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Temperature variation magnifies chlorpyrifos toxicity differently between larval and adult mosquitoes

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Abstract

To improve risk assessment there is increasing attention for the effect of climate change on the sensitivity to contaminants and vice versa. Two important and connected topics have been largely ignored in this context: (i) the increase of daily temperature variation (DTV) as a key component of climate change, and (ii) differences in sensitivity to climate change and contaminants between developmental stages. We therefore investigated whether DTV magnified the negative effects of the organophosphate insecticide chlorpyrifos on mortality and heat tolerance and whether this effect was stronger in aquatic larvae than in terrestrial adults of the mosquito *Culex pipiens*. Exposure to chlorpyrifos at a constant temperature imposed mortality and reduced the heat tolerance in both larvae and adult males, but not in adult females. This provides the first evidence that the TICS ("toxicant-induced climate change sensitivity") concept can be sex-specific. DTV had no direct negative effects. Yet, consistent with the CITS ("climate-induced toxicant sensitivity") concept, DTV magnified the toxicity of the pesticide in terms of larval mortality. This was not the case in the adult stage indicating the CITS concept to be dependent on the developmental stage. Notably, chlorpyrifos reduced the heat tolerance of adult females only in the presence of DTV, thereby providing support for the reciprocal effects between DTV and contaminants, hence the coupling of the TICS and CITS concepts. Taken together, our results highlight the importance of integrating DTV and the developmental stage to improve risk assessment of contaminants under climate change.

Key words: complex life cycle; daily temperature fluctuation; global warming; multistressor; pesticide; upper thermal tolerance

1. Introduction

Chemical contaminants and global climate change are major challenges for life on our planet (European Environment Agency, 2015). Thus, understanding the combined effects of chemical contaminants and key aspects of global climate change is a major research focus in ecotoxicology (e.g. Moe et al., 2013; Noyes and Lema, 2015; Van den Brink et al., 2018). There is increasing support for two concepts reflecting the interplay between contaminants and climate change. On the one hand, environmental factors associated with climate change can alter the susceptibility to chemical contaminants, the so-called "climate-induced toxicant sensitivity" concept (CITS, Noyes et al., 2009; Noyes and Lema, 2015). For many pesticides (organophosphates and carbamates) this takes the form of a higher toxicity at a higher mean temperature (Hooper et al., 2013). On the other hand, chemical exposure can increase the susceptibility to climate change, the so-called "toxicant-induced climate change sensitivity" concept (TICS, Noyes et al., 2009; Noyes and Lema, 2015). In line with the TICS concept, many studies showed that exposure to contaminants can reduce the heat tolerance of animals (e.g. Janssens et al., 2018; Patra et al., 2007). Notably, both concepts may not be independent. Recently, it has indeed been shown that exposure to pesticides at higher temperatures may shape the magnitude of the decrease in heat tolerance (Op de Beeck et al., 2018).

Despite the increasing awareness that both the CITS and TICS concepts are crucial for ecological risk assessment of pesticides in a warming planet (e.g. Moe et al., 2013; Noyes and Lema, 2015), two major aspects have been largely ignored. A first limitation is that the majority of studies equalled climate change to increases in mean temperature. However, the

Abbreviations: [1]CITS climate-induced chemical toxicant sensitivity, [2]CPF chlorpyrifos, [3]CTmax critical thermal maximum, [4]DTV daily temperature variation, [5]L4 last larval stage, [6]PERANOVAs permutational analyses of variance, [7]TICS toxicant-induced climate change sensitivity

expected increase in daily temperature variation (DTV) is another key factor of global climate change (Vázquez et al., 2017). Many studies have shown important effects of DTV on life history traits (reviewed in Colinet et al., 2015). Recent progress in ecology even revealed that increases in DTV can pose greater risk to species than increases in mean temperature (e.g. Sheldon and Dillon, 2016; Vasseur et al., 2014). Yet, the role of DTV in changing the toxicity of chemical contaminants has been less addressed. The few studies addressing this topic in ecotoxicology did find that the toxicity of various contaminants was higher under temperature variation (fluoxetine: Barbosa et al., 2017; chlorothalonil and bifenthrin: Willming et al., 2013; pyraclostrobin: Willming and Maul, 2016; chlorpyrifos: Verheyen et al., 2019; Verheyen and Stoks, 2019), presumably due to energetic costs associated with exposure to DTV (Colinet et al., 2015). Notably, only two of these studies (Verheyen et al., 2019; Verheyen and Stoks, 2019) did expose animals to two DTF levels at the same mean temperature, one matching the current DTF level and a second one matching the predicted increase in DTF level, which is crucial to better integrate DTF in risk assessment under climate change.

A second limitation of current CITS and TICs studies are that they focus on a single developmental stage, hence may not capture the full impact on organisms. Many taxa such as amphibians and insects have complex life cycles with distinct developmental stages that occupy different habitats, and accordingly may experience different thermal regimes (Stoks and Córdoba-Aguilar, 2012), hence differ in thermal sensitivity (Bowler and Terblanche, 2008; Kingsolver et al., 2011). Whether the strength of the interplay between global change and toxicants also differs between developmental stages is, however, largely unknown. Daily temperature variation (DTV) and developmental stage may play an interactive role when shaping the TICS and CITS concepts. Indeed, for the many taxa with an aquatic larval stage and a terrestrial adult stage one can expect that high DTV has a stronger effect on the larvae

than on the adults. This is because air temperatures fluctuate more strongly compared to water temperatures (Jacobs et al., 2008). As a result, adults experience stronger selection imposed by high DTV compared to larvae in nature, and therefore adults are expected to have developed a higher ability to cope with high DTV and heat. If true, one could, for example, expect a stronger effect of DTV on the sensitivity to contaminants in aquatic larvae than in terrestrial adults. Despite the direct relevance for risk assessment, we lack information whether the reciprocal effects of DTV and contaminants differ between larvae and adults.

In this study, we explicitly integrated DTV and the developmental stage in the TICS and CITS concepts by addressing the following questions in a semi-aquatic insect: (Q1) Do aquatic larvae have a lower heat tolerance than adults? (Q2) Does DTV affect mortality and increase heat tolerance? (Q3) Does pesticide exposure reduce heat tolerance (TICS)? (Q4) Does DTV increase pesticide sensitivity (CITS)? (Q5) Are the CITS and TICS concepts interconnected? In other words, does DTV shape how a pesticide reduces the heat tolerance? Moreover, for questions 2-5 we specifically tested whether the responses differed between developmental stages. To answer these questions, the single and combined effects of DTV at a single constant mean temperature and pesticide exposure were determined on mortality and on heat tolerance (CTmax). We used two levels of DTV: a small, currently frequent DTV level and a high DTV level that is expected to become more frequent in the future (details see 2.1). As pesticide, we chose chlorpyrifos (CPF) which is one of the most commonly used organophosphate insecticides in agriculture worldwide (Eaton et al., 2008; Gómez-Canela et al., 2017). CPF is one of the priority substances in the European Water Framework Directive (2000/60/EC) and is in the top ten of chemicals with a high risk for aquatic organisms in surface waters in the UK (Johnson et al., 2017). As study animals mosquitoes were chosen as these have a complex life cycle with an aquatic larval stage and terrestrial adult stage, and are major components of aquatic and terrestrial food webs (Becker et al., 2010). More

specifically, we chose *Culex pipiens* biotype *molestus* (Forskål, 1775), whose larvae live in shallow ponds and lakes where temperature variation can be considerable (Jacobs et al., 2008). Mosquito larvae are important prey species in terms of biomass both in aquatic and in terrestrial food webs (Becker et al., 2010).

2. Materials & methods

2.1. Origin of the lab culture and pre-experimental rearing

The experiment was started from a lab culture of *C. pipiens* originated from three natural ponds in Germany with a mean summer water temperature of ca. 20°C (see Appendix S1 in Tran et al., 2016). The amplitude of daily temperature variations in the experiment was based on the daily minimum and maximum air temperatures during the summer months (from June to September) for the period 1998 to 2017 of the three sites of origin of the studied lab culture. These data were extracted from the German Climate Data Centre (https://www.dwd.de/EN/climate_environment/cdc/cdc_node.html). During summer, ca. 34% of the days have a DTV (difference between daily maximum and minimum temperatures) around 7°C (within the range 5-9°C) and ca. 21% have a DTV around 14°C (within the range 12-16°C). From these data a constant (0°C), small (7°C) and a large (14°C) DTV at a mean of 20°C were chosen for this study. Under global climate change increases in DTV are to be expected (Vasseur et al., 2014), hence the DTV of 14°C is expected to become the more frequent DTV level in the future.

Before the start of the experiments, larvae of *C. pipiens* were reared in 2 L white containers (18.0 cm x 13.3 cm x 12.1 cm) filled with 1 L aerated tap water at a density of ca. 100 larvae (2 egg clutches) per container. The larvae were placed in a temperature-controlled room with a 14:10 h light:dark regime and a water temperature of 19.84 °C (SD: 1.00 °C). Larvae were fed *ad libitum* three times a week with 3 mL of a 20 g/L mixture

146 of Olvarit® 7 cereal flakes (46%), wheat germs (51%) and Supradyn® vitamins (3%) (Tran et
147 al., 2016) which equals 0.257 mg of food per larva per day. Since *ad libitum* food increased
148 turbidity, two third of the medium of the rearing containers was replaced by new
149 dechlorinated tap water after 7 days.

150 *2.2. General experimental strategy*

151 To test the combined effects of daily temperature variation (DTV) and pesticide exposure on
152 mortality and heat tolerance we crossed three DTV treatments [constant (= 0 °C), 7 °C and
153 14 °C DTV] with two pesticide treatments (solvent control, chlorpyrifos). Note that our aim
154 was to test for an effect of DTV at a given mean temperature and not to test for an effect of
155 mean temperatures. More information about how the DTV regimes were realized in the
156 incubators can be found in Appendix A1. The experiments lasted six days with a four-day
157 pre-pesticide-exposure period (only exposure to the DTV treatment without exposure to the
158 pesticide) followed by a two-day pesticide-exposure period (in combination with the DTV
159 treatment). All experiments were run at a 14:10 h light:dark regime.

160 We ran per DTV-by-pesticide treatment combination 31-36 replicate vials for the
161 larval stage (total of 4,140 larvae), 23-27 replicate vials for the adult males (total of 912
162 males) and 17-19 replicate vials for the adult females (total of 624 females). Exact sample
163 sizes are shown in Figure 1.

164 *2.3. Chlorpyrifos concentrations*

165 Separate experiments were performed for larvae and adults, because of their different
166 vulnerability to CPF (see Appendix B1) asking for different absolute CPF concentrations. Both
167 the larval and adult experiments were, however, set up in such a way that they allow a direct
168 comparison of the CITS and TICS concepts between stages. To allow a direct comparison of
169 effects of DTV on pesticide sensitivity between the larval and the adult stage, CPF

concentrations were used that caused 50% mortality in the larvae (0.65 µg/L) and in the adult males (9.5 µg/L) after the two-day pesticide-exposure period ($LC_{50,48h}$). These values were based on range-finder experiments (see Appendix B1). At the LC_{50} there is still room to detect synergistic/antagonistic interactions with DTV. For example, 4°C warming increased the mortality with ca. 5% in the study species (Tran et al., 2018), when additive this would give 55% mortality in the presence of the pesticide, still allowing a further 45% increase in mortality possible, hence the possibility to detect a synergism. Note, that no mortality was expected for the adult females at the LC50 value for the adult males (see Appendix B1). As in the field, adult male and female mosquitoes likely encounter the same pesticide concentrations, the same concentration was used for both sexes. Note that since no food was provided during the pesticide-exposure period, the main uptake route for both developmental stages is likely through the body surface (Buchwalter et al., 2004). In addition, some volatilization may have occurred (Racke, 1993), hence some uptake through respiration in the adults.

Chlorpyrifos (CPF) was bought from Sigma-Aldrich (St. Louis, Missouri, USA). The CPF solution was prepared by using a stock solution of 100 µg/mL CPF dissolved in ethanol, which was kept in the dark at 4 °C. From this stock solution, a second stock solution was made of 10 µg/mL for the larvae and of 20 µg/mL for the adults in Milli-Q water. Chlorpyrifos samples were measured at the start and after 48 hours of the pesticide-exposure period (see Appendix C1). In the control treatment, a solvent control of ethanol was used similar to the ethanol concentration used in the CPF treatment (16.67 µL/L for the larvae and 105.56 µL/L for the adults). There were no differences in survival or growth rate of L4 larvae and survival of adults of *C. pipiens* between the solvent control and the water control (Tran et al., unpublished data).

194 *2.4. Larval exposure experiment*

195 For larval exposure experiments, 20 randomly selected larvae across all containers (less than
196 24h in the fourth larval stage, L4) were transferred to a 210 mL glass vial filled with 100 mL
197 of aerated tap water. During the first four days (pre-pesticide-exposure period), the larvae
198 were fed *ad libitum* with 310 µL of the 20 g/L food mixture (0.310 mg of food per larva per
199 day) and the medium was not refreshed. Thereafter, the larvae were exposed for two days to
200 the pesticide treatment. The larvae were not fed during the pesticide treatment, following
201 OECD guidelines (OECD, 2011).

202 *2.5. Adult exposure experiment*

203 For the adult exposure experiment, sets of 100 L4 larvae (less than 24h in the L4 stage) were
204 placed in 2 L containers filled with 1 L aerated tap water. Larvae were fed daily *ad libitum*
205 until pupation (as during the first four days of the exposure period in the larval experiment).
206 We checked daily for pupae that were removed and replaced by other L4 larvae to maintain
207 the same density. All pupae were placed separately in 100 mL transparent cups with 50 mL
208 aerated water covered with a net (mesh size: 1 mm). Newly emerged adults of the same sex
209 were placed per six in a 210 mL glass vial. The sex was determined based on the morphology
210 of the proboscis (tubular mouthpart for feeding) and the antennae of the adults. The walls of
211 the vial and its lid were covered with Whatman™ No. 1 qualitative filter paper soaked in a
212 6% D-glucose solution as food source. The lid had 10 holes (diameter: 1 mm) for aeration.
213 The vials with the adult mosquitoes were placed for six days on the temperature treatment.
214 Males and females were kept separately. After four days, the adults were transferred to
215 another similar vial for the two-day pesticide-exposure period. The pesticide exposure
216 protocol of the adults was based on the insecticide resistance protocol of the World Health
217 Organization (2013). The vial and its lid were covered with the same filter paper, which was
218 soaked in a solvent or CPF solution in the absence of food.

2.6. Response variables

Mortality was quantified for the pre-pesticide-exposure period (days 1-4) and the pesticide-exposure period (days 5-6). After the pesticide exposure, the animals were subjected to a heat tolerance test where their critical thermal maximum (CTmax) was quantified. CTmax is considered a good proxy for evaluating an organism's vulnerability to global warming (Huey et al., 2012). CTmax for the larvae is defined as the temperature when larvae lie motionless at the water surface, lose their self-righting ability, and do not react when gently tapped with a plastic stick. CTmax for the adults is defined as the temperature when adults lie motionless at the bottom of the vial, lose their self-righting ability, and cannot stand on their legs. A ramping method was used where the animals were heated at a constant rate (0.3°C per min) up to a temperature at which animals no longer showed any body movement or muscular spasms (Verberk and Bilton, 2011). This heating rate is within the range commonly used for testing heat tolerance of aquatic insects (e.g. Verberk and Bilton, 2011, for mosquitoes: Dallas and Rivers-Moore, 2012) and terrestrial insects (Terblanche et al., 2011). We had to use different protocols for the measurement of heat tolerance because larvae are aquatic and adults are terrestrial. CTmax was quantified following Op de Beeck et al. (2017) for the aquatic L4 larvae, and following Sniegula et al. (2017) for the terrestrial adults (see details in Appendix D1). CTmax trials were randomized across treatments in the water bath for larvae and in the incubator for adults, and were performed by the same observer for both developmental stages. To avoid different starting temperatures of the CTmax trials between DTV treatments, we ran all trials when all DTV treatments were at the mean temperature of 20°C (between 11 AM and 1 PM). Thus, none of the animals (irrespective of DTV treatment) were experiencing ramping up or down of the temperatures at the start of the CTmax trials. We tested in total 447 larvae, 425 adult males and 397 adult females. Exact numbers of individuals tested per treatment for each developmental stage are shown in Figure 2.

244 *2.7. Statistical analyses*

245 All statistical analyses were performed in R v3.4.0 (Core Team R, 2017). We used the
246 packages lme4 v1.1-18.1 (Bates et al. 2015), car v3.0-2 (Fox and Weisberg, 2011), lsmeans
247 v2.27-62 (Lenth, 2016), afex v0.22-1 (Singmann et al., 2017) and drc 3.0-1 (Ritz et al.,
248 2015).

249 Due to the large differences in mortality after exposure to CPF between larvae, adult
250 males and adult females (see Appendix B1), the mortality and CTmax after the pesticide-
251 exposure period were analysed separately for each group. In addition, a combined analysis
252 with all three groups was performed in the absence of CPF to test for group differences in
253 mortality during the pre-pesticide-exposure period and in CTmax for the solvent control
254 during the pesticide-exposure period. In this combined analysis, an extra fixed factor
255 ('group') with three levels (larvae, adult males and adult females) was added.

256 Mortality during the first four days (pre-pesticide-exposure period) and during the last
257 two days (pesticide-exposure period) was scored as 0 (alive) and 1 (dead) for each larva or
258 adult within a vial. We separately tested for effects of the DTV treatment during the first four
259 days, and for effects of DTV and chlorpyrifos (CPF) exposure during the last two days on
260 mortality using generalized linear mixed models with a binomial error structure and the logit
261 link. To avoid pseudoreplication, rearing vial was added to the models as a random factor.

262 The effects of DTV and CPF exposure on CTmax were initially analysed using a set
263 of linear mixed models with a normal error structure and the identity link. In both
264 developmental stages, body mass of the animals was added as a covariate. Date was added as
265 a random effect to control for possible temporal patterns. To avoid pseudoreplication, rearing
266 vial was added as a random factor. Because the assumptions of normality and homogeneity of
267 variances were not met, permutational analyses of variance (PERANOVAs) were performed
268 (Anderson, 2001). The number of permutations was set at 5,000. Significant interactions and

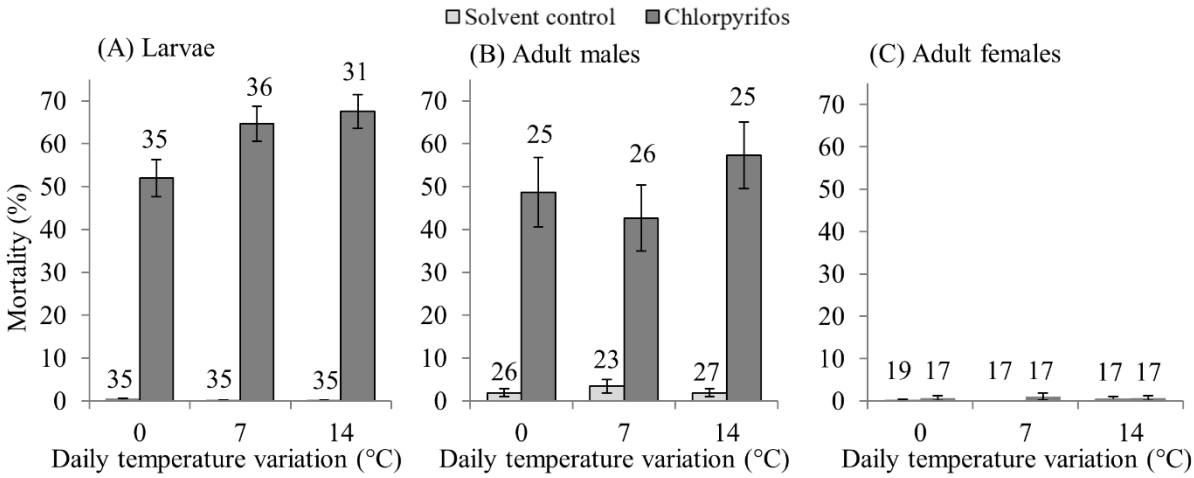
effects of the different DTV levels were further analysed by comparing least-square means with false discovery rate (fdr) correction using the function contrasts in the lsmeans package.

3. Results

3.1. Mortality

During the pre-pesticide-exposure period, mortality was very low in all three groups and somewhat higher in adult males than in larvae (Combined analysis, Group: $\chi^2_2 = 13.28$, $P = 0.0013$; mean ± SE, larvae: 1.06% ± 0.29%, adult females: 2.52% ± 0.83%, and adult males: 2.63% ± 0.83%). DTV had no effect on mortality (DTV: $\chi^2_2 = 1.95$, $P = 0.38$); this was consistent among the three groups (DTV × group: $\chi^2_4 = 3.49$, $P = 0.48$).

During the pesticide-exposure period, chlorpyrifos (CPF) increased mortality in larvae and in males but not in females (Table 1A, Fig. 1). Note that we indeed used the same relative concentration of CPF causing ca. 50% mortality in larvae and adult males at the constant temperature (0°C DTV). The main effect of DTV was not significant in larvae, adult males or adult females (Table 1A, Fig. 1). Larval mortality in the solvent control was still near zero and did not depend on the DTV treatment (Contrasts: all P-values ≥ 0.43). However, larval mortality in the presence of CPF was ca. 15% higher at 7°C and 14°C DTV (ca. 65%) than at 0°C DTV (ca. 50%); this was confirmed by highly significant contrast tests (both P-values < 0.001) and supported by a trend for a CPF × DTV interaction ($P = 0.079$, Table 1A, Fig. 1A). In contrast, DTV did not change the toxicity of CPF in adult males and females (CPF × DTV, Table 1A, Fig. 1).



289

290 Figure 1. Mortality during the pesticide-exposure period of L4 larvae (A), adult males (B) and females
291 (C) of the mosquito *Culex pipiens* as a function of daily temperature variation (DTV) and chlorpyrifos
292 exposure. Numbers above the bars represent the number of replicate vials. Given are means ± 1 SE.
293 All DTV treatments had a mean of 20°C; at 0° DTV the temperature was constant 20°C. The $LC_{50,48h}$
294 of chlorpyrifos for the larvae (0.65 µg/L) and for the adult males (9.5 µg/L) after the two-day
295 pesticide-exposure period was used. Adult females were exposed to the same concentration as adult
296 males.

297 *3.2. Heat tolerance*

298 The combined analysis indicated that in the solvent control the heat tolerance was lower in
299 adult males compared to adult females (Contrast: $P = 0.035$), while larvae did not differ in
300 heat tolerance compared to both adult males and adult females (Contrasts: both P-values ≥
301 0.29, Group: $\chi^2_2 = 8.10$, $P = 0.014$).

302 CPF reduced the heat tolerance in each group (Table 1B, Fig. 2). In larvae, CPF
303 exposure reduced the heat tolerance at the constant temperature (0°C DTV) with ca. 9%
304 (Contrast: $P < 0.001$; Fig. 2A). The CPF-induced reduction of larval heat tolerance was
305 stronger under constant temperature (0°C DTV) and at 7°C DTV than at 14°C DTV (CPF
306 × DTV, Table 1B, Contrasts: both P-values ≤ 0.0075; Fig. 2A). DTV did not cause a
307 difference in larval heat tolerance in the solvent control (Contrasts: all P-values ≥ 0.76). In

adult males, CPF exposure reduced the heat tolerance at 0°C DTV with ca. 8% (Fig. 2B); the effect of CPF was not shaped by the DTV treatment (CPF × DTV, Table 1B, Fig. 2B). There was a main effect of DTV with males having a higher heat tolerance at 7°C and 14°C DTV than at 0°C DTV (DTV, Table 1B, Fig. 2B, Contrasts: both *P*-values < 0.011). In adult females, CPF reduced the heat tolerance at 7°C and 14°C DTV with ca. 6% (Contrasts: both *P*-values < 0.001; Fig. 2C), but not at 0°C DTV (Contrast: $P = 0.30$) (CPF × DTV, Table 2). In the solvent control, 14°C DTV tended to increase female heat tolerance compared to 0°C DTV (Contrast: $P = 0.081$).

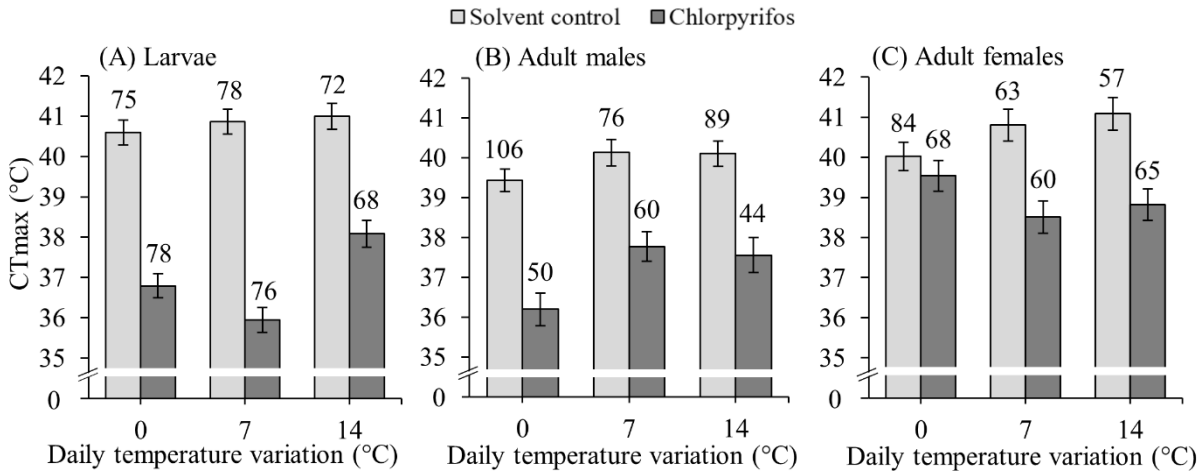


Figure 2. Heat tolerance measured as CTmax of the L4 larvae (A), adult males (B) and adult females (C) of the mosquito *Culex pipiens* as a function of daily temperature variation (DTV) and chlorpyrifos exposure. Numbers above the bars represent the number of individuals tested. Given are means ± SE. All DTV treatments had a mean of 20°C; at 0° DTV the temperature was constant 20°C. The $LC_{50,48h}$ of chlorpyrifos for the larvae (0.65 µg/L) and for the adult males (9.5 µg/L) after the two-day pesticide-exposure period was used. Adult females were exposed to the same concentration as adult males.

Table 1. Results of the generalized linear mixed models (mortality, A) and the general linear mixed models (heat tolerance, B) testing for the single and combined effects of daily temperature variation (DTV) and chlorpyrifos exposure (CPF) on larvae, adult males and adult females of the mosquito *Culex pipiens*. *P*-values indicated in bold are significant ($P < 0.05$).

	(A) Mortality			(B) Heat tolerance		
	χ^2-value	Df	*P*-value	χ^2-value	Df	*P*-value
Larvae						
DTV	0.84	2	0.66	14.2	2	**< 0.001**
CPF	184.63	1	**< 0.001**	228.54	1	**< 0.001**
DTV × CPF	5.07	2	0.079	10.53	2	**0.0038**
Adult males						
DTV	0.41	2	0.82	12.25	2	**0.0022**
CPF	144.20	1	**< 0.001**	79.17	1	**< 0.001**
DTV × CPF	3.56	2	0.17	1.69	2	0.40
Adult females						
DTV	0.15	2	0.93	0.70	2	0.71
CPF	0.00	1	0.99	32.51	1	**< 0.001**
DTV × CPF	0.21	2	0.90	9.34	2	**0.0094**

4. Discussion

(Q1) Do aquatic larvae and adults differ in heat tolerance?

Adult males had a somewhat lower heat tolerance than adult females, while the heat tolerance did not differ between larvae and adults. Thus, there was a small difference in heat tolerance between sexes, but not between developmental stages. This contrasts with our initial hypothesis that because the aquatic environment is thermally more buffered (Jacobs et al., 2008), larvae may have a lower ability to deal with heat stress than adults. Moreover, as larvae mainly occupy temporary ponds, which are thermally homogenous (show no temperature stratification, Brönmark and Hansson, 2017), it is less likely that they will be able to select thermally more favourable microclimates. In the terrestrial adults such behavioural plasticity may be more likely. Our finding also contrasts with the general pattern that heat tolerance is lower in later developmental stages (Bowler and Terblanche, 2008),

which, however, was based on animals that were terrestrial in both the larval and adult stage (e.g. butterflies: Kingsolver et al., 2011; Klockmann et al., 2017; fruit flies: Tucic, 1979; beetles: Knapp and Nedvěd, 2013).

(Q2) Does DTV affect mortality and heat tolerance, does this differ between developmental stages?

Fluctuating temperatures caused no mortality in the larvae and adults, despite temperatures reaching up to 27°C for three hours each 24h cycle at the high DTV of 14°C. Under DTV, animals can cope with higher temperatures since they can recover during the cold portion of each DTV cycle (Colinet et al., 2015). Other studies did report lethal effects of DTV on larvae of other mosquito species, yet at higher mean temperatures (e.g. Carrington et al., 2013; Paaijmans et al., 2013). For example, in *Anopheles stephensi* a DTV of 12°C was highly lethal at a mean of 32°C, but not at means below 30°C (Paaijmans et al., 2013).

The effect of fluctuating temperatures on heat tolerance differed between developmental stages in the solvent control (as supported by the contrasts), possibly reflecting the higher DTV regimes in terrestrial habitats (Jacobs et al., 2008). Indeed, in the solvent control DTV caused no difference in larval heat tolerance, but both 7°C and 14°C DTV increased the male heat tolerance, and 14°C DTV tended to increase the female heat tolerance. This is partly in line with the general pattern of increased thermal tolerance under DTV in insects (Colinet et al., 2015). For example, a higher heat tolerance was reported in *Drosophila melanogaster* exposed to a DTV of 5°C at a mean of 24°C compared to those kept at constant 24°C (Bozinovic et al., 2011). The underlying reasons for a higher heat tolerance under DTV are still debated, one mechanism could be the upregulation of heat shock proteins under DTV (Colinet et al., 2015, but see Fischer et al., 2011).

(Q3) Does pesticide exposure reduce heat tolerance (TICS), does this differ between developmental stages?

Pesticide exposure at the constant temperature reduced the heat tolerance of larvae (ca. 9%) and adult males (ca. 8%), but not of adult females. Hence, the TICS concept was not dependent upon developmental stage but upon sex, likely because CPF was much less toxic to females. Indeed the chosen CPF concentration did not cause any mortality in females, while in males approximately 50% of the individuals died. Such sex-specific sensitivity to pesticides, including organophosphate insecticides, have been reported in other insects (e.g. *Drosophila*: Smirle et al., 2017; midges: Hahn et al., 2001; moths: De Lame et al., 2001) and have been explained by sex differences in baseline activity levels of acetylcholinesterase and general esterases, and in the sensitivity of these enzymes to organophosphates (De Lame et al., 2001).

A CPF-induced reduction in larval and male heat tolerance is as expected by the TICS concept (Noyes and Lema, 2015) and documented in other aquatic animals (e.g. damselfly larvae: Janssens et al., 2018; Op de Beeck et al., 2018, 2017; fish: Patra et al., 2007). Heat tolerance is determined by the mismatch between the oxygen demand (that increases) and the oxygen supply (that decreases) with increasing temperatures (Verberk et al., 2016). Under toxicant exposure, the heat tolerance is expected to decrease because the oxygen demand increases and the oxygen supply decreases, thereby causing the mismatch to occur already at less extreme high temperatures (Sokolova, 2013). Indeed, under stress conditions like pesticide exposure, the basal metabolism, hence also oxygen consumption, increases to meet the demands for additional energy for activation of the mechanisms for protection and damage repair (Sokolova, 2013). At the same time, pesticide exposure may reduce oxygen uptake or the internal capacity to transport oxygen to tissues. For example, CPF exposure caused gill damage in the freshwater crab *Zilchiopsis collastinensis* (Negro and Collins, 2017) and in the brackish water fish *Lates calcarifer* (Marigoudar et al., 2018). Note this may

cause oxygen limitation also in air-breathing mosquito larvae despite oxygen supply being less of a problem compared to other aquatic organisms (Verberk et al., 2016).

(Q4) Does DTV increase pesticide sensitivity (CITS), does this differ between developmental stages?

Despite DTV not being lethal itself, it increased the CPF-induced mortality of larvae with ca. 15%. Given DTV is predicted to increase under global climate change (Vázquez et al., 2017), this is an important overlooked aspect of the CITS concept (Noyes and Lema, 2015). Our results provide further support to recent observations that contaminants may become more toxic under fluctuating temperatures (Barbosa et al., 2017; Verheyen et al., 2019; Verheyen and Stoks, 2019; Willming et al., 2013; Willming and Maul, 2016). Exposure to DTV is assumed to be energetically costly because of the higher energetic costs incurred during the warming part compared to the energetic savings during the cooling part of a DTV cycle (Colinet et al., 2015). Subsequently, in the presence of a second stressor like CPF, the exposed larvae likely have less energy to invest in detoxification and damage repair (Congdon et al., 2001). In such cases where a contaminant is combined with an environmental stressor that is energetically costly, an increased toxicity has been theoretically expected and matched to empirical findings of synergistic interactions (Liess et al., 2016). Furthermore, under DTV animals experience temperatures up to 23.5°C (DTV 7°C) and 27°C (DTV 14°C) for several hours during a 24h cycle. CPF is in general more toxic at higher temperatures (e.g. Dinh Van et al., 2014; for the study species: Tran et al., 2018) which can be explained by the faster biotransformation into more toxic metabolites (Harwood et al., 2009).

All studies showing increased pesticide toxicity under DTV were done on aquatic (stages of) invertebrates (the water flea *Daphnia*: Barbosa et al., 2017; midge larvae: Willming et al., 2013; the amphipod *Hyalella azteca*: Willming et al., 2013; Willming and

Maul, 2016, damselfly larvae: Verheyen et al., 2019; Verheyen and Stoks, 2019, mosquito larvae: current study). In contrast, DTV did not increase the CPF-induced mortality in the here studied terrestrial adults of *C. pipiens*. This matches our prediction that DTV would have less impact on toxicity in terrestrial adults. This can be expected because air temperatures fluctuate more strongly compared to water temperatures (Jacobs et al., 2008), hence terrestrial stages can be expected to be more adapted to deal with high DTV compared to aquatic stages. This suggests that the here documented dependence of the CITS concept on the developmental stage might be general in semi-aquatic insects.

(Q5) Are the CITS and TICS concepts interconnected, does this differ between developmental stages?

A key finding was that exposure to DTV changed how CPF reduced the heat tolerance, and this differently in larvae and adult females. This provides the first evidence that the CITS and TICS concepts are not only interconnected, but that this interconnection is also dependent on the developmental stage. In adult females, the negative effect of CPF on heat tolerance was only present under fluctuating temperatures. In other words, this shows that DTV can magnify the 'toxicant-induced climate change sensitivity', thereby adding a new pathway how the CITS and TICs concepts may be tightly linked (for the pathway working through changes in mean temperature: Op de Beeck et al., 2018). This challenges the typical approach of testing both concepts in isolation, thereby implicitly assuming their independence. This DTV-increased toxicity of CPF may also be explained by the higher energetic costs of dealing with fluctuating compared to constant temperatures (Colinet et al., 2015). This would indeed strengthen the CPF-induced mismatch between oxygen supply and demand, hence more easily cause a reduction in CTmax.

It is surprising that the CPF-induced reduction in heat tolerance in larvae was stronger under constant temperature and low DTV compared to high DTV. Possibly, at high DTV the

larvae reduced energetic costs by moving less or by reducing metabolic rate (Kern et al., 2015), thereby partly buffering the negative effect of CPF on CTmax compared to the other DTV treatments. In line with this, *Platyplectrum ornatum* tadpoles reduced activity of metabolic enzymes such as citrate synthase and cytochrome c oxidase when reared under high DTV, but not under small DTV (Kern et al., 2015). The activity of both enzymes indicates the potential metabolic energy since they are rate-limiting enzymes in the mitochondrial respiration (Kern et al., 2015). In addition, HSP synthesis may have been induced when CPF exposure was combined with high DTV. These conjectures, however, would need behavioural and physiological measurements to be explicitly evaluated in our study species.

4.1. Conclusions and implications

By integrating effects of daily temperature variation (DTV) and pesticide exposure on mortality and heat tolerance in both developmental stages of a semi-aquatic insect, we addressed two connected knowledge gaps at the interface of global change ecology and ecotoxicology. First, we demonstrated that DTV magnified the toxicity of the pesticide in terms of larval mortality, thereby proving DTV to be an important component of the CITS concept. Moreover, only under DTV there was a CPF-induced reduction in heat tolerance of adult females, indicating an overlooked link between the CITS and TICS concepts. This urges caution for risk assessment as testing the effects of CPF on heat tolerance in adult females under a constant temperature would not have revealed an effect. Future work should examine to what extent the expected increases in both mean temperatures and DTV under global warming interact and jointly shape the CITS and TICS concepts. Second, we provided the first evidence that the strength of the CITS and TICS concepts and their integration can be strongly different between developmental stages and sexes. Taken together, our results highlight the importance of integrating daily temperature variation and developmental stages

to improve risk assessment of contaminants under global climate change (e.g. Moe et al., 2013; Noyes and Lema, 2015; Rasmussen et al., 2018; Van den Brink et al., 2018).

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