Investigating multiple stressor effects at the molecular level

Transcriptomic profiling of freshwater macroinvertebrate species exposed to anthropogenic stressors

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This thesis is dedicated to all the creatures that were sacrificed

for the sake of science.

Summary

Freshwater ecosystems are simultaneously exposed to multiple anthropogenic stressors, resulting in a global decline of freshwater biodiversity. Identifying the drivers of stream ecosystem deterioration is therefore urgently required to establish effective environmental protection strategies. This is, however, challenging because the impact of stressors depends on their abiotic and biotic context: co-occurring stressors can weaken or amplify each other, resulting in antagonistic or synergistic stressor interactions, respectively. Further, stressors can induce indirect effects e.g., through alterations of biotic interactions, which in turn induce trophic cascades. This context-dependency can only be resolved when the environmental complexity is decomposed experimentally. Semi-natural mesocosm field experiments have gained momentum in multiple stressor research because they allow the controlled manipulation of environmental stressor levels while incorporating sufficient natural complexity to obtain realistic estimates of stressor effects. In these experiments, stressor effects are often quantified based on specimen abundance changes following stressor exposure. Yet, relying only on changes in specimen abundances can be misleading because organisms have physiological protective pathways, allowing them to withstand unfavorable habitat conditions for some time. In fact, sublethal stressor effects are likely to be missed during the short time frame of an ecological experiment. In contrast, physiological endpoints have a higher temporal resolution. Specifically, changes in gene expression are one of the most immediate physiological responses to environmental stimuli. Therefore, this thesis addresses the context-dependency of multiple stressor dynamics from a functional genomics perspective through the integration of RNA-sequencing and ecological experiments. As such, the studies in this thesis focus on the molecular basis of stressor-induced physiological response mechanisms in four freshwater macroinvertebrates: the caddisfly *Lepidostoma basale*, the mayfly *Ephemera danica* and the amphipods *Gammarus fossarum* and *Gammaurs pulex*.

These focal taxa are non-model organisms i.e., they belong to taxonomically underexplored groups in the field of genomics. For RNA-sequencing data obtained from non-model organisms, appropriate analyses pipelines must be purpose built. Therefore, I established in Chapter 2 a data processing workflow which generates high-quality *de novo* transcriptomes and reliable expression estimates

and provide the rationale behind the data analysis strategy employed in the subsequent ecological studies.

In the following study (Chapter 3), a mesocosm field experiment was performed to assess the impact of increased fine sediment load, reduced flow velocity and increased salinity on the gene expression profile of the amphipod *G. fossarum*. Because stressor effects were already quantified based on specimen abundance changes of gammarids in the same experiment, it was possible to differentiate between stressor effects which were consistent across ecological scales and stressor effects which were only detected at the transcriptomic level due to the high temporal resolution of expression data. Reduced flow velocity compensated the transcriptional profile of increased fine sediment, an antagonistic stressor interaction that was also detected at the population level. In addition, increased salinity induced by far the strongest transcriptomic response, contrasting stressor effects at the specimen abundance level where no salinity-induced change was observed.

In the following two chapters, RNA-sequencing was used to quantify exposure effects of the insecticide chlorantraniliprole, a globally applied control agent against butterfly pest species, in different abiotic and biotic contexts: in Chapter 4, chlorantraniliprole-induced transcriptional alterations were studied under well-controlled experimental conditions. Using an indoor experimental setup allowed an accurate correlation between insecticide exposure and changes in gene expression in the caddisfly *L. basale*, the mayfly *E. danica* and the amphipod *G. pulex*. Further, it was possible to test whether biotic interaction between the two leaf-shredding species *G. pulex* and *L. basale* modulates their insecticide-induced expression profiles. In Chapter 5, a mesocosm field experiment was used to study multiple stressor effects of chlorantraniliprole and increased fine sediment load on the gene expression profiles of *L. basale* and *G. pulex* under near-natural conditions. Both studies complement each other, allowing to identify patterns and transcriptional stress responses, which arise independent from the abiotic and biotic environment: *G. pulex* was only weakly affected by the insecticide stressor in both experiments (Chapter 4 and 5). In contrast, the aquatic insects *E. danica* (Chapter 4) and *L. basale* (Chapter 4 and 5) showed strong transcriptional alterations in response to chlorantraniliprole exposure. This suggests that the impact of chlorantraniliprole on the transcriptomic profile of

non-target taxa depends on the phylogenetic distance between the studied species and butterflies.

Further, the indoor experiment showed that biotic interaction modulated the insecticide-induced transcriptional stress response of *L. basale*, which was presumably the weaker competitor due to the strong insecticide-induced effect in this species (Chapter 4), whereas the mesocosm experiment revealed mainly antagonistic interactions between the insecticide stressor and increased fine sediment (Chapter 5).

In conclusion, the studies in this thesis provide important insights how multiple stressor exposure affects freshwater organisms. The chemical stressor had the most pronounced effects on the transcriptomic profiles of the selected macroinvertebrate species. In combination with non-chemical stressors, antagonism was the dominant type of stressor interaction. While all study species exhibited some form of metabolic depression in response to stressor exposure, some physiological responses appeared to be stressor specific: increased salt concentration affected the expression of ion transporter genes in *G. fossarum*, whereas chlorantraniliprole exposure resulted in the differential expression of genes involved in the insect development in *E. danica* and *L. basale*. The latter was not expected based on the described mode of action of chlorantraniliprole. These mechanistic insights of stressor effects and the high temporal resolution of expression data highlight the potential of transcriptomics in the field of multiple stressor research.

Content

Chapter 1

Introduction

Introduction

Multiple stressors in freshwaters

Pristine freshwater ecosystems are hotspots of biodiversity. Although standing and running freshwaters cover less than 1% of the Earth's surface area, approximately 10% of all described species are associated with these habitats (Shiklomanov 1993; Balian et al. 2008). Biodiversity is fundamental for the maintenance of ecosystem function and ecosystem services (Loreau et al. 2001), which comprise in freshwaters e.g., energy and organic matter cycling as well as pollutant attenuation (Costanza et al. 1997), allowing to sustain one of the most essential goods provided by nature: clean drinking water.

Despite being fundamental for mankind, freshwater ecosystems like rivers and streams are threatened by anthropogenic activities (Dudgeon et al. 2006; Ormerod et al. 2010; Vörösmarty et al. 2010; Dudgeon 2019). Stream ecosystems are confronted with e.g., water pollution (Malaj et al. 2014; Liess et al. 2021), salinization (Cañedo-Argüelles et al. 2013; Herbert et al. 2015), hydro-morphological degradation (Nilsson and Berggren 2000; Grill et al. 2019), increased nutrient input (Weijters et al. 2009; Schäfer et al. 2012), stressors linked to climate change such as increased temperature (Meyer et al. 1999; Woodward et al. 2010) or the introduction of alien species, which alter the structure of native food webs (Strayer 2010). These stressors often exert pressure on stream ecosystems simultaneously (Ormerod et al. 2010; Schäfer et al. 2012; Dudgeon 2019; Liess et al. 2021). Simultaneous stressor occurrences arise because stressors have the same source such as e.g., intensive land use (Allan 2004; Vander Laan et al. 2013), but also because stressors are transported by hydrological processes within the dendritic stream network (Crosa et al. 2006; Gozzard et al. 2011; Malaj et al. 2014). Therefore, stressors which entered the stream from spatially separated point sources can admix and accumulate at lower stream reaches (Nguyen et al. 2023). Especially chemical toxicants, which persist in the environment, contribute to a diffuse 'stressor background' that is omnipresent in the stream (Halbach et al. 2021; Liess et al. 2021). As such, the impact of initially isolated stressors becomes integrated at the catchment scale due to the connectivity and hierarchical structure of stream ecosystems (e.g., Malaj et al. 2014). This results in complex spatio-temporal stressor exposure dynamics, which are further shaped by weather conditions and seasonal fluctuations (Schulz and Liess 1999; Crosa et al. 2006; Halbach et al. 2021),

rendering the multiple stressor exposure scenario as the new default for freshwater biota. Unsurprisingly, freshwater systems are highly vulnerable to anthropogenic stressors and face greater species decline rates than the ones observed for the most affected terrestrial habitats (Ricciardi and Rasmussen 1999; Strayer and Dudgeon 2010; Reid et al. 2019). Identifying the consequences of environmental stressor exposure for freshwater organisms and thus the drivers of stream ecosystem deterioration is therefore urgently required in order to develop and implement effective management and protection strategies (Hering et al. 2015). However, quantifying the impact of individual stressors in natural streams is not only challenging due to the vast variety of stressors which are simultaneously present, but especially because co-occurring stressors can modulate their effects in complex ways, resulting in unexpected, non-linear ecological response patterns (Christensen et al. 2006; Dieleman et al. 2012; Jackson et al. 2016). Such stressor interactions are defined as deviations of the cumulative effects from assumptions derived from additivity, i.e., when joint effects are larger (synergism) or smaller (antagonism) than anticipated based on the individual stressor effects (Piggott et al. 2015c). For instance, streams in agricultural catchments are typically exposed to organic toxicants such as pesticides and fine sediment deposition at the same time, because these stressors enter the stream together with surface run-off from adjacent fields following precipitation (Schulz and Liess 1999; Stehle and Schulz 2015). If the impact of these stressors is quantified e.g., based on absolute or relative specimen abundance changes of a given taxon following stressor exposure, and both fine sediment and the pesticide individually evoke an abundance change of 5 specimens, the expected cumulative effect of both stressors would be an abundance change of 10 specimens under the assumption of stressor additivity. If the cumulative abundance change is smaller or larger, the stressors weaken or amplify each other, respectively (see Piggott et al. 2015c for detailed discussion). In the given example, the mechanism underlying the stressor interaction between pesticide exposure and increased fine sediment load might be a mutual alteration of the physico-chemical properties of the co-occurring stressors: pesticides tend to adsorb to fine sediment particles, a mechanism reducing the bioavailability of the dissolved pesticide in the water, yet leading to an enriched pesticide phase in the sediment (Leonard et al. 2001; Liu et al. 2004; Stehle and Schulz 2015). Accordingly, the pesticide exposure dynamics will vary for organismic groups with different ecological requirements, since an adsorption of the pesticide to

fine sediment particles would reduce the pesticide exposure concentrations for free-swimming organisms (i.e., antagonistic interaction) but would intensify these for sediment-dwelling organisms (i.e., synergistic interaction). Unfortunately, these complex dynamics are further modulated by indirect stressor effects (Jackson et al. 2021). At the population and community level, indirect effects typically arise when stressors generate trophic cascades, which in turn induce abundance changes of organisms that are not directly affected by the stressor itself (e.g., Hallmann et al. 2014). For instance, specimen abundances change in response to top-down or bottom-up regulatory mechanisms in the food web i.e., alterations of herbivory or predation dynamics (Rogers et al. 2016; Bruder et al. 2017; Rodrigues et al. 2018). The ability of biotic interactions to modify stressor effects is important to consider, especially since biotic interactions between native species are not necessarily perceived as stressor (i.e., not human-induced), although predation or competition can exert strong stress on individuals and mitigate their ability to cope with abiotic stressors such as pesticides (Coors and De Meester 2008; Knillmann et al. 2012; Kattwinkel and Liess 2014; Janssens and Stoks 2017). Since biotic factors such as competition or predation can result in the same compositional change of communities as abiotic drivers, the presence or absence of a species can only be explained when we account for both, biotic and abiotic constraints (Cadotte and Tucker 2017).

The complexity of natural systems and the multidimensionality of multiple stressor impacts lead to a context-dependency of stressor effects, which is currently poorly understood. In fact, the correlation between measurements of environmental stressors with biological response variables is often inconsistent and not reproducible (e.g., Ryan 1991; Dewson et al. 2007a; Dieleman et al. 2012; Kefford et al. 2012a), preventing to derive causal relationships. Due to the complex spatial and temporal stressor exposure patterns, stressor interactions and indirect effects, it appears impossible to measure all confounding factors which may affect a biological response variable in the field. Considering that stressor interactions are common (Jackson et al. 2016) and might even be the rule rather than the exception (Dieleman et al. 2012), it is questionable whether stressor effects can be reliably quantified in the field, if their effects are shaped by higher-order interactions with hidden stressors. At the same time, simplified lab experiments are highly controlled, and effects can be accurately quantified, but it is unknown to which degree the obtained results can be extrapolated to natural ecosystems (Townsend et al. 2008; Stewart et al. 2013; Boyle et al. 2016).

To move from mere descriptions to a deeper understanding of multiple stressor effects, we need to advance current approaches to evaluate the impact of multiple stressor dynamics (Segner et al. 2014; Schäfer and Piggott 2018; Simmons et al. 2021).

Advancing multiple stressor research

Assessing stressor effects under controlled conditions while incorporating natural complexity

Considering the numerous confounding factors which influence biological response variables, an assessment of effects is likely to be imprecise or even impossible in the field. While we cannot control for the different layers of complexity of multiple stressor dynamics in natural streams, we can conduct controlled experiments under adjustable conditions. Given the context-dependency of stressor effects, experimental settings are required that incorporate sufficient natural complexity to simulate a field realistic stressor exposure scenario, while allowing the controlled manipulation of environmental stressor levels (Townsend et al. 2008; Stewart et al. 2013). A possible solution addressing this trade-off is offered by the ExStream system (Piggott et al. 2015b), an innovative mesocosm field experimental setup enabling the study of multiple stressor effects under near-natural conditions. The ExStream system comprises circular experimental units i.e., mesocosms, which are constantly provided with water from an adjacent stream, thereby ensuring natural light, temperature and water chemistry conditions in the experiment. The mesocosm substratum and flow velocity resemble the natural conditions present in the study stream. Moreover, within each mesocosm, various structures such as leaf litter tubes, stones, and wood sticks are incorporated in order to create different microhabitats with food resources for the biological community exposed to different stressor treatments. Since stressor effects are modulated by other stressors, the freshwater organisms inhabiting the mesocosms are confronted with multiple stressors in a well replicated, full-factorial design, allowing to disentangle single and joint stressor effects and to specifically address stressor interactions.

Assessing stressor effects across ecological scales

A key advantage of the ExStream system is that stressor effects can be assessed based on various response variables across ecological levels. Identifying how stressor effects are propagated between different levels of biological organization is essential if we aim for a holistic understanding of multiple stressor dynamics from individuals to ecosystems (Simmons et al. 2021). However, most ExStream studies focused on stressor induced structural alterations of biological communities (e.g., Beermann et al., 2018a, 2018b; Elbrecht et al., 2016; Magbanua et al., 2016; Nuy et al., 2018; Piggott et al., 2015) and, to a lesser extent, on stressor impact on ecosystem functions such as organic matter decomposition (e.g., Magbanua et al. 2013; Piggott et al. 2015a; Bruder et al. 2016).

In contrast, stressor effects at the individual level such as physiological reactions received, so far, little attention in ExStream studies. In fact, physiological stress responses are typically estimated in highly controlled experiments (e.g., Issartel 2010; Pestana et al. 2014; Janssens and Stoks 2017; Verheyen and Stoks 2020), neglecting the complexity of multiple stressor dynamics present under natural conditions. This is concerning given that stressor effects within individuals represent the most fundamental level on which stressors can act (Biagianti-Risbourg et al. 2013), thereby driving stressor effects at higher ecological levels such as population decline, which in turn may propagate to structural and functional alterations of the community composition, which lead to trophic cascades and finally compromise ecosystem function (Jackson et al. 2021) (Fig. 1.1). Since acclimatization (i.e., physiological compensation) to changing environmental conditions defines an organism's ecological amplitude, stressor effects are expected to be propagated to higher ecological levels if these exceed an organism's physiological coping ability. This, in turn, implies that the identification of stressor-induced physiological reactions allows to detect subtle stressor effects *before* these translate into changes of specimen abundances due to migration or mortality: assuming that organisms try to withstand unfavorable habitat conditions for a given period of time, sublethal effects such as decreased growth rates or reduced reproductive output might be missed if stressor effects are solely quantified based on changes in specimen abundances, although they could lead to pronounced effects on higher ecological levels if stressors persist.

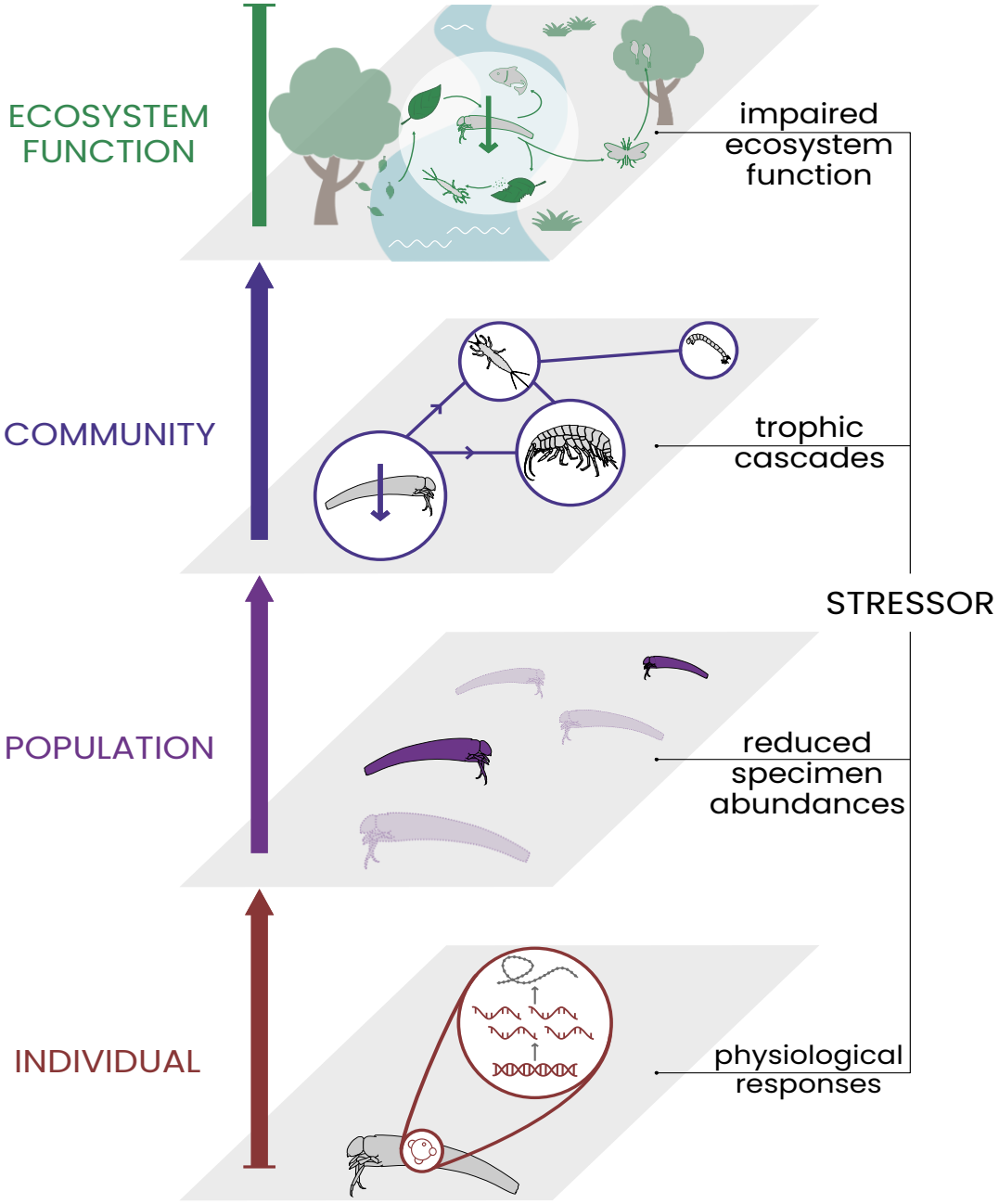

Fig. 1.1: Ecological scales at and across which stressors operate. At the base level, stressors act on the metabolism and physiology within individuals. Since physiological responses are ultimately controlled by gene expression networks, the transcriptional plasticity represents the molecular basis for an individual's ability to cope with environmental stress. Since a physiological compensation of stressor effects is limited, stressors exceeding the fundamental ecological niche of an individual will be propagated to higher ecological levels. For example, salinization of a river induces osmotic stress in leaf-shredding caddisfly larvae. Although the increased ion concentration might be sublethal, the physiological compensatory mechanism within the individuals (e.g., osmoregulation) is associated with metabolic costs, resulting in energy allocation from growth and reproduction. The long-term consequence will be a population decline, i.e., reduced specimen abundances. Since species are linked by trophic interactions and organized in networks, such stressor effects at the population level can induce trophic cascades. Depending on the traits of the affected species, ecosystem function such as the shredder-mediated conversion of energy and organic matter might be compromised by stressors which have adverse effects on an organism's physiology.

Considering the limited temporal resolution of population level data is especially important due to the relatively short time frame of ecological experiments i.e., typically days to weeks (e.g., Magbanua et al. 2013; Lavtižar et al. 2015; Rodrigues et al. 2017, 2018; Chará-Serna and Richardson 2018), highlighting how physiological stress responses can complement the assessment of multiple stressor effects.

Identifying mechanistic targets of stressors

Including stressor-induced physiological responses provides the opportunity to detect sublethal stressor effects before they are reflected at the population level i.e., induce changes in specimen abundances. Further, the assessment of stressor-induced physiological response mechanisms provides insights in the stressor's mode of action within individuals (Simmons et al. 2021). Identifying the mechanism through which stressors act on individuals, populations, communities and finally ecosystems will improve our understanding of the context dependency of stressor effects because stressor interactions are likely to arise if stressors operate on the same mechanistic target (Velasco et al. 2019; Simmons et al. 2021). For example, physiological stressor responses are coordinated by cellular signaling pathways; if two stressors trigger the same regulatory pathway (i.e., the stressor's target), the metabolic costs associated with downstream physiological protective responses might be mitigated, reducing the magnitude of stressor effects (antagonism) (Velasco et al. 2019; Delnat et al. 2020). In contrast, when energetic trade-offs emerge between dependent physiological stress responses, the presence of one stressor may impair the protection against the other, potentially inducing synergistic effects (Velasco et al. 2019; Delnat et al. 2020).

Integrating ecological field experiments and high-throughput transcriptomic profiling in multiple stressor research

Project concept and innovation

In this project, we addressed the challenges outlined above and studied multiple stressor effects and their interactions of globally relevant anthropogenic stressors on freshwater macroinvertebrates using the ExStream system in combination with an indoor experiment. Complementing stressor effects derived from a semi-natural experimental setup with results obtained under highly

controlled conditions allows to determine consistent stressor effects, which arise independently from the biotic and abiotic context present in the experiment. The key innovation of the studies presented in this thesis is the integration of functional genomics data obtained from high-throughput transcriptomic sequencing (RNA-sequencing) in the assessment of multiple stressor effects. As such, this thesis focuses on multiple stressor effects on the gene expression profiles of selected freshwater macroinvertebrate taxa, but the work is part of a project which aimed to identify multiple stressor effects across ecological scales. Assessing stressor effects at the transcriptional level is our attempt to address the knowledge gap concerning multiple stressor effects at the individual scale. Due to the transcriptomic sensitivity to environmental stimuli, the temporal resolution of gene expression data sets is high (López-Maury et al. 2008; Bahrami and Drabløs 2016). Since RNA-sequencing allows an unbiased, global characterization of the present transcriptome in a biological sample, transcriptomic profiling can inform about mechanistic targets of stressors at the molecular level (Gonzalez and Pierron 2015). Accordingly, the integration of transcriptomics within the framework of a semi-natural field experiment allows studying the molecular basis of stressor-induced physiological response pathways under a field realistic exposure scenario. Further, we explored for the first time whether RNA-sequencing can capture the signal of interspecific competition between macroinvertebrate species relying on the same nutritional resources and how this biotic interaction alters stressor induced expression profiles.

Selected stressors

In this thesis, multiple stressor effects of fine sediment deposition, pesticide pollution, salinization and reduced flow velocity were assessed. All selected stressors represent major threats to stream ecosystems and are often associated with intensive land use and/or industrialization (Allan 2004; Cañedo-Argüelles et al. 2013; Grill et al. 2019; Liess et al. 2021). Therefore, they frequently occur concurrently in anthropogenically impacted catchments, but little is known about their combined effects and interactions. Specifically, no study to date has investigated multiple stressor effects of these stressors on the gene expression profiles of stream macroinvertebrates under field realistic exposure conditions.

Fine sediment erodes from exposed soils in agricultural fields and enters the stream during surface run-off (Wood and Armitage 1997; Allan 2004). Increased particle load leads to an increased water turbidity, resulting in negative effects for filter-feeding organisms (Lemly 1982), as well as photosynthetically active primary producers or visual predators due to decreased light penetration (Ryan 1991). Further, increased concentrations of suspended particles can physically damage aquatic biota due to abrasion (Jones et al. 2012). Fine sediment load can impact osmoregulation by clogging of respiratory organs (Lemly 1982; Jones et al. 2012) and/or induce oxygen depletion in the stream water (Henley et al. 2000; Pretty et al. 2006). Settled fine sediment fills gravel interstices (Beschta and Jackson 1979), thereby homogenizing the stream substratum, and can in extreme cases form an impermeable barrier that prevents interstitial water circulation (Schälchli 1992; Henley et al. 2000) and vertical migration of freshwater invertebrates (Jones et al. 2012; Vadher et al. 2015). As such, increased fine sediment load reduces the availability of habitats and food resources for macroinvertebrates (Lemly 1982; Jones et al. 2012). In multiple stressor experiments, many of them performed using the ExStream system, fine sediment was identified as the most pervasive stressor for the macroinvertebrate community (Piggott et al. 2012; Magbanua et al. 2013; Elbrecht et al. 2016; Beermann et al. 2018a; Chará-Serna and Richardson 2018). These observations were consistent across experiments and stressor combinations, highlighting the significance of fine sediment load as a stressor for freshwater macroinvertebrates.

Pesticides are toxic substances designed to harm target pest species, which can also have deleterious effects on non-target taxa such as freshwater organisms when they enter the environment (Liess and Schulz 1999; Beketov et al. 2013; Liess et al. 2021). For the macroinvertebrate community, which comprises many insect species in its taxonomic spectrum (Balian et al. 2008), insecticides constitute the largest risk. The toxicity of insecticides is typically evaluated under controlled conditions and based on a few species models well established in ecotoxicology (Calow and Forbes 2003). Although this improves the reproducibility and comparability of these studies, it also implies that exposure effects remain unknown for the majority of non-target aquatic biota and that an insecticide's toxicity is determined without considering the complex interplay between insecticides and other environmental variables. We selected the new

insecticide chlorantraniliprole as model compound, since only a few studies are available for this substance (Hashimoto et al. 2020). Chlorantraniliprole, which belongs to the family of anthranilic diamides, has been reported to be highly potent against lepidopteran pests, while showing an exceptional safety for non-target organisms during ecotoxicological risk assessment tests (Lahm et al. 2007; Sattelle et al. 2008). Therefore, the compound is globally applied and was detected in surface waters from e.g., Asia (Zhang et al. 2012), Europe (Liess et al. 2021), and the U.S. (Sandstrom et al. 2022). Especially in the Europe Union, anthranilic diamides are expected to gain even further relevance after the ban of several neonicotinoids (Schmidt-Jeffris and Nault 2016; Raby et al. 2022). Chlorantraniliprole serves as an agonist of the insect ryanodine receptor, a calcium channel located in the membrane of the sarcoplasmic reticulum in muscle cells (Lahm et al. 2007). Binding of chlorantraniliprole induces a conformational change of the ryanodine receptor, which subsequently results in the release of calcium from its intracellular storages (Lahm et al. 2007). The calcium ions bind to troponin, the protein complex responsible for stabilizing the activated state of actin filaments i.e., exposing their myosin binding sites (Sweeney and Hammers 2018). As such, a chlorantraniliprole-induced depletion of the intracellular calcium storages leads to uncontrolled muscle contraction, paralysis, feeding cessation and ultimately the death of the insect (Lahm et al. 2007). Although the mode of action of chlorantraniliprole is well defined, the physiological response mechanisms in aquatic non-target taxa are largely unknown.

Salinization of rivers represents an emerging threat to freshwater biodiversity (Cañedo-Argüelles et al. 2013; Herbert et al. 2015). Increased salinity is associated with declines in richness of salt-sensitive taxa (Piscart et al. 2006a; Kefford et al. 2011; Cañedo-Argüelles et al. 2012) and turnover of benthic invertebrate assemblage along increasing salt concentration gradients (Piscart et al. 2006b; Kefford et al. 2012b). The main sources of increased salt concentrations are agricultural derived saline run-off due to irrigation, mining activities, road run-off containing de-icing salts as well as industrial and urban wastewater discharge (Coring and Bäthe 2011; Cañedo-Argüelles et al. 2013; Herbert et al. 2015; Tiwari and Rachlin 2018; Thorslund et al. 2021). Increased salinity in freshwater systems changes the physical chemistry of stream habitats (Herbert et al. 2015), inducing osmotic pressure in aquatic biota which are physiologically adapted to their freshwater environment (Evans and Kültz 2020).

Increased ion concentrations directly interfere with the cellular physiology (Lignot et al. 2000; Evans and Kültz 2020), inducing osmoregulatory compensatory mechanisms which are associated with metabolic costs, thereby decreasing an organism's fitness (Cañedo-Argüelles et al. 2018).

Reduced flow velocities are the result of hydro-morphological alterations such as damming and water regulation (i.e., channelization) of running waters (Nilsson and Berggren 2000; Dewson et al. 2007a). Worldwide, less than a quarter of all large river systems flow without human construction into the sea (Grill et al. 2019). Hydrological stressors such as low flow velocities can have strong impacts on macroinvertebrate communities (Dewson et al. 2007b; Elbrecht et al. 2016; Beermann et al. 2018a), but often, the individual responses are taxon-specific (Dewson et al. 2007a; James et al. 2009). Presumably, this is because hydrological changes interfere with the physico-chemical properties of stream habitats: for instance, water temperature, pH, electrical conductivity and sedimentation rates typically increase (Dewson et al. 2007a), whereas oxygen concentrations can decrease in slow flowing waters (James et al. 2009). These habitat alterations have varying impacts on different organismic groups, depending on their biological and ecological traits (Dewson et al. 2007a). Identifying the physiological mechanisms induced by slow flow conditions could as such help to differentiate between effects evoked by e.g., thermal or osmotic stress.

Selected study species

To assess the impact of stressors at the level of individuals requires the selection of focal species. The benthic macroinvertebrate fauna is a good environmental descriptor due to the high sensitivity to habitat degradation of many taxa (Kenney et al. 2009). In this project, four macroinvertebrate species were selected as representatives of major arthropod orders: the mayfly (Ephemeroptera) *Ephemera danica* Müller, 1764, the caddisfly (Trichoptera) *Lepidostoma basale* (Kolenati, 1848) and the two amphipod (Amphipoda) crustaceans *Gammarus fossarum* Koch in Panzer, 1836, and *Gammarus pulex* (Linnaeus, 1758). As such, the selected test species comprise a wide phylogenetic and ecological spectrum.

The order Ephemeroptera is one of the oldest lineages of flying insects and its representatives colonize all continents except Antarctica (Jacobus et al. 2019).

Ephemeropterans are hemimetabolous insects which spend the majority of their lives as larvae (Bauernfeind and Humpesch 2001). The larval stages are always aquatic and often represent a large portion of the biomass in streams (Bauernfeind and Humpesch 2001; Jacobus et al. 2019). *E. danica* is a semivoltine mayfly species occurring across Europe (Buffagni et al. 2009; Schmidt-Kloiber and Hering 2015). The sand-burrowing larvae prefer rather slow flowing stream sections with sandy sediments rich in organic matter (Buffagni et al. 2009; Schmidt-Kloiber and Hering 2015). *E. danica* larvae form tubular burrows, from which they actively filter fine particulate organic matter from the water or collect detritus deposited in the sediment (Schmedtje and Colling 1996; AQEM expert consortium 2002; Schmidt-Kloiber and Hering 2015). The larval development of *E. danica* comprises 24-36 molting cycles until the final larval stage transforms in the subimago (Bauernfeind and Humpesch 2001). This fully-winged but pre-mature developmental stage of mayflies is unique among extant insects (Bauernfeind and Humpesch 2001). The subimago leaves the stream and finally completes the transformation to the reproductively active imago in the riparian vegetation (Bauernfeind and Humpesch 2001). As in all mayflies, the adult life of *E. danica* comprises only a few days (typically 2-4 days) (Bauernfeind and Humpesch 2001). Because mating is only possible during this short period of time, the terrestrial mayfly imago is adapted to maximize dispersal and reproduction but does not possess functional mouthparts or a functional digestive system (Jacobus et al. 2019). Therefore, *E. danica* imagos depend on the energy reserves acquired during their larval stage for their energy demanding courtship behavior and mating (Winkelmann and Koop 2006). Accordingly, the habitat quality of streams is important to maintain viable populations of *E. danica*.

Caddisflies are holometabolous insects whose larval stages live in aquatic environments, whereas the adults are terrestrial (Holzenthal et al. 2015). During an intermediate developmental stage, the pupae, caddisflies undergo a complete metamorphosis (Holzenthal et al. 2015). The order Trichoptera represents one of the most diverse orders of aquatic insects and their species richness is only surpassed by dipterans, which include many terrestrial species as well (Mackay and Wiggins 1979; Holzenthal et al. 2007). All other primarily aquatic insect orders combined comprise less species than the order Trichoptera (Mackay and Wiggins 1979; Holzenthal et al. 2007). A key adaptation linked to the success of trichopterans is the use of silk produced by the larval labial glands (Mackay and

Wiggins 1979). The silk is used for case construction, as well as net spinning in case-free caddisflies to capture food items (Mackay and Wiggins 1979). As such, this evolutionary invention, which caddisflies share with their sister order Lepidoptera, enabled trichopterans to occupy a diversity of ecological niches in their aquatic environment (Holzenthal et al. 2007). However, despite their high species diversity, caddisflies are lost at greater extinction rates than other freshwater insect orders (Sánchez-Bayo and Wyckhuys 2019).

The genus *Lepidostoma* is the most species-rich genus in the Lepidostomatidae, a family widely distributed over the northern hemisphere (Holzenthal et al. 2007). *Lepidostoma* sp. can be the dominant shredder taxon in forested stream sections (Andrushchenko et al. 2017), and a loss of these populations can have pronounced effects on the stream ecosystem. For instance, it was shown that litter decomposition rates in streams could not be maintained when *Lepidostoma* sp. was removed from the community (Ruesink and Srivastava 2001). Since these organisms are early colonizers after environmental disturbance, they play fundamental roles not only in the maintenance but also in the recovery of ecosystem function (Whiles et al. 1993). *L. basale* shows a strong ecological association with wood: larvae accumulate in high densities in leaf packs or inhabit stream areas with submerged wood or roots to find shelter from high velocities (Hoffmann 2000). Despite the low nutritional value of wood, *L. basale* is a facultative xylophagous shredder (Hoffmann and Hering 2000), therefore exploiting nutritional resources which are avoided by other shredder organisms. Due to their large abundances and the high wood ingestion quantities required to ensure sufficient caloric uptake, *L. basale* has a strong impact on organic matter flux dynamics in streams colonized by this species (Hoffmann 2000).

The genus *Gammarus* represents the amphipod genus with the highest number of palearctic epigean freshwater species (Väinölä et al. 2008). Gammarids are widely distributed hololimnic organisms (Karaman and Pinkster 1977) and can be the dominant macroinvertebrate taxon in stream ecosystems in terms of biomass and abundances (MacNeil et al. 1997). Because gammarids frequently accumulate in leaf packs and can sustain themselves from allochthonous leaf litter, they are classified as shredder organisms (MacNeil et al. 1997). Additionally, they show a great feeding plasticity and are known for cannibalism and intraguild predation (MacNeil et al. 1997, 1999), as well as predation on other macroinvertebrates (Kelly et al. 2002). The ability to assimilate a diversity of

food items clearly contributes to the success of gammarids in streams where nutritional sources seasonally change, or when new habitats are colonized (MacNeil et al. 1997). *G. fossarum* and *G. pulex* are widespread across Europe (Karaman and Pinkster 1977). Although their distribution area largely overlaps in central Europe, *G. fossarum* is restricted to south-eastern parts of Europe, whereas *G. pulex* inhabits also streams in the north-west of the continent (Goedmakers 1972; Karaman and Pinkster 1977; Schmidt-Kloiber and Hering 2015). *G. fossarum* typically inhabits headwater stream reaches that are characterized by lower water temperatures and faster current velocities, whereas *G. pulex* is less tolerant towards high flow velocities, therefore occurring mainly in the lower stream reaches (Kinzelbach and Claus 1977). Both species can coexist in transition zones, although *G. pulex* is often the stronger competitor (Kinzelbach and Claus 1977), especially under environmentally challenging conditions (Meijering 1991). Given the zonation of occurrence patterns of *G. fossarum* and *G. pulex* at the landscape and local scale, both species are important elements of distinct aquatic communities. Assessing the impact of multiple stressor exposure on both species individually is therefore highly important. Moreover, gammarids are known for their cryptic diversity (Katouzian et al. 2016). Especially within *G. fossarum*, several genetically distinct clades have been reported (e.g., Weiss et al. 2014). These clades can differ in their habitat requirements (Eisenring et al. 2016) as well as in their sensitivity towards toxicants such as pesticides (Feckler et al. 2012), highlighting the potentially high differential susceptibility towards environmental stressors even between morphologically indistinguishable cryptic lineages.

All selected study organisms constitute integral parts of aquatic food webs, thereby linking various trophic levels, and are involved in fundamental ecosystem processes. Streams depend on allochthonous organic matter input from the adjacent riparian habitat (Fisher and Likens 1973; Vannote et al. 1980). Through assimilation, shredder organisms like *L. basale*, *G. fossarum* and *G. pulex* provide access to these nutritional sources for higher trophic levels (Cummins 1973). Further, detritus feeders like *E. danica* profit from the shredder-mediated conversion of coarse particulate organic matter to fine particulate organic matter (Cummins 1973; Wallace and Webster 1996). Accordingly, shredders function as consumers and secondary producers. When the sand-burrowing *E. danica* feeds on detritus either by actively filtering fine particles from the water or by

collecting detritus from the sediment, the species contributes to stream water purification as well as bioturbation and bioirrigation of benthic habitats (Jacobus et al. 2019). Since adult insects represent important prey taxa for terrestrial predators, caddisflies and mayflies contribute to the organic matter and energy recirculation between aquatic and terrestrial ecosystems (Baxter et al. 2005). The abundances and thus the contributions to ecosystem processes of these merolimnic taxa undergo seasonal fluctuations linked to the developmental cycle of aquatic insects, as opposed to hololimnic organisms like *G. pulex* and *G. fossarum*, which can reproduce throughout the year (Roux 1970; Goedmakers 1981) and remain in their aquatic environment throughout their life.

While all study species are of significant importance for the integrity of aquatic food webs, they are also highly vulnerable to habitat degradation and water pollution, and their distribution patterns are constrained by oxygen concentration, fluctuations in pH and specifically acidification (Goedmakers 1981; Meijering 1991; Bonada et al. 2004; Schröder et al. 2015; Turley et al. 2016; Vilenica et al. 2017). Mayflies and caddisflies are typically amongst the most sensitive macroinvertebrate taxa to environmental disturbances (e.g., Weijters et al. 2009; Magbanua et al. 2010) such as elevated fine sediment levels, which result in decreased species richness and population declines due to increased drift rates or mortality (e.g., Elbrecht et al. 2016; Beermann et al. 2018a; Davis et al. 2018). Specifically, *Lepidostoma* sp. has been reported being susceptible to fine sediment, although for *L. basale* the association with woody debris seems to be more important than its preference for coarse substratum (Turley et al. 2016). Gammarids show a similar preference for coarse gravel substratum (Dahl and Greenberg 1996), as opposed to the sand-burrowing *E. danica* (Schmedtje and Colling 1996; Buffagni et al. 2009; Schmidt-Kloiber and Hering 2015), and tend to avoid increased fine sediment levels (Beermann et al. 2018a). All species are sensitive to increased salt concentrations (Georgiadis 1977; Bonada et al. 2004; Schröder et al. 2015) and pesticide pollution, yet data regarding chlorantraniliprole-induced effects on the selected species are lacking: *L. basale* and *E. danica* are both classified as 'SPEcies At Risk' based on the SPEAR index, which defines the pesticide vulnerability of a species based on its physiological sensitivity to pesticides and their recovery potential due to their ecological traits (Liess and Von Der Ohe 2005; Schmidt-Kloiber and Hering 2015). Likewise, *G. pulex* and *G. fossarum* are both sensitive to pesticide exposure (Liess and Schulz

1999; Adam et al. 2010; Lebrun et al. 2020), despite being not classified as SPEAR (Liess and Von Der Ohe 2005; Schmidt-Kloiber and Hering 2015).

While *E. danica* and *L. basale* naturally occur in moderate to slow flowing stream sections (Graf et al. 2008; Buffagni et al. 2009; Schmidt-Kloiber and Hering 2015), *G. pulex* and *G. fossarum* prefer faster current velocities (Schmedtje and Colling 1996; Peeters et al. 1998; Schmidt-Kloiber and Hering 2015). Especially *G. fossarum* usually inhabits fast flowing stream sections, but its preference (or tolerance) for higher flow velocities might rather represent a strategy to reduce competition pressure with other *Gammarus* species (Karaman and Pinkster 1977). Consequently, it is crucial to resolve multiple stressor effects on the selected species in order to identify the most pressing environmental stressors, before their impact leads to population declines which can disrupt aquatic and terrestrial food webs.

Aims and scope

The aim of this thesis was to quantify the effects of globally important stressors and their interactions on the gene expression profiles of selected key macroinvertebrate species and to uncover metabolic pathways responding to the environmental stressors. As such, the studies of this thesis address the question how stressor-specific transcriptomic responses are influenced by the presence of other environmental factors such as abiotic stressors and biotic interaction.

RNA-sequencing is a promising method to address the limited knowledge of multiple stressor effects at the individual level, allowing to study the molecular basis of stressor-induced physiological response mechanisms (Gonzalez and Pierron 2015), which are expected to drive stressor effects at higher ecological levels (Fig. 1.1). However, since the selected species are non-model organisms and the specimens were obtained from wild (i.e., genetically polymorphic) populations, the bioinformatic processing of their transcriptomic data is challenging and data analysis workflows must be purpose-built. Therefore, I benchmarked in Chapter 2 the performance of bioinformatic processing software in order to establish an RNA-sequencing data analysis pipeline, which is appropriate for non-model arthropods exposed to environmental stressors. As such, Chapter 2 provides the rationale behind the analysis strategy employed to process the RNA-sequencing data sets generated during the course of this project. The following three chapters (Chapter 3 - 5) address the context-dependency of stressor effects and focus on stressor-induced physiological response mechanism

in the focal species. In Chapter 3, multiple stressor effects of increased fine sediment levels, reduced flow velocity and increased salinity on the transcriptional response of *G. fossarum* were assessed. This data set provided the opportunity to compare transcriptomic stressor effects with stressor effects derived from abundance data of gammarids from the same experiment (Beermann et al. 2018a), allowing to discern effects that are exclusively detected at the transcriptomic level and stressor effects which were propagated to higher ecological levels. The obtained results indicated that RNA-sequencing data can be used to reliably detect multiple stressor effects and their interactions, which were consistent across ecological scales. Moreover, stressor effects were detected which were not reflected in specimen abundance changes of gammarids within the time frame of the experiment, highlighting the high temporal resolution of expression data. Based on these encouraging results, we used transcriptomic data to assess the impact of chlorantraniliprole exposure in different abiotic and biotic contexts, aiming to identify a core transcriptional program associated with the insecticide stressor. The study presented in Chapter 4 focuses on exposure effects of chlorantraniliprole on the gene expression profiles of *G. pulex*, *L. basale* and *E. danica*. Since biotic interactions, which are inherently part of natural stream communities, can interfere with an organism's ability to cope with insecticide stress (Coors and De Meester 2008; Janssens and Stoks 2017), the effect of biotic interaction in terms of interspecific competition between the two native shredders *L. basale* and *G. pulex* was included in the experimental design of this study. These data sets were generated during a controlled indoor experiment, allowing to quantify expression changes as a function of increasing insecticide concentration along a phylogenetic gradient of non-target taxa and to test how interspecific competition modifies the insecticide-induced expression profiles. As such, the obtained differential expression results serve as effect baseline for the last study (Chapter 5), in which multiple stressor effects of chlorantraniliprole and increased fine sediment on the transcriptomic response of *G. pulex* and *L. basale* were quantified under near-natural conditions. In the final chapter of this thesis (Chapter 6), I provide a synthesis of stressor effects which are consistent across experimental settings (i.e., different stressor combinations, indoor and outdoor experiments), different species (i.e., *L. basale*, *E. danica*, *G. pulex*, *G. fossarum*) and ecological scales (i.e., changes in gene expression and specimen abundances), and discuss limitations and future prospects of multiple stressor research.

Chapter 2

Establishing a bioinformatic processing pipeline for RNA-sequencing data from non-model arthropods

This chapter has not been published but the analyzed data were published in the following articles:

RNA-sequencing data from *G. fossarum*: Brasseur et al. (2022) - *BMC Genomics* (https://doi.org/10.1186/s12864-022-09050-1)

RNA-sequencing data from *E. danica*: (Brasseur et al. 2023b) - *Environmental Pollution* (https://doi.org/10.1016/j.envpol.2023.122306)

Summary

RNA-sequencing has greatly improved our understanding of the transcriptomic regulation of fundamental biological processes such as development, cell differentiation, or pathological mechanisms underlying diseases. Although the method significantly matured within the last decade, technical artifacts and the heterogeneity inherent to expression data still make the bioinformatic processing of these complex, high-dimensional data sets challenging (Finotello and Di Camillo 2015; Kukurba and Montgomery 2015; Conesa et al. 2016; Freedman et al. 2021). Further, the performance of bioinformatic algorithms used to process RNA-sequencing data can vary between organismic groups due to differences in their genomic architecture and transcriptional complexity (Zhao et al. 2011). Countless tools were developed for RNA-sequencing data analysis, each claiming superior performance over existing software solutions (e.g., Grabherr et al. 2011; Li and Dewey 2011; Peng et al. 2013; Patro et al. 2017; Bushmanova et al. 2019). However, for most non-model organism data, no literature evidence exists which software might be most suited to handle the taxon-specific characteristics of these data sets. Since only appropriately processed data can provide reliable differential expression results, I evaluated the performance of bioinformatic processing software in order to establish an analysis workflow that is appropriate for the analysis of Illumina short-read RNA-sequencing data obtained from two non-model arthropod species exposed to environmental stressors: the amphipod *Gammarus fossarum* data set from Brasseur et al. (2022) (Chapter 3) and the mayfly *Ephemera danica* data set from Brasseur et al. (2023b) (Chapter 4).

To perform differential expression analyses, RNA-sequencing read data must be transformed to counts, the digital representation of expression (Finotello and Di Camillo 2015). Counts are derived from transcript or gene abundance estimations (Finotello and Di Camillo 2015). Abundance estimations can be inferred from read alignments against a genomic or transcriptomic reference. An alignment-based abundance estimation is precise, but requires substantial computing time (Kanitz et al. 2015; Bray et al. 2016). More recently developed, ultra-fast mapping algorithms, which only approximate the read origin in the reference, claim to achieve a significant speed improvement at an accuracy comparable to alignment-based abundance estimation methods (Bray et al. 2016; Patro et al. 2017). Still, both approaches require a genomic or transcriptomic reference, which is often not available for non-model organisms. In these cases, a reference transcriptome can be assembled *de novo* from RNA-sequencing reads (e.g.,

Grabherr et al. 2011; Peng et al. 2013; Bushmanova et al. 2019), but the performance of assembly programs can differ between data sets (Hölzer and Marz 2019). Since technical artifacts are present in raw RNA-sequencing reads, quality control steps should be performed prior to assembly to prevent the incorporation of non-biological sequences in the transcriptome (Martin and Wang 2011; Nguyen et al. 2018). Such technical artifacts comprise sequencing adaptors and errors, as well as homopolymer stretches at the end of the sequencing read (Schröder et al. 2010; Martin and Wang 2011). Homopolymer stretches of adenine (A) or thymine (T) can arise due to mRNA enrichment protocols using oligo(dT) primers (Pickrell et al. 2010; Wilhelm et al. 2010), or represent sequencing artifacts from Illumina platforms using a two-color chemistry (Andrews 2016). The latter can result in overcalling of the guanine (G) base with high base call quality scores, which may hamper effective quality trimming (Andrews 2016). Although the best trimming strategy for RNA-sequencing data is up to debate (Fabbro et al. 2013; MacManes 2014), the computational implementation of quality trimming is straightforward. In contrast, the *de novo* transcriptome assembly and transcript abundance quantification represent major computational challenges (Martin and Wang 2011; Finotello and Di Camillo 2015) which are differently addressed by individual software approaches. In this chapter, I evaluated the impact of two different data pre-processing strategies on the quality of *de novo* transcriptome assemblies, the performance of three *de novo* transcriptome assemblers and the consistency of two transcript quantification approaches, thereby addressing the following three questions in Chapter 2:

(i) Does homopolymer removal in RNA-sequencing read data prior to quality trimming affect the quality of *de novo* transcriptome assemblies?

(ii) Which of the tested *de novo* assemblers (Trintiy, rnaSPAdes, IDBA-tran) generates high quality transcriptomes from RNA-sequencing data obtained from non-model aquatic arthropods exposed to environmental stressors?

(iii) Does the performance of an alignment-free transcript quantification algorithm (salmon) differ from the performance of an alignment-based quantification algorithm (RSEM)?

While homopolymer trimming had no considerable effect on the quality metrics of the generated transcriptome assemblies, the choice of the assembler showed a strong impact: IDBA-tran was substantially less sensitive than the two other assemblers and produced the most fragmented transcriptome assemblies. The low remapping rates of reads against IDBA-tran assemblies suggested that the input read data was not effectively leveraged by this algorithm. In contrast, Trinity and rnaSPAdes both generated comprehensive and contiguous *de novo* transcriptome assemblies, although Trinity appeared to be slightly more sensitive. This increased sensitivity, however, was associated with a higher redundancy in Trinity-generated assemblies compared to assemblies produced with rnaSPAdes.

When the quality of the transcriptome assembly was high, remapping rates were consistently above 90%. Although RSEM and salmon rely on different transcript quantification approaches (Li and Dewey 2011; Patro et al. 2017), both algorithms produced in general consistent isoform abundance estimations and generated highly correlated count data sets. Still, the alignment-free transcript quantification algorithm salmon was slightly more sensitive than the alignment-based approach of RSEM, increasing the average mapping rate to ~98%.

Taken together, this comparative evaluation indicated that the presence of homopolymers did not compromise the quality of the generated *de novo* transcriptome assemblies. Still, excessive homopolymers at the end of sequencing reads are expected to represent technical artifacts, therefore the RNA-sequencing data processing pipeline used in the following chapters includes the homopolymer removal step, followed by quality trimming of reads. The pre-processed reads are subsequently assembled with either Trinity or rnaSPAdes, and transcript abundance estimation was performed with either RSEM or salmon. Excitingly, I found that, irrespective of the choice of the assembler (i.e., Trinity or rnaSPAdes) and the transcript quantification algorithm, the *de novo* pipeline was able to generate count data sets which exhibit expression signals that are consistent with the expression signal derived from a genome-guided transcriptome analysis, highlighting the value and reliability of the *de novo* RNA-sequencing data analysis approach.

Establishing a bioinformatic pipeline for RNA-sequencing data from non-model arthropods

Introduction

Massively parallel sequencing of complementary DNA, termed RNA-sequencing, has revolutionized gene expression research (Wang et al. 2009). Compared to former hybridization-based microarrays, RNA-sequencing enables profiling of all RNA molecules present in a sample at single-base resolution without any *a priori* knowledge about the underlying genome (Wang et al. 2009; Finotello and Di Camillo 2015). This key advantage and the development of sophisticated bioinformatic algorithms has made transcriptomic profiling accessible for non-model organism research (Ekblom and Galindo 2011; Geniza and Jaiswal 2017).
However, RNA-sequencing produces high-dimensional data sets whose analysis is not straightforward (Conesa et al. 2016; Eldem et al. 2017). The properties of data sets from different experiments and organisms can vary due to technical (e.g., different library preparation methods, sequencing platforms) and biological reasons (e.g., organism-specific differences in GC content, transcriptome diversity) (Shi et al. 2021; García-Nieto et al. 2022). Despite the heterogeneity of expression data sets, bioinformatic algorithms are inevitably developed and evaluated using gold-standard data i.e., simulated data and/or model organism data (e.g., Bushmanova et al., 2019; Chandramohan et al., 2013; Teng et al., 2016; Voshall and Moriyama, 2018). For data obtained from non-model organisms, the performance of bioinformatic processing software needs to be evaluated and analysis workflows must be purpose-built since no algorithm fits all data sets (Kanitz et al. 2015; Teng et al. 2016; Hölzer and Marz 2019).

RNA-sequencing data analysis

If the aim of a study is to infer differential expression patterns in a non-model species, a standard bioinformatic processing pipeline for RNA-sequencing data comprises three main steps prior to statistical testing (Kukurba and Montgomery 2015):

1. Pre-processing of raw sequencing reads i.e., quality control.
2. Reconstruction of a transcriptomic reference i.e., *de novo* transcriptome assembly.
3. Transcript abundance estimation.

Reconstructing a representative transcriptome and the accurate quantification of transcript abundances are fundamental prerequisites for a robust differential expression analysis. Therefore, I evaluated the performance of different assembly and quantification software using RNA-sequencing data from non-model arthropods generated during this project. Both steps, assembly and quantification, represent major bioinformatic challenges, which can be, in principle, traced back to the same cause: relying on sequencing reads which are relatively short (~ 75-150 bp) compared to the length of the original biological sequences they derived from (Li et al. 2010; Grabherr et al. 2011) e.g., 3,058 bp on average estimated in *Drosophila melanogaster* (Adams et al. 2000). Since the quality of the input data is fundamental to ensure accurate downstream analysis results (Fabbro et al. 2013), the impact of quality trimming on the assembled transcriptomes was also assessed. While the implementation of quality trimming is computationally straightforward, the best trimming strategy for RNA-sequencing data is up to debate (e.g., Fabbro et al., 2013; MacManes, 2014; Williams et al., 2016). Therefore, I compared two different trimming strategies instead of different trimming software.
In the following, I outline the specific challenges associated with the three main processing steps. Based on literature evidence, I pre-selected three state-of-the-art assemblers and two quantification tools, and shortly synthesize the key differences between the algorithms, which are likely to affect their performance. The programs were chosen due to their reported high performance and the diversity of the underlying algorithms.

Quality control

Quality control is important to account for technical artifacts in raw sequencing reads. The most common error of Illumina platforms are nucleotide substitutions, which typically accumulate towards the 3' end of the read (Fox et al. 2014; Stoler and Nekrutenko 2021). This systematic error distribution is exploited by widely-used trimming tools like Cutadapt (Martin 2011) or its wrapper script TrimGalore! (https://github.com/FelixKrueger/TrimGalore), which start at the 3' end of the read and perform the quality cut-off when an increase in base quality is detected (Cutadapt manual). Another frequently observed technical artifact in sequencing data are homopolymers at the end of the reads (Schröder et al. 2010). In RNA-sequencing data sets, these homopolymers arise due to e.g., mRNA enrichment using oligo(dT)-primers, which result in mononucleotide stretches of

adenine (A) or thymine (T) at the end of the read (Pickrell et al. 2010). Additionally, libraries sequenced on Illumina NovaSeq or NextSeq platforms are biased towards high confidence overcalling of the guanine (G) base due to their two-color chemistry: during base calling, the absence of a fluorescence emission signal is interpreted as incorporation of a G (Van Pelt-Verkuil et al. 2019). In case of a premature termination of the read extension, high-quality but erroneous homopolymer stretches are appended to the read (Andrews 2016). If these Gs at the 3' end mask the quality profile leveraged by trimming tools, they might hamper effective trimming of lower quality bases (Andrews 2016). Accordingly, removing low-complexity reads prior to quality trimming might be necessary to improve the outcome of downstream processing steps such as the *de novo* transcriptome assembly.

De novo transcriptome assembly

Since for most non-model organisms no high-quality genome or transcriptome exist, a transcriptome must be assembled *de novo* from the cleaned read data (Conesa et al. 2016; Geniza and Jaiswal 2017). While the computational challenge inherent to short read data is not specific to transcriptome assemblies, the following biases unique to expression data must be addressed specifically: the coverage of different transcripts varies by several orders of magnitude and even different isoforms can show highly dynamic expression patterns (Grabherr et al. 2011). Shared sequence information, e.g., between alternatively spliced isoforms, introduces ambiguity, which is difficult to resolve (Grabherr et al. 2011). Many assembly algorithms were developed (e.g., Bushmanova et al., 2019; Grabherr et al., 2011; Peng et al., 2013), and their performance was evaluated extensively (e.g., Clarke et al., 2013; Hölzer and Marz, 2019; Voshall and Moriyama, 2018; Wang and Gribskov, 2017). Although these comparative studies agree that no optimal algorithm exists for all data sets, the Trinity (Grabherr et al. 2011) and rnaSPAdes (Bushmanova et al. 2019) assemblers are typically among the best performing programs (Clarke et al. 2013; Wang and Gribskov 2017; Voshall and Moriyama 2018; Hölzer and Marz 2019). Yet, both assemblers can require substantial computational resources i.e., time and memory consumption (Zhao et al. 2011; Lu et al. 2013; Hölzer and Marz 2019). The assembler IDBA-tran (Peng et al. 2013) instead requires significantly less memory than the two other programs and was shown to be generally fast, but often scored only in the midfield of assembly performance (Hölzer and Marz 2019). Since performance

but also computational efficiency are important characteristics to consider in the analysis of complex data sets (Zhao et al. 2011; Hölzer and Marz 2019), I included all three programs in this evaluation.
All selected algorithms use the *de Bruijn* graph assembly approach for sequencing reads (Fig. 2.1), which can be divided in the following steps (Martin and Wang 2011):

1. Decomposing the reads into seeds with a fixed length k (k-mer). Unique k-mers represent the *de Bruijn* graph nodes.
2. Consecutively overlapping k-mers are connected by edges (overlap = k-1).
3. Variable sites induce branching of the graph structure.
4. Contigs are derived by traversing all possible path combinations through the *de Bruijn* graph.
5. Contigs that are well supported by the read data are reported as assembled isoforms.

Although the same fundamental strategy is used, each assembly program relies on different assumptions and thresholds to construct, correct, and traverse the *de Bruijn* graph (Grabherr et al. 2011; Peng et al. 2013; Bushmanova et al. 2019). Trinity involves three functional submodules, Inchworm, Chrysalis and Butterfly (Grabherr et al. 2011). Inchworm creates a k-mer catalog (with k = 25), followed by the reconstruction of linear contigs based on overlapping (k-1)-mers through a greedy extension (Grabherr et al. 2011). The Inchworm module reports only the full-length sequence for a dominant isoform, but related contigs share sequence information by partially overlapping k-mers (Grabherr et al. 2011). Chrysalis clusters related contigs and constructs a *de Brujin* graph for each cluster (Grabherr et al. 2011). Butterfly operates on these graphs, collapsing the unbranched parts and identifying the path through the graph which is best supported by the read data (Grabherr et al. 2011). The construction of thousands of *de Bruijn* graphs, each ideally representing one gene and its transcriptional complexity (Grabherr et al. 2011), is a specific trait of Trinity, which was directly manufactured for RNA-sequencing data.

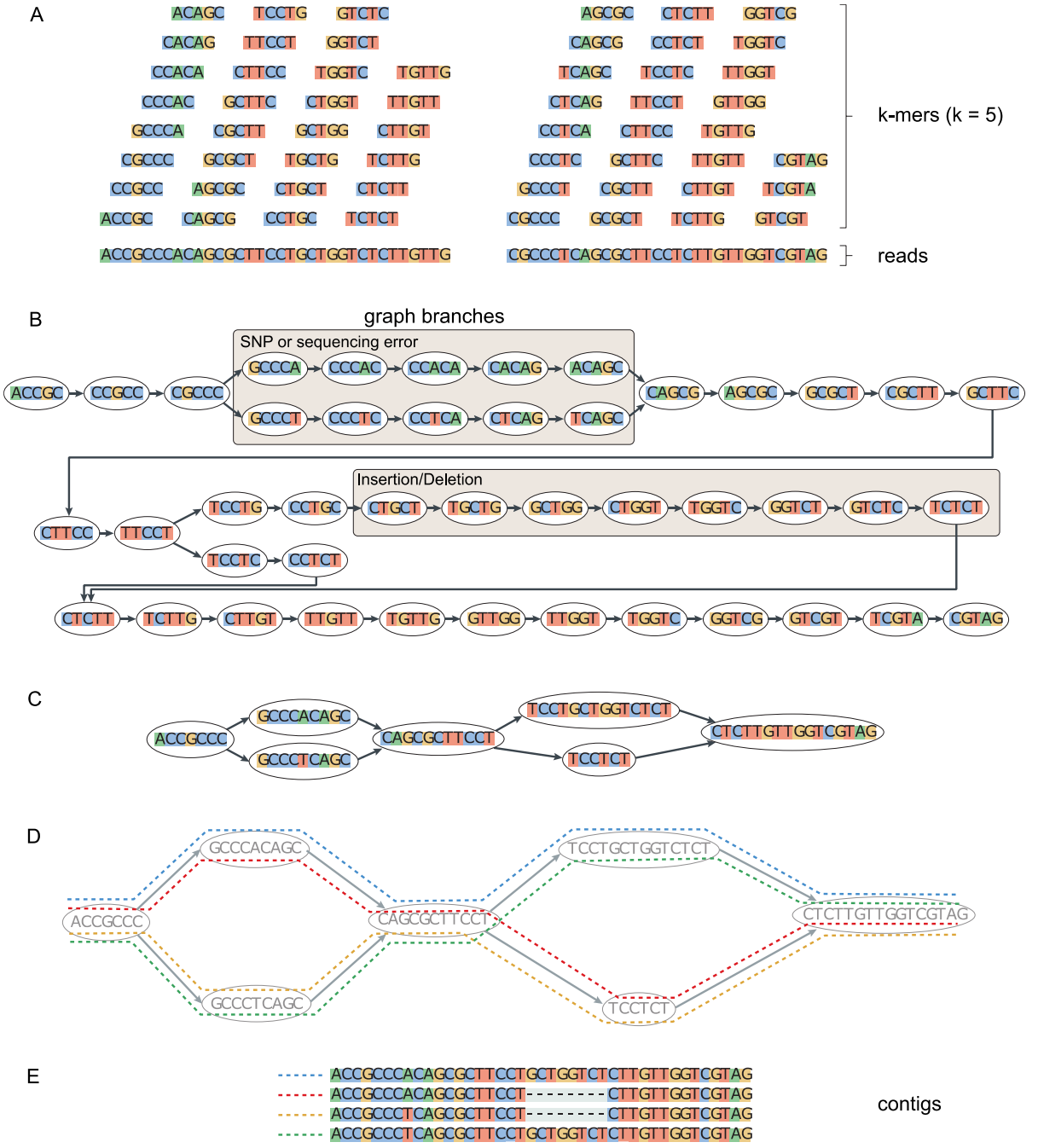

Fig. 2.1: *De Bruijn* graph-based sequence reconstruction. A) The reads are decomposed into overlapping substrings of length k (k-mers). Each k-mer overlaps the next k-mer by k-1 bases. B) During the graph construction, each unique k-mer is assigned to a node and consecutively overlapping k-mers are connected through edges. The graph branches at positions where similar sequences diverge (e.g., due to a sequencing error or a single nucleotide polymorphism). C) Following the removal of branches which are likely the result of sequencing errors, the nodes forming linear unbranched k-mer sequences are collapsed. D) The final reconstruction of contigs, is performed via traversing all possible paths across the graph. E) The path combinations which are well supported by the read data (e.g., coverage information) are reported as final contigs, which ideally represent transcripts. The figure was taken from Martin and Wang (2011) and modified by M. Brasseur.

In contrast, rnaSPAdes and IDBA-tran, which are extensions of the genome assemblers SPAdes (Bankevich et al. 2012) and IDBA (Peng et al. 2010), respectively, build a single *de Bruijn* graph that is subsequently decomposed (Peng et al. 2013; Bushmanova et al. 2019). In rnaSPAdes, the construction of the assembly graph is followed by a simplification step, including repeat resolution and scaffolding based on read alignments to the graph (Bushmanova et al. 2019). rnaSPAdes relies on an iterative assembly graph construction based on two values of k, which are dynamically determined based on the read length of the input data (Bushmanova et al. 2019). The multi k-mer strategy is similarly adopted by IDBA-tran, which starts with a small k to construct a graph that is iteratively updated by graphs formed based on larger k (Peng et al. 2013). In contrast to rnaSPAdes, the default values of k are fixed: the version of IDBA-tran used in this study initializes the assembly with k_{min} = 20, increases k by increments of 10 in each iteration, until k_{max} = 60. During each iteration, the previously generated contigs are treated as input reads for the following graph construction (Peng et al. 2013). Based on coverage information, the *de Bruijn* graph is modularized in components containing related contigs (Peng et al. 2013). A feature of IDBA-tran is the implementation of a probabilistic framework to model the error probability of a k-mer or a contig (Peng et al. 2013). Through progressive removal of erroneous contigs, connected subcomponents i.e., isoforms from the same gene, are finally resolved (Peng et al. 2013).

Quantification

Identifying the true origin of RNA-sequencing reads in a transcriptomic or genomic reference is a non-trivial task. Ideally, the perfect algorithm would resolve the true loci of which all reads were derived, but repetitive regions, sequencing errors and in particular shared sequence information between paralogs, alternatively spliced isoforms and antisense transcripts introduce ambiguity (Li et al. 2010). Consequently, a substantial number of reads map equally well to multiple locations (i.e., multi-mapping reads) in the reference (Mortazavi et al. 2008; Li et al. 2010).

Inferring transcript abundances comprises two steps: identifying the transcriptomic source of all reads in a given sample, followed by transcript abundance estimation (e.g., Li and Dewey, 2011; Nicolae et al., 2011; Patro et al., 2017). Two computational strategies exist to target the first part of the quantification problem: alignment-based (e.g., Li and Dewey, 2011; Nariai et al.,

2014; Nicolae et al., 2011) or alignment-free (e.g., Bray et al., 2016; Patro et al., 2017) algorithms. Alignment-based algorithms are typically very accurate, but their precise read alignment in a base-per-base fashion requires substantial computing time when the data set is complex (Kanitz et al. 2015; Bray et al. 2016). Alignment-free alternative algorithms are substantially faster (Patro et al. 2014; Bray et al. 2016; Zhang et al. 2017); they exploit the idea that an approximation of the read origin is sufficient, termed mapping, if the aim is expression quantification (Bray et al. 2016; Zhang et al. 2016) and search for a partial but exact overlap between a read and the reference (Bray et al. 2016; Srivastava et al. 2016; Patro et al. 2017).

The program RSEM is guided by read alignments and is often the best performing algorithm in benchmarking studies (Kanitz et al. 2015; Bray et al. 2016; Teng et al. 2016; Zhang et al. 2017). Despite being very accurate, RSEM is slow (Kanitz et al. 2015; Zhang et al. 2017). In contrast, the mapper salmon was shown to be substantially faster and almost as accurate as RSEM (Teng et al. 2016; Zhang et al. 2017). Both programs frame the expression estimation problem in a maximum likelihood context (Li and Dewey 2011; Patro et al. 2017). Apart from the expression parameter, their models implement parameters to correct for the non-uniform distribution of reads along a transcript, which arise during the generation of RNA-sequencing data e.g., due to differences in sequence composition and fragment length distribution (Li and Dewey 2011; Patro et al. 2017). Further, both rely on the expectation-maximization (EM) algorithm (Dempster et al. 1977) to iteratively assign reads to transcripts, proportional to the current transcript abundances (Li and Dewey 2011; Patro et al. 2017). RSEM estimates the probability that a read derived from a specific transcript given the observed read alignment data (Li and Dewey 2011). In other words, RSEM estimates transcript abundances which maximize the likelihood of the estimated expression values (Li et al. 2010; Li and Dewey 2011). During iterations of the EM algorithm, fractions of reads are proportionally assigned to their possible origins according to the relative expression of a transcript (expectation step) (Li and Dewey 2011). Then, the relative expression of a transcript is re-estimated based on the allocated read count (maximization step) (Li and Dewey 2011).

In the likelihood model of salmon, the expression parameter is defined as nucleotide fractions, which depend on transcript abundances (Li et al. 2010; Patro et al. 2017). Salmon tries to identify the nucleotide fractions of transcripts which maximize the probability that the nucleotides were sampled from a

particular transcript given the expression data (Patro et al. 2017). The bias model of salmon, which accounts for the non-uniform distribution of reads along a transcript, computes the probability that a particular read was generated given the specific transcript sequence i.e., its composition (Patro et al. 2017). During iterations of the EM-algorithm, read counts which are weighted by the bias model are proportionally assigned to the transcripts from which they were putatively sampled until model convergence (Patro et al. 2017).

Methods

The analyzed species repertoire of the RNA-sequencing data sets obtained during this project comprised two crustacean species of the order Amphipoda (*Gammarus* sp.) and aquatic larvae of the insect orders Trichoptera and Ephemeroptera. For this benchmarking study, two data sets were selected as representatives for crustaceans and insects: the RNA-sequencing data obtained from *Gammarus fossarum*, published in Brasseur et al., (2022) (Chapter 3) and from *Ephemera danica*, published in Brasseur et al., (2023) (Chapter 4). Libraries of both data sets contained pooled RNA extracts of two (*G. fossarum*) or three (*E. danica*) specimens and were generated with different library preparation protocols and sequencing strategies (Table 2.1). A detailed description of the library preparation protocols is given in the respective studies.

Table 2.1: Library preparation strategies of the RNA-sequencing data sets used in this benchmarking study.

	G. fossarum	*E. danica*
No. of libraries	32	36
RNA extraction	TRIzol + QIAGEN columns	GITC + magnetic beads
Library preparation	mRNA enrichment; unstranded	mRNA enrichment; stranded
Sequencing platform	HiSeq X	Illumina NovaSeq 6000
Read length	150 bp (paired-end)	150 bp (paired-end)

Bioinformatic processing

Quality of raw sequencing read data was checked with FastQC (Andrews 2010). Homopolymers were trimmed with a custom C++ (Stroustrup 1997) program, written by Christoph Mayer. Adapter removal and trimming of low-quality bases was performed with the cutadapt v3.2 wrapper script TrimGalore! v0.6.6 in paired-end mode, applying a base quality cutoff Phred value of 20 and retaining only reads with a minimum length of 25 bp. The length filtering was performed

in order to supply the same read data set to all assemblers, because Trinity only considers reads with a minimum length of 25 bp due to the fixed length of k = 25. *De novo* transcriptomes were assembled with Trinity v2.9.0 and v2.13.3, rnaSPAdes v3.15.0 and IDBA-tran v1.1.3. All assemblers were run in paired-end mode. Assemblies of *E. danica* were generated in strand-specific mode, except using IDBA-tran, which has no specific mode for stranded data. For the other parameters, the default settings were used, except for parameters specifying memory consumption and multi-threading. In total, six assemblies were produced for each species. Per species, two assemblies were generated with the same assembler, using either RNA-sequencing reads that were homopolymer trimmed prior to quality trimming or RNA-sequencing reads which were only subjected to quality trimming.

The cleaned sequencing reads used to generate the individual assemblies were either mapped with salmon v1.9.0 or aligned with bowtie2 v2.3.5.1 (Langmead and Salzberg 2012) followed by quantification with RSEM v1.3.3. Salmon was run in default mode except for parameters specifying different library preparation protocols (unstranded *G. fossarum* data: --libType IU; stranded *E. danica* data: --libType ISR) and parameters correcting for sequence-specific mapping biases (--validateMappings, --seqBias, --gcBias) as well as parameters controlling multi-threading. Similarly, RSEM was run with default parameters except the ones specifying multi-threading, paired-end data (--paired-end) and in the case of *E. danica,* strandedness (--forward-prob 0).

In total, 12 species-specific data sets were generated to evaluate the impact of the trimming strategy and the choice of the assembly and quantification software.

Evaluation

The quality of the transcriptome assemblies was evaluated based on predefined metrics, covering reference-free and reference-dependent quality elements. Reference-free metrics refer to technical aspects of the assembly, including basic statistics (min., max., mean, median contig length), remapping rates of reads used to assemble the transcriptome and the ExN50 statistic. The ExN50 value, proposed by the Trinity developers, is a modified version of the N50 value that is limited to the topmost highly expressed transcripts accounting for x% of the total expression (for further details, see the Trinity GitHub repository, accessed on 04.11.2023). These metrics were obtained with scripts shipped with Trinity and bash shell commands.

While useful, reference-free metrics cannot inform about biologically correct sequence composition, as opposed to reference-dependent metrics (Eldem et al. 2017; Voshall and Moriyama 2018). For non-model organisms lacking genomic resources, the gene content of an assembly can be evaluated based on homology searches (Voshall and Moriyama 2018). To obtain the coverage of full-length proteins in an assembly, BLASTX v2.9.0 (Altschul et al. 1990) searches (e-value < 1e-20) of the assembled transcripts were performed against all known proteins in the Swissprot/Uniprot database (The UniProt Consortium et al. 2021). A full-length protein was reported in an assembly, if a BLASTX match covered at least 90% of the protein's length.

Another reference-dependent evaluation is the assembly scanning for benchmarking universal single copy orthologs (BUSCOs) (Waterhouse et al. 2018). BUSCOs are expected to be highly conserved, therefore representing a known set of genes even in non-model organisms (Simão et al. 2015; Waterhouse et al. 2018). BUSCO recovery rates were obtained from the BUSCO v4 software and database (Manni et al. 2021). For *G. fossarum*, the arthropod BUSCO reference data set was used, comprising 1,013 BUSCOs. The insect reference BUSCO data set used for *E. danica* contained 1,367 BUSCOs.

To explore the consistency of expression estimates obtained from RSEM and salmon, the Pearson correlation coefficient between their generated count data sets was calculated. Only homopolymer trimmed Trinity and rnaSPAdes data sets were included here. Because the count data sets include for each sample all transcripts, a correlation of the expression estimates of all transcripts was not possible due to the large number of comparisons (no. of transcripts*no. of samples). Therefore, I randomly selected 100 transcripts for this correlation analysis.

All selected evaluation metrics provide important information about the quality of the generated transcriptomes and as such, the performance of the selected algorithms. However, the true underlying transcriptome and expression values remain unknown. Since a reference genome is available for *E. danica* (NCBI BioProject PRJNA171755), I compared expression estimates obtained from the *de novo* approach with a count data set from a genome-guided analysis. The aim of this comparison was to assess the ability of a *de novo* analysis pipeline to retrieve the expression signal reliably. Only count data sets that were homopolymer trimmed and generated with Trinity or rnaSPAdes were included in this comparison.

For the genome-guided analysis, homopolymer trimmed RNA-sequencing reads were aligned against the *E. danica* reference genome with HISAT2 v2.2.1 (Kim et al. 2019) as follows: first, splice sites and exons were extracted from the genomic feature annotation and the genome was indexed. Next, all libraries were individually mapped against the reference genome in strand-specific mode (--rna-strandness RF) with a maximum intron length of 350,000. The maximum intron length was initially estimated via mapping 10 million reads from a randomly selected library against the genome using bbmap v38.82 (Bushnell 2014). Alignments were reported for downstream transcriptome assembly (--dta) and transformed to coordinate sorted bam files with samtools v1.10 (Danecek et al. 2021). Transcript reconstruction and expression estimation was conducted with Stringtie v2.2.1 (Pertea et al. 2015) in stranded library mode (--fr) with a minimum isoform fraction of 0.01. Final counts were derived from coverage estimates using the prepDE.py script, which is shipped with Stringtie, using an average read length of 150 bp.
To infer the expression signal in the different count data sets in an unsupervised manner, the count data was normalized with DESeq2 v1.34.0 (Love et al. 2014) and principal component analyses (PCAs) were performed using the R package PCAtools v2.6.0 (Blighe and Lun 2021).

All bioinformatic processing steps were performed on a Linux based HPC server, in a Snakemake v7.20.0 (Köster and Rahmann 2012) workflow. Visualization of results was conducted in RStudio v2022.07.2 (RStudio Team 2022) with R v4.2.2 (R Core Team 2022) using the R package ggplot2 v3.4.1 (Wickham 2017) and ggridges v0.5.4 (Wilke 2022). InkScape v0.92.4 (Inkscape Project 2019) was used to digitize the silhouette of *G. fossarum* and *E. danica* and to add the generated icons to the result figures.

Results and Discussion

The bioinformatic processing of RNA-sequencing data comprises three major steps: data preprocessing, *de novo* assembly and transcript quantification (Kukurba and Montgomery 2015). The strongest effect was, by far, introduced by the choice of the assembler. This observation is consistent across all evaluation metrics, irrespective of the species, whereas the applied trimming strategy and the choice of the quantification algorithms had no considerable impact on the data sets.

The choice of the trimming approach

Theoretically, low-complexity reads can result in chimeric contigs, which were misassembled based on artificially shared sequence information (Bushmanova et al. 2019), or introduce read mapping biases (Li et al. 2010). In practice, however, no obvious benefit of the homopolymer removal was reflected in the data and most metrics were highly consistent between the differently trimmed data sets (Fig. 2.2 - 2.4). Slightly less bases and contigs were assembled when homopolymers were removed (Table 2.2, 2.3), reflecting the marginally reduced number of input reads due to the additional homopolymer removal step. Only the shape of the ExN50 curve of Trinity assemblies slightly changed when the homopolymer removal step was performed (Fig. 2.2). This variation is probably rather related to random components of Trinity or differentially handled borderline cases during different runs of the assembly process (Haas et al. 2013), than induced by the trimming approach. Importantly, these within-Trinity variations are mainly present for N50 values estimated from the subset of transcripts accounting for less than < 60% of the total expression, which appears to be more prone to random fluctuations. The impact of the trimming strategy is presumably limited because modern Illumina data is typically of high quality i.e., the majority of base quality scores ≥ 30 (Illumina website, accessed on 04.11.2023), and because assembly programs correct for or discard low-complexity sequences (Grabherr et al. 2011; Bushmanova et al. 2019).

Table 2.2: Basic statistics of the different assemblies generated with the *Gammarus fossarum* RNA-sequencing data. QT = Quality trimming, H + QT = Homopolymer removal + quality trimming.

Assembler	Trimming strategy	Assembled bases [Mb]	No. of contigs	Contig length [bp]				No. of full-length proteins
				Min.	Max.	Mean	Median	
Trinity	QT	998.5	1,393,435	180	33,568	717	376	9,171
	H + QT	980.2	1,372,461	168	32,242	714	376	9,153
rnaSPAdes	QT	838.9	1,045,534	73	28,548	802	378	8,648
	H + QT	817.1	1,025,640	73	28,545	797	380	8,689
IDBA-tran	QT	539.9	1,054,917	200	21,562	512	352	4,533
	H + QT	532.9	1,043,527	200	21,562	511	352	4,504

Table 2.3: Basic statistics of the different assemblies generated with the *Ephemera danica* RNA-sequencing data. QT = Quality trimming, H + QT = Homopolymer removal + quality trimming.

Assembler	Trimming strategy	Assembled bases [Mb]	No. of contigs	Contig length [bp]				No. of full-length proteins
				Min.	Max.	Mean	Median	
Trinity	QT	743.7	1,142,363	174	32,439	651	367	8,628
	H + QT	738.4	1,131,805	178	44,525	652	367	8,620
rnaSPAdes	QT	646.5	664,539	93	32,452	973	427	7,998
	H + QT	634.9	649,827	186	32,452	977	429	7,963
IDBA-tran	QT	308.3	647,645	200	18,511	476	342	4,063
	H + QT	305	636,905	200	18,511	479	344	4,066

The choice of the assembler

The remapping rates of the Trinity and rnaSPAdes assemblies were consistently above 90%, suggesting that most of the read data was leveraged. Both programs generated assemblies with decent contiguity, reflected in ExN50 values peaking on average at 1,910/2,630 bp (Trinity/rnaSPAdes) and at 2,056/2,756 bp (Trinity/rnaSPAdes) for *G. fossarum* and *E. danica* assemblies, respectively. While rnaSPAdes produced the most contiguous assemblies, Trinity consistently reconstructed the largest number of contigs as well as transcripts corresponding to full-length proteins (Table 2.2, 2.3). The higher sensitivity but reduced contiguity of Trinity compared to rnaSPAdes is related to the k-mer selection approach: Trinity assembles the read data based on 25-mers (Grabherr et al. 2011); these short k-mers are beneficial to resolve rare transcripts because the required overlap is small, but are also more likely shared by unrelated contigs, thereby increasing the number of chimeras (Zhao et al. 2011; Wang and Gribskov 2017). A larger value of k improves the assembly in terms of contiguity and correctness but comes at the cost of missing lowly expressed transcripts (Zhao et al. 2011; Wang and Gribskov 2017). In rnaSPAdes, a robust assembly, derived from the larger k-mer size (i.e., k = 73 if read length = 150 bp), is extended by rare transcripts that were only resolved with the smaller k-mer size (i.e., k = 49 if read length = 150 bp). As such, the multi k-mer approach of rnaSPAdes aids in retrieving the full transcriptomic spectrum (Chen et al. 2011; Zhao et al. 2011) but Trinity still appears to be more sensitive. Consistently, a slightly larger

number of duplicated BUSCOs was detected in the *G. fossarum* transcriptomes created with Trinity compared to the transcriptomes generated with rnaSPAdes. This increased sensitivity can decrease the ExN50 metric of Trinity, since rare transcripts are more likely to be partially assembled. Yet, both assemblers recovered a large portion of complete BUSCOs in general (Fig. 2.3) and performed overall well on all data sets.

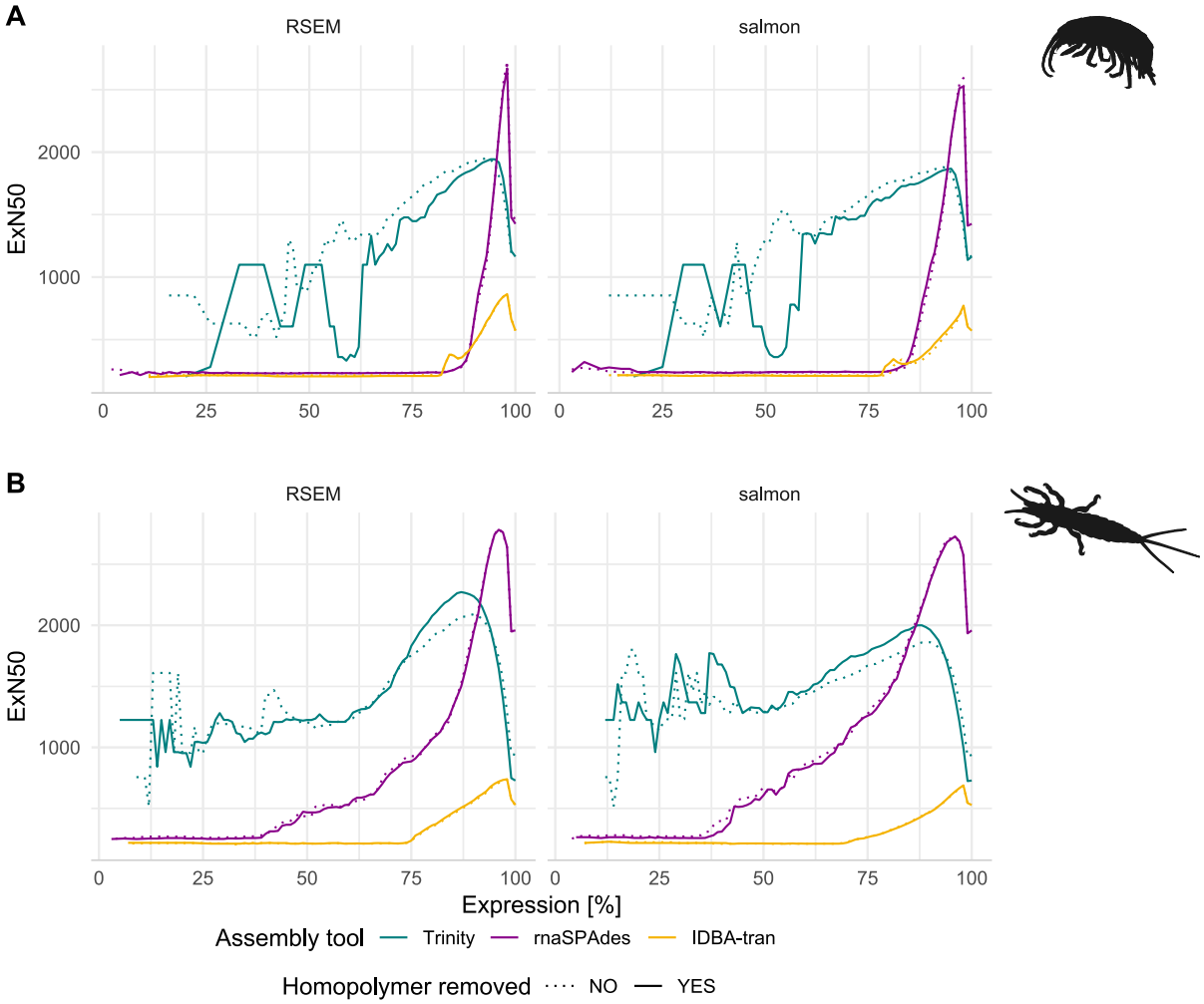

Fig. 2.2: ExN50 values of *G. fossarum* (top) and *E. danica* (bottom) assemblies, obtained from abundance estimation using RSEM (left) and salmon (right). These transcripts are expected to be well supported by the read data and therefore accurately assembled. The different trimming strategies are indicated by dashed and solid lines. The different colors indicate the assembly program.

In contrast, the read remapping rate for assemblies created with IDBA-tran was low (consistently < 60%) and the fewest transcripts corresponding to full-length proteins were detected in these assemblies (Table 2.2, 2.3), implying that IDBA-tran did not effectively exploit the input data. The IDBA-tran assemblies showed

considerably smaller ExN50 values (peaking on average at 864 bp and 712 bp for *G. fossarum* and *E. danica* assemblies, respectively), indicating partially assembled contigs which result in a fragmented transcriptome. Consistently, the largest numbers of fragmented and missing BUSCOs were observed when IDBA-tran was used (Fig. 2.3).

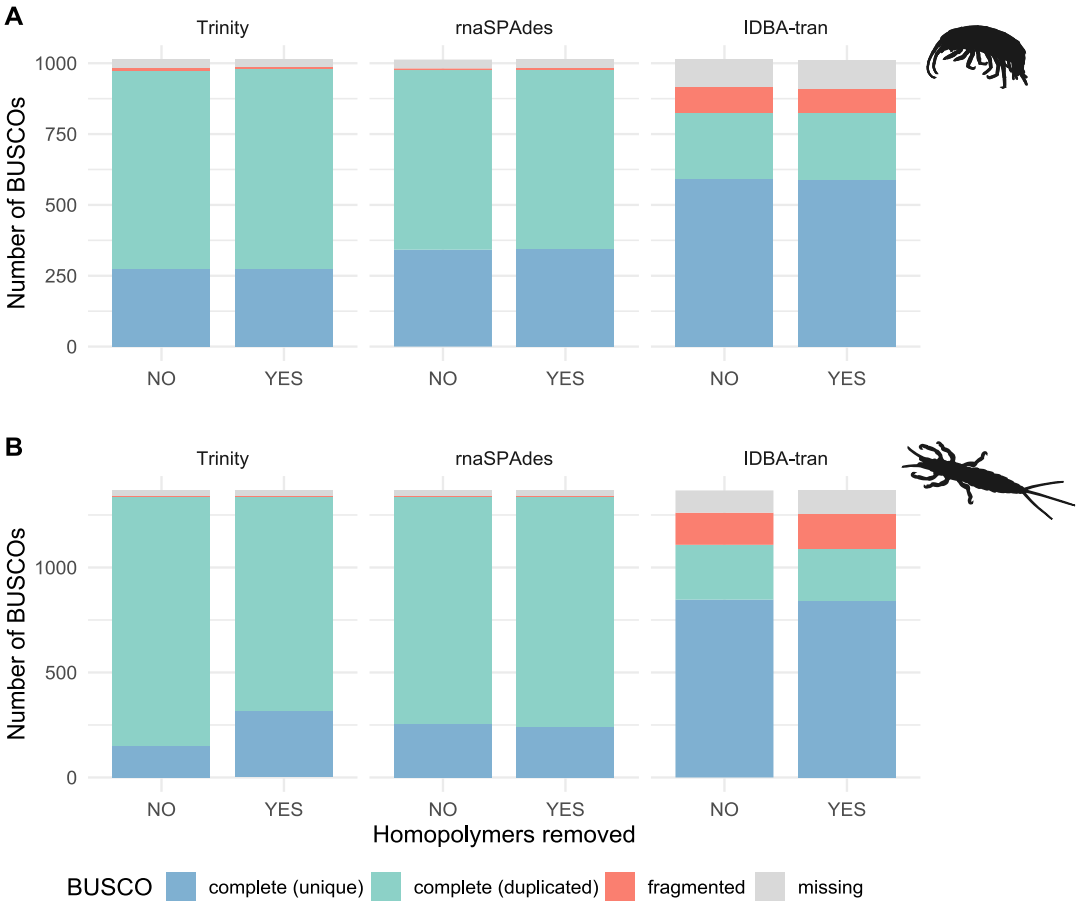

Fig. 2.3: BUSCO recovery in *G. fossarum* (top) and *E. danica* (bottom) assemblies. Due to alternative splicing, transcriptome assemblies typically contain a large proportion of duplicated BUSCOs.

The choice of the quantification method

Both RSEM and salmon were able to identify the origin of reads for at least 90% of the read data, if the assembly quality was sufficiently high (Fig. 2.4). Further, the shape of the ExN50 curves in well-supported data regions were highly consistent between the two quantification methods (Fig. 2.2). Salmon produced slightly higher read mapping rates than RSEM in both species data sets (Fig. 2.4), indicating that salmon is slightly less strict when defining read mapping thresholds. Overall, the estimated transcript counts of RSEM and salmon were in

good agreement and highly correlated (Fig. 2.5); only for the 100 randomly selected transcripts of the *G. fossarum* data assembled with rnaSPAdes, RSEM estimated higher abundances than salmon (Fig. 2.5B). While it is not possible to differentiate which transcript counts are correct, a systematic error is presumably less of concern for differential expression analyses, which focus on relative expression changes (i.e., fold change between samples).

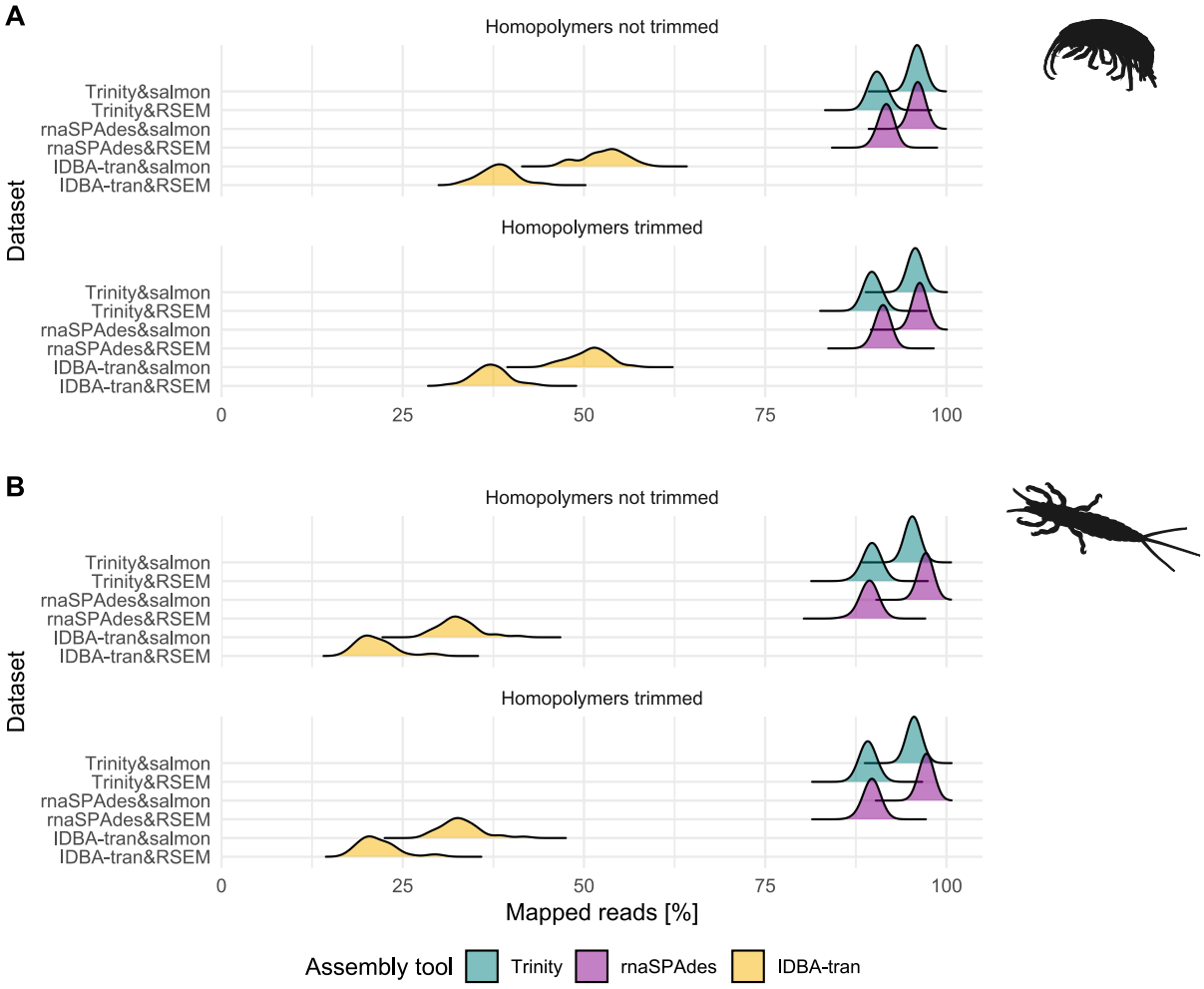

Fig. 2.4: Density distributions of mapping rates of reads used to assemble the transcriptomes of *G. fossarum* (top) and *E. danica* (bottom).

Implications – how to process data and which software to use for assembly and quantification?

Although no effect of the trimming strategy was observed, homopolymers that represent technical artifacts should be removed from the sequencing data to retain only biologically valid reads, and to reduce the amount of data that needs to be processed. Further, a stronger impact of quality trimming might arise if the quality of the data is low or the sequencing depth is shallow.

Based on the evaluation metrics, IDBA-tran appears to be unsuited for the assembly of RNA-sequencing data obtained from the species under investigation. The two other assemblers performed generally well on the data, although small differences between the generated assemblies were observed, which needs to be considered. Methodological imperfections such as incomplete stranding (Zeng and Mortazavi 2012) or sequencing errors (Stoler and Nekrutenko 2021) introduce artificial sequence diversity. When a highly sensitive assembler such as Trinity reconstructs these technical artifacts in separate contigs, non-biological redundancy will be introduced in the assembly. Further, Trinity is known to separately assemble allelic variants within and between organisms (Freedman et al. 2021), which are reported as different isoforms. For instance, Trinity reported in all assemblies that were generated during this project nearly identical sequences as different isoforms, which only differed in length or by single nucleotide polymorphism.

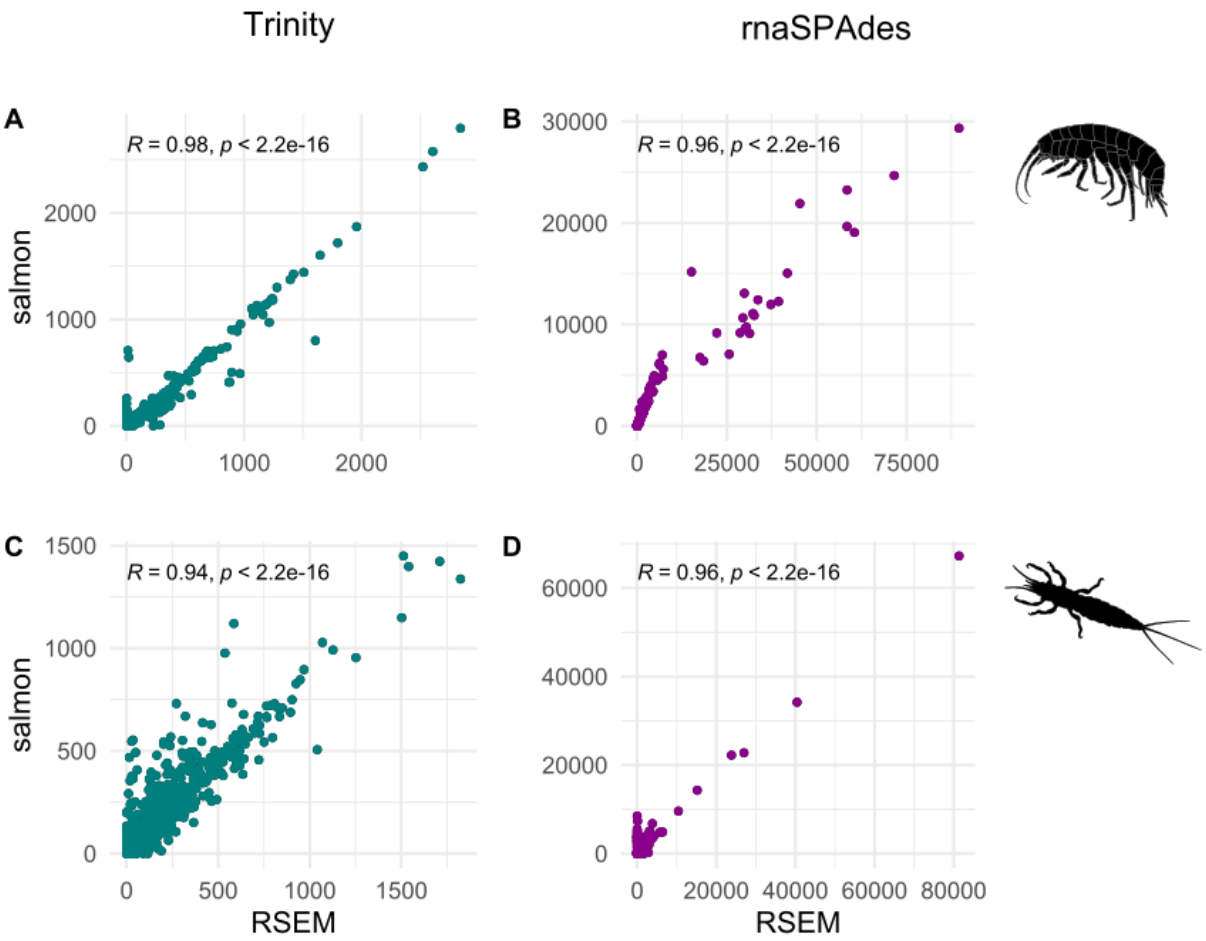

Fig. 2.5: Correlation between counts estimated with RSEM and salmon, for 100 randomly selected genes in *G. fossarum* (A,B) and *E. danica* (C,D). P-values were obtained from a t-test.

Taking the sensitivity of Trinity into account is therefore especially relevant when RNA-sequencing data are obtained from pooled libraries of wild (i.e., genetically diverse) organisms such as *G. fossarum*, which is known for its cryptic diversity (Weiss et al. 2014).

Consequently, rnaSPAdes which performed best in terms of contiguity and produced less redundant assemblies can be a more appropriate choice, although the lower sensitivity might come at the expense of missing rare i.e., lowly expressed transcripts. Further, the principle of contiguity does not perfectly apply to transcriptome assemblies which are per definition dynamic, and larger (Ex)N50 values do not necessarily reflect the true underlying biology but potentially reward assemblers that over-assemble sequences (Voshall and Moriyama 2018).

It should be noted that the here applied evaluation metrics can be used to identify assemblies of poor quality, as the ones produced by IDBA-tran, but that a distinction between 'good' assemblies is not straightforward. The same applies to the evaluation of quantification tools: high remapping rates are important since the aim is to leverage the read data as efficiently as possible. However, an accurate quantification should be favored instead of a sensitive one. Excitingly, the ordination-based inferences of the expression signal were consistent between count data sets generated by the *de novo* pipelines and the count data generated using a genomic reference (Fig. 2.6), suggesting that, despite the numerous approximations made by *de novo* transcriptome assemblers and transcript quantification algorithms, the retrieved biological signal is valid and robust.

Therefore, I conclude that both Trinity and rnaSPAdes can be used for the assembly of the RNA-sequencing data sets generated from non-model arthropods exposed to environmental stressors. Since the performance of assemblers can be unsatisfactory, as it was observed for IDBA-tran in this study, several assemblies should be generated and evaluated. The final choice of the assembler might further depend on its gene model i.e., how assemblers define isoform-to-gene relationships, when transcript counts are summarized to gene counts in order to perform differential expression at the gene level. Finally, post-assembly improvement might be necessary to reduce redundancy, such as assembly thinning or clustering (Raghavan et al. 2022).

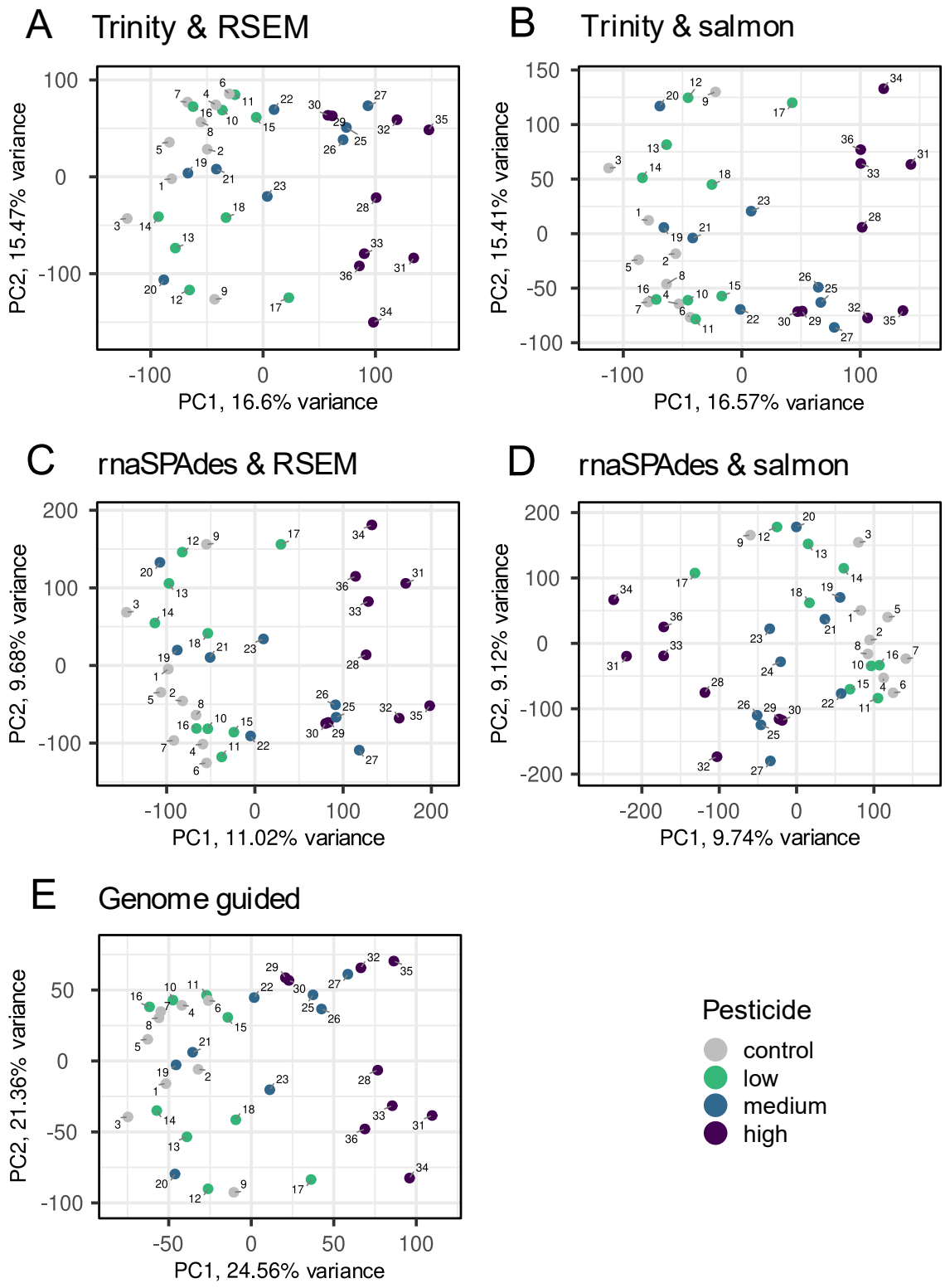

Fig. 2.6: Principal component analyses based on the *E. danica* count data sets generated with *de novo* assembly pipelines (A-D) and the genome-guided transcript reconstruction and quantification (E). The patterns are consistent between all data sets, but the orientation of the axes can be inverted. Due to lower redundancy in the expression data obtained from genome-guided bioinformatic processing, the first two principal components account for higher proportions of explained variance. This expression data set was obtained from an experiment in which the insecticide chlorantraniliprole was applied in three different concentrations (low, medium, high). For further information see Chapter 4 (Brasseur et al. 2023b).

Both RSEM and salmon are suited to estimate expression from the here analyzed transcriptomic data sets. While it is not possible to make a qualitative performance ranking based on the evaluation metrics, salmon requires significantly less computing time (e.g., for the *E. danica* data set ~ 2 days) than RSEM (e.g., for the *E. danica* data set ~ 10 days). This highly increased efficiency of salmon is not only enabled by its alignment-free mapping approach, but also by a reduction of the data complexity: while the initial inference problem is represented in the read data space, salmon groups reads based on alignment information in equivalence classes (Patro et al. 2017). As such, the number of classes grows with the complexity of the transcriptome, not with the number of input reads. Considering that RNA-sequencing data sets become increasingly complex, most researchers will presumably shift to computational solutions which can scale with the amount of data produced.

Conclusion

In this chapter, I established a bioinformatic pipeline to process the RNA-sequencing data sets generated during this project which comprises (i) a homopolymer removal step prior to quality trimming, (ii) a *de novo* transcriptome assembly using either Trinity or rnaSPAdes (depending on the individual assembler performance for each specific data set) and (iii) transcript abundance estimations using either RSEM or salmon. Because these assembly and quantification programs performed well and produced consistent results, the established analysis workflow should enable a high comparability between the expression results presented in the following chapters, while taking specific data set properties into account.

In general, a transcript reconstruction and transcript abundance estimation can be more robust and accurate if it is guided by a genome assembly compared to the *de novo* transcriptome assembly and subsequent abundance estimation, because a reference genome mitigates the challenges associated with the highly uneven coverage of expression data: the genome-guided transcript reconstruction allows to resolve ambiguities, resulting in less redundant transcriptomes and more uniquely assigned reads to their genomic or transcriptomic origin during abundance estimation. Moreover, genome assemblies can be constantly improved by performing additional sequencing experiments, whereas a *de novo* transcriptome assembly represents a dynamic snapshot of the analyzed RNA-sequencing data set. However, we are currently

far away from a scenario in which high-quality genomes (i.e., completely assembled *and* well annotated) are accessible for most of the taxonomic groups. Until then, the *de novo* approach is a valid and reliable analysis strategy for RNA-sequencing data sets obtained from non-model organism.

Chapter 3

Impacts of multiple anthropogenic stressors on the transcriptional response of *Gammarus fossarum* in a mesocosm field experiment

This study has been published under open-access in *BMC Genomics*:

Brasseur, M.V., Beermann, A.J., Elbrecht, V., Grabner, D., Peinert-Voss, B., Salis, R., Weiss, M., Mayer, C., Leese, F., 2022. Impacts of multiple anthropogenic stressors on the transcriptional response of Gammarus fossarum in a mesocosm field experiment. *BMC Genomics* 23, 816. https://doi.org/10.1186/s12864-022-09050-1

Summary

Anthropogenic stressors pose a high risk to aquatic biota such as macroinvertebrates, inducing decreased species densities and community shifts towards more tolerant taxa (Piscart et al. 2006a; Matthaei et al. 2010; Piggott et al. 2012; Macaulay et al. 2021). However, quantifying the impact of stressors solely based on changes in specimen abundances in ecological experiments that typically run for days or a few weeks (e.g., Magbanua et al. 2013; Lavtižar et al. 2015; Rodrigues et al. 2017, 2018; Chará-Serna and Richardson 2018) might be misleading since organisms have physiological coping mechanisms to counteract stressor effects (Biagianti-Risbourg et al. 2013), allowing them to withstand unfavorable habitat conditions, at least for a given period of time. Consequently, strong (physiological) stress might not be reflected in abundance data within the limited time frame of an ecological study. Integrating physiological reactions such as changes in gene expression allows to detect stressor effects with a high temporal resolution (Bahrami and Drabløs 2016) and provides insights into stressor-induced physiological response mechanisms (Gonzalez and Pierron 2015). Therefore, we used transcriptome-wide sequencing data obtained from an ExStream field experiment conducted in 2014 (Beermann et al. 2018a) to quantify single and combined effects of increased fine sediment deposition, reduced flow velocity and increased salinity on the gene expression profile of the amphipod *Gammarus fossarum*. The selected stressors are strongly associated with intensive land use, urbanization and industrialization (Allan 2004; Cañedo-Argüelles et al. 2013; Thorslund et al. 2021), therefore exerting pressure on freshwater biota in anthropogenically impacted catchments simultaneously: fine sediment is transported via surface runoff from exposed soils in agriculturally used areas (Henley et al. 2000). Salts enter streams via road run-off (Tiwari and Rachlin 2018), sewage or industrial wastewater discharge (Coring and Bäthe 2011), as well as agricultural derived run-off due to dissolved fertilizers (Vander Laan et al. 2013) or saltwater irrigation (Thorslund et al. 2021). Water abstraction for crop irrigation, which is expected to peak during periods when flow is naturally low (e.g., during summer), results in reduced discharge, thus impairing the transport efficiency of the stream (Dewson et al. 2007a). If sedimentation rates increase and/or the occurrence of chemical stressors is prolonged, complex stressor interactions can arise (e.g., Matthaei et al. 2010; Beermann et al. 2018a). RNA-sequencing offers the opportunity to quantify gene expression levels and to profile transcript sequences at the same time, enabling

to study the molecular basis of the physiological stress responses induced by these major anthropogenic stressors. However, transcriptomic data obtained from outbred organisms under experimental conditions which incorporate natural complexity are inherently characterized by expression noise due to high biological variability between samples (Todd et al. 2016). Since no study to date has addressed the challenging topic of identifying multiple stressor effects through integration of functional genomics in the framework of near-natural field experiments, the question arises whether RNA-sequencing data can provide reliable estimates of multiple stressor effects and their interactions. Since a traditional assessment of stressor effects i.e., based on specimen abundance change, was already performed (Beermann et al. 2018a), we were able to compare multiple stressor effects at the transcriptomic level with stressor effects derived from population level data, allowing to address the following two research questions in chapter 3:

> (i) How does single and combined stressor exposure of increased fine sediment deposition, reduced flow velocity and increased salinity affect the transcriptional profile of *G. fossarum*?
>
> (ii) How consistent are stressor effects and their interactions derived from transcriptomic sequencing data with stressor effects derived from specimen abundance change of *Gammarus* sp.?

We found that stressor exposure resulted in a strong transcriptional suppression in *G. fossarum* and interpreted this as a stressor-induced metabolic depression, because many of the downregulated genes were annotated with metabolic functions and energy consuming cellular processes. Metabolic depression is a common physiological stress response in many invertebrates (e.g., Chen and Stillman 2012; Rodrigues et al. 2017; Andrade et al. 2022; Shi et al. 2022), which could reflect an allocation of energy to vitally essential processes, which is required to cope with environmental stress.

The only exception to this expression trend characterized by a downregulation of genes was observed in gammarids exposed to reduced flow velocity. These individuals displayed a transcriptional activity compared to specimens haltered at control conditions. Interestingly, the reduced flow velocity treatment was also associated with an increase in specimen abundances of gammarids (Beermann et

al. 2018a). Taken together, these results suggested that under the conditions simulated in the ExStream system, reduced flow velocity did not act as a stressor, neither on the transcriptional nor on the specimen abundance level. Further, we observed that the reduced flow velocity treatment compensated the transcriptomic stress response induced by increased fine sediment deposition. This antagonistic stressor interaction was again in line with observations based on specimen abundance data (Beermann et al. 2018a), indicating that RNA-sequencing can reliably detect stressor effects and their interactions. In addition to these stressor effects which were consistent across ecological scales, we identified increased salinity as the most pervasive stressor at the transcriptional level, contrasting specimen abundance patterns of gammarids which did not reveal a detectable salt-induced effect (Beermann et al. 2018a). Specifically, increased salinity treatments resulted in the differential expression of detoxification enzymes and ion transporter genes, which control the membrane permeability of sodium, potassium or chloride. As such, the results presented in this chapter illustrate how transcriptomic sequencing data can be used to provide new mechanistic insights in responses of freshwater organisms to multiple anthropogenic stressors, which would remain unnoticed if only specimen abundance changes are considered.

Personal contributions

I curated all RNA-sequencing data sets and performed all analyses i.e., deposition of the RNA-sequencing data at the European Nucleotide Archive, bioinformatic processing, functional annotation, statistical testing for differential expression and overrepresentation of gene ontology terms as well as clustering analyses. I interpreted all data, visualized all results, and wrote the first draft of the manuscript, to which all co-authors contributed.

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Impacts of multiple anthropogenic stressors on the transcriptional response of *Gammarus fossarum* in a mesocosm field experiment

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Abstract

Background: Freshwaters are exposed to multiple anthropogenic stressors, leading to habitat degradation and biodiversity decline. In particular, agricultural stressors are known to result in decreased abundances and community shifts towards more tolerant taxa. However, the combined effects of stressors are difficult to predict as they can interact in complex ways, leading to enhanced (synergistic) or decreased (antagonistic) response patterns. Furthermore, stress responses may remain undetected if only the abundance changes in ecological experiments are considered, as organisms may have physiological protective pathways to counteract stressor effects. Therefore, we here used transcriptome-wide sequencing data to quantify single and combined effects of elevated fine sediment deposition, increased salinity and reduced flow velocity on the gene expression of the amphipod *Gammarus fossarum* in a mesocosm field experiment.

Results: Stressor exposure resulted in a strong transcriptional suppression of genes involved in metabolic and energy consuming cellular processes, indicating that *G. fossarum* responds to stressor exposure by directing energy to vitally essential processes. Treatments involving increased salinity induced by far the strongest transcriptional response, contrasting the observed abundance patterns where no effect was detected. Specifically, increased salinity induced the expression of detoxification enzymes and ion transporter genes, which control the membrane permeability of sodium, potassium or chloride. Stressor interactions at the physiological level were mainly antagonistic, such as the combined effect of increased fine sediment and reduced flow velocity. The compensation of the fine sediment induced effect by reduced flow velocity is in line with observations based on specimen abundance data.

Conclusions: Our findings show that gene expression data provide new mechanistic insights in responses of freshwater organisms to multiple anthropogenic stressors. The assessment of stressor effects at the transcriptomic level and its integration with stressor effects at the level of specimen abundances significantly contribute to our understanding of multiple stressor effects in freshwater ecosystems.

Keywords: Antagonistic stressor interaction, Flow alteration, Gene expression, Metabolic depression, Salinisation, Sedimentation, Transcriptomic stress

Background

Freshwater ecosystems are globally affected by anthropogenic activities, leading to changes in physicochemical and hydromorphological conditions with deleterious effects on the associated biota [1]. Intensive land-use can be a major driver of running water degradation due to

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flow modifications, contamination with organic toxicants or high nutrient concentrations [2, 3]. Despite an increasing number of studies showing the negative impact of land-use on riverine systems [4–7], agricultural stressor effects are still difficult to predict, since they often coincide with a variety of other stressors such as climate stressors [8] or biological invasion [9]. Because rivers are highly connected ecosystems, the impact of anthropogenic activities is integrated across the whole catchment [10]. This leads to high temporal and spatial variations in stressor occurrences and complex interaction patterns, which are in addition mediated through food web interactions [11]. For example, decreased discharge due to water abstraction or damming directly affects the community composition of streams by decreasing species richness and shifting communities towards more tolerant taxa [12]. Because transport efficiency directly depends on flow velocity, effects of other stressors such as fine sediment deposition might be magnified indirectly [10]. Likewise, if more water evaporates during summer and is increasingly used for irrigation purposes, discharge decreases while salinity increases. Such stressor interactions lead to deviations of observed effects from the expected effects of the individual stressors. When combined stressor effects exceed the sum or product of single stressor effects (i.e. stronger effect on the response variable than predicted), they synergistically interact. Vice versa, when combined effects are lower than expected, these stressors show an antagonistic interaction (see [13] for a detailed discussion about the concepts).

Ecological and physiological models often explain deviations of multiple stressor responses from the expected additive effects through the activation of different protective pathways based on the limited energy budget available to an organism. If the evoked protective pathways are shared by two stressors, energetic costs can be mitigated, resulting in combined stressor effects which might be smaller than expected by an additive model (antagonism). If, however, distinct but dependent pathways are activated by the two stressors, energetic trade-offs can arise which result in higher stress responses than anticipated for an additive model [14].

In the present study, we focused on single and combined effects of reduced flow velocity, increased fine sediment load and increased salt (sodium chloride) concentration. These important agricultural and hydrological stressors often occur together and can therefore interact. Several studies aimed to disentangle single and combined effects of these stressors [6, 15, 16] or combined effects with other stressors [7, 17, 18]. These studies focused on community level effects, particularly on stream macroinvertebrates, which are important bioindicator organisms. For instance, reduced flow velocity was shown to decrease species densities and richness of EPT taxa [19]. Sediment erosion can result in macroinvertebrate community turnovers due to a loss of structural heterogeneity because open interstitial spaces are filled up [20, 21]. Increased ion concentrations due to salinisation occur in areas with high return flows from e.g. fertilisers, road salt, or from mining activities, but can also be the result of irrigation or of salt-water intrusions [22, 23]. Salinisation leads to altered chemical water conditions such as pH, affecting osmoregulation, oxygen consumption or growth rates in aquatic biota [24] and can result in community shifts towards more salt tolerant taxa [25].

The assessment of multiple stressor effects in communities is of central interest in ecological research. Often the change in abundance is the key parameter assessed. However, the time scales addressed in ecological studies are often days or weeks and organisms may withstand the stress conditions due to physiological acclimation for these short periods. Thus, despite strong physiological stress, the observed abundance data may not indicate the negative stressor effect. In contrast, changes in gene expression represent an immediate physiological response to environmental conditions and can reflect stressor effects on short temporal scales. Further, the transcriptional plasticity determines the limits of an organism's ecological amplitude. Stressor effects exceeding this physiological compensatory mechanism are expected to be reflected on a higher ecological level (e.g. in altered species abundance or distribution range). Therefore, complementing observational data with gene expression data can provide a more holistic picture on stressor effects acting on biodiversity.

Here, we quantified the gene expression profile of a key freshwater macroinvertebrate species, the amphipod *Gammarus fossarum* Koch, in Panzer 1836 clade 11 [26] using RNA-sequencing (RNA-seq). *G. fossarum* prefers headwaters and upper reaches with higher flow velocities [27] and is sensitive to acidification, hypoxia and organic pollution [28]. Since these organisms often dominate stream invertebrate communities and possess an intermediate position in food webs [29, 30], a loss of these populations would have pronounced effects on stream ecosystems.

Based on the induced gene expression profiles in response to i) increased sodium chloride concentration (hereafter referred to as 'salinity treatment'), ii) reduced flow velocity ('flow treatment') and iii) elevated fine sediment level ('sediment treatment'), we assessed single and combined stressor effects using a mesocosm field experiment. Specifically, we aimed to:

1 Identify differentially expressed genes and molecular pathways involved in the response to single and multiple stressor exposure, and
2 Identify and quantify stressor interactions (synergistic/antagonistic).

We also compare the gene expression patterns to results obtained from abundance data generated in the same experiment [15] and discuss prospects and limitations of gene expression data for multiple stressor research on freshwater organisms.

Results

Sequencing of the 64 transcriptome samples yielded 17,236,422 ± 2,005,378 (mean ± S.D.) paired-end reads, ranging from 13,283,344 to 22,704,046 read pairs. After homopolymer removal and quality filtering, 17,101,459 ± 1,989,353 reads per sample were retained. The *G. fossarum* transcriptome comprised 1,197,198 transcripts from 983,707 'genes' with an E90N50 transcript length of 2,003 bp. All RNA-seq samples showed consistently high re-mapping rates (88.46 ± 1.84%). We detected 96% complete BUSCOs, indicating that the reference transcriptome was nearly complete (Table 1).

Increased salinity induces the strongest transcriptomic response

After gene level summarisation and filtering, 73,479 genes were retained and tested for differential expression. Across all treatment combinations, 613 genes were identified as differentially expressed. Overall, the expression profiles were dominated by downregulation of the affected genes (Fig. 1).

Only under reduced flow velocity, more upregulated (56 genes) than downregulated (19 genes) genes were detected (Fig. 1) and the highest number of exclusively upregulated genes was detected for this treatment (Fig. 2). Reduced flow velocity in combination with increased sediment levels resulted in the lowest number of differentially expressed genes (41 genes), followed by the single stressor treatments low flow velocity and increased fine sediment (both induced the differential regulation of 75 genes).

However, in combination with increased salinity, reduced flow velocity induced the strongest gene regulatory response (285 genes of which 139 were exclusively detected in this treatment combination). Interestingly, 54 of the downregulated genes in the salinity + flow treatment were also differentially downregulated when only fine sediment was added but not in any of the other stressor combinations (Fig. 2). Treatments involving increased salinity had the most pervasive effects on the gene expression profile: a total of 528 genes (86%) responded in the treatments that included increased salinity, of which 442 were exclusively regulated under increased salt concentrations. All salinity treatments, except the combination of increased salinity and added fine sediment, formed a cluster based on Euclidean distances obtained from shrunken LFC values (Fig. 1).

Table 1 Trinity de novo assembly metrics

Basic assembly statistics	
Trinity contigs (isoforms)	1,197,198
Trinity genes	983,707
Median contig length	354
Contig N50	855
E90N50 (25,952 genes)	2003
average remapping rate ± S.D. [%]	88.46 ± 1.84
BUSCO	C:96.4% [S:38.1%, D:58.3%], F:0.7%, M:2.9%

BUSCO analysis was performed with the arthropod BUSCO dataset (1,013 BUSCOs); *C* = Complete, *S* = Single, *D* = Duplicated, *F* = Fragmented, *M* = Missing

Stressor interactions are mainly antagonistic

For 139 differentially expressed genes, a significant interaction was detected in at least one treatment combination (see Additional file 1, Table S1). Due to the interaction of increased salinity with reduced flow velocity, 109 genes responded differently than expected based on the individual effects (Table 2). When increased salinity co-occurred with added fine sediment, 31 genes showed a significant interaction. Under combined stressor exposure of reduced flow velocity and elevated fine sediment, 27 antagonistic gene expression patterns were identified. Finally, we found 7 genes that showed a three-way interaction when all stressors were applied (Table 2).

Clustering of genes that showed an interaction between increased salt concentration and reduced flow resulted in three distinct groupings (Fig. 3a-c). The first two clusters were characterised only by A + interactions, i.e., genes that were less upregulated than expected due to the combined effect of reduced flow and increased salinity. The third cluster comprised genes, which were either stronger (S-) or weaker downregulated (A-) than predicted based on single stressor effects. Likewise, the two clusters formed by genes which responded differently to added fine sediment when either increased salinity (Fig. 3d) or reduced flow velocity (Fig. 3e) were applied, encompassed only A- interactions. In these cases, the downregulation of genes induced by added fine sediment decreased when this stressor was combined with one of the other stressors.

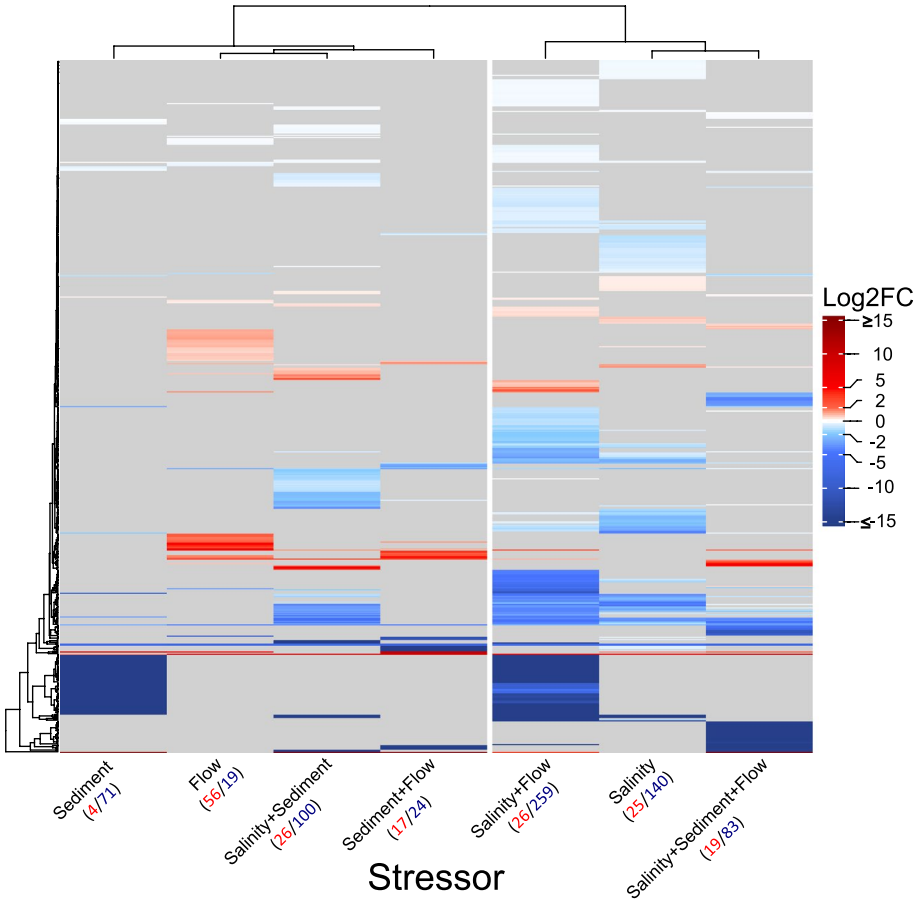

Fig. 1 Heatmap showing the differential expression of genes in response to single and combined stressor exposure. Grey bars indicate changes in expression, which are not significant (adjusted *p*-values ≥ 0.1). Numbers in red and blue denote upregulated and downregulated genes, respectively

Metabolic suppression appears to be a physiological stress response in *G. fossarum*

Transdecoder predicted 269,426 coding sequences, which were included in the annotation. In total 43,826 genes were successfully annotated, corresponding to only 5% of the whole assembly. From the set of genes that were included in the differential expression analysis, 20% were annotated with GO terms.

All stressor treatment combinations except the reduced flow velocity treatment induced a strong metabolic suppression in *G. fossarum*. The downregulated genes were annotated with GO terms suggesting gene involvement in biosynthetic processes, organisation of cellular components or inter- and intracellular signalling. While traversing the DAG towards more specific GO terms, it became evident that these terms either refer to (i) e gy metabolism (e.g., GO:0045333, 'cellular respiration'; GO:0046034, 'ATP metabolic process') or (ii) regulation of gene expression and polypeptide synthesis (e.g., GO:0043043, 'peptide biosynthetic process'; GO:0043604, 'amide biosynthetic process'). On the contrary, terms referring to the organonitrogen metabolism were annotated to upregulated genes in the reduced flow velocity treatment.

This stressor induced metabolic suppression was further supported by the functional enrichment analyses. Genes downregulated due to fine sediment addition, increased salinity, fine sediment addition in combination with reduced flow or increased salinity in combination with reduced flow were enriched for mitochondrial processes and ATP synthesis, as well as catabolic processes (e.g., GO:0005980, 'glycogen catabolic process'; GO:0006635, 'fatty acid beta-oxidation'). Linked to the lowered metabolic activity, we detected several functionally enriched GO terms referring to reproduction or growth in response to reduced flow velocity (GO:0022414, 'reproductive process') and reduced flow in combination with added fine sediment (GO:0008340, 'determination of adult lifespan'). Many overrepresented GO terms comprised biological

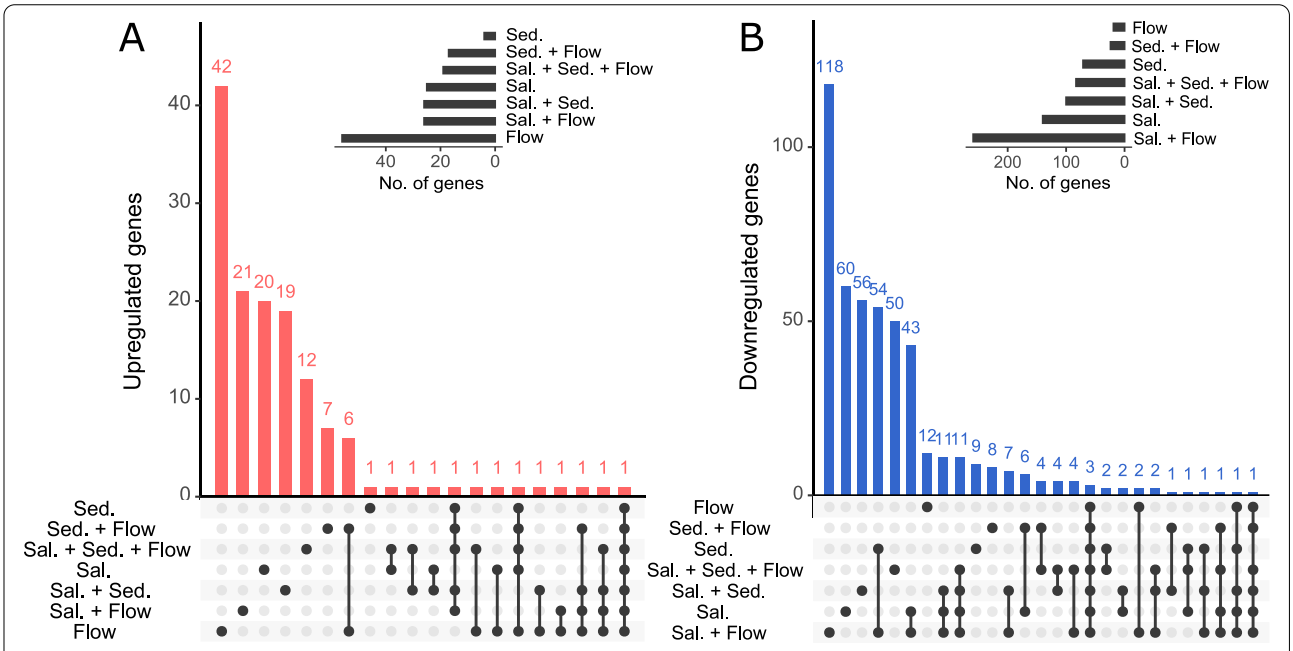

Fig. 2 UpSet plots, summarising unique and shared differentially expressed genes due to exposure to different treatments. Black dots in panel matrices correspond to different Venn diagram sections: connected dots indicate intersections of sets of differentially expressed genes; singular dots represent unique sets of differentially expressed genes. Vertical barplots summarise the number of upregulated (a) and downregulated (b) genes. Horizontal bars indicate the total number of regulated genes in each treatment combination. The set union of all upregulated and downregulated genes detected in salinity treatments represents 443 salinity genes, of which one gene was upregulated when increased salinity was combined with reduced flow but downregulated when all three stressors were applied (=442 exclusively regulated genes)

Table 2 Number of genes, which have an additional LFC due to the interaction between stressors

Stressor interaction	Salinity*Flow	Salinity*Sediment	Sediment*Flow	Salinity*Sediment*Flow
Positive synergistic	-	-	-	-
Negative synergistic	13	-	-	-
Positive antagonistic	85	3	7	2
Negative antagonistic	11	28	20	5
Sum	109	31	27	7

processes involved in DNA organisation, transcription and translation. These terms were enriched in the sets of genes downregulated in all salinity treatments and in response to increased fine sediment alone (see Additional file 1, Table S2).

Additionally, the gene expression profiles were characterised by downregulation of cellular stress responses (e.g. GO:0006457, 'protein folding'; GO:0034605, 'cellular response to heat'), which were attributed to the downregulation of putative heat-shock proteins (HSPs) in response to increased fine sediment or increased salinity in combination with reduced flow velocity.

The functional enrichment analyses testing salinity-specific genes further supported the general stress response involving metabolic processes, protein biosynthesis and cellular stress responses (see Additional file 2, Fig. S1, S2).

The genes which showed a negative synergistic interaction comprised a component of the mitochondrial cytochrome b-c1 complex (TRINITY_DN186_c1_g1) and the HSP83 (TRINITY_DN179_c0_g1). Further, three genes were annotated as uncharacterised proteins of which one likely represents a further chaperone (TRINITY_DN314351_c0_g1) and three appear to be involved in protein biosynthesis (TRINITY_DN14015_c3_g1, TRINITY_DN15558_c0_g1, TRINITY_DN315110_c0_g1).

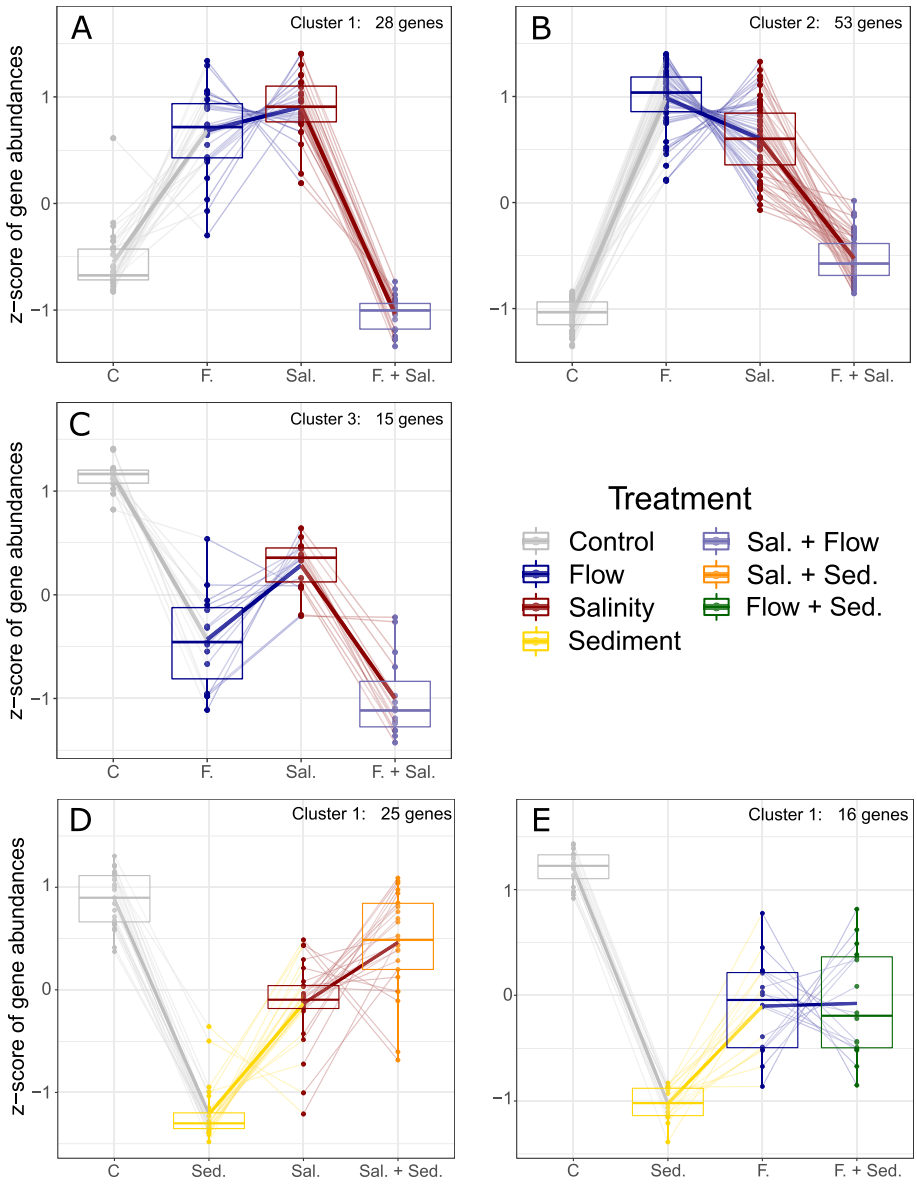

Fig. 3 Clustering of genes, which showed an interaction between two stressors. The expression significantly differed from expectations based on the single stressor effects due to the interaction between salinity and flow (a-c), sediment and salinity (d) or sediment and flow (e). The z-score represents the scaled and centred gene expression. Positive and negative z-scores indicate expression values above and below the average across samples, respectively. Pale lines connect expression values of individual genes, bold lines the average expression values

Discussion

Most stressor treatments resulted in a strong downregulation of the majority of genes in *G. fossarum*. When confronted with unfavourable environmental conditions, organisms are expected to allocate energy from processes like reproduction, growth or locomotion to vitally essential processes. This is supported by the downregulation of genes involved in these metabolic processes in response to the stressor treatments in the present study. It might seem counterintuitive that many downregulated genes were functionally enriched for biological processes involved in protein biosynthesis. In fact, processes involved in gene expression such as mRNA splicing are energetically costly since the polymerisation of pre-mRNA and the subsequent processing of RNA by the spliceosome is ATP-dependent [31]. When organisms are confronted with adverse habitat conditions, the energetic trade-off between transcriptional plasticity and suboptimal overexpression must be minimised in favour of maintaining homeostasis. Our findings imply that this

could comprise cellular stress compensatory mechanisms such as the expression of HSPs. HSPs are not only relevant for protein refolding under cellular stress but are essential for folding newly synthesised proteins. When the stressor induced metabolic suppression leads to a general decrease in protein biosynthesis, less HSPs are required to counteract cellular proteotoxic stress.

Single and combined stressor effects

Added fine sediment and reduced flow velocity induced the lowest number of differentially expressed genes. *Gammarus* tends to avoid habitats with increased fine sediment load, reflected in decreased abundances and increased drift propensities of these organisms at elevated sediment levels [15] and were shown to prefer habitats with coarse substratum over less complex habitats characterised by sand [32, 33]. If gammarids can inhabit stream bed habitats with their preferred particle size, they require less oxygen [34] and show increased growth rates [33]. During periods with high energetic maintenance costs, less energy can be allocated to processes, which are not required to maintain homeostasis. Our results suggest that this is at least partly controlled via sediment-induced energetic downregulation of specific metabolic genes and genes associated with protein biosynthesis. A similar metabolic depression was detected in other invertebrates like mussels [35] or shrimps [36] exposed to abiotic stress such as temperature, indicating that a reduction of cellular metabolic activity is a general strategy of different organismic groups to cope with stressful conditions.

In contrast, reduced flow velocity induced gene expression as this was the treatment with the highest number of upregulated genes. Compared to other native *Gammarus* species such as *G. pulex* or *G. roeselii*, *G. fossarum* is known to inhabit faster flowing stream sections [27] but habitat preferences are often linked to several factors such as reduced interspecific competition or risk of predation [12]. In fact, gammarids were shown to prefer moderate velocities, probably due to increased energetic costs associated with high flow velocities [32]. Gammarids have been found to be more active under lower flow velocities [37], which might reflect that more energy can be allocated to locomotion under moderate flow velocity.

Increased salinity alone and in combination with other stressors induced the largest number of differentially regulated genes in *G. fossarum*. Salt stress is known to regulate the expression of enzymes involved in detoxification of superoxide radicals e.g. superoxide dismutases or glutathione S-transferases [38, 39]. We found two putative glutathione transferase enzyme genes, which were exclusively regulated under increased salinity concentrations (namely TRINITY_DN1113_c0_g1, upregulated when only salinity was increased and TRINITY_DN613487_c0_g1, downregulated when increased salinity was combined with reduced flow velocity). Further, salinity stress induces oxidative stress in mitochondria of aquatic organisms through the production of reactive oxygen species [38]. In the present study, several GO terms were enriched for molecular functions involved in ATP metabolisms and one biological process involved in mitochondrial double-strand repair mechanisms in the set of salinity-specific genes. Freshwater crustaceans are hyperosmoregulators that maintain extracellular and intracellular ion homeostasis against an osmotic gradient. The passive loss of salts is regulated through expression of ion transporters like the Na^+/K^+-ATPase [40] or $Na^+/K^+/2Cl^-$ co-transporters [41]. Changes in the expression of genes encoding these ion transporters were for example reported for *Daphnia* during salinity stress [31]. In our study, two genes which are annotated to encode subunits of the Na^+/K^+-ATPase were downregulated in response to salinity and flow or when all three stressors were applied (TRINITY_DN67768_c1_g1, TRINITY_DN103746_c0_g1), but also in treatments involving sediment and flow manipulations (TRINITY_DN103746_c0_g1). The differentiation between metabolic processes involved in particular physiological stress response or those involved in general energy allocation requires further research. This is especially important for a deeper understanding of multiple stressor interactions when using RNA-seq data.

Comparison to stressor effects derived from abundance data

In our experiment, the fine sediment induced expression profile disappeared when flow velocity was additionally reduced. This compensatory mechanism is consistent with the observations made by [15] based on abundance data, which showed that gammarids were more abundant under low flow conditions and their drift propensities decreased. The transcriptional activity of *G. fossarum* indicates that reduced flow velocity is tolerated by gammarids, or even preferred under the experimental conditions. Under natural conditions, other physicochemical parameters such as oxygen content or water temperature change during low flow periods. For example, gammarids seek for drying refugia within the interstitial spaces in the streambed when streams become intermittent. If fine sediment load prevents the hiding in the interstitial or hamper vertical movement of organisms, as found by [42], the combined effects of increased fine sediment and reduced flow velocity are expected to be stronger in natural streams. Further, the transcriptional profiling identified increased salinity as the most pervasive stressor. This

is opposed to the patterns obtained from abundance data from the same experiment [15], where no effect due to salinity was detected for gammarids, neither in single nor in combined stressor treatments. The high temporal resolution of expression data provides insights in organismic stress responses which are not indicated by abundance data and an integration of these ecological endpoints provide a more holistic picture about multiple stressor effects. If salinity remains increased but gammarids are unable to maintain the physiological stress response for a longer period of time, drift and mortality rates are expected to increase. If the metabolic suppression can be maintained by *G. fossarum* in order to cope with increased salinity and the related cellular toxic effects, then this probably affects specimen body sizes, biomass, and fecundity. Therefore, irrespective of whether or not the stress induced physiological state can be maintained, the long-term effect of increased salinity is likely to have adverse consequences on the whole population.

We detected many more non-additive interactions than observed in multiple stressor effects based on specimen abundances in [15]. Inherently, this is related to the larger number of tested response variables (i.e., genes) compared to community data, reflecting the fine resolution of transcriptomic data on both temporal and mechanistic scales. The fact that these interactions are mostly antagonistic shows that the observed expression patterns do not predictively scale along intensifying stressor gradients. The self-regulatory plasticity of the transcriptome, which involves many regulatory pathways, signal transduction cascades and posttranscriptional or posttranslational modifications appear to play a fundamental role in physiological coping strategies.

Limitations of transcriptomic profiling in non-model organisms

RNA-seq has become a powerful tool to study gene regulatory mechanisms. Although it is nowadays commonly used in biological sciences, most applications are still limited to biomedical sciences or when using highly controlled experimental setups. Under experimental conditions with many degrees of freedom (as in our mesocosm experiment, which aims to reflect near-natural conditions), an accurate detection of a true biological signal is more challenging because gene dispersion estimates will be inflated due to introduced variation that is unrelated to the tested treatments, preventing these genes to be detected as differentially regulated. The low signal-to-noise ratio, which is a common problem for RNA-seq data [43, 44], can not only have statistical but also biological or technical reasons. For example, very distinct transcriptomes will be sequenced together when working with non-model organisms collected from the field that are genetically highly diverse. If a reference genome is absent, the bioinformatic processing (especially the de novo assembly and subsequent mapping of reads) of these sequencing libraries can yield inadequate gene abundance estimations due to over-splitting of genes into too many contigs. Functional transcriptomic profiling in non-model species such as *G. fossarum* is further complicated by the scarce annotation data. Crustaceans are highly underrepresented in genomic resource databases, as has also been found by [45, 46]. In the current study, only 20% of the genes that were tested for differential expression were annotated. Assuming that more conserved genes will be more often annotated because their annotation appears to be more reliable, functional enrichment tests tend to identify more conserved gene functions, although this is not necessarily the main underlying physiological response. This highlights the need for ongoing research and effort in the field of functional genomics in non-model organisms.

Conclusion

Anthropogenic stressors, like salinisation, an elevated fine sediment level or reduced flow velocity, degrade stream ecosystems and negatively affect key macroinvertebrates such as *G. fossarum*. We found a reduced transcriptional activity of many genes in response to stressor exposure, probably due to increased energetic maintenance costs. The compensatory effects between added fine sediment and reduced flow velocity are consistent with specimen abundance and drift data, indicating that RNA-seq data can detect stressor effects, which are similarly propagated to higher ecological levels. The transcriptomic profiling further indicated that increased salinity was by far the most pervasive stressor at the physiological level. This, however, is not reflected in abundance data of gammarids, suggesting that the differential downregulation due to increased salt concentration is part of a physiological acclimation strategy, which enables *G. fossarum* to remain at affected sites. Our data show that transcriptomic data provide important information and enhance our understanding of multiple-stressor effects from genes to ecosystems.

Methods

Experimental setup

To identify and disentangle the individual and combined effects of the stressors increased salinity, reduced flow velocity and elevated fine sediment levels, an ExStream mesocosm field experiment [18] was conducted at the Felderbach (Germany, North Rhine-Westphalia, 51°20′59.09″N, 7°10′14.03″E, 136 m a.s.l) from 8 March to 22 April 2014 (a full experimental description is given in [15]). Stream water was pumped in four

header tanks for 46 days (24-day colonisation period, 22-day manipulative period). Each header tank supplied water to 16 mesocosms (diameter 25 cm, volume 3.5 l) via gravity. This ensures the same light regime, water temperature and chemistry in the experiment and the natural stream system. All mesocosms were filled with 300 ml fine sediment (< 2 mm), 900 g gravel (2–30 mm), 4 stones (> 30 mm) and three big stones, reflecting the natural composition of the streambed, and two leaf litter bags filled with 2.5 g dried alder leafs. Colonisation of mesocosms by stream organisms with a diameter < 4 mm occurred passively via drift and was complemented by macroinvertebrates that were actively collected in the stream one week prior to the start of the manipulative period. During the manipulative period, stream organisms were exposed to multiple stressor combinations in a 2x2x2 factorial design with eight replicates per treatment, comprising three factors (salinity, fine sediment, and flow velocity) with two levels each: ambient salinity (18.2 mg/l, SD ± 4.1) versus increased salinity (312.2 mg/l, SD ± 78.5) in terms of chloride concentration, natural flow velocity (16.5 cm/s, SD ± 0.1) versus reduced flow velocity (9.6 cm/s, SD ± 0.1) and ambient levels of fine sediment (300 ml, < 2 mm) versus added fine sediment (750 ml, < 2 mm). The chosen stressor conditions for flow velocity and sediment cover were shown to be realistic for streams in agricultural areas and the increased salinity concentration is expected to be reached based on chloride thresholds implemented into german law (see [15] for further details).

At the end of the experiment, 145 amphipods (2–4 specimens per mesocosm) were sampled from the channel substratum, irrespective of their sex or life stage. The organisms were snap frozen in liquid nitrogen and stored at -80 °C until nucleic acid extraction.

RNA extraction, library preparation and sequencing

Frozen samples were disrupted with a disposable pestle and homogenised in 500 µl TRIzol reagent, allowing the dissociation of nucleoprotein complexes. After incubation, 200 µl chloroform were added and samples were centrifuged. RNA was isolated from the aqueous phase and precipitated with absolute ethanol. RNA was then purified using the RNeasy Plus Micro Kit (QIAGEN). Briefly, the samples were transferred to RNeasy mini-spin columns and washed with 700 µl RW1 buffer, followed by a wash step with 500 µl RPE buffer and finally with 500 µl 80% ethanol. The RNA was eluted in RNase-free water and sample integrity and concentration were assessed with a Fragment Analyzer using the RNA 15 nt kit (Agilent), following the manufacturer instructions. Based on sample quality, the extracts of two individuals per experimental unit (channel) were pooled for sequencing. For two channels, only one specimen was used. Library preparation and sequencing was done separately for each experimental unit, resulting in 64 RNA-seq libraries. RNA extracts were sent to Macrogen and cDNA libraries were prepared with the TruSeq RNA Sample Prep v2 kit. Libraries were 150 bp paired-end sequenced on an Illumina NovaSeq 6000.

DNA extraction and barcoding

DNA was isolated from the lower organic phase and ethanol precipitated. The DNA pellet was washed 1–2 times with 500 µl 0.1 M sodium citrate in 10% ethanol (pH 8.5), followed by a final wash step with 1 ml 75% ethanol. The DNA was resolubilised in 150–300 µl 8 mM NaOH and pH was adjusted to ~ 8 using HEPES.

Species identity of amphipods was confirmed via DNA barcoding. The 658 bp barcoding fragment of the cytochrome-c-oxidase subunit 1 (COI) gene was amplified using the primer pair LCO1490-JJ/HCO2198-JJ [47]. Per reaction, 2.5 µl buffer (10x), 2.5 µl dNTPs (2 mM), 2.5 µl $MgCl_2$ (25 mM), 0.125 µl HCO2198-JJ (100 µM), 0.125 µl LCO1490-JJ (100 µM), 0.2 µl *Taq* polymerase (5 U/µl, VWR), 14.04 µl PCR water and 1 µl DNA were used. PCR cycling conditions were 2 min of initial denaturation at 94 °C, followed by 33 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 30 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 5 min. PCR products were purified for Sanger sequencing using the exonuclease *ExoI* (20 U/µl, Thermo Scientific™) and alkaline phosphatase *FastAP* (1 U/µl, Thermo Scientific™). Bidirectional Sanger sequencing was performed at Eurofins Genomics. COI barcodes were compared to NCBI (www.ncbi.nlm.nih.gov/) and only individuals identified as *G. fossarum* (n = 130) were used for sequencing, irrespective of the mitochondrial clade they were assigned to.

Assembly & read abundance estimation

Raw reads were homopolymer trimmed with a custom C++ script and quality trimmed with the cutadapt v 3.2 [48] rapper script TrimGalore! v 0.6.6 (https://github.com/FelixKrueger/TrimGalore) in paired-end mode with a quality cut-off Phred > 20, retaining only reads with a minimum length of 25 bp. De novo assembly of reads was performed with Trinity v 2.9.0 [49], based on four library replicates per treatment. To reduce redundancy in the transcriptome, contigs were clustered at 96% similarity with CD-HIT-EST v 4.8.1 [50]. Transcript abundances were estimated via mapping of quality trimmed reads against the clustered transcriptome using RSEM v1.3.3 [51] ith bowtie2 v 2.3.5.1 [52] as mapper. Assembly quality and completeness was assessed by searching for arthropod benchmarking universal single-copy orthologs

(BUSCOs) (BUSCO v 4 arthropoda_odb10, comprising 1,013 BUSCOs, [53]). Furthermore, we determined remapping rates of all sequencing libraries (8 libraries per treatment) and the corresponding E90N50 statistics, which represents the N50 value for the subset of genes that account for 90% of the total expression.

Statistical analyses

Estimated transcript counts were summarised to gene level with the R package tximport [54], adjusting isoform abundances according to gene length. For gene inferences, we used the Trinity assumptions about isoform-gene relations. Since no reference genome for *G. fossarum* is available, the term 'gene' is used loosely here. Only genes with at least 10 normalised counts in at least 8 samples were included in downstream analyses. Gene counts were modelled with the R package DESeq2 [55] sing the design ~ Salinity*Sediment*Flow. To account for hidden unwanted variation, surrogate variables were constructed from normalised counts (full model: ~ Salinity*Sediment*Flow, reduced model: ~ 1) using the sva package [56]. All significant surrogates were incorporated in an updated model as covariates.

Statistical inferences of individual and combined stressor effects were conducted using the Wald test statistic, applying a significant threshold of <0.1 after FDR correction for multiple testing. LFC values were shrunken with the adaptive shrinkage estimator from the ashr R package [57].

To identify stressor interactions, we classified interactions as synergistic if the combined stressor effects were larger, and as antagonistic if they were smaller than the product of the single stressor effects. More specifically, a positive synergistic interaction (S+) is found when the observed gene expression was more positive (stronger upregulated) than expected; a negative synergistic (S-) interaction indicates that a stronger downregulation is observed than expected compared to the addition of main effects. Positive antagonistic (A+) interactions are defined as 'less upregulated than expected', whereas a negative antagonistic (A-) interaction refers to genes that are 'less down regulated than expected' due to the interaction effect.

Functional annotation and enrichment

TransDecoder (https://github.com/TransDecoder/) was used to identify coding DNA sequences and the putative peptides were searched for protein domains using hmmer v3.3 (http://hmmer.org/) and the Pfam database [58]. Further, they were queried against the Swissprot/Uniprot database [59] using blastp [60] with 1e-5 as e-value cut-off. Homology inferences were integrated in the final prediction of proteins.

Predicted proteins were iteratively annotated via blastp (e-value 1e-5) searches against (i) the *Daphnia pulex* proteome (similarity $\geq 40\%$) (http://wfleabase.org) and (ii) he invertebrate Uniprot Swissprot/TrEMBL database (similarity $\geq 50\%$). Annotations from the invertebrate database were only included if no annotation was obtained from the *D. pulex* proteome, and the best protein hit (highest bitscore) was retained.

Gene ontology (GO) terms [61, 62] were obtained from UniProt IDs of proteins that were successfully mapped to isoforms. To obtain a general overview about the annotation data set, ancestor terms were derived from the GO.db [63] R package and sorted according to their position in the directed acyclic graph (DAG) with GOxploreR [64]. Functional enrichment analyses of GO terms associated with biological processes were performed with topGO [65] using Fisher's Exact test and the weight01 algorithm. All genes which were tested for differential expression were used as gene universe and enrichment analyses were performed separately for up- and downregulated genes. In order to test for enriched GO terms in the set of genes which were exclusively regulated due to increased salinity (hereafter referred to as 'salinity specific'), GO enrichment analyses for biological processes and molecular functions were performed using all genes which were exclusively differentially expressed in response to increased salinity, either in a single or combined stressor treatment.

Clustering and visualisation

Genes which showed significant interactions between two stressor treatments were clustered according to their expression profiles using DEGreport [66]. The variance stabilised counts of these genes were corrected with a frozen surrogate variable analysis [56] and the minimum cluster size was set to 10. Significant clusters of expression profiles and GO term enrichment results were visualised with ggplot2 [67]. Upset barplots were generated with UpSetR [68]. Heatmaps were created with functions from the R package ComplexHeatmap [69] and dendrograms were based on Euclidean distances obtained from shrunken LFC values.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-022-09050-1.

Additional file 1.

Additional file 2.

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Authors' contributions
FL, AJB and DG designed the study and acquired funding. VE conducted the field experiment. RS and BPV performed the lab work. MVB performed the analyses and wrote the manuscript with helpful input of CM and FL. All authors contributed to the manuscript and approved the final version.

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Availability of data and materials
The RNA-seq libraries have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under project accession number PRJEB56296.

Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
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Consent for publication
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Competing interest
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Publisher's note

Chapter 4

Transcriptomic sequencing data illuminate insecticide-induced physiological stress mechanisms in aquatic non-target invertebrates

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Summary

Pesticides are dominant stressors of freshwater systems (Vörösmarty et al. 2005; Malaj et al. 2014; Liess et al. 2021). Although numerous studies have proven the threats associated with pesticide contamination for freshwater biodiversity (e.g., Liess and Von Der Ohe 2005; Beketov et al. 2013; Liess et al. 2021), the physiological mechanisms through which pesticides affect aquatic organisms are largely unknown. The study presented in the previous chapter showed that RNA-sequencing can provide reliable estimates of stressor effects and how transcriptomic data can be used to obtain insights in stressor induced molecular response mechanisms. Encouraged by these promising results, we used RNA-sequencing in this study to profile transcriptomic stress responses evoked by chlorantraniliprole exposure across a wide taxonomic range of non-target freshwater macroinvertebrates. Chlorantraniliprole is supposed to be highly selective for its target taxon i.e., typically terrestrial Lepidoptera (butterflies) (Lahm et al. 2007), but its effects on aquatic non-target taxa are poorly studied (Hashimoto et al. 2020). To test the validity of the insecticide's assumed selectivity, we assessed the impact of chlorantraniliprole exposure on the gene expression profiles of the caddisfly *Lepidostoma basale*, the mayfly *Ephemera danica* and the amphipod *Gammarus pulex*. Assuming that the reported selectivity of chlorantraniliprole for butterflies is driven by molecular mechanisms that are conserved between members of evolutionary lineages, we expected a differential sensitivity of the test species towards the insecticide stressor, depending on their phylogenetic distance to butterflies. Further, the leaf-shredding species *L. basale* and *G. pulex* rely on the same nutritional resources. If interspecific competition leads to a decreased caloric uptake in one of the species, the energetic requirements of the insecticide-induced physiological stress responses might not be met in the weaker competitor. Here, we explored whether RNA-sequencing can capture the expression signal linked to the interfering metabolic demands between insecticide-induced transcriptional stress responses and different levels of interspecific competition. Because the ExStream system is designed to simulate a field realistic multiple stressor exposure scenario, the experimental setting does not allow to control for the complexity of biotic interactions contributing to natural communities. Therefore, we performed a highly controlled indoor experiment to address the following three research questions in this chapter:

(i) How does exposure to the insecticide chlorantraniliprole affect the transcriptional profile of *L. basale*, *E. danica* and *G. pulex*?

(ii) Does the sensitivity of the three studied species to the insecticide stressor correlate with their phylogenetic distance to the insecticide's target taxon Lepidoptera?

(iii) Does interspecific competition between *L. basale* and *G. pulex* modulate their insecticide-induced gene expression profiles?

In line with our expectations, we detected strong insecticide-induced transcriptional changes in the two insect species and a limited effect of chlorantraniliprole exposure on the gene expression profile of the amphipod *G. pulex*. This implies that molecular stress receptor mechanisms, which determine the cellular sensitivity towards chlorantraniliprole, are conserved between different taxonomic groups of insects such as butterflies, caddisflies and mayflies. Further, we observed that under interspecific competition, the transcriptomic stress response of *L. basale* was impeded and fewer genes were differentially expressed, supporting our hypothesis that biotic interaction interferes with the ability of organisms to cope with environmental stress.
The functional annotation data of genes differentially expressed in *L. basale* following chlorantraniliprole exposure revealed a stimulation of its immune system, as well as a suppression of mitochondrial processes, presumably in muscle cells. However, a disruption of the calcium homeostasis, the insecticide's described mode of action in butterflies, was not clearly reflected in the functional profile of genes regulated in *L. basale* and *E. danica*. In fact, both insect species exhibited strong expression changes of genes involved in their developmental program: the hemimetabolous mayfly *E. danica* responded to chlorantraniliprole exposure with a suppression of genes regulating the chitin metabolisms and thus molting and growth. In contrast, the holometabolous caddisfly *L. basale* displayed a stimulated expression of genes associated with growth and metamorphosis. These distinct responses of the developmental program could lead to taxon-specific shifts in emergence patterns i.e., prolonged development in mayflies, as opposed to a potential premature emergence in caddisflies. In both scenarios could an insecticide-induced alteration of the emergence phenology of aquatic insects result in the disruption of food-web dynamics in streams and the linked riparian habitats (Hallmann et al. 2014; Cavallaro et al. 2018; Ohler et al. 2023).

Personal contributions

The initial study was conceptualized by Florian Leese, Christoph Mayer and Ralf Schäfer. I conceptualized the final experimental design together with Florian Leese and was responsible for the technical implementation and maintenance of the experiment. I performed all the lab work related to biological sample processing, which included RNA extraction, purification and quality control of RNA extracts. For further sample processing (i.e, mRNA enrichment and reverse transcription of RNA to cDNA) and sequencing, the samples were sent to the West German Genome Centre (WGGC) Bonn. I curated all RNA-sequencing data sets and performed all analyses i.e., deposition of the RNA-sequencing data at the European Nucleotide Archive, bioinformatic processing, functional annotation, statistical testing for differential expression and overrepresentation of gene ontology terms. I interpreted all data, visualized the experimental overview and all results, and wrote the first draft of the manuscript, to which all co-authors contributed.

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Transcriptomic sequencing data illuminate insecticide-induced physiological stress mechanisms in aquatic non-target invertebrates☆

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Transcriptomic stress response

ABSTRACT

Pesticides are major agricultural stressors for freshwater species. Exposure to pesticides can disrupt the biotic integrity of freshwater ecosystems and impair associated ecosystem functions. Unfortunately, physiological mechanisms through which pesticides affect aquatic organisms are largely unknown. For example, the widely-used insecticide chlorantraniliprole is supposed to be highly selective for target pest species, i.e. Lepidoptera (butterflies), but its effect in aquatic non-target taxa is poorly studied. Using RNA-sequencing data, we quantified the insecticide effect on three aquatic invertebrate species: the caddisfly *Lepidostoma basale*, the mayfly *Ephemera danica* and the amphipod *Gammarus pulex*. Further, we tested how the insecticide-induced transcriptional response is modulated by biotic interaction between the two leaf-shredding species *L. basale* and *G. pulex*. While *G. pulex* was only weakly affected by chlorantraniliprole exposure, we detected strong transcriptional responses in *L. basale* and *E. danica*, implying that the stressor receptors are conserved between the target taxon Lepidoptera and other insect groups. We found in both insect species evidence for alterations of the developmental program. If transcriptional changes in the developmental program induce alterations in emergence phenology, pronounced effects on food web dynamics in a cross-ecosystem context are expected.

1. Introduction

Intensive land-use has been identified as a major driver of stream ecosystem degradation (Dudgeon, 2019; Vörösmarty et al., 2010). One of the main agricultural stressors is pesticide pollution (Beketov et al., 2013; Dudgeon, 2019; Malaj et al., 2014). Despite the broad agreement that contamination with pesticides is of special concern, their environmental risk assessment and management is currently failing, and regulatory threshold concentrations are frequently exceeded (Liess et al., 2021; Peters et al., 2013; Stehle and Schulz, 2015). Pesticides tend to accumulate in the environment, together with chemicals from urban and industrial sources, resulting in permanent exposure of aquatic biota to a heterogenous mixture of toxicants (Sandstrom et al., 2022).

Quantifying the impact of pesticide exposure on biological communities is challenging. Pesticide effects can be indirectly mediated through food web interactions and are further modulated by co-occurring abiotic and biotic stressors (Chará-Serna and Richardson, 2018; Coors and De Meester, 2008; Delnat et al., 2020; Verheyen and Stoks, 2020). For instance, complex interactions between carbaryl exposure, parasitism and predation risk were detected for life-history traits of *Daphnia magna* (Coors and De Meester, 2008). In a mesocosm experiment, exposure to the insecticide chlorpyrifos reduced leaf decomposition rates although the macroinvertebrate community composition was not affected. Instead, the alteration of this important ecosystem function was presumably mediated by invertebrate feeding inhibition (Chará-Serna and Richardson, 2018), implying that sublethal stressor effects are only reflected in population abundance changes over longer time scales. Including more sensitive endpoints such as physiological parameters in the experimental quantification of stressor impacts allows an earlier detection. However, the physiological stressor response

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of the assessed toxicants can additionally be modulated by interactions with other stressors. For example, temperature stress enhanced the negative effects of chlorpyrifos in the damselfly *Ischnura elegans* (Verheyen and Stoks, 2020) but reduced the amount of oxidative stress induced by chlorpyrifos in another damselfly, *Enallagma cyathigerum* (Janssens and Stoks, 2017).

These allegedly ambiguous biological responses to pesticide exposure highlight our limited mechanistic understanding of the toxicant impacts and the challenges we face in accurately forecasting ecological effects. Assessing toxicant effects at the molecular level using RNA sequencing data provides an unbiased identification of the underlying regulatory pathways induced by xenobiotic stress (Gonzalez and Pierron, 2015). Due to the transcriptomic plasticity and sensitivity to environmental changes, even subtle effects can be quantified that would not immediately translate in structural alterations of biological communities (Brasseur et al., 2022). Since the limit of physiological plasticity is ultimately defined at the transcriptional level, gene regulatory stress responses are expected to determine environmental stressor effects at the organismal and population level (Delnat et al., 2020).

Here, we address the limited knowledge about physiological mechanisms induced by pesticide exposure in aquatic non-target invertebrates. We used transcriptomic sequencing data to quantify the effect of the novel insecticide chlorantraniliprole on three freshwater invertebrate species: the mayfly *Ephemera danica* Müller, 1764, the caddisfly *Lepidostoma basale* Kolenati, 1848, and the amphipod *Gammarus pulex* Linnaeus, 1758.

Chlorantraniliprole belongs to the anthranilic diamides, which have rapidly replaced organophosphates and neonicotinoids due to their potency and advertised selectivity for insect pest species (Lahm et al., 2009; Sattelle et al., 2008). Binding of chlorantraniliprole to its agonist, the insect ryanodine receptor, disrupts the cellular calcium homeostasis, resulting in uncontrolled muscle contraction, feeding cessation, paralysis and eventually the death of the insect (Lahm et al., 2007; Sattelle et al., 2008). Despite its frequent use, the exposure-induced molecular mechanisms in aquatic non-target invertebrates remain elusive.

Chlorantraniliprole was originally designed for controlling butterfly pest species (Lahm et al., 2007). Assuming that its molecular target i.e., the ryanodine receptor, is more conserved between more closely related taxa, the insecticide's toxicity is expected to decrease with increasing phylogenetic distance. While butterflies are the sister order of caddisflies, mayflies are phylogenetically more distant. Amphipods, which are crustaceans, show the highest genetic divergence to butterflies. Accordingly, the three test species represent a phylogenetic gradient of non-target taxa belonging to major arthropod orders. All species are dominant in stream ecosystems (Bauernfeind and Humpesch, 2001; MacNeil et al., 1997; Morse et al., 2019), play fundamental roles in aquatic food webs and significantly contribute to the organic matter decomposition in streams (Cummins, 1973; MacNeil et al., 1997). Still, they are characterized by different ecological requirements: the sand-burrowing *E. danica* larvae feed on organic matter by ingesting sediment, whereas the shredder species *L. basale* and *G. pulex* mainly feed on leaf material (Cummins, 1973; Cummins and Klug, 1979). While *E. danica* is expected to profit from small particles of organic matter produced by the shredders, the utilization of shared resources likely induces competition between the two shredder species *L. basale* and *G. pulex*. If this leads to a decreased caloric uptake in the weaker competitor, a disruption of the energy metabolism may be the consequence of intraspecific competition. Because physiological compensatory mechanisms such as insecticide-induced stress responses depend on the energy budget available to an organism, metabolic stress is likely to affect an organism's ability to cope with environmental stress. Therefore, we included the effect of biotic interaction in our experimental design and tested how potential interspecific competition between the two shredders changes their insecticide-induced expression profiles.

To the best of our knowledge, this is the first study that systematically assesses the transcriptomic stress response of the insecticide chlorantraniliprole across a wide taxonomic range of aquatic non-target invertebrates. Further, we are not aware of any other studies which examined whether the effects of interspecific competition can be quantified using RNA-sequencing data. We expect that the intensity of the insecticide-induced cellular stress is determined by the phylogenetic distance of the test species to butterflies due to the different degrees of molecular pathway conservation. Therefore, we hypothesize that

(i) the transcriptional response is most pronounced in the caddisfly *L. basale*, followed by the mayfly *E. danica* and the amphipod *G. pulex*.

Due to the molecular effect mechanism of chlorantraniliprole and the subsequent induced physiological responses (i.e., uncontrolled muscle contraction, feeding cessation), we further hypothesize

(ii) genes involved in maintaining the calcium homeostasis to be differentially expressed (DE), as well as the induction of regulatory pathways associated with muscular processes and bioenergetic responses.

If competing for the same resources induces additional stress in the two shredder species *L. basale* and *G. pulex*, we expect a modulation of the insecticide effect. Our third hypothesis thus postulates that

(iii) biotic interaction impacts on the insecticide-induced gene expression profiles, at least in *L. basale*, which is expected to be the most sensitive to chlorantraniliprole exposure.

2. Methods

2.1. Indoor experiment

Stream water was taken from the stream Bieber (50°13′21.3″N, 9°15′56.5″E), a fine substrate dominated siliceous highland stream (Hessian Ministry of the Environment) in Hesse, Germany. The Bieber belongs to the Rhine-Main-Observatory (https://deims.org/9f9ba137-342d-4813-ae58-a60911c3abc1), a long-term ecological research site (Mirtl et al., 2018). The stream water was transported to the lab and distributed over 4 header tanks (50 L each). Each header tank supplied the water to 9 channels (length = 25 cm), i.e., the experimental units. The water was subsequently collected in a reservoir and pumped back into the header tanks, resulting in a closed water circuit (Fig. 1A). The experiment was performed for 10 days from August 27th to September 6th, 2021. To account for possible fluctuations in nitrate and nitrite concentrations in the natural stream water, the system was calibrated for 1 week (day −6 to day 0) without invertebrates (Fig. 1B). On day −3, the channels were filled with substrate from the Bieber, which was air dried for 5 days beforehand to reduce biological and chemical contamination. Each channel contained 150 g gravel <1 cm, 150 g gravel 1–3 cm and three large flat stones to resemble the natural steam bed. The flow rate within the channels was calibrated to 1 L/min daily from day −1 onwards and the water in the header tanks was constantly oxygenated. A room temperature of 18 °C was maintained throughout the whole experimental period, which is slightly lower than the average stream temperature (22.2 ± 0.5 °C) during the time of the experiment. Water temperature and oxygen content in the header tanks and reservoirs were measured with the MultiLine P4 probe (WTW, Washington, D.C., United States) at day −1 and at day 4. The light:dark regime was a natural photoperiod i.e., not artificially controlled.

Macroinvertebrates (108 *E. danica*, 240 *G. pulex* and 1,440 *L. basale*) were sampled at day 0 from the Bieber (50°09′41.0″N, 9°18′12.0″E) and transferred to the lab. We exposed the three test species to three different concentrations of chlorantraniliprole. Each channel comprised 3 *E. danica* specimens and additionally either (i) 60 *L. basale* specimens, (ii) 10 *G. pulex* specimens or (iii) 60 *L. basale* and 10 *G. pulex* specimens

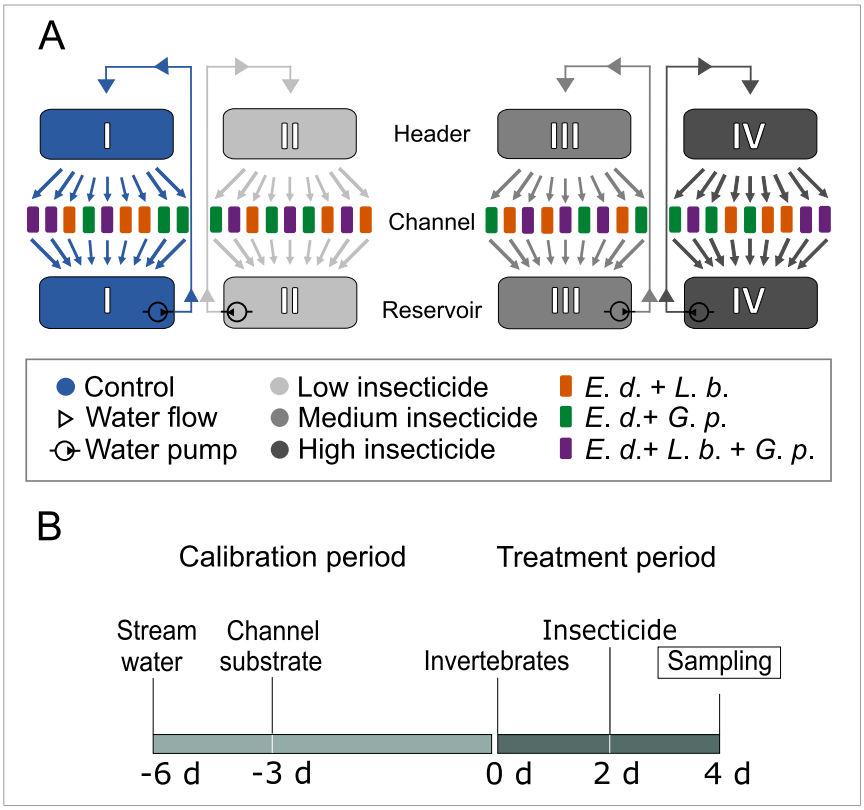

Fig. 1. Experimental setup. A: Four closed stream water circuits (I-IV) were used to test the insecticide effect on *E. danica*, *L. basale* and *G. pulex* and additionally the effect of biotic interaction on the two leaf-shredding species. Three different insecticide levels i.e., low, medium and high exposure concentration were targeted, referring to nominal chlorantraniliprole concentrations of 0.2 (low), 2 (medium) and 20 µg/L (high). All channels contained 3 *E. danica* specimens and in addition either 60 *L. basale* specimens (*E. d.* + *L. b.*), 10 *G. pulex* specimens (*E. d.* + *G. p.*) or the combination of all three species (*E. d.* + *L. b.* + *G. p.*). The channels represent the experimental units, therefore, the insecticide effect in *E. danica* was estimated based on 9 replicates per treatment (n = 36). In the two shredder species, the two treatment variables were manipulated in a 4x2 full-factorial design, and each unique treatment combination was present in triplicates (n = 24). B: The experiment comprised a one-week calibration period (day −6 to day 0) followed by a treatment period for 4 days (day 0 to day 4). During the treatment period, macroinvertebrates were allowed to acclimatize for 48 h (starting at day 0, 03:00 p.m.) before they were exposed to the insecticide (starting at day 2, 03:00 p.m.). Specimens were sampled after 48 h of exposure at day 4.

(Fig. 1). The numbers of the shredder organisms differed to account for the difference in biomass between the two species. Since *E. danica* was present in all channels, the effect of biotic interaction between *E. danica* and the other species was not taken into account here. Instead, only the species composition of the two shredder species was iterated over the experimental units, allowing to test the effect of biotic interaction in terms of intraspecific competition. As food resource for the shredder organisms, two alder (*Alnus glutinosa*, (L.) Gaertn) leaf discs (diameter = 2 cm) per channel were provided. Alder leaves were sampled from the stream's riparian area at day 0. The organisms were allowed to acclimatize for 48 h (day 0–2). Then, the water in three header tanks was spiked with the commercial product Coragen (batch no.: MAY19CL13A, DuPont, Wilmington, Delaware, United States) to three nominal chlorantraniliprole concentrations of 0.2, 2 and 20 µg/L. Coragen contains 18.4% chlorantraniliprole (CAS no.: 500008-45-7), which is the only active compound reported by DuPont (Coragen data safety sheet). Still, other ingredients of the Coragen formulation such as the solvent likely evoke gene regulatory responses as well, which cannot be separated from the effect of chlorantraniliprole in this study.

Lethal concentrations of chlorantraniliprole (i.e., LC_{50} after 48 h) for aquatic invertebrates reported by the European Food Safety Authority (EFSA) range from 11.6 µg/L in *D. pulex* to 85.9 µg/L for *Chironomus riparius* (EFSA, 2013), but sublethal effects (i.e., reduced glutathione content) can be observed at concentrations as low as 0.2 µg/L (Rodrigues et al., 2017). Accordingly, the test organisms were exposed to a concentration range which was expected to induce weak, sublethal effects in the lowest insecticide treatment up to very strong effects in the highest insecticide treatment. Since measured concentrations in surface waters up to 28 µg/L were reported in the past (Zhang et al., 2012), our exposure concentrations are environmentally relevant. In fact, the acute exposure concentrations in natural systems could have been even higher, considering the substance's sensitivity to photodegradation (Lewis et al., 2016) and its tendency to accumulate in sediment and soil (EFSA, 2013). The organisms were exposed for 48 h (day 2–4) and water samples for chlorantraniliprole concentration measurements were taken at the start and the end of the exposure period. We aimed to pool RNA from 3 *E. danica*, 3 *G. pulex* and 10 *L. basale* specimens per channel but one *E. danica* larva died and from another larva a subimago emerged during the experiment. Thus, two channels were only represented by 2 individuals. Further, the high insecticide concentration treatment induced paralysis or mortality in the caddisflies, which we could not confidently distinguish in several cases. To avoid including dead specimens in the downstream laboratory processing, we only sampled 8 caddisflies in two channels with high insecticide concentration. All specimens were directly preserved at −80 °C.

2.2. Chlorantraniliprole analysis

To qualify chlorantraniliprole concentrations, water samples were pre-concentrated via solid-phase extraction using Oasis HLB 6 cc 500 mg extraction cartridges (Waters Corp., Milford, Massachusetts, United States). After conditioning the cartridges with 5 mL of methanol and equilibrating with 10 mL ultrapure water, an aliquot of ~500 mL (exact volume was recorded) was loaded to the cartridge at a flow rate of ~7 mL/min. Subsequently, the cartridges were dried with nitrogen for 2 h. The samples were eluded with 6 mL methanol:ethyl acetate (1:1, v/v, methanol LC grade, ethyl acetate) and 2 mL methanol. The eluates were then dried to 50 µL at room temperature under a gentle nitrogen flow and reconstituted with 450 µL ultrapure water. The resulting extracts were centrifuged (4000 rpm, 30 min) and the supernatants were used for chemical analysis. To correct for potential evaporation and extraction losses as well as matrix effects, selected standards (i.e., with 0.2 and 2 µg/L chlorantraniliprole) in ultrapure water as well as in stream water from the Bieber were treated identically. The samples were quantified using an LC-HRMS system from Exactive Orbitrap (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The chromatographic

separation was achieved with an Atlantis T3 5 μm 3.0 × 150 mm column (Waters Corp., Milford, Massachusetts, United States). A calibration row of chlorantraniliprole (PESTANAL analytical standards, Merck, Darmstadt, Hesse, Germany) was linear from 2 μg/L (analytical limit of quantification (LOQ) in water calculated as 0.004 μg/L) to 500 μg/L. Details on gradient settings are given in Schreiner et al. (2020).

2.3. RNA extraction, library preparation and sequencing

E. danica and *G. pulex* specimens were individually homogenized, whereas up to 5 *L. basale* specimens per channel were pooled for homogenization. Whole specimens were homogenized in 1 mL (*E. danica*, *G. pulex*) or 400 μL (*L. basale*) guanidinium thiocyanate (GITC) lysis buffer (Oberacker et al., 2019) with 1% β-mercaptoethanol (Acros organics, Waltham, Massachusetts, United States). Tissue disruption was performed with the MiniBeadbeater-96 system and zirconia beads (both BioSpec Products, Bartlesville, Oklahoma, United States) (diameter: 2 and 1 mm, 1:1) for 5 min at 2400 rpm, followed by centrifugation for 3 min at 16,000×*g*. Magnetic beads (Cytiva Lifesciences, Marlborough, Massachusetts, United States) in Tris-EDTA-min (TE-min) buffer (10 mM Tris, 1 mM EDTA) were added to 350 μL lysate and nucleic acids were precipitated with molecular grade isopropanol (1:1, v/v, aqueous solutions, isopropanol; Fisher Scientific, Waltham, Massachusetts, United States), following incubation for 10 min. Then, the beads were allowed to settle to a magnet and the supernatant was discarded. The samples were washed three times with 150 μL 80% ethanol (molecular grade). Finally, the nucleic acids were eluted in 55 μL TE-min buffer and stored at −80 °C. The success of the extraction was checked on a 1.5% agarose gel. Subsequently, the extracts of all individuals from one experimental replicate (i.e., channel) were pooled per species, resulting in 24 *L. basale*, 24 *G. pulex* and 36 *E. danica* libraries.

The genomic DNA was digested for 15 min at 37 °C with 2 Units DNase (Ambion, Waltham, Massachusetts, United States) per library. Next, RNA binding buffer (Oberacker et al., 2019) and magnetic beads in TE-min buffer (4:1, v/v, alcoholic RNA binding buffer, aqueous solutions i.e., digested extract and beads in TE-min) were added to the digests. Following incubation for 5 min, the beads were allowed to settle to a magnet, and the supernatant was discarded. The RNA was washed twice with 100 μL 80% ethanol (molecular grade) and finally solubilized in 55 μL TE-min buffer. Ingredients of the GITC lysis buffer and the RNA binding buffer can be found under https://bomb.bio/protocols/ (protocol 8.2).

Sample concentration and quality were assessed on a Fragment Analyzer system using the 15 nt RNA kit (both Agilent, Santa Clara, California, United States). All 84 purified libraries were sent to the West German Genome Centre (WGGC) Bonn for further library preparation and sequencing. For *E. danica* libraries, 800 ng total RNA were used for library preparation. For *G. pulex* and *L. basale*, 500 ng total RNA were used as input. Poly-A selection for mRNA enrichment and strand-specific cDNA library preparation were performed with the NEBNext Ultra II Directional RNA kit (New England Biolabs, Ipswich, Massachusetts, United States). Samples were paired-end sequenced (2x150 bp) on NovaSeq 6000 platforms (Illumina, San Diego, California, United States).

2.4. Bioinformatics

Raw RNA-sequencing reads were homopolymer trimmed with a custom C++ (Stroustrup, 1997) program, followed by removal of adapters and low-quality bases (Phred <20) using the cutadapt v3.2 (Martin, 2011) wrapper script TrimGalore! v0.6.6 (https://github.com/FelixKrueger/TrimGalore) in paired-end mode. All reads with a length of ≥25 nucleotides were retained and used for subsequent *de novo* transcriptome assembly. The transcriptome assemblies of the individual insect species were created with Trinity v2.13.2 (Grabherr et al., 2011). For the assembly of the *G. pulex* transcriptome, rnaSPADES (Bushmanova et al., 2019) performed better than Trinity (mainly in terms of the ExNy statistic) and the final assembly was generated with rnaSPADES v3.15.0. All transcriptomes were generated in strand-specific mode. The resulting contigs were clustered at 98% sequence similarity with CD-HIT-EST v4.8.1 (Fu et al., 2012; Li and Godzik, 2006) to reduce redundancy in the assemblies. Quality and completeness of the assemblies were assessed based on read re-mapping rates, the ExNy statistic from the Trinity toolkit, and the detection rate of Benchmarking Universal Single Copy Orthologs (BUSCOs). BUSCO analyses were performed with the BUSCO software v4.0.6 and database (Manni et al., 2021) based on the BUSCO set for insects (*L. basale*, *E. danica*) and arthropods (*G. pulex*). Transcript counts were estimated by mapping the quality trimmed reads against the respective transcriptome using the light-weight alignment tool salmon v1.9.0 (Patro et al., 2017). Salmon was run in strand-specific mode, specifying the flags for GC bias correction and sequence specific bias correction. To improve reproducibility and scalability, bioinformatic sample processing was performed in a Snakemake v7.20.0 (Köster and Rahmann, 2012) workflow.

Functional annotation of *de novo* transcriptomes was performed on protein level. Coding sequences were identified with TransDecoder v5.5.0 (https://github.com/TransDecoder/), including homology searches to SwissProt/UniProt (The UniProt Consortium et al., 2021) and Pfam (Mistry et al., 2021) databases as open reading frame retention criteria (e-value < $1e^{-5}$). DIAMOND v2.0.15 (Buchfink et al., 2021) searches of the predicted protein sequences against the eggNOG v5.0.2 (Huerta-Cepas et al., 2019) database were performed with the eggNOG-mapper v2.1.9 (Cantalapiedra et al., 2021) to retrieve gene ontology (GO) terms and the final annotations using 'Arthropoda' as target taxon. Hits were required to show >50% sequence identity, a bit score >50 and an e-value < $1e^{-5}$. In case of multiple hits, the annotation with the highest bit score was retained with a Python 3 (Van Rossum and Drake, 2009) script.

2.5. Statistical analyses

All statistical analyses and visualizations were performed in R v4.1.2 (R Core Team, 2022). Transcript counts were summarized to gene level with the tximport package (Soneson et al., 2015), correcting for differences in isoform length. The inference of the isoform-to-gene relation is based on assumptions made by the assembler, resulting in a loose usage of the term gene.

DESeq2 (Love et al., 2014) was used to fit negative binomial generalized linear models with two different model designs: ~Insecticide (*E. danica*) and ~Biotic interaction*Insecticide (*L. basale*, *G. pulex*). The predictor 'Insecticide' represents the measured chlorantraniliprole concentrations at day 4 (i.e., day of specimen sampling after exposure), whereas 'Biotic interaction' is a categorical predictor with two levels, single shredder species vs. both shredder species. Since the 'Insecticide' predictor measures on a continuous scale, we tested for genes changing their expression linearly when increasing the insecticide concentration. Accordingly, the reported log2 fold changes refer to expression changes per unit (i.e., 1 μg/L) increase in chlorantraniliprole concentration. For *E. danica*, this refers to the general insecticide effect, whereas the insecticide-induced gene expression in *L. basale* and *G. pulex* was modelled with and without the effect of biotic interaction. Further, we tested how intraspecific competition itself affects the gene expression profiles in the two shredder species. Only genes with at least 12 normalized counts in at least 9 (*E. danica*) or 6 (*L. basale*, *G. pulex*) samples were included in the downstream analysis. The different sample numbers account for the different numbers of replicates used to estimate the insecticide effect in *E. danica* and the shredder species. One sample from the *E. danica* data set (sample 24, medium concentration) was excluded due to a low read mapping rate against the *E. danica* genome in a preliminary analysis. To control for hidden sources of variation unrelated to the specified predictors, surrogate variables were constructed with the SVA package (Leek et al., 2012) (reduced model: ~1, full

models: ~Insecticide or ~ Biotic interaction*Insecticide). All significant surrogates were included as covariates in the respective models. The Wald statistic was used to test for differential gene expression, applying a significant threshold of <0.05 after FDR correction (adjusted p-value). When gene counts are low, large fold changes can arise due to imprecise estimation of effect sizes. To account for effect size inflation in weakly supported genes, effect sizes were shrunk with the adaptive shrinkage estimator from the ashr R package (Stephens, 2016).

Functional enrichment of GO terms was conducted with the weight01 algorithm implemented in the topGO package (Alexa et al., 2006). Enrichment tests of biological process terms were performed separately for insecticide exposure induced upregulated and downregulated genes in (i) *E. danica*, in (ii) *L. basale* without biotic interaction, and in (iii) *L. basale* under biotic interaction. All annotated genes were used as the gene universe and Fisher's exact test was performed to identify statistically significant overrepresentation (p-value <0.05). Due to the limited effect of the treatment on *G. pulex*, no functional enrichment analysis was performed.

2.6. Visualization

Principal component analyses (PCAs) were conducted using the PCAtools package (Blighe and Lun, 2021). PCAs were performed based on variance-stabilized gene counts. The gene counts were either adjusted for surrogates by the frozen SVAs using the function fsva() from the SVA R package or remained unadjusted for the PCAs. Frozen SVAs correct for expression signals in the count data introduced by latent factors (i.e., the constructed surrogate variables) through linear regression, providing an adjusted data set where the data variation related to the latent factors is removed.

Heatmaps were produced with ComplexHeatmap (Gu et al., 2016) and rows (i.e., genes) were clustered based on Euclidean distances. For *E. danica*, only one contrast was performed, therefore, the differential gene expression results were not visualized in a heatmap but in a volcano plot. The volcano plot and all functional enrichment visualizations were produced with ggplot2 (Wickham, 2016).

3. Results and Discussion

Using the Coragen formulation, we achieved three different insecticide exposure levels close to the targeted nominal concentrations (Table S1). The measured chlorantraniliprole concentrations slightly increased from day 2 to day 4, probably because the insecticide was not completely dissolved at the beginning of the exposure period. At the time of specimen sampling (day 4), the concentrations were 0.3 (low insecticide treatment), 4.93 (medium insecticide treatment) and 19.5 μg/L (high insecticide treatment). No chlorantraniliprole contamination was found in the control treatment or in the natural stream water.

On average, sequencing yielded 21,021,078 ± 2,097,154 (mean ± s.d.), 21,232,715 ± 2,380,913 and 25,240,494 ± 3,891,720 reads for *L. basale*, *G. pulex* and *E. danica*, respectively. BUSCO recovery rates and library remapping rates of >90% indicated complete and representative transcriptome assemblies (Table S2).

3.1. Insecticide-induced gene regulatory pathways differ between organismic groups

In line with our expectations, we detected a relatively weak transcriptional response evoked by the insecticide treatment in the amphipod *G. pulex* compared to the strong insecticide-induced effect in both insect species (Fig. 2). Experimental units containing *G. pulex* exposed to the insecticide clustered in the same ordination space as control samples (Fig. 2A). Although at least the treatment with the highest insecticide concentration appears to be associated with the first PCA axis, this sample group is characterized by a large within-group variability. Accordingly, we detected only a few DE genes that changed expression patterns linearly with increasing insecticide concentration. Without biotic interaction, slightly more upregulated (35) than downregulated (28) genes were identified. When L. *basale* was present, the insecticide exposure induced a distinct transcriptional profile in *G. pulex* (Fig. 2B).

In contrast, chlorantraniliprole showed a pronounced effect on the gene expression profiles of the two insect species. The highest insecticide concentration treatment was strongly associated with the first PCA axes, accounting for 28% (Figs. 2C) and 18% (Fig. 2E) variance in the adjusted *L. basale* and *E. danica* data sets, respectively. We identified in both insect species thousands of DE genes (Fig. 2D and F), supporting our first hypothesis that the induced transcriptional stress response is stronger in caddisflies and mayflies, which are more closely related to the target taxon of chlorantraniliprole, i.e., Lepidoptera, than the amphipod crustacean *G. pulex*.

The transcriptional response in the mayfly *E. danica* was characterized by a strong genetic suppression. From 4,701 DE genes, 3,279 (70%) genes were downregulated in response to the insecticide exposure (Fig. 2F). These downregulated genes appear to be involved in various metabolic pathways comprising immunity, neuromuscular and developmental processes and especially the chitin metabolism (Fig. 3, Table S3). Chitin serves as the major polysaccharide in the cuticle of insects (Kramer et al., 1995). As such, chitin plays a key role in the insect development, which comprises several molting cycles to accommodate the increase in body size. Molting requires the synthesis of epidermal cells to form a novel cuticle (Charles, 2010), explaining the overrepresentation of biological process terms referring to cell-cell adhesion and the metabolism of sphingomyelin and other lipids, which constitute important cell membrane molecules (Kraft, 2017). Sublethal exposure to different insecticides, including chlorantraniliprole, has been reported to impede molting and development in insects by affecting chitin synthesis, the formation of novel epidermis and the separation of old cuticle (Chen et al., 2020; Meng et al., 2018, 2020; Wang et al., 2018). Albeit this has only been observed for terrestrial species, our data indicate that chlorantraniliprole can similarly disrupt the chitin metabolism in aquatic non-target insects such as the mayfly *E. danica*, which could lead to tissue damage, molting failure or developmental abnormalities.

In contrast to *E. danica*, the number of upregulated and downregulated genes was approximately equal in the caddisfly *L. basale*. In total, 5,512 genes responded to the insecticide exposure when *G. pulex* was absent as a competitor (Fig. 2D). The enrichment analyses for biological process terms annotated to these genes suggest that the insecticide treatment induced a suppression of the energy metabolism, mainly mediated via downregulation of genes involved in mitochondrial processes (Fig. S2, Table S4). The set of upregulated genes was significantly associated with numerous morphogenetic developmental process terms (e.g., GO:0008586: 'imaginal disc-derived wing vein morphogenesis', GO:0007526: 'larval somatic muscle development', GO:0007552: 'metamorphosis') and, to a lesser extent, with terms referring to immunity (Fig. S2, Table S4).

We hypothesized that the insecticide treatment induces the differential expression of genes involved in maintaining the calcium homeostasis (hypothesis ii). Based on the functional enrichment data, we cannot infer a transcriptional response directly related to a disruption of the calcium homeostasis in any of the species. Instead, the alteration of developmental processes appeared to be a key physiological response mechanism, although distinct gene regulatory networks were involved: in *E. danica*, the insecticide might induce a developmental suppression, mediated via the chitin metabolism, whereas in *L. basale*, a stimulation of developmental genes was observed. This leads to two competing hypotheses concerning the effect mechanisms of chlorantraniliprole in non-target taxa. If the calcium homeostasis is not disturbed, the insecticide-induced regulation of developmental processes is the result of a mechanistic target other than the alteration of the ryanodine receptor conformation. Developmental processes in insects are regulated by their neuroendocrine system. Many insecticides exhibit endocrine

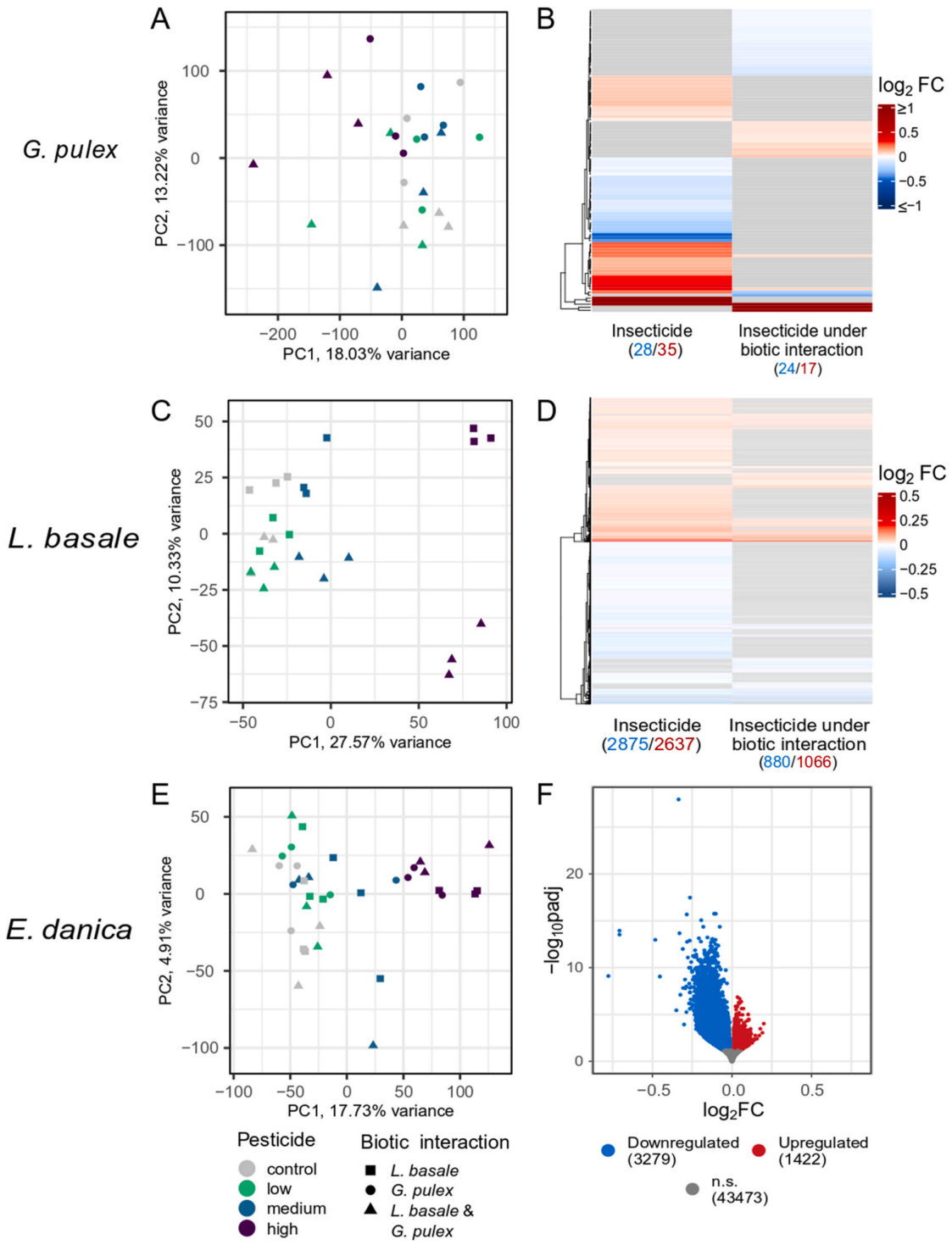

Fig. 2. Gene expression patterns obtained from unsupervised ordination analyses and statistical tests for insecticide-induced differential expression for *G. pulex* (A, B), *L. basale* (C, D) and *E. danica* (E, F). PCA biplots were produced based on SVA corrected variance stabilized gene counts. The log2 fold changes (log_2FC) indicate the expression change per unit increase (i.e., 1 μg/L) in chlorantraniliprole concentration. The blue and red colored numbers below the heatmaps refer to the number of downregulated and upregulated genes, respectively, with an adjusted p-value <0.05. The y-axis in the volcano plot represents the adjusted p-values on the negative decadic logarithmic scale (-log_{10}padj). n.s.: not significant.

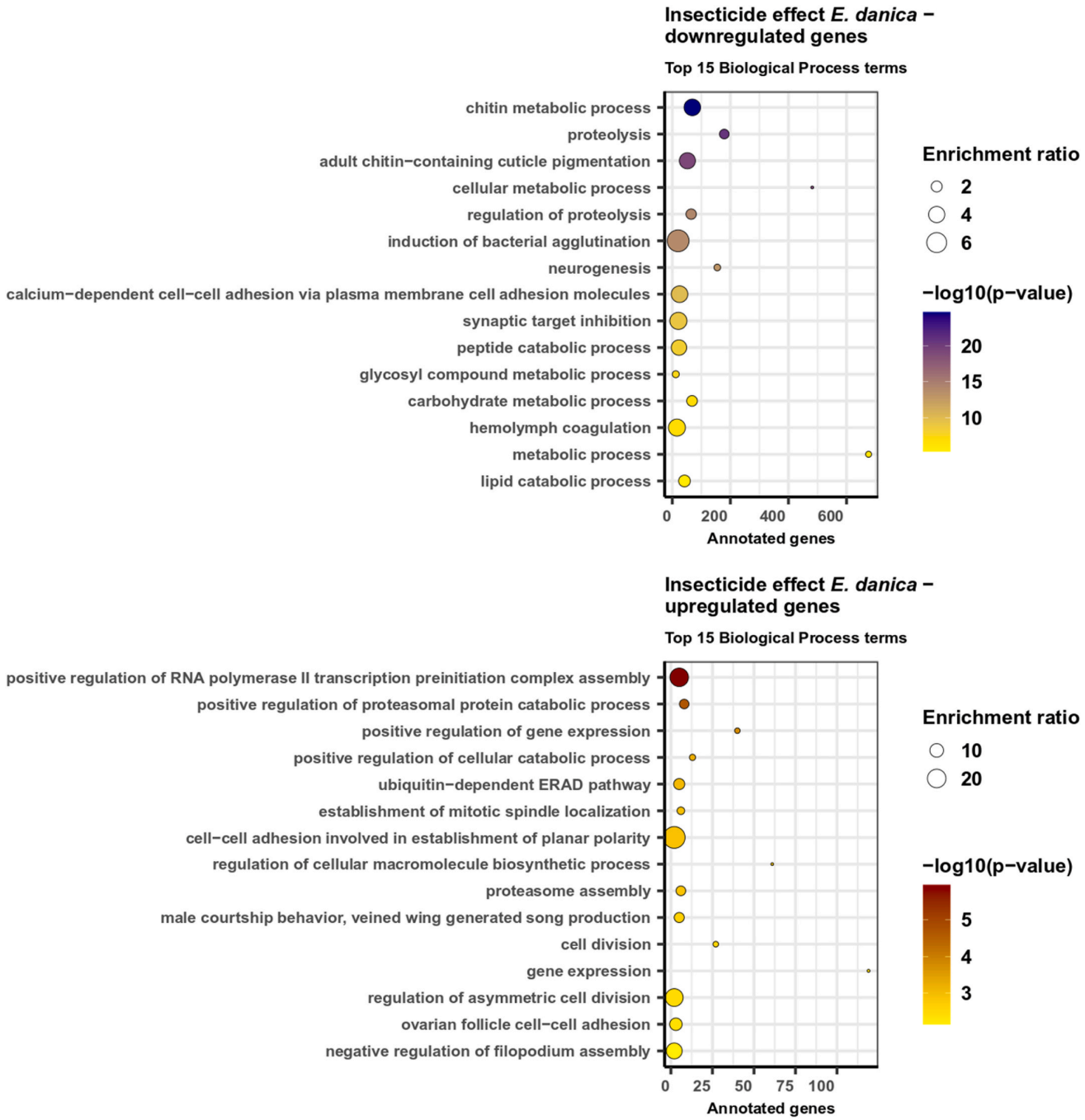

Fig. 3. Functional enrichment analyses for the insecticide-induced gene expression in the mayfly *E. danica*. Shown are the most significant biological process terms. The downregulated genes were predominantly annotated to metabolic functions, pointing towards a suppression of the chitin metabolism. The set of upregulated genes are presumably mainly involved in the regulation of the transcriptional program and protein biosynthesis. 'Annotated genes' indicate the number of term observations in the whole transcriptome. 'Enrichment ratio' refers to the number of annotations found in the set of DE genes relative to the expected number by chance. P-values were obtained from Fisher's exact test.

activity and/or modulate neuronal processes of hormone synthesis (Crane et al., 2022; Peric-Mataruga et al., 2006), although endocrine disruption may not be assumed based on their described mode of action. Such off-target effects are of special concern because the exposure consequences in natural systems are unpredictable.

Alternatively, the observed differential expression patterns of genes involved in the insect development are the product of a disturbed calcium homeostasis. Calcium is one of the most important cellular signalling molecules (Clapham, 2007), with known mediatory function in developmental and molting processes in insects (Gu et al., 1998). Whether the observed gene expression responses are evoked by alterations of the intracellular calcium level or by distinct regulatory pathways, requires further research.

Interestingly, the enrichment analysis of the downregulated genes in *L. basale* included muscular process terms when *G. pulex* was present as a competitor in the biotic interaction treatment (Fig. S3, Table S5).

Notably, most of the downregulated genes were similarly downregulated without biotic interaction and the intersection of DE genes was generally large: from 1,946 genes, 63% were also regulated without biotic interaction (Fig. 2D). We argue that these downregulated genes involved in muscular processes represent a core transcriptional stress response induced by the insecticide, since this subset of genes is downregulated in *L. basale*, irrespective whether the competing shredder species is present or not. Further, the suppression of mitochondrial and muscular processes is mechanistically not exclusive. The functional enrichment analysis aims to identify significant overrepresentation of specific gene annotations (i.e., more observations of an annotation than expected by chance) and consequently depends on the completeness of the transcriptome annotation. Testing a smaller set of DE genes can decrease the sensitivity simply because less annotation information is available. It is possible that the reduction of muscular processes is achieved by a downregulation of mitochondrial genes in muscle cells, but that the latter metabolic pathway can only be resolved in the larger set of downregulated genes.

The dominant genetic downregulation and the enrichment for neuromuscular processes identified in *E. danica*, as well as the suppression of mitochondrial and muscular processes in *L. basale* support at least partially our second hypothesis that the insecticide treatment induced regulatory pathways associated with muscular and bioenergetic processes. However, we cannot fully accept this hypothesis, since the gene expression data do not clearly indicate the regulation of genes involved in maintaining the calcium homeostasis.

3.2. Biotic interaction can shape the insecticide-induced expression profile

Without insecticide exposure, the effect of intraspecific competition at the transcriptional level was weak in both shredder species. In *G. pulex*, 28 genes were detected as DE (16 downregulated, 12 upregulated), whereas in *L. basale*, only 11 genes were DE (4 downregulated, 7 upregulated). At the same time, biotic interaction modulated the insecticide-induced expression profile in *L. basale*. The substantially reduced number of insecticide responsive genes in this species as well as the shift in the functional perspective of the downregulated genes from mitochondrial to muscular processes supports our third hypothesis that biotic interaction influences the insecticide-induced expression profile. This together with the limited effect of biotic interaction alone in both species have an important implication: while none of the shredder species experienced a physiological stress solely due to the presence of the competing shredder, the effect of competition became more pronounced, when the organisms were confronted with environmental conditions that were physiologically challenging. In our treatment scenario, *G. pulex* is likely to be the stronger competitor due to the minimal insecticide effect in this species and is therefore not confronted with a resource limitation. In contrast, the strong insecticide-induced transcriptomic response in *L. basale* is probably energetically demanding since gene expression or physiological stress responses in general are ATP-dependent. Consequently, an insufficient nutrient uptake could hamper the physiological adaptability of *L. basale*, which in turn would indirectly increase the toxicity of chlorantraniliprole. As such, biotic interactions which act as additional stressor can result in synergistic or antagonistic stressor interactions with pesticides, as reported previously (e.g., Coors and De Meester, 2008; Janssens and Stoks, 2017).

Cells which are at the limit of their physiological capabilities do not show a constant gene expression behaviour, limiting the ability to detect them as DE (de Jong et al., 2019). Accordingly, the substantially reduced number of insecticide-responsive genes in *L. basale* under biotic interaction does not necessarily imply reduced insecticide effects but could rather hint towards inflated gene dispersion (i.e., variance in expression within treatment replicates). Large gene dispersion accompanied with rather small effect sizes may have further prevented the detection of subtle changes in expression due to the biotic interaction treatment alone. Future experiments with an increased number of biological replicates would allow a more sensitive analysis in that regard (Schurch et al., 2016). Likewise, more pronounced effects may arise when the biotic interaction treatment is applied for more than 48 h or when a more complex species interaction scenario is tested.

3.3. Implications for biological communities in natural systems

We identified a reduced metabolic activity and found evidence for alterations of developmental processes in both insect species. Such transcriptional changes could affect metamorphosis and might be responsible for temporal shifts in emergence patterns of aquatic insects. Environmental stress and specifically insecticide exposure has been reported to alter insect emergence dynamics (Cavallaro et al., 2018; Ohler et al., 2023; Rogers et al., 2016). For instance, bifenthrin can accelerate or delay emergence, depending on the exposure concentration (Rogers et al., 2016). Earlier insect emergence was also observed following exposure to several neonicotinoids (Cavallaro et al., 2018).

In our study, changes in the developmental program were achieved by different genetic response pathways. In the mayfly *E. danica*, the metabolic depression, especially of the chitin metabolism, could eventually result in reduced energy reserves and delayed metamorphosis. Imagos of *E. danica* do not possess functional mouthparts (Bauernfeind and Humpesch, 2001), thus relying on energy reserves which were acquired during their larval stage. Their courtship behavior is not only energetically consuming but also synchronized in swarming events (Bauernfeind and Humpesch, 2001). Therefore, changes in emergence phenology that are accompanied with reduced energy reserves are likely to disrupt the reproductive success of populations in agricultural streams.

Conversely, the insecticide treatment stimulates molecular pathways regulating development in the caddisfly *L. basale*, which could induce premature emergence. While such earlier emergence has been attributed to an alteration of food supply in response to insecticide contamination (Dewey, 1986; Lowell et al., 1995), our data clearly suggest that insecticides directly interfere with the developmental program at a transcriptional level.

If phenological plasticity enhances survival and reproductive success by decreasing generation times in dynamic environments, increased emergence rates could represent a strategy to cope with environmental stress. Consistently, insect species with shorter generation times are favored in agricultural streams, presumably due to their higher recovery potential (Larsen and Ormerod, 2010; Ohler et al., 2023). Without the inclusion of further ecological endpoints, we cannot clearly differentiate to which degree an alteration of the developmental program is the result of adverse effects such as endocrine disruption directly induced by the insecticide, or whether this represents a strategy to mitigate environmental stress. Although this requires further research, long-term stressor exposure clearly comes at the expense of physiological stress responses and is likely to induce effects at the organismal and population level. Since aquatic insects are relevant for the organic matter transition between aquatic and the linked terrestrial ecosystem, this could induce structural and functional alteration of aquatic and terrestrial food webs (Ohler et al., 2023).

4. Conclusion

Transcriptomic profiling can provide valuable insights into physiological mechanisms of important environmental stressors such as pesticides. The observed physiological effects of insecticides can strongly vary between members of different evolutionary lineages. Since these effects are further modulated by biotic interactions, the assessed impact of pesticide contamination in natural streams depends on the community composition at given sites. Considering this context dependency is crucial when insecticide effects obtained from ecological studies with limited biological complexity are extrapolated to the ecosystem scale.

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Author statement

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Declaration of competing interest

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

Data availability

All RNA-seq data sets are deposited at the European Nucleotide Archive at EMBL-EBI under project accession numbers PRJEB61461 (*Lepidostoma basale*), PRJEB61444 (*Ephemera danica*) and PRJEB61462 (*Gammarus pulex*). All bioinformatic processing scripts are publicy available at GitHub: https://github.com/marievalerie/CAP-BioticInteraction.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122306.

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Chapter 5

Multiple stressor effects of insecticide exposure and increased fine sediment deposition on the gene expression profiles of two freshwater invertebrate species

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Summary

Insecticides or their metabolites often accumulate in the environment, resulting in an omnipresent, heterogeneous mixture of organic toxicants in streams (Halbach et al. 2021; Liess et al. 2021; Sandstrom et al. 2022). Consequently, insecticide pollution frequently coincides with other stressors, particularly agriculturally derived ones such as an increased fine sediment load (Schulz and Liess 1999; Stehle and Schulz 2015). Due to the adsorption dynamics between dissolved organic compounds like pesticides and fine particulate matter (Leonard et al. 2001; Liu et al. 2004), the effect of pesticide exposure is potentially modulated by the presence of fine sediment (Chará-Serna and Richardson 2018). In addition, disparate stressor effects and interactions may arise for different species, depending on their specific ecological and physiological traits which define external and internal stressor exposure, respectively (Van Straalen 1993). Accordingly, to obtain realistic estimates of the impact of pesticide pollution, the exposure effects must be studied in the presence of other stressors while integrating ecological complexity in the experimental setting. In this study we addressed the context dependency of multiple stressor effects by using the ExStream mesocosm system and assessed the effect of chlorantraniliprole exposure and increased fine sediment on the transcriptomic response of the amphipod *Gammarus pulex* and the caddisfly *Lepidostoma basale*. The selected mesocosm field experiment approximates the natural stressor exposure scenario more accurately than the previously presented indoor system, yet expression estimates are presumably noisier due to more degrees of freedom in the experiment. Therefore, we compared the differential expression patterns and gene regulatory pathways induced by the insecticide chlorantraniliprole under conditions where insecticide exposure was maximized (indoor experiment) with insecticide effects obtained under near-natural conditions and addressed the two following research questions in Chapter 5:

(i) How does single and combined stressor exposure of increased fine sediment deposition and different concentration levels of the insecticide chlorantraniliprole affect the transcriptional profile of *L. basale* and *G. pulex*?

(ii) How consistent are insecticide effects obtained from controlled experimental conditions (i.e., the indoor system used in Chapter 4) with

insecticide effects obtained from experiments incorporating natural complexity (i.e., the ExStream system used in this study)?

In line with the observations from Chapter 4, the insecticide stressor treatment showed a pronounced effect on the transcriptome of *L. basale*. Again, the caddisfly responded to chlorantraniliprole exposure with an upregulation of developmental and immune genes and a downregulation of genes involved in the energy metabolism and cell cycle, while the functional profile of chlorantraniliprole responsive genes did not provide clear evidence for a disruption of the calcium homeostasis.
In contrast to *L. basale*, *G. pulex* exhibited only minor transcriptional changes in response to chlorantraniliprole exposure. Because this is in agreement with the results obtained from the previous indoor experiment, we attributed the species-specific insecticide sensitivity mainly to differential toxicodynamics of chlorantraniliprole between *L. basale* and *G. pulex* due to distinct biological traits at the molecular level, which determined the intrinsic sensitivity of both species. However, differential toxicokinetics i.e., internal and external insecticide exposure patterns, have certainly contributed to the overall difference in species vulnerability. While internal differences in insecticide exposure are related to the organism's physiology (Van Straalen 1993) e.g., the degree of cuticle sclerotization, the external stressor exposure can be reduced by organisms through e.g., seeking refugia in other mesocosm compartments. *L. basale* might have been restricted in such stressor avoidance behavior due to its specific habitat demands (Schmidt-Kloiber and Hering 2015), which therefore contributed to a stronger external stressor exposure compared to the more flexible *G. pulex*. Further, the expression data of both species suggested that the toxicokinetics of chlorantraniliprole are shaped by the presence of fine sediment: in *L. basale*, the insecticide effect was most pronounced when chlorantraniliprole was applied as single stressor in its highest concentration, and slightly declined when fine sediment was present as additional stressor. Consistently, we detected solely antagonistic stressor interactions between insecticide exposure and increased fine sediment deposition in the expression data of *L. basale*. Similarly, a predominant antagonistic nature of stressor interactions was observed for *G. pulex*, suggesting that the insecticide's bioavailability (and thus uptake) decreased in the presence of increased fine sediment levels.

Surprisingly, the overall effect associated with fine sediment addition was limited in both species. We explained this by different mechanistic targets of chemical and non-chemical stressors, whereby the latter may not directly interfere with cells at the molecular level. We concluded that multiple stressors effects are shaped by various factors such as molecular mechanisms of stress perception and species-specific biological and ecological traits. These findings have important implications for the environmental risk assessment of pesticides since (i) the toxicity of pesticides is largely determined in ecotoxicological assays which are limited in their species repertoire and ecological complexity, and (ii) pesticide effects are not necessarily covered by their described mode of action. In the case of the caddisfly *L. basale*, an insecticide-induced disruption of the developmental program accompanied with decreased metabolic activity could result in population declines, which in turn may affect aquatic and terrestrial food webs as well as ecosystem functions such as shredder-mediated conversion of organic matter.

Personal contributions

The study was conceptualized by Florian Leese, Christoph Mayer and Ralf Schäfer. The ExStream system was set up and maintained by Domnik Buchner, Leoni Mack and myself. Dominik Buchner helped me to establish a nucleic acid extraction protocol in the lab. I performed all the lab work related to biological sample processing, which included nucleic acid extraction, DNA barcoding, RNA purification and quality control of RNA extracts. For further sample processing (i.e., mRNA enrichment and reverse transcription of RNA to cDNA) and sequencing, the samples were sent to the West German Genome Centre (WGGC) Bonn. I curated all RNA-sequencing data sets and performed all analyses i.e., deposition of the RNA-sequencing data at the European Nucleotide Archive, bioinformatic processing, functional annotation, statistical testing for differential expression and overrepresentation of gene ontology terms. I interpreted all data, visualized the experimental overview and all results, and wrote the first draft of the manuscript, to which all co-authors contributed.

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RESEARCH

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Multiple stressor effects of insecticide exposure and increased fine sediment deposition on the gene expression profiles of two freshwater invertebrate species

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Abstract

Background Freshwater ecosystem degradation and biodiversity decline are strongly associated with intensive agricultural practices. Simultaneously occurring agricultural stressors can interact in complex ways, preventing an accurate prediction of their combined effects on aquatic biota. Here, we address the limited mechanistic understanding of multiple stressor effects of two globally important stressors, an insecticide (chlorantraniliprole), and increased fine sediment load and assessed their impact on the transcriptomic profile of two stream macroinvertebrates: the amphipod *Gammarus pulex* and the caddisfly *Lepidostoma basale*.

Results We identified mainly antagonistic stressor interactions at the transcriptional level, presumably because the insecticide adsorbed to fine sediment particles. *L. basale*, which is phylogenetically more closely related to the insecticide's target taxon Lepidoptera, exhibited strong transcriptional changes when the insecticide stressor was applied, whereas no clear response patterns were observed in the amphipod *G. pulex*. These differences in species vulnerability can presumably be attributed to molecular mechanisms determining the cellular affinity toward a stressor as well as differential exposure patterns resulting from varying ecological requirements between *L. basale* and *G. pulex*. Interestingly, the transcriptional response induced by insecticide exposure in *L. basale* was not associated with a disruption of the calcium homeostasis, which is the described mode of action for chlorantraniliprole. Instead, immune responses and alterations of the developmental program appear to play a more significant role.

Conclusions Our study shows how transcriptomic data can be used to identify multiple stressor effects and to explore the molecular mechanisms underlying stressor-induced physiological responses. As such, stressor effects assessed at the molecular level can inform about modes of action of chemicals and their interplay with non-chemical stressors. We demonstrated that stressor effects vary between different organismic groups and that insecticide effects are not necessarily covered by their described mode of action, which has important implications for environmental risk assessment of insecticides in non-target organisms.

Keywords Agricultural stressor, Antagonistic interaction, Chlorantraniliprole, Pesticide, Sedimentation, Synergistic interaction, Transcriptomic stress

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Background

Anthropogenic activities lead to stream degradation worldwide. One of the most pressing factors is intensive land use, which poses a variety of stressors to stream ecosystems and their biodiversity [1]. Agricultural streams are exposed to stressors such as increased fine sediment deposition [2], reduced discharge [3], or contamination with organic substances like pesticides [4], which are applied at adjacent fields for crop protection. Predicting the impact of simultaneously present stressors on stream ecosystems is challenging, as their combined effects can strongly deviate from expectations which were derived based on individual effects assuming stressor additivity. In fact, stressors often do not operate independently but rather mitigate (antagonistic interaction) or amplify (synergistic interaction) each other [5]. These interactions can arise from a wide variety of mechanisms such as physico-chemical properties of stressors and complex spatial and temporal exposure patterns and might be further modulated indirectly via species interaction and trophic cascades [6, 7].

For instance, precipitation and flood events lead to elevated fine sediment levels in streams due to sediment erosion and surface runoff. The subsequent water turbidity negatively affects visual predators [8] and primary production [9], resulting in top-down or bottom-up effects in the food web, respectively. Sedimentation further decreases the structural complexity of microhabitats because interstitial spaces in the streambed and hyporheic zone are clogged [10, 11]. In agricultural areas, increased fine sediment load is accompanied by chemical stressors like pesticides, which also enter the stream via surface runoff, i.e., simultaneously with sediment input [12, 13]. Since contaminants such as heavy metals or pesticides attach to small particles, sedimentation can either represent a major source of chemical pollution [14, 15] or an reduce its bioavailability and mitigate the toxic effects of a pollutant [16].

Although it is crucial to understand the environmental impact of these globally important stressors, few studies have addressed multiple stressor effects of pesticides and fine sediment on freshwater invertebrates [e.g., 17–19]. Only one focused on a substance relevant for invertebrates, the insecticide chlorpyrifos [19]. Based on specimen abundance information, i.e., population level data, all studies identified increased fine sediment load as the major stressor for macroinvertebrate communities, while no or minor effects were attributed to pesticides [17–19]. However, relying solely on abundance information can be misleading, as organisms can remain at affected sites due to physiological coping mechanisms at the individual level and even a strong physiological stress might not be immediately reflected in population abundance data [20]. For instance, although the chlorpyrifos exposure in [19] did not induce mortality of macroinvertebrates, the authors found evidence for indirect effects such as feeding inhibition, reflected in reduced organic matter decomposition [19], emphasizing the need for an integration of physiological endpoints. Because such physiological responses are regulated by gene expression networks, studying stressor effects at the transcriptomic scale can provide significant insights into the mechanistic stress responses of an organism long before abundances change [20].

To the best of our knowledge, no study to date has looked at the impact of the individual and joint application of an insecticide and increased fine sediment load at the transcriptional level within freshwater invertebrates. In the current study, we addressed this research gap and assessed single and combined effects of increased fine sediment deposition and the insecticide chlorantraniliprole on the gene expression profiles of the caddisfly *Lepidostoma basale* (Kolenati, 1848), and the amphipod crustacean *Gammarus pulex* (Linnaeus, 1758).

Chlorantraniliprole is an anthranilic diamide and designed for crop protection against lepidopteran pest species [21]. It selectively binds to the insect ryanodine receptor, a non-voltage-gated calcium channel controlling the intracellular calcium homeostasis, resulting in uncontrolled calcium release in neurons and muscle cells [22]. Although chlorantraniliprole was initially reported to be highly selective for target pest species [22, 23], it was shown to be acutely toxic for aquatic macroinvertebrates at concentrations, which are observed for insecticides in agricultural streams [4, 13, 24].

For the species under investigation, no toxicity information is available, but ecotoxicological studies indicated that caddisflies are more sensitive to chlorantraniliprole than amphipods (for the caddisfly *Chimarra aterrima* and the amphipod *Gammarus pseudolimnaeus* LC_{50} values of 11.7 µg/L and 35.1 µg/L were determined, respectively [25]). Despite its fast photolysis [26], chlorantraniliprole is persistent in the environment and has been found to accumulate in sediments [24]. Therefore, both the dissolved and the adsorbed phase pose a relevant risk to aquatic biota, but the exposure phase-dependent physiological responses are largely unknown. Exploring their molecular basis could provide insights into mechanisms underlying the different sensitivity toward chlorantraniliprole between caddisflies and amphipods, which both possess key positions in aquatic food webs [27, 28].

We used an innovative mesocosm setup, the ExStream system [29], that allows to integrate ecological complexity when studying the interplay between chlorantraniliprole and increased fine sediment load. *L. basale*

and *G. pulex* have slightly different habitat preferences which may determine their susceptibility to the applied stressors. For instance, the clinger organism *L. basale* is strongly associated with wood and typically attaches to woody structures in streams [30]. As such, *L. basale* does not directly rely on a structural heterogeneity of the channel substratum and might thus be less impacted by fine sediment deposition. In contrast, *G. pulex* tends to use the interstitium as shelter for predation or during low flow periods in natural streams [31]. Moreover, sheltering of *G. pulex* may reduce its exposure to the insecticide stressor. If this avoidance strategy is, however, prevented by fine sediment clogging the interstitium, stressor interactions could arise.

Fine sediment deposition has been shown to induce a downregulation of genes associated with the energy metabolism in the amphipod *Gammarus fossarum* [20]. Since metabolic depression is a common physiological response to environmental stressors in different groups of invertebrates [32, 33], our first hypothesis is that

(i) both species react with energy allocation and metabolic suppression in response to the fine sediment stressor.

Stressor-induced molecular response pathways, which determine the cellular affinity toward a stressor, are expected to be more conserved between more closely related taxa. Since chlorantraniliprole is designed as pest control agent against lepidopteran species, which represents the sister order of caddisflies (Trichoptera), our second hypothesis is that

(ii) he insecticide has a more pronounced effect on the gene expression profile of *L. basale* than on *G. pulex*.

Due to the described mode of action of chlorantraniliprole, we further hypothesize that

(iii) chlorantraniliprole induces a differential expression of genes involved in maintaining the calcium homeostasis and therefore in neuromuscular processes, at least in *L. basale*.

Methods

Outdoor mesocosm experiment

We aimed to disentangle single and joint exposure effects of the insecticide chlorantraniliprole and increased fine sediment on the transcriptional profile of *L. basale* and *G. pulex* under semi-natural conditions. The ExStream system (Fig. 1A; ExStream Systems Ltd., Dunedin New Zealand) was set up next to the the Bieber, a fine substrate dominated siliceous low mountain stream (Hessian Ministry of the Environment) in Hesse, Germany (50°09′38.9"N, 9°17′58.6"E; 213 m a.s.l). The river belongs to the Rhine-Main-Observatory (https://deims.org/9f9ba137-342d-4813-ae58-a60911c3abc1), a long-term ecological research (LTER) site [34, 35]. The experiment was conducted from August 09 to September 19, 2020 and comprised a 21-day colonization period followed by a 21-day stressor period (Fig. 1B). Stream water was pumped continuously into four header tanks, each supplying 16 circular mesocosms (diameter = 25 cm, vol. = 3.5 L, area = 450 cm^2) permanently with fresh stream water. The stream water flow within the mesocosms was calibrated daily to 2 L/min. Each mesocosm was filled with substrate collected from the Bieber stream bed: 600 g gravel < 1 cm, 600 g gravel 1–3 cm, 300 g stones > 3 cm, and 3 large flat stones. This composition reflected stream bed areas where flow velocities resembled the ones realized in the mesocosms (~ 10 cm/s). The water temperature was assessed throughout the whole experiment in 5 min intervals using HOBO pendant loggers (Onset, Bourne, United States). Other physico-chemical parameters (oxygen concentration, pH, conductivity) were measured once a week with a MultiLine P4 probe (WTW, Washington, D.C., United States).

Each mesocosm comprised one unglazed ceramic tile (35 × 35 mm) for biofilm accumulation, one T-shaped tube (length: 10.4 cm; opening diameter: 5 cm) filled with 2.67 ± 0.12 g (mean ± S.D.) alder leaves (*Alnus glutinosa*, (L.) Gaertn.), and one 3 g pack alder sticks (Fig. 1C). Alder sticks were sampled from the stream's riparian area, air-dried, and cut in 8 cm long pieces. Alder leaves were collected in autumn 2018 in a biosphere reserve (at 49°14′24"N, 7°53′24"E) shortly before senescence, subsequently quality sorted, air-dried, and stored in the dark at room temperature.

Colonization period

Natural drift colonization of the experimental system was possible during the whole experiment for aquatic organisms < 4 mm via the water pumps. This passive drift colonization was supplemented by active sampling of the macroinvertebrate community one week prior to the start of the manipulative period (day -7). Eight kick-net samples, each comprising two pool and two riffle microhabitats, were taken along a 50 m river section 250 m upstream of the experimental setup (50°09′41.0"N, 9°18′12.0"E), and specimens obtained from one kick-net sample were randomly distributed across eight mesocosms. After specimens were supplied to each mesocosm once, the procedure was repeated but kick-net samples were taken from a different 50 m river section (50°09′41.0"N, 9°18′14.0"E). During the subsequent acclimatization phase (days -7

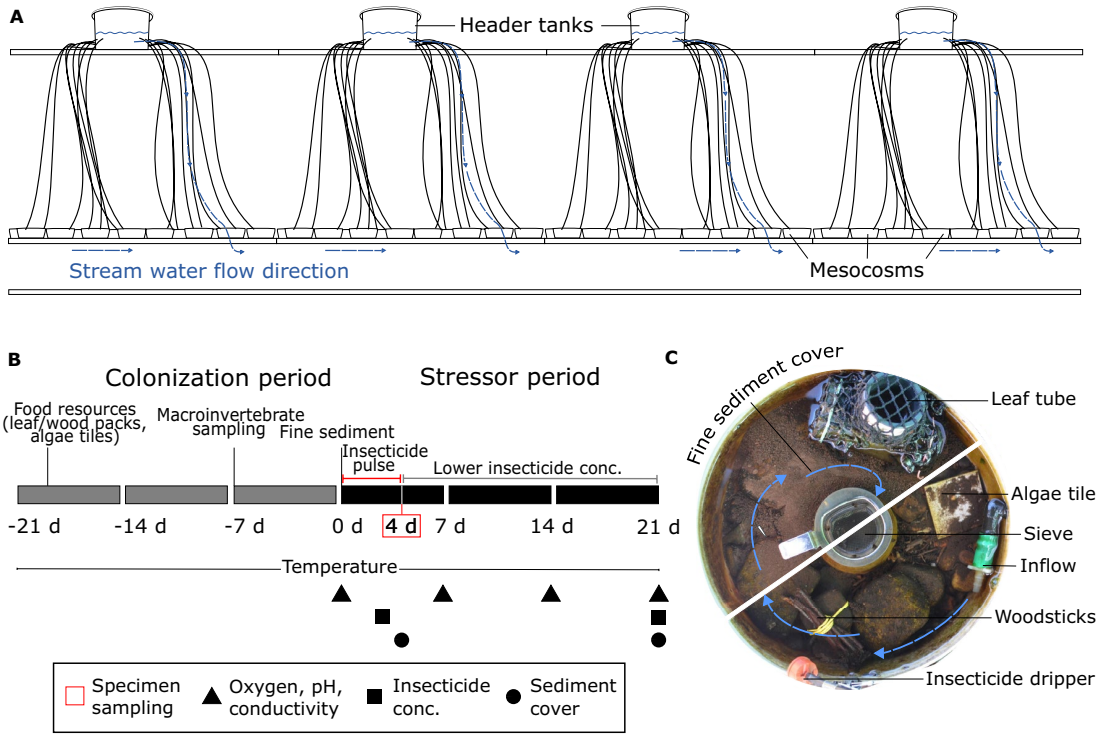

Fig. 1 Overview of the field experiment. **A** Stream water was pumped into header tanks and supplied to 64 mesocosms. The macroinvertebrate communities living in the mesocosms were exposed to the stressors in a 4×2 factorial design (4 pesticide levels, 2 fine sediment levels) with 8 replicates per treatment. **B** Time course of the experiment. A 21-day colonization period was followed by a 21-day stressor period. For this study, specimens were sampled after the insecticide pulse (day 4, red square). **C** Stream water entered the mesocosms via the inflow, flowed in a clockwise orientation, and left the system through the central circular opening. The sieve in the outflow allowed sampling of drifting organisms to estimate stressor effects for the macroinvertebrate community

to 0), all drifting organisms were captured with a sieve (mesh size: 2 mm) placed in the central circular opening (Fig. 1C) and reintroduced in the mesocosms.

Stressor period

During the stress period, the macroinvertebrate community was exposed to three different concentrations of the insecticide chlorantraniliprole and increased fine sediment in a full-factorial design with 8 replicates (i.e., mesocosms) per treatment. Control mesocosms received no stressor treatment. The stressors were applied in a randomized block design, i.e., each header tank supplied stream water to two experimental replicates of each unique treatment combination. Fine sediment (< 2 mm particle size) was collected from the adjacent field and 450 mL were manually added to each mesocosm that was exposed to increased fine sediment levels. This led to approximately 90% fine sediment coverage, which has been shown to be realistic for streams in areas with intensive agriculture [2] and to induce stressor responses of the macroinvertebrate community [36, 37]. The fine sediment level was constant throughout the whole manipulative period. The height of the sediment layer was measured on day 4 and day 21 at three distinct points in each mesocosm (Additional file 2: Table S1).

We used the commercially available product Coragen (batch no.: MAY19CL13A, DuPont, Wilmington, Delaware, United States) to apply chlorantraniliprole in three different concentration levels, i.e., low, medium and high concentration treatments. Notably, other ingredients of the formulation Coragen likely evoke gene regulatory responses as well, which cannot be separated from the effect of chlorantraniliprole in this study. A dosing pump delivered an insecticide stock solution to three dripper lines. Each dripper line then supplied the insecticide directly to the respective mesocosms. The different insecticide levels were targeted by different pumping pulses of the dosing pump, thereby delivering different volumes of the insecticide stock solution to the dripper lines and finally to the mesocosms. During the first 4 days of the stressor period, we aimed for nominal concentrations of 0.2 µg/L, 2 µg/L, and 20 µg/L, but expected to detect lower concentrations in our experiment due to potential

adsorption and photodegradation of chlorantraniliprole (DT_{50} = 0.31 days) [24]. The insecticide stressor levels were selected based on results obtained from ecotoxicological studies reporting lethal concentrations (i.e., LC_{50} after 48 h) for freshwater macroinvertebrates such as the cladoceran *Daphnia pulex* (LC_{50} = 11.6 µg/L), the mayfly *Centroptilum triangulifer* (LC_{50} = 11.6 µg/L), the caddisfly *C. aterrima* (LC_{50} = 11.7 µg/L), and the amphipod *G. pseudolimnaeus* (LC_{50} = 35.1 µg/L) [24, 25]. Sublethal effects were reported at concentrations as low as 0.2 µg/L in the caddisfly *Sericostoma personatum* [38]. Since environmental concentrations of chlorantraniliprole of up to 28 µg/L were reported in the past [39], our nominal concentration gradient represented a field realistic exposure scenario with stressor effects expected to range from weak (0.2 µg/L) to strong (20 µg/L).

When fine sediment and insecticides enter the natural stream during rainfall, aquatic organisms are shortly confronted with an intense stressor exposure ('stressor pulse'), followed by a reduced but persistent stressor occurrence ('base exposure'). To resemble these natural dynamics, the insecticide concentrations were lowered by the factor of 10 after four days for the remaining 17 days. The insecticide concentration in different treatment combinations was measured from water samples taken at day 3 (Additional file 2: Table S2) and day 21. Water samples (500 mL) were collected from the outflow of eight randomly selected mesocosms, each representing one unique treatment combination.

Sampling

In this study, we focused on two species and sampled the specimens at day 4 to identify the stressor-induced transcriptional response after the stressor pulse. Because the experiment was continued for further 17 days, we avoided any sampling-induced disturbance of the mesocosms. Therefore, we were restricted to sample the specimens directly from the water column within the mesocosm or from the large flat stones. During this sampling procedure, we were not able to sample gammarids in one increased fine sediment mesocosm. From each of the remaining mesocosms, three *G. pulex* and five *L. basale* specimens were sampled, irrespective of sex or developmental stage. In total, 189 gammarids (3×63 mesocosms) and 320 caddisflies (5×64 mesocosms) were sampled, directly preserved on dry ice, and stored at -80 °C.

Lab procedures

Nucleic acid extraction

Specimens were individually disrupted with zirconia beads (diameter: 2 and 1 mm, 1:1) in 400 µL (*L. basale*) or 1 mL (*G. pulex*) guanidinium thiocyanate (GITC) lysis buffer [40] with 1% β-mercaptoethanol (Acros organics, Waltham, Massachusetts, United States) using the MiniBeadbeater-96 (BioSpec Products, Bartlesville, Oklahoma, United States) for 5 min at 2,400 rpm. After tissue disruption, the samples were centrifuged for 3 min at 16,000×g. Then, magnetic beads (Cytiva Lifesciences, Marlborough, Massachusetts, United States) in Tris–EDTA-min (TE-min) buffer (10 mM Tris, 1 mM EDTA; both Diagonal, Münster, Germany) were added to the lysate and nucleic acids were precipitated with molecular grade isopropanol (1:1, v/v, aqueous solutions, isopropanol; Thermo Fisher Scientific, Waltham, Massachusetts, United States). After incubation for 10 min, the samples were washed three times with 80% ethanol (Thermo Fisher Scientific, Waltham, Massachusetts, United States), finally solubilized in TE-min buffer and stored at − 80 °C until further use.

DNA barcoding

The extracted nucleic acids were used as template for the amplification of the cytochrome c oxidase subunit one barcoding fragment using HCO2198-JJ/LCO1490-JJ (Eurofins Genomics, Konstanz, Germany) primers [41]. Per sample, the reaction contained 5 µL DreamTaq (Thermo Fisher Scientific, Waltham, Massachusetts, United States) master mix, 0.03 µL of each HCO2198-JJ and LCO1490-JJ (100 µM), 2.9 µl molecular grade water (Thermo Fisher Scientific, Waltham, Massachusetts, United States), and 1 µL nucleic acid extract. The initial denaturation of DNA was conducted for 3 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 46 °C for 30 s, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 5 min. Amplicons were purified with 1 unit ExoI and 10 units FastAP (both Thermo Fisher Scientific, Waltham, Massachusetts, United States). Unidirectional sequencing was performed at Eurofins Genomics (Konstanz, Germany) and barcode sequences were queried against the Barcode of Life Data System database [42]. Through this, all trichopterans were verified to be *L. basale* and all amphipods *G. pulex*.

RNA purification

Nucleic acid extracts were digested with 2 units DNase (Ambion, Waltham, Massachusetts, United States) in order to remove genomic DNA. Then, the samples were mixed with RNA binding buffer [40] and magnetic beads in TE-min buffer (4:1, v/v, alcoholic RNA binding buffer, aqueous solutions, i.e., digested extract and beads in TE-min). The RNA was washed twice with 80% ethanol and finally resolubilized in TE-min buffer. The concentration and quality of the cleaned RNA samples were assessed on a Fragment Analyzer system using the 15 nt RNA kit (both Agilent, Santa Clara, California, United States).

Based on sample quality, RNA extracts of each mesocosm were pooled per species. Accordingly, a sequencing library represented one mesocosm, comprising the pooled RNA extracts of either *G. pulex* or *L. basale* specimens. RNA extracts obtained from *G. pulex* specimens that showed a low quality or quantity were excluded from downstream processing. Therefore, 35 *G. pulex* libraries contained pooled RNA extracts from all three specimens, 26 libraries included RNA from two specimens, and 2 libraries contained RNA of only one specimen. All *L. basale* libraries consisted of 5 specimens. Protocols to prepare the GITC lysis buffer and the RNA binding buffer can be found under https://bomb.bio/protocols/ (protocol 8.2).

Library preparation and sequencing

All 127 RNA libraries were sent to the West German Genome Center (WGGC) for further library preparation and sequencing. mRNA enrichment was conducted with 800 ng total RNA as input. The poly-A selection and cDNA library construction were performed using the NEBNext Ultra II Directional RNA kit (New England Biolabs, Ipswich, Massachusetts, United States). Libraries were paired-end sequenced (150 bp) on a NovaSeq 6000 (Illumina, San Diego, California, United States).

Chlorantraniliprole analysis

To qualify chlorantraniliprole concentrations, water samples were pre-concentrated via solid-phase extraction using Oasis HLB 6 cc 500 mg extraction cartridges (Waters Corp., Milford, Massachusetts, United States). After conditioning the cartridges with 5 mL of methanol (Carl Roth, Karlsruhe, Germany) and equilibrating with 10 mL ultrapure water, an aliquot of ~ 500 mL (exact volume was recorded) was loaded to the cartridge at a flow rate of ~ 7 mL/min. Subsequently, the cartridges were dried with nitrogen (Carl Roth, Karlsruhe, Germany) for 2 h. The samples were eluded with 6 mL methanol:ethyl acetate (1:1, v/v, methanol LC grade, ethyl acetate; Carl Roth, Karlsruhe, Germany) and 2 mL methanol. The eluates were then dried to 50 µL at room temperature under a gentle nitrogen flow and reconstituted with 450 µL ultrapure water. The resulting extracts were centrifuged (4000 rpm, 30 min) and the supernatants were used for chemical analysis. To correct for potential evaporation and extraction losses and matrix effects, selected standards in ultrapure water as well as in stream water from the Bieber were treated identically. The samples were quantified using an Exactive (LCHRMS) Orbitrap system (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The chromatographic separation was achieved with an Atlantis T3 5 µm 3.0 × 150 mm column (Waters Corp., Milford, Massachusetts, United States). A calibration row of chlorantraniliprole (PESTANAL analytical standards, Merck, Darmstadt, Hesse, Germany) was linear from 3 µg/L (analytical limit of quantification (LOQ) in water calculated as 0.007 µg/L) to 500 µg/L. Details on gradient settings were given in [43].

Data analysis

Raw sequencing libraries were homopolymer trimmed using a custom C + + [44] program followed by quality and adapter trimming with the Cutadapt v3.2 [45] wrapper script TrimGalore! v0.6.6 (https://github.com/FelixKrueger/TrimGalore) in paired-end mode, retaining only bases with Phred > 20 and reads with a length ≥ 25. The quality trimmed read data were used for de novo transcriptome assembly with Trinity v2.13 [46] and rnaSPADES v3.15.0 [47] for *L. basale* and *G. pulex*, respectively. Assemblers were run in default modes, except for parameters which control memory usage and multi-threading. Details about the assembly evaluation are provided in Additional file 1. Quality trimmed reads were mapped against the transcriptomes using bowtie2 [48] and transcript abundances were estimated with RSEM v1.3.3 [49]. The number of reads obtained after sequencing and quality trimming as well as results of the transcriptome assembly evaluation are presented in Additional file 2: Table S3.

The functional annotation of the generated transcriptomes was performed on protein level. Protein coding sequences were identified with TransDecoder v5.5.0 (https://github.com/TransDecoder/). HMMER v3.3 (http://hmmer.org/) was used to search for protein domains in the putative protein sequences based on the Pfam database [50]. Blastp v2.9.0 [51] searches for the putative protein sequences were conducted (e-value < 1e-5) against the Swissprot/Uniprot database [52] for protein prediction. Gene ontology (GO) terms [53, 54] were retrieved from the eggNOG v5.0.2 database [55] with the eggNOG-mapper v2.1.9 [56]. DIAMOND v2.0.15.153 [57] searches were performed to query the final protein sequences against the database, using 'Arthropoda' as target taxon. Hits were required to have an e-value ≤ 1e−5, sequence identity ≥ 50% and a bitscore ≥ 50 to be included in the final annotation. For *G. pulex*, the annotation data were scarce, i.e., less than 20% of all genes tested for differential expression were annotated with GO terms. Scarce annotation data are a general problem for functional profiling of non-model organisms and crustaceans have been found to be poorly represented in databases [58, 59]. Therefore, we iteratively supplemented the annotation of the *G. pulex* transcriptome with annotations retrieved from the protein sets of (i) the amphipod *Hyalella azteca* (GenBank

accession no. GCA_000764305.4), (ii) the cladoceran *D. pulex* (https://wfleabase.org), and (iii) the invertebrate Uniprot/Swissprot TrEMBL database. Blastp hits were considered valid if they showed an e-value ≤ 1e−5, sequence identity ≥ 50% and a bitscore ≥ 50 and were only included if no annotations were obtained from the previous annotation round.

Differential expression analyses and clustering of insecticide-responsive L. basale genes

Transcript abundances were summarized to gene level estimates using tximport v1.22.0 [60]. Isoform-to-gene relationships were inferred based on assembler assumptions; therefore, 'genes' are approximated, and the term is used loosely here. Only genes with ≥ 20 normalized counts in at least eight samples were included. Gene counts were modeled in DESeq2 v1.34.0 [61] specifying the model design ~ Sediment*Insecticide. Variance stabilized counts were extracted, and a surrogate variable analysis (SVA) was performed with the sva R package v3.42.0 [62] (full model: ~ Sediment*Insecticide; reduced model: ~ 1). Including surrogates in the model allows to account for data variation that is related to unmeasured sources (e.g., expression differences between sexes or developmental stages). All significant surrogates were incorporated as covariates in an updated DESeq2 model. The Wald test was used to identify differentially expressed genes with a | log2FC | > 0 (FDR adjusted p-values < 0.05). Effect sizes were shrunk with the adaptive shrinkage estimator from the R package ashr v2.2.54 [63].

The differential expression analyses revealed, by far, the strongest treatment effect in the expression data set from *L. basale* exposed to high insecticide concentrations. This is partly related to the low insecticide concentrations achieved in our experiment (Additional file 2: Table S2) but might be also an artifact of the low signal-to-noise ratio inherent to RNA-sequencing data obtained from wild organisms in a semi-natural setting. To explore the expression data in an unsupervised manner, we clustered insecticide-responsive genes in *L. basale* according to their expression profiles along an increasing insecticide concentration gradient. This co-expression analysis can inform about subtle changes in expression (i.e., small effect sizes) beyond statistical testing. Clustering was performed separately for genes which were differentially expressed in (i) the single stressor high insecticide concentration treatment and in (ii) the treatment combining high insecticide concentration and increased fine sediment using DEGreport v1.30.3 [64].

Stressor interactions

To identify genes showing a stressor interaction, DESeq2 was used to test the interaction terms for being non-zero (FDR adjusted *p*-values < 0.05, obtained from the Wald test). The reported effect sizes of these contrasts represent an additional log2FC which is specifically attributed to the interaction between stressors. These genes were then classified in genes showing a synergistic and antagonistic stressor interaction when the combined stressor effect was larger and smaller than expected based on the individual stressor effects, respectively. The expectation was derived from the null model of additive stressor effects, i.e., the sum of the individual log2FC. In the gene regulatory context, a positive synergistic interaction (S+) is defined as a stronger upregulation than expected based on the assumption of additivity, whereas a negative synergistic (S−) interaction indicates a stronger downregulation than expected. Vice versa, positive antagonistic (A+) interactions denote 'less upregulated than expected,' whereas a negative antagonistic (A−) interaction refers to genes, which are 'less downregulated than expected.' For instance, a significant interaction was identified for a given gene, indicating that this gene changed its expression due to the interaction of two stressors. If this gene shows a log2FC = 1 in both individual stressor treatments, the combination of stressors is expected to evoke a log2FC = 2 under the scenario of additivity. If, however, the combined stressor treatment induced a log2FC > 2, a 'stronger upregulation than expected' was observed, and a positive synergistic interaction is reported. If a log2FC < 2 was observed, the gene was 'less upregulated than expected,' and a positive antagonistic interaction is reported.

Functional enrichment

The functional enrichment analysis aims to identify gene ontology (GO) terms, which are more frequently observed in a specific set of genes than expected by chance. We used the elim algorithm implemented in the R package topGO v2.50.0 [65] and Fisher's exact test (*p*-value < 0.05) to identify overrepresented biological process terms.

The functional enrichment was performed for each set of differentially expressed genes from the different stressor treatments. All genes that were initially tested for differential expression were used as the gene universe. Upregulated and downregulated genes were tested separately.

In order to identify functional submodules within the insecticide-responsive genes of *L. basale*, we further performed a functional enrichment for the identified clusters

from the high insecticide concentration treatments. The subset of genes belonging to each cluster was tested for overrepresentation of biological process terms against the set of genes from which the cluster was derived, i.e., either genes differentially expressed due to (i) the high insecticide concentration treatment alone or (ii) the combination of high insecticide concentration and increased fine sediment.

Visualization

Variance stabilized gene counts were corrected with a frozen SVA [62] and a principle component analysis (PCA) was performed with PCAtools v2.6.0 [66]. Heatmaps were created with ComplexHeatmap v2.14.0 [67] and treatments were clustered based on Euclidean (*G. pulex*) and Canberra (*L. basale*) distances. Clusters of co-expressed genes and functional enrichment results were visualized with ggplot2 v3.4.1 [68]. To determine how cluster identity changes due to the presence of increased fine sediment, the intersection of genes from (**i**) and (ii as visualized in a chord diagram with the circlize library v0.4.15 [69]. All statistical analyses and data visualizations were performed in R v4.1.2 [70].

Results

Chlorantraniliprole concentration

Measured chlorantraniliprole concentrations in the water samples differed strongly from the nominal concentrations: low insecticide concentrations ranged from < 0.007 µg/L to 0.14 µg/L (nominal concentration: 0.02 µg/L), medium insecticide concentrations from 0.009 µg/L to 0.65 µg/L (nominal concentration: 2 µg/L), and high insecticide concentrations from 0.13 µg/L to 2.73 µg/L (nominal concentration: 20 µg/L) (Additional file 2: Table S2). In a complementary study [71], we performed a similar experiment (i.e., exposing *L. basale* and *G. pulex* specimens from the same populations to different concentrations of chlorantraniliprole using the formulation Coragen) in an indoor setting under highly controlled conditions and observed gene expression patterns consistent to the results reported in this study. Given the match between nominal and measured concentrations under highly controlled conditions, we attribute the here observed discrepancies between nominal and measured insecticide concentrations mainly to our application approach using a dosing pump that applied micro-pulses of the insecticide stock solution to the distributing tubes. Compared to the constant concentrations under controlled conditions, this micro-pulse approach led to highly variable short-term concentrations, a limitation inherent to the semi-natural experimental setting. Accordingly, the insecticide stressor levels cannot be treated quantitatively and constitute only relative exposure levels. However, the signal in the expression data suggests that we achieved different exposure levels that were relatively consistent in their effects within the treatments. Therefore, we retain a qualitative ranking of the insecticide levels, i.e., low, medium, and high exposure concentrations. Additional explanation is given in Additional file 1.

Treatment-induced gene expression patterns in L. basale and G. pulex

We observed a strong physiological response in the caddisfly *L. basale* obtained from mesocosms in which high chlorantraniliprole concentrations were applied. The PCA revealed a separation of these from the remaining treatments along the first PCA axis, which accounted for 13% variance (Fig. 2A). This differentiation was most pronounced when the insecticide was applied as single stressor and slightly declined when increased fine sediment was added as second stressor. Accordingly, we detected the strongest differential expression response in *L. basale* exposed to high insecticide concentration alone (5,237 genes), followed by the combined exposure of high insecticide concentration and increased fine sediment (1,080 genes) (Fig. 3A). Consistent to these results, only antagonistic stressor interactions (A + /A−) between increased fine sediment and insecticide exposure were identified in *L. basale* (Table 1, Additional file 2: Table S5).

The transcriptional response of *G. pulex* to the applied stressor combinations was less pronounced compared to the caddisfly: the PCA revealed no clear pattern for *G. pulex*, and the first axis explained only 4% data variance (Fig. 2B). Relatively few genes were differentially expressed, and the detected expression patterns were rather inconsistent. The highest number of differentially expressed genes were found when increased fine sediment was applied as single stressor, inducing a differential expression of 125 genes, of which the majority (73 genes) were downregulated (Fig. 3B). Among these downregulated genes, we identified the mitochondrial ND4 and COX6A2 gene. In *G. pulex*, 13 genes were stronger downregulated than anticipated based on the single stressor effects (Table 1, Additional file 2: Table S4). Among these genes showing a synergistic interaction, one encoded the NADH dehydrogenase subunit 4 protein.

Functional enrichment of L. basale genes responding to high insecticide concentration treatments

We could only infer robust functional enrichment results in the set of differentially expressed genes obtained from

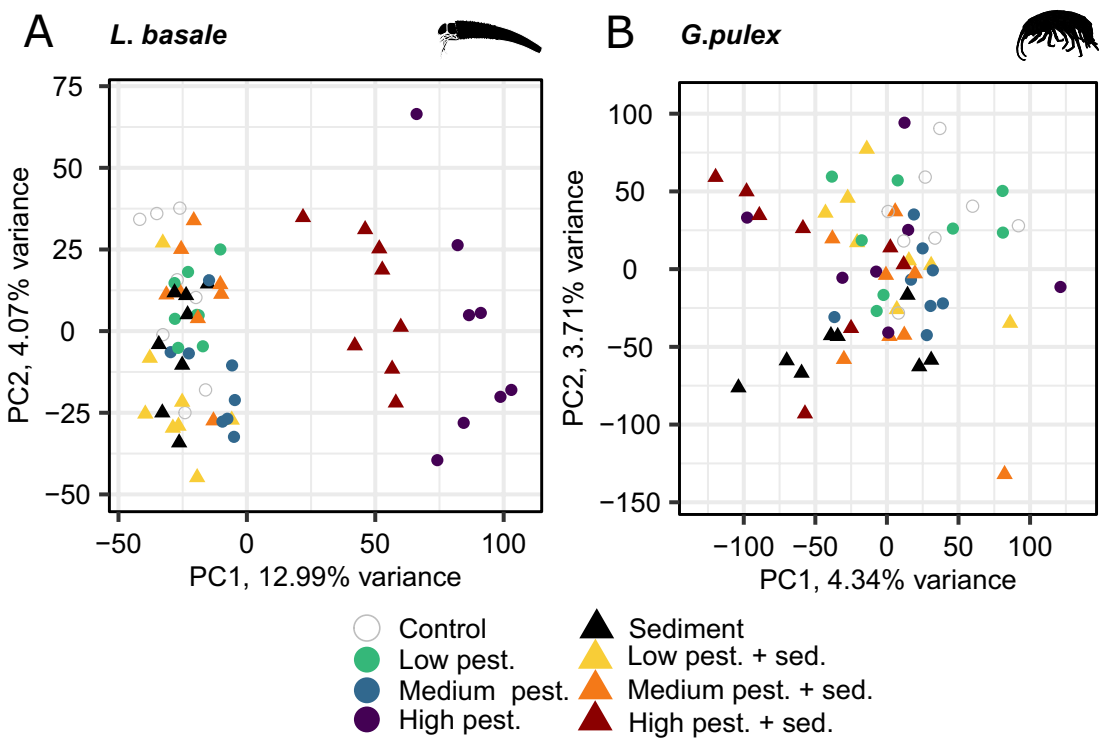

Fig. 2 Biplot of the first two principal component axes for **A** *L. basale* and **B** *G. pulex*

L. basale exposed to high insecticide concentrations. Due to the limited effect of the remaining treatment combinations, only a few overrepresented terms were detected in the corresponding sets of differentially expressed genes, and these terms were often represented by a single gene (Additional file 2: Table S7). Such enrichment results are unlikely to allow a biologically meaningful interpretation. In the *G. pulex* enrichment results (Additional file 2: Table S6), this problem is aggravated by scarce annotation data. Therefore, we focused on the functional profile of genes that were regulated in *L. basale* due to high insecticide concentration exposure, applied as single stressor treatment and in combination with increased fine sediment, as well as the functional profiles of the identified clusters.

Genes which were suppressed in the high insecticide concentration treatment applied as single stressor were significantly associated with the cellular machinery of protein biosynthesis (Additional file 2: Table S7): overrepresented biological process terms described the accession, replication and repair of DNA (e.g., 'chromosome organization,' 'DNA biosynthetic process,' 'cellular response to DNA damage stimulus'), transcription (e.g., 'mRNA polyadenylation,' 'alternative mRNA splicing, via spliceosome'), and RNA metabolism (e.g., 'RNA processing,' 'RNA phosphodiester bond hydrolysis') as well as translation (e.g., 'ribosome assembly,' 'translational elongation') and post-translational protein modification (e.g., 'protein deneddylation,' 'N-terminal protein amino acid acetylation'). In addition, mitochondrial gene expression (e.g., 'mitochondrial translation') and processes (e.g., 'mitochondrial electron transport, ubiquinol to cytochrome c,' 'mitochondrial respiratory chain complex assembly') appeared to play a dominant role in the set of downregulated genes. Further, we found evidence for the suppression of the cell cycle (e.g., 'chromosome segregation,' 'cell cycle G2/M phase transition'). Clustering of the genes that were differentially expressed in this treatment resulted in six significant groupings (Fig. 4A–F), of which three clusters represented downregulated genes (clusters 1, 4, and 5). The most prominently enriched terms, i.e., terms linked with protein biosynthesis and cell cycle, were consistently detected in all of these three clusters (Additional file 2: Table S8, Additional file 1: Fig. S2), indicating that the co-expression networks have similar functional profiles. In addition, we detected some terms referring to neuromuscular processes in cluster 4 (Fig. 4D) and cluster 5 (Fig. 4E), but these were annotated to only a few genes and referred in several cases to neuromuscular development (e.g., 'neuromuscular junction development, skeletal muscle fiber'; Additional file 2:

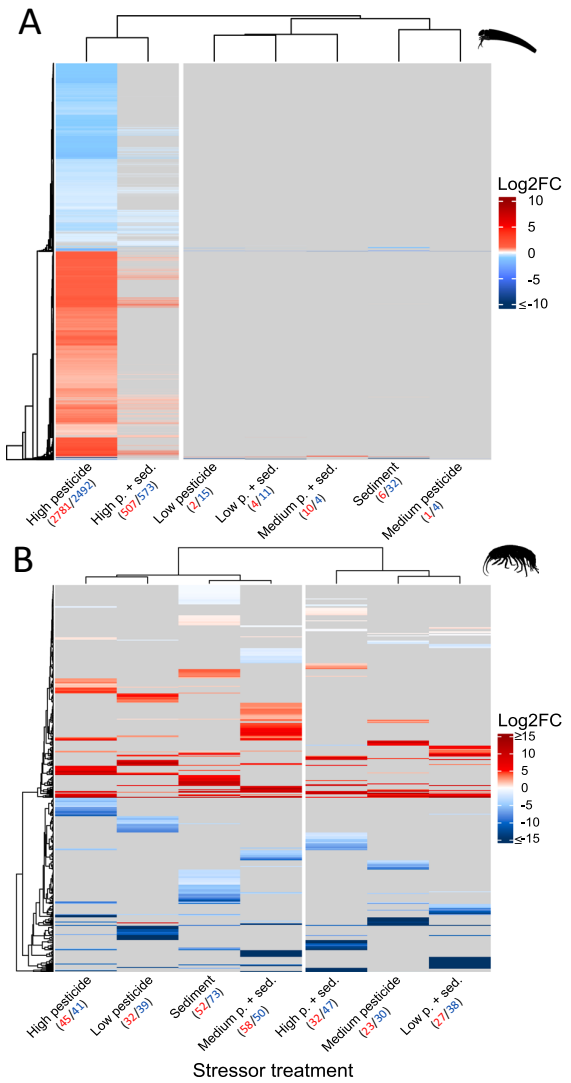

Fig. 3 Heatmap of differentially expressed genes in *L. basale* (**A**) and *G. pulex* (**B**). Gray bars represent genes with no significant differences in expression between the treatment and control conditions (adjusted p-value ≥ 0.05). Red and blue numbers represent up- and downregulated genes, respectively. Fold changes are reported at the binary logarithmic scale (Log2FC)

Table S8, Additional file 1: Fig. S2). The addition of fine sediment did not significantly alter the functional profile of downregulated genes (Additional file 2: Tables S7, S9, Additional file 1: Fig. S3), and most of the genes remained co-expressed (Fig. 4K). However, the presence of fine sediment slightly modified the average expression trend of the largest cluster (single stressor treatment cluster 1, Fig. 4A) from a linear expression decrease toward an expression pattern characterized by higher gene expression under medium concentration than under low insecticide concentration (combined stressor treatment cluster 1, Fig. 4G).

Genes which were stimulated in response to high insecticide concentration could be broadly categorized into two functional groups. These functional categories were observed in both treatment combinations and all derived clusters (Additional file 2: Tables S7–S9, Additional file 1: Figs. S2, S3): the first group comprised genes involved in molecular response pathways to adverse, exogenous stimuli: specifically, we detected a strong overrepresentation of not only terms describing the insect immune system (e.g., 'Toll signaling pathway,' 'regulation of innate immune response,' 'humoral immune response') but also many general terms associated with physiological responses to xenobiotics (e.g., 'response to antibiotic,' 'response to DDT') or stress conditions (e.g., 'response to starvation,' 'response to oxidative stress') (Additional file 2: Table S7). The second dominant functional enrichment category was related to the insect larval development (e.g., 'instar larval or pupal development,' 'determination of adult lifespan,' 'larval salivary gland morphogenesis'), including neuromuscular development (e.g., 'larval somatic muscle development,' 'nephrocyte differentiation'), and endocrine metabolism (e.g., 'cellular hormone metabolic process,' 'juvenile hormone biosynthetic process'). In the combined stressor treatment, we observed a similar overrepresentation of terms related to the perception of (adverse) abiotic and biotic stimuli with special focus on the insect immune system, as well as terms referring to the developmental cycle of insects (Additional file 2: Table S7). This suggests again that the addition of fine sediment induced no pronounced shift in the functional perspective of upregulated genes. Further, fine sediment addition induced no change in the expression profiles of most upregulated genes: the largest cluster derived from the single stressor treatment (cluster 2, Fig. 4B) comprised genes which similarly aggregated together in cluster 4 of the combined stressor treatment (Fig. 4K). These genes exhibited a linear increase in expression with increasing insecticide concentration (Fig. 4B, J).

Discussion

Stressor effects of fine sediment deposition and insecticide exposure

Although increased fine sediment applied as a single stressor induced the largest number of differentially expressed genes in *G. pulex*, the overall effect in terms of the number of differentially expressed genes due to this treatment was limited in both species. This might be surprising, considering the literature evidence that fine sediment load is often among the most pressing stressors for freshwater organisms [17–19, 36], but could be explained

Table 1 Number of genes that showed antagonistic (A+/A−) or synergistic (S+/S−) stressor interactions

Interaction type	Low insecticide*sediment		Medium insecticide*sediment		High insecticide*sediment	
	L. basale	*G. pulex*	*L. basale*	*G. pulex*	*L. basale*	*G. pulex*
A-	12	53	13	37	80	22
A+	8	16	3	22	275	34
S-	–	6	-	5	–	2
S+	–	–	–	–	–	–
Total	20	75	16	64	355	58

Positive synergistic (S+) and positive antagonistic (A+) interactions were reported when the upregulation of a gene was stronger and weaker, respectively, than assumed based on additive stressor effects. Negative synergistic (S−) and negative antagonistic (A−) interactions were reported when the downregulation of a gene was stronger and weaker, respectively, than expected based on additivity

by the different mechanistic targets of chemical and non-chemical stressors. While chemical stressors interact with receptors, thereby inducing physiological response cascades, increased fine sediment levels do not directly interfere with cells at the molecular level but rather alter physico-chemical conditions (e.g., light, oxygen) of microhabitats [10, 11]. Therefore, immediate effects on the transcriptome may be more difficult to detect. Similar observations were made in a previous ExStream experiment in which fine sediment levels, flow velocity, and salinity were manipulated for 22 days and macroinvertebrate community responses as well as the transcriptional stress response of the amphipod *G. fossarum* were assessed [20, 36]. During this experiment, fine sediment deposition was identified as the most pervasive stressor for the macroinvertebrate community [36], but the strongest transcriptional effects were observed in response to the chemical stressor treatment, i.e., increased ion concentrations [20]. These results further suggest that the limited effect of fine sediment observed in this study is not a result of insufficient stressor exposure time. Since the quantity of added fine sediment is comparable to previous ExStream experiments [36, 37], we further argue that the amount of added fine sediment was sufficient to act as stressor for stream macroinvertebrates. However, it is possible that the fine sediment stressor did not alter habitat characteristics in the mesocosms that are specifically relevant for the two study species *L. basale* and *G. pulex*. Because an ecological dimension shaping stressor effects is inherently part of a semi-natural setup such as the ExStream system, stressor effects must be discussed in the ecological context of both species: fine sediment load directly increases water turbidity, thereby potentially affecting macroinvertebrate taxa that directly rely on primary production or visual predators [8, 9]. Further, the subsequent deposition leads to a homogenization of the channel substratum [10, 11]. For the leaf-shredding organisms *L. basale* and *G. pulex*, the food availability is unlikely affected since leaf material can be found in sufficient quantities in the leaf packs within the mesocosms, as has also been reported by [72]. Another consequence of particle load in the water is impeded respiration when gills are covered [73]. While this may affect *G. pulex*, in which we detected a slightly stronger fine sediment effect, caddisflies such as *L. basale* largely rely on cutaneous respiration [74] and might therefore be less impacted by water turbidity. A similar observation was made in a recent multiple stressor study, in which fine sediment addition induced functional shifts of the macroinvertebrate community by favoring organismic groups which rely on integumentary rather than branchial respiration [72].

In line with our expectations, we detected strong transcriptional changes in the caddisfly *L. basale* when the insecticide stressor was applied, as opposed to *G. pulex* in which we could not infer a clear physiological response associated with chlorantraniliprole exposure. We can only speculate to which degree the observed differential species vulnerability is the result of differences in toxicant uptake between the species, driven by biological and ecological traits, and varying intrinsic sensitivity. However, we argue that the overall high toxicological sensitivity of *L. basale* is inherently driven by predispositions present at the molecular and the cellular level, which is in line with ecotoxicological studies reporting higher toxicity of chlorantraniliprole for aquatic insects than for amphipods [25] and our indoor experiment [71]: first, the stressor receptors at the molecular level likely show a higher affinity toward the insecticide in caddisflies than in amphipods. This is, for instance, illustrated by the divergence of the ryanodine receptor amino acid sequences between insects and crustaceans (Additional file 1: Fig. S1). Second, water-borne insecticide exposure at the cellular level might be stronger in caddisfly larvae than in amphipods due to the varying degree of cuticle sclerotization. These differential toxicodynamics might be further influenced by differences in ecological requirements, which may have enhanced insecticide exposure

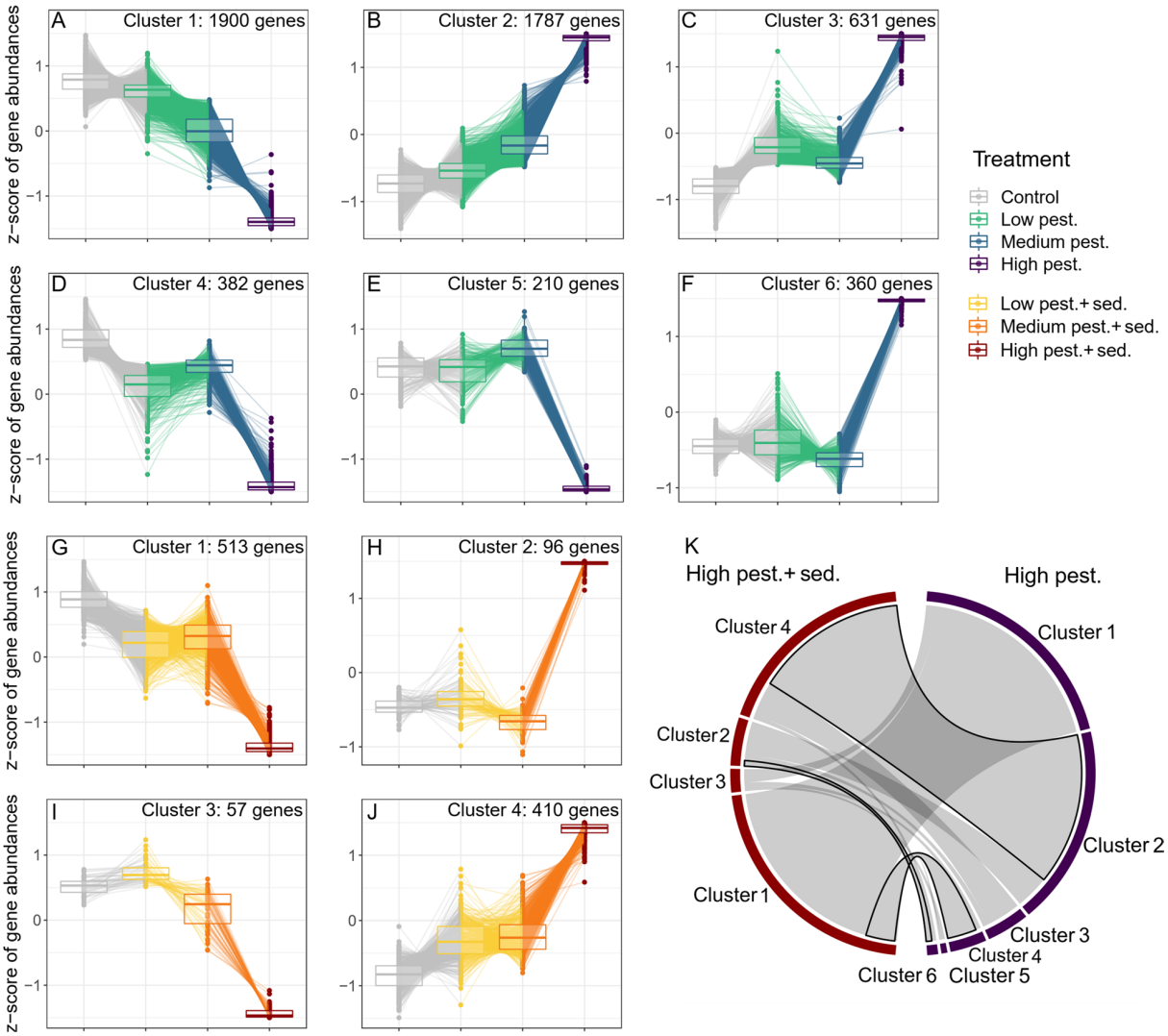

Fig. 4 Clustering of insecticide-responsive genes according to their expression profile along an increasing insecticide concentration gradient. **A**–**F** Differentially expressed genes in *L. basale* under high insecticide concentration. **G**–**J** Differentially expressed genes in *L. basale* under high insecticide concentration combined with increased fine sediment. Z-scores represent the scaled and centered gene expression value. **K** Cluster identities of differentially expressed genes in the single (purple arc, right) and the two-stressor treatment (red arc, left). Black lines indicate groups of genes for which the average expression pattern is similar between the two stressor scenarios. The purple and red arcs only contain the genes that are differentially expressed in both treatments

in the mesocosm for *L. basale* compared to *G. pulex*: the strong association of *L. basale* to wood [30] narrows its ecological niche, thereby potentially reducing its ability to find shelter from the insecticide stressor in less affected microhabitats within the mesocosm. As a more flexible and relatively mobile species, *G. pulex* could have effectively avoided the insecticide stressor by withdrawal to microhabitats where contaminant exposure is minimized. Apparently, fine sediment deposition had no significantly adverse impact on this avoidance behavior.

We conclude that fine sediment addition did not induce strong transcriptional stress responses in *G. pulex* and *L. basale* in our experiment. Therefore, we reject our first hypothesis that fine sediment addition induces a metabolic depression in the two invertebrate species. In contrast, applying the insecticide stressor in its highest concentration resulted in a highly pronounced effect on the transcriptome of *L. basale*, whereas no considerable transcriptional changes were detected in *G. pulex*. In favor of our second hypothesis, we attribute

the species-specific insecticide sensitivity to the degree of molecular pathway conservation between butterflies, caddisflies, and amphipods and conclude that the magnitude of stressor effects depends on molecular stress receptor mechanisms. These comprise different mechanistic targets of stressors at the molecular level as well as the cellular affinity toward stressors, which differs between evolutionary lineages. In addition, we acknowledge that ecological determinants such as varying stressor exposure or the ability to avoid stressors are likely to shape stressor effects under natural conditions [75], which we aimed to simulate in our mesocosm experiment.

Consistent stressor effects, antagonism and synergism

Identifying stressor interactions is crucial to improve our understanding of the ecological impact of co-occurring stressors in natural systems. At the same time, an identification of consistent stressor effects is similarly important [76], as they provide the complementary perspective on multiple stressor dynamics. Despite the strong discrepancies between the stressor effects on *L. basale* and *G. pulex*, we can report the following consistent effects at the inter- and intraspecific level: (i) a limited effect of fine sediment on the transcriptomic response, (ii) a downregulation of mitochondrial genes in response to environmental stressors such as fine sediment deposition (*G. pulex*) and insecticide pollution (*L. basale*), and (iii) edominant antagonistic nature of stressor interactions between increased fine sediment and insecticide exposure. Within *L. basale* (iv), the suppression of mitochondrial processes as well as the upregulation of developmental and immunity-related genes evoked by insecticide exposure persisted, independent of the expression profile of genes or the mitigating effect by increased fine sediment addition. The associated genes were consistently co-expressed in both treatment scenarios, indicating that these genes are part of key gene regulatory networks, which contribute to the protective pathways that allow *L. basale* to cope with sublethal insecticide effects.

We detected only antagonistic gene expression changes in *L. basale,* and fine sediment addition substantially reduced the number of insecticide-responsive genes. These observations suggest that the bioavailability of chlorantraniliprole is reduced by fine sediment addition. While other studies reported food-related uptake as the most relevant source of pesticide exposure for freshwater organisms [77], our findings imply that one of the main exposure sources is dissolved insecticide in the aqueous phase and adsorption to fine sediment particles decreases the insecticide concentration in the water. While this pattern might be specific for a shredder organism like *L. basale*, other types of stressor interactions could arise for organisms that show a strong ecological association to sediment or soil.

Insecticide-induced molecular response mechanisms in *L. basale* – a comparison between indoor and outdoor experiments

The insecticide mode of action is clearly described: binding of chlorantraniliprole to the insect ryanodine receptor releases calcium from its intracellular storages, resulting in uncontrolled muscle contraction, paralysis, and eventually the death of the target pest organism [22]. In *L. basale*, several biological process terms referring to calcium homeostasis/signaling and neuromuscular processes were detected as functionally enriched in the high insecticide concentration treatments. However, these terms often refer to (embryonic or larval) neuromuscular development (single stressor treatment: clusters 3–6; two-stressor treatment: clusters 2, 3) (Additional file 1: Figs. S2, S3, Additional file 2: Tables S8–S9) than to processes directly associated to the calcium homeostasis or involved in muscle contraction and were in many instances annotated to only a few genes. Therefore, we conclude that these genes play a subordinate role in the molecular response mechanisms of *L. basale* exposed to the insecticide stressor and reject our third hypothesis.

Instead, our data suggest that the caddisfly reacts to the highest insecticide concentration treatments with (i) a su ession of the protein biosynthesis, particularly of genes involved in mitochondrial processes, (ii) an increase in expression of genes involved in immunity, and (iii) a ulation of the genetic developmental program. Since these results are highly consistent with the expression results obtained from [71], in which we exposed *L. basale* to an insecticide concentration gradient of 0.3—19.5 µg/L (Additional file 1), we argue that the observed effects are not a result of the relatively low insecticide concentrations achieved during this experiment. However, in the indoor experiment, the stimulation of immunity genes was less pronounced than in this study, and we detected additionally an overrepresentation of muscular terms under higher insecticide concentrations [71]. Taken together, we propose the following molecular response mechanism: lower insecticide concentrations evoke rather general physiological stress responses, which comprise a metabolic depression (reflected in suppression of the protein biosynthesis, the cell cycle, and reduced mitochondrial activity) or the stimulation of cellular pathways associated with the perception of external stimuli and general stress responses, including the immune system. The expression of innate insect immune genes such as antimicrobial peptides can be induced by non-immune stressors [78] and a similar stimulation

of immune gene expression in response to chlorantraniliprole exposure was observed in other non-target insects such as the honeybee *Apis mellifera* [79] and the fruit fly *Drosophila melanogaster* [80]. Other arthropods such as the amphipod *H. azteca* showed a similar differential expression of immune genes following insecticide exposure [81], indicating a signaling overlap between immunity and molecular stress perception which is conserved across evolutionary lineages. For instance, reactive oxygen species (ROS) which are excessively generated during mitochondrial stress [82] are linked to the activation of innate immune pathways in vertebrates [83] and invertebrates [78, 84]. Cellular oxidative stress could further contribute to the activation of unspecific stress responses such as cellular detoxification pathways, which were induced following chlorantraniliprole exposure in freshwater macroinvertebrates such as the non-biting midge *Chironomus riparius* [85] or the caddisfly *S. vittatum* [38]. Since cellular detoxification mechanisms and (undirected) immune responses are expected to come at the expense of increased energetic maintenance costs, even low insecticide exposure concentrations could affect growth rates or emergence patterns, as has been found for, e.g., *C. riparius* exposed to chlorantraniliprole [85].

Under higher insecticide concentrations, as the ones achieved in the indoor system, we observed in addition to the suppression of mitochondrial processes an inhibition of muscular processes [71]. Considering that muscle tissue is rich in mitochondria, these two mechanisms are not exclusive and could be explained by a disruption of mitochondrial processes specifically in muscle cells. Further, alterations of the developmental program became more prominent under stronger insecticide stress, whereas the induction of immune genes played a subordinate role. Changes in the developmental program could be the result of endocrine disruption, an off-target effect known for many insecticides [86], or represent a physiological coping strategy to mitigate toxic effects [87, 88]. Due to the importance of calcium as a signaling molecule, an alternative explanation might be an insecticide-induced alteration of intracellular calcium levels (for further discussion, see [71]). However, the disruption of the calcium homeostasis is clearly not reflected in our data and further research is required.

Conclusions

Our results show that multiple stressor effects are shaped by various factors such as molecular mechanisms of stress perception. Ecological requirements, which lead to different stressor exposure patterns, could further contribute to variation of stressor effects in different organismic groups. Although the molecular response pathways clearly diverge between amphipods and caddisflies, we observed a predominantly antagonistic stressor interaction in both species. Furthermore, we found evidence that the downregulation of mitochondrial gene expression represents a conserved mechanism to cope with environmental stress. Suppression of the respiratory chain will decrease the energy budget available to an organism, resulting in negative long-term effects at the population level. Consequently, biotic communities in freshwater systems will be affected differently by stressor exposure, depending on, e.g., community composition or molecular targets of chemical stressors. The latter are not always known since insecticides are complex chemical mixtures and effects are not only induced by the active compound, adding further layers of complexity to multiple stressor dynamics in natural systems. Our findings underpin the need of gene expression analyses to supplement multiple stressor research with endpoints informing about stressor-induced physiological mechanisms and have important implications for results obtained from ecotoxicological assays, which typically test pure substances and are limited in time and biotic complexity.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-023-00785-6.

Additional file 1. Transcriptome assembly evaluation, chlorantraniliprole concentration measurement and additional figures. Fig. S1. Maximum-Likelihood tree inferred from ryanodine receptor amino acid sequences from representatives of the insecticide target group Lepidoptera, the insect *L. basale*, amphipods, and more distantly related crustaceans. **Fig. S2**. Functional enrichment for *L. basale* high insecticide concentration gene clusters. **Fig. S3.** Functional enrichment for *L. basale* high insecticide concentration + increased fine sediment gene clusters.

Additional file 2. Additional tables.

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Author contributions

CM, FL, and RBS conceptualized the study and acquired funding. FL, MVB, DB, and LM planned the experiment and MVB, DB, and LM performed the field work. MVB processed the samples in the lab with support from DB. VCS measured the pesticide concentrations in water samples. MVB performed the analyses, visualized the results, and wrote the manuscript. All the authors contributed to the manuscript and approved the final version.

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Availability of data and materials
All RNA-seq libraries of *Lepidostoma basale* and *Gammarus pulex* generated during this study were archived in the European Nucleotide Archive (ENA) at EMBL-EBI under project accession numbers PRJEB57954 (https://www.ebi.ac.uk/ena/browser/view/PRJEB57954) and PRJEB57955 (https://www.ebi.ac.uk/ena/browser/view/PRJEB57955), respectively.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interest.

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Publisher's Note

Chapter 6

Discussion

Discussion

Multiple stressor effects at the transcriptomic level

Multiple stressor effects are difficult to predict because stressor effects are shaped by interactions with other stressors (Christensen et al. 2006; Jackson et al. 2016). By integrating RNA-sequencing and mesocosm experiments in the assessment of multiple stressor effects, the studies presented in this thesis unveiled stressor interactions at the transcriptional level between fine sediment deposition, increased salinity and reduced flow velocity (Chapter 3), as well as between fine sediment deposition and the insecticide chlorantraniliprole (Chapter 5). Additional challenges in the assessment of multiple stressor effects arise because the impact of individual stressors differs between organismic groups such as aquatic insects and amphipods, which displayed a diverging sensitivity towards chlorantraniliprole (Chapter 4 and 5). This differential vulnerability can be attributed to taxon-specific physiological predispositions, but is also shaped by the different ecological requirements of the studied species (Chapter 4 and 5). Since species are linked by trophic relationships, stressors can further induce indirect effects when their impact modulates functional connections within the food web (Segner et al. 2014) (Fig. 1.1.). We found that RNA-sequencing can capture the signal of biotic interaction (Chapter 4), implying that interspecific resource competition modulates transcriptional stress responses and that the assessed impact of stressors can depend on the biotic environment present in the experimental setting.

Notwithstanding the strong context-dependency of stressor effects, this thesis reveals stressor effects and physiological response mechanisms which are consistent across different stressor types (i.e., chemical, non-chemical), organismic groups (i.e., caddisflies, mayflies and amphipods), experimental settings (i.e., indoor and outdoor systems), and ecological scales (i.e., changes in gene expression and specimen abundances).

The investigated chemical stressors induce the strongest transcriptomic responses

In both ExStream experiments (Chapter 3 and 5), the chemical stressors induced by far the strongest change in gene expression: *G. fossarum* responded to treatments involving increased salinity with the differential expression of hundreds of genes, whereas exposure to the non-chemical stressors (i.e.,

increased fine sediment, reduced flow velocity) resulted only in dozens of differentially expressed genes (Chapter 3). Consistently, the insecticide chlorantraniliprole induced transcriptional alterations of thousands of genes in the caddisfly *L. basale*, whereas only a few genes responded to the addition of fine sediment (Chapter 5). A possible explanation for the stronger impact of chemical stressors is given by different mechanistic targets of stressors. Chemical stressors interfere with cells directly at the molecular level: chlorantraniliprole is supposed to target the insect ryanodine receptor, leading to a disruption of the calcium homeostasis (Lahm et al. 2007), whereas increased ion concentrations induce cellular osmotic stress due to passive gain and loss of ions and water, respectively (Evans and Kültz 2020). In contrast, non-chemical stressors primarily alter habitat conditions like e.g., fine sediment deposition, which directly reduces the structural heterogeneity of the benthic substratum (Wood and Armitage 1997). Depending on the fine sediment composition (e.g., organic content, ionic composition), increased fine sediment load can induce changes in physico-chemical parameters such as O_2 saturation or pH (Ryan 1991), but these changes in environmental variables are locally constrained to areas where the fine sediment is deposited. In semi-natural experimental settings, such as the ExStream system, organisms can avoid unfavorable habitat conditions by e.g., seeking refugia in other channel compartments (e.g., Beermann et al. 2018a). Such behavioral escape mechanisms are probably less protective against chemical stressors, which are dissolved in the water and thus omnipresent. Accordingly, exposure to non-chemical stressors might be more subtle and less specific, making transcriptomic stress responses therefore more difficult to detect.

Although increased salt concentrations and insecticide exposure represent both chemical stressors, their effects at the cellular level are different, and the induction of distinct physiological response mechanisms is therefore not unexpected: the insecticide chlorantraniliprole stimulated the expression of genes involved in immunity and development in *L. basale* (Chapter 4 and 5). These results are in line with observations from previous studies reporting a chlorantraniliprole-induced expression of innate immune genes in *Drosophila melanogaster* (Gao et al. 2021), as well as an accelerated development of aquatic insects following exposure to various insecticides (Cavallaro et al. 2018). In contrast, increased ion concentrations evoked the differential regulation of genes contributing to detoxification as well as ion transporter genes in *G. fossarum* (Chapter 3). Despite these rather stressor-specific physiological responses, the

studies presented in this thesis showed that both chemical stressors induced various kinds of reduced metabolic (Chapter 3 – 5), suggesting that a metabolic depression represents a general physiological response linked to environmental stressor exposure in the studied species.

Metabolic depression is a common physiological stress response in the study species

Transcriptomic profiling revealed that the environmental stressors investigated in this thesis induced a reduced metabolic activity in freshwater macroinvertebrates: the amphipod *G. fossarum* exposed to increased salinity, reduced flow velocity and increased fine sediment deposition responded primarily with a downregulation of genes involved in the protein biosynthesis or other energy consuming cellular processes (Chapter 3). Likewise, the mayfly *E. danica* displayed a suppressed transcriptional activity of genes involved in the chitin metabolism following chlorantraniliprole exposure (Chapter 4). In the caddisfly *L. basale*, chlorantraniliprole exposure induced a suppression of the protein biosynthesis, in particular of mitochondrial genes, thereby directly interfering with the energy metabolism of this organism (Chapter 4 and 5). Similarly, we identified synergistic downregulations of mitochondrial genes in the amphipod *G. pulex* due to the combined effect of insecticide exposure and fine sediment deposition, although the treatment effects were relatively weak in this species (Chapter 5).

Reduced metabolic activity is a common physiological response evoked by environmental stressors in aquatic invertebrates (Chen and Stillman 2012; Rodrigues et al. 2017; Andrade et al. 2022; Shi et al. 2022). However, the exact underlying physiological mechanisms are manifold, depending on the specific stressor-species combination, and can represent adaptive response as well as adverse stressor effects. For instance, the filter-feeding mussel *Mytilus galloprovinciales* presumably avoids accumulation of Lanthanum in its tissue by reducing its metabolic activity (Andrade et al. 2022). In contrast, water contamination with trace metals can reduce the metabolic capacity of aquatic organisms by disrupting the electron transfer in mitochondria (Kurochkin et al. 2011) or by damaging gills, thereby impairing respiration and oxygen uptake (Issartel 2010).

In general, environmental stressor exposure interferes with an organism's energy metabolism via (i) increasing energetic costs associated with maintaining homeostasis, (ii) increasing energetic costs due to the activation of protective and repair mechanisms, (iii) decreasing foraging abilities and therefore compromising food uptake or (iv) stressors interfere with ATP synthesis (Sokolova 2013). Due to the limited energy budget available to an organism, increased energetic costs are expected to induce energy allocation to vitally essential processes. Given that protein biosynthesis can demand 20% or more of the metabolic energy expended under resting conditions (Hawkins 1991), decreasing the rate of protein biosynthesis, as has been observed in *G. fossarum*, *E. danica* and *L. basale*, could represents an energy-saving mechanism when organisms face unfavorable environmental conditions (Hand and Hardewig 1996). However, the chlorantraniliprole-induced inhibition of mitochondrial gene expression, which was observed in *L. basale* (Chapter 3 and 4) and in several pest species (Gao et al. 2018; Meng et al. 2019), is expected to reduce ATP synthesis. This would, in turn, impair the capacity of the energy metabolism, blurring the distinction between adaptive and adverse transcriptional stress responses. Chlorantraniliprole is known to induce oxidative stress in aquatic invertebrates (Rodrigues et al. 2015, 2017) due to e.g., excessive generation of reactive oxygen species (ROS) (Cui et al. 2017). If chlorantraniliprole exposure generated ROS in the mitochondria of *L. basale*, a downregulation of mitochondrial genes and processes could represent an adaptive response in order to mitigate oxidative stress. Alternatively, a decreased mitochondrial activity might be the consequence of oxidative damage, and the reduced expression of mitochondrial genes would reflect adverse stressor effects.

A similar ambiguity needs to be resolved in the case of the insecticide-induced downregulation of the chitin metabolism in *E. danica*, since phenological plasticity could represent a strategy to mitigate environmental stress. However, the inhibition of genes involved in the chitin synthesis following insecticide exposure (i.e., chlorantraniliprole and teflubenzuron) disrupted the ecdysis and impaired pupation in the Colorado potato beetle *Leptinotarsa decemlineata* (Meng et al. 2018) and the silkworm *Bombyx mori* (Chen et al. 2020), strongly suggesting adverse effects of a chlorantraniliprole-mediated suppression of the chitin metabolism. To finally resolve whether the alteration of the developmental program in the non-target taxon *E. danica* represents an adaptive strategy or is the result of endocrine disruption, a frequently observed off-target effect of

many insecticides (Crane et al. 2022), requires further research. Nonetheless, a reduced metabolic activity is likely unsustainable in the long-term and negative effects at higher ecological levels are expected to arise, irrespective of the adaptive potential of the stressor-induced transcriptional alterations.

The investigated stressor combinations act mainly antagonistic at the transcriptional level

We detected mainly antagonistic stressor interactions between increased salinity, reduced flow velocity and fine sediment deposition in *G. fossarum* (Chapter 3) as well as between the insecticide chlorantraniliprole and fine sediment deposition in *G. pulex* and *L. basale* (Chapter 5). These observations can be partly explained by the physico-chemical interplay between stressors. The antagonism observed in the expression data from *L. basale* exposed to the insecticide chlorantraniliprole and increased fine sediment is presumably the result of reduced insecticide exposure concentrations due to adsorbance of chlorantraniliprole to fine sediment particles (EFSA 2013). Furthermore, two other factors may have contributed to the observed tendency towards antagonistic stressor interactions: first, transcriptional stress responses are often non-specific and cells respond to various environmental challenges with the induction of general protective pathways (López-Maury et al. 2008; Delnat et al. 2020). Non-specific stress responses are presumably adaptive, since cells cannot account for all potential adverse stimuli encountered in the present environment (López-Maury et al. 2008). In addition, shared protection allows to mediate the associated energetic costs (López-Maury et al. 2008; Velasco et al. 2019), which would be reflected in expression changes that do not scale linearly with increasing stressor exposure. Such a cross-tolerance consequently results in antagonistic upregulations of protective pathways (Velasco et al. 2019; Delnat et al. 2020). For instance, a similar predominant antagonistic nature of stressor interactions was reported by Delnat et al. (2020), who investigated multiple stressor effects of the insecticide chlorpyrifos and increased temperature on the gene expression profile of the mosquito *Culex pipiens molestus*. Consistently, Jackson et al. (2016) reported that antagonism is the most common type of stressor interaction at the organismal level. Interestingly, antagonism was also the dominant type of stressor interaction at the community level, especially when functional performance parameters were included as endpoints, suggesting that stress-tolerant species compensate the functional loss of stress-sensitive species

(Jackson et al. 2016). Taken together, these findings suggest that antagonistic stressor interactions might arise due to constraints inherent to the biological system such as the limited energy budget available to organisms (i.e., at the individual level) or functional redundancy (i.e., at the community level).

Second, the selected null model of stressor additivity determines how stressor interactions are classified (Schäfer and Piggott 2018). At the transcriptomic level, synergistic stressor interactions might be reported when single stressors do not surpass a certain threshold required to induce a transcriptional response, while their combined exposure evokes a (measurable) change in expression. For instance, synergistic stressor interactions at the transcriptomic level were detected for multiple stressor effects of elevated pCO_2 levels and hypoxia in fish (Cline et al. 2020) or increased temperature and low salinity in oysters (Ertl et al. 2019). Notably, these studies assessed synergistic stressor interactions qualitatively, i.e., multiple stressor treatments inducing the differential expression of genes that did not respond to individual stressor exposure were classified as synergism. Such a qualitative assessment can misclassify additive effects as synergism (Delnat et al. 2020), when actually the statistical power is insufficient to detect small effect sizes in individual stressor treatments. Further, a qualitative classification cannot detect stressor interactions, which modify the expression of genes that are already differentially expressed in single stressor treatments i.e., changes in expression, that are larger or smaller under co-exposure than anticipated based on expression changes evoked by the individual stressors (Delnat et al. 2020). These cases can only be detected if stressor interactions are quantitatively identified (Delnat et al. 2020) as in this thesis, where the effect sizes of interaction terms were tested for being non-zero (for further explanation see Chapter 5). Important to note here is that the null model of stressor additivity applied to the expression data sets from *G. fossarum* (Chapter 3), *G. pulex* and *L. basale* (Chapter 5) is based on the cumulative sum of count data at the binary logarithmic scale. As such, only stressor interactions are detected if the combined stressor effects deviate from the product of individual stressor effects at the original count data scale, changing the null model from additive to multiplicative. Arguably, the multiplicative null model can result in different interaction classifications than the additive null model (Schäfer and Piggott 2018), because the cumulative effect predicted by the multiplicative null model is larger than the cumulative effect predicted by the additive null model.

Advantages, limitations and future prospects of multiple stressor research

Transcriptomics in multiple stressor research

Comparing multiple stressor effects of increased salinity, reduced flow velocity and increased fine sediment levels on the transcriptomic profile of *G. fossarum* with stressor effects derived from abundance changes of gammarids showed that RNA-sequencing data can reliably identify stressor effects and their interactions. Further, the high temporal resolution of the expression data allowed to unveil stressor effects, which were not translated into changes of specimen abundances within the time frame of the experiment. Apart from its high sensitivity, the most significant advantage of RNA-sequencing is its global perspective on the transcribed part of the genome without the need for any evidence-based gene selection *a priori*. As such, RNA-sequencing enables to study the molecular mechanism underlying physiological stress responses in understudied taxonomic groups. For instance, the characterization of the molecular responses evoked by chlorantraniliprole exposure in aquatic insect larvae is highly important, since the effects were not expected based on the insecticide's clearly defined mode of action. Despite these advances provided by transcriptomic profiling, the sensitivity of RNA-sequencing data obtained from non-model organisms is limited because the bioinformatic processing of short-read data is challenging and sequencing of non-model organisms from typically outbred populations provides noisy expression data (Todd et al. 2016). The latter is inherently linked to the allelic diversity present in wild populations, but also to the transcriptomic plasticity between e.g., different sexes or developmental stages, which can impair precise estimation of stressor effects during statistical modeling. Fortunately, the methodological challenges associated with RNA-sequencing are currently already addressed and should be solvable in the near future e.g., through technological advances in long-read sequencing platforms, allowing to sequence entire transcripts (Wang et al. 2021). In particular, the expected increase in reference genomes brought by dedicated global initiatives such as the Earth BioGenome Project (Lewin et al. 2018), which aims to generate high-quality genomic resources for all described eukaryotic families, is promising. These reference genomes will greatly improve transcriptomic profiling in non-model organisms and will make gene expression studies more comparable, because genome-guided transcript reconstruction and abundance quantification are more accurate compared to RNA-sequencing data analyses based on *de novo* assembled transcriptomes, which are, by nature, dynamic snapshots of the transcribed part

of the genome. However, we need to face the fact that the generation of reference genomes is a highly resource demanding and ongoing process, and first draft assemblies do not represent an organism's genome comprehensively. Even the human reference genome is continuously updated since its initial release in 2000 (Nurk et al. 2022), raising concerns regarding the ambitious aim of the Earth BioGenome Project to generate 9,000 reference genomes within a decade (Lewin et al. 2018). Important to note here is that an assembly is the first major steps towards a reference genome, but functional annotation data is still scarce for most non-model organisms and genomic reference databases are far away from being complete. This is illustrated e.g., by the fact that from the seven amphipod genome assemblies deposited at NCBI (https://www.ncbi.nlm.nih.gov/genome/; accessed on 04.11.2023), only two are annotated. In fact, most annotation data come from a few, well-studied model species. Accordingly, functional profiling based on sequence homology inferences is inherently biased towards more conserved genomic regions, simply because more reliable annotation data is available. Since we can only identify the regulation of molecular pathways, if the genes contributing to these pathways are annotated, the absence of a signal does not allow the conclusion that a given gene regulatory network is not affected by a stressor. For instance, it is currently not possible to differentiate whether the observed insecticide-induced differential expression of developmental genes in aquatic insect larvae represent an off-target effect of chlorantraniliprole (i.e., decoupled from its mode of action) or if they are the result of altered intracellular calcium levels. Filling the gaps in genomic reference databases will certainly improve our ability to precisely profile stressor-induced molecular response mechanisms, but having a high-quality, (almost) completely annotated reference genome available is for most non-model organism researchers still a long-term future perspective. This is, however, not discouraging but encouraging: RNA-sequencing is already a powerful method for transcriptomic profiling in non-model organisms, but the data harbor much more potential than is currently exploited. Despite these exciting prospects, the most pressing question related to multiple stressor research remains: which consequences arise from transcriptomic stressor effects for individuals, populations, communities and ecosystem function? This question can only be addressed through the integration of various ecological response variables in the assessment of multiple stressor impact.

Multiple stressor effects across ecological scales

The aim of this thesis was to characterize the molecular basis of physiological responses evoked by multiple environmental stressors in freshwater macroinvertebrates. Since stressors operate across ecological scales, which are intrinsically connected (Fig. 1.1), the obtained findings need to be set in context with stressor effects at higher ecological levels. Through this, we can distinguish between stressor-induced transcriptional changes that reflect adverse effects and those that are adaptive. Further, identifying stressor effects that are consistent across ecological scales improves our ability to predict multiple stressor dynamics (Simmons et al. 2021). It was possible to show that 'positive' effects of reduced flow velocity and its antagonistic interaction with increased fine sediment on *G. fossarum* were similarly reflected at the transcriptional and population level (Chapter 3). A positive effect of reduced flow velocity was also observed in another ExStream experiment manipulating fine sediment levels, flow velocity and nutrient concentrations, whereby slow flow led to an increase of gammarid abundances, but only at ambient nutrient levels (Elbrecht et al. 2016). In contrast, the salinity-induced transcriptomic responses in *G. fossarum* might have played a role in a physiological acclimatization strategy, since they were not translated into changes of specimen abundances (Beermann et al. 2018a).

At first, the rather limited effect of fine sediment deposition on the gene expression profiles of amphipods and the caddisfly *L. basale* was surprising, given that increased fine sediment load was often identified as the most pervasive stressor for the macroinvertebrate community in previous multiple stressor experiments (Piggott et al. 2012; Magbanua et al. 2013; Elbrecht et al. 2016; Beermann et al. 2018a; Chará-Serna and Richardson 2018). Actually, however, increased fine sediment appears to be not a strong stressor for the tested species: in Beermann et al. (2018a) *Gammarus* sp. abundances decreased in channels where fine sediment was applied, mainly due to increased drift rates and, to a lesser extent, through evading of gammarids into the leaf-litter compartment. However, these effects disappeared at higher taxonomic resolution provided by molecular methods, allowing to differentiate between *G. fossarum* and *G. roeselii* (Beermann et al. 2021). For *L. basale*, less information is available: although *Lepidostoma* sp. was shown to be susceptible to fine sediment, the presence of woody debris appears to be more important for *L. basale* than its preference for coarse substratum (Turley et al. 2016). In general, strong negative effects of fine

sediment on the population dynamics of different caddisfly taxa such as *Hydropsyche* sp. (Beermann et al. 2018a), *Psilochorema* sp. (Magbanua et al. 2013) or *Pycnocentrodes* sp. (Matthaei et al. 2010) were reported in multiple stressor experiments. Unfortunately, *L. basale* was never part of the studied communities, highlighting one of the strongest limitations of current multiple stressor research: including only structural endpoints in the assessment of stressor impact prevents a generalization of stressor effects, because the composition of biological communities is different across spatial scales (Liess and Von Der Ohe 2005; Juvigny-Khenafou et al. 2021). Therefore, the assessed stressor impact might be restricted to regional species pools (Kefford et al. 2012a). In fact, extrapolating stressor effects only based on phylogenetic relationships can be misleading, since even different mitochondrial lineages within the same species can display distinct stressor effects, highlighting further the need for a high taxonomic resolution (Macher et al. 2016; Beermann et al. 2021). Integrating trait data in the assessment of multiple stressor research is therefore urgently required since this allows to transfer stressor effects from one community to another: for instance, increased fine sediment was shown to have no effects or even favor organismic groups that have similar ecological traits as *L. basale* i.e., univoltine, clinger, shredder, tegumentary respiration mode (Juvigny-Khenafou et al. 2021).

Integrating a functional perspective in the assessment of stressor impact on the community level would not only help to derive general stressor effects but would also improve our mechanistic understanding of multiple stressor dynamics. Stressors are effectors, which generate impact on biological receptors (Segner et al. 2014). These biological receptors can be molecules, individuals, populations, communities or even ecosystems, and are linked through functional pathways i.e., organized in networks (Segner et al. 2014). For instance, the expression of genes is controlled by regulatory signaling pathways, while food webs describe functional links between species in terms of trophic relationships. Important to note is that stressor effects largely depend on the properties (i.e., 'functions' or 'traits') of biological receptors, which in turn drive stressor effects at higher ecological levels (Segner et al. 2014). We could show that such differences between biological receptors at the molecular level contribute to the differential sensitivity towards the insecticide chlorantraniliprole between aquatic insect larvae and crustaceans (Chapter 4 and 5). Depending on the function of these species in the food web and how stressors modulate their trophic relationships,

particular ecosystem functions might be compromised. An excellent example is given by the study of Rodrigues et al. (2018), who investigated chlorantraniliprole-induced changes in the macroinvertebrate community composition as well as ecosystem functions, and how biotic stressors (i.e., an invasive primary producer and invasive predator) alter biological responses to chemicals. By focusing on both, density-mediated and trait-mediated stressor effects, the authors could identify mechanisms through which chlorantraniliprole and invasive species impacted the macroinvertebrate community and thus, the ecosystem function: chlorantraniliprole exposure strongly affected shredders and grazers, while the non-native predator mainly decreased abundances of collectors and grazers due to its specific predation strategy. Due to the reduced grazer densities, both stressors had a positive effect on primary production. While antagonistic stressor interactions were observed between chlorantraniliprole and the presence of the non-native predator, presumably due to reduced exposure of prey taxa as a consequence of a chlorantraniliprole-induced immobility, synergistic effects between chlorantraniliprole and the presence of the non-native plant were specifically detected for grazers. The authors explained this with the reduced nutritional value of leaves from the non-native primary producer, which compromised the energy demanding detoxification response evoked by chlorantraniliprole exposure.

The need to integrate a functional perspective is intuitive at the level of gene expression: the number of differentially expressed genes provides first insights into an organism's overall sensitivity towards a stressor, but only the functional annotation of the regulated genes informs about the physiological response mechanism. The same principle applies to stressor effects assessed at the population and community level and the given example from Rodrigues et al. (2018) illustrates how valuable the integration of trait data is; however, many community level studies focus on density-mediated stressor effects such as community turnover or changes in alpha diversity linked to stressor exposure (e.g., Matthaei et al. 2010; Piggott et al. 2012; Elbrecht et al. 2016; Beermann et al. 2018a; Chará-Serna and Richardson 2018). Identifying stressor-induced changes in the taxonomic composition typically reveals proximate stressor effects (i.e., how stressors shape community assemblages), but not ultimate causes (i.e., the underlying mechanisms). One important exception to that are insecticides, which are designed to target specific phylogenetic groups like butterflies in the case of chlorantraniliprole (Lahm et al. 2007): when the

properties of biological receptors are similar due to a shared evolutionary history rather than overlapping ecological niches, phylogenetic relationships can be indicative for the vulnerability towards stressors, reflected in the differential sensitivity of aquatic insects and amphipods to chlorantraniliprole exposure (Chapter 4 and 5). This context-dependency can be resolved, when we consider the mechanisms through which stressors act: to predict the effect of stressors, which alter habitat conditions, ecological trait data should be integrated, because the impact of the stressors will depend on the ecological profile of the species. Similarly, the integration of phylogenetic relationships is highly relevant if stressors such as pesticides target specific phylogenetic groups. For instance, chlorantraniliprole shows a higher binding efficiency to the ryanodine receptor of insects than decapods or vertebrates (Qi and Casida 2013). Consistently, ecotoxicological tests reported a higher sensitivity of mayflies and caddisflies for chlorantraniliprole than for amphipods (APVMA 2008). Currently, only a few studies exist which evaluated the impact of chlorantraniliprole on macroinvertebrate communities in a controlled experimental setting while integrating natural complexity (e.g., Rodrigues et al. 2018; Stenert et al. 2018). As for most pesticides, the majority of results are obtained from highly controlled ecotoxicological studies (e.g., Adam et al. 2010; Pestana et al. 2014; Lavtižar et al. 2015; Rodrigues et al. 2017; Hunn et al. 2019; Verheyen and Stoks 2020) or field surveys which investigate exposure effects of pesticide mixtures present in natural streams (e.g., Liess and Schulz 1999; Liess and Von Der Ohe 2005; Schäfer et al. 2012; Ohler et al. 2023). Hence, it is yet to be determined how the stimulation of the developmental program in *L. basale* and its suppression in *E. danica* in response to chlorantraniliprole exposure correspond to alterations in the population dynamics of these species. At the moment, these findings provide a possible mechanistic explanation, why stream contamination with insecticides leads to changes in emergence phenology of aquatic insects (Ohler et al. 2023) and should be used to generate evidence-based hypotheses.

Future prospects

This thesis revealed important first insights into the physiological response mechanisms of freshwater macroinvertebrates that are exposed to globally relevant anthropogenic stressors. Yet, the functional annotation of the generated *de novo* transcriptomes can be biased towards more conserved genomic regions

due to incomplete reference databases. Further, mRNA levels are not necessarily correlated with protein abundances (Chen et al. 2002; Pascal et al. 2008; Ghazalpour et al. 2011), highlighting the need to experimentally validate the induction of the here reported physiological response pathways. A possible way to achieve this could be the integration of proteomic data, enabling to study gene expression changes at the protein level. A comparative analysis of transcriptomic and proteomic dynamics would improve our understanding how molecular mechanisms shape the phenotype of an organism (Diz et al. 2012). Resolving adaptive stressor-induced gene expression changes would be further possible through gene silencing approaches such as RNA interference, where mRNA is enzymatically degraded (e.g., Wang et al. 2018; Gao et al. 2021), or steric blocking of transcripts which prevents their translation (e.g., Bryant et al. 2017). While all these approaches can be used to infer the impact of stressor-induced gene expression changes at the individual level, it is crucial to understand how stressors operate across ecological scales. Field experiments like the ExStream system, which allow the assessment of multiple stressors at various ecological endpoints in a controlled but realistic setting, are well suited to address these questions. Specifically, the here obtained results generate the following hypotheses, which could be tested:

(i) Stressors which induce the strongest physiological response show the strongest impact at higher ecological levels such as population decline and trophic cascades. Such consistent stressor effects would be expected if stressor-induced transcriptional changes represent adverse effects.

(ii) Stressor exposure evokes transcriptional changes, but no effects are detected at higher ecological levels. Such contradictory patterns would be expected, if the differential gene expression represents a physiological coping strategy which enables organisms to compensate for environmental stress. However, the potentially limited temporal resolution of population level data must be taken into account.

(iii) Antagonistic upregulation and synergistic downregulation of protective pathways induce synergism at the organismal level because the general stress defense response will be insufficient to buffer multiple stressor effects (Delnat et al., 2020).

To improve our mechanistic understanding of multiple stressor effects, we need to focus on the properties of biological receptors (Segner et al. 2014). Since RNA-

sequencing provides information about transcript abundances and allows to profile RNA molecules at single base resolution, the method is well suited to investigate physiological response mechanisms. However, observations such as the limited effect of fine sediment at the transcriptomic level should be verified by community ecologists who need to assess both, structural and functional alterations of the community composition in response to multiple stressor exposure.

Furthermore, stressor combinations should be tested for interactions not only because these stressors have the same source such as e.g., intensive agriculture. Given that the simultaneous occurrence is necessary for stressors to interact, but not sufficient, future research should focus on stressor combinations when a shared mechanistic target between stressors is hypothesized (Simmons et al. 2021). In this regard, identifying multiple stressor effects of increased temperature and chemical stressors would be highly relevant: higher temperatures result in increased water evaporation and reduced discharge, and might therefore enhance the exposure effects of chemical stressors. Furthermore, and maybe more important, increased temperatures strongly interferes with metabolic rates and increases biological activity (Brown et al. 2004). Given that chemical stressors such as pesticides interfere with the energy metabolism of macroinvertebrates, complex stressor interactions are likely to arise (e.g., Janssens and Stoks 2013, 2017; Verheyen and Stoks 2020). Such mechanistic assumptions should be used to develop more sophisticated null-models of stressor additivity (Schäfer and Piggott 2018). Identifying the impact of these stressors across ecological scales, and specifically at the physiological level through e.g., transcriptomics, will inform about stressor interactions beyond the physico-chemical interplay between stressors.

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Supplementary material

Chapter 3: The supplementary material for the study *Impacts of multiple anthropogenic stressors on the transcriptional response of Gammarus fossarum in a mesocosm field experiment* (Brasseur et al. 2022) can be downloaded from: https://doi.org/10.1186/s12864-022-09050-1

Chapter 4: The supplementary material for the study *Transcriptomic sequencing data illuminate insecticide-induced physiological stress mechanisms in aquatic non-target invertebrates* (Brasseur et al. 2023b) can be downloaded from: https://doi.org/10.1016/j.envpol.2023.122306

Chapter 5: The supplementary material for the study *Multiple stressor effects of insecticide exposure and increased fine sediment deposition on the gene expression profiles of two freshwater invertebrate species* (Brasseur et al. 2023a) can be downloaded from: https://doi.org/10.1186/s12302-023-00785-6

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