Negative bioenergetic responses to pesticides in damselfly larvae are more likely when it is hotter and when temperatures fluctuate

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Abstract: To make more realistic predictions about the current and future effects of pesticides, we need to better understand physiological mechanisms associated with the widespread higher toxicity of many pesticides under increasing mean temperatures and daily temperature fluctuations (DTFs). One overlooked, yet insightful, mechanism are bioenergetic responses as these provide information about the balance between energy gains and costs. Therefore, we studied how the bioenergetic responses to the insecticide chlorpyrifos were affected by a higher mean temperature and a higher DTF in *Ischnura elegans* damselfly larvae. To quantify bioenergetic responses we measured energy availability (Ea), energy consumption (Ec) and total net energy budget (cellular energy allocation, CEA). Exposure to chlorpyrifos considerably reduced CEA values when a high mean temperature was combined with a high DTF (up to -18%). Notably, chlorpyrifos had little effect on CEA at a constant 20 °C, meaning that the bioenergetic impact of chlorpyrifos would have been underestimated if we had only tested under standard testing conditions. The chlorpyrifos-induced reductions in CEA under warming were driven by reductions in Ea (up to -16%, mainly through large reductions in sugar and fat contents) while Ec was unaffected by chlorpyrifos. Treatment groups with a lower CEA value showed a higher mortality and a lower growth rate, indicating bioenergetic responses are contributing to the higher toxicity of chlorpyrifos under warming. Our study highlights the importance of evaluating the effects of pesticides under an increase in both mean temperature and DTF to improve the ecological risk assessment of pesticides under global warming.

Keywords: "Climate-induced toxicant sensitivity" (CITS) concept; Multiple stressors; Net energy budget; Organophosphate pesticide; Spatial gradient; Temperature variability

Introduction

The ecological risk assessment of pesticides is currently failing as aquatic biodiversity is declining at pesticide concentrations regarded as safe by legislation (Beketov et al., 2013; Peters et al., 2013). One reason may be that the effects of toxicants on organisms are standardly evaluated under optimal laboratory conditions. However, many pesticides become more toxic at higher mean temperatures, which is captured by the "climate-induced toxicant sensitivity" concept (Hooper et al., 2013; Moe et al., 2013; Noyes et al., 2009; Noyes and Lema, 2015). Therefore, one key challenge for the ecological risk assessment of pesticides is to include the effects of temperature (and global warming) on pesticide toxicity (Landis et al., 2014; Noyes and Lema, 2015; Van den Brink et al., 2018). This is important both for current risk assessment, as large temperature differences exist across spatial gradients, and for future risk assessment as under global warming temperatures will rise (Landis et al., 2014).

Current risk assessment of pesticides ignores a key aspect of temperature in nature: daily temperature fluctuations (DTF, Colinet et al., 2015). Increases in these fluctuations may result in daily exposures to very high temperatures, and thereby more strongly affect fitness and population dynamics than increases in mean temperatures (Paaijmans et al., 2013; Vasseur et al., 2014). This is especially the case when DTF increases are occurring at higher mean temperatures (Verheyen and Stoks, 2019a). As for mean temperatures, DTFs can greatly differ in magnitude across spatial gradients (Wang and Dillon, 2014), and are predicted to increase under global warming (Colinet et al., 2015; IPCC, 2013; Vázquez et al., 2017). Notably, there is increasing evidence that DTFs may make pesticides more toxic (Delnat et al., 2019b; Verheyen et al., 2019; Verheyen and Stoks, 2019b; Willming et al., 2013; Willming and Maul, 2016). For example, while a given environmentally realistic concentration of the organophosphate insecticide chlorpyrifos was not toxic at a constant temperature of 20 °C, it did increase mortality fivefold

under DTFs in damselfly larvae (Verheyen and Stoks, 2019b). To increase the realism of current and future risk assessment it is therefore crucial to study the combined effects of a mean temperature and DTF on pesticide toxicity (Verheyen et al., 2019)

To better understand interactions between pesticides and natural stressors, it is important to consider effects on physiological traits (Côté et al., 2016; Gunderson et al., 2016; Hooper et al., 2013; Jackson et al., 2016). There is particularly poor knowledge about how pesticide toxicity is affected by DTF through physiological changes (but see Verheyen and Stoks, 2019a; Willming et al., 2013). Against this background, bioenergetic responses (i.e. energy budgets and energy allocation) are especially important as these provide information about the 'energy gain and loss' balance (Sokolova, 2013). When stressors cause energy imbalances, this can have important fitness consequences (e.g. lower growth rate and higher mortality: Sokolova, 2013). Nevertheless, bioenergetic responses to combined exposure to a pesticide and an increase in mean temperature or DTF have never been studied.

An important bioenergetic response variable at the cellular level that captures the energy budget of organisms, is the cellular energy allocation (CEA). The CEA reflects the net energy budget that is available for an organism by taking the ratio of energy stored in reserve molecules (energy available, Ea) over the energy that is consumed (energy consumption, Ec) (De Coen and Janssen, 2003). The CEA can be used as an indicator for effects on higher levels of biological organization as it has been shown to positively correlate with organismal growth rate (Goodchild et al., 2019; Verslycke et al., 2004). CEA has been often used as a biomarker for toxicant stress (e.g. De Coen and Janssen, 1997, 2003; Novais et al., 2013; Smolders et al., 2004; Verslycke et al., 2004) but less often for natural stressors (but see for temperature stress: Gandar et al., 2017; Kühnhold et al., 2017; and for salinity stress: Verslycke and Janssen, 2002). It is still unknown

how DTFs will affect CEA, but this might be a crucial underlying pathway in how DTFs affect pesticide toxicity. Indeed, DTFs are assumed to be energetically costly (Colinet et al., 2015), and natural stressors that reduce the energy budget have been modelled to increase contaminant toxicity (Liess et al., 2016).

The aim of this study was to examine how an increase in mean temperature and in DTF shape the bioenergetics response at the cellular level to a pesticide in an aquatic insect. We studied the widely used organophosphate insecticide chlorpyrifos (Eaton et al., 2008) that is well known to become more toxic at higher mean temperatures (e.g., Delnat et al., 2019a; Dinh Van et al., 2014) and under DTF (Delnat et al., 2019b; Verheyen et al., 2019; Verheyen and Stoks, 2019b; Willming et al., 2013). This priority pollutant (European Water Framework Directive 2000/60/EC, Ojec, 2000) is one of the top ten pollutants measured in UK surface waters that pose greatest risk to aquatic organisms (Johnson et al., 2017). Effects were tested on damselfly larvae that show intermediate sensitivity to chlorpyrifos compared to other aquatic invertebrates (Rubach et al. 2011). Damselfly larvae cannot escape exposure to warming and pesticide exposure because of their obligate aquatic life (Stoks et al., 2015). The study species *Ischnura elegans* prefers shallow freshwater ponds, including edge-to-field water bodies, in which they encounter large DTFs and may be exposed to high pesticide pulses. Warm-adapted low-latitude and cold-adapted high-latitude populations (Debecker and Stoks, 2019) were used to test for spatial differences in the effects of the stressors. In a companion study we have shown that both increases in mean temperature and in DTF, and especially their combination made chlorpyrifos more toxic in terms of growth and mortality (Verheyen and Stoks, 2019c). To determine the cellular energy allocation (CEA), we quantified the energy availability by measuring the key energy reserve molecules (proteins, sugars and fat), and the energy consumption by measuring

the electron transport system (ETS) activity at the mitochondrial level (following De Coen and Janssen, 2003). Our main hypotheses were: (1) exposure to chlorpyrifos to reduce the cellular energy allocation (CEA) by reducing energy availability (Ea) and increasing energy demands (i.e. consumption, Ec), (2) the chlorpyrifos-induced reduction in CEA to be stronger at the combination of a high mean temperature and a high DTF (Moe et al., 2013), and (3) low-latitude larvae to be less sensitive to chlorpyrifos under warming (high mean temperature and high DTF) as they are adapted to higher temperatures (Verheyen and Stoks, 2019a). Following Sokolova (2013) we also tested whether bioenergetic responses contributed to the observed toxicity patterns in terms of mortality and growth reduction that we reported in the companion study. Our hypothesis was that treatment groups with a lower CEA to show a higher mortality and a lower growth rate (cfr Goodchild et al., 2019)

Materials and methods

Study populations

From mid-June to mid-July 2017, 30 mated females of *I. elegans* were collected in each of three high-latitude populations and three low-latitude populations in the species' European range (Gosden et al., 2011). The low-latitude populations were situated in southern France and the high-latitude populations were situated in Denmark/Sweden. The low-latitude populations were situated in southern France (Bassin de Réaltor: 43°28'11.1"N; 05°19'44.1"E, La Durance: 43°43'52.5"N; 05°44'53.0"E and St. Martin de Crau: 43°37'57.8"N; 04°46'55.1"E). The high-latitude populations were situated in Denmark (Roskilde: 55°39'09.8"N; 12°08'01.7"E) and southern Sweden (Hovgardsdammarna: 57°14'24.3"N;12°08'28.2"E, and Kalmar Dämme: 56°40'04.6"N; 16°17'46.5"E). All six populations were located at shallow water bodies (< 1m depth) at least 100 m from agricultural fields. This reduces the possibility of direct exposure to pesticides (Declerck et al., 2006; Hua et al., 2015). In general, pesticide use and consequently

runoff are in Europe higher low-latitude sites compared to high-latitude sites (Kattwinkel et al., 2011). Therefore, the low-latitude sites may experience more immigration of damselflies coming from sites with pesticide exposure.

Mated females were placed separately in plastic cups (7.5 cm height, 3.5 cm diameter) containing wet filter paper for oviposition. After transfer of the eggs to the laboratory (KU Leuven, Belgium), they were incubated at a water temperature of 22 °C and a 14h:10h light-dark cycle. Hatchlings were daily fed *Artemia* nauplii ad libitum. Ten days after hatching, larvae were put separately in plastic cups (5 cm height, 6 cm diameter) containing 90 mL dechlorinated tap water and fed ad libitum with nauplii of *Artemia* during weekdays (Monday-Friday). The same photoperiod (14h:10h light-dark cycle) was maintained during the entire experiment.

General experimental setup

To test for the single and combined effects of the thermal regimes and the pesticide chlorpyrifos, and whether this differs between the two latitudes of origin, a full factorial experiment was conducted. The design had six thermal regimes consisting of two mean temperatures (20 and 24 °C) and three levels of daily temperature fluctuations (DTFs: constant = 0 °C, low = 5 °C and high = 10 °C), that were fully crossed with four pesticide treatments (solvent control = 0 µg/L chlorpyrifos, low = 0.75 µg/L chlorpyrifos, medium = 1.50 µg/L chlorpyrifos and high = 2.25 µg/L chlorpyrifos). This gives a total of 48 treatment combinations: 6 thermal regimes × 4 pesticide treatments × two latitudes (high and low). Details and motivation of the chosen mean temperatures and DTF levels can be found in Appendix S1. While the six thermal regimes directly started after the separation of the 10-day old hatchlings, the 6-day pesticide treatment started one day after the larvae molted into the final instar (last larval instar) at their respective thermal regime. Accordingly, larvae had been acclimated for min. 53 days to one of the thermal

regimes before being introduced to the pesticide treatment. Sample sizes per treatment combination varied between 24 and 26 larvae (total of 1198 larvae). For each latitude, we randomly selected one larva from a random subset of 24-26 mothers per treatment combination thereby taking into account that the larvae per treatment combination were distributed as equally as possible among the three populations of that latitude. Because of this procedure and because of the large number of females per latitude (n=90) relative to the number of larvae used per treatment combination (n=24-26) it is highly unlikely that our setup would have introduced any bias caused by differential representation of larvae of the different females.

Pesticide exposure

Larvae started the 6-day pesticide-exposure period one day after they molted into the final instar. Larvae were exposed individually in glass jars (200 mL) which were filled with 100 mL medium: either the solvent control or one of the three chlorpyrifos concentrations (0.75, 1.50 and 2.25 µg/L). All media were daily refreshed ('static renewal'). The chlorpyrifos concentrations (0.75, 1.50 and 2.25 µg/L) were chosen based on a previous study on *I. elegans* larvae where 1.50 µg/L and 3 µg/L chlorpyrifos reduced growth and increased mortality in a dose-dependent way (Dinh Van et al., 2014). Concentrations below 0.75 µg/L cause minor effects on growth rate while concentrations above 2.25 µg/L cause 50% mortality at 20 °C and are therefore less ideal to address our objectives (Op de Beeck et al., 2017, personal observations). This was confirmed in a companion study where at constant 20 °C the low chlorpyrifos concentration (0.75 µg/L) caused ~5.5% mortality, the medium chlorpyrifos (1.50 µg/L) caused ~15% mortality, and the high chlorpyrifos (2.25 µg/L) caused ~21 % mortality (Verheyen and Stoks, 2019c). Larvae of the study species may be continuously exposed to high chlorpyrifos concentrations (> 2.25 µg/L) for more than a week in edge-to-field water bodies. This results from a combination of the

following factors: (i) the recommended application doses may result in peak concentrations of more than 700 µg/L (Moore et al., 2002), (ii) the high application frequency for widely applied pesticides (multiple times per growing season, Van Drooge et al., 2001), and (iii) the relative long persistence of chlorpyrifos in artificial ponds (ca 3% remains after 10 days, Mazanti et al., 2003).

To obtain the chlorpyrifos concentrations, chlorpyrifos powder (Sigma-Aldrich, purity > 99%) was first dissolved in absolute ethanol (100%) to obtain a chlorpyrifos stock solution (1 mg/mL). This chlorpyrifos stock solution was further diluted, first with Milli-Q water and second with dechlorinated tap water to reach the desired exposure concentrations of chlorpyrifos (0.75, 1.50, 2.25 µg/L) (see Appendix S2 for more details). The same amount of absolute ethanol (100%) as in the highest chlorpyrifos treatment (2.25 µL/L) was applied to the solvent control treatment. This ethanol concentration is >5× lower than the no effect concentration (NOEC) for aquatic invertebrates (United Nations Environment Program, 2004), and therefore unlikely to cause effects on the study species. Details about the measured chlorpyrifos concentrations and water quality parameters during the experiment are given in Appendix S2.

Response variables

After the 6-day pesticide-exposure period, larvae were weighed (wet mass) to the nearest 0.01 mg by using an electronic balance (Mettler Toledo® AB135-S, Ohio, USA). Before weighing, larvae were gently blotted dry by using tissue paper. This provides reliable wet masses, which strongly correlate with the dry masses (Stoks et al., 2005). After weighing, larvae were frozen at -80 °C to measure physiological parameters related to the cellular energy allocation (CEA): the available energy reserves (Ea) and energy consumption (Ec). To estimate the available energy reserves the total protein, sugar and fat contents were measured (De Coen and Janssen, 2003).

198 The energy consumption was assessed by quantifying the activity level of the electron transport
199 system (ETS, the cellular respiration rate) (De Coen and Janssen, 2003).

200 The bioenergetic response variables were quantified on the body supernatant using
201 spectrophotometry based on established protocols for damselfly larvae (Van Dievel et al., 2019;
202 Verheyen et al., 2018). The bodies were first homogenized in Phosphoric Buffered Saline (50
203 mmol/L PBS, pH 7.4) buffer (90% of the final mass x 15 µL) and then centrifuged to obtain the
204 supernatant. The total protein content was quantified by using the Bradford (1976) method. The
205 total sugar content (glucose and glycogen: Hahn and Delinger 2007) was measured using a
206 protocol based on the glucose kit of Sigma-Aldrich USA (Stoks et al. 2006) and the assay to
207 quantify the total lipid content was based on a protocol of Marsh and Weinstein (1966). We
208 measured the activity of the electron transport system (ETS) to assess metabolic rate by using the
209 protocol of De Coen and Janssen (2003), which was modified for damselflies (Janssens and
210 Stoks, 2013). Detailed protocols of the bioenergetic response variables can be found in Appendix
211 S3.

212 The total net energy budget or the CEA of each larva was calculated as Ea/Ec (Pestana et
213 al., 2009; Van Dievel et al., 2019; Verslycke et al., 2004). The whole body Ea was calculated as
214 the sum of energy present in the different energy reserves (proteins, sugars and lipids). The three
215 energy reserve biomolecules were spectrophotometrically quantified and converted into
216 energetic equivalents by using the corresponding energy of combustion values: 24,000 mJ/mg
217 protein, 17,500 mJ/mg glycogen and 39,500 mJ/mg lipid (De Coen and Janssen, 2003). The Ec
218 was assessed by measuring the ETS activity (De Coen and Janssen, 2003). To calculate Ec, the
219 total consumed oxygen was converted into energetic equivalents by using an oxyenthalpic

equivalent of 480 kJ/mol O_2 for an average protein, sugar, and lipid mixture (De Coen and Janssen, 2003).

Statistical analyses

The main effects of mean temperature, DTF, pesticide treatment and latitude, and all their interactions were analyzed by using separate linear mixed models per response variable with a normal error distribution and the identity link ('lme4' package v1.1-21; Bates et al., 2015; 'afex' package v0.23-0; Singmann et al., 2017). Population nested in latitude was added to each model as a random factor. We initially also added the interaction of this term with pesticide, but this was never significant (all $P > 0.33$) indicating that populations within a given latitude responded in the same way to the pesticide. Non-normal distributed response variables (total protein and fat content, Ea, and CEA) were square-root transformed to meet the model assumption of normality.

To explore relationships between CEA and life history (mortality and growth rate) at the treatment group level (n = 48 treatment combinations), we correlated treatment group means for CEA obtained in current study with those for life history obtained in the companion study (Verheyen and Stoks, 2019c). We did so using Pearson's product moment correlation.

All response variables were analyzed using R v3.5.3. for Windows (R Core Team, 2018). Wald chi-square statistics and p-values for fixed effects were calculated using the 'car' package (v3.0-2; Fox and Weisberg, 2011). Least-square means contrasts obtained from the 'lsmeans' package (v2.30-0; Lenth, 2016) were used to further analyze treatment interactions. Contrasts related to the effects of chlorpyrifos are visualized in the figures. The p-values associated with the contrasts were false discovery rate corrected. Only effects of the pesticide and its interactions on the bioenergetic response variables are reported in the results section to keep the manuscript focused. The extended results can be found in Appendix S4 and the results

on individual categories of energy storage molecules (proteins, sugars and fat) are presented in Appendix S5.

Results

Energy availability (Ea)

While the main effect of chlorpyrifos on the energy availability was highly significant ($P < 0.001$), chlorpyrifos mainly reduced Ea at a mean of 24 °C combined with 5 °C and 10 °C DTF (Mean T × Pesticide, Mean T × DTF × Pesticide), and more likely so in low-latitude larvae (Mean T × DTF × Pesticide × Latitude, Table 1, Fig. 1). At a mean of 20 °C, chlorpyrifos had little effect on Ea in high-latitude larvae (contrasts all $P > 0.052$; only the high chlorpyrifos decreased Ea with 15.1% at 0 °C DTF: $P = 0.001$), yet reduced Ea with 13.1% in low-latitude larvae at the medium (1.50 µg/L) and tended to reduce Ea with 10.5% at the high (2.25 µg/L) chlorpyrifos concentration at 10 °C DTF (contrasts: medium chlorpyrifos, $P = 0.020$; high chlorpyrifos, $P = 0.072$, other $P > 0.27$). At a mean of 24 °C and in high-latitude larvae, medium chlorpyrifos decreased Ea with 13.0% at 0 °C DTF (contrast: $P = 0.010$, other $P > 0.12$) and tended to decrease Ea with 9.6% at 5 °C DTF (contrast: $P = 0.061$), while high chlorpyrifos decreased Ea at 5 (-16.5%) and 10 °C (-15.5%) DTF (contrasts: both $P < 0.003$, other $P > 0.14$). At a mean of 24 °C and in low-latitude larvae, energy levels were similar across chlorpyrifos levels in the absence of DTF (0 °C DTF, contrasts: all $P > 0.20$), while medium and high chlorpyrifos decreased Ea at 5 (-12.4% for medium; -15.5% for high chlorpyrifos) and 10 °C (-12.6% for medium; -13.4% for high chlorpyrifos) DTF (contrasts: all $P < 0.017$, other $P = 0.82$). Notably, exposure to low chlorpyrifos only reduced Ea (-14.6%) in low-latitude larvae at 24 °C in the presence of high (10 °C) DTF (contrast: $P = 0.005$).

Energy consumption (Ec)

Exposure to chlorpyrifos did not affect the energy consumption in the larvae: neither its main effect nor its interactions were significant (Table 1, Fig. 2).

Cellular energy allocation (CEA)

While the main effect of chlorpyrifos on the cellular energy allocation was highly significant ($P < 0.001$, Table 1, Fig. 3), whether chlorpyrifos reduced the CEA jointly depended on the mean temperature, the DTF level and the latitude of origin (Mean T × DTF × Pesticide × Latitude, Table 1, Fig. 3). At a mean of 20 °C, CEA was not reduced by chlorpyrifos in any of the thermal treatment combinations in low-latitude larvae (contrasts: all $P > 0.19$), while in high-latitude larvae it was only reduced (-15.2%) by high chlorpyrifos in the absence of DTF (contrast: $P = 0.038$; other $P > 0.096$). At a mean of 24 °C and in high-latitude larvae, CEA was only reduced by high chlorpyrifos, and this at each DTF level (-9.9% at 0 °C DTF, -15.7% at 5 °C DTF, -14.8% at 10 °C DTF; contrasts, all $P < 0.068$). At a mean of 24 °C and in low-latitude larvae, however, CEA tended to be reduced (-13.6%) by medium chlorpyrifos (contrast: $P = 0.068$) and was reduced (-14.6%) by high chlorpyrifos (contrast: $P = 0.049$) at low (5 °C) DTF (other $P > 0.31$). Notably, when 24 °C was combined with the high (10 °C) DTF level, the chlorpyrifos-induced reduction in CEA was stronger and already occurred when low-latitude larvae were exposed to low chlorpyrifos (-18.8% at low chlorpyrifos, -16.7% at medium chlorpyrifos, -17.4% at high chlorpyrifos; contrasts, all $P < 0.010$).

Relationships between CEA and life history

Treatment groups that had lower mean CEA levels showed a higher mean mortality (Pearson r = -0.57, t = -4.71, $P < 0.001$, Figure 4a), and a lower mean growth rate (Pearson r = 0.29, t = 2.07, $P = 0.044$, Figure 4b).

Discussion

As expected, exposure to chlorpyrifos considerably reduced the total net energy budget (CEA values) of the larvae. Notably, this reduction was strongly dependent on the combination of mean temperature and DTF. As further expected, the emerging pattern was that the chlorpyrifos-induced reduction in CEA mainly occurred at the high mean temperature in combination with fluctuating temperatures. Indeed, of the 10 contrasts testing for an effect of chlorpyrifos on CEA that showed a trend or were significant, seven occurred at 24 °C in the presence of DTF and only one at 20 °C in the absence of DTF. The chlorpyrifos-induced reductions in CEA under warming were driven by reductions in energy availability (Ea), mainly caused by reductions in sugar and fat contents (Appendix S5). Energy consumption (Ec), however, was not affected by the pesticide or its interactions with the thermal conditions. Overall, the negative effects of chlorpyrifos on CEA were occurring at lower concentrations in low-latitude than in high-latitude larvae.

Effects of chlorpyrifos under warming on energy availability (Ea)

Exposure to high chlorpyrifos reduced the energy availability (Ea) mainly at the high mean temperature of 24 °C combined with the presence of either low (5 °C) or high (10 °C) DTF. Our results match the general finding that many pesticides (including chlorpyrifos: Dinh Van et al., 2014) become more toxic at higher mean temperatures (Noyes et al., 2009; Noyes and Lema, 2015). Furthermore, our results are in line with an increasing number of studies showing that DTFs can increase pesticide toxicity (Willming and Maul, 2016, including chlorpyrifos: Delnat et al., 2019; Verheyen et al., 2019; Verheyen and Stoks, 2019a; Willming et al., 2013). Two major mechanisms may explain the higher impact of chlorpyrifos at higher constant temperatures: an increase in pesticide uptake (Hooper et al., 2013); and an increase in metabolic conversion of the original molecule (chlorpyrifos) to the more toxic metabolite (Harwood et al.,

2009; i.e. the chlorpyrifos oxon: Buchwalter et al., 2004). The higher impact of the pesticide under DTFs may also be explained via these two mechanisms because temperatures daily reach up to 25 °C (at mean of 20 °C) and even 29 °C (at mean of 24 °C) for several hours in the high (10 °C) DTF treatment. Both mechanisms may have shaped the bioenergetic responses to chlorpyrifos under warming. Indeed, under conditions that cause chlorpyrifos to be more toxic, a higher allocation of energy away from storage toward investment in defense and repair is to be expected. For example, in response to chlorpyrifos animals have been shown to invest in the production of stress proteins (e.g. Janssens et al., 2014; Scheil et al., 2010), and to upregulate levels of the detoxification enzyme cytochrome P450 monooxygenase (CytP450; e.g. Verheyen and Stoks, 2019a; Verheyen and Stoks, 2019c). Moreover, the food intake may have been lower under chlorpyrifos exposure (e.g. Dinh Van et al., 2014; Pestana et al., 2009; Ribeiro et al., 2001) further contributing to a lower Ea.

The strong chlorpyrifos-induced reductions in Ea when the high mean temperature (24 °C) was combined with the high (10 °C) DTF were driven by chlorpyrifos-induced reductions in sugar and fat contents (see Appendix S5). Protein content, however, was little affected by chlorpyrifos and was only reduced by high chlorpyrifos when the high mean temperature was combined with the high DTF. This matches the general pattern that sugar and fat reserves are more sensitive to stressors (as both are readily available energy sources in many aquatic invertebrates), than protein reserves (Giesy and Graney, 1989; Smolders et al., 2003). Protein levels were also only reduced by high pollutant concentrations in *Daphnia magna*, while they could even increase at low concentrations (De Coen and Janssen, 2003); these low levels of stress might trigger protein synthesis (e.g. for detoxification, Smolders et al., 2003). Pollutant-induced reductions in sugar and fat contents are common and have been found before in other

invertebrates (De Coen and Janssen, 2003; Ribeiro et al., 2001; Smolders et al., 2004), also in response to chlorpyrifos exposure (Verslycke et al., 2004).

Effects of chlorpyrifos under warming on energy consumption (Ec)

Pollutant exposure is often expected (Sokolova, 2013) and shown (e.g., Van Dievel et al., 2019; Verslycke et al., 2004) to increase energy consumption (Ec). Nevertheless, we did not observe any effects of chlorpyrifos on Ec (measured as the ETS activity), also not under the hotter thermal conditions. An increased energy consumption under pollutant exposure is, however, not general. For example, chlorpyrifos exposure decreased ETS activity in the mosquito *Culex pipiens* (Delnat et al., 2019a), and exposure to tributyltin did not affect ETS in the polychaete *Hediste diversicolor* (Stomperudhaugen et al., 2009). Likely, pollutant effects on energy consumption are concentration-dependent, and especially at high concentrations no upregulation may be possible and instead animals may even undergo metabolic depression (Rodrigues et al., 2017; Storey, 2015).

In contrast, the thermal regimes did have a strong effect on ETS activity. Indeed, Ec was strongly reduced by the high mean temperature (at 0 °C and 5 °C DTF) and by the high DTF level (at 20 °C). While in most studies ETS activity increases with raising mean temperatures (e.g. Simcic and Brancelj, 1997; Van Dievel et al., 2017), higher temperatures reduced the metabolic rate in snails to compensate for the costs of living at these high temperatures (i.e. 'metabolic compensation', Marshall and McQuaid 2010). This may also explain why ETS activity was lower under high DTF, as already observed in the study species (Verheyen and Stoks, 2019a) and in other species (e.g. shrimps: Tian et al., 2004; sea cucumbers: Dong and Dong, 2006).

Effects of chlorpyrifos under warming on cellular energy allocation (CEA)

A key finding was that the net energy budget (CEA) of the damselfly larvae was lowered by high chlorpyrifos, and this mainly at 24 °C when combined with high DTF. This matches the higher chlorpyrifos-induced mortality under the combination of the high mean temperature and the presence of DTFs in the companion study (Verheyen and Stoks, 2019c). Notably, we could show the expected pattern that treatment groups with a lower CEA showed a higher mortality and a lower growth rate (cfr Goodchild et al., 2019). Together, this indicates that the bioenergetic responses to chlorpyrifos under warming are contributing to the observed toxicity patterns in terms of mortality and growth reduction. This gives further support to the idea that an energy imbalance may have important fitness consequences, for example a lowered growth rate and a higher mortality (Sokolova, 2013).

The chlorpyrifos-induced CEA pattern was driven by reductions in Ea, while Ec had no or little contribution. This matches other studies on aquatic invertebrates that also found that a pollutant-induced reduction in CEA was mainly explained by reductions in Ea, while Ec contributed little (e.g. De Coen and Janssen, 2003; Muyssen and Janssen, 2001). This stressor-induced energy imbalance (lowered CEA) implies that maintenance costs (energy losses) were higher than the amount of gained energy (energy gains).

In contrast with our hypothesis, the negative effects of chlorpyrifos on CEA (and Ea) occurred at lower concentrations in low-latitude than in high-latitude larvae when 24 °C was combined with 10 °C DTF. Possibly, survival selection played a role since low chlorpyrifos increased mortality more in high-latitude larvae than in low-latitude larvae (Verheyen and Stoks, 2019c). Although chlorpyrifos-induced reductions in Ea at 24 °C with 10 °C DTF were slightly larger in high-latitude larvae compared to low-latitude larvae, we did not find this result for

CEA. This means that local thermal adaptation to both higher mean temperatures and higher DTFs (as shown in Verheyen and Stoks, 2019a) could not buffer the negative impact of chlorpyrifos on CEA in low-latitude larvae. The absence of evidence for latitude-associated thermal adaptation in shaping the vulnerability of the study species to pollutants under warming has been found before and was explained by differences in voltinism between high- and low-latitude populations (e.g. Debecker et al., 2017; Dinh Van et al., 2014). Specifically, low-latitude larvae possibly allocated more energy towards growth during the pre-exposure period, as they are multivoltine (multiple generations a year) and thus faster growing than semivoltine (one generation every two years) high-latitude larvae (Corbet et al., 2006), making them less tolerant to pollutants during the exposure period. It should also be noted that high-latitude larvae in general had a higher total net energy budget (CEA) by having more available energy (Ea) through higher levels of energy reserves (proteins, sugars and fat). As such they may have experienced less resistance to decrease these reserves.

Conclusions

Despite their relevance as biomarker and as mechanism underlying life history responses to pollutants (Adams and Greeley, 2000; De Coen and Janssen, 1997, 2003; Goodchild et al., 2019; Sokolova, 2013), bioenergetic responses to pollutants under warming had never been studied. This is especially important as toxic effects of pollutants are expected to be magnified when combined with natural stressors that are energetically costly (Liess et al., 2016). Against this background, we demonstrated that chlorpyrifos reduced the net energy budget mainly at the high mean temperature under DTF, thereby matching the higher toxicity of chlorpyrifos in terms of mortality and growth reduction under these thermal conditions (Verheyen et al., 2019; Verheyen and Stoks, 2019c). Moreover, treatment groups with a lower CEA showed a higher mortality and

a lower growth rate. This indicates that bioenergetic responses are contributing to the higher toxicity of chlorpyrifos under warming, hence deepens our mechanistic insights in the "climate-induced toxicant sensitivity" concept (Moe et al., 2013; Noyes and Lema, 2015).

Our findings are highly relevant to increase the realism of both current and future risk assessment of pesticides under warming. Indeed, the bioenergetic impact of chlorpyrifos toxicity would have been underestimated if we had only evaluated the effects of chlorpyrifos on *I. elegans* larvae under the standard temperature conditions of a constant water temperature of 20 °C. Notably, under the IPCC (2013) RCP8.5 warming scenario (leading to a mean temperature of 24 °C with 10 °C DTF in our study region) chlorpyrifos had the most toxic effects. This highlights the importance of evaluating the impact of pollutants on organisms under the more realistic future scenario of a combination of an increase in mean temperature and in DTF.

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Authors' Contributions

The experimental setup was conceived and designed by both authors. JV ran the experiments and analyzed the data. The manuscript was written by both authors who both gave final approval for publication.

Notes

The authors declare no conflict of interest.

Supporting Information

Appendix S1: Details about the thermal regimes

Appendix S2: Details about the chlorpyrifos concentrations and water quality parameters

Appendix S3: Detailed protocols of the bioenergetic response variables

Appendix S4: Extended results

Appendix S5: Results on energy storage molecules

References

Adams, S.M., Greeley, M.S., 2000. Ecotoxicological indicators of water quality: Using multi-response indicators to assess the health of aquatic ecosystems. Water. Air. Soil Pollut. 123, 103–115. https://doi.org/10.1093/carcin/14.4.637

Bates, D., Mächler, M., Bolker, B., Walker, S.C., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48.

Beketov, M.A., Kefford, B.J., Schäfer, R.B., Liess, M., 2013. Pesticides reduce regional biodiversity of stream invertebrates. Proc. Natl. Acad. Sci. 110, 11039–11043. https://doi.org/10.1073/pnas.1305618110

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein-dye landing. Anal. Biochem. 72, 248–254. https://doi.org/10.1006/abio.1976.9999

Buchwalter, D.B., Sandahl, J.F., Jenkins, J.J., Curtis, L.R., 2004. Roles of uptake, biotransformation, and target site sensitivity in determining the differential toxicity of chlorpyrifos to second to fourth instar *Chironomous riparius* (Meigen). Aquat. Toxicol. 66,

149–157. https://doi.org/10.1016/j.aquatox.2003.08.004

Colinet, H., Sinclair, B.J., Vernon, P., Renault, D., 2015. Insects in fluctuating thermal environments. Annu. Rev. Entomol. 60, 123–140. https://doi.org/10.1146/annurev-ento-010814-021017

Corbet, P.S., Suhling, F., Soendgerath, D., 2006. Voltinism of odonata: A review. Int. J. Odonatol. 9, 1–44. https://doi.org/10.1080/13887890.2006.9748261

Côté, I.M., Darling, E.S., Brown, C.J., 2016. Interactions among ecosystem stressors and their importance in conservation. Proc. R. Soc. B Biol. Sci. 283, 20152592. https://doi.org/10.1098/rspb.2015.2592

De Coen, W., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. J. Aquat. Ecosyst. Stress Recover. 6, 43–55. https://doi.org/10.1023/A:1008228517955

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22, 1632–1641. https://doi.org/10.1002/etc.5620220727

Debecker, S., Dinh, K. V., Stoks, R., 2017. Strong delayed interactive effects of metal exposure and warming: Latitude-dependent synergisms persist across metamorphosis. Environ. Sci. Technol. 51, 2409–2417. https://doi.org/10.1021/acs.est.6b04989

Debecker, S., Stoks, R., 2019. Pace of life syndrome under warming and pollution: integrating life history, behavior and physiology across latitudes. Ecol. Monogr. 89, e01332. https://doi.org/10.1002/ecm.1332

Declerck, S., De Bie, T., Ercken, D., Hampel, H., Schrijvers, S., Van Wichelen, J., Gillard, V., Mandiki, R., Losson, B., Bauwens, D., Keijers, S., Vyverman, W., Goddeeris, B., De Meester, L., Brendonck, L., Martens, K., 2006. Ecological characteristics of small farmland ponds: Associations with land use practices at multiple spatial scales. Biol. Conserv. 131, 523–532. https://doi.org/10.1016/j.biocon.2006.02.024

Delnat, V., Janssens, L., Stoks, R., 2019a. Whether warming magnifies the toxicity of a pesticide is strongly dependent on the concentration and the null model. Aquat. Toxicol. 211, 38–45. https://doi.org/10.1016/j.aquatox.2019.03.010

Delnat, V., Tran, T.T., Janssens, L., Stoks, R., 2019b. Daily temperature variation magnifies the toxicity of a mixture consisting of a chemical pesticide and a biopesticide in a vector mosquito. Sci. Total Environ. 659, 33–40. https://doi.org/10.1016/j.scitotenv.2018.12.332

Dinh Van, K., Janssens, L., Debecker, S., Stoks, R., 2014. Temperature- and latitude-specific individual growth rates shape the vulnerability of damselfly larvae to a widespread pesticide. J. Appl. Ecol. 51, 919–928. https://doi.org/10.1111/1365-2664.12269

Dong, Y., Dong, S., 2006. Growth and oxygen consumption of the juvenile sea cucumber *Apostichopus japonicus* (Selenka) at constant and fluctuating water temperatures. Aquac. Res. 37, 1327–1333. https://doi.org/10.1111/j.1365-2109.2006.01570.x

Eaton, D.L., Daroff, R.B., Autrup, H., Bridges, J., Buffler, P., Costa, L.G., Coyle, J., McKhann, G., Mobley, W.C., Nadel, L., Neubert, D., Schulte-Hermann, R., Spencer, P.S., 2008. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. Crit. Rev. Toxicol. 38, 1–125. https://doi.org/10.1080/10408440802272158

Fox, J., Weisberg, S., 2011. An {R} Companion to Aplied Regression, Second. ed. Sage

Publications, Thousand Oaks CA.

Gandar, A., Laffaille, P., Canlet, C., Tremblay-Franco, M., Gautier, R., Perrault, A., Gress, L., Mormède, P., Tapie, N., Budzinski, H., Jean, S., 2017. Adaptive response under multiple stress exposure in fish: From the molecular to individual level. Chemosphere 188, 60–72. https://doi.org/10.1016/j.chemosphere.2017.08.089

Giesy, J.P., Graney, R.L., 1989. Recent developments in and intercomparisons of acute and chronic bioassays and bioindicators. Hydrobiologia 188/189, 21–60.

Goodchild, C.G., Simpson, A.M., Minghetti, M., Durant, S.E., 2019. Bioenergetics-adverse outcome pathway: linking organismal and suborganismal energetic endpoints to adverse outcomes. Environ. Toxicol. Chem. 38, 27–45. https://doi.org/10.1002/etc.4280

Gosden, T.P., Stoks, R., Svensson, E.I., 2011. Range limits, large-scale bioeographic variation and localised evolutionary dynamics in a polymorphic damselfly. Biol. J. Linn. Soc. 102, 775–785. https://doi.org/10.1111/j.1095-8312.2011.01619.x

Gunderson, A.R., Armstrong, E.J., Stillman, J.H., 2016. Multiple stressors in a changing world: The need for an improved perspective on physiological responses to the dynamic marine environment. Ann. Rev. Mar. Sci. 8, 357–378. https://doi.org/10.1146/annurev-marine-122414-033953

Harwood, A.D., You, J., Lydy, M.J., 2009. Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: toxicokinetic confirmation. Environ. Toxicol. Chem. 28, 1051–1058. https://doi.org/10.1897/08-291.1

Hooper, M.J., Ankley, G.T., Cristol, D.A., Maryoung, L.A., Noyes, P.D., Pinkerton, K.E., 2013. Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks. Environ. Toxicol. Chem. 32, 32–48.

https://doi.org/10.1002/etc.2043

Hua, J., Jones, D.K., Mattes, B.M., Cothran, R.D., Relyea, R.A., Hoverman, J.T., 2015. The contribution of phenotypic plasticity to the evolution of insecticide tolerance in amphibian populations. Evol. Appl. 8, 586–596. https://doi.org/10.1111/eva.12267

IPCC, 2013. Climate Change 2013: The physical science basis. Contribution of working group I to the fifth assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK.

Jackson, M.C., Loewen, C.J.G., Vinebrooke, R.D., Chimimba, C.T., 2016. Net effects of multiple stressors in freshwater ecosystems: A meta-analysis. Glob. Chang. Biol. 22, 180–189. https://doi.org/10.1111/gcb.13028

Janssens, L., Dinh Van, K., Stoks, R., 2014. Extreme temperatures in the adult stage shape delayed effects of larval pesticide stress: a comparison between latitudes. Aquat. Toxicol. 148, 74–82. https://doi.org/10.1016/j.aquatox.2014.01.002

Janssens, L., Stoks, R., 2013. Synergistic effects between pesticide stress and predator cues: Conflicting results from life history and physiology in the damselfly *Enallagma cyathigerum*. Aquat. Toxicol. 132–133, 92–99. https://doi.org/10.1016/j.aquatox.2013.02.003

Johnson, A.C., Donnachie, R.L., Sumpter, J.P., Jürgens, M.D., Moeckel, C., Pereira, M.G., 2017. An alternative approach to risk rank chemicals on the threat they pose to the aquatic environment. Sci. Total Environ. 599–600, 1372–1381. https://doi.org/10.1016/j.scitotenv.2017.05.039

Kattwinkel, M., Jan-Valentin, K., Foit, K., Liess, M., 2011. Climate change, agricultural insecticide exposure, and risk for freshwater communities. Ecol. Appl. 21, 2068–2081.

https://doi.org/10.1890/10-1993.1

Kühnhold, H., Kamyab, E., Novais, S., Indriana, L., Kunzmann, A., Slater, M., Lemos, M., 2017. Thermal stress effects on energy resource allocation and oxygen consumption rate in the juvenile sea cucumber, *Holothuria scabra* (Jaeger, 1833). Aquaculture 467, 109–117. https://doi.org/10.1016/j.aquaculture.2016.03.018

Landis, W.G., Rohr, J.R., Moe, S.J., Balbus, J.M., Clements, W., Fritz, A., Helm, R., Hickey, C., Hooper, M., Stahl, R.G., Stauber, J., 2014. Global climate change and contaminants, a call to arms not yet heard? Integr. Environ. Assess. Manag. 10, 483–484. https://doi.org/10.1002/ieam.1568

Lenth, R. V., 2016. Least-squares means: The R package lsmeans. J. Stat. Softw. 69, 1–33. https://doi.org/10.18637/jss.v069.i01

Liess, M., Foit, K., Knillmann, S., Schäfer, R.B., Liess, H.D., 2016. Predicting the synergy of multiple stress effects. Sci. Rep. 6, 1–8. https://doi.org/10.1038/srep32965

Marsh, J.B., Weinstein, D.B., 1966. Simple charring method for determination of lipids. J. Lipid Res. 7, 574–576.

Marshall, D.J., McQuaid, C.D., 2010. Warming reduces metabolic rate in marine snails: adaptation to fluctuating high temperatures challenges the metabolic theory of ecology. Proc. R. Soc. B 278, 281–288. https://doi.org/10.1098/rspb.2010.1414

Mazanti, L., Rice, C., Bialek, K., Sparling, D., Stevenson, C., Johnson, W., Kangas, P., Rheinstein, J., 2003. Aqueous-phase disappearance of atrazine, metolachlor, and chlorpyrifos in laboratory aquaria and outdoor macrocosms. Arch. Environ. Contam. Toxicol. 44, 67–76.

Moe, S.J., De Schamphelaere, K., Clements, W.H., Sorensen, M.T., Van den Brink, P.J., Liess,

M., 2013. Combined and interactive effects of global climate change and toxicants on populations and communities. Environ. Toxicol. Chem. 32, 49–61. https://doi.org/10.1002/etc.2045

Moore, M., Schulz, R., Cooper, C., Smith, S., Rodgers, J., 2002. Mitigation of chlorpyrifos runoff using constructed wetlands. Chemosphere 46, 827–835.

Muyssen, B.T.A., Janssen, C.R., 2001. Multigeneration zinc acclimation and tolerance in *Daphnia Magna*: Implications for water-quality guidelines and ecological risk assessment. Environ. Toxicol. Chem. 20, 2053–2060. https://doi.org/10.1897/1551-5028(2001)020<2053:mzaati>2.0.co;2

Novais, S.C., Soares, A.M.V.M., De Coen, W., Amorim, M.J.B., 2013. Exposure of *Enchytraeus albidus* to Cd and Zn - Changes in cellular energy allocation (CEA) and linkage to transcriptional, enzymatic and reproductive effects. Chemosphere 90, 1305–1309. https://doi.org/10.1016/j.chemosphere.2012.09.030

Noyes, P.D., Lema, S.C., 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. Curr. Zool. 61, 669–689. https://doi.org/10.1093/czoolo/61.4.669

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

Ojec, D., 2000. 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy. Off. J. Eur. Communities 22, 1–73.

Op de Beeck, L., Verheyen, J., Olsen, K., Stoks, R., 2017. Negative effects of pesticides under

587 global warming can be counteracted by a higher degradation rate and thermal adaptation. J.
588 Appl. Ecol. 54, 1847–1855. https://doi.org/10.1111/1365-2664.12919
589 Paaijmans, K.P., Heinig, R.L., Seliga, R.A., Blanford, J.I., Blanford, S., Murdock, C.C., Thomas,
590 M.B., 2013. Temperature variation makes ectotherms more sensitive to climate change.
591 Glob. Chang. Biol. 19, 2373–2380. https://doi.org/10.1111/gcb.12240
592 Pestana, J.L.T., Loureiro, S., Baird, D.J., Soares, A.M.V.M., 2009. Fear and loathing in the
593 benthos: Responses of aquatic insect larvae to the pesticide imidacloprid in the presence of
594 chemical signals of predation risk. Aquat. Toxicol. 93, 138–149.
595 https://doi.org/10.1016/j.aquatox.2009.04.008
596 Peters, K., Bundschuh, M., Schäfer, R.B., 2013. Review on the effects of toxicants on freshwater
597 ecosystem functions. Environ. Pollut. 180, 324–329.
598 https://doi.org/10.1016/j.envpol.2013.05.025
599 R Core Team, 2018. R: A language and environment for statistical computing. R Found. Stat.
600 Comput. https://doi.org/ISBN 3-900051-07-0
601 Ribeiro, S., Sousa, J.P., Nogueira, A.J.A., Soares, A.M.V.M., 2001. Effect of endosulfan and
602 parathion on energy reserves and physiological parameters of the terrestrial isopod
603 *Porcellio dilatatus*. Ecotoxicol. Environ. Saf. 49, 131–138.
604 https://doi.org/10.1006/eesa.2001.2045
605 Rodrigues, A.C.M., Gravato, C., Quintaneiro, C., Bordalo, M.D., Golovko, O., Žlábek, V.,
606 Barata, C., Soares, A.M.V.M., Pestana, J.L.T., 2017. Exposure to chlorantraniliprole affects
607 the energy metabolism of the caddisfly *Sericostoma vittatum*. Environ. Toxicol. Chem. 36,
608 1584–1591. https://doi.org/10.1002/etc.3684
609 Scheil, V., Zürn, A., Köhler, H.-R., Triebskorn, R., 2010. Embryo development, stress protein

(Hsp70) responses, and histopathology in zebrafish (*Danio rerio*) following exposure to nickel chloride, chlorpyrifos, and binary mixtures of them. Environ. Toxicol. 25, 83–83. https://doi.org/https://doi.org/10.1002/tox.20477

Simcic, T., Brancelj, A., 1997. Electron transport system (ETS) activity and respiration rate in five *Daphnia* species at different temperatures. Hydrobiologia 360, 117–125. https://doi.org/10.1023/A:1003117221455

Singmann, H., Bolker, B., Westfall, J., Aust, F., 2017. Afex: Analysis of factorial experiments.

Smolders, R., Bervoets, L., De Coen, W., Blust, R., 2004. Cellular energy allocation in zebra mussels exposed along a pollution gradient: Linking cellular effects to higher levels of biological organization. Environ. Pollut. 129, 99–112. https://doi.org/10.1016/j.envpol.2003.09.027

Smolders, R., De Boeck, G., Blust, R., 2003. Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 22, 890–899. https://doi.org/10.1897/1551-5028(2003)022<0890:CICEBA>2.0.CO;2

Sokolova, I.M., 2013. Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. Integr. Comp. Biol. 53, 597–608. https://doi.org/10.1093/icb/ict028

Stoks, R., De Block, M., Van De Meutter, F., Johansson, F., 2005. Predation cost of rapid growth: Behavioural coupling and physiological decoupling. J. Anim. Ecol. 74, 708–715. https://doi.org/10.1111/j.1365-2656.2005.00969.x

Stoks, R., Debecker, S., Dinh Van, K., Janssens, L., 2015. Integrating ecology and evolution in aquatic toxicology: insights from damselflies. Freshw. Sci. 34, 1032–1039. https://doi.org/10.1086/682571.

633 Stomperudhaugen, E.S., Hanssen Øverås, N.H., Langford, K., De Coen, W., Smolders, R.,
634 Hylland, K., 2009. Cellular energy allocation in Hediste diversicolor exposed to sediment
635 contaminants. J. Toxicol. Environ. Heal. - Part A 72, 244–253.
636 https://doi.org/10.1080/15287390802539178
637 Storey, K.B., 2015. Regulation of hypometabolism: insights into epigenetic controls. J. Exp.
638 Biol. 218, 150–159. https://doi.org/10.1242/jeb.106369
639 Tian, X., Dong, S., Wang, F., Wu, L., 2004. The effects of temperature changes on the oxygen
640 consumption of juvenile Chinese shrimp *Fenneropenaeus chinensis* Osbeck. J. Exp. Mar.
641 Bio. Ecol. 310, 59–72. https://doi.org/10.1016/j.jembe.2004.04.002
642 United Nations Environment Program, 2004. Screening information dataSet: Initial assessment
643 report ethanol cas no: 64-17-5.
644 Van den Brink, P.J., Boxall, A.B.A., Maltby, L., Brooks, B.W., Rudd, M.A., Backhaus, T.,
645 Spurgeon, D., Verougstraete, V., Ajao, C., Ankley, G.T., Apitz, S.E., Arnold, K., Brodin,
646 T., Cañedo-Argüelles, M., Chapman, J., Corrales, J., Coutellec, M.A., Fernandes, T.F.,
647 Fick, J., Ford, A.T., Giménez Papiol, G., Groh, K.J., Hutchinson, T.H., Kruger, H.,
648 Kukkonen, J.V.K., Loutseti, S., Marshall, S., Muir, D., Ortiz-Santaliestra, M.E., Paul, K.B.,
649 Rico, A., Rodea-Palomares, I., Römbke, J., Rydberg, T., Segner, H., Smit, M., van Gestel,
650 C.A.M., Vighi, M., Werner, I., Zimmer, E.I., van Wensem, J., 2018. Toward sustainable
651 environmental quality: Priority research questions for Europe. Environ. Toxicol. Chem. 37,
652 2281–2295. https://doi.org/10.1002/etc.4205
653 Van Dievel, M., Janssens, L., Stoks, R., 2019. Additive bioenergetic responses to a pesticide and
654 predation risk in an aquatic insect. Aquat. Toxicol. 212, 205–213.
655 https://doi.org/10.1016/j.aquatox.2019.05.010

656 Van Dievel, M., Stoks, R., Janssens, L., 2017. Beneficial effects of a heat wave: higher growth
657 and immune components driven by a higher food intake. J. Exp. Biol. 220, 3908–3915.
658 https://doi.org/10.1242/jeb.158899
659 Van Drooge, H., Groeneveld, C., Schipper, H., 2001. Data on application frequency of pesticide
660 for risk assessment purposes. Ann. Occup. Hyg. 45, S95–S101.
661 Vasseur, D.A., Delong, J.P., Gilbert, B., Greig, H.S., Harley, C.D.G., McCann, K.S., Savage, V.,
662 Tunney, T.D., O'Connor, M.I., 2014. Increased temperature variation poses a greater risk to
663 species than climate warming. Proc. R. Soc. B 281, 20132612.
664 https://doi.org/10.1098/rspb.2013.2612
665 Vázquez, D.P., Gianoli, E., Morris, W.F., Bozinovic, F., 2017. Ecological and evolutionary
666 impacts of changing climatic variability. Biol. Rev. 92, 22–42.
667 https://doi.org/https://doi.org/10.1111/brv.12216
668 Verheyen, J., Delnat, V., Stoks, R., 2019. Increased daily temperature fluctuations overrule the
669 ability of gradual thermal evolution to offset the increased pesticide toxicity under global
670 warming. Environ. Sci. Technol. 53, 4600–4608. https://doi.org/10.1021/acs.est.8b07166
671 Verheyen, J., Stoks, R., 2019a. Temperature variation makes an ectotherm more sensitive to
672 global warming unless thermal evolution occurs. J. Anim. Ecol. 88, 624–636.
673 Verheyen, J., Stoks, R., 2019b. Current and future daily temperature fluctuations make a
674 pesticide more toxic: Contrasting effects on life history and physiology. Environ. Pollut.
675 248, 209–218. https://doi.org/10.1016/j.envpol.2019.02.022
676 Verheyen, J., Stoks, R., 2019c. Shrinking body size and physiology contribute to geographic
677 variation and the higher toxicity of pesticides in a warming world. Environ. Sci. Technol.
678 53, 11515–11523. https://doi.org/10.1021/acs.est.9b03806

679 Verheyen, J., Temmerman, K., De Block, M., Stoks, R., 2018. Voltinism-associated differences
680 in winter survival across latitudes: integrating growth, physiology, and food intake.
681 Oecologia 186, 919–929. https://doi.org/10.1007/s00442-018-4079-5
682 Verslycke, T., Janssen, C.R., 2002. Effects of a changing abiotic environment on the energy
683 metabolism in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea). J. Exp.
684 Mar. Bio. Ecol. 279, 61–72. https://doi.org/10.1016/S0022-0981(02)00339-8
685 Verslycke, T., Roast, S.D., Widdows, J., Jones, M.B., Janssen, C.R., 2004. Cellular energy
686 allocation and scope for growth in the estuarine mysid *Neomysis integer* (Crustacea:
687 Mysidacea) following chlorpyrifos exposure: A method comparison. J. Exp. Mar. Bio. Ecol.
688 306, 1–16. https://doi.org/10.1016/j.jembe.2003.12.022
689 Wang, G., Dillon, M.E., 2014. Recent geographic convergence in diurnal and annual temperature
690 cycling flattens global thermal profiles. Nat. Clim. Chang. 4, 988–992.
691 https://doi.org/10.1038/nclimate2378
692 Willming, M.M., Maul, J.D., 2016. Direct and indirect toxicity of the fungicide pyraclostrobin to
693 *Hyalella azteca* and effects on leaf processing under realistic daily temperature regimes.
694 Environ. Pollut. 211, 435–442. https://doi.org/10.1016/j.envpol.2015.11.029
695 Willming, M.M., Qin, G., Maul, J.D., 2013. Effects of environmentally realistic daily
696 temperature variation on pesticide toxicity to aquatic invertebrates. Environ. Toxicol. Chem.
697 32, 2738–2745. https://doi.org/10.1002/etc.2354
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Table 1. Results of linear mixed models testing for the effects of pesticide (Pest), mean temperature (Mean T), daily temperature fluctuation (DTF) and latitude (Lat) on bioenergetic response variables in final instar *Ischnura elegans* damselfly larvae. Bold *P*-values are significant ($P < 0.05$), underlined *P*-values indicate a trend ($P < 0.10$).

		Energy availability (Ea)		Energy consumption (Ec)		Cellular energy allocation (CEA)	
Effect	df	χ^2	*P*	χ^2	*P*	χ^2	*P*
Pest	3	81.81	**<0.001**	5.41	0.14	78.76	**<0.001**
Mean T	1	20.27	**<0.001**	43.33	**<0.001**	0.53	0.47
DTF	2	15.55	**<0.001**	14.16	**<0.001**	2.91	0.23
Lat	1	39.59	**<0.001**	5.34	**0.021**	12.07	**<0.001**
Mean T × DTF	2	7.85	**0.020**	26.55	**<0.001**	2.23	0.33
Pest × Mean T	3	7.89	**0.048**	2.93	0.40	1.83	0.61
Pest × DTF	6	5.69	0.46	4.88	0.56	2.12	0.91
Mean T × Lat	1	22.40	**<0.001**	13.42	**<0.001**	2.78	<u>0.095</u>
DTF × Lat	2	7.39	**0.025**	3.26	0.20	2.94	0.23
Pest × Lat	3	3.18	0.37	1.41	0.70	1.14	0.77
Pest × Mean T× DTF	6	14.34	**0.026**	2.69	0.85	8.09	0.23
Mean T × DTF × Lat	2	10.03	**0.0066**	0.86	0.65	5.40	<u>0.067</u>
Pest × Mean T × Lat	3	2.81	0.42	4.88	0.18	1.60	0.66
Pest × DTF × Lat	6	4.11	0.66	7.29	0.29	8.61	0.20
Pest × Mean T × DTF × Lat	6	13.06	**0.042**	3.16	0.79	14.07	**0.029**

Figure legends

Figure 1. Effects of chlorpyrifos on energy availability in *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and latitude. Nominal chlorpyrifos concentrations are 0.75 µg/L (low), 1.50 µg/L (medium) and 2.25 µg/L (high). Means are square-root transformed and given ± 1 SE. Significant (false discovery rate corrected *P*-value < 0.05) effects of chlorpyrifos compared to the solvent control at the same DTF level are indicated by *; trends (false discovery rate corrected *P*-value < 0.10) are indicated by •.

Figure 2. Effects of chlorpyrifos on energy consumption in *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and latitude. Nominal chlorpyrifos concentrations are 0.75 µg/L (low), 1.50 µg/L (medium) and 2.25 µg/L (high). Means are given ± 1 SE.

Figure 3. Effects of chlorpyrifos on cellular energy allocation in *Ischnura elegans* damselfly larvae as a function of mean temperature, daily temperature fluctuation (DTF), and latitude. Nominal chlorpyrifos concentrations are 0.75 µg/L (low), 1.50 µg/L (medium) and 2.25 µg/L (high). Means are square-root transformed and given ± 1 SE. Significant (false discovery rate corrected *P*-value < 0.05) effects of chlorpyrifos compared to the solvent control at the same DTF level are indicated by *; trends (false discovery rate corrected *P*-value < 0.10) are indicated by •.

Figure 4. The relationship between cellular energy allocation (CEA) and life history at the treatment group level: (a) mortality, and (b) growth rate. Each symbol represents a treatment combination (n = 48).

Figures

Figure 1

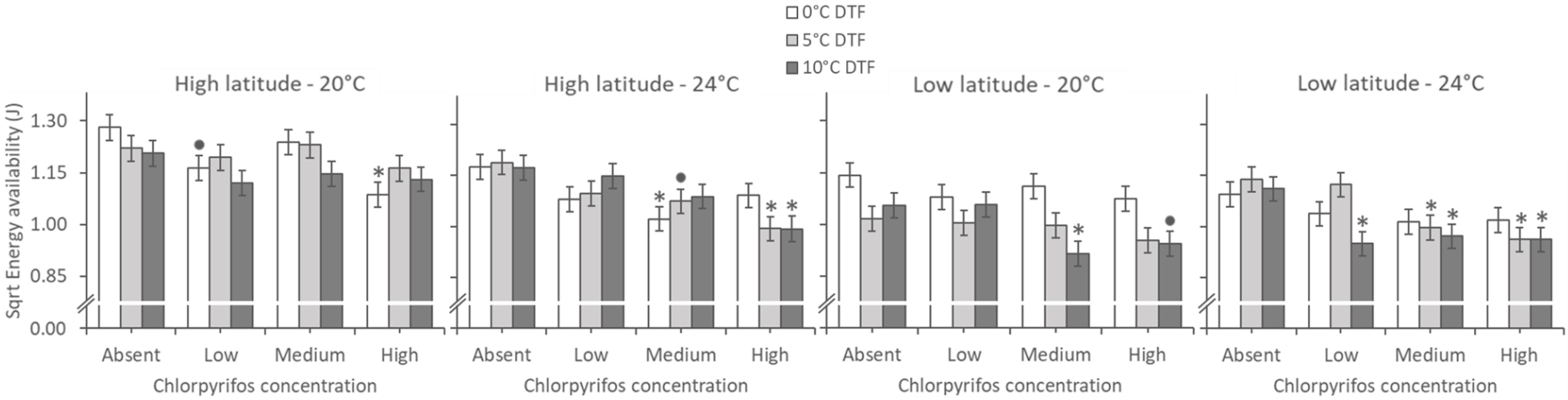


Figure 2

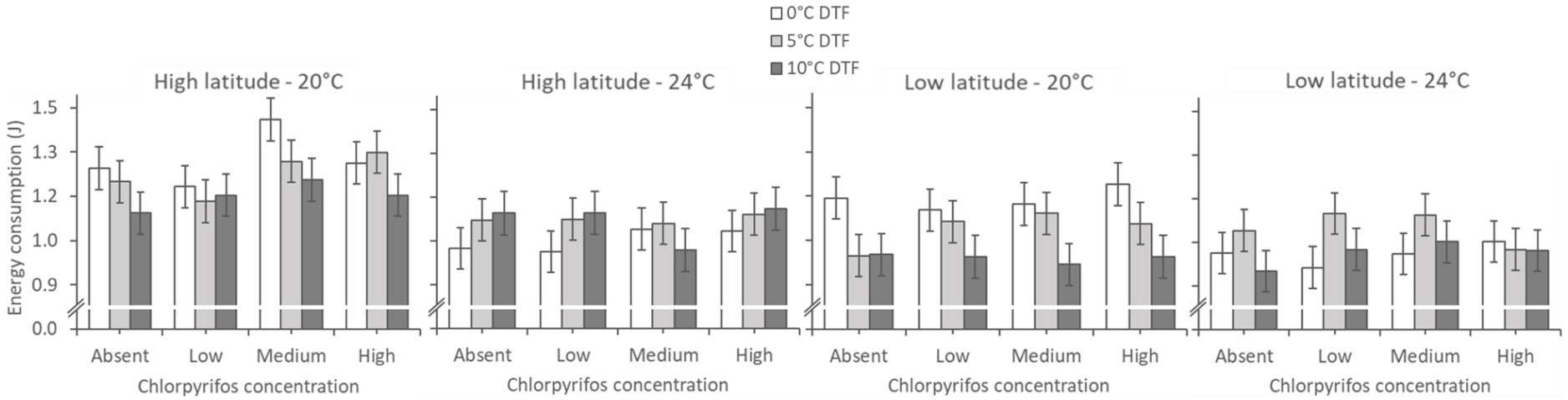

0°C DTF
5°C DTF
10°C DTF
High latitude - 20°C
High latitude - 24°C
Low latitude - 20°C
Low latitude - 24°C
Energy consumption (J)
1.5
1.3
1.2
1.0
0.9
0.0
Absent
Low
Medium
High
Chlorpyrifos concentration

Figure 3

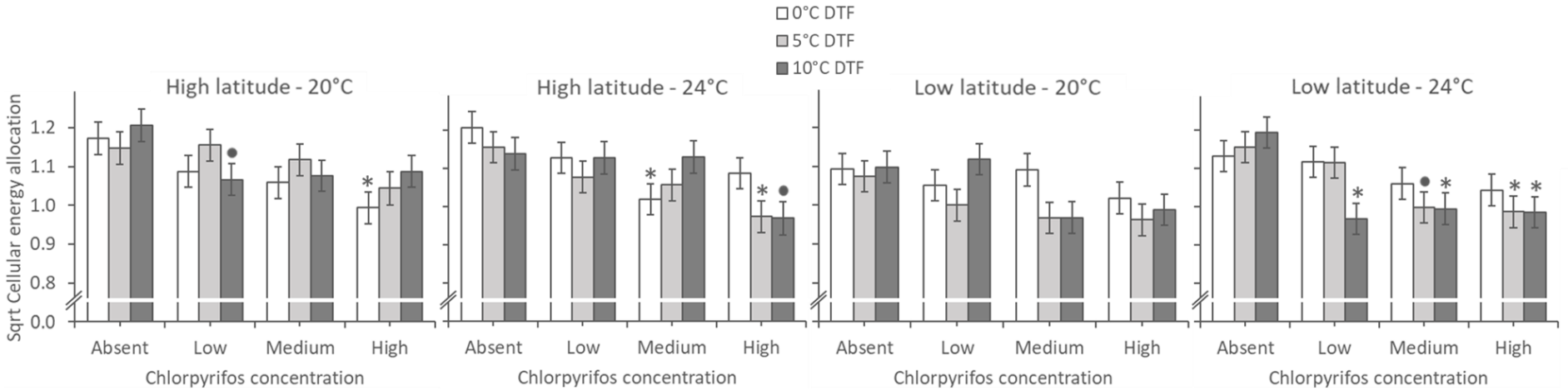
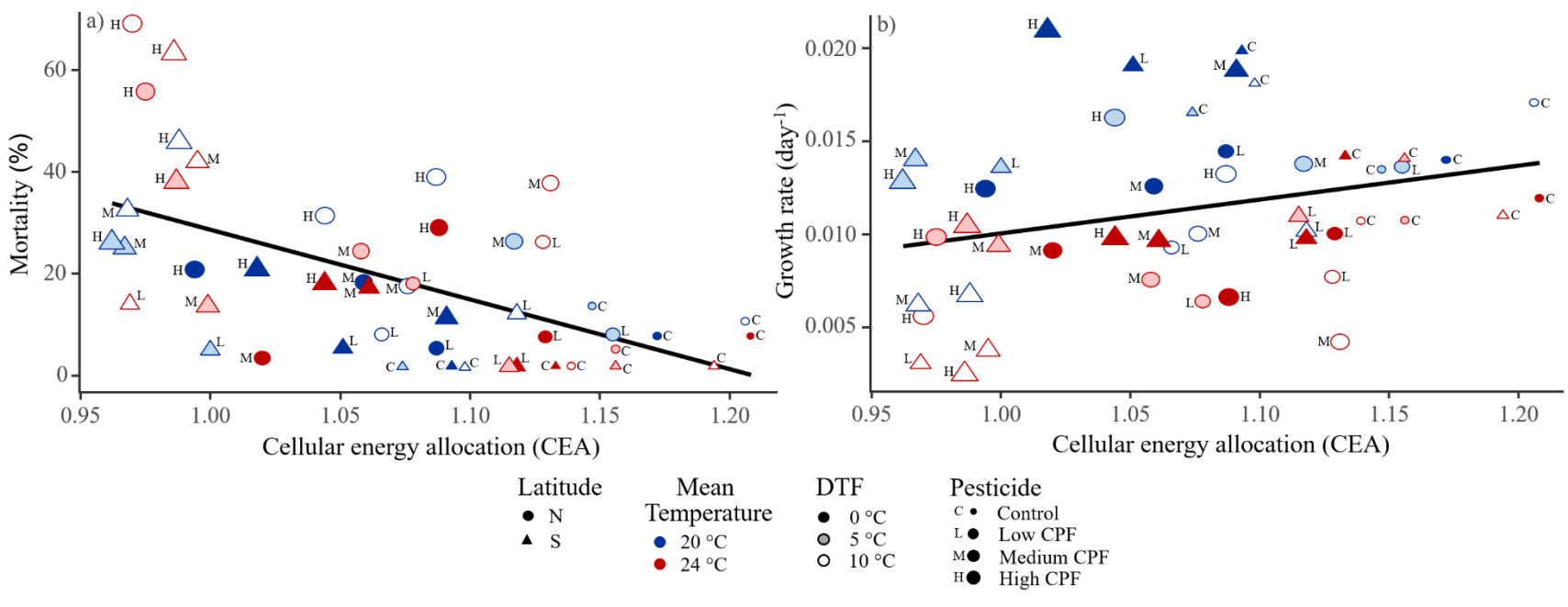

0°C DTF
5°C DTF
10°C DTF
High latitude - 20°C
High latitude - 24°C
Low latitude - 20°C
Low latitude - 24°C
Sqrt Cellular energy allocation
0.0
0.8
0.9
1.0
1.1
1.2
Absent
Low
Medium
High
Chlorpyrifos concentration

Figure 4


a)
Mortality (%)
Cellular energy allocation (CEA)
b)
Growth rate (day-1)
Cellular energy allocation (CEA)
Latitude
N
S
Mean Temperature
20 °C
24 °C
DTF
0 °C
5 °C
10 °C
Pesticide
Control
Low CPF
Medium CPF
High CPF