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Metabolomics reveals changes in metabolite profiles due to growth and metamorphosis during the ontogeny of the northern damselfly

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ABSTRACT

Many insects have complex life cycles where a drastic ontogenetic change happens between the larval stages and the adult stage, i.e. metamorphosis. Damselflies (order Odonata, suborder Zygoptera) are widely distributed and ecologically important semi-aquatic insects with a complex life cycle. Phenotypic changes over damselfly ontogeny have been documented, however, if and how metabolite profiles are also changing is currently unknown. Here we used a metabolomics methodology to gain insights into the metabolic changes during the life cycle of the Northern damselfly (*Coenagrion hastulatum*). Hatchlings of wild-caught damselflies were reared in the laboratory and metabolomics analyses using liquid chromatography and gas chromatography coupled to mass spectrometry were carried out at three larval stages and on adult damselflies. Additionally, a subset of larvae was exposed to wastewater effluent to assess how metabolite profiles responded to an environmental stressor. A total of 212 compounds belonging to several classes (e.g. amino acids, fatty acids, sugars) were annotated. Across metamorphosis, we found that damselflies shifted from protein catabolism to lipid catabolism. Wastewater effluent exposure resulted in ontogenetic stage-dependent changes of individual metabolites, but not to a marked extent. Overall, our study is one of the first to describe changes of metabolite profiles during ontogeny of an insect, and it provides a first step towards a greater understanding of the physiological changes occurring during general insect—but especially damselfly—ontogeny.

1. Introduction

An increasing number of studies are reporting that insects are declining across the world, and the reason(s) for this is currently not well understood (Knops et al., 1999; Biesmeijer et al., 2006; Hallmann et al., 2017; Córdoba-Aguilar and Rocha-Ortega, 2019; Wagner, 2020). In some cases it correlates well with climate change (Hallmann et al., 2017), in other studies decline is apparent regardless of habitat type, climate change, land use, or habitat characteristics (Lister and Garcia, 2018). However, several studies have highlighted that increased exposure to wastewater effluent is one of the contributing factors to the observed decline (e.g. Lister and Garcia 2018; Córdoba-Aguilar and Rocha-Ortega, 2019). The lack of evidence for why insects are declining is partly because these declines were demonstrated fairly recently, and partly because the tools needed to study subtle physiological effects of environmental factors (e.g. chemicals, temperature) have to be expanded to the tools closest to phenotype. Thanks to recent advances in analytical techniques that allow screening of metabolites, i.e. small molecules required for growth and maintenance of a cell, via chromatographic analyses, we can now study the physiological changes associated with a variety of conditions (Dettmer et al., 2007; Khodayari et al., 2013). This metabolomics approach has been employed in several entomology studies to investigate the role of metabolites associated with insect behaviour and phenotype (Snart et al., 2015), as well as physiological responses to challenging environmental conditions such as temperature stress (Robert Michaud et al., 2008; Chou et al., 2017; Hidalgo et al., 2019) and hypoxia (Hines et al., 2007; Agbo et al., 2013; Venter et al., 2018). The overarching strength of metabolomic outcomes

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is that they reflect the end stage of cellular biochemical cascades in response to environmental factors and other signalling events, and thereby provide insights into the phenotype and functional status of an organism (Bundy et al., 2009). Hence, the metabolomics methodology offers data on metabolite profiles and pathways that are most relevant to phenotype among the omics techniques.

One group of insects especially suited for studying effects of environmental factors are the damselflies (class Insecta, order Odonata, suborder Zygoptera). Damselflies are abundant on every continent except Antarctica and are highly suitable as sentinel species because they are highly resilient and easily captured. Damselflies are semi-aquatic invertebrates. They start as aquatic predatory larvae and then emerge into the terrestrial environment – and as such are affected by both aquatic and terrestrial conditions during their ontogeny (Janssens and Stoks, 2017). Their complex life cycle comprises a drastic ontogenetic shift via metamorphosis between larval stages (that are adapted for growth and development) and the adult stage (Wilbur, 1980; Stoks and Córdoba-Aguilar, 2011). Typically, ontogenetic changes are associated with a change in life-history traits, behaviour, morphology, and physiology. Changes in behaviour and physiology associated with the damselfly life cycle have been well documented, e.g. anti-predator responses (Richardson and Anholt, 1995; Brodin et al., Johansson, 2006), as well as thermal sensitivity during damselfly (Nilsson-Örtman et al., 2013) and other insects' life cycles (Kingsolver and Buckley, 2020). Physiological changes due to environmental stress often occur prior to changes in traditional life history traits (Viant, 2007; Janssens and Stoks, 2013; Jeong and Simpson, 2019). Moreover, seemingly subtle physiological changes can induce major effects on individuals and, as a consequence, can have cascading effects up to the population level (Brodin et al., 2014). Damselfly larvae are a very important ecological group because they play a key role in aquatic food webs, both preying on smaller invertebrates and serving as prey for bigger invertebrates and fish (Boroń and Mirosławski, 2009; Jonsson et al., 2014; Janssens and Stoks, 2017). Thus, any factor impacting the phenotype of damselflies, has the potential to impact both aquatic and terrestrial ecosystems (Janssens and Stoks, 2017).

The overall aim of this study was to identify physiological changes over damselfly ontogeny by elucidating variations in metabolite profiles to enhance our understanding of the species' physiology. A change in metabolite profiles reflective of activated metabolic pathways is expected across ontogeny since substrates and enzyme activity is likely to shift in association with other changes (e.g. in life-history traits, behaviour, etc). We used mass spectrometry (MS) analytical protocols with both liquid and gas chromatography (LC and GC) methods for the separation of molecules to measure baseline metabolite profiles in three larval stages and in the adult stage of the northern damselfly (*Coenagrion hastulatum*). In addition, to test how ontogeny affects the damselfly's ability to maintain homoeostasis under environmental stress, we also measured metabolite abundances in a subset of damselflies that were exposed to treated wastewater effluent as larvae in a pilot study under the assumption that the response is larval stage-dependent. Wastewater effluent represents an external, environmentally realistic and important challenge for a wide range of aquatic organisms, because it contains a complex mixture of anthropogenic pollutants suggested to play a role in the insect decline (e.g., insecticides and heavy metals) (Feldhaar and Otti, 2020). We propose that metabolomics studies of metabolites close to phenotype in non-model organisms, such as damselflies, can deepen our understanding of both their physiology, as well as their resilience to different environmental challenges.

2. Materials and methods

A subset of metabolites in fatty acid metabolic pathways (oxylipins) has previously been investigated in lab-reared damselflies (*Coenagrion hastulatum*) at different ontogenetic stages (Späth et al., 2021). Here, we expanded on the metabolomics methodology and covered a larger set of metabolites by the use of GC–MS and LC-MS analyses of the same individuals.

2.1. Rearing of damselflies

Four females, in copula, of the northern damselfly (*Coenagrion hastulatum*) were collected at a local lake in Umeå municipality, Sweden and brought to a laboratory at Umeå University for egg laying (for full rearing protocol, see Späth et al. (2021). The eggs were then submerged in aerated aquariums filled with aged tap-water until hatching. After hatching, the larvae (N = 108) were placed individually in small aquaria (10 × 10 cm) containing aerated aged tap water. The larvae were fed three times per week with size-matched zooplankton obtained from a local pond as long as weather conditions allowed, then daily with brine shrimp (*Artemia salina*) and zooplankton cultivated in-house. Larvae were checked daily for survival and change of developmental stage, i.e., instar, indicated by exoskeleton shedding. Northern damselflies undergo a total of eleven instars from hatching to emergence. Duration of their life cycle ranges from one to four years, depending on climatic conditions (Norling, 1984). The instar at which sampling for analysis took place was randomly preselected for each individual. In total, three instars (L-5, L-3, L-1) were selected together with the final stage (L-0), where the larva emerged as an adult (Fig. 1). When the larvae had molted into the preselected instar, they were placed individually in 2 mL microcentrifuge tubes and stored at −20 °C until analysis (n = 21, 24, 21, 27 for L-5, L-3, L-1, L-0, respectively). Adults were frozen when found (without provision of food), maximum 24 h after emergence. As a pilot study to measure the effects of an environmental challenge on metabolite profiles, a subset of larvae had their aerated water replaced by treated wastewater effluent (obtained from a local wastewater treatment plant; see Supplementary text for details) for the duration of the larval stage prior to the preselected stage, e.g., larvae selected for L-3 were exposed from the time they reached L-4 until they molted into L-3 (n = 6, 3, 6 for L-3, L-1, L-0, respectively). On average, this resulted in exposure duration of 22, 16 and 34 days for L-3, L-1 and L-0, respectively (effluent exposure had no influence on the number of days the larvae spent transitioning from one stage to another; however larval mass was significantly lower in effluent exposed larvae at larval stage L-3; Student's t-test, $p < 0.05$, data not shown).

2.2. Sample preparation

Extractions were performed from whole-body individuals as previously described (Späth et al., 2021). 1.5 mL acetonitrile/water (90/10), containing internal standard (IS) mix A (Table S1 in the Electronic Supplementary Material) were added to each larva. Homogenisation was performed by shaking each sample for 3 min at 30 Hz in a mixer mill (MM400, Retsch Technology, Haan, Germany) with stainless steel beads (3 mm of diameter). Then the samples underwent centrifugation for 10 min at 14,000 RPM, the supernatant was collected, and the pellet re-extracted with 1.5 mL 90% ACN (not containing IS) a second time. Supernatants derived from each sample were combined and aliquots of 300 and 150 μL transferred to LC and GC vials, respectively. After evaporation under vacuum (MiniVac system, Farmingdale, NY, USA), samples were stored at −20 °C until analysis.

2.3. LC-MS analysis

Prior to LC-MS analysis, samples were reconstituted in 20 μL of methanol/water (50/50) containing IS mix B (Table S1). 2 μL was injected onto an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an Acquity UPLC HSS T3 C18 column (2.1 × 50 mm, 1.8 μm) and a 2.1 mm × 5 mm, 1.8 μm VanGuard precolumn (Waters Corporation, Milford, MA, USA). Compounds were eluted with a 10.8 min gradient of ACN/isopropanol (75/25) (Table S2). Compounds were detected with an Agilent 6550

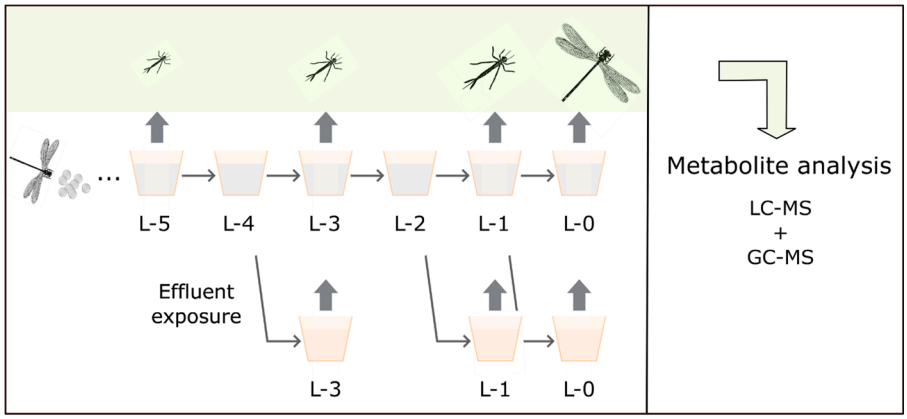

Fig. 1. Experimental set-up. Damselfly larvae were reared individually until they reached their preselected stage (L-5, L-3, L-1, or L-0) at which the metabolite analyses were carried out.

quadrupole time-of-flight mass spectrometer (Q-TOF) equipped with an Agilent Jet stream Electrospray Ion Source operating in positive or negative ionisation mode (separate injections), see Electronic Supplementary Material for settings.

LC-MS data processing was performed using the Agilent Masshunter Profinder software (version 10.0, Agilent Technologies Inc., Santa Clara, CA, USA). Several in-house LC-MS libraries built up by authentic standards run on the same system with the same chromatographic and MS settings, were used for identification purposes using the software's Batch Targeted extraction feature. The libraries covered a wide range of compound classes, including amino acids, carnitines, fatty acids, lysophospholipids, nucleotides, peptides, and steroids. However, since the libraries included a significant number of compounds present in samples from species other than insects (for example humans, plants, bacteria, etc.), it was expected that not all metabolites present in the libraries could be identified in our samples.

2.4. GC–MS analysis

Prior to GC–MS analysis, 50 μL of methanol containing IS mix C (Table S1) was added to the vials with the dried extracts and solvents were evaporated. Derivatisation was then performed according to the protocol in the Electronic Supplementary Material. 0.5 μL of the derivatised extract was injected splitless by an L-PAL3 autosampler (CTC Analytics AG, Switzerland) into an Agilent 7890B gas chromatograph equipped with a 10 m × 0.18 mm fused silica capillary column with a chemically bonded 0.18 μm Rxi-5 Sil MS stationary phase (Restek Corporation, U.S.). The column effluent was introduced into the ion source of a Pegasus BT Q-TOF (Leco Corp., St Joseph, MI, USA), see Electronic Supplementary Material for settings.

GC–MS data files were exported from the ChromaTOF software in NetCDF format to MATLAB R2016a (Mathworks, Natick, MA, USA), where all data pre-treatment procedures such as baseline correction, chromatogram alignment, and peak deconvolution were performed. The extracted mass spectra were identified by comparisons of their retention index and mass spectra with libraries of retention time indices and mass spectra (Schauer et al., 2005). Identification of compounds was based on comparison with mass spectra libraries (in-house database) as well as retention index using the NIST MS 2.0 software.

2.5. Statistical analysis

Multivariate analysis of the combined LC–MS and GC–MS data set was performed using SIMCA software version 14.1 (Sartorius AB, Umeå, Sweden). Variables (metabolites) were scaled to unit variance (autoscaled and mean-centered) in the calculated models. Unsupervised principal component analysis (PCA) was first carried out to identify patterns in the data and check for trends and outliers (Smilowitz et al., 2013). Then, supervised Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was performed to identify the most important metabolites (ranked by variable importance in the projection, VIP) in discriminating between the developmental stages. The OPLS-DA models were evaluated in terms of their goodness of fit (R2Xcum, R2Ycum) and goodness of prediction (Q2cum). R2Xcum is the cumulative modelled variation in X, R2Ycum is the cumulative variation in X correlated to Y and Q2cum estimates the cumulative predictive ability of the model. In addition, to assess the degree of overfitting of the OPLS-DA models, p-values were determined by cross-validated analysis of variance (CV-ANOVA) and permutation analyses were carried out. A shared and unique structures (SUS) plot was built by plotting scaled loadings (p(corr)) from two OPLS-DA models to visualise shared and unique variables across the models (Wheelock and Wheelock, 2013). Possible pathways involved in metamorphosis between final larval stage L-1 and adults (L-0) were assessed by pathway analysis using MetaboAnalyst 4.0, a web tool for metabolomics data analysis (http://www.metaboanalyst.ca/) (Chong et al., 2019).

To identify metabolites affected by exposure to wastewater effluent, sparse projection to latent structures discriminant analysis (sPLS-DA) was carried out using the mixOmics package in R (Rohart et al., 2017). Each platform (i.e., GC–MS, LC-MS) and each developmental stage were analysed separately (i.e. L-3, L-1, L-0). The performance and tuning functions implemented in sPLS-DA identified the number of components and the metabolites loading on those components to maximise covariance between the exposed and unexposed groups. Within each model and each component, any metabolite with a VIP score >1 was followed up with a linear model to identify statistical differences among treatments (Gorrochategui et al., 2016). VIPs were provided by the MixOmics package (Rohart et al., 2017) which recommends that predictors with VIP > 1 are the most relevant for explaining Y, we therefore used this threshold. All contrasts were corrected for multiple testing using Benjamini-Hochberg for each sPLS-DA.

3. Results and discussion

3.1. Baseline metabolite profiles across damselfly developmental stages

A total of 125 compounds were annotated using LC-MS analysis of extracts from three larval stages and the adult stage. These compounds included amino acids (14), carnitines (35), lysophospholipids (28), nucleotides (5), peptides (15), steroids (1), fatty acids (15), sugars (1), and 11 metabolites that did not fall into the above categories (Table S3). Using GC–MS, 126 compounds were detected of which 87 were annotated. Annotated compounds included amino acids (26), fatty acids (15), nucleotides (3), sterols (3), sugars (20), sugar acids (9), and 11

metabolites that did not fall into the above categories (Table S3). Only compounds detected in all stages were included in statistical analysis.

First, unsupervised multivariate analysis (PCA) was carried out to get an overview of the variation within the data (combined LC-MS and GC–MS dataset; non-exposed individuals). The first two principal components (PC) explained 55% of the variability in the combined dataset (93 observations, 251 variables). The PCA t1/t2-score plot (Fig. 2) revealed overlapping and unique metabolite profiles for the four life stages. For the three larval stages a linear trend was observed from the earliest to the latest stage (L-5 to L-3 to L-1), indicating an increase of total metabolite concentrations, which can be related to growth. Accordingly, PCA showed clustering patterns of the damselfly larvae according to mass along PC1 (Fig. S1A), which was confirmed in a linear regression of insect mass against PC1 scores (Fig. S1B) indicating that PC1 is due to increased mass and an increase in metabolite detection. A clear separation was found for the adult damselflies, as their profiles differed to the greatest extent from damselfly larval stages, suggesting differentiation of metabolite profiles due to metamorphosis. Our previous study targeting fatty acid metabolites (oxylipins) in the same individuals showed a different pattern, with the two intermediate stages (L-3 and L-1) displaying similar oxylipin profiles, whereas the earliest stage (L-5) and adult stage (L-0) were more dispersed in the score plot (Späth et al., 2021). These discrepancies in metabolite profiles were due to different pathways being explored with different analytical platforms and highlight the importance of standardised metabolomics protocols in order to facilitate comparisons of metabolic pathways between studies.

Secondly, supervised multivariate analysis (OPLS-DA) maximising separation between the groups revealed differential metabolites across damselfly larval stages (L-5 vs L-3, L-3 vs L-1, L-5 vs L-1) and between larval and adult stage (L-1 vs L-0), with p-values from cross-validated analysis of variance (CV-ANOVA) ranging from 2.35×10^{-32} to 5.55×10^{-9} and sufficient predictive ability Q2cum > 0.6 (see Table S4 for model parameters). Permutation tests (Fig. S2) indicated that the models were not influenced by overfitting.

Metabolites deemed to be important for differentiating between ontogenetic stages were ranked according to the VIP scores. A total of 105, 119, 126, and 103 metabolites, in the L-5 vs L-3, L-3 vs L-1, L-5 vs L-1, and L-1 vs L-0 models, respectively had VIP scores >1 (see Supplementary Data File). Among these, metabolites from several categories including amino acids, fatty acids, nucleotides, and sugars were identified as the most important metabolites in the models.

To investigate the changes of these metabolites during the life stages, the scaled loadings (p(corr)) from two OPLS-DA models, L-5 vs L-1, representing growth, and L-1 vs L-0, representing metamorphosis, were plotted in a SUS plot (Fig. 3). This enabled the distinction of shared and unique features of the two models (Wheelock and Wheelock, 2013). Metabolites with shared patterns plotted along the diagonals, whereas metabolites with unique patterns plotted along the respective axes (see Fig. 3 and Supplementary Data File for metabolites p(corr) > 0.5). Hence, metabolites playing a unique role in larval development were plotted along the y-axis. Most metabolite abundances were increasing over the larval stages (e.g. Lyso-PC (16:0), Fig. 3G), which is in accordance with the patterns observed in the PCA. Only two metabolites (glutamate in Fig. 3B and β-alanine) were down-regulated during larval growth. Unique effects of metamorphosis plotted along the x-axis and were mostly metabolites that were upregulated in L-0 (e.g. threonate, Fig. 3D). An example of a down-regulated compound after metamorphosis was N-acteyl galactosamine (Fig. 3E).

MetaboAnalyst pathway analysis was used to assess metabolic pathways correlated with metamorphosis since it represented a specific shift as opposed to merely growth during the larval stages. According to the MetaboAnalyst assessment, top pathways were the D-glutamine and D-glutamate metabolism and alanine, aspartate and glutamate metabolism (Fig. 4). However, biosynthesis of aminoacyl-tRNA as well as valine, leucine and isoleucine were the only significantly enriched pathways after adjusting for multiple testing by the Holm-Bonferroni test ($p < 0.05$) (see Supplementary Data File).

Taken together, the results suggest that metamorphosis induced a shift mainly in amino acid metabolism implying that amino acid metabolism plays a key role in the early life stages of damselflies. Amino acids

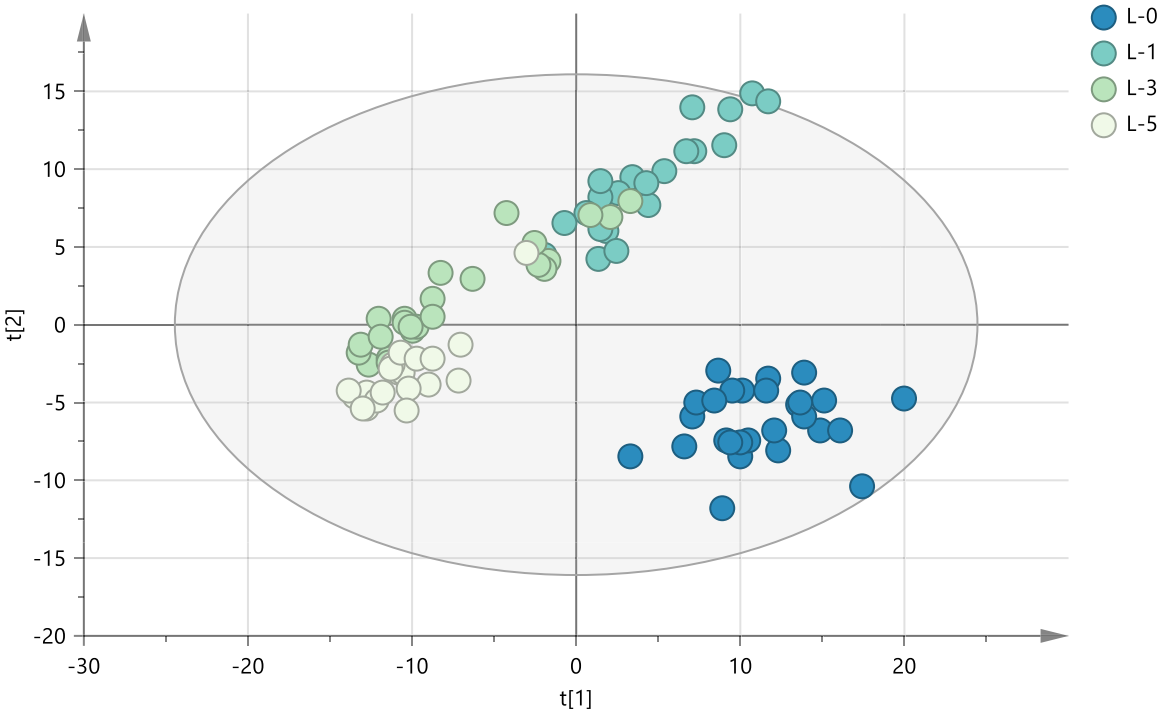

Fig. 2. Principal component analysis (PCA) score plots of damselfly metabolite profiles. Each observation was coloured according to ontogenetic stage L-5, L-3, L-1, and L-0, respectively. Variation explained by PC1 and PC2 were 38% and 17%, respectively. The first component t1 explains the largest variation, t2 explains second largest variation.

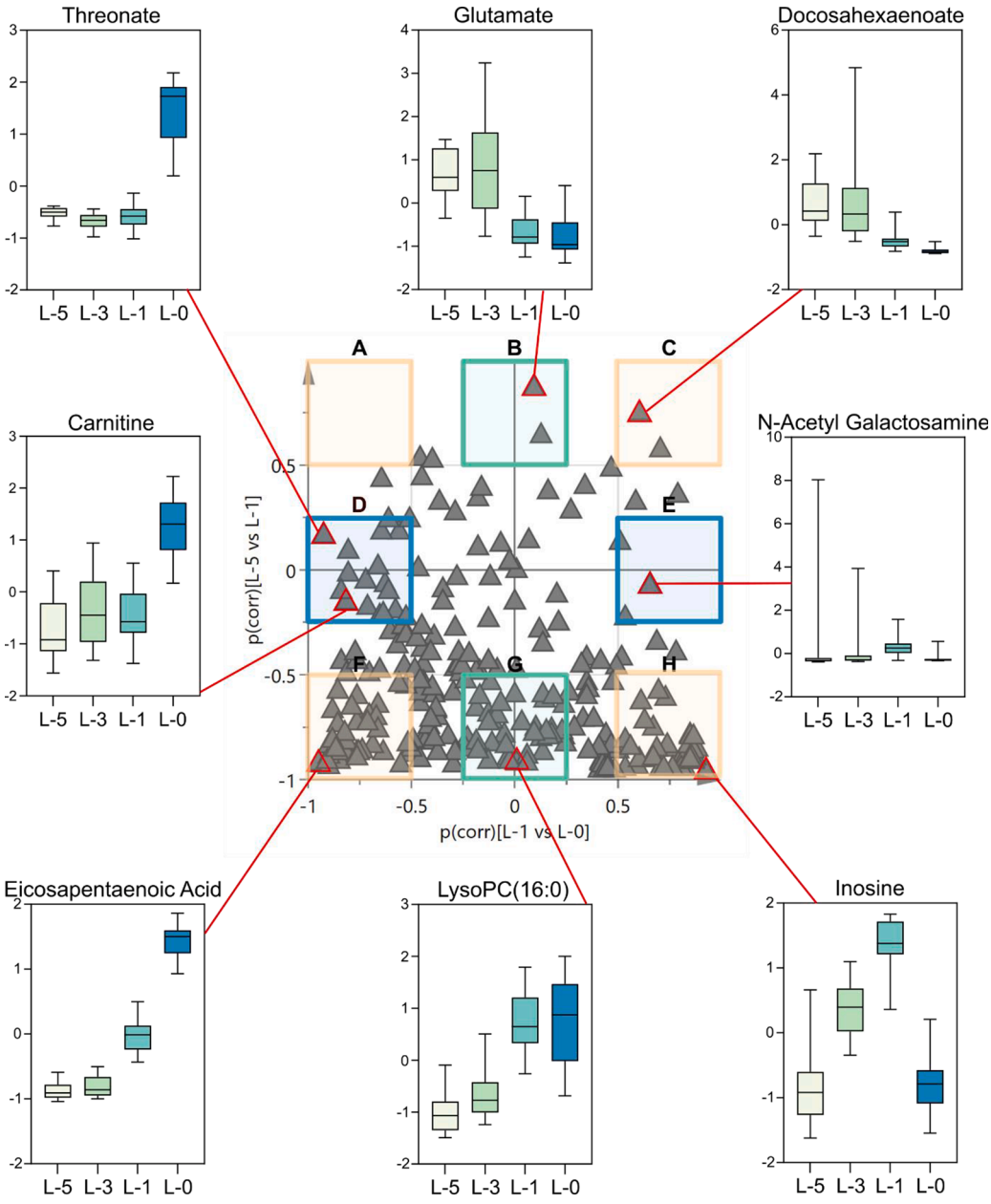

Fig. 3. Shared-and-unique-structures (SUS) plot of two Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) models for instars L-5 vs L-1, representing growth, and L-1 vs L-0, representing metamorphosis and box plots of exemplary metabolites (red triangles in the SUS plot) at the four instars L-5, L-3, L-1, and L-0. Box plots show metabolite levels that were auto-scaled and mean-centered). Shared structures (orange): Metabolites that were equally important for both models: metabolites up- (F) and down-regulated (C) in both models; metabolites up-regulated during larval development and down-regulated after metamorphosis (H) – and vice-versa, displayed by none of the metabolites (A). Unique structures: Metabolites unique for larval development (green): up- (G) and down-regulated (B) during larval development. Metabolites unique for metamorphosis (blue): up- (D) and down-regulated (E) after metamorphosis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

are commonly used as neurotransmitters and for protein synthesis by invertebrates and other insects (Schuster et al., 1991; Walker and van der Donk, 2016). There is limited knowledge on metabolic shifts associated with invertebrate metamorphosis. However, two recent studies have shown that amino acids, i.e. glutamate and aspartate, play a role in metamorphosis in other species (ascidian embryos Hirai et al., 2018; Pacific oyster, *Crassostrea gigas* Vogeler et al., 2019), which is in line with our findings.

Moreover, catabolism of proteins during the progression of larval stages and a domination of lipid catabolism closer to metamorphosis was recorded in yet another species, *Farfantepenaeus paulensis* (Lemos and Phan, 2001). The same pattern was reflected in our data, where for instance levels of the amino acid glutamate were low closer to metamorphosis (Fig. 3B), while eicosapentaenoic acid (EPA) was higher (Fig. 3F). Other upregulated metabolites over larval development and at metamorphosis included a range of fatty acids beyond EPA, as well as carnitines (consisting of fatty acids and responsible for their transport for energy production, see Fig. 3) showing the same trend as EPA (see Supplementary Data File). Furthermore, we found lower levels of several amino acids and peptides after metamorphosis, these are building blocks for proteins (Supplementary Data File). Therefore, our results corroborate previous findings suggesting a shift from protein catabolism to lipid catabolism through metamorphosis. Such metabolic shift could possibly be linked to morphological and behavioural changes necessary for the transition from the benthic habitat to land, accompanied by a change of energy requirements, which was similarly discussed by Lemos and Phan (2001) in the context of another species (*Farfantepenaeus paulensis*). For example, lipids are an important energy source for flying insects (Arrese and Soulages, 2010). However, more research is needed to verify these assumptions.

This study is a first attempt to associate shifts in global metabolite profiles during the ontogeny of the northern damselfly (*Coenagrion hastulatum*) to physiologically relevant metabolic pathways, and as such demonstrates proof-of-principle for ontogeny metabolomics study of this species. Metabolomics in developmental studies has revealed stage-dependent metabolite profiles throughout the life cycle of *Drosophila melanogaster* (An et al., 2017) and pearl oyster (Zhang et al., 2021). Furthermore, metabolic reorganisation was detected at metamorphic

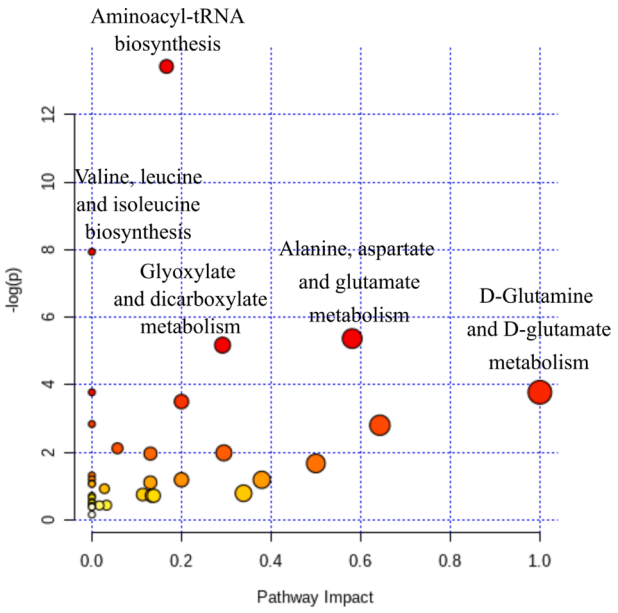

Fig. 4. Pathway analysis of damselflies before and after metamorphosis. The x-axis represents the pathway impact score (0–1) and the y-axis (−log(p-value)) marks the pathway enrichment score. Each circle marks a pathway, larger sizes and darker colours suggest higher pathway impact and higher pathway enrichment (lower p-values).

climax in frogs (Zhu et al., 2020). Commonalities between underlying patterns were few, suggesting that metabolic shifts during developmental stages are species-specific. However, standardised analytical protocols in future metabolomics studies are needed to validate the findings. Assigning function to upregulated or downregulated metabolites is beyond the current study design in which whole-body samples and the metabolism database of another species (*Drosophila melanogaster*) was used as reference, in lack of a *Coenagrion hastulatum* database. The availability of insect metabolism platforms is scarce, and there is limited knowledge about the generality of metabolic pathways between species, which underline the need for more research in this area.

3.2. Metabolite response to an environmental stressor

Linear models were used to investigate significant changes in metabolite profiles (at three different stages) of larvae grown in wastewater effluent instead of aerated water for the duration of one larval stage prior to the stage that was analysed. Overall, effluent exposure resulted in a change of metabolite profiles depending on the respective ontogenetic stage. Out of the 251 detected compounds, a total of 10, 26, and 4 metabolites were significantly affected in larval stage L-3, L-1, and the adult stage L-0, respectively (Table 1, corrected using Benjamini-Hochberg). Effluent exposure resulted in elevated metabolite levels, ranging from a 1.2 – 18-fold change, with only one exception, campesterol, that was reduced after exposure in the final instar (0.72-fold change). Affected metabolites varied greatly between larval stages. Only β-D-glucopyranose (glucose) was affected by the exposure in more than one larval stage (L-3 and L-1) and three un-annotated compounds (NAs) in L-1 and L-0, respectively. Based on 36 metabolites with VIP > 1 in the most affected larval stage L-1, pathway analysis revealed that biosynthesis of phenylalanine, tyrosine and tryptophan was the most impacted pathway (Fig. S3 and Supplementary Data File). An earlier study (Van Praet et al., 2014) correlated pollutant load with reduced body mass, which is in line with our results. Even though larvae development focuses mainly on growth, there are some physiological differences between the different instars, e.g. changes occur in the sensory system

Table 1
Significant* effects of wastewater effluent exposure on LC-MS and GC–MS metabolites in damselflies at three ontogenetic stages (L-3, L-1, L-0). Fold change > 1 means that the intensity of the metabolite was higher in the exposed damselflies than in the non-exposed individuals of the same stage. Larvae were exposed for the duration of one larval stage prior to the analysed one. *Corrected for multiple comparisons using Benjamini-Hochberg.

Stage	Metabolite	Fold change	Method
L-3	Beta-D-Glucopyranose	6.4	GC–MS
	2-Pentadecanoyl-GPC (15:0)	4.4	LC-MS
	LysoPC(16:1)	3.9	LC-MS
	LysoPC(14:0)	3.8	LC-MS
	LysoPC(20:5)	3.1	LC-MS
	2-Arachidonoyl-GPC (20:4)	2.9	LC-MS
	Pantothenic Acid	2.5	LC-MS
	Isovalerylcarnitine	2.1	LC-MS
	2-Oxoisocaproic acid	1.9	LC-MS
	Tryptophan	1.3	LC-MS
L-1	N-Acetyl-Galactosamine	18	GC–MS
	NA 34	8.7	GC–MS
	NA 14	7.6	GC–MS
	Proline [+CO2]	5.7	GC–MS
	NA 06	4.3	GC–MS
	Beta-D-Glucopyranose	3.9	GC–MS
	NA 19	3.2	GC–MS
	Phenylalanyltryptophan	2.6	LC-MS
	Beta-Alanine	2.6	GC–MS
	Adipic Acid	2.3	GC–MS
	NA 27	2.3	GC–MS
	NA 31	2.3	GC–MS
	Octanoylcarnitine	2.2	LC-MS
	NA 25	2.1	GC–MS
	Homocysteine	1.9	GC–MS
	Squalene	1.9	GC–MS
	Eicosapentaenoic Acid	1.7	GC–MS
	NA 08	1.7	GC–MS
	3,6-Anhydro-D-Galactose	1.4	GC–MS
	Homoserine	1.4	GC–MS
	Phenylalanine	1.4	GC–MS
	Glycerol-2-Phosphate	1.3	GC–MS
	Ribose	1.3	GC–MS
	Tyrosine	1.3	GC–MS
	Uracil	1.3	GC–MS
	NA 05	1.2	GC–MS
L-0	NA 27	2.6	GC–MS
	NA 31	2.6	GC–MS
	NA 25	2.1	GC–MS
	Campesterol	0.73	GC–MS

(Chapman, 2013). Several studies have shown instar specific toxicity which further demonstrates the physiological differences and that usually the early life stages are more sensitive (Jin et al., 2014), but not always (Khan and Hamed, 2005). While implications of these disruptions are hard to foresee at the individual level, a change in amino acid composition of invertebrate communities could potentially affect the fitness of higher consumers (Dwyer et al., 2017).

However, overall, the results suggest that the primary metabolism is robust to realistic environmental challenges, such as wastewater effluent that we investigated in this study, because the majority of metabolites were unaffected by the effluent exposure, as opposed to the findings on oxylipins where close to 60% of the detected fatty acid metabolites were responsive to a significant extent to effluent exposure in at least one of the stages (Späth et al., 2021). This is a plausible finding, since organisms benefit from maintaining homeostasis, i.e. a relatively stable internal state reflected in the primary metabolism, while specific pathways such as the fatty acid metabolism containing for instance enzymatic drug targets may be sensitive to pharmaceuticals and other anthropogenic pollution. In this pilot study of exposure effects, relatively few individuals were included, therefore effects on the primary metabolism of wastewater effluent exposure cannot be excluded. More studies with larger sample sizes are needed to investigate damselflies

reared long term (from eggs to adult stage) across different exposure scenarios to identify potential biomarkers of environmental pollution.

4. Conclusions

This study provides novel insights into the metabolite profiles (baseline metabolism) of damselflies during different stages of ontogeny and shows that the physiological characteristics of damselflies are not stable over development. LC-MS and GC–MS based metabolomics successfully identified a number of amino acids, fatty acids, peptides, sugars and intermediates of cellular metabolism that were up- or down-regulated during the damselfly ontogeny. Metamorphosis induced a specific shift of metabolites from protein catabolism to lipid catabolism. Furthermore, the study indicates limited effects on metabolite profiles in response to an environmental challenge, i.e. wastewater effluent exposure, and the effects were dependent on the respective ontogenetic stage. Overall, our study is the first to describe changes of metabolite profiles during ontogeny in any insect and provides a first step towards a more detailed understanding of physiological changes during insect ontogeny in general and damselfly ontogeny in particular.

5. Data statement

The metabolomics and metadata reported in this paper are available via the Metabolights repository www.ebi.ac.uk/metabolights/MTBLS2378, study identifier MTBLS2378.

CRediT authorship contribution statement

Jana Späth: Conceptualization, Investigation, Formal analysis, Writing – original draft, Visualization. **Tomas Brodin:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Erin McCallum:** Formal analysis, Writing – review & editing. **Daniel Cerveny:** Investigation. **Jerker Fick:** Conceptualization, Funding acquisition. **Malin L. Nording:** Supervision.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2021.104341.

References

Agbo, S.O., Keinänen, M., Keski-Saari, S., Lemmetyinen, J.h., Akkanen, J., Leppänen, M. T., Mayer, P., Kukkonen, J.V.K., 2013. Changes in Lumbriculus Variegatus Metabolites under Hypoxic Exposure to Benzo(a)Pyrene, Chlorpyrifos and Pentachlorophenol: Consequences on Biotransformation. Chemosphere 93 (2), 302–310. https://doi.org/10.1016/j.chemosphere.2013.04.082.

An, P.N., Thuy, M.Y., Fukusaki, E., 2017. Metabolic profiling of drosophila melanogaster metamorphosis: a new insight into the central metabolic pathways. Metabolomics 13 (3), 29. https://doi.org/10.1007/s11306-017-1167-1.

Arrese, E.L., Soulages, J.L., 2010. Insect fat body: energy, metabolism, and regulation. Annu. Rev. Entomol. 55 (1), 207–225. https://doi.org/10.1146/annurev-ento-112408-085356.

Biesmeijer, J.C., Roberts, S.P.M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A.P., et al., 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313 (5785), 351–354. https://doi.org/10.1126/science.1127863.

Boroń, Marta, Janusz Mirosławski. 2009. Using Insects (Damselflies: Azure Damselfly – Coenagrion Puella) as Biomarkers of Environmental Pollution: 8.

Brodin, T., Mikolajewski, D.J., Johansson, F., 2006. Behavioural and life history effects of predator diet cues during ontogeny in damselfly Larvae. Oecologia 148 (1), 162–169. https://doi.org/10.1007/s00442-005-0334-7.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., Jonsson, M., 2014. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. Phil. Trans. R. Soc. B 369 (1656), 20130580. https://doi.org/10.1098/rstb.2013.0580.

Bundy, J.G., Davey, M.P., Viant, M.R., 2009. Environmental metabolomics: a critical review and future perspectives. Metabolomics 5 (1), 3–21. https://doi.org/10.1007/s11306-008-0152-0.

Chapman, R.F., 2013. The Insects Structure and Function, 5th ed.

Chong, J., Wishart, D.S., Xia, J., 2019. Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. Curr. Protocols Bioinf. 68 (1), e86 https://doi.org/10.1002/cpbi.86.

Chou, H., Pathmasiri, W., Deese-Spruill, J., Sumner, S., Buchwalter, D.B., 2017. Metabolomics reveal physiological changes in mayfly larvae (Neocloeon Triangulifer) at ecological upper thermal limits. J. Insect Physiol. 101 (August), 107–112. https://doi.org/10.1016/j.jinsphys.2017.07.008.

Córdoba-Aguilar, A., Rocha-Ortega, M., 2019. Damselfly (Odonata: Calopterygidae) population decline in an urbanizing Watershed. J. Insect Sci. 19 (30) https://doi.org/10.1093/jisesa/iez063.

Dettmer, K., Aronov, P.A., Hammock, B.D., 2007. Mass spectrometry-based metabolomics. Mass Spectrom. Rev. 26 (1), 51–78. https://doi.org/10.1002/mas.v26:110.1002/mas.20108.

Dwyer, G.K., Stoffels, R.J., Rees, G.N., Shackleton, M.E., Silvester, E., 2017. A predicted change in the amino acid landscapes available to freshwater carnivores. Freshwater Sci. 37 (1), 108–120. https://doi.org/10.1086/696128.

Feldhaar, H., Otti, O., 2020. Pollutants and their interaction with diseases of social Hymenoptera. Insects 11 (3), 153. https://doi.org/10.3390/insects11030153.

Gorrochategui, E., Jaumot, J., Lacorte, S., Tauler, R., 2016. Data analysis strategies for targeted and untargeted LC-MS metabolomic studies: overview and workflow. TrAC, Trends Anal. Chem. 82 (September), 425–442. https://doi.org/10.1016/j.trac.2016.07.004.

Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., de Kroon, H., Lamb, E. G., 2017. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PLoS ONE 12 (10), e0185809. https://doi.org/10.1371/journal.pone.0185809.

Hidalgo, K., Beaugeard, E., Renault, D., Dedeine, F., Lécureuil, C., 2019. Physiological and biochemical responses to thermal stress vary among genotypes in the parasitic Wasp Nasonia Vitripennis. J. Insect Physiol. 117 (August), 103909 https://doi.org/10.1016/j.jinsphys.2019.103909.

Hines, A., Oladiran, G.S., Bignell, J.P., Stentiford, G.D., Viant, M.R., 2007. Direct sampling of organisms from the field and knowledge of their phenotype: key recommendations for environmental metabolomics. Environ. Sci. Technol. 41 (9), 3375–3381. https://doi.org/10.1021/es062745w.

Hirai, S., Hotta, K., Okado, H., 2018. Developmental roles and evolutionary significance of AMPA-type glutamate receptors. BioEssays 40 (9), 1800028. https://doi.org/10.1002/bies.201800028.

Janssens, L., Stoks, R., 2013. Synergistic effects between pesticide stress and predator cues: conflicting results from life history and physiology in the Damselfly Enallagma Cyathigerum. Aquat. Toxicol. 132–133 (May), 92–99. https://doi.org/10.1016/j.aquatox.2013.02.003.

Janssens, L., Stoks, R., 2017. Stronger effects of roundup than its active ingredient glyphosate in Damselfly Larvae. Aquat. Toxicol. 193 (December), 210–216. https://doi.org/10.1016/j.aquatox.2017.10.028.

Jeong, T.-Y., Simpson, M.J., 2019. Daphnia Magna metabolic profiling as a promising water quality parameter for the biological early warning system. Water Res. 166 (December), 115033 https://doi.org/10.1016/j.watres.2019.115033.

Jin, T., Lin, Y.-Y., Jin, Q.-a., Wen, H.-b., Peng, Z.-Q., 2014. Sublethal Effect of Avermectin and Acetamiprid on the Mortality of Different Life Stages of Brontispa Longissima (Gestro) (Coleoptera: Hispidae) and Its Larvae Parasitoid Asecodes Hispinarum Bouček (Hymenoptera: Eulophidae). Crop Prot. 58 (April), 55–60. https://doi.org/10.1016/j.cropro.2013.12.025.

Jonsson, M., Fick, J., Klaminder, J., Brodin, T., 2014. Antihistamines and Aquatic Insects: Bioconcentration and Impacts on Behavior in Damselfly Larvae (Zygoptera). Sci. Total Environ. 472 (February), 108–111. https://doi.org/10.1016/j.scitotenv.2013.10.104.

Khodayari, S., Moharramipour, S., Larvor, V., Hidalgo, K., Renault, D., Palli, S.R., 2013. Deciphering the metabolic changes associated with diapause syndrome and cold acclimation in the two-spotted spider Mite Tetranychus Urticae. PLoS ONE 8 (1), e54025. https://doi.org/10.1371/journal.pone.0054025.

Kingsolver, J.G., Buckley, L.B., 2020. Ontogenetic variation in thermal sensitivity shapes insect ecological responses to climate change. *Curr Opin Insect Sci*, Glob Change Biol Mol Physiol Sect 41 (October), 17–24. https://doi.org/10.1016/j.cois.2020.05.005.

Knops, J.M.H., Tilman, D., Haddad, N.M., Naeem, S., Mitchell, C.E., Haarstad, J., Ritchie, M.E., Howe, K.M., Reich, P.B., Siemann, E., Groth, J., 1999. Effects of plant species richness on invasion dynamics, disease outbreaks, insect abundances and diversity. Ecol. Lett. 2 (5), 286–293. https://doi.org/10.1046/j.1461-0248.1999.00083.x.

Lemos, D., Phan, V.N., 2001. Ontogenetic variation in metabolism, biochemical composition and energy content during the early life stages of Farfantepenaeus Paulensis (Crustacea: Decapoda: Penaeidae). Mar. Biol. 138 (5), 985–997. https://doi.org/10.1007/s002270000516.

Lister, B.C., Garcia, A., 2018. Climate-driven declines in arthropod abundance restructure a rainforest food web. Proc. Natl. Acad. Sci. 115 (44), E10397–E10406. https://doi.org/10.1073/pnas.1722477115.

Nilsson-Örtman, V., Stoks, R., De Block, M., Johansson, F., 2013. Latitudinal patterns of phenology and age-specific thermal performance across six coenagrion damselfly species. Ecol. Monogr. 83 (4), 491–510. https://doi.org/10.1890/12-1383.1.

Norling, U., 1984. The Life Cycle and Larval Photoperiodic Responses of Coenagrion Hastulatum (Charpentier) in Two Climatically Different Areas (Zygoptera: Coenagrionidae). Odonatologica 13 (3), 429–449.

Praet, V., Nander, L.D., Bruyn, M.D., Jonge, L.V., Stoks, R., Bervoets, L., 2014. Can Damselfly Larvae (Ischnura Elegans) Be Used as Bioindicators of Sublethal Effects of Environmental Contamination? Aquat. Toxicol. 154 (September), 270–277. https://doi.org/10.1016/j.aquatox.2014.05.028.

Khan, Rashid Ahmed, Hamed, M., 2005. "Toxicity of Different Groups of Insecticides Against First, Second and Third Instar Larvae of Cotton Bollworm, Helicoverpa Armigera (Hub.). Noctuidae, Lepidoptera, p. 3.

Richardson, J.M.L., Anholt, B.R., 1995. Ontogenetic Behaviour Changes in Larvae of the Damselfly Ischnura Verticalis (Odonata: Coenagrionidae). Ethology 101 (4), 308–334. https://doi.org/10.1111/j.1439-0310.1995.tb00368.x.

Robert Michaud, M., Benoit, J.B., Lopez-Martinez, G., Elnitsky, M.A., Lee, R.E., Denlinger, D.L., 2008. Metabolomics Reveals Unique and Shared Metabolic Changes in Response to Heat Shock, Freezing and Desiccation in the Antarctic Midge, Belgica Antarctica. J. Insect Physiol. 54 (4), 645–655. https://doi.org/10.1016/j.jinsphys.2008.01.003.

Rohart, F., Gautier, B., Singh, A., Lê Cao, K.-A., Schneidman, D., 2017. MixOmics: An R Package for 'omics Feature Selection and Multiple Data Integration. PLoS Comput. Biol. 13 (11), e1005752. https://doi.org/10.1371/journal.pcbi.1005752.

Schauer, Nicolas, Dirk Steinhauser, Sergej Strelkov, Dietmar Schomburg, Gordon Allison, Thomas Moritz, Krister Lundgren, et al. 2005. "GC–MS Libraries for the Rapid Identification of Metabolites in Complex Biological Samples." *FEBS Lett.* 579 (6): 1332–37. 10.1016/j.febslet.2005.01.029.

Schuster, C.M., Ultsch, A., Schloss, P., Cox, J.A., Schmitt, B., Betz, H., 1991. Molecular cloning of an invertebrate glutamate receptor subunit expressed in drosophila muscle. Science 254 (5028), 112–114. https://doi.org/10.1126/science.1681587.

Smilowitz, J.T., Zivkovic, A.M., Wan, Y.-J., Watkins, S.M., Nording, M.L., Hammock, B. D., Bruce German, J., 2013. Nutritional lipidomics: molecular metabolism, analytics, and diagnostics. Mol. Nutr. Food Res. 57 (8), 1319–1335. https://doi.org/10.1002/mnfr.201200808.

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

Stoks, R., Córdoba-Aguilar, A., 2011. Evolutionary ECOLOGY of Odonata: A complex life cycle perspective. Annu. Rev. Entomol. 57 (1), 249–265. https://doi.org/10.1146/annurev-ento-120710-100557.

Späth, Jana, Brodin, Tomas, Cerveny, Daniel, Lindberg, Richard, Fick, Jerker, Nording, Malin L., 2021. Oxylipins at Intermediate Larval Stages of Damselfly Coenagrion Hastulatum as Biochemical Biomarkers for Anthropogenic Pollution. Environmental Science and Pollution Research, January. https://doi.org/10.1007/s11356-021-12503-x.

Venter, L., Loots, D.T., Mienie, L.J., Peet, J., van Rensburg, J., Mason, S., Vosloo, A., Lindeque, J.Z., 2018. Uncovering the metabolic response of abalone *Haliotis Midae* to environmental hypoxia through metabolomics. Metabolomics 14 (4), 49. https://doi.org/10.1007/s11306-018-1346-8.

Viant, M.R., 2007. Metabolomics of aquatic organisms: The New 'Omics' on the Block. Mar. Ecol. Prog. Ser. 332 (March), 301–306. https://doi.org/10.3354/meps332301.

Vogeler, S., Wikfors, G.H., Li, X., Veilleux, D., Miller-Ezzy, P., Joyce, A., 2019. Larval metamorphosis in four bivalve species in response to NMDA receptor ligands: The NMDA Receptor Pathway as Potential Regulator of Bivalve Transition to Spat. Aquaculture 511 (September), 634173. https://doi.org/10.1016/j.aquaculture.2019.05.058.

Wagner, D.L., 2020. Insect declines in the anthropocene. Annu. Rev. Entomol. 65 (1), 457–480. https://doi.org/10.1146/annurev-ento-011019-025151.

Walker, M.C., van der Donk, W.A., 2016. The many roles of glutamate in metabolism. J. Ind. Microbiol. Biotechnol. 43, 419–430. https://doi.org/10.1007/s10295-015-1665-y.

Wheelock, Å.M., Wheelock, C.E., 2013. Trials and tribulations of 'omics data analysis: assessing quality of SIMCA-based multivariate models using examples from pulmonary medicine. Mol. BioSyst. 9 (11), 2589–2596. https://doi.org/10.1039/C3MB70194H.

Wilbur, H.M., 1980. Complex Life Cycles. Annu. Rev. Ecol. Syst. 11 (1), 67–93. https://doi.org/10.1146/annurev.es.11.110180.000435.

Zhang, J., Xiong, X., Deng, Y., Zheng, Z., Yang, C., Xiaodong, D.u., 2021. Integrated application of transcriptomics and metabolomics provides insights into the larval metamorphosis of pearl oyster (Pinctada Fucata Martensii). Aquaculture 532 (February), 736067. https://doi.org/10.1016/j.aquaculture.2020.736067.

Zhu, W., Chang, L., Zhao, T., Wang, B., Jiang, J., 2020. Remarkable metabolic reorganization and altered metabolic requirements in frog metamorphic climax. Front. Zool. 17 (1), 30. https://doi.org/10.1186/s12983-020-00378-6.