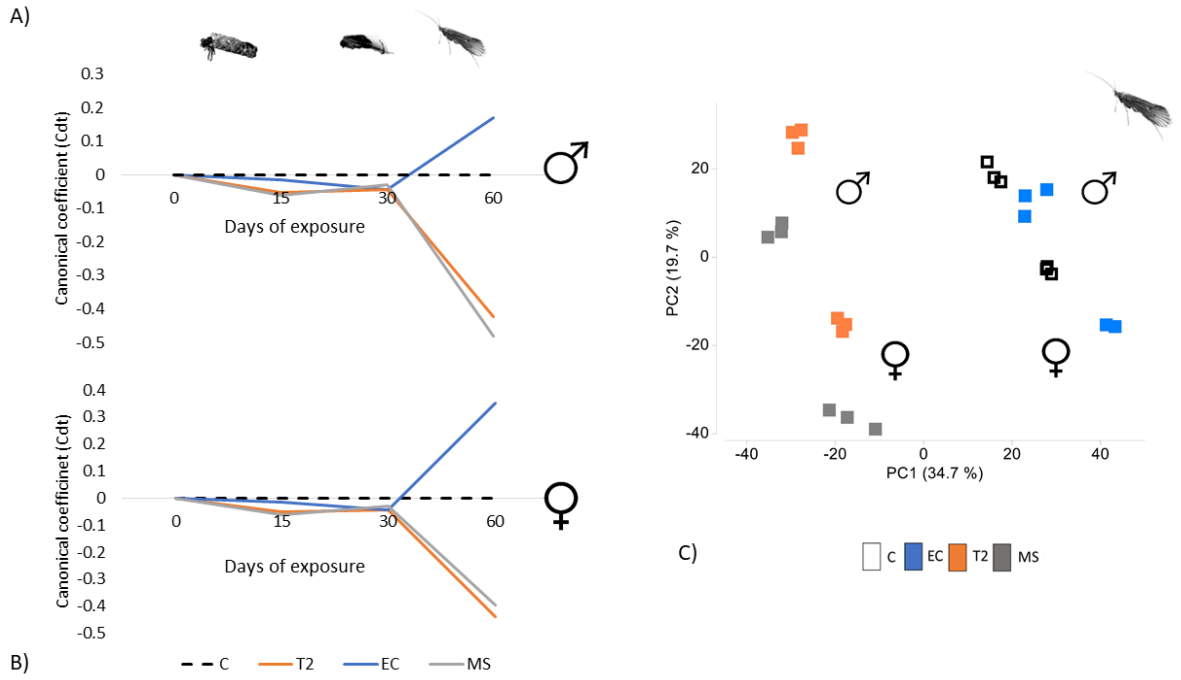


Figure 6. Lipidome profiles of the caddisfly *Micropterna nycterobia;* PRC of changes in the lipidome profiles of caddisfly life stages and A) male and B) female adults exposed to treatments in relation to control. Numbers on x-axis denote days of exposure. C) PCA plot showing separation adult *M. nycterobia* separated by sex in different treatments. Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC); Day 0 –pre-exposure sample.

Principal component (PCA) analysis based on non-target lipidome profiles of all life stages of *M. nycterobia* showed clear separation of three major life stages, larvae, pupae and adults (Fig. S8). The total variance accounted by the three clusters was ~68.8 % (variance from [PC1, PC2, PC3]≈[47.7 %, 14.8 %, 6.3 %]). Clustering of particular treatments was particularly evident in separate analyses of each life stage. The highest degree of clustering based on treatments was inferred for adults, with the first principle component axis (PC1 = 34.7 %) separating adults based

on increased water temperature (T2 and MS treatments), whereas the second axis separated males from females (PC2 = 19.7 %, Fig. 6C).

Within the lipidome, most significant changes in abundance to stressor treatments were noticed in glycerophospholipids (67%) followed by fatty acyls (12%), sphingolipids (12%), glycerolipids (5%), prenol lipids (2%) and sterol lipids (1%) (Fig. S9, Table S21).

4. DISCUSSION

Water pollution has far-reaching consequences for the ecosystem health and functioning, and it is important to understand these impacts, especially in a context of climate change. Here we used simplified freshwater food web exposed to a mixture of PhACs and EDCs and increased water temperature to broaden our understanding of the impacts of climate change and pollution on freshwater ecosystems.

4.1. Warming and pollution with PhACs & EDCs triggered a mild response in moss

A mild response of the moss to both individual stressors (increased water temperature and contamination with ECs) and their combination suggests mosses' resilience. It has been shown that plants have specific temperature and pollution thresholds that trigger or inhibit certain physiological processes, allowing them to respond to stressful conditions (Firmansyah and Argosubekti, 2020; Gorovits et al., 2020; Sun et al., 2018; Zezulka et al., 2013). One of the most common physiological responses includes production of heat shock proteins (HSPs), and lack of observable change in regulation of HSPs may suggest that the thermal and pollution threshold necessary to trigger stress response in the moss was not reached. This observation is in agreement with established thermal threshold of plants of minimum 5 °C (Firmansyah and Argosubekti, 2020). Even though changes in metabolomic and lipidomic profiles among treatments were significant, one should keep in mind that changes in metabolite and lipid composition can be related to stress response but also developmental stages (Lu et al., 2019; Mikami and Hartmann, 2004). In addition, the biotic stress induced by the feeding of *M. nycterobia* larvae on the moss might have masked the impact of the pollution and increased

476 temperature. Plants can prioritize their responses to address individual stressors when exposed
477 to multiple abiotic and/or biotic stressors (Rejeb et al., 2014; Suzuki et al., 2014). We argue that
478 the moss in the current experiment might have prioritized biotic stress over abiotic stressors and
479 activated different defence mechanisms to mitigate larval feeding. However, we could not test
480 for the latter, since feeding of *M. nycterobia* larvae on the moss was also present in control
481 treatments. Moreover, research has shown that different stress combinations activate different
482 pathways and signals thus making it harder to predict multiple stressor effects (Rejeb et al., 2014;
483 Suzuki et al., 2014; Vescio et al., 2022).

484

485 4.2. Single stressor impacts: effects of warming and pollution with PhACs and EDCs on *M.*
486 *nycterobia*

487 In the current experiment, we recorded a body mass loss in *M. nycterobia* that is in line with the
488 usual life cycle patterns of the holometabolous caddisflies, with larvae having the highest body
489 weight and adults the lowest (Huryn and Wallace, 2000). Increased temperature during insect
490 development typically leads to reduced adult body size, negatively influencing fecundity and
491 longevity (Mirth and Riddiford, 2007), this effect was however, not observed in the current study.
492 Our observations are in line with data on chronic exposure to pesticides in the caddisfly
493 *Limnephilus lunatus,* where reduced body weight of adults was observed only if younger instar
494 larvae were exposed, and not the fifth-instars (Liess and Schulz, 1996; Schulz and Liess, 2001,
495 1995).

496 *M. nycterobia* inhabits clean crenal and rhithral sections and is therefore expected to be sensitive
497 to presence of contaminants (Graf et al., 2023). Indeed, sensitivity is displayed through intense
498 change in regulation of both metabolites and lipids in respect to control, however, the
499 contaminants seem to have a more significant impact on metabolome than temperature. More
500 precisely, the most pronounced metabolome response was post-metamorphosis, yet changes in
501 metabolite regulation are already notable in larvae at D15. Notably, the sampled caddisflies'
502 metabolome contained the biogenic amine octopamine, a significant neurotransmitter,
503 neuromodulator, and neurohormone influencing various physiological functions, behaviour and

endocrine activity (Farooqui, 2012) Changes in octopamine levels due to PhACs and EDCs during metamorphosis could affect not only the subsequent life stage but potentially extend across multiple generations.

Observed difference in dynamics of regulation of metabolites *vs* lipids can be related to physiological roles of metabolites and lipids which response tends to differ depending on the type of stress and the metabolic pathways involved (Kainz and Fisk, 2009; Snart et al., 2015). Generally, lipids serve as a long-term energy source and are stored in lipid droplets, which can be mobilized to provide energy during times of starvation, embryogenesis, prolonged periods of flight and stress (Arrese and Soulages, 2010; Kainz and Fisk, 2009). Here, metabolites were more regulated than lipids in response to stress probably in order to maintain cellular homeostasis and ensure energy reserves (lipids) for the emerging adults (Arrese and Soulages, 2010). Glycerolipids such as triglycerides are stored in the core of the lipid droplets surrounded by glycerophospholipids (Arrese and Soulages, 2010). As glycerophospholipids were mostly affected by experimental treatments, this further supports the fact that lipid reserves were preserved for adults. However, changes in glycerophospholipids of insects can also be related to development and metamorphosis (Bashan et al., 2002; Cargill et al., 1985; Duarte, 2019) as well as food source (Hanson et al., 1985; Torres-Ruiz et al., 2010).

The lack of strong effects of increased water temperature on metabolite regulation, as well as absence of heat shock protein HSP70 expression, is most likely due to adaptation of *M. nycterobia* to thermal stress regularly occurring in intermittent streams (Qin et al., 2003). This finding suggests that *M. nycterobia* and possibly other intermittent habitats indicators may have metabolic flexibility to cope with thermal stress, allowing them to survive extreme climatic events characterised by fluctuating temperature regimes. In addition, the regulation of lipidome of *M. nycterobia* aquatic stages exhibited intriguing resilience to both increased water temperature and pollution with PhACs and EDCs. The limited impact on lipid regulation suggests that the stress response threshold triggering significant lipid mobilisation may not have been surpassed. Instead, the priority for the aquatic stages was directed towards lipid accumulation rather than mobilization in response to stress challenges (Arrese and Soulages, 2010; Hoppeler et al., 2018). Emphasis on lipid accumulation was evident through the observed increase in total lipid content

from larval to pupal stages, serving as a crucial energy source to sustain adult insects during non-feeding periods and fuel their flights (Arrese and Soulages, 2010; Hoppeler et al., 2018). While the anticipation of intense lipidome activity in adult insects is well-founded, it is surprising that so distinct regulatory mechanisms are operating in response to different stressors. However, lipidomic profiles of multiple stressor treatment were in both males and females congruent with those of increased temperature, implying the dominant impact of increased water temperature on regulation of lipids in aquatic insects.

Increased water temperature accelerated development of *M. nycterobia,* resulting in earlier adult emergence and lower total lipid content of all life stages in treatments with increased temperature. Similarly, the mayfly larvae chronically exposed to increased temperature used lipids and amino acids as alternative energy sources to support their growth and maintenance costs, ultimately resulting in reduced total lipid content (Chou et al., 2018). Furthermore, sensitivity of the temporal emergence patterns of aquatic insects was already discussed as toxicological endpoint for exposure to pesticides (Schulz and Liess, 2001), as timing of aquatic insect emergence plays a crucial role for riparian predators (review in Bundschuh et al., 2020). More precisely, insectivorous birds almost exclusively obtain the omega-3 long-chain polyunsaturated fatty acids from emerging aquatic insects, thus shifts in the relative availability and phenology of aquatic insects in response to a changing climate are likely to have major fitness consequences for their breeding success (Shipley et al., 2022). Hence, such shifts can have cascading effects on cross-ecosystem energy flow. This is of particular importance in intermittent water bodies, like those inhabited by *M. nycterobia,* where mass emergence during short periods is typical and riparian food webs are highly dependent on the aquatic subsidies (McIntosh et al., 2017).

4.3. Multiple stressor impacts of warming and PhACs and EDCs on aquatic insects

A synergistic interactive effect was observed, leading to a decrease in total lipid content and significant variation in lipid profiles in adults of both sexes under the MS treatment. *Micropterna nycterobia*, a specialist in intermittent rivers, exhibits a behaviour where adults leave the water

bodies upon emergence and migrate to cooler mountainous regions or nearby caves for a few months until their gonads develop (Waringer and Graf, 2011). In autumn, after copulation, they lay eggs in re-established surface flow. Therefore, the observed decrease in lipid reserves in adults (8.9% and 10.7% decrease in total lipid content in males and females, respectively) could have a major impact on their reproduction and population dynamics. Lipids serve as crucial energy reserves for insects, especially during non-feeding life stages and long-distance flights (Arrese and Soulages, 2010; Downer and Matthews, 1976). Maintaining metapopulation dynamics is particularly important for inhabitants of intermittent water bodies (Datry et al., 2017). Various pollutants, such as fungicides and copper, can decrease the lipid content of limnephilid caddisflies (Konschak et al., 2019). However, this effect was not observed in the current study as PhACs and EDCs did not influence the lipid content of *M. nycterobia*. Furthermore, the negative effects of pollutants on lipids can be exacerbated by elevated water temperatures (Yoon et al., 2022). However, in our study, increased water temperature primarily influenced the lipids of caddisflies, and its adverse effects were amplified by pollution with PhACs and EDCs. Additionally, warming indirectly contributed to the further deterioration of environmental conditions in the T2 and MS treatments, as evidenced by increased conductivity within these two treatments.

In terms of the timing of the strongest stress response, our results differ from a previous experiment involving the same caddisfly species, where the effects of ECs and microplastic particles were most pronounced in the first 15 days of exposure, both in single and combined stressor treatments (Grgić et al., 2023). Apart from the dominant impact of increased temperature, these differences may also be attributed to variations in the mixture of ECs used, as different compounds can have varying effects on biota (Muñoz et al., 2015; Nilsen et al., 2019). Nevertheless, our study indicates that the adverse synergistic effects of warming events and freshwater contamination with ECs could intensify throughout the life cycle of aquatic insects, potentially leading to developmental impairments and disturbances in population dynamics (Kazmi et al., 2022). However, in most environmental health assessment and monitoring programs, only the aquatic stages of aquatic insects are considered (Water Framework Directive (WFD) 2000/60/EC). Furthermore, the multiple stressor effects observed in the current study not

590 only resulted in reduced resource quality for aquatic food webs but also affected the quality of
591 emergence, thus impacting the riparian food webs at the aquatic-terrestrial interface (Bundschuh
592 et al., 2020).

593

594 4.5. Differential response to stressor impacts of male and female caddisflies

595 Our study reveals sex-specific responses to both single and multiple stressors, with females
596 exhibiting more pronounced impacts on the metabolome while males show greater effects on
597 the lipidome. In the majority of insects, there is sexual dimorphism in lipid content due to distinct
598 roles played by lipids, such as egg production in females and flight behaviour in males (Lease and
599 Wolf, 2011). Certain aquatic insect species, characterized by specific male flight behaviour (Lease
600 and Wolf, 2011) or swarming (Sartori et al., 1992), experience negative impacts on population
601 fitness when males have decreased lipid content. Furthermore, this study identifies 4-
602 hydroxyestradiol, an endogenous metabolite of 17β-estradiol, from *M. nycterobia*, which has
603 been shown to have significant lipid-modulating effects in rats (Wang and Zhu, 2017).
604 Additionally, in *Drosophila*, sexual dimorphism has been observed in metabolic genes and
605 mechanisms involved in triglyceride homeostasis (Wat et al., 2020).

606 Likewise, the findings of this study highlight sex-dependent variation in metabolites induced by
607 stress, consistent with a previous study involving *M. nycterobia* exposed to microplastic particles
608 and a mixture of personal care products (Grgić et al., 2023). Moreover, female insects generally
609 exhibit a stronger stress response across different taxa and under various stress conditions,
610 including parasite infections, predation, food quality, chemical stress, and the impacts of climate
611 change (Lindsey and Altizer, 2009; Slos et al., 2009; Stillwell and Davidowitz, 2010). This is likely
612 due to the different evolutionary roles of male and female insects, which have led to the
613 development of distinct stress-defence mechanisms. The fitness of females is closely linked to
614 their life expectancy and the number of offspring they produce.

615 Therefore, our study emphasizes the variability in the impacts of both single and multiple
616 stressors on various traits, different life stages, and sexes within a single species. Consequently,
617 it underscores the importance of developing a more comprehensive understanding of the

sensitivity of freshwater organisms to the adverse effects of single and multiple stressors, particularly when addressing the management of freshwater ecosystems in the context of global change (Schäfer and Piggott, 2018).

Acknowledgment

We thank Dr Ruđer Novak (Faculty of Medicine, University of Zagreb) for providing assistance in mass spectrometry analysis. This study is part of the outcomes of the Croatian Science Foundation project MUSE (PZS-2019-02-9479 and DOK-2020-01-6998 to A.P.) and KLIMA-4HR project (KK.05.1.1.02.0006). M.R. acknowledges additional support from the Croatian Science Foundation (IP-2018-01-2298). Two anonymous reviewers are thanked for significantly improving the quality of earlier version of the manuscript.

LITERATURE

Arrese, E.L., Soulages, J.L., 2010. Insect Fat Body: Energy, Metabolism, and Regulation. Annu. Rev. Entomol. 55, 207–225. https://doi.org/10.1146/annurev-ento-112408-085356

Barbosa, M., Inocentes, N., Soares, A.M.V.M., Oliveira, M., 2017. Synergy effects of fluoxetine and variability in temperature lead to proportionally greater fitness costs in Daphnia: A multigenerational test. Aquat. Toxicol. 193, 268–275. https://doi.org/10.1016/j.aquatox.2017.10.017

Bashan, M., Akbas, H., Yurdakoc, K., 2002. Phospholipid and triacylglycerol fatty acid composition of major life stages of sunn pest, Eurygaster integriceps (Heteroptera: Scutelleridae). Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 132, 375–380. https://doi.org/10.1016/S1096-4959(02)00045-3

Bhangare, D., Rajput, N., Jadav, T., Sahu, A.K., Tekade, R.K., Sengupta, P., 2022. Systematic strategies for degradation kinetic study of pharmaceuticals: an issue of utmost importance

644 concerning current stability analysis practices. J. Anal. Sci. Technol. 13, 7.
645 https://doi.org/10.1186/s40543-022-00317-6

646 Blackman, R.C., Altermatt, F., Foulquier, A., Lefébure, T., Gauthier, M., Bouchez, A.,
647 Stubbington, R., Weigand, A.M., Leese, F., Datry, T., 2021. Unlocking our understanding of
648 intermittent rivers and ephemeral streams with genomic tools. Front. Ecol. Environ. 19,
649 574–583. https://doi.org/10.1002/fee.2404

650 Bravo, D.N., Araújo, M.B., Lasanta, T., Moreno, J.I.L., 2008. Climate change in Mediterranean
651 mountains during the 21st century. Ambio 37, 280–285.
652 https://doi.org/10.1579/00410.1579/0044-7447(2008)37[280:ccimmd]2.0.co;2

653 Bundschuh, M., Zubrod, J.P., Wieczorek, M. V., Schulz, R., 2020. Studying Effects of
654 Contaminants on Aquatic-Terrestrial Subsidies: Experimental Designs Using Outdoor and
655 Indoor Mesocosms and Microcosms, in: Contaminants and Ecological Subsidies. Springer
656 International Publishing, Cham, pp. 279–296. https://doi.org/10.1007/978-3-030-49480-
657 3_12

658 Cargill, A.S., Cummins, K.W., Hanson, B.J., Lowry, R.R., 1985. The role of lipids as feeding
659 stimulants for shredding aquatic insects. Freshw. Biol. 15, 455–464.
660 https://doi.org/10.1111/j.1365-2427.1985.tb00215.x

661 Cetinić, K.A., Grgić, I., Previšić, A., Rožman, M., 2022. The curious case of methylparaben:
662 Anthropogenic contaminant or natural origin? Chemosphere 294, 133781.
663 https://doi.org/10.1016/j.chemosphere.2022.133781

664 Chou, H., Pathmasiri, W., Deese-spruill, J., Sumner, S.J., Jima, D.D., Funk, D.H., Jackson, J.K.,
665 Sweeney, B.W., Buchwalter, D.B., 2018. The Good, the Bad, and the Lethal: Gene
666 Expression and Metabolomics Reveal Physiological Mechanisms Underlying Chronic
667 Thermal Effects in Mayfly Larvae (Neocloeon triangulifer). Front. Ecol. Evol. 6, 1–11.
668 https://doi.org/10.3389/fevo.2018.00027

669 Clarke, K.R., Gorley, R.N., 2015. PRIMER v7 :, PRIMER-E Ltd Registered.

Cogo, G.B., Martínez, J., Santos, S., Graça, M.A.S., 2020. Caddisflies growth and size along an elevation/temperature gradient. Hydrobiologia 847, 207–216. https://doi.org/10.1007/s10750-019-04082-3

Conti, L., Schmidt-Kloiber, A., Grenouillet, G., Graf, W., 2014. A trait-based approach to assess the vulnerability of European aquatic insects to climate change. Hydrobiologia 721, 297–315. https://doi.org/10.1007/s10750-013-1690-7

Cruzeiro, C., Ramos, A., Loganimoce, E.M., Arenas, F., Rocha, E., Cardoso, P.G., 2019. Genotoxic effects of combined multiple stressors on Gammarus locusta haemocytes: Interactions between temperature, pCO2 and the synthetic progestin levonorgestrel. Environ. Pollut. 245, 864–872. https://doi.org/10.1016/j.envpol.2018.11.070

Datry, T., Corti, R., Heino, J., Hugueny, B., Rolls, R.J., Ruhí, A., 2017. Habitat Fragmentation and Metapopulation, Metacommunity, and Metaecosystem Dynamics in Intermittent Rivers and Ephemeral Streams, in: Intermittent Rivers and Ephemeral Streams. Elsevier, pp. 377–403. https://doi.org/10.1016/B978-0-12-803835-2.00014-0

DeCourten, B.M., Brander, S.M., 2017. Combined effects of increased temperature and endocrine disrupting pollutants on sex determination, survival, and development across generations. Sci. Rep. 7, 9310. https://doi.org/10.1038/s41598-017-09631-1

Dinh, K. V., Konestabo, H.S., Borgå, K., Hylland, K., Macaulay, S.J., Jackson, M.C., Verheyen, J., Stoks, R., 2022. Interactive Effects of Warming and Pollutants on Marine and Freshwater Invertebrates. Curr. Pollut. Reports 8, 341–359. https://doi.org/10.1007/s40726-022-00245-4

Dorić, V., Ivković, M., Baranov, V., Pozojević, I., Mihaljević, Z., 2023. Extreme freshwater discharge events exacerbated by climate change influence the structure and functional response of the chironomid community in a biodiversity hotspot. Sci. Total Environ. 879. https://doi.org/10.1016/j.scitotenv.2023.163110

Downer, R.G.H., Matthews, J.R., 1976. Patterns of Lipid Distribution and Utilisation in Insects. Am. Zool. 16, 733–745. https://doi.org/10.1093/icb/16.4.733

Duarte, P.M., 2019. Unravelling the lipid profile of coastal insects from Ria de Aveiro, Portugal: implications for sustainable aquaculture. University of Aveiro. https://doi.org/ria.ua.pt/bitstream/10773/28378/1/Pedro%20Martins%20Duarte.pdf

Duchet, C., Grabicová, K., Kolar, V., Lepšová, O., Csercsa, A., Zdvihalova, B., Randák, T., Boukal, D., 2023. Combined effects of climate warming and environmentally relevant concentrations of pharmaceutical active compounds on a freshwater community. bioRxiv 244, 2023.03.31.535078.

Ebele, A.J., Abou-Elwafa Abdallah, M., Harrad, S., 2017. Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. Emerg. Contam. 3, 1–16. https://doi.org/10.1016/j.emcon.2016.12.004

Erasmus, J.H., Lorenz, A.W., Zimmermann, S., Wepener, V., Sures, B., Smit, N.J., Malherbe, W., 2021. A diversity and functional approach to evaluate the macroinvertebrate responses to multiple stressors in a small subtropical austral river. Ecol. Indic. 131, 108206. https://doi.org/10.1016/j.ecolind.2021.108206

Farooqui, T., 2012. Review of octopamine in insect nervous systems. Open access insect physiol. 1. https://doi.org/10.2147/OAIP.S20911

Finn, D.S., Johnson, S.L., Gerth, W.J., Arismendi, I., Li, J.L., 2022. Spatiotemporal patterns of emergence phenology reveal complex species-specific responses to temperature in aquatic insects. Divers. Distrib. 28, 1524–1541. https://doi.org/10.1111/ddi.13472

Firmansyah, Argosubekti, N., 2020. A review of heat stress signaling in plants. IOP Conf. Ser. Earth Environ. Sci. 484, 012041. https://doi.org/10.1088/1755-1315/484/1/012041

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509. https://doi.org/10.1016/S0021-9258(18)64849-5

Gorovits, R., Sobol, I., Akama, K., Chefetz, B., Czosnek, H., 2020. Pharmaceuticals in treated wastewater induce a stress response in tomato plants. Sci. Rep. 10, 1856.

https://doi.org/10.1038/s41598-020-58776-z

Grabicova, K., Grabic, R., Blaha, M., Kumar, V., Cerveny, D., Fedorova, G., Randak, T., 2015. Presence of pharmaceuticals in benthic fauna living in a small stream affected by effluent from a municipal sewage treatment plant. Water Res. 72, 145–153. https://doi.org/10.1016/j.watres.2014.09.018

Graf, W., Murphy, J., Dahl, J., Zamora-Munoz, C., Lopez-Rodriguez M.J. Schmidt-Kloiber., A., 2023. Dataset "Trichoptera". www.freshwaterecology.info - the taxa and autecology database for freshwater organisms, version 8.0 (accessed on 06.06.2023) [WWW Document]. https://doi.org/https://www.freshwaterecology.info/index.php

Grgić, I., Cetinić, K.A., Karačić, Z., Previšić, A., Rožman, M., 2023. Fate and effects of microplastics in combination with pharmaceuticals and endocrine disruptors in freshwaters: Insights from a microcosm experiment. Sci. Total Environ. 859. https://doi.org/10.1016/j.scitotenv.2022.160387

Halsch, C.A., Shapiro, A.M., Fordyce, J.A., Nice, C.C., Thorne, J.H., Waetjen, D.P., Forister, M.L., 2021. Insects and recent climate change. Proc. Natl. Acad. Sci. U. S. A. 118, 1–9. https://doi.org/10.1073/PNAS.2002543117

Hanson, B.J., Cummins, K.W., Cargill, A.S., Lowry, R.R., 1985. Lipid content, fatty acid composition, and the effect of diet on fats of aquatic insects. Comp. Biochem. Physiol. Part B Comp. Biochem. 80, 257–276. https://doi.org/10.1016/0305-0491(85)90206-8

Hering, D., Schmidt-Kloiber, A., Murphy, J., Lücke, S., Zamora-Muñoz, C., López-Rodríguez, M.J., Huber, T., Graf, W., 2009. Potential impact of climate change on aquatic insects: A sensitivity analysis for European caddisflies (Trichoptera) based on distribution patterns and ecological preferences. Aquat. Sci. 71, 3–14. https://doi.org/10.1007/s00027-009-9159-5

Hershkovitz, Y., Dahm, V., Lorenz, A.W., Hering, D., 2015. A multi-trait approach for the identification and protection of European freshwater species that are potentially vulnerable to the impacts of climate change. Ecol. Indic. 50, 150–160.

https://doi.org/10.1016/j.ecolind.2014.10.023

Heye, K., Lotz, T., Wick, A., Oehlmann, J., 2019. Interactive effects of biotic and abiotic environmental stressors on carbamazepine toxicity in the non-biting midge Chironomus riparius. Water Res. 156, 92–101. https://doi.org/10.1016/j.watres.2019.03.007

Hoppeler, F., Winkelmann, C., Becker, J., Pauls, S.U., 2018. Larval growth and metabolic energy storage of Micropterna lateralis (Trichoptera: Limnephilidae) in an intermittent stream: glycogen dominates in final instars. Hydrobiologia 806, 175–185. https://doi.org/10.1007/s10750-017-3354-5

Huryn, A.D., Wallace, J.B., 2000. LIFE HISTORY AND PRODUCTION OF STREAM INSECTS. Annu. Rev. Entomol. 83–110. https://doi.org/10.1146/annurev.ento.51.110104.151107

Jarvis, A.L., Bernot, M.J., Bernot, R.J., 2014. The effects of the pharmaceutical carbamazepine on life history characteristics of flat-headed mayflies (Heptageniidae) and aquatic resource interactions. Ecotoxicology 23, 1701–1712. https://doi.org/10.1007/s10646-014-1309-4

Kainz, M.J., Fisk, A.T., 2009. Integrating lipids and contaminants in aquatic ecology and ecotoxicology, in: Lipids in Aquatic Ecosystems. Springer New York, New York, NY, pp. 93–114. https://doi.org/10.1007/978-0-387-89366-2_5

Katajamaa, M., Miettinen, J., Orešič, M., 2006. MZmine: Toolbox for processing and visualization of mass spectrometry based molecular profile data. Bioinformatics 22, 634–636. https://doi.org/10.1093/bioinformatics/btk039

Kazmi, S.S.U.H., Wang, Y.Y.L., Cai, Y.E., Wang, Z., 2022. Temperature effects in single or combined with chemicals to the aquatic organisms: An overview of thermo-chemical stress. Ecol. Indic. 143, 109354. https://doi.org/10.1016/j.ecolind.2022.109354

Konschak, M., Zubrod, J.P., Baudy, P., Englert, D., Herrmann, B., Schulz, R., Bundschuh, M., 2019. Waterborne and diet-related effects of inorganic and organic fungicides on the insect leaf shredder Chaetopteryx villosa (Trichoptera). Aquat. Toxicol. 206, 33–42. https://doi.org/10.1016/j.aquatox.2018.10.021

776 Krajick, K., 2004. All Downhill From Here? Science (80-.). 303, 1600–1602.
777 https://doi.org/10.1126/science.303.5664.1600

778 Kraufvelin, P., 1998. Model ecosystem replicability challenged by the “soft” reality of a hard
779 bottom mesocosm. J. Exp. Mar. Bio. Ecol. 222, 247–267. https://doi.org/10.1016/S0022-
780 0981(97)00143-3

781 Lagesson, A., Fahlman, J., Brodin, T., Fick, J., Jonsson, M., Byström, P., Klaminder, J., 2016.
782 Bioaccumulation of five pharmaceuticals at multiple trophic levels in an aquatic food web -
783 Insights from a field experiment. Sci. Total Environ. 568, 208–215.
784 https://doi.org/10.1016/j.scitotenv.2016.05.206

785 Lease, H.M., Wolf, B.O., 2011. Lipid content of terrestrial arthropods in relation to body size,
786 phylogeny, ontogeny and sex. Physiol. Entomol. 36, 29–38.
787 https://doi.org/10.1111/j.1365-3032.2010.00767.x

788 Liess, M., Schulz, R., 1996. Chronic effects of short-term contamination with the pyrethroid
789 insecticide fenvalerate on the caddisfly Limnephilus lunatus. Hydrobiologia 324, 99–106.
790 https://doi.org/10.1007/BF00018170

791 Lindsey, E., Altizer, S., 2009. Sex differences in immune defenses and response to parasitism in
792 monarch butterflies. Evol. Ecol. 23, 607–620. https://doi.org/10.1007/s10682-008-9258-0

793 López-Doval, J.C., Kukkonen, J.V.K., Rodrigo, P., Muñoz, I., 2012. Effects of indomethacin and
794 propranolol on Chironomus riparius and Physella (Costatella) acuta. Ecotoxicol. Environ.
795 Saf. 78, 110–115. https://doi.org/10.1016/j.ecoenv.2011.11.004

796 Lu, Eiriksson, Thorsteinsdóttir, Simonsen, 2019. Valuable Fatty Acids in Bryophytes—
797 Production, Biosynthesis, Analysis and Applications. Plants 8, 524.
798 https://doi.org/10.3390/plants8110524

799 Macadam, C.R., England, J., Chadd, R., 2022. The vulnerability of British aquatic insects to
800 climate change. Knowl. Manag. Aquat. Ecosyst. 2022-Janua, 3.
801 https://doi.org/10.1051/kmae/2022003

Maenpaa, K., Kukkonen, J., 2006. Bioaccumulation and toxicity of 4-nonylphenol (4-NP) and 4-(2-dodecyl)-benzene sulfonate (LAS) in Lumbriculus variegatus (Oligochaeta) and Chironomus riparius (Insecta). Aquat. Toxicol. 77, 329–338. https://doi.org/10.1016/j.aquatox.2006.01.002

Mandaric, L., Celic, M., Marcé, R., Petrovic, M., 2015. Introduction on Emerging Contaminants in Rivers and Their Environmental Risk, in: Handbook of Environmental Chemistry. pp. 3–25. https://doi.org/10.1007/698_2015_5012

McIntosh, A.R., Leigh, C., Boersma, K.S., McHugh, P.A., Febria, C., García-Berthou, E., 2017. Food Webs and Trophic Interactions in Intermittent Rivers and Ephemeral Streams, in: Intermittent Rivers and Ephemeral Streams. Elsevier, pp. 323–347. https://doi.org/10.1016/B978-0-12-803835-2.00012-7

Mikami, K., Hartmann, E., 2004. Lipid Metabolism in Mosses, in: New Frontiers in Bryology. Springer Netherlands, Dordrecht, pp. 133–155. https://doi.org/10.1007/978-0-306-48568-8_8

Mirth, C.K., Riddiford, L.M., 2007. Size assessment and growth control: how adult size is determined in insects. BioEssays 29, 344–355. https://doi.org/10.1002/bies.20552

Muñoz, I., López-Doval, J.C., De Castro-Català, N., Kuzmanovic, M., Ginebreda, A., Sabater, S., 2015. Effects of Emerging Contaminants on Biodiversity, Community Structure, and Adaptation of River Biota, in: Handbook of Environmental Chemistry. pp. 79–119. https://doi.org/10.1007/698_2015_5013

Nilsen, E., Smalling, K.L., Ahrens, L., Gros, M., Miglioranza, K.S.B., Picó, Y., Schoenfuss, H.L., 2019. Critical review: Grand challenges in assessing the adverse effects of contaminants of emerging concern on aquatic food webs. Environ. Toxicol. Chem. 38, 46–60. https://doi.org/10.1002/etc.4290

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: Environmental contaminants in a warming world. Environ. Int. 35, 971–986. https://doi.org/10.1016/j.envint.2009.02.006

Nukazawa, K., Arai, R., Kazama, S., Takemon, Y., 2018. Projection of invertebrate populations in the headwater streams of a temperate catchment under a changing climate. Sci. Total Environ. 642, 610–618. https://doi.org/10.1016/j.scitotenv.2018.06.109

Oksanen, J., Simpson, G.L., Blanchet, F.G., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., Durand, S., Beatriz, H., Evangelista, A., Friendly, M., Hannigan, G., Hill, M.O., Lahti, L., Mcglinn, D., Ribeiro, E., Smith, T., Stier, A., Ter, C.J.F., Weedon, J., 2020. Title Community Ecology Package. Vegan Community Ecol. Packag. R Packag. version 2.5-7. https://doi.org/https://github.com/vegandevs/vegan

Pestana, J.L.T., Novais, S.C., Lemos, M.F.L., Soares, A.M.V.M., 2014. Cholinesterase activity in the caddisfly Sericostoma vittatum: Biochemical enzyme characterization and in vitro effects of insecticides and psychiatric drugs. Ecotoxicol. Environ. Saf. 104, 263–268. https://doi.org/10.1016/j.ecoenv.2014.03.012

Piggott, J.J., Townsend, C.R., Matthaei, C.D., 2015. Reconceptualizing synergism and antagonism among multiple stressors. Ecol. Evol. 5, 1538–1547. https://doi.org/10.1002/ece3.1465

Polazzo, F., Roth, S.K., Hermann, M., Mangold-Döring, A., Rico, A., Sobek, A., Van den Brink, P.J., Jackson, M.C., 2022. Combined effects of heatwaves and micropollutants on freshwater ecosystems: Towards an integrated assessment of extreme events in multiple stressors research. Glob. Chang. Biol. https://doi.org/10.1111/gcb.15971

Previšić, A., Rožman, M., Mor, J.R., Acuña, V., Serra-Compte, A., Petrović, M., Sabater, S., 2020. Aquatic macroinvertebrates under stress: Bioaccumulation of emerging contaminants and metabolomics implications. Sci. Total Environ. 704, 135333. https://doi.org/10.1016/j.scitotenv.2019.135333

Previšić, A., Vilenica, M., Vučković, N., Petrović, M., Rožman, M., 2021. Aquatic Insects Transfer Pharmaceuticals and Endocrine Disruptors from Aquatic to Terrestrial Ecosystems. Environ. Sci. Technol. 55, 3736–3746. https://doi.org/10.1021/acs.est.0c07609

Qin, W., Tyshenko, M.G., Wu, B.S., Walker, V.K., Robertson, R.M., 2003. Cloning and characterization of a member of the hsp70 gene family from Locusta migratoria, a highly thermotolerant insect. Cell Stress Chaperones 8, 144. https://doi.org/10.1379/1466-1268(2003)008<0144:CACOAM>2.0.CO;2

Rejeb, I., Pastor, V., Mauch-Mani, B., 2014. Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms. Plants 3, 458–475. https://doi.org/10.3390/plants3040458

Rožman, M., Acuña, V., Petrović, M., 2018. Effects of chronic pollution and water flow intermittency on stream biofilms biodegradation capacity. Environ. Pollut. 233, 1131–1137. https://doi.org/10.1016/j.envpol.2017.10.019

Sanderson, H., 2002. Pesticide studies: Replicability of micro/mesocosms. Environ. Sci. Pollut. Res. 9, 429–435. https://doi.org/10.1007/BF02987597

Sarafian, M.H., Gaudin, M., Lewis, M.R., Martin, F.P., Holmes, E., Nicholson, J.K., Dumas, M.E., 2014. Objective set of criteria for optimization of sample preparation procedures for ultra-high throughput untargeted blood plasma lipid profiling by ultra performance liquid chromatography-mass spectrometry. Anal. Chem. 86, 5766–5774. https://doi.org/10.1021/ac500317c

Sartori, M., Keller, L., Thomas, A.G.B., Passera, L., 1992. Flight energetics in relation to sexual differences in the mating behaviour of a mayfly, Siphlonurus aestivalis. Oecologia 92, 172–176. https://doi.org/10.1007/BF00317360

Schäfer, R.B., Piggott, J.J., 2018. Advancing understanding and prediction in multiple stressor research through a mechanistic basis for null models. Glob. Chang. Biol. 24, 1817–1826. https://doi.org/10.1111/gcb.14073

Schulz, R., Liess, M., 2001. Toxicity of Aqueous-Phase and Suspended Particle-Associated Fenvalerate: Chronic Effects After Pulse-Dosed Exposure of Limnephilus Lunatus (Trichoptera). Environ. Toxicol. Chem. 20, 185. https://doi.org/10.1897/1551-5028(2001)020<0185:toapas>2.0.co;2

Schulz, R., Liess, M., 1995. Chronic effects of low insecticide concentrations on freshwater caddisfly larvae. Hydrobiologia 299, 103–113. https://doi.org/10.1007/BF00017562

Serra-Compte, A., Maulvault, A.L., Camacho, C., Álvarez-Muñoz, D., Barceló, D., Rodríguez-Mozaz, S., Marques, A., 2018. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (Mytilus galloprovincialis). Environ. Pollut. 236, 824–834. https://doi.org/10.1016/j.envpol.2018.02.018

Shipley, J.R., Twining, C.W., Mathieu-Resuge, M., Parmar, T.P., Kainz, M., Martin-Creuzburg, D., Weber, C., Winkler, D.W., Graham, C.H., Matthews, B., 2022. Climate change shifts the timing of nutritional flux from aquatic insects. Curr. Biol. 32, 1342-1349.e3. https://doi.org/10.1016/j.cub.2022.01.057

Slos, S., De Meester, L., Stoks, R., 2009. Food level and sex shape predator-induced physiological stress: immune defence and antioxidant defence. Oecologia 161, 461–467. https://doi.org/10.1007/s00442-009-1401-2

Snart, C.J.P., Hardy, I.C.W., Barrett, D.A., 2015. Entometabolomics: applications of modern analytical techniques to insect studies. Entomol. Exp. Appl. 155, 1–17. https://doi.org/10.1111/eea.12281

Späth, J., Fick, J., McCallum, E., Cerveny, D., Nording, M.L., Brodin, T., 2022. Wastewater effluent affects behaviour and metabolomic endpoints in damselfly larvae. Sci. Rep. 12, 1–14. https://doi.org/10.1038/s41598-022-10805-9

Stillwell, R.C., Davidowitz, G., 2010. Sex differences in phenotypic plasticity of a mechanism that controls body size: implications for sexual size dimorphism. Proc. R. Soc. B Biol. Sci. 277, 3819–3826. https://doi.org/10.1098/rspb.2010.0895

Stubbington, R., Bogan, M.T., Bonada, N., Boulton, A.J., Datry, T., Leigh, C., Vander Vorste, R., 2017. The Biota of Intermittent Rivers and Ephemeral Streams: Aquatic Invertebrates, Intermittent Rivers and Ephemeral Streams: Ecology and Management. Elsevier Inc. https://doi.org/10.1016/B978-0-12-803835-2.00007-3

Sun, C., Dudley, S., Trumble, J., Gan, J., 2018. Pharmaceutical and personal care products-induced stress symptoms and detoxification mechanisms in cucumber plants. Environ. Pollut. 234, 39–47. https://doi.org/10.1016/j.envpol.2017.11.041

Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R., 2014. Abiotic and biotic stress combinations. New Phytol. 203, 32–43. https://doi.org/10.1111/nph.12797

ter Braak, C.J.F., Šmilauer, P., 2012. Canoco reference manual and user's guide: software for ordination, version 5.0. Microcomputer Power.

Tijani, J.O., Fatoba, O.O., Petrik, L.F., 2013. A Review of Pharmaceuticals and Endocrine-Disrupting Compounds: Sources, Effects, Removal, and Detections. Water, Air, Soil Pollut. 224, 1770. https://doi.org/10.1007/s11270-013-1770-3

Torres-Ruiz, M., Wehr, J.D., Perrone, A.A., 2010. Are net-spinning caddisflies what they eat? An investigation using controlled diets and fatty acids. J. North Am. Benthol. Soc. 29, 803–813. https://doi.org/10.1899/09-162.1

Van Geest, J.L., Poirier, D.G., Sibley, P.K., Solomon, K.R., 2010. Measuring bioaccumulation of contaminants from field-collected sediment in freshwater organisms: A critical review of laboratory methods. Environ. Toxicol. Chem. 29, 2391–2401. https://doi.org/10.1002/etc.326

Vescio, R., Caridi, R., Laudani, F., Palmeri, V., Zappalà, L., Badiani, M., Sorgonà, A., 2022. Abiotic and Herbivory Combined Stress in Tomato: Additive, Synergic and Antagonistic Effects and Within-Plant Phenotypic Plasticity. Life 12, 1804. https://doi.org/10.3390/life12111804

Veseli, M., Rožman, M., Vilenica, M., Petrović, M., Previšić, A., 2022. Bioaccumulation and bioamplification of pharmaceuticals and endocrine disruptors in aquatic insects. Sci. Total Environ. 838. https://doi.org/10.1016/j.scitotenv.2022.156208

von Schiller, D., Acuña, V., Aristi, I., Arroita, M., Basaguren, A., Bellin, A., Boyero, L., Butturini, A., Ginebreda, A., Kalogianni, E., Larrañaga, A., Majone, B., Martínez, A., Monroy, S., Muñoz, I., Paunović, M., Pereda, O., Petrovic, M., Pozo, J., Rodríguez-Mozaz, S., Rivas, D.,

Sabater, S., Sabater, F., Skoulikidis, N., Solagaistua, L., Vardakas, L., Elosegi, A., 2017. River ecosystem processes: A synthesis of approaches, criteria of use and sensitivity to environmental stressors. Sci. Total Environ. 596–597, 465–480. https://doi.org/10.1016/j.scitotenv.2017.04.081

Wang, P., Zhu, B.-T., 2017. Unique effect of 4-hydroxyestradiol and its methylation metabolites on lipid and cholesterol profiles in ovariectomized female rats. Eur. J. Pharmacol. 800, 107–117. https://doi.org/10.1016/j.ejphar.2017.02.032

Waringer, J., Graf, W., 2011. Atlas of Central European Trichoptera Larvae. Erik Mauch Verlag, Dinkelscherben.

Wat, L.W., Chao, C., Bartlett, R., Buchanan, J.L., Millington, J.W., Chih, H.J., Chowdhury, Z.S., Biswas, P., Huang, V., Shin, L.J., Wang, L.C., Gauthier, M.-P.L., Barone, M.C., Montooth, K.L., Welte, M.A., Rideout, E.J., 2020. A role for triglyceride lipase brummer in the regulation of sex differences in Drosophila fat storage and breakdown. PLOS Biol. 18, e3000595. https://doi.org/10.1371/journal.pbio.3000595

Webb, B.W., Hannah, D.M., Moore, R.D., Brown, L.E., Nobilis, F., 2008. Recent advances in stream and river temperature research. Hydrol. Process. 22, 902–918. https://doi.org/10.1002/hyp.6994

Wilkinson, J., Hooda, P.S., Barker, J., Barton, S., Swinden, J., 2017. Occurrence, fate and transformation of emerging contaminants in water: An overarching review of the field. Environ. Pollut. 231, 954–970. https://doi.org/10.1016/j.envpol.2017.08.032

Wrona, F.J., Prowse, T.D., Reist, J.D., Hobbie, J.E., Lévesque, L.M.J., Vincent, W.F., 2006. Climate change effects on aquatic biota, ecosystem structure and function. Ambio 35, 359–369. https://doi.org/10.1579/0044-7447(2006)35[359:CCEOAB]2.0.CO;2

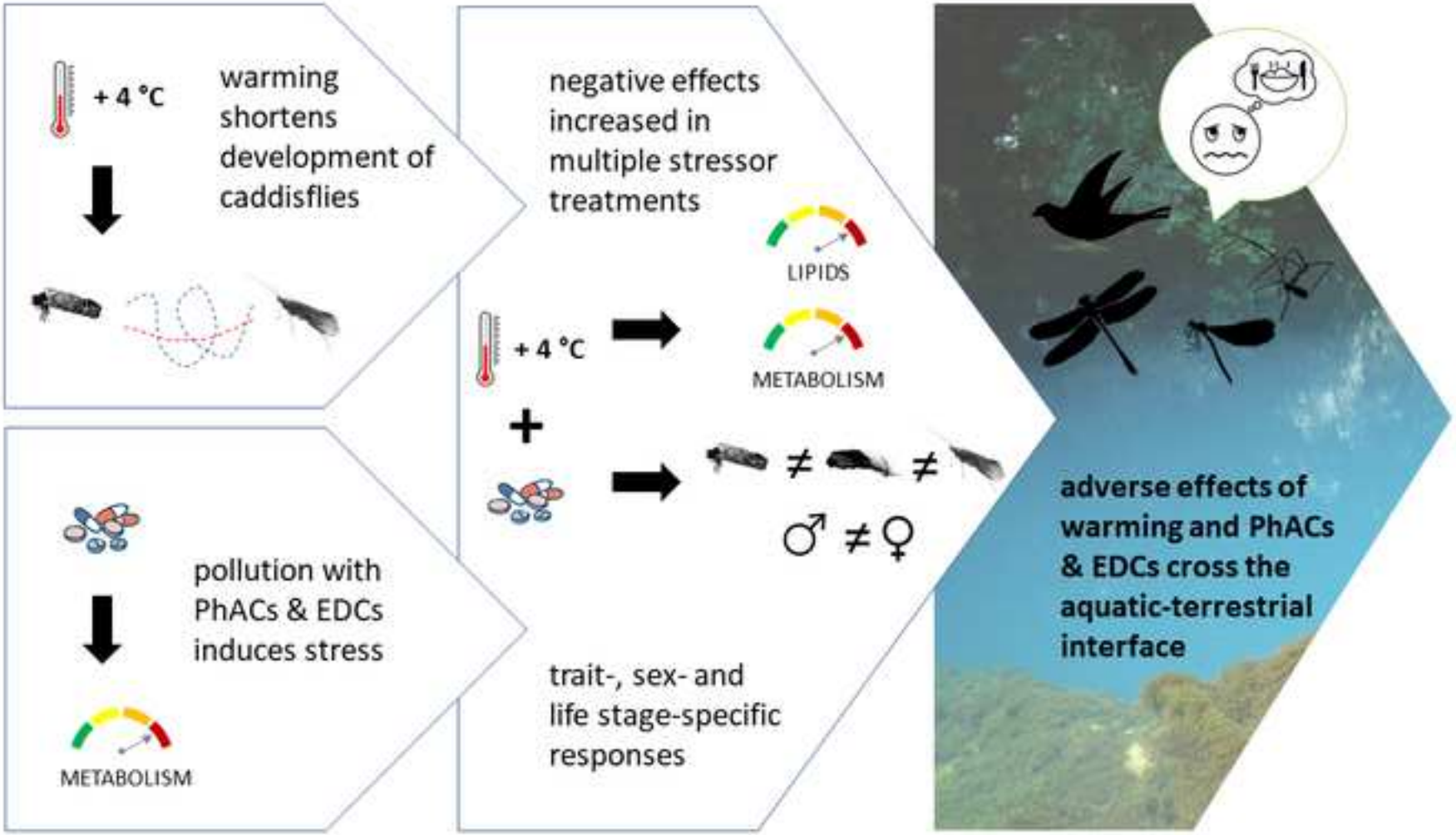
Yoon, D.S., Byeon, E., Kim, D.H., Lee, M.C., Shin, K.H., Hagiwara, A., Park, H.G., Lee, J.S., 2022. Effects of temperature and combinational exposures on lipid metabolism in aquatic invertebrates. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 262, 109449. https://doi.org/10.1016/j.cbpc.2022.109449

962 Zezulka, Š., Kummerová, M., Babula, P., Váňová, L., 2013. Lemna minor exposed to
963 fluoranthene: Growth, biochemical, physiological and histochemical changes. Aquat.
964 Toxicol. 140–141, 37–47. https://doi.org/10.1016/j.aquatox.2013.05.011

965

966

Graphical Abstract



Highlights

1. Warming was a dominant stressor for development-related traits of the caddisfly
2. Negative effects of warming on lipids were increased by presence of PhACs&EDCs
3. Pollution with PhACs & EDCs has higher impact on caddisfly metabolism than warming
4. Trait-, sex- and life stage-specific responses to multiple stressors were observed
5. Impacts of warming and PhACs & EDCs cross the aquatic-terrestrial interface

Manuscript (double-spaced and continuously LINE and PAGE numbered)-for final publication

Click here to view linked References

Pharmaceuticals and endocrine disrupting compounds modulate adverse effects of climate change on resource quality in freshwater food webs

Iva, Kokotović[1]; Marina, Veseli[1]; Filip, Ložek[1,2]; Zrinka, Karačić[3]; Marko, Rožman[3]; Ana, Previšić[1]

[1] Department of Biology, Zoology, Faculty of Science, University of Zagreb, Zagreb, Croatia

[2] South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic

[3] Ruđer Bošković Institute, Zagreb, Croatia

Iva Kokotović: iva.kokotovic@biol.pmf.hr

Marina Veseli: marina.veseli@biol.pmf.hr

Filip Ložek: lozekf@frov.jcu.cz

Zrinka Karačić: zrinka.karacic@irb.hr

Marko Rožman: marko.rozman@irb.hr

Ana Previšić: ana.previsic@biol.pmf.hr

*Corresponding author:

Ana Previšić

Department of Biology, Zoology, Faculty of Science, University of Zagreb

Horvatovac 102a, 10000 Zagreb, Croatia

ana.previsic@biol.pmf.hr

These two authors contributed equally: Ana Previšić and Marko Rožman

Abstract

Freshwater biodiversity, ecosystem functions and services are changing at an unprecedented rate due to the impacts of vast number of stressors overlapping in time and space. Our study aimed at characterizing individual and combined impacts of pollution with pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) and increased water temperature (as a proxy for climate change) on primary producers and first level consumers in freshwaters. We conducted a microcosm experiment with a simplified freshwater food web containing moss (Bryophyta) and shredding caddisfly larvae of *Micropterna nycterobia* (Trichoptera). The experiment was conducted with four treatments; control (C), increased water temperature +4 °C (T2), emerging contaminants' mix (EC = 15 PhACs & 5 EDCs), and multiple stressor treatment (MS = EC + T2). Moss exhibited an overall mild response to selected stressors and their combination. Higher water temperature negatively affected development of *M. nycterobia* through causing earlier emergence of adults and changes in their lipidome profiles. Pollution with PhACs and EDCs had higher impact on metabolism of all life stages of *M. nycterobia* than warming. Multiple stressor effect was recorded in *M. nycterobia* adults in metabolic response, lipidome profiles and as a decrease in total lipid content. Sex specific response to stressor effects was observed in adults, with impacts on metabolome generally more pronounced in females, and on lipidome in males. Thus, our study highlights the variability of both single and multiple stressor impacts on different traits, different life stages and sexes of a single insect species. Furthermore, our research suggests that the combined impacts of warming, linked to climate change, and contamination with PhACs and EDCs could have adverse consequences on the population dynamics of aquatic insects. Additionally, these findings point to a potential decrease in the quality of resources available for both aquatic and potentially terrestrial food webs.

Keywords:

climate change, pollution, caddisflies, sex specific stress response, aquatic-terrestrial subsidies, ecosystem subsidies

Highlights:

- warming was a dominant stressor for development-related traits of the caddisfly
- negative effects of warming on lipids were increased by presence of PhACs&EDCs
- pollution with PhACs & EDCs has higher impact on caddisfly metabolism than warming
- trait-, sex- and life stage-specific responses to multiple stressors were observed
- impacts of warming and PhACs & EDCs cross the aquatic-terrestrial interface

1. INTRODUCTION

Freshwater ecosystems are susceptible to various anthropogenic stressors (e.g. chemical pollution, climate change, habitat loss, invasive species) which mostly coincide. Pharmaceuticals (PhACs) and endocrine disrupting compounds (EDCs) are a diverse group of pollutants designed to be biologically active at low doses, targeting specific metabolic and molecular pathways in humans and animals, thus posing risk for aquatic organisms even at low environmental concentrations (Ebele et al., 2017; Tijani et al., 2013; Wilkinson et al., 2017). Moreover, EDCs encompass a wide range of chemicals (e.g. personal care products, cleaning products, food preservatives, etc.) that interfere with normal function(s) of the endocrine system (Ebele et al., 2017). PhACs and EDCs were shown to affect aquatic insects in many ways, causing changes in growth and development (Jarvis et al., 2014), biomass (López-Doval et al., 2012), enzymatic activity (Pestana et al., 2014), metabolome composition (Grgić et al., 2023; Previšić et al., 2020; Späth et al., 2022), behavior (Jarvis et al., 2014; Späth et al., 2022) and survival rate (López-Doval et al., 2012; Maenpaa and Kukkonen, 2006). Furthermore, PhACs and EDCs have the potential to bioaccumulate in aquatic insects (Grabicova et al., 2015; Lagesson et al., 2016; Previšić et al., 2021; Veseli et al., 2022) and to cross ecosystem boundaries through emerging aquatic insects and thus contaminate terrestrial habitats (Previšić et al., 2021; Veseli et al., 2022). Moreover, in freshwater ecosystems these compounds rarely occur one at a time but rather in complex mixtures of several different emerging contaminants, thus making it harder to investigate their effects (Wilkinson et al., 2017).

Climate change, with its long-term shifts in global weather patterns, poses significant challenges to freshwater ecosystems. Within freshwater ecosystems, aquatic insect groups, such as caddisflies, mayflies, and stoneflies, play a crucial role as inter-habitat linkages between aquatic and terrestrial ecosystems, facilitating the flow of energy and nutrients (Huryn and Wallace, 2000). Additionally, they serve as valuable bioindicators for assessing the health of freshwater environments (Water Framework Directive (WFD) 2000/60/EC). Their vulnerability to climate change is influenced by their specific biological traits and ecological preferences, with a considerable proportion of them belonging to cold-adapted taxa (Conti et al., 2014; Hershkovitz

et al., 2015). The impact of climate change, characterized by rising temperatures and changes in precipitation levels (Webb et al., 2008), is particularly profound on these cold-adapted taxa in higher altitudes, making them highly vulnerable to warming (Krajick, 2004; Macadam et al., 2022). As a consequence of climate change, temperature increase affects various aspects of aquatic insects' lives. It directly impacts their growth, development, and body size (Cogo et al., 2020), as well as their emergence patterns (Finn et al., 2022). The anticipated rise in the frequency and extent of intermittent rivers and streams, which periodically cease flow or even completely dry, is a direct consequence of global climate change and the increasing human demand for freshwater resources (Blackman et al., 2021). This trend holds significant implications for aquatic insect communities, as considerably altered environmental conditions not only impact their geographic distribution (Cogo et al., 2020 and references therein), but also their population dynamics (Nukazawa et al., 2018), and community structure (Dorić et al., 2023).

Global warming is likely to exacerbate impact of other anthropogenic stressors (Wrona et al., 2006). For instance, as temperature rises, the solubility and mobility of PhACs and EDCs in water can increase, leading to higher concentrations and potentially greater toxicity (Kazmi et al., 2022; Noyes et al., 2009). Temperature change may also alter degradation rates of some chemical contaminants, with increasing temperature usually shortening their half-life and reducing the overall risk (Bhangare et al., 2022; Noyes et al., 2009). While accelerated decomposition reduces the concentration of the parent compound, it increases the concentration of the degradation products, which in some cases could be even more toxic for aquatic organisms (Noyes et al., 2009). Warmer water usually increases the metabolic rate of aquatic organisms, thus leading to potentially increased uptake of these chemicals (Kazmi et al., 2022). In addition to increasing uptake, warming can also affect behaviour and physiology of aquatic organisms making them more susceptible to stressors (Polazzo et al., 2022). Toxicity of chemicals in water can depend on variety of abiotic (e.g. photolysis, hydrolysis, etc.) and biotic processes (e.g. biotransformation, biodegradation, etc.) (von Schiller et al., 2017). Moreover, the impact of warming on the toxicity of PhACs and EDCs in water is complex and can depend on a variety of factors, including the type of the chemical (Serra-Compte et al., 2018), aquatic species (Duchet et al., 2023, preprint), and life-stage tested (DeCourten and Brander, 2017). Due to the increase in temperature caused by

116 climate change, the temperature-dependent toxicity of PhACs and EDCs in water will become a
117 matter of growing significance that warrants assessment.

118 It is generally very challenging to gain empirical understanding of the effects of climate change
119 in comparison with other stressors (Halsch et al., 2021), as well as their combined effects (Dinh
120 et al., 2022). Consequently, there have been only a handful of studies investigating single and
121 combined effects of increased water temperature and PhACs and EDCs in aquatic invertebrates
122 (e.g. Barbosa et al., 2017; Cruzeiro et al., 2019; Heye et al., 2019). More specifically,
123 carbamazepine and higher temperatures increased *Chironomus riparius* mortality (Heye et al.,
124 2019); fluoxetine combined with higher temperature reduced reproductive success and
125 population growth in *Daphnia magna* (Barbosa et al., 2017), whereas levonorgestrel and
126 increased temperature did not cause DNA damage in *Gammarus locusta* cells (Cruzeiro et al.,
127 2019). To address this knowledge gap, we conducted a study aimed at characterizing effects of
128 increased water temperature and exposure to PhACs and EDCs on primary producers (non-
129 vascular macrophytes; moss) and first level consumers (shredding aquatic insects) in freshwaters.
130 Considering the importance of aquatic insects as fundamental links between aquatic and
131 terrestrial food webs and reliable bioindicators to pollution (Erasmus et al., 2021; Muñoz et al.,
132 2015) we chose caddisflies as our model organisms. Caddisflies are a species-rich and ecologically
133 diverse insect order, that is well-suited to reflect effects of various stressors on aquatic
134 ecosystems (Hering et al., 2009). Despite of the high ecological plasticity and thermal tolerance
135 of intermittent rivers specialists (Stubbington et al., 2017) we hypothesized that the individual
136 and combined stressor effects of both, increased water temperature and PhACs and EDCs, will
137 trigger a stress response in an intermittent river caddisfly, *Micropterna nycterobia* (McLachlan,
138 1875). Chronic *in situ* exposure to pollution with ECs results in measurable changes of metabolite
139 levels in relatively pollution-tolerant caddisfly larvae (Previšić et al., 2020), whereas increased
140 temperature results in depletion of lipids and reduces developmental period of mayfly larvae
141 (Chou et al., 2018). Moreover, variable impacts on emergence and survival were observed in
142 dragonflies and an aquatic heteropteran exposed to increased water temperature, PhACs and
143 their combination (Duchet et al., 2023, preprint). Hence, we conducted a 78-day microcosm
144 experiment composed of a simplified freshwater food web exposed to a mixture of PhACs and

EDCs and increased water temperature as a proxy for climate change using the randomized factorial design. We analyzed the physiological changes in caddisflies and moss via non-targeted metabolomics and lipidomics, i.e. by evaluating alterations in the metabolite and lipid profiles of non-model organisms across their life cycles, as well as changes in emergence patterns, body weight and total lipid content.

2. MATERIALS AND METHODS

2.1 Microcosm Experiment: Experimental Design and Sample Collection

We conducted the microcosm experiment with a simplified freshwater food web containing nonvascular macrophytes (Bryophyta), hereafter moss and caddisfly larvae *Micropterna nycterobia* (McLachlan, 1875) (Limnephilidae, Trichoptera) feeding mainly as shredders. Trichoptera larvae, moss (*Cinclidotus aquaticus* (Hedw.) Bruch & Schimp and *Rhynchostegium riparioides* (Hedw.) Cardot), water, sand and stones collected from the pristine Krčić River (N44.027321 E16.318936), minimally impacted by anthropogenic activity, were used for the experiment. Microcosm setup followed Previšić et al., 2021 with modifications in number of microcosms, temperature regime and mixture of moss species. Upon collection, 12 microcosms (aquaria 30 × 20 × 15 cm) were installed with 3 L of water,10 tablespoons of sand, 3 large stones (> 10 cm),10 small stones (2–5 cm), 3 tufts of moss (6–8 cm in diameter and plants up to 15 cm in length) and 5th instar *M. nycterobia* larvae (ca. 30 larvae per aquarium). Constant oxygen levels were kept using aquaria air pumps, and to minimize evaporation, each microcosm was covered with a glass cover. Natural light and day-night regime was supplemented with the artificial light (with sunlight's spectrum) in the regime of 12 hours of light followed by 12 hours without light. All microcosms were acclimatized for 10 days at 10 °C under aforementioned conditions preceding the start of the experiment. The experiment was conducted in the randomized factorial design, with treatments as follows: Control (C), Increased temperature (T2), ECs mix (EC), multiple stressor treatment = ECs mix + increased temperature (EC + T2 = MS), with three replicates of each. Subsequently, 6 of them were exposed to a mixture of 20 ECs over a 78-days period. The composition of the ECs mixture was selected based on occurrence of particular

compounds in freshwaters in Europe (Mandaric et al., 2015), and included 15 PhACs; azaperol, acetaminophen, thiabendazole, levamisol hydrochloride, dexamethasone, ketoprofen, naproxen, ranitidine hydrochloride, soltalol hydrochloride, valsartan, diphenhydramine, clopidogrel hydrogen sulfate, hydrochlorothiazide, sertraline hydrochloride, cimetidine and 5 EDCs; benylparaben, ethylparaben, propylparaben, estriol, estradiol-β. The volume of water was kept constant by adding fresh dechlorinated tap water (ca. 100 mL every week), and the concentration of each compound was kept at a pseudo-constant concentration of 500 ng L^{-1}. Taking into consideration knowledge gained from our previous experiments (Cetinić et al., 2022; Grgić et al., 2023; Previšić et al., 2021), three stock solutions were prepared and 100 µL of the solution was added each day, every three days or once a month, depending on the compound. In this way, abiotic attenuation (sorption and/or (photo)degradation) was taken in consideration and the nominal concentration of each compound was maintained, more details provided in Supporting information (SI 1). As different temperature regimes needed to be followed, aquaria were placed in two different incubators (POL-EKO APARATURA, Poland). “Natural” temperature regime (T1) mimicked the regime of the Krčić spring reach before the drying phase (10 °C) and temperature was successively increased 0.5 °C every 15 days. Increased temperature regime (T2) followed the same pattern but with temperature increased by 4 °C, in accordance with patterns of reduced flow observed in the selected river coupled with projections of temperature increase due to climate change in the Mediterranean montane regions (Bravo et al., 2008).

Biota sampling included initial (after the acclimatization period - day 0 (D0)) and several consecutive collections (ca. day 15 (D15), day 30 (D30) and day 60 (D60) of exposure) including different life stages (larvae, pupae and adult stage), in accordance with the life cycle of holometabolous caddisflies. At each sampling date, we collected replicate samples from each microcosm, consisting of 2 g of moss and 3−5 Trichoptera larvae/pupae. However, these samples were pooled per treatment to mitigate the potential extreme effects of individual microcosms (Kraufvelin, 1998). The complexity and extended duration of micro and mesocosm test systems exacerbate issues like "aquarium individuality," where initially identical replicates diverge due to random effects, introducing significant variability and complicating the identification of statistically significant deviations (Kraufvelin, 1998; Sanderson, 2002). Additionally, the weight

reduction of adult aquatic insects during metamorphosis (Huryn and Wallace, 2000) poses a challenge, leading to high tissue demand. Consequently, analytical replicates for each treatment per sampling date were created after homogenizing the pooled samples (details under 2.2, Biota Sample Processing). The chosen approach aims to strike a balance between providing ecologically meaningful results with non-model organisms and minimizing methodological constraints. Trichoptera larvae were kept in clean aquaria for 24 h to allow for gut clearance prior to collection (Van Geest et al., 2010). Additionally, emerging adult *M. nycterobia* were collected as they emerged (daily), and sex of each individual was determined. These samples were pooled depending on sex for subsequent analyses due to tissue requirements and to match the overall sampling scheme, according to temperature treatments. Emergence following the „natural" temperature regime (T1) started ca. 2 weeks later than in T2, thus samples were pooled for dates 45-60 and 60-75 days of exposure. Samples in elevated temperature regime (T2) were pooled for dates: 30-37 and 38-47 days of exposure. We combined adult *M. nycterobia* from 45-60 days of exposure in T1 and 38-47 days of exposure in T2 to one time point (day 60 of exposure) in order to have approximately the same time period of exposure to selected stressors. All samples were freeze-dried upon collection and stored at −80 °C until further processing.

Oxygen concentration (mg L^{-1}), oxygen saturation (%), pH and electrical conductivity (mS cm^{-1}) were measured in every microcosm at the beginning of the experiment (D0) and subsequently every two weeks (D15, D30, D45 and D60) using Hach HQ40D portable multi-parameter probe (Hach, Germany).

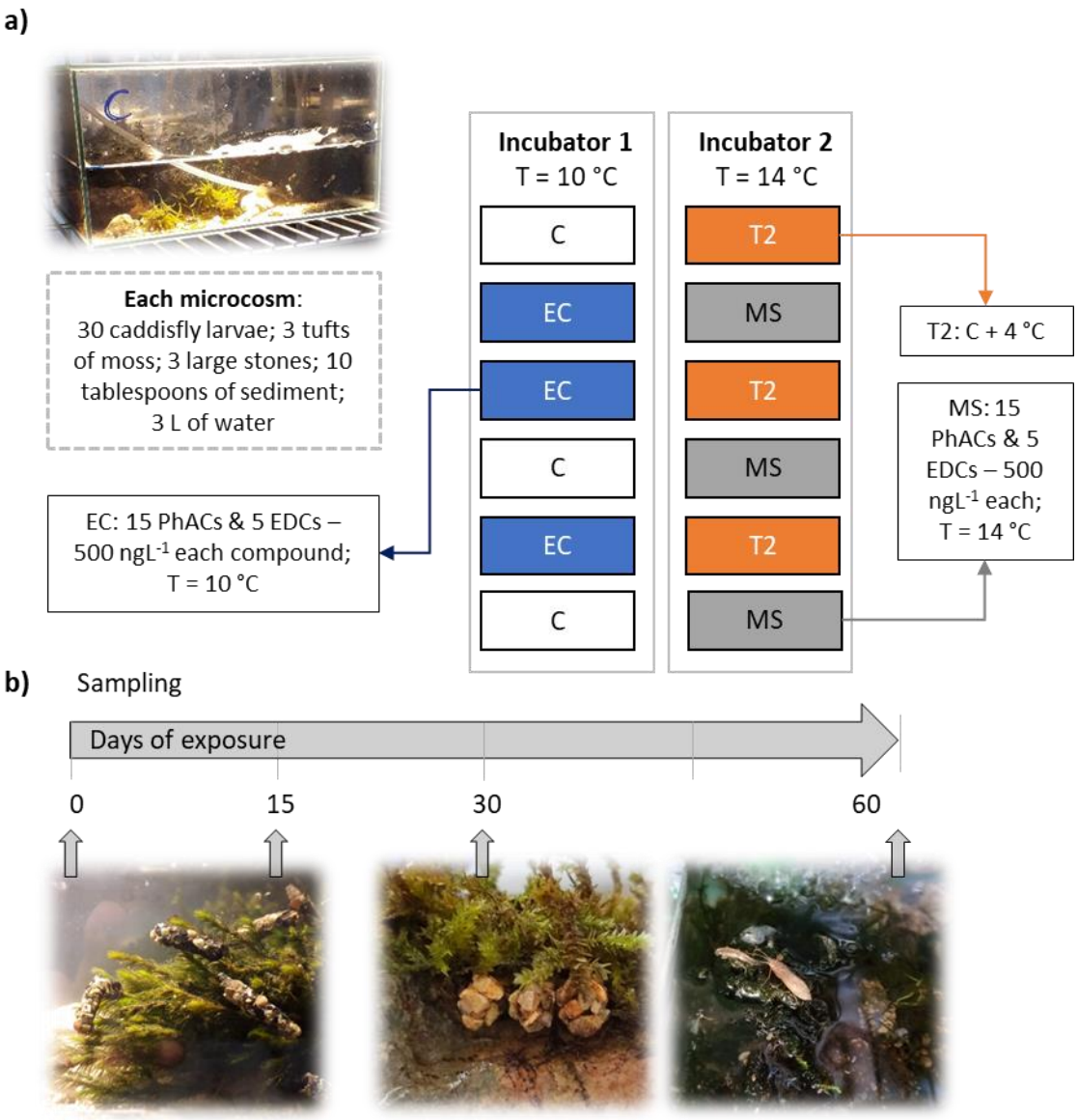


Figure 1. A) Experiment design with the following treatments: C - Control (T1 = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (EC + T2) and b) sampling scheme with caddisfly larvae, pupae and adults sampled at various days of exposure.

2.2. Biota sample processing: extractions of metabolites and lipids

Within this study, the term “metabolome” refers to the complete set of low molecular weight molecules or metabolites present within an organism, while “lipidome” is used to describe the complete lipid profile within an organism (i.e. all lipids).

Biota samples processing, metabolite and protein extraction and metabolite profiling analyses followed (Grgić et al., 2023). In order to detect Hsp70 proteins, we performed Western blot analysis of protein samples, the details are provided in Supporting Information (SI 2). As there was no detection of the aforementioned proteins no results are shown.

The Folch lipid extraction method (Folch et al., 1957) was used and performed following the protocol of (Sarafian et al., 2014) with modifications. Briefly, for lipidome profiling and determination of the total lipid content (TLC), each sample (30 mg) was dissolved in 600 µL $CHCl_3$: MeOH (2:1 v:v). Samples were vortexed at medium speed (IKA® Vortex Genius 3, Germany) for 5 min. After 10 min of incubation at room temperature, samples were cooled at -20 °C for 10 min and additional lysis was done via ultrasonic probe (Sonoplus HD4050, Bandelin electronic GmbH, Germany) for 1 min at 50% of intensity. Samples were stored overnight at −20 °C to improve protein precipitation and then centrifuged at 14 000g for 20 min (Tehtnica-Centric 200R, Slovenia). The supernatant was collected (600 µL) in a previously weighed tube, filtered through a PVDF filer (MILLEX® - GV Syringe Filter 0.22 µm Hydrophilic PVDF, 13 mm, Sterile) and evaporated to dryness. The tube was weighed to determine the TLC.TLC was determined using the following equation; TLC = weight of "full" tube – empty tube. Samples were dissolved in 200µL IPA : ACN : H2O (2:1:1, v:v:v) for subsequent LCMSMS non-target analysis.

For both metabolome and lipidome analysis, set of quality control samples was prepared by taking small aliquot of the each sample solution from the entire set and pooling them together. Subaliquots of this pooled sample are regarded as set of quality control samples.

2.3 Non-target metabolome and lipidome analysis

Non-target analyses of the metabolom and lipidome samples were performed using a high-resolution mass spectrometry system; LTQ-Orbitrap VelosTM (Thermo Fisher Scientific, USA) coupled with an ultra-performance liquid chromatography (UPLC) system (Ultimate 3000 RSLCnano system, Dionex, Amsterdam, Netherlands). Instrument parameters and UPLC gradients are provided in Supporting Information (SI). Data extraction, chromatographic deconvolution and final alignment were done using the MZmine program (Katajamaa et al., 2006). Steps and settings

used in the MZmine program are provided in Supporting information (SI 3). The exported .csv files were further filtered and sorted using modified parts of Bqunat script written in Mathematica (Wolfram Research Inc.,Campaign, IL, USA) (Rožman et al., 2018). Data were cleaned by removing of all blank-related features. Feature was considered as blank related if an intensity ratio sample:blank was < 10. Quality acceptance criteria for each metabolite were: detection rate > 70%, relative standard deviation < 30% and dispersion ratio < 40%. Based on the exact mass match, metabolite and lipid identification was performed in http://ceumass.eps.uspceu.es/ and by searching Metlin, Kegg, LipidMaps, PubChem, and HMDB databases. It is worth noting that the metabolites and lipids reported here are only the metabolites that were putatively annotated.

2.4 Statistical Analysis

2.4.1 Body weight changes & total lipid content of *Micropterna nycterobia* and non-vascular macrophytes, and physico-chemical water parameters

The effects of experimental treatments on body weight (evaluated individually in *M. nycterobia* specimens; N = 12 per treatment per collecting date) and total lipid content (TLC; evaluated in composite samples of *M. nycterobia* pooled per life stage and treatment; N = 3 per treatment per collection date) of different life stages of *M. nycterobia* were analysed using Generalized Linear Models (GZLMs) constructed in IBM SPSS Statistics 27.0 (IBM Corporation). Additionally, for adults the differences between the sexes were determined. Normal distribution linear scale response was used for all data except for body weight of adults where gamma scale response with log link was used as the data did not achieve normal distribution. Maximum likelihood estimate was used for parameter estimation. Pairwise contrasts of estimated means were performed using Wald's statistics.

The changes in total lipid content in moss, as well as changes in the physico-chemical water properties, between different treatments over time were tested using repeated measures ANOVA using the IBM SPSS Statistics 27.0 (IBM Corporation). Pairwise comparisons were

conducted with Bonferroni adjustment for multiple comparisons. Obtained data were analyzed and visualized using Principal Component Analysis (PCA) in Primer 7.

2.4.2. Data analysis of non-target metabolomic and lipidomic profiles of *Micropterna nycterobia* and non-vascular macrophytes

Obtained metabolomic and lipidomic data matrices were forth root transformed and analyzed using Principal Component Analysis (PCA) and Principal Response Curves (PRC) in package vegan (version 2.5-7) RStudio version 4.1.2 (Oksanen et al., 2020). Additionally, significance of the results was tested using the Monte Carlo test in the 'permute' package, with 99 permutations, more specifically significance of the 1st canonical axis of the PRC and significance of sampling date/insect stage was tested. Due to the missing moss sample D30 EC, Redundancy Analysis (RDA) was performed instead of PRC for moss metabolome and lipidome data using CANOCCO software (version 5.11,ter Braak and Šmilauer, 2012). We used treatment as the categorical explanatory variable, metabolites and lipids as response, respectively and time as a covariate. The RDA significances were tested using Monte Carlo test with 999 permutations. Values of MZ masses of the metabolome dataset were normalized and processed by Principal Component Analysis (PCA) in Rstudio. The PCA was visualized in Primer 7 (Clarke and Gorley, 2015). The first 100 variables (MZ masses) that contribute most to separation along certain PCs were selected for identification of metabolites.

3. RESULTS

3.1. Physico-chemical properties of water during microcosm experiment

Oxygen concentration (mg L-1) and oxygen saturation (%) throughout the experiment did not differ significantly among treatments, they were impacted only by the day of measurement (repeated measures ANOVA, Table S1, S2; and PCA analysis, the total variance accounted by the two components shown was ~69 %; variance from [PC1, PC2]≈[46.4 %, 22.6 %]; Fig. S1). Day of measurement and interactive effects; temperature x ECs, temperature x time and ECs x time caused changes in pH levels (repeated measures ANOVA, Table S3). Specifically, pH was higher

on D15 and D60 in treatments with ECs mixture (EC and MS). Additionally, on D30 and D45 in treatments with elevated water temperature (T2 and MS) pH was higher and lower, respectively (repeated measures ANOVA, Table S3). Electrical conductivity differed between treatments, i.e. treatments with increased water temperature (T2 and MS) had higher conductivity compared to the "natural" temperature regime (C and ECs) (repeated measures ANOVA, Table S4). Moreover, electrical conductivity was generally rising throughout the whole experiment in all treatments, thus it was also impacted by the day of exposure (repeated measures ANOVA, Table S4).

3.2. Total lipid content, metabolome and lipidome profiles of non-vascular macrophytes

Principal component analysis (PCA) based on non-target metabolic profiles and lipidome profile of moss revealed clustering primarily based on the duration of the experiment rather than treatment (Fig. 2A&B). In the analysis of the metabolome and lipidome, the total variance accounted by the first two components was ~45.5 % (variance from [PC1, PC2]≈[31.9 %, 13.6 %] and ~41,5 % (variance from [PC1, PC2]≈[28.8 %, 12.7 %]), respectively. In both analyses, the first principal component (PC) axis separated the profiles between D0 and D15 from D60, with D30 lacking a consistent grouping patterns (Fig. 2A&B; note however, that D30 EC samples are missing, as they were lost during the processing). In addition, RDA analyses shows that metabolome and lipidome profiles differed significantly among treatments (RDA: pseudo-F = 3.0, p = 0.002, explained variability = 12.1 %; Fig. S2A and RDA: pseudo-F = 2.2, p = 0.004, explained variability = 7.7 %; Fig. S2B, respectively). Majority of metabolite groups (17 in total) showing the most significant changes in abundance to stressor treatments were terpenoids, terpenes class (50%) followed by lipids and fatty acids (29%), amino acids, peptides and proteins (11%), carbonyl compounds (5%), organic acids, carboxylic acids and monocarboxylic acids (5%) (Fig. S3, Table S5). Within the lipidome, most significant changes in abundance of 51 lipids to stressor treatments were noticed in glycerophospholipids (51%) followed by glycerolipids (20%), sterol lipids (12%), sphingolipids (8%), prenol lipids (8%) and fatty acyls (2%) (Fig. S4, Table S6). The lowest total lipid content in all treatments was measured on D 15, however, no significant

changes in TLC was observed related to days of exposure or treatments (Fig. S5, repeated measures ANOVA, Table S7).

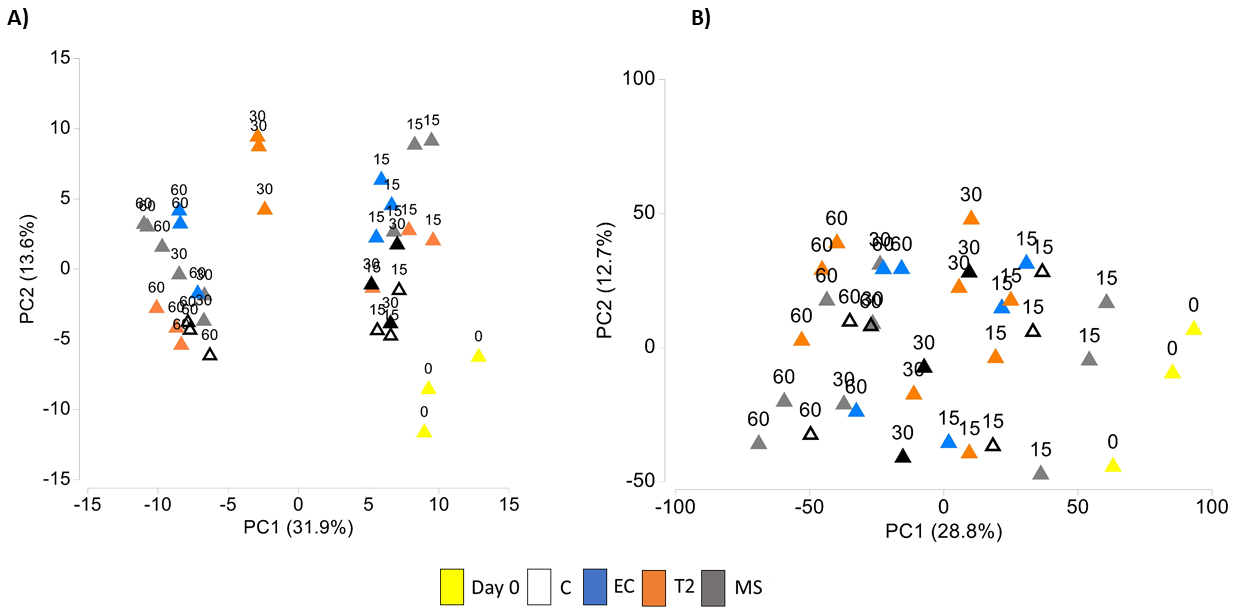


Figure 2. PCA plot showing separation based on A) metabolome and B) lipidome of moss in different treatments. Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC); Day 0 – pre-exposure sample.

3.3. Phenology (emergence patterns) of the caddisfly *Micropterna nycterobia*

Increased water temperature caused earlier emergence of both males and females, resulting in approximately three-week shift in peak emergence between the normal (C and EC) and elevated temperature (T2 and MS) treatments (Fig. 3). Overall, more males emerged throughout the experiment, however, the majority of pupae that did not emerge by the end of experiment were females. Only minor differences between emergence patterns of males and females were observed within treatments (Fig. 3).

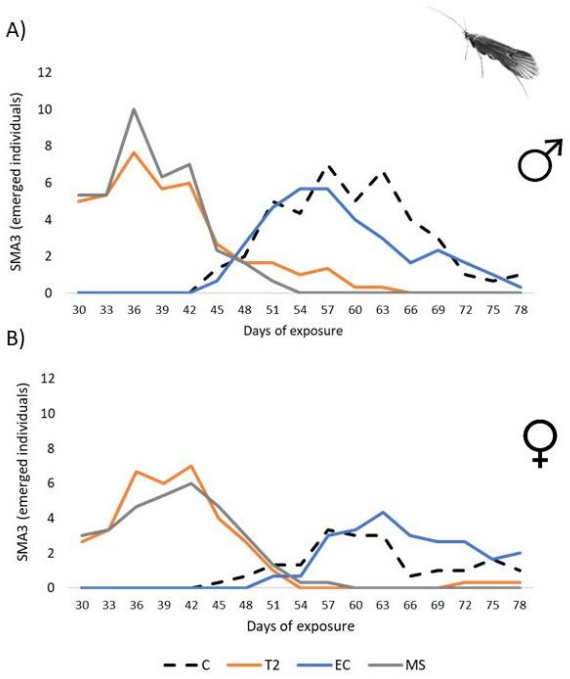
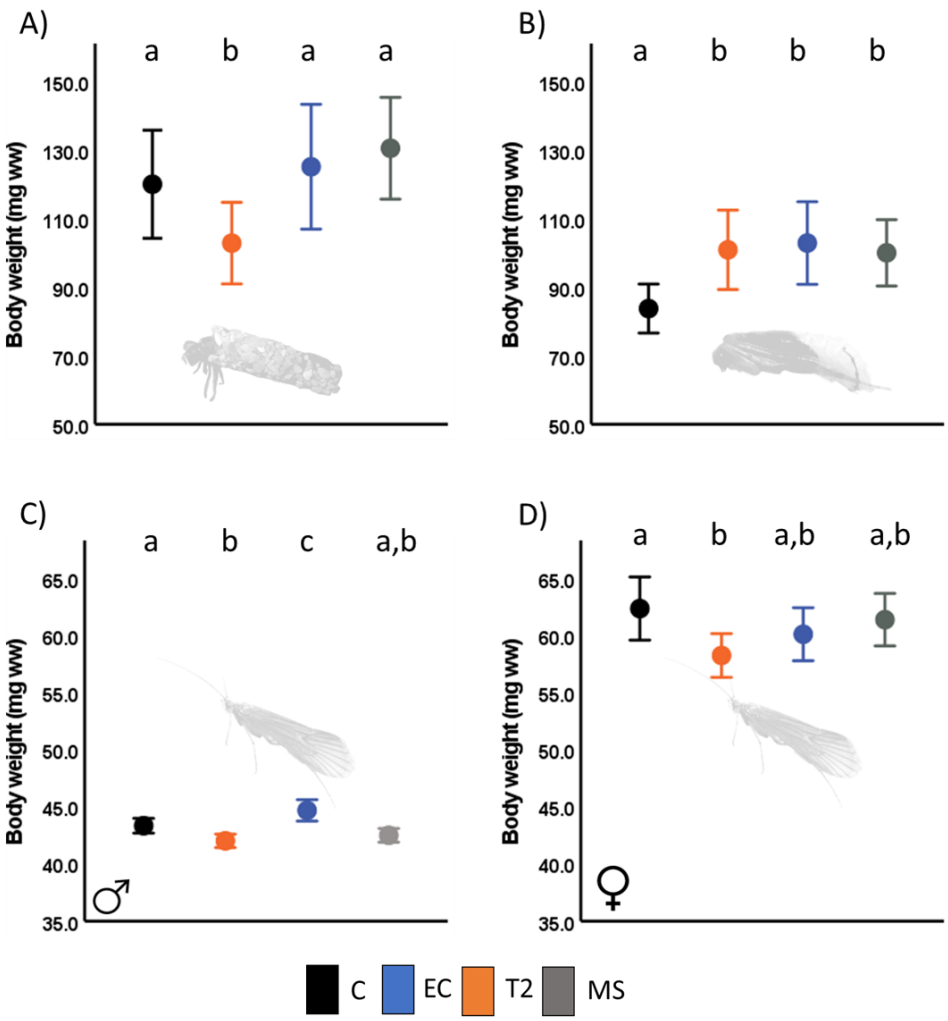


Figure 3. Three-day simple moving average (SMA3) of emerged A) male and B) female adults of *Micropterna nycterobia* across treatments. Abbreviations of treatments are as follows: C - Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC).

3.4. Body weight and total lipid content changes of the caddisfly *Micropterna nycterobia*

Overall, a loss in body mass was recorded in *M. nycterobia* throughout the life cycle, with larvae having the highest body weight and adults the lowest (Fig. 4A-D). A statistically significant drop in body weight was observed in larvae in T2 compared to control (4A, Table S8). All experimental treatments increased the body weight of pupae compared to control (Fig. 4B, Table S9). Adult females had significantly higher body weight compared to males (Fig. 4C & 4D, Table S10), however, in adults of both sexes a statistically significant drop in body weight was observed in T2 compared to control (Fig. 4C & 4D, Table S11 and S12).



370

371 Figure 4. Model predictions illustrating the effect of treatment on body weight of A) larvae B)
372 pupae C) adult males and D) adult females of *M.* nycterobia. Mean values of twelve replicates are
373 presented with 95% confidence intervals. Different letters indicate significant differences among
374 treatments ($p < 0.05$). Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 -
375 Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC).

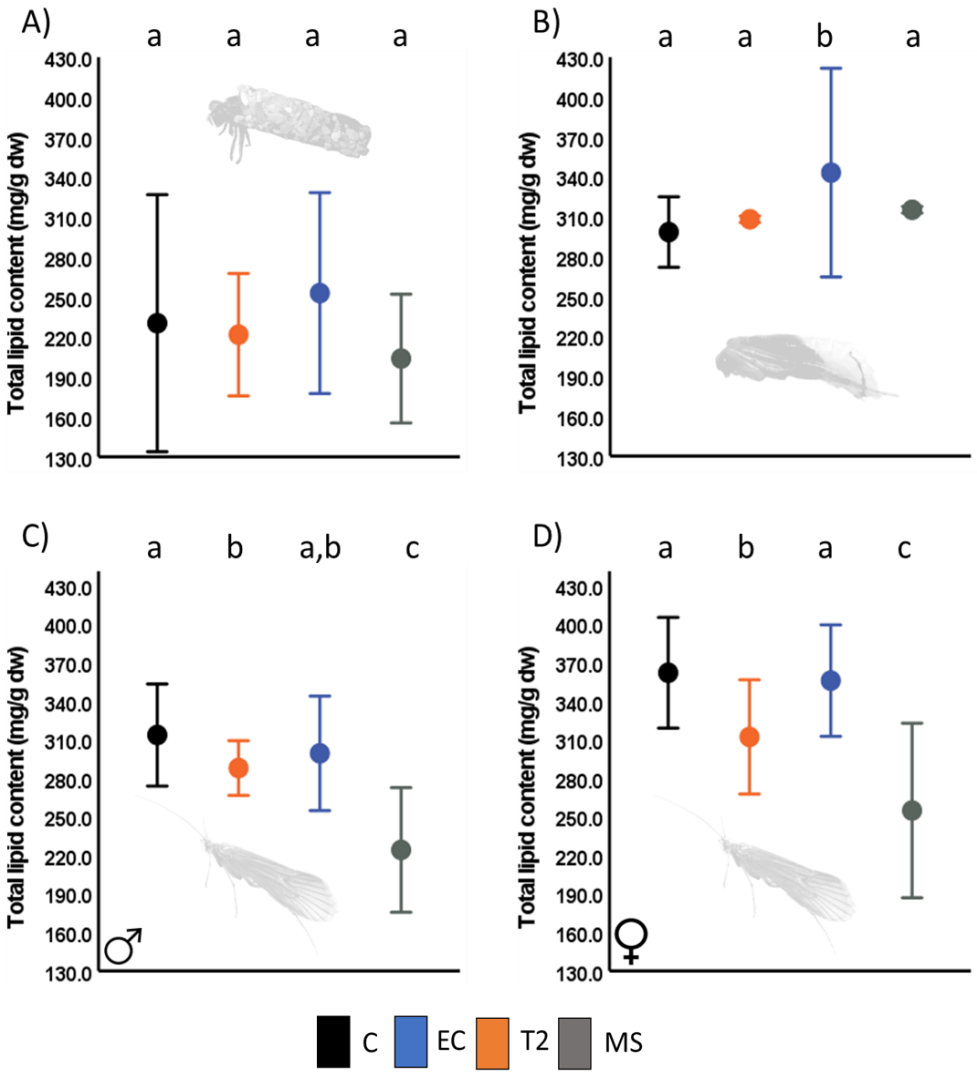


Figure 5. Model predictions illustrating the effect of treatment on total lipid content of A) larvae B) pupae C) adult males and D) adult females of *M. nycterobia*. Mean values of three replicates are presented with 95% confidence intervals. Different letters indicate significant differences among treatments ($p < 0.05$). Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC).

Changes in the TLC were more pronounced post-metamorphoses (Fig. 5A-D). Experimental treatments had no effect on TLC on larvae (Fig. 5A, Table S13), while pupae exhibited an increase in the EC treatment compared to control (Fig. 5B, Table S14). Total lipid content of adults was

lower compared to control in treatments with increased temperature (T2 and MS) (Fig. 5C & 5D, Table S15 & S16). Moreover, the greatest drop in TLC of adults was observed in the MS treatment ((Fig. 5C & 5D, Table S15 & S16), corresponding with the negative synergistic effect according to (Piggott et al., 2015). Females had significantly higher total lipid content compared to males (Figure 5C & 5D, Table S17).

3.5. Metabolome profiles of the caddisfly *Micropterna nycterobia*

Principal Response Curves (PRC) of non-target metabolome profile of larvae, pupae and adult caddisflies show that the metabolome of all caddisfly life stages was mostly affected by the ECs mixture (EC and MS), as evident by the highest deviation from the control (Fig. 5A & B). Deviations were observed in larvae at D15 already, and further increased in pupae and adults. The MS treatment showed highest impact on metabolome of both male and female adult caddisflies (Fig. 5A & B). Additionally, 62.6 % of total variance in males and 59.5 % in females could be attributed to time, whereas 22.1 % in males and 25.8 % in females could be attributed to the treatment (including its interaction with time, Table S18). The first PRC axis was significant (males – F (1, 32) = 18.714 $p < 0.05$, females – F (1, 32) = 28.172, $p < 0.05$) and based on the Monte Carlo tests per sampling date, the treatment regime had a significant influence on all sampling dates.

Principal component (PCA) analysis based on non-target metabolic profiles of all life stages of *M. nycterobia* revealed clear separation of pre-metamorphosis larvae and post-metamorphosis pupae and adult imagines (Fig. S6, PC1). However, when particular life stages were analyzed separately, the PCA also revealed separation into distinct groups based on treatments. In larvae, for instance, PC1 axis separating larvae in ECs and MS treatments from D0, C and T2 treatments accounted for 37.7 % of variability (Fig. 4C; variance from [PC1, PC2]≈[37.7 %, 15 %]).

Majority of metabolite groups (18 in total) showing the most significant changes in abundance to stressor treatments in *M. nycterobia* were lipids and fatty acids (39%) followed by amino acids (22%), quinones (6%), acylcholine (6%), adrenergic agents (6%), lactones (6%), alcohols (5%), pyridines (5%), alkaloids (5%) (Fig. S7, Table S19.

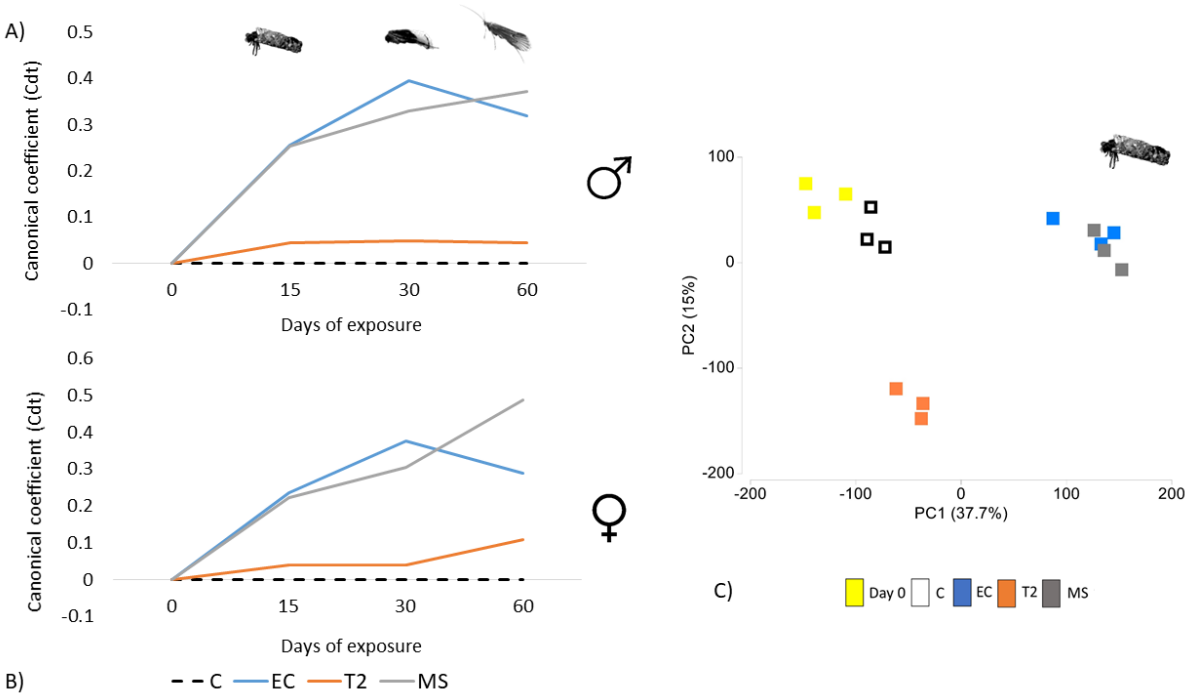


Figure 5. Metabolome profiles of the caddisfly *Micropterna nycterobia;* PRC of changes in the metabolic profiles along the caddisfly life cycle in A) male and B) female adults exposed to treatments in relation to control. Numbers on x-axis denote days of exposure. C) PCA plot showing separation based on metabolome of *M. nycterobia* larvae in different treatments at 15 days of exposure. Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC); Day 0 – pre-exposure sample.

3.5. Lipidome profiles of the caddisfly *Micropterna nycterobia*

Principal Response Curves (PRC) of non-target lipidome profile of larvae, pupae and adult caddisflies shows the highest deviation from the control in male and female adults, with an opposing individual stressor effect (EC vs T2; Fig. 6A & B). In MS treatments, increased temperature was the dominant stressor for both sexes, however, there were differences between sexes, as male caddisflies lipidome was more affected in T2 and MS treatments, whereas female caddisflies lipidome was more affected in the EC (Fig. 6A & 5B). Moreover, table S20 shows that 79.8 % of total variance in male and 68.4 % in female caddisflies can be attributed

to time, whereas 13.3 % in male and 17.3% in female can be attributed to the treatment (including its interaction with time). The first PRC axis was significant (males - F (1, 32) = 17.475 $p < 0.05$, females – F (1, 32) = 14.583, $p < 0.05$) and based on the Monte Carlo tests per sampling date, the treatment regime had a significant influence on all sampling dates.

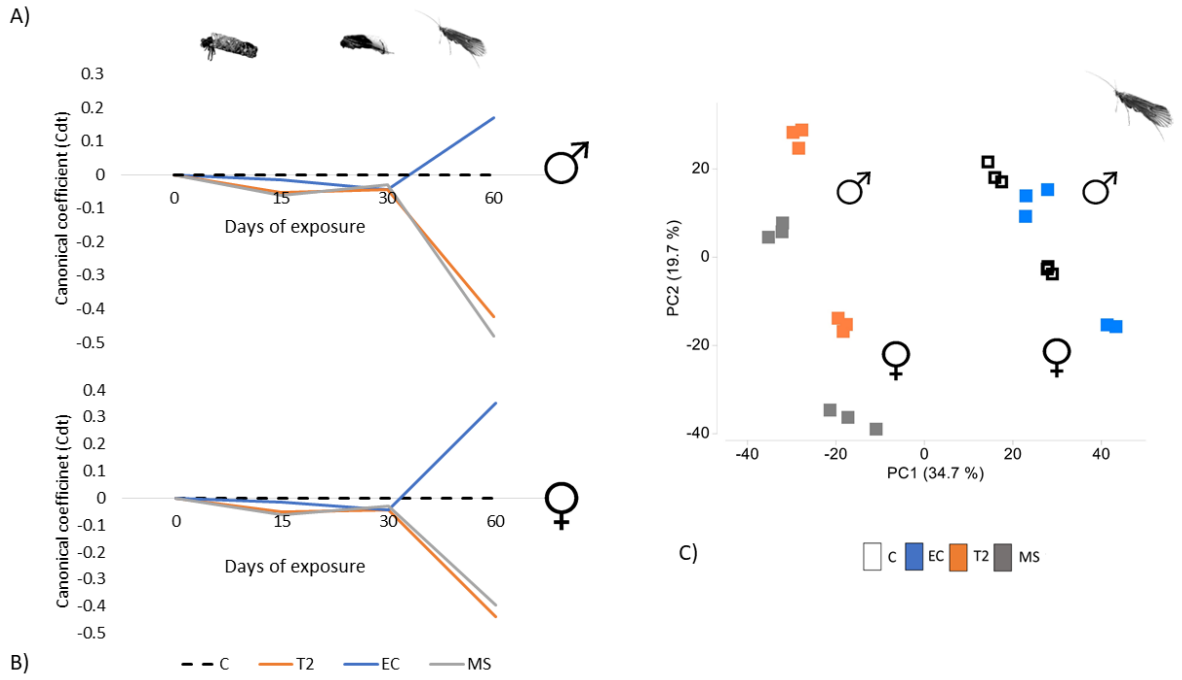


Figure 6. Lipidome profiles of the caddisfly *Micropterna nycterobia;* PRC of changes in the lipidome profiles of caddisfly life stages and A) male and B) female adults exposed to treatments in relation to control. Numbers on x-axis denote days of exposure. C) PCA plot showing separation adult *M. nycterobia* separated by sex in different treatments. Abbreviations of treatments are as follows: C – Control (T = 10 °C), T2 - Increased temperature (14 °C), EC - ECs mix, MS - multiple stressor treatment (T2 + EC); Day 0 –pre-exposure sample.

Principal component (PCA) analysis based on non-target lipidome profiles of all life stages of *M. nycterobia* showed clear separation of three major life stages, larvae, pupae and adults (Fig. S8). The total variance accounted by the three clusters was ~68.8 % (variance from [PC1, PC2, PC3]≈[47.7 %, 14.8 %, 6.3 %]). Clustering of particular treatments was particularly evident in separate analyses of each life stage. The highest degree of clustering based on treatments was inferred for adults, with the first principle component axis (PC1 = 34.7 %) separating adults based

on increased water temperature (T2 and MS treatments), whereas the second axis separated males from females (PC2 = 19.7 %, Fig. 6C).

Within the lipidome, most significant changes in abundance to stressor treatments were noticed in glycerophospholipids (67%) followed by fatty acyls (12%), sphingolipids (12%), glycerolipids (5%), prenol lipids (2%) and sterol lipids (1%) (Fig. S9, Table S21).

4. DISCUSSION

Water pollution has far-reaching consequences for the ecosystem health and functioning, and it is important to understand these impacts, especially in a context of climate change. Here we used simplified freshwater food web exposed to a mixture of PhACs and EDCs and increased water temperature to broaden our understanding of the impacts of climate change and pollution on freshwater ecosystems.

4.1. Warming and pollution with PhACs & EDCs triggered a mild response in moss

A mild response of the moss to both individual stressors (increased water temperature and contamination with ECs) and their combination suggests mosses' resilience. It has been shown that plants have specific temperature and pollution thresholds that trigger or inhibit certain physiological processes, allowing them to respond to stressful conditions (Firmansyah and Argosubekti, 2020; Gorovits et al., 2020; Sun et al., 2018; Zezulka et al., 2013). One of the most common physiological responses includes production of heat shock proteins (HSPs), and lack of observable change in regulation of HSPs may suggest that the thermal and pollution threshold necessary to trigger stress response in the moss was not reached. This observation is in agreement with established thermal threshold of plants of minimum 5 °C (Firmansyah and Argosubekti, 2020). Even though changes in metabolomic and lipidomic profiles among treatments were significant, one should keep in mind that changes in metabolite and lipid composition can be related to stress response but also developmental stages (Lu et al., 2019; Mikami and Hartmann, 2004). In addition, the biotic stress induced by the feeding of *M. nycterobia* larvae on the moss might have masked the impact of the pollution and increased

474 temperature. Plants can prioritize their responses to address individual stressors when exposed
475 to multiple abiotic and/or biotic stressors (Rejeb et al., 2014; Suzuki et al., 2014). We argue that
476 the moss in the current experiment might have prioritized biotic stress over abiotic stressors and
477 activated different defence mechanisms to mitigate larval feeding. However, we could not test
478 for the latter, since feeding of *M. nycterobia* larvae on the moss was also present in control
479 treatments. Moreover, research has shown that different stress combinations activate different
480 pathways and signals thus making it harder to predict multiple stressor effects (Rejeb et al., 2014;
481 Suzuki et al., 2014; Vescio et al., 2022).

482

483 4.2. Single stressor impacts: effects of warming and pollution with PhACs and EDCs on *M.*
484 *nycterobia*

485 In the current experiment, we recorded a body mass loss in *M. nycterobia* that is in line with the
486 usual life cycle patterns of the holometabolous caddisflies, with larvae having the highest body
487 weight and adults the lowest (Huryn and Wallace, 2000). Increased temperature during insect
488 development typically leads to reduced adult body size, negatively influencing fecundity and
489 longevity (Mirth and Riddiford, 2007), this effect was however, not observed in the current study.
490 Our observations are in line with data on chronic exposure to pesticides in the caddisfly
491 *Limnephilus lunatus,* where reduced body weight of adults was observed only if younger instar
492 larvae were exposed, and not the fifth-instars (Liess and Schulz, 1996; Schulz and Liess, 2001,
493 1995).

494 *M. nycterobia* inhabits clean crenal and rhithral sections and is therefore expected to be sensitive
495 to presence of contaminants (Graf et al., 2023). Indeed, sensitivity is displayed through intense
496 change in regulation of both metabolites and lipids in respect to control, however, the
497 contaminants seem to have a more significant impact on metabolome than temperature. More
498 precisely, the most pronounced metabolome response was post-metamorphosis, yet changes in
499 metabolite regulation are already notable in larvae at D15. Notably, the sampled caddisflies'
500 metabolome contained the biogenic amine octopamine, a significant neurotransmitter,
501 neuromodulator, and neurohormone influencing various physiological functions, behaviour and

endocrine activity (Farooqui, 2012) Changes in octopamine levels due to PhACs and EDCs during metamorphosis could affect not only the subsequent life stage but potentially extend across multiple generations.

Observed difference in dynamics of regulation of metabolites *vs* lipids can be related to physiological roles of metabolites and lipids which response tends to differ depending on the type of stress and the metabolic pathways involved (Kainz and Fisk, 2009; Snart et al., 2015). Generally, lipids serve as a long-term energy source and are stored in lipid droplets, which can be mobilized to provide energy during times of starvation, embryogenesis, prolonged periods of flight and stress (Arrese and Soulages, 2010; Kainz and Fisk, 2009). Here, metabolites were more regulated than lipids in response to stress probably in order to maintain cellular homeostasis and ensure energy reserves (lipids) for the emerging adults (Arrese and Soulages, 2010). Glycerolipids such as triglycerides are stored in the core of the lipid droplets surrounded by glycerophospholipids (Arrese and Soulages, 2010). As glycerophospholipids were mostly affected by experimental treatments, this further supports the fact that lipid reserves were preserved for adults. However, changes in glycerophospholipids of insects can also be related to development and metamorphosis (Bashan et al., 2002; Cargill et al., 1985; Duarte, 2019) as well as food source (Hanson et al., 1985; Torres-Ruiz et al., 2010).

The lack of strong effects of increased water temperature on metabolite regulation, as well as absence of heat shock protein HSP70 expression, is most likely due to adaptation of *M. nycterobia* to thermal stress regularly occurring in intermittent streams (Qin et al., 2003). This finding suggests that *M. nycterobia* and possibly other intermittent habitats indicators may have metabolic flexibility to cope with thermal stress, allowing them to survive extreme climatic events characterised by fluctuating temperature regimes. In addition, the regulation of lipidome of *M. nycterobia* aquatic stages exhibited intriguing resilience to both increased water temperature and pollution with PhACs and EDCs. The limited impact on lipid regulation suggests that the stress response threshold triggering significant lipid mobilisation may not have been surpassed. Instead, the priority for the aquatic stages was directed towards lipid accumulation rather than mobilization in response to stress challenges (Arrese and Soulages, 2010; Hoppeler et al., 2018). Emphasis on lipid accumulation was evident through the observed increase in total lipid content

from larval to pupal stages, serving as a crucial energy source to sustain adult insects during non-feeding periods and fuel their flights (Arrese and Soulages, 2010; Hoppeler et al., 2018). While the anticipation of intense lipidome activity in adult insects is well-founded, it is surprising that so distinct regulatory mechanisms are operating in response to different stressors. However, lipidomic profiles of multiple stressor treatment were in both males and females congruent with those of increased temperature, implying the dominant impact of increased water temperature on regulation of lipids in aquatic insects.

Increased water temperature accelerated development of *M. nycterobia,* resulting in earlier adult emergence and lower total lipid content of all life stages in treatments with increased temperature. Similarly, the mayfly larvae chronically exposed to increased temperature used lipids and amino acids as alternative energy sources to support their growth and maintenance costs, ultimately resulting in reduced total lipid content (Chou et al., 2018). Furthermore, sensitivity of the temporal emergence patterns of aquatic insects was already discussed as toxicological endpoint for exposure to pesticides (Schulz and Liess, 2001), as timing of aquatic insect emergence plays a crucial role for riparian predators (review in Bundschuh et al., 2020). More precisely, insectivorous birds almost exclusively obtain the omega-3 long-chain polyunsaturated fatty acids from emerging aquatic insects, thus shifts in the relative availability and phenology of aquatic insects in response to a changing climate are likely to have major fitness consequences for their breeding success (Shipley et al., 2022). Hence, such shifts can have cascading effects on cross-ecosystem energy flow. This is of particular importance in intermittent water bodies, like those inhabited by *M. nycterobia,* where mass emergence during short periods is typical and riparian food webs are highly dependent on the aquatic subsidies (McIntosh et al., 2017).

4.3. Multiple stressor impacts of warming and PhACs and EDCs on aquatic insects

A synergistic interactive effect was observed, leading to a decrease in total lipid content and significant variation in lipid profiles in adults of both sexes under the MS treatment. *Micropterna nycterobia*, a specialist in intermittent rivers, exhibits a behaviour where adults leave the water

559 bodies upon emergence and migrate to cooler mountainous regions or nearby caves for a few
560 months until their gonads develop (Waringer and Graf, 2011). In autumn, after copulation, they
561 lay eggs in re-established surface flow. Therefore, the observed decrease in lipid reserves in
562 adults (8.9% and 10.7% decrease in total lipid content in males and females, respectively) could
563 have a major impact on their reproduction and population dynamics. Lipids serve as crucial
564 energy reserves for insects, especially during non-feeding life stages and long-distance flights
565 (Arrese and Soulages, 2010; Downer and Matthews, 1976). Maintaining metapopulation
566 dynamics is particularly important for inhabitants of intermittent water bodies (Datry et al.,
567 2017). Various pollutants, such as fungicides and copper, can decrease the lipid content of
568 limnephilid caddisflies (Konschak et al., 2019). However, this effect was not observed in the
569 current study as PhACs and EDCs did not influence the lipid content of *M. nycterobia*.
570 Furthermore, the negative effects of pollutants on lipids can be exacerbated by elevated water
571 temperatures (Yoon et al., 2022). However, in our study, increased water temperature primarily
572 influenced the lipids of caddisflies, and its adverse effects were amplified by pollution with PhACs
573 and EDCs. Additionally, warming indirectly contributed to the further deterioration of
574 environmental conditions in the T2 and MS treatments, as evidenced by increased conductivity
575 within these two treatments.

576 In terms of the timing of the strongest stress response, our results differ from a previous
577 experiment involving the same caddisfly species, where the effects of ECs and microplastic
578 particles were most pronounced in the first 15 days of exposure, both in single and combined
579 stressor treatments (Grgić et al., 2023). Apart from the dominant impact of increased
580 temperature, these differences may also be attributed to variations in the mixture of ECs used,
581 as different compounds can have varying effects on biota (Muñoz et al., 2015; Nilsen et al., 2019).
582 Nevertheless, our study indicates that the adverse synergistic effects of warming events and
583 freshwater contamination with ECs could intensify throughout the life cycle of aquatic insects,
584 potentially leading to developmental impairments and disturbances in population dynamics
585 (Kazmi et al., 2022). However, in most environmental health assessment and monitoring
586 programs, only the aquatic stages of aquatic insects are considered (Water Framework Directive
587 (WFD) 2000/60/EC). Furthermore, the multiple stressor effects observed in the current study not

588 only resulted in reduced resource quality for aquatic food webs but also affected the quality of
589 emergence, thus impacting the riparian food webs at the aquatic-terrestrial interface (Bundschuh
590 et al., 2020).

591

592 4.5. Differential response to stressor impacts of male and female caddisflies

593 Our study reveals sex-specific responses to both single and multiple stressors, with females
594 exhibiting more pronounced impacts on the metabolome while males show greater effects on
595 the lipidome. In the majority of insects, there is sexual dimorphism in lipid content due to distinct
596 roles played by lipids, such as egg production in females and flight behaviour in males (Lease and
597 Wolf, 2011). Certain aquatic insect species, characterized by specific male flight behaviour (Lease
598 and Wolf, 2011) or swarming (Sartori et al., 1992), experience negative impacts on population
599 fitness when males have decreased lipid content. Furthermore, this study identifies 4-
600 hydroxyestradiol, an endogenous metabolite of 17β-estradiol, from *M. nycterobia*, which has
601 been shown to have significant lipid-modulating effects in rats (Wang and Zhu, 2017).
602 Additionally, in *Drosophila*, sexual dimorphism has been observed in metabolic genes and
603 mechanisms involved in triglyceride homeostasis (Wat et al., 2020).

604 Likewise, the findings of this study highlight sex-dependent variation in metabolites induced by
605 stress, consistent with a previous study involving *M. nycterobia* exposed to microplastic particles
606 and a mixture of personal care products (Grgić et al., 2023). Moreover, female insects generally
607 exhibit a stronger stress response across different taxa and under various stress conditions,
608 including parasite infections, predation, food quality, chemical stress, and the impacts of climate
609 change (Lindsey and Altizer, 2009; Slos et al., 2009; Stillwell and Davidowitz, 2010). This is likely
610 due to the different evolutionary roles of male and female insects, which have led to the
611 development of distinct stress-defence mechanisms. The fitness of females is closely linked to
612 their life expectancy and the number of offspring they produce.

613 Therefore, our study emphasizes the variability in the impacts of both single and multiple
614 stressors on various traits, different life stages, and sexes within a single species. Consequently,
615 it underscores the importance of developing a more comprehensive understanding of the

sensitivity of freshwater organisms to the adverse effects of single and multiple stressors, particularly when addressing the management of freshwater ecosystems in the context of global change (Schäfer and Piggott, 2018).

Acknowledgment

We thank Dr Ruđer Novak (Faculty of Medicine, University of Zagreb) for providing assistance in mass spectrometry analysis. This study is part of the outcomes of the Croatian Science Foundation project MUSE (PZS-2019-02-9479 and DOK-2020-01-6998 to A.P.) and KLIMA-4HR project (KK.05.1.1.02.0006). M.R. acknowledges additional support from the Croatian Science Foundation (IP-2018-01-2298). Two anonymous reviewers are thanked for significantly improving the quality of earlier version of the manuscript.

LITERATURE

Arrese, E.L., Soulages, J.L., 2010. Insect Fat Body: Energy, Metabolism, and Regulation. Annu. Rev. Entomol. 55, 207–225. https://doi.org/10.1146/annurev-ento-112408-085356

Barbosa, M., Inocentes, N., Soares, A.M.V.M., Oliveira, M., 2017. Synergy effects of fluoxetine and variability in temperature lead to proportionally greater fitness costs in Daphnia: A multigenerational test. Aquat. Toxicol. 193, 268–275. https://doi.org/10.1016/j.aquatox.2017.10.017

Bashan, M., Akbas, H., Yurdakoc, K., 2002. Phospholipid and triacylglycerol fatty acid composition of major life stages of sunn pest, Eurygaster integriceps (Heteroptera: Scutelleridae). Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 132, 375–380. https://doi.org/10.1016/S1096-4959(02)00045-3

Bhangare, D., Rajput, N., Jadav, T., Sahu, A.K., Tekade, R.K., Sengupta, P., 2022. Systematic strategies for degradation kinetic study of pharmaceuticals: an issue of utmost importance

642 concerning current stability analysis practices. J. Anal. Sci. Technol. 13, 7.
643 https://doi.org/10.1186/s40543-022-00317-6

644 Blackman, R.C., Altermatt, F., Foulquier, A., Lefébure, T., Gauthier, M., Bouchez, A.,
645 Stubbington, R., Weigand, A.M., Leese, F., Datry, T., 2021. Unlocking our understanding of
646 intermittent rivers and ephemeral streams with genomic tools. Front. Ecol. Environ. 19,
647 574–583. https://doi.org/10.1002/fee.2404

648 Bravo, D.N., Araújo, M.B., Lasanta, T., Moreno, J.I.L., 2008. Climate change in Mediterranean
649 mountains during the 21st century. Ambio 37, 280–285.
650 https://doi.org/10.1579/00410.1579/0044-7447(2008)37[280:ccimmd]2.0.co;2

651 Bundschuh, M., Zubrod, J.P., Wieczorek, M. V., Schulz, R., 2020. Studying Effects of
652 Contaminants on Aquatic-Terrestrial Subsidies: Experimental Designs Using Outdoor and
653 Indoor Mesocosms and Microcosms, in: Contaminants and Ecological Subsidies. Springer
654 International Publishing, Cham, pp. 279–296. https://doi.org/10.1007/978-3-030-49480-
655 3_12

656 Cargill, A.S., Cummins, K.W., Hanson, B.J., Lowry, R.R., 1985. The role of lipids as feeding
657 stimulants for shredding aquatic insects. Freshw. Biol. 15, 455–464.
658 https://doi.org/10.1111/j.1365-2427.1985.tb00215.x

659 Cetinić, K.A., Grgić, I., Previšić, A., Rožman, M., 2022. The curious case of methylparaben:
660 Anthropogenic contaminant or natural origin? Chemosphere 294, 133781.
661 https://doi.org/10.1016/j.chemosphere.2022.133781

662 Chou, H., Pathmasiri, W., Deese-spruill, J., Sumner, S.J., Jima, D.D., Funk, D.H., Jackson, J.K.,
663 Sweeney, B.W., Buchwalter, D.B., 2018. The Good, the Bad, and the Lethal: Gene
664 Expression and Metabolomics Reveal Physiological Mechanisms Underlying Chronic
665 Thermal Effects in Mayfly Larvae (Neocloeon triangulifer). Front. Ecol. Evol. 6, 1–11.
666 https://doi.org/10.3389/fevo.2018.00027

667 Clarke, K.R., Gorley, R.N., 2015. PRIMER v7 :, PRIMER-E Ltd Registered.

668 Cogo, G.B., Martínez, J., Santos, S., Graça, M.A.S., 2020. Caddisflies growth and size along an
669 elevation/temperature gradient. Hydrobiologia 847, 207–216.
670 https://doi.org/10.1007/s10750-019-04082-3

671 Conti, L., Schmidt-Kloiber, A., Grenouillet, G., Graf, W., 2014. A trait-based approach to assess
672 the vulnerability of European aquatic insects to climate change. Hydrobiologia 721, 297–
673 315. https://doi.org/10.1007/s10750-013-1690-7

674 Cruzeiro, C., Ramos, A., Loganimoce, E.M., Arenas, F., Rocha, E., Cardoso, P.G., 2019. Genotoxic
675 effects of combined multiple stressors on Gammarus locusta haemocytes: Interactions
676 between temperature, pCO2 and the synthetic progestin levonorgestrel. Environ. Pollut.
677 245, 864–872. https://doi.org/10.1016/j.envpol.2018.11.070

678 Datry, T., Corti, R., Heino, J., Hugueny, B., Rolls, R.J., Ruhí, A., 2017. Habitat Fragmentation and
679 Metapopulation, Metacommunity, and Metaecosystem Dynamics in Intermittent Rivers
680 and Ephemeral Streams, in: Intermittent Rivers and Ephemeral Streams. Elsevier, pp. 377–
681 403. https://doi.org/10.1016/B978-0-12-803835-2.00014-0

682 DeCourten, B.M., Brander, S.M., 2017. Combined effects of increased temperature and
683 endocrine disrupting pollutants on sex determination, survival, and development across
684 generations. Sci. Rep. 7, 9310. https://doi.org/10.1038/s41598-017-09631-1

685 Dinh, K. V., Konestabo, H.S., Borgå, K., Hylland, K., Macaulay, S.J., Jackson, M.C., Verheyen, J.,
686 Stoks, R., 2022. Interactive Effects of Warming and Pollutants on Marine and Freshwater
687 Invertebrates. Curr. Pollut. Reports 8, 341–359. https://doi.org/10.1007/s40726-022-
688 00245-4

689 Dorić, V., Ivković, M., Baranov, V., Pozojević, I., Mihaljević, Z., 2023. Extreme freshwater
690 discharge events exacerbated by climate change influence the structure and functional
691 response of the chironomid community in a biodiversity hotspot. Sci. Total Environ. 879.
692 https://doi.org/10.1016/j.scitotenv.2023.163110

693 Downer, R.G.H., Matthews, J.R., 1976. Patterns of Lipid Distribution and Utilisation in Insects.
694 Am. Zool. 16, 733–745. https://doi.org/10.1093/icb/16.4.733

695 Duarte, P.M., 2019. Unravelling the lipid profile of coastal insects from Ria de Aveiro, Portugal:
696 implications for sustainable aquaculture. University of Aveiro.
697 https://doi.org/ria.ua.pt/bitstream/10773/28378/1/Pedro%20Martins%20Duarte.pdf

698 Duchet, C., Grabicová, K., Kolar, V., Lepšová, O., Csercsa, A., Zdvihalova, B., Randák, T., Boukal,
699 D., 2023. Combined effects of climate warming and environmentally relevant
700 concentrations of pharmaceutical active compounds on a freshwater community. bioRxiv
701 244, 2023.03.31.535078.

702 Ebele, A.J., Abou-Elwafa Abdallah, M., Harrad, S., 2017. Pharmaceuticals and personal care
703 products (PPCPs) in the freshwater aquatic environment. Emerg. Contam. 3, 1–16.
704 https://doi.org/10.1016/j.emcon.2016.12.004

705 Erasmus, J.H., Lorenz, A.W., Zimmermann, S., Wepener, V., Sures, B., Smit, N.J., Malherbe, W.,
706 2021. A diversity and functional approach to evaluate the macroinvertebrate responses to
707 multiple stressors in a small subtropical austral river. Ecol. Indic. 131, 108206.
708 https://doi.org/10.1016/j.ecolind.2021.108206

709 Farooqui, T., 2012. Review of octopamine in insect nervous systems. Open access insect physiol.
710 1. https://doi.org/10.2147/OAIP.S20911

711 Finn, D.S., Johnson, S.L., Gerth, W.J., Arismendi, I., Li, J.L., 2022. Spatiotemporal patterns of
712 emergence phenology reveal complex species-specific responses to temperature in aquatic
713 insects. Divers. Distrib. 28, 1524–1541. https://doi.org/10.1111/ddi.13472

714 Firmansyah, Argosubekti, N., 2020. A review of heat stress signaling in plants. IOP Conf. Ser.
715 Earth Environ. Sci. 484, 012041. https://doi.org/10.1088/1755-1315/484/1/012041

716 Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification
717 of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.
718 https://doi.org/10.1016/S0021-9258(18)64849-5

719 Gorovits, R., Sobol, I., Akama, K., Chefetz, B., Czosnek, H., 2020. Pharmaceuticals in treated
720 wastewater induce a stress response in tomato plants. Sci. Rep. 10, 1856.

https://doi.org/10.1038/s41598-020-58776-z

Grabicova, K., Grabic, R., Blaha, M., Kumar, V., Cerveny, D., Fedorova, G., Randak, T., 2015. Presence of pharmaceuticals in benthic fauna living in a small stream affected by effluent from a municipal sewage treatment plant. Water Res. 72, 145–153. https://doi.org/10.1016/j.watres.2014.09.018

Graf, W., Murphy, J., Dahl, J., Zamora-Munoz, C., Lopez-Rodriguez M.J. Schmidt-Kloiber., A., 2023. Dataset "Trichoptera". www.freshwaterecology.info - the taxa and autecology database for freshwater organisms, version 8.0 (accessed on 06.06.2023) [WWW Document]. https://doi.org/https://www.freshwaterecology.info/index.php

Grgić, I., Cetinić, K.A., Karačić, Z., Previšić, A., Rožman, M., 2023. Fate and effects of microplastics in combination with pharmaceuticals and endocrine disruptors in freshwaters: Insights from a microcosm experiment. Sci. Total Environ. 859. https://doi.org/10.1016/j.scitotenv.2022.160387

Halsch, C.A., Shapiro, A.M., Fordyce, J.A., Nice, C.C., Thorne, J.H., Waetjen, D.P., Forister, M.L., 2021. Insects and recent climate change. Proc. Natl. Acad. Sci. U. S. A. 118, 1–9. https://doi.org/10.1073/PNAS.2002543117

Hanson, B.J., Cummins, K.W., Cargill, A.S., Lowry, R.R., 1985. Lipid content, fatty acid composition, and the effect of diet on fats of aquatic insects. Comp. Biochem. Physiol. Part B Comp. Biochem. 80, 257–276. https://doi.org/10.1016/0305-0491(85)90206-8

Hering, D., Schmidt-Kloiber, A., Murphy, J., Lücke, S., Zamora-Muñoz, C., López-Rodríguez, M.J., Huber, T., Graf, W., 2009. Potential impact of climate change on aquatic insects: A sensitivity analysis for European caddisflies (Trichoptera) based on distribution patterns and ecological preferences. Aquat. Sci. 71, 3–14. https://doi.org/10.1007/s00027-009-9159-5

Hershkovitz, Y., Dahm, V., Lorenz, A.W., Hering, D., 2015. A multi-trait approach for the identification and protection of European freshwater species that are potentially vulnerable to the impacts of climate change. Ecol. Indic. 50, 150–160.

https://doi.org/10.1016/j.ecolind.2014.10.023

Heye, K., Lotz, T., Wick, A., Oehlmann, J., 2019. Interactive effects of biotic and abiotic environmental stressors on carbamazepine toxicity in the non-biting midge Chironomus riparius. Water Res. 156, 92–101. https://doi.org/10.1016/j.watres.2019.03.007

Hoppeler, F., Winkelmann, C., Becker, J., Pauls, S.U., 2018. Larval growth and metabolic energy storage of Micropterna lateralis (Trichoptera: Limnephilidae) in an intermittent stream: glycogen dominates in final instars. Hydrobiologia 806, 175–185. https://doi.org/10.1007/s10750-017-3354-5

Huryn, A.D., Wallace, J.B., 2000. LIFE HISTORY AND PRODUCTION OF STREAM INSECTS. Annu. Rev. Entomol. 83–110. https://doi.org/10.1146/annurev.ento.51.110104.151107

Jarvis, A.L., Bernot, M.J., Bernot, R.J., 2014. The effects of the pharmaceutical carbamazepine on life history characteristics of flat-headed mayflies (Heptageniidae) and aquatic resource interactions. Ecotoxicology 23, 1701–1712. https://doi.org/10.1007/s10646-014-1309-4

Kainz, M.J., Fisk, A.T., 2009. Integrating lipids and contaminants in aquatic ecology and ecotoxicology, in: Lipids in Aquatic Ecosystems. Springer New York, New York, NY, pp. 93–114. https://doi.org/10.1007/978-0-387-89366-2_5

Katajamaa, M., Miettinen, J., Orešič, M., 2006. MZmine: Toolbox for processing and visualization of mass spectrometry based molecular profile data. Bioinformatics 22, 634–636. https://doi.org/10.1093/bioinformatics/btk039

Kazmi, S.S.U.H., Wang, Y.Y.L., Cai, Y.E., Wang, Z., 2022. Temperature effects in single or combined with chemicals to the aquatic organisms: An overview of thermo-chemical stress. Ecol. Indic. 143, 109354. https://doi.org/10.1016/j.ecolind.2022.109354

Konschak, M., Zubrod, J.P., Baudy, P., Englert, D., Herrmann, B., Schulz, R., Bundschuh, M., 2019. Waterborne and diet-related effects of inorganic and organic fungicides on the insect leaf shredder Chaetopteryx villosa (Trichoptera). Aquat. Toxicol. 206, 33–42. https://doi.org/10.1016/j.aquatox.2018.10.021

774 Krajick, K., 2004. All Downhill From Here? Science (80-.). 303, 1600–1602.
775 https://doi.org/10.1126/science.303.5664.1600

776 Kraufvelin, P., 1998. Model ecosystem replicability challenged by the “soft” reality of a hard
777 bottom mesocosm. J. Exp. Mar. Bio. Ecol. 222, 247–267. https://doi.org/10.1016/S0022-
778 0981(97)00143-3

779 Lagesson, A., Fahlman, J., Brodin, T., Fick, J., Jonsson, M., Byström, P., Klaminder, J., 2016.
780 Bioaccumulation of five pharmaceuticals at multiple trophic levels in an aquatic food web -
781 Insights from a field experiment. Sci. Total Environ. 568, 208–215.
782 https://doi.org/10.1016/j.scitotenv.2016.05.206

783 Lease, H.M., Wolf, B.O., 2011. Lipid content of terrestrial arthropods in relation to body size,
784 phylogeny, ontogeny and sex. Physiol. Entomol. 36, 29–38.
785 https://doi.org/10.1111/j.1365-3032.2010.00767.x

786 Liess, M., Schulz, R., 1996. Chronic effects of short-term contamination with the pyrethroid
787 insecticide fenvalerate on the caddisfly Limnephilus lunatus. Hydrobiologia 324, 99–106.
788 https://doi.org/10.1007/BF00018170

789 Lindsey, E., Altizer, S., 2009. Sex differences in immune defenses and response to parasitism in
790 monarch butterflies. Evol. Ecol. 23, 607–620. https://doi.org/10.1007/s10682-008-9258-0

791 López-Doval, J.C., Kukkonen, J.V.K., Rodrigo, P., Muñoz, I., 2012. Effects of indomethacin and
792 propranolol on Chironomus riparius and Physella (Costatella) acuta. Ecotoxicol. Environ.
793 Saf. 78, 110–115. https://doi.org/10.1016/j.ecoenv.2011.11.004

794 Lu, Eiriksson, Thorsteinsdóttir, Simonsen, 2019. Valuable Fatty Acids in Bryophytes—
795 Production, Biosynthesis, Analysis and Applications. Plants 8, 524.
796 https://doi.org/10.3390/plants8110524

797 Macadam, C.R., England, J., Chadd, R., 2022. The vulnerability of British aquatic insects to
798 climate change. Knowl. Manag. Aquat. Ecosyst. 2022-Janua, 3.
799 https://doi.org/10.1051/kmae/2022003

Maenpaa, K., Kukkonen, J., 2006. Bioaccumulation and toxicity of 4-nonylphenol (4-NP) and 4-(2-dodecyl)-benzene sulfonate (LAS) in Lumbriculus variegatus (Oligochaeta) and Chironomus riparius (Insecta). Aquat. Toxicol. 77, 329–338. https://doi.org/10.1016/j.aquatox.2006.01.002

Mandaric, L., Celic, M., Marcé, R., Petrovic, M., 2015. Introduction on Emerging Contaminants in Rivers and Their Environmental Risk, in: Handbook of Environmental Chemistry. pp. 3–25. https://doi.org/10.1007/698_2015_5012

McIntosh, A.R., Leigh, C., Boersma, K.S., McHugh, P.A., Febria, C., García-Berthou, E., 2017. Food Webs and Trophic Interactions in Intermittent Rivers and Ephemeral Streams, in: Intermittent Rivers and Ephemeral Streams. Elsevier, pp. 323–347. https://doi.org/10.1016/B978-0-12-803835-2.00012-7

Mikami, K., Hartmann, E., 2004. Lipid Metabolism in Mosses, in: New Frontiers in Bryology. Springer Netherlands, Dordrecht, pp. 133–155. https://doi.org/10.1007/978-0-306-48568-8_8

Mirth, C.K., Riddiford, L.M., 2007. Size assessment and growth control: how adult size is determined in insects. BioEssays 29, 344–355. https://doi.org/10.1002/bies.20552

Muñoz, I., López-Doval, J.C., De Castro-Català, N., Kuzmanovic, M., Ginebreda, A., Sabater, S., 2015. Effects of Emerging Contaminants on Biodiversity, Community Structure, and Adaptation of River Biota, in: Handbook of Environmental Chemistry. pp. 79–119. https://doi.org/10.1007/698_2015_5013

Nilsen, E., Smalling, K.L., Ahrens, L., Gros, M., Miglioranza, K.S.B., Picó, Y., Schoenfuss, H.L., 2019. Critical review: Grand challenges in assessing the adverse effects of contaminants of emerging concern on aquatic food webs. Environ. Toxicol. Chem. 38, 46–60. https://doi.org/10.1002/etc.4290

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: Environmental contaminants in a warming world. Environ. Int. 35, 971–986. https://doi.org/10.1016/j.envint.2009.02.006

827 Nukazawa, K., Arai, R., Kazama, S., Takemon, Y., 2018. Projection of invertebrate populations in
828 the headwater streams of a temperate catchment under a changing climate. Sci. Total
829 Environ. 642, 610–618. https://doi.org/10.1016/j.scitotenv.2018.06.109

830 Oksanen, J., Simpson, G.L., Blanchet, F.G., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H.,
831 Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., Durand, S.,
832 Beatriz, H., Evangelista, A., Friendly, M., Hannigan, G., Hill, M.O., Lahti, L., Mcglinn, D.,
833 Ribeiro, E., Smith, T., Stier, A., Ter, C.J.F., Weedon, J., 2020. Title Community Ecology
834 Package. Vegan Community Ecol. Packag. R Packag. version 2.5-7.
835 https://doi.org/https://github.com/vegandevs/vegan

836 Pestana, J.L.T., Novais, S.C., Lemos, M.F.L., Soares, A.M.V.M., 2014. Cholinesterase activity in
837 the caddisfly Sericostoma vittatum: Biochemical enzyme characterization and in vitro
838 effects of insecticides and psychiatric drugs. Ecotoxicol. Environ. Saf. 104, 263–268.
839 https://doi.org/10.1016/j.ecoenv.2014.03.012

840 Piggott, J.J., Townsend, C.R., Matthaei, C.D., 2015. Reconceptualizing synergism and
841 antagonism among multiple stressors. Ecol. Evol. 5, 1538–1547.
842 https://doi.org/10.1002/ece3.1465

843 Polazzo, F., Roth, S.K., Hermann, M., Mangold-Döring, A., Rico, A., Sobek, A., Van den Brink, P.J.,
844 Jackson, M.C., 2022. Combined effects of heatwaves and micropollutants on freshwater
845 ecosystems: Towards an integrated assessment of extreme events in multiple stressors
846 research. Glob. Chang. Biol. https://doi.org/10.1111/gcb.15971

847 Previšić, A., Rožman, M., Mor, J.R., Acuña, V., Serra-Compte, A., Petrović, M., Sabater, S., 2020.
848 Aquatic macroinvertebrates under stress: Bioaccumulation of emerging contaminants and
849 metabolomics implications. Sci. Total Environ. 704, 135333.
850 https://doi.org/10.1016/j.scitotenv.2019.135333

851 Previšić, A., Vilenica, M., Vučković, N., Petrović, M., Rožman, M., 2021. Aquatic Insects Transfer
852 Pharmaceuticals and Endocrine Disruptors from Aquatic to Terrestrial Ecosystems. Environ.
853 Sci. Technol. 55, 3736–3746. https://doi.org/10.1021/acs.est.0c07609

Qin, W., Tyshenko, M.G., Wu, B.S., Walker, V.K., Robertson, R.M., 2003. Cloning and characterization of a member of the hsp70 gene family from Locusta migratoria, a highly thermotolerant insect. Cell Stress Chaperones 8, 144. https://doi.org/10.1379/1466-1268(2003)008<0144:CACOAM>2.0.CO;2

Rejeb, I., Pastor, V., Mauch-Mani, B., 2014. Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms. Plants 3, 458–475. https://doi.org/10.3390/plants3040458

Rožman, M., Acuña, V., Petrović, M., 2018. Effects of chronic pollution and water flow intermittency on stream biofilms biodegradation capacity. Environ. Pollut. 233, 1131–1137. https://doi.org/10.1016/j.envpol.2017.10.019

Sanderson, H., 2002. Pesticide studies: Replicability of micro/mesocosms. Environ. Sci. Pollut. Res. 9, 429–435. https://doi.org/10.1007/BF02987597

Sarafian, M.H., Gaudin, M., Lewis, M.R., Martin, F.P., Holmes, E., Nicholson, J.K., Dumas, M.E., 2014. Objective set of criteria for optimization of sample preparation procedures for ultra-high throughput untargeted blood plasma lipid profiling by ultra performance liquid chromatography-mass spectrometry. Anal. Chem. 86, 5766–5774. https://doi.org/10.1021/ac500317c

Sartori, M., Keller, L., Thomas, A.G.B., Passera, L., 1992. Flight energetics in relation to sexual differences in the mating behaviour of a mayfly, Siphlonurus aestivalis. Oecologia 92, 172–176. https://doi.org/10.1007/BF00317360

Schäfer, R.B., Piggott, J.J., 2018. Advancing understanding and prediction in multiple stressor research through a mechanistic basis for null models. Glob. Chang. Biol. 24, 1817–1826. https://doi.org/10.1111/gcb.14073

Schulz, R., Liess, M., 2001. Toxicity of Aqueous-Phase and Suspended Particle-Associated Fenvalerate: Chronic Effects After Pulse-Dosed Exposure of Limnephilus Lunatus (Trichoptera). Environ. Toxicol. Chem. 20, 185. https://doi.org/10.1897/1551-5028(2001)020<0185:toapas>2.0.co;2

Schulz, R., Liess, M., 1995. Chronic effects of low insecticide concentrations on freshwater caddisfly larvae. Hydrobiologia 299, 103–113. https://doi.org/10.1007/BF00017562

Serra-Compte, A., Maulvault, A.L., Camacho, C., Álvarez-Muñoz, D., Barceló, D., Rodríguez-Mozaz, S., Marques, A., 2018. Effects of water warming and acidification on bioconcentration, metabolization and depuration of pharmaceuticals and endocrine disrupting compounds in marine mussels (Mytilus galloprovincialis). Environ. Pollut. 236, 824–834. https://doi.org/10.1016/j.envpol.2018.02.018

Shipley, J.R., Twining, C.W., Mathieu-Resuge, M., Parmar, T.P., Kainz, M., Martin-Creuzburg, D., Weber, C., Winkler, D.W., Graham, C.H., Matthews, B., 2022. Climate change shifts the timing of nutritional flux from aquatic insects. Curr. Biol. 32, 1342-1349.e3. https://doi.org/10.1016/j.cub.2022.01.057

Slos, S., De Meester, L., Stoks, R., 2009. Food level and sex shape predator-induced physiological stress: immune defence and antioxidant defence. Oecologia 161, 461–467. https://doi.org/10.1007/s00442-009-1401-2

Snart, C.J.P., Hardy, I.C.W., Barrett, D.A., 2015. Entometabolomics: applications of modern analytical techniques to insect studies. Entomol. Exp. Appl. 155, 1–17. https://doi.org/10.1111/eea.12281

Späth, J., Fick, J., McCallum, E., Cerveny, D., Nording, M.L., Brodin, T., 2022. Wastewater effluent affects behaviour and metabolomic endpoints in damselfly larvae. Sci. Rep. 12, 1–14. https://doi.org/10.1038/s41598-022-10805-9

Stillwell, R.C., Davidowitz, G., 2010. Sex differences in phenotypic plasticity of a mechanism that controls body size: implications for sexual size dimorphism. Proc. R. Soc. B Biol. Sci. 277, 3819–3826. https://doi.org/10.1098/rspb.2010.0895

Stubbington, R., Bogan, M.T., Bonada, N., Boulton, A.J., Datry, T., Leigh, C., Vander Vorste, R., 2017. The Biota of Intermittent Rivers and Ephemeral Streams: Aquatic Invertebrates, Intermittent Rivers and Ephemeral Streams: Ecology and Management. Elsevier Inc. https://doi.org/10.1016/B978-0-12-803835-2.00007-3

907 Sun, C., Dudley, S., Trumble, J., Gan, J., 2018. Pharmaceutical and personal care products-
908 induced stress symptoms and detoxification mechanisms in cucumber plants. Environ.
909 Pollut. 234, 39–47. https://doi.org/10.1016/j.envpol.2017.11.041

910 Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R., 2014. Abiotic and biotic stress
911 combinations. New Phytol. 203, 32–43. https://doi.org/10.1111/nph.12797

912 ter Braak, C.J.F., Šmilauer, P., 2012. Canoco reference manual and user's guide: software for
913 ordination, version 5.0. Microcomputer Power.

914 Tijani, J.O., Fatoba, O.O., Petrik, L.F., 2013. A Review of Pharmaceuticals and Endocrine-
915 Disrupting Compounds: Sources, Effects, Removal, and Detections. Water, Air, Soil Pollut.
916 224, 1770. https://doi.org/10.1007/s11270-013-1770-3

917 Torres-Ruiz, M., Wehr, J.D., Perrone, A.A., 2010. Are net-spinning caddisflies what they eat? An
918 investigation using controlled diets and fatty acids. J. North Am. Benthol. Soc. 29, 803–813.
919 https://doi.org/10.1899/09-162.1

920 Van Geest, J.L., Poirier, D.G., Sibley, P.K., Solomon, K.R., 2010. Measuring bioaccumulation of
921 contaminants from field-collected sediment in freshwater organisms: A critical review of
922 laboratory methods. Environ. Toxicol. Chem. 29, 2391–2401.
923 https://doi.org/10.1002/etc.326

924 Vescio, R., Caridi, R., Laudani, F., Palmeri, V., Zappalà, L., Badiani, M., Sorgonà, A., 2022. Abiotic
925 and Herbivory Combined Stress in Tomato: Additive, Synergic and Antagonistic Effects and
926 Within-Plant Phenotypic Plasticity. Life 12, 1804. https://doi.org/10.3390/life12111804

927 Veseli, M., Rožman, M., Vilenica, M., Petrović, M., Previšić, A., 2022. Bioaccumulation and
928 bioamplification of pharmaceuticals and endocrine disruptors in aquatic insects. Sci. Total
929 Environ. 838. https://doi.org/10.1016/j.scitotenv.2022.156208

930 von Schiller, D., Acuña, V., Aristi, I., Arroita, M., Basaguren, A., Bellin, A., Boyero, L., Butturini,
931 A., Ginebreda, A., Kalogianni, E., Larrañaga, A., Majone, B., Martínez, A., Monroy, S.,
932 Muñoz, I., Paunović, M., Pereda, O., Petrovic, M., Pozo, J., Rodríguez-Mozaz, S., Rivas, D.,

Sabater, S., Sabater, F., Skoulikidis, N., Solagaistua, L., Vardakas, L., Elosegi, A., 2017. River ecosystem processes: A synthesis of approaches, criteria of use and sensitivity to environmental stressors. Sci. Total Environ. 596–597, 465–480. https://doi.org/10.1016/j.scitotenv.2017.04.081

Wang, P., Zhu, B.-T., 2017. Unique effect of 4-hydroxyestradiol and its methylation metabolites on lipid and cholesterol profiles in ovariectomized female rats. Eur. J. Pharmacol. 800, 107–117. https://doi.org/10.1016/j.ejphar.2017.02.032

Waringer, J., Graf, W., 2011. Atlas of Central European Trichoptera Larvae. Erik Mauch Verlag, Dinkelscherben.

Wat, L.W., Chao, C., Bartlett, R., Buchanan, J.L., Millington, J.W., Chih, H.J., Chowdhury, Z.S., Biswas, P., Huang, V., Shin, L.J., Wang, L.C., Gauthier, M.-P.L., Barone, M.C., Montooth, K.L., Welte, M.A., Rideout, E.J., 2020. A role for triglyceride lipase brummer in the regulation of sex differences in Drosophila fat storage and breakdown. PLOS Biol. 18, e3000595. https://doi.org/10.1371/journal.pbio.3000595

Webb, B.W., Hannah, D.M., Moore, R.D., Brown, L.E., Nobilis, F., 2008. Recent advances in stream and river temperature research. Hydrol. Process. 22, 902–918. https://doi.org/10.1002/hyp.6994

Wilkinson, J., Hooda, P.S., Barker, J., Barton, S., Swinden, J., 2017. Occurrence, fate and transformation of emerging contaminants in water: An overarching review of the field. Environ. Pollut. 231, 954–970. https://doi.org/10.1016/j.envpol.2017.08.032

Wrona, F.J., Prowse, T.D., Reist, J.D., Hobbie, J.E., Lévesque, L.M.J., Vincent, W.F., 2006. Climate change effects on aquatic biota, ecosystem structure and function. Ambio 35, 359–369. https://doi.org/10.1579/0044-7447(2006)35[359:CCEOAB]2.0.CO;2

Yoon, D.S., Byeon, E., Kim, D.H., Lee, M.C., Shin, K.H., Hagiwara, A., Park, H.G., Lee, J.S., 2022. Effects of temperature and combinational exposures on lipid metabolism in aquatic invertebrates. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 262, 109449. https://doi.org/10.1016/j.cbpc.2022.109449

960 Zezulka, Š., Kummerová, M., Babula, P., Váňová, L., 2013. Lemna minor exposed to
961 fluoranthene: Growth, biochemical, physiological and histochemical changes. Aquat.
962 Toxicol. 140–141, 37–47. https://doi.org/10.1016/j.aquatox.2013.05.011

963

Click here to access/download
Supplementary Material
FoS Micropterna MS_Supplementary material_R1_for submission.docx

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.

CRediT authorship contribution statement

Iva Kokotović: Data curation, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Marina Veseli:** Investigation, Methodology. **Filip Ložek:** Investigation, Methodology, Visualization, Writing - original draft. **Zrinka Karačić:** Investigation, Methodology. **Marko Rožman:** Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Ana Previšić:** Conceptualization, Investigation, Methodology, Funding acquisition, Writing - original draft, Writing - review & editing.