Current and future daily temperature fluctuations make a pesticide more toxic: contrasting effects on life history and physiology

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Abstract: There is increasing concern that climate change may make organisms more sensitive to chemical pollution. Many pesticides are indeed more toxic at higher mean temperatures. Yet, we know next to nothing about the effect of another key component of climate change, the increase of daily temperature fluctuations (DTFs), on pesticide toxicity. Therefore, we tested the effect of the pesticide chlorpyrifos under different levels of DTF (constant = 0°C, low = 5°C (current maximum level) and high = 10°C (predicted maximum level under global warming)) around the same mean temperature on key life history and physiological traits of *Ischnura elegans* damselfly larvae in a common-garden experiment. At all levels of DTF, chlorpyrifos exposure was stressful: it reduced energy storage (fat content) and the activity of its target enzyme acetylcholinesterase, while it increased the activity of the detoxification enzyme cytochrome P450 monooxygenase. Notably, chlorpyrifos did not cause mortality or reduced growth rate at the constant temperature (0°C DTF), yet increased mortality 6x and reduced growth rate with ca. 115 % in the presence of DTF. This indicates that daily short-term exposures to higher temperatures can increase pesticide toxicity. Our data suggest that when 5°C DTF will become more common in the studied high-latitude populations, this will increase the toxicity of CPF, and that a further increase from 5° DTF to 10°C DTF may not result in a further increase of pesticide toxicity. Our results highlight the biological importance of including daily temperature fluctuations in ecological risk assessment of pesticides and as an extra dimension in the climate-induced toxicant sensitivity concept.

Capsule: Chlorpyrifos only induced mortality and reduced growth rate in the presence of daily temperature fluctuations and not when the temperature was kept constant.

Keywords: Global climate change; Temperature variability; Organophosphate pesticide; Ecological risk assessment; Multiple stressors

Introduction

There is increasing concern that the current ecological risk assessment (ERA) of pollutants is failing to protect biodiversity (Beketov et al., 2013; Rohr et al., 2016). Pesticide concentrations that were considered environmentally safe by the European legislation are causing up to 42% biodiversity loss in European surface waters (Beketov et al., 2013). One major reason for this failure of ERA is that standard ecotoxicology testing does not explicitly take environmental stressors into account, while even individual environmental stressors can increase the toxicity of pesticides by a factor of up to 100 (Liess et al., 2016).

Temperature is a widespread and potent environmental stressor in freshwater ecosystems (Meyer et al., 1999) that interacts with toxic chemicals. Effects of temperature are receiving more and more attention because of climate change. Both toxic chemicals and climate change are recently identified by the European Environment Agency as two areas of major concern for environmental impacts (Van den Brink et al., 2018). Moreover, how these two stressors interact and operate at multiple levels of biological organization, and how this can be integrated in ERA is a top priority research question identified by the European SETAC horizon-scanning workshop (Van den Brink et al., 2018). Many pesticides (including the organophosphate chlorpyrifos: De Silva et al. 2009; Harwood et al. 2009) become more toxic to organisms under higher temperatures and thus under climate change (Noyes et al., 2009; Noyes and Lema 2015). This scenario, where exposure to a climate change related stressor alters the toxicity of pesticides is called “climate-induced toxicant sensitivity” (CITS) (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). The first major mechanism underlying CITS is a higher metabolic conversion of the pesticide to a more toxic metabolite at higher temperatures (Harwood et al., 2009; Noyes et al., 2009). The second major mechanism underlying CITS is that at higher

temperatures, ectotherms have a higher metabolism that causes a higher respiration and food intake to meet the higher metabolic costs (Hallman and Brooks, 2015; Noyes et al., 2009). This results in an increased toxicity when the higher toxicant uptake and especially the higher metabolic conversion overrule any increased detoxification and excretion rates at higher temperatures (Noyes et al., 2009).

While the CITS scenario has much empirical support, experimental studies have almost exclusively tested the effects on toxicity of higher mean temperatures (Holmstrup et al., 2010; Noyes et al., 2009). However, another important aspect of climate change is an increase in the magnitude of daily temperature fluctuations (DTFs, Colinet et al. 2015; Vázquez et al. 2015). Notably, DTFs may have more severe effects for species than increases in mean temperatures (Vasseur et al., 2014). The very limited evidence indicates that the toxicity of pesticides under a constant temperature regime may not correspond to the actual pesticide toxicity that organisms encounter in their natural habitat where large DTFs can occur (Barbosa et al., 2017; Willming et al., 2013; Willming and Maul, 2016; for metals: Hallman and Brooks, 2015). For example, the pyrethroid insecticide bifenthrin is more lethal to *Chironomus dilutes* midges and the organochlorine fungicide chlorothalonil is more lethal to the amphipod *Hyalella azteca* under a DTF of 10°C compared to a constant water temperature of 24°C (Willming et al., 2013). This indicates that it might be important to integrate DTFs as an extra dimension to the CITS concept to better assess the ecological impact of pesticides. Our knowledge of how DTFs increase toxicity of pesticides is, however, in its infancy and studies so far mainly focused on life history and largely ignored physiology (except for cholinesterase activity, Willming et al. 2013). Two of these studies (Hallman and Brooks, 2015; Willming and Maul, 2016) did expose animals to a currently experienced DTF level and a predicted increase in DTF level, which is needed to better

integrate DTF in ERA under climate change. However, in both studies DTF levels had different mean temperatures than the constant temperature treatment, precluding disentangling the effects of DTF and mean temperature.

The aim of this study was to investigate how current and future daily temperature fluctuations (DTFs) around the same mean temperature influence the toxicity of the organophosphate pesticide chlorpyrifos within a single generation in an aquatic insect. We thereby looked at effects on life history and physiology. As study organism, we chose larvae of the damselfly *Ischnura elegans*. Since damselfly larvae have an obligate aquatic life during which they cannot escape exposure to neither warming (Hassall and Thompson, 2008) or toxicants (Liess and Von Der Ohe, 2005), they are particularly vulnerable to both stressors. Furthermore, *I. elegans* larvae are typically exposed to DTFs in the shallow freshwater ponds they prefer. We chose to study high-latitude populations as these currently experience rather low DTF levels (maximum 5°C), which however, are expected to double (maximum 10°DTF) by 2100 under the IPCC (2013) scenario RCP8.5. As model pesticide we chose the insecticide chlorpyrifos (CPF), this organophosphate is frequently used worldwide (Eaton et al., 2008). CPF belongs to the top ten chemicals found in surface waters in the UK that have the highest risk to aquatic organisms (Johnson et al., 2017) and is considered as a priority pollutant by the European Water Framework Directive (2000/60/EC). We performed a static renewal experiment, where the medium was daily refreshed, to keep the CPF concentrations constant among temperature treatments to study how DTF changes the toxicity of the same CPF concentration. It is already shown that an increase in mean temperature elevated CPF toxicity in the study species when the concentration is kept constant (Dinh Van et al., 2014).

To get more insights in whether CPF exposure was also more stressful under DTF at the physiological level, we measured the following important condition-related traits in damselflies (Rolff and Joop, 2002; Stoks and Córdoba-Aguilar, 2012): total fat content, the major energy storage molecule, and activity levels of the enzyme phenoloxidase (PO), a key enzyme in immune function of insects. We also quantified a set of physiological traits that are linked to CPF toxicity: the activity of acetylcholine esterase (AChE) which is the target enzyme of CPF (Domingues et al., 2010), the activity of cytochrome P450 monooxygenase (Cyt P450) as this is an important detoxification enzyme for xenobiotics (including CPF, Rakotondravelo et al. 2006), and lipid peroxidation (measured as malondialdehyde: MDA) to assess oxidative damage to lipids (Monaghan et al., 2009).

Materials and methods

Study populations and rearing

We sampled *I. elegans* at three shallow lakes in the high-latitude region of the species' range (Gosden et al., 2011). Two populations were located in Denmark (Laesoe 57°15'12.14"N - 10°54'19.75"E and Roskilde 55°39'09.80"N - 12°08'01.68"E), and one population in southern Sweden (Lund 55°44'5.4"N - 13°9'13.4"E). Based on the surrounding land use these populations were likely not influenced by pesticide exposure because agricultural area was situated at > 100m distance from each pond (Hua et al., 2015).

We did the exposure experiment with the first generation (F1) larvae obtained from field-collected mothers. In each population ca. 25 mated females were sampled in the early summer of 2015 (end of June – early July). We kept the females individually in plastic cups (7.5 cm height, 3.5 cm diameter), to which a wet filter paper was added for oviposition. Filter papers that carried eggs were transported to the laboratory in Leuven (Belgium). Immediately after they arrived in

the laboratory, the eggs were placed in a temperature-controlled room to keep the water temperature at 20°C with a 14:10h light-dark cycle. Ten days after the F1 larvae hatched, larvae were reared in groups of 15 larvae in 2L plastic boxes. After two months, larvae were separated and placed individually in plastic cups (7.5 cm height, 3.5 cm diameter) filled with dechlorinated tap water (100 mL). Larvae were kept in the same temperature-controlled room as where the eggs were incubated to keep the same rearing conditions in terms of water temperature and photoperiod. We fed the larvae *Artemia* nauplii ad libitum, five days per week (Monday-Friday).

To mimic fall and winter conditions, cups with larvae were placed in an outdoor cage next to the laboratory building at the end of September. During this period temperatures ranged between 4 and 12°C, which spans the temperatures also encountered in the study populations during this period (Flake model, Simmons et al. 2007). In mid-January, larvae were placed back inside in incubators where water temperature was kept at 10°C. We then further raised the water temperature in gradual steps over nine days to 22°C at 4 February. From the end of September till 4 February, larvae were fed the same amount *Artemia* nauplii as before but only three days a week (Monday, Wednesday and Friday). From 4 February onwards, we raised feeding again to five days a week (Monday-Friday). We checked every second day for larvae that reached the last larval stage (final instar). At that moment, they were transferred to clean plastic cups (7.5 cm height, 3.5 cm diameter) with 100 mL dechlorinated tap water for the experiment.

Experimental design

We ran a full factorial experiment on the F1 larvae with six treatment combinations: three daily temperature fluctuations (DTFs: constant = 0°C, low = 5°C, high = 10°C) × two pesticide treatments (solvent control, chlorpyrifos) to study the single and combined effects of DTFs and pesticide exposure on high-latitude populations of *I. elegans* damselfly larvae (Fig. 1a). The DTF

148 treatment started one day after larvae moulted into the final instar ('pre-exposure period'), the
149 pesticide treatment started 7 days later and lasted for 6 days ('exposure period'). Larvae were fed
150 *Artemia* nauplii ad libitum once a day during the entire 13-day experiment. We started between
151 42 and 51 larvae per DTF treatment in the pre-exposure period, and between 21 and 30 larvae
152 per DTF-by-CPF combination during the pesticide-exposure period (total of 142 larvae). Per
153 combination larvae were as equally distributed as possible across populations. The number of
154 larvae that started per treatment in both periods differed depending on mortality during the
155 pesticide-exposure period, since we wanted to end up with 20 replicates per treatment
156 combination at the end of the pesticide-exposure period to quantify larval growth rate across the
157 exposure period and physiological traits at the end of the exposure period (see detailed overview
158 in Figure S1 in Appendix 3).
159 All DTF treatments had the same mean of 20°C that matches the mean summer water
160 temperature of shallow lakes inhabited by the study species in southern Scandinavia (De Block et
161 al., 2013; Debecker and Stoks, 2019). The low daily temperature fluctuation (DTF) of 5°C
162 corresponds to the maximum DTF that occurs in shallow (< 1m) clear freshwater bodies in the
163 study region in July. DTF data were based on the freshwater lake model (Flake, Simmons et al.
164 2007) and temperature data measured in shallow freshwater bodies at high latitude (unpublished
165 data). Since under global warming daily temperature fluctuations are expected to increase in
166 magnitude (Colinet et al., 2015; IPCC, 2013), we also included a high DTF of 10°C based on
167 model predictions (following Paaijmans et al. 2013). We derived the future maximum DTF at the
168 high latitude as the difference between the daily maximum and minimum temperature data
169 predicted for 2080 under the RCP 8.5 scenario (IPCC, 2013). Therefore, we used the BCC_CSM
170 1.1 (Beijing Climate Center Climate System Model 1.1) and the Delta Method IPCC AR5 and

171 applied a spatial resolution of 2.5 min (downloaded data from http://www.ccafs-
172 climated.org/Data). To obtain the predicted DTF by 2080, we imported the modeled temperature
173 data in ArcGIS Pro 2.2. The DTF regimes were programmed in temperature-controlled
174 incubators. Throughout the whole experiment, water temperatures were logged every 10 minutes
175 in each temperature treatment by Hobo onset data loggers (TidbiT v2 Temp logger). The
176 resulting thermal regimes during the experiment are given in figure 1b. The photoperiod was set
177 at 14:10h (L:D).

178 Based on a range finding experiment, where we exposed final instar larvae for six days to
179 a range of CPF concentrations (0, 1, 2, 3 and 4 µg/L) with daily renewal of the medium, we
180 selected a CPF concentration of 2 µg/L (see results in Appendix S1). This was the lowest
181 concentration that caused a growth reduction in the *I. elegans* larvae at 10°C DTF. This CPF
182 concentration is very high, but CPF peak concentrations can reach 100 µg/L due to runoff in
183 edge-of-field waterbodies (Bernabò et al., 2011). In these waterbodies, the study species can be
184 very abundant (Dijkstra, 2006).

185 First, we prepared a stock solution of 1 mg/mL by dissolving CPF powder (Sigma-
186 Aldrich, purity >99%) in absolute ethanol (100%). The stock solution was renewed monthly and
187 stored in an amber glass bottle in a dark cold room (4°C). To obtain a concentration of 2 µg/mL
188 we diluted the stock solution with Milli-Q water and we further diluted this solution with
189 dechlorinated tap water to obtain the experimental CPF concentration of 2 µg/L. In the control
190 treatment, we used a solvent control where the same amount of absolute ethanol (100%) was
191 added (2 µL/L). Ethanol concentrations up to 5 µL/L have been shown to cause no effects on
192 larval survival and growth rate in coenagrionid damselflies (L. Janssens, unpublished data).
193 Moreover, the used ethanol concentration (2 µL/L) is circa 50 times lower than the concentration

recommended by OECD (100 µL/L; OECD 2000). Before the 6-day CPF-exposure period started, larvae were transferred individually from the plastic rearing cups to glass jars (200 mL) which were filled with 100 mL of the control or the CPF medium. The medium was daily renewed (static renewal). To determine the CPF concentrations in the vials, we took water samples at the start (3 replicates) and after 24h of the exposure period (3 replicates per DTF treatment), this is directly before renewal of the medium. Samples were analyzed at KU Leuven using UPLC MS/MS with Triple Quadrupole Mass Spectrometry. More details about analytical quantification of CPF concentrations can be found in Appendix S2. The mean CPF concentration in the vials at the start of the exposure period was 1.919 µg/L (SE: 0.796). After 24 hours, the concentration lowered to 0.933 µg/L (SE: 0.029) at the constant (0°C), to 1.010 µg/L (SE: 0.027) at the low (5°C) and to 1.174 µg/L (SE: 0.152) at the high (10°C) DTF treatment. During the pesticide-exposure period, we took water samples at the start (3 replicates) and after 24h of the exposure period (3 replicates per DTF treatment) in both the solvent control and pesticide treatment to measure the following physico-chemical parameters: dissolved oxygen (mg/L), pH, conductivity (µS/cm) and hardness (mg/L $CaCO_3$) (see results in Appendix S2).

Response variables

We quantified effects on life history traits (mortality and growth rate), condition-related traits (phenoloxidase activity and total fat content), and physiological traits related to the pesticide's mode-of-action (the activity of acetylcholinesterase) and detoxification (cytochrome P450), and the resulting damage (oxidative damage to lipids).

We checked daily for mortality during both the pre-exposure and pesticide-exposure periods. No mortality was associated with recording the mass of the larvae. To estimate larval growth rate during both periods, each larva was weighed to the nearest 0.01 mg (using an

electronic balance from Mettler Toledo® AB135-S, Ohio, USA) at three time points in the experiment: at the start of the pre-exposure period, and at the start and end of the pesticide-exposure period. To obtain reliable wet mass data, larvae were gently blotted dry by using tissue paper; wet mass data obtained this way highly correlate with the dry mass (Stoks et al., 2005). We calculated larval growth rates for both periods as [ln (final mass) – ln (initial mass)] divided by the length of the period. All larvae were frozen (-80°C) at the last day of the experiment for further physiological analyses. Sample sizes for larval growth rate varied between 42 and 51 larvae per DTF treatment during the pre-exposure period (total of 142 larvae), to arrive at 20 larvae per DTF-by-pesticide combination during the pesticide-exposure period due to mortality in the pesticide-exposure period (total of 120 larvae). See Figure S1 in Appendix S3 for a detailed schematic overview of the sample sizes. We spectrophotometrically quantified all physiological traits on the body supernatants, except for acetylcholinesterase (AChE) activity which was measured on the head supernatants (see Appendix S4 for detailed protocols). Sample sizes for the physiological traits ranged from 17 to 20 larvae (see figures for exact sample sizes of all response variables).

Statistics

Data were analyzed statistically using R 3.4.0. for Windows (R Core Team, 2014). For all response variables with a normal error structure, general linear mixed models (GLMM) with a normal error structure and identity link were used from the 'lme4' package (Bates et al., 2015). For the pre-exposure period, we tested the effects of daily temperature fluctuation (DTF) on larval growth rate. We did not analyze the effects of DTF on mortality since there was no mortality in any of the three DTF treatments. For the pesticide-exposure period, we tested the effects of DTF, the pesticide treatment, and their interaction in separate GLMMs for larval

240 growth rate and each physiological trait. Moreover, as we had two repeated measurements of
241 growth rate per larva (during the pre-exposure and during the exposure periods) we also ran an
242 overarching repeated-measures analysis of variance (RM-ANOVA) with a normal error structure
243 and identity link. In this model we added larva as random factor to take into account the
244 successive (repeated) measurements of growth rate of the same larvae in both periods. In each
245 model we included female (mother of the larvae) nested in population and population as random
246 factors. While these random factors never reached significance (all $P > 0.09$), they were kept in
247 the models. The package 'lmerTest' was used to calculate p-values for random effects
248 (Kuznetsova et al., 2017). We also included the sex of the larvae as a fixed factor (without
249 interactions with DTF and CPF) in each model. Results of the full models (including sex and its
250 interactions with DTF and CPF) are reported in Appendix S5 since there were almost no effects
251 of sex and to keep the manuscript easy to read. The 'car' package was used to calculate Wald
252 chi-square statistics and p-values for fixed effects (Fox and Weisberg, 2011). Least-square means
253 were compared to further analyze significant interactions between the DTF and pesticide
254 treatments by using Tukey posthoc tests from the 'lsmeans' package (Lenth, 2016), and p-values
255 were False Discovery Rate (FDR) corrected. Total fat content and MDA levels were square root
256 transformed to meet the ANOVA assumption of normality. After transformation, both models
257 met the ANOVA assumptions.

258 Effects on the binary response variable mortality were initially tested using a generalized
259 linear mixed model with a binomial error distribution and logit-link function. However, during
260 the pesticide-exposure period there was almost no larval mortality in four (out of six) treatment
261 combinations (including the three solvent control treatments, see results). This caused quasi
262 complete separation of 0's and 1's in the data set. This generates large parameter estimates with

very large (to infinite) standard errors and very large p-values ($p \approx 1$), a phenomenon that is called the Hauck-Donner effect (Fox et al., 2015; Venables and Ripley, 2002). Therefore, the effect of pesticide exposure on mortality was analyzed separately for each DTF treatment by using G-tests (Sokal and Rohlf, 2001). This allowed more focused testing of the pesticide being lethal at each of the DTFs. We performed a (FDR) correction for multiple testing (n=3 G-tests).

Results

Life history

No mortality occurred during the pre-exposure period, and mortality was low (ca. 5%) in the solvent control during the pesticide-exposure period. Overall, exposure to chlorpyrifos caused an increase in larval mortality (Pesticide: $\chi^2_1 = 5.67$, $P = 0.017$; Fig. 2). Separate G-tests per DTF treatment showed that pesticide exposure increased larval mortality to ca. 30% at 5°C DTF ($G = 5.19$, $df = 1$, $P = 0.034$) and 10°C DTF ($G = 6.95$, $df = 1$, $P = 0.025$), but not when at the constant temperature (0°C DTF)) where it remained at ca. 5% ($G = 0$, $df = 1$, $P = 1$).

During the pre-exposure period, larval growth rate was lower at 10°C DTF compared to 0°C and 5°C DTF (Tukey tests: both $P = 0.045$), while there was no difference in growth rate between 0°C and 5°C DTF (Tukey test: $P = 0.76$) (DTF effect, Table 1; Fig. 3a). During the pesticide-exposure period, chlorpyrifos decreased larval growth rate more than 100 %, but only at 5°C and 10°C DTF (Tukey tests: both $P < 0.001$) and not at 0° DTF (Tukey test: $P = 0.14$) (DTF × Pesticide, Table 1, Fig. 3b). These results were confirmed by a three-way interaction between DTF, pesticide exposure and period in the overarching RM-ANOVA (Table 2). Exposure to chlorpyrifos in the pesticide-exposure period only decreased larval growth rate compared to the pre-exposure period at 5°C and 10°C DTF (Tukey tests: both $P < 0.001$) and not

285 at 0°C DTF (Tukey test: $P = 0.11$). In the absence of chlorpyrifos, there was no difference in
286 larval growth rate between the two periods (Tukey tests: all $P > 0.097$).

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288 Pesticide exposure reduced the total fat content but did not affect the PO activity (Pesticide
289 effect, Table 1; Fig. 4a-b). DTF or its interaction with CPF did not affect both condition-related
290 traits (Table 1, Fig. 4a-b).

291 Exposure to chlorpyrifos resulted in a lower AChE activity and a higher cytochrome
292 P450 monooxygenase activity (Pesticide effect, Table 1; Fig. 5a-b). There were no effects of
293 DTF and its interaction with CPF on both enzyme activities (Table 1). Under pesticide exposure,
294 MDA levels were lower (Pesticide effect, Table 1; Fig. 5c). MDA levels were not affected by
295 DTF or its interaction with CPF (Table 1).

296 **Discussion**

297 Exposure to the pesticide chlorpyrifos (CPF) at the constant temperature (0°C DTF) did not
298 affect mortality and growth rate, yet caused changes in physiology. Exposure to daily
299 temperature fluctuations (DTFs) in the absence of CPF had little effect, except for the highest
300 DTF of 10°C reducing growth rate during the pre-exposure period. Nevertheless, and as
301 expected, the toxicity of CPF was strongly influenced by the temperature regime: both DTFs
302 caused an increase in CPF toxicity compared to a constant temperature at the same mean
303 temperature of 20°C. Indeed, a striking finding was that exposure to CPF increased mortality and
304 decreased larval growth rate compared to the control treatment, but only when DTFs were
305 present. This highlights the biological importance of including daily temperature fluctuations that
306 organisms encounter in their natural habitat (Colinet et al., 2015) when assessing the toxicity of
307 pesticides in ecological risk assessment. Notably, the predicted maximum DTF level of 10°C by

2080 under climate change did not further increase the pesticide toxicity compared to the current maximum DTF level of 5°C.

A key finding was that daily temperature fluctuations, both low (5°C) and high (10°C), increased CPF toxicity at the lethal level and the sublethal level (lower growth rate) in *I. elegans* larvae compared to the constant temperature regime around the same mean of 20°C. This occurred while CPF did not have any effects on the mortality or larval growth rate under the constant temperature regime of 20°C. In other words, the CPF concentration that was used in this experiment would have been regarded a NOEC ('No Observed Effect Concentration') if we had only tested the species under the standard conditions of constant 20°C. These standard conditions are typically used in ecotox laboratory testing, while at the current DTF (going up to 5°C in this region), this CPF concentration may cause considerable mortality (6x higher than the solvent control). Our study adds to the few others showing an increased toxicity of pesticides under DTF (Barbosa et al., 2017; Willming et al., 2013; Willming and Maul, 2016; for metals: Hallman and Brooks 2015). The increased mortality in the presence of DTF matches the study of Willming et al. (2013), where a DTF of 10°C made the pyrethroid insecticide bifenthrin more lethal to *Chironomus dilutes* midges and the organochlorine fungicide chlorothalonil more lethal to the amphipod *Hyalella azteca*, compared to when the water temperature was kept constant at 24°C. Finally, stronger negative sublethal effects of the pharmaceutical fluoxetine under fluctuating temperatures have also been found on the reproductive success and population growth rate of the water flea *Daphnia magna* (Barbosa et al., 2017).

The mechanisms causing an increased toxicity under DTF are not understood but may partly match those causing the widespread pattern that pesticides such as organophosphates are more toxic at higher mean temperatures (Noyes et al., 2009; Noyes and Lema, 2015). The higher

toxicity of many pesticides at higher constant mean temperatures has been explained by two major mechanisms. First, a higher metabolic activity at higher temperatures is expected to result in an increased uptake of pesticides (Hooper et al. 2013), especially via the caudal gills in aquatic insects (Buchwalter et al., 2003). Second, the most important mechanism according to Harwood et al. (2009), is a higher metabolic conversion of the original molecule to a more toxic metabolite, specifically the conversion of CPF to the more toxic CPF-oxon (Buchwalter et al., 2004). Possibly, these processes also contributed to the higher toxicity of CPF under fluctuating temperatures. Our results indeed extend the observation that CPF is more toxic (in terms of both mortality and growth reduction) at the higher mean temperature of 24°C than at a mean of 20°C in the here studied high-latitude populations of *I. elegans* (Dinh Van et al., 2014). During a 24h cycle, larvae were exposed to higher temperatures up to 22.5°C (DTF 5°C) and up to 25°C (DTF 10°C) for several hours. Our data suggest that these daily short-term exposures to higher temperatures are enough to increase pesticide toxicity.

Notably, CPF toxicity was not stronger at 10°C compared to 5°DTF. Possibly, there was a more pronounced acclimatization response in larvae exposed to 10°C DTF than to 5°C DTF triggered by the more stressful DTF level of 10°C as indicated by the observation that only 10°C DTF caused a growth reduction on the pre-exposure period (larvae in the 5°C DTF treatment had the same growth rate that those reared at 0°C DTF). A stronger acclimatization response at 10°DTF may have resulted in 5°C and 10°C DTF causing the same increase in CPF toxicity. Acclimatization responses to DTF have been documented (e.g. Bozinovic et al., 2013; Meráková and Gvozdík, 2009). Furthermore, the absence of a stronger effect of 10°C DTF compared to 5° DTF on growth rate in CPF-exposed larvae during the exposure period is possibly due to the already negative growth rate at 5°C DTF. CPF-exposed larvae may have avoided an even

stronger mass loss than at 5°C by re-allocating energy to growth away from other processes such as investment in immune function.

Processes that reduce pesticide toxicity may also increase at higher temperatures, yet these were apparently absent or overruled as the net effect was an increased CPF toxicity under DTF. Indeed, at higher temperatures one could expect higher degradation rates of the pesticide in the water (Hooper et al., 2013), higher elimination rates outside the body (Harwood et al., 2009), and higher detoxification rates in the organism (Laetz et al., 2014). In our study, the degradation rate probably did not have a big impact since the medium was daily refreshed to keep the CPF concentrations constant among treatments. Moreover, we detected no difference in detoxification rates between DTF treatments, at least in terms of the activity of the phase one detoxification enzyme cytochrome P450. Moreover, since an increased metabolic conversion to the more toxic CPF oxon metabolite plays a major role in the higher CPF toxicity at higher temperatures (Harwood et al., 2009), any increased elimination rates of CPF seem to be of minor importance.

While CPF at the mean temperature did not affect life history traits, it did affect all physiological traits (except for PO activity) and this irrespective of the DTF treatment. As expected, acetylcholinesterase (AChE), the target enzyme of CPF that is involved in neurotransmission (Domingues et al., 2010), was inhibited in CPF-exposed larvae. The CPF-induced inhibition of AChE is a general response in freshwater insects (Domingues et al., 2010), including *I. elegans* larvae (Dinh Van et al., 2014). Also the energy storage was reduced under CPF exposure, thereby matching similar findings in other aquatic invertebrates (Janssens and Stoks 2013; Jeon et al. 2013), including the study species (Dinh Van et al., 2016). Several non-exclusive mechanisms may explain this reduction in energy storage (Campero et al., 2007; Xuereb et al., 2009): a reduced feeding rate (Ribeiro et al. 2001); the inhibition of the enzyme

AChE (Newman, 1998), and the diversion of energy away from energy storage towards maintenance (Congdon et al., 2001) and/or detoxification and repair (Sokolova and Lannig, 2008). In line with the latter explanation, we observed an increased activity of the detoxification enzyme cytochrome P450 in larvae exposed to CPF (for other studies on invertebrates: Rakotondravelo et al. 2006; Chang et al. 2017). The increased cytochrome P450 activity may be also one of the reasons why MDA levels (our measure of oxidative damage to lipids) were unexpectedly reduced when larvae were exposed to CPF. Pesticides, including CPF (Kavitha and Rao, 2008), can generate reactive oxygen species (ROS), leading to oxidative stress and eventually damage (e.g. lipid peroxidation), if not counterbalanced by antioxidant defense mechanisms (Valavanidis et al., 2006). Besides the increase in cytochrome P450, CPF-exposed larvae may have upregulated antioxidant enzymes to neutralize the increased ROS and consequently reduce MDA levels. For example, increased activity levels of CAT and GPx have been found after CPF exposure in the freshwater gastropod *Planorbarius corneus* (Cacciatore et al., 2015) and in the freshwater snail *Lanistes carinatus* (Khalil, 2015). In general, reductions in MDA levels when animals are exposed to pesticides are rare, but for example observed in fish (*Prochilodus lineatus*) exposed to the herbicide atrazine (Santos and Martinez, 2012).

In contrast with the effects of life history, the CPF effects on the physiological traits were already present at the constant temperature and not stronger in the presence of DTF. Likely the 25% higher mortality when CPF was combined with 5°C-10°C DTF caused survival selection, thereby removing the weakest larvae with the strongest AChE inhibition and strongest reduction in fat storage. Notably, a similar decoupling of life history and physiological responses has been observed in *I. elegans* larvae in response to a 4°C higher mean temperature. Indeed, while the effect of CPF was stronger on mortality and growth at a constant temperature of 24°C

compared to 20°C, this was not the case for the physiological traits (activity levels of AChE and GST) (Dinh Van et al., 2014). Only one other ecotox study tested for effects of DTF on physiology. Willming et al. (2013) showed that while malathion inhibited the ChE activity in *D. magna*, this inhibition was equally strong under DTF, however, DTF resulted in overall higher ChE levels.

Conclusions and implications

Our data suggest that when 5°C DTF becomes more common in the studied high-latitude populations, it is likely to contribute to an increased toxicity of pesticides such as CPF. Our data also suggest that a further increase from 5°C DTF to 10°C DTF may not result in a further increase of pesticide toxicity. As no other ecotox studies imposed two levels of DTF and kept the mean temperature the same, more studies are needed that expose organisms to a range of DTF levels, to evaluate whether this reflects a general threshold pattern. We could demonstrate these effects of DTF on CPF toxicity without exposure of the previous generations to CPF (cfr. Barbosa et al., 2017). Multigenerational exposure experiments to CPF may further increase our insights in the potentiating effect of DTF on pesticide toxicity. Understanding how climate change affects the impact of chemicals is pivotal to improve risk assessment in a warming world (Bednarska et al., 2013; Moe et al., 2013; Stahl et al., 2013). Against this background, our results highlight the importance of integrating daily temperature fluctuations as an extra dimension to the CITS (climate-induced toxicant sensitivity, Hooper et al. 2013) concept at the interface of global change biology and ecotoxicology.

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649 **Table 1.** Results of linear mixed models testing for the effects of daily temperature fluctuation (DTF) on growth rate during the pre-
650 exposure period and testing for the effects of daily temperature fluctuation (DTF) and pesticide exposure (Pest) on growth rate, and a
651 set of physiological traits during the pesticide-exposure period in *Ischnura elegans* damselfly larvae. Sex was added as fixed factor
652 (without interactions) to each model. Bold *P*-values are significant (< 0.05).

	Pre-exposure period			Pesticide-exposure period											
	Life history trait			Life history trait			Condition-related traits				Other physiological traits					
	Growth rate			Growth rate			Total fat content		PO activity		AChE activity		CytP450 activity		MDA levels	
Effect	df	χ^2	*P*	df	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
DTF	2	7.24	**0.027**	2	19.47	< **0.001**	1.54	0.46	0.95	0.62	0.68	0.71	0.54	0.76	0.77	0.68
Pest	/	/	/	1	3.19	0.074	5.76	**0.016**	0.91	0.34	6.68	**0.01**	4.27	**0.039**	7.16	**0.007**
Sex	2	0.26	0.88	1	0.24	0.63	0.053	0.82	3.29	0.07	0.36	0.55	0.81	0.37	4.15	**0.042**
DTF × Pest	/	/	/	2	6.48	**0.039**	0.54	0.76	0.99	0.61	1.36	0.51	2.46	0.29	0.53	0.77

653

Table 2. Results of the repeated-measures analyses of variance testing for the effects of daily temperature fluctuation (DTF), pesticide exposure (Pest), and Period (pre-exposure period and exposure period) on growth rate in *Ischnura elegans* damselfly larvae. Sex was added as fixed factor to the model. Bold *P*-values are significant (< 0.05).

	Growth rate		
Effect	χ^2	df	*P*
DTF	26.39	2	**< 0.001**
Pest	5.83	1	**0.016**
Period	2.59	1	0.11
Sex	0.75	2	0.69
DTF × Pest	8.31	2	**0.016**
DTF × Period	21.87	2	**< 0.001**
Pest × Period	0.00	1	0.96
DTF × Pest × Period	14.22	2	**< 0.001**

660 **Figure legends**

661 **Figure 1.** (a) Overview of the experimental setup and (b) the three realized thermal regimes over
662 a 24 hour period, measured in the water using Hobo Onset data loggers. The figure (b) shows
663 three daily temperature fluctuations (constant: 0°C DTF, low: 5°C DTF and high: 10°C DTF)
664 around the same mean temperature of 20°C. The grey area around the black curves (means of ca.
665 35 days) gives the 95% confidence intervals.

666 **Figure 2.** Mean (± 1 SE) percentage mortality of *Ischnura elegans* during the pesticide exposure
667 period as a function of daily temperature fluctuation (DTF) and pesticide exposure. Different
668 letters indicate significant differences between treatment combinations based on FDR-corrected
669 Tukey tests.

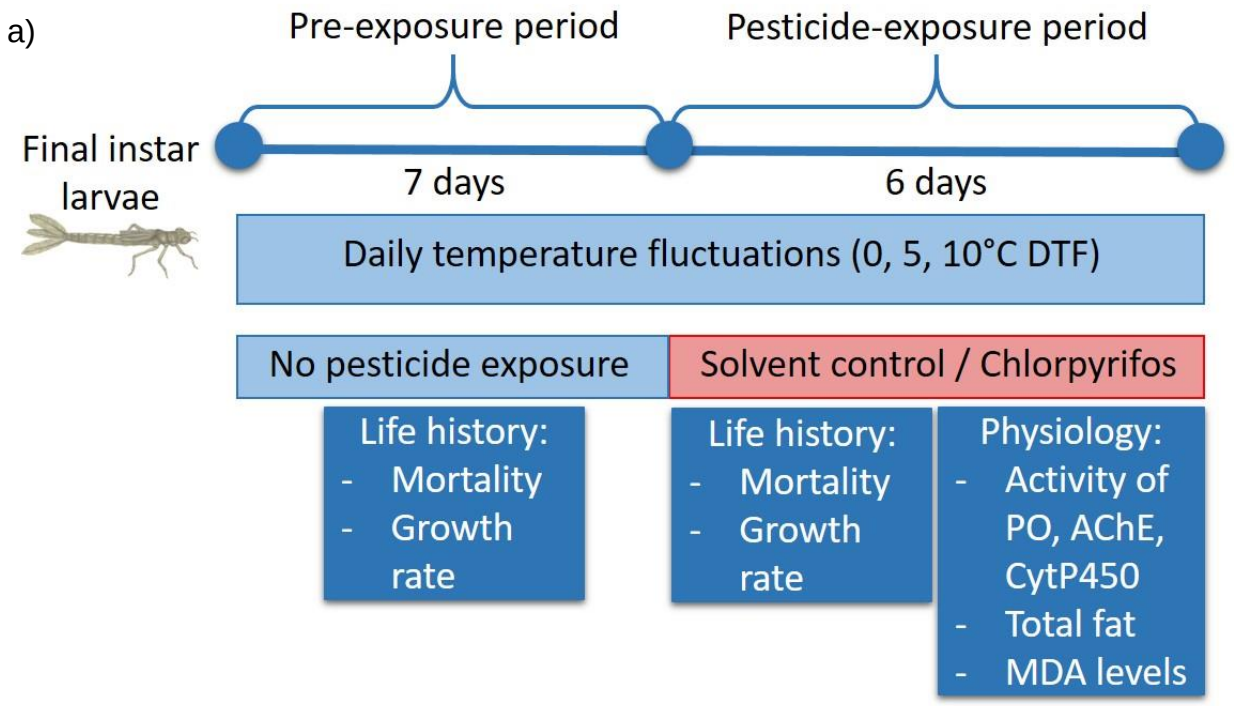
670 **Figure 3.** Mean (± 1 SE) larval growth rate of *Ischnura elegans* (a) during the pre-exposure
671 period as a function of daily temperature fluctuation (DTF), and (b) during the pesticide-
672 exposure period as a function of daily temperature fluctuation (DTF) and pesticide exposure.
673 Numbers indicate sample sizes per treatment combination. Different letters indicate significant
674 differences between treatment combinations based on FDR-corrected Tukey tests.

675 **Figure 4.** Mean (±1 SE) levels of two condition-related traits of *Ischnura elegans* at the end of
676 the pesticide-exposure period as a function of daily temperature fluctuation (DTF) and pesticide
677 exposure: (a) phenoloxidase (PO) activity, and (b) total fat content. Numbers indicate sample
678 sizes per treatment combination. Different letters indicate significant differences between
679 treatment combinations based on FDR-corrected Tukey tests.

680 **Figure 5.** Mean (± 1 SE) activity levels of the enzymes acetylcholinesterase (AChE) (a),
681 cytochrome P450 (Cyt P450) (b) and mean (±1 SE) levels of oxidative damage to lipids
682 (measured as levels of malondialdehyde, MDA) (c) of *Ischnura elegans* at the end of the

683 pesticide-exposure period as a function of daily temperature fluctuation (DTF) and pesticide
684 exposure. Numbers indicate sample sizes per treatment combination. Different letters indicate
685 significant differences between treatment combinations based on FDR-corrected Tukey tests.
686

687 **Figure 1**



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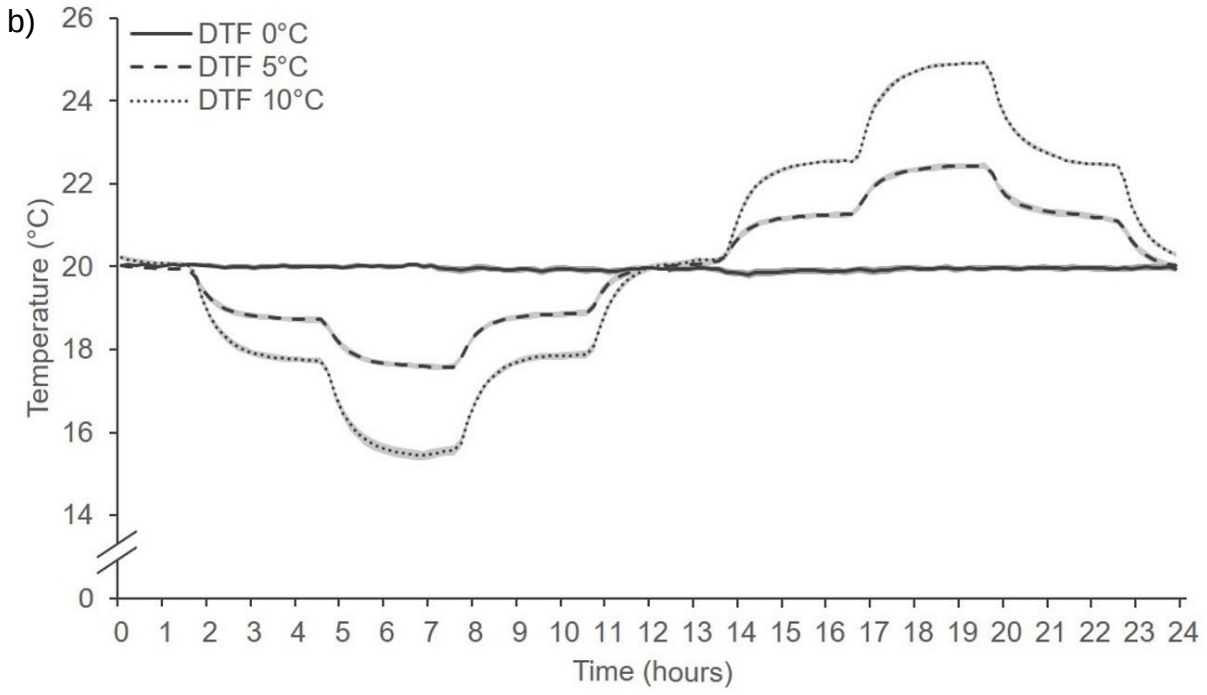

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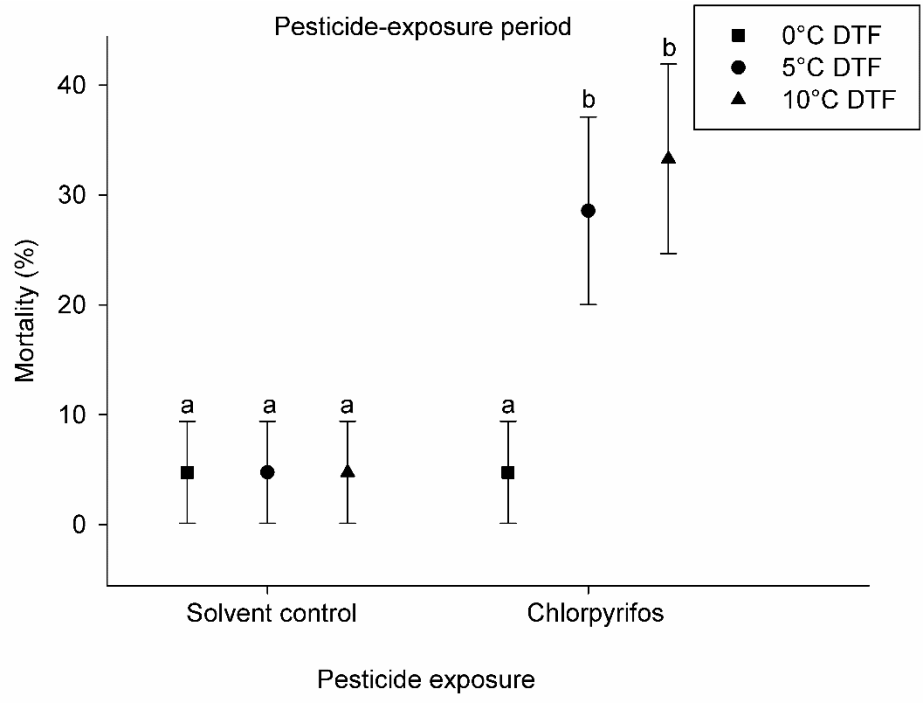
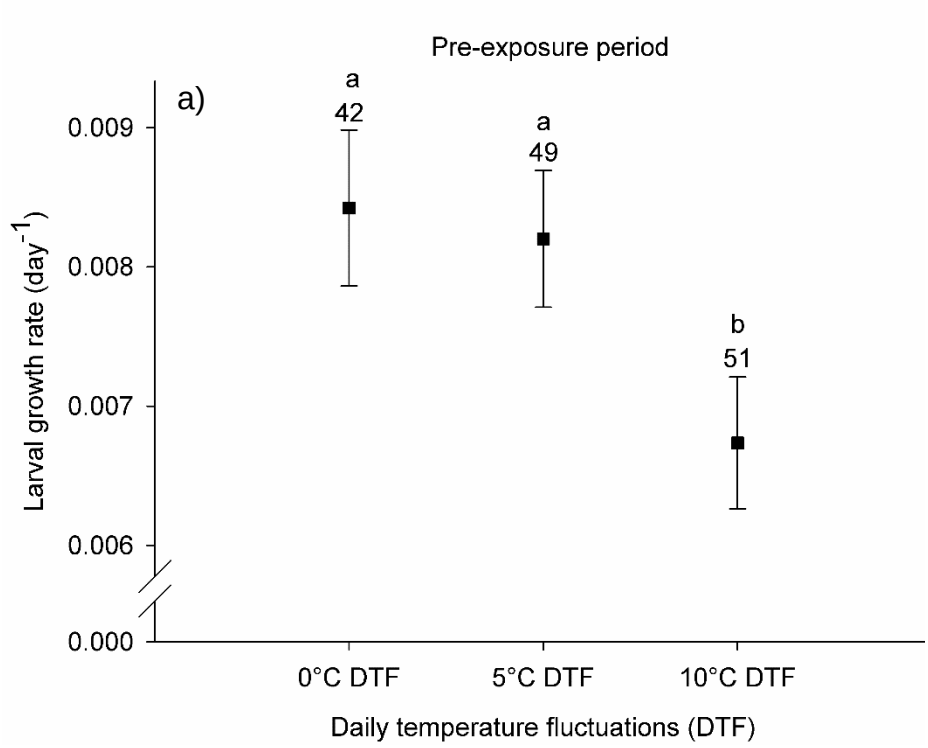
690 **Figure 2**



691

692 **Figure 3**



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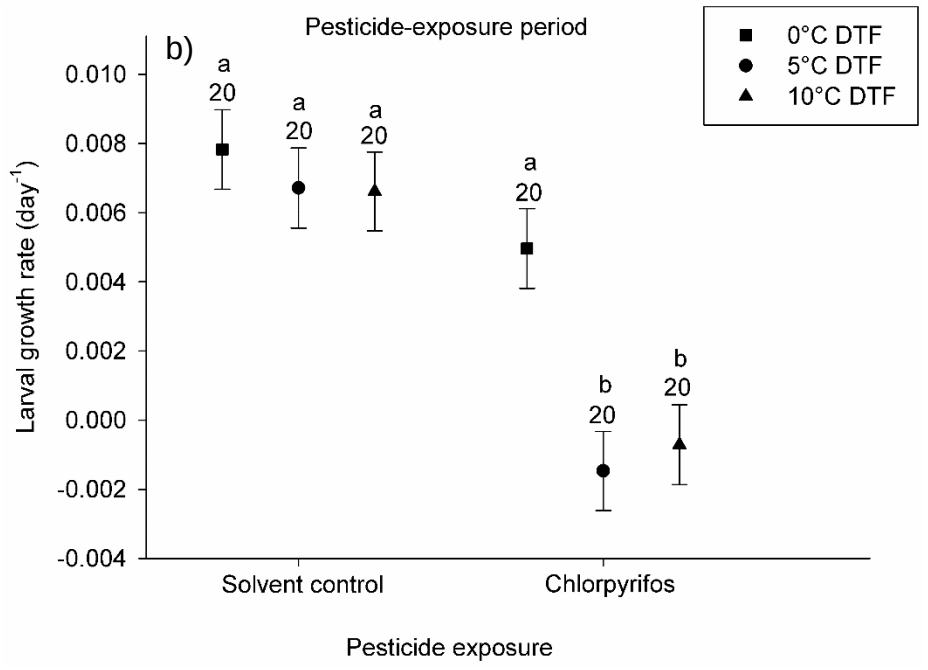
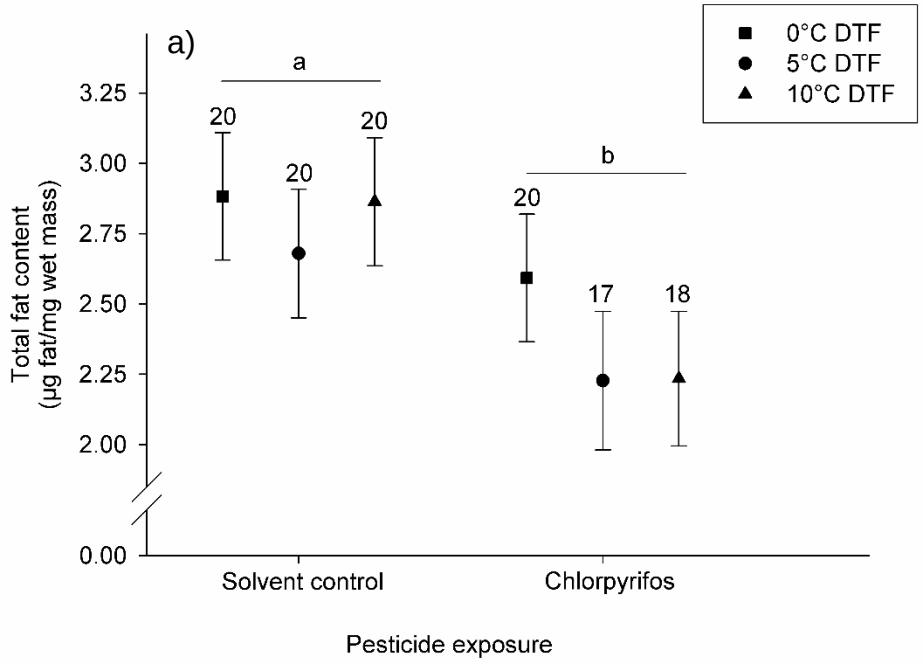


694

695

696

Figure 4


a)
0°C DTF
5°C DTF
10°C DTF
a
b
20
20
20
20
17
18
3.25
3.00
2.75
2.50
2.25
2.00
0.00
Total fat content
(µg fat/mg wet mass)
Solvent control
Chlorpyrifos
Pesticide exposure

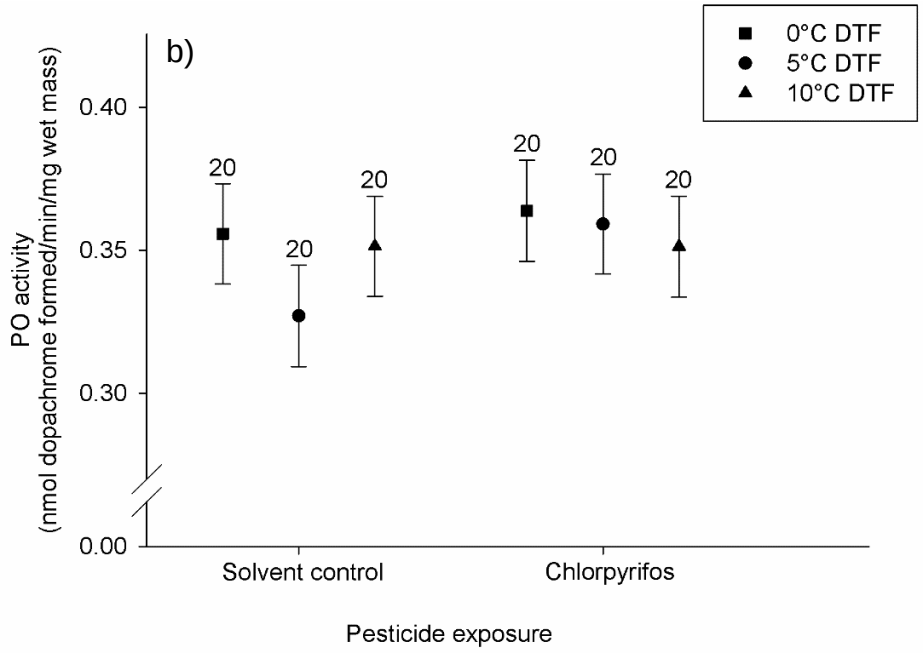
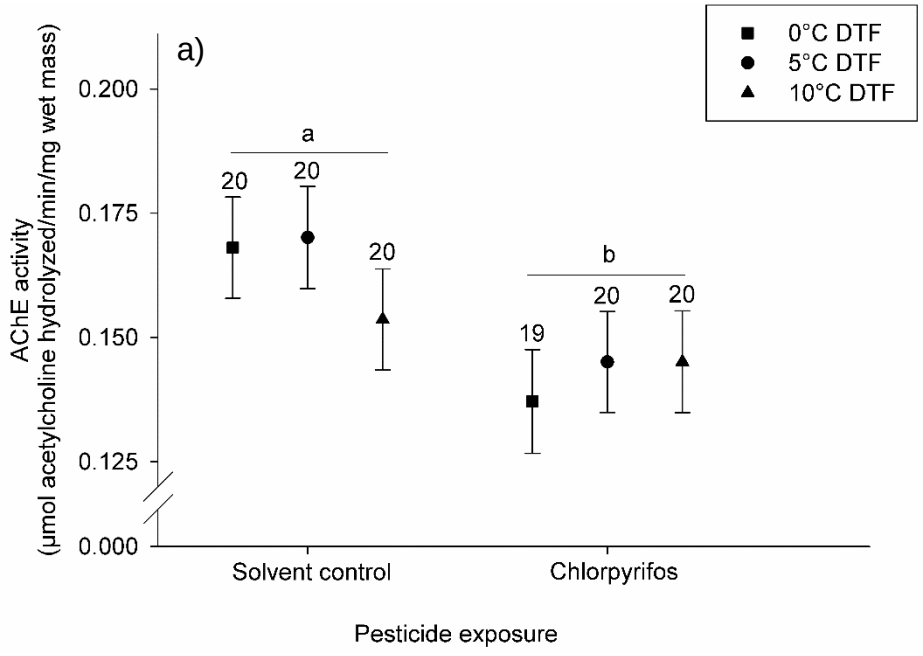
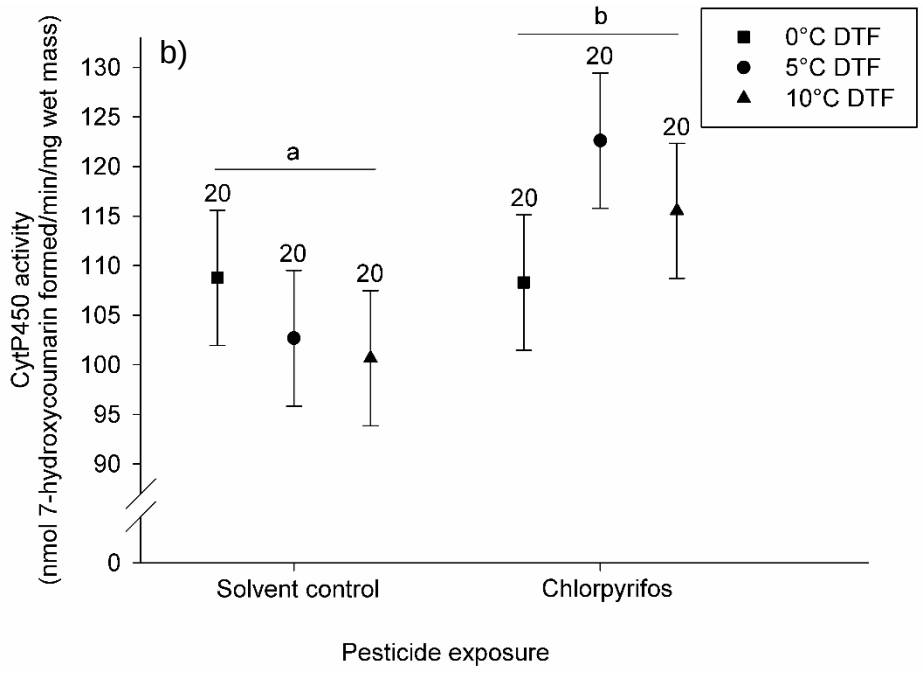
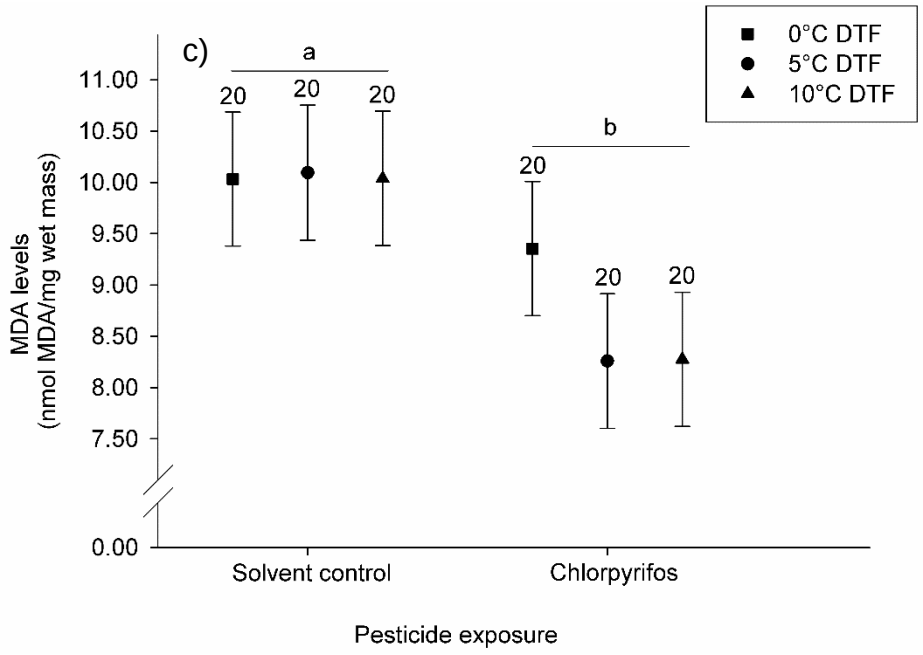

b)
0°C DTF
5°C DTF
10°C DTF
20
20
20
20
20
20
0.40
0.35
0.30
0.00
PO activity
(nmol dopachrome formed/min/mg wet mass)
Solvent control
Chlorpyrifos
Pesticide exposure

Figure 5


a)
0°C DTF
5°C DTF
10°C DTF
AChE activity
(µmol acetylcholine hydrolyzed/min/mg wet mass)
0.200
0.175
0.150
0.125
0.000
a
b
20
20
20
19
20
20
Solvent control
Chlorpyrifos
Pesticide exposure


b)
0°C DTF
5°C DTF
10°C DTF
CytP450 activity
(nmol 7-hydroxycoumarin formed/min/mg wet mass)
130
125
120
115
110
105
100
95
90
0
a
b
20
20
20
20
20
20
Solvent control
Chlorpyrifos
Pesticide exposure


c)
a
b
20
20
20
20
20
20
0°C DTF
5°C DTF
10°C DTF
MDA levels
(nmol MDA/mg wet mass)
11.00
10.50
10.00
9.50
9.00
8.50
8.00
7.50
0.00
Solvent control
Chlorpyrifos
Pesticide exposure

705

706