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Whether warming magnifies the toxicity of a pesticide is strongly dependent on the concentration and the null model

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Abstract

How global warming changes the toxicity of contaminants is a research priority at the intersection of global change biology and ecotoxicology. While many pesticides are more toxic at higher temperatures this is not always detected. We studied whether deviations from this general pattern can be explained by concentration-dependent interaction effects and by testing the interaction against the inappropriate null model. We exposed larvae of the mosquito *Culex pipiens* to three concentrations of the pesticide chlorpyrifos (absence, low and high) in the absence and presence of 4°C warming. Both the low and high chlorpyrifos concentration were lethal and generated negative sublethal effects: activity of acetylcholinesterase (AChE) and total fat content decreased, and oxidative damage to lipids increased, yet growth rate increased. Warming was slightly lethal, yet had positive sublethal effects: growth rate, total fat content and metabolic rate increased, and oxidative damage decreased. For four out of seven response variables the independent action model identified the expected synergistic interaction between chlorpyrifos and warming. Notably, for three variables (survival, AChE and fat content) this was strongly dependent on the chlorpyrifos concentration, and for two of these (AChE and fat content) not associated with a significant interaction in the general(ized) linear models. For survival and fat content, warming only potentiated chlorpyrifos (CPF) toxicity at the low CPF concentration, while the opposite was true for AChE. Our results highlight that taking into account concentration-dependence and appropriate null model testing is crucial to improve our understanding of the toxicity of contaminants in a warming world.

Keywords: climate change, 'climate-induced toxicant sensitivity' (CITS) concept, multiple stressors, null model, pesticide, synergistic interaction

1. Introduction

How stressors interact may determine the fate of populations and is important to prioritize conservation measures (Côté et al., 2016; Liess et al., 2016; Schäfer and Piggott, 2018). Nevertheless, and despite widespread co-exposure of organisms (Liess et al., 2016), combinations of contaminants and natural stressors are still largely ignored in ecological risk assessment (Goussen et al., 2016). The interaction type between stressors is determined relative to a null model that predicts the combined effect assuming the stressors are independent (Côté et al., 2016; Schäfer and Piggott, 2018). An interaction between stressors that results in a lesser combined effect than that predicted by a null model is an antagonism, while an interaction between stressors that results in a stronger combined effect of stressors than that predicted by a null model is a synergism (Côté et al., 2016; Schäfer and Piggott, 2018). Recent studies highlighted that general linear models may fail to identify the correct interaction type and the need to consider the appropriate null model to derive valid conclusions about the interaction type between stressors (Côté et al., 2016; Griffen et al., 2016; Schäfer and Piggott, 2018). For example, for the same magnitude of the combined response of two stressors the additive null model (used in general lineral models, GLMs) can falsely identify an antagonistic or additive

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Abbreviations: AChE – acetylcholinesterase, CITS - 'climate-induced toxicant sensitivity' concept, CPF – chlorpyrifos, DTNB - dithiobis-2-nitro-benzoic acid, ETS - electron transport system, GLM – General Linear Model, GLMM - Generalized Linear Mixed Model, IA – Independent Action model, INT - p-iodonitrotetrazolium, IPCC - Intergovernmental Panel on Climate Change, L4 - last larval instar, MDA – malondialdehyde, PBS-buffer - phosphate buffer saline, RCP - Representative Concentration Pathway, ROS – Reactive Oxygen Species, TBARS - thiobarbituric acid reactive substance, UPLC MS/MS - Ultra performance liquid chromatography - tandem mass spectrometer, WHO – World Health Organization

effect, while the multiplicative null model (used in the independent action model, IA) can identify a synergism (Fig. 1). Nevertheless, the use of correct null model has been often ignored in the context of combinations of contaminants and natural stressors (but see e.g. Coors, A. & De Meester, 2008; Korkaric et al., 2015; Pestana et al., 2009; Yu et al., 2015).

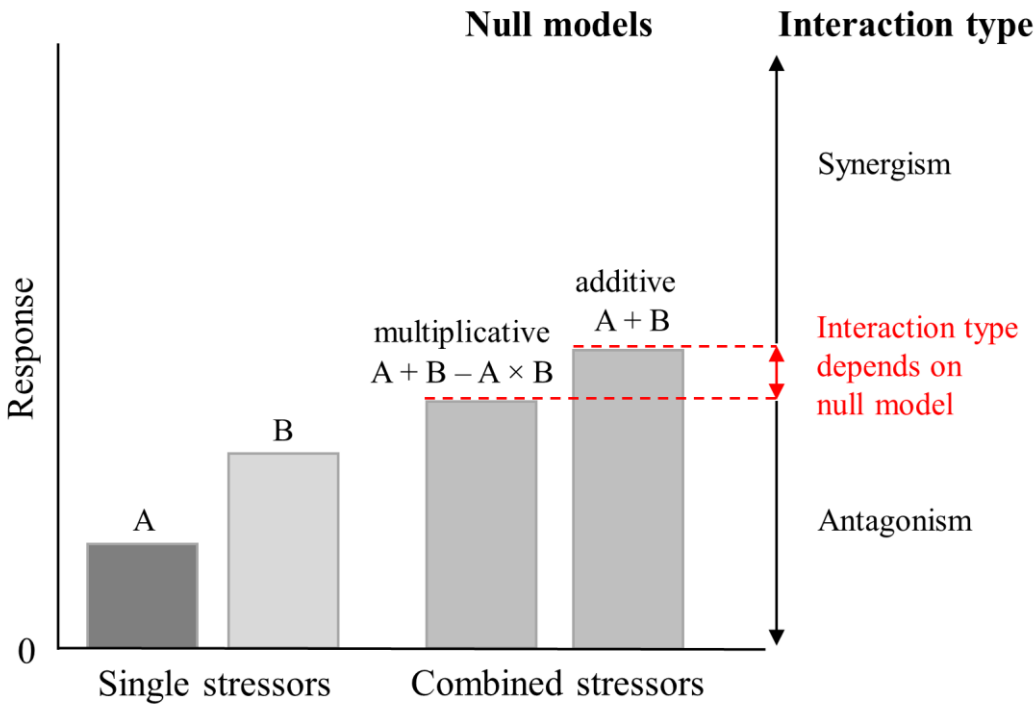

Figure 1. Hypothetical scenario illustrating how identification of the interaction type between two stressors A and B depends on the null model against which the interaction is tested (adapted from Figure 1 of Côté et al., 2016). The combined effect of stressors A and B can be additive (no interaction), synergistic (stronger effect than predicted by a null model) or antagonistic (weaker effect than predicted by a null model). In the range of combined responses indicated in red, the multiplicative null model used by the independent action (IA) model identifies a synergism, while the additive null model used by general linear models identifies an antagonism or an additive effect (at the upper limit of the red zone). Note that in the latter case the GLM would not detect a significant interaction while the IA model would detect a significant synergism.

Especially how global warming changes the toxicity of contaminants has been highlighted as a research priority (Van den Brink et al., 2018). The emerging general pattern of a higher toxicity of metals and many pesticides under warming (Heugens et al., 2001; Holmstrup et al., 2010) has been formalized into the 'climate-induced toxicant sensitivity' concept (CITS; Moe et al., 2013; Noyes and Lema, 2015) and challenges risk assessment of contaminants in a warming world (Landis et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). Yet, there are empirical studies not supporting the concept even within groups of contaminants that typically show the predicted synergism (e.g. Kimberly and Salice, 2014; Perschbacher, 2005; Scheil and Köhler, 2009). Increasing our understanding of such deviations is crucial to improve risk assessment of contaminants in a warming world.

One possible reason for not identifying the expected synergism between a contaminant and warming is the misidentification of the interaction effect due to the usage of a wrong null model (Côté et al., 2016; Schäfer and Piggott, 2018). Given the mode of action of contaminants and warming is typically different, the multiplicative null model as implemented in the independent action model is recommended and not the additive model as implemented in standard general linear models (Schäfer and Piggott, 2018). As illustrated in Figure 1, conclusions for the same combined stress response may match the expected synergism between contaminants and warming when using the appropriate multiplicative null model, yet may apparently deviate from the CITS concept when using the inappropriate additive null model.

A second possible reason of deviations of the CITS concept may be concentration dependence of the interaction between contaminants and warming. Despite the well-known pattern that interactions between contaminants may critically depend on the concentration (e.g. Maazouzi et al., 2016; Pacheco et al., 2018), this has been much less considered for interactions between a contaminant and a natural stressor (but see e.g. Korkaric et al., 2015; Yu et al., 2015). This is partly because the majority of multi-stressor studies only considered two levels per

stressor (Griffen et al., 2016). Very few studies indeed tested the combined effects of multiple contaminant concentrations and temperature (but see e.g. Kimberly and Salice, 2014; Seeland et al., 2013; Vellinger et al., 2012). Moreover, to the best of our knowledge, none of these studies explicitly considered how the interaction type between the contaminant and warming can be concentration-dependent, thereby potentially causing a deviation from the CITS concept.

In this study we tested whether the interaction type between a pesticide and warming differed between a low and high pesticide concentration and whether this may cause deviations from the CITS concept. In addition, given that many tests of the CITS concept make conclusions based on significance of the contaminant-by-warming interaction in a general(ized) linear model, rather than the more appropriate independent action model (Schäfer and Piggott, 2018; Figure 1), we evaluated whether this could generate apparent deviations from the CITS concept. Studies on the CITS concept typically focused on effects on life history (mainly mortality), yet additional insights may be gained by also evaluating effects on physiology (e.g. Op de Beeck et al., 2017b). Physiology may inform about the mechanisms underlying the patterns in life history, but also identify interaction types not captured by life history (e.g. Janssens and Stoks, 2013a; Karl et al., 2011). We therefore tested effects on both life history and physiology with as general CITS-based hypothesis that warming would magnify the negative effects of the pesticide (Heugens et al., 2001; Holmstrup et al., 2010; Noyes and Lema, 2015).

As pesticide, the organophosphate chlorpyrifos (CPF) was chosen because this is an important contaminant in aquatic systems. CPF has been identified as priority pollutant by the European Water Framework Directive (2000/60/EC), and is listed in the top ten chemicals that have the highest risk to aquatic organisms in UK surface waters (Johnson et al., 2017). CPF inhibits acetylcholinesterase, an important enzyme in the nervous system, thereby causing muscle spasms and eventually death (Eaton et al., 2008; Gupta, 2011). The Northern house

mosquito *Culex pipiens* (Linaeus, 1758) form molestus was used as study species. Mosquitoes play an important role as prey species in both the aquatic and the terrestrial food webs (Becker et al., 2010), and therefore are important non-target species to be considered in ecological risk assessment of pesticides in temperate pond food webs (e.g. Rubach et al., 2012). Several studies showed that chlorpyrifos is more toxic at higher temperatures in aquatic insects (e.g. Dinh Van et al., 2014; Janssens and Stoks, 2013; Lydy et al., 1999), including the study species (Tran et al., 2018), making it especially relevant to study the impact of CPF under warming.

2. Materials and methods

2.1. Experimental design

We tested the single and combined effects of warming and pesticide exposure using a full factorial design with two temperature treatments (20°C vs 24°C) crossed with three pesticide treatments (solvent control, low CPF and high CPF). The rearing temperature of 20°C represents the current mean summer water temperature of ponds in Germany where the mosquito culture originates (Tran et al., 2016), while 24°C matches the expected mean temperature by 2100 under the 4°C warming scenario RCP 8.5 (IPCC, 2013). The mosquito culture was maintained for more than 10 generations in the laboratory at ca. 20°C and a light:dark period of 14:10 hours prior to the experiment. The temperature treatment started in the egg stage, while animals were exposed to the pesticide for two days in the last larval instar (L4). Based on the guidelines by WHO (2005), larvae were exposed in the L4 stage in groups of 30 since this is the most resistant stage. We started 21-62 vials per treatment combination (total of 226 vials and 6,780 mosquito larvae). More vials were started in the treatments with CPF, especially at high CPF, to obtain enough larvae that survived the exposure period for the measurements of physiology. Exact numbers of replicate vials per treatment combination are shown in Figure 2A.

156 The mosquito larvae were kept until their molt in L4 in white 2 L containers
157 (18.0 x 13.3 x 12.1 cm^3, made of polypropylene) filled with 1 L dechlorinated tap water.
158 Containers were randomly allocated to a temperature treatment (20°C or 24°C) at a 14:10 h
159 light:dark photoperiod in incubators (2 incubators per temperature). We kept a set of ca. 130
160 larvae (from 2 egg clutches) in each 2 L container. Larvae were fed daily with 1.6 mL of a
161 20 g/L mixture of Olvarit® 7 cereal flakes (46%), wheat germs (51%) and Supradyn® vitamins
162 (3%). This equals 0.32 mg of food/day/larva which is ad libitum (Op de Beeck et al., 2016).
163 The pesticide treatment was started within 16h after larvae molted into the last larval instar
164 (L4). At that moment, larvae were placed per 30 in 210 mL glass vials filled with 100 mL
165 medium. Vials were randomly attributed to one of the three pesticide treatments. These vials
166 were placed in the same incubators as the 2 L containers at a 14:10 h light:dark photoperiod
167 and kept at the same temperature as before (20°C or 24°C). Larvae were counted daily during
168 the two-day pesticide exposure period and we adjusted food rations to keep the food level
169 constant per larva (at 0.32 mg of food/day/larvae) to avoid possible density effects. During the
170 two-day pesticide exposure period we daily renewed the medium (after 24h and after 48h) to
171 keep the CPF concentrations constant between temperature treatments. Two hours after the
172 latest pesticide renewal, five L4 larvae per vial were placed on tissue paper to remove the
173 water on the outer surface of the larvae. They were weighed to the nearest 0.01 mg using an
174 electronic balance (AB135-S, Mettler Toledo), transferred to a single Eppendorf tube and
175 immediately frozen using dry ice. The samples were stored at -80°C till physiological
176 analysis.

177 *2.2. Pesticide concentrations*

178 Chlorpyrifos (CPF, purity grade > 99%) was purchased at Sigma-Aldrich (St. Louis,
179 Missouri, USA). Based on a range-finding experiment (see Appendix A) we selected two
180 concentrations that gave ca. 15% (0.37 µg/L, 1.48 nmol/L) and ca 50% mortality (0.44 µg/L,

1.76 nmol/L) after 48 hours with two exposure medium renewals (after 24h and 48h). These concentrations of CPF are ecologically relevant as they can be encountered in edge-to-field water bodies (Bernabò et al., 2011). The CPF solution was prepared by using a stock solution of 100 µg/mL CPF dissolved in absolute ethanol which was kept in the dark at 4°C. From this stock solution, a second stock solution of 1 µg/mL was made in Milli-Q water. All pesticide treatments, including the solvent control, contained 3.7 µL ethanol/L. In a pilot experiment, ethanol concentrations up to 500 µL/L did not affect survival and growth of larvae of the study species (Tran et al., unpublished data).

During the two-day pesticide exposure period the pesticide medium was daily renewed. We took samples from 3-4 vials of the CPF treatments at the start and after 24 hours (before the next renewal of the medium) on both temperatures. CPF concentrations were quantified at the Division of Soil and Water Management of the KU Leuven using UPLC MS/MS with Triple Quadrupole Mass Spectrometry. The initial low CPF concentration was 0.3925 ± 0.0248 µg/L (mean ± SE, N = 4 vials) and the initial high CPF concentration was 0.5458 ± 0.0266 (N = 3 vials). After 24 hours at 20°C, the low CPF concentration was 0.0953 ± 0.0120 µg/L and the high CPF concentration was 0.1022 ± 0.0283 µg/L (N = 4 vials). After 24 hours at 24°C, the low CPF concentration was 0.0903 ± 0.0153 µg/L and the high CPF concentration was 0.1061 ± 0.0092 µg/L (N = 4 vials).

2.3. Life history response variables

Survival during the two-day pesticide exposure period was scored as 1 (alive) and 0 (dead) for each larva within a vial. Growth rate was estimated per vial as the increase in body mass during the two-day pesticide exposure period. Growth rate was quantified as [ln(end mass)-ln(start mass)]/(2 days). Per vial, the start mass was set as the average mass of extra sets of five pooled larvae that did not enter the experiment, but had the same age as the larvae of the vial when they molted into the L4 stage. The end mass per vial was taken as the average mass

after the two-day during pesticide exposure of two sets of five pooled larvae of that vial (or based on one set when not enough survivors were present in that vial).

2.4. Physiological response variables

Five physiological response variables were measured. Acetylcholinesterase (AChE), the target enzyme inhibited by organophosphates such as CPF (Domingues et al., 2010), was measured based on a modified protocol of Jensen et al. (1997). Total fat content, the major long-term energy storage in insects (Azeez et al., 2014), was quantified based on a modified protocol of Marsh and Weinstein (1966). The activity of the mitochondrial electron transport system (ETS), an estimate of metabolic rate, was determined based on the modified protocol of De Coen and Janssen (1997). Finally, two physiological variables related to oxidative stress were quantified. The concentration of the superoxide anion (O_2^-), a highly toxic reactive oxygen species, was measured based on the protocol of Oracz et al. (2007). As a measure of oxidative damage to lipids, the level of malondialdehyde (MDA) was determined based on a modified protocol of Miyamoto et al. (2011). A detailed description of the protocols can be found in Appendix B.

2.5. Statistical analyses

All statistical analyses were performed in R v3.3.2. (Core Team R, 2017) with the packages lme4 v1.1-13 (Bates et al., 2015), car v2.1-5 (Fox and Weisberg, 2002), afex v0.18-0 (Singmann et al., 2017) and lsmeans v2.26-3 (Lenth, 2016).

We tested for effects of temperature, chlorpyrifos (CPF) and their interaction on individual survival (alive/dead) using a generalized linear mixed model with a binomial error structure and the logit link. To take into account that animals from the same vial were not independent we added vial to the model as a random factor.

To test if temperature, CPF and their interaction had an effect on growth rate and the five physiological response variables, we used general linear models (GLM) with a normal

error structure and the identity link. To meet the model assumptions, the total fat content, the superoxide anion concentration and the AChE activity were ln-transformed. When there was a significant effect of the CPF treatment (which had three levels) or a significant interaction between temperature and CPF, we performed Tukey HSD post hoc tests to explore which treatment levels differed.

To formally identify the interaction type (additive, synergism or antagonism) between the two stressors with a different mode of action we used the independent action (IA) model as recommended by Schäfer and Piggott (2018). Furthermore, the IA model takes explicitly into account that a given individual killed by one stressor can no longer be killed by the other stressor (see Table 1 in Schäfer and Piggott, 2018). We determined the interaction type separately for the low and high CPF concentrations given that the interaction type might be concentration-dependent. We applied the IA model following the procedure of Coors & De Meester (2008). For details of this procedure, see Appendix C.

3. Results

3.1. Life history

Overall, warming reduced survival but this was much less pronounced in the solvent control (Tukey: $P = 0.052$) than at low and high CPF (Tukey: both $P \leq 0.032$) (Temperature and Temperature × CPF, Table 1, Figure 2A). CPF exposure reduced survival with ca. 15% at low CPF (Tukey: $P < 0.001$) and with ca. 50% at high CPF (Tukey: $P < 0.001$) (CPF, Table 1). Warming increased the CPF-induced mortality only at low CPF (Tukey: $P < 0.001$) but not at high CPF (Tukey: $P = 0.32$) (Temperature × CPF, Table 1). The IA model indicated that warming and CPF interacted synergistically at low CPF, but additively at high CPF (Appendix D Table D.1A).

The growth rate was higher under warming and higher when exposed to high CPF (Tukey: $P < 0.001$), but not when exposed to low CPF (Tukey: $P = 0.71$) (Temperature and CPF, Table 1, Figure 2B). There was no significant interaction for growth between temperature and CPF (Temperature × CPF, Table 1). Also the IA model identified the interaction between CPF and temperature as additive for both low and high CPF (Appendix D Table D.1B).

Table 1. Results of the general(ized) linear (mixed) models testing for the effects of temperature and exposure to chlorpyrifos (CPF) on two life history variables and five physiological variables of larvae of the mosquito *Culex pipiens*. The *P*-values indicated in bold are significant ($P < 0.05$). Df is the abbreviation of the degree of freedom.

	Survival			Growth rate		
	χ^2-value	Df	*P*-value	*F*-value	Df	*P*-value
Temperature	6.895	1	**0.0086**	9.40	1, 190	**0.0025**
CPF	733.576	2	**<0.001**	18.03	2, 190	**<0.001**
Temperature × CPF	11.985	2	**0.0025**	0.039	2, 190	0.96
	AChE activity			Total fat		
	F-value	Df	*P*-value	*F*-value	Df	*P*-value
Temperature	1.21	1	0.27	59.73	1, 84	**<0.001**
CPF	5.53	2	**0.0055**	6.26	2, 84	**0.0029**
Temperature × CPF	0.61	2	0.54	0.27	2, 84	0.77
	ETS activity			Superoxide anion conc.		
	F-value	Df	*P*-value	*F*-value	Df	*P*-value
Temperature	0.88	1	0.35	0.003	1, 84	0.96
CPF	8.40	2	**<0.001**	1.69	2, 84	0.19
Temperature × CPF	3.47	2	**0.036**	0.33	2, 84	0.72
	MDA level					
	F-value	Df	*P*-value			
Temperature	18.35	1	**<0.001**			
CPF	7.07	2	**0.00146**			
Temperature × CPF	0.010	2	0.99			

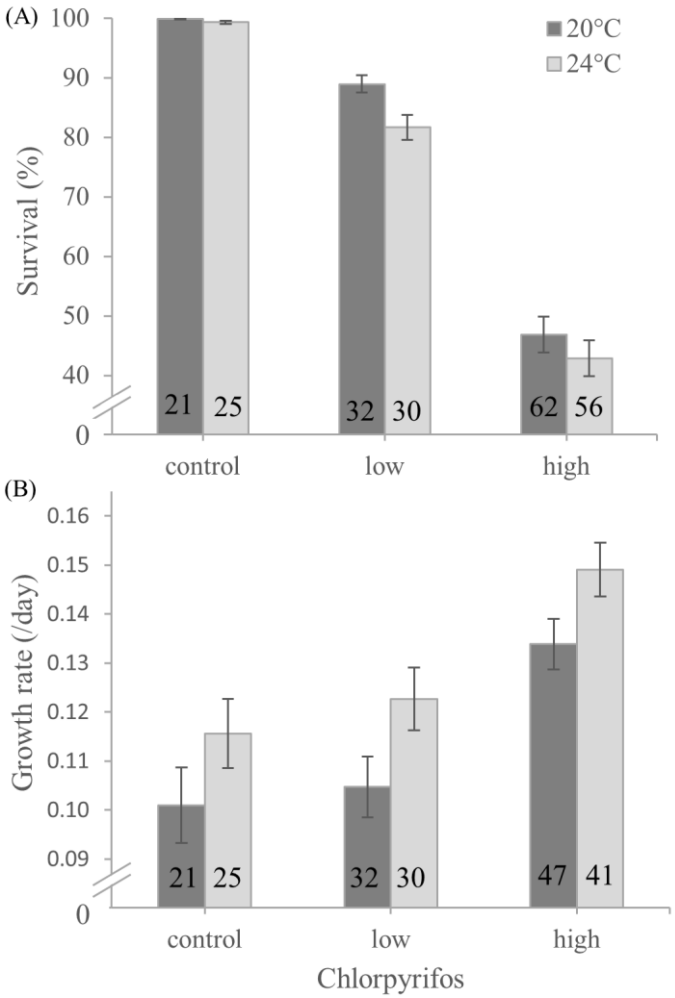

Figure 2. Single and combined effects of the pesticide chlorpyrifos (CPF) and warming on life history variables of larvae of the mosquito *C. pipiens*: (A) survival, and (B) growth rate. Means are given ± 1 SE. Numbers inside the bars of the life history variables indicate the number of replicate vials (each vial contained 30 L4 larvae).

3.2. Physiology

Warming did not affect the activity of acetylcholinesterase (AChE) (Temperature, Table 1, Figure 3A). Overall, CPF exposure resulted in a lower AChE activity (CPF, Table 1), this was only marginally significant at low CPF (Tukey: $P = 0.060$) and more obvious at high CPF (Tukey: $P = 0.005$)(Figure 3A). There was no significant Temperature × CPF interaction in the GLM (Table 1). While the IA model indicated an additive effect between temperature and low CPF, it identified a synergistic interaction between temperature and high CPF (reflecting a

279 stronger CPF-induced reduction in AChE at 24°C than at 20°C) (Appendix D Table D.1C,
280 Figure 3A).

281 Warming increased the total fat content (Temperature, Table 1, Figure 3B). Instead,
282 exposure to both low and high CPF reduced the total fat content (CPF, Table 1, Figure 3B,
283 Tukey: both $P \leq 0.023$). The Temperature × CPF interaction was not significant (Table 1).
284 However, the IA model indicated a synergistic interaction between temperature and low CPF
285 (reflecting a stronger CPF-induced reduction in fat content at 24°C than at 20°C), yet an additive
286 effect at high CPF (Appendix D Table D.1D, Figure 3B).

287 Warming increased the activity of the electron transport system (ETS) only in the
288 solvent control (Tukey: $P = 0.042$) but not in the presence of CPF (Tukey: both $P \geq 0.52$)
289 (Temperature × CPF, Table 1, Figure 3C). Both low and high CPF exposure reduced ETS
290 activity, but only at 24°C (Tukey: both $P \leq 0.049$) and not at 20°C (Tukey: both $P \geq 0.41$)
291 (Temperature × CPF, Table 1). The IA model showed a synergistic interaction for both low and
292 high CPF (reflecting a stronger CPF-induced reduction in ETS at 24°C than at 20°C;
293 Appendix D Table D.1E, Figure 3C).

294 Neither the effects of warming and CPF nor their interaction were significant for the
295 superoxide anion concentration (Table 1, Figure 3D). Accordingly, the IA model identified an
296 additive effect between temperature and CPF at both CPF concentrations (Appendix D
297 Table D.1F).

298 Warming reduced the malondialdehyde (MDA) level (Temperature, Table 1,
299 Figure 3E). Instead, exposure to both low and high CPF increased MDA (CPF, Table 1, Tukey:
300 both $P \leq 0.026$). There was no significant Temperature × CPF interaction (Table 1). The IA
301 model identified an additive effect between temperature and CPF at both CPF concentrations
302 (Appendix D Table D.1G).

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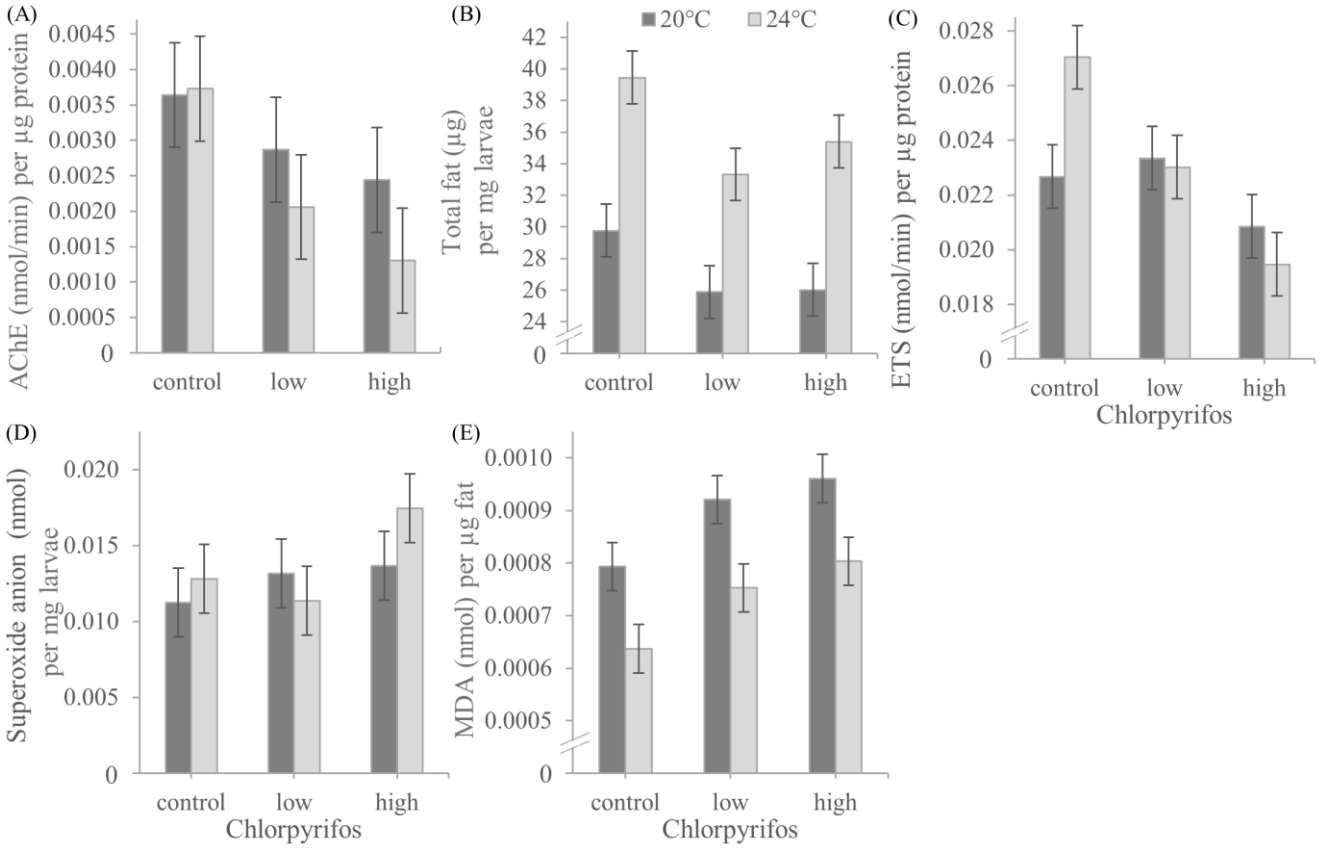

Figure 3. Single and combined effects of the pesticide chlorpyrifos (CPF) and warming on physiological variables of larvae of the mosquito *C. pipiens*: (A) acetylcholinesterase (AChE) activity, (B) total fat content, (C) ETS activity, (D) superoxide anion concentration, and (E) MDA level. Means are given ± 1 SE. The physiological variables are based on 15 replicate vials.

4. Discussion

Warming and especially chlorpyrifos (CPF) were lethal as stressors. In the absence of warming, CPF in general also was stressful by generating negative sublethal effects in the survivors: it decreased AChE activity and total fat content, and increased oxidative damage to lipids (measured as MDA), yet it increased growth rate. In the absence of the pesticide, warming had positive sublethal effects in the survivors: it increased growth rate, total fat content and ETS activity, and decreased the MDA level. We found partial support for the CITS concept. For five

317 out of seven response variables the independent action (IA) model identified the expected
318 synergistic interaction between CPF and warming. Yet, not for all variables and three of the
319 identified synergistic interactions were dependent on the concentration of CPF, and not always
320 associated with a significant interaction in the linear models.

321 *4.1. Effects of warming in the absence of the pesticide*

322 A temperature increase of 4°C, as expected by 2100 under IPCC (2013) scenario RCP8.5,
323 slightly reduced the survival of *C. pipiens*. This is consistent with previous results on thermal
324 adaptation in the study species since 20°C corresponds with the mean summer water
325 temperatures of the field sites in Germany where the lab culture originates from (Tran et al.,
326 2018, 2016). In contrast, in the absence of the pesticide warming had positive (associated with
327 presumably higher fitness values) effects in the survivors by increasing growth rate, total fat
328 content and ETS activity, and decreasing the MDA level. The increase in growth rate under
329 warming might be an adaptive strategy to shorten the life cycle. This suggests that despite 24°C
330 being slightly lethal, this temperature was closer to the optimal temperature for growth than
331 20°C. This reflects the general pattern that many temperate ectotherms have a thermal optimum
332 that is higher than the mean ambient temperature (Deutsch et al., 2008). Moreover, thermal
333 optima may differ between traits (here survival vs growth rate) (Sinclair et al., 2016). The higher
334 growth rate at 24°C may on its turn explain the higher metabolic activity (measured as ETS),
335 as growth rate and metabolic rate are often positively coupled (Downs et al., 2016). An increase
336 in metabolic rate can cause increases in reactive oxygen species (ROS) and oxidative damage
337 (Lushchak, 2011), yet in our study warming did not increase the superoxide anion concentration
338 and even reduced the level of MDA. In larvae of the damselfly *Enallagma cyathigerum*
339 warming did also not increase levels of ROS and oxidative damage which could be explained
340 by the observed upregulation of two antioxidant enzymes (Janssens and Stoks, 2017; see also
341 Tu et al., 2018). Possibly, in our study warming created an over-compensatory antioxidant

response resulting in lower MDA levels (see e.g. Costantini et al., 2010; Sohal and Weindruch, 1996).

4.2. Effects of chlorpyrifos in the absence of warming

Survival at 20°C decreased with ca. 15% when exposed to low CPF (measured concentration = 0.39 µg/L) and with ca. 50% when exposed to high CPF (measured concentration = 0.55 µg/L). The here documented $LC_{50,48h}$ is more than two times higher than the 0.2 µg/L reported for *C. pipiens* by Rubach et al. (2012). CPF also was stressful by generating negative sublethal effects in the survivors: it decreased AChE activity and total fat content, and increased oxidative damage to lipids (measured as MDA). These sublethal effects indicate that the survivors will likely still suffer negative post-exposure effects on fitness. For example, lower lipid levels in *Culex* sp. have been associated with delayed oogenesis (Shin et al., 2012), and a decreased female survival (Vrzal et al., 2010). Furthermore, a higher oxidative damage to lipids has been shown to cause a shorter adult lifespan in another semi-aquatic insect (Janssens and Stoks, 2018). The only positive effect of CPF exposure was the increased growth rate at the high CPF concentration, which may indicate an escape response toward the terrestrial adult stage. Similar escape responses where semi-aquatic insects accelerated growth and development to avoid aquatic exposure to CPF have been documented in the study species (Delnat et al., 2019), and in other taxa such as damselfly larvae (Janssens and Stoks, 2013b) and fiddler crabs (Weis and Mantel, 1976). However, note that this increased growth rate did not occur at a low CPF concentration which can be explained on the one hand by the magnitude of the stressor not being high enough to cause this escape response or on the other hand that survival selection retained only survivors of high quality. Consistent with its mode of action (Eaton et al., 2008), under CPF exposure AChE was inhibited. This, together with the CPF-induced increase in oxidative damage (measured as MDA), may have contributed to the increased mortality. Pesticides such as CPF are known to increase oxidative damage, for example in fish (Zahran et

al., 2018) and in semi-aquatic insects (Janssens and Stoks, 2017; Op de Beeck et al., 2017a). The increase in oxidative damage in CPF-exposed larvae could not be explained by higher superoxide anion levels as these remained constant. The latter is in contrast with the well-documented increase in ROS production in CPF-exposed animals (e.g. Cacciatore et al., 2015; Itziou et al., 2011; Jin et al., 2015; Patetsini et al., 2013). Possibly, the CPF-induced increase in MDA levels was due to an increase in other ROS (such as hydrogen peroxide and hydroxyl radicals). Moreover, even without an increase in ROS levels, CPF-induced reductions in the activity levels of antioxidant enzymes (e.g. Janssens and Stoks, 2017; Marigoudar et al., 2018), may have shifted the balance between ROS and antioxidant defense toward a state of oxidative stress resulting in an increase in MDA. Finally, CPF exposure caused a reduced energy storage as measured by a lowered total fat content (see e.g. also Arambourou and Stoks, 2015; Dinh Van et al., 2016). This might be explained by a reallocation of energy to detoxification processes (Campero et al., 2007).

4.3. Interactive effects between chlorpyrifos and warming

For four response variables (survival, ETS, fat content and AChE) the IA model identified a synergistic interaction indicating CPF being more toxic under warming. This matches the general CITS pattern that many pesticides, including organophosphates, are more toxic at higher temperatures (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). This confirms previous studies on aquatic animals such as fish (Patra et al., 2015; Philippe et al., 2018), damselfly larvae (Dinh Van et al., 2014) and the study species (Tran et al., 2018). This has been explained by a higher uptake and, specifically for CPF, also a faster conversion to the more toxic oxon-metabolite at higher temperatures (Buchwalter et al., 2003; Lydy et al., 1999).

We could demonstrate that whether the interaction between CPF and warming was synergistic depended for three variables (survival, AChE activity and fat content) on the CPF concentration. Notably, for survival and total fat content, warming only potentiated CPF

toxicity at the low and not at the high CPF concentration. This switch from a synergistic to an additive interaction with increasing CPF concentration is intriguing. Likely, because of the considerably higher mortality at the high (ca. 50%) compared to at the low (ca. 15%) CPF concentration, more of the weakest larvae were already removed by the single exposure to high CPF, so that the additional warming stress had little extra effect on survival and fat content (see also Côté et al., 2016; Vinebrooke et al., 2004). Yet, for AChE the opposite switch occurred: from an additive interaction between low CPF and warming toward a synergistic interaction between high CPF and warming. To become toxic, chlorpyrifos needs to be bioactivated through transformation by enzymes into the chlorpyrifos-oxon metabolite that permanently inhibits AChE (Eaton et al., 2008; Flaskos, 2012). The combination of a higher biotransformation at higher temperatures and the presence of more CPF that can be converted to the metabolite at the high CPF concentration apparently resulted in a more than additive increase in the chlorpyrifos-oxon, hence inhibition of AChE. Note that the physiological measurements (including AChE) and growth could only be measured in survivors which could explain discrepancies between the patterns of survival and those of the other end points since these survivors showed a higher tolerance against CPF. Whatever the underlying reasons, our results indicate that the presence of a synergistic interaction between a pesticide and warming may not be general and strongly depend on the pesticide concentration. Note that even though the difference between both concentrations is small, they caused a considerable difference in survival (42%) in this study in this study reflecting the steepness of the dose-response curve (see Appendix A). This concentration-dependence is important as it may explain differences between studies in whether warming magnifies the toxicity of pesticides or not. Such concentration-dependent interaction effects have been observed before when contaminants were combined with other natural stressors. For example, there was an additive effect on mortality in the African clawed frog (*Xenopus laevis*) between UVB radiation and low

endosulfan concentrations, yet at the highest endosulfan concentration the interaction became antagonistic (Yu et al., 2015).

Notably, for two variables (AChE activity and fat content) a synergistic interaction type was detected by the IA model while there was no significant Temperature × CPF interaction in the linear models (both $P > 0.50$). This can be explained because the IA model and the general linear models (GLMs) differ in their null model against which an interaction is tested (Schäfer and Piggott, 2018). As visualized in Figure 1, the additive null model used in general lineral models indeed may detect an additive effect, while the multiplicative null model used in the independent action model (IA) detects a synergistic interaction effect (Côté et al., 2016). Similar to our results, Yu et al. (2015) also found no interaction effect between UVB radiation and α-cypermethrin for larval size of the African clawed frog based on the GLM, while the IA model identified a synergistic interaction effect between both stressors. Two alternative reasons for a mismatch between the GLMs and the IA model can be excluded in our study. First, the ln-transformation of both variables, which may affect significance of interaction terms in GLMs, did not play a role. Also for the non-transformed variables no significant interaction effect was found in the GLMs (both $P \geq 0.54$). Second, for both variables the synergism was only detected at one CPF concentration, which may have made it less likely to detect an overall interaction in the GLMs. Yet, also in separate GLMs per concentration, no significant temperature × CPF interactions were present (for AChE: both $P \geq 0.28$; for fat content: both $P > 0.58$). While there is a long tradition to explicitly use the IA model to detect and identify interactions between contaminants with a different mode of action (Schäfer and Piggott, 2018) this has been largely ignored when analyzing the combinations of a contaminant with a natural stressor (but see e.g. Coors & De Meester, 2008; Korkaric et al., 2015; Yu et al., 2015). Our results demonstrate that not explicitly applying the IA model may result in the failure to detect synergisms between a pesticide and warming.

4.4. Conclusions

Our study provides partial support for the CITS concept, and more importantly it identified two important, likely widespread reasons that may cause (apparent) deviations. First, we provided proof-of-principle that the synergistic interaction between temperature and CPF can be concentration-dependent. Both concentrations we used caused mortality, integrating also non-lethal concentrations in future experiments would be rewarding by extending the range of concentrations toward levels where no survival selection occurs. Second, we demonstrated that the often used approach to test the CITS concept based on the significance of the contaminant-by-warming interaction in a GLM may be misleading, thereby supporting the recent and more general call to use appropriate null models when testing interactions between stressors (Schäfer and Piggott, 2018). Our results highlight that taking into account concentration-dependence and appropriate null model testing is crucial to improve our understanding of the toxicity of contaminants in a warming world.

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