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Title: Daily temperature variation magnifies the toxicity of a mixture consisting of a chemical pesticide and a biopesticide in a vector mosquito

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Abstract: While many studies on the toxicity of pesticides looked at the effects of a higher mean temperature, effects of the realistic scenario of daily temperature variation are understudied. Moreover, despite the increasing interest for the toxicity of pesticide mixtures how this is influenced by temperature has been largely ignored. We tested whether daily temperature variation (DTV) magnifies the toxicity of two pesticides with a different mode of action, the organophosphate pesticide chlorpyrifos (CPF) and the biopesticide *Bacillus thuringiensis* var. *israelensis* (Bti), and of their mixture in the vector mosquito *Culex pipiens*. Single exposure to CPF and Bti increased mortality and reduced female development time, and exposure to CPF also increased female wing length. DTV was not lethal and did not change the toxicity of the individual pesticides. Yet, a key novel finding was that high DTV increased the mortality of the mixture by changing the interaction between both pesticides from additive to synergistic. Given that in nature daily temperature variation is omnipresent, this is important both for vector control and for ecological risk assessment. The higher toxicity of the mixture at high DTV compared to the typically used constant test temperatures in the laboratory urges caution when evaluating the environmental impact of pesticide mixtures.

Capsule: We provide the first evidence that daily temperature variation can magnify the toxicity of a pesticide mixture by changing the interaction type between the pesticides.

Key words: antagonism; climate change; global warming; interaction effect; synergism; integrated vector management

Highlights:

- The influence of temperature on the toxicity of mixtures has largely been ignored
- Mosquitoes were exposed to two pesticides under daily temperature variation (DTV)
- DTV did not increase the toxicity of the single pesticides

- DTV increased the toxicity of the pesticide mixture
- DTV is omnipresent in nature, hence important for risk assessment of mixtures

Abbreviations: [1]Bti - *Bacillus thuringiensis* var. *israelensis*, [2]CPF – chlorpyrifos, [3]DTV – daily temperature variation, [4]DTV-0 = constant temperature, [5]DTV-7 = daily temperature variation of 7 °C, [6]DTV-14 = daily temperature variation of 14 °C, [6]IVM – integrated vector management, [7]L4 – fourth and final larval stage, [8]OECD - Organisation for Economic Co-operation and Development, [9]UPLC-MS/MS - Ultra performance liquid chromatography - tandem mass spectrometer, [10]WHOPES – World Health Organisation Pesticide Scheme

1. Introduction

Many studies have documented how the toxicity of pesticides such as organophosphates and carbamates increases at higher mean temperatures (reviewed in Moe et al., 2013; Noyes et al., 2009; Noyes & Lema, 2015). In contrast, another key aspect of global warming, the increase in daily temperature variation (DTV; Colinet et al., 2015), has received much less attention in ecotoxicology. The very few studies on this topic, however, convincingly showed that increased DTV around the same mean temperature may, just as increases in mean temperature, magnify the toxicity of pollutants (the pesticide chlorothalonil: Willming, Qin, & Maul, 2013; the pharmaceutical fluoxetine: Barbosa et al., 2017).

Despite the growing interest in pesticide mixtures (e.g. Cedergreen, 2014; Hasenbein, Lawler, & Connon, 2017; Hernández, Gil, & Lacasaña, 2017; Nowell et al., 2018), it is largely unknown how thermal regimes shape their toxicity. One notable exception is the study of Laetz et al. (2014), that documented that the synergistic effect in a mixture of two organophosphates (ethoprop and malathion) was stronger at a higher mean temperature in the coho salmon

Oncorhynchus kisutch. Yet, no studies tested how DTV affects how pesticides within a mixture interact and thereby determines mixture toxicity. When combined, pesticides may interact in three ways (Cedergreen, 2014; Gunderson et al., 2016): (i) additive, where the combined effect equals the sum of the effects of the individual pesticides, (ii) synergistic, where the effect is larger than the additive effect and (iii) antagonistic, where the effect is smaller than the additive effect. The emerging view is that both synergistic and antagonistic effects in binary mixtures are rare and mostly occur at high concentrations (Cedergreen, 2014; Kamo and Yokomizo, 2015; Rizzati et al., 2016; Rodea-Palomares et al., 2015). Evaluating the presence of synergistic effects between stressors is of key importance for nature management and risk assessment (Côté et al., 2016; Holmstrup et al., 2010). Given that synergisms can be expected when energetically costly stressors are combined (Liess et al., 2016), and that DTV imposes net energetic costs as animals face higher temperatures during their daily thermal cycle (Colinet et al., 2015), one can expect DTV to increase the toxicity of pesticide mixtures.

While effects of thermal regimes on the toxicity of single pesticides and their mixtures are of large concern for populations of non-target organisms (Dinh Van et al., 2014; Relyea, 2009), they may at the same time improve the efficacy of the control of targeted pest species (e.g. Gomes, Nunomura, Marinotti, & Tadei, 2016). Given the build-up of resistance, vector control strategies that rely on the use of single chemical insecticides are becoming increasingly inefficient (Ranson and Lissenden, 2016), and this despite the increasing need for vector control under global warming (Ogden and Lindsay, 2016). Integrated vector management (IVM) promotes simultaneous use of different vector control tools and is therefore advocated to improve the efficacy, cost-effectiveness, sustainability and ecological soundness of disease-vector control to prevent transmission of vector-borne diseases (WHO, 2012). The efficacy of

IVM based on pesticide mixtures (Bourguet et al., 2013) may obviously benefit from synergistic interactions between the pesticides in the mixture used.

Synergistic interactions between biopesticides and chemical pesticides are a particularly promising IVM approach to control vector mosquitoes as these could potentially result in the combination of efficacy of control, slowdown of resistance build-up and lower ecological damage. One such promising biopesticide is Bti, the bacterium *Bacillus thuringiensis* var. *israelensis* (Becker et al., 2010). Bti is specifically harmful to dipterans and is the most widely used biopesticide to control mosquitoes in Europe (Becker et al., 2010; Bellini et al., 2014; WHOPES, 2016). The review of Cedergreen (2014) revealed that synergistic pesticide mixtures, while rare in general, often included an organophosphate. In line with this, a synergism was detected when Bt or Bti were combined with the organophosphate temephos to kill *Aedes aegypti* and *Anopheles stephensi* mosquitoes (de Andrande and Modolo, 1991; Narkhede et al., 2017). However, an additive effect between Bti and the organophosphate malathion was found in the mosquito *Culex pipiens* (Mansour et al., 2012). How DTV affects the toxicity of biopesticides has never been studied.

In this study, we tested whether DTV magnifies the toxicity of two pesticides with a different mode of action, the organophosphate pesticide chlorpyrifos (CPF) and the biopesticide Bti, and of their mixture in a vector mosquito. Therefore, we setup up a full factorial design: 3 DTV levels (constant, low, high) × 2 CPF treatments (absent, present) × 2 Bti treatments (absent, present). We expected that the toxic effects of the single pesticides are strengthened in the presence of DTV (Barbosa et al., 2017; Willming et al., 2013). In addition, we tested whether there is an interaction between the chemical pesticide CPF and the biopesticide Bti within the mixture, and whether this interaction is magnified in the presence of DTV. As study species we used the Northern house mosquito *Culex pipiens* (Linaeus, 1758) form *molestus*. This species is part of the *C. pipiens* complex, which are important vectors of the West Nile and St. Louis encephalitis viruses (Farajollahi et al., 2011). In Europe and in the USA, this species is the most common mosquito vector species in urban areas (Fonseca et al., 2004) which makes it an important target for vector control (Kilpatrick, 2011). As chemical pesticide, we chose CPF as this is one of the most used chemical insecticides in vector control management (Eaton et al., 2008; WHOPES, 2016).

2. Material and methods

2.1 Study species and rearing

A continuous lab culture of *C. pipiens* initiated from a culture maintained at the Helmholtz Centre for Environmental Research (UFZ, Germany) was used in this experiment. The original field population was also situated in Germany where it encountered a mean summer water temperature of ca. 20°C (Tran et al. 2016). The mosquitoes were reared from egg to third instar (L3) larvae in white 2 L containers (18.0 x 13.3 x 12.1 cm^3) filled with 1 L aerated tap water. Containers were placed in a temperature-controlled room under standard conditions of 14:10 h light:dark and a water temperature of 19.84 °C (STD: 1.00 °C). We kept ca. 100 larvae (from 2-3 egg clutches) in each 2 L container. Larvae were fed three times a week with 3 mL of a 20 g/L mixture of Olvarit® 7 cereal flakes (46%), wheat germs (51%) and Supradyn® vitamins (3%) (Op de Beeck et al., 2016). This equals 0.257 mg of food/day/larva which is ad libitum.

2.2 Experimental design

To test the single and combined effects of daily temperature variation (DTV) and pesticide exposure we used a full factorial design with three DTV treatments with the same mean temperature of 20 °C (DTV-0 = constant temperature, DTV-7 = low daily temperature variation of 7 °C, and DTV-14 = high daily temperature variation of 14 °C) crossed with two CPF treatments (CPF absent and present) and with two Bti treatments (Bti absent and present). This gave a full factorial 3 DTV × 2 CPF × 2 Bti experimental design. Note that the treatment combination where both CPF and Bti are present, is the mixture treatment. Note also that our aim was not to test for an effect of mean temperature but instead to test for an effect of DTV at a given mean temperature. Therefore, and to keep the experiment feasible, we ran all DTV treatments at the same mean temperature (see e.g. also Alto et al., 2017; Carrington et al., 2013; Willming et al., 2013), here 20 °C the mean summer water temperature at the site of origin of the study population. This design perfectly suits our aim to test the effect of realistic DTV scenarios.

We started 27-36 vials per treatment combination (total of 377 vials and 7,540 mosquito larvae). The exact number of replicates of each treatment combination per response variable is indicated above the bars in the figures. The experiment was performed in the last larval instar (L4) during which larvae were continuously exposed to a DTV treatment, while the pesticide exposure treatment was imposed for only two days. This resulted in three experimental steps: (i) 4-day exposure to DTV, (ii) 2-day exposure to DTV and the pesticide treatment and (iii) exposure to DTV until metamorphosis (Figure 1).

We started the DTV treatment within 24h after larvae molted into the last larval instar (L4). At that moment, larvae were placed per 20 in 210 mL glass vials filled with 100 mL pre-aerated water. Vials with larvae were placed in incubators at a 14:10 h light:dark photoperiod with a light intensity of 1730 lux (±SE: 132 lux, *N* = 4) measured with a Testo 0500 Lux-meter. All treatments received the same photoperiod and light intensity, and also experienced the same mean temperature, only the daily temperature cycle differed between the three DTV treatments.

After four days, we quantified mortality and started the pesticide treatment. Per DTV treatment, vials were randomly attributed to one of the four pesticide treatment combinations. At that moment, we refreshed the medium with 100 mL of one of the four pesticide solutions. Two days later, mortality was quantified again and the survivors were transferred to a new 210 mL glass vial filled with 100 mL pre-aerated water while they stayed on their DTV treatment. We daily counted larvae and adjusted food rations to keep the food level constant per larva (at 0.313 mg of food/day/larva, Beketov & Liess, 2007); except during the two-day pesticide exposure period when they did not receive any food following OECD guidelines (OECD, 2011).

After the 2-day pesticide exposure, we kept monitoring for delayed mortality, and recorded emergence success and development time (from egg hatching until metamorphosis). Total mortality was calculated from the start of the L4 stage until metamorphosis. Successfully emerged adults were sexed and frozen to measure the wing lengths of the females afterwards with StreamPix v7 (Norpix, Inc., Canada) and ImageJ v1.51k (Rasband, W., National institute of health, USA). Only the wing lengths of the females were measured, since this is a good proxy for their fecundity (Costanzo et al., 2011). Note that all end points (total mortality, development time and adult wing length) 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.

2.3 DTV treatment

At DTV-7 water temperatures fluctuated daily between 16.5 °C and 23.5 °C, while at DTV-14 water temperatures fluctuated between 13 °C and 27 °C. The water temperatures were measured every 15 minutes in a single vial per incubator during the experiment using HOBO temperature loggers. The water temperatures are shown in the appendix Figure A.1. The mean temperature of

20 °C in the two regimes with fluctuating temperatures (DTV-7 and DTV-14) was reached at 11h30 and 23h30; there was an increase in temperature from 8h30 to 20h30, and a decrease in temperature from 20h30 to 8h30 in steps of 3 hours.

2.4 Pesticide treatment

Chlorpyrifos (CPF, purity grade > 99%) was purchased at Sigma-Aldrich (St. Louis, Missouri, USA) and *Bacillus thuringiensis israelensis* (Bti) was bought as Vectobac WG *Bacillus thuringiensis* serotype H-14 (37.4%) at Technic 3D (Paris, France). Based on a pilot study we chose for both pesticides a concentration that caused moderate (ca. 30%) mortality, hence that still allowed to detect sublethal effects: 70 µg/L for Bti and 0.65 µg/L for CPF. Both concentrations are ecologically relevant. According to WHOPES (2016) guidelines to control mosquitoes, the recommended application dosage for natural water bodies ranges from 12.5 to 75 mg/m² for Vectobac WG and from 1.1 to 2.5 mg/m² for CPF. If a depth of 0.5 m is assumed for a natural body, which corresponds with the shallow ponds preferred by *Culex pipiens* (Becker et al., 2010), this gives a recommended dosage of 25-150 µg/L for Bti and of 2.2-5 µg/L for CPF. 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 solutions (including the Bti treatment) contained 104.5 µL ethanol/L. There were no differences in survival or growth rate of L4 larvae of *C. pipiens* between the solvent control and the water control (results not shown). The Bti solution was prepared daily from a new stock solution of 1 mg/mL Vectobac dissolved in Milli-Q water. From this stock solution, a second stock solution of 100 µg/mL was made in Milli-Q water.

During the 2-day pesticide exposure period we gave one pesticide pulse at the start when refreshing the medium. We took water samples from three vials of the CPF treatments at the start and at the end of the pesticide exposure period which were analyzed at KU Leuven using UPLC-MS/MS with Triple Quadrupole Mass Spectrometry. The initial CPF concentration was 0.456 ± 0.006 µg/L (mean ± SE, $N = 3$). After two days, the CPF concentration was 0.148 ± 0.013 µg/L at DTV-0, 0.143 ± 0.011 µg/L at DTV-7 and 0.099 ± 0.008 µg/L at DTV-14 ($N = 3$). This suggests that, if anything, CPF degradation across the 2-day exposure period was slightly higher at DTV-14.

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). The figure was made in Microsoft Excel 2016.

Total mortality from the start of the L4 stage until metamorphosis was scored as 0 (alive) and 1 (dead) for each larva within a vial. We tested for effects of the DTV (daily temperature variation), CPF (chlorpyrifos) and Bti treatments on total mortality using a generalized linear mixed model with a binomial error structure and the logit link. To test if the DTV, CPF and Bti treatments had an effect on female wing length and female development time, we used general linear mixed models with a normal error structure and the identity link. All models included all possible interactions between the treatment factors. We only report the results on female wing length (proxy for fecundity, Costanzo et al., 2011) and female development time as females are the most important sex for population dynamics (Livdahl and Sugihara, 1984). For total mortality, wing length and development time 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 random factor. If there was a significant effect of the DTV treatment (which had three levels) or a significant interaction between the DTV, CPF and/or Bti treatments, we performed Tukey HSD post hoc tests to further explore which DTV levels differed.

To identify which type of interaction was present between CPF and Bti in the mixture, the independent action model was used as advocated when both chemicals have a different mode of action and when analyzing mortality (Bliss, 1939; Faust et al., 2000; Schäfer and Piggott, 2018). Given that DTV may change the interaction type we determined this type per DTV level. We followed the procedure explained in detail in Coors & De Meester (2008). First, the observed effects of the single pesticides in absolute units were transformed to proportional effects using: $E_i = \frac{(e_i - e_{control})}{(e_{max} - e_{control})}$ where e_i is the single-pesticide effect in absolute units, $e_{control}$ is the effect of the pesticide-free control in absolute units and e_{max} is the maximum possible effect of that single pesticide. For total mortality 100% was used as e_{max}, while for the female development time (46 days) and the female wing length (4.279 mm) the maximum values in the dataset were used as e_{max}. Second, the predicted joint effects were calculated using the formula $E_{mix} = 1 - \prod^{i} (1 - E_i)$. Third, the predicted joint effect E_{mix} was transformed back to absolute units to allow comparison with the observed joint effects using the formula $e_{mix} = E_{mix} * (e_{max} - e_{control}) + e_{control}$. When the predicted joint effect was not in the 95% confidence interval of the observed joint effect in absolute units, this was taken as evidence for a significant deviation of observed from predicted joint effects. A significantly stronger observed joint effect compared to the predicted joint effect indicated a synergistic interaction, while a significantly weaker observed joint indicated an antagonistic interaction.

3. Results

3.1 Total mortality

Both single CPF exposure and single Bti exposure increased the total mortality from the start of the L4 stage until metamorphosis (Figure 2A, Table 1). There was a significant 3-way DTV × CPF × Bti interaction (Figure 2A, Table 1): DTV did not increase mortality in the solvent control (Tukey: all $P > 0.74$), in the single Bti exposure (all $P > 0.67$) and in the single CPF exposure (all $P > 0.24$), but DTV-14 increased mortality in the CPF-Bti mixture (all $P < 0.001$). According to the independent action model the CPF-Bti mixture was additive at DTV-0 and DTV-7, while it was synergistic at DTV-14 (Figure 2A, Table 2).

3.2 Development time of females

Both single CPF exposure and single Bti exposure reduced the development time of females (Figure 2B, Table 1). There was a significant 3-way DTV × CPF × Bti interaction (Figure 2B, Table 1): DTV-14 decreased the development time in the solvent control (Tukey: all $P \leq 0.001$), in the single Bti exposure (all $P \leq 0.001$) and in the CPF-Bti mixture (all $P \leq 0.0.39$), but not in the single CPF exposure ($P > 0.12$). According to the independent action model the CPF-Bti mixture was antagonistic at DTV-7, while it was additive at DTV-0 and DTV-14 (Figure 2B, Table 2).

3.3 Wing length of females

Single CPF exposure but not single Bti exposure increased the female wing length (Figure 2C, Table 1). There was a significant 3-way DTV × CPF × Bti interaction (Figure 2C, Table 1). This indicated that DTV did not affect the female wing length in the single Bti exposure (Tukey: all $P > 0.16$) and in the single CPF exposure (all $P > 0.11$), but DTV-14 decreased wing length in the solvent control (all $P \leq 0.023$) and increased the wing length in the CPF-Bti mixture ($P = 0.018$). According to the independent action model the CPF-Bti mixture was antagonistic for female wing length at DTV-7, while it was additive at DTV-0 and DTV-14 (Figure 2C, Table 2).

4. Discussion

As expected, single exposure to each pesticide caused mortality and shaped the sublethal traits in the survivors. Unexpectedly, daily temperature variation (DTV) did not make the single pesticides more toxic. Yet, a key finding was that DTV did increase the toxicity of the mixture by changing the interaction type between both pesticides for mortality from additive to synergistic.

4.1 Effects of the pesticides and their mixture at the constant temperature

As expected, exposure to 0.65 µg/L of the chemical pesticide CPF or to 70 µg/L of the biopesticide Bti increased the mortality of *C. pipiens*. In addition, both pesticides also reduced the female development time. Similarly, CPF exposure reduced the development time in the damselfly *Enallagma cyathigerum* when enough food was available (Janssens & Stoks, 2013). Earlier metamorphosis when exposed to pesticides in the larval stage may be an adaptive strategy to escape the pesticide exposure in the water. Despite that a shorter development time gives less time to acquire resources (Dmitriew, 2011), the female wing length increased when exposed to CPF. The beneficial sublethal effects of CPF may be explained by survival selection retaining only survivors of high quality. In line with our results, females of the mosquito *Aedes aegypti* that survived exposure to the insecticide Spinosad also had a larger wing length (Antonio et al., 2009).

Consistent with the emerging view on mixture toxicity (Cedergreen, 2014; Kamo and Yokomizo, 2015; Rizzati et al., 2016; Rodea-Palomares et al., 2015), no endpoint showed a synergistic interaction between CPF and Bti in their binary mixture when the temperature was kept constant (DTV-0°C). On the other hand, in the few studies that did document synergistic interactions in mixtures, organophosphates are overrepresented (Cedergreen, 2014; see also de Andrande and Modolo, 1991 and Narkhede et al., 2017). Two main mechanisms behind synergisms between organophosphates and other pesticides have been suggested (Cedergreen, 2014): (i) a change in the uptake rates and transport to the target site where one pesticide causes an increase in oxygen consumption due to metabolization leading to an increased respiration, which increases the uptake of the other pesticide, and (ii) a change in metabolic enzyme activities where one pesticide can increase the activation to a more toxic metabolite of the other pesticide or can decrease the detoxification by enzymes. For example, the mixture of the organophosphate temephos and the biopesticide Bt increased the level of alkaline phosphatase that increases the toxicity of Bt compared to the control and single Bt exposure (Narkhede et al., 2017). The here documented additive effects between CPF and Bti at the constant temperature, however, does correspond with the additive effect between another organophosphate, malathion, and Bti for mortality in *Culex pipiens* (Mansour et al., 2012).

4.2 Effects of daily temperature variation (DTV) in the pesticide-free control

The imposed levels of DTV did not affect the mortality in the pesticide-free control. This indicates that at a mean of 20 °C even a DTV of 14 °C was in general not harmful. At DTV-14, temperatures up to 27 °C are reached for 3 hours during each 24h cycle. While constant exposures to 24 °C have been shown to increase mortality in the study species (Tran et al., 2016), it is known that under DTV animals may recover during the colder periods of each cycle and therefore are able to endure short periods of otherwise harmful temperatures (Colinet et al., 2015). In line with this, a DTV of 12 °C did not increase total mortality around a mean of 26 °C but did so around a mean temperature of 32 °C in the mosquito *Anopheles stephensi* (Paaijmans et al., 2013). Similarly, a DTV of 18 °C around a mean of 26 °C caused an increase in larval mortality in the mosquito *Aedes aegypti* (Carrington et al., 2013).

While the low DTV of 7 °C also had no sublethal effects on *C. pipiens*, the high DTV of 14 °C affected development time and wing length. The high DTV decreased the female development time suggesting that at a mean of 20 °C the thermal performance curve for development rate is concave upward. This indeed would imply that the increases in development rate at temperatures higher than the mean are larger than the decreases at temperatures lower than the mean, resulting in a net increase of development rate under DTV (Colinet et al., 2015). Similarly, in *A. stephensi* a DTV of 12 °C around a low mean temperature (16 and 18 °C) also resulted in shorter development times (Paaijmans et al., 2010). In contrast, a high DTV of 18 °C caused an increase in development time of the mosquito *A. aegypti* (Carrington et al., 2013). However, the latter was around a higher mean temperature than in our study (26 °C compared to 20 °C). Furthermore, the high DTV of 14 °C reduced female wing length, matching the general pattern of reductions in body size at higher DTV (Colinet et al., 2015). Given that the wing length is a good proxy for fecundity in *C. pipiens* (Costanzo et al., 2011), this indicates that a large DTV is expected to decrease fecundity. This may be explained by the energetic costs of coping with the daily experienced stressful upper temperatures associated with the high DTV (Colinet et al., 2015).

4.3 Effects of DTV on the toxicity of pesticides and their mixture

Our expectation of DTV magnifying the toxic effects of single exposure to CPF or Bti was not upheld. This expectation was based on the fact that during the daily thermal cycle higher temperatures are reached under DTV, and both CPF (e.g. Dinh Van et al., 2014; Hooper et al., 2013) and Bti (e.g. Duque and Navarro-Silva, 2006; Stevens et al., 2004) have been shown to be more toxic at higher temperatures. Several mechanisms have been suggested for the higher toxicity of pollutants at higher temperatures, including an increase in uptake and metabolic activity (Noyes et al., 2009). Synergistic interactions between chemical and environmental stressors are in general expected when there are energetic costs when coping with each single stressor (Liess et al., 2016). It can therefore also be expected that DTV magnifies the effect of a pollutant because DTV may generate energetic costs when organisms deal with the higher temperatures during the cycle (Colinet et al., 2015). Accordingly, the study of Willming et al. (2013) reported an increased mortality imposed by the chemical pesticides chlorothalonil (in *Hyalella azteca*) and bifenthrin (in *Chironomus dilutes*) under DTV. Yet, in line with our findings, a DTV of 7.5 °C did not magnify the negative effect of the pharmaceutical fluoxetine on survival in *D. magna* (Barbosa et al., 2017). The latter was explained by prioritization of survival over reproduction in the presence of the combined stressors (DTV and CPF), as DTV did magnify the negative effect of fluoxetine on reproduction (Barbosa et al., 2017). While the high DTV likely also caused energetic costs in our study (as suggested by the reduced wing length), the costs may have been too low for DTV to magnify the effect of Bti alone or of CPF alone.

A key novel finding of our study was that the high DTV did increase the mortality imposed by the pesticide mixture which was associated with a change in the interaction type between both pesticides in the mixture from additive at a constant temperature into synergistic at high DTV. Notably, this occurred despite that the high DTV did not increase the toxicity of one of the individual pesticides. Note that, given the CPF concentrations at the end of the experiment were, if anything, lower at high DTV (possibly due to a higher photodegradation), our finding of a higher toxicity of the mixture is conservative. The specific mechanisms behind the interactions between daily temperature variation and pesticides are not understood (e.g. Barbosa et al., 2017; Willming et al., 2013). The energetic costs associated with coping with the higher temperatures during the daily cycle (Colinet et al., 2015) may have only generated a synergism when energy levels of the larvae are very low as can be expected in the mixture when larvae had to defend against both pesticides. Energetic costs may also explain why at a higher mean temperature the synergism between two organophosphates was stronger in the coho salmon *Oncorhynchus kisutch* (Laetz et al., 2014). A general explanation for why combinations of toxicants and environmental stressors (such as DTV) may work synergistically is provided by the Stress Addition Model (Liess et al., 2016). According to this model, each organism has the capacity to handle a certain level of stress. When stress levels become too high (as happens when stressors are combined), individuals with a stress capacity below the given total stress level will die. The SAM shows theoretically how a mechanism where both stressors jointly reduce the common stress capacity of organisms, translates into a synergistic interaction for mortality. This stress capacity can be seen as the energy-limited tolerance to stress (sensu Sokolova, 2013). Thus, environmental stress (in our case a high DTV), by being energetically costly (Colinet et al., 2015), can increase the toxicant sensitivity of individuals thereby creating a synergistic interaction effect between CPF and Bti in their mixture at high DTV (Liess et al., 2016). In addition, it may also be possible that under DTV, specifically at the higher temperatures during the daily cycle, there was a higher uptake of both pesticides contributing to their synergism. While our study was not designed to explain the type of interaction, this dependence of the interaction type between two pesticides on DTV is intriguing and asks for mechanistic follow-up studies at the physiological level. Based on our hypothesized mechanism, particularly measuring levels of energy storage and energy consumption may be rewarding. Whatever the underlying reason, the appearance of this synergistic interaction between CPF and Bti only at high DTV might suggest that interaction effects in binary mixtures are not so rare as based on meta-analyses (e.g. Cedergreen, 2014). Indeed, while daily temperature fluctuations are common in nature, no other mixture studies considered natural daily temperature variation in their experimental design.

Intriguingly, DTV had a different effect on the interaction type between both pesticides for the sublethal response variables. Indeed, it caused an antagonistic interaction for both development time and wing length. In general, differences in interaction effects between the same stressors for different end points are widespread, yet poorly understood (Côté et al., 2016). Development time decreased and wing length increased when exposed to the CPF-Bti mixture at a constant temperature which corresponded to the sum of the response to the single pesticide exposures (additive effect). However, these energetically costly responses apparently were not possible anymore to the same extent in the presence of the DTV stressor, likely because of a higher total energetic cost. This might explain the antagonistic effect between CPF and Bti at a low DTV for both sublethal endpoints. Noteworthy, at high DTV the interaction type became additive again. This may be explained by survival selection associated with the synergistic effect between CPF and Bti at high DTV for total mortality. Indeed the higher mortality in the mixture at high DTV may have removed the weakest individuals with lower energy budgets.

5. Conclusions

Despite the increasing concern of warming magnifying the toxicity of single pesticides (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015), and the increasing numbers of studies on the toxicity of pesticide mixtures (Cedergreen, 2014; Hernández et al., 2017), how temperature influences the interaction between two pesticides in a mixture has been largely ignored. The very limited evidence shows that both a higher mean temperature (Laetz et al., 2014) and a higher daily temperature variation (this study) may increase the toxicity of pesticide mixtures. The finding that DTV can modify the interaction effect between pesticides in a mixture is important for vector control and ecological risk assessment. It indicates that DTV may not only change life history traits of vector species (Paaijmans et al., 2010; this study), but also the efficiency of pesticide mixtures used in vector control. Our data suggest that laboratory-based individual toxicity of chlorpyrifos or Bti obtained at constant temperatures may not be affected by the DTV that is present under natural conditions, yet their mixture toxicity may become higher under DTV. Applying realistic levels of DTV may therefore be important when designing laboratory tests for developing pest management strategies based on pesticide mixtures. Future studies are needed to test the generality of our findings across a broader range of concentrations and for other mixtures, and especially for vector control multigenerational tests will be very valuable. Furthermore, mechanistic studies unraveling the mechanisms causing the interaction effects in the presence of DTV will further enable making generalizations. The higher toxicity of the mixture at high DTV compared to the typically used constant test temperatures in the laboratory urges caution when evaluating both the vector control efficiency and the environmental impact of pesticide mixtures as in nature daily temperature variation is omnipresent.

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Table 1. Results of the generalized linear mixed model (total mortality) and the general linear mixed models (female development time and female wing length) testing for the effects of exposure to chlorpyrifos (CPF) and to *Bacillus thuringiensis* var. *israelensis* (Bti) under different levels of daily temperature variation (DTV) in *Culex pipiens*. *P*-values indicated in bold are significant ($P \leq 0.05$).

	Total mortality			Female development time			Female wing length		
	χ^2	df	*P*	χ^2	df	*P*	χ^2	df	*P*
DTV	18.32	2	**<0.001**	57.36	2	**<0.001**	1.11	2	0.58
CPF	1449.32	1	**<0.001**	174.18	1	**<0.001**	79.86	1	**<0.001**
Bti	104.93	1	**<0.001**	42.84	1	**<0.001**	2.30	1	0.13
DTV×CPF	23.62	2	**<0.001**	5.05	2	0.080	13.40	2	**0.001**
DTV×Bti	9.67	2	**0.008**	1.90	2	0.39	6.41	2	**0.041**
CPF×Bti	0.51	1	0.47	1.10	1	0.30	0.02	1	0.90
DTV×CPF×Bti	10.43	2	**0.005**	6.53	2	**0.038**	5.99	2	**0.050**

Table 2. Results of the independent action models identifying the interaction type per DTV (daily temperature variation) treatment for (A) the total mortality, (B) the female development time, and (C) the female wing length. If the predicted value is not within the range of the 95% confidence interval of the observed value, there is a synergism or an antagonism (indicated in bold).

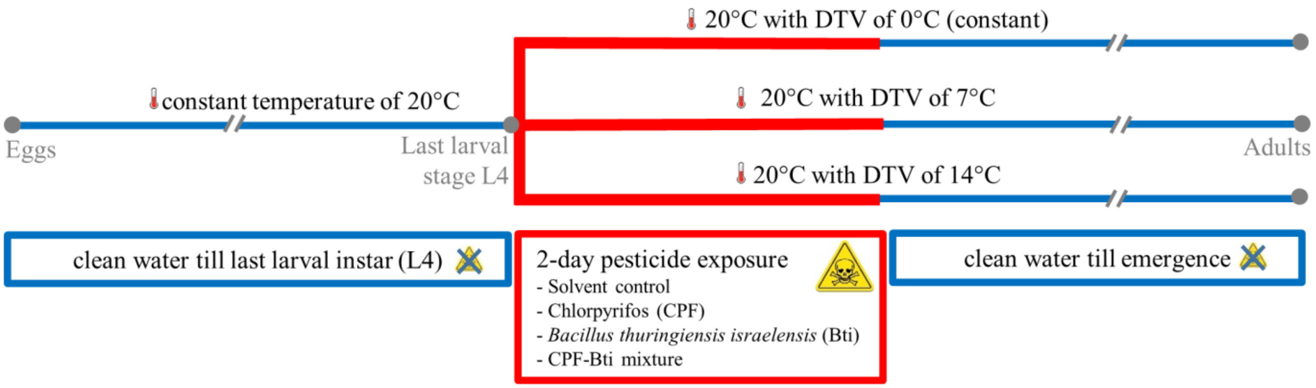
	Predicted	Observed	95%CI Observed	Interaction effect
(A) Total Mortality				
DTV of 0°C	84.49	83.33	[76.91, 89.76]	Additive
DTV of 7°C	86.09	85.96	[78.63, 93.30]	Additive
DTV of 14°C	86.79	94.19	[92.31, 96.07]	**Synergistic**
(B) Development Time				
DTV of 0°C	28.62	29.17	[28.38, 29.97]	Additive
DTV of 7°C	28.07	30.15	[29.09, 31.22]	**Antagonistic**
DTV of 14°C	26.90	27.00	[26.14, 27.86]	Additive
(C) Wing Length				
DTV of 0°C	3.64	3.70	[3.63, 3.76]	Additive
DTV of 7°C	3.75	3.66	[3.57, 3.74]	**Antagonistic**
DTV of 14°C	3.75	3.80	[3.71, 3.90]	Additive

Figure legends

Figure 1. Schematic overview of the experimental design.

Figure 2. Effects of chlorpyrifos (CPF), *Bacillus thuringiensis* var. *israelensis* (Bti) and the CPF-Bti mixture under different levels of daily temperature variation (DTV) in *Culex pipiens*: (A) total mortality, (B) female development time, and (C) female wing length. Means are given with their 95% confidence interval. Numbers inside the bars indicate the number of vials (total mortality) or number of adults (development time and wing length). Besides the observed values for the CPF-Bti mixture also the predicted values of the mixture based on the independent action model are shown. An asterisk (*) above the observed mixture values for a given DTV indicates a significant difference ($P < 0.05$) with the predicted mixture values based on the independent action model, hence a deviation from additivity. Interaction types for the CPF-Bti mixture per DTV level are indicated as additive, synergistic or antagonistic.

650 Figure 1.



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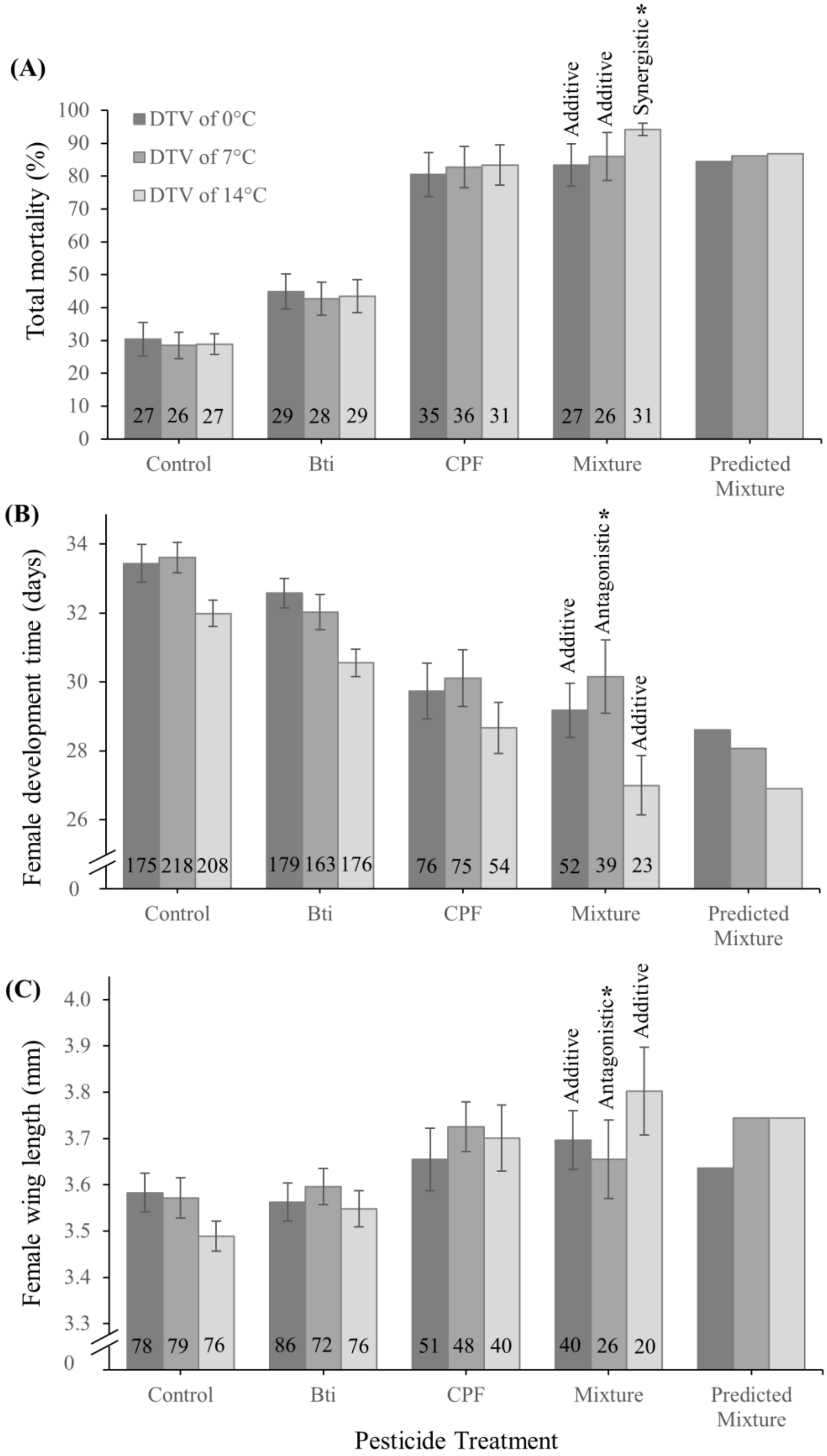
652 Figure 2.



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