KU LEUVEN

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THE IMPACT OF NATURAL AND ANTHROPOGENIC CONDITIONS ON PHYSIOLOGY AND STOICHIOMETRY IN DAMSELFLIES

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Summary

In nature organisms encounter many environmental conditions, which could be perceived as beneficial or stressful. Understanding how different levels of biological organization respond to these environmental conditions is a key challenge in stress ecology, that asks for an integrated approach including life history, physiology and behaviour. Moreover, in this context it is very relevant to study body stoichiometry (C(carbon):N(nitrogen):P(phosphorus) composition), as it can link different biological levels, from physiology to ecosystem functioning. Several theories have been put forward to predict stoichiometric changes caused by environmental conditions with growth rate, body size and metabolic rate and efficiency as important drivers. Moreover, ecological stoichiometry links the elemental body composition to the most important macromolecules (proteins, fat, sugar, RNA). Therefore, changes in these macromolecules are expected to cause changes in body stoichiometry. In this thesis I studied whether and how single and combined environmental conditions affected the assumed drivers of body stoichiometry and included the proposed underlying mechanisms. Moreover, I investigated the effects on nutrient release and the cascading impact on primary production. Specifically, I studied the effects of three environmental conditions: temperature, predation risk and pesticide exposure. I did this by executing a series of common-garden experiments and one outdoor mesocosm study, using *Ischnura elegans* (from different latitudes, giving me the possibility to determine the role of thermal evolution in the response to a temperature increase) and *Enallagma cyathigerum* damselflies as model organisms.

While extreme temperatures increased mortality (Chapters I, III), both mild and extreme temperatures positively influenced growth rate (Chapters I-III, V). The growth rate hypothesis (GRH) predicts that faster growing organisms have a higher P and N, due to an increase in P-rich RNA together with a higher synthesis of N-rich proteins. I found that most of the time the observed increased growth rate was associated with an increase in N, yet only in Chapter V also the protein content increased. For P the results were more variable, with an increase in Chapter V (associated with a RNA increase) and a trend for a decrease in Chapter II.

Summary

In contrast to temperature, predation risk and pesticide exposure were both stressful for the damselflies, as indicated by the lower survival and/or growth rates. When environmental conditions are stressful, the general stress paradigm (GSP) is used to predict the changes in elemental body composition. This theory states that under stress, the metabolic rate will increase, thereby allocating more energy (i.e. C-rich fat and sugars) towards maintenance and defence and away from growth (i.e. N-rich proteins) and the associated P-rich RNA. This is predicted to result in lower body N and P and higher body C, hence an increased C:N ratio. In my thesis, predation risk did cause an increase in C:N ratio (chapter IV-V), but this was not associated with an increase in fat and sugar (which decreased), or a decrease in proteins. Predation risk exposure resulted in an increased egestion of N (Chapter VII). In line with the general consideration that N is a limited nutrient for algae growth, the increased egestion of N resulted in an increased algae growth (Chapter VII). When exposed to the pesticide, the body C, N and P contents increased, also here without an increase of the associated macromolecules (Chapters VI-VII). In the excreta C increased, but as expected this had no effect on algae growth (Chapter VII).

When looking at the combined effect of the environmental conditions, the predation risk effect was stronger at the higher temperature (Chapter V) and not affected by the presence of the pesticide (Chapter VI-VII). The latter may be the result of a difference in perception of the stress levels, whereby the pesticide was considered as much more stressful by the animals, leaving little room for the predation risk to cause additional stress.

My results indicate that elemental body composition is strongly affected by environmental conditions, and that current theories (GRH and GSP) fail to predict the stoichiometric response to changes in these conditions. Moreover I showed that changes in elemental body composition are hardly linked to changes in macromolecules. Therefore, I can conclude that the current theories are too simplistic to make reliable predictions. Good additional parameters to include are linked with morphological defence, life history strategies and behavioral adjustments. Moreover, more research in regard to combined stressor effects on stoichiometry and the cascading effects on ecosystem functioning is needed.

Samenvatting

In hun natuurlijke omgeving worden organismen blootgesteld aan verschillende omgevingscondities. Deze condities kunnen worden ervaren als gunstig of stressvol. Het begrijpen van de impact van deze omgevingscondities op verschillende biologische niveaus, is één van de belangrijkste uitdagingen in de stressecologie. Het vraagt om een geïntegreerde benadering, waarbij zowel levensgeschiedeniskenmerken, fysiologie en gedrag in rekening moeten worden gebracht. Hiervoor kan het ook zeer relevant zijn om de lichaamsstoichiometrie [i.e. de C(koolstof):N(stikstof):P(fosfor) samenstelling] te bestuderen, aangezien stoichiometrie verschillende biologische niveaus, van fysiologie tot het functioneren van ecosystemen, met elkaar verbindt. Verschillende theorieën trachten de invloed van de omgevingscondities op de lichaamsstoichiometrie te voorspellen, via veranderingen in groeisnelheid, lichaamsgrootte, metabolisme of metabolische efficiëntie van de organismen (de *drivers*). Bovendien worden in de ecologische stoichiometrie de individuele elementen (C, N en P) rechtstreeks gekoppeld aan de belangrijkste macromoleculen (eiwitten, vet, suiker, RNA). Daarom wordt er verwacht dat veranderingen in deze macromoleculen aanleiding geven tot veranderingen in de lichaamsstoichiometrie. In dit proefschrift onderzocht ik of en hoe blootstelling aan individuele en gecombineerde omgevingscondities de verschillende *drivers* en hun onderliggende werkingsmechanisme beïnvloedde. Daarnaast onderzocht ik ook de impact van deze omgevingscondities op de excretie van nutriënten en hoe dit de primaire productie veranderde. Concreet heb ik drie verschillende omgevingscondities bestudeerd: temperatuurstijging, predatierisico en pesticideblootstelling. Dit deed ik via verschillende laboratoriumexperimenten en een semi-natuurlijk mesocosm-experiment met waterjuffers (*Ischnura elegans* en *Enallagma cyathigerum*). Doordat ik waterjuffers afkomstig van verschillende latitude gebruikte, kon ik bovendien de rol van thermische evolutie in de respons op een temperatuurstijging bepalen.

Terwijl extreem hoge temperaturen de mortaliteit verhoogden (Hoofdstukken I, III), hadden zowel milde als extreme temperaturen een positief effect op de groeisnelheid (Hoofdstukken I-III, V). De *growth rate hypothesis* (GRH) voorspelt dat

sneller groeiende organismen een hoger P en N gehalte hebben, als gevolg van een toename in P-rijk RNA en een verhoogde synthese van N-rijke eiwitten. Ook in mijn resultaten ging de hogere groeisnelheid gepaard met een toename in N, maar enkel in hoofdstuk V nam ook het eiwitgehalte toe. Voor P waren de resultaten variabeler, met een toename in Hoofdstuk V (geassocieerd met een toename in RNA) en een trend voor een afname in Hoofdstuk II.

In tegenstelling tot de temperatuurstijging, waren blootstelling aan predatierisico en pesticide beide stressvol voor de waterjuffers, zoals bleek uit de lagere overleving en / of groeisnelheid. Wanneer de omgevingsomstandigheden stressvol zijn, wordt het *general stress paradigm* (GSP) gebruikt om de veranderingen in de lichaamsstoichiometrie te voorspellen. Deze theorie stelt dat onder stress de snelheid van het metabolisme zal toenemen, waardoor meer energie (i.e. C-rijke vetten en suikers) geïnvesteerd wordt in het metabolisme in plaats van in groei (i.e. N-rijke eiwitten) en het hiermee geassocieerde P-rijke RNA. Dus onder stress zal het N en P gehalte in het lichaam dalen en het C gehalte stijgen, waardoor ook de C:N verhouding stijgt. In mijn proefschrift veroorzaakte het predatierisico inderdaad een toename in de C:N verhouding (hoofdstuk IV-V), maar dit ging niet gepaard met een toename in vet en suiker (deze namen zelfs af) of een afname in eiwitten. Verder resulteerde bloostelling aan predatierisico in een verhoogde excretie van N, (Hoofdstuk VII). Aangezien N vaak een limiterend nutrient is voor algengroei, resulteerde de verhoogde excretie van N in een hogere algengroei (Hoofdstuk VII).Bij blootstelling aan het pesticide verhoogden het C, N en P gehalte van het lichaam, maar ook hier was dit niet geassocieerd met veranderingen in de macromoleculen (Hoofdstukken VI-VII). In de fecale pellets nam enkel het C gehalte toe, bijgevolg was er geen effect op de algengroei (Hoofdstuk VII).

Wanneer predatierisico gecombineerd werd met één van de andere omgevingscondities, bleek het effect van predatierisico sterker te zijn bij een hogere temperatuur (Hoofdstuk V), maar werd het niet beïnvloed door de aanwezigheid van het pesticide (Hoofdstuk VI-VII). Dit laatste kan het gevolg zijn van een verschil in perceptie van de stressniveaus, waarbij het pesticide door de dieren als veel stressvoller

werd beschouwd, waardoor er weinig ruimte was voor het predatierisico om extra stress te veroorzaken.

In het algemeen geven mijn resultaten aan dat lichaamsstoichiometrie sterk wordt beïnvloed door omgevingscondities, en dat de huidige theorieën (GRH en GSP) niet in staat zijn om de veranderingen in stoichiometrie accuraat te voorspellen. Daarnaast kon ik aantonen dat de stoichiometrische veranderingen nauwelijks gekoppeld waren met veranderingen in de macromoleculen. Hieruit kan ik concluderen dat de huidige theorieën te simplistisch zijn om betrouwbare voorspellingen te doen. Bijkomende parameters, zoals levensgeschiedenisstrategieën, gedrag en morfologische veranderingen zouden mee in rekening gebracht moeten worden. Daarnaast is ook extra onderzoek nodig met betrekking tot gecombineerde stressoreffecten op stoichiometrie en de cascade-effecten ervan op het functioneren van ecosystemen.

Abbreviations

ANOVA	analysis of variance
C	carbon
CAT	catalase
CEA	cellular energy allocation
Ea	energy availability
Ec	energy consumption
ERA	ecological risk assessment
ETS	electron transport system
GRH	growth rate hypothesis
GSP	general stress paradigm
GST	glutathione-S-transferase
IPCC	Intergovernmental Panel on Climate Change
MANOVA	multivariate analysis of variance
N	nitrogen
P	phosphorus
PBS	phosphate-buffered saline
PCA	principal component analysis
PO	phenoloxidase
SE(M)	standard error (of mean)
SOD	superoxide dismutase
TPC	thermal performance curve
TRC	thermal response curve
UPLC MS/MS	ultra performance liquid chromatography – tandem mass spectrometer

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General introduction

In nature, organisms face many environmental conditions both in isolation and combined that are often stressful. Understanding and predicting how these environmental conditions and their interactions affect different levels of biological organization is a key challenge in stress ecology. This problem asks for an integrated approach including life history, physiology and behaviour (Sterner & Elser, 2002; Hawlena & Schmitz, 2010a; Dalton & Flecker, 2014; Trakimas *et al.*, 2019).

The general aim of this thesis was to study the impact of environmental conditions (temperature, predation risk and pesticide exposure) on traits linked to the energy metabolism using damselflies as model organism. While, many studies focused on the impact of stressors on life history traits (e.g. Relyea & Mills, 2001; Côté *et al.*, 2016), I investigated how these effects were mediated by looking at different levels (life history, behaviour, physiology and stoichiometry). Special attention was given to bioenergetic responses both at the organismal and cellular level.

I had a special focus on body stoichiometry, as this organismal trait can translate effects of environmental conditions on organisms into effects at the ecosystem level. Although, the effect of stressors on body composition has been studied before (e.g. Liess *et al.*,2013; Dalton & Flecker, 2014; Zhang *et al.*, 2016), my research added new insights to this field by also incorporating the macromolecules assumed to underlie the changes in body composition. Moreover, I did not only focus on the mechanisms that traditionally are put forward to explain changes in stoichiometry (e.g. growth rate hypothesis, general stress paradigm) but also proposed and tested two other mechanisms. More specifically, I tested the contributions of cuticular chitin and melanin to the body composition and its responses to environmental conditions. Finally, I started exploring the relationships between the different levels of organization including between elements and macromolecules, between life history and macromolecules, and between elements and an ecosystem function. This way I could obtain an integrated view of the impact of stressors on an organism from the element to the ecosystem.

In this introductory chapter I will present the core ideas and concepts of the thesis, including information on the used environmental conditions and the concept of

ecological stoichiometry. I will introduce the different traits I measured and present the study species. At the end of this chapter I will give an overview of the outline of the thesis.

Single and multiple stressors

General concept

In nature, organisms encounter many environmental conditions. Sometimes these conditions can have beneficial effects on the organisms. For example in ectothermic species, temperature increases could increase their growth rate (Deutsch *et al.*, 2008; Nilsson-Örtman *et al.*, 2012) or upregulate their immune function (Prokkola *et al.*, 2013). Yet, these environmental conditions can also be perceived as stressful. Stress is defined as an internal state outside an organism's normal operating range (van Straalen, 2003; Steinberg, 2012). Stress is not absolute, and must therefore be defined in reference to the normal functioning of an organism. This means that what is extremely stressful for one organism, can be normal for another one (van Straalen, 2003). Once an organism perceives an environmental condition as stressful, it will elicit a stress response. This stress response is defined as a cascade of internal changes (van Straalen, 2003) with organisms switching their focus from growth and reproduction to defence and maintenance (Hawlena & Schmitz, 2010a). This switch will have no or only little structural or functional consequences in the organisms if the stressor is encountered during a short-term period. However, long-term exposure to a stressor prolongs the activation of the stress response (Adamo & Baker, 2011). This can have severe costs such as decreased growth rates, depletion of energy storage and the build-up of toxic compounds (Hawlena & Schmitz, 2010a). While stress is mainly studied at the organismal level, stress responses are mostly studied on the cellular and biochemical level (van Straalen, 2003).

Organisms can encounter several types of external, potentially stressful factors. These are often categorized based on their nature of origin. Firstly, there is a distinction between natural and anthropogenic stressors. Natural stressors are factors which are naturally present in the environment for instancesalinity, temperature or predators.

Anthropogenic stressors on the other hand, such as contaminants, are present in the environment due to human actions. Moreover, the human actions can also affect natural stressors, for example temperature increase caused by climate change. This makes a strict distinction between the two types of stressors difficult. Secondly, there is a difference between biotic and abiotic stressors. Biotic stress is caused by interacting with other organisms, for example predation, competition and parasitism. Abiotic stressors, on the other hand, are non-living factors such as pH, temperature, conductivity or contaminants.

Organisms can be exposed to a single stressor, however, in nature it is much more likely they encounter multiple stressors simultaneously. Exposure to a single stressor will elicit defence mechanisms. These defence mechanism can be energetically costly, resulting in a lower energy budget available for, for example, the production of new tissue. Moreover, the reduced energy availability could also increase sensitivity to an additional stressor (Sokolova, 2013). However, some defence mechanisms can be used against several stressors and therefore result in cross-resistance (e.g. Raghavendra *et al.*, 2010; Bubliy *et al.*, 2012). For example, the upregulation of stress proteins is an appropriate response against heat stress, pesticide exposure and predation risk (Sørensen *et al.*, 2003). This indicates that multiple stressors can interact with each other, making it difficult to predict the effects of a multiple stressors based on the effects of single stressors (Newman & Unger, 2003; Jackson *et al.*, 2016; Liess *et al.*, 2016). Interactions can be categorized into three major types: additive, antagonistic and synergistic. An additive effect occurs when the combined effect of the multiple stressors equals the sum of the single stressors. When the effect of the combined stressors is larger than the sum of the single stressor effects, then the two stressors enhance each other and the effect is synergistic. When the effect of the two stressors is smaller than the expected additive effects, the effect is antagonistic (Gunderson *et al.*, 2016). A special type of antagonistic interaction is the reversal interaction (Jackson *et al.*, 2016). In this case the combined effect is in the opposite direction compared to what is expected based on the effects of the single stressors. The interaction types are schematically presented in Figure 1.

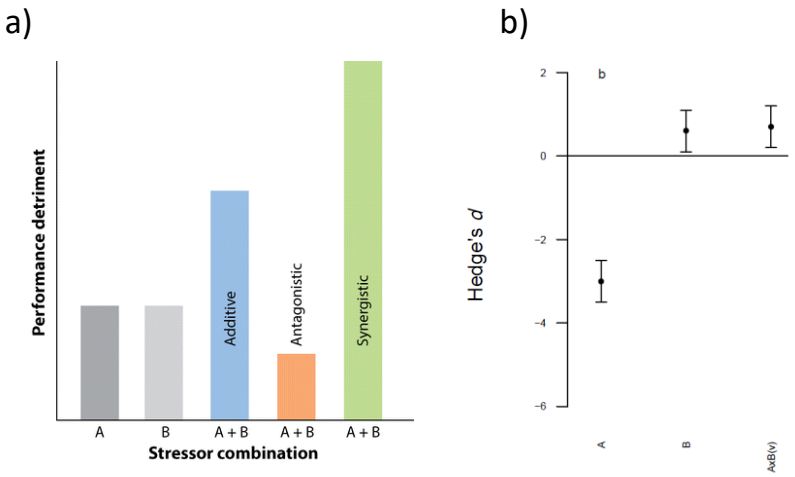

Figure 1. Schematic representation of (a) additive, antagonistic and synergistic interactions between two stressors (from Gunderson *et al.*, 2016) and (b) a reversal interaction (Jackson *et al.*, 2016).

It is very relevant to study responses to stressors in organisms with a complex life cycle, i.e. animals who have discrete larval and adult stages with stage-specific behavioural, morphological and physiological traits (Moran, 1994). Especially, since the sensitivity to stressors can be stage-specific (Bowler & Terblanche, 2008; Kingsolver *et al.*, 2011; Zhao *et al.*, 2017; Müller, 2018; Rasmussen *et al.*, 2018; Tran *et al.*, 2018). In many taxa with a complex life cycle, including amphibians and aquatic insects, animals cross habitat boundaries during their life cycle with an aquatic larval stage followed by an adult terrestrial stage (Rowe & Ludwig, 1991; Stoks & Córdoba-Aguilar, 2012). Therefore, stressors experienced in the larval aquatic stage may affect the terrestrial adult stage (e.g. Baxter *et al.*, 2005; Dreyer *et al.*, 2015). Stressors thereby have the potential to interact across metamorphosis (e.g. Rohr & Palmer, 2005; Janssens *et al.*, 2014a).

Single stressors

Temperature

Temperature is a key environmental variable affecting all levels of biological organization (Bale *et al.*, 2002; Woodward *et al.*, 2010). Especially for ectotherms the environmental temperature is important, since their body temperature conforms with the ambient temperature. Therefore the ambient temperature affects the rates of their

biochemical and physiological processes (Angilletta, 2009; Harrison *et al.*, 2012). Whether temperature has positive or negative effects on an organism's performance, depends on its location on the thermal performance curve (TPC, Figure 2) (Angilletta, 2009). A thermal performance curve describes the relationship between temperature and the organism's performance. It generally has an accelerating rising part until an optimal temperature is reached, followed by a steep decline until the thermal maximum temperature is reached (Kingsolver & Gomulkiewickz, 2003; Angilletta, 2009; Sinclair *et al.*, 2016).

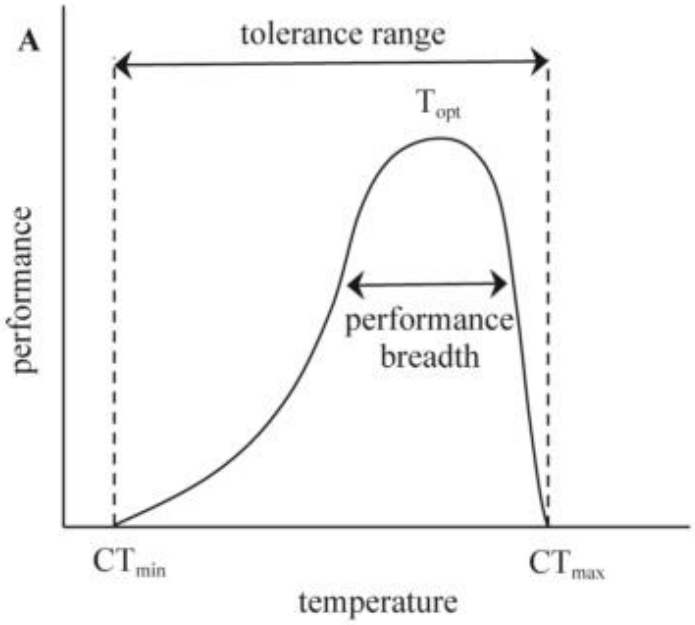

Figure 2. Thermal performance curve (TPC) for a typical ectothermic animal. The critical thermal minimum (CTmin) and maximum (CTmax), optimal performance temperature (Topt), near-optimal performance breadth, and overall temperature tolerance range are indicated (from Noyes & Lema, 2015).

One of the biggest challenges of the 21st century is dealing with global warming. Global warming is mainly caused by human-induced emissions of greenhouse gasses into the atmosphere (IPCC, 2013). The last 100 years, the average temperature on earth has increased with 0.85 [0.65-1.06] °C (IPCC, 2014). Recent climate models predict that without additional effort to decrease the emission of greenhouse gasses the increase in temperature will range from 3.7 to 4.8°C by 2100 (e.g. RCP8.5; IPCC, 2014). This increase in temperature could have direct effects on both the fauna and flora on earth.

Besides increases in the average temperature on earth, global warming is also characterized by more extreme weather events such as heat waves (IPCC, 2013). Heat waves are predicted to occur more frequent, be more intense and of longer duration (Meehl & Tebaldi, 2004; Jentsch *et al.,* 2007; IPCC, 2013). For Western Europe, for example, the frequency of hot days has been tripled between 1880 and 2005 (Rebetez *et al.,* 2009). These extreme weather events will not only harm natural ecosystems but also have severe socio-economic consequences (Meehl & Tebaldi, 2004), for example weather-related fatalities (Robinson, 2001). Therefore, the ability of species to deal with heat waves, will be key to determine whether they can survive under global warming (Thompson *et al.,* 2013; Vasseur *et al.,* 2014; Ma *et al.,* 2015).

Organisms can respond to global warming by adapting to the temperature rise both through a plastic response and through thermal evolution. A key tool to indirectly study gradual thermal evolution is to use a space-for-time substitution (Fukami & Wardle, 2005). In this approach, future temporal dynamics are extrapolated from current spatial variation in local adaptation (Fukami & Wardle, 2005). To predict the effect of global warming, assuming gradual thermal evolution, the phenotype of populations which are currently adapted to warmer sites (e.g. low latitude) are used as proxies for the future phenotype of populations which are currently adapted to a colder site (e.g. high latitude). Latitudes should be selected so that the temperature difference matches the predicted temperature increases by global warming IPCC scenarios (2014).

Predation

Predators are omnipresent in nature (Kerfoot & Sih, 1987) and may considerably affect prey traits and their population dynamics, thereby shaping community structure and ecosystem functions (Werner & Peacor, 2003; Preisser & Bolnick, 2008; Preisser *et al.,* 2005). While these effects have been attributed to the direct consumptive predation (Werner & Paecor, 2003; Preisser *et al.,* 2005), the last decade also nonconsumptive predation effects are receiving increased attention (Creel & Christianson, 2008; Peckarsky *et al.,* 2008; Kimbro *et al.,* 2017; Moll *et al.,* 2017; Gehr *et al.,* 2018). This nonconsumptive predation is referring to the fear imposed by predators, i.e. predation risk, which may cause stress (Slos & Stoks, 2008; Clinchy *et al.,* 2013) and thereby reduce the prey's fitness. Moreover, recent evidence indicates that, compared to

consumptive predation, nonconsumptive predation may equally or even stronger affect prey traits (Siepielski *et al.*, 2014) and population dynamics (Peacor *et al.*, 2012). Hereby, nonconsumptive predation effects may also have the potential to change community structure (Peacor *et al.*, 2012; Zanette *et al.*, 2011) and ecosystem functions (Hawlena *et al.*, 2012). While most studies on nonconsumptive predation focus on life history, morphology and behaviour (Benard, 2004), less attention went to the physiological stress responses (Preisser & Bolnick, 2008). Nevertheless, prey typically show a set of adaptive physiological stress responses to avoid being killed by the predator (Sapolsky, 2002; Hawlena & Schmitz, 2010a; Adamo & Baker, 2011; Boonstra, 2013). More specifically, prey release stress hormones upon exposure to predation risk. These stress hormones will, amongst other changes, increase the metabolic rate andprepare for the 'flight-or-fight' response. To fuel the increased metabolic rate, prey will mobilise carbohydrates. Moreover, they will allocate resources to maintenance away from new tissue production (Hawlena & Schmitz, 2010a).

Pesticides: Chlorpyrifos

A major threat for aquatic biodiversity is the ongoing contamination of aquatic systems with pesticides (Schwarzenbach *et al.*, 2006; Malaj *et al.*, 2014). Due to processes such as runoff, these pesticides can be transferred to aquatic systems (Bloomfield *et al.*, 2006). As a result, non-target organisms are exposed to these substances (Liess *et al.*, 2008). In Europe, the pesticide concentrations found in nature are mostly sublethal (Beketov *et al.*, 2013). Yet, these sublethal pesticide concentrations may negatively affect the population dynamics of non-target species.

One of the most frequently used pesticides worldwide is chlorpyrifos (Eaton *et al.*, 2008). It is a priority substance in the European Water Framework Directive (2000/60/EC), which compiles substances that present a significant risk to aquatic habitats. Moreover, chlorpyrifos is in the top ten of the most risky chemicals to aquatic organisms in surface waters in the UK (Johnson *et al.*, 2017). Chlorpyrifos is an organophosphate insecticide, which functions as an inhibitor of the enzyme acetylcholine esterase, thereby preventing the breakdown of the neurotransmitter acetylcholine at the synaptic cleft (Costa, 2006; Christensen *et al.*, 2009). This results in the accumulation of acetylcholine at the cholinergic nerve ending, causing

hyperstimulation of the neural cells (Christensen *et al.,* 2009). This can lead to epileptic attacks and eventually even to neuronal dead (Hoffman *et al.,* 2003; Rush *et al.,* 2010). Before being able to exert its toxic mechanism, chlorpyrifos needs to be metabolically bioactivated by cytochrome P450 enzymes to chlorpyrifos-oxon. It is this chlorpyrifos-oxon that binds to acetylcholine esterase and causes the toxic effects (Costa 2006; Eaton *et al.,* 2008). In Europe, chlorpyrifos concentrations ranging between 1 and 100 µg/l have been detected in water bodies close to agricultural lands (Bernabò *et al.,* 2011). These aquatic systems may receive several peak pesticide pulses per season during the growing season of the crops (Van Drooge *et al.,* 2001).

Multiple stressors

In nature, organisms are almost always exposed to multiple stressors simultaneously (Sih *et al.,* 2004). Since stressors can interact with each other, thereby weakening or enhancing each other's effects, it is also important to consider these combined effects. In this thesis, I studied two different combinations of stressors: (i) the combination of predation risk and temperature (Chapter V), and (ii) the combination of predation risk and pesticide exposure (Chapters VI, VII).

While there is a growing interest in the importance of nonconsumptive predation effects (Preisser *et al.,* 2005; Peckarsky *et al.,* 2008; Clinchy *et al.,* 2013), the way how these effects change under global warming is largely unknown (but see Stoks *et al.,* 2017). However, investigating the interplay between predation risk and temperature is necessary to understand how populations, communities and ecosystem functions will respond to the ongoing global warming (Traill *et al.,* 2010; Angert *et al.,* 2013; Blois *et al.,* 2013). The few studies that assessed the interaction between predation risk and temperature documented that predation risk could constrain the capacity of prey to deal with the physiological stress imposed by warming (Culler *et al.,* 2014; Miller *et al.,* 2014; Schmitz *et al.,* 2016).

Currently, the ecological risk assessment (ERA) of pesticides seems ineffective to protect freshwater ecosystems. Sublethal pesticide concentrations, which are considered safe by the European legislation (Beketov *et al.,* 2013; Köhler & Triebskorn, 2013), can still cause declines in the aquatic biodiversity (Beketov *et al.,* 2013). In this

context, the stressor combination of pesticides and predation risk is getting increased attention as this combination may drastically magnify the impact of sublethal pesticide concentrations (e.g. Relyea & Mills, 2001; Trekels *et al.*, 2011; Janssens & Stoks, 2013a). However, synergistic effects between predation risk and pesticides are not general (e.g. Coors & De Meester, 2008; Pestana *et al.*, 2009; Qin *et al.*, 2011).

Interactions between stressors are mostly investigated at the level of life history (e.g. Relyea & Mills, 2001; Campero *et al.*, 2007; Trekels *et al.*, 2011). This is because life history is thought to be directly translated into fitness consequences. To gain more insight into the underlying mechanisms shaping the interaction, it is also important to assess effects at the physiological level (Côté *et al.*, 2016; Jackson *et al.*, 2016; Kaunisto *et al.*, 2016). Especially since these physiological traits can be highly responsive to stressors (Sokolova, 2013). Therefore, "hidden interactions" can be present at the physiological level, which will not be detected when only studying effects on life history. Moreover, physiological traits, such as traits related to the bioenergetic responses, can provide information on key processes in the organism's energy acquisition and expenditure, which are pivotal to increase our understanding of the impact of multiple stressors (Sovolova, 2013).

Ecological stoichiometry

General concepts

All living material on earth consists of more than 20, mostly not substitutable, elements (Hessen *et al.*, 2013). Of these, carbon (C), nitrogen (N) and phosphorus (P) are the three main elements (Sterner & Elser, 2002). Ecological stoichiometry investigates the elemental composition of organisms and their ecological interactions in ecosystems (Elser *et al.*, 1996). More specifically, Sterner and Elser (2002) defined ecological stoichiometry as "the balance of multiple chemical substances in ecological interactions and processes, or the study of this balance". In other words, ecological stoichiometry is a framework that links an organism's metabolic demand with the relative supply of elements in the environment. It postulates a crucial relationship between the balance of elements and their role in determining growth and reproduction of organisms as well as

in ecological interactions (Meunier *et al.*, 2017). Historically, ecological stoichiometry originates from the work done by Alfred C. Redfield. Redfield discovered that there was a relationship between the C:N:P ratios of marine plankton and the dissolved nutrient in the water they were living in (Redfield *et al.*, 1963). Nowadays, this relationship of C_{106}:N_{16}:P_1 is well-known as the Redfield ratio. Over the last three decades the field of ecological stoichiometry expanded greatly and the scope of the studies extended to cellular responses (e.g. N and P regulation of growth, metabolism and genomic responses), up to small scale effects on organism, population and community level, up to biogeochemical couplings at the ecosystem and global level (Hessen *et al.*, 2013; Sperfeld *et al.*, 2017).

In my thesis, I will mainly focus on the cellular and organismal level. At the cellular level, the framework of ecological stoichiometry links the individual elements to macromolecules or other biochemical compounds (Sterner & Elser, 2002). More specifically, C is related to fat and sugars, N to proteins and nucleotides and P to RNA and phospholipids (Sterner & Elser, 2002). Therefore, the relative investment in these macromolecules can be used to predict the stoichiometry in cells. Moreover, this approach is also useful to investigate the effects of environmental variables on the body stoichiometry of organisms and, subsequently, the flow of elements (Sperfeld *et al.*, 2017). At the organismal level, a well-known theory describes the relationship between growth rate and body stoichiometry, i.e. the growth rate hypothesis (GRH). The GRH states that "differences in organismal C:N:P ratios are caused by differential allocation to RNA, which is necessary to meet the protein synthesis demands of rapid rates of biomass growth and development" (Sterner & Elser, 2002). More specifically, since rRNA is P-rich, faster growing animals are expected to have much more body P and a lower C:P and N:P ratio (Elser *et al.*, 2000; Sterner & Elser, 2002; Watts *et al.*, 2006). In addition, the increased synthesis of N-rich proteins, the building blocks of new tissue, is expected to increase the body N content, hence decrease the C:N ratio, of faster growing animals (Watts *et al.*, 2006) (see also full-lined boxes on Figure 3).

Stoichiometric changes

Organisms can regulate their internal state in response to the external world to maintain homeostasis (Sterner & Elser, 2002). To make this possible, organisms may apply different mechanisms (reviewed in Hessen *et al.,* 2013), such ase food selection (Raubenheimer & Jones, 2006), adjustments of the elemental intake (Plath & Boersma, 2001), regulation of assimilation, egestion and excretion (Urabe, 1993; DeMott *et al.,* 1998), or combinations of the mechanisms mentioned above (Anderson *et al.*, 2005; Suzuki-Ohno *et al.,* 2012). Yet, the elemental composition of organisms is not completely fixed, and variation exists (Sterner & Elser, 2002; Evans-White *et al.,* 2005; Jeyasingh & Weider, 2007; Vrede *et al.,* 2011; El-Sabaawi *et al.,* 2012). Important determinants causing variation in the elemental composition are for example growth rate, body size and metabolic efficiency (Elser *et al.,* 1996, 2000; Sterner & Elser, 2002; Woods *et al.,* 2003; Sibly *et al.,* 2012). As a consequence, environmental variables affecting these determinants are expected to ultimately change organisms' body C:N:P ratios.

Several environmental conditions have been shown to shape the C:N:P ratios of organisms and communities: temperature (Liess *et al.,* 2013; Schmitz, 2013; Zhang *et al.,* 2016), predation (Costello & Michel, 2013; Dalton & Flecker, 2014; Zhang *et al.,* 2016), pollution (Janssens *et al.,* 2017) and eutrophication (De Senerpont Domis *et al.,* 2014). Depending on the effects of these environmental conditions on growth rate, body size and metabolic efficiency, different mechanisms have been put forward to predict body stoichiometric changes. For example, increasing temperature can increase an organism's growth rate (Angilletta, 2009; Nilsson-Örtman *et al.,* 2012) and following the GRH, faster growing organisms are predicted to have higher body P content and a lower C:P ratio (Cross *et al.,* 2015). In addition, since proteins contain about 17% of N (Elser *et al.,* 1996), the increase protein synthesis is predicted to increase the N content of faster growing organisms. In contrast, temperature can also increase the metabolic efficiency of organisms (i.e. thermodynamic principles, Angilletta, 2009). A higher metabolic efficiency results in a higher protein synthesis per ribosome (Farewell & Neidhardt, 1998). As a result, animals would not need more P to increase their protein

synthesis (reviewed in Woods *et al.*, 2003), causing an increased N:P ratio (Toseland *et al.*, 2013).

If the environmental conditions, such as extreme temperatures (surpassing the thermal optimum), predation risk or pesticides exposure, are stressful, they generally cause a decreased growth rate. A mechanism linking stressors to stoichiometric changes is the general stress paradigm (GSP; Hawlena & Schmitz, 2010a). This theory asserts that under stressful environmental conditions animals will increase their metabolic rate and allocate more energy [C -rich biomolecules i.e. fat and sugars] towards maintenance systems and away from the production of new tissues [N-rich proteins]. To maintain internal homeostasis, this will lead to the release of excess nutrients, mostly N. To create more C-rich sugars to fuel the increased metabolism, gluconeogenesis (the breakdown of N-rich proteins in C-rich biomolecules) is predicted to increase. Besides this increased gluconeogenesis, animals are expected to reduce the assimilation of N-rich molecules under stress. Consequently, these physiological adjustments are expected to result in a higher C content and lower N contents leading to increased body C:N ratio (Hawlena & Schmitz, 2010a; Schmitz, 2013). See dashed-lined boxes in Figure 3 for a visualization of this mechanism.

Note that environmental conditions can also induce changes in organism's morphology, behaviour, fecundity,… , which could enhance or dampen the expected changes in body stoichiometry based on the mechanisms mentioned above. For example, Costello and Michel (2013) found that *Hyla versicolor* tadpoles developed a bigger tail muscle under predation risk. As a result the N content increased, thereby counteracting the prediction of the GSP. Furthermore, Zhang *et al.* (2016) documented a reduction in the body C:N ratio of *Daphnia magna* under predation risk due to an increased investment in N-rich eggs.

Importance of stoichiometry changes

An organism’s urge to keep the internal key elements and stoichiometric ratios in balance can have strong ‘downstream’ effects through nutrient cycling (Hessen & Anderson, 2008; Hawlena & Schmitz, 2010a; Sistla & Schimel, 2012) on ecosystem functions such as decomposition (Güsewell & Gessner, 2009; Hawlena *et al.*, 2012). For

example, Hawlena *et al.* (2012) showed that a 4% higher C:N content of grasshopper carcasses can slow down bacterial plant litter decomposition rate by a threefold. Another way changes in stoichiometry can cascade through the food web, is by altering the nutritional quality [match between the elemental compositions of resources and consumers (Sterner & Elser, 2002)] of prey for predators (Abrams, 1992; Schmitz, 2013).

Organisms with a complex life cycle, including amphibians and semi-aquatic insects such as midges and odonates, cross habitat boundaries during their life (Rowe & Ludwig, 1991; Stoks & Córdoba-Aguilar, 2012). Thereby, the terrestrial adults could play an important role in the nutrient transfer from water to land (Baxter *et al.*, 2005; Dreyer *et al.*, 2015). Indeed, the emerging adults make up high quality fluxes, containing a high N and P supply per unit C, that terrestrial predators can exploit (Dreyer *et al.*, 2012, 2015). In addition, environmental conditions experienced in the larval aquatic stage may, through shaping the body stoichiometry of the terrestrial adult, change the nutritional value of the adults and hence change the elemental composition of aquatic subsidies to the terrestrial ecosystem (Sitters *et al.*, 2015). Despite its potential importance for coupling aquatic and terrestrial habitats, carry-over effects of environmental variables, such as warming and predation risk, on body stoichiometry across metamorphosis have been rarely studied (but see Norlin *et al.*, 2016 for a study on an amphibian).

Traits under investigation

To evaluate the impact of the different stressors, I used a multi-trait approach. Hereby, I looked at effects on survival, life history, stoichiometry, physiology and behaviour.

In Figure 3, I present a schematic overview of the main response variables I studied in this thesis.

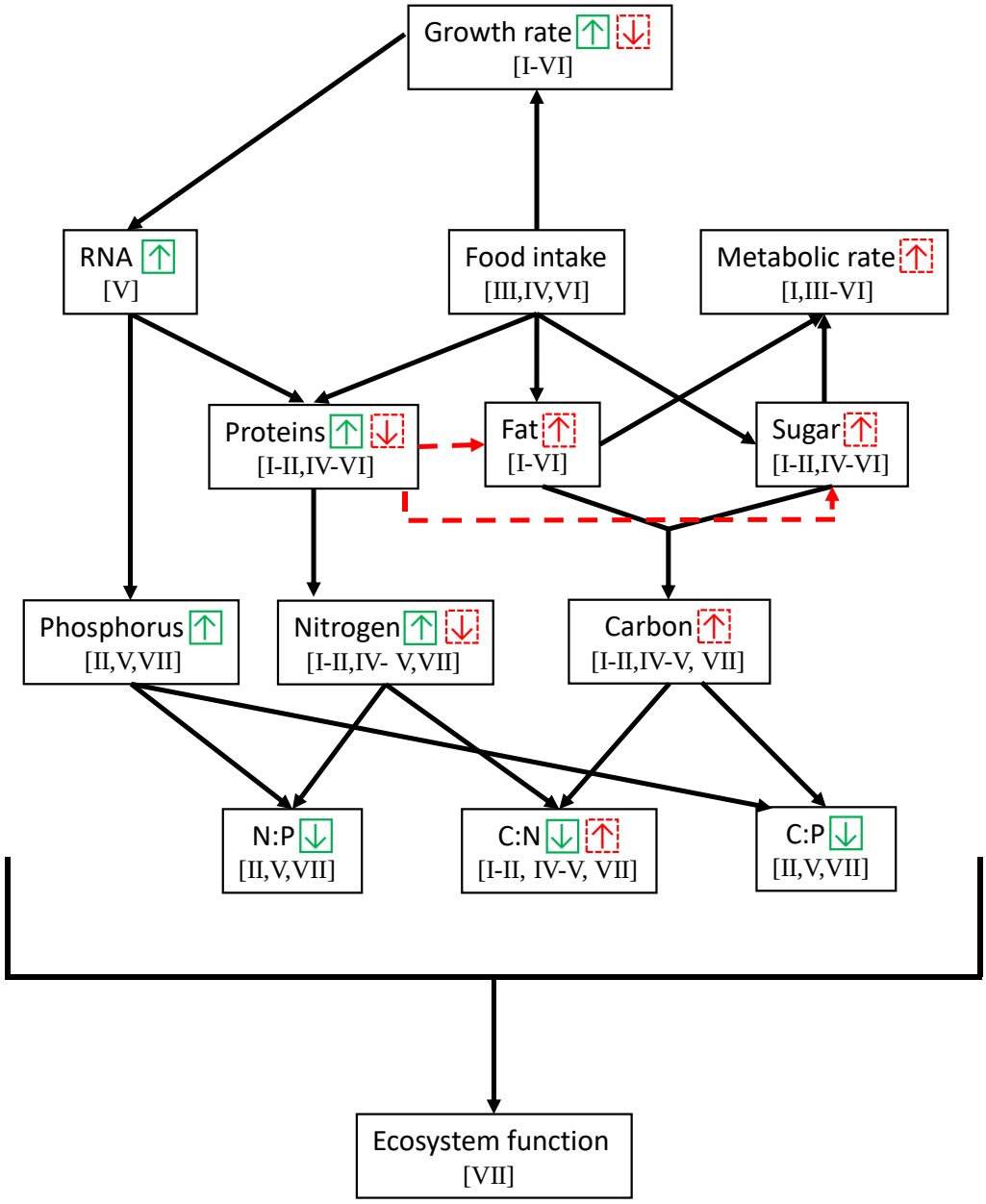

Figure 3. A schematic overview of the main response variables studied in this thesis and the hypothetical relationships between these variables. The arrows in the boxes present the predictions of the two main stoichiometric theories, with full-lined boxes the growth rate hypothesis (Elser *et al.*, 2000; Watts *et al.*, 2006) and in dashed-lined boxes the general stress paradigm, with the dotted lines presenting the expected gluconeogenesis (Hawlena & Schmitz, 2010a). An upward (downward) arrow indicates an increase (decrease) of the response variable. The Latin numbers represent chater numbers.

Life-history traits

For all stressors I studied responses in life history traits. The main focus was on larval growth rate. Together with development time, growth rate determines the mass at emergence, which on its term affects reproductive success (Stoks & Córdoba-Aguilar, 2012).

Nonconsumptive predation may impose considerable stress on prey organisms (Clinchy *et al.,* 2013), which could increase mortality (Stoks, 2001; McCauley *et al.*, 2011; Siepielski *et al.,* 2014; Gehr *et al.,* 2018), but also have important sublethal negative effects, such as a growth reduction (Benard, 2004). This is also true for pesticides, including chlorpyrifos, whereby several studies observed chlorpyrifos-induced mortality (e.g. Rubach *et al.,* 2012; Dinh Van *et al.,* 2014; Arambourou & Stoks, 2015) and decreased growth rates (e.g. Janssens & Stoks, 2013b; Arambourou & Stoks, 2015; Dinh Van *et al.*, 2016; Janssens *et al.,* 2017). Yet, higher temperatures may increase or decrease growth rate and development rate in ectotherms depending on whether or not temperature is experienced as stressful (transcends the thermal optima for these traits) (Angilletta, 2009; Tattersall *et al.,* 2012).

Bioenergetics

Bioenergetics describe the amount of available energy, the rate at which it can be gained and used and the capacity to store energy. In other words it describes the energy acquisition and its partitioning among processes such as growth, reproduction etc. Bioenergetic responses can be divided in whole-organism responses (food intake and digestive physiology) and cellular responses (cellular energy allocation) (Sokolova, 2013).

Organismal level

At the organismal level growth rate is a result of behaviour (food intake) and digestive physiology. The digestive physiology exists of the assimilation efficiency, the proportion of food that is not lost as faecal excreta, and the conversion efficiency, the percentage of the assimilated food that is converted into biomass (McPeek *et al.,* 2001).

The product of these two components is defined as the growth efficiency. For all stressors I studied the bioenergetic responses at the organismal level.

All three studied stressors have been associated with reductions in food intake or digestive efficiency: temperature (e.g. Heilmayer *et al.*, 2004; Rall *et al.*, 2010; Lemoine & Burkepile, 2012; Janssens *et al.*, 2014b), predation risk (e.g. McPeek *et al.*, 2001; Trussell *et al.*, 2006; Campero *et al.*, 2007) and pesticide (e.g. Ribeiro *et al.*, 2001; Campero *et al.*, 2007; Pestana *et al.*, 2009; Dinh Van *et al.*, 2014). Yet, to meet the increased energy demands caused by the upregulation of defence mechanisms and detoxification or to escape from the stressor, increases in food intake and digestive efficiency under stress can also be expected. Indeed, both for temperature (e.g. Thompson, 1978; Culler *et al.*, 2014;), predation risk (e.g. Stoks, 2001; Janssens & Stoks, 2013a; Thaler *et al.*, 2012; Culler *et al.*, 2014) and pesticides (e.g. Campero *et al.*, 2007; Janssens & Stoks, 2013b) such increases have been reported.

Cellular level

At the cellular level, the amount of energy that organisms can allocate to important fitness processes such as growth and reproduction is described by the cellular energy allocation (CEA) or the total net energy budget. The CEA of an organism is determined by the energy reserves available (Ea) and the energy consumption (Ec), quantified as electron transport system (ETS) activity (De Coen & Janssen, 2003; Verslycke *et al.*, 2004). Recently, a positive correlation between the CEA and organismal growth rates has been shown (Goodchild *et al.*, 2019). This indicates that the CEA could be used as an indicator for effects at higher biological levels (De Coen & Janssen, 2003). Notably, the CEA approach may be more accurate in indicating stress effects compared to the scope for growth method, which is based on assimilation and whole-animal respiration (Verslycke *et al.*, 2004). Although the CEA is described as a general biomarker, it has mainly been used for pollutant stress (e.g. De Coen & Janssen, 1997, 2003; Smolders *et al.*, 2004; Verslycke *et al.*, 2004; Novais *et al.*, 2013; Aderemi *et al.*, 2018). A few studies applied the CEA as an indicator for thermal stress (e.g. Wang *et al.*, 2012; Ferreira *et al.*, 2015, 2016; Gandar *et al.*, 2017; Kühnhold *et al.*, 2017), but to our knowledge there are no studies using CEA as a biomarker for predation stress.

The amount of energy reserves is considered a measurement of the overall condition of an organism. Energy reserves are considered as an important resistance against starvation and could further be linked to adult survival and mating success (Stoks & Córdoba-Aquilar, 2012). Both warming (e.g. Janssens *et al.,* 2014a), predation risk (e.g. Stoks *et al.,* 2005a) and pesticide exposure (e.g. Frontera *et al.,* 2011) have been shown to decrease the amount of energy reserves. Generally, energy reserves are quantified as the protein, fat and carbohydrate contents of an organism. For insects the fat content is considered as the most important reserve, since it has the highest caloric content per unit weight (Gnaiger, 1983; Arrese & Soulages, 2010). Fat is a long-term energy storage and can be used to meet the energy demand during diapause, to provide energy for the developing embryo, and to fuel prolonged periods of flight (Arrese & Soulages, 2010). Carbohydrates are used as a short-term energy storage. Insect store glucose in a polymeric form, glycogen. Yet, when needed, they can rapidly transform glycogen again into glucose (Steele, 1982). This is important as glucose is a central molecule of the metabolism (Sterner & Elser, 2002). Therefore, to create more glucose, for example to fuel their elevated metabolism, organisms can increase gluconeogenesis, or the breakdown of proteins to glucose (Hawlena & Schmitz, 2010a).

The metabolic rate of animals is often considered as the 'pace maker' for growth and other processes (Glazier, 2015). It is the amount of energy consumed by an animal during a given period, and can be estimated by heat production, O_2 consumed, or CO_2 produced. More specifically, it represents the rate at which an animal converts chemical energy to heat and mechanical work (Careau *et al.,* 2014). Since oxygen consumption itself is a function of the ETS, the activity of enzymes of this respiratory chain at the mitochondrial level have been proposed as a valid alternative for the whole-animal metabolic rate (De Coen & Janssen, 2003). Generally, the metabolic rate of animals increases exponentially with temperature (Gillooly *et al.,* 2001; Brown *et al.,* 2004). Also the activation of a stress response increases the metabolic rate of animals (Hawlena & Schmitz, 2010a). In line with this, several studies documented an increased metabolic rate when animals were exposed to one of the studied stressors: temperature (e.g. Simčič *et al.,* 2014; Kühnhold *et al.,* 2017), predation (e.g. Slos & Stoks, 2008; Thaler *et al.,* 2014), pesticide (e.g. De Coen & Janssen, 1997; Verslycke *et al.,* 2004). However, under

high or chronic stress, when animals need to reduce their energy expenditure, also a metabolic depression can occur (Storey, 2015; Dinh Van *et al.,* 2016).

Elemental composition

In this thesis there will be considerable attention for the elemental composition, more specifically the C, N and P contents and ratios, of the damselflies and the associated macromolecules. Carbon has a high binding energy (expressed as kcal mol^{-1}), suggesting that an important feature of C is its ability to store energy. Both fat and sugars are important energy reserve molecules including in insects and they are especially C-rich. More specifically, fat can consist for 70% of C and sugars for 37% (Sterner & Elser, 2002). The amount of fat and sugar is highly variable but it can represent 25% of the organism's dry mass (Sterner & Elser, 2002; Vrede *et al.,* 2004). Therefore, changes in the amount of fat and sugar can affect the C content of an organism. Nitrogen is an important element in proteins. More specifically, proteins contain about 17% N by mass and often make up a large fraction of total organismal N (Elser *et al.,* 1996; Sterner & Elser, 2002). Nucleotides and nucleic acids are the two most P-rich molecules of organisms. Especially RNA contains about 10% P by mass and often accounts for most of the total organismal P (Sterner & Elser, 2002). All the three stressor in this thesis are known to influence the RNA:DNA ratio of animals: i.e. temperature (e.g. Zhang *et al.,* 2016, 2018a), predation risk (e.g. Zhang *et al.,* 2016), pesticide (e.g. Revankar & Shyama, 2009)

In insects, two other macromolecules could also be important contributors to the C and N contents, namely the chitin and melanin in their cuticle. Chitin is a structural polysaccharide and is on average 7% N by mass. Although this is only 0.35% of the total N content of an organism, it could influence the N content and N:P ratio of organisms, especially in the cuticle (Sterner & Elser, 2002). Melanin is an N-rich polymer and on average has a C:N content of 9:1 (Chedekel *et al.,* 1992). Moreover, the studied stressors could affect both chitin and melanin content: i.e. temperature (True, 2003; Amore *et al.,* 2017), predation risk (Rabus *et al.,* 2013; Duong & McCauley, 2016), pesticide: (e.g. Zhang *et al.,* 2008).

Immune function

An important immune-related trait in insect, is the activity of the enzyme phenoloxidase (PO). This enzyme is part of the prophenoloxidase cascade, which catalyses the production of melanin (Sugumaran, 2002; González-Santoyo & Córdoba-Aquilar, 2012). Melanin also has an important function in invertebrates immunity, since it had the ability to encapsulate pathogens (Siva-Jothy *et al.*, 2005). More specifically, melanin is deposited around pathogens, thereby cutting the pathogen of from available nutrients and preventing further distribution (Gillespie *et al.*, 1997; Sugumaran, 2002). The prophenoloxidase cascade also produces several other molecules such as proteases, cytotoxin quinones, reactive oxygen and nitrogen species, which are highly reactive and toxic to pathogens and are involved in cell signalling. When these substances are produced in excess, they can become harmful to the host. Therefore, a tight regulation of prophenoloxidase cascade is needed (González-Santoyo & Córdoba-Aquilar, 2012). Given that high levels of PO and melanin are costly to maintain (e.g. Siva-Jothy & Thompson, 2002; Siva-Jothy *et al.*, 2005; De Block & Stoks, 2008; Slos *et al.*, 2009), immune function can be considered as a measure of overall condition, rather than only an indicator of immunocompetence (González-Santoyo & Córdoba-Aquilar, 2012). Due to the high maintenance cost exposure to warming (e.g. Karl *et al.*, 2011; Seppälä & Jokela, 2011; Dinh Van *et al.*, 2016), predation risk (e.g. Buchanan, 2000; Mikolajewski *et al.*, 2005) and pesticides (e.g. Galloway & Handy, 2003) can result in a reduced immune activity.

Ecosystem functioning: primary production

Ecosystem functions include all processes that facilitate energy transfer along food webs, and the major processes that allow the cycling of elements (Traill *et al.*, 2010). In this thesis I focus on primary production, since almost all life on earth directly or indirectly depends on it. Primary production or the synthesis or organic compounds from aqueous carbon dioxide mostly occurs through the process of photosynthesis. In aquatic ecosystems this is mainly done by phytoplankton such as algae. The growth and reproduction of phytoplankton species is mostly limited by the availability of P and N (Tilman *et al.*, 1982; Hecky & Kiham, 1988; Schindler & Eby, 1997; Elser *et al.*, 2007).

Moreover, enrichment of these nutrients significantly increases primary production (Elser *et al.,* 2007). Organisms directly influence nutrient supply, and hence indirectly primary production, trough nutrient excretion (Sterner, 1990; Vanni, 2002; Knoll *et al.*, 2009).

Study species

Damselflies (Odonata: Zygoptera) are well-studied and important model organisms in both the fields of ecology and evolution (Corbet, 1999; Stoks & Córdoba-Aguilar, 2012). More recently, they also gain popularity as study organisms in the fields of stress ecology and ecotoxicology (Stoks *et al.,* 2015). Because they are important intermediate predators in aquatic ecosystems, stressors affecting damselfly larvae will very likely affect the whole ecosystem. Moreover, given that damselflies have a complex life cycle with an aquatic larval stage focussing on growth, and a terrestrial adult stage focussing on dispersal and reproduction (Corbet, 1999), they can couple aquatic and terrestrial ecosystems (Stoks & Córdoba-Aguilar, 2012). Therefore, stressors affecting the aquatic food web have the potential to cascade to the terrestrial food web (Knight *et al.*, 2005; Greig *et al.*, 2012; Kraus *et al.*, 2014). Since damselfly larvae are restricted to a certain pond, they cannot escape local stressors, such as warming, predation risk and pollution. This makes them very vulnerable for such stressors.

In Chapters I-III, I used the blue-tailed damselfly, *Ischnura elegans* (Vander linden 1820) (Zygoptera: Coenagrionidae) as study species (Figure 4) This damselfly is a common species in Europe with a distribution from Spain, Italy and Greece in the south to southern Scandinavia in the North (Dijkstra, 2006; Gosden *et al.*, 2011). In Chapter I, I studied populations originating from two latitudes (France and Sweden). Between the two latitudes there is a difference in voltinism, with southern populations being multivoltine (3-4 generations per year) and northern populations being semivoltine (1 generation every two year) (Corbet *et al.*, 2006). Previous studies in our research group have documented patterns of local thermal adaptation for growth, behaviour and physiology (e.g. Shama *et al.,* 2001; Stoks *et al.*, 2012; De Block *et al.*, 2013; Debecker & Stoks, 2019).

Figure 4. Upper panels: *Ischnura elegans* adult and larvae, lower panel: *Enallagma cyathigerum* adult and larvae (photo credit: adults – Robby Stoks, larvae - Christophe Brochard).

I used the common bluet damselfly, *Enallagma cyathigerum* (Charpentier 1840) (Zygoptera: Coenagrionidae) (Figure 4), as a study species in Chapters III-VII to study the impact of predation risk, pesticides and warming. The bluet damslelfy prefers fishless ponds with large invertebrates as main predators and shows a physiological stress response when exposed to predation risk (e.g. Slos & Stoks, 2008). Although most damselflies are sensitive for pesticides, this species is particularly vulnerable for pollution, making it from a nature conservation perspective extremely relevant to study. The common bluet damselfly often co-occurs with the blue-tailed damselfly. However, while the common bluet prefers larger and deeper water bodies with more stable water temperatures, the blue-tailed occurs more in smaller, shallow water bodies with a lot of temperature fluctuations. This difference in habitat preference makes it interessting to study the difference in temperature sensibility to warming between the two species.

Outline of the thesis

In this thesis I studied the effects of two natural stressors (temperature and predation risk) and an anthropogenic stressor (pesticide exposure) on damselflies. In a first part, I focused on the effects of the single stressors on life history, stoichiometry, physiology and behaviour. In a second part, I focused on the effects of predation risk combined with temperature or pesticide exposure. More specifically, I studied both the interaction effects in the damselfly larvae and the effects on nutrient cycling.

PART 1: Single stressors

In Chapters I-III, I focused on the effects of temperature on life history, stoichiometry and physiology. Chapter I focused on a wide temperature range to reconstruct the thermal response curve of the body C:N ratio. To mechanistically understand the effects, I also studied the thermal responses of the underlying macromolecules, and life history and metabolic rate. In addition, to investigate latitude-associated thermal evolution I used *I. elegans* larvae from two latitudes.

Chapter II focused on the effects of mild warming. Using outdoor mesocosms I studied how a 4°C temperature increase (as predicted by IPCC scenario 8.5) in the larval stage shaped the adult body stoichiometry across metamorphosis. I thereby evaluated to what extent warming effects on body stoichiometry could be explained by changes in growth rate, energy storage molecules and body size.

In contrast to the mild warming in Chapter II, I exposed damselfly larvae to extreme warming in Chapter III. Hereby, I studied how a heat wave influences growth rate and investment in immune components. To gain more insight in the heat wave effects, I also quantified traits related with energy uptake, energy consumption and energy storage. To explore the consistency of heat wave responses in damselflies, I studied this in larvae of *I. elegans* and *E. cyathigerum*.

Chapter IV contrasted short-term and long-term exposure to predation risk and focused on the stoichiometric effects. I related these effects to life history, physiology and behaviour.

PART 2: Multiple stressors

Chapter V investigated the combined impact of mild (+ 4°C) warming and predation risk on growth rate and body stoichiometry. To advance the mechanistic understanding, I quantified all variables associated with the growth rate hypothesis and the general stress paradigm.

Chapter VI studied the combined impact of exposure to the pesticide chlorpyrifos and predation risk on life history. Moreover, I investigated bioenergetic responses at the organismal level and on the cellular level.

Chapter VII dealt with the impact of combined chlorpyrifos exposure and predation risk on both body stoichiometry and the elemental composition of the faecal pellets in *E. cyathigerum* larvae. Moreover, I studied how changes in the faecal pellet composition could translate into effects in ecosystem function, by investigating the impact on algae growth.

PART 1

Single stressors

Chapter I

Latitude-associated evolution and drivers of thermal response curves in body stoichiometry

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Slightly modified version

I

Abstract

Trait-based studies are needed to understand the plastic and genetic responses of organisms to warming. A neglected organismal trait is elemental composition, despite its potential to cascade into effects on the ecosystem level. Warming is predicted to shape elemental composition through shifts in storage molecules associated with responses in growth, body size, and metabolic rate. Our goals were to quantify thermal response patterns in body composition, and to obtain insights in their underlying drivers and their evolution across latitudes. We reconstructed the thermal response curves (TRCs) for body elemental composition [C(carbon), N(nitrogen), and the C:N ratio] of damselfly larvae from high- and low-latitude populations. Additionally, we quantified the TRCs for survival, growth and development rates and body size to assess local thermal adaptation, as well as the TRCs for metabolic rate and key macromolecules (proteins, fat, sugars, and cuticular melanin and chitin) as these may underlie the elemental TRCs. All larvae died at 36°C. Up to 32°C, low-latitude larvae increased growth and development rates and did not suffer increased mortality. Instead, growth and development rates of high-latitude larvae were lower and levelled off at 24°C, and mortality increased at 32°C. This latitude-associated thermal adaptation pattern matched the 'hotter-is-better' hypothesis. With increasing temperatures, low-latitude larvae decreased C:N, while high-latitude larvae increased C:N. These patterns were driven by associated changes in N contents while C contents did not respond to temperature. Consistent with the temperature-size-rule and the thermal melanism hypothesis, body size and melanin levels decreased with warming. While all traits and associated macromolecules (except for metabolic rate that showed thermal compensation) assumed to underlie thermal responses in elemental composition showed thermal plasticity, these were largely independent and none could explain the stoichiometric TRCs. Our results highlight that thermal responses in elemental composition cannot be explained by traditionally assumed drivers, asking for a broader perspective including the thermal dependence of elemental fluxes. Another key implication is that thermal evolution can reverse the plastic stoichiometric thermal responses, hence reverse how warming may shape food web dynamics through changes in body composition at different latitudes.

Introduction

There is increasing evidence that global warming can affect species' traits and thereby their local persistence and food web dynamics (Sinclair *et al.,* 2016; Gibert, 2019). A powerful conceptual tool for describing the thermal sensitivity of species' traits is the thermal performance curve (TPC), the relationship between a performance trait and a temperature gradient (Sinclair *et al.,* 2016). TPCs typically have an accelerating rising part until maximum performance is reached, followed by a fast decelerating part until the critical maximum temperature where performance is zero (Angilletta, 2009; Sinclair *et al.,* 2016).

Species may not only show plastic but also genetic responses to global warming (Merilä & Hendry, 2014). Such responses may cause TPCs to shift as a result of adaptation to the local thermal conditions (Conover *et al.,* 2009; Sinclair *et al.,* 2016). Shifts in TPCs typically take one of two forms (Conover *et al.,* 2009; Tüzün & Stoks, 2018). A 'horizontal shift' occurs when warm-adapted populations perform better at higher temperatures compared to cold-adapted populations; and vice versa. This pattern is assumed to be caused by the combination of local thermal adaptation, where maximum performances are achieved at temperatures the populations are adapted to, and a trade-off between performance at high and low temperatures (Angilletta, 2009; Kingsolver, 2009). A 'vertical shift' occurs when a population outperforms others across a temperature range. The most common scenario for life history traits is that populations inhabiting colder areas outperform those from warmer areas, and is explained by stronger time constraints experienced in colder environments (so-called countergradient variation, Conover & Schultz, 1995; Conover *et al.,* 2009). Apart from these two main modes of shifting, TPCs can also show a combination of vertical and horizontal shifts. The 'hotter-is-better' hypothesis predicts warm-adapted populations to reach higher maximum performance than cold-adapted populations at higher temperatures; this is explained by constraints on biochemical rates imposed by low temperatures (Kingsolver, 2009). One powerful method to obtain insight in the evolution of TPCs under global warming is a space-for-time substitution (Fukami & Wardle, 2005; Verheyen *et al.,* 2019). The current TPC of warm-adapted populations (e.g. at low

latitudes) is thereby used to predict the future TPC of populations currently living at colder temperatures (e.g. at high latitudes) under gradual thermal evolution (Sinclair *et al.*, 2016; Tüzün & Stoks, 2018).

While performance traits are crucial for understanding effects on organismal fitness, insight into effects of warming on ecosystems also requires the study of other organismal traits. Body stoichiometry has been identified as a key organismal trait in this context. It can mediate the effect of environmental factors on ecosystem processes such as primary production, secondary production, and nutrient cycling (Sterner & Elser, 2002; Hawlena & Schmitz, 2010a; Hawlena *et al.*, 2012; Schmitz, 2013; Leroux, 2018). Effects of warming on body elemental composition have recently been demonstrated in several animal species (e.g. Liess *et al.*, 2013; Schmitz, 2013; Janssens *et al.*, 2015, Chapter V; Norlin *et al.*, 2016; Zhang *et al.*, 2016). Yet, these studies only considered two temperatures, precluding a general 'thermal response curve' (TRC) perspective.

Three non-exclusive mechanisms have been put forward to predict and explain the effects of warming on body elemental composition (Cross *et al.*, 2015). First, building on the growth rate hypothesis (Elser *et al.*, 1996) it has been argued and empirically shown that rapid growth is associated with higher N contents (Watts *et al.*, 2006; Janssens *et al.*, 2015, Chapter V). This is expected because growth requires increased allocation to ribosomes, and cells with relatively high ribosome content also may be N-rich due to contributions of ribosomal proteins, RNAs and protein synthetic products (Watts *et al.*, 2006). Therefore, if warming causes an increase in growth rate, this should be associated with an increased synthesis of proteins, hence an increase in the body N content (shown by Janssens *et al.*, 2015, Chapter V, but see Liess *et al.*, 2013; Norlin *et al.*, 2016). Second, ectotherms generally become smaller under warming (temperature-size-rule, Atkinson, 1994), and a synthesis of empirical studies showed that smaller animals contain relatively less proteins (Woods *et al.*, 2003). Hence, warming can therefore be expected to decrease the body N content, as supported by the review of Woods *et al.* (2003). A third mechanism is based on the metabolic rate of organisms. Organisms use C-rich macromolecules (i.e. fat and sugars) to fuel their metabolic rate. Therefore, if warming increases the metabolic rate, this is expected to be

coupled with an increase in C-rich macromolecules (Schmitz, 2013). Moreover, through gluconeogenesis organisms can thereby break down N-rich proteins to obtain more C-rich molecules. To maintain homeostasis, they should excrete the excess N (Hawlena & Schmitz, 2010). Together, this is expected to result in a higher body C:N ratio under warming, as has been shown in grasshoppers (Schmitz, 2013). However, if warming causes a metabolic depression (Storey, 2015), organisms would need less C-rich molecules to fuel their metabolic rate, resulting in a decreased body C content.

Recent studies indicated that traditionally studied macromolecules (fat, sugars and proteins) upon which the above three mechanisms rely, may not fully capture how environmental factors shape the body elemental composition (e.g. Janssens *et al.*, 2015, Chapter V; Van Dievel *et al.*, 2016, Chapter IV; Zhang *et al.*, 2016). Moreover, it has been shown that body contents of C, N and P may not covary with the body content of these macromolecules (Wilder & Jeyasingh, 2016). We therefore suggest two additional candidate macromolecules that may contribute to warming-induced changes in body composition in insects: melanin and chitin, important components of the cuticle. Given their amount in the body is small compared to the traditionally studied macromolecules, they are thought to contribute little to the C content. Yet, because of their high N content, they are expected to influence the total body N content (Chedekel *et al.*, 1992; Sterner & Elser, 2002). Moreover, both chitin and melanin have been functionally linked to temperature (True, 2003; Amore *et al.*, 2017). Therefore, it is relevant to study their response to temperature in the context of ecological stoichiometry. Chitin is a structural polysaccharide that may reduce heating rates (Amore *et al.*, 2017). Melanin is a N-rich polymer, that is involved in thermoregulation (True, 2003). According to the thermal melanism hypothesis, darker individuals (with more melanin) have a heating advantage in colder environments, while light-coloured individuals are better in avoiding overheating in hot environments (True, 2003; Zeuss *et al.*, 2014).

In the current study, we performed a common-garden experiment to reconstruct the thermal response curves of the body elemental composition (C, N and C:N ratio) in larvae of the damselfly *Ischnura elegans*. To evaluate support for the three mechanisms hypothesized to cause temperature-induced changes in body elemental composition (see above), we also quantified TRCs for following traits: (i) life history (survival, growth

and development rates, and body size), (ii) metabolic rate, (iii) the macromolecules proteins, fat, and sugars, and (iv) the cuticle components melanin and chitin. Moreover, we compared the TPCs between high- and low-latitude populations to assess thermal adaptation and the potential effect of gradual evolution in shaping the TRCs at the high latitude in a warming world by using a space-for-time substitution.

Given that the proposed mechanisms underlying thermal responses of body elemental composition are based on thermal effects on growth rate, body size, and metabolic rate, and how these shape the level of the macromolecules proteins, fat and/or sugars (see above), we expect the TRCs of these traits to be closely associated. Based on the first mechanism, we predict that a warming-induced increase in growth rate is associated with a higher protein content, and hence a higher N content (Sterner & Elser, 2002; Watts *et al.,* 2006). Based on the temperature-size rule (Atkinson, 1994), we predict that a temperature increase would cause a smaller body size, resulting in lower protein, hence N contents. Based on the third mechanism, we predict that a temperature-driven increase in metabolic rate leads to an increase of C-rich molecules and excretion of excess N, hence an increased C content and C:N ratio (Schmitz, 2013). Finally, as two additional mechanisms, we predict that a temperature increase would result in a lower melanin content (True 2003; Zeuss *et al.*, 2014), hence a lower C:N ratio and N content. Yet, in contrast it may also result in a thicker chitin (Amore *et al.,* 2017), hence higher C:N and N content. Underlying all these predictions are following assumed links between levels of macromolecules and body elemental composition that we will test: higher protein levels translate in higher body N levels, higher fat and sugar levels contribute to higher body C levels, and higher cuticular chitin and melanin levels translate in higher body C:N ratio and N levels.

Based on the higher time constraints in the low-latitude populations (associated with a higher number of generations per year, see further), we predict a vertical shift of the TPC for growth/development rate, with the low-latitude larvae outperforming high-latitude larvae across the temperature gradient (Conover & Schultz, 1995; Conover *et al.,* 2009). In addition, thermal adaptation may result in growth and development rates levelling off at lower temperatures, and mortality increasing more at higher temperatures in high-latitude larvae than in low-latitude larvae. We further predict that warming

decreases the body size of the larvae, and the magnitude of this pattern to be independent of latitude (based on Klok & Harrison, 2013). Based on a meta-analysis (Zeuss *et al.*, 2014), we expect a higher melanin content in high-latitude larvae. Because of lower heating rates, we predict that low-latitude larvae have a higher cuticular chitin content (Amore *et al.*, 2017). These expected latitudinal differences in life history and melanin content may drive latitudinal differences in the elemental and macromolecular body composition following the mechanisms and directions explained above.

Materials and methods

Study populations and pre-experimental rearing

We studied *I. elegans* from three populations each in the low-latitude (southern France) and high-latitude (Denmark and Sweden) parts of the range in Europe (Gosden *et al.*, 2011) (for details see Appendix 1). High-latitude populations of the species are semivoltine (2 years per generation), and low-latitude populations are multivoltine (>2 generations per year) (Corbet *et al.*, 2006). We collected 10 mated females per population. Ten days after egg hatching, larvae were placed individually in plastic 200 mL vials filled with dechlorinated tap water. At that moment, the thermal treatments started (see below). During this period, larvae were fed six days a week ad libitum with *Artemia* nauplii.

Experimental setup

To reconstruct the latitude-specific thermal response curves for life history (survival, growth and development rate, body size), metabolic rate, elemental body composition (C, N and C:N) and macromolecules (proteins, fat, sugars, cuticular chitin and melanin) we setup a common-garden experiment with six rearing temperatures. We assigned 35 to 75 individuals per population to each temperature (total of 1756 larvae).

Ten days after hatching, larvae were assigned to one of the six temperature treatments: 17, 20, 24, 28, 32 and 36°C. Water temperature in the vials were measured twice a day. Measured mean temperatures (in °C) closely matched this range: 17.30 (SE: 0.03), 20.11 (SE: 0.03), 23.98 (SE: 0.02), 27.91 (SE: 0.02), 31.93 (SE: 0.03) and 35.81 (SE: 0.05). Larvae typically encounter temperatures between 17-24°C during the spring-

summer main growing season at both latitudes (Debecker & Stoks, 2019). The mean summer water temperatures in shallow lakes inhabited by the study species are 20°C at the high latitude and 24°C at the low latitude (Debecker & Stoks, 2019). According to global warming scenario RCP8.5, the average temperature will increase with 4°C by 2100 (ICPP, 2013), leading to mean summer water temperatures of 24°C at the high latitude and of 28°C at the low latitude. Moreover, we also added higher temperatures which may be encountered during warmer periods, including heat waves. Simulations of the Lake Model Flake (2009) using model settings suitable for damselfly larvae (based on Nilsson-Örtman *et al.*, 2012) indicated that water temperatures of 30°C and higher are frequently encountered during summer at the low latitude but never at the high latitude. At neither latitude the water temperatures reach 36°C. Since under global warming heat waves are expected to become more intense (IPCC, 2013), 36°C can be considered a future extreme temperature at the low latitude.

We used a stepwise temperature increase of 4°C every 24h to reach the rearing temperatures of 28, 32 and 36°C. Larvae were fed twice per day with *Artemia* nauplii ad libitum for six days per week. Once the larvae moulted into the final instar they were fed daily. After seven days in the final instar, the larvae were frozen (-80°C) for further analyses.

Response variables

We daily checked for mortality. When the damselfly larvae moulted into their final instar (F0) we measured their head width, a good proxy for body size in odonate larvae (Benke, 1970). We photographed the head using a stereomicroscope with a camera operated by the imaging software StreamPix version 7.3.0.0 (NorPix, Inc., Montreal, Canada). We quantified the head width to the nearest 0.001 mm from enlarged images of the head (30× magnification) using ImageJ 1.50e (National Institutes of Health, Bethesda, Maryland, USA). Reference photographs of a glass scale were used for calibration. When the larvae were one week into their final instar, they were weighed to the nearest 0.01 mg using an electronic balance (Mettler Toledo® AB135-S, Columbus, OH, USA). We calculated the growth rate as ln(wet mass) divided by the development

time (the number of days between hatching and one week in F0) (as in Johansson *et al.*, 2001). The development rate was calculated as the inverse of the development time.

We quantified the elemental composition, the macromolecules and the metabolic rate on a subset of 20 larvae per treatment combination (total of 200 larvae). A detailed description of the protocols is presented in the Supporting information (Appendix 2). First, we measured the C and N contents with an element analyser (Carlo Erba 1108, Thermo Fisher, Waltham, USA) using leucine for calibration. The C and N contents were expressed as % of dry mass and the C:N ratio was expressed as a molar ratio. Next, we quantified the macromolecular contents and the metabolic rate. We quantified protein, fat and sugar contents using established protocols for damselfly larvae (Stoks *et al.*, 2006a). All macromolecules were expressed as µg per mg wet mass. As a measure of metabolic rate, we measured the activity of the electron transport system (ETS) based on the protocol of De Coen and Janssen (2003). The ETS activity was expressed as nmol O_2 consumed per min and per mg wet mass.

Statistical analyses

In a first step, we performed a multivariate analysis of variance (MANOVA) to test for the effects of temperature, latitude, and their interactions on the complete set of 12 response variables (except survival). We included both the linear and quadratic terms of temperature (indicated as Temperature^2) to capture nonlinear responses to temperature. We separately evaluated survival (dead vs. alive) with a generalized linear mixed effect model including temperature, latitude, and their interaction, and with a binominal error structure and a logit-link function. Since all larvae reared at 36°C died, we excluded this temperature from the model.

In a next step, we explored the relationship between the different response variables, as well as reduced the number of response variables for further univariate analyses. For this, we performed a principal component analysis (PCA) with varimax rotation on the complete set op 12 response variables (except survival). Subsequently, separate univariate linear mixed effect models were performed using the scores of each PC dderived from the PCA as response variables and with the same fixed-effect structure

as in the MANOVA. We also performed univariate models for each original response variable to confirm the findings based on the PC axes (reported in Appendix 3).

To take into account that the three populations at a given latitude are not independent replicates, we included population nested in latitude as a random factor to all models. As an additional step, we tested for potential differences in thermal responses between the three population at a given latitude, using (generalized) linear models with population, temperature (linear and quadratic terms), and their interactions included as fixed effects. Full results of these analyses are presented in Appendix 4.

We performed all statistical analyses in R v3.4.0 (R Development Core Team, 2017). We used the package 'lme4' (Bates *et al.,* 2015) to run the (generalized) linear mixed effects models, and the package 'car' (Fox & Weisberg, 2011) to calculate the F-statistics and *P*-values for fixed effects using the Kenward–Roger method. All models were fitted using restricted maximum likelihood.

Results

Multivariate analyses

The MANOVA of the 12 original life history, physiological, elemental and macromolecular variables showed strong effects of temperature (Temperature: Pillai's trace = 0.73, $F_{1.179} = 44.13$, $P < 0.001$, Temperature²: Pillai's trace = 0.27, $F_{1.179} = 5.56$, $P < 0.001$) and latitude (Pillai's trace = 0.71, $F_{1.179} = 36.09$, $P < 0.001$). The Temperature × Latitude interaction (Pillai's trace = 0.56 $F_{1.179} = 19.30$, $P < 0.001$) indicated that the two latitudes differed in their multivariate response to temperature.

The PCA revealed that the 12 response variables were largely independent (biplots are presented in Appendix 5). Eight PC axes (each explaining >9% of the variance) were needed to jointly explain 90% of the variance, with many axes only having high loadings from a single variable (Table 1). PC1 reflected life history with larger values indicating faster growth and development rates, and smaller body size. PCs 2 and 3 reflected body elemental composition with higher PC2-values indicating higher C:N and lower N contents, while higher PC3-values indicated higher C contents. Higher PC4-values were associated with higher fat and sugar contents, while higher PC5-values

were associated with higher protein contents. PC6 and PC7 had high loadings of melanin and chitin contents, respectively. Finally, higher PC8-values indicated higher metabolic rates.

Table 1. Factor loadings of the principal component analyses of the set of life history (growth rate, development rate and body size), elemental (C and N contents, C:N ratio), macromolecular (protein, fat, sugar, cuticular melanin and cuticular chitin contents), and physiology (metabolic rate) traits. High factor loadings are indicated in bold.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Growth rate	**0.98**	-0.11	-0.09	0.02	-0.01	-0.13	-0.05	0.02
Development rate	**0.96**	-0.13	-0.12	0.02	0.01	-0.17	-0.08	0.02
Head width	**-0.58**	0.03	0.08	-0.07	-0.08	0.34	0.16	-0.01
Metabolic rate	0.03	0.04	0.05	0.08	0.06	0.03	-0.16	**0.98**
C content	-0.18	0.26	**0.91**	0.15	0.12	0.08	0.11	0.06
N content	0.15	**-0.93**	-0.11	-0.21	-0.14	-0.09	-0.10	-0.02
C:N ratio	-0.18	**0.77**	0.51	0.21	0.16	0.10	0.12	0.04
Protein content	0.01	0.18	0.12	0.13	**0.96**	0.00	0.00	0.07
Fat content	0.08	0.26	0.19	**0.50**	0.25	0.00	-0.14	0.15
Sugar content	0.04	0.29	0.15	**0.91**	0.13	0.04	-0.09	0.08
Melanin content	-0.31	0.13	0.09	0.04	0.01	**0.91**	0.15	0.04
Chitin content	-0.12	0.14	0.11	-0.10	0.00	0.14	**0.94**	-0.18
Cumulative variance	20%	35%	45%	55%	64%	73%	81%	90%

Life history

At the highest rearing temperature (36°C), all larvae of both latitudes died within the first three weeks. At the other temperatures, the effects of temperature differed between latitudes (Temperature × Latitude, Temperature² × Latitude, Table 2; Fig. 1). In the high-latitude larvae, survival first increased with increasing temperatures, and then decreased giving an inverse U-shape pattern (Fig. 1). Instead, survival remained constantly high across temperatures in the low-latitude larvae.

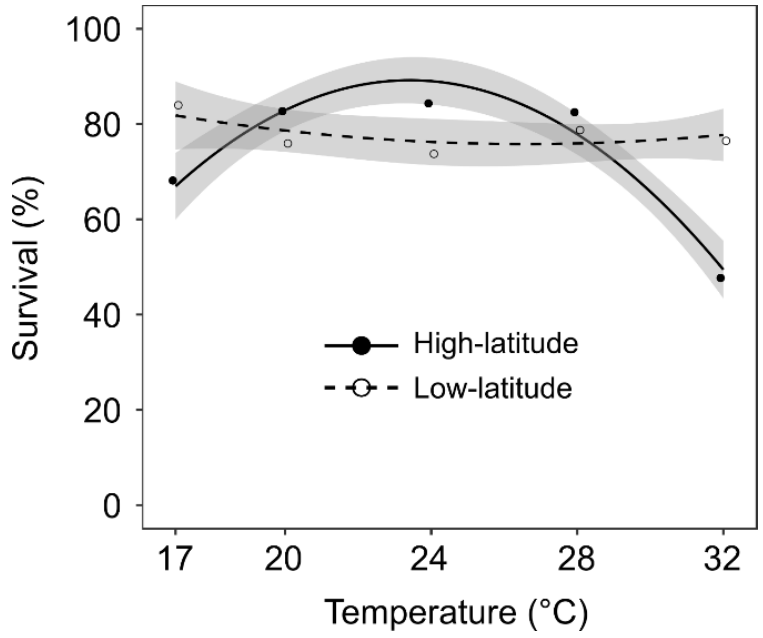

Figure 1. Thermal performance curves of survival of high- and low-latitude *Ischnura elegans* larvae. Grey bands around the curves represent 95% confidence intervals. Note that survival was 0% at 36 °C for both latitudes.

The temperature response curves of PC1 (+ growth and development rates, - body size) had a concave downward shape (Temperature², Table 2; Fig. 2a, Fig. S1a-c). PC1 increased more with temperature in low-latitude than in high-latitude larvae, where PC1 clearly levelled off at higher temperatures (Temperature × Latitude, Table 2; Fig. 2a).

Table 2. Results of the (generalized) linear mixed models testing for the effects of temperature and latitude on survival, and the principal components extracted from the set of life history (growth rate, development rate and body size), elemental (C and N contents, C:N ratio), macromolecular (protein, fat, sugar, cuticular melanin and cuticular chitin contents), and physiology (metabolic rate) traits in *Ischnura elegans* larvae.

Response variable	Effect	df_1, df_2	F	*P*
Survival	Temperature	1,1480	35.50	**<0.001**
	$Temperature^2$	1,1480	20.50	**<0.001**
	Latitude	1,1480	7.02	**0.008**
	Temperature x Latitude	1,1480	35.27	**<0.001**
	$Temperature^2$ x Latitude	1,1480	33.50	**<0.001**
PC1	Temperature	1,188	283.80	**<0.001**
	$Temperature^2$	1,189	24.06	**<0.001**
	Latitude	1,4	137.70	**<0.001**
	Temperature x Latitude	1,188	125.11	**<0.001**
	$Temperature^2$ x Latitude	1,189	2.15	0.144
PC2	Temperature	1,188	1.76	0.187
	$Temperature^2$	1,189	0.02	0.876
	Latitude	1,4	8.77	**0.042**
	Temperature x Latitude	1,188	9.09	**0.003**
	$Temperature^2$ x Latitude	1,189	1.19	0.276
PC3	Temperature	1,188	0.08	0.771
	$Temperature^2$	1,189	0.87	0.353
	Latitude	1,4	32.35	**0.005**
	Temperature x Latitude	1,188	0.29	0.592
	$Temperature^2$ x Latitude	1,189	1.07	0.302
PC4	Temperature	1,188	9.58	**0.003**
	$Temperature^2$	1,189	0.01	0.912
	Latitude	1,4	3.89	0.121
	Temperature x Latitude	1,188	4.43	**0.037**
	$Temperature^2$ x Latitude	1,189	0.81	0.368
PC5	Temperature	1,188	16.61	**<0.001**
	$Temperature^2$	1,189	0.06	0.798
	Latitude	1,4	0.45	0.538
	Temperature x Latitude	1,188	11.06	**0.001**
	$Temperature^2$ x Latitude	1,189	0.24	0.624
PC6	Temperature	1,188	10.20	**0.002**
	$Temperature^2$	1,189	5.02	**0.026**
	Latitude	1,4	5.39	0.081
	Temperature x Latitude	1,188	18.93	**<0.001**
	$Temperature^2$ x Latitude	1,189	0.17	0.684
PC7	Temperature	1,188	0.0071	0.933
	$Temperature^2$	1,189	9.34	**0.003**
	Latitude	1,4	3.27	0.145

	Temperature x Latitude	1,188	2.25	0.135
	Temperature^2 x Latitude	1,189	4.00	**0.047**
PC8	Temperature	1,188	0.85	0.357
	Temperature^2	1,189	0.53	0.468
	Latitude	1,4	3.43	0.138
	Temperature x Latitude	1,188	2.00	0.158
	Temperature^2 x Latitude	1,189	0.34	0.563

Body elemental composition

PC2 (- N content, + C:N ratio) increased linearly (hence N decreased and C:N increased) with increasing temperature for the high-latitude larvae, while there was no temperature effect for the low-latitude larvae (Temperature × Latitude, Table 2; Fig. 2b, Fig. S2b-c). PC2 was higher for the high-latitude larvae compared to the low-latitude larvae. The univariate ANOVAs on the original variables confirmed that the N content of the high-latitude larvae decreased linearly, and their C:N ratio increased linearly. Yet, these ANOVAs also identified the opposite stoichiometric response pattern in the low-latitude larvae: the N content of the low-latitude larvae increased linearly and their C:N ratio slightly decreased linearly with increasing temperature (Table S1; Fig. S2b-c).

Temperature did not affect PC3 (+ C content). High-latitude larvae had a higher PC3 (more C) than low-latitude larvae (Table 2; Fig. 2c, Fig. S2a).

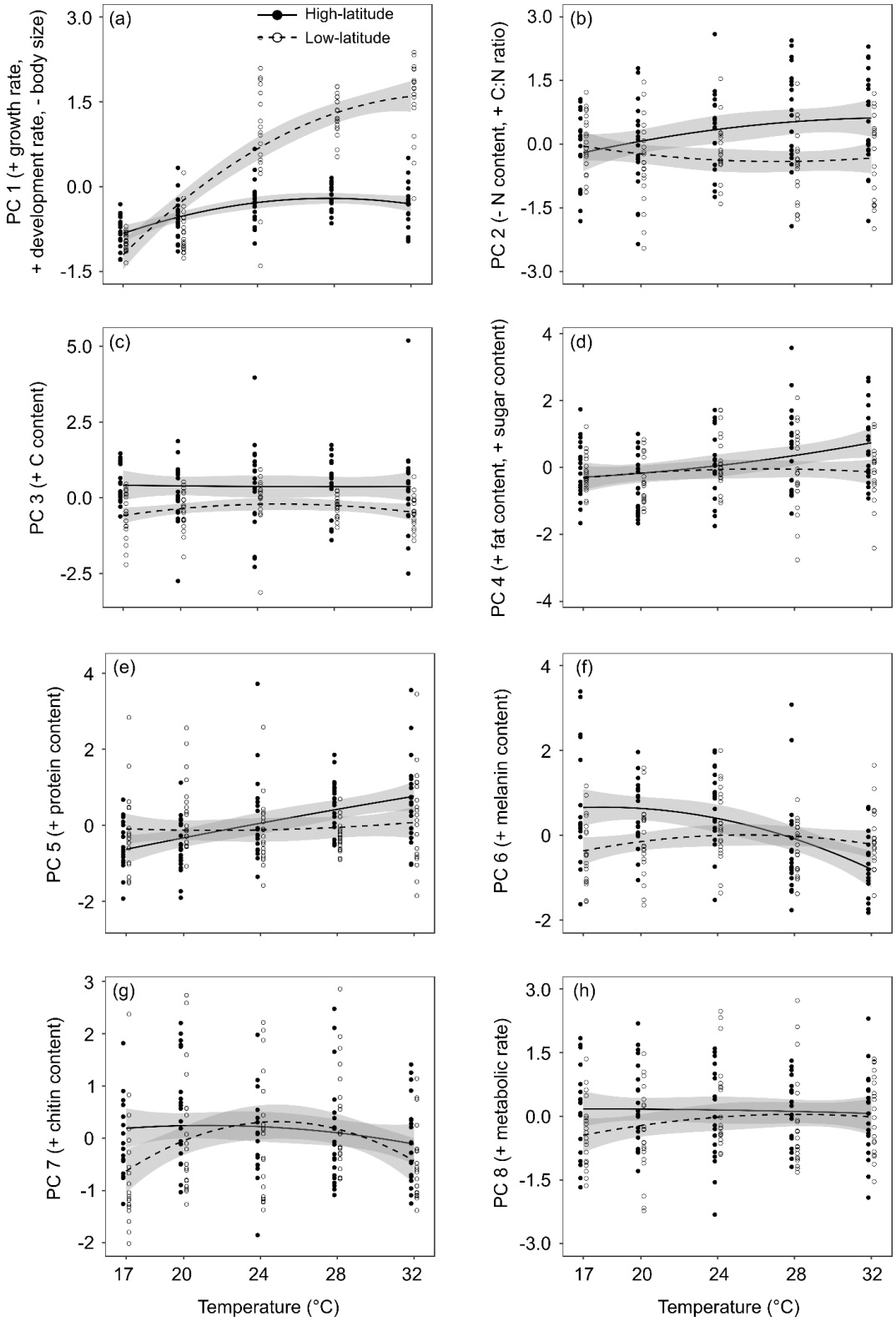

Figure 2. Thermal response curves of the principal component axes extracted from the set of life history, elemental, macromolecular and physiology traits of high- and low-latitude *Ischnura elegans* larvae. Shown are results for PC1 (+ growth rate, + development rate, – body size), PC2 (- N content, + C:N ratio), PC3 (+ C content), PC4 (+ fat content, + sugar content), PC5 (+ protein content), PC6 (+ melanin content), PC7 (+ chitin content), and PC8 (+ metabolic rate). See Table 1 for the factor loadings on the PC axes. Grey bands around the curves represent 95% confidence intervals.

Macromolecular composition

Both PC4 (+ fat and sugar contents) and PC5 (+ protein content) showed a linear thermal response that differed between the two latitudes (Temperature × Latitude, Table 2; Fig. 2d–e). In the low-latitude larvae, temperature had no effect on the two PCs. In the high-latitude larvae, warming markedly increased PC4 and PC5 (hence the levels of all three macromolecules, Fig. S3a-c).

The thermal response curve of PC6 (+ melanin content) had a concave shape (Temperature and Temperature², Table 2; Fig. 2f, Fig. S3d). The linear temperature effect differed between the latitudes (Temperature × Latitude, Table 2; Fig. f2): PC6 decreased at high temperatures for the high-latitude larvae, but there was no temperature effect for the low-latitude larvae. This interaction also indicated the melanin content was at low temperatures higher in high-latitude than in low-latitude larvae. The thermal response curve of PC7 (+ chitin content) was quadratic but only in low-latitude larvae: PC7 first increased with increasing temperatures, and then decreased giving an inverse U-shape pattern (Temperature² × Latitude, Table 2; Fig. 2g, Fig. S3e). There was no quadratic temperature effect in the high-latitude larvae.

Physiology

PC8 (+ metabolic rate) was not affected by temperature, nor did it differ between the two latitudes (Table 2; Fig. 2h, Fig. S1d).

Discussion

Temperature influenced all response variables (except the C content and the metabolic rate), and most of the thermal response curves had shifted between latitudes, indicating widespread evolution of thermal plasticity. Thermal response patterns were largely consistent among the three studied populations per latitude (Appendix 4), indicating we captured general latitudinal patterns of adaptation in thermal response curves. We will first discuss latitude-associated thermal adaptation in life history, and then discuss how each of the mechanisms put forward to predict and explain thermal patterns in body elemental composition failed to explain the observed patterns.

Latitude-associated thermal adaptation

The TPCs for life history indicated local thermal adaptation: while the growth and development rates of low-latitude larvae (that experience higher environmental temperatures) were highest at higher temperatures (up to 32°C), the high-latitude larvae already reached the highest levels at 24-28°C. Moreover, survival decreased for the high-latitude larvae but remained high for the low-latitude larvae at the higher temperatures (up to 32°C). In addition, the performance of the low-latitude larvae was generally higher than the high-latitude larvae over the temperature range. Together, these two elements indicate a shift in TPCs following the hotter-is-better hypothesis (Kingsolver, 2009).

A recent synthesis of empirical studies showed widespread support for the hotter-is-better hypothesis (Sørensen *et al.*, 2018). Moreover, this pattern seems stronger for life history traits, such as the here studied growth and development rates (e.g. Frazier *et al.*, 2006; Knies *et al.*, 2009; Angilletta *et al.*, 2010; Dillon & Frazier, 2013). At the basis of this hypothesis is the general thermodynamic principle of increased rates of biological processes at higher temperatures. This is expected to result in warm-adapted populations to have higher maximum performance than cold-adapted populations at their respective optimum temperatures (Kingsolver & Huey, 2008; Kingsolver, 2009). In other words, the 'hotter-is-better' pattern we observed indicates that our study species, as many other insects (Dillon & Frazier, 2013), did not fully compensate its life history for the negative effects of low temperatures on biological rates (Kingsolver & Huey, 2008; Angilletta, 2009). Notably, this thermodynamic effect contrasts with our observation that larvae showed perfect thermal acclimation across the temperature range for metabolic rate (Seebacher *et al.*, 2018). Empirical evidence for temperature compensation of metabolic rate is mixed, with studies both in favor and against it (reviewed in Sørensen *et al.*, 2018).

For both latitudes, the optimal growth and developmental temperatures were above the environmental temperature. This pattern is not uncommon (Angilletta *et al.*, 2010, for damselfly species: Nilsson-Örtman *et al.*, 2012; Van Dievel *et al.*, 2017, Chapter III). Shifting the optimal temperature above the average summer temperature (20°C for the high latitude and 24°C for the low latitude) may be adaptive both to exploit

short infrequent periods of higher temperatures (Kingsolver, 2000), and to avoid negative fitness effects during transient exposure to extreme temperatures (Martin & Huey, 2008).

Mechanism 1: Changes in body elemental composition mediated through growth rate

Building on the growth rate hypothesis (Sterner & Elser, 2002), a higher growth rate under warming should be associated with a higher protein content driven by new tissue production, hence a higher N content (Watts *et al.*, 2006; Janssens *et al.*, 2015, Chapter V). At neither latitude we found support for this mechanism. In high-latitude larvae the growth rate first increased with warming and then levelled off, while the protein content kept increasing with increasing temperature. Possibly, the further increase of proteins at the higher temperatures reflected a higher production of stress proteins (for the study species: Janssens *et al.*, 2014a, Lancaster *et al.*, 2016). Moreover, the consistent increase in protein content with warming contrasted with the consistent decrease in N content.

In low-latitude larvae the growth rate consistently increased with warming (up to 32°C). In accordance with the first mechanism, also the N content of the low-latitude larvae increased with temperature, resulting in a slight decrease of the C:N ratio with increasing temperature (Figure S1a in Appendix 3). This matches the recently identified pattern that faster developing *Gryllus integer* crickets also showed a higher N content and a lower C:N ratio (Trakimas *et al.*, 2019). Yet, in contrast to the first mechanism, the protein content of the low-latitude damselfly larvae did not change under warming. In general, the key assumption of this growth-mediated mechanism was not met. Indeed, the loadings on separate PC axes indicated no strong relationship between the body contents of proteins and N.

Mechanism 2: Changes in body elemental composition mediated through body size

In line with the temperature-size-rule (Atkinson, 1994), warming reduced the body size of the damselfly larvae. Furthermore, the magnitude of the thermal response was similar between the latitudes, matching the general pattern in arthropods (reviewed in Klok & Harrison, 2013). Yet, the second mechanism could not explain the thermal responses in body elemental composition through the assumed lower protein, hence lower N contents

of smaller animals (Woods *et al.*, 2003). Indeed, only the high-latitude larvae showed the associated decrease in N content under warming which was, however, not associated with a decrease in protein content. Similarly, El-Sabaawi *et al.* (2012) documented that body size could not predict the body elemental composition of guppies.

Mechanism 3: Changes in body elemental composition mediated through metabolic rate

The third mechanism predicts that warming should increase the C content and lower the N content because of an increase in metabolic rate that asks for a higher investment in C-rich sugar and fat contents and a higher excretion of N (Schmitz, 2013). Yet, in contrast with this mechanism, warming had no effect on the metabolic rate (measured as the ETS activity). As for the metabolic rate, temperature also had no effect on the C content. Moreover, only in high-latitude larvae did the C-rich fat and sugar contents increase with temperature. In general, the key assumption of this mechanism was not met. Indeed, the body contents of sugars and fat, and the C content were not strongly associated and loaded on different PC axes.

Mechanisms 4 and 5: Changes in body elemental composition mediated through cuticular melanin and chitin

As predicted by the thermal melanism hypothesis (True, 2003; Zeuss *et al.*, 2014), the cuticular melanin content decreased with increasing temperatures. While PC6 only showed this in the high-latitude larvae, direct analysis of the melanin content showed this also in the low-latitude larvae (Figure S3d). A light-coloured body is advantageous at higher temperatures because a high body reflectance prevents overheating (True, 2003; Zeuss *et al.*, 2014). A darker coloration is beneficial at lower temperatures because the lower reflectance of the cuticle enables dark-coloured ectotherms to heat up faster. In line with this, high-latitude larvae, which are adapted to colder temperatures (Stoks & De Block, 2011), also had more melanin in their cuticle at lower temperatures when compared to the low-latitude larvae. This matches the general pattern that high-latitude species have a darker cuticle (Zeuss *et al.*, 2014).

The cuticular chitin content showed a concave temperature response in low-altitude larvae. The lower chitin content at the lower temperatures might be adaptive as

it may increase the heating rate of ectotherms (Arrese & Soulages, 2010). Yet, this may not explain the reduced chitin content at higher temperatures which may increase the risk of overheating (Amore *et al.,* 2017). This decrease of chitin content at the higher temperatures was also identified in high-latitude larvae by the direct analysis (Figure S3e). Possibly, it was energetically too costly to maintain a high chitin synthesis at the highest temperatures.

The observed thermal response patterns in cuticle melanin and chitin, molecules with a high C:N content (Chedekel *et al.,* 1992; Sterner & Elser, 2002), are unlikely to have contributed to the thermal response patterns in body elemental composition. The decreased melanin and chitin contents at higher temperatures could have balanced the increased C-rich fat and sugar contents at higher temperatures in the high-latitude larvae. However, since the fat and sugar contents of low-latitude larvae did not change with temperature, it could not explain why the C content of the low-latitude larvae did not respond to temperature. In addition, also the increased N content of the low-latitude larvae with warming is in contrast with the decrease in melanin and chitin contents with warming. Especially, since there was no effect of temperature on the protein content of the low-latitude larvae. Possibly, warming altered the N excretion rates (e.g. Liess *et al.,* 2015). For the high-latitude larvae, the decrease in N content with warming followed the decreases in cuticular melanin and chitin contents. Yet, despite this congruent pattern it is unlikely to be causal for two reasons. First, at the individual level the PC analysis indicated that the N content was not related with the cuticular melanin and chitin contents. Second, the contributions of cuticular melanin and chitin were likely too low to alter the total body C and N contents (see Appendix 6: Table S3).

Conclusions and ways forward

An emerging insight is that to understand and predict the impact of global warming on species and food webs we need to study species' trait responses (Sinclair *et al.,* 2016; Fox, 2018; Gibert, 2019). Against this background, we for the first time reconstructed TRCs of stoichiometric traits; organismal traits that have the potential to mediate effects of warming on ecosystem functions (Schmitz, 2013; Leroux, 2018). A key finding was that the TRCs of the elemental body composition could not be explained by the

traditional drivers (growth rate, body size, and metabolic rate, Cross *et al.*, 2015; Schmitz, 2013), nor by the here proposed additional drivers (cuticular melanin and chitin contents). Instead, our results showed that the proposed mechanisms and the associated macromolecules were largely independent of the body elemental composition. This is an important observation since ecological stoichiometry assumes that the elemental composition of organisms is related to their macromolecular composition (Sterner & Elser, 2002). Yet, the few studies explicitly investigated this relationship, also found no or weak links (Wilder & Jeyasingh, 2016; Zhang *et al.*, 2018b). To advance insights in the (de)coupling between elemental and macromolecular body contents it may be important to study in more detail the macromolecular content of the exoskeleton (Wilder *et al.*, 2019) as this makes up an important part of the total body mass in insects (Lease & Wolf, 2010). Furthermore, better characterizing the macromolecules may be important. For example, the N content of amino acids varies between 9-35% which may cause strong variation in the N content of proteins (Sterner & Elser, 2002). Another promising avenue to obtain better mechanistic insights is to focus also on the fluxes of key elements and how these are shaped by warming. A recent conceptual framework indeed indicated that the thermal dependency of the assimilation and ingestion rates of key elements may determine the stoichiometric thermal response curves (Schmitz & Rosenblatt, 2017).

Studying TRCs of elemental composition within replicated populations of two strategically chosen latitudes generated important novel insights at the intersection of global warming and ecological stoichiometry. Our study thereby added to the increasing awareness of the importance of both spatial (Leroux, 2018) and evolutionary patterns (Leal *et al.*, 2017) in elemental composition of organisms. Our results revealed that the change in body composition was linear across the wide temperature range (from 17°C to 32°C), yet in opposite directions between latitudes. This indicates that latitude-associated thermal evolution can reverse the plastic thermal stoichiometric response, hence how warming may shape food web dynamics through changes in body elemental composition at different latitudes. Specifically, our results suggest that in the absence of thermal evolution, 4°C warming to a mean of 24°C by 2100 at the high latitude will lead to a plastic 4.2% increase in the C:N ratio (Fig. S2c). Yet, based on a space-for-time

approach (Fukami & Wardle, 2005; Verheyen *et al.*, 2019) using the current body elemental composition of the low-latitude larvae at 24°C (the temperature to which they are adapted to), the opposite response is to be expected: a 3.4% decrease in C:N. This may have far reaching consequences for ecosystem functioning as changes in C:N of similar magnitude have been associated with a threefold change in nutrient cycling (Hawlena *et al.*, 2012). Integrating stoichiometric traits into the trait sets typically studied in global warming studies may address the call for better mechanistic insights in global change ecology (Schmitz, 2013). Yet, as we highlighted, unravelling the mechanisms shaping the thermal stoichiometric response curves themselves remains a challenge.

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Author contributions: MVD and RS conceived the ideas and designed the experiment; MVD collected the data; MVD and NT analyzed the data; MVD, NT and RS wrote the manuscript.

Appendix 1: A detailed description of the study populations and pre-experimental rearing

We studied *I. elegans* from three populations each in the low-latitude (southern France) and high-latitude (Denmark and Sweden) parts of the range in Europe (Gosden *et al.*, 2011). The north-south distance between these latitudes is ca. 1500 km. At each latitude three random populations were sampled. At the low latitude, we collected at the French sites Saint-Martin-de-Crau (43°37'56.77"N, 04°58'20"E), Camaret-sur-aigues (44°08'56"N, 04°51'17"E) and Cabriès (43°28'1.85"N, 05°19'35.51"E). At the high latitude, we collected at the Swedish sites Lund (55°44'5.4"N, 13°9'13.4"E) and Uppsala (59°50'37.1"N, 17°39'59.7"E), and in the Danish site Laesø (57°15'21.14"N, 10°54'19.75"E). High-latitude populations of the species are semivoltine (2 years per generation), and low-latitude populations are multivoltine (>2 generations per year) (Corbet *et al.*, 2006). All study populations inhabited shallow lakes with abundant aquatic vegetation.

In the summer of 2015, we collected 10 mated females per population (total of 60 females). Females were placed individually in plastic cups with wet filter paper for oviposition. Eggs of each female were transferred in 50 ml plastic vails filled for 30 ml with water to the laboratory in Belgium and incubated at a water temperature of 20°C and a photoperiod of 14L:10D. The water was refreshed daily and newly hatched larvae were kept together in group per female. Ten days after hatching, larvae were placed individually in plastic 200 mL vials filled with dechlorinated tap water. At that moment, the thermal treatments started (see below). During this period, larvae were fed six days a week ad libitum with *Artemia* nauplii.

Appendix 2: A detailed description of the used protocols

For the quantification of the metabolic rate, the elemental composition and the macromolecules we first homogenised the larvae and diluted the homogenate five times in milli-Q water. Then, 40 µL of the sample was transferred to tin cups and dried and weighed (60°C, 24 h). Afterwards the C and N contents of the samples were measured with an element analyser (Carlo Erba 1108,Thermo Fisher Benelux, Eke, Belgium) using leucine for calibration. The C and N contents were expressed as % of dry mass. The C:N ratio was expressed as a molar ratio taking into account the molar mass of C (12 g/mol) and N (14 g/mol). The rest of the sample was used for the assays of metabolic rate and macromolecules (see below).

To quantify the metabolic rate (ETS activity) and the macromolecules we first centrifuged the rest of the sample for 8 min (13,000 rpm, 4 °C). Next, we transferred all the supernatant to a new Eppendorf tube. This supernatant was used for the determination of the ETS activity and the fat, sugar and protein contents. The pellet that remained after transferring the supernatant was used to quantify the melanin and chitin contents.

To quantify the ETS activity and the protein, fat and sugar contents we first took 30 µL of the supernatant and diluted this three times with phosphate-buffered saline (PBS). The ETS activity was measured based on the protocol of De Coen and Janssen (2003). ETS activity at the mitochondrial level is directly linked to O_2 consumption and is considered a good proxy for metabolic rate (De Coen & Janssen, 2003). A 348-well microtiter plate was filled with 15 µL buffered substrate solution (0.13 mol/L Tris-HCl, 0.3% Triton X-100, 1.7 mmol/L NADH, 250 µmol/L NADPH, pH 8.5) and 5 µL of the diluted supernatant. To start the reaction we added 10 µL INT (8 mmol/L p-iodonitrotetrazolium). The increase in absorbance was measured at 490 nm (at 20°C, TECAN infinite M200 spectrophotometer, Männedorf, Switzerland) during 10 minutes with readings every 30 seconds. We used the Lambert-Beer formula to calculate the concentration of formazan (extinction coefficient 15 900 mol/L cm). Afterwards, we converted this concentration to cellular oxygen consumption based on the theoretical stoichiometric relationship that for each 2 µmol of formazan formed, 1 µmol of O_2 was

consumed in the ETS system. ETS measurements were done in quadruplicate and the means were used for statistical analyses. The ETS activity was expressed as nmol O_2 consumed per min and per mg wet mass.

We quantified protein, fat and sugar contents using established protocols for damselfly larvae (Stoks *et al.*, 2006a). We determined the protein content using the Bradford (1976) method. 160 µL milli-Q water and 1 µL of the diluted supernatant were added to a 96-well microtiter plate. Then, we added 40 µL Biorad protein dye and mixed the sample. We incubated the plate for 5 min at 30°C and subsequently measured the absorbance at 595 nm (at 25°C). The protein content was measured in quadruplicate and we used the average absorbance to calculate the protein content based on a standard curve of known bovine serum albumin concentrations. For the quantification of the fat content we used a modified version of the protocol of Marsh and Weinstein (1966). In 2 mL glass tubes we mixed 8 µL of the supernatant with 56 µl H_2O_4 (100%). Afterwards, we heated the tubes for 20 minutes at 150°C and subsequently added 64 µL milli-Q water. A 384-well microtiter plate was filled with 30 µL of the sample and we measured the absorbance at 490 nm (at 25°C). Fat content was measured in triplicate and we converted the averaged absorbance per larva to its fat content using a standard calibration curve of glyceryl tripalmitate. To measure the total sugar content (glucose + glycogen) we used the protocol of Stoks *et al.* (2006a) based on the glucose kit from Sigma Aldrich (St. Louis, Missouri, USA). First, we transformed all glycogen into glucose. Therefore, we mixed 32.5 µL milli-Q water, 12.5 µL of the supernatant and 5 µL amyloglucosidase (Sigma A7420) in a 96-well microtiter plate. Then, the plate was incubated for 30 min at 37°C to transform the glycogen to glucose. The total sugar concentrations were measured by adding 100 µL of glucose assay reagent (Sigma G3293) to each well and incubating the plate for 20 min at 30°C. Afterwards, we measured the absorbance at 340 nm (25°C). The glucose content was measured in duplicate and we converted the averaged absorbance per larva to calculate the glucose content based on a standard curve of known concentrations of glucose. All these macromolecules were expressed as µg per mg wet mass.

We quantified the cuticular components based on protocols by Zhou *et al.* (2012) for melanin and by Katano *et al.* (2016) for chitin. We first dried the remaining sample

(60°C, 24h). Then, we added 100 µL 5N HCl and incubated the sample for 2h at 70°C. To quantify the melanin content, we added 120 µL 1 N NaOH / 10% DMSO to 30 µL of the sample. This was then incubated for 2h at 80 °C and centrifuged for 10 min (13000g, 4°C). Next, we transferred 30 µL to a 384-well microtiter plate and we measured the absorbance at 380 nm. The melanin content was measured in duplicate and the means per larva were used to calculate the melanin content using a standard calibration curve of synthetic melanin (Sigma-Aldrich®). To quantify the chitin content we added 20 µL 5N NaOH and 100 µL molybdenum blue-reagent to 20 µL of the sample. Then, we incubated the Eppendorf tube for 30 min at 70°C. After this incubation period, we centrifuged the tubes for 1 min (3000g, 4°C) and we added 30 µL of the supernatant in a 384-well microtiter plate. We measured the absorbance at 340 nm (25°C). The chitin content was measured in triplicate and the average absorbance was used to calculate the chitin content based on a standard calibration curve with known concentrations of chitin obtained from shrimp shells (Sigma-Aldrich®). The chitin and melanin contents were expressed as µg per mg wet mass.

Appendix 3: Detailed analyses and results of the individual traits

Statistical analyses

We analysed the effects of temperature (both linear and quadratic terms), and latitude and their interactions on all response variables using linear mixed models. To take into account that populations at a given latitude are not independent, population nested in latitude was included as a random factor.

Results

Life history

Thermal performance curves for growth rate and development rate had a similar concave downward shape at both latitudes, except for the development rate of the low-latitude larvae, which increased linearly (Table S1; Fig. S1a-b). The increase in growth rate and development rate with temperature was stronger in low-latitude than in high-latitude larvae (Table S1; Fig. S1a-b).

The head width of the larvae, as a proxy for body size, decreased with temperature. This decrease had a linear and quadratic component which did not differ between latitudes (Table S1; Fig. S1c). The high-latitude larvae were consistently larger than the low-latitude larvae across temperatures (Table S1; Fig. S1c).

Physiology

The ETS activity, as a proxy for metabolic rate, was not affected by temperature, nor did it differ between the two latitudes (Table S1; Fig. S1d).

Table S1. Results of the linear mixed models testing for the effects of temperature and latitude on life history, physiology, elemental and macromolecular composition in *Ischnura elegans* larvae.

Response variable	Effect	df_1, df_2	F	*P*
Life history				
Growth rate	Temperature	1,480	534.94	**<0.001**
	Temperature^2	1,480	16.26	**<0.001**
	Latitude	1,4	32.33	**0.005**
	Temperature x Latitude	1,480	147.36	**<0.001**
	Temperature^2 x Latitude	1,480	0.87	0.811
Development rate	Temperature	1,480	579.73	**<0.001**
	Temperature^2	1,480	4.77	**0.029**
	Latitude	1,4	41.60	**0.003**
	Temperature x Latitude	1,480	138.94	**<0.001**
	Temperature^2 x Latitude	1,480	0.87	0.352
Body size	Temperature	1,473	280.46	**<0.001**
	Temperature^2	1,473	8.35	**0.004**
	Latitude	1,4	9.15	**0.039**
	Temperature x Latitude	1,473	2.06	0.151
	Temperature^2 x Latitude	1,473	0.80	0.371
Physiology				
Metabolic rate	Temperature	1,188	2.93	0.088
	Temperature^2	1,189	0.15	0.697
	Latitude	1,4	4.50	0.101
	Temperature x Latitude	1,88	0.84	0.361
	Temperature^2 x Latitude	1,189	0.097	0.756
Elemental composition				
C content	Temperature	1,189	0.19	0.662
	Temperature^2	1,190	1.22	0.271
	Latitude	1,4	93.18	**<0.001**
	Temperature x Latitude	1,189	1.50	0.222
	Temperature^2 x Latitude	1,190	0.79	0.377
N content	Temperature	1,189	1.12	0.291
	Temperature^2	1,190	0.0091	0.924
	Latitude	1,4	28.60	**0.006**
	Temperature x Latitude	1,189	17.83	**<0.001**
	Temperature^2 x Latitude	1,190	0.60	0.441
C:N ratio	Temperature	1,189	1.54	0.215
	Temperature^2	1,190	0.43	0.514
	Latitude	1,4	81.78	**<0.001**
	Temperature x Latitude	1,189	14.55	**<0.001**
	Temperature^2 x Latitude	1,190	0.03	0.862
Macromolecules				
Protein content	Temperature	1,190	24. 10	**<0.001**
	Temperature^2	1,191	0.0026	0.959
	Latitude	1,4	4.82	0.093

	Temperature x Latitude	1,190	15.94	**<0.001**
	$Temperature^2$ x Latitude	1,191	0.23	0.633
Fat content	Temperature	1,190	27.59	**<0.001**
	$Temperature^2$	1,191	0.10	0.748
	Latitude	1,4	14.40	**0.019**
	Temperature x Latitude	1,190	8.56	**0.004**
	$Temperature^2$ x Latitude	1,191	0.0029	0.957
Sugar content	Temperature	1,190	20.12	**<0.001**
	$Temperature^2$	1,191	0.0084	0.927
	Latitude	1,4	17.47	**0.014**
	Temperature x Latitude	1,190	7.39	**0.007**
	$Temperature^2$ x Latitude	1,191	0.20	0.654
Melanin content	Temperature	1,190	18.42	**<0.001**
	$Temperature^2$	1,191	6.09	**0.014**
	Latitude	1,4	40.30	**0.003**
	Temperature x Latitude	1,190	3.07	0.081
	$Temperature^2$ x Latitude	1,191	0.24	0.624
Chitin content	Temperature	1,190	0.16	0.688
	$Temperature^2$	1,191	9.69	**0.002**
	Latitude	1,4	12.72	**0.023**
	Temperature x Latitude	1,190	2.53	0.113
	$Temperature^2$ x Latitude	1,191	2.30	0.131

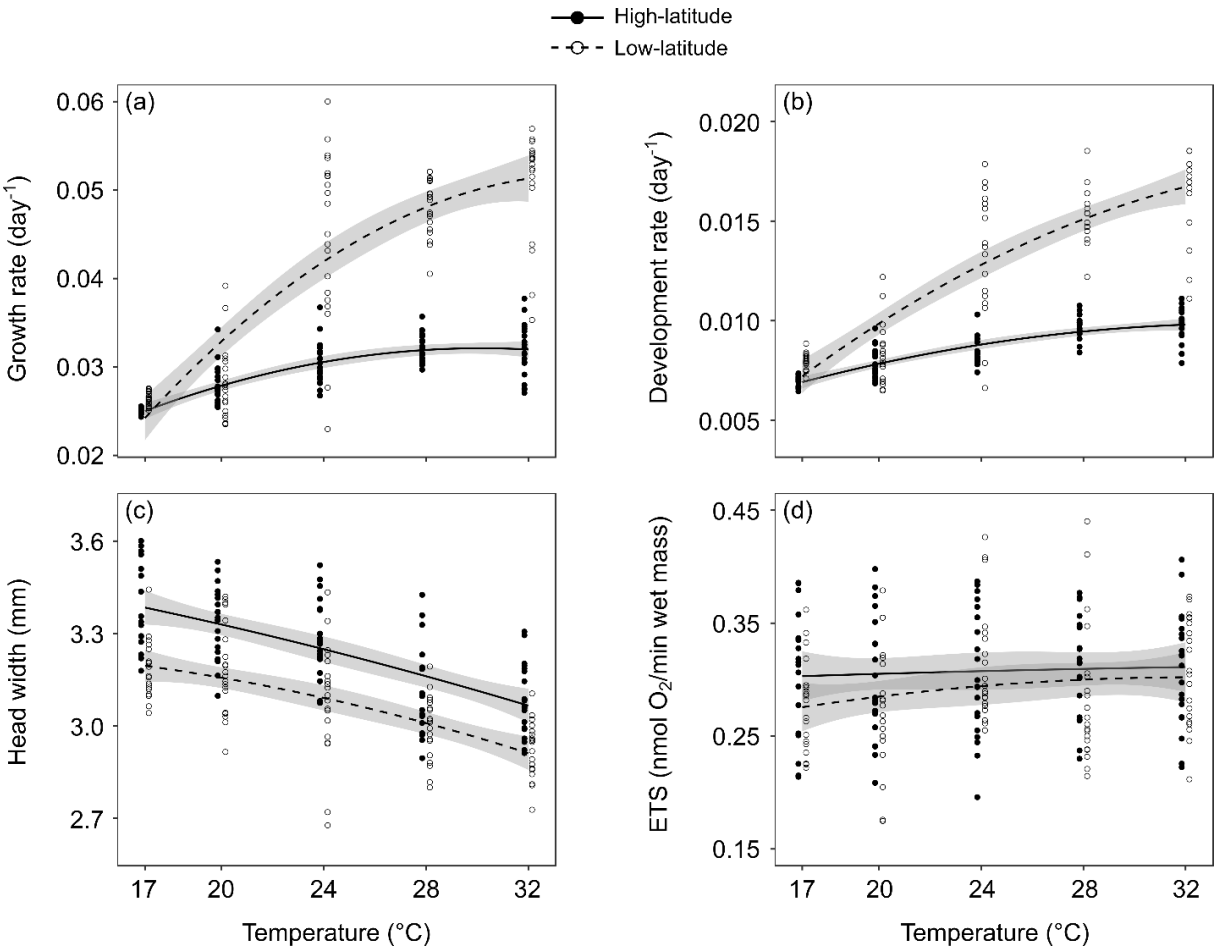

Figure S1. Thermal response curves of life history and physiology traits of high- and low-latitude *Ischnura elegans* larvae: (a) growth rate, (b) development rate, (c) head width, and (d) ETS activity. Grey bands around the curves represent 95% confidence intervals.

Elemental composition

Temperature did not affect the C content, and high-latitude larvae had a higher C content than low-latitude larvae (Table S1; Fig. S2a). With increasing temperature, the N content of the high-latitude larvae decreased linearly, while the N content of the low-latitude larvae increased linearly (Table S1; Fig. S2b). This resulted in opposite linear patterns for the C:N ratio which with increasing temperatures markedly increased in the high-latitude larvae and slightly decreased in the low-latitude larvae (Table S1; Fig. S2c). The Temperature × Latitude interactions for N and C:N also indicated that with increasing temperatures, the N content became lower and the C:N ratio became higher in the high- compared to the low-latitude larvae (Fig. S2b-c).

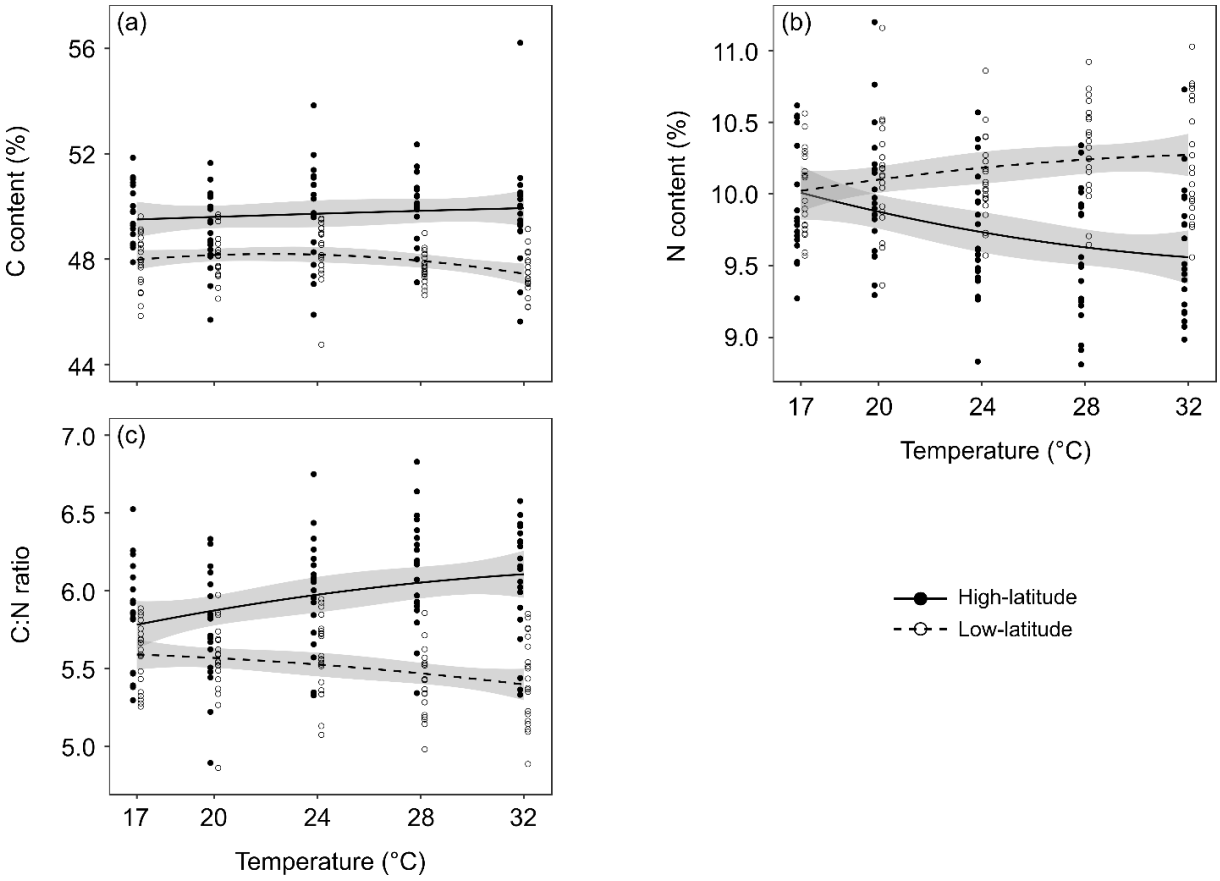

Figure S2. Thermal response curves of body elemental composition of high- and low-latitude *Ischnura elegans* larvae: (a) C content, (b) N content, and (c) molar C:N ratio. Grey bands around the curves represent 95% confidence intervals.

Macromolecular composition

Protein, sugar and fat contents showed linear but not quadratic responses to temperature, and these linear thermal responses strongly differed between the two latitudes (Table S1; Fig. S3a-c). In the low-latitude larvae, temperature had no effect on these macromolecules. In the high-latitude larvae, warming markedly increased the protein, sugar and fat contents.

Warming reduced the cuticular melanin content in the same way at the two latitudes (Table S1; Fig. S3d). The melanin content was higher in high-latitude than in low-latitude larvae (Table S1; Fig. S3d). The cuticular chitin content showed a similar inverse U-shaped pattern at both latitudes (Table S1; Fig. S3e). The chitin content was higher in high-latitude than in low-latitude larvae (Fig. S3e).

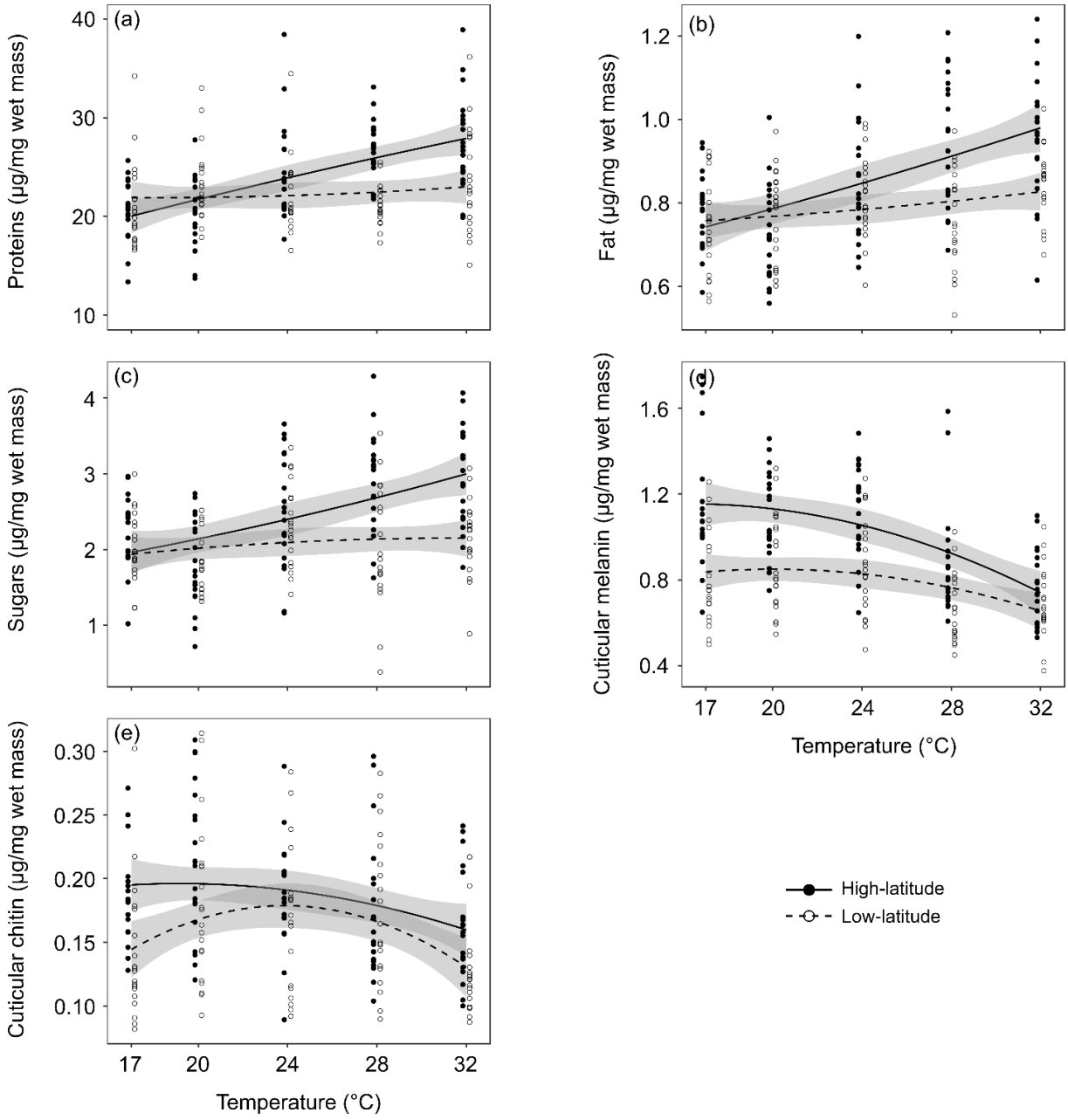

Figure S3. Thermal response curves of five macromolecules of high- and low-latitude *Ischnura elegans* larvae: (a) protein, (b) fat, (c) sugar, (d) cuticular melanin, and (e) chitin contents. Grey bands around the curves represent 95% confidence intervals.

Appendix 4: Detailed analyses and results of the variation between populations within each latitude

Statistical analyses

We tested for potential differences in thermal responses between the three populations of a given latitude using linear models with population, temperature (linear and quadratic terms), and their interactions included as fixed effects. For survival (dead vs. alive) we used a generalized linear model, including the same fixed-effect structure, but with a binominal error structure and a logit-link function. Since all larvae at 36°C died, we excluded this temperature from the model.

Results

The three different populations within a latitude showed in general a consistent thermal response as indicated by the non-significant Temperature × Population and Temperature^2 × Population interaction terms (Table S2; Fig. S4-S5). However, there were two exceptions where one low-latitude population showed a different response to the Temperature^2 term compared to the other two low-latitude populations (Table S2; Fig. S5a, S5h). The low-latitude population originating from Cabriès (pink TPC in Fig. S5a) showed a stronger increase in PC1 (+ growth rate, + development rate, - body size) with temperature compared to the other two populations from low latitudes. Nevertheless, all three low-latitude populations showed a stronger thermal response than the three high-latitude populations. For PC8 (+ metabolic rate), the low-latitude larvae from Saint-Martin-de-Crau (light blue color TPC in Fig. S5h) showed an inverse U-shape, whereas the other two populations from the low latitude did not react to temperature.

Table S2. Results of the (generalized) linear models testing for the effects of temperature and population on life history (survival, growth rate, development rate and body size), elemental (C and N contents, C:N ratio), macromolecular (protein, fat, sugar, cuticular melanin and cuticular chitin contents), and physiological (metabolic rate) traits in *Ischnura elegans* larvae.

Response variable	Effect	df_1, df_2	F	*P*
High latitude populations				
Survival	Temperature	1,720	42.79	**<0.001**
	Temperature^2	1,720	53.79	**<0.001**
	Population	2,720	4.55	**0.011**
	Temperature x Population	2,720	1.63	0.196
	Temperature^2 x Population	2,720	0.45	0.637
PC1	Temperature	1,89	34.01	**<0.001**
	Temperature^2	1,89	14.08	**<0.001**
	Population	2,89	1.36	0.263
	Temperature x Population	2,89	0.14	0.869
	Temperature^2 x Population	2,89	0.31	0.734
PC2	Temperature	1,89	7.71	**0.007**
	Temperature^2	1,89	0.30	0.583
	Population	2,89	3.30	**0.041**
	Temperature x Population	2,89	0.13	0.874
	Temperature^2 x Population	2,89	0.25	0.775
PC3	Temperature	1,89	0.0072	0.933
	Temperature^2	1,89	0.0036	0.952
	Population	2,89	0.65	0.522
	Temperature x Population	2,89	1.44	0.243
	Temperature^2 x Population	2,89	0.49	0.614
PC4	Temperature	1,89	11.45	**0.001**
	Temperature^2	1,89	0.24	0.621
	Population	2,89	1.56	0.215
	Temperature x Population	2,89	1.34	0.267
	Temperature^2 x Population	2,89	0.55	0.580
PC5	Temperature	1,89	31.65	**<0.001**
	Temperature^2	1,89	0.05	0.819
	Population	2,89	1.77	0.176
	Temperature x Population	2,89	2.82	0.065
	Temperature^2 x Population	2,89	1.35	0.263
PC6	Temperature	1,89	22.98	**<0.001**
	Temperature^2	1,89	2.60	0.110
	Population	2,89	0.17	0.839
	Temperature x Population	2,89	0.13	0.881
	Temperature^2 x Population	2,89	2.12	0.126
PC7	Temperature	1,89	1.29	0.259

	Temperature^2	1,89	0.59	0.443
	Population	2,89	0.0001	1.000
	Temperature x Population	2,89	0.108	0.345
	Temperature^2 x Population	2,89	2.04	0.136
PC8	Temperature	1,89	0.01	0.680
	Temperature^2	1,89	0.0012	0.972
	Population	2,89	1.68	0.192
	Temperature x Population	2,89	1.41	0.248
	Temperature^2 x Population	2,89	1.34	0.267
Low latitude populations				
Survival	Temperature	1,748	0.27	0.605
	Temperature^2	1,748	1.12	0.290
	Population	2,748	2.83	0.060
	Temperature x Population	2,748	1.08	0.342
	Temperature^2 x Population	2,748	0.38	0.682
PC1	Temperature	1,91	285.84	**<0.001**
	Temperature^2	1,91	14.70	**<0.001**
	Population	2,91	0.47	0.623
	Temperature x Population	2,91	1.30	0.277
	Temperature^2 x Population	2,91	7.41	**0.001**
PC2	Temperature	1,91	1.56	0.215
	Temperature^2	1,91	0.94	0.336
	Population	2,91	0.081	0.922
	Temperature x Population	2,91	0.72	0.490
	Temperature^2 x Population	2,91	0.025	0.975
PC3	Temperature	1,91	0.75	0.389
	Temperature^2	1,91	4.32	**0.040**
	Population	2,91	0.061	0.941
	Temperature x Population	2,91	2.46	0.091
	Temperature^2 x Population	2,91	2.13	0.125
PC4	Temperature	1,91	0.64	0.424
	Temperature^2	1,91	0.59	0.445
	Population	2,91	0.42	0.659
	Temperature x Population	2,91	0.50	0.609
	Temperature^2 x Population	2,91	0.22	0.799
PC5	Temperature	1,91	0.35	0.555
	Temperature^2	1,91	0.32	0.575
	Population	2,91	0.31	0.105
	Temperature x Population	2,91	2.62	0.078
	Temperature^2 x Population	2,91	2.06	0.134
PC6	Temperature	1,91	0.78	0.379
	Temperature^2	1,91	2.16	0.145
	Population	2,91	1.73	0.184

	Temperature x Population	2,91	1.13	0.326
	Temperature^2 x Population	2,91	1.57	0.212
PC7	Temperature	1,91	1.04	0.309
	Temperature^2	1,91	10.81	**0.001**
	Population	2,91	0.02	0.979
	Temperature x Population	2,91	0.23	0.793
	Temperature^2 x Population	2,91	0.77	0.468
PC8	Temperature	1,91	2.76	0.100
	Temperature^2	1,91	0.86	0.356
	Population	2,91	0.24	0.783
	Temperature x Population	2,91	0.70	0.501
	Temperature^2 x Population	2,91	3.34	**0.040**

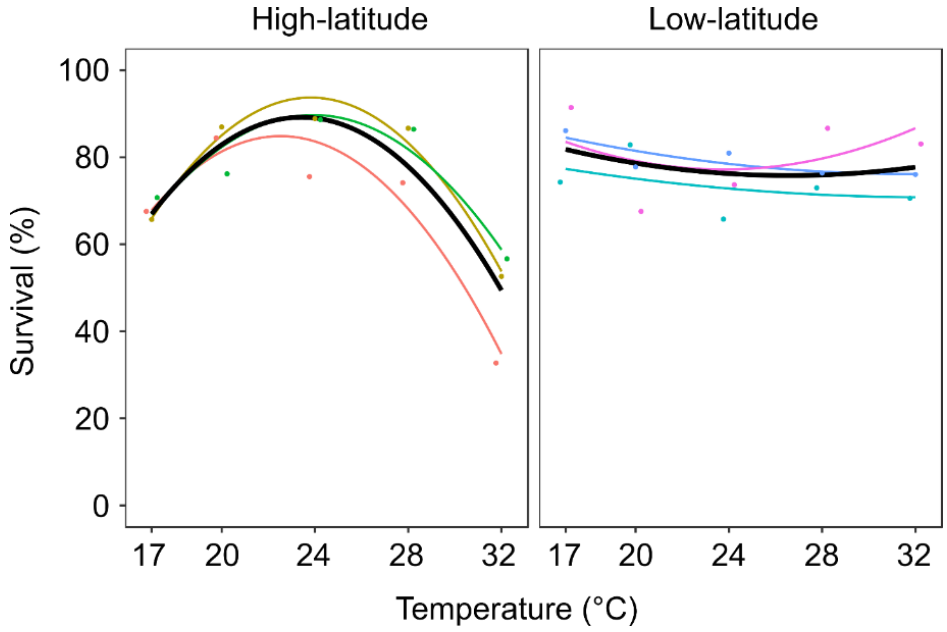

Figure S4. Thermal response curves of larval survival of the three high- and low-latitude populations of *Ischnura elegans*. The black line shows the mean response curve of the three populations at a given latitude.

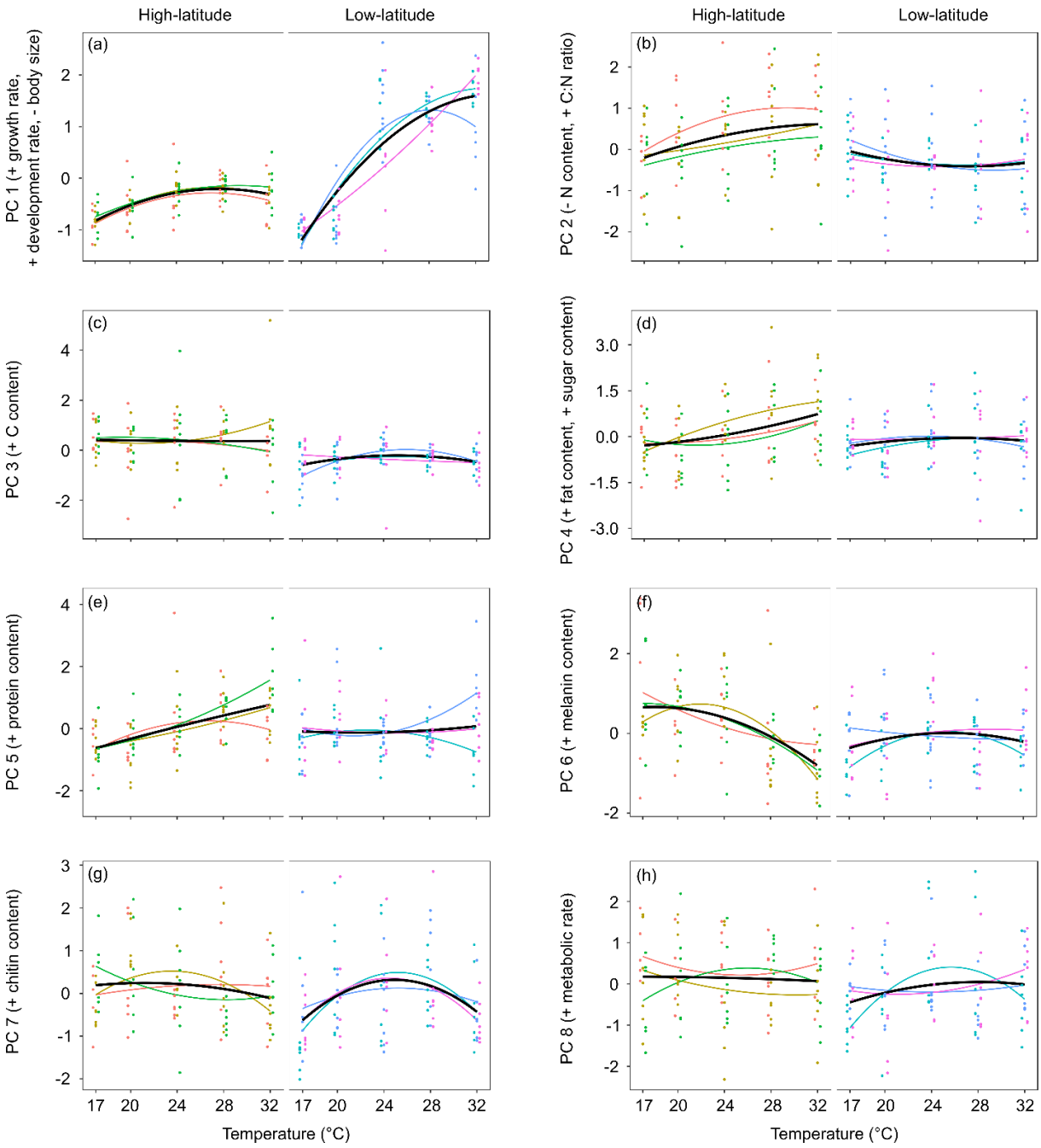

Figure S5. Thermal response curves of the three high- and low-latitude populations of *Ischnura elegans*. Shown are results for PC1 (+ growth rate, + development rate, – body size), PC2 (- N content, + C:N ratio), PC3 (+ C content), PC4 (+ fat content, + sugar content), PC5 (+ protein content), PC6 (+ melanin content), PC7 (+ chitin content) and PC8 (+ metabolic rate). The black lines represent the mean response curve of the three populations at a given latitude.

Appendix 5: Biplots of the Principal Component Analysis

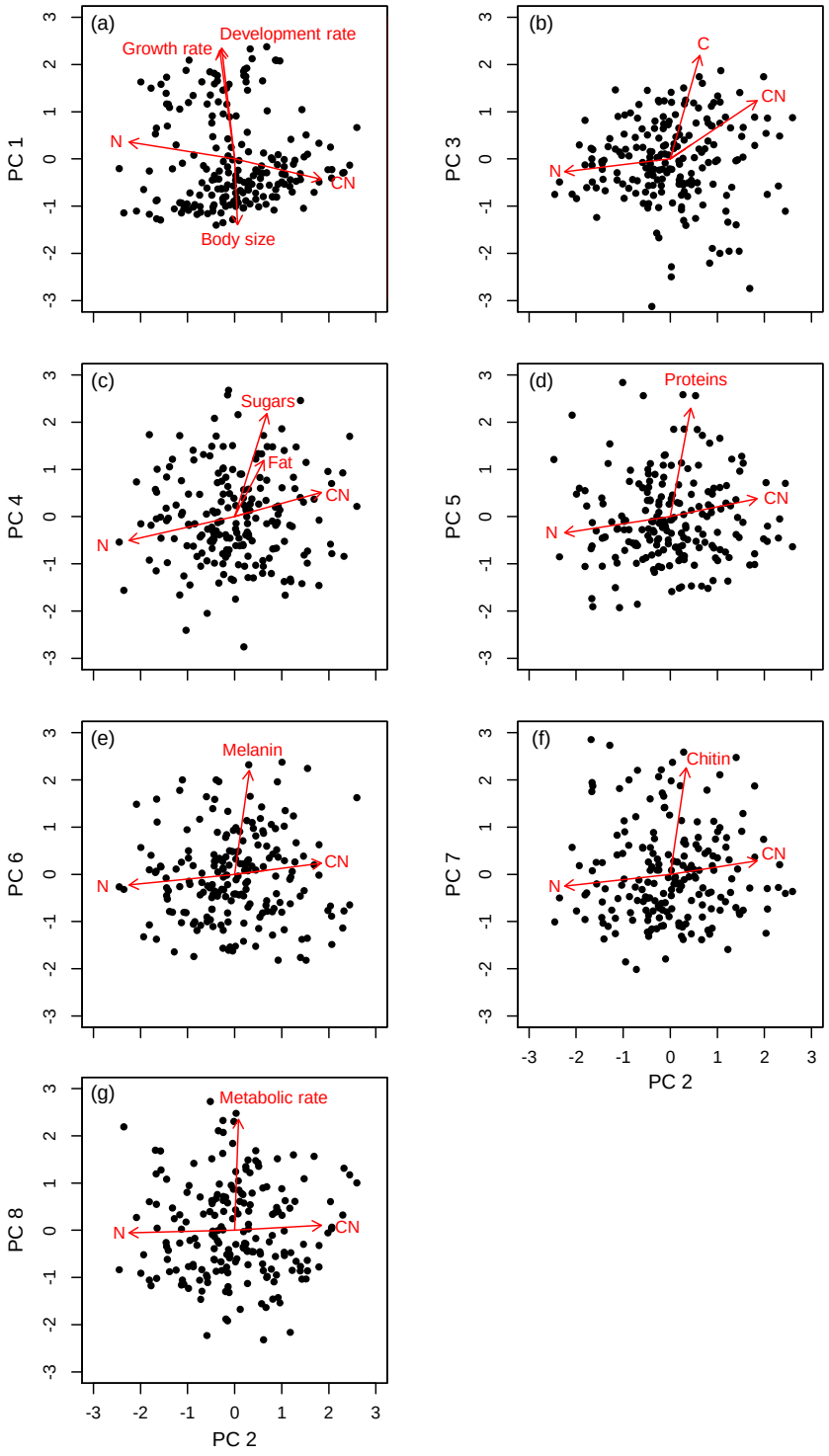

Figure S6. PCA biplots illustrating the relationship between PC2 (N content and the C:N ratio) of *Ischnura elegans* and (a) PC1 (growth rate, development rate and body size), (b) PC3 (C content), (c) PC4 (fat content and sugar content), (d) PC5 (protein content), (e) PC6 (melanin content), (f) PC7 (chitin content) and, (g) PC8 (metabolic rate).

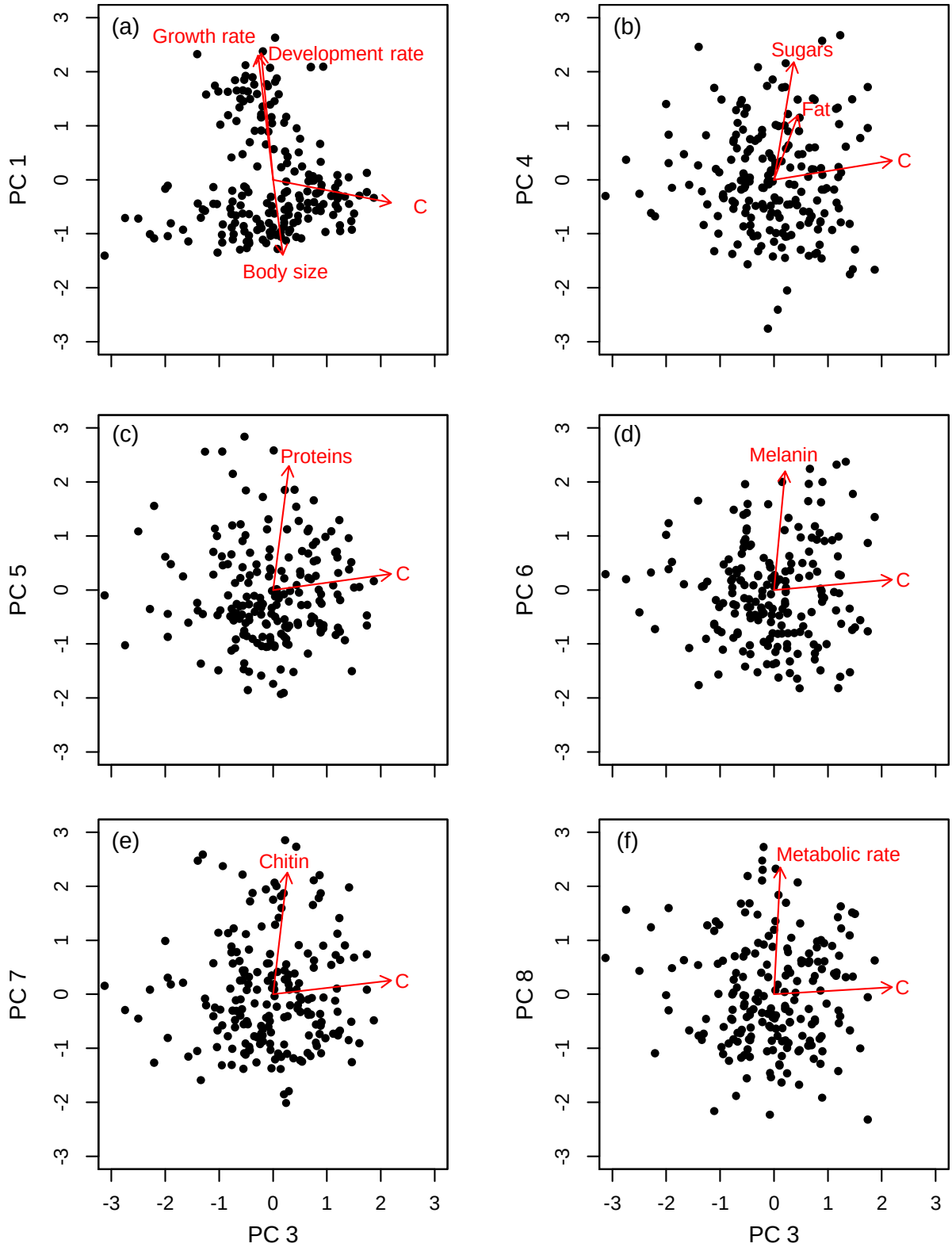

Figure S7. PCA biplots illustrating the relationship between PC3 (Ccontent) of *Ischnura elegans* and (a) PC1 (growth rate, development rate and body size), (b) PC4 (fat content and sugar content), (c) PC5 (protein content), (d) PC6 (melanin content), (e) PC7 (chitin content) and, (f) PC8 (metabolic rate).

Appendix 6: Contribution of the macromolecules to the total body C and N contents

Estimation procedure

The contribution of the macromolecules to the total body C and N contents was estimated by combining published values of their elemental composition (Table S3) and the in current study quantified contributions of their dry mass to the total body dry mass.

For proteins, we assumed an average N composition of 17% and a C composition between 46 and 53% (Sterner & Elser, 2002; Vrede *et al.*, 2004). To estimate the contribution of fat to the total C and N pools we focused on triglycerols and phospholipids, two major lipid classes. Triglycerols contain no N, and their C content varies between 54.2 and 75%. Phospholipids contain 1.6% N, and between 51 and 65% C (Sterner & Elser, 2002; Vrede *et al.*, 2004). Therefore, we calculated the contribution of fat to the total C and N pool assuming fat contains 51-75% C and 1.6% N. Since in the present study we measured the total lipid content, and storage lipids like triglycerols contribute more to total body dry mass (on average 25%) than phospholipids (on average less than 6%) (Sterner & Elser, 2002), the contribution of fat to the total N pool is likely overestimated.

We measured the total glucose content (free glucose + glycogen) as an estimate of the total sugar content. Glucose contains 37% C, and 0% N (Sterner & Elser, 2002). Following Vrede *et al.,* (2004) sugars on average contain 54.2% C. Therefore, we calculated the contribution of sugars to the total C pool assuming their C content varies between 37 and 54.2%.

Chitin is an important structural polysaccharide that consists of 41.4% C and 6.9% N (Sterner & Elser, 2002). Melanin is a polymer composed of various percentages of three or four monomeric units with different elemental composition. Melanin contains between 44.3 and 65.3% C, and between 7.3 and 9.5% N (Chedekel *et al.,* 1992; Sun *et al.,* 2016).

Table S3. The estimated elemental composition of macromolecules and the contribution of the macromolecules to the total body dry mass and the total body C and N contents. Dm = dry mass per larva.

Macromolecules	C (% of dm)	N (% of dm)	Dry mass (mg)	Contribution to C (%)	Contribution to N (%)
Proteins	46 - 53	17	1.00	16.5 – 19.0	29.9
Fat	54.2 – 70	1.6	0.06	1.7 - 2.3	0.3
Sugars	37 – 54.2	0	0.02	0.5 – 0.7	0
Cuticular chitin	41.4	6.9	0.02	0.4	0.3
Cuticular melanin	44.3 -65.3	7.5 – 9.5	0.006	0.1 – 0.2	0.1 – 0.2

Chapter II

Warming shapes the body stoichiometry of an insect across metamorphosis

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Unpublished manuscript

II

Abstract

How warming changes the body stoichiometry is largely unknown, yet ecologically important. In the many animals with a semi-aquatic lifestyle, warming in the aquatic larval stage may potentially shape the body stoichiometry of the terrestrial adults thereby alter carry-over effects from aquatic toward terrestrial habitats. Using outdoor mesocosms we studied in damselflies how a realistic warming scenario (+ 4°C) in the larval stage shaped the adult body stoichiometry across metamorphosis. We thereby evaluated to what extent warming effects on body stoichiometry could be explained by changes in growth rate, metabolic efficiency, energy storage molecules (proteins, sugars and fat) and body size. In both sexes warming increased the larval growth rates. In males, warming increased the C(carbon) body content which was driven by an increase in C-rich fat and sugars. In females, warming increased N(nitrogen) and tended to reduce the P(phosphorus) content, resulting in increased C:P and N:P ratios as predicted by an increased metabolic efficiency at higher temperatures. However, female stoichiometric changes were not associated with changes in energy storage molecules. Sex-specific stoichiometric responses to warming are likely widespread and we hypothesize these are linked to sex-specific life history strategies, with males prioritizing flight endurance (hence C) and females investing more energy in egg production and longevity and the associated defence mechanisms (hence N). The carry-over effects of warming in the larval stage on the body stoichiometry of the adult stage can change the nutrient value of the adult damselflies for their terrestrial predators and change the elemental composition of aquatic subsidies to the terrestrial ecosystem.

Introduction

Temperature is a key environmental variable affecting all levels of biological organization (Bale *et al.*, 2002; Woodward *et al.*, 2010). Almost all aquatic habitats are experiencing increasing temperatures as a consequence of global warming, and further temperature increases are predicted to occur during the next century (IPCC, 2013). It has been well studied how warming affects life history in ectothermic animals (Gillooly *et al.*, 2001; Angilletta, 2009; Harrison *et al.*, 2012). Yet, few studies looked how a realistic warming scenario may alter an organism's elemental body composition (but see Liess *et al.*, 2013; Schmitz, 2013; Janssens *et al.*, 2015, Chapter V; Norlin *et al.*, 2016; Zhang *et al.*, 2016). Understanding how and to what extent warming influences body stoichiometry is important, since such changes may affect the nutritional value [match between the elemental compositions of resources and consumers (Sterner & Elser, 2002)] of prey organisms for their predators (Abrams, 1992; Schmitz, 2013). Moreover, changes in body stoichiometric ratios have the potential to scale up to ecosystem functioning linked to elemental cycling (Hawlena & Schmitz, 2010a; Hawlena *et al.*, 2012). For example, it has been shown that a 4% higher C(carbon):N(nitrogen) content of grasshopper carcasses can slow down bacterial plant litter decomposition rate by threefold (Hawlena *et al.*, 2012).

It is especially relevant to study the effects of warming in organisms with a complex life cycle, i.e. animals who have discrete larval and adult stages that differ in behaviour, morphology and physiology (Moran, 1994). In many taxa with a complex life cycle, including amphibians and semi-aquatic insects such as midges and odonates, animals cross habitat boundaries during their life cycle with an aquatic larval stage followed by an adult terrestrial stage (Rowe & Ludwig, 1991; Stoks & Córdoba-Aguilar, 2012). Therefore, these animals play an important role in the nutrient transfer from water to land (Baxter *et al.*, 2005; Dreyer *et al.*, 2015). Especially since the emerging adults contain a relatively high N and P(phosphorus) supply per unit C, and terrestrial predators may rely on these fluxes (Dreyer *et al.*, 2012, 2015). However, warming experienced in the larval aquatic stage may, through shaping the body stoichiometry of the terrestrial adult, change the nutritional value of the adults for their terrestrial predators and change

the elemental composition of aquatic subsidies to the terrestrial ecosystem (Sitters *et al.*, 2015). Despite its potential importance for coupling aquatic and terrestrial habitats, carry-over effects of warming on body stoichiometry across metamorphosis have been rarely studied (but see Norlin *et al.*, 2016 for a study on an amphibian).

Four mechanisms have been put forward to explain how warming will affect the body stoichiometry, based on growth rate, body size and metabolic efficiency (Cross *et al.*, 2015). Notably, these mechanisms differ in their predictions in terms of how C, N and P or their ratios should change under warming. A first mechanism builds on the idea that under beneficial warming (temperatures not surpassing the thermal optimum) there will be an increase in growth rate (Angilletta 2009; Nilsson-Örtman *et al.*, 2012). Faster growth rates are associated with increased levels of P-rich ribosomal RNA to increase the production of N-rich proteins, important building blocks of tissues (i.e. the growth rate hypothesis, Sterner & Elser, 2002; Cross *et al.*, 2015). Therefore, fast growing animals are expected to have lower C:P (Sterner & Elser, 2002; Elser *et al.*, 2003). In line with this, warming increased the growth rate of *Enallagma cyathigerum* damselfly larvae, which was associated with higher RNA and protein contents, resulting in lower C:P and C:N ratios (Janssens *et al.*, 2015, Chapter V). A second mechanism focusses on the increased efficiency of biochemical and physiological processes under beneficial warming (i.e. thermodynamic principles; Angilletta, 2009). This increased metabolic efficiency results in a higher protein synthesis per ribosome, rather than an increase in ribosome number (Farewell & Neidhardt, 1998). Therefore, animals would need less P (reviewed in Woods *et al.*, 2003), resulting in an increased C:P ratio. Moreover, since an increased synthesis of proteins can be accomplished by less ribosomes, the body N:P ratio is predicted to increase under warming (Toseland *et al.*, 2013). A third mechanism builds on the observation that despite increases in growth rate, ectotherms generally become smaller under warming ('temperature-size rule', Atkinson, 1994). Because smaller animals contain relatively less proteins, warming can therefore be expected to decrease the body N content (reviewed in Woods *et al.*, 2003). A fourth mechanism assumes a decrease in growth rate under warming (when temperatures are surpassing the thermal optimum) and is based on the general stress paradigm (Hawlena & Schmitz, 2010a; Schmitz, 2013). This theory asserts that under stressful environmental conditions

(including warming) animals will increase their metabolic rate and allocate more energy [C-rich biomolecules i.e. fat and sugars] towards maintenance and away from growth, hence the production of new tissues [N-rich proteins] and the associated P-rich RNA. To maintain internal homeostasis, this will lead to the release of excess N and P. To create more C-rich sugars to fuel the increased metabolism, gluconeogenesis (the breakdown of N-rich proteins in C-rich biomolecules) is predicted to increase. Consequently, these physiological adjustments result in a higher C content and lower N and P contents, leading to increased body C:N and C:P ratios (Hawlena & Schmitz, 2010a; Schmitz, 2013). In accordance, *Melanopulus femurrubrum* grasshoppers showed a 17% higher C:N ratio under warming (Schmitz, 2013).

We here studied whether a realistic warming scenario experienced during the aquatic larval stage can shape the body stoichiometric composition in the terrestrial adult stage of a semi-aquatic insect. We hereby evaluate which of the four mechanisms (see above) best fits the warming-induced changes in stoichiometric body composition across metamorphosis. To interpret any changes in body stoichiometry we measured the assumed underlying changes in growth rate, energy storage molecules (i.e. proteins, sugars and fat) and body size. As study species we chose the damselfly *Ischnura elegans* for which the effects of temperature on life history have been well studied (e.g. Shama *et al.*, 2011; Stoks *et al.*, 2012). Odonates integrate aquatic and terrestrial habitats and therefore could play an important role in the nutrient flux. Moreover, given that adult tissues are built with resources obtained during the larval stage (Seifert & Scheu, 2012), warming could affect the nutritional value of terrestrial adult. This is important since odonates are an important food source for terrestrial predators (e.g. birds) (Corbet, 1999), and the biomass transport from water to land by odonates can be considerable. For example, Popova *et al.* (2016) estimated that in Novasibirsk oblast (Siberia, Russia) the biomass transport of odonates across habitats was on average 0.7-4.1 g dry mass /(m^2 year), which was 4-5 times larger than the biomass of Diptera.

Material and Methods

Experimental setup

To evaluate the effect of increased temperature on adult body composition (elemental stoichiometry and energy storage molecules) under realistic outdoor conditions, we reared larval damselflies to adult emergence in heated and unheated (ambient) outdoor mesocosms. The experimental setup consisted of two temperature treatments: in the unheated mesocosms larvae experienced the natural ambient temperature and its daily fluctuations, whereas in the heated mesocosms temperatures also fluctuated but were at each moment 4°C (4.23 ± 0.08, mean ± SE; Appendix 1) higher than in the unheated mesocosms. The 4°C temperature difference was chosen to match the predicted temperature increase by 2100 under IPCC scenario RCP8.5 (IPCC, 2013). There were eight replicated mesocosms for each temperature treatment, hence 16 mesocosms in total.

The mesocosms were green plastic 210 L containers that were placed at an outdoor experimental area in Heverlee (Belgium). At 1 October 2014, they were filled with 180 L water which consisted of 60 L filtered pond water and 120 L tap water. Leaf litter (*Quercus* spp. and *Tilia* spp.) was added as organic substrate. All mesocosms were covered with a net (1 mm mesh size) to prevent colonization by other aquatic insects. We randomly assigned mesocosms to the temperature treatments. To manipulate mesocosm temperatures, we installed one heater (300 W, Eheim Jager Heater, Deizisau, Germany) in each heated mesocosm. Heaters were connected to temperature sensors (PT100, Omega Engineering, Inc., CT, USA) that continuously measured the ambient water temperature in the unheated mesocosms. Whenever a temperature difference between ambient and heated mesocosms that deviated from 4°C was detected, a signal was sent to the heaters to re-establish the 4°C temperature difference.

One month after filling the mesocosms with water (6 November 2014), we inoculated each mesocosm with 35 larvae of the damselfly *Ischnura elegans* (total of 560 larvae). Larvae were collected in a shallow pond in the nature reserve De Maten in Genk (Belgium). Larvae were sorted into three size classes: 2-3 cm (final instars, F_0), 1-2 cm (F_1-F_2 instars) and <1 cm (< F_2 instars). Equal numbers of each size class were

randomly added to the mesocosms. To reduce the handling stress and because the smaller instars cannot be sexed, we have no information on the initial sex ratios. Additionally, we inoculated a mixture of zooplankton and mayfly larvae of the collection pond to each mesocosm as food supply for the damselfly larvae.

Starting from April 2015, we checked the mesocosms daily for emergence of adult damselflies. Freshly emerged adults were brought to the laboratory, where their wet mass was quantified the next day. Afterwards, they were frozen (-80°C) for physiological analyses. For this we selected six adult damselflies per mesocosm (49 females and 47 males, total of 96 individuals) at the peak of the emergence curve per mesocosm. Note that the focus in the current study is on body stoichiometry, while an accompanying study focussed on warming-induced effects on flight performance (Tüzün *et al.*, 2018).

Response variables

We calculated the larval growth rate as the ln-transformed mass at emergence divided by development time (see e.g. Johansson *et al.*, 2001). For the mass at emergence, we weighed each adult damselfly to the nearest 0.01 mg using an electronic microbalance (Mettler Toledo, AB135-S, Columbus, OH, USA).

To quantify the body C:N:P ratios and the energy storage molecules, we followed established protocols for damselflies (Janssens *et al.*, 2015, Chapter V, Van Dievel *et al.*, 2016, Chapter IV). In a first step, adults were homogenized and diluted 5 times in milli-Q water. The samples were centrifuged for 5 min (13.2 g, 4°C) and afterwards 25 µL of the supernatant was diluted three times in PBS buffer to measure the energy storage molecules. The rest of the wet homogenates were used to quantify C:N:P ratios.

To quantify the C:N:P ratios we first divided the homogenate in two parts, with ¼ of the homogenate used for the C and N analyses and the other ¾ of the homogenate for the P analyses. For the measurement of the C and N content we first transferred the samples to tin cups and dried them for 24 h at 60 °C. Then, the C and N contents were quantified with an elemental analyser (Carlo Erba 1108, Thermo Scientific, Waltham, USA) using leucine for calibration. The dry mass of this subsample was used to estimate the total dry mass of the adult damselflies. For the P content, we filled a glass tube with

the other subsample together with 1 mL HNO_3 (70%). The glass tubes were heated at 150°C for approximately 45 minutes to let the HNO_3 evaporate. Afterwards, we diluted the digest to 10 mL with milli-Q water. The P content was quantified using inductively coupled plasma mass spectrometry (Aqilent 7700x ICP-MS, Biocompare, South San Francisco, California, USA). In the figures we expressed the elemental C, N and P contents as % of dry mass. The C:N, C:P and N:P ratios were expressed as molar ratios by taking into account the molar mass.

The energy storage molecules were assayed spectrophotometrically using a TECAN infinite M200 spectrophotometer (Männedorf, Switzerland). The protein content was measured based on the Bradford (1976) method. We added 1 µL supernatant and 160 µL milli-Q water to a well of a 96-well microtiter plate. Then, we added 40 µL Biorad protein dye and mixed the sample very well. The plate was incubated for 5 min at 30°C and the absorbance was measured at 595 nm (in quadruplicate). We determined the protein concentrations using a standard curve of known concentrations of bovine serum albumin. The fat content was determined using a modified protocol of Marsh and Weinstein (1966). We added 8 µL of the supernatant and 56 µL H_2SO_4 (100%) in a 2 mL glass tube. These tubes were heated for 20 min at 150°C. Then we added 64 µL milli-Q water and mixed the sample. We filled wells of a 384-well microtiter plate with 30 µL of the sample and measured the absorbance at 470 nm (in duplicate). Fat content was calculated based on a standard curve of glyceryl tripalmitate. We quantified the total sugar content (glucose + glycogen) using the protocol described in Stoks *et al.* (2006a), based on the glucose kit from Sigma Aldrich. First, we converted all glycogen to glucose by mixing 50 µL milli-Q water, 20 µL supernatant and 10 µL amyloglucosidase (1 unit/10 µL; Sigma A7420) in wells of a 96-well microtiter plate. We incubated the plate for 30 min at 37°C. To determine the glucose content, we added 160 µL of glucose assay reagent (Sigma G3293) to each well and incubated the plate for 20 min on 30°C. Afterwards, we measured the absorbance at 340 nm (in duplicate). The glucose concentrations were obtained using a standard curve of known concentrations of glucose. All energy storage molecules were expressed as µg per mg dry mass.

Stoichiometry of zooplankton and mayfly prey

To test for a potential effect of the prey species in the mesocosms on the stoichiometry of the damselflies, we quantified the body C:N:P ratios of the zooplankton and mayfly larvae. Therefore, we sampled in each mesocosm the zooplankton and mayfly prey between 14 and 18 March 2015. To take quantitative samples, we first mixed the water column and then took a 5L depth-integrated water sample using a PVC tube sampler. The content of the tube sampler was poured over a sieve (mesh size 60 µm) and stored at -20°C. In the laboratory, we sorted the zooplankton and mayfly larvae using a stereomicroscope (Olympus, SZX-ILLK200, Berchem, Belgium). Per mesocosm we took the dry mass of the pooled zooplankton sample and the pooled mayfly sample to the nearest 0.1µg after drying 48h at 60°C. Since the dry mass of the mayfly samples of four mesocosms of the ambient temperature treatment was too low to measure C:N:P, we combined these per two. This resulted in eight mesocosm samples for each of the warming x prey type combinations, except for 6 mesocosm samples for the mayfly larvae in the ambient treatment. We quantified the C:N:P ratios of these samples as described above.

Statistical analyses

We tested for effects of the temperature treatment on the different response variables using linear mixed-effect models. Since sexes typically differ in their response to temperature in insects (e.g. Fischer & Fiedler, 2000; Westerman & Monteiro, 2016), including damselflies (De Block & Stoks, 2003), we evaluated the effects of temperature separately for males and females. We had 8 replicated mesocosms per temperature treatment and from each mesocosm we sampled six adult damselflies. To explicitly take into account that sets of adults came from the same mesocosm, hence to avoid pseudoreplication, we included the mesocosm identity (nested in the temperature treatment) as a random factor. The C, N and P contents were analysed in two batches. To correct for a potential effect of batch, we also included batch as a random factor. As energy storage molecules (Woods *et al.,* 2003; Ardia *et al.,* 2012) and elemental contents (Elser *et al.,* 1996; Ventura & Catalan, 2005) increase with body mass, we included body mass as a covariate in all models. Statistically correcting for mass by adding it as

a covariate is recommended above dividing by body mass (Beaupre & Dunham, 1995). Note that to increase the comparability with other studies we report on the figures the energy storage molecules per mg dry mass and the C, N and P contents as % of the dry mass.

The effect of the temperature treatment on the zooplankton and mayfly prey stoichiometry was analysed using linear mixed-effect models with prey category and temperature as fixed effects. We added mass of the prey sample as a covariate in the models. Again, we added mesocosm identity (nested in the temperature treatment) as a random factor to the models. All statistical analyses were performed with the program R v.3.4.0 (R Development Core Team, 2015), using the package 'lme4' to run linear mixed models (Bates *et al.*, 2015) and the package 'car' to calculate Wald chi-square statistics and p-values for the fixed effects (Fox & Weisberg, 2011).

Results

Temperature effects on males

Male damselfly larvae from the heated mesocosms had a higher growth rate compared to those from the ambient mesocosms (χ_1^2 = 14.41, P < 0.001; Fig. 1), but warming did not affect their mass at emergence (χ_1^2 = 2.78, p = 0.095; Fig. 1). Males emerging from the heated mesocosms had a higher mass-corrected C content (χ_1^2 = 5.73, P = 0.017; Fig. 2a), which was associated with higher mass-corrected fat (χ_1^2 = 8.55, P = 0.004; Fig. 3b) and sugar (χ_1^2 = 9.51, P = 0.002; Fig. 3c) contents. The larval temperature treatment did not affect the other stoichiometric variables (all P > 0.147; Fig. 2b-f) or the protein content of adult males (χ_1^2 = 0.32, P = 0.571; Fig. 3a).

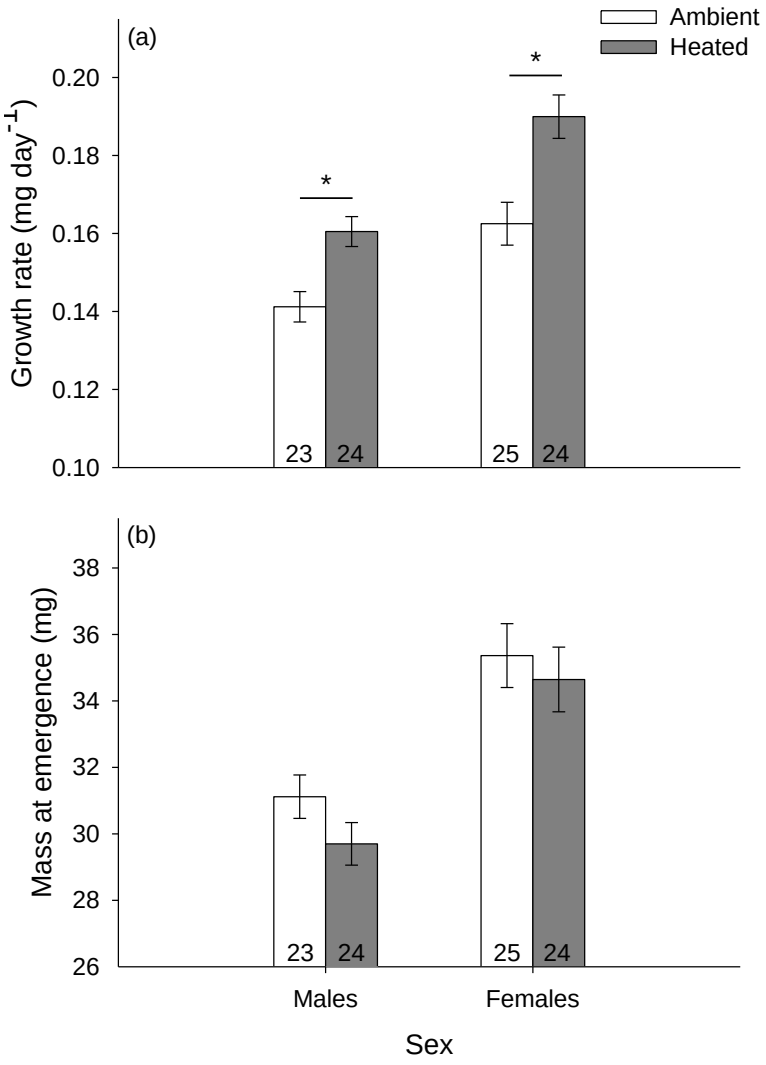

Figure 1. Mean larval growth rate (a) and mass at emergence (b) of *Ischnura elegans* as a function of the larval temperature treatment and sex. Given are least-squares means (± 1 s.e.m.). Within each sex, significant ($P \leq 0.05$) effects of the warming treatment are indicated by *. Numbers in bars represent sample sizes.

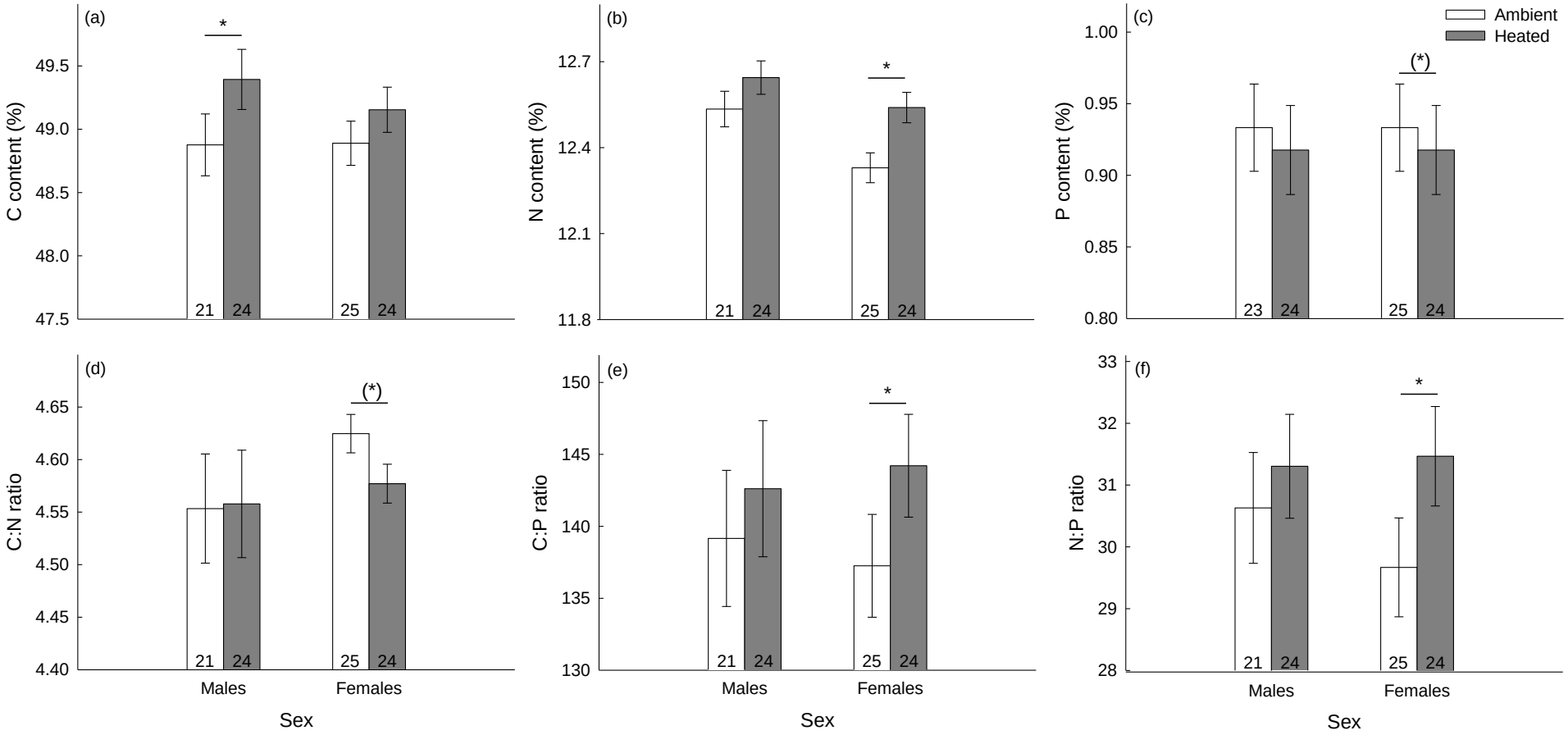

Figure 2. Mean body stoichiometric composition of *Ischnura elegans* adults as a function of the larval temperature treatment and sex: C (a), N (b), and P (c) contents as % of dry mass and the C:N (d), C:P (e) and N:P (f) molar ratios. Given are least-squares means (± 1 s.e.m.). Within each sex, significant ($P \leq 0.05$) effects of the warming treatment are indicated by * and trends ($P \leq 0.07$) by (*). Numbers in bars represent sample sizes.

Temperature effects on females

Female larvae had a higher growth rate in the heated mesocosms (χ^2_1 = 14.29, P < 0.001; Fig. 1), yet there was no effect of temperature on the mass at emergence (χ^2_1 = 0.32, p = 0.57; Fig. 1). Adult females emerging from the heated mesocosms had a higher N content (χ^2_1 = 8.13, P = 0.004) and tended to have a higher C content (χ^2_1 = 2.95, P = 0.086) and lower P content (χ^2_1 = 3.42, P = 0.064)) compared to the females from the ambient temperature mesocosms. These changes in the stoichiometric elements resulted in females emerging from the heated mesocosms to show a trend for a decreased C:N ratio (-1.0 % reduction, χ^2_1 = 3.66, P = 0.056) and to have significantly increased C:P (+4.8 % increase, χ^2_1 = 4.64, P = 0.031) and N:P (+5.7 % increase, χ^2_1 = 6.19, P = 0.013) ratios (Fig. 2d-f). Yet, there was no effect of the larval temperature treatment on the mass-corrected energy storage molecules in adult females (all P > 0.137; Fig. 3).

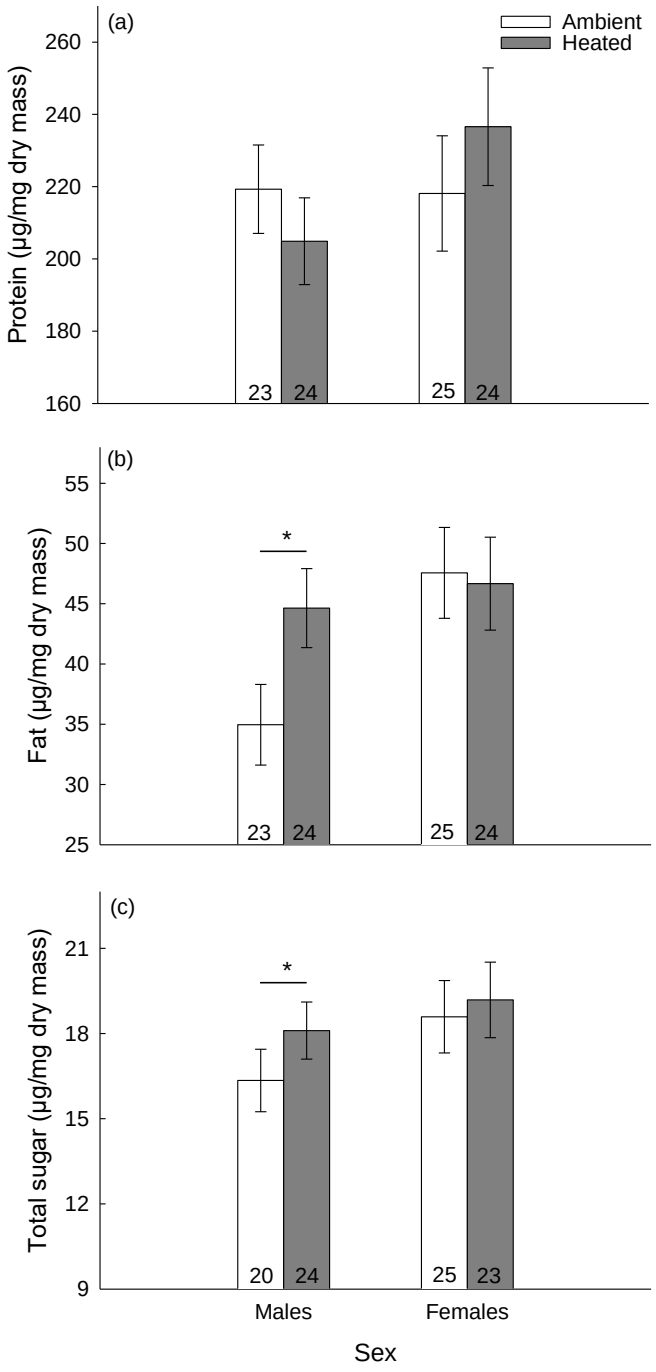

Figure 3. Mean levels of energy storage molecules of *Ischnura elegans* adults as a function of the larval temperature treatment and sex: proteins (a), fat (b) and total sugars (c) per mg dry mass. Given are least-squares means (± 1 s.e.m.). Within each sex, significant ($P \leq 0.05$) effects of the warming treatment are indicated by *. Numbers in bars represent sample sizes.

Temperature effects in stoichiometry of zooplankton and mayfly prey

Temperature had no effect on the number of zooplankton and mayfly prey recovered in the mesocosms (χ_1^2= 0.56, *P* = 0.45; Fig. 4) nor did it affect the body stoichiometry (C, N and P contents, and their ratios) of the prey (all *P* > 0.362; Fig. 5). The mayfly larvae had a 24.9% higher C (χ_1^2 = 24.34, *P* < 0.001; Fig. 5a), a 30.9% higher N (χ_1^2 =38.113, *P* < 0.001; Fig. 5b), and a 36.8% lower P content (χ_1^2 = 11.79, *P* = 0.005; Fig. 5c) than the zooplankton. This resulted in a 8% lower C:N ratio in the mayfly larvae compared to the zooplankton (χ_1^2 = 6.67, *P* = 0.010; Fig. 5d), a 37.4% higher C:P ratio (χ_1^2 = 18.02, *P* < 0.001; Fig 5e) and a 43.2% higher N:P ratio (χ_1^2 = 26.96, *P* < 0.001; Fig. 5f).

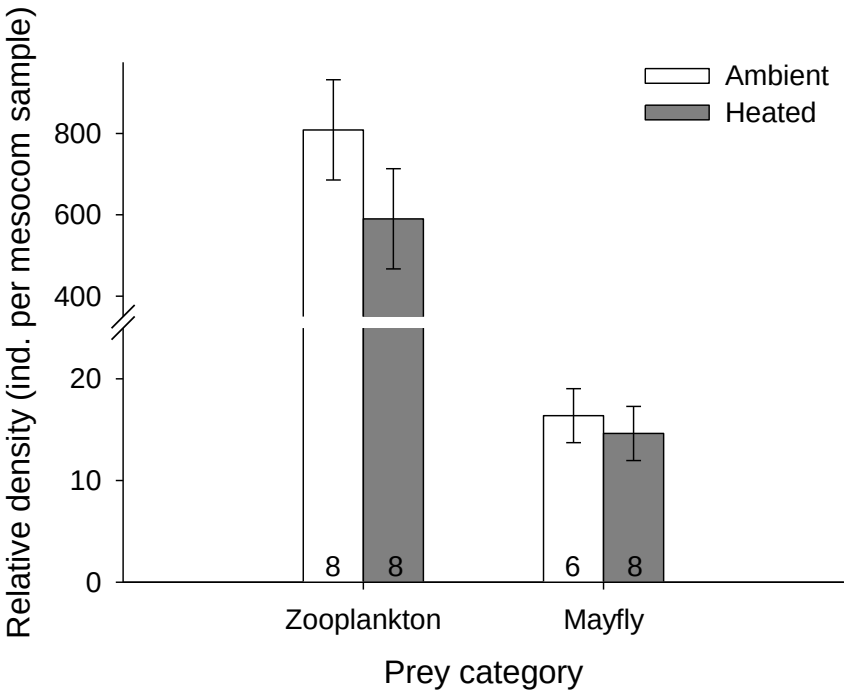

Figure 4. Mean relative density of the zooplankton and mayfly prey as a function of the mesocosm temperature treatment. Given are least-squares means (± 1 SE). Numbers in bars represent sample sizes.

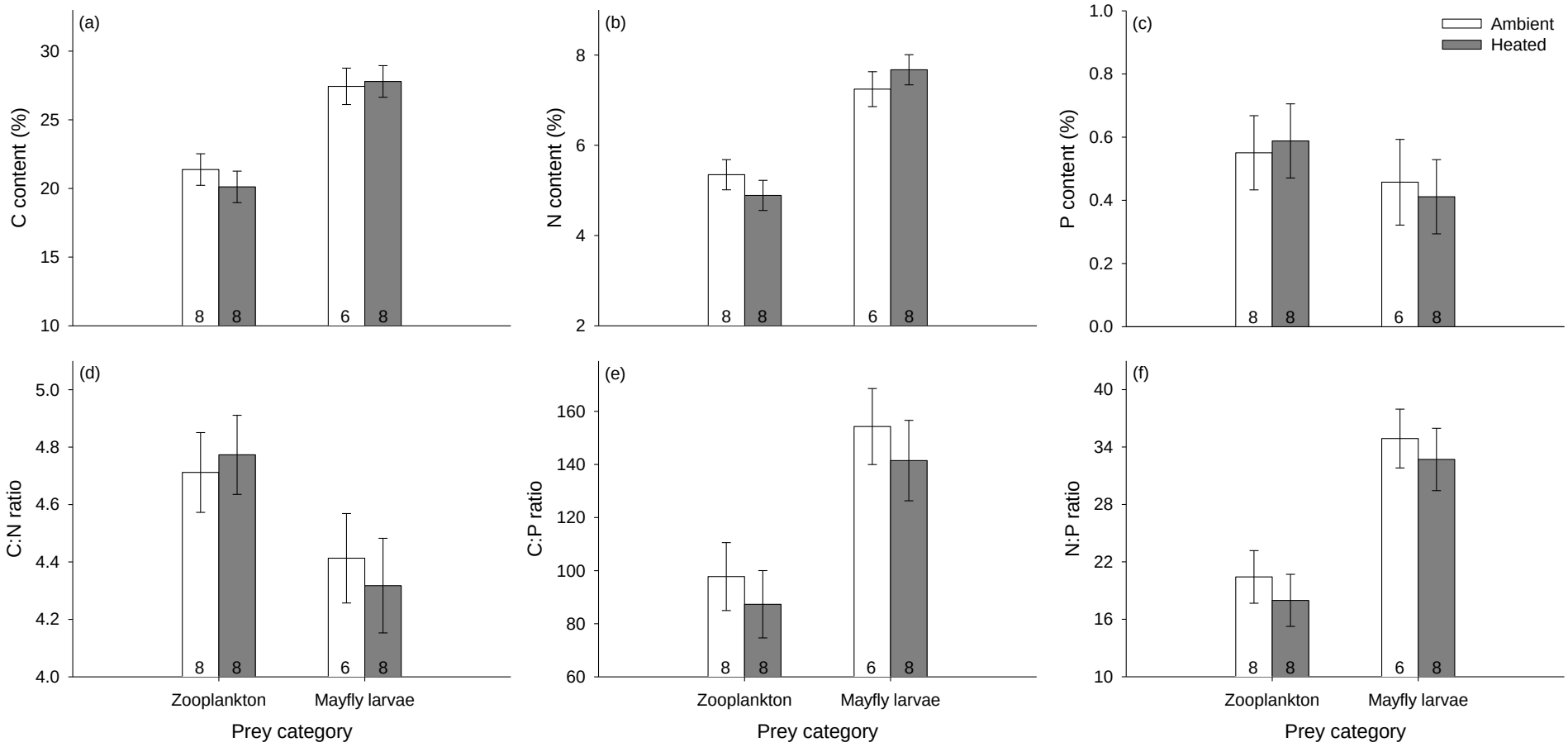

Figure 5. Mean body stoichiometric composition of the zooplankton and mayfly prey as a function of the mesocosm temperature treatment: C (a), N (b), and P (c) contents as % of dry mass and the C:N (d), C:P (e) and N:P (f) molar ratios. Given are least-squares means (± 1 s.e.m.). Numbers in bars represent sample sizes.

Discussion

As expected, the realistic 4°C warming scenario imposed in the larval stage carried over to the adult stage of the damselflies where it shaped the energy storage molecules (fat and sugars) and all stoichiometric variables. Notably, warming effects on body stoichiometry were different between sexes. Stoichiometric changes in body composition of the damselflies under warming were likely not mediated by their prey because the prey items present in the mesocosms did not change body stoichiometry under warming. Possibly, the zooplankton prey were able to adapt to the temperature increase, as they went through multiple generations during the experimental period. The mayflies, however, only had one generation, precluding such adapatation to warming. Yet, in previous research on mayflies only little effects of a temperature increase on their abundance or size were shown (McKee & Atkinson, 2002). The low sensitivity of mayflies to warming together with the possibility of adapation of the zooplankton, could explain the absence of warming-induced changes in body composition of the prey. Moreover, the densities of both prey types did not differ between unheated and heated mesocosms suggesting no warming-induced switch in prey choice occurred.

When contrasting our results with the four proposed mechanisms to explain how warming may change the body stoichiometry (see introduction), we did not found full support for any of them. For both sexes the imposed warming scenario increased their growth rate. Yet, despite the increase in growth rate, there was no support for the mechanism of faster growth rates driving increases in the P and N contents, and as such reducing the C:P (Growth rate hypothesis, Sterner & Elser, 2002; Elser *et al.*, 2003; Cross *et al.*, 2015). Indeed, in males P and N contents were not affected by warming, and although the N content in females increased, the P content tended to decrease. However, hereby females follow the mechanism whereby the P content would decrease under warming because of an increased metabolic efficiency resulting in an increased C:P ratio (Woods *et al.*, 2003) and an increased body N:P ratio (Toseland *et al.*, 2013). In females, there were indeed trends for a reduction in the P content and for an increase in the C:P ratio, and the N:P ratio significantly increased. Yet, the associated underlying increase in protein synthesis could not explain the observed pattern. Indeed, the protein

content did not increase under warming in females, possibly since it was already high at the ambient temperature (see below). Further, warming did not reduce the mass at emergence (i.e. measure for body size, Forster *et al.*, 2012), also precluding this mechanism (Woods *et al.*, 2003) to play a role. Finally, since warming was beneficial (growth rates increased and did not decrease under warming) also the general stress paradigm (Schmitz, 2013) cannot be used to explain the effects of warming on body stoichiometry.

We will further generate hypotheses how sex differences in life history could have contributed to the observed sex-specific responses to warming in storage molecules and in body stoichiometry. Note however that the damselflies in our study were not yet sexually mature. However, preparation for reproduction starts already in the larval stage (Baker *et al.*, 1992; Corbet, 1999). In males, the higher temperature led to an increase in the C content which could be explained by an associated increase in C-rich fat and sugar contents. The warming-induced increase in storage molecules is relevant for an increased flight endurance which is under sexual section in male damselflies that show scramble competition (Gyulavári *et al.*, 2014; Therry *et al.*, 2014; Tüzün *et al.*, 2017). Increases in the concentrations of fat and sugar under warming have been observed before in butterflies (Karl & Fischer, 2008) and in another damselfly species (Janssens *et al.*, 2015, Chapter V). Moreover, at higher temperatures damselfly larvae increase their food intake and growth efficiency (Stoks *et al.*, 2012; Culler *et al.*, 2014), resulting in a higher energy input which could be allocated to increase the fat and sugar contents. This could also explain the warming-induced increase in growth rate.

Females already had high energy storage levels at the ambient rearing temperature and did not further increase them under warming, which is in contrast with the response to warming in males. One possibility is that energy storage might be more important to females than to males as females invest more energy in reproduction than males (Trivers, 1972). This may be mechanistically explained by the fact that females eat more compared to males (for *Ischnura* damselfly species: Debecker *et al.*, 2016). We hypothesize that females did not further increase storage levels under warming as these were already high, and also benefit from investing in energetically costly defence mechanisms (which animals often increase at higher temperatures, Adamo & Lovett,

2011; Karl *et al.*, 2011). We hypothesize therefore, that the trend for an increase in C and the increase in N under warming was partly linked to the widespread pattern of a higher investment in immune function in females (Rolff, 2002; Nunn *et al.*, 2009), as observed before in *Coenagrion puella* damselflies (Joop & Rolff, 2004). An important component of the insect's immune function is melanin, which is a N-rich polymer (Chedekel *et al.*, 1992) with a relatively high C:N (9:1) ratio. An increased melanin production under warming has already been observed in female *Tenebrio molitor* mealworm beetles (Prokkola *et al.*, 2013). The stronger increase in N than C under warming in females can also be linked to an increased egg production under warming (Adamo & Lovett, 2011), which requires a substantial amount of N (e.g. Joern & Behmer, 1997). However, female's oogenesis also acquires P, yet our results do not support the expected higher P content in females compared to males (Markow *et al.*, 1999). Moreover, the P content tended to decrease under warming. This is in line with the metabolic efficiency theory, which states that the warming-induced efficiency of the biochemical reactions of the ribosomes result in the need of less P.

Conclusions

By for the first time explicitly considering all four mechanisms put forward to explain how warming should shape body stoichiometry we could show there was no full support for neither mechanism. Our results urge caution when basing tests of these mechanisms only by measuring the stoichiometry and not the macromolecules associated with these mechanisms. Indeed, females largely followed the changes in body elemental composition as predicted by an increased metabolic efficiency (Woods *et al.*, 2003, Toseland *et al.*, 2013), yet this was not associated with the assumed underlying changes in protein contents. Another important novel finding was that stoichiometric responses to warming were sex-specific and that changes in biomolecules could explain the changes in body stoichiometry in males but not in females. This rejection of current ideas is important to move the field of ecological stoichiometry forward. To this end, we formulated testable hypotheses linked to the different life history strategies in males and females to explain the observed patterns that may guide future work. Notably, our results add to the rare studies documenting effects of warming across metamorphosis

(see also Norlin *et al.,* 2016). This is important as many organisms, including many semi-aquatic insects, have a complex life cycle, thereby coupling aquatic and terrestrial habitats (Marcarelli *et al.,* 2011; Sitters *et al.,* 2015). Therefore, the here documented changes in the body composition of damselflies may have important consequences for their nutritional value for terrestrial predators (e.g. birds, Corbet, 1999; Sitters *et al.,* 2015), and potentially change the elemental composition of aquatic subsidies to the terrestrial ecosystem (Hawlena & Schmitz, 2010a; Schmitz, 2013).

Acknowledgements

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Author contributions: NT, LODB and RS conceived and designed the experiment. NT and LODB performed the experiment. MVD carried out the physiological analyses. MVD, RS and LJ analysed the data and wrote the manuscript. All authors approved the final manuscript

Appendix 1: Main daily water temperature

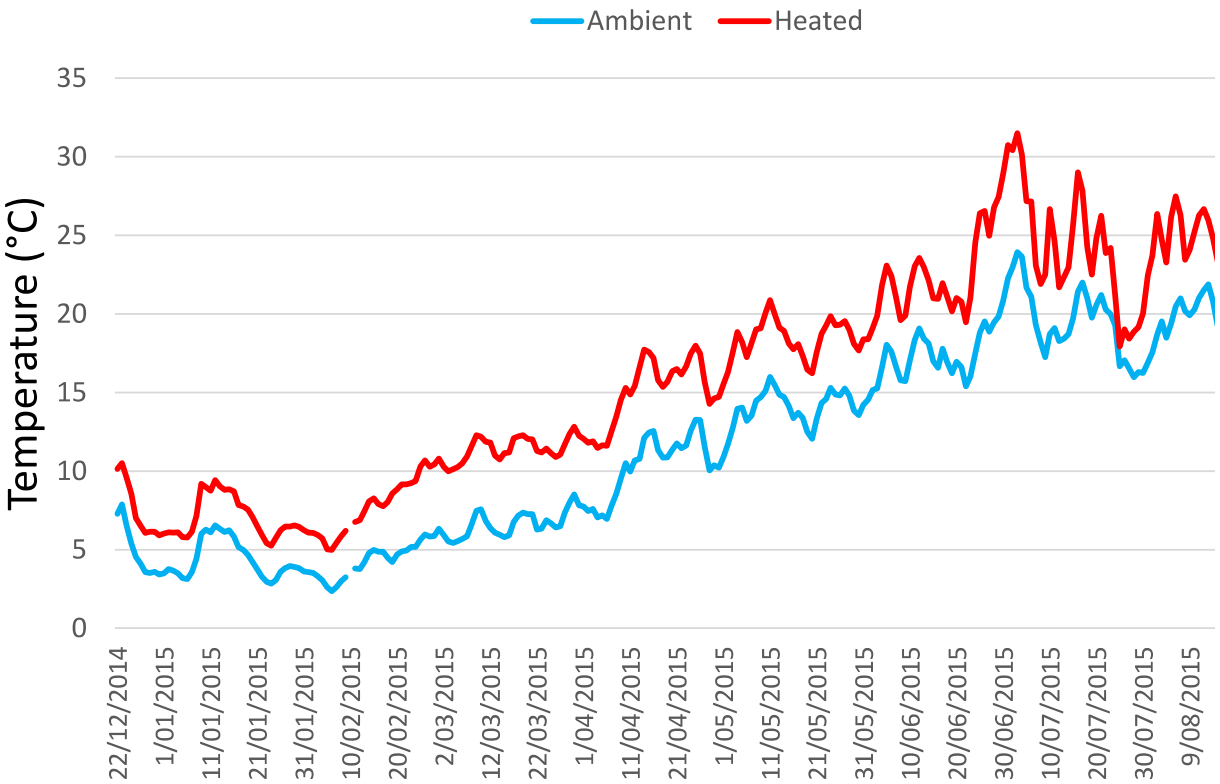

Figure S1. Mean daily water temperatures in the ambient and heated mesocosm during the experiment

Chapter III

Beneficial effects of a heat wave: higher growth and immune components driven by a higher food intake

Marie Van Dievel, Robby Stoks and Lizanne Janssens

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Slightly modified version

III

Abstract

While heat waves will become more frequent and intense under global warming, the ability of species to deal with extreme weather events is still poorly understood. We investigated how a heat wave influenced growth rate and investment in two immune components (phenoloxidase activity and melanin content) in the larvae of two damselfly species, *Ischnura elegans* and *Enallagma cyathigerum*. Larvae were kept at 18°C or at a simulated long heat wave at 30°C. To explain effects of the heat wave we quantified traits related to energy uptake (food intake and growth efficiency), energy expenditure (metabolic rate measured as activity of the electron transport system, ETS) and investment in energy storage (fat content). The two species differed in life strategy with *I. elegans* having a higher growth rate, growth efficiency, ETS activity, and fat content. In line with its preference for cooler water bodies, the heat wave was only lethal for *E. cyathigerum*. Yet, both species benefited from the heat wave in terms of an increased growth rate despite a higher ETS activity. This could be explained by a higher food intake which also may have contributed to the increased investments in energy storage and in the immune components under the heat wave. This mediatory role of food intake indicates the critical role of food availability and behaviour in shaping the impact of heat waves. Our results highlight the importance of including the assumed underlying behavioural and physiological variables to unravel and predict the impact of extreme climate events on organisms.

Introduction

Global warming is affecting many ecosystems worldwide. While higher temperatures are often thought to negatively affect the performance of ectotherms, the mild temperatures increases predicted by recent global warming scenarios (IPCC, 2013) are often beneficial for temperate organisms (Deutsch *et al.*, 2008, Nilsson-Örtman *et al.*, 2012). Yet, besides mild increases in mean temperatures, global warming will also be characterized by more extreme weather events, such as heat waves. Under global warming, heat waves are predicted to become more frequent, more intense and of longer duration (Meehl & Tebaldi, 2004; Jentsch *et al.*, 2007; IPCC, 2013). Exposure to extreme temperatures typically reduces performance and recent studies suggest that the impact of heat waves may overrule the beneficial effects of mild increases in mean temperatures (reviewed in Lawson *et al.*, 2015; Vázquez *et al.*, 2015). However, this picture may be too simplistic as non-lethal heat waves also have been shown to increase performance (e.g. Adamo & Lovett, 2011; Arambourou & Stoks, 2015). Therefore, to gain more insight in the impact of global warming on ectotherm performance, it is pivotal to predict and understand the impact of heat waves on ectotherm populations. This is especially true since the ability of species to survive global warming may depend largely on their ability to deal with extreme temperatures (Thompson *et al.*, 2013; Vasseur *et al.*, 2014; Ma *et al.*, 2015).

Whether a temperature increase will have positive rather than negative consequences for organisms depends on whether the elevated temperatures surpass the thermal optimum (Angilletta, 2009). Heat wave temperatures typically exceed optimal temperatures and are therefore often associated with mortality and steep declines in performance (Vasseur *et al.*, 2014; Ma *et al.*, 2015). This has for example been shown in several insects (Asin & Pons, 2001; Chang *et al.*, 2007; Gillespie *et al.*, 2012; Bauerfeind & Fischer, 2014). Similarly, under extreme high temperatures reductions in growth rate have been suggested (Lemoine & Burkepile, 2012), a key performance trait frequently studied in thermal research (Schulte *et al.*, 2011) and directly relevant for biotic interactions (Stoks *et al.*, 2017). Yet, several studies that explicitly simulated heat waves or exposed insects to extreme temperatures (6 to 10°C above normal) did not find

a decrease in growth rate (e.g. Adamo & Lovett, 2011; Arambourou & Stoks, 2015; Dinh Van *et al.*, 2016; Klockmann *et al.,* 2016, but see Kingsolver & Woods, 1997).

As growth rates are the result of behaviour (food intake) and physiology (growth efficiency: the efficiency to assimilate and convert ingested food into biomass), heat wave effects on growth rate may be mediated by effects on both components. At high temperatures both food intake and growth efficiency have been shown to decrease (Heilmayer *et al.,* 2004; Lemoine & Burkepile, 2012), yet not in all taxa (e.g. Culler *et al.,* 2014; Schmitz *et al.,* 2016). Furthermore, a higher food intake may not necessarily result in a higher growth rate as an important part of the energy obtained through food intake may not be converted to body mass but to other functions such as metabolic rate (Clarke & Fraser, 2004; Rall *et al.,* 2010; Lemoine & Burkepile, 2012) and investment in energy storage (Kooijman, 1995). Therefore, to obtain a full understanding of the effects of a heat wave on growth rate, it is beneficial to include also metabolic rate and energy storage, besides food intake and growth efficiency. For example, at high temperatures metabolic rate can increase more than food intake (Rall *et al.,* 2010; Lemoine & Burkepile, 2012), resulting in decreased growth (Lemoine & Burkepile, 2012). Intriguingly, also the opposite may occur, and it was recently shown that exposure to a heat wave may reduce metabolic rate (Dinh Van *et al.,* 2016), reflecting the well-known phenomenon of metabolic depression under high stress levels (Storey, 2015).

Another key trait closely linked to fitness that may be impaired by heat waves is immune function (Roth *et al.,* 2010; Karl *et al.,* 2011; Seppälä & Jokela, 2011; Dittmar *et al.,* 2014; Dinh Van *et al.,* 2016). Given that investment in immune function is energetically costly (e.g. Siva-Jothy & Thompson, 2002; De Block & Stoks, 2008), the same behavioural and physiological mechanisms driving growth reductions under heat waves may underlie impairment of immune function during heat waves. A suppression of immune function may have important fitness consequences as it reduces pathogen resistance. This is especially important as disease susceptibility, pathogen abundance and virulence are expected to increase under global warming (e.g. Maynard *et al.,* 2015). Yet importantly, negative effects of heat waves on immune function are not general and

some studies detected no or even a positive effect (Adamo & Lovett, 2011; Bauerfeind & Fischer, 2014; Arambourou & Stoks, 2015).

In the current study we investigated how a heat wave influenced growth rate and immune components in the larvae of two damselfly species. We were particularly interested in how effects of the heat wave on growth rate and immune components were mediated and therefore quantified food intake, growth efficiency, metabolic rate and potential trade-offs with investment in energy storage. Damselfly larvae are important intermediate predators in aquatic food webs that are particularly sensitive to global warming (Hassall & Thompson, 2008). We studied the damselfly *Ischnura elegans* because temperatures of 30°C had no lethal effects and even positively affected growth and physiological traits (immune function, fat content and flight muscle mass) (Arambourou & Stoks, 2015; Arambourou *et al.*, 2017). This suggests that exposure to 30°C is for most performance traits still in the optimal temperature range for this species. This may be because this species is often abundant in small, shallow water bodies (Dijkstra, 2006) that are more affected by heat waves. To start exploring consistency of responses to heat waves across damselfly species we also studied larvae of the damselfly *Enallagma cyathigerum.* While both species may co-occur, *E. cyathigerum* prefers larger, deeper water bodies (Dijkstra, 2006). Since there are smaller temperature fluctuations in these deeper and cooler water bodies, we hypothesise that *E. cyathigerum* larvae are more sensitive to heat waves. Understanding the sensitivity of species to extreme temperatures is important to predict which species will suffer more from global warming (Domisch *et al.*, 2011; Rosset & Oertli, 2011).

Material and Methods

Collecting and housing

In the summer of 2014 we collected mated females of both species. For each species we randomly selected two populations in Flanders (Belgium), in the core of their distribution (Dijkstra, 2006). Both species were collected in “Torfbroek” (50°55'32.5" N, 4°32'21.4" E); *I. elegans* was additionally collected in “Oud-Heverlee-Zuid” (50°50'30.34" N, 4°39'31.91" E) and *E. cyathigerum* in “Bergerven” (51°03'58.9284"

N, 5°41'29.9796" E). All populations are located in protected nature areas in Belgium. Females were transported to the laboratory for egg laying. Freshly hatched larvae were first kept in groups to increase survival (De Block & Stoks, 2003). Ten days after hatching, larvae were placed individually in plastic 200 mL cups filled with conditioned tap water (aged tap water with straw and grass). Prior to the experiment, larvae were fed *Artemia* nauplii *ad libitum* (mean average daily dose ± 1 SE = 205 ± 54, n = 10 daily portions) for six days per week. Larvae were checked three times a week for moults into the penultimate instar. When larvae moulted into the penultimate instar, they entered the heat wave experiment.

Heat wave treatment

To assess the effects of a simulated heat wave, we set up a laboratory experiment where larvae were reared during their last two instars at a water temperature of 18°C or 30°C. The temperature of 18°C is the average water temperature during the months May and June in Belgium (Lake Model Flake, 2009, http://www.flake.igb-berlin.de/index.shtml). During these two months most *I. elegans* and *E. cyathigerum* larvae in Belgium are in their penultimate or final instars (based on De Knijf *et al.,* 2006). The growth of these two instars was quantified in this study because they show the largest mass increase. The temperature of 30°C was chosen to reflect a heat wave temperature in Belgium. Indeed, the Royal Meteorological Institute of Belgium (KMI, 2017, http://www.meteo.be) defines a period consisting of minimum five consecutive days with at least 25°C, of which at least three days are 30°C or higher as a heat wave. On average, there are yearly 27.9 days with air temperatures of 25°C or higher, and 3.9 days with air temperatures of 30°C or higher in Belgium (KMI, 2017, http://www.meteo.be). According to the Lake Flake model (Lake Model Flake, 2009, http://www.flake.igb-berlin.de/index.shtml), using model settings suitable for damselfly larvae (based on Nilsson-Örtman *et al.*, 2012), the maximum daily water temperatures almost never reach 30°C in Belgium. Therefore, long-term exposure to 30°C as in our study can be considered an extreme future heat wave in Belgium, as global warming predicts an increase in the duration of heat waves (Meehl & Tebaldi, 2004; Jentsch *et al.*, 2007; IPCC, 2013).

Experimental setup

The heat wave period started one day after the larvae moulted into the penultimate instar and ended one week after the larvae moulted into their final instar. This period lasted ca. one month, corresponding with 20% of the larval stage of the here studied damselfly species. At the start of the heat wave period, we transferred the larvae to a new plastic 100 mL cup filled with aerated conditioned water that was randomly placed in a water bath (2-3 water baths per temperature treatment). To initiate the heat wave, we placed the larvae first at 24°C for 24 h, after which the temperature was increased to 30°C, this to avoid a shock effect and to more realistically mimic the start of a heat wave. From that moment, we fed the larvae seven days a week with a higher daily dose of *Artemia* nauplii (mean average daily dose ± 1 SE = 686 ± 28, n = 49 daily portions) to meet the higher energy demands of final instar larvae. At the end of the heat wave period, larvae were individually stored in a -80°C freezer for further analyses. For both species we tested between 23 and 32 larvae per heat wave treatment (total of 104 larvae).

Response variables

We daily checked for survival. We quantified traits related to growth (growth rate, food intake, growth efficiency), metabolic rate (activity of the electron transport system, ETS), energy storage (fat content), and investment in immune function (the activity of phenoloxidase activity, PO, and melanin content). PO, a key enzyme involved in insect immune function, is part of the prophenoloxidase cascade, which catalyses the production of melanin (González-Santoyo & Córdoba-Aguilar, 2012). Melanin has an important function in the invertebrate immunity (Siva-Jothy *et al.*, 2005), as it is deposited around pathogens, thereby cutting the pathogen off from available nutrients and preventing further distribution (Gillespie *et al.*, 1997). The prophenoloxidase cascade also produces several other molecules like cytotoxic quinones, reactive oxygen and nitrogen species, which are highly reactive and toxic to pathogens (González-Santoyo & Córdoba-Aguilar, 2012).

We quantified growth rate as the increase in wet mass over the 7-day heat wave period in the final instar of the larvae. Wet masses were taken to the nearest 0.01 mg (Mettler Toledo® AB135-S, Ohio, USA) after gently blotting the larvae dry with tissue

paper. The daily growth rate was calculated as [ln(final wet mass) – ln(initial wet mass)] / 7 days (McPeek, 2004). During the heat wave period we determined for each larva its total food intake and growth efficiency based on McPeek *et al.* (2001) and Campero *et al.* (2007). To estimate the total dry mass of food given to a larva, we daily collected three food portions of *Artemia* and stored these in 70% ethanol. The number of *Artemia* in these three daily collected food portions did not differ between days ($F_{1,25} = 0.50$, $p = 0.49$), illustrating food rations were constant through time. As we daily fed the damselfly larvae with freshly-hatched *Artemia* from the same batch of cysts, size differences of *Artemia* between food portions or days are unlikely and would have been randomized across treatments.

Per larva, the uneaten food was daily collected two hours after feeding the larvae. Before the feeding period started we first transferred the larvae to a new cup with clean conditioned water. This avoided the build-up of detritus and faeces in the rearing cups interfering with the quantification of the amount of uneaten food. Faeces produced during the 2h feeding period, were carefully removed with fine tweezers before we collected the food samples. This way we avoided that any detritus or faeces were collected together with the food remains. To collect the uneaten *Artemia* we poured the water of the cup over a sieve (mesh size 64µm). Then we rinsed the sieve with 70% ethanol and collected the *Artemia* and ethanol in plastic vials. At the end of the 7-day period, we pooled the seven daily samples of uneaten food per larva and poured them over a dried and pre-weighed filter paper. These filters were dried at 60°C for at least 48h before being weighed to the nearest 0.01 mg. The three daily collected food portions were treated in the same way. We calculated the mass of the uneaten *Artemia* and the daily food portions by subtracting the final mass with the mass of the pre-weighed filter. The total food intake per larva was calculated as the difference between the total dry mass of the given food and the total dry mass of the uneaten food across the 7-day period. The growth efficiency was calculated as the gain in dry mass of a larva divided by its total food intake (McPeek *et al.*, 2001; Campero *et al.*, 2007). To obtain the gain in dry mass we converted larval wet mass into dry mass using the conversion equation for *Enallagma* and *Ischnura* damselfly larvae: dry mass = 0.1497 × wet mass (McPeek *et al.*, 2001).

For the quantification of the metabolic rate, energy storage and the investment in immune function, we first homogenised the larvae using a pestle and diluted them 15 times in phosphate buffer (pH 7.4, 50 nm PBS) and then centrifuged the sample for 5 minutes (10,000 rpm, 4°C). The obtained supernatant was used for the physiological analyses.

We quantified the activity of the electron transport system (ETS) as a proxy of the metabolic rate (De Coen & Janssen, 2003). The measurement of ETS activity was based on the protocol of De Coen and Janssen (2003) adapted for damselflies (Janssens and Stoks, 2013a). A 384-well microtiter plate was filled with 5 µL supernatant, 15 µL buffered substrate solution (0.13 mol L^{-1} Tris HCl, pH 8.5, 15% polyvinyl pyrrolidone, 153 µmol L^{-1} $MgSO_4$ and 0.2% Triton X-100) and 10 µL INT (8 mmol L^{-1} p-iodonitrotetrazolim) to start the reaction. We monitored the increase in absorbance at 490 nm (TECAN infinite M200 spectrophotometer, Männedorf, Switzerland) and 20°C over a period of 5 minutes (measurements every 20 s at 20°C). We used the formula of Lambert-Beer to convert absorbances into the concentration of formazan (extinction coefficient 15.9 $M^{-1}cm^{-1}$). Then we converted formazan concentrations to cellular oxygen consumption based on the stoichiometric relationship that for each 2 µmol of formazan formed, 1 µmol of O_2 was consumed in the ETS system. The ETS activity was measured in quadruplicate and expressed as nmol O_2 min^{-1}.

The quantification of the fat content was based on a modified version of the protocol of Bligh and Dyer (1959). We mixed eight µL of the supernatant with 56 µl H_2O_4 (100%) in 2 mL glass tubes. Then the tubes were heated for 20 minutes at 150°C and afterwards 64 µL milli-Q water was added. We filled a 384-well microtiter plate with 30 µL of the sample and we measured the absorbance at 490 nm (at 25°C). Fat content was measured in triplicate and we converted the averaged absorbance per larva to its fat content using a standard calibration curve of glyceryl tripalmitate. Fat content was expressed as mg per individual.

The PO activity was measured using a modified protocol of Stoks *et al.* (2006a). PO catalyses the transformation of phenols into quinones, which polymerize non-enzymatically into melanin. We mixed 10 µL supernatant with 65 µl PBS in a 96-well

microtiter plate. We then added 5 µL chymotrypsin (1 mg mL^{-1}) and incubated the plate for 5 minutes in room temperature. During this incubation period all pro-enzyme proPO present was converted into PO. Subsequently, we added 120 µL L-DOPA and we measured the absorbance at 490 nm over a period of 45 minutes (measurements every 30 s at 30°C). The PO activity was quantified in duplicate as the slope of the linear part (400-1100 s) of the reaction curve. The PO activity was expressed per mg protein.

We quantified the melanin content based on the protocol of Zhou *et al.* (2012). First, we mixed the homogenate again with the pellet and transferred 100 µl of this mixture to an Eppendorf tube. We added 25 µl of 5N NaOH / 50% DMSO and then incubated the tubes for two hours at 80°C. After this incubation period we centrifuged the samples for ten minutes (7,800 rpm). Afterwards, we filled a 384-well microtiter plate with 30 µL of the sample and we measured the absorbance at 480 nm (at 25°C). The melanin content was measured in triplicate. We converted the averaged absorbance per larva to melanin contents using a standard calibration curve. The total melanin content was expressed as mg per individual.

Statistical analyses

We evaluated the effects of species and the heat wave treatment on the different response variables using separate linear mixed models. To correct for a potential effect of population, we added population as a random effect in all models, although it never had an effect. We added body mass as a covariate in all models, except for growth rate and PO activity. We further analysed significant interactions by comparing least-square means using Tukey posthoc tests. Survival was tested using a Fisher's exact test. Growth efficiency did not meet the assumption of normality, not even after transformations. Based on large (>3) absolute values of the Studentized residuals we identified three data points as outliers. Therefore, we ran a non-parametric rank *F*-test test (Quinn & Keough, 2002, p.196) on the entire data set (including the outliers) by first ranking the growth efficiency data and then performing a linear mixed model on the ranked values. The ranking of the data gives less weight to the outliers. To evaluate two other potential functions in which PO is involved, cuticle hardening (Hopkins & Kramer, 1992; Sugumaran, 2002) and body darkness through the production of the pigment melanin

(True, 2003), we added growth rate and melanin content as covariates to the PO model. All statistical analyses were performed in the program R v3.2.2 (R Core Team, 2015). We used the R package 'lme4' (Bates *et al.*, 2015) for running the linear mixed models and the 'car' package was used to compute Wald chi-square statistic and *p*-values for fixed effects (Fox & Weisberg, 2011).

Results

Species effects

Overall, *I. elegans* larvae had a higher growth rate compared to *E. cyathigerum* larvae (Table 1, Fig. 1A). Both species ingested the same amount of food, but *I. elegans* larvae had a higher growth efficiency (Table 1, Fig. 1B-C). Both the ETS activity and the fat content was higher in *I. elegans* than in *E. cyathigerum* (Table 1, Fig. 2). *I. elegans* larvae had also a higher melanin content (Table 1, Fig. 3).

Heat wave exposure

Heat wave exposure did not impose mortality in *I. elegans* (survival at 18°C: 90%, at 30°C: 91%, Fisher's Exact test: $P = 1.00$), but it did so in *E. cyathigerum* (survival at 18°C: 94%, at 30°C: 75%, $P < 0.001$). Heat wave exposure resulted in an increased growth rate for both species compared to 18°C (Table 1, Fig. 1A). This was associated with a higher food intake under the heat wave, while growth efficiency was not affected (Table 1, Fig. 1B-C).

Table 1. Results of the linear mixed models testing for the effects of species and the heat wave on growth rate, food intake, growth efficiency, ETS (electron transport system) activity, fat content, PO (phenoloxidase) activity and melanin content in *Ischnura elegans* and *Enallagma cyathigerum* larvae. Significant *P* values ($P < 0.05$) are printed in bold.

	Growth rate			Food intake			Growth efficiency			ETS			Fat content			PO			Melanin		
Effect	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
Species (S)	1	10.77	**0.0010**	1	0037	0.84	1	11.70	**< 0.001**	1	4.46	**0.035**	1	4.32	**0.038**	1	0.83	0.36	1	19.09	**< 0.001**
Heat wave (H)	1	37.89	**< 0.001**	1	21.48	**< 0.001**	1	1.79	0.18	1	25.85	**< 0.001**	1	16.38	**< 0.001**	1	0.94	0.33	1	34.47	**< 0.001**
S x H	1	0.98	0.32	1	2.89	0.089	1	0.0057	0.94	1	0.17	0.68	1	0.60	0.44	1	4.11	**0.043**	1	4.07	**0.044**

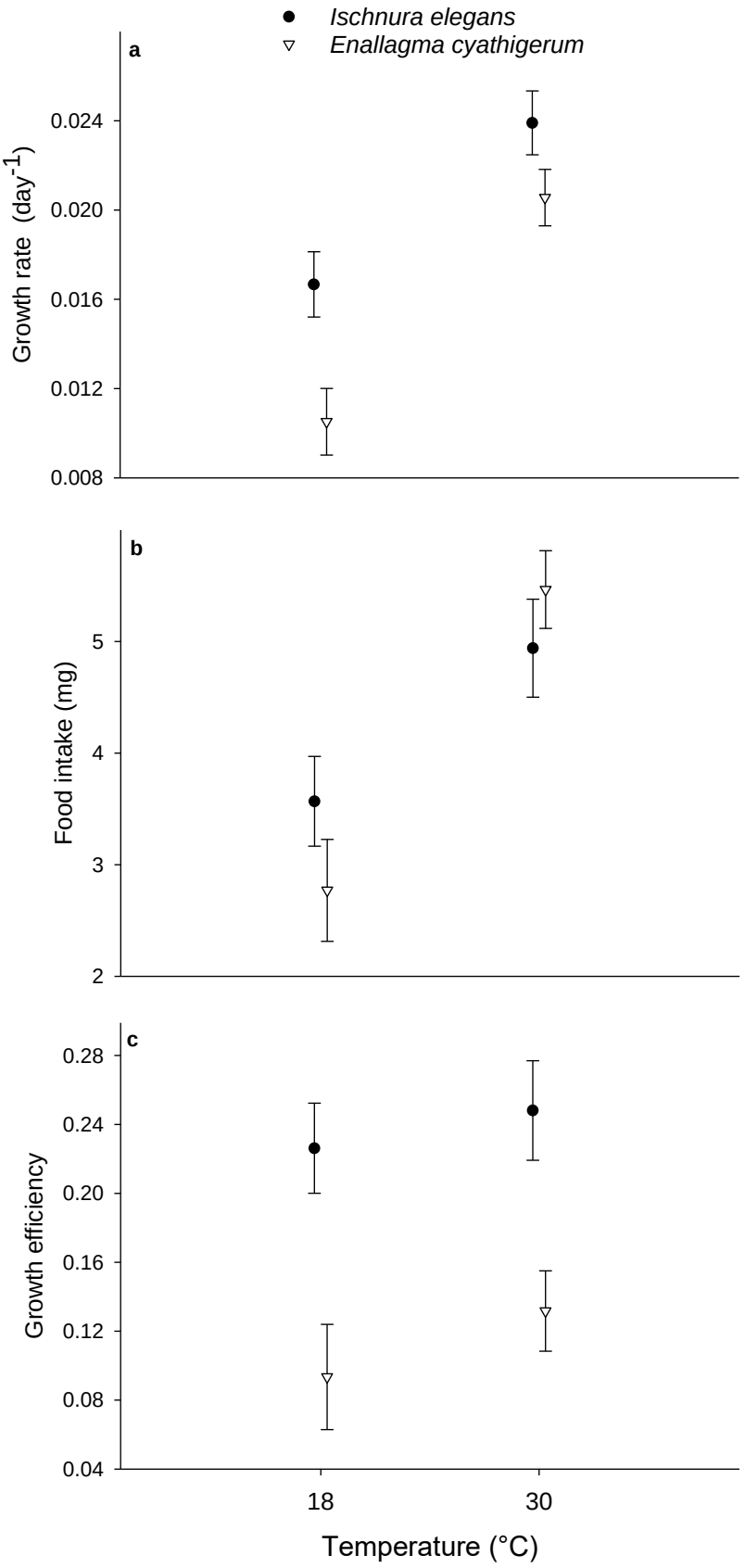

Figure 1. Mean levels of growth-related variables of *Ischnura elegans* and *Enallagma cyathigerum* damselfly larvae as a function of temperature: (a) growth rate, (b) food intake, and (c) growth efficiency. Given are the least-squares means (± 1 s.e.m.).

Exposure to the heat wave increased ETS activity and fat content in both species (Table 1, Fig. 2). For the PO activity, there was a Species x Heat wave interaction (Table1, Fig. 3A), indicating that the heat wave only resulted in an increased PO activity for *I. elegans* larvae (Tukey posthoc test, heat wave effect for *I. elegans: P* = 0035; for *E. cyathigerum*: *P* = 0.49). The larval growth rate did negatively covary with their PO activity (slope = -0.31 with SE = 0.11, χ_1^2 = 7.94, *P* = 0.0048). Exposure to the heat wave increased the melanin content in both species and this increase was larger for *E. cyathigerum* larvae (Species x Heat wave interaction, Table 1, Fig. 3B). The melanin content at 30°C was, however, still higher in *I. elegans* than in *E. cyathigerum*. The melanin content showed no covariation with the PO activity (χ_1^2 = 1.21, *P* = 0.27).

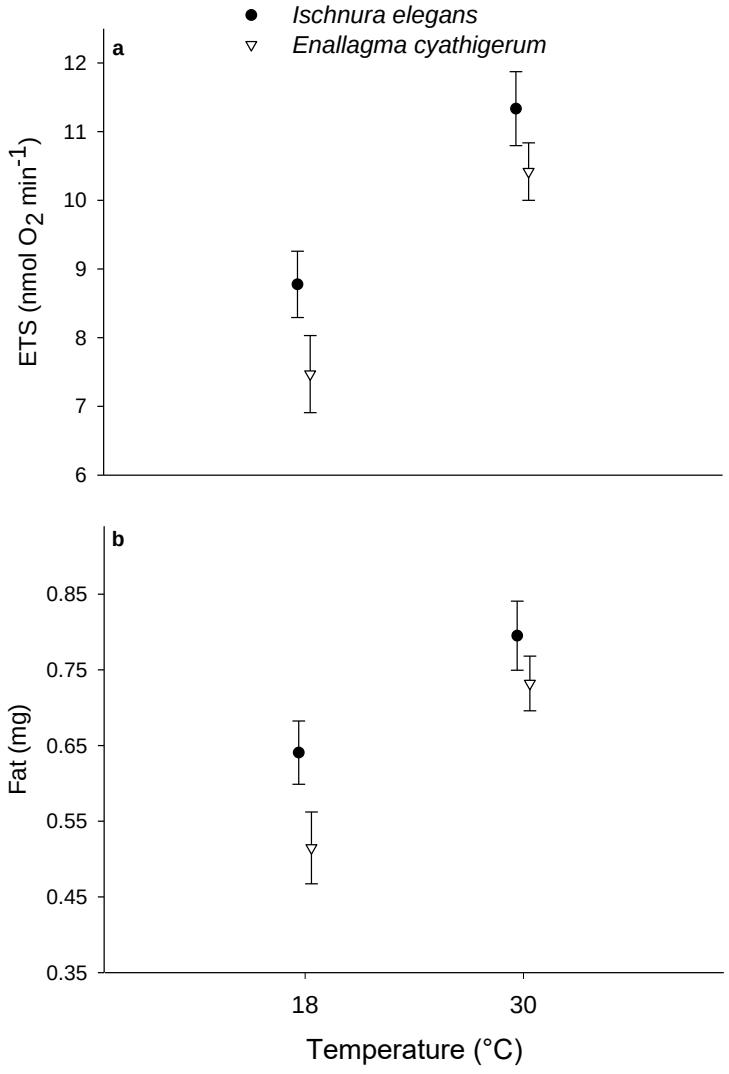

Figure 2. Mean levels of metabolic rate and investment in energy storage of *Ischnura elegans* and *Enallagma cyathigerum* damselfly larvae as a function of temperature: (a) ETS activity and (b) fat content. Given are the least-squares means (± 1s.e.m.).

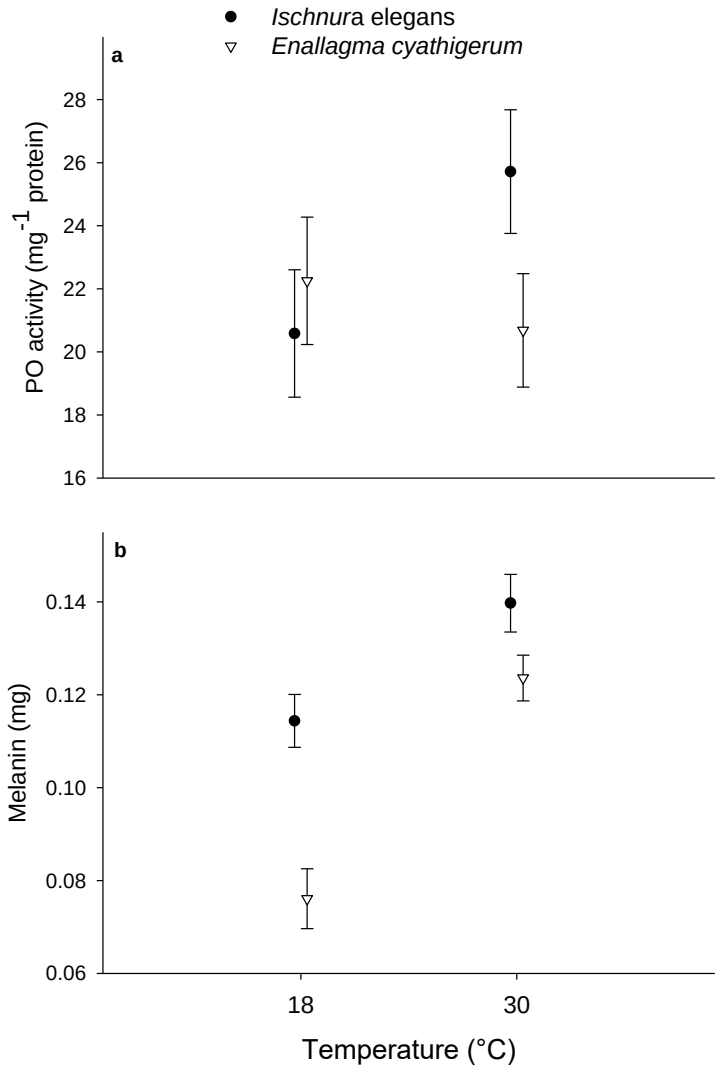

Figure 3. Mean levels of larval investment in immune function of *Ischnura elegans* and *Enallagma cyathigerum* damselfly larvae as a function of temperature: (A) PO activity and (B) melanin content. Given are the least-squares means (± 1s.e.m.).

Discussion

Exposure to heat wave temperatures can have negative effects on performance and can even cause mortality (Garrabou *et al.*, 2009; Petter *et al.*, 2014; Mislan & Wethey, 2015) as observed before in damselflies (Chang *et al.*, 2007). We detected a striking species difference with the heat wave only being lethal in *E. cyathigerum* and not in *I. elegans*. However, for both *I. elegans* and *E. cyathigerum* larval performance measured as growth rate was higher under the simulated heat wave of 30°C compared to 18°C, the mean water temperature the studied larval instars would experience in natural populations in Belgium. Given that larval growth of both species was also higher at 30°C compared to

the intermediate temperature of 24°C (M. Van Dievel, unpublished results), our data indicate the heat wave was beneficial in terms of growth rate (at least for the survivors) compared to the temperatures larvae would otherwise experience in natural populations. Beneficial effects of the heat wave were also detected for two other fitness-related traits: the investments in energy storage (measured as fat content) and in immune components [PO activity (for *I. elegans*) and melanin content]. The beneficial effects of the heat wave on these traits were associated with an increase in food intake. We first discuss the general heat wave effects that were observed for both species and then focus on species differences

General heat wave effects

Exposure to the extreme heat wave increased the metabolic rate (measured as ETS activity) of the damselfly larvae, likely causing higher cellular maintenance costs (Lemoine & Burkepile, 2012). Increases in metabolic rate with temperature may generate growth reductions when metabolic rate increases faster than food consumption under high temperature (Rall *et al.,* 2010; Lemoine & Burkepile, 2012). Instead, we documented larger increases in food intake (39% for *I. elegans* and 97% for *E. cyathigerum*) than in metabolic rate (29% for *I. elegans* and 39% for *E. cyathigerum*) under the heat wave. This may explain why larvae of both damselfly species were able to increase their growth rate under the extreme heat wave. The higher increase in food intake than in metabolic rate may also explain the increased investment in energy storage. A higher fat content under a heat wave has been documented before in the study species *I. elegans* (Arambourou & Stoks, 2015) and in other insect taxa (butterflies: Karl *et al.,* 2011; crickets: Adamo *et al.,* 2012; but see Fischer *et al.,* 2014; Dinh Van *et al.,* 2016).

Similar to our results, food intake of the damselfly *E. vesperum* was also higher at 30°C (Culler *et al.,* 2014). Likewise, a study on *I. elegans* showed food intake to increase up to the highest temperature tested (27.5°C, Thompson, 1978). This pattern of increasing food intake up to extreme temperatures may be related to the tropical origin of Odonates (Pritchard *et al.,* 1996). An increase in metabolic rate under a heat wave was also suggested in a study on a tropical butterfly (Karl *et al.,* 2011). It, however,

contrasts with research showing a metabolic depression to overcome a short- to medium-term exposure to extreme temperatures (Pörtner & Farrel, 2008; Dinh Van *et al.,* 2016). The latter is thought to occur to reduce energy depletion (Marshall & McQuaid, 2010). Likely, since the animals in the current study were fed ad libitum during the heat wave and increased their food intake they accumulated enough energy to cope with the higher energy demand associated with the increasing metabolic rate at the high temperature (Rall *et al.,* 2010; Lemoine & Burkepile, 2012; Culler *et al.,* 2014).

Although we documented positive effects on growth and fat storage under the simulated heat wave, there could be hidden costs. These can be both directly driven by the heat wave (for example, a reduction in reproduction, Zhang *et al.,* 2015) or indirectly caused by the increases in growth rate and metabolic rate (for example, an increased production of reactive oxygen species causing oxidative damage, Mangel & Munch, 2005).

Species differences

In line with previous studies comparing *Ischnura* and *Enallagma* damselfly larvae (McPeek, 1996, 1998; Stoks *et al.,* 2005a; Siepielski *et al.,* 2011), *I. elegans* grew faster and had a higher metabolic rate than *E. cyathigerum*. As shown before when comparing both genera (McPeek *et al.*, 2001, McPeek, 2004), the higher growth rate in *Ischnura* was not caused by a higher food intake but a higher efficiency of converting ingested food in biomass. This may also explain the observed higher fat and melanin contents in *I. elegans* larvae. The higher melanin contents could be associated with their presence in small, shallow water bodies (Dijkstra, 2006), where they are more exposed to UV. Damselfly larvae have been shown to increase melanin content in response to UV exposure (Debecker *et al.,* 2016).

While the response to the extreme heat wave was for most non-lethal response traits similar in both species, the heat wave only caused mortality in *E. cyathigerum*. This higher sensitivity to the heat wave was expected based on the species preference for deeper and cooler water bodies that have smaller temperature fluctuations. That for most traits *E. cyathigerum* did react in the same way to the heat wave as *I. elegans* may be due to two non-exclusive reasons. First, for these other traits the heat wave may have

still been in the optimal range of temperatures; traits indeed may differ strongly in their thermal optimum (Sinclair *et al.*, 2016). Second, survival selection may have occurred whereby the most sensitive *E. cyathigerum* larvae (that would have shown reduced performance values under the heat wave) were eliminated before they could be measured.

Both species differed in their response to the heat wave with regard to two traits related to investment in immune function. The heat wave only resulted in the energetically costly (González-Santoyo & Córdoba-Aguilar, 2012; for damselflies: De Block & Stoks, 2008) higher PO activity in *I. elegans* larvae. As for the investment in energy storage, the increased investment in immune function may reflect the higher food intake. In addition, the higher PO activity could be the result of a changed trade-off pattern with unmeasured traits. An increased PO activity in response to heat wave temperatures has been observed before in another insect order (crickets: Adamo & Lovett, 2011), and in the study species *I. elegans* (Arambourou & Stoks, 2015). Although PO is a key enzyme of the immune system in insects, it is also involved in other functions like hardening of the cuticle and pigment synthesis (González-Santoyo & Córdoba-Aguilar, 2012). Yet, it is unlikely that in our study the other functions of PO caused the response to the heat wave, as we observed a negative correlation between growth rate and PO activity and no association between PO and melanin content. The former reflects the well-known trade-off between growth rate and PO activity (for damselflies: De Block & Stoks, 2008).

Also the melanin content increased under the heat wave; while this increase was stronger in *E. cyathigerum*, the melanin content was still higher in *I. elegans* at 30°C. Melanin is another important component of an insect's immune function that is involved in the melanotic encapsulation response where it neutralizes pathogens and parasites (Gillespie *et al.*, 1997; Sugumaran, 2002). While melanin may also play a role in thermoregulation (Roulin, 2014), this cannot explain the here observed increase under the heat wave temperature as we would then have expected lighter animals with less melanin.

Studies focusing on effects of heat waves on immune function have found mixed results with some studies showing an immunosuppression while other studies detected no or even a positive effect of heat waves on immune function (see introduction). This is an important topic as a higher investment in immune function might be important during heat waves as pathogens may develop faster with increasing temperature (Karvonen *et al.*, 2010). Our results showed increases of the two studied immune components under a long-term heat wave, suggesting an overall higher immune function. Yet, we cannot exclude that other unmeasured immune components (such as the number of hemocytes, Siva-Jothy *et al.*, 2005) were suppressed by the heat wave. Indeed, trade-offs between different immune components have been reported, and some studies found opposing results of a heat wave on different immune components (Roth *et al.*, 2010; Karl *et al.*, 2011; Seppälä & Jokela, 2011). This may also explain the stronger heat wave-induced increase of melanin in *E. cyathigerum*, without an effect on the PO activity.

Conclusions

Understanding how extreme temperatures influence species is important to determine which species can survive under global warming (Domisch *et al.*, 2011; Rosset & Oertli, 2011). The emerging pattern of the impact of heat waves on species is that of strong opposing fitness effects on different species with some species suffering (e.g. decreased immune function: Fischer *et al.*, 2014; Dinh Van *et al.*, 2016) and other species benefiting (e.g. increased fat content and/or immune function: Adamo & Lovett, 2011; Arambourou & Stoks, 2015). We added to this intriguing pattern by providing a mechanistic understanding of the beneficial effect of an extreme heat wave on growth rate, energy storage and two immune components. Exposure to the simulated heat wave (30°C) positively affected several performance traits in both *I. elegans* and *E. cyathigerum* larvae. This is especially striking as we exposed both species to a long and extreme heat wave while previous studies that reported no or a negative effect of a heat wave used a shorter exposure period (e.g. Fischer *et al.*, 2014; Dinh Van *et al.*, 2016). Furthermore, it has been shown that a longer exposure period can impose stronger effects (Leicht *et al.*, 2013). Especially, the fact that *I. elegans* larvae could maintain

high performance over a prolonged experimental heat wave suggests that this species was able to acclimatize to the increased temperature. The larvae did so by increasing their food intake and therefore were able to cope with the higher energy demands associated with the increased metabolic rate at the high temperature. In the more sensitive *E. cyathigerum* that showed mortality under the heat wave, survival selection removing the larvae with the lowest performance at 30°C likely also a played role in causing positive effects of the heat wave. The dependence of the positive effects of the heat wave on the higher food intake supports the view that the impact of heat waves on fitness may critically depend on food availability (Adamo *et al.*, 2012). Our results highlight the importance of not only studying fitness-related response variables but also the assumed underlying behavioural and physiological variables to unravel and predict the impact of extreme climate events on organisms.

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Author contributions: MVD, RS and LJ conceived and designed the experiment. MVD conducted the experiment. MVD, RS and LJ analysed the data and wrote the manuscript. All authors approved the final manuscript.

Chapter IV

Short- and long-term behavioural, physiological and stoichiometric responses to predation risk indicate chronic stress and compensatory mechanisms

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Slightly modified version

IV

Abstract

Prey organisms are expected to use different short- and long-term responses to predation risk to avoid excessive costs. Contrasting both types of responses is important to identify chronic stress responses and possible compensatory mechanisms in order to better understand the full impact of predators on prey life history and population dynamics. Using larvae of the damselfly *Enallagma cyathigerum*, we contrasted the effects of short- and long-term predation risk, with special focus on consequences for body stoichiometry. Under short-term predation risk, larvae reduced growth rate, which was associated with a reduced food intake, increased metabolic rate and reduced glucose content. Under long-term predation risk, larvae showed chronic predator stress as indicated by persistent increases in metabolic rate and reduced food intake. Despite this, larvae were able to compensate for the short-term growth reduction under long-term predation risk by relying on physiological compensatory mechanisms, including reduced energy storage. Only under long-term predation risk did we observe an increase in body C:N ratio, as predicted under the general stress paradigm (GSP). Although this was caused by a predator-induced decrease in N content, there was no associated increase in C content. These stoichiometric changes could not be explained by GSP responses because, under chronic predation risk, there was no decrease in N-rich proteins or increase in C-rich fat and sugars; instead glycogen decreased. Our results highlight the importance of compensatory mechanisms and the value of explicitly integrating physiological mechanisms to obtain insights in the temporal dynamics of nonconsumptive effects, including effects on body stoichiometry.

Introduction

As prey organisms often face long-term exposure to predators, coping with predators is a central problem for them (Boonstra, 2013). It is increasingly clear that the impact of predators not only works through direct killing but that nonconsumptive fear effects, causing changes in behaviour, life history and physiology, may be as important in shaping prey population dynamics (Preisser *et al.,* 2005; Creel & Christianson, 2008; Zanette *et al.,* 2014). These nonconsumptive effects may scale up and affect community structure (Peacor *et al.,* 2012) and ecosystem functions (Hawlena *et al.,* 2012). Despite the potential far-reaching implications of nonconsumptive effects, their temporal dynamics and specifically the differential effects of short-term and long-term exposure to predation risk are little understood (Thaler *et al.,* 2012; Boonstra, 2013).

Under short-term exposure to predators, prey organisms show a series of adaptive physiological responses, summarised under the general stress paradigm (GSP) (Hawlena & Schmitz, 2010a). These responses increase survival by increasing metabolic rate, and mobilising and shunting energy to the brain and other organs essential to survive the threatening episode (Sapolsky, 2002). This results in less allocation of energy toward growth and reproduction (Hawlena & Schmitz, 2010a). Under long-term exposure to predators, prey organisms may experience chronic stress, i.e. prolonged activation of a short-term physiological stress response (Adamo & Baker, 2011). The presence of chronic predator stress responses is not general and is poorly understood (Boonstra, 2013): it occurs in some prey taxa (e.g. Hik *et al.,* 2001; Sheriff *et al.,* 2011) and not in others (e.g. Creel *et al.,* 2009; Steiner & Van Buskirk, 2009). Chronic stress responses have largely been studied in vertebrates, but invertebrate responses are less well known (Preisser, 2009; but see Stoks *et al.,* 2006b; Adamo & Baker, 2011; Hawlena & Schmitz, 2010b; Hawlena *et al.,* 2011; Thaler *et al.,* 2012). While the prolonged use of the stress response may be necessary for survival, it may have several costs including decreased growth rates, depletion of energy storage and build-up of toxic compounds (Hawlena & Schmitz, 2010a). Importantly, certain species can avoid some of the negative effects of chronic stress, indicating that these costs are not inevitable and that compensatory mechanisms may exist (Thaler *et al.,* 2012). Studies directly comparing short- and long-

term responses to predation risk are rare (but see Thaler *et al.*, 2012 for a notable exception), yet necessary to identify chronic stress responses and the presence of compensatory mechanisms.

Growth rate is a life history trait closely related to fitness (Dmitriew, 2011) and that is often reduced under short-term exposure to predation risk (e.g. Stoks & McPeek, 2003; McPeek, 2004; Culler *et al.*, 2014). Growth reductions are much less common under long-term exposure to predation risk. For example, Benard (2004) reported that of 16 studies that explicitly tested for an effect of long-term exposure to predation risk on growth rate, only four detected a reduction and the remaining 12 detected no significant effect. This may be explained by the absence of chronic predator stress responses (Boonstra, 2013) or the presence of compensatory mechanisms to avoid growth reductions under chronic predator stress (Thaler *et al.*, 2012). Prey organisms may rely on several compensatory mechanisms to avoid growth reductions under chronic predation risk. These include behavioural mechanisms such as an increased food intake and physiological mechanisms such as increased assimilation efficiency.

In a recent fascinating extension of the known nonconsumptive effects induced by predators it was demonstrated that the predator-induced physiological changes under the general stress paradigm may affect the elemental body composition of the prey (Hawlena & Schmitz, 2010a, b). These changes in prey body stoichiometry may have far reaching consequences for nutrient cycling (Hawlena & Schmitz, 2010b) including ecosystem functions such as detritus decomposition (Hawlena *et al.*, 2012). According to the general stress paradigm, the breakdown of nitrogen-rich (N-rich) proteins to produce more carbon-rich (C-rich) fat and sugars, to fuel heightened respiratory demands under predation risk, and the excretion of excess N, will result in a changed body composition with a lower N content, a higher C content and a higher C:N ratio (Hawlena & Schmitz, 2010a). Despite the importance of predator-induced changes in proteins, fat and sugars for driving changes in body stoichiometry, the few studies on predator-induced effects on body stoichiometry (Hawlena & Schmitz, 2010b; Costello & Michel, 2013; Dalton & Flecker, 2014) did not quantify these biomolecules.

Moreover, the temporal dynamics of this nonconsumptive effect is unknown and the few studies so far only considered long-term exposure to predation risk.

We here compare short- and long-term effects of exposure to predation risk to test for chronic predator stress responses and compensatory mechanisms to avoid long-term growth reductions in an invertebrate prey. As study species we used larvae of the damselfly *Enallagma cyathigerum* whose short-term exposure to predation risk is well characterized, including reduced food intake (Stoks *et al.*, 2005b), increased metabolic rate and reduced growth (Slos & Stoks, 2008). As advocated by Boonstra (2013) we followed a multivariate approach and studied several manifestations related to predation risk, both behavioural (food intake) and physiological (metabolic rate and energy storage levels). Moreover, we aimed to directly relate the predator-induced effects to temporal changes in the elemental body composition by examining the C and N contents and the C:N body ratio of the damselfly larvae. Based on the framework of the GSP (Hawlena & Schmitz, 2010a) we generally predict that under predation risk larvae will (1) increase their metabolic rate; (2) increase their content of carbon-rich biomolecules to fuel this elevated metabolic rate, thereby increasing their body C-content; (3) invest less in body tissue (growth) and break down nitrogen-rich proteins (gluconeogenesis), thereby decreasing their body N-content, which will result in (4) an increased body C:N ratio.

Materials and methods

Collecting and housing

In May-June 2014 we collected *Enallagma cyathigerum* larvae from ponds located in Kalmhoutse Heide (51°24'34.60" N, 4°26'32.28" E) and Bergerven (51°03'58.9284" N, 05°41'29.9796" E), which are two protected nature areas in Belgium. Large larvae of the dragonfly *Anax imperator*, which are important predators of *Enallagma* larvae (Stoks *et al.*, 2005b) were present in both ponds. In the laboratory, we kept the larvae individually in plastic 200 ml cups, filled with a mixture of filtered pond water and dechlorinated tap water. The cups were placed in incubators at 20 °C and a 14:10 L:D photoperiod. Prior to the experiment, larvae were fed daily with *Artemia* nauplii *ad*

libitum. When the larvae moulted into the final instar (on average 18 days after capture), they were used in the experiment.

Experimental setup

To study how exposure to predation risk affects growth rate and key physiological components related to the general stress paradigm, and how these responses are altered over time, we set up a full factorial experiment with two levels of predation risk (absent and present) crossed with three exposure durations (three, six and nine days). We consider three days as a short-term exposure period because we know that a predator-induced growth reduction occurs after three days of exposure (M. Van Dievel unpublished results, and similar studies on other species of *Enallagma* during a four day exposure period McPeek *et al.*, 2001; McPeek, 2004; Stoks *et al.*, 2005a). Six and especially nine days can be considered as long-term exposure times for the study species as they make up ca. 25 % and ca. 40 %, respectively, of the duration of the final instar, when most of the increase in larval mass occurs. In addition, previous research on damselflies did not detect a predator-induced growth reduction for an exposure period of ten days (Slos *et al.*, 2009). We manipulated predation risk by exposing half of the larvae to both visual and chemical predator cues, thereby mimicking the predator cues that damselfly larvae naturally encounter. *Enallagma* larvae are known to respond to both visual and chemical predator cues (Mortensen & Richardson, 2008). We tested between 23 and 31 larvae per treatment combination (total of 167 larvae).

At the start of the exposure experiment we placed larvae individually in a glass vial (100 ml) filled with 50 ml dechlorinated tap water. Four vials of the same predation risk treatment were placed together in a larger container (750 ml). We daily reshuffled the vials among containers of the same predation risk treatment. Because damselfly larvae are cannibalistic and impose predator stress to each other (De Block & Stoks, 2004), the walls of the glass vials for the treatment without predation risk were made opaque using tape. As vials received light from above, this did not affect the light intensity in the vials. For the treatment with predation risk we placed a large *Anax* dragonfly larva in the larger container. This way, damselfly larvae received visual predator cues from conspecifics and from the *Anax* predator. Chemical cues were prepared daily by homogenising one *Enallagma* larva in 20 ml water from a container (300 ml) in which a large *Anax* larva had eaten an *Enallagma* larva. One ml of this

predator medium was added daily to the vials of the treatment with predation risk. During the experiment all containers were placed in a water bath at 20 °C (14:10 L:D). Larvae were daily fed *ad libitum* by giving them 25 *Daphnia magna* of a standardized size (*Daphnia* that were retained by a 1 mm mesh sieve). Uneaten food was removed daily when refreshing the medium and new *Daphnia* were provided.

Response variables

For all larvae, we quantified growth rate as the increase in wet mass during the exposure period. After gently blotting the larvae dry with tissue paper, we weighed each larva to the nearest 0.01 mg at the start and the end of the exposure period. The daily growth rate was calculated as [ln(final wet mass) – ln(initial wet mass)] / exposure period (three, six or nine days) (McPeek, 2004).

For all larvae that experienced the 9-day exposure period, we estimated daily food intake separately for the three successive periods of three days. This way we obtained three repeated estimates of food intake of individual larvae. For logistical reasons we did not estimate daily food intake for the larvae of the 3-day and 6-day exposure periods. Daily food intake was estimated per period of three days as the sum of the dry mass of *Daphnia* eaten across each 3-day period divided by three days. Total dry mass of *Daphnia* eaten was calculated as the difference of the total dry mass of the *Daphnia* added to a vial and the total dry mass of the uneaten *Daphnia* recovered from a vial. To estimate the individual dry mass of the *Daphnia* fed to the larvae we daily collected three samples of 10 *Daphnia* individuals, transferred the samples to small aluminium foil cups and dried these at 60 °C for at least 48 h. Afterwards, we weighed each *Daphnia* sample on a microbalance (Thermo Cahn C-35) to the nearest 0.1 µg. Uneaten *Daphnia* were also transferred daily to aluminium foil cups, pooled per three days in one cup, dried and weighed as above.

At the end of the exposure period larvae were frozen and stored individually in eppendorf tubes at -80 °C for physiological analyses. We measured metabolic rate on 23-29 larvae per treatment combination and measured energy reserves on a subset of these (15-19 per treatment combination). When predator-induced effects differed among periods, this difference was largest between the 3- and 9-day exposure periods (i.e. for

growth and glycogen content). Therefore, we only measured the C and N contents for these two exposure periods (15 larvae per predation risk level, total of 60 larvae).

To estimate the metabolic rate we measured the electron transport system (ETS) activity at the mitochondrial level. The activity of ETS is directly linked to oxygen consumption and was measured based on the protocol of De Coen and Janssen (2003) adjusted for damselfly larvae by Janssens and Stoks (2013a). We diluted the body of the larvae 15 times in a homogenisation buffer (0.1 M Tris-HCl, pH 8.5, 15 % polyvinyl pyrrolidone, 153 µM $MgSO_4$ and 0.2 % Triton X-100) and centrifuged it during 5 minutes (13.2 g, 4 °C). To measure the ETS activity we filled a 384 well microtiter plate with 15 µl buffered substrate solution (0.13 M Tris-HCl, 0.3 % Triton X-100, 1.7 mM NADH, 250 µM NADPH, pH 8.5) and 5 µl supernatant. The reaction was started by adding 10 µl (8 mM) p-iodonitrotetrazolium (INT), an artificial electron acceptor. The reduction of INT causes the formation of formazan, which was monitored as the increase in absorbance at 490 nm and 20 °C over a period of 5 minutes (measurements every 30 seconds). ETS activity was determined as the slope of the linear part of the reaction curve. The samples were measured in duplicate and the means were used for the statistical analyses. For the larvae that were also used to determine energy reserves and C:N, we took 20 µl homogenate (see below) and diluted it 3 times in the homogenisation buffer. From then onwards they followed the same protocol as described above.

For the quantification of the energy reserves we measured the total fat, sugars (glucose and glycogen) and protein contents. We homogenised larvae using a pestle and diluted the homogenate 5 times in milli-Q water. The sample was centrifuged for 5 minutes (13.2 g, 4 °C). We took 35 µl of the resulting supernatants and diluted this 3 times in milli-Q water. The remaining sample was used to measure the body C and N contents and the C:N ratio. All samples were measured in duplicate and the means were used for the statistical analyses. Energy reserves were expressed as µg per mg wet mass.

The fat content was quantified based on the protocol of Bligh and Dyer (1959). We filled a 2 ml glass tube with a mixture of 8 µl of the supernatant and 56 µl sulphuric acid (100 %). The tubes were heated for 20 minutes at 150 °C. Afterwards, we added 64 µl milli-Q water and the whole sample was mixed. We filled a transparent 384 well

microtiter plate with 30 µl of the sample and measured absorbance at 470 nm. Fat contents were calculated using a standard curve of glyceryl tripalmitate.

We determined the sugar (glucose and glycogen) content using an adapted protocol from Stoks *et al.* (2006a) based on the glucose kit of Sigma-Aldrich USA. We mixed 50 µl milli-Q water, 20 µl of the supernatant and 10 µl amyloglucosidase (Sigma A7420) in a 96 well microtiter plate. The plate was incubated for 30 minutes at 37 °C. This way all glycogen was transformed into glucose. We measured the glucose levels by adding 160 µl of glucose assay reagent (Sigma G3293) to each well and incubated the plate for 20 minutes at 30 °C. After this incubation period we measured absorbance at 340 nm. To measure only the free glucose we mixed 60 µl milli-Q water and 20 µl of the supernatant in a 96 well microtiter plate. Thereafter, we followed the same procedure as above. The glucose content was calculated based on a standard curve of known concentrations of glucose and their absorbance. The difference in glucose content between the two measurements is equal to the amount of glucose stored in glycogen.

The protein content was measured based on the Bradford (1976) method. We added 160 µl milli-Q water and 1 µl of the supernatant in a 96 well microtiter plate. Then we added 40 µl Biorad Protein Dye and mixed the sample. We incubated the plate for 5 minutes at 30 °C and subsequently measured absorbance at 595 nm. The protein content was calculated based on a standard curve of known protein concentrations.

To determine the internal C and N contents and the C:N ratio we dried (60 °C, 24h) and weighed the rest of the homogenate in tin cups. Afterwards, we quantified the C and N content using an element analyser (Carlo Erba 1108). We expressed the C:N ratio as molar ratios. Because we had data on both wet and dry mass for this subset of larvae, we could use them to evaluate the possibility that predator-induced mass changes were due to changes in water content rather than changes in tissue content. We calculated the water content as the difference between total wet mass and total dry mass and expressed it as a percentage of the total wet mass.

Statistical analyses

All statistical analyses were performed in STATISTICA v12. We used ANOVA to test for the effects of predation risk and exposure period on the different response variables.

When there was a predation risk × exposure period interaction, we tested for an effect of predation risk within each exposure period using linear contrasts. In the figures we present both uncorrected p-values for these contrasts and p-values corrected for multiple testing using the false discovery rate procedure outlined in Benjamini and Hochberg (1995). For food intake, which was measured only in the larvae exposed for 9 days, we had successive measures of the same larva every 3 days. These were treated as repeated observations and we tested for an effect of predation risk and exposure period on food intake using a repeated measures ANOVA. One measurement of the treatment without predation risk showed a negative value for food intake and was removed from the dataset.

We considered three covariates related to experimental procedures that may have affected outcomes, but that were not of direct interest to our hypotheses. Whenever these covariates and their interactions were not significant they were removed from the final models, when significant they are reported in the statistical tables. Because we sampled larvae from two populations we initially included the population of origin in each ANOVA. This covariate was significant for fat, protein and water content and retained in these models (Table 1). To correct for the two slightly different homogenization buffers used to quantify ETS activity, we included the ETS homogenization buffer as a variable in the model analysing ETS activity. When analysing food intake, we included larval wet mass as a covariate, but this had no effect and was removed from the final analysis.

The stoichiometric data did not meet the assumption of normality, not even after transformations, owing to several outlying data points. Both for the 3- and 9-days exposure periods we identified three data points as outliers for N content based on the absolute value of their studentized residuals (> 2) and these were confirmed by the interquartile range method (1.5 times the interquartile range above the third quartile or under the first quartile) (Crawley, 2007; Manoj & Senthamarai Kannan, 2013). For both periods, two of the three outliers for N were also (the only) outliers for the C content. After excluding these outliers, parametric assumptions were met and we used ANOVA to test for the effects of predation risk and exposure period. Additionally, we also ran nonparametric analyses on the entire dataset (including the outliers) by first ranking the

data and then conducting ANOVAs on the ranks (rank F-tests, Quinn & Keough, 2002, p. 196). The ranking of the data gives less weight to the outliers. Both analyses gave very similar results (Table 2).

According to the general stress paradigm (Hawlena & Schmitz, 2010a) the prey body stoichiometric composition will change in a predictable way under predation risk: the C content will increase, the N content will decrease and the resulting C:N ratio will increase. Based on these theoretical grounds and on empirical studies reporting decreases in N content and increases in C:N ratio (Hawlena & Schmitz, 2010b; Hawlena *et al.,* 2012; Janssens *et al.,* 2015, Chapter V), we used one-tailed, planned linear contrasts to test for the effect of predation risk within each exposure period.

Results

Growth rate and food intake

Growth rates were lower in the presence of predation risk only for the 3-day exposure period, as indicated by the significant predation risk × exposure period interaction (Table 1, Fig. 1a). Growth rate depended on time and was higher for the 3-day exposure period. Over time, food intake was consistently lower under predation risk (Table 1, Fig. 1b). Food intake depended on time and was highest for the 3-day exposure period.

Table 1. Results of ANOVA testing for effects of predation risk and exposure period on life history, behaviour and physiology in *E. cyathigerum* larvae. Given that we had repeated observations of the same larva for food intake, food intake was analysed using a repeated-measures ANOVA

Response variable	Effect	df	MS	F	*P*
Growth rate	Predation risk (Pr)	1	0.0031	4.46	0.036
	Exposure period (Ex)	2	0.0023	37.81	<0.001
	Pr × Ex	2	0.00023	3.36	0.037
	Error	161	0.000069		
Food intake	Predation risk (Pr)	1	0.78	4.25	0.045
	Error between-effects	46	0.18		
	Exposure period (Ex)	2	0.86	4.54	0.013
	Pr × Ex	2	0.11	0.56	0.57
	Error within-effects	92	0.19		
ETS activity	Predation risk (Pr)	1	0.00069	4.34	0.039
	Exposure period (Ex)	2	0.00040	2.52	0.084
	Pr × Ex	2	0.000013	0.085	0.092
	ETS method	1	0.016	102.85	<0.001
	Error	151	0.00016		
Energy storage					
Fat content	Predation risk (Pr)	1	187.60	1.17	0.28
	Exposure period (Ex)	2	2612.20	16.24	<0.001
	Pr × Ex	2	38.20	0.24	0.79
	Population of origin	1	640.30	3.98	0.049
	Error	98	160.80		
Glucose content	Predation risk (Pr)	1	27.95	5.12	0.025
	Exposure period (Ex)	2	35.27	6.47	0.0023

	Pr × Ex	2	5.52	1.013	0.37
	Error	99	5.45		
Glycogen content	Predation risk (Pr)	1	11.83	14.27	<0.001
	Exposure period (Ex)	2	2.94	3.54	0.033
	Pr × Ex	2	2.46	2.97	0.056
	Error	99	0.83		
Protein content	Predation risk (Pr)	1	8.17	1.29	0.26
	Exposure period (Ex)	2	67.22	10.66	<0.001
	Pr × Ex	2	12.13	4.37	0.15
	Population of origin	1	27.57	4.37	0.039
	Error	98	6.31	1.92	
Water content	Predation risk (Pr)	1	0.90	0.12	0.73
	Exposure period (Ex)	1	65.6	8.70	0.0047
	Pr × Ex	1	0.50	0.060	0.81
	Population of origin	1	45.60	6.04	0.017
	Error	55			

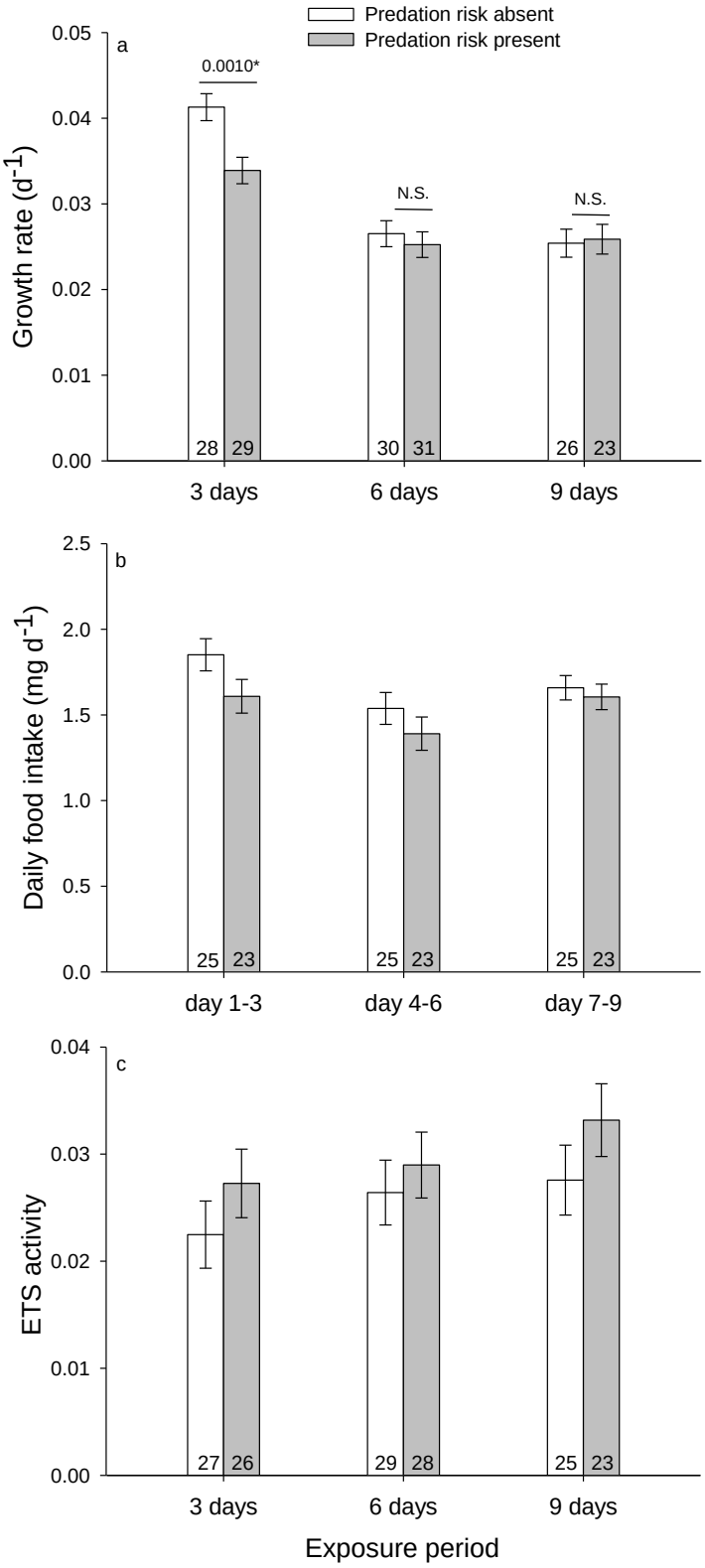

Figure 1. Mean (± 1 SE) (a) growth rate daily, (b) food intake and (c) electron transport system (ETS) activity of *E. cyathigerum* larvae as a function of predation risk and exposure period. In case of a significant predation risk × exposure period interaction *P*-values of linear contrasts testing for the effect of predation risk within each exposure period and the significance levels corrected for multiple testing using the False Discovery Rate are presented (*$P \leq 0.05$). Numbers in bars represent sample sizes. Note that food intake was quantified over successive period of three days for the larvae in the 9-day exposure period.

Physiological response variables

The ETS activity was consistently higher in larvae exposed to predation risk. The duration of the exposure period did not affect ETS activity and there was no interaction between predation risk and exposure period for ETS activity (Table 1, Fig. 1c).

Of the four energy storage molecules, the fat and protein content did not differ between predation risk treatments, nor was there a predation risk × exposure period interaction (Table 1, Fig. 2). The glucose levels were consistently lower in the presence of predation risk. Glycogen levels were also lower in the presence of predation risk, but only after an exposure period of nine days as suggested by the significant interaction term (Table 1, Fig. 2c). All storage molecules increased with exposure period, except glycogen levels, which increased only in larvae reared without predation risk (predation risk × exposure period, Table 1, Fig. 2c).

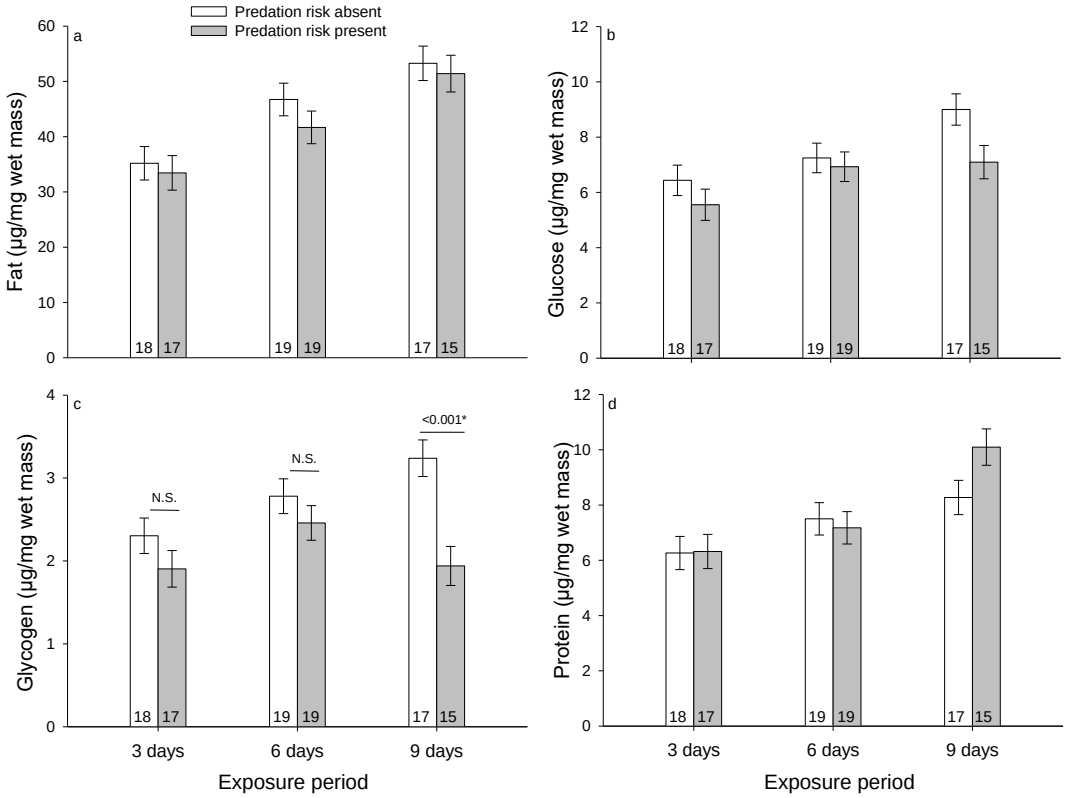

Figure 2. Mean (± 1 SE) levels of energy storage molecules: (a) total fat, (b) glucose, (c) glycogen and (d) protein content of *E. cyathigerum* larvae as a function of predation risk and exposure period. In case of a significant predation risk × exposure period interaction *P*-values of linear contrasts testing for the effect of predation risk within each exposure period and the significance levels corrected for multiple testing using the False Discovery Rate are presented (*$P \leq 0.05$). Numbers in bars represent sample sizes.

The water content of the larvae was not dependent on predation risk, and slightly decreased with longer exposure periods (Table 1). After the 3-day exposure period, the mean water content (± 1 SE) was 85.05 ± 0.68 % in the absence of predation risk, and 85.59 ± 0.86 % in the presence of predation risk. After the 9-day exposure period, the mean water content (± 1 SE) was 82.99 ± 0.72 % in the absence of predation risk, and 83.19 ± 0.67 % in the presence of predation risk.

The two-way ANOVA (excluding outliers) and the rank F-tests (including outliers) on the stoichiometric variables gave similar results (Table 2). Both tests indicated that exposure to predation risk had no overall effect on the C content, and that there was no predation risk × exposure period interaction, which was confirmed by the non-significant linear contrasts within each exposure period (Fig. 3a). While both tests suggested an overall effect of predation risk on the N content, the predation risk × exposure period interaction had low power (ANOVA: 0.15, rank F-test: 0.05). The linear contrasts indicated a predator-induced reduction in N content after nine days but not after three days (Fig. 3b). Further, while both tests suggested no effect of predation risk on the C:N ratio, the predation risk × exposure period interaction had low power (ANOVA: 0.22, rank F-test: 0.30). The linear contrasts indicated a predator-induced increase in C:N content after nine days but not after three days (Fig. 3c). All three stoichiometric variables varied with time, with a higher C content, lower N content and higher C:N ratio at day 9 than at day 3.

Table 2. Results of ANOVA and associated rank F-test testing for effects of predation risk and exposure period on body stoichiometry in *E. cyathigerum* larvae

		ANOVA				Rank F-test			
Response variable	Effect	df	MS	F	*P*	df	MS	F	*P*
C content	Predation risk (Pr)	1	1.20	2.30	0.13	1	640.27	2.51	0.12
	Exposure period (Ex)	1	6.30	12.20	0.0010	1	3053.07	11.96	0.0010
	Pr × Ex	1	0.10	0.20	0.68	1	3.27	0.013	0.91
	Error	52	0.50			56	255.33		
N content	Predation risk (Pr)	1	0.28	6.40	0.015	1	763.27	2.80	0.099
	Exposure period (Ex)	1	0.33	7.60	0.0083	1	1706.67	6.27	0.015
	Pr × Ex	1	0.038	0.90	0.36	1	273.07	1.00	0.32
	Error	50	0.044			56	272.36		
C:N ratio	Predation risk (Pr)	1	0.0080	0.0080	0.27	1	96.27	0.72	0.40
	Exposure period (Ex)	1	0.31	0.31	<0.001	1	10140	75.94	<0.001
	Pr × Ex	1	0.010	0.010	0.23	1	281.67	2.11	0.15
	Error	50	0.0070			56	133.52		

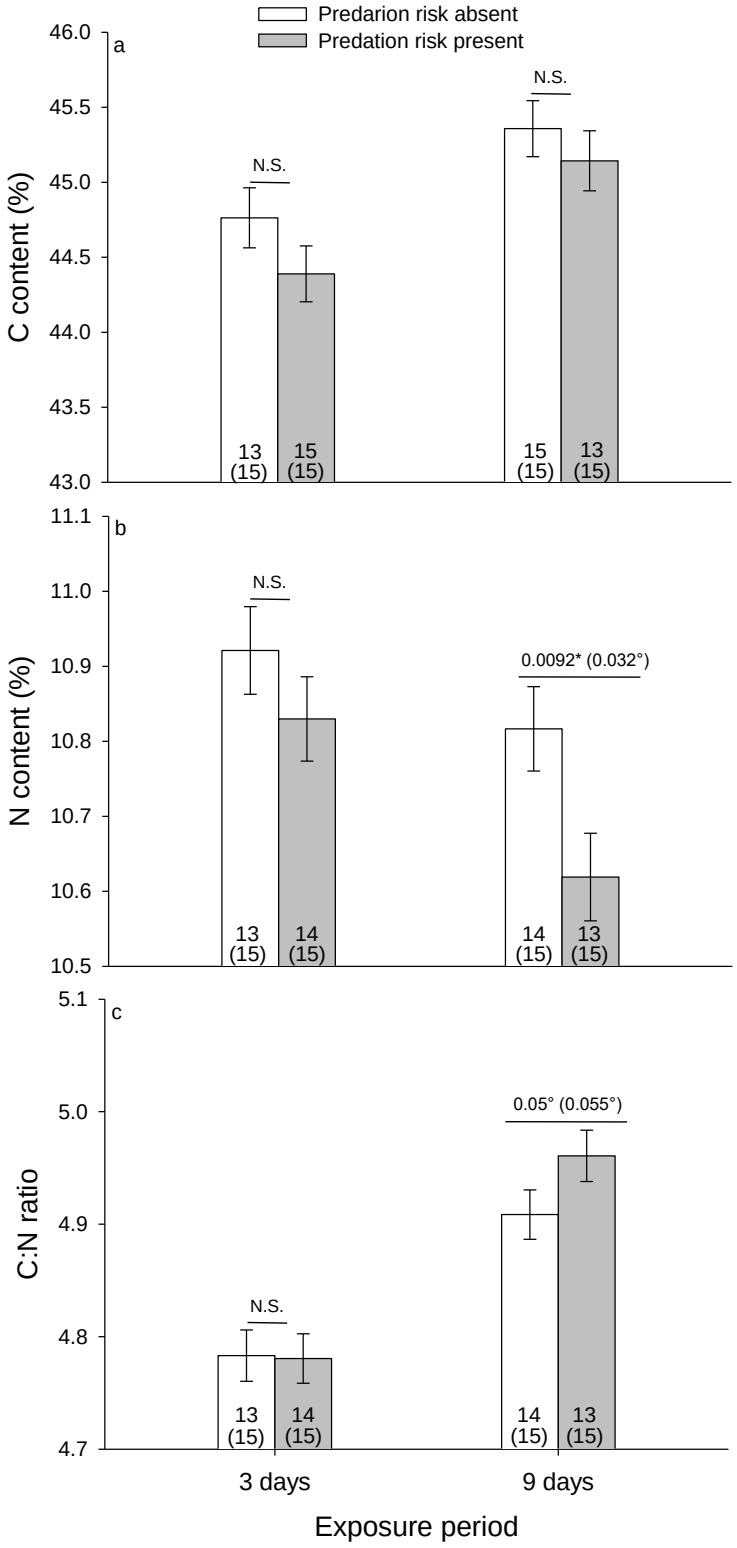

Figure 3. Mean (± 1 SE) (a) C content, (b) N content and (c) the C:N ratio of *E. cyathigerum* larvae as a function of predation risk and exposure period. *P*-values for one-tailed linear contrasts testing for an effect of predation risk within each exposure period are given for the parametric ANOVA (excluding outliers) and within brackets for the rank F-test (including outliers). Uncorrected *P*-values and the significance levels corrected for multiple testing using the False Discovery Rate are presented (°$P < 0.10$ and *$P \leq 0.05$). Numbers in bars represent sample sizes without outliers (within brackets with outliers).

Discussion

Most response variables, except fat, protein and C content, were affected by predation risk during at least one exposure period and the directions of change were as expected *a priori*: growth rate, food intake and N contents decreased while the ETS activity and C:N ratio increased. In contrast to the general stress paradigm, glucose and glycogen levels decreased under predation risk. Notably, we found a strong time-dependence in the predator-induced changes in growth rate, glycogen and N contents, and C:N ratio while the other predator-induced responses were persistent through time (reduced food intake and glucose content; increase in ETS activity).

Short-term effects of predation risk

During the short-term exposure period, *E. cyathigerum* larvae reduced growth rate under predation risk. This is a well-documented life history response in many taxa (Abrams & Rowe, 1996) including *Enallagma* larvae (e.g. McPeek *et al.*, 2001; McPeek, 2004; Stoks *et al.*, 2005a). Predator-induced growth reductions can be mediated both by behaviour (reduced food intake) and by physiology (McPeek *et al.*, 2001; Stoks & McPeek, 2003; Stoks *et al.*, 2005c; Trussell *et al.*, 2006, 2008), and our results suggest both played a role in our study. In line with theoretical expectations (Werner & Anholt, 1993), larvae reduced food intake under short-term predation risk to avoid detection by predators. This response has been observed in many taxa (Lima, 1998), including *Enallagma* larvae (McPeek *et al.*, 2001; Stoks *et al.*, 2003; for *E. cyathigerum*: Stoks *et al.*, 2005b).

Physiological responses also influenced the short-term growth reduction under predation risk. Under predation risk, prey organisms typically show a series of physiological responses including an increase in metabolic rate (e.g. respiration) to increase short-term survival by mobilizing and shunting energy to the brain and other organs (e.g. muscles) essential to surviving the threat (Sapolsky, 2002; Hawlena & Schmitz, 2010a). The flight response of damselfly larvae, escape swimming, is energetically costly (Strobbe *et al.*, 2010). The observed increase in ETS activity is consistent with this short-term physiological stress response. Similar increases in respiration rate under short-term predation risk have been observed in many taxa (e.g.

Steiner & Van Buskirk, 2009; Manzur *et al.,* 2014; Thaler *et al.,* 2014), including the study species (Slos & Stoks, 2008). These physiological responses impose a direct energetic cost by shunting less energy toward growth (Slos & Stoks, 2008; Hawlena & Schmitz, 2010a). In addition, indirect energetic costs may have resulted from upregulation of cellular metabolism. Indeed, an increased metabolic rate is expected to challenge cellular homeostasis and is typically associated with increased levels of stress proteins, including Hsp70 (Sørensen *et al.,* 2003) whose levels covary negatively with individual growth rates in damselfly larvae (Stoks & De Block, 2011). In line with these assumed costs we observed a reduction in glucose content.

Long-term effect of predation risk and compensatory mechanisms

In contrast with the short-term exposure period, there was no reduction in growth rate under predation risk during longer exposures. This matches the pattern that growth reductions are typically found in studies imposing short-term (e.g. McPeek *et al.,* 2001, McPeek, 2004), but not long-term predation risk (e.g. Benard, 2004; Slos *et al.,* 2009). Growth compensation under long-term predation risk is especially striking in this study as both the behavioural (reduced food intake) and the physiological (increased metabolic rate as measured by ETS activity) mechanisms associated with the short-term growth reduction were still operating. The long-term reduction in food intake contrasts with the predation-risk allocation hypothesis (Lima & Bednekoff, 1999), which predicts reduced behavioural responsiveness in prey organisms exposed to chronic predation risk in order to avoid starvation. Similar long-term reductions in food intake have been observed in other invertebrates, including crickets (Adamo & Baker, 2011). The persistently increased ETS activity in larvae exposed to predator cues indicates that these larvae experienced chronic predator stress. Similarly, Hawlena and Schmitz (2010b) reported an increased metabolic rate, measured as respiration, in grasshoppers facing chronic exposure to predatory spiders.

The compensatory mechanisms to reverse the short-term growth reduction when under chronic predator stress in this study are likely to be physiological because food intake was consistently reduced under predation risk. Changes in water content cannot explain this result because water content did not change under predation risk. The

reduced glycogen content under the 9-day exposure period may reflect a re-allocation of energy toward growth. Because the growth reduction under predation risk had disappeared by the 6-day exposure period when no reduction in energy storage was detected, it seems likely that several other non-exclusive mechanisms were involved. Larvae may have increased their assimilation efficiency under predation risk, as shown in caterpillars (Thaler *et al.*, 2012) and in larvae of other coenagrionid damselfly species (McPeek, 2004). This was unlikely in terms of nitrogen, but more likely in terms of carbon to compensate for elevated metabolism and reduced food intake. Larvae may also have increased the conversion of assimilated food into biomass, for example, by reducing investment in immune function under predation risk, as observed in damselfly larvae (Stoks *et al.*, 2006b). Alternatively, larvae may have invested more in the exoskeleton as a morphological defence, as in some mayfly larvae (Dahl & Peckarsky, 2002).

Of the other chronic stress studies in invertebrates, only the study of Thaler *et al.* (2012) on *Manduca sexta* caterpillars also explicitly tested for compensatory mechanisms to avoid growth reduction. To our knowledge, this is also the only other study of an invertebrate comparing short- and long-term effects of exposure to predation risk (for a rare example on vertebrates, see Steiner & Van Buskirk, 2009). In contrast to our study, Thaler *et al.* (2012) did not detect a predator-induced growth reduction in the short- or long-term, suggesting that other compensatory mechanisms were at work. During short exposure, caterpillars relied on an increased assimilation efficiency (partly by moulting earlier in another instar) to compensate for a reduced food intake. During long exposure, the caterpillars also relied on behavioural compensation (transient increase in food intake) yet also suffered reduced assimilation efficiency. Unfortunately, energy storage of the caterpillars was not measured in the long-term and reduced investment in energy storage (as shown in this study) may have been a compensatory mechanism. Despite some obvious differences, both our study and the one by Thaler *et al.* (2012) highlight the use of temporally dynamic strategies to avoid a growth reduction in invertebrate prey under chronic predation risk.

Effects on body stoichiometry

Although we did not detect predator-induced changes in N content or C:N ratio after the short-term exposure, N content decreased and the C:N ratio increased under chronic predation risk. These stoichiometric changes are expected by the GSP because of increased gluconeogenesis (the breakdown of N-rich proteins to generate more C-rich glucose) and excretion of excess N (Hawlena & Schmitz, 2010a), and this has been experimentally demonstrated in grasshoppers exposed to predators for five weeks (Hawlena & Schmitz, 2010b). In contrast to the GSP, we did not detect an increase in C content or C-rich molecules (fat and sugars) under predation risk, but did find a decrease in C-rich glycogen at day 9. No decrease in N-rich protein content was observed.

Our results indicate that mechanisms other than the changes in fat, sugars and protein contents predicted by the GSP (Hawlena & Schmitz, 2010a) were driving the predator-induced decrease in N content and increase in C:N in our study species. Costello and Michel (2013) argued that predator-induced morphological defences can enhance or mask the alterations in C:N predicted by the GSP. A predator-induced thicker exoskeleton, as documented in mayflies (Dahl & Peckarsky, 2002), may have played a role in our study. The major molecule in the insect exoskeleton, chitin, has a high C:N (5.1) ratio (Sterner & Elser, 2002) and therefore has the potential to increase C:N ratio of the whole body. We have no data on chitin contents and this hypothesis requires testing. However, it cannot be the only mechanism because C content did not increase in our study. Indeed, the predator-induced increase in C:N was entirely driven by an decrease in N content. The N content may have been manipulated more directly by a lowered extraction from the food and/or an elevated excretion of N (Sterner & Elser, 2002). The latter mechanism is included in the GSP as a result of the gluconeogenesis of proteins (Hawlena & Schmitz, 2010a), yet gluconeogenesis did not seem to occur in our study as total protein content remained unchanged under predation risk.

Understanding the temporal dynamics of nonconsumptive effects imposed by predators, including the presence and the nature of compensatory mechanisms to deal with chronic predation risk, is important as these may affect prey populations more than direct consumption (Preisser *et al.*, 2005). Like short-term stress responses, chronic stress responses may be adaptive and continue to promote fitness (Boonstra, 2013). This

could occur if prey or their offspring benefit from the long-term stress response in terms of survival, for example, by persistent increases in escape performance (Adamo & Baker, 2011; Hawlena *et al.*, 2011). Our results suggest that invertebrate prey organisms also may have evolved mechanisms to cope with chronic predator stress and to avoid a long-term growth reduction.

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Author contributions: MVD, LJ and RS conceived and designed the experiment. MVD and LJ performed the experiment. MVD, LJ and RS analysed the data. LJ, MVD and RS wrote the manuscript.

PART 2

Multiple stressors

Chapter V

Warming reinforces nonconsumptive predator effects on prey growth, physiology and body stoichiometry

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*Joint first authors

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Slightly modified version

V

Abstract

While nonconsumptive effects of predators may strongly affect prey populations, little is known how future warming will modulate these effects. Such information would be especially relevant with regard to prey physiology and resulting changes in prey stoichiometry. We investigated in *Enallagma cyathigerum* damselfly larvae the effects of a 4°C warming (20°C vs. 24°C) and predation risk on growth rate, physiology and body stoichiometry, for the first time including all key mechanisms suggested by the general stress paradigm (GSP) on how stressors shape changes in body stoichiometry. Growth rate and energy storage were higher at 24°C. Based on thermodynamic principles and the growth rate hypothesis, we could demonstrate predictable reductions in body C:P under warming and link these to the increase in P-rich RNA; the associated warming-induced decrease in C:N may be explained by the increased synthesis of N-rich proteins. Yet, under predation risk, growth rate instead decreased with warming and the warming-induced decreases in C:N and C:P disappeared. As predicted by the GSP, larvae increased body C:N and C:P at 24°C under predation risk. Notably, we did not detect the assumed GSP-mechanisms driving these changes: despite an increased metabolic rate there was neither an increase of C-rich biomolecules (instead fat and sugar contents decreased under predation risk), nor a decrease of N-rich proteins. We hypothesize that the higher C:N and N:P under predation risk are caused by a higher investment in morphological defence. This may also explain the stronger predator-induced increase in C:N under warming. The expected higher C:P under predation risk was only present under warming and matched the observed growth reduction and associated reduction in P-rich RNA. Our integrated mechanistic approach unravelled novel pathways of how warming and predation risk shape body stoichiometry. Key findings that (1) warming effects on elemental stoichiometry were predictable and only present in the absence of predation risk and that (2) warming reinforced the predator-induced effects on C:N:P, are pivotal in understanding how nonconsumptive predator effects under global warming will shape prey populations.

Introduction

Understanding the interplay between temperature and biotic interactions is essential for fully anticipating how populations, communities, and ecosystem functions will respond to global warming (Traill *et al.,* 2010; Angert *et al.,* 2013; Blois *et al.,* 2013). While mild warming may positively affect fitness in many ectotherms (Deutsch *et al.,* 2008), these positive effects of warming on prey populations may no longer hold in the presence of predators (De Block *et al.,* 2013; Barton & Ives, 2014). The ability of populations to survive locally under global warming will therefore not only depend on their ability to deal with the temperature increase itself, for example through physiological adjustments (Chown & Gaston, 2008), but also on their ability to deal with temperature-induced changes in the interactions with predators (Traill *et al.,* 2010). While there is a growing appreciation for the importance of nonconsumptive effects imposed by predators, which can often outweigh the importance of direct feeding (Preisser *et al.,* 2005), the way nonconsumptive predator effects change under warming is largely unknown (but see, e.g. Miller *et al.,* 2014). This is especially relevant for physiological stress effects imposed by predators as these can shape prey stoichiometry and thereby scale up to ecosystem functions linked to elemental cycling (Hawlena & Schmitz, 2010a; Hawlena *et al.,* 2012). Studying how warming and predator-induced stress jointly shape prey stoichiometry may therefore provide crucially important mechanistic insights for forecasting future consequences of global warming (Schmitz, 2013).

According to metabolic ecology (Sibly *et al.,* 2012), mild warming likely will affect prey stoichiometry through its positive effects on growth rate. Based on thermodynamic principles, reaction rates inevitably increase with absolute temperatures because the kinetic energy of a system increases with absolute temperature. Therefore, animals reared at higher nonlethal temperatures typically have higher growth rates (Nilsson-Örtman *et al.,* 2012). Further, the growth rate hypothesis (Elser *et al.,* 1996) asserts that faster growth rates will require the allocation of resources to P-rich ribosomal RNA to increase the synthesis of N-rich proteins, resulting in decreased body ratios of C:P and N:P (Sterner & Elser, 2002). Taken together, we therefore expect reductions in C:P and N:P under mild warming.

Central to our understanding of how predator stress shapes prey stoichiometry is the general stress paradigm (GSP, Hawlena & Schmitz, 2010a). According to the GSP, prey under predation risk will increase their metabolic rate, thereby mobilize energy for predator escape and divert energy toward costly defence mechanisms away from production of new tissues. This is associated with an increased gluconeogenesis (creation of C-rich biomolecules from N-rich proteins) and the excretion of excess nutrients, mostly N and P, resulting in increased body ratios of C:N and C:P (Hawlena & Schmitz, 2010a). While a predator-induced increase in C:N has been demonstrated in *Melanoplus femurrubrum* grasshoppers (Hawlena & Schmitz, 2010b), the very few follow-up studies in other taxa showed deviating patterns and highlighted additional behavioral and morphological responses to predation risk that may overwhelm or counteract the stoichiometric changes predicted by the GSP (Costello & Michel, 2013; Dalton & Flecker, 2014).

To gain insight in how mild warming and predation risk affected body nutrient condition we investigated the combined impact of a mild (4°C) temperature increase and predation risk on prey physiology with special attention for changes in body stoichiometry. We studied this in *Enallagma* damselfly larvae, among the most extensively studied invertebrates with regard to physiological responses to predators (e.g. McPeek *et al.*, 2001; Stoks *et al.*, 2005a, 2006a; Slos & Stoks, 2008; Culler *et al.*, 2014, Janssens & Stoks, 2014). We predicted decreased C:P and N:P under a mild temperature increase (growth rate hypothesis; Elser *et al.*, 1996; Sterner & Elser, 2002; Sibly *et al.*, 2012), and as temperature-induced growth increases have been shown to be less pronounced under predation risk in the damselfly *Enallagma vesperum* (Culler *et al.*, 2014), we expected less strong effects of warming on body stoichiometry under predation risk. We predicted increased C:N and C:P under predation risk (GSP; Hawlena & Schmitz, 2010a), and as the effects of predation risk predicted by the GSP are driven by increases in metabolic rate, we expected stronger effects of predation risk on prey growth, physiology, and stoichiometry under mild warming (see also Laurila *et al.*, 2008; Kuehne *et al.*, 2012; Culler *et al.*, 2014; but see Touchon & Warkentin, 2011). To advance our mechanistic understanding, we quantified all variables associated with the assumed key mechanisms put forward by these theories: individual growth rates,

RNA:DNA ratios (as a proxy for protein synthesis; Karasov & Martinez del Rio, 2007), the activity of the electron transport system (ETS, as a proxy for metabolic rate; De Coen & Janssen, 2003), and energy storage molecules (fat, sugars, and proteins). This multifaceted approach provided unique information how mild warming and predation risk shape body stoichiometry.

Materials and methods

Collection and housing

Mated females (n = 20) of the damselfly *Enallagma cyathigerum* were collected in Het Stappersven (Kalmthout, Belgium), a fishless lake with *Anax* dragonfly larvae as top predators, and transferred to the laboratory for egg laying. After hatching, larvae were placed individually in 200 mL cups in temperature-controlled water baths set at 20°C and 24°C (photoperiod 14:10 light : dark). Importantly, 20°C is the mean summer water temperature in shallow lakes occupied by the study species in Flanders, yet water temperatures of 24°C are frequently observed during summer. The 4°C temperature difference corresponds with the predicted surface temperature increase by 2100 under IPCC scenario RCP8.5 (IPCC, 2013). Damselfly larvae were fed ad libitum with *Artemia* nauplii five days a week (mean daily dose ± SE: 673 ± 53 nauplii, n = 10 daily doses).

Experimental setup

To test for the single and combined effects of rearing temperature and predation risk on growth rate and associated physiological variables, we set up a full factorial experiment with two temperature treatments (20°C and 24°C) crossed with two predation risk treatments (absence and presence). While the temperature treatment started when the eggs hatched, the predation risk treatments were imposed during a 7-day exposure period starting when larvae molted into their final instar. Larvae entered the exposure period after 144 ± 1 d (mean ± SE) at the 20°C rearing temperature and after 135 ± 1 d at the 24°C rearing temperature. During the exposure period, larvae were placed individually in glass vials (100 mL) at their respective rearing temperature. Sets of four vials were placed together in a larger outer container (750 mL). To avoid any bias due

to larvae being associated with a specific container (set of conspecific larvae and predator), we randomly redistributed vials among containers of the same temperature-by-predation risk combination on a daily basis (see McPeek, 2004). Throughout the exposure period, larvae were daily fed ad libitum with *Artemia* nauplii (1224 ± 108 nauplii/d, n = 10 daily doses). The number of larvae tested at each treatment combination was 45 (total of 180 larvae).

Predation risk was manipulated using visual and chemical predator cues, reflecting the cocktail of predator cues that damselfly larvae encounter in nature. To ensure visual predator cues, a large *Anax* dragonfly larva, important predators of damselfly larvae (Stoks *et al.*, 2005b), was placed in the outer container of the treatment with predator cues. Additionally, larvae could see the conspecific larvae in the other vials in the container (damselfly larvae are cannibalistic; De Block & Stoks, 2004). When no complete sets of four larvae could be made for the treatments with predation risk at a given temperature, we added vials with ‘‘dummy larvae,’’ final instar conspecific larvae not included in the experiment. To avoid visual predator cues in the treatment without predation risk, the walls of these vials were made non-transparent using tape (this did not affect light levels in the vials). For the chemical predator cues, we homogenized one *E. cyathigerum* larva in 20 mL of water from an aquarium filled with 300 mL aged tap water in which a large *Anax* dragonfly larva had eaten one *E. cyathigerum* larva. We daily added 1 mL of this predator medium to each vial of the treatments with predator cues, to the other vials we daily added 1 mL of aged tap water.

Response variables

To quantify growth rate, we weighed each larva to the nearest 0.01 mg at the start and at the end of the 7-day exposure period. Growth rate was calculated as [ln(final mass)_ ln(initial mass)]/7 days (McPeek *et al.*, 2001) for all 180 larvae. After determining final mass, the larvae were directly frozen on dry ice and stored at -80°C for physiological analyses. Given that not all physiological variables could be measured on the same larva, we worked with three randomly chosen sets of larvae.

A first set of larvae (20 per treatment combination, total of 80 larvae) was used to quantify RNA:DNA ratios based on the protocol by Vrede *et al.* (2002). Larvae were

homogenized and diluted 15 times in extraction buffer (50 mmol/L EDTA, 0.05% SDS in 50 mmol/L Tris). In a first step, we measured the total amount of RNA and DNA by filling a 96-well black microtiter plate with 100 µL sample and 2 µL ethidium bromide (100 µg/mL). After an incubation on ice for 15 minutes, fluorescence was measured using a spectrophotometer (Infinite M2000; TECAN0029, Grodig, Austria) at an excitation/emission wavelength of 535:595 nm. In a second step, we measured the total amount of DNA by filling a 96-well black microtiter plate with 100 µL of the sample and 1 µL of RNase (20 mg/mL; which break downs the RNA). After an incubation of 60 minutes at room temperature, 2 µL ethidium bromide (100 µg/mL) was added. After an incubation on ice for 15 minutes, fluorescence was measured. Subtracting the amount of DNA from the total amount of RNA and DNA, resulted in the RNA concentration in the samples. RNA and DNA concentrations were measured in triplicate and the means per larva were used for the statistical analyses.

A second set of larvae (10 per treatment combination, total of 40 larvae) was used to quantify electron transport system (ETS) activity based on the protocol of De Coen and Janssen (2003). Larvae were homogenized and diluted 15 times in a homogenization buffer (100 mmol/L Tris-HCl, pH 8.5, 15% polyvinyl pyrrolidone, 153 µmol/L MgSO4 and 0.2% Triton X-100). A 96-well microtiter plate was filled with 150 µL buffered substrate solution (0.13 mol/L Tris HCl, 0.3% Triton X- 100, 1.7 mmol/L NADH, 250 µmol/L NADPH, pH 8.5) and 50 µL of the supernatant. We started the reaction by adding 100 µL INT (8 mmol/L p-iodonitrotetrazolium) and followed the increase in absorbance at 490 nm (Infinite M2000; TECAN) and 20°C during 5 minutes with readings every 30 seconds. Using the Lambert-Beer formula, we calculated the concentration of formazan (extinction coefficient 15 900 mol L^{-1} cm^{-1}) and afterward converted this to cellular oxygen consumption based on the theoretical stoichiometric relationship that for each 2 µmol of formazan formed, 1 µmol of O2 was consumed in the ETS system. Measurements were done in duplicate. The means were used for statistical analyses and expressed as nmol O2/min.

A third set of larvae (15 per treatment combination, total of 60 larvae) was used to quantify energy reserves and C:N:P ratios. Larvae were homogenized and diluted five times in milli-Q water. Afterward, 35 µL of the supernatant was further diluted three

times with milli-Q water and used to measure energy reserves. The rest of the homogenate was used to quantify C:N:P ratios. Fat content was measured following Janssens and Stoks (2014). We mixed 8 µL of the body supernatant and 56 µL sulfuric acid (100%) in a glass tube. The tubes were heated for 20 minutes at 150°C. Afterward, we added 64 µL of milli-Q water. We filled a 384-wellmicrotiter plate with 30 µL of the sample and measured absorbance at 340 nm (Infinite M2000, TECAN). Fat concentrations were calculated using a standard curve of glyceryl tripalmitate. Measurements were done in triplicate and the means per larva were used for statistical analyses. For total sugar content (glucose + glycogen), we used the protocol described in Stoks *et al.* (2006a) based on the glucose kit from Sigma Aldrich (St. Louis, Missouri, USA). In a first step, all glycogen was transformed to glucose. Therefore, we mixed 50 µL milli-Q water, 20 µL body supernatant, and 10 µL amyloglucosidase (1 unit/10 µL; Sigma A7420) in a 96-well microtiter plate. After 30 minutes of incubation at 37°C, all glycogen is transformed to glucose. We measured the glucose levels by adding 160 µl of glucose assay reagent (Sigma G3293) to each well. After another incubation period of 20 minutes at 30°C we measured absorbance at 340 nm (Infinite M2000, TECAN). We calculated sugar concentration based on a standard curve of known concentrations of glucose and their absorbance. Measurements were done in duplicate and the means per larva were used for statistical analyses. The results for glucose and glycogen concentrations were very similar, therefore we only report the total sugar content in the Results and Discussion sections. Protein content in the body homogenates was measured using the Bradford method (Bradford, 1976). Measurements were done in triplicate and the means per larva were used for statistical analyses. Fat content, total sugar content and protein content were expressed as µg per mg dry mass.

For the quantification of C:N:P ratios, we divided the homogenate in two parts: one-quarter of the sample was used for C and N analyses and three-quarters of the sample for P analyses. For the quantification of the C and N content, samples were placed in tin cups and dried for 24 h (60°C), where after C and N were quantified using an elemental analyzer (Carlo Erba 1108; Thermo Fisher Benelux, Eke, Belgium). For P analysis, we mixed the sample with 1000 µL HNO_3 (70%) in a glass tube (based on Van Moorleghem *et al.,* 2013). The tubes were heated for 15 minutes at 150°C. Afterward,

the digests were diluted to 10 mL with milli-Q water and analyzed using inductively coupled plasma mass spectrometry (Agilent 7700x ICP-MS; Biocompare, South San Francisco, California, USA). The measurements (n = 8) of the Spectrapure Standard SPS-SW2 Batch 128 (Spectrapure Standards, Oslo, Norway) showed 1.41% deviation. C:N, C:P, and N:P were expressed as molar ratios. All measured physiological variables were within the range observed for aquatic invertebrates, including damselfly larvae (Appendix A: Table A1).

Statistical analyses

We used two-way ANOVAs to test for the effects of temperature and predation risk on the different response variables. We compared means using linear contrasts, which were corrected for multiple testing using the false discovery rate procedure as outlined in Benjamini and Hochberg (1995). Given the interest for temperature as well as for predation risk, their two-way interactions will be described from the perspective of each factor separately. All tests were done in STATISTICA v12 (StatSoft, Tulsa, Oklahoma, USA). Values of $P < 0.05$ were considered significant. For all variables, the assumptions of ANOVA (normal distribution and homogeneity of variances) were met without the need for transformations.

Results

Life history, RNA:DNA, and ETS

Rearing temperature and predation risk interacted for growth rate, the RNA:DNA ratio and ETS activity, but their interaction patterns differed (Table 1, Fig. 1). All three variables were affected by temperature, yet these effects strongly depended upon predation risk. While growth rate increased at 24°C in the absence of predation risk, it decreased at 24°C when predation risk was present. The RNA:DNA ratio increased at 24°C but only in larvae not exposed to predation risk. ETS activity increased at 24°C, especially in larvae exposed to predation risk.

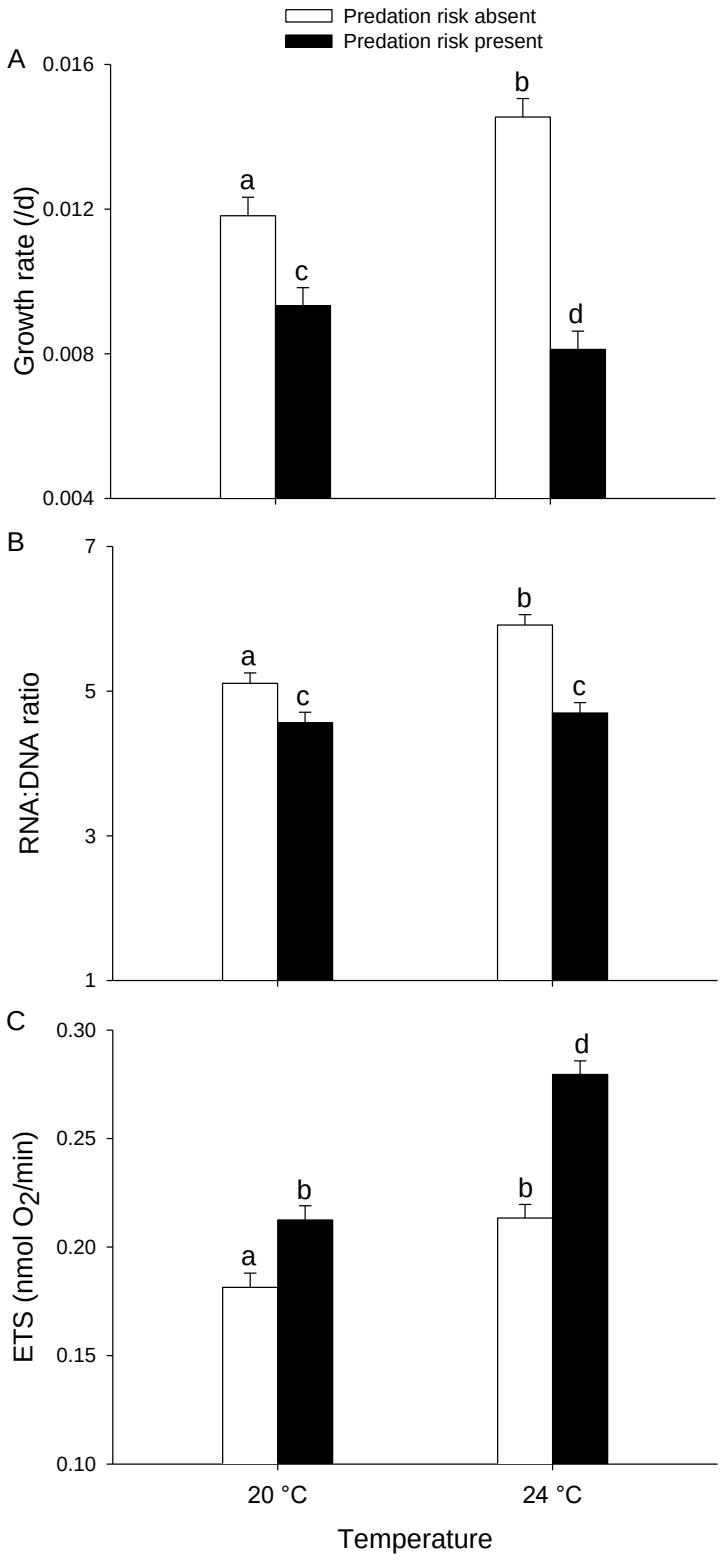

Figure 1. (A) Growth rate, (B) RNA:DNA ratios, and (C) oxygen consumption (electron transport system [ETS activity] activity) of *Enallagma cyathigerum* larvae as a function of rearing temperature and predation risk exposure. Bars show least-squares means + SE. Means having different letters were significantly different ($P < 0.05$; based on linear contrasts corrected for multiple testing using the false discovery rate procedure).

Table 1. Results of ANOVAs testing for the effects of rearing temperature and predation risk on life history and physiology in the damselfly *Enallagma cyathigerum.*

	Temperature (T)			Predation risk (P)			T x P		
	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Growth rate	1, 176	2.24	0.14	1, 176	76.99	< 0.001	1, 176	15.02	< 0.001
RNA:DNA	1, 76	10.65	0.0017	1, 76	37.31	< 0.001	1, 76	5.45	0.022
ETS	1, 36	59.76	< 0.001	1, 36	57.6	< 0.001	1, 36	7.50	0.0097
Energy storage									
Fat	1, 56	16.87	< 0.001	1, 56	32.08	< 0.001	1, 56	6.62	0.013
Sugars	1, 56	7.92	0.0068	1, 56	120.87	< 0.001	1, 56	12.98	< 0.001
Protein	1, 56	8.40	0.0054	1, 56	0.12	0.73	1, 56	0.39	0.54
Body stoichiometry									
C:N	1, 56	15.24	< 0.001	1, 56	56.34	< 0.001	1, 56	5.91	0.018
C:P	1, 56	3.11	0.062	1, 56	27.66	< 0.001	1, 56	4.59	0.034
N:P	1, 56	0.79	0.52	1, 56	5.15	0.029	1, 56	0.11	0.74

All three variables were affected by predation risk and these effects were stronger at 24°C. Growth rate was lower under predation risk and this predator-induced growth reduction was approximately twice as strong at 24°C (44%) as at 20°C (21%). Exposure to predator cues resulted in a reduction of the RNA:DNA ratio and this predator-induced reduction was twice as strong at 24°C (21%) as at 20°C (11%). Exposure to predator cues resulted in an increased ETS activity, and this increase was approximately twice as strong at 24°C (31%) as at 20°C (17%).

Energy storage

All three storage molecules had higher levels at 24°C than at 20°C in the absence of predation risk (Table 1, Fig. 2). Yet, in the presence of predator cues this was only true for protein content. Instead, fat content and total sugar content did not increase with temperature under predation risk (temperature × predation risk, Table 1). Exposure to predator cues resulted in lower fat and sugar contents and these reductions were stronger at 24°C (reductions in fat content: 34% at 24°C vs. 17% at 20°C; reductions in sugar content: 39% at 24°C vs. 24% at 20°C, Fig. 2A and B). Neither predation risk, nor the interaction between rearing temperature and predation risk affected the protein content (Table 1).

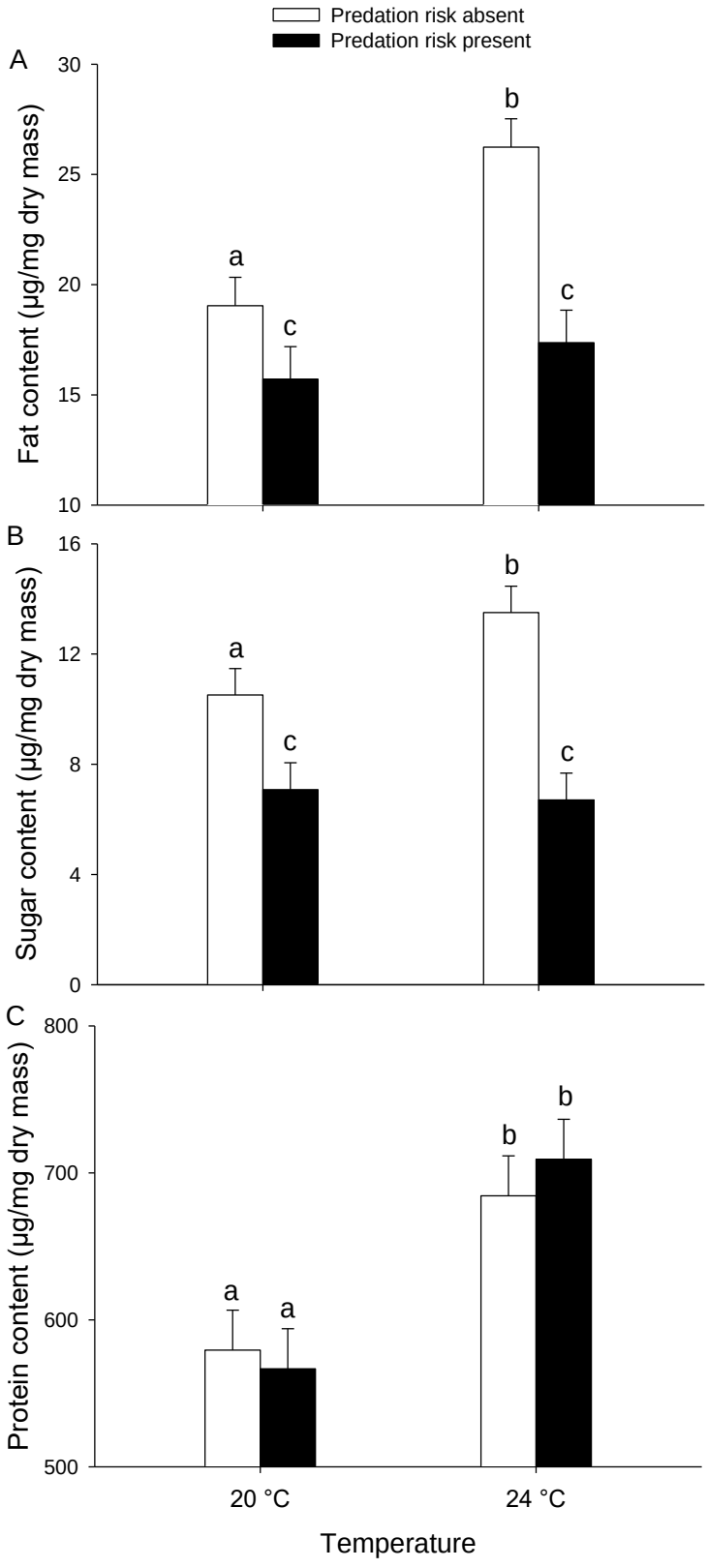

Figure 2. Levels of energy storage molecules: (A) fat content, (B) sugar content, and (C) protein content of *E. cyathigerum* larvae as a function of rearing temperature and predation risk exposure. Bars show least-squares means + SE. Means having different letters were significantly different ($P < 0.05$; based on linear contrasts corrected for multiple testing using the false discovery rate procedure).

Body stoichiometry

Both the C:N and the C:P ratios were lower at 24°C than at 20°C, yet only in the absence of predation risk (temperature × predation risk, Table 1, Fig. 3A and B). In the presence of predation risk, the temperature had no effect on these ratios. Animals exposed to predator cues had an approximately two times stronger increase of C:N ratios at 24°C (14%) than at 20°C (6%), while the predator-induced increase in C:P ratio (~16%) was only present at 24°C (Fig. 3A and B).

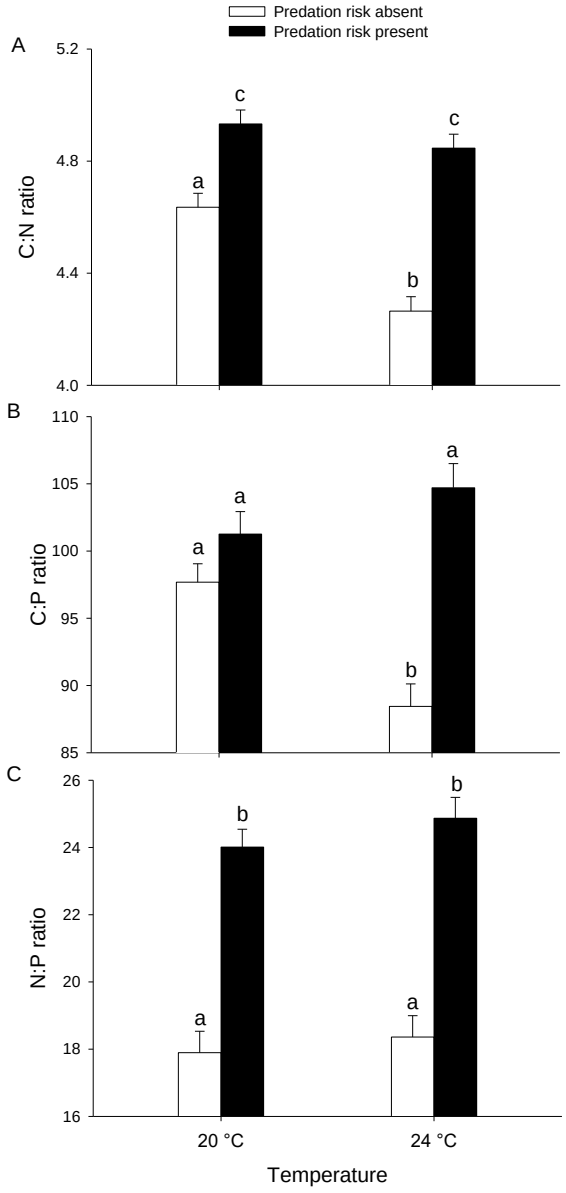

Figure 3. (A) C:N ratios, (B) C:P ratios, and (C) N:P ratios of *E. cyathigerum* larvae as a function of rearing temperature and predation risk exposure. Bars show least-squares means + SE. Means having different letters were significantly different ($P < 0.05$; based on linear contrasts corrected for multiple testing using the false discovery rate procedure).

The N:P ratio was higher in larvae exposed to predation risk (Table 1, Fig. 3C). Neither the rearing temperature, nor the interaction between rearing temperature and predation risk affected the N:P ratio.

Discussion

Both temperature and predation risk strongly affected growth rate, RNA:DNA ratio, ETS activity, energy storage and the C:N:P signature of the damselfly larvae. Mild warming in the absence of predation risk induced the predicted reduction in C:P (Elser *et al.*, 1996; Sterner & Elser, 2002; Sibly *et al.*, 2012). Opposite to our predictions, warming did not decrease N:P, instead, warming reduced C:N. The effects of warming on most end points were, as predicted, less pronounced in the presence of predation risk. Predation risk induced the expected increases in C:N and C:P at 24°C (Hawlena & Schmitz, 2010a). In addition, also N:P increased under predation risk. Finally, the impact of predation risk was, as expected, stronger under mild warming (Laurila *et al.*, 2008; Kuehne *et al.*, 2012; Culler *et al.*, 2014). We will integrate the patterns of all variables measured with special focus on the explanation of response patterns in body stoichiometry for these four scenarios.

Temperature effects in the absence of predation risk

In accordance with thermodynamic principles, larval growth rate and energy storage were higher at 24°C than at 20°C. This fits the observation in other damselflies (e.g. Stoks *et al.*, 2012; Culler *et al.*, 2014) and other insects (Karl & Fischer, 2008) that food intake and growth efficiency increase in this temperature range. This can, in turn, explain the increases in growth rate and in energy storage. Following the metabolic theory of ecology (Gillooly *et al.*, 2001), metabolic rate (as measured by ETS activity) was also higher in larvae reared at the higher temperature.

Integrated changes in growth rate, RNA:DNA and protein content can explain the observed stoichiometric changes under mild warming in the absence of predation risk. In line with the growth rate hypothesis (Elser *et al.*, 1996), the increased growth rate at 24°C was associated with a higher RNA:DNA ratio and lower C:P. A lower C:P is expected because of an increased allocation of resources to P-rich ribosomal RNA to

increase the synthesis of proteins (Karasov & Martinez del Rio, 2008), as here indicated by the increased RNA:DNA ratio. This is in line with other studies showing higher RNA:DNA ratios in faster growing animals (e.g. Weider *et al.,* 2004; Elser *et al.,* 2006). While often not explicitly addressed by the growth rate hypothesis (but see, e.g. Watts *et al.,* 2006; Chen *et al.,* 2010; Reef *et al.,* 2010), we also observed a lower C:N in the faster growing animals at 24°C. This can be explained because rapid growth requires increased allocation to ribosomes (hence also ribosomal proteins), RNA and protein synthetic products (Watts *et al.,* 2006). This might explain the pattern we observed in damselflies, as we did indeed find higher protein levels in the faster growing larvae at 24°C. Higher growth rates have been shown to be inversely correlated with C:N in several other taxa (e.g. clovers [Chen *et al.,* 2010], fruit flies [Watts *et al.,* 2006], trees [Reef *et al.,* 2010]). Note that the observed increases in C-rich fat and sugar storage molecules under warming were overruled by the increases in N and P. Furthermore, the increases in N and P balanced out, resulting in no change in the N:P ratio under mild warming.

The very few studies that experimentally tested for an effect of temperature on body stoichiometry did not quantify the underlying physiological mechanisms. Liess *et al.* (2013) documented similar decreases in C:N and C:P when *Rana temporaria* tadpoles were reared from the egg stage at 23°C compared to 18°C. In contrast with our study, tadpole growth rate was, however, lower at the higher temperature. The higher N content at the higher temperature was therefore assumed to be the result of an increased protein synthesis in relation to thermal tolerance; the higher P content at the higher temperature was hypothesized to be the result of morphological changes (Liess *et al.,* 2013). Schmitz (2013) instead documented a higher C:N in *Melanoplus femurrubrum* grasshoppers kept in outdoor mesocosms warmed 2.5–3.0°C above ambient. This was assumed to be driven by a shift of nutrient demands from N-rich to C-rich food and an excretion of excess nitrogen in response to increased metabolism under thermal stress. Compared to our study, the opposite change in C:N at higher temperature in the study of Schmitz (2013) may be because the high temperature was considered as stressful and the grasshoppers could change food preference.

Temperature effects in the presence of predation risk

When mild warming was combined with predation risk, the damselfly larvae showed the highest metabolic rate (as measured by ETS activity), which likely increased energetic costs (Lemoine & Burkepile, 2012) to such extent that the positive effect of temperature on growth rate changed into a negative effect and that the temperature-induced increases in RNA:DNA ratio and in fat and total sugar contents disappeared. As an important consequence, while the growth rate had the highest values at the high temperature in the control larvae, it reached the lowest values when the high temperature was combined with predation risk. In line with this, the temperature-induced growth increase in the damselfly *Enallagma vesperum* was less pronounced under predation risk, which was also attributed to a higher metabolic rate (Culler *et al.*, 2014). The disappearance of the positive temperature effects on growth rate and RNA:DNA ratios combined with an increased morphological defence (see Effects of predation risk in the absence of warming) can explain why the decrease in C:P under mild warming in the absence of predation risk disappeared when mild warming was combined with predation risk.

Effects of predation risk in the absence of warming

As predicted by theory (Abrams & Rowe, 1996) and widely documented (Benard, 2004), larvae decreased growth rate under predation risk. This can be explained by a combination of a lower food intake and a lower efficiency to convert food into body mass (e.g. McPeek *et al.*, 2001; Stoks *et al.*, 2005c; Trussell *et al.*, 2006; Miller *et al.*, 2014). According to the general stress paradigm (GSP; Hawlena & Schmitz, 2010a), when exposed to predation risk, prey increase metabolic rate, and thereby mobilize energy for predator escape and divert more energy toward the upregulation of costly defence mechanisms and less toward production of new tissues (hence growth). This may explain the observed increase in ETS activity (as a measure of metabolic activity), and reductions in energy storage (fat and total sugars; see also Stoks *et al.*, 2005a, 2006; Thaler *et al.*, 2012) and in RNA:DNA ratio under predation risk.

The observed predator-induced increase in body C:N is as expected by the GSP (Hawlena & Schmitz, 2010a; Costello & Michel, 2013) and documented before in *M.*

femurrubrum grasshoppers (Hawlena & Schmitz, 2010b; Hawlena *et al.*, 2012). To meet the energetic demands of the predator-induced stress response, prey are expected to increase their production of C-rich glucose through the breakdown of the N-rich proteins (gluconeogenesis) and release the excess nutrients, mostly N and P (Hawlena & Schmitz, 2010a). Yet, despite indications of a general stress response in our study species when exposed to predator cues (Slos & Stoks, 2008), we did not detect an increased investment in C-rich biomolecules (instead the fat and sugar contents were lower in larvae exposed to predation risk), neither a decreased protein content. Note that the only studies that explicitly tested for changes in N excretion under predation risk reported, in contrast with the GSP prediction, reduced N excretion in guppies (Dalton & Flecker, 2014) and in caterpillars (Thaler *et al.*, 2012), indicating that also other mechanisms are shaping predator-induced patterns in these biomolecules. One recently invoked mechanism by Dalton and Flecker (2014) that may have contributed to the here observed patterns in these biomolecules, is the physiology of food restriction associated with the lowered food intake under predation risk. Animals faced with food restriction prefer to mobilize glycogen and lipid stores for energy production and spare the resource stores (i.e., proteins) most needed for future physiological activities (Wang *et al.*, 2006). In support of this mechanism, larvae of the study species decrease food intake under predation risk (Stoks *et al.*, 2005b; Janssens & Stoks, 2014), and food-restricted damselfly larvae show reduced levels of fat and sugars (Stoks *et al.*, 2006a). This food restriction mechanism may overrule the GSP response and has been assumed to contribute to the predator-induced reduction in C:N in guppies (Dalton & Flecker, 2014).

Although the pattern of predator-induced increase in body C:N in our study species is as predicted by the GSP, this pattern cannot be fully explained by the GSP (more particularly by the changes in biomolecules). We hypothesize that the higher C:N under predation risk is caused by a higher investment in the exoskeleton, which mainly consists of chitin, a polysaccharide with a high C:N (5.1) compared to other biomolecules (Sterner & Elser, 2002). Such a morphological response is assumed to be a widespread structural defence strategy to reduce predator attack efficiency (Rabus *et al.*, 2013) and has been observed in aquatic insects (e.g. mayfly larvae; Dahl &

Peckarsky, 2002). Future work on predator-induced effects on body stoichiometry would therefore benefit from explicitly measuring cuticle thickness and chitin content. Notably, Costello and Michel (2013) attributed the absence of a change in C:N under predation risk in *Hyla versicolor* tadpoles to strong morphological defences (i.e., increased size of the protein-rich tail muscles). Taken together, this suggests a major role of morphological defence mechanisms in shaping C:N under predation risk and thereby overwhelming or counteracting some key GSP-mechanisms. Together with Costello and Michel (2013), we show that also body N:P increased under predation risk. They hypothesized this was the result of an increase in protein content linked to more N-rich muscle tissue. In our study, the protein content, however, was not influenced by predation risk. Moreover, also the predator-induced decrease in RNA:DNA would, if anything, work to reduce the N:P content. The above invoked higher investment in the exoskeleton (and higher chitin content) in the presence of predation risk may also explain the higher N:P. Indeed, chitin contains nitrogen but no phosphorus (Sterner & Elser, 2002). Therefore, a thicker exoskeleton in the presence of predation risk would also result in a higher N:P ratio.

Effects of predation risk under mild warming

As expected the predator-induced reductions in growth rate and the associated RNA:DNA ratio and the reductions in fat and total sugar contents were stronger under mild warming. This can be explained because these predator-induced changes are generated by predator-induced increases in metabolic rate (GSP; Hawlena & Schmitz, 2010a) while metabolic rates were the highest under the combination of predation risk and mild warming. In accordance with our findings, stronger predator-induced growth reductions under mild warming have been observed in Chinook salmon (*Oncorhynchus tshawytscha*; Kuehne *et al.*, 2012) and *E. vesperum* damselfly larvae (Culler *et al.*, 2014), in both cases attributed to increased metabolic demands at the higher temperature.

In line with the GSP, we also found an increased C:P under predation risk, but only at 24°C. The only other study on this also demonstrated a higher C:P in tadpoles exposed to predation risk (Costello & Michel, 2013). They assumed this could be

explained by the GSP as the result of an increased gluconeogenesis (i.e., production of C-rich glucose) and the excretion of P. In our study, however, this explanation is unlikely given the lower sugar content under predation risk. Instead, in the condition with increased C:P (predation risk at 24°C), the growth reduction and the associated lowered RNA:DNA ratio were the strongest, which can explain the decreased P content, hence the higher C:P under predation risk at 24°C. The predator-induced increase in C:N, which was stronger under warming, could not be explained by the GSP as we did not observe increases in fat and total sugar content nor a decrease in protein content in this condition. Instead, the above suggested increase in chitin content might also explain the stronger predator-induced increase in C:N at 24°C as morphological defences against predators have been shown to be more pronounced at higher temperatures (e.g. Laurila *et al.*, 2008).

Synthesis and conclusions

By combining a mild 4°C temperature increase (matching IPCC [2013] warming scenario RCP 8.5) and predation risk, we obtained three novel insights directly relevant for understanding the impact of global warming on prey organisms. First, the effects of warming did strongly depend upon predation risk. In accordance with thermodynamic principles (Sibly *et al.*, 2012) larvae reared at the higher temperature performed better (had higher growth rates and energy reserves). Yet, when larvae were exposed to predation risk, the positive effect of the higher temperature on the growth rate reversed into a negative effect, and the increase in energy reserves disappeared. This indicates that laboratory studies should be used with caution when making predictions under global warming, as in nature prey organisms typically experience predation risk. Second, our results indicate that the negative impact of predation risk on performance (growth rate and energy storage) is expected to increase under global warming in this study system. Predation risk is increasingly appreciated as an important driver of population dynamics (Preisser *et al.*, 2005) and even community composition (Peacor *et al.*, 2012). While recent studies stressed the importance of increased consumptive predation under global warming (e.g. De Block *et al.*, 2013), we here highlight another pathway through which warming may indirectly affect ecological communities: by

changing nonconsumptive effects of predators (see also Miller *et al.,* 2014). This mechanism may contribute to the observed stronger topdown effects by predators on their prey at higher temperatures (e.g. Kratina *et al.,* 2012; O'Gorman *et al.,* 2012).

Third, how temperature (Schmitz, 2013) and predation risk (Hawlena & Schmitz, 2010a, b) shape body stoichiometry may profoundly affect the functioning of food webs, particularly elemental cycling, and is therefore key to understand the impact of global warming in natural systems. For example, soil samples that received carcasses of grasshoppers with a 4% higher C:N body content due to exposure to predators, showed a threefold decrease in the mineralization of plant litter (Hawlena *et al.,* 2012). By integrating metabolic ecology and the growth rate hypothesis, we could demonstrate predictable changes in body stoichiometry in the absence of predation risk. Given the generality of the underlying mechanisms this likely provides a useful predictive framework for many other ectotherms. Notably, we extended the very few studies that experimentally studied the separate effects of temperature and predation risk on body stoichiometry by also focusing on their combined effects and by quantifying the full set of assumed driving key biomolecules (i.e., RNA:DNA, fat, sugars, and proteins). Building on the inspiring and innovative studies of Hawlena and Schmitz (2010a,b) and Schmitz (2013), our integrated approach provided novel insights about the interplay of warming and nonconsumptive effects on prey stoichiometry and the underlying mechanisms. Current study together with the very few other ones that looked at the effects of warming (Liess *et al.,* 2013) and predation risk (Costello & Michel, 2013; Dalton & Flecker, 2014) highlighted the interplay of the physiological GSP-responses with behavioural changes affecting food restriction and morphological defences. Further validating this predictive mechanistic framework is an important challenge linking stress ecology and ecosystem functioning and will be pivotal in understanding how nonconsumptive predator effects under global warming will shape prey populations.

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Author contribution: LJ and RS conceived and designed the experiment. LJ and MVD preformed the experiment. LJ, MVD and RS analysed the data and wrote the manuscript.

Appendix 1: Comparison with literature data

Table S1. Comparison of the values for the physiological variables in our study with some relevant examples from the literature. Where possible comparisons were made with aquatic insects. Given are 95% ranges of data values (based on means ± 2 SE). Dry mass abbreviated as dm.

Parameter	Taxon	95% range of data values	Reference
RNA:DNA			
	Damselfly (*Enallagma cyathigerum*)	4.0 – 6.4	This study
	Bivalve (*Crassostrea angulata*)	2.0 – 6.0	Chicharo *et al.*, 2001
	Bivalve (*Ruditapes decussatus*)	4.0 – 8.0	Chicharo *et al.*, 2001
	Fish (*Paralichtys olivaceus*)	3.0 – 6.0	Gwak *et al.*, 2003
Fat content			
	Damselfly (*Enallagma cyathigerum*)	15 – 27 µg/mg dm	This study
	Damselfly (*Enallagma aspersum, Ischnura verticalis*)	2 – 28 µg/mg dm	Stoks *et al.*, 2005a
	Caterpillar (*Manduca sexta*)	18 – 27 µg/mg dm	Thaler *et al.*, 2012
Sugar content			
	Damselfly (*Enallagma cyathigerum*)	2 – 18 µg/mg dm	This study
	Damselfly (*Enallagma aspersum, Ischnura verticalis*)	3.5 – 5.5 µg/mg dm	Stoks *et al.*, 2005a
	Caterpillar (*Manduca sexta*)	18 – 48 µg/mg dm	Thaler *et al.*, 2012
Protein content			
	Damselfly (*Enallagma cyathigerum*)	550 – 710 µg/mg dm	This study
	Damselfly (*Enallagma aspersum, Ischnura verticalis*)	500 – 700 µg/mg dm	Stoks *et al.*, 2005a
	Oyster (*Crassostrea gigas*)	300 – 590 µg/mg dm	Delaporte *et al.*, 2006

C:N			
	Damselfly (*Enallagma cyathigerum*)	3.9 – 5.2	This study
	Odonata	4.0 – 6.0	Cross *et al.*, 2003
	Zygoptera	3.0 – 7.0	Frost *et al.*, 2003
	Benthic insects	4.1 – 7.3	Evans-White *et al.*, 2005
	Diptera	4.0 – 8.0	Liess & Hillebrand, 2005
	Trichoptera	4.0 – 8.0	Liess & Hillebrand, 2005
	Odonata	4.4 – 6.2	Lauridsen *et al.*, 2012
	Mayflies (Heptageniidae)	5.6 – 6.4	Mehler *et al.*, 2013
	Guppy (*Poecilia reticulata*)	4.0 – 8.0	Dalton & Flecker, 2014
C:P			
	Damselfly (*Enallagma cyathigerum*)	80 – 113	This study
	Odonata	140 – 260	Cross *et al.*, 2003
	Zygoptera	100 – 220	Frost *et al.*, 2003
	Benthic insects	237 – 299	Evans-White *et al.*, 2005
	Diptera	50 – 350	Liess & Hillebrand, 2005
	Trichoptera	80 – 220	Liess & Hillebrand, 2005
	Odonata	136 – 286	Lauridsen *et al.*, 2012
	Mayflies (Heptageniidae)	62 – 361	Mehler *et al.*, 2013
N:P			
	Damselfly (*Enallagma cyathigerum*)	16 – 27	This study
	Odonata	35 – 55	Cross *et al.*, 2003
	Zygoptera	24 – 40	Frost *et al.*, 2003
	Benthic insects	6 – 86	Evans-White *et al.*, 2005
	Diptera	15 – 65	Liess & Hillebrand, 2005
	Trichoptera	10 – 40	Liess & Hillebrand, 2005
	Odonata	23 – 59	Lauridsen *et al.*, 2012
	Mayflies (Heptageniidae)	11 – 63	Mehler *et al.*, 2013

Chapter VI

Additive bioenergetic responses to a pesticide and predation risk in an aquatic insect

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Slightly modified version

VI

Abstract

Ignoring natural stressors such as predation risk may contribute to the failure of ecological risk assessment of pesticides to protect freshwater biodiversity. To better understand combined effects of multiple stressors, bioenergetic responses are important as these inform about the balance between energy input and consumption, and provide a unifying mechanism to integrate the impact of multiple stressors with different modes of action. We studied in *Enallagma cyathigerum* damselfly larvae the single and combined effects of exposure to the pesticide chlorpyrifos and predation risk on life history (survival and growth rate) and bioenergetic response variables at the organismal level (assimilation and conversion efficiency) and the cellular level (cellular energy allocation CEA, energy storage Ea, and energy consumption Ec). Chlorpyrifos exposure almost halved the survival of the damselfly larvae, while predation risk had no effect on survival. Both exposure to the pesticide and to predation risk reduced larval growth rates. This was caused by a reduced conversion efficiency under chlorpyrifos exposure, and by a reduced assimilation efficiency under predation risk. Both chlorpyrifos and predation risk reduced the CEA because of a decreased Ea, and for chlorpyrifos also an increased Ec. The lower Ea was driven by reductions in the fat and glycogen contents. Effects of the pesticide and predation risk were consistently additive and for most variables the strongest response was detected when both stressors were present. The absence of any synergisms may be explained by the high mortality and hypometabolism caused by the pesticide. Our results indicate that CEA can be a sensitive biomarker to evaluate effects of not only contaminants but also natural stressors, such as predation risk, and their combined impact on organisms.

Introduction

A major threat for aquatic biodiversity is the ongoing contamination of aquatic systems with pesticides (Schwarzenbach *et al.,* 2006; Malaj *et al.,* 2014). Moreover, the current ecological risk assessment (ERA) of pesticides seems ineffective to protect freshwater ecosystems. Indeed, the aquatic biodiversity is declining at pesticide concentrations that are considered as safe by legislation (Beketov *et al.*, 2013; Peters *et al.,* 2013). One major weakness of ERA is that traditional ecotoxicological tests evaluate the sensitivity of organisms under optimal laboratory conditions. However, in nature organisms often face additional environmental stressors. These stressors could reduce the overall body condition of an organism and thereby they may increase the impact of pesticides (Holmstrup *et al.,* 2010; Liess *et al.,* 2016). Therefore, examining and understanding the impact of pesticides under more natural conditions is important to develop a more realistic ERA (Holmstrup *et al.,* 2010; Liess *et al.*, 2016).

A widespread and important environmental stressor in aquatic systems is predation (Kerfoot & Sih, 1987). The mere perception of cues associated with predation may impose considerable stress on prey organisms (Clinchy *et al.,* 2013), thereby generating not only important sublethal negative effects, such as a growth reduction (Benard, 2004), but also mortality (Stoks, 2001; McCauley *et al.*, 2011; Siepielski *et al.,* 2014). Given the omnipresence of predation risk in nature, the traditionally laboratory tests of ERA are expected to underestimate the effects on the total fitness of prey organisms. Especially since predation risk has the potential to magnify the effects of pesticide exposure (e.g. Relyea & Mills, 2001; Trekels *et al.,* 2011; Janssens & Stoks, 2013a). Synergistic effects between predation risk and pesticides are, however, not general (e.g. Coors & De Meester, 2008; Pestana *et al.*, 2009; Qin *et al.*, 2011). Moreover, it is still poorly understood how interactions between natural stressors and pesticides are generated (Relyea & Hoverman, 2006; Qin *et al.,* 2011; Côté *et al.,* 2016; Liess *et al.,* 2016).

Traditionally, multistressor studies focus on life history traits (Holmstrup *et al.,* 2010; Côté *et al.,* 2016). To get insight into the underlying mechanisms shaping interactions between stressors, it is needed to also evaluate physiological traits (Côté *et*

al., 2016; Jackson *et al.,* 2016; Kaunisto *et al.,* 2016). Specifically, stressor effects on energy allocation and energy budgets are pivotal to increase our understanding of the impact of multiple stressors (Sokolova, 2013). This is because bioenergetic responses give information about the balance between energy input and consumption. Stressor-induced imbalances can lead to a reduction in growth and reproduction (Sokolova, 2013). Moreover, bioenergetic responses provide a unifying mechanism to integrate the impact of multiple stressors with different modes of action (Baas *et al.,* 2010; Sokolova, 2013).

An important bioenergetic response variable at the cellular level is the cellular energy allocation (CEA). The CEA estimates the available net energy as the difference between the energy stored in reserve molecules and the energy consumption. The latter is quantified as the electron transport system (ETS) activity at the mitochondrial level (De Coen & Janssen, 2003). The CEA has been shown to be positively correlated with organismal growth rates (Verslycke *et al.,* 2004; reviewed in Goodchild *et al.,* 2019). This illustrates the possibility of using bioenergetic responses as indicators for effects at higher biological levels (De Coen & Janssen, 2003). Notably, CEA can be more accurate in indicating stress effects compared to the scope for growth approach, based on assimilation and respiration rates measured at the organismal level (Verslycke *et al.,* 2004). CEA was designed as a general indicator of stress. Yet, it has mainly been applied to quantify pollutant stress (e.g. De Coen & Janssen, 1997, 2003; Smolders *et al.,* 2004; Verslycke *et al.,* 2004; Novais *et al.,* 2013; Aderemi *et al.,* 2018) and less often for environmental stressors such as temperature (e.g. Gandar *et al.,* 2017; Kühnhold *et al.,* 2017) and salinity (e.g. Verslycke & Janssen, 2002).

In current study, the single and combined effects of exposure to a pesticide and predation risk on life history (survival and growth rate) and bioenergetic response variables were investigated. The bioenergetic responses were studied both at the organismal and at the cellular level. At the organismal level, two traits directly related to growth rate were investigated: assimilation efficiency and conversion efficiency. Assimilation efficiency is the efficiency of energy uptake from the ingested food, and conversion efficiency is the degree of which assimilated energy is converted into body mass (hence allocated to growth). At the cellular level, the CEA was quantified by

measuring both the energy reserves (protein, fat and glycogen contents) and the energy consumption (measured as the electron transport activity, ETS). Larvae of the damselfly *Enallagma cyathigerum* were used as study organisms. Both pesticide exposure (Janssens & Stoks, 2013a; Dinh Van *et al.*, 2014) and predation risk (McPeek, 2004; Slos & Stoks, 2008; Culler *et al.*, 2014; Janssens *et al.*, 2015, Chapter V) can negatively affect damselfly larvae. Because damselfly larvae are important intermediate predators in aquatic food webs, the effects of these stressors can cascade through the food web (Stoks & Córdoba-Aquilar, 2012). As pesticide we chose chlorpyrifos, a widely used organophosphate (Eaton *et al.*, 2008) that is within the top ten of the most risky chemicals to aquatic organisms in surface waters in the UK (Johnson *et al.*, 2017). Chlorpyrifos is listed as a priority pollutant by the European Water Framework Directive (2000/60/EC).

Materials and methods

Collecting and housing

We collected *E. cyathigerum* larvae in a pond located in Bergerven (51°03'58.9284"N, 05°41'29.9796"E) in Belgium. Bergerven is a nature reserve without agriculture, which makes it unlikely the pond was ever exposed to pesticides (Coors *et al.*, 2009). In the laboratory, we kept single larvae in 100 mL plastic cups filled with a mixture of dechlorinated tap water (50%) and filtered pond water (50%). Afterwards, when water from the cups evaporated, we gradually added dechlorinated tap water to the cups. The cups were placed in incubators at 20°C with a 14h :10h light: dark photoperiod. The larvae were fed 6 days a week with *Artemia* nauplii (mean±1SE, daily dose: 247±81 nauplii, n=12 daily portions). Once the larvae moulted into the penultimate instar, they were fed three living larvae of *Chironomus riparius* per week. When the larvae moulted into the final instar, the experimental treatments started. In Belgium, final instars are present in spring-summer (based on De Knijf *et al.*, 2006). This corresponds to the application period of many pesticides (Van Drooge *et al.*, 2001).

Experimental design

To assess the single and combined effects of the pesticide chlorpyrifos and predation risk, we set up a full factorial experiment with two pesticide treatments (chlorpyrifos absent or present) crossed with two predation risk treatments (predation cues absent or present). The damselfly larvae were exposed to both stressors for nine days (based on Van Dievel *et al.,* 2016, Chapter IV). At the start of the experiment the larvae were transferred to glass vials (100 mL) filled with 50 mL medium (see below). We daily renewed the medium. Six water samples were taken to measure physico-chemical parameters, with mean pH levels in the vial varying between 8.15 - 8.27, hardness between 161.33 - 164.67 mg/L $CaCO_3$, dissolved oxygen levels between 7.69 - 7.84 mg/L and conductivity levels between 603 – 607 µS/cm. Throughout the 9 day exposure period the larvae were fed in total four living *C. riparius* larvae. The first chironomid larvae was given at day 1. Afterwards, damselfly larvae received a single chironomid every other day. We thereby simulated a low food level. This is ecologically relevant since in the field damselfly larvae are often food limited (for *Enallagma* damselfly larvae: McPeek, 1998). Moreover, under food-limited conditions energy-mediated effects of stressors are more easily detected (Karl *et al.,* 2011). The experiment was performed in incubators at 20°C with a 14h:10h light: dark photoperiod. At the end of the experiment the damselfly larvae were frozen and stored at -80°C for physiological analyses. We started between 51 and 110 larvae per treatment combination (total of 312 larvae). More larvae were started in treatments where more mortality occurred in order to arrive at 50-52 survivors per treatment combination for physiological analyses. Exact numbers of larvae started per treatment combination are shown in Figure 1.

For the treatment combinations with the pesticide, we exposed the larvae to 2 µg/L chlorpyrifos. We chose this concentration because it is mildly lethal for *E. cyathigerum* (M. Van Dievel, unpublished results). Although this concentration is high, it falls within the range of chlorpyrifos concentrations found in edge-to-field water bodies (Schulz, 2004; Bernabò *et al.,* 2011). We daily renewed the medium to guarantee continuous exposure to chlorpyrifos throughout the 9-day period (static renewal experiment). We made a 1 mg/mL stock solution by dissolving chlorpyrifos powder (Sigma-Aldrich, purity 99%) in absolute ethanol (100%). The stock solution was made

in an amber glass bottle and stored in the dark at 4°C. We renewed the stock solution monthly. We daily prepared the chlorpyrifos exposure solution by diluting the stock concentration with milli-Q water to a concentration of 10 µg/L. We added 200 µL of the latter solution to 999.8 mL dechlorinated tap water to obtain the exposure concentration of 2 µg/L. The same amount of ethanol was added in the solvent control as in the chlorpyrifos treatment (2 µg/L). This dose of ethanol does not affect growth and behaviour of the study species (Janssens & Stoks, 2013b). We measured the chlorpyrifos concentration in a pooled sample of the medium of 10 vials at the start of the experiment, and after 24h (before renewal of the medium) using UPLC MS/MS with Triple Quadrupole Mass Spectrometry in the Division of Soil and Water Management at the KU Leuven. The initial chlorpyrifos concentration was 2.14 µg/L, and after 24h the chlorpyrifos concentration was reduced to 1.28 µg/L in the vials without predation risk and to 1.34 µg/L in the vials with predation risk.

We manipulated predation risk by exposing half of the larvae to chemical cues of the dragonfly *Anax imperator*. Larvae of this dragonfly are important predators of *Enallagma* larvae (Stoks *et al.,* 2005b), and were collected in the pond of origin. The chemical cues were daily prepared by homogenizing one *E. cyathigerum* larva in 10 mL water from a container (300 mL) in which a large *A. imperator* larva had eaten an *E. cyathigerum* larva. We daily added 1 mL of predator cues to the vials of the predation risk treatments (Janssens *et al.*, 2015, Chapter V; Van Dievel *et al.,* 2016, Chapter IV). Note that we only used chemical cues, as these have been shown to be sufficient to elicit behavioural anti-predator responses in *Enallagma* damselfly larvae (Mortensen & Richardson, 2008).

Response variables

During the 9-day exposure period we daily checked survival. At the organismal level, we determined traits related to growth (growth rate, assimilation efficiency and conversion efficiency) and related to the cellular energy allocation (CEA): energy reserves available (Ea) and energy consumption (Ec). The available energy reserves were estimated based on the total protein, fat and glycogen contents. We used the

activity of the electron transport system (ETS) as proxy for the cellular energy consumption (De Coen & Janssen, 2003).

Growth rate was quantified as the increase in wet mass over the 9-day exposure period. Both at the start and the end of the exposure period we weighed the larvae to the nearest 0.01 mg using an electronic balance. We calculated the growth rate as [ln(final mass) – ln(initial mass)] / 9 days (McPeek *et al.*, 2001). To calculate a mass budget we quantified the total dry masses of food eaten and of faecal pellets produced. All damselfly larvae, with the exception of one, ate all four *C. riparius* larvae given. We converted the amount of food eaten to total dry mass based on the mean dry mass of a *C. riparius* larva (mean ± 1SE = 1.06 ± 0.09 mg, n = 10 individuals). This was determined by weighing *C. riparius* larvae after drying them for >24h at 60°C. We daily removed the faecal pellets of each damselfly larva and stored these in separate aluminium foil containers per larva. At the end of the exposure period, the set of faecal pellets produced by a larva was dried for >24h at 60°C and weighed to the nearest 0.001 mg. The amount of assimilated food was calculated as the difference between the total dry mass of the ingested *C. riparius* larvae and the total dry mass of the faecal pellets produced by an individual damselfly larva. The assimilation efficiency was estimated as the amount of assimilated food divided by the total dry mass of food eaten (McPeek *et al.*, 2001). The conversion efficiency was calculated as dry mass gain of a larva divided by the amount of assimilated food. To determine the dry mass gain, we converted larval wet mass into dry mass using the equation derived for *Enallagma* larvae: dry mass = 0.1497 × wet mass (McPeek *et al.*, 2001). Note that since previous studies with damselflies documented that neither chlorpyrifos exposure (Janssens *et al.*, 2014a) nor predation risk (Van Dievel *et al.*, 2016, Chapter IV) affected the body water content, we assumed that the dry/wet ratio was unaffected by the stressors.

We used a random subset of 25 larvae per treatment combination (total of 100 larvae) to determine the CEA following De Coen and Janssen (2003). The CEA, or the total net energy budget of an organism, was calculated as Ea/Ec (Verslycke *et al.*, 2004). The Ea was calculated as the sum of energy present in proteins, glycogen and fat. For this, we quantified the dry mass of these biomolecules and converted these into energetic equivalents by multiplying them with the corresponding energy of combustion values:

24,000 mJ/mg protein, 17,500 mJ/mg glycogen and 39,500 mJ/mg fat (De Coen & Janssen, 2003). The Ec was estimated as the ETS activity. To obtain these values we first homogenized the larvae with a pestle and diluted the homogenates 5 times in milli-Q water. We then centrifuged the sample for 5 minutes (10,000g, 4°C). We took 35 µL of the supernatant and we further diluted this three times in phosphate-buffered saline (pH 7.4, 50 mmol L^{-1} PBS). All energy reserves and the ETS activity were spectrophotometrically quantified (Infinite M2000, TECAN). Detailed assays are given in Appendix 1.

Briefly, we determined the protein content following Bradford (1976). The protein content was calculated based on a standard curve of known bovine serum albumin concentrations. The measurements were done in quadruplicate and the means per larva were used for statistical analyses. The total fat content was based on a modified version of the protocol of Marsh and Weinstein (1966). We calculated the fat content using a standard curve of glyceryl tripalmitate. The fat content was measured in triplicate and we used the mean per larva for the statistical analyses. The glycogen content was measured using a modified protocol of Stoks *et al.* (2006a) based on the glucose kit of Sigma-Aldrich (St. Louis, Missouri, USA). To determine the glycogen content we first converted all glycogen to glucose. Secondly, we determined only the free glucose. The glucose levels were calculated based on a standard curve of known glucose concentrations. The glycogen content was estimated as the difference of the two glucose measurements. Measurements were done in duplicate and the mean per larva was used for the statistical analyses.

We quantified the ETS activity based on the protocol of De Coen and Janssen (2003) that was adapted for damselflies by Janssens and Stoks (2013a). The cellular respiration rate (E_c) was determined using the theoretical stoichiometric relationship that for 2 µmol formazan formed, 1 µmol of O_2 was consumed in the ETS system. We converted the amount of consumed oxygen into energetic equivalents using the oxyenthalpic equivalents for an average protein, glycogen and fat mixture of 480 kJ/mol O_2 (De Coen & Janssen, 2003).

Statistical analyses

We tested the effects of chlorpyrifos exposure, predation risk and their interaction on all response variables using linear models. Survival (binary: dead vs. alive) was evaluated using a generalized linear model with a binomial error structure and the logit link function. All other response variables were analysed using linear models with a normal error structure and the identity link function. As energy molecules increase with body mass (Widder & Bidwell, 2006; Ardia, *et al.*, 2012), we included body mass as a covariate in all CEA models. Statistically correcting for mass by adding it as a covariate is recommended above dividing by body mass (Beaupre & Dunham, 1995). We tested for homogeneity of variances using Levene's tests and for normality with Shapiro–Wilk's tests. In case of non-normal distributed variables (Ea, Ec, protein budget), these were log-transformed.

All statistical analyses were performed in R v3.4.0 (R development core Team, 2015). We used the package 'lme4' (Bates *et al.*, 2015) to run the (generalized) linear models. F-statistics and *P*-values for fixed effects were obtained in the package 'car' (Fox & Weisberg, 2011), thereby using the Kenward–Roger method for denominator degrees of freedom approximation. All models were fitted with restricted maximum likelihood.

Results

In the absence of both chlorpyrifos and predation risk almost all damselfly larvae survived (98%; Fig. 1). Exposure to chlorpyrifos decreased larval survival with 45% (Table 1; Fig. 1). Exposure to predation risk had no effect on the survival of the larvae, nor was there an interaction between chlorpyrifos exposure and predation risk (Table 1; Fig. 1).

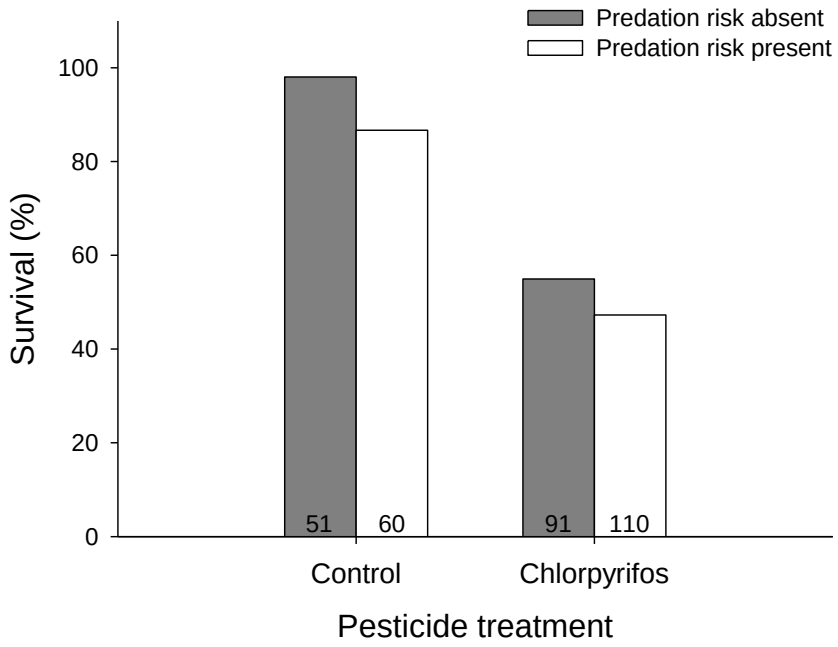

Figure 1. Survival (%) of *Enallagma cyathigerum* damselfly larvae as a function of exposure to chlorpyrifos and predation risk. Exact numbers of larvae started per treatment combination are indicated in the bars.

Table 1. Results of the linear models testing for the effects of chlorpyrifos and predation risk on survival, growth-related traits, cellular energy allocation and energy reserves in *Enallagma cyathigerum* larvae. Significant values are indicated in bold.

Response variable	Effect	df_1, df_2	F	*P*
Survival	Chlorpyrifos	1,308	61.05	**<0.001**
	Predation risk	1,308	3.31	0.070
	Chlorpyrifos x Predation risk	1,308	3.28	0.071
Growth-related traits				
Growth rate	Chlorpyrifos	1,200	6.88	**0.009**
	Predation risk	1,200	5.03	**0.026**
	Chlorpyrifos x Predation risk	1,200	0.37	0.544
Food intake	Chlorpyrifos	1,200	1.00	0.318
	Predation risk	1,200	0.96	0.328
	Chlorpyrifos x Predation risk	1,200	0.96	0.328
Assimilation efficiency	Chlorpyrifos	1,196	11.42	**<0.001**
	Predation risk	1,196	4.39	**0.037**
	Chlorpyrifos x Predation risk	1,196	0.60	0.440
Conversion efficiency	Chlorpyrifos	1,197	4.04	**0.046**
	Predation risk	1,197	01.60	0.208
	Chlorpyrifos x Predation risk	1,197	0.51	0.474
Cellular energy allocation				
Available energy	Chlorpyrifos	1,95	5.90	**0.017**
	Predation risk	1,95	3.74	0.056
	Chlorpyrifos x Predation risk	1,95	0.88	0.350

Energy consumption	Chlorpyrifos	1,95	13.96	**<0.001**
	Predation risk	1,95	0.05	0.823
	Chlorpyrifos x Predation risk	1,95	0.83	0.364
Cellular energy allocation	Chlorpyrifos	1,96	59.96	**<0.001**
	Predation risk	1,96	11.41	**0.001**
	Chlorpyrifos x Predation risk	1,96	0.13	0.717
Energy reserves				
Proteins	Chlorpyrifos	1,95	0.50	0.481
	Predation risk	1,95	0.34	0.550
	Chlorpyrifos x Predation risk	1,95	3.51	0.064
Fat	Chlorpyrifos	1,95	13.36	**<0.001**
	Predation risk	1,95	4.32	**0.040**
	Chlorpyrifos x Predation risk	1,95	0.80	0.372
Glycogen	Chlorpyrifos	1,95	12.99	**<0.001**
	Predation risk	1,95	14.32	**<0.001**
	Chlorpyrifos x Predation risk	1,95	0.00	0.996

Both exposure to chlorpyrifos (-12%) and to predation risk (-10%) reduced the growth rate of the larvae (Table 1; Fig. 2a). There was no chlorpyrifos × predation risk interaction for any growth-related variable (Table 1). Chlorpyrifos exposure increased the assimilation efficiency, yet decreased the conversion efficiency (Table 1; Fig. 2b-c). Predation risk decreased the assimilation efficiency, but did not affect the conversion efficiency (Table 1; Fig. 2b-c).

When exposed to chlorpyrifos, the damselfly larvae had ca 13% less available energy (Table 1; Fig. 3a). This was due to reductions in fat and glycogen contents while the protein content was not affected (Table 1; Fig 4a). In addition, energy consumption increased under chlorpyrifos exposure with ca. 21% (Table 1; Fig. 3b). This resulted in a 32% reduction of CEA under chlorpyrifos exposure (Table 1; Fig. 3c). Under predation risk larvae had a marginally non-significant ($P = 0.056$) reduction (- 12%) in the amount of available energy, which was associated with significantly lowered fat and glycogen contents (Table 1; Fig. 3c, Fig. 4). This resulted in a significant lower CEA (ca. 24%) under predation risk (Table 1; Fig. 3c). There was no significant interaction between chlorpyrifos and predation risk for any of the traits related to CEA (Table 1).

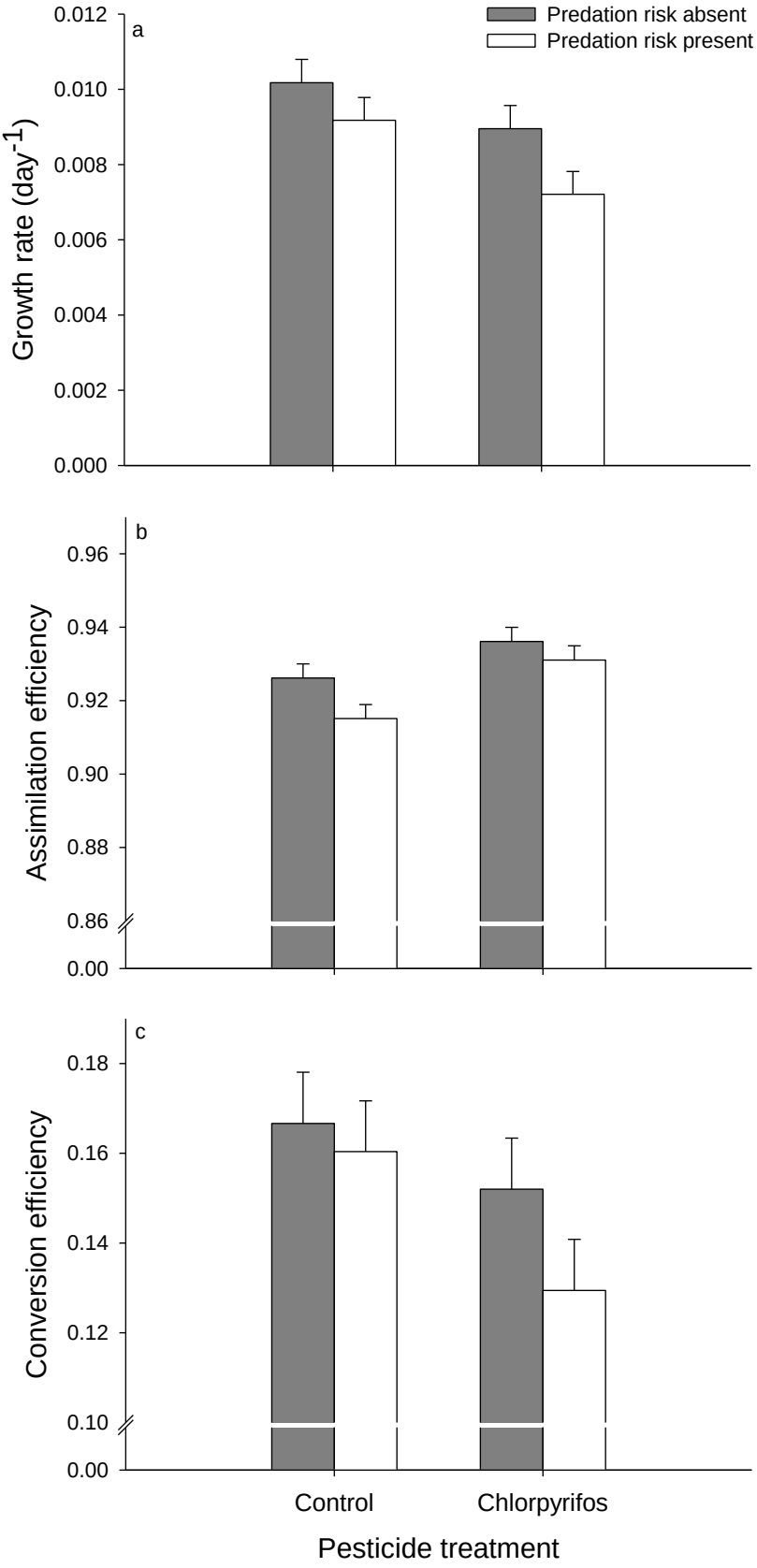

Figure 2. Growth-related variables of *Enallagma cyathigerum* damselfly larvae as a function of exposure to chlorpyrifos and predation risk: (a) growth rate, (b) assimilation efficiency, and (c) conversion efficiency. Given are the least-squares means (+ 1 s.e).

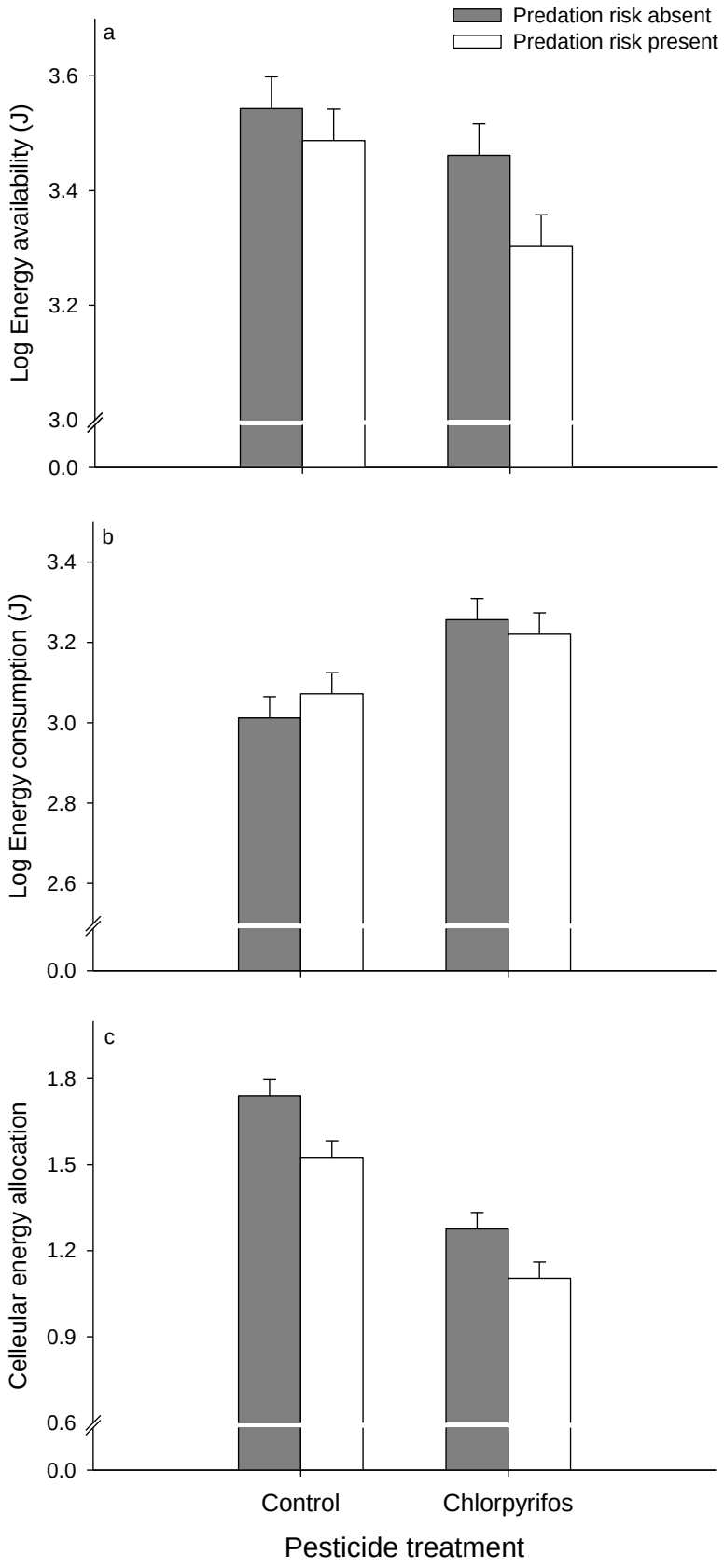

Figure 3. Levels of variables related to cellular energy allocation (CEA) of *Enallagma cyathigerum* damselfly larvae as a function of exposure to chlorpyrifos and predation risk: (a) Energy availability, (b) energy consumption, and (c) cellular energy allocation. Given are the least-squares means (+ 1 s.e).

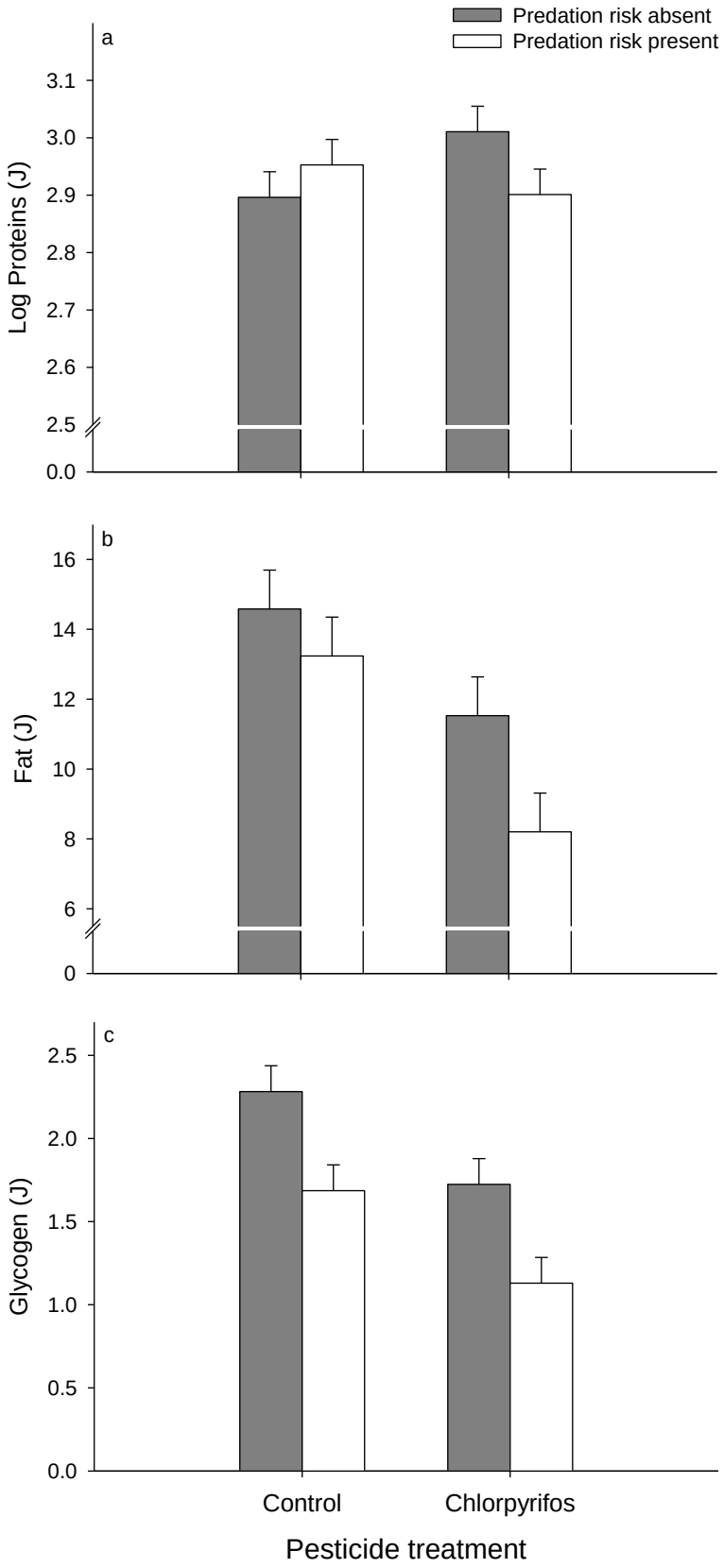

Figure 4. Levels of energy reserve molecules of *Enallagma cyathigerum* damselfly larvae as a function of exposure to chlorpyrifos and predation risk: (a) protein, (b) fat, and (c) glycogen contents. Given are the least-squares means (+ 1 s.e).

Discussion

Effects of survival

Exposure to the here used dose of chlorpyrifos (2.14 µg/L) almost halved the survival of the *E. cyathigerum* damselfly larvae. The toxicity of chlorpyrifos results from the inhibition of acetylcholinesterase, which disturbs the signal transmission in the nervous system, eventually causing mortality (Stenersen, 2004; Domingues *et al.*, 2010). Moreover, during this process reactive oxygen species can be generated, which could lead to oxidative stress (e.g. Patetsini *et al.*, 2013; Cacciatore *et al.*, 2015), as demonstrated in *E. cyathigerum* larvae (Janssens & Stoks, 2017). In line with this, considerable mortality and reductions in acetylcholinesterase at similar concentrations of chlorpyrifos have been documented in the study species (Janssens & Stoks, 2013b) and a related damselfly species (Dinh Van *et al.*, 2014).

There is accumulating proof that 'fear' caused by predation risk can be lethal as shown in other damselfly species (Stoks, 2001, Siepielski *et al.*, 2014) and other taxa (McCauley *et al.*, 2011; Gehr *et al.*, 2018). Nevertheless, we did not observe an increased mortality when the larvae were exposed to chemical predator cues. Pathways how fear may cause mortality include a reduction in food intake resulting in starvation and/or an increased susceptibility to pathogens (McCauley *et al.*, 2011). Yet, in our study all larvae ate the same amount of food and were not exposed to pathogens. In addition, severe physiological fear-induced stress may also cause death (Creel *et al.*, 2009; Preisser, 2009). Yet, our results did not show an increase in Ec under predation risk, suggesting no strong fight-or-flight physiological stress response (Hawlena & Schmitz, 2010a).

Effects on growth-related traits

Both exposure to the pesticide and to predation risk reduced larval growth rates, and this in an additive way. This additive effect supports previous experiments both in the study species (Janssens & Stoks, 2013a, but see Campero *et al.*, 2007 for a synergism in a related damselfly species), and in other aquatic insects (the midge *C. riparius*: Pestana

et al., 2009; corixid species: Trekels *et al.,* 2012). Growth reductions under these stressors have been often associated with a reduced food intake (Pesticide: e.g. Ribeiro *et al.,* 2001; Pestana *et al.,* 2009; Dinh Van *et al.,* 2014, predation risk: e.g. McPeek *et al.,* 2001; Trussell *et al.,* 2006; Van Dievel *et al.,* 2016, Chapter IV). In the present study, the stress-induced reductions in growth, however, occurred despite no reduction in food intake indicating a role for digestive physiology. This matches previous studies that reported growth reductions not mediated by food intake in response to pesticide exposure (e.g. Campero *et al.,* 2007) and predation risk (e.g. Stoks, 2001; Janssens & Stoks, 2013a; Culler *et al.,* 2014).

The observed growth reduction under pesticide exposure was driven by a reduced efficiency to convert assimilated food into biomass. This may be caused by investment in energetically costly processes, like defence and detoxification, that may require allocation of resources away from growth (Sibly & Calow, 1989; Lika & Kooijman, 2003). For example, under chlorpyrifos exposure organisms have been shown to upregulate levels of the protective stress protein Hsp70 (e.g. Scheil *et al.,* 2010; Janssens & Stoks, 2013a; Janssens *et al.,* 2014) and the activity of the detoxification enzyme glutathione S-transferase (GST) (e.g. Cinzia *et al.,* 2006; Janssens & Stoks, 2013b; Kim *et al.,* 2016) and cytochrome P450 monooxygenase (Verheyen & Stoks, 2019). Notably, the pesticide-induced reduction in conversion efficiency overruled the increase in assimilation efficiency. A higher assimilation of energy out of the ingested food under pesticide exposure may be a compensatory mechanism to gain more energy to cope with chemical stressors. Such compensatory increase in assimilation efficiency has been observed in response to salinity in clams (Zhuang, 2005, 2006) and in response to pesticides in a related damselfly (Campero *et al.,* 2007). In addition, since almost half of the damselfly larvae died under chlorpyrifos exposure, the survivors may have been those able to upregulate the digestive enzymes needed for such compensatory response (Chapman, 1998).

The predator-induced growth reduction was driven by a reduction in assimilation efficiency, while conversion efficiency did not change. These results are in contrast with other studies showing predation risk to increase assimilation efficiency in damselfly larvae (McPeek, 2004; Stoks *et al.,* 2005c; Culler *et al.,* 2014). The latter was

hypothesized to be the result of differences in food intake. The authors suggested that predator-exposed larvae ate smaller amounts of food at once, so that their guts were less full and as a result greater extraction of resources could occur. Yet, in all these studies damselfly larvae were fed *ad libitum*, while we here simulated the realistic scenario of *Enallagma* damselfly larvae to be food-limited (McPeek, 1998). Since the damselfly larvae in our study only received and consumed one *C. riparius* larvae at a time, no difference in the fullness of the guts across the conditions is expected. Moreover, these example studies had a shorter exposure period (4 days) compared to our 9-day exposure period. Thaler *et al.* (2012) demonstrated that *Manduca sexta* caterpillars initially (day 3 of the exposure period) increased assimilation efficiency under predation risk, yet had the same efficiency as the control caterpillars at day 6 and even decreased their assimilation efficiency at day 9. They hypothesized that prey can alter the timing of physiological processes (like assimilation) to maximize performance and this way can overcome short-term threats such as predation risk. However, in the long-term this could come with costs. In contrast to chlorpyrifos, no mortality and hence no survival selection occurred under predation risk. Therefore, also the larvae that could not keep activity of the digestive enzymes high survived and were included in the measurements.

Effects on cellular energy allocation (CEA)

The total net energy budget of the larvae, quantified as CEA, was considerably reduced both under exposure to chlorpyrifos (-31%) and under predation risk (-24%). The underlying contributions of changes in the amount of Ea and in Ec, however, differed between both stressors. Both chlorpyrifos exposure and predation risk (marginally non-significant) decreased Ea of the damselfly larvae, which was driven by reductions in the fat and glycogen contents. Possibly, the larvae accumulated less energy reserves as they had to invest in costly defence mechanisms to cope with the pesticide or predation risk. For example, as shown for chlorpyrifos also under predation risk Hsp70 levels (in the study species: Slos & Stoks, 2008) and GST activity (in a related damselfly species: Van Praet *et al.*, 2014, see also Hylander *et al.*, 2012) may increase. While the upregulation of defence proteins could increase the total protein content, we did not observe this. Possibly, any upregulation of defence proteins was balanced by a stress-induced

increase in gluconeogenesis (the breakdown of proteins into sugar) (Hawlena & Schmitz, 2010a).

Only for chlorpyrifos exposure we observed an increased energy consumption, as indicated by the increased ETS activity. Pesticide-induced increases in ETS activity have been observed before (e.g. Verslycke *et al.,* 2004) and probably related with the upregulation of defence and detoxification mechanisms. Against our expectations, and in contrast to other studies (Janssen *et al.*, 2015, Chapter V; Van Dievel *et al.*, 2016, Chapter IV), we did not observe an increase in ETS activity under predation risk. Possibly, the stress imposed by predation risk was less strong compared to chlorpyrifos, as suggested by no or weaker effects on survival, growth and CEA. In the present study larvae were only exposed to chemical predation cues (based on Mortensen & Richardson, 2008), whereas previous work on *E. cyathigerum* damselfly larvae that showed an upregulation of oxygen consumption used a combination of both chemical and visual cues (Slos & Stoks, 2008; Janssens *et al.*, 2015, Chapter V). Furthermore, the exposure period in the previous studies was shorter (between 5 and 7 days) and effects on metabolic rate may disappear under long-term exposure to predation risk (Steiner & Van Buskirk, 2009). The 9 days exposure period in the present study can be considered as long-term exposure, since for the study species it comprises ca 40% of the duration of the final instar. Note that, we simulated the realistic situation were damselfly larvae are food limited (for *Enallagma* larvae: McPeek, 1998). In such condition, effects of predation risk on foraging are likely less important compared to physiological responses. Yet, in natural systems without food shortage prey organisms may also show a reduced activity and food intake under predation risk. Because foraging and feeding (capture and handling prey and also digestion) are energy consuming processes this may alter bioenergetics responses (less activity leading to less energy consumption, less food intake altering energy reserves).

The interaction type between the pesticide and predation risk

We did not detect a synergistic interaction between pesticide exposure and predation risk. Nevertheless, for most variables the strongest response was detected in the combined stressor condition. This indicates that post-exposure effects on prey fitness

and population dynamics would still be underestimated by only taking the pesticide exposure into account for ERA. Recent meta-analyses on multistressor interactions showed high variability in the interaction type between stressors (Côté *et al.*, 2016, Jackson *et al.*, 2016). Many studies documented synergistic interactions between pesticides and predation risk, for example for survival (e.g. Relyea & Mills, 2001; Relyea 2003, 2004; Maul *et al.*, 2006). Yet, a considerable number found additive effects (e.g. Relyea 2003; Qin *et al.*, 2011; Trekels *et al.*, 2012; this study). The underlying mechanisms shaping the interaction type are still poorly understood (Qin *et al.*, 2011; Todgham & Stillman, 2013, Côté *et al.*, 2016). According to the Stress Addition Model by Liess *et al.* (2016) synergisms can be expected when each stressor in the combination is energetically costly. Yet, we did not detect synergistic interactions despite the food-limited conditions and the reductions in the amount of available energy by both stressors.

Two mechanisms may have contributed to the absence of a synergism between predation risk and pesticide exposure in the current study. First, the pesticide itself already caused high mortality in the larvae, thereby likely removing the most sensitive animals. Therefore, the additional stress due to simultaneous exposure to predation risk may have had no additional effect on survival. This is in accordance with the dominance model by Schäfer and Piggott (2018) and precludes the detection of synergistic effects. Similarly, a high carbaryl dose caused high mortality in tadpoles regardless of whether predation risk was present, while predation risk increased mortality of a low carbaryl dose 2-4 times (Relyea & Mills, 2001). Besides survival, also for the measured sublethal endpoints we consistently observed additive effects. This may also be explained by survival selection filtering out only the strongest larvae that were better able to avoid a synergistic effect (Côté *et al.*, 2016). Second, the larvae that survived the high chlorpyrifos level may have undergone metabolic depression (Pörtner & Farrell, 2008; Storey 2015). For example, a similar chlorpyrifos exposure has been shown to reduce the ETS activity of *Ischnura elegans* larvae (Dinh Van *et al.*, 2016). This may have masked energy expenditure by compensating for the increase in metabolic rate due to detoxification.

Conclusions

Effects of the pesticide chlorpyrifos and predation risk for life history and bioenergetic response variables were consistently additive. The absence of synergisms may be explained by the high mortality and associated survival selection by the pesticide, and metabolic reduction. Future work would benefit from studying the bioenergetic responses to range of sublethal and lethal pesticide concentrations and investigate how predation risk mediates these effects. Our results indicate that CEA can not only be a sensitive biomarker to evaluate toxic effects of contaminants, but also be a valuable biomarker for natural stressors. Moreover, by integrating energy reserves and energy consumption, CEA fits the recommendation to use the energy balance as a common denominator to determine the combined effects of stressors (Sokolova, 2013). We advocate CEA as an important tool to integrate and understand combined effects of pesticides and natural stressors that also may provide an important connection in bioenergetic adverse outcome pathways (Goodchild *et al.*, 2019) to better link ecotoxicology research and risk assessment (Ankley *et al.*, 2010).

Acknowledgements

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Author contributions: MDV and RS conceived and designed the experiment. MVD performed the experiment and analysed the data. MVD, LJ and RS contributed to the manuscript.

Appendix 1: Detailed description of assay protocols

Material and methods

We determined the protein content based on the method described by Bradford (1976). For this, we mixed 160 µL milli-Q water and 1µL of the supernatant with 40 µL Biorad protein dye in a 96-well microtiter plate. Then the plate was incubated for 5 minutes at 30°C and afterwards the absorbance was measured at 595 nm (in quadruplicate at 25°C). The protein content was calculated based on a standard curve of known protein concentrations.

The fat content was based on a modified version of the protocol of Marsh and Weinstein (1966). In 2 mL glass tubes we added 8 µL supernatant and 56 µL H_2SO_4 (100%). The tubes were heated for 20 minutes at 150°C. Afterwards we added 64 µL milli-Q water and mixed the sample. 30 µL of the sample was added to a 384-well microtiter plate and we measured absorbance at 490 nm (in triplicate at 25°C). Based on a standard curve of glyceryl tripalmitate we calculated the fat content.

The glycogen content was measured using a modified protocol of Stoks *et al.* (2006a) based on the glucose kit of Sigma-Aldrich (St. Louis, Missouri, USA). To determine the glycogen content we first had to transform all glycogen to glucose. Therefore, we mixed 12.5 µL milli-Q water with 25 µL supernatant and 5 µL amyloglucosidase (1 unit/10 µL; Sigma A7420) in a 96-well microtiter plate. Then we incubated the plate for 30 minutes at 37°C. Then we added 100 µL glucose assay reagent and incubated the plate for another 20 minutes at 30°C. After 20 minutes we measured the glucose levels at 340 nm (in duplicate at 25°C). Secondly, we determined only the free glucose by adding 37.5 µL milli-Q water, 12.5 µL supernatant and 100 µL glucose assay reagent in a 96-well microtiter plate. Again, after an incubation period of 20 minutes at 30°C we measured the free glucose levels at 340 nm (in duplicate at 25°C). The glucose levels were calculated based on a standard curve of known concentrations of glucose. The glycogen content was equal to the difference of the two glucose measurements.

We quantified the ETS activity as a proxy for oxygen consumption based on the protocol of De Coen and Janssen (2003). A 384-well microtiter plate was filled with 5 µL supernatant and15 µL buffered substrate solution (0.13 M TRIS-HCL; 0.3% Trition X-100, 1.7 mM nicotinamide adenine dinucleotide, 250 mM nicotinamide adenine dinucleotide phosphate, pH 8.5) and 10 µL (8 mM) p-iodonitrotetrazolium (INT). The reduction of INT leads causes the formation of formazan, which was measured as the increase in absorbance at 490 nm (in triplicate at 25°C) during 10 minutes (measurements every 30 seconds). Following the Lambert-Beer formula, we calculated the concentration formazan (extinction coefficient 15900 mol L^{-1} cm^{-1}) .

Chapter VII

Pesticide exposure and predation risk shape egestion of elements and primary production

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Unpublished manuscript

VII

Abstract

Understanding how pesticides and natural stressors shape ecosystem functions remains a major challenge for stress ecology. A largely overlooked way how stressors may affect nutrient cycling and primary production is through effects on body stoichiometry and the excretion of elements. We investigated how exposure to the pesticide chlorpyrifos and predation risk, an abundant natural stressor in aquatic systems, altered the stoichiometry of the bodies and the egested faecal pellets of *Enallagma cyathigerum* damselfly larvae and how this further cascaded into effects on primary production (algae growth). Chlorpyrifos exposure and predation risk affected both elemental composition of bodies and faecal pellets, and this in an additive way. Chlorpyrifos exposure increased body C(carbon), N(nitrogen), and P(phosphorous) contents, and increased the C content of the faecal pellets. Predation risk induced an increase of the N content of the bodies and the faecal pellets. This resulted in a decreased C:N ratio of the bodies and faecal pellets. The changes in the composition of the faecal pellets caused by predation risk but not by chlorpyrifos exposure increased algae growth under control conditions. This is consistent with the general finding that algae growth is often N limited. Our results provide an important proof-of-principle how a stressor may shape nutrient cycling and subsequently primary productivity.

Introduction

Pesticides are a major threat in aquatic ecosystems (Schwarzenbach *et al.*, 2006; Malaj *et al.*, 2014) that may strongly reduce aquatic biodiversity (Beketov *et al.*, 2013) and affect ecosystem functions (McMahon *et al.*, 2012; Peters *et al.*, 2013, Rodrigues *et al.*, 2018). Our understanding how pesticides affect ecosystem functions remains, however, limited (McMahon *et al.*, 2012, Peters *et al.*, 2013). Moreover, pesticides may interact with natural stressors in affecting organisms (Liess *et al.*, 2016). How these combined effects translate into effects on ecosystem functions has been identified as a priority question to improve ecological risk assessment of pesticides (Van den Brink *et al.*, 2018).

A key ecosystem function in water bodies is nutrient cycling (Costanza *et al.*, 1997; DeAngelis, 1992), whereby nutrients are transformed from one chemical form to another, and/or transported between organisms, habitats or ecosystems (Vanni, 2002). This cycling is important to guarantee the ecosystem function primary production, such as the production of algal biomass (Vanni, 2002; Liess & Hillebrand, 2004). In aquatic systems, nitrogen (N) and P (phosphorus) are key nutrients driving primary production (Tilman *et al.*, 1982; Elser *et al.*, 2007; Dodds & Whiles, 2010), whose cycling can be animal-mediated (Vanni, 2002; Atkinson *et al.*, 2017). Hereby, animals control the existing stocks and supply rates of nutrients through direct consumption but also through nutrient release (nutrient excretion/egestion) (Vanni, 2002; Knoll *et al.*, 2009). In freshwaters, nutrient release has been shown to play a significant role in the organic matter budgets (Cuffney *et al.*, 1990).

A largely overlooked way how pesticides may affect nutrient cycling and primary production is through their effects on body stoichiometry and the excretion of elements. Recently, two studies documented that exposure to pesticides can alter the elemental body composition of animals. Exposure of the water flea *Daphnia magna* to lindane resulted in a decreased body C(carbon):N ratio due to a decrease in C content (Ek *et al.*, 2015), while exposure of the damselfly *Enallagma cyathigerum* to chlorpyrifos caused both reductions in C and N contents, resulting in no net change in the C:N ratio (Janssens *et al.*, 2017). These pesticide-induced changes in body stoichiometry likely will also be

associated with a changed excretion of elements. Yet, the expected links between pesticide-induced changes in body stoichiometry and nutrient excretion, and eventual effects on primary production await experimental testing.

In aquatic systems, animals typically are not only exposed to pesticides but also to natural stressors, of which predation is widespread (Kerfoot & Sih, 1987). The fear imposed by predators has the potential to magnify the effects of pesticide exposure (e.g. Relyea & Mills, 2001; Trekels *et al.*, 2011; Janssens & Stoks 2013a, but see e.g. Coors & De Meester, 2008; Pestana *et al.*, 2009; Qin *et al.*, 2011). These studies were, however, mainly limited to effects on life history, physiology and behaviour. To our knowledge, only one study looked at the combined effects of pesticide exposure and predation risk on body stoichiometry (Janssens *et al.*, 2017), but did not explore how these stressors may shape nutrient cycling and primary production. Notably, the fear imposed by predation itself can affect elemental body composition and nutrient release, and thereby nutrient cycling (Hawlena & Schmitz, 2010b; Schmitz *et al.*, 2010; Hawlena *et al.*, 2012; Dalton & Flecker, 2014). For example, a predator-induced 4% higher C:N content of grasshopper carcasses has been shown to slow down plant litter decomposition rate by a threefold (Hawlena *et al.*, 2012).

A predictive framework to understand how stressors may affect nutrient cycling is the general stress paradigm (GSP, Hawlena & Schmitz, 2010a,b). The GSP states that stressed animals increase their metabolism and allocate resources to defence mechanisms and maintenance. To fuel these processes animals will increase the production of C(carbon)-rich biomolecules (partially through the breakdown of N-rich proteins into glucose). Moreover, they will invest less in the production of new tissues and reproduction, resulting in a decreased need of N and P. To maintain homeostasis, they will excrete the excess N and P (Hawlena & Schmitz, 2010a,b). Although both pesticide exposure (e.g. Ek *et al.*, 2015; Janssens *et al.*, 2017) and predation risk (e.g. Hawlena & Schmitz 2010a,b; Dalton & Flecker, 2014; Janssens *et al.*, 2015, Chapter V; Van Dievel *et al.*, 2016, Chapter IV) have been shown to cause changes in body elemental composition, these changes are not always in accordance with the GSP predictions. As most of these studies only considered body elemental composition, also

explicitly measuring elemental content of the excretes may provide more complete insights in the stoichiometric response patterns to these stressors.

We tested how exposure to a pesticide and predation risk shape the body composition and excretion of the key elements C, N and P. Subsequently, we investigated how both stressors, through affecting the release of nutrients, affected primary production by measuring algae growth. As study species we used *Enallagma cyathigerum* damselfly larvae. Damselflies are important intermediate predators in aquatic food webs. Moreover, both pesticide exposure (Janssens & Stoks, 2013a; Dinh Van *et al.*, 2014) and predation risk (McPeek, 2004; Slos & Stoks, 2008; Janssens *et al.*, 2015, Chapter V) are known to negatively impact damselfly larvae, and therefore the effects of these stressors could cascade through the food web (Stoks & Córdoba-Aguilar, 2012; Stoks *et al.*, 2015). Damselfly larvae egest faecal pellets which have been shown to play an important role in nutrient cycling (Ngai & Srivastava, 2006). We studied effects of the widely used pesticide chlorpyrifos (Eaton *et al.*, 2008). Chlorpyrifos is within the top ten of most risky chemicals to aquatic organisms in surface waters in the UK (Johnson *et al.*, 2017). It is listed as a priority pollutant by the European Water Framework Directive (2000/60/EC).

Material and methods

Collecting and housing

In the autumn of 2017, we collected *E. cyathigerum* larvae from a pond in the nature reserve Bergerven (51°03'58.9284"N, 05°41'29.9796"E) in Belgium. Given the surrounding land use, direct exposure of this population to pesticides is unlikely. In the laboratory, the larvae were placed individually in 100 mL plastic cups filled with a mixture of dechlorinated tap water and filtered pond water. The cups were placed in incubators at 20°C with a 14:10 light: dark photoperiod. Six days a week the larvae were fed *Artemia* nauplii (mean±1SE, daily dose: 247±81 nauplii, n=12 daily portions). Once the larvae moulted into the penultimate instar, we fed them three living larvae of *Chironomus riparius* per week, originating from a lab culture.

Experimental setup

To test the effects of pesticide exposure and predation risk on the elemental composition of the larval bodies and faecal pellets, and subsequently on primary production we set up a full factorial experiment with all four combinations of two pesticide (chlorpyrifos absent or present) and two predation risk (predation cues absent or present) treatments. When the larvae entered the final instar, they were randomly attributed to one of the four treatment combinations for nine days. We quantified the C:N:P composition of the larvae at the end of the exposure period. The faecal pellets produced during the experiment were collected to analyse their C:N:P composition and to quantify their effect on primary production (algae biomass increase) under control conditions (no pesticide and no predation risk) in a follow-up 'algae growth experiment'.

At the start of the exposure period, the larvae were transferred to 100 mL glass vials filled with 50 mL medium. Half of them were exposed to a nominal concentration of 2 µg/L chlorpyrifos. This concentration has lethal effects in *E. cyathigerum* (Van Dievel *et al.,* 2019, Chapter VI). Although the chosen concentration is high, it is within the range of chlorpyrifos concentrations observed in edge-to-field water bodies after pesticide run-off (Schulz, 2004; Bernabò *et al.,* 2011). We daily renewed the medium. To obtain a chlorpyrifos concentration of 2 µg/mL, we first prepared a 1 mg/mL stock solution by dissolving chlorpyrifos powder (Sigma-Aldrich, purity 99%) in absolute ethanol (100%). This stock solution was made in an amber glass bottle and stored in the dark at 4°C. After four weeks, this stock solution was renewed. The chlorpyrifos exposure solution was daily prepared by diluting the stock concentration with milli-Q water to a concentration of 10 µg/L. We added 200 µL of this solution to 999.8 mL dechlorinated tap water to obtain the exposure concentration of 2 µg/L. We added the same amount of ethanol to the solvent control as in the chlorpyrifos treatment (2 µg/L). This dose of ethanol does not affect growth and behaviour of the study species (Janssens & Stoks, 2013b). We measured the chlorpyrifos concentration in three replicated pooled samples of the medium of 10 vials at the start of the experiment, and after 24h (before renewal of the medium) using UPLC MS/MS with Triple Quadrupole Mass Spectrometry in the Division Soil and Water Management at the KU Leuven. The mean initial chlorpyrifos concentration was 2.14 µg/L (SE = 0.40), and after 24h the

chlorpyrifos concentration was reduced to 1.28 µg/L (SE = 0.07) in the vials without predation risk and to 1.34 µg/L (SE = 0.29) in the vials with predation risk.

To manipulate predation risk, half of the larvae were exposed to chemical cues of larvae of the dragonfly *Anax imperator*, important predators of *Enallagma* larvae (Stoks *et al.,* 2005b). Damselfly larvae have been shown to respond behaviourally and physiologically to *Anax* chemical cues (Mortensen & Richardson, 2008; Van Dievel *et al.,* 2019, Chapter VI). To obtain the chemical cues, we homogenized one *E. cyathigerum* larva in 20 mL of water from an aquarium filled with 300 mL aged tap water in which a large *Anax* dragonfly larva had eaten a larva of *E. cyathigerum*. One mL of this predator medium was added to the vials of the predation risk treatment; the other vials received 1 mL of aged tap water. During the 9 day exposure period larvae were fed four *C. riparius* larvae.

Together with the renewal of the medium, we daily collected faecal pellets using fine tweezers. To remove any residue of the pesticide and/or predation cues, we gently rinsed the pellets first in ethanol and then in distilled water. Afterwards, we transferred the faecal pellets in separate aluminium foil cups per larva. The faecal pellets were dried for at least 24h at 60°C and weighed to the nearest 0.001 mg. We collected the faecal pellets of 50-52 larvae per treatment combination. Since the dry mass of the pooled set of faecal pellets of a single larva was too low for further analyses (see below), we combined the faecal pellets of 5-7 larvae. To obtain matched data, we applied the same pooling approach for the damselfly larvae. This resulted in 8-9 replicates per treatment combination.

Stoichiometric response variables

To quantify the C:N:P composition of the larvae and their faecal pellets, we first weighed and separately homogenized the larvae and the faecal pellets with a pestle and diluted them five times in milli-Q water. We used 75 µL of the faecal pellet homogenate for the algae growth experiment (see below), the rest was used for the C:N:P measurements. We divided the C:N:P samples in two subsamples: ¼ for the C and N analyses, and ¾ for the P analyses. To determine the C and N contents we first transferred the subsamples to tin cups and dried these for 24 h at 60 °C. Then, the C and

N contents were quantified with an elemental analyser (Carlo Erba 1108, Thermo Scientific, Waltham, USA) using leucine for internal calibration. To determine the P content, we filled a glass tube with the subsample together with 1 mL HNO_3 (70%). The glass tubes were heated at 150 °C for approximately 45 minutes to let the HNO_3 evaporate. Afterwards, the digest was diluted to 10 mL with milli-Q water. The P content was quantified using inductively coupled plasma mass spectrometry (Aqilent 7700x ICP-MS, Biocompare, South San Francisco, California, USA). The elemental C, N and P contents were expressed as % of dry mass. The C:N, C:P and N:P ratios were expressed as molar ratios by taking into account the molar masses of C (12 g/mol), N (14 g/mol) and P (31 g/mol).

Algae growth experiment

To investigate how pesticide exposure and predation risk affected primary production through changes in the faecal pellet composition, we used the faecal pellets obtained in the exposure experiment as growth medium for *Scenedesmus obliquus* algae. Algae growth trials were always run under control conditions, hence in the absence of chlorpyrifos and predator cues (see rinsing step above). To avoid effects of the stressors being mediated by the mass of the pellets produced, we diluted the pooled set of faecal pellets relative to their mass. Any treatment effects on algae growth could therefore only be mediated through the composition of the faecal pellets. Each faecal pellet sample was tested in three algae growth trials (three technical replicates) and the mean used for statistical analyses.

For each replicate run, we mixed 25 µL of the faecal pellet sample with 965 µl mineral water of the brand SPA Reine® and 1×10^6 *S. obliquus* algae cells in a 24-well microtiter plate. We first autoclaved the faecal pellet samples. This was done to avoid potential effects of chlorpyrifos and predation risk on the microbes in the faecal pellets, which could alter leaching mineralization of the pellets. As medium for the algae growth we used bottled mineral water Spa Reine® that has a chemical composition suitable for algae growth (C. Zhang, personal observation, based on Toumi *et al.*, 2013). The microtiter plates were placed in an incubator at 20°C with a 24h light regime. To keep the algae in suspension, we placed the plate on a shaker (Unimax 1010, Heildolph

Instruments, Schwabach, Germany) at 200 rpm. We quantified algae growth based on the first 7 days; after 7 days the algae no longer showed exponential growth. We determined the algal densities using a spectrophotometer (Infinite M2000, TECAN) at 420 nm. We converted the absorbance of the algae into the number of algae cells per millilitre using a standard curve of known algae concentrations. This standard curve was based on counts with an Attunew Acoustic Focusing Cytometer (Applied BiosystemsTM by Life TechnologiesTM, Carlsbad, CA). The algae growth rate was calculated as [ln(N2) − ln(N1)]/ 7 days, with N1 and N2 the number of algae cells per millilitre at day 1 and day 7, respectively. We corrected for algae growth in the medium without any additional nutrients leached from the faecal pellets, by substracting the growth rate of the algae in the SPA Reine® control.

Statistical analyses

We analysed the effects of chlorpyrifos exposure, predation risk and their interaction on all response variables using linear models with a normal error structure and the identity link function. We investigated the relationships between the elemental composition of the larvae and their faecal pellets, and between the faecal pellets and algae growth using pearson correlations. We tested for homogeneity of variances using Levene's tests and for normality with Shapiro–Wilk's tests. All statistical analyses were performed in R v3.4.0 (R development core Team, 2017). We used the package 'lme4' (Bates *et al.*, 2015) to run the linear models and the package 'car' (Fox & Weisberg, 2011) to calculate the F-statistics and *P*-values.

Results

Correlations between stoichiometric composition of bodies and fecal pellets

The N content of the faecal pellets was positively correlated with the N content of the bodies, which resulted in a positive correlation between their C:N ratios. However, these relations were weak (R^2 = 0.17- 0.18, Table 1). There were no significant correlations between the other stoichiometric variables of the bodies and the faecal pellets (Table 1).

Table 1. Correlations between elemental composition of the faecal pellets with the body elemental composition of *Enallagma cyathigerum* damselfly larvae.

	Body composition (N = 33)		
Faecal pellet composition	R	R^2	*P*
C content	0.23	0.053	0.203
N content	0.42	0.18	**0.015**
P content	0.010	0.00	0.987
C:N ratio	0.41	0.17	**0.019**
C:P ratio	-0.13	0.018	0.464
N: P ratio	-0.012	0.15	0.941

Elemental composition

Chlorpyrifos exposure increased the larval body contents of C (+ 13%), N (+ 6%) and P (+ 12%), yet this had no significant effect on their ratios (Table 2; Fig. 1a-f). Predation risk increased the body N content (+ 5%), but it had no effect on the C and P contents. This resulted in a decreased C:N ratio (- 3%), yet there was no significant change in the C:P and N:P ratios (Table 2; Fig. 1d-f). For none of the three elements or ratios there was a significant Chlorpyrifos × Predation risk interaction (Table 2).

Table 2. Results of the linear models testing for the effects of chlorpyrifos exposure and predation risk on the stoichiometry of *Enallagma cyathigerum* larvae and their faecal pellets, and algae growth on these pellets.

Response variable	Effect	df_1, df_2	F	*P*
Body stoichiometry				
C content	Chlorpyrifos	1,28	25.59	**<0.001**
	Predation risk	1,28	0.0078	0.930
	Chlorpyrifos x Predation risk	1,28	0.98	0.170
N content	Chlorpyrifos	1,28	20.82	**<0.001**
	Predation risk	1,28	8.21	**0.008**
	Chlorpyrifos x Predation risk	1,28	0.023	0.881
P content	Chlorpyrifos	1,28	5.32	**0.028**
	Predation risk	1,28	0.081	0.778
	Chlorpyrifos x Predation risk	1,28	1.32	0.259
C:N ratio	Chlorpyrifos	1,28	1.40	0.246
	Predation risk	1,28	6.80	**0.014**
	Chlorpyrifos x Predation risk	1,28	0.55	0.462
C:P ratio	Chlorpyrifos	1,28	1.23	0.278
	Predation risk	1,28	0.14	0.712
	Chlorpyrifos x Predation risk	1,28	0.25	0.622
N:P ratio	Chlorpyrifos	1,28	1.19	0.284
	Predation risk	1,28	0.56	0.461
	Chlorpyrifos x Predation risk	1,28	0.84	0.368
Faecal pellet stoichiometry				
C content	Chlorpyrifos	1,29	6.45	**0.017**
	Predation risk	1,29	0.94	0.341
	Chlorpyrifos x Predation risk	1,29	0.0066	0.936
N content	Chlorpyrifos	1,29	3.26	0.081
	Predation risk	1,29	5.24	**0.029**
	Chlorpyrifos x Predation risk	1,29	1.26	0.270
P content	Chlorpyrifos	1,29	1.88	0.180
	Predation risk	1,29	1.48	0.233
	Chlorpyrifos x Predation risk	1,29	0.16	0.689
C:N ratio	Chlorpyrifos	1,29	0.058	0.812
	Predation risk	1,29	4.45	**0.043**
	Chlorpyrifos x Predation risk	1,29	1.27	0.269
C:P ratio	Chlorpyrifos	1,29	0.19	0.667
	Predation risk	1,29	1.86	0.183
	Chlorpyrifos x Predation risk	1,29	0.20	0.661
N:P ratio	Chlorpyrifos	1,29	0.050	0.825
	Predation risk	1,29	3.69	0.065
	Chlorpyrifos x Predation risk	1,29	0.033	0.858

Primary production				
Algae growth	Chlorpyrifos	1,29	0.061	0.807
	Predation risk	1,29	9.19	**0.005**
	Chlorpyrifos x Predation risk	1,29	0.70	0.410

Chlorpyrifos exposure increased the C content (+ 9%) of the faecal pellets, but had no effect on their N and P contents; nor did it cause significant changes in the stoichiometric ratios (Table 2; Fig. 2a-f). Exposure to predation risk increased the N content (+ 12%) of the faecal pellets, but had no effect on their C and P contents (Table 2; Fig. 2a-c). This increase in N under predation risk, resulted in faecal pellets with a 4% lower C:N ratio (Table 2; Fig. 2d-f). For none of the stoichiometric elements or ratios the interaction between chlorpyrifos exposure and predation risk was significant (Table 2).

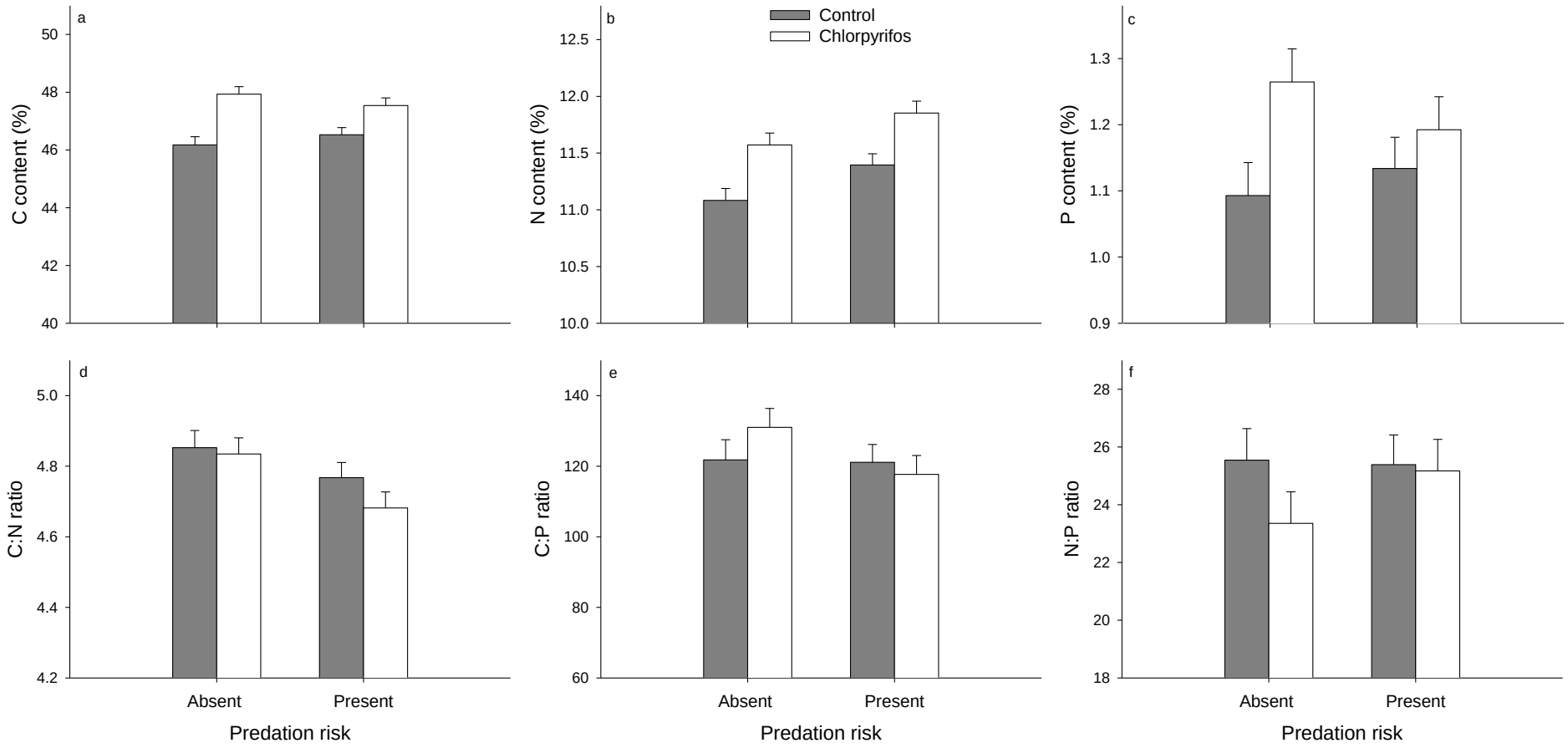

Figure 1. Elemental composition of *Enallagma cyathigerum* damselfly larvae as a function of exposure to chlorpyrifos and predation risk: (a) C content, (b) N content, (c) P content, (d) C:N ratio, (e) C:P ratio, and (f) N:P ratio. Given are the least-squares means (+ 1 s.e.).

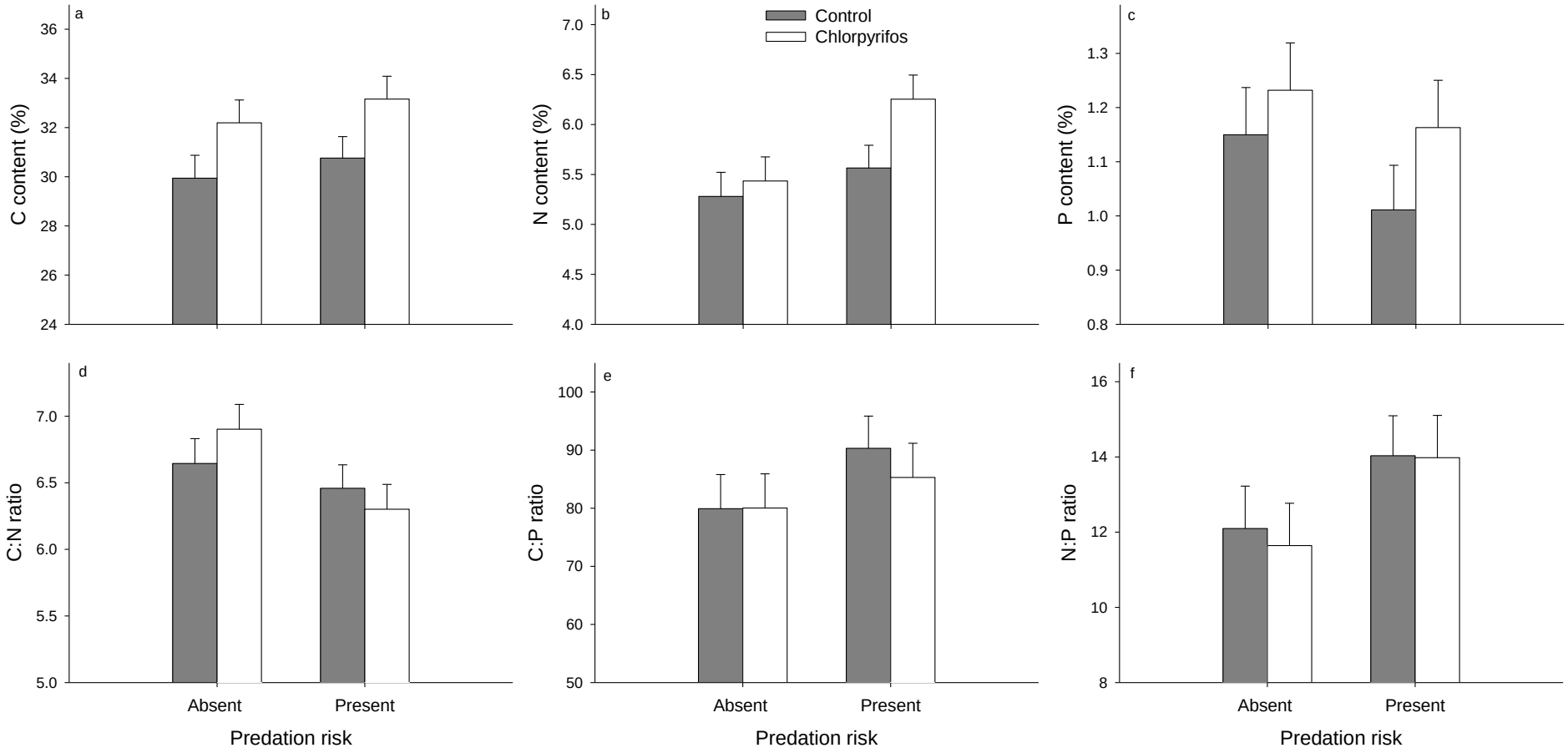

Figure 2. Elemental composition of the faecal pellets of *Enallagma cyathigerum* damselfly larvae as a function of exposure to chlorpyrifos and predation risk: (a) C content, (b) N content, (c) P content, (d) C:N ratio, (e) C:P ratio, and (f) N:P ratio. Given are the least-squares means (+ 1 s.e.).

Algae growth

The growth rate of the algae was 84 % higher when they were growing on a medium with faecal pellets originating from larvae exposed to predation risk (Table 2; Fig. 3). The growth of the algae was not affected when grown on pellets from larvae that had been exposed to chlorpyrifos (Table 2; Fig. 3).

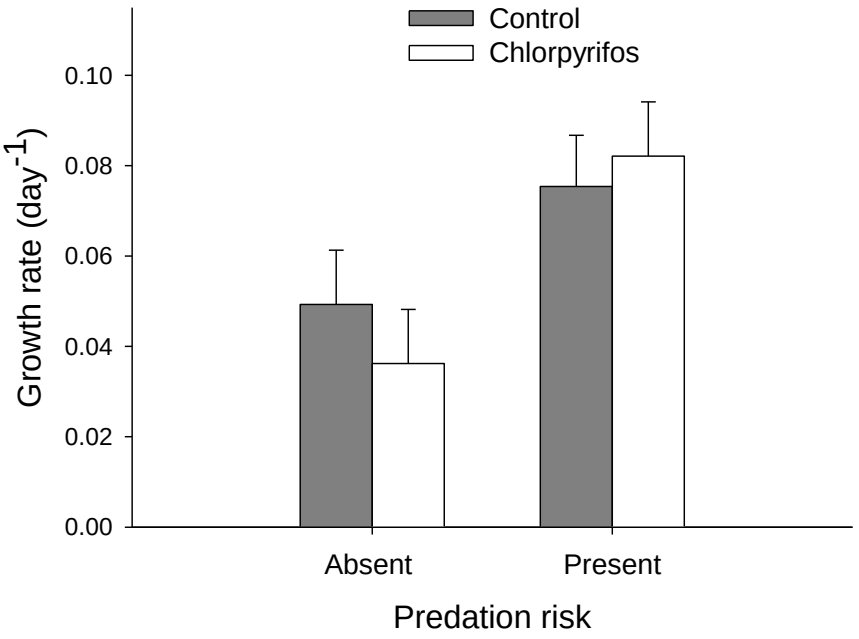

Figure 3. Growth rate of *Scenedesmus obliquus* algae under control conditions (absence of chlorpyrifos and absence of predation risk) on a medium with faecal pellets originating from *Enallagma cyathigerum* damselfly larvae that had been exposed to combinations of chlorpyrifos and predation risk. The algae growth rate was corrected for the growth rate of the algae in the SPA Reine® control that did not receive additional nutrients from faecal pellets. Given are the least-squares means (+ 1 s.e).

Discussion

We investigated how exposure to a pesticide and predation risk altered the stoichiometry of the bodies and faecal pellets of *E. cyathigerum* larvae, and whether this had cascading effects on primary production. Key findings were that chlorpyrifos increased the excretion of C, yet this did not affect algae growth. Predation risk, on the other hand, increased the egestion of N, which did have a positive effect on algae growth.

Elemental composition of the damselfly larvae

Both exposure to the pesticide and to predation risk had effects on the elemental composition of the damselfly larvae and this is an additive way. The general stress paradigm (GSP, Hawlena & Schmitz, 2010a), that was developed to predict stressor-induced changes in elemental body composition of organisms, could not fully explain the observed changes. In accordance with the GSP, the C content of the larvae increased under chlorpyrifos exposure. This could be caused by an increase in C-rich energy storage molecules to fuel the predicted increase in metabolic rate under stress (Hawlena & Schmitz, 2010a). Under chlorpyrifos exposure increases in metabolic rate have indeed been observed (Verslycke *et al.*, 2004; Van Dievel *et al.*, 2019, Chapter VI), yet in a companion study the fat and sugar contents decreased under chlorpyrifos exposure (Van Dievel *et al.*, 2019, Chapter VI). Possibly, the increase in C may have been associated with a higher investment in the exoskeleton. By making the cuticle thicker and hence less permeable, the uptake rate of pesticides decreases (Zhang *et al.*, 2008). Chitin is the main component of the cuticle and contains much C and N (Sterner & Elser, 2002). Therefore, the increase in chitin content could potentially also have contributed to the increase in N content. Note that a thicker exoskeleton is also a defence strategy to reduce predator attack efficiency (Rabus *et al.*, 2013). Yet, the increase in C was only observed under chlorpyrifos exposure and not under predation risk. Possibly, the stress imposed by predation risk was not strong enough to induce this defence mechanism (see also Van Dievel *et al.*, 2019, Chapter VI). In contrast to the GSP, both chlorpyrifos exposure and predation risk increased the N content of the larvae. The expected decrease in N may have been overruled by the stress-induced upregulation of stress proteins (Sørensen *et*

al., 2003). The increased synthesis of N-rich stress proteins has been documented both for chlorpyrifos (e.g. Scheil *et al.,* 2010; for the study species: Janssens and Stoks, 2013a; Janssens *et al.,* 2014a) and predation risk (for the study species: Slos & Stoks, 2008). Moreover, the increased synthesis of stress proteins may also explain the increased P content under chlorpyrifos exposure because of an upregulation of P-rich RNA (Lindquist, 1986; Goto *et al.,* 1998). Whatever the underlying reasons for the changes in element contents, exposure to chlorpyrifos eventually did not affect the stoichiometric body ratios while predation risk resulted in a lower body C:N ratio.

The two other studies testing for effects of pesticide exposure on body stoichiometry (Ek *et al.,* 2015; Janssens *et al.,* 2017) showed contrasting results. *Daphnia magna* water fleas exposed to the pesticide lindane showed a decrease in body C:N ratio, which was explained by less incorporation of carbon in structural biomass and higher use of carbon for the synthesis of compounds involved in detoxification, increased respiration and oxidized products (Ek *et al.,* 2015). In line with our results, the other study exposing *E. cyathigerum* larvae to chlorpyrifos (Janssens *et al.,* 2017) exposure also showed no effect on the C:N ratio. However, Janssens *et al.* (2017) found that this was due to decreases in both C and N contents, associated with chlorpyrifos-induced decreases in C-rich fat and sugars, and in N-rich proteins. Although in a companion study we found fat and sugar contents decreased under chlorpyrifos exposure (Van Dievel *et al.,* 2019, Chapter VI), we did not observe the expected decrease in C content. Neither did we observe that chlorpyrifos-exposed *E. cyathigerum* larvae had a lower protein content. Possibly, in the study by Janssens *et al.* (2017) the decrease in proteins due to gluconeogenesis out weighted the upregulation of stress proteins because larvae likely experienced a lower stress level imposed by chlorpyrifos compared to current study resulting in a lower upregulation of the stress proteins. Indeed, while previous study used a sublethal concentration of 1 µg/L we used 2 µg/L chlorpyrifos.

Elemental composition of the excreta

We documented weak but positive correlations between the N content and C:N ratio of the damselfly larvae and their faecal pellets. In contrast, most studies reported a negative

correlation between the elemental composition of the bodies and the excreta (Vanni, 2002; Atkinson *et al.*, 2013; Atkinson *et al.*, 2017). Yet, this pattern is not general (see e.g. Torres & Vanni, 2007; Benstead *et al.*, 2010). For example, Benstead *et al.* (2010) only found weak relations (negative for N, positive for P) between the elemental contents of shrimp bodies and their faecal pellets. They argued that this was due to the small ranges in the values of the stoichiometric variables in their study (e.g. 0.7-1.2 %P) as compared to the study by Vanni (2002) (e.g. <1 to > 4 %P).

In contrast to the predictions of the GSP (Hawlena & Schmitz, 2010a), the damselfly larvae excreted more C when they were exposed to the pesticide chlorpyrifos. This may seem counterintuitive given the associated chlopryrifos-induced increase in body C content. Notably, from the three studied elements, C showed the highest increase in body content under chlorpyrifos exposure. This may suggest that to maintain homeostasis (Sterner & Elser, 2002; Persson *et al.*, 2010) the larvae excreted excess C.

While larvae exposed to predation risk excreted more N, the increase was non-significant for larvae exposed to chlorpyrifos. We argued that the increase in body N content was likely associated with the upregulation of stress proteins, yet it is still possible that the larvae transformed their structural proteins into energy storage molecules. To meet the energetic demands imposed by stress responses and upregulation of defence mechanisms (Hawlena & Schmitz, 2010a), animals have indeed been predicted to break down N-rich proteins into C-rich glucose, thereby excreting the excess N (Hawlena & Schmitz, 2010a).

Neither chlorpyrifos nor predation risk affected the P content of the faecal pellets. Yet, as hypothesized for C, an increased release of P to maintain homeostasis under chlorpyrifos exposure could have been expected because chlorpyrifos-exposed larvae showed an increase in P content. Possibly, this effect was masked due to the high variation in the P measurements (coefficient of variation = 22%, compared to 9% and 13% for C and N contents, respectively).

To our knowledge, only a few studies explicitly tested stressor-induced changes in element excretion, and none of these considered pollutants. Predation risk reduced N excretion in guppies (Dalton & Flecker, 2014) and in caterpillars (Thaler *et al.*, 2012).

Dalton and Flecker (2014) argued that predation risk may reduce food intake and that the resulting food restriction, may cause mobilization of glycogen and lipid stores for energy production (Wang *et al.*, 2006), thereby reducing amino acid catabolism and lowering the production of N waste as NH_3 (Dalton & Flecker, 2014). Food restriction may thereby overrule the GSP-mechanisms, as observed in predator-exposed guppies (Dalton & Flecker, 2014).

Algae growth

Studies on cascading effects of stressors at the organismal level are few, especially those going up to ecosystem functioning, and mainly limited to terrestrial systems. For example, detritivorous collembolans decreased their activity under predation risk, resulting in lower soil N content because of a decreased interaction with N-fixers, and a lower soil CO_2 flux attributed to a predator-induced decrease in the stimulation of bacteria and fungi by the collembolans (Sitvarin & Rypstra, 2014). In another example, Hawlena *et al.* (2012) documented that grasshoppers reared in the presence of predatory spiders showed a 4% increase in body C:N, and soil samples that received the grasshopper carcases showed a threefold decrease in the mineralization of plant litter. They argued that the small shift in C:N resulted from an increase in N, which primed the activities of decomposer microorganisms. These results indicated a causal link between predator-induced changes in body stoichiometry and altered nutrient cycling (Hawlena *et al.*, 2012).

We here explored for the first time the link between two stressors, nutrient cycling and primary production in an aquatic system. A key finding was that the growth rate of the algae was indeed affected when they were growing in a medium with faecal pellets originating from larvae exposed to predation risk. The stressor-induced patterns on algae growth are likely caused by the different nutrient availability through leaching in the growth medium mediated by the different elemental composition of the faecal pellets. While both stressors may also affect the total mass of faecal pellets produced, we corrected for this by using mass-specific dilutions. In accordance to the general finding that algal growth is often N-limited (e.g. Tilman *et al.*, 1982; Elser *et al.*, 2007), the higher N content of the faecal pellets originating from the predation risk treatment

resulted in an increased algae growth. Chlorpyrifos exposure increased the egestion of C, which is generally not a limiting nutrient for algae, which may explain it did not affect algae growth.

To our knowledge, only few studies considered the direct effect of the excreta and/or egestion on primary production (e.g. Liess & Haglund, 2007; Rubio *et al.*, 2016). However, none of these studies looked directly at the nutrient composition of the excretion products nor included a stressor treatment. Rubio *et al.* (2016) found that algae growth was higher in the presence of faecal pellets of *Pterygoplivhthys disjunctivus* fish. In contrast, Liess and Haglund (2007) found that faecal pellets of *Theodoxus fluviatilis* snails did not increase primary production. They hypothesized that the faecal pellets contained an insufficient amount of N, since the nutrient ratios of the periphyton showed that the algae growth was N-mimited.

Conclusions

A challenge in ecotoxicology (Peters *et al.*, 2013) and stress ecology in general (Steinberg, 2012) is to understand and predict whether and how effects of stressors at the organismal level affect ecosystem functions. We here studied for the first time how pesticide exposure and predation risk may affect nutrient cycling and thereby shape primary production. Both stressors affected the release of key elements in the faecal pellets, yet only predation risk thereby shaped algae growth. This is an important proof-of-principle of an ignored pathway how a widespread stressor may shape ecosystem functions. To advance current insights, follow-up studies are needed at larger temporal and spatial scales in mesocosms that also analyse the dissolved organic molecules in the faecal pellets and the growth medium of the algae.

Acknowledgements

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Author contributions: MVD, LJ and RS conceived and designed the experiment, MVD performed the experiment. MVD, LJ and RS analysed the data and wrote the manuscript.

General discussion

In this thesis I focused on the effects of three environmental conditions (temperature, predation risk and pesticide exposure) on damselflies. I here will first discuss the impact of exposure to a single environmental condition on life history, behaviour, physiology and stoichiometry. Schematic overviews of the observed single-exposure effects are presented in Figures 1-3 and summarized in Table 1. In a second step, I will discuss the combined impact of warming and predation risk and of pesticide exposure and predation risk; the observed effects are summarized in Table 2. Afterwards, I will discuss limitations of the current thesis and provide suggestions for future research. I will conclude by giving the important take home messages.

Single stressors

Organisms encounter many environmental conditions, which could be beneficial for them or which could be perceived as stressful. I focused on three widespread and important environmental conditions: temperature, predation and pesticide exposure. While exposures to predation risk and chlorpyrifos clearly imposed stress, this was less straightforward for temperature (see below).

In several Chapters I sampled multiple populations of the same species in order to obtain a sufficient amount of animals and to be able to meaningfully compare different species or latitudes. When choosing the populations, I made sure the different sites were as similar as possible. Moreover, I took potential effects of the population of origin into account by including population as a random factor in the statistical models. For most variables, there was indeed no difference between the sampled populations in the measured traits and none of the significant population effects (see Chapter I and IV) affected the main outcomes. For example in Chapter I one of the low-latitude populations showed a different response to the Temperature^2 term for PC1 compared to the other populations of the same latitude. Nevertheless, all three low-latitude popumations clearly showed a stronger thermal response compared to the three high-latitude populations.

Temperature

Whether temperature has positive or negative effects on an organism's responses, depends on its location on the thermal response curve (Angilletta, 2009). Exposure to extreme temperatures can have negative effects on performance and can even cause mortality (Garrabou *et al.,* 2009; Petter *et al.,* 2015; Mislan & Wethey, 2015), whereas mild warming could be beneficial for animals (Deutsch *et al.,* 2008; Nilsson-Örtman *et al.,* 2012).

Mortality

In this thesis I could show that high, but realistic (30°C-32°C) temperatures induced mortality in damselfly larvae and that differential patterns in survival could be linked to the habitat preference of the species or population under study. In Chapter III, a temperature of 30°C was only lethal for *E. cyathigerum* larvae, while it did not cause mortality in *I. elegans* larvae. This could be explained by the preference of *E. cyathigerum* larvae for larger, deeper water bodies, with small temperatures fluctuations. *I. elegans* larvae on the other hand, prefer more shallow water bodies where they experience higher temperature fluctuations. A similar impact of the habitat of origin on the heat tolerance, but this time at the population level, was shown in Chapter I. In contrast to southern *I. elegans* larvae, almost half of the northern larvae died at 32°C. Similar as for *E. cyathigerum* larvae, these northern larvae never experience temperatures up to 30°C (Lake Model Flake, 2009), potentially making them more sensitive to warming.

Growth rate and growth-related traits

In general higher temperatures had positive effects on growth rate, even at lethal temperatures (Chapter I-III, V, see Figure 1), indicating that the optimal temperature for growth was above the environmental temperature (see also Angilletta *et al.,* 2010; Nilsson-Örtman *et al.,* 2012). This was true both for *Enallagma* and all *Ischnura* populations (from French, Belgian and Swedish latitudes) (Chapter I-III, V). Shifting the optimal temperature above the environmental temperature may be adaptive to exploit short infrequent periods of higher temperatures (Kingsolver, 2000), and to avoid negative effects during transient exposure to extreme temperatures (Martin & Huey, 2008). Following the metabolic theory of ecology (Gillooly *et al.,* 2001), metabolic rate

was higher in larvae exposed to higher temperatures (Chapter III, V, but see Chapter I, Figure 1). This increase probably causes higher cellular maintenance costs (Lemoine & Burkepile, 2012). To compensate for these costs, energy uptake should increase faster than the metabolic rate (Rall *et al.*, 2010; Lemoine & Burkeplile, 2012). In accordance, there was a larger increase in food intake compared to metabolic rate under warming (Chapter III, Figure 1), and a temperature-induced increase in growth efficiency (see also Karl & Fisher, 2008; Stoks *et al.*, 2012; Culler *et al.*, 2014). Together, the increased food intake and growth efficiency may explain the observed higher investment in energy storage under warming (Chapter I-III, V, Figure 1). However, in food limited environments, where the energy demands are likely to increase faster than the energy uptake with increasing temperature, this beneficial effect on energy storage is expected to be smaller or even absent. In an extreme example this can lead to starvation and population extinction (Boukal *et al.*, 2019).

Elemental composition

Several studies have shown (often contrasting) temperature-induced changes in elemental body composition (e.g. Cotner *et al.*, 2006; Liess *et al.*, 2013; Schmitz, 2013; Zhang *et al.*, 2016, 2018a) and also in my thesis it turned out to be a sensitive, yet variable predictor for warming. For beneficial warming, an often used framework is the growth rate hypothesis (GRH) (Elser *et al.*, 1996). The GRH predicts an increased growth rate under warming which is associated with a higher RNA:DNA ratio (lower C:P and N:P); together with an increased synthesis of N-rich proteins (Elser *et al.*, 1996, Figure 1). These predictions corresponded with the response in *E. cyathigerum* larvae in Chapter V following a temperature increase of 4°C (IPCC scenario RCP 8.5, IPCC, 2013).

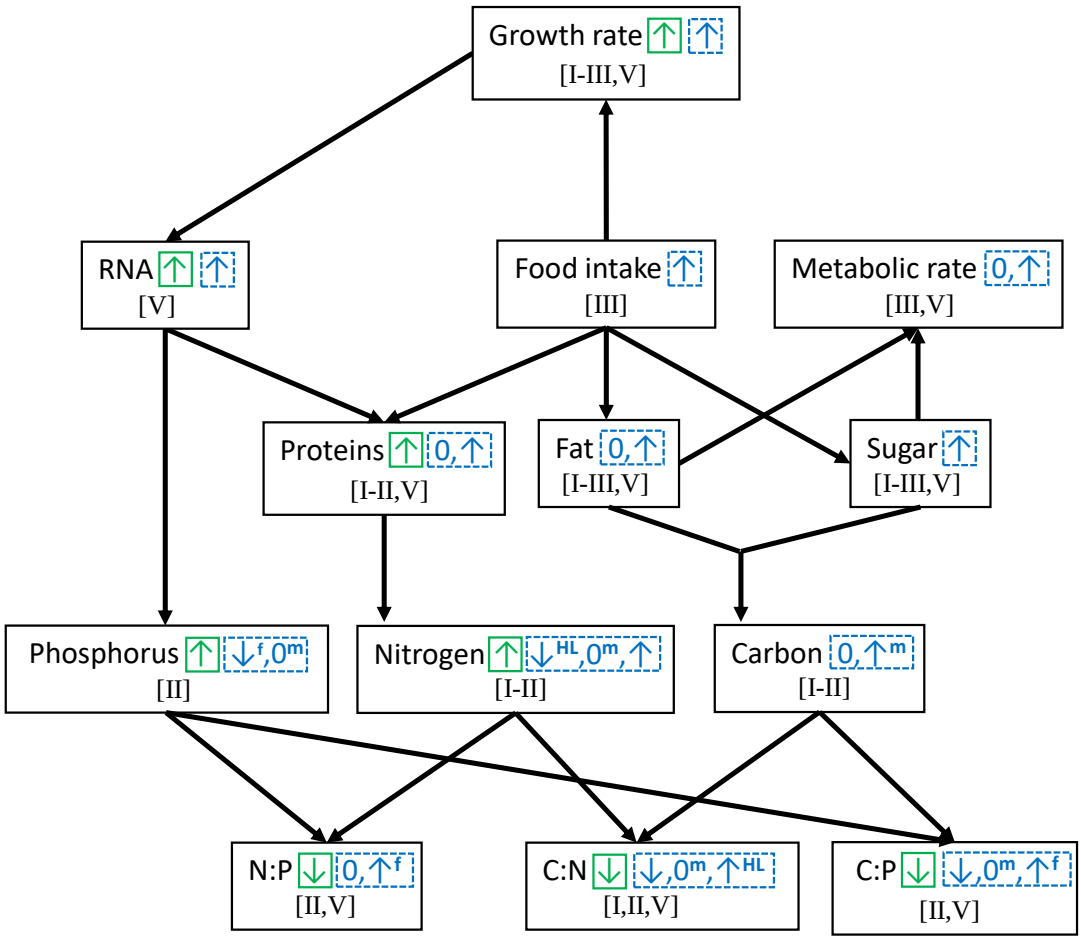

Figure 1. Schematic overview of the responses to warming and their link to the growth rate hypothesis. The arrows in the full-lined boxes present the predictions following the growth rate hypothesis (Elser *et al.*, 1996; Watts *et al.*, 2006). The arrows in the dashed-lined boxes present the observed results. An upward (downward) arrow indicates an increase (decrease) or 0 that no change was observed under warming. A superscript indicates that the result was only present in a specific sex or latitude, with m: males; f: female and HL: high-latitude. The Latin number indicates the chapter in which the result was found.

However, as mentioned above, the stoichiometric response to warming is variable (see Table 1) and shows deviations from the predictive framework (e.g. Zhang *et al.*, 2016, 2018b). Similarly, in Chapters I and II, I did not observe the by the GRH predicted increase in P under warming and the increase in N was not associated with an increased growth rate (see Figure 1). Three confounding factors may have played a role. Firstly, for the results obtained in Chapter II, I performed a mesocosm experiment with more natural conditions, while the experiment presented in Chapter V was a laboratory study under standardized conditions. It could be that the natural fluctuations in temperature and photoperiod, the different feeding conditions and prey items and

presence of conspecifics in the mesocosm could have interacted with the warming effects on the elemental composition of the damselflies, resulting in deviations from the predictions by the GRH. A second confounding factor is the developmental stage and the sex of the individuals. Indeed, while Chapter V focused on damselfly larvae, Chapter II I studied the carry-over effects of a temperature increase experienced in the larval stage on adult elemental composition and this separately for males and females. More specifically, under mild warming males invested in C-rich fat and sugars, which are needed for an increased flight endurance (important for mating in male damselflies, e.g. Tüzün *et al.,* 2017). Females on the other hand, invested more in N which seemed to be associated with a higher investment in immune function (e.g. melanin: Prokkola *et al.,* 2013) and egg production (Adamo & Lovett, 2011) under warming. The P content of females tended to decrease (as observed for *E. coli* (Cotner *et al.,* 2006)), which can be explained by an increased metabolic efficiency under warming (Farewell & Neidhardt, 1998). Thirdly, the latitude of origin linked with thermal adaptation (Chapter I) could have played a role. While Chapter V studied Belgian *I. elegans* larvae, Chapter I quantified thermal response curves for high-latitude and low-latitude *I. elegans* populations. At the high-latitude, the N content consistently decreased and as a result the C:N ratio consistently increased with temperature. At the low latitude, exactly the opposite linear stoichiometric responses occurred.

Predation risk

There is increasing interest in the nonconsumptive effects of predators on their prey as these may be as important as consumptive effects in shaping prey population dynamics (Preisser *et al.,* 2005; Creel & Christianson, 2008; Zanette *et al.,* 2014). Furthermore, nonconsumptive effects may scale up to and affect community structure (Peacor *et al.,* 2012) and ecosystem functions (Hawlena *et al.,* 2012). I studied the nonconsumptive effects of predation on life history, behaviour, physiology and stoichiometry of *E. cyathigerum* larvae (Chapter IV-VII).

Mortality

While the 'fear' of predation risk has been shown to induce mortality in prey (McCauley *et al.,* 2011; Gehr *et al.,* 2018) including damselflies (Stoks, 2001; Siepielski *et al.,*

2014), I did not observe such pattern. Pathways how fear may cause mortality include for example a reduction in food intake resulting in starvation (McCauley *et al.*, 2011). In addition also severe predator-induced physiological stress may cause mortality. Although I detected both a reduced food intake (Chapter IV) and physiological stress (see below, Chapter IV-VI) under predation risk, these effects were not strong enough to cause mortality.

Growth rate and growth-related traits

As predicted by theory (Abrams & Rowe, 1996) and widely documented (Benard, 2004), damselfly larvae decreased their growth rate under predation risk (Chapters IV-VI, Figure 2). A combination of a reduced food intake and efficiency to convert food into biomass has been proposed as a possible mechanism (e.g. McPeek *et al.*, 2001; Stoks *et al.*, 2005b; Trussell *et al.*, 2006; Miller *et al.*, 2014). This reduced allocation of energy towards growth is expected due to the upregulation of energetically costly defence mechanisms, such as the upregulation of Hsp70 levels (e.g. Slos & Stoks, 2008; Janssens & Stoks, 2013a). In accordance, predation risk-exposed larvae had a lower food intake (Chapter IV, Figure 2), yet no reduced conversion efficiency (Chapter VI). The assimilation efficiency was, however, reduced under predation risk (Chapter VI), which means that these larvae obtained less energy from their food. Together, both a reduced food intake and assimilation efficiency could have caused the observed reduction in energy storage in the presence of predation risk (Chapter IV-VI, Figure 2; see also Stoks *et al.*, 2005b; Thaler *et al.*, 2012).

Important to acknowledge is that the response to predation risk is not static, but can vary in function of the exposure duration (Thaler *et al.*, 2012). In Chapters V and VI, the chronic exposure to predation risk (seven and nine days, respectively), caused the expected decrease in growth rate. However, in Chapter IV growth rate was reduced after the short-term exposure period of three days and not after six or nine days. This was surprising, since after nine days of exposure to predation risk the larvae showed a reduced food intake and increased energy consumption. Possibly, larvae reallocated energy for defence or immune function (e.g. Stoks *et al.*, 2006b) towards growth as a strategy to escape from an unfavourable environment. Since damselflies have a complex

life cycle, they can escape predation risk encountered during the larval phase by emerging into terrestrial adults.

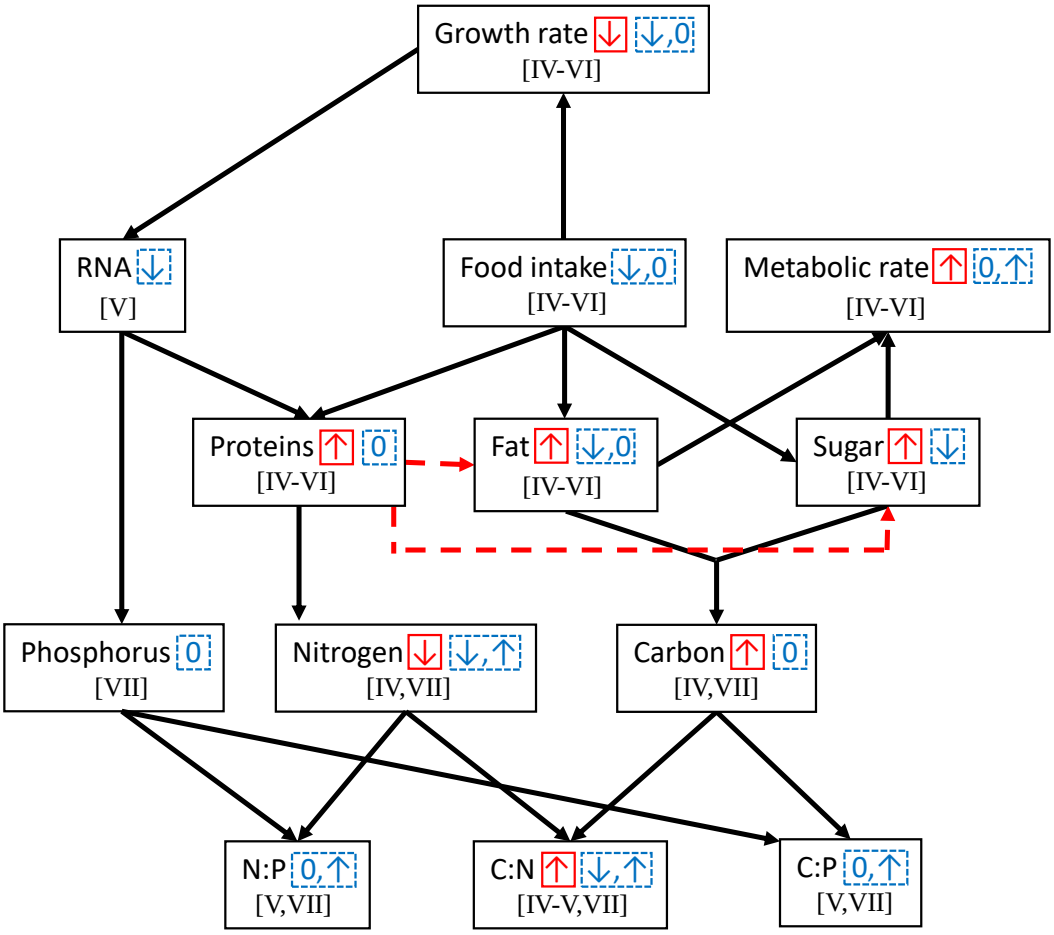

Figure 2. Schematic overview of the responses to predation risk and their link to the general stress paradigm. The arrows in the full-lined boxes present the predictions following the general stress paradigm (Hawlena & Schmitz, 2010a). Note that the dotted lines represent the expected gluconeogenesis process. The arrows in the dashed-lined boxes present the observed results. An upward (downward) arrow indicates an increase (decrease) or 0 that no change was observed under predantion risk. The Latin number indicates the chapter in which the result was found.

Elemental composition

In several chapters I focused on the predator-induced physiological stress response, summarized under the general stress paradigm (GSP) (Hawlena & Schmitz, 2010a). According to the GSP, under exposure to predation risk, prey increase their metabolic rate, thereby mobilizing energy for predator escape and diverting energy away from growth toward the upregulation of defence mechanisms (see also above). In line with the GSP, ETS activity (a proxy for metabolic rate) increased when animals were exposed to predator cues (Chapters IV, V, Figure 2). To meet the higher energetic demands prey

are expected to mobilize C-rich molecules such as fat and sugar. To obtain even more glucose, prey can convert N-rich proteins into C-rich macromolecules (gluconeogenesis) and release the excess nitrogen (Hawlena & Schmitz, 2010a, see dotted red lines Figure 2). However, in contrast to these predictions, the levels of C-rich biomolecules did not increase, neither did the protein content decrease (Chapters IV-VI, Figure 2).

Changes in elemental body composition in response to predation risk have been reported before (e.g. Hawlena & Schmitz, 2010b), but often do not follow the predictions of the GSP (e.g. Dalton & Flecker, 2014; Zhang *et al.,* 2016, see Table 1). In line with the GSP (Hawlena & Schmitz, 2010a), the body C:N ratio increased under predation risk (Chapters IV, V, Figure 2). However, although the C-rich macromolecules decreased under predation risk, this was not associated with a decreased C content (Chapters IV, VII). Moreover, predation risk did not affect the protein content of the damselfly larvae (chapter IV-VI, Figure 2), yet the N content did change (Chapters IV, V, VII, Figure 2) when exposed to predation risk. Therefore, my results suggest that other mechanisms can drive the stoichiometric effects under predation risk, including an increased investment in predator-induced morphological defences (e.g. Costello & Michel, 2013). It is plausible that under predation risk damselfly larvae increased the thickness of their exoskeleton to reduce the attack efficiency of the predator (e.g. Rabus *et al.,* 2013). This may be associated with more chitin, a polysaccharide with a relative high C:N (5:1) (Sterner & Elser, 2002), resulting in an increased body C:N. Other non-exclusive explanations are that under predation risk the excretion of N (Sterner & Elser, 2002; Thaler *et al.*, 2012; Dalton & Flecker, 2014) or the investment in egg production changed (e.g. Zhang *et al.,* 2016). There was some evidence for the excretion mechanism since animals exposed to predation risk increased the excretion of N (Chapter VII). A differential investment in eggs is probably less important in my thesis, given I studied the effects of predation risk in the larval stage.

Pesticide exposure

Mortality

Freshwater ecosystems are suffering declines in biodiversity attributed to the contamination by pesticides. Exposure to the pesticide chlorpyrifos (2 µg/L) was highly stressful for *E. cyathigerum* larvae, as indicated by the high mortality (49%) (Chapter VI). Although the applied concentration is relatively high, it is realistic for water bodies near agricultural areas where concentrations ranging from 10 to 100 µg/L have been documented (Bernabò *et al.,* 2011). Also other studies on damselflies have shown considerable mortality at concentrations ranging from 1-3 µg/L (Janssens & Stoks, 2013b; Dinh Van *et al.,* 2014), suggesting that damselflies are sensitive species for chlorpyrifos pollution.

Growth and growth-related traits

In line with previous studies (e.g. Widder & Bidwell, 2006; Huynh & Nugegoda, 2012) chlorpyrifos exposure reduced the growth rate (Chapter VI, Figure 3). As shown previously (Campero *et al.,* 2007), this was driven by a reduced conversion efficiency in pesticide-exposed larvae. This might be the result of an upregulation of energetically costly defence and detoxification mechanisms such as Hsp70 (e.g. Scheil *et al.,* 2010; Janssens & Stoks, 2013b) and glutathione S-transferase (GST) (e.g. Cinzia *et al.,* 2006; Janssens & Stoks, 2013b; Kim *et al.,* 2014). The chlorpyrifos-induced shift in allocation of energy away from growth and toward defence was confirmed at the cellular level. More specifically, pesticide exposure caused an increase in metabolic rate and decreases in fat and sugar contents, eventually resulting in a decreased total net energy budget of the larvae (Verslycke *et al.,* 2004) (Chapter VI, Figure 3).

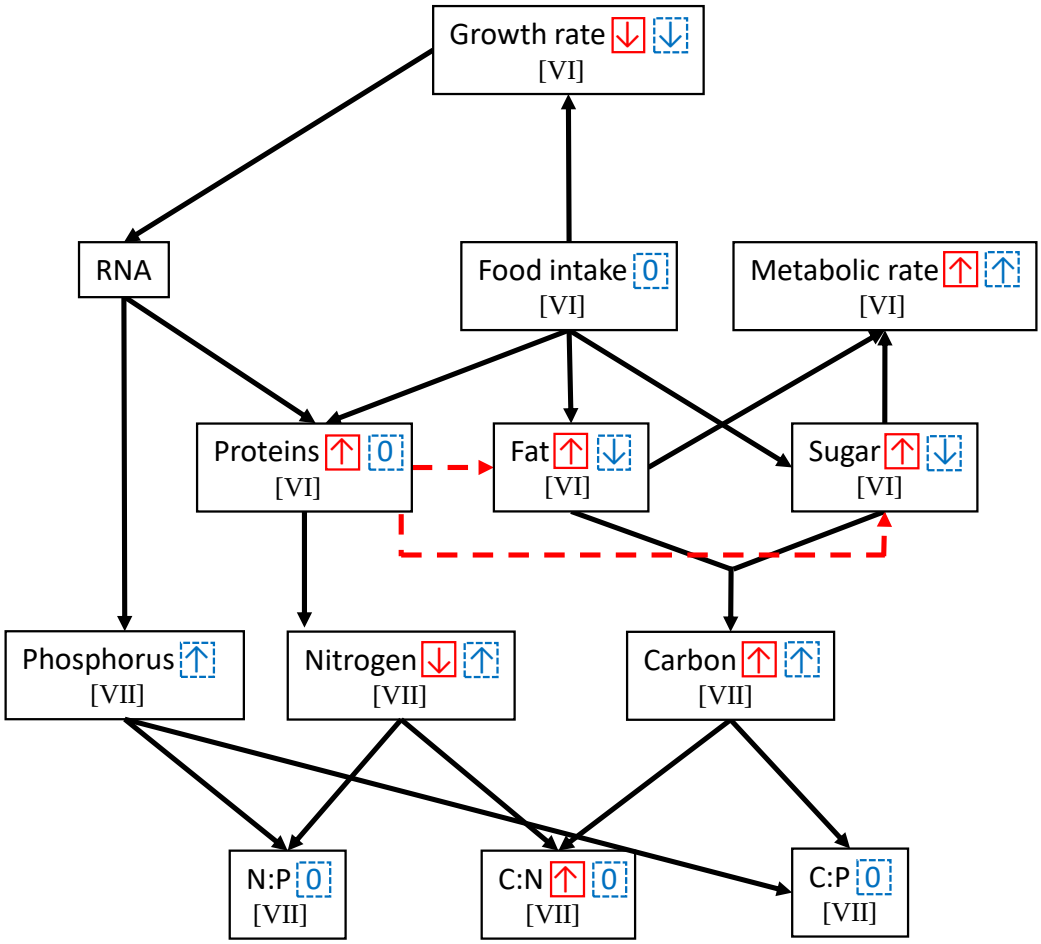

Figure 3. Schematic overview of the responses to the pesticide chlorpyrifos and their link to the general stress paradigm. The arrows in the full-lined boxes present the predictions following the general stress paradigm (Hawlena & Schmitz, 2010a). Note that the dotted lines represent the expected gluconeogenesis process. The arrows in the dashed-lined boxes present the observed results. An upward (downward) arrow indicates an increase (decrease) or 0 that no change was observed under exposure to the pesticide chlorpyrifos. The Latin number indicates the chapter in which the result was found.

Elemental composition

Although the GSP was developed to predict changes in elemental composition, it can be applied for all stressors that induce an increase in metabolic rate to fuel energetically costly defence processes (Hawlena & Schmitz, 2010a), including pesticides (for experimental studies: Ek *et al.*, 2015; Janssens *et al.*, 2017). Some patterns in my thesis, and the two other pesticide studies (Ek *et al.*, 2015; Janssens *et al.*, 2017), supported the GSP predictions (see Table1), although the underlying mechanisms were often different (see Figure 3). In line with the GSP, the C content of exposed larvae increased (Chapter VII). However, this was not associated with an increase in C-rich macromolecules.

Possibly, animals tried to reduce the uptake of chlorpyrifos by thickening (hence making it less permeable, Zhang *et al.,* 2008) their exoskeleton. When done by incorporating more chitin, a molecule with a relative high C:N (5:1) content (Sterner & Elser, 2002), this may increase the C and potentially the N content. An increased chitin content could also have contributed to the chlorpyrifos-induced increase in N content, next to the pesticide stress-induced upregulation of stress proteins (Sørensen *et al.,* 2003). In addition, an increased stress protein synthesis could potentially also explain the observed increase in P content under chlorpyrifos exposure (Chapter VII). More specifically, the synthesis of stress proteins has been associated with an upregulation of P-rich RNA (Lindquist, 1986; Goto *et al.,* 1998).

Not only the elemental body composition changed under chlorpyrifos exposure, also the elemental composition of the excreta changed with an increased release of C and a trend for an increased release of N in pesticide-exposed animals. The latter is in accordance with the GSP and could be attributed to increased gluconeogenesis (Hawlena & Schmitz, 2010a). The increase in C in the excreta might seem surprising, but is likely caused by the need to maintain internal homeostasis (Sterner & Elser, 2002; Persson *et al.,* 2010). Indeed, also in the body the increase in C was the strongest, leading to an imbalance between the different elements, which likely was managed by excreting the excess nutrients (in this case C).

Table 1. Summarizing table of the available studies on the effects of warming, predation risk and pesticide exposure on body stoichiometry.

Environmental condition	Study species	C, N, P contents		C:N:P ratios		References
Temperature						
		High-latitude	*Low-latitude*	*High-latitude*	*Low-latitude*	
17°C – 36°C	*Ischnura elegans*	C% – N% ↓	C% – N% ↑	C:N ↑	C:N ↓	Chapter I
		Males	*Females*	*Males*	*Females*	
Ambient and Ambient +4°C	*Ischnura elegans*	C% ↑ N% – P% –	C% (↑) N% ↑ P% (↓)	C:N – C:P – N:P –	C:N (↓) C:P ↑ N:P ↑	Chapter II
20°C and 24°C	*Enallagma cyathigerum*			C:N ↓ C:P ↓ N:P –		Chapter V
18°C and 23°C	*Rana temporaria* (Arctic and Boreal)	C% ↓ N% ↑ (Arctic); ↓(Boreal) P% ↑				Liess *et al.,* 2013
20°C and 24°C (as stressor)	*Daphnia magna*	C% ↓ N% ↓ P% –		C:N ↑ C:P – N:P –		Zhang *et al.,* 2019
20°C and 24°C (as stressor)	*Daphnia magna*	C% ↓ N% ↓ P% ↓		C:N ↓ C:P – N:P ↑		Zhang *et al.,* 2016
Ambient and Ambient +2.5-3°C (as stressor)	*Melanoplus femerrubrum*			C:N ↑		Schmitz, 2013
Seasonal increase	*Daphnia* populations			C:P ↓		Prater *et al.,* 2018
Predation risk						
Nonconsumptive	*Enallagma cyathigerum*	C% – N% ↓		C:N ↓		Chapter IV

Nonconsumptive	*Enallagma cyathigerum*		C:N ↑ C:P ↑ N:P –	Chapter V
Nonconsumptive	*Enallagma cyathigerum*	C% – N% ↑ P% –	C:N ↓ C:P – N:P –	Chapter VII
Nonconsumptive	*Daphnia magna*	C% ↓ N% ↓ P% ↓	C:N ↓ C:P – N:P ↑	Zhang *et al.,* 2016
Nonconsumptive	*Melanoplus femerrubrum*	C% ↑ N% ↓	C:N ↑	Hawlena & Schmitz, 2010b
Nonconsumptive	*Poecilia retuculata*	C% ↓ N% –	C:N ↓	Dalton & Flecker, 2014
Nonconsumptive	*Hyla versicolor*	C% ↑ N% – P% –	C:N – C:P ↑ N:P ↑	Costello & Michel, 2013
Nonconsumptive	*Enallagma cyathigerum*	C% ↓ N% ↓ P% ↓	C:N – C:P ↑ N:P ↑	Janssens *et al.,* 2017
Pesticide				
Chlorpyrifos	*Enallagma cyathigerum*	C% ↑ N% ↑ P% ↑	C:N – C:P – N:P –	Chapter VII
Chlorpyrifos	*Enallagma cyathigerum*	C% ↓ N% ↓ P% ↓	C:N – C:P – N:P –	Janssens *et al.,* 2017
Lindane	*Daphnia magna*		C:N ↓	Ek *et al.,* 2015

Interactions between predation risk and temperature or pesticide exposure

Interactive effects between multiple environmental conditions are omnipresent and are important to take into account when assigning the effects of the environmental conditions in nature (Gunderson *et al.*, 2016; Jackson *et al.*, 2016). There are three main types of interactions: additive, synergistic and antagonistic, but it is difficult to predict what kind of interaction will occur. One example of a confounding factor is the trait type: in general stressors show additive effects at the individual or population level, while synergistic effects most often occur at the physiological level (Côté *et al.*, 2016; for a case study see e.g. Janssens & Stoks, 2013a). Another factor influencing the type of interaction is the magnitude of the stressors: when one of the stressors is causing a very strong stress response, it is likely that the second stressor will have no additional impact, resulting in the combined stressor effect to be equal to that of the dominant stressor (Schäfer & Piggott, 2018). The exact mechanisms causing interactions between environmental conditions are not yet understood, but it is suggested that energetic trade-offs play a role (Qin *et al.*, 2011). In my thesis I studied the combined impact of warming and predation risk and of pesticide exposure and predation risk, with a focus on life history and physiology linked to bioenergetics and stoichiometry (see Table 2).

Table 2 Schematic overview of the multiple stressor effects observed in this thesis with 0 = additive, + = synergism, - = antagonism: revered interaction. The Latin number indicates the chapter in which the result was found.

	Predation risk × Temperature		Predation risk × Pesticide	
Response variable	Result	Chapter	Result	Chapter
Life history				
Survival	0	V	0	VI
Growth rate	-	V	0	VI
Bioenergetics				
Food intake				
ETS activity	+	V	0	VI
Protein content	0	V	0	VI
Fat content	-	V	0	VI
Sugar content	-	V	0	VI
Stoichiometry				
C content			0	VII
N content			0	VII
P content			0	VII
C:N ratio	-	V	0	VII
C:P ratio	-	V	0	VII
N:P ratio	0	V	0	VII

When *E. cyathigerum* damselfly larvae were jointly exposed to predation risk and mild warming, predation risk effects were stronger at the higher temperature. Both mild warming and predation risk increased the metabolic rate (Chapter V), with the predation risk induced increase being strongest at 24°C (synergism; Chapter V). Since an increase in metabolic rate is energetically costly (Lemoine & Burkepile, 2012), this cost was also the highest under the combined exposure. Indeed, the predator-induced reductions in growth rate, RNA:DNA and energy storage were stronger under warming (Chapter V). In line with this, stronger predator-induced growth reductions under mild warming have been observed before in Chinook salmon (Kuehne *et al.*, 2012) and in the damselfly *Enallagma versperum* (Culler *et al.*, 2014). Both studies also attributed this to the higher

metabolic demands under combined exposure. Further, the strong reductions in growth rate and RNA:DNA ratio could explain the observed highest increase in body C:P ratio when exposed to both predation risk and warming. My results indicate that effects of predation risk might enhance under global warming, possibly resulting in shifts in ecological communities (see also Miller *et al.*, 2014), including a stronger top-down control by predators of their prey under warming (e.g. Kratina *et al.*, 2012).

In contrast to several previous studies that documented synergistic interactions when combining predation risk and pesticide exposure (e.g. Relyea & Mills, 2001; Trekels *et al.*, 2011; Janssens & Stoks, 2013a; but see e.g. Pestana *et al.*, 2009), I only observed additive effects. One of the key factors influencing the effects of contaminants and the possible interactions with other stressors is the dose (e.g. Eggen *et al.*, 2004; Kahru & Dubourguier, 2010; Ritz, 2010). I used a relatively high chlorpyrifos concentration, which killed almost half of the damselfly larvae regardless of the predation risk exposure. According to the dominance model by Schäfer and Piggot (2018), the stronger effects of one stressor could mask the effects of a second stressor. Moreover, it is likely that the weaker larvae (that died, hence could not be tested for physiology) would have shown a stronger physiological response to the pesticide and potentially a synergistic interaction (such as observed for example by Janssens & Stoks (2013a) who used a non-lethal pesticide concentration). It is indeed suggested that synergistic interactions are potentially more easily detected in weaker individuals, based on their energetic status (e.g. Relya & Mills, 2001; Qin *et al.*, 2011).

Limitations of current study and suggestions for future research

I looked at single and combined effects of several environmental factors (temperature, predation risk and pesticide exposure), and this at different levels of organization (organismal, cellular and ecosystem function), and for different trait types (life history, behaviour, physiology and stoichiometry). I thereby tried to link the experimental results with predictions from theoretical models (GSP, GRH, hotter-is-better-hypothesis). Several limitations and suggestions for future research can be formulated based on my thesis.

In Chapter I, I used the space-for-time approach to assess the gradual influence of gradual thermal evolution on body composition. Yet, this approach has some intrinsic limitations. For example, the current warm-adapted populations had plenty of time to evolve, while future adaptive evolution under global warming probably has to occur in a shorter time period. Moreover, there is also no guarantee that the current cold-adapted populations contain enough genetic variation to allow further evolution. Further, besides temperature the space-for-time substitution does not take any other important characteristics (e.g. photoperiod) into account that differ between the regions. Although it is important to keep this limitation in mind when drawing general conclusions, the space-for-time substitution has been proven valid for studying microevolutionary processes such as those related to global warming (reviewed in Verheyen *et al.*, 2019).

The pesticide dose used was lethal for my study species. Therefore, when combined with an additional stressor (predation risk), the second stressor was not able to add to the combined effect. This resulted in the combined effect being equal to the pesticide effect in isolation (as predicted by the dominance model by Schäfer & Piggott, 2018), thereby precluding the detection of a synergistic interaction with predation risk (as shown before for example by Relyea & Mills, 2001). Future work would benefit from studying responses to multiple chlorpyrifos concentrations, ranging from sublethal to lethal concentrations to investigate how predation risk mediates these effects in a concentration-dependent way. For example, it recently has been shown that the interaction type between a pesticide and daily temperature fluctuations depends on pesticide concentration (Delnat *et al.*, 2019).

Not only the number and magnitude of stressors organisms are exposed to may vary, but also the exposure duration, ranging from acute short-term and chronic long-term stress. Given that the stress response is energetically costly (Hawlena & Schmitz, 2010a; Thaler *et al.*, 2012), it is therefore expected that organisms will respond differently to a short- versus long-term stress exposure (as show by Thaler *et al.*, 2012 for predation risk). In my thesis I also studied in one chapter (Chapter IV) both acute and chronic exposure to predation risk. Hereby I detected that the predator-induced responses differed depending on the exposure time, and that this was associated with the energetic status. Also the presence of pesticides in nature can be acute or chronic and

they also affect the energetic status of organism. As a consequence, exposure duration dependent differences in stress response are also expected under pesticide exposure and would be very relevant for future risk assessment to implement.

Besides exposure time, also development stage can be important, for example due to differential investment in life strategies, leading to differential needs of nutrients. For example in damselflies the larval stage is meant for growth, while in the adult stage the focus is on dispersal and reproduction (Stoks & Cordoba-Aguilar, 2012). In this thesis I always (except in Chapter II) studied the last larval stage. There are several arguments why this is a relevant stage to study. Methodological reasons include the fact that this stage is easy to recognize, lasts long enough to study chronic stressor effects and that animals in this stage are large enough to obtain enough tissue to do physiological analyses. In addition, there are also several ecological reasons. During the final larval stage most of the mass increase takes place, making it a sensitive stage to study the impact of energy-related stressors. Moreover, damselfly larvae of both study species occupy this stage in spring to summer, which corresponds to the period that potential heat waves could occur and the period that pesticides are applied. Yet, despite the larval stage being very relevant to study, damselflies have a complex life cycle, whereby the larval aquatic stage is followed by a terrestrial adult stage. As a result, damselflies contribute to the nutrient flux from water to land, what makes their adult traits also interesting to study. In chapter II, I studied how mild warming during the aquatic larval stage altered the body stoichiometry of the terrestrial adults. I found that the effects of warming on body stoichiometry differed from these observed in the larval stage (quantified in Chapter V). However, the design between both studies differed (mesocosm study in Chapter II vs laboratory study in Chapter V), making it difficult to draw any conclusions about the difference in stoichiometric response between the larval and adult stage. Therefore, it would be interesting to quantify both larval and adult body stoichiometry (and the underlying energy-related traits) in the same study to gain more insight in the effects of metamorphosis and the potential difference in stress response between the two stages.

For both predation risk and pesticide exposure, I argued, based on previous studies, that the reduction in energy availability and the associated decrease in energy

allocation to growth is caused by the upregulation of defence mechanisms. However, I did not actually quantify these defence mechanisms. It would be very interesting to measure defence mechanisms such as the expression levels of Hsp70, the activity of detoxification enzymes (e.g. GST) and enzymes related with oxidative stress (e.g. SOD and CAT) and directly look for correlations with growth, bioenergetic and stoichiometric responses.

A well-known concept in ecological stoichiometry is "you are what you eat" (Frost *et al.,* 2005; Persson *et al.,* 2010). For example, many studies documented that low-quality food (i.e. high C:P ratio) could affect body stoichiometry, and this in many different taxa (crustacean: e.g. Meunier *et al.,* 2012, 2016, mollusks: e.g. Fink & von Elert, 2006, insects: e.g. Perkins *et al.,* 2004, fish: e.g. Vrede *et al.,* 2011, birds: e.g. Velthuis *et al.,* 2017). In my thesis I used several different food types, and often did not allow the damselfly larvae to select food, which potentially could have contributed to the inconsistency of stoichiometric results. Therefore, it would be interesting to quantify and take into account the body composition of the supplied food.

Stoichiometric responses to stressors have the possibility to cascade through the food web (e.g. Hawlena *et al.,* 2012). I did a first test on a small scale how exposure of damselfly larvae to a pesticide and predation risk may affect an ecosystem system, by looking at how stress-induced changes in stoichiometric composition of the excreta affected primary production (algal growth, Chapter VII). Although I could demonstrate stressor-induced changes in algal growth in microcosms, follow-up studies in larger mesocosms are needed. Not only further research on primary production is needed, but also on other processes such as decomposition rates. Furthermore, given the complex life cycle of damselflies, it would be interesting to study how stressor-induced changes in elemental composition of prey (hence nutritional value) would affect the population dynamics of their predators both in aquatic and terrestrial systems and especially their potential coupling.

Take-home messages

I do have two important take-home messages based on my PhD thesis: one with regard to stoichiometric responses and another one related to energetic traits, and more specifically the CEA, in the stress response.

Stoichiometric mechanisms

In general, only few studies looked at the stoichiometric responses to environmental conditions and they mainly focussed on the growth rate hypothesis or the general stress paradigm. However, several theories have been put forward to predict the stoichiometric changes and in this thesis I made a first attempt to consider them all. Important drivers expected to mediate stoichiometric changes are growth rate, body size and metabolic rate and efficiency (Elser *et al.,* 1996; Woods *et al.,* 2003; Cross *et al.,* 2015). Moreover, since ecological stoichiometry links the elemental body composition of organisms to the most important macromolecules (proteins, fat, sugar, RNA) (Sterner & Elser, 2002), changes in the macromolecules are expected to determine the stoichiometric changes. Yet, although these theories use macromolecules as surrogates for the elemental composition (Sterner & Elser, 2002; Hawlena & Schmitz, 2010a), they did not empirically test whether these were actually correlated.

My results indicate that all theories (e.g. GRH and GSP) fail to predict the stoichiometric response to changes in environmental conditions, and that changes in elemental body composition are poorly linked to changes in macromolecules (Chapter I, see also Wilder & Jeyasingh, 2016; Zhang *et al.,* 2018b). These observations are in accordance with the very few other studies investigating both body stoichiometry and underlying macromolecules (Zhang *et al.*, 2016, 2018b; Janssens *et al.*, 2017). In general, the studies reporting how environmental conditions affect body stoichiometry provide mixed results, making it difficult to draw general conclusions.

Several confounding factors have been hypothesized, which could contribute to the stoichiometric changes, but that are now not included in the theory. For example, stoichiometric response could strongly depending on the latitude of origin, linked with thermal adaptation (Chapter I), sex (Chapter II) and development stage (Chapter II). But

also other traits should be included in the theory such as fecundity (e.g. Zhang *et al.*, 2016), morphology (e.g. Costello & Michel, 2013) and behaviour (Guariento *et al.*, 2018; Trakimas *et al.*, 2019) and nutrient supply (Frost *et al.*, 2005; Persson *et al.*, 2010) to provide a more comprehensive view on the stoichiometric responses. To summarize, this illustrates that the current theoretical models that are based on the main macromolecules and the excretion rates of elements, are too incomplete to make reliable predictions. Therefore, an extensive analysis of as many molecules as possible, could already give more information about the observed stoichiometric changes. I made a first step by also investigating chitin and melanin, two molecules functionally linked to temperature and important for the morphological defence against stressors.

Cellular energy allocation

The cellular energy allocation corresponds to the net energy budget of an organism and can be used as a general indicator of stress. Currently it is, however, mostly and most successfully used for pollutant stress, whereby a decrease in CEA is an indicator for stress (e.g. De Coen & Janssen, 1997, 2003; Smolder *et al.*, 2004; Novais *et al.*, 2013; Aderemi *et al.*, 2018). A few studies looked at the impact of other stressors on the CEA (e.g. Wang *et al.*, 2012; Ferreira *et al.*, 2016; Kühnhold *et al.*, 2017), but especially studies focusing on multiple stressors are scarce (e.g. Gandar *et al.*, 2017). Although I only calculated the CEA once (Chapter VI), I studied the underlying components, energy availability and energy consumption, for the three environmental conditions used in this thesis. I documented that these components were sensitive and reliable predictors for the effects of the environmental conditions on growth. This was in line with the recent documented positive correlation between CEA and growth rate (reviewed by Goodchild *et al.*, 2019). Besides with growth rate, the CEA also correlates with other individual (e.g. body size) and population (e.g. intrinsic growth rate) traits (De Coen & Janssens, 2003). Moreover, the CEA has been shown to be a more accurate biomarker to indicate stress compared to the scope for growth, which is more variable and based on assimilation and whole-body respiration (Verslycke *et al.*, 2004). Together, these are strong arguments for using the CEA as indicator for higher biological levels (De Coen & Janssen, 2003). In addition, by studying and integrating both energy consumption and

availability, the CEA fits the recommendation to use energy balances as a common denominator to determine (combined) effects of stressors (Sokolova, 2013).

In general, my results underline that the CEA is a valuable and sensitive biomarker to evaluate the impact of environmental conditions. This was not only the case for pollutant stress, but also for other environmental conditions like temperature and, for the first time, predation risk. These are environmental conditions with a very different mode of action and it is therefore likely that CEA will also be useful as indicator for changes in other environmental conditions not studied in this thesis such as parasites, UV-radiation, nanometals…

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