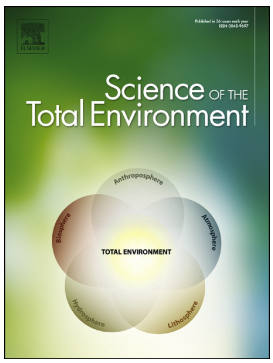

Pesticide-induced metabolic changes are amplified by food stress

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Pesticide-induced metabolic changes are amplified by food stress

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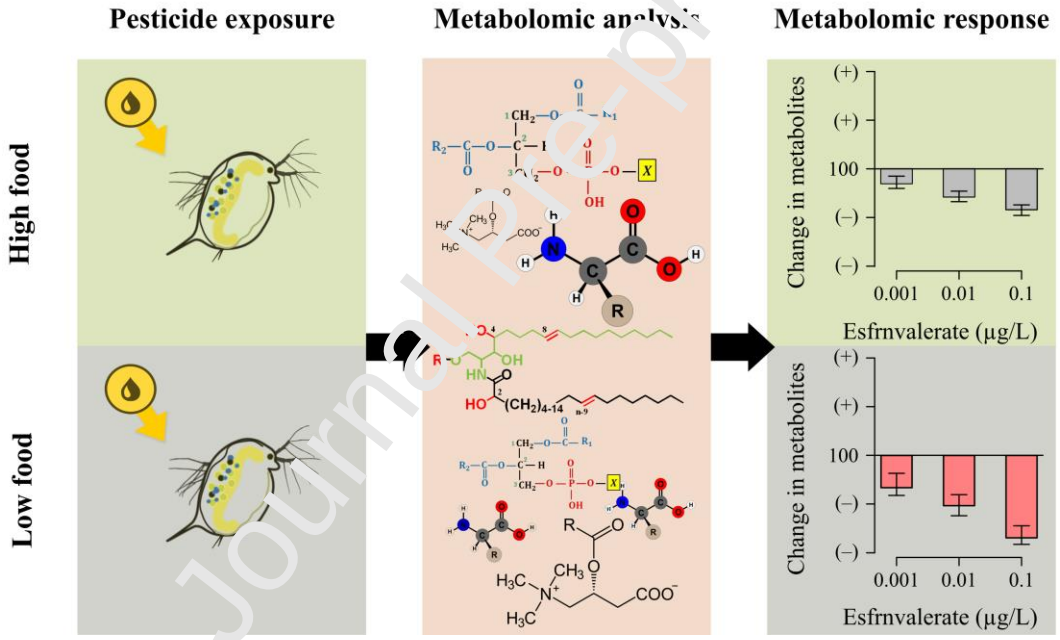
Abstract

In natural ecosystems, long-term detrimental effects of pesticides may occur at very low concentrations, below those considered safe by the governmental risk assessment. Mechanisms potentially responsible for this unexpected sensitivity include environmental stress-factors such as food deficiency. To understand this so called “effect-paradox”, we investigated how food stress interacts with insecticide-induced biochemical fingerprints. Therefore, we measured metabolomic perturbations in *Daphnia magna* following a 24h exposure to esfenvalerate under high and low food conditions. In total, 160 metabolites covering the groups of amino acids, fatty

acids, lipids and sugars were analyzed. At 0.001µg/L esfenvalerate – a factor of >200 below the acute lethal concentration (LC_{50}) – the endogenous metabolome was significantly affected. Further, the effect under low food conditions was considerably stronger compared to high food conditions. Individual metabolites showed up to 7-fold stronger effects under low food conditions. In general, the metabolomic changes were largely dose-specific and increased over seven days after contamination. We conclude that the metabolic profiles are altered for at least seven days after a pulse exposure, and therefore might be a key process to understanding population level changes at ultra-low pesticide concentrations in the field.



Graphical abstract

Keywords: Metabolic changes; *Daphnia magna*; Pyrethroid; Interaction; Food stress

Introduction

Over the past decades, the large-scale use of agricultural pesticides has raised concerns about their presence in the environment (Liess et al., 1999) and their effects on ecosystem structure and functions (Landis et al., 2003; Liess and Schulz, 1999). In fact, several studies have shown a decline in aquatic and terrestrial biodiversity due to pesticides (Beketov et al., 2013; Fox, 2013). Such pesticide effects have been reported far below the concentrations that are considered to be

safe by regulatory authorities (Liess and Von Der Ohe, 2005; Schafer et al., 2012) which led to the identification of the "effect paradox (Liess et al., 2019b). The underestimation of pesticide effects in field studies include higher sensitivity of individuals to pesticides under suboptimal conditions (Liess et al., 2016). Such environmental factors, as food availability, temperature and ecological interactions (predation, competition etc.) may enhance the potential effects of contaminants (Morrison et al., 2007; Viant, 2007). Lab investigations have also identified pesticide effects under ultra-low concentrations starting at around 100 times below the LC_{50} (Liess et al., 2019a). For example, Liess and Schulz (1999) observed delayed effects of a pyrethroid Fenvalerate on caddisflies at more than three orders of magnitude below the LC_{50}. Similarly, Siddique et al. (2020) and Cold and Forbes (2004) showed long term effects on key life traits in *Gammarus pulex* at very low pesticide concentrations. However, the underlying mechanisms of these unexpected effects at ultra-low pesticide concentrations are unclear. It is suggested that the sub-organismic responses may provide early warning for general stress at individual or higher levels (De Coen and Janssen, 2003; Hanson and Lagadic, 2005). Therefore, it is imperative to link the molecular actions of pesticides to their possible interference with biological processes for a better understanding of pesticide toxicity, specifically at ultra-low concentrations.

Numerous promising techniques such as proteomics, transcriptomics and metabolomics have already been developed for characterizing the sub-organismic responses to environmental stressors (Escher et al., 2017; Jemec et al., 2010). Since food stress is supposed to directly affect the metabolism, the analysis of endogenous metabolites might provide information on the molecular adaptation under stress. Metabolomics is a well-established –omics technique, widely applied to analyze endogenous metabolites within a cell, tissue or biofluid (Bundy et al., 2004;

Jones et al., 2008; Wu et al., 2011). Metabolomics can provide a comprehensive evaluation of a biological response of living organisms under stressed conditions (Bundy et al., 2008; Garreta-Lara et al., 2016), and adds to the base of knowledge on the potential effects of contaminants that are of great environmental concern (Bundy et al., 2008; Park et al., 2019; Van Aggelen et al., 2009). This technique has been employed for a wide range of organisms to characterize the effects of toxicants (Kovacevic et al., 2016; Sotto et al., 2017; Wu et al., 2013) and environmental stressors (Garreta-Lara et al., 2018; Kullgren et al., 2013).

The drawback of metabolomics is that it detects the consequence of molecular adaptation or adverse effects rather than the regulation which is controlled on the transcriptional level. But on the other hand, the direct link to the phenotype results in the high sensitivity of metabolic changes as a response to external stressors. Recent investigations have reported metabolomic changes in freshwater invertebrates following an exposure to various toxicants (Kovacevic et al., 2016; Maity et al., 2012; Nagato et al., 2016; Russo et al., 2018) and environmental stressors (Garreta-Lara et al., 2016; Smolders et al., 2005; Wagner et al., 2015). Since the studies on toxic effects are generally based on experiments without environmental stress, none of the studies employed metabolomics so far to investigate the interaction between chemical and environmental stress. Therefore, more information is needed on metabolomic changes under conditions prevalent in the field (including chemical- and environmental stressors).

The pyrethroid insecticide esfenvalerate is widely applied in agriculture and has frequently been detected in the environment (Bacey et al., 2005; Cooper et al., 2003; Munze et al., 2017). Exposure to esfenvalerate may cause detrimental effects on the aquatic organisms. However, under suboptimal environmental conditions, these effects can be even more pronounced. Food limitation is one of the environmental stressors that may intensify the effect of contaminants

(Hammock et al., 2015). For pesticides, Shahid et al. (2019) reported a synergistic interaction between the pyrethroid esfenvalerate and food stress. Similarly, Pieters et al. (2005) reported an increased effect of Fenvalerate on the survival and growth of *Daphnia magna* under low food conditions. For heavy metals, Liess et al. (2001) reported a synergistic interaction between copper and food stress. Therefore, in the present investigation, we aim to explore the association between exposure to very low concentrations of the pyrethroid esfenvalerate and metabolic changes in *Daphnia magna*, under high and low food conditions. These key metabolic profiles can reveal the combined effects of chemical and environmental factors in the laboratory, which potentially lead to delayed/synergistic effects in the field.

Materials and methods

We studied lethal effects as well as metabolic changes in *Daphnia magna* related to the exposure of a pyrethroid insecticide esfenvalerate under high and low food conditions. In all experiments, we exposed *Daphnia magna* to low concentrations of esfenvalerate: 0.001, 0.01 and 0.1 µg/L for 24h and quantified metabolite contents at different time points (after 24h and on 4^{th} and 7^{th} day after exposure) using LC-MS-MS.

Culture of test organisms

Test organisms were obtained from a clone "Aachen V" cultured at the Department of System Eco-toxicology, UFZ Leipzig. The daphnid culture was maintained in standard conditions, at a constant temperature of 20.0±1°C and a controlled photoperiod cycle of 16 hours light/8 hours darkness (Sebens, 1982). The daphnids were cultured in 2L glass beakers (20 animals per beaker) with 1800 mL volume of ADaM (Artificial Daphnia Medium) (Klüttgen et al., 1994). The culture medium was renewed three times per week and daphnids were fed with a suspension of green algae *D. subspicatus* expressed in cells/ind/day. The quantity of food was 0.5×10^9 cells

cells/ind/day in the first week, which was then increased to 0.75×10^9 cells/ind/day. Additionally, organisms were also fed with yeast (0.6 mg/L) once per week.

During experiments, the organisms used in the high food treatment were fed with 0.5×10^9 cells/ind/day in the first week, 1.15×10^9 cells/ind/day in the second week, and 1.35×10^9 cells/ind/day in the third and fourth weeks. Whereas, organisms in the low food treatments were fed with 0.5×10^7 cells/ind/day in the first week, 1.15×10^7 cells/ind/day in the second week, and 1.35×10^7 cells/ind/day in the third and fourth weeks (Shahid et al., 2019).

Exposure to esfenvalerate and food stress

We selected a pyrethroid esfenvalerate (CAS 66230-04-4, purity: 99.8%) for pesticide exposure that was purchased from Sigma–Aldrich, Germany. We prepared the stock solution by diluting 5mg of esfenvalerate in 10mL of dimethyl sulfoxide (DMSO) solvent. The DMSO concentration was always kept below 0.02% [vol/vol] that is two orders of magnitude lower than the LOEC (Lowest observed-effect concentration; 2%) (Bowman et al., 1981) and under the solvent limit suggested by Organisation of Economic Cooperation and Development (OECD) guidelines (OECD, 2000).The stock solution was then also diluted in ADaM to the required test concentrations. Briefly, we applied four esfenvalerate concentrations: control group, 0.001 µg/L (1/500 of LC_{50}), 0.01 µg/L (1/50 of LC_{50}) and 0.1 µg/L (1/5 of LC_{50}) and two food levels (i.e. high and low). After a 7 days period of acclimatization to the corresponding food conditions (low and high food), test organisms were exposed to esfenvalerate for 24 h following the OECD Guidelines for Testing of Chemicals (2004). The applied esfenvalerate concentrations were somewhat lower than those concentrations frequently recorded in the field ranging from trace concentrations to 0.166 µg/L (Bacey et al., 2005; Munze et al., 2017) or even 0.76 µg/L (Cooper et al., 2003).

For both the control and pesticide concentrations, 120 daphnids were tested with 30 individual per beaker containing 1800 mL of the test solution. Thus, four glass beakers per concentration were prepared. The temperature of all experiments was maintained at 20.0 ± 1 °C under a photoperiod of 16/8 h (Sebens, 1982). After a pulse exposure of 24 h, 30 surviving organisms from each concentration and control treatments were collected in 1.5 mL Eppendorf tubes and stored at –80 °C for metabolite analysis. The remaining living daphnids were transferred into beakers with uncontaminated medium, and fed with a respective amount of green algae. On day 4 and 7, again 30 surviving organisms from each treatment were collected for metabolite analysis. The experiment was performed in nine replicates over a period of nine months. To verify exposure concentrations, we analyzed the test medium for all treatments throughout the experiment. The average measured concentrations of esfenvalerate showed an acceptable deviation (±20%).

Sample preparation

Daphnia magna individuals were stored in Eppendorf tubes at -80°C. For sample preparation, 1mL of acetonitrile/water (1:1) and 3 metal beads were added to each sample, and homogenized for 10 minutes using TissueLyser (30/s) model). Then, samples were centrifuged for 15 minutes (15,000 rpm at 5°C), dried in a vacuum centrifuge (V-AQ at 30°C for 4 h) and diluted in 1 mL of methanol/acetonitrile (1:1). Subsequently, these samples were treated with ultrasound under cooling with ice for 1 h and centrifuged again for 15 min (15,000 rpm at 5°C). Afterwards, supernatant was transferred into new Eppendorf tubes and dried again in a vacuum centrifuge (V-AQ at 30°C for 2 h).

Targeted Metabolomics

The metabolomic analyses were carried out with the Absolute***IDQ***® p150 Kit (Biocrates Life Science AG, Austria). The Kit identifies and quantifies 163 metabolites from 5 compound classes (acyl-carnitines, amino acids, biogenic amines, glycerophosphol- and sphingolipids, and hexoses. The kit preparation was carried out following the manufacturer's instructions. Recently, Russo et al. (Russo et al., 2018) successfully employed this metabolomics kit for analysing pesticide-induced metabolic changes in aquatic invertebrates.

The LC-MS/MS analysis was carried out by MRM acquisition using a Waters Acquity UPLC System (Waters, Eschborn, Germany) coupled with QTRAP 5500 (AB Sciex, Darmstadt, Germany). The following MS parameters were used: Ion Source: Turbo Spray, Curtian Gas: 20psi, CAD Gas: Medium, Ion Spray Voltage: 5500 V, Temperature: 500°C, Ion Source Gas 1: 40psi, Ion Source Gas 2: 50 psi. The analyses were performed by an isocratic FIA-MS-MS method with two runs (positive and negative ionisation mode) respectively as three min runs. Running solvent was a mixture of water and 5mM ammonium acetate in methanol (v/v) was used. Mass spectra were analyzed with Analyst Software version 1.6.2 and validated by the MetVal tool from the MetIDQ Software tool delivered by Biocrates Life Science AG. An automatic quality assessment was conducted by comparing the obtained values for blanks, internal standards and quality controls. Raw data were processed by the integrated MetIDQ software (Biocrates, Innsbruck, Austria) which streamlines data analysis by automated calculation of metabolite concentrations providing quality measures and quantification. The Kit has been validated according to the FDA Guidance for Industry.

Materials

Water: Milipore, PITC: Fluka (for proteine sequence analysis), Pyridine: Fluka (p.a.), Methanol (Merck KGaA, Darmstadt, Germany, hypergrade for LC-MS), Acetonitril: Merck, Lichrosolv for LC/MS, Ammonium Acetate (Honeywell - Fluka, Seelze, Germany)

Data analysis

The data analyses were conducted using the statistical software R studio for windows (version 1.0.44) (RStudio, 2016) and R (version 3.0.3) (R Core Team, 2017). To increase the reliability of analyses, the experiment was performed in nine replicates. We exposed organisms to four different concentrations of esfenvalerate at two food levels, and analyzed 160 metabolites at four different time points. Thus, a data set of about 21,120 observations was analyzed (Tables S1-S4). The LC_{50} and 95% confidence intervals were calculated using the log-logistic model (Ritz and Streibig, 2005) with the five-parameter log-logistic function LL.5. We applied linear mixed-effects (LME) models to investigate the effect of esfenvalerate on (i) the overall metabolite content of the exposed daphnids and (ii) the content of each metabolite class separately. For the overall metabolite content, different pesticide concentrations were compared using the post hoc analysis with specific custom contrasts. To identify the effect of esfenvalerate on individual metabolites, we compared the concentration of each metabolite in pesticide-exposed *Daphnia magna* and in non-exposed control group using paired sample *t*-tests with nine experimental replications as the grouping factor. A comparison of high and low food treatments was conducted using two sample *t*-tests. To improve the normal distribution of residuals, the data were ln(x) transformed prior to the analyses.

RESULTS

Pesticide effect on overall metabolite content

We observed a significant effect of esfenvalerate on the overall metabolite contents of exposed organisms (Figure 1). The metabolic alterations were significantly stronger under low food conditions (Figure 1 b, d, Table S2). Under high food conditions, 24 h exposure to 0.01 µg/L and 0.1 µg/L of esfenvalerate caused a 10% and 6 % reduction of the overall metabolite content respectively (Figure 1a; $p < 0.01$). Under low food conditions, a dose-dependent down-regulation of metabolite content was observed (Figure 1b). The lowest concentration reduced 5% of the overall metabolite content ($p < 0.01$) followed by the medium (8%; $p < 0.001$) and the highest concentration (13%; $p < 0.001$). Further, the effect at lowest and highest concentration was significantly stronger than the effect observed under high food conditions (Table S2, two sample *t*-test; $p < 0.01$).

After a recovery time of 48 h (day 4), both an up- and down-regulation of metabolites was observed (Figure 1c, d). Under high food conditions, the lowest and medium concentrations increased the metabolite content by 7 and 6% respectively (Figure 1c; $p < 0.01$). While the highest pesticide concentration resulted in a significant reduction in overall metabolite content (11%; $p < 0.001$). Under low food conditions, we observed up-regulation at 0.001 µg/L (10%$p < 0.001$) and down-regulations at 0.1 µg/L (35%; $p < 0.001$). Remarkably, the effect at 0.1 µg/L was three times higher than the effect observed under high food conditions (two sample *t*-test; $p < 0.001$).

Furthermore, on day 7, the reduction in metabolite content was notably increased with an increasing concentration of esfenvalerate (Figure 1e). Exposure to 0.1 µg/L resulted in a 19% reduction in metabolic content ($p < 0.001$) followed by 0.01 µg/L (9%; $p < 0.001$) and 0.001µg/L (7%; $p < 0.01$).

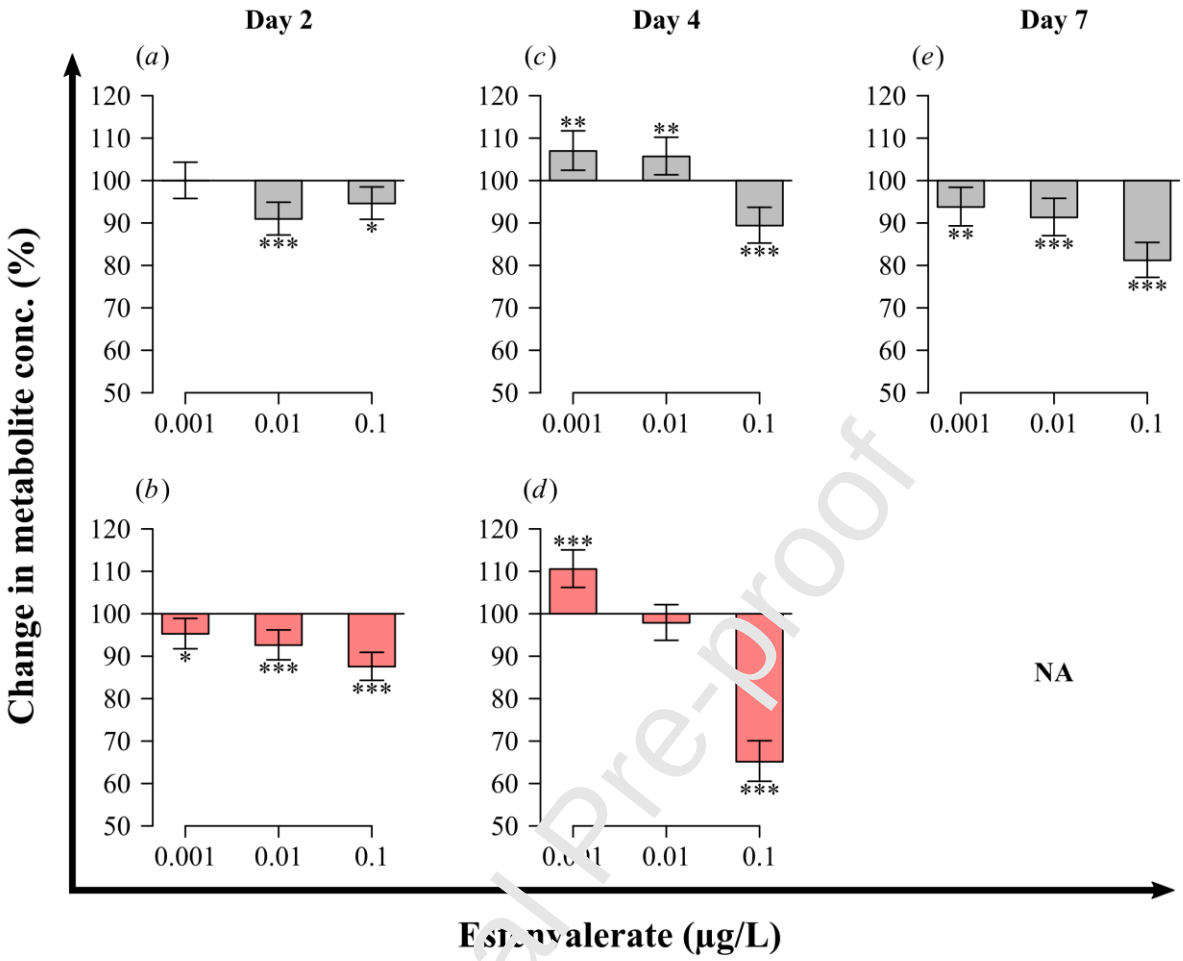


Figure 1 – Percent change in the overall metabolite content of *D. magna* exposed to esfenvalerate in relation to the respective control groups. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h under (a, c, e; grey) high and (b, d; red) low food conditions. Metabolites were measured (a, b) directly after an exposure of 24h, (c, d) on day 4 (after a recovery time of 48h) and (e) on day 7. Changes are presented with 95 % confidence intervals and statistical significance is indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

Effect of pesticide exposure on metabolite classes

To understand the effect of pesticide exposure in respect to the underpinning mechanisms, we identified changes in different metabolite classes. On day 2 and on day 4, we observed significant changes in two metabolite classes i.e., amino acids and glycerophospholipids under

high food conditions (Figure 2a, 3a). Whereas under low food conditions, we even observed significant changes in five classes i.e., amino acids, glycerophospholipids, sphingolipids, acylcarnitines and sugars (Figure 2b, 3b). Furthermore, the effects under low food conditions were significantly stronger compared to those under high food conditions (Table S3).

Under high food conditions, exposure to 0.1 and 0.01 µg/L significantly reduced the contents of amino acids by 17% and 13% respectively (Figure 2a, $p < 0.001$). Whereas, 0.001 µg/L marginally reduced the amino acids content (6%; $p = 0.07$). Similarly, exposure to 0.1 µg/L and 0.01 µg/L significantly reduced the contents of glycerophospholipid by 9% each ($p < 0.001$). Whereas, the lowest concentration marginally increased the glycerophospholipid content (5%; $p = 0.07$).

Under low food conditions, 0.1 and 0.01 µg/L each resulted in an 18% reduction in the amino acids content (Figure 2b, $p < 0.001$) followed by 0.001 µg/L (11%; $p < 0.001$). The decreased glycerophospholipid content was recorded at 0.001 µg/L (6%; $p < 0.05$) and 0.1 µg/L (15%; $p < 0.001$). However, reductions in sphingolipids and sugars were only observed at 0.1 µg/L (sphingolipids: 13%; $p < 0.1$, sugars: 28%; $p < 0.05$), and reduction in acylcarnitines was only recorded at 0.01 µg/L (11%; $p < 0.01$).

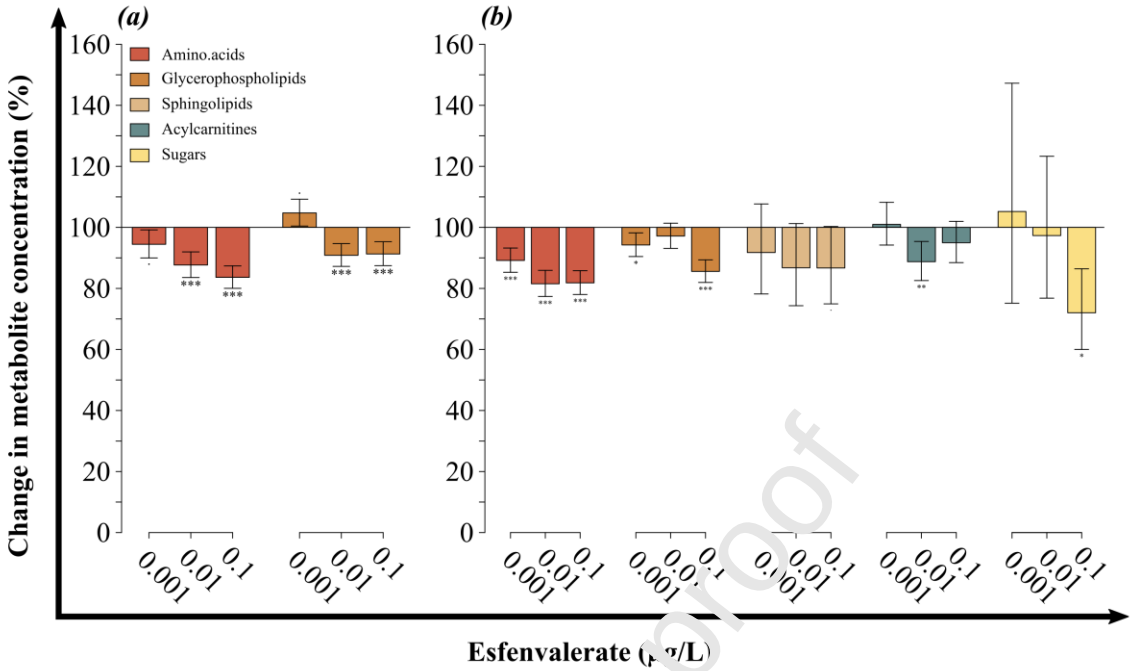


Figure 2 – Percent change in the content of different metabolite classes of *D. magna* exposed to esfenvalerate in relation to the respective control groups. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h. Metabolites were measured directly after an exposure of 24h under (a) high and (b) low food conditions. Only metabolite classes with significant perturbations are reported. Changes are presented with 95 % confidence intervals and statistical significance is indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

On day 4, there was an even greater reduction in the amino acids content of organisms exposed to 0.001 and 0.1µg/L ($p < 0.01$). Under high food conditions (Figure 3a), we observed down-regulation at 0.001 µg/L (13%; $p < 0.01$) and 0.1 µg/L (38%; $p < 0.001$). In contrast, exposure to 0.001 µg/L and 0.01 µg/L significantly increased the glycerophospholipid content by 17% and 19% respectively ($p < 0.001$). A reduction was only recorded at 0.1 µg/L (10%; $p < 0.001$). Under low food conditions (Figure 3b), the metabolite content of amino acids down-regulated at medium concentration (13%; $p < 0.01$) and the highest concentration (13%; $p < 0.01$). Similar to high food conditions, glycerophospholipids showed up-regulation at low and medium

concentrations (0.001µg/L: 16%; $p < 0.001$, 0.01µg/L: 5%; $p = 0.08$), and down-regulation at 0.1 µg/L (17%; $p < 0.001$). A significant reduction in sphingolipids, acylcarnitines and sugars was only observed at the highest concentration 0.1 µg/L (sphingolipids; 46%, acylcarnitines; 24%, sugars; 70%; $p < 0.01$).

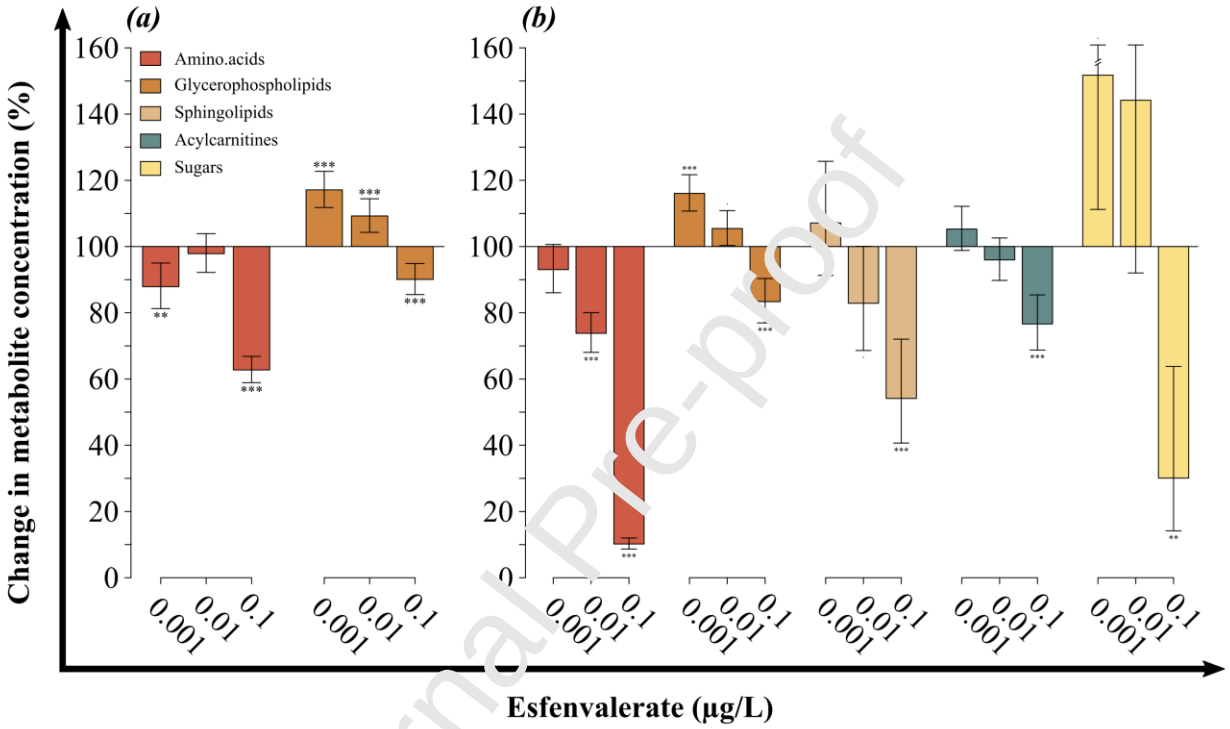


Figure 3 – Percent change in the content of different metabolite classes of *D. magna* exposed to esfenvalerate in relation to the respective control groups. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h. Metabolites were measured on day 4 (after a recovery time of 48h) under (a) high and (b) low food conditions. Only metabolite classes with significant perturbations are reported. Changes are presented with 95 % confidence intervals and statistical significance is indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

On day 7, we only presented the results for high food conditions as the number of replicates under low conditions was not sufficient due to such high mortality. We observed a significant reduction in the contents of four metabolite classes including amino acids, glycerophospholipids,

sphingolipids and acylcarnitines (Figure 4). In amino acids and glycerophospholipids, the reduction was recorded at all concentrations ($p < 0.01$). The reduction in amino acids content was increased considerably with an increasing concentration of esfenvalerate. The highest concentration 0.1 µg/L resulted in a 19% reduction in metabolic content ($p < 0.001$) followed by a medium concentration 0.01 µg/L (9%; $p < 0.001$) and the lowest concentration 0.001µg/L (7%; $p < 0.01$). However, reduction in sphingolipids and acylcarnitines was only observed at the highest concentration (0.1 µg/L).

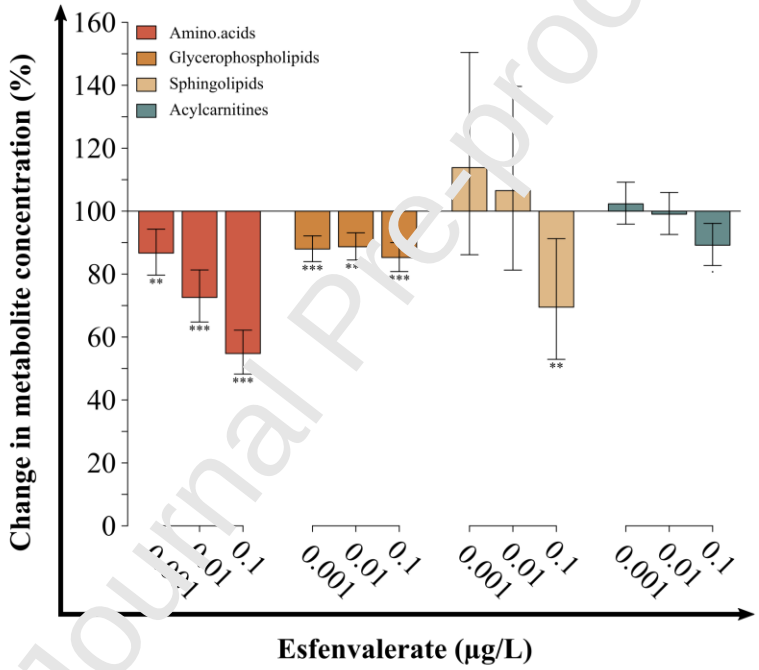


Figure 4 – Percent change in the content of different metabolite classes of *D. magna* exposed to esfenvalerate in relation to the respective control group. Daphnids were exposed to 0.001 µg/L, 0.01 µg/L and 0.1 µg/L of esfenvalerate for 24 h. Metabolites were measured on day 7 only under high food conditions. Changes are presented with 95 % confidence intervals and statistical significance is indicated with asterisks: "." $p < 0.1$ "*" $p < 0.05$ "**" $p < 0.01$ and "***" $p < 0.001$.

The effect of pesticide exposure on individual metabolites

Out of 160 metabolites, we revealed significant changes in 14 metabolites under high food conditions and 15 metabolites under low food conditions. In total, 13 metabolites from amino acids (arginine, glutamine, glycine, methionine, phenylalanine, descriptionproline, serine, threonine, tyrosine, leucine, histidine, ornithine and tryptophan), one long-chain phospholipid phosphatidylcholine diacyl C42:4 (PC aa C42:4), one acylcarnitine (hydroxyvalerylcarnitine C5-OH (C3-DC-M)) and one sugar showed significant down-regulation (Figure S1) at the highest concentration (0.1 µg/L). However, under low food conditions, phosphatidylcholine diacyl C42:4 and sugar also showed a significant change at the lowest concentration (0.001 µg/L). In general, the effect on day 4 was significantly stronger compared to day 2 (p <0.05, Figure S2, S3). Furthermore, the effect under low food conditions regarding individual metabolites was up to 7-fold stronger than under high food conditions (Figure S3).

DISCUSSION

Several investigations have reported significant effects of pesticides even at three to four orders of magnitude below the acute LC_{50} (Liess and Von Der Ohe, 2005; Schafer et al., 2012; Siddique et al., 2020). Reasons for these low effect concentrations and the unexplained variance of field effects on non-target invertebrates include an increased sensitivity of individuals to pesticides due to multiple stress conditions (Liess et al., 2016; Liess et al., 2019a). Aquatic organisms are often impacted by multiple stressors including agrochemicals and environmental conditions (Dinh et al., 2016; Hammock et al., 2015; Pieters et al., 2005). It is suggested that the presence of a large number of different toxicants in the field, coupled with environmental stress, compared to the null model of concentration addition (CA), could potentially result in synergistic interactions – contributing to unexpectedly high sensitivity of field populations (Liess et al., 2020). A meta-study showed that environmental stress severely enhances the toxicity of single pesticides (Liess

et al., 2016). Examples include food stress, competition, pathogens, salinity, elevated temperature or UVB radiation. Accordingly, we investigated how the food supply interacts with insecticide-induced biochemical fingerprints. Since food stress is supposed to directly affect the metabolism, we analyzed different metabolite classes that are associated with energy, and may affect survival, growth and reproduction. We hypothesized that the food stress can amplify the pesticide-induced metabolomic changes even at very low concentrations. In the present study, esfenvalerate exposure incurred significant effects on the overall metabolite contents of *Daphnia magna* at a concentration two orders of magnitude below the acute LC_{50}. Further, the effect was considerably stronger under low food conditions (Figure 1, Table S2). It is known that the detoxification mechanisms require a substantial investment of energy resources, therefore, starving organisms show a higher sensitivity to toxicants (Sibly, 1999).

Amino acids

Under both food conditions, we observed a significant decrease in amino acids content. Amino acids have been reported to play a significant role in the growth, reproduction and energy metabolism of *Daphnia magna* (Koch et al., 2011; Yebra and Hernández-León, 2004). During stress conditions, *Daphnia magna* may carry out gluconeogenesis to sustain the glucose levels required for survival. This process is characterized by the conversion of glucogenic amino acids (e.g. arginine, glutamine, glycine, methionine, proline, serine, tyrosine, histidine, tryptophan) into intermediates of pyruvate (Kokushi et al., 2012). Therefore, the down-regulation of glucogenic amino acids may be caused by their incorporation into energy production without their regeneration through proteolysis. Moreover, a general reduction can be attributed to the increased synthesis of antioxidant proteins in response to toxicant stress (Knops et al., 2001; Smolders et al., 2005). Both, the food limitation and pyrethroid esfenvalerate are known to cause

oxidative stress in organisms (Pascual et al., 2003; Yang et al., 2020). Moreover, the interaction between food limitation and toxicant can increase the combined effect of both stressors (Heugens et al., 2006; Shahid et al., 2019). A recent review concluded that the multiple stressors including both biotic and abiotic conditions may increase the oxidative stress in aquatic organisms (Vasquez et al., 2020). Therefore, the decrease of amino acids in the present study might imply that the esfenvalerate exposure caused more severe oxidative stress in *D. magna* especially under low food conditions. With regard to oxidative stress, the changes in glutamine, cysteine and glycine are of specific interest, since they constitute the building blocks for glutathione which is the major antioxidant in insects that is rapidly consumed under oxidative stress. Consequently, in the first phase of oxidative stress an increase in glutathione synthesis is often observed. A down-regulation in amino acids content has already been reported in fish *Clarias batrachus* exposed to chlorpyrifos Narra et al. (2011) and clam *Ruditapes philippinarum* exposed to arsenic (Wu et al., 2013). Lin et al. (2011) also reported down-regulation in amino acids of mice exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Contrary to our findings, Kovacevic et al. (2016) and Martin-Park et al. (2017) reported an increase in amino acid content in organisms exposed to pesticides. They considered an increase in amino acids levels as a result of protein breakdown, releasing free amino acids for energy metabolism under stress conditions (Gillis and Ballantyne, 1996).

Glycerophospholipids

In the present study, glycerophospholipids showed significant up- and down-regulations under both food conditions. When we analyzed the individual metabolites, we identified a significant reduction of long-chain phospholipids phosphatidylcholine diacyl C42:4 (PC aa C42:4) under low food conditions. Both the up- and down- regulation of glycerophospholipids in rats have

been associated with a high exposure to fipronil insecticide (Moser et al., 2015). Perturbations in glycerophospholipids have also been reported in *D. magna* exposed to different flame retardants (Scanlan et al., 2015; Stanley et al., 2013). Recently, Fuertes et al. (2018) reported the dynamics of glycerophospholipids in *Daphnia magna* exposed to juvenoids and bisphenol A. Glycerophospholipids are an integral component of most of the cellular membranes (Ecker and Liebisch, 2014; Hishikawa et al., 2014), involved in many vital functions, such as survival, growth, development and reproduction (Fuertes et al., 2018; Herrmansson et al., 2011; Kerr and Colucci, 2011). They also modulated the energy metabolism, neural activities and intracellular signaling pathways (Castro-Gomez et al., 2015; Farooqui et al., 2000). One of the two acyl chains in diacyl phospholipids can be arachidonic acid (20:4) that is involved in the synthesis of prostaglandin and the detoxification response. Therefore, a greater consumption of arachidonic acid – a building block of phospholipids – may cause a down-regulation of glycerophospholipids. Additionally, the observed change of glycerophospholipids in this study might be resulted from esfenvalerate-induced perturbation of neural cell membranes. Pyrethroid insecticides pose toxic effects by disrupting the voltage-dependent sodium channels in the nervous system, leading to a knockdown of the normal functioning of the nervous system and eventually to death (Davies et al., 2007). In this study, we assume that different doses of esfenvalerate triggered different defense mechanisms, resulting in an up- and down-regulation of metabolites. For instance, recently Shi et al. (2018) reported dose-dependent responses of earthworms exposed to different concentrations of a flame-retardant hexabromocyclododecane. Russo et al. (2018) also observed different levels of glycerophospholipids at different clothianidin concentrations and at different time points.

Sphingolipids

Sphingolipids exhibited up-regulation at the lowest concentration only under low food conditions, but down-regulated at the highest concentration under both food conditions. Like glycerophospholipids, sphingolipids are important constituents of neural membranes, and participate in a variety of indispensable metabolic, neurological, and intracellular signaling processes (Cole et al., 2012; Hermansson et al., 2011). Therefore, perturbation in sphingolipids could be attributed to the disturbance of neural cell membranes induced by the exposure to esfenvalerate. Moser et al. (Moser et al., 2015) also reported changes in sphingolipids levels as the result of fipronil exposure.

Acylcarnitines

Acylcarnitines are widely known to be involved in energy production through the β-oxidation of fatty acids and are therefore considered as a widely used marker for metabolic disorders in mammals (Indiveri et al., 2011; Rodriguez-Sanchez et al., 2015). In the present study, acylcarnitines significantly decreased under both food levels at the highest concentrations, suggesting a potential alteration in mitochondrial metabolism, energy production, and oxidative stress (Reuter and Evans, 2012). Recent investigations have suggested that the acylcarnitines also play a significant role in modulating neurotransmission (Jones et al., 2010). Therefore, down-regulation might be attributed to the potential neurotoxicity of esfenvalerate. Recently, Martin-Park et al. (Martin-Park et al., 2017) reported a significant decrease in acylcarnitine levels and associated it with the exposure to pyrethroid insecticide. However, organisms exposed to permethrin showed an up-regulation of acylcarnitines. By contrast, Russo et al. (2018) reported up-regulation at low concentrations, and down-regulation at high concentration of clothianidin. In the present study, we also observed an up-regulation of acylcarnitines at low concentrations, but it was not significant.

Sugars

A change in sugar was only recorded under low food conditions. Nutritional stress can induce significant changes in the metabolite composition of organisms (Wagner et al., 2015). In our study, this appeared to be the case of high stress, where low food increased the toxic pressure by interacting with esfenvalerate. Under high stress conditions, all available energy and metabolic capacity is dedicated to maintain the survival of an organism until the return of favorable conditions (Sokolova et al., 2012). Recently, Zhang, et al. (Zhang et al., 2020; Zhang et al., 2018) also reported a decrease in sugar levels of *Daphnia magna* exposed to multiple stress. In our study, a significant decrease in sugar contents indicate the metabolic efforts of the organism for recovery after exposure to esfenvalerate under low food conditions. However, most of the organisms could not survive until day 7 especially at higher concentrations. Therefore, we can assume that the perturbations of sugar in *Daphnia magna* were caused by the interaction of insecticide exposure and inadequate nutrition.

We can suggest glucogenic amino acids, phosphatidylcholine diacyl C42:4 and hydroxyvalerylcarnitine C5-OH (C3-DC-M) as putative biomarkers of metabolic dysregulation, oxidative stress and inflammation caused by esfenvalerate exposure. However, further studies are necessary to explore the consistency of metabolomic responses across different toxicants, concentrations and species to identify the role of individual metabolites in the toxicity of esfenvalerate.

CONCLUSIONS

Exposure to esfenvalerate, even well below the regulatory acceptable concentrations, was observed to manifest significant metabolic effects. Further, the effect was considerably stronger under low food conditions. An interaction between food- and chemical stress was mainly

responsible for high stress, and thereby increased the energy demand for survival. Altogether, a strong depletion of energy reserves – due to food stress – can directly translate into lower fitness and may explain changes in the freshwater ecosystem structure in the field. Further, the metabolomic changes might be a possible key to explain such unexpected effects.

AUTHOR CONTRIBUTIONS

Conceptualization: NS, ML and MvB; Study design: NS and URK; Investigation: NS and AS; Metabolomic analysis: NS and URK; Formal analysis: NS and ML; Writing Original Draft: NS; Review & Editing: All.

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Declaration of competing interests

☒ 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.

Highlights

- Endogenous metabolome was significantly affected even at very low pesticide exposure.
- Interaction between food stress and pesticides incurred stronger metabolic alterations.
- Metabolomic changes increased over seven days after contamination.