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Title: Daily temperature variation lowers the lethal and sublethal impact of a pesticide pulse due to a higher degradation rate

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Abstract: Daily temperature variation (DTV) is an important warming-related stressor that may magnify pesticide toxicity. Yet, it is unknown whether the pesticide impact under DTV is partly ameliorated by a faster pesticide degradation caused by cyclically higher temperatures under DTV. As synergisms may be more likely under energy-limiting conditions, the impact of the pesticide chlorpyrifos was tested under DTV on the mosquito *Culex pipiens* in the absence and presence of interspecific competition with the water flea *Daphnia magna*. Chlorpyrifos exposure at a constant temperature without interspecific competition caused considerable mortality, decreased development time, and increased pupal mass of *C. pipiens*. Competition with *D. magna* had negative sublethal effects, but it did not affect the toxicity of chlorpyrifos. In contrast, the presence of *C. pipiens* decreased the impact of chlorpyrifos on *D. magna* probably due to corporal absorption of chlorpyrifos by *C. pipiens*. A key finding was that chlorpyrifos no longer caused lethal effects on *C. pipiens* under DTV, despite DTV on its own being mildly lethal. Additionally, chlorpyrifos exposure under DTV decreased development time less and had no effect anymore on pupal mass compared to chlorpyrifos exposure at a constant temperature. Similarly, the negative chlorpyrifos impact on adult survival of *D. magna* was less under DTV than at the constant temperature. This could be explained by a faster chlorpyrifos degradation under DTV. This antagonism between pesticide exposure and DTV is likely widespread because organisms experience DTV, many pesticides are applied in pulses, and pesticide degradation is faster at higher temperatures.

Key words: Antagonistic interaction; Biotic interactions; ‘Climate-induced toxicant sensitivity’ (CITS); Ecological Risk Assessment (ERA); Interspecific competition; Temperature fluctuations

1. Introduction

In nature, organisms are increasingly facing multiple stressors and their ability to cope with these often interacting stressor combinations is of key importance for their persistence (Côté et al., 2016; Galic et al., 2018). Toxicants and warming are two widespread stressors that may negatively affect aquatic organisms (Galic et al., 2018; Wang et al., 2019). Moreover, warming may increase the toxicity of many chemical compounds such as metals, and organophosphate and carbamate pesticides (Holmstrup et al., 2010). This has been encapsulated in the climate-induced toxicant sensitivity (CITS) concept (Moe et al., 2013; Noyes et al., 2009). The wide support for the CITS concept is mostly based on studies testing for toxicity at a higher mean temperature. However, also the predictable daily temperature variation (DTV) is another important warming-related variable that should be considered. DTV is omnipresent in nature, is expected to further increase under global warming (Colinet et al., 2015; Paaijmans et al., 2010; Verheyen and Stoks, 2019a) and may increase the toxicity of toxicants such as pesticides (e.g., Barbosa et al., 2017; Verheyen et al., 2019; Verheyen and Stoks, 2019a, 2019b; Willming et al., 2013).

When studying the impact of warming on toxicants the focus is mostly on the increase in toxicity, yet the net impact of toxicants under warming may be counteracted by an accelerated breakdown (Hooper et al., 2013). In a rare example study, the lethal impact of

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Abbreviations: CPF – chlorpyrifos, CITS – climate-induced toxicant sensitivity, DTV – daily temperature variation, ERA – ecological risk assessment, fdr – false discovery rate, IA – independent action, L4 – fourth and final larval stage, $LC_{50,72h}$ – lethal concentrations whereby 50% of the population is dead after 72 h, MDR – model deviation ratio, NOEC – No Observed Effect Concentration, OECD – Organisation for Economic Co-operation and Development, UPLC-MS/MS - Ultra performance liquid chromatography - tandem mass spectrometer.

chlorpyrifos on the aquatic larvae of the damselfly *Ischnura elegans* was smaller at a higher mean temperature because of a higher degradation and lower accumulation of the pesticide after multiple pulse exposures (Op de Beeck et al., 2017). This accelerated breakdown is often not included in empirical CITS studies which typically keep the concentration of the toxicant constant. While integrating this mechanism in CITS studies is important to arrive at a more realistic ecological risk assessment (ERA) of pesticides under warming (Noyes and Lema, 2015; Van den Brink et al., 2018), this has never been done in the context of DTV. This mechanism can be expected to counteract the increased toxicity under DTV as the higher temperatures encountered during each daily temperature cycle may potentially accelerate pesticide degradation. In line with this idea, pesticide degradation occurred to a much greater extent at fluctuating temperatures under outdoor conditions compared to constant temperatures in the laboratory (Sundaram and Sundaram, 1995).

Next to temperature, biotic interactions such as interspecific competition are key to improve the realism of the ERA of pesticides (Rico et al., 2016). Interspecific competition is a major structuring factor in aquatic communities and induces energetic costs by reducing food availability (Kroeger et al., 2013b, 2014). Synergistic interactions between toxicants and natural stressors can be expected when there are energetic costs of coping with each stressor individually (Liess et al., 2016). In line with this, interspecific competition has indeed been shown to increase the immediate pesticide impact (Kroeger et al., 2013a, 2013b; Shuman-Goodier et al., 2017) and to delay the recovery of populations after pesticide exposure (Dolciotti et al., 2014; Knillmann et al., 2013; Liess et al., 2013). Nevertheless, these effects are not consistent and other studies have found no effect of interspecific competition on the direct impact of pesticides (Distel and Boone, 2010; Van den Brink et al., 2017). Interspecific competition is especially relevant to consider in a CITS context as organisms typically have a higher metabolic cost at higher temperatures requiring a higher foraging effort, causing

higher encounter rates, and a higher food intake (Boukal et al., 2019; Hallman and Brooks, 2015). While never studied explicitly, it can be expected that the effect of interspecific competition also increases under DTV because DTV typically increases the energetic costs of organisms (Colinet et al., 2015).

We tested the single and combined effects of chlorpyrifos exposure, interspecific competition with the water flea *Daphnia magna* and DTV on the mosquito *Culex pipiens*. As pesticide, chlorpyrifos was used as it is one of the most commonly used organophosphate insecticides in agriculture worldwide (Eaton et al., 2008; Gómez-Canela et al., 2017), and is listed in the top ten of chemicals with a high risk for aquatic organisms in surface waters in the UK (Johnson et al., 2017). As focal study species, the Northern house mosquito *C. pipiens* biotype *molestus* (Forskål, 1775) was used. Mosquitoes are important prey in terms of biomass in aquatic and in terrestrial food webs (Becker et al., 2010). Larvae of this species live in shallow ponds and lakes where DTV can be considerable (Jacobs et al., 2008). We have shown before that chlorpyrifos toxicity can increase under DTV in this species (Delnat et al., 2019). As competitor species, the water flea *Daphnia magna* was used as both *Daphnia* and *Culex* are filter-feeders sharing the same trophic level and food niche (Blaustein and Chase, 2007). *D. magna* is a well-established model organism in ecology, evolution, and ecotoxicology (Miner et al., 2012; OECD, 2004).

By imposing a realistic exposure scenario where we applied a single pulse of the pesticide without renewal of the medium, we allowed pesticide degradation to play a role in how DTV could change the net pesticide impact. Our first hypothesis is that a faster degradation of the pesticide under DTV may occur because of the higher temperatures encountered during each daily cycle, thereby leading to a weakened impact of chlorpyrifos under DTV (Hooper et al., 2013; Op de Beeck et al., 2017; Sundaram and Sundaram, 1995).

Our second hypothesis is that a stronger impact of chlorpyrifos in the presence of interspecific competition may occur because of its energetic costs (Dolciotti et al., 2014).

2. Material and methods

2.1 Study species and laboratory cultures

No permits or animal care protocols by the Animal Ethics Committee of the University of Leuven were required for this experiment since the study organisms are invertebrates. For both study species we kept continuous laboratory cultures for >10 generations in rooms at 20 °C, a photoperiod of 14:10 h light:dark, and for the mosquitoes a relative air humidity of >70% (see details of the rearing in Appendix A). Note that we only used a single clone (genotype; clone M55) of the water flea *Daphnia magna* that originated from Langerodevijver (Huldenberg, Belgium); the use of a single clone in all trials maximized standardization across treatments.

2.2 Experimental design

To test the single and combined effects of pesticide exposure, daily temperature variation (DTV) and competition with *D. magna* on the mosquito *C. pipiens* a full factorial design was used with 2 chlorpyrifos treatments (absent, present, see 2.3) × 2 DTV treatments (absent, present, see 2.4) × 2 competition treatments ('*Culex* alone', '*Culex* with *Daphnia*') (Figure 1). In addition, a third level of the competition treatment ('*Daphnia* alone') and its four combinations with the two chlorpyrifos treatments and the two DTV levels was also included to obtain information on the effects of the chlorpyrifos and DTV treatments on the competitor (Figure B.1 in Appendix B). These additional treatment combinations did not include mosquito larvae and, therefore, their results are reported in Appendix B. While the DTV treatment was imposed on the mosquitoes from egg hatching onwards, the chlorpyrifos pulse exposure was applied when they molted in the final larval stage (L4). This mimics a

realistic scenario where mosquitoes are exposed their entire larval life to DTV, but only to a short-term pesticide pulse. Based on the guidelines by WHO (2005), the L4 stage was chosen for the pesticide exposure as this is typically the most robust larval stage in mosquitoes. Any effects in this stage would therefore also be present and likely more pronounced in the younger larval instars. This has indeed been shown for chlorpyrifos in another dipteran, the midge *Chironomus riparius* (Buchwalter et al., 2004). Twenty-four hours after the start of the chlorpyrifos treatment, we initiated the competition treatment with the water fleas by adding four mature *D. magna* in the glass vials with the L4 mosquito larvae ('*Culex* with *Daphnia*') or not ('*Culex* alone') (following Becker and Liess, 2015). This way, the *D. magna* were not exposed to chlorpyrifos during the first 24 h, hence they experienced lower chlorpyrifos concentrations (because of degradation). This simulates competition with a more chlorpyrifos-tolerant *D. magna* clone as competitor. Pesticide tolerance has been often documented in *Daphnia* sp. (e.g., Jansen et al., 2011; Simpson et al., 2017). The experiment ended when mosquitoes entered the non-feeding pupal stage (~19 days). We started 12-15 vials with 10 mosquito larvae per treatment combination (total of 112 vials with 1,120 mosquito larvae). The exact number of replicate vials for each treatment combination is indicated in Figure 4A.

To start the experiment, mosquitoes were allocated to a given DTV treatment and reared from the egg stage in white 2 L containers (18.0 cm x 13.3 cm x 12.1 cm) filled with 1 L aerated tap water. In each 2 L container ~130 larvae (from 2-3 egg clutches) were kept and fed daily with 250,000 cells/mL of the green algae *Chlamydomonas reinhardtii*. Once the last larval instar (L4) was reached, mosquito larvae were added per ten in 210 mL glass vials filled with 180 mL medium (solvent control or chlorpyrifos, see 2.3). The medium was not refreshed to allow natural breakdown of chlorpyrifos. We daily added 500,000 cells/mL of the green algae *C. reinhardtii* as food per vial. The *D. magna* that were used in the

experiment had initially been reared per five starting from the neonate stage (<24 h old). These neonates were reared in 210 mL vials filled with 180 mL aerated tap water at one of the two DTV treatments (matching the DTV treatment of *Culex* in the competition trials). They were also daily fed with 500,000 cells/mL of the green algae *C. reinhardtii* per vial.

Throughout the experiment, all animals were reared in incubators under standard conditions of 14:10 h light:dark. The light intensity measured with a Testo 0500 Lux-meter was 1730 lux (SE = 132 lux, N = 4). All treatments received the same light intensity, photoperiod, and mean temperature, only the daily temperature cycle differed between the two DTV treatments (see 2.4).

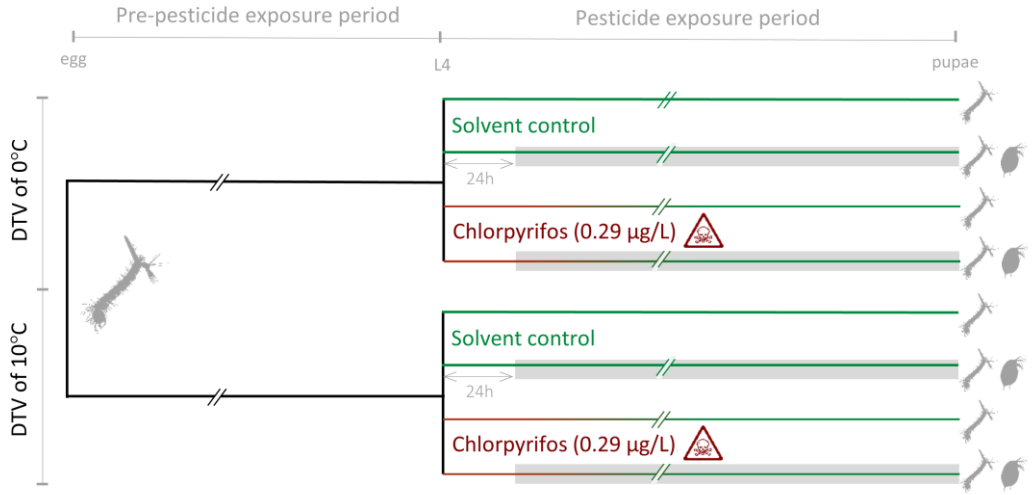

Figure 1. A visualization of the experimental scheme for the *Culex pipiens* larvae. Larvae were continuously reared at a constant temperature (DTV of 0 °C) or fluctuating temperatures (DTV of 10 °C) at the same mean temperature of 20 °C. In the final L4 instar they were exposed to the solvent control of a chlorpyrifos pulse, and 24 h later the interspecific competition treatment started. The gray bands indicate periods with interspecific competition with *Daphnia magna.*

184 *2.3 Pesticide treatment*

185 Chlorpyrifos (purity grade > 99%) was purchased at Sigma-Aldrich (St. Louis, Missouri,
186 USA). To determine the dose-response curve at a constant temperature of 20 °C, we exposed
187 sets of 10 L4 (final larval stage) larvae in 210 mL glass vials filled with 180 mL pesticide
188 medium with the following 15 nominal concentrations: 0, 0.16, 0.18, 0.20, 0.22, 0.24, 0.26,
189 0.28, 0.30, 0.32, 0.34, 0.36, 0.38, 0.40, and 0.60 µg/L. Based on this dose-response
190 experiment, a nominal chlorpyrifos concentration of 0.29 µg/L was chosen that when applied
191 as a single pulse caused ~50% mortality after 72 hours in the L4 mosquito larvae ($LC_{50,72h}$;
192 see results). This concentration of chlorpyrifos is ecologically relevant as peak concentrations
193 in surface waters (Stehle & Schulz, 2015: personal communication: 95%CI = [0.07 µg/L,
194 0.69 µg/L]). Details of the preparation of the chlorpyrifos solution can be found in Appendix
195 A.

196 One pesticide pulse was given at the start of the L4 stage. At the start and after 24 h,
197 water samples were taken from three vials of each of the 2 DTV × 3 Competition treatment
198 combinations with chlorpyrifos. Samples were analyzed at KU Leuven using UPLC-MS/MS
199 with Triple Quadrupole Mass Spectrometry.

200 *2.4 DTV treatment*

201 For both DTV levels, the mean water temperature was 20 °C. The culture of *C. pipiens* was
202 originally derived from field sites in Germany where the mean summer water temperature is
203 ~20 °C (Tran et al., 2016). Water temperatures at the DTV level of 10 °C fluctuated daily
204 between 15 °C and 25 °C using a constant DTV cycle (see Figure C.1 in Appendix C). A
205 DTV of 10 °C frequently occurs at the field sites in Germany during the summer (June-
206 September). Based on air temperature data in the period 1997-2017 from the German Climate
207 Data Centre (https://www.dwd.de/EN/climate_environment/cdc/cdc_node.html), ~45% of the
208 days have a DTV (difference between daily maximum and minimum temperatures) around 10

°C (within the range 8-12 °C) in the source region. Under global climate change, increases in DTV are expected (Vázquez et al., 2017), hence the DTV of 10 °C is expected to become more frequent in the future.

To obtain a daily cycle, the temperatures were adjusted every three hours in steps of 2.5 °C. The water temperatures in the experimental vials were measured every 15 minutes during the experiment using HOBO temperature loggers. The water temperatures are shown in Figure C.1 in Appendix C.

2.5 Response variables

Survival of *C. pipiens* and *D. magna* was checked daily throughout the experiment whereby dead animals were removed. *D. magna* juveniles were removed daily by sieving the medium to maintain constant interspecific competition levels in all replicate vials. To correct for any possible effects of sieving, all vials were sieved simultaneously, also those without *D. magna*. We expressed total survival across the experiment as the percentage of the initial number of animals per vial that survived. Mosquito pupae were collected within 24 hours and were dried at 60 °C. Subsequently, the dry mass was determined using a Cahn C-35 microbalance to the nearest 0.001 mg. Development time from the L4 till the pupal stage was recorded. Note that all response variables (total survival, development time and pupal dry mass) are time-integrated 'accumulated' measures of the effects of the treatments across the entire experiment, hence are not linked to a specific moment in the daily temperature cycle.

2.6 Statistical analyses

All statistical analyses were performed in R v3.6.1 (R Development Core Team, 2017) with the packages lme4 v1.1-21 (Bates et al., 2015), car v3.0-6 (Fox and Weisberg, 2018), afex v0.26-0 (Singmann et al., 2017), emmeans v1.4-5 (Lenth, 2016) and drc v3.0.1 (Ritz et al., 2015).

Based on the results of the dose-response experiment, dose-response curves of chlorpyrifos were fitted after 24, 48, 72, 96, 120, 144, and 168 hours using a log-logistic function (see results in Appendix D). The fit of the dose response curve after 72 h was tested using a lack-of-fit test where a non-significant test indicates a good fit to the data (Ritz and Martinussen, 2011). This dose-response curve was used to determine the $LC_{50,72h}$ value with 95% confidence interval.

To test the first hypothesis of a higher degradation of chlorpyrifos under DTV, a general linear model was used to test for the effect of the DTV and interspecific competition treatments on the chlorpyrifos concentration after 24 h. Since the interaction between DTV and interspecific competition was not significant ($F_{2,12} = 0.30$, $P = 0.74$), and the model without the interaction also had a lower AIC score, the DTV × Competition interaction was removed from the model testing for effects on the chlorpyrifos concentration after 24 h.

When analyzing total survival from the start of the L4 stage until pupation (so not after a fixed time after the start of the pesticide exposure), we coded survival of each larva within a vial as 0 (alive) or 1 (dead). We tested for effects of chlorpyrifos exposure, DTV, and interspecific competition with *D. magna* on total survival of *C. pipiens* using a generalized linear mixed model with a binomial error structure and the logit link. To get more detailed information on when mortality mainly occurred, the effects of these three stressors and their interactions on survival after 48 h and 72 h were also analyzed (Appendix E). To test if the chlorpyrifos, DTV and competition treatments had an effect on the development time and the pupal mass, we used general linear mixed models with a normal error structure and the identity link. Pupal mass was corrected for development time by adding it as a covariate. For total survival, development time and pupal mass, we used individuals as the unit of replication, yet took into account that animals from the same vial were not independent by adding vial to the models as a random factor. If there was a significant

interaction, we performed contrasts with false discovery rate (fdr) correction for pairwise posthoc comparisons using the emmeans package in R to further explore which treatment levels differed.

To explicitly determine the interaction type for total survival between chlorpyrifos and DTV or interspecific competition, we used the independent action (IA) model (see Appendix F). We did so separately in the absence and presence of the other stressor. To estimate the strength of the interaction effect, the MDR (model deviation ratio) was calculated as the predicted combined survival based on the IA model divided by the observed combined survival (Shahid et al., 2019). For synergistic (antagonistic) interactions MDR values are higher (lower) than one.

3. Results

3.1 Chlorpyrifos dose-response curve and experimental concentrations

The lack-of-fit test of the dose response curve indicated a good fit to the data ($F_{11,109} = 1.45$, $P = 0.16$). The dose-response curve was steep with <10% mortality up to 0.282 µg/L (95% CI [0.275; 0.289]) and >90% mortality starting at 0.309 µg/L (95% CI [0.302; 0.315]) (Figure 2). The $LC_{50,72h}$ value of chlorpyrifos was 0.295 µg/L (95% CI [0.292; 0.298]) based on nominal concentrations.

The measured initial chlorpyrifos concentration was 0.145 ± 0.008 µg/L (mean ± SE, $N = 3$). Chlorpyrifos levels after 24 h are shown in Figure 3 were ~10% lower at a DTV of 10 °C than at the constant temperature (DTV of 0 °C) (DTV: $F_{1,14} = 4.87$, $P = 0.045$; per treatment combination: $N = 3$). The chlorpyrifos concentration after 24 h was ~13% lower in the '*Culex* alone' (Contrast '*Culex* alone vs *Daphnia* alone': $P = 0.043$) and '*Culex* with *Daphnia*' (Contrast '*Culex* with *Daphnia* vs *Daphnia* alone': $P = 0.072$) competition treatment compared to the '*Daphnia* alone' treatment (Competition: $F_{2,14} = 4.39$, $P = 0.033$).

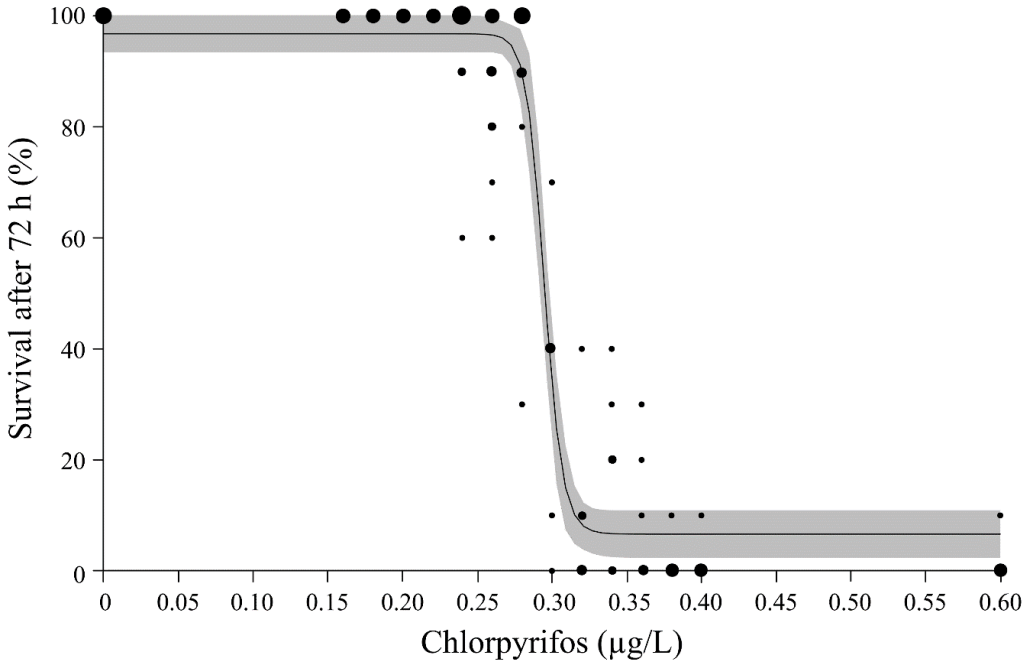

Figure 2. Dose-response curve for the effect of chlorpyrifos (nominal concentrations) on survival after 72h in the mosquito *Culex pipiens* at a constant temperature of 20 °C. The gray area gives the 95% confidence interval and the dots visualize the observed survival percentage for a given vial. The size of the dots indicates the number of replicate vials (smallest dot: 1 vial; largest dot: 10 vials).

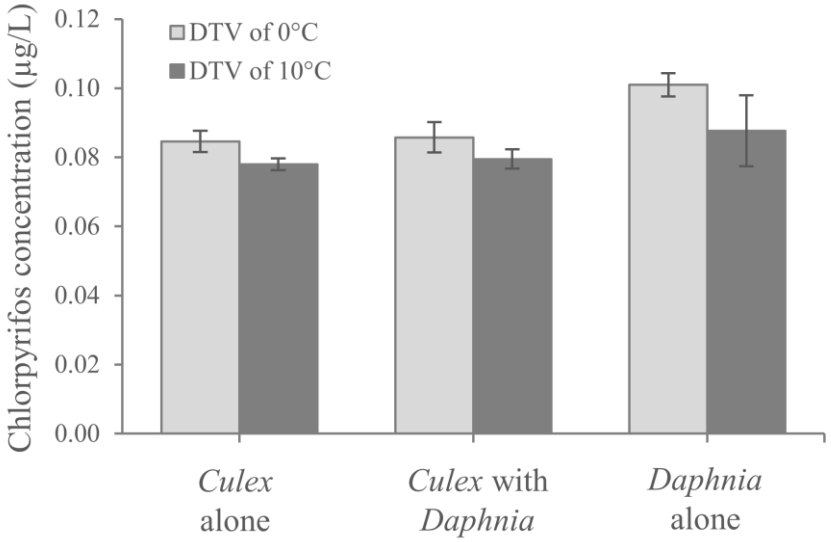

Figure 3. Measured chlorpyrifos concentrations 24 hours after the application of the pesticide pulse for each of the six treatment combinations of interspecific competition and daily temperature variation (DTV). Each mean concentration is given ±1 standard error.

3.2 Total survival of Culex pipiens

Chlorpyrifos (CPF) exposure decreased the total survival from the start of the L4 stage to pupation by ~47% at the constant temperature (Contrast 'chlorpyrifos-constant vs solvent-constant': $P < 0.001$), while there was no effect of chlorpyrifos on total survival under DTV (Contrast 'chlorpyrifos-DTV vs solvent control-DTV': $P = 0.93$) (CPF × DTV, Table 1, Figure 4A). DTV decreased the total survival by ~8% in the pesticide-free solvent control (Contrast 'solvent control-DTV vs solvent control-constant': $P < 0.001$), but increased the total survival by ~43% in the presence of chlorpyrifos (Contrast 'chlorpyrifos-DTV vs chlorpyrifos-constant': $P < 0.001$) (CPF × DTV, Table 1, Figure 4A). There was no significant effect of competition with *D. magna*, nor did competition influence the effects of chlorpyrifos and DTV on total survival (Table 1, Figure 4A).

The interaction between chlorpyrifos and DTV was antagonistic for total survival both in the absence of competition with *D. magna* (Predicted mean total survival when additive effect = 48.19%; 95% CI observed effect = [83.24, 92.76]; MDR = 0.55) and in the presence of competition with *D. magna* (Predicted mean total survival when additive effect = 44.77%; 95% CI observed effect = [83.59, 94.87]; MDR = 0.50). The interaction between chlorpyrifos and interspecific competition was additive for total survival both at a constant temperature (Predicted mean total survival when additive effect = 52.96%; 95% CI observed effect = [37.63, 61.04]; MDR = 1.07) and under DTV (Predicted mean total survival when additive effect = 87.76%; 95% CI observed effect = [83.59, 94.87]; MDR = 0.98).

Table 1. Results of the generalized linear mixed model (total survival) and the general linear mixed models (development time and pupal mass) testing for effects of exposure to chlorpyrifos (CPF), daily temperature variation (DTV) and interspecific competition with *Daphnia magna* on *Culex pipiens*. *P*-values indicated in bold are significant ($P < 0.05$).

	Total survival			Development time			Pupal mass		
	χ^2	df	*P*-value	χ^2	df	*P*-value	χ^2	df	*P*-value
CPF	58.89	1	< **0.001**	283.09	1	< **0.001**	100.35	1	< **0.001**
DTV	1.46	1	0.23	39.21	1	< **0.001**	5.30	1	**0.021**
Competition	0.024	1	0.88	63.80	1	< **0.001**	89.56	1	< **0.001**
CPF × DTV	60.25	1	< **0.001**	110.58	1	< **0.001**	92.84	1	< **0.001**
CPF × Competition	0.023	1	0.88	5.18	1	**0.023**	0.016	1	0.90
DTV × Competition	0.14	1	0.70	0.027	1	0.87	5.00	1	**0.025**
CPF × DTV × Competition	0.10	1	0.75	0.41	1	0.52	0.16	1	0.69

3.3 Development time of Culex pipiens

Chlorpyrifos shortened the development time more (~31%) at the constant temperature than under DTV (~7%) (CPF × DTV, Table 1, Figure 4B, Contrasts 'chlorpyrifos-constant vs solvent control-constant' and 'chlorpyrifos-DTV vs solvent control-DTV': both *P*-values < 0.001). Chlorpyrifos decreased the development time slightly more in the presence of competition (~21%) than in the absence of competition (~18%) (CPF × Competition, Table 1, Figure 4B, Contrasts 'chlorpyrifos-with competition vs solvent control-with competition' and 'chlorpyrifos-without competition vs solvent control-without competition': both *P*-values < 0.001).

DTV slightly decreased the development time by ~5% in the solvent control (Contrast 'solvent control-DTV vs solvent control-constant': $P = 0.0012$), but increased the development time by ~21% when exposed to chlorpyrifos (Contrast 'chlorpyrifos-DTV vs chlorpyrifos-constant': $P < 0.001$) (CPF × DTV, Table 1, Figure 4B). Competition with *D. magna* increased the development time of *C. pipiens* more in the solvent control (~11% increase) than in the presence of chlorpyrifos (~8%) (Contrasts 'solvent control-with competition vs solvent control-without competition' and 'chlorpyrifos-with competition vs chlorpyrifos-without competition: both *P*-values < 0.001) (CPF × Competition, Table 1, Figure 4B). There was no significant three-way interaction (Table 1, Figure 4B).

3.4 Pupal mass of Culex pipiens

Chlorpyrifos exposure increased the pupal mass by ~27% at a constant temperature (Contrast 'chlorpyrifos-constant vs solvent control-constant': $P < 0.001$), while chlorpyrifos had no effect on the pupal mass under DTV (Contrast 'chlorpyrifos-DTV vs solvent control-DTV': $P = 0.37$) (CPF × DTV, Table 1, Figure 4C). DTV increased the pupal mass by ~12% in the solvent control (Contrast 'solvent control-DTV vs solvent control-constant': $P < 0.001$), but decreased the pupal mass by ~16% when exposed to chlorpyrifos (Contrast 'chlorpyrifos-DTV vs chlorpyrifos-constant': $P < 0.001$) (CPF × DTV, Table 1, Figure 4C). Moreover, DTV decreased the pupal mass by ~7% in the presence of competition (Contrast 'DTV-with competition vs constant-with competition': $P = 0.0022$), but not in the absence of competition (Contrast 'DTV-without competition vs constant-without competition': $P = 0.94$) (DTV × Competition, Table 1, Figure 4C). Competition decreased the pupal mass, but less at the constant temperature (~11% decrease) than under DTV (~17%) (Contrasts 'constant-with competition vs constant-without competition' and 'DTV-with competition vs DTV-without

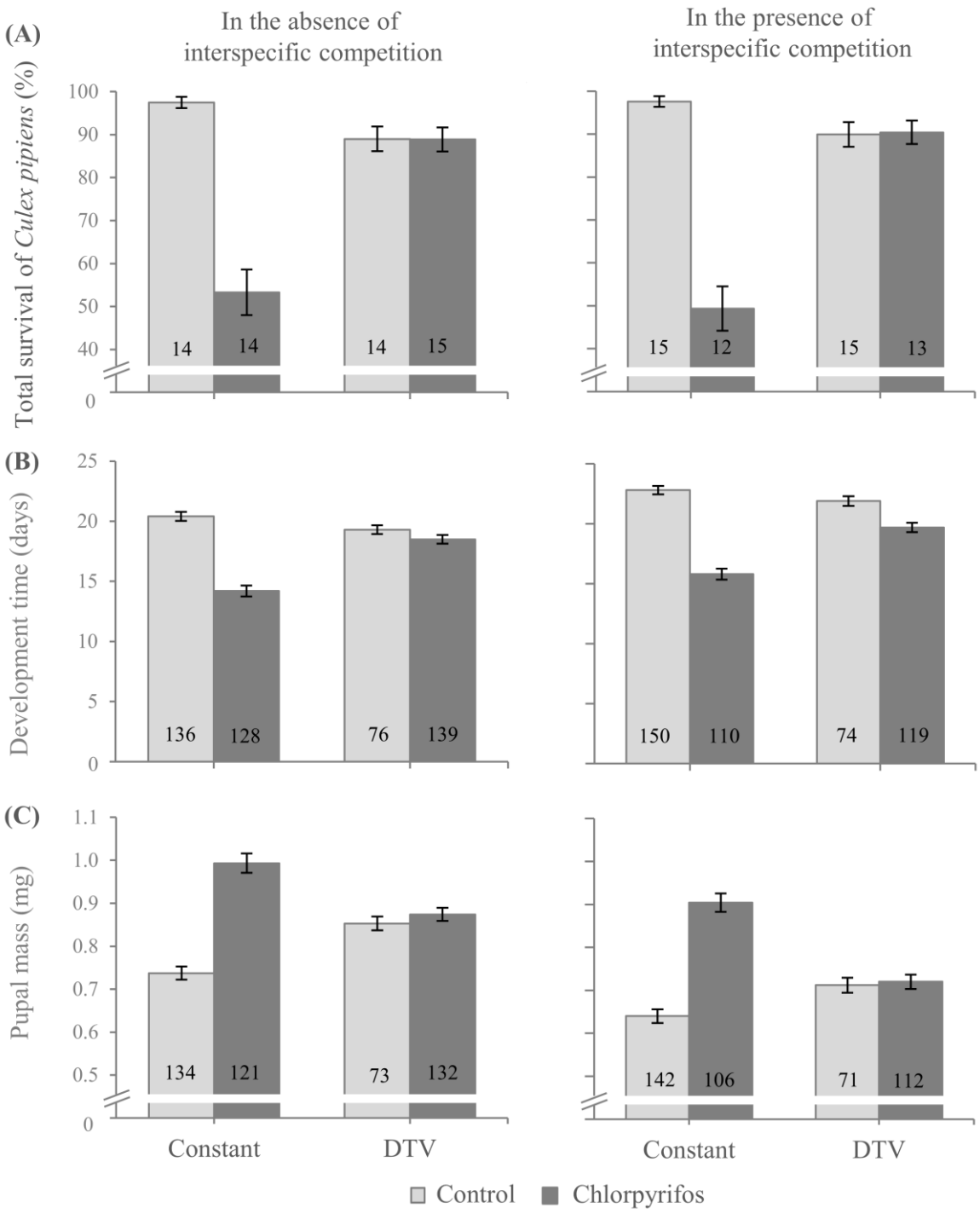

Figure 4. Effects of chlorpyrifos (CPF) on a lethal and the two sublethal response variables of the mosquito *Culex pipiens* in function of daily temperature variation (DTV) and competition with the water flea *Daphnia magna*: (A) total survival from start L4 until pupa, (B) development time from start L4 until pupa, and (C) pupal mass. Means are given with their

355 standard errors. Numbers inside the bars indicate the number of (A) vials or (B,C) individual
356 mosquitoes.
357 competition': both *P*-values < 0.001). Competition with *D. magna* did not influence the
358 effects of chlorpyrifos on pupal mass nor the interaction between chlorpyrifos and DTV (CPF
359 × Competition, CPF × DTV × Competition, Table 1, Figure 4C).

360 **4. Discussion**

361 Chlorpyrifos exposure at a constant temperature without interspecific competition caused
362 considerable mortality, decreased development time, and increased pupal mass of *C. pipiens*.
363 In line with our first hypothesis, a key finding was that chlorpyrifos no longer caused lethal
364 effects under daily temperature variation (DTV), despite single exposure to DTV on its own
365 being mildly lethal. Additionally, chlorpyrifos caused smaller (development time) to no
366 (pupal mass) sublethal effects when combined with a DTV of 10 °C. Hereby, a faster
367 degradation of chlorpyrifos under DTV was an important mechanism counteracting any
368 increase in toxicity of the pesticide under DTV. By showing that DTV can reduce the impact
369 of a pesticide pulse, we provide a new important addition to the CITS concept (Moe et al.,
370 2013; Noyes et al., 2009) whereby not only the effects of global warming on pesticide
371 toxicity but also on the pesticide impact is of importance for future risk assessment of
372 pesticides (Amiard-Triquet et al., 2015; Hooper et al., 2013). In contrast with our second
373 hypothesis, competition with the water flea *D. magna*, although it had negative sublethal
374 effects, did not increase the toxicity of chlorpyrifos. Since our focus was on how DTV and
375 interspecific competition could influence the impact of chlorpyrifos on *C. pipiens*, the effects
376 of single exposure to chlorpyrifos, to DTV or to interspecific competition are discussed in
377 Appendix G.

4.1 Effects of DTV on the impact of chlorpyrifos

In line with our first hypothesis, DTV decreased the impact of chlorpyrifos whereby no effect of chlorpyrifos exposure was present anymore on total survival and pupal mass under DTV. Additionally, chlorpyrifos caused a lower decrease of development time under DTV than at the constant temperature. This resulted in an antagonistic interaction effect for total survival between chlorpyrifos and DTV. This contrasts with the emerging pattern that DTV may magnify the negative impact of toxicants in aquatic invertebrates. This synergism between DTV and pesticides has been shown for the pesticides chlorothalonil in the amphipod *Hyalella azteca* and bifenthrin in the midge *Chironomus dilutus* (Willming et al., 2013), and for chlorpyrifos in damselfly larvae (Verheyen et al., 2019; Verheyen and Stoks, 2019b, 2019c) and in the study species (Delnat et al., 2019). For example, chlorpyrifos increased mortality six-fold under DTV in larvae of the damselfly *I. elegans*, while it did not cause mortality at the constant temperature (Verheyen and Stoks, 2019c). In the latter study, the pesticide medium was renewed daily during the six-day pesticide period, thus any potential differential degradation of the pesticide between the temperature treatments was counteracted by changing the medium when giving multiple pulses (Verheyen and Stoks, 2019c). An increase in toxicity under DTV might be expected based on the fact that during the daily thermal cycle higher temperatures are reached under DTV, and chlorpyrifos has been shown to be more toxic at higher temperatures (e.g., Dinh Van et al., 2014; Hooper et al., 2013). The higher toxicity of pesticides such as chlorpyrifos at higher temperatures might be caused by an increase in metabolic activity leading to an increase in uptake (Buchwalter et al., 2003; Hooper et al., 2013) and by a higher metabolic conversion of the original toxicant to a more toxic metabolite (Buchwalter et al., 2004; Harwood et al., 2009).

Although the toxicity of a toxicant at a constant concentration might increase under DTV, the net impact of this toxicant might still decrease if the increased toxicity is overruled

by a faster degradation rate. In a rare example study on the larvae of the damselfly *I. elegans*, a higher constant temperature of 24 °C caused a faster degradation of chlorpyrifos leading to a lower concentration compared to the lower constant temperature of 20 °C (Op de Beeck et al., 2017). We here for the first time extend this pattern for DTV. In the current study, the faster degradation rate of chlorpyrifos under DTV, leading to a ~8% lower concentration, may explain that the same initial pulse concentration did no longer cause mortality. Important to note is that even a small decrease in concentration can cause a much lower mortality due to the steep dose-response curve of chlorpyrifos for *C. pipiens* (see Figure 2, but note this is based on nominal concentrations precluding a direct comparison of lethal levels observed in the experiment). To illustrate this better, we provide a numerical example: the nominal chlorpyrifos concentration of 0.29 µg/L caused 50% mortality after 72h ($LC_{50,72h}$), while even a 3% lower nominal chlorpyrifos concentration of 0.28 µg/L only caused ~10% mortality. Similarly, the chlorpyrifos impact on the adult survival of *D. magna* was slightly less under DTV than at the constant temperature (Appendix B). The lower chlorpyrifos concentration under DTV (whereby a maximum temperature of 25 °C was daily encountered for three hours) supports the hypothesis by Hooper et al. (2013) that the expected elevated toxicity at higher temperatures might be tempered by the higher hydrolysis rate of organophosphate pesticides. This further supports the importance of considering not only the direct effect of warming on pesticide toxicity, but also on pesticide exposure to understand the net impact of pesticide exposure under warming (Hooper et al., 2013; Op de Beeck et al., 2017).

Note that our finding of a higher chlorpyrifos degradation under DTV was based on measurements 24h after the pulse application, while we reported a lower pesticide-induced 'time-integrated' total mortality (from L4 until pupa) under DTV. Yet, this does not invalidate our interpretation of a higher pesticide degradation under DTV causing the lower pesticide-induced total mortality under DTV. Pesticide-induced mortality was rare during the

first 24 h after the pulse and was mainly observed in the 48-72h interval after the pulse (Appendix D). Chlorpyrifos typically causes delayed effects (not within 24h after a single pulse application), because it first has to be transformed into the toxic oxon form (e.g., Xuereb et al., 2009). Notably, the same survival pattern as for total survival was already present when analyzing survival after 48 h and 72 h (CPF × DTV, Table E.1 and Figure E.2 in Appendix E). The measured pattern of a higher concentration of chlorpyrifos 24h after the pulse in the DTV treatment than in the constant temperature treatment is likely to, if anything, persist through time (and not reverse) while at the same time pesticide levels will further decrease after 24h because of ongoing degradation. Moreover, chlorpyrifos concentrations after 24h were already quite low (< 0.086 µg/L) whereby their contribution to the survival pattern of *C. pipiens* would be very low. As chlorpyrifos concentrations are highest directly after the pulse, the observed degradation pattern after 24h is therefore the most likely driver of the total survival pattern and the sublethal patterns. Note that survival selection caused by DTV prior to the L4 stage (hence prior to the pesticide exposure) is an unlikely driver of the total survival pattern as there was no differential mortality of the mosquito larvae between DTV treatments before the pesticide exposure (see details Appendix H).

4.2 Effects of interspecific competition on the impact of chlorpyrifos

Competition with *D. magna* had negative sublethal effects on the mosquito larvae (increased development time by 9% and decreased pupal mass by 11%), suggesting it was energetically costly. Nevertheless, interspecific competition did not change the lethal impact and even slightly improved the sublethal impact of the pesticide which is in contrast with our second hypothesis. Indeed, in the presence of *D. magna* the likely adaptive shortening of development time under chlorpyrifos exposure was 2% larger. This was in contrast with our expectation that interspecific competition would increase the mortality caused by chlorpyrifos

453 based on the assumption that there are energetic costs when coping with each single stressor
454 (Liess et al., 2016). In line with our results, several other studies that tested for effects of
455 interspecific competition on the acute sensitivity to toxicants also did not detect any effects of
456 interspecific competition on the negative impact of pesticides (Del Arco et al., 2015;
457 Knillmann et al., 2012; Van den Brink et al., 2017). For example, no effect of interspecific
458 competition by rotifers on the impact of the fungicide carbendazim on *D. magna* was
459 detected, likely because rotifers were immediately outcompeted by *D. magna* and were
460 possibly even damaged by being swept in the branchial chambers of the daphnids (Del Arco
461 et al., 2015). While some studies did report the expected pattern whereby interspecific
462 competition increased the negative impact of pesticides (Kroeger et al., 2013a, 2013b;
463 Shuman-Goodier et al., 2017), also the opposite pattern has been reported (Distel and Boone,
464 2010; Van den Brink et al., 2017). For example, the survival of American toads *Bufo*
465 *americanus* was 22% higher in the presence of both carbaryl and the northern leopard frog
466 (*Rana pipiens*) than when facing either stressor alone (Distel and Boone, 2010). This could be
467 explained be a greater corporeal absorption of carbaryl by the leopard frog tadpoles, reducing
468 the concentration to which toad tadpoles were exposed (Distel and Boone, 2010). Corporal
469 absorption of chlorpyrifos by *C. pipiens*, may also explain the lower chlorpyrifos impact on
470 the intrinsic population growth rate, survival, and size of second brood of *D. magna* in the
471 presence of *C. pipiens* in our study (Appendix B). Indeed, the chlorpyrifos concentration after
472 24 h, thus at the start of the *Daphnia* pesticide exposure period, was ~13% lower in the
473 '*Culex* alone' and '*Culex* with *Daphnia'* treatments compared to the '*Daphnia* alone'
474 treatment.

5. Conclusions

By showing that DTV can reduce the impact of a pesticide pulse, we provide a new important addition to the CITS concept (Moe et al., 2013; Noyes et al., 2009). The here observed antagonism between pesticide exposure and DTV is likely widespread because nearly all organisms experience DTV, many pesticides are applied in pulses (Van Drooge et al., 2001), and pesticide degradation is faster at higher temperatures (Hooper et al., 2013; Op de Beeck et al., 2017). As this is a highly controlled laboratory experiment, it would be interesting for future studies to test our hypothesis in more realistic settings such as in an outdoor mesocosm experiment. Our findings support the hypothesis by Hooper et al. (2013) that the expected elevated toxicity at higher temperatures might be tempered by the higher hydrolysis rate of organophosphate pesticides, and extend it to DTV. Under a scenario of multiple pulse exposures, that is not unlikely given pesticide application schedules in agriculture (Dabrowski et al., 2002), this higher degradation rate under DTV is likely to play even a larger role as it will result in less accumulation. Note, that the here identified counteracting mechanism does not imply that the general view of a higher pesticide toxicity under warming is not valid. Instead it points to the need of considering not only the direct effect of warming on pesticide toxicity, but also on pesticide exposure which is crucial to correctly assess the predicted environmental concentration (PEC), a crucial component in ecological risk assessment (Amiard-Triquet et al., 2015). DTV is only recently considered in ecotoxicology, yet the accumulating evidence that it may increase the toxicity of pesticides (Barbosa et al., 2017; Verheyen et al., 2019; Verheyen and Stoks, 2019b, 2019c; Willming et al., 2013), together with current evidence that it may also accelerate pesticide degradation and thereby reduce the net impact of a pesticide, makes it an important abiotic factor to consider in ecological risk assessment. As climate models predict DTV to further increase under global

499 warming (Vázquez et al., 2017), DTV will prove especially important to assess the impact of
500 pesticides in a warming world.

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507 ## 7. Data availability

508 Data will be available on Mendeley data: xxx.

509 ## 8. References

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