Environmental Pollution 292 (2022) 118333

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Multigenerational effects modify the tolerance of mosquito larvae to chlorpyrifos but not to a heat spike and do not change their synergism☆

Shandong Meng *, Vienna Delnat, Robby Stoks

Laboratory of Evolutionary Stress Ecology and Ecotoxicology, University of Leuven, Belgium

ARTICLE INFO

Keywords:
Global change
Multigenerational effects
Multiple stressors
Synergistic interaction
Temperature extremes

ABSTRACT

While interactions with global warming and multigenerational effects are considered crucial to improve risk assessment of pesticides, these have rarely been studied in an integrated way. While heat extremes can magnify pesticide toxicity, no studies tested how their combined effects may transmit to the next generation. We exposed mosquito larvae in a full factorial, two-generation experiment to a heat spike followed by chlorpyrifos exposure. As expected, the heat spike magnified the chlorpyrifos-induced lethal and sublethal effects within both generations. Only when preceded by the heat spike, chlorpyrifos increased mortality and reduced the population growth rate. Moreover, chlorpyrifos-induced reductions in heat tolerance (CTmax), acetylcholinesterase (AChE) activity and development time were further magnified by the heat spike. Notably, when parents were exposed to chlorpyrifos, the chlorpyrifos-induced lethal and sublethal effects in the offspring were smaller, indicating increased tolerance to chlorpyrifos. In contrast, there was no such multigenerational effect for the heat spike. Despite the adaptive multigenerational effect to the pesticide, the synergism with the heat spike was still present in the offspring generation. Generally, our results provide important evidence that short exposure to pulse-like global change stressors can strongly affect organisms within and across generations, and highlight the importance of considering multigenerational effects in risk assessment.

1. Introduction

Pesticides are threatening non-target organisms in freshwater ecosystems, and their current ecological risk assessment is failing, partly because it does not include other stressors that have intensified in recent years (Beketov et al., 2013; Topping et al., 2020; Schulz et al., 2021). One such major stressor is global warming, whereby particularly simultaneous exposure to higher mean temperatures has been well documented to modulate, and in most cases increase, the toxicity of many pesticides (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). Vice versa, exposure to pesticides often has been shown to reduce the heat tolerance (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013), which is another key reason why the effects of pesticides on populations of aquatic organisms may be underestimated in a warming world.

While the focus has been mainly on simultaneous exposure to a pesticide and warming, evidence is accumulating that also sequential exposure may cause synergisms (e.g. Meng et al., 2020a; overview in Gunderson et al., 2016). Sequential exposure is especially relevant when studying pulse-like stressors such as pesticide pulses and heat spikes. In general, when stressors are applied in close succession, synergistic interactions can be expected because the delayed effects of the first stressor may weaken the ability of organisms to deal with the second stressor (Gunderson et al., 2016). In general, the impact of heat extremes in ecotoxicology has been much less studied compared to the impact of increases in mean temperatures. Nevertheless, heat spikes are increasing in frequency, duration and intensity, and this is expected to continue under global warming (IPCC, 2014; Stillman, 2019). Moreover, the impact of heat extremes may be much stronger on populations compared to the impact of increased mean temperatures (Vasseur et al., 2014; Ma et al., 2015).

There is increasing evidence that multigenerational effects, whereby exposure to stressors of the parents affects the overall fitness of the offspring and/or their ability to deal with stressors over several generations (Bell and Hellmann, 2019; Bonduriansky et al., 2012; Salinas et al., 2013), may be important in ecotoxicology (Shaw et al., 2017; Head, 2019) and global warming research (Salinas and Munch, 2012; Donelson et al., 2018). Multigenerational effects can cause a negative

☆ This paper has been recommended for acceptance by Da Chen.

* Corresponding author. Deberiotstraat 32, 3000, Leuven, Belgium.
E-mail address: shandong.meng@kuleuven.be (S. Meng).

https://doi.org/10.1016/j.envpol.2021.118333
Received 23 May 2021; Received in revised form 3 October 2021; Accepted 7 October 2021
Available online 9 October 2021

impact of stressors experienced in the parental generation in offspring generations that were never exposed to these stressors (Guillaume et al., 2016; Blanc et al., 2021). Moreover, multigenerational effects of a parental stressor may reduce the tolerance of the offspring to the same stressor. Such maladaptive multigenerational effects have been shown both for warming (e.g. Tran et al., 2018) and for pesticide exposure (e.g. Sánchez et al., 2000), and can be expected when the parents do not adapt to a stressor (Uller, 2008). When ignored, maladaptive multigenerational effects may underestimate the total impact of stressors on populations. Yet, multigenerational effects may also be adaptive and overall increase the fitness in the offspring generation (Kopp and Matuszewski, 2014; Oppold et al., 2015) and/or the ability of the offspring to deal with the same stressor (for warming: e.g. Shama et al., 2014; for pesticides: e.g. Oppold et al., 2015). Despite the frequent interplay between the effects of pesticide pulses and warming, combined multigenerational effects of these stressors have, however, been rarely studied (but see e.g. Tran et al., 2018) and never for heat spikes.

In this study, we conducted a two-generation experiment to test the within- and multigenerational single and combined effects of a heat spike and exposure to a pesticide on a mosquito species. Specifically, we investigated the single and combined effects of exposure to a heat spike followed by exposure to the pesticide chlorpyrifos in both the parental and the offspring generations with a full factorial design. We thereby tested whether and how the legacy from exposure to a stressor in the parents affected the fitness of the offspring in general and their tolerance to these stressors, with special attention for the interactive effect between stressors. The study was conducted with the mosquito *Culex pipiens* form *molestus* (Forskal, 1775). This species is commonly distributed in Europe and North America (Fonseca et al., 2004), and its aquatic larvae inhibit shallow ponds where heat extremes frequently occur (Jacobs et al., 2008). The aquatic larvae of mosquitoes can reach a high biomass, thus can serve as important food sources in freshwater ecosystems (Becker et al., 2010). The organophosphate chlorpyrifos lists in the top ten hazardous substances that cause high risk for aquatic ecosystems (Johnson et al., 2017), and although it has been banned in some countries recently, it is still used in large amounts worldwide (Rahman et al., 2021). Chlorpyrifos has been shown to be more toxic under higher mean temperatures (e.g. Lydy et al., 1999; Tran et al., 2018) and after a heat spike (Meng et al., 2020a), and to reduce the heat tolerance (Meng et al., 2021). We therefore expected that within a generation the heat spike would increase the toxicity of chlorpyrifos, including a stronger reduction in heat tolerance. Depending on multigenerational effects of warming and pesticide exposure being (mal)adaptive we expected these to reduce or increase the synergism in the offspring generation.

2. Materials and methods

2.1. Experimental setup

We set up a full factorial experiment in both the parental and the offspring generations to test how the legacies of a heat spike and/or chlorpyrifos exposure in the parental generation modulate the single and combined effects of a heat spike and chlorpyrifos exposure in the offspring generation (Fig. 1). In the parental generation, there were four treatment combinations with two heat spike levels (absence vs presence) crossed with two chlorpyrifos concentrations (absence vs presence). Each of these four combinations was then split into the same four treatment combinations in the next generation, thus resulting in 16 treatment combinations in the offspring generation. Both the heat spike and the chlorpyrifos exposure were applied in L4 (= final instar) larvae, the most robust larval stage, thereby following the guidelines by WHO (2005). We first exposed the larvae to the heat spike and subsequently to chlorpyrifos as this has the strongest impact on the study species (Meng et al., 2020a). Larvae were exposed in sets of 30 per jar. There were 24 replicate jars for each of the four treatment combinations in the parental generation (in total 96 jars; 2880 larvae), and 21–25 replicate jars for each of the 16 treatment combinations in the offspring generation (in total 394 jars, 11 820 larvae). For the heat spike treatments, 20 °C was chosen as the temperature control as this matches the mean summer water temperature of the shallow water bodies in Germany where the mosquito culture originated from, and 30 °C was set for the heat spike treatment since this temperature can exist for several days in the original habitat (Tran et al., 2016).

We started the parental generation by collecting 24 sets of six egg clutches (in total 144 egg clutches) from a lab culture. The newly (<24 h) hatched L1 (= first instar) larvae from each set of six egg clutches were split into three white 2 L containers containing 1 L dechlorinated tap water at a density of 120 larvae per container. This resulted in 24 sets of three white 2 L containers. Containers were placed in a temperature-controlled room at 20 °C. When larvae entered L4, we pooled the newly

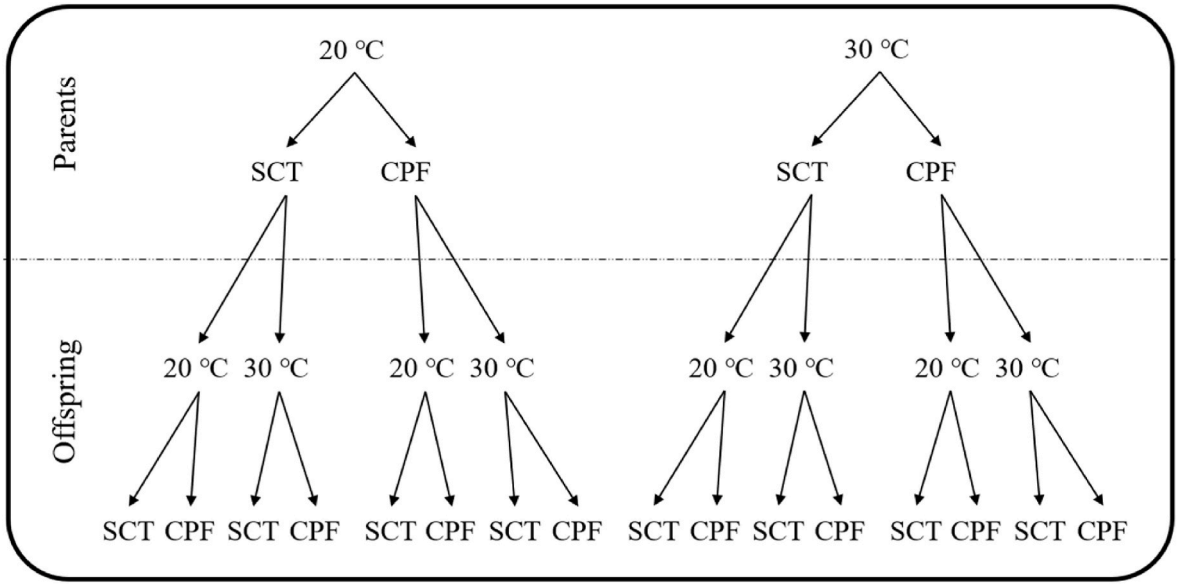

Fig. 1. Scheme of the bifactorial transgenerational experimental design to test how legacies of the heat spike and/or the exposure to chlorpyrifos in parents affect the sensitivity of offspring when dealing with the heat spike and chlorpyrifos. In both generations, L4 larvae were first exposed to 20 °C (temperature control) or 30 °C (heat spike) for 48 h, then to a solvent control (SCT) or chlorpyrifos solution (CPF) for another 48 h.

(<24 h) moulted L4 larvae from each set of three white 2 L containers, and then randomly distributed them into four glass jars (210 mL) that were placed in water baths. Each jar was filled with 125 mL dechlorinated tap water and contained 30 larvae. So, for each of the 24 sets, we started four jars with 30 L4 larvae that per set experienced the same heat spike treatment. Half (12) of these sets were randomly selected and kept constantly at 20 °C, the other half experienced a heat spike. To impose the heat spike, the jars were first heated from 20 to 30 °C in 5 h, and kept at 30 °C for 38 h, then cooled from 30 to 20 °C in 5 h. The total duration of the heat spike was 48 h with a realistic ramping rate of 2 °C per hour (Cambronero et al., 2018). The chlorpyrifos treatment started immediately after the heat spike ended, when all larvae were at 20 °C. Two of the four jars from each set were randomly exposed to 0.32 μg/L chlorpyrifos, the other two to the solvent control (ethanol). This chlorpyrifos concentration was based on a pre-trial (see Appendix A for details). The duration of chlorpyrifos exposure was 48 h with the medium refreshed after 24 h. When the chlorpyrifos exposure finished, the surviving larvae were transferred to new jars with dechlorinated tap water and kept there till metamorphosis. The adults from each couple of two jars from the same set with the same pesticide treatment were moved to one insectary using an aspirator. This way, we installed 48 insectaries (12 for each of the four treatment combinations) in the parental generation.

For the offspring generation we followed the same experimental procedure as for the parental generation. To start the offspring generation, we used two sets of six egg clutches from each insectary (in total 48 sets and 576 egg clutches). Of the two sets obtained from a single insectary, one set was exposed as four groups of 30 larvae per jar to 20 °C and the other set as four groups of 30 larvae per jar to 30 °C during the heat spike period at the start of the L4 stage. Hence, per insectary we started 8 jars. The 2-day chlorpyrifos exposure started when the 2-day heat spike treatments ended as in the parental generation.

The used concentration (0.32 μg/L) is ecologically relevant as it falls within the range of chlorpyrifos concentrations measured in European surface waters: 95% CI = [0.07 μg/L, 0.69 μg/L] (Stehle and Schulz, 2015). The actual concentrations in the experimental jars at the start of the exposure were 0.156 ± 0.003 (mean ± 1SE) μg/L and 24 h later (just before the renewal of the medium) 0.053 ± 0.003 μg/L. Concentrations were measured by using UPLC-MS/MS with Triple Quadrupole Mass Spectrometry based on four pooled samples of the medium from 11 jars. The chlorpyrifos solution was prepared from a stock solution of 100 μg/mL by dissolving chlorpyrifos powder (purity grade > 99%, Sigma-Aldrich, Missouri, USA) in absolute ethanol, and stored at 4 °C in dark. The same amount of ethanol (3.2 μL/L) as in the chlorpyrifos treatment was included in the solvent control. During the exposure to the heat spike and chlorpyrifos, we took water samples from the experimental jars at the start and 24 h later before the medium was refreshed to measure the physio-chemical parameters: pH, dissolved oxygen (mg/L), hardness (mg/L $CaCO_3$) and conductivity (μS/cm) (see Appendix B for details). During the entire experiment, larvae were fed daily with a high amount of food mixture (0.313 mg/larva) (Beketov and Liess, 2007) containing Supradyn vitamins (3%), wheat germs (51%), and Olvarit 7 cereal flakes (46%).

2.1.1. Life history traits

During the total exposure period to the heat spike and pesticide, we daily recorded the number of dead larvae in each jar and calculated the mortality across the 4 days. When the 4-d exposure ended, one set of five larvae per jar was collected and weighed to the nearest 0.01 mg using a balance (AV135-S, Mettler Toledo, Columbus, OH, USA), and stored at −80 °C for the physiological measurement. To calculate growth rate, the mean wet mass of the five pooled larvae for physiology was used as end mass (Me). At the start of the 4-d exposure period, extra groups of five pooled larvae from each set of three white 2 L containers were weighed and the mean value was used as start mass (Ms). The growth rate across the 4-d exposure was calculated per jar as [ln(Me)-ln(Ms)]/4 days. Development time was recorded per larva from the molt in L4 till adult metamorphosis, and the mean of all larvae per jar was used for analyses. After metamorphosis, we collected three females per jar and took the mean of their wing lengths as estimate of adult female size per jar. The left wing was removed and photographed under a microscope (Olympus B × 51) at a magnification 20 × using a digital camera (Basler, AG, Ahrensburg, Germany) which was connected to a computer via Streampix software (Norpix, Inc., Montreal, Canada). Pictures were analyzed with ImageJ (Rasband, 1997–2014) to determine the wing length. As an estimate of population growth rate, the composite index of population performance (r') was calculated based on Livdahl and Sugihara (1984) (see Appendix C for details).

2.1.2. Heat tolerance

The heat tolerance was estimated as CTmax, the critical thermal maximum. At the end of the 4-d exposure period, we obtained one mean CTmax estimate per jar based on measuring three randomly selected larvae following the protocol of Meng et al. (2020b). Specifically, larvae were individually placed in 50 mL cups filled with dechlorinated tap water and placed in a water bath. The water bath was heated at a rate of 0.3 °C per minute by a heater (TC120 optima immersion thermostat, Cambridgeshire, UK). This ramping rate is commonly used for measuring CTmax in aquatic invertebrates (Cambronero et al., 2018; Verberk and Bilton, 2013). The temperature when the larva started floating motionlessly at the water surface was recorded as the CTmax. Thereafter, larvae were allowed to recover at 20 °C for 20 min, the recovered larvae were weighed to the nearest 0.01 mg for mass correction of CTmax. The larvae that died during the CTmax measurement (5 out of 1500, 1.12%) were excluded from the analyses.

2.1.3. Physiology

We measured the activity of acetylcholinesterase (AChE), the target enzyme of chlorpyrifos (Domingues et al., 2010), following the protocol in Appendix B of Delnat et al. (2019). The measurements were conducted based on the pooled sets of five larvae per jar, and one mean value per jar was obtained from three technical replicates (see Appendix D for detailed protocols).

2.2. Data analyses

The main and interactive effects of the heat spike and chlorpyrifos exposure on the response variables (one value per jar) were analyzed separately using general linear mixed models. To take into account the non-independence of the four jars per set of larvae, we added “set” as a random factor to the models when analyzing response variables in the parental generation. Similarly, to take into account that we started eight jars per insectary, we added “insectary” as random factor in the offspring generation. Mortality was expressed as percentage per jar and was arcsine square root transformed to meet the assumption of normality. The body mass was added as covariate when analyzing CTmax, and sex was added when analyzing the development time. In the offspring generation, we included both the heat spike treatment of the parents (Heat_P) and the offspring (Heat_O), and chlorpyrifos exposure treatments of the parents (CPF_P) and the offspring (CPF_O), and their interactions. We used the independent action (IA) model to formally assess the interaction type for mortality, as this model takes into account the situation that an individual killed by one stressor cannot be killed again by the second stressor (Bliss, 1939; Schäfer and Piggott, 2018) (see Appendix E for details). An interaction is synergistic when the observed 95% confidence interval of mortality caused by the combined effects of the heat spike and chlorpyrifos is significantly higher than the predicted mean of mortality based on the sum of the single effects.

All analyses were conducted in R v4.0.2 (R Core Team, 2020). We used the packages “lme4” (v1.1-21, Bates et al., 2015) to fit the models, “afex” (v0.25–1, Singmann et al., 2017) to set effect coding, “car” (v3.0-3, Fox and Weisberg, 2018) to calculate the wald chi-square statistics and the p-values, and “emmeans” (v2.30–0, Lenth et al., 2019) to

get contrasts (false discovery rate corrected for the associated *p*-values). Contrast analyses were done whenever there was a significant interaction between the heat spike and chlorpyrifos exposure to test whether the effect of chlorpyrifos was significant in each of the heat spike treatments separately. Therefore, all contrasts were made against the solvent control.

3. Results

3.1. Mortality

In the parental generation, chlorpyrifos caused mortality, especially in larvae pre-exposed to the heat spike (10.8% vs 0.6%) (Table 1, Fig. 2A). This synergism was confirmed by the IA model (Appendix E). The heat spike itself (in the solvent control) did not cause mortality (contrast: $P = 0.717$). In the offspring generation, both the heat spike (1.0%) and chlorpyrifos exposure (1.2%) in isolation slightly increased mortality (Table 2, Fig. 3A). Moreover, the chlorpyrifos-induced mortality was much higher after the heat spike (25.0%). This synergism was confirmed by the IA model (Appendix E). Furthermore, when parents were exposed to chlorpyrifos, the mortality induced by chlorpyrifos in their offspring was less (11.6% vs 15.9%).

3.1.1. CTmax

In the parental generation, chlorpyrifos reduced CTmax, especially in larvae pre-exposed to the heat spike (−3.4% vs −1.5%) (Table 1, Fig. 2B). The heat spike itself did not affect CTmax. In the offspring generation, chlorpyrifos also decreased CTmax, especially after the heat spike (−5.7% vs −1.4%) (Table 2, Fig. 3B). In addition, when parents were exposed to chlorpyrifos, the chlorpyrifos-induced reduction in CTmax in their offspring was less (−2.5% vs −4.2%).

3.1.2. Growth rate

In the parental generation, chlorpyrifos generally increased the larval growth rate (+10.3%), while the heat spike itself did not (Table 1, Fig. 2C). In the offspring generation, chlorpyrifos also increased the growth rate but only in offspring pre-exposed to the heat spike and whose parents were not exposed to chlorpyrifos (+16.1%) (Table 2, Fig. 3C).

3.1.3. AChE activity

In the parental generation, chlorpyrifos decreased the AChE activity, especially in larvae pre-exposed to the heat spike (−57.9% vs −34.6%) (Table 1, Fig. 2D). The heat spike itself (in the solvent control) did not affect the AChE activity (contrast: $P = 0.413$). In the offspring generation, chlorpyrifos also reduced the AChE activity, and again especially after the heat spike (−56.6% vs −37.1%) (Table 2, Fig. 3D). The chlorpyrifos-induced reduction in AChE activity was smaller when parents were exposed to chlorpyrifos than they were not (−42.6% vs −50.8%). In addition, the heat spike decreased the AChE activity but only when the parents had been exposed to chlorpyrifos.

3.1.4. Wing length

In the parental generation, chlorpyrifos reduced female wing length but only in larvae pre-exposed to the heat spike (Table 1, Fig. 2E). The heat spike itself did not affect wing length. In the offspring generation, neither the heat spike nor chlorpyrifos affected female wing length. Yet, parental exposure to chlorpyrifos generally reduced female wing length in the offspring; this was most pronounced when the offspring were exposed to both stressors (Table 2, Fig. 3E).

3.1.5. Development time

In the parental generation, chlorpyrifos decreased the development time, especially in larvae pre-exposed to the heat spike (−11.5% vs −2.9%) (Table 1, Fig. 2F). The heat spike itself also reduced the development time. Similarly, in the offspring generation, both the heat spike (−8.8%) and chlorpyrifos decreased the development time, and the chlorpyrifos-induced reduction in development time was again more pronounced after the heat spike (−14.0% vs −1.2%) (Table 2, Fig. 3F). When the parents were exposed to either chlorpyrifos or the heat spike, the development time was less reduced by chlorpyrifos in the offspring. In addition, the development time was slightly less reduced by the heat spike when the parents were exposed to both the heat spike and chlorpyrifos.

3.1.6. The composite index of population performance (r')

In the parental generation, chlorpyrifos reduced the composite index of population performance (*r'*) but only after the heat spike (−5.8%, Table 1, Fig. 2G). The heat spike itself did not affect *r'*. Also in the offspring generation, chlorpyrifos only reduced the composite index of population performance (*r'*) after the heat spike (−7.8%). Furthermore, when the parents were exposed to chlorpyrifos, chlorpyrifos no longer reduced *r'*. The heat spike itself decreased *r'* when parents were not exposed to the heat spike.

4. Discussion

Our results provide widespread evidence that the preceding heat spike magnified the lethal and sublethal effects of chlorpyrifos within a generation. Indeed, chlorpyrifos caused higher mortality, and the chlorpyrifos-induced reductions in heat tolerance (CTmax), AChE activity and development time were magnified in larvae pre-exposed to the heat spike. In addition, wing length and the estimate of population growth rate *r'* were only reduced by chlorpyrifos when combined with the heat spike. A key finding was that when the parents were already exposed to chlorpyrifos, the chlorpyrifos-induced lethal and sublethal effects were less pronounced in the offspring, indicating adaptive multigenerational effects in response to chlorpyrifos exposure. This

Table 1
The main and interactive effects of the heat spike (Heat) and chlorpyrifos (CPF) exposure on life history, heat tolerance and physiological variables in the parental generation. Bold *P*-values represent significant effects ($P < 0.05$).

	Mortality			CTmax			Growth rate			AChE		
	χ^2	Df	*P*	χ^2	Df	*P*	χ^2	Df	*P*	χ^2	Df	*P*
Heat	26.47	1	**< 0.001**	0.33	1	0.566	0.50	1	0.478	7.78	1	**0.005**
CPF	152.66	1	**< 0.001**	32.59	1	**< 0.001**	5.54	1	**0.019**	92.75	1	**< 0.001**
Heat × CPF	63.16	1	**< 0.001**	5.91	1	**0.015**	0.03	1	0.874	3.90	1	**0.048**
Mass				0.56	1	0.454						

	Wing length			Development time			*r'*		
	χ^2	Df	*P*	χ^2	Df	*P*	χ^2	Df	*P*
Heat	0.20	1	0.656	11.41	1	**< 0.001**	0.01	1	0.912
CPF	0.65	1	0.420	24.35	1	**< 0.001**	5.68	1	**0.017**
Sex				630.72	1	**< 0.001**			
Heat × CPF	4.12	1	**0.042**	4.31	1	**0.038**	10.91	1	**< 0.001**

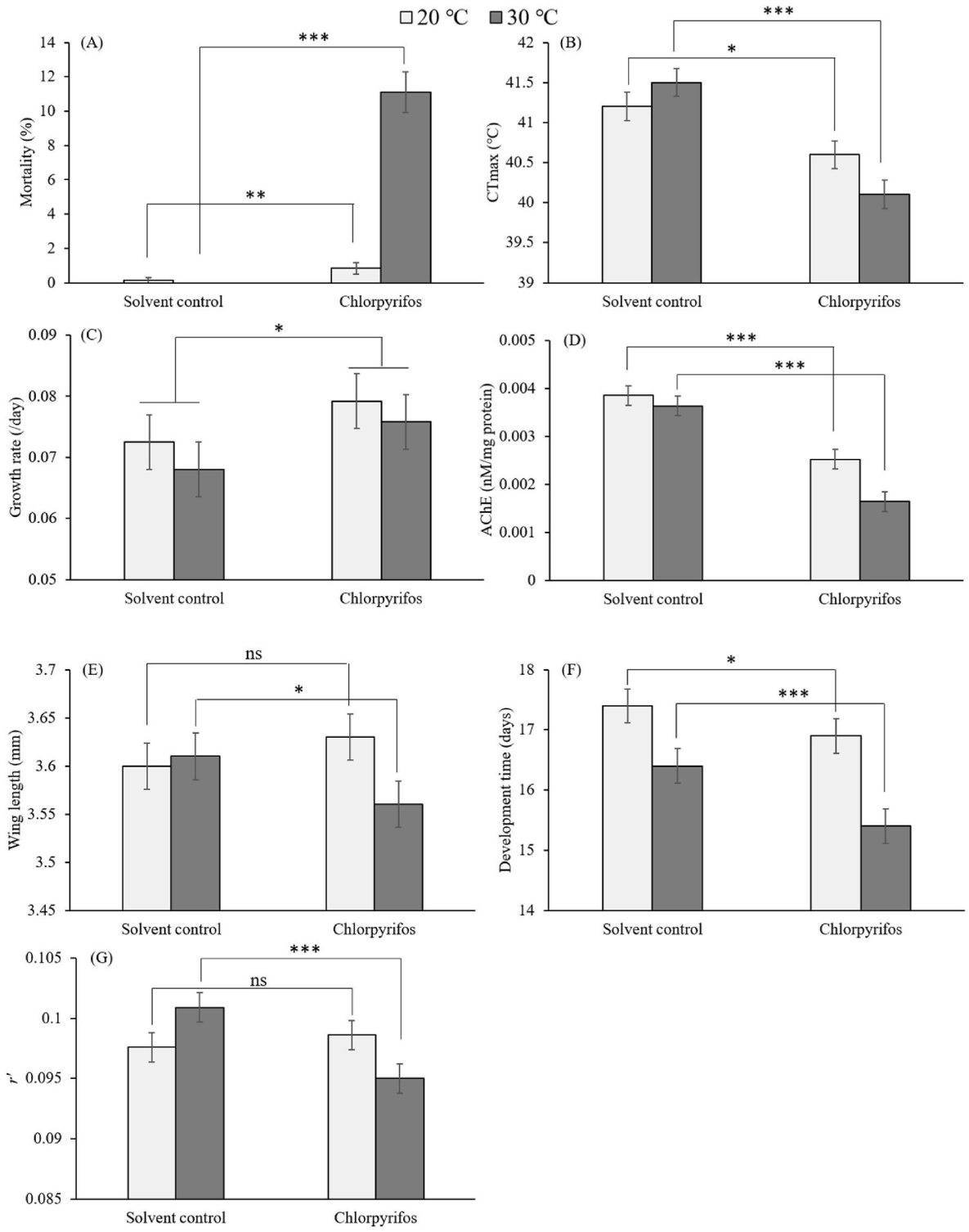

Fig. 2. The single and combined effects of the heat spike and chlorpyrifos exposure on life history, heat tolerance and physiological variables in the parental generation of the mosquito *Culex pipiens*. (A) mortality (mortality is zero in the solvent control at 30 °C), (B) heat tolerance (CTmax), (C) growth rate, (D) acetylcholinesterase activity (AChE), (E) wing length, (F) development time and (G) the composite index of population performance (*r'*). Means are given with one standard error. *P*-values associated with contrast analyses are coded as follows: 'ns': $P > 0.05$; '*': $P < 0.05$; '**': $P < 0.01$; '***': $P < 0.001$.

adaptive multigenerational effect was not found for parental heat spike exposure. Despite the adaptive multigenerational effect to the pesticide, the synergism with the heat spike was still present in the offspring generation.

While both generations responded overall very similar to the heat spike and the pesticide, one obvious difference between generations was that the mortality was overall about twice higher in the offspring than in the parental generation. This may be explained by the higher density that the parents were exposed to as larvae compared to the grandparents. The grandparental generation (as the entire lab culture) was reared during the entire larval stage at a density of ~80 larvae/L. While the parental generation was reared from the L1 stage at a density of 120

Table 2
The main and interactive effects of the heat spike and chlorpyrifos exposure in the parental (Heat_P and Heat_P) and in the offspring (Heat_O and CPF_O) generations on life history, heat tolerance and physiological variables in the offspring generation. Bold *P*-values represent significant effects ($P < 0.05$).

	Mortality			CTmax			Growth rate			AChE		
	χ^2	Df	*P*	χ^2	Df	*P*	χ^2	Df	*P*	χ^2	Df	*P*
Heat_P	13.9	1	0.394	0.78	1	0.376	0.46	1	0.496	0.25	1	0.620
CPF_P	0.11	1	0.344	0.65	1	0.420	0.00	1	0.974	0.50	1	0.481
Heat_O	263.87	1	**< 0.001**	33.41	1	**< 0.001**	0.28	1	0.594	13.37	1	**< 0.001**
CPF_O	342.27	1	**< 0.001**	284.38	1	**< 0.001**	3.86	1	**0.049**	297.80	1	**< 0.001**
Heat_P × CPF_P	1.63	1	0.825	0.51	1	0.477	0.14	1	0.703	0.00	1	0.989
Heat_P × Heat_O	4.35	1	0.258	0.23	1	0.629	0.11	1	0.739	0.20	1	0.652
CPF_P × Heat_O	0.28	1	0.860	0.99	1	0.319	0.46	1	0.498	7.73	1	**0.005**
Heat_P × CPF_O	8.66	1	0.778	1.23	1	0.267	1.14	1	0.285	0.25	1	0.619
CPF_P × CPF_O	3.93	1	**0.016**	7.50	1	**0.006**	4.52	1	**0.034**	4.84	1	**0.028**
Heat_O × CPF_O	0.41	1	**< 0.001**	92.90	1	**< 0.001**	0.06	1	0.799	12.28	1	**< 0.001**
Heat_P × CPF_P × Heat_O	3.04	1	0.416	0.00	1	0.951	1.07	1	0.301	0.03	1	0.859
Heat_P × CPF_P × CPF_O	0.76	1	0.446	0.63	1	0.426	3.09	1	0.078	0.10	1	0.758
Heat_P × Heat_O × CPF_O	4.99	1	0.790	0.20	1	0.659	0.78	1	0.377	0.00	1	0.999
CPF_P × Heat_O × CPF_O	1.29	1	0.775	0.26	1	0.608	3.87	1	**0.049**	0.09	1	0.769
Heat_P × CPF_P × Heat_O × CPF_O	2.25	1	0.398	0.33	1	0.569	0.00	1	0.975	0.38	1	0.536
Mass				0.02	1	0.888						

	Development time			Wing length			*r'*		
	χ^2	Df	*P*	χ^2	Df	*P*	χ^2	Df	*P*
Heat_P	1.01	1	0.316	0.16	1	0.687	0.50	1	0.479
CPF_P	0.08	1	0.780	4.46	1	**0.035**	0.34	1	0.561
Heat_O	458.83	1	**< 0.001**	1.05	1	0.305	3.44	1	0.064
CPF_O	51.24	1	**< 0.001**	2.18	1	0.140	16.33	1	**< 0.001**
Sex	1642.96	1	**< 0.001**						
Heat_P × CPF_P	0.16	1	0.69	0.29	1	0.592	0.10	1	0.754
Heat_P × Heat_O	0.01	1	0.944	0.59	1	0.441	3.98	1	**0.046**
CPF_P × Heat_O	0.36	1	0.551	0.81	1	0.367	0.07	1	0.788
Heat_P × CPF_O	8.87	1	**0.003**	0.86	1	0.353	0.65	1	0.420
CPF_P × CPF_O	6.16	1	**0.013**	0.00	1	0.972	6.32	1	**0.012**
Heat_O × CPF_O	22.47	1	**< 0.001**	0.67	1	0.414	13.09	1	**< 0.001**
Heat_P × CPF_P × Heat_O	5.80	1	**0.016**	0.01	1	0.909	0.45	1	0.504
Heat_P × CPF_P × CPF_O	1.32	1	0.250	0.00	1	0.997	0.42	1	0.517
Heat_P × Heat_O × CPF_O	0.07	1	0.795	0.00	1	0.981	0.31	1	0.580
CPF_P × Heat_O × CPF_O	0.00	1	0.996	3.93	1	**0.047**	0.18	1	0.675
Heat_P × CPF_P × Heat_O × CPF_O	1.74	1	0.187	0.61	1	0.435	0.32	1	0.571
Mass									

larvae/L, and from the L4 stage at a density of 240 larvae/L. So the higher density during the parental generation could have caused the parents to produce weaker offspring even in the absence of any stressor. Note, this would not bias the patterns in the multigenerational effects of the heat spike and chlorpyrifos since larvae of all treatments within the same generation followed the same rearing procedure at the same density. Given the variation in larval densities in natural populations, multigenerational effects caused by larval densities might be an important avenue for future research.

4.1. *Within-generation effects of the heat spike and chlorpyrifos exposure*

In both generations chlorpyrifos caused only minor mortality, unless larvae were first exposed to the heat spike. This synergistic interaction between the heat spike and chlorpyrifos exposure confirms previous results in the study species (Meng et al., 2020a) and is in line with the "climate change induced toxicant sensitivity" (CITS) concept (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). This synergism may be explained by the heat spike that reduced the energy budget of the larvae as we observed before in the study species (Meng et al., 2020a). This reduced energy budget would leave less energy available for chlorpyrifos detoxification, defense and repair. Exposure to a heat spike may reduce the energy budget by impairing ATP synthesis (Harada et al., 2019; Stillman, 2019). Related to this, the stronger chlorpyrifos-induced reduction in AChE activity when combined with the heat spike may also have contributed to the synergism for mortality (Domingues et al., 2010). The stronger pesticide-induced mortality after a heat spike, combined with a stronger reduction in wing length which is a validated proxy of female fecundity in the study species (Costanzo et al., 2014), resulted in the negative effect of the pesticide on the estimate of population growth rate (*r'*) only being present in larvae that first experienced a heat spike.

Chlorpyrifos exposure reduced the heat tolerance estimated as CTmax, which is consistent with the "toxicant induced climate change sensitivity" (TICS) concept (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013). Chlorpyrifos-induced reductions in heat tolerance have been recorded in the study species (e.g. Meng et al., 2020a,b,2021) and other aquatic insects (e.g. damselfly larvae: Verheyen et al., 2019). The observed absolute reduction in CTmax caused by chlorpyrifos (ca. 1.5 °C) is likely biologically relevant. For example, it matches the observed difference in CTmax between latitudes in another semi-aquatic insect that shows latitude-associated thermal adaptation, and low-latitude populations can better deal with heat waves than high-latitude populations (Janssens et al., 2021). CTmax occurs when the oxygen demand can no longer be met by the oxygen supply (Verberk et al., 2016). Chlorpyrifos exposure is expected to shift this mismatch to a lower temperature by both increasing oxygen demand (e.g., Narváez et al., 2016) and by reducing oxygen supply caused by the impaired respiratory system (e.g., Negro and Collins, 2017; Marigoudar et al., 2018). Interestingly, although the previous heat spike did not significantly affect CTmax, it magnified the chlorpyrifos-induced reduction of CTmax. Likely, the preceding heat spike increased the energy, hence oxygen, demand for repair mechanisms thereby further lowering the temperature at which the mismatch between oxygen demand and supply occurred. A similar pattern was also found in the study species by Meng et al. (2020a).

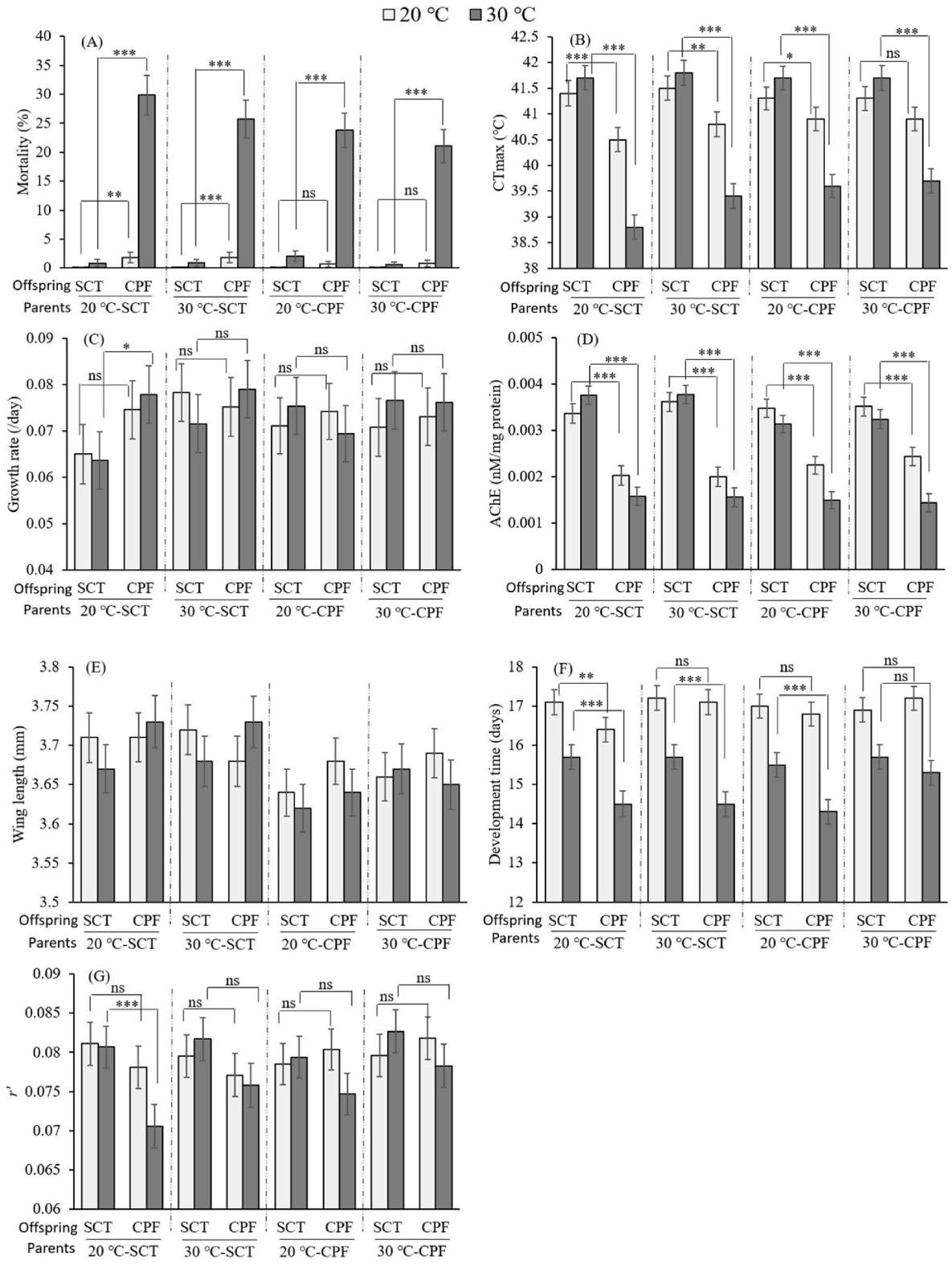

Fig. 3. The single and combined effects of the heat spike and chlorpyrifos exposure in the parental and the offspring generations on life history, heat tolerance and physiological variables in the offspring generation of the mosquito *Culex pipiens*. (A) mortality, (B) heat tolerance (CTmax), (C) growth rate, (D) acetylcholinesterase activity (AChE), (E) wing length, (F) development time and (G) the composite index of population performance (*r'*). Means are given with one standard error. SCT = solvent control, CPF = chlorpyrifos exposure. *P*-values associated with contrast analyses are coded as follows: 'ns': $P > 0.05$; '*': $P < 0.05$; '**': $P < 0.01$; '***': $P < 0.001$.

In contrast with the negative effects on survival and heat tolerance, chlorpyrifos exposure accelerated growth and shortened the development time, and the latter was further magnified by the heat spike. Chlorpyrifos-induced accelerations of growth and development have been recorded several times in the study species (Meng et al., 2020a&b; Delnat et al., 2019) and other aquatic insects (in damselfly larvae: Janssens and Stoks, 2013), and may be an adaptive response for semi-aquatic organisms to escape exposure to toxicants in their aquatic habitat (Rohr et al., 2011). Alternatively, this is also in line with the phenomenon that sublethal pesticides exposure can cause hormetic effects, positively influencing life history traits (Margus et al., 2019; Brevik et al., 2018). The absolute reduction in development time (ca. 1 d) can have a strong impact on fitness as it allows an earlier metamorphosis into the terrestrial adult stage. It thereby may make the difference between being able or not to avoid being exposed to another pesticide pulse in their pond, and to escape death by drying out of the temporary ponds the species often inhabits.

4.1.1. *Multigenerational effects of the heat spike and chlorpyrifos exposure*

Overall, parental exposure to either the heat spike or chlorpyrifos did not cause lethal and sublethal effects on themselves (in the absence of the other stressor) in the offspring generation. Similar absences of a main multigenerational effect on offspring were, for example, also described in zebrafish when parents were exposed to coumarin 47 (Blanc et al., 2020), and in the ladybird beetle *Coccinella septempunctata* when parents were exposed to tolfenpyrad (Chi et al., 2021). In contrast, other studies showed negative (e.g. Tran et al., 2018) or positive (e.g. Lim et al., 2021) effects of stressors in the parental generation on offspring survival. The timing and exposure duration to the stressors experienced by the parents are critical for the expression of multigenerational effects (Donelson et al., 2018). Particular critical periods are early development, like embryogenesis, and prior to and during reproduction (Feil and Fraga, 2012; Burton and Metcalfe, 2014). Therefore, our result may be explained by the fact that we applied a short-term exposure (2 d vs. the entire life cycle) in the relatively less sensitive final larval stage.

A key finding was that the lethal and sublethal effects of chlorpyrifos were reduced in the offspring generation when parents also had been exposed to chlorpyrifos. This indicates that exposure of parents to chlorpyrifos caused adaptive responses increasing the tolerance of the offspring when dealing with the same stressor. This effect was likely not driven by survival selection, since the mortality caused by chlorpyrifos in the parents can be neglected (less than 1 out of 30 larvae per jar). The observation that the activity of AChE was also reduced less by chlorpyrifos in the offspring whose parents had also been exposed to the pesticide, may partly explain the observed multigenerational pattern in mortality. In addition, parental exposure to chlorpyrifos reduced the accelerated growth and development, and the decrease in population growth rate caused by chlorpyrifos in the offspring, also suggesting that parental exposure to chlorpyrifos reduced the sensitivity of offspring to chlorpyrifos.

Remarkably, parental exposure to chlorpyrifos also reduced the chlorpyrifos-induced negative effects on CTmax. This adds a new multigenerational dimension to the TICS concept (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013). CTmax has been shown to be a good proxy of the ability to tolerate increases in mean temperatures (Åsheim et al., 2020) as well as extreme heat (Kaspari et al., 2015; Jørgensen et al., 2019). This adaptive multigenerational effects of chlorpyrifos on CTmax may help the larvae to deal better with thermal stress in a warming, polluted world. As discussed above, parental exposure to chlorpyrifos increased the tolerance to chlorpyrifos of the offspring, thus may have reduced the chlorpyrifos-induced shift toward lower temperatures of the mismatch between oxygen demand and supply (e.g. less energy needed for detoxification, and less affected energy supply), contributing to a smaller chlorpyrifos-induced reduction in CTmax.

Adaptive multigenerational effects have been reported before, and can be explained by in utero exposure to stressors of the developing embryo (Heard and Martienssen, 2014; Lim and Brunet, 2013). Remarkably, different from previous studies that applied stressors in the most sensitive ontogenetic stages (e.g. Lim et al., 2021; reviewed by Donelson et al., 2018; for toxicants: e.g. Reátegui-Zirena et al., 2017; Oppold et al., 2015) or across the entire life cycle (e.g. Shama et al., 2014; for toxicants: e.g. Marshall, 2008), we here provide evidence that even a short-term exposure to a pesticide in the relatively less sensitive final larval stage can generate multigenerational effects and partly offset the negative effects of a pesticide in the offspring. This may be important at the population level as we could demonstrate that our estimate of population growth rate was not affected by the pesticide in the offspring when also the parents had been exposed.

In contrast, parental exposure to the heat spike did not change the sensitivity to the heat spike in the offspring. This may be partly explained by the fact the heat spike itself had less effect on the mosquito larvae within a generation. However, this does not exclude the possibility of a multigenerational effect after exposure to a heat spike for more than two generations, an exposure scenario that is, however, much less likely for heat spikes. Another important finding was that the synergistic interaction between the heat spike and the pesticide in the offspring generation was not affected by the parental exposure history to these stressors. This occurred despite the adaptive multigenerational increased tolerance to the pesticide in offspring whose parents also had been exposed to the pesticide. One reason for this may be that the overall mortality level, hence likely also stress level, was higher in the offspring generation. In addition, given that generating the adaptive multigenerational effects for chlorpyrifos can be energetically costly, this may leave less energy available for tolerating the heat spike which has a different mode of action from chlorpyrifos. This phenomenon is suggested by the observation that the heat spike-induced mortality in offspring whose parents were exposed to chlorpyrifos was somewhat higher (2.1% vs. 0.8%).

5. Conclusions

While multigenerational effects of stressors are receiving increased attention in ecotoxicology (Shaw et al., 2017; Brevik et al., 2018), multigenerational studies are largely limited to a single stressor (but see Tran et al., 2018), and rarely involve transient exposure to a stressor. While heat extremes and pesticide exposure are two key threats to aquatic ecosystems (Heino et al., 2009; Schulz et al., 2021), and heat extremes can strongly alter pesticide toxicity (Meng et al., 2020a&b), no study so far tested how their combined effects may transmit to the next generation and alter the sensitivity of offspring to these stressors. We detected adaptive multigenerational effects of parental exposure to chlorpyrifos in terms of increased tolerance to chlorpyrifos in the offspring. This finding has implications for current risk assessment as it suggests that the effects of a toxicant can be underestimated in field-collected animals in case their parents already experienced the toxicant. This can be expected in animals with a short generation time, such as mosquitoes, that live in edge-to-field water bodies where several pulses of pesticides are applied during the crop growing season (Van Drooge et al., 2001). Furthermore, our results also add a new multigenerational dimension to the TICS concept (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013): parental exposure to a pesticide reduced the negative impact of the pesticide on heat tolerance in the offspring. The heat spike magnified the chlorpyrifos-induced lethal and sublethal effects within both generations (CITS concept, Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). This synergism, however, did not change in the offspring generation despite the adaptive multigenerational effect to the pesticide. Despite the importance of temporal aspects in multi-stressor studies (Orr et al., 2020), multigenerational effects on interaction patterns are rare (but see Tran et al., 2018), yet much needed to understand and predict how interaction

patterns may change across generations. Generally, our results provide important evidence that short exposure to pulse-like global change stressors can strongly affect organisms within and across generations, and highlight the importance of considering multigenerational effects in risk assessment.

Credit author statement

Shandong Meng: Conceptualization, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Vienna Delnat:** Methodology, Formal analysis, Writing – review & editing. **Robby Stoks:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Geert Neyens and Ria Van Houdt for assisting during the experiment. S.M. is funded by the China Scholarship Council (Grant 201706300124) and V.D. by a postdoctoral mandate (PDM) of the KU Leuven. This study was financially supported by FWO (Research Grant G.0524.17) and KU Leuven (Research Grant C16/17/002).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.118333.

References

Åsheim, E.R., Andreassen, A.H., Morgan, R., Jutfelt, F., 2020. Rapid-warming tolerance correlates with tolerance to slow warming but not growth at non-optimal temperatures in zebrafish. J. Exp. Biol. 223 (23).
Bates, D., Machler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using Lme4. J. Stat. Software 67, 1–48.
Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C., Kaiser, A., 2010. Mosquitoes and Their Control. Springer Science & Business Media.
Beketov, M.A., Liess, M., 2007. Predation risk perception and food scarcity induce alterations of life-cycle traits of the mosquito *Culex pipiens*. Ecol. Entomol. 32 (4), 405–410.
Beketov, M.A., Kefford, B.J., Schäfer, R.B., Liess, M., 2013. Pesticides reduce regional biodiversity of stream invertebrates. Proc. Natl. Acad. Sci. Unit. States Am. 110 (27), 11039–11043.
Bell, A.M., Hellmann, J.K., 2019. An integrative framework for understanding the mechanisms and multigenerational consequences of transgenerational plasticity. Annu. Rev. Ecol. Evol. Syst. 50, 97–118.
Blanc, M., Cormier, B., Hyötyläinen, T., Krauss, M., Scherbak, N., Cousin, X., Keiter, S.H., 2020. Multi-and transgenerational effects following early-life exposure of zebrafish to permethrin and coumarin 47: impact on growth, fertility, behavior and lipid metabolism. Ecotoxicol. Environ. Saf. 205, 111348.
Blanc, M., Antczak, P., Cousin, X., Grunau, C., Scherbak, N., Rüegg, J., Keiter, S.H., 2021. The insecticide permethrin induces transgenerational behavioral changes linked to transcriptomic and epigenetic alterations in zebrafish (*Danio rerio*). Sci. Total Environ. 779, 146404.
Bliss, C.I., 1939. The toxicity of poisons applied jointly 1. Ann. Appl. Biol. 26 (3), 585–615.
Bonduriansky, R., Crean, A.J., Day, T., 2012. The implications of nongenetic inheritance for evolution in changing environments. Evol. Appl. 5 (2), 192–201.
Brevik, K., Lindström, L., McKay, S.D., Chen, Y.H., 2018. Transgenerational effects of insecticides—implications for rapid pest evolution in agroecosystems. Curr. Opin. Insect Sci. 26, 34–40.
Burton, T., Metcalfe, N.B., 2014. Can environmental conditions experienced in early life influence future generations? Proc. Biol. Sci. 281 (1785), 20140311.
Cambronero, M.C., Beasley, J., Kissane, S., Orsini, L., 2018. Evolution of thermal tolerance in multifarious environments. Mol. Ecol. 27 (22), 4529–4541.
Chi, W., Mingyuan, H., Fengshou, D., Jun, X., Xiaohu, W., Bing, C., et al., 2021. The influence of tolfenpyrad on fitness, development, and reproduction in parents and offspring of *Coccinella septempunctata*. Ecotoxicol. Environ. Saf. 210, 111875.
Costanzo, K.S., Muturi, E.J., Lampman, R.L., Alto, B.W., 2014. The effects of resource type and ratio on competition with *Aedes albopictus* and *Culex pipiens* (Diptera: Culicidae). J. Med. Entomol. 48 (1), 29–38.
Delnat, V., Janssens, L., Stoks, R., 2019. Whether warming magnifies the toxicity of a pesticide is strongly dependent on the concentration and the null model. Aquat. Toxicol. 211, 38–45.
Domingues, I., Agra, A.R., Monaghan, K., Soares, A.M., Nogueira, A.J., 2010. Cholinesterase and glutathione-S-transferase activities in freshwater invertebrates as biomarkers to assess pesticide contamination. Environ. Toxicol. Chem. 29 (1), 5–18.
Donelson, J.M., Salinas, S., Munday, P.L., Shama, L.N., 2018. Transgenerational plasticity and climate change experiments: where do we go from here? Global Change Biol. 24 (1), 13–34.
Feil, R., Fraga, M.F., 2012. Epigenetics and the environment: emerging patterns and implications. Nat. Rev. Genet. 13 (2), 97–109.
Fonseca, D.M., Keyghobadi, N., Malcolm, C.A., Mehmet, C., Schaffner, F., Mogi, M., et al., 2004. Emerging vectors in the *Culex pipiens* complex. Science 303 (5663), 1535–1538.
Fox, J., Weisberg, S., 2018. An R Companion to Applied Regression. Sage publications.
Guillaume, A.S., Monro, K., Marshall, D.J., 2016. Transgenerational plasticity and environmental stress: do paternal effects act as a conduit or a buffer? Funct. Ecol. 30 (7), 1175–1184.
Gunderson, A.R., Armstrong, E.J., Stillman, J.H., 2016. Multiple stressors in a changing world: the need for an improved perspective on physiological responses to the dynamic marine environment. Ann. Rev. Marine Sci. 8, 357–378.
Harada, A.E., Healy, T.M., Burton, R.S., 2019. Variation in thermal tolerance and its relationship to mitochondrial function across populations of *Tigriopus californicus*. Front. Physiol. 10, 213.
Head, J., 2019. An ecotoxicological perspective on transgenerational epigenetic inheritance. Environ. Toxicol. Chem. 38 (6), 1149–1151.
Heard, E., Martienssen, R.A., 2014. Transgenerational epigenetic inheritance: myths and mechanisms. Cell 157 (1), 95–109.
Heino, J., Virkkala, R., Toivonen, H., 2009. Climate change and freshwater biodiversity: detected patterns, future trends and adaptations in northern regions. Biol. Rev. 84 (1), 39–54.
Hooper, M.J., Ankley, G.T., Cristol, D.A., Maryoung, L.A., Noyes, P.D., Pinkerton, K.E., 2013. Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks. Environ. Toxicol. Chem. 32 (1), 32–48.
IPCC, 2014. Climate Change 2013: the Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press.
Jacobs, A.F., Heusinkveld, B.G., Kraai, A., Paaijmans, K.P., 2008. Diurnal temperature fluctuations in an artificial small shallow water body. Int. J. Biometeorol. 52 (4), 271–280.
Janssens, L., Stoks, R., 2013. Fitness effects of chlorpyrifos in the damselfly *Enallagma cyathigerum* strongly depend upon temperature and food level and can bridge metamorphosis. PLoS One 8 (6), e68107.
Janssens, L., Verberk, W., Stoks, R., 2021. The Pace-Of-Life Explains whether Gills Improve or Exacerbate Pesticide Sensitivity in a Damselfly Larva. Environmental Pollution, p. 117019.
Johnson, A.C., Donnachie, R.L., Sumpter, J.P., Jürgens, M.D., Moeckel, C., Pereira, M.G., 2017. An alternative approach to risk rank chemicals on the threat they pose to the aquatic environment. Sci. Total Environ. 599, 1372–1381.
Jørgensen, L.B., Malte, H., Overgaard, J., 2019. How to assess *Drosophila* heat tolerance: unifying static and dynamic tolerance assays to predict heat distribution limits. Funct. Ecol. 33 (4), 629–642.
Kaspari, M., Clay, N.A., Lucas, J., Yanoviak, S.P., Kay, A., 2015. Thermal adaptation generates a diversity of thermal limits in a rainforest ant community. Global Change Biol. 21 (3), 1092–1102.
Kopp, M., Matuszewski, S., 2014. Rapid evolution of quantitative traits: theoretical perspectives. Evol. Appl. 7 (1), 169–191.
Lenth, R., Singmann, H., Love, J., Buerkner, P., Herve, M., 2019. Emmeans: Estimated Marginal Means, Aka Least-Squares Means, Version 1.3. 4.
Lim, J.P., Brunet, A., 2013. Bridging the transgenerational gap with epigenetic memory. Trend. Gen. 29 (3), 176–186.
Lim, Y.K., Dang, X., Thiyagarajan, V., 2021. Transgenerational Responses to Seawater pH in the Edible Oyster, with Implications for the Mariculture of the Species under Future Ocean Acidification. Science of The Total Environment, p. 146704.
Livdahl, T.P., Sugihara, G., 1984. Non-linear interactions of populations and the importance of estimating per capita rates of change. J. Anim. Ecol. 573–580.
Lydy, M.J., Belden, J.B., Ternes, M.A., 1999. Effects of temperature on the toxicity of M-parathion, chlorpyrifos, and pentachlorobenzene to *Chironomus tentans*. Arch. Environ. Contam. Toxicol. 37 (4), 542–547.
Ma, G., Rudolf, V.H., Ma, C.S., 2015. Extreme temperature events alter demographic rates, relative fitness, and community structure. Global Change Biol. 21 (5), 1794–1808.
Margus, A., Piiroinen, S., Lehmann, P., Tikka, S., Karvanen, J., Lindström, L., 2019. Sublethal pyrethroid insecticide exposure carries positive fitness effects over generations in a pest insect. Sci. Rep. 9 (1), 1–10.
Marigoudar, S.R., Mohan, D., Nagarjuna, A., Karthikeyan, P., 2018. Biomarker and histopathological responses of *Lates calcarifer* on exposure to sub lethal concentrations of chlorpyrifos. Ecotoxicol. Environ. Saf. 148, 327–335.
Marshall, D.J., 2008. Transgenerational plasticity in the sea: context-dependent maternal effects across the life history. Ecology 89 (2), 418–427.
Meng, S., Delnat, V., Stoks, R., 2020a. The exposure order strongly modifies how a heat spike increases pesticide toxicity. Environ. Sci. Technol. 54 (18), 11476–11484.

Meng, S., Delnat, V., Stoks, R., 2020b. Mosquito larvae that survive a heat spike are less sensitive to subsequent exposure to the pesticide chlorpyrifos. Environ. Pollut. 265, 114824.
Meng, S., Tran, T.T., Delnat, V., Stoks, R., 2021. Transgenerational exposure to warming reduces the sensitivity to a pesticide under warming. Environ. Pollut. 284, 117217.
Moe, S.J., De Schamphelaere, K., Clements, W.H., Sorensen, M.T., Van den Brink, P.J., Liess, M., 2013. Combined and interactive effects of global climate change and toxicants on populations and communities. Environ. Toxicol. Chem. 32 (1), 49–61.
Narváez, C., Ríos, J.M., Píriz, G., Sanchez-Hernandez, J.C., Sabat, P., 2016. Subchronic exposure to chlorpyrifos affects energy expenditure and detoxification capacity in juvenile Japanese quails. Chemosphere 144, 775–784.
Negro, C.L., Collins, P., 2017. Histopathological effects of chlorpyrifos on the gills, hepatopancreas and gonads of the freshwater crab *Zilchiopsis collastinensis*. Persistent effects after exposure. Ecotoxicol. Environ. Saf. 140, 116–122.
Noyes, P.D., Lema, S.C., 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. Current Zoology 61 (4), 669–689.
Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., et al., 2009. The toxicology of climate change: environmental contaminants in a warming world. Environ. Int. 35 (6), 971–986.
Oppold, A., Kreß, A., Bussche, J.V., Diogo, J.B., Kuch, U., Oehlmann, J., et al., 2015. Epigenetic alterations and decreasing insecticide sensitivity of the Asian tiger mosquito *Aedes albopictus*. Ecotoxicol. Environ. Saf. 122, 45–53.
Orr, J.A., Vinebrooke, R.D., Jackson, M.C., Kroeker, K.J., Kordas, R.L., Mantyka-Pringle, C., et al., 2020. Towards a unified study of multiple stressors: divisions and common goals across research disciplines. Proceedings of the Royal Society B 287 (1926), 20200421.
R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rahman, H.U., Asghar, W., Nazir, W., Sandhu, M.A., Ahmed, A., Khalid, N., 2021. A Comprehensive Review on Chlorpyrifos Toxicity with Special Reference to Endocrine Disruption: Evidence of Mechanisms, Exposures and Mitigation Strategies. Science of The Total Environment, p. 142649.
Reátegui-Zirena, E.G., Fidder, B.N., Olson, A.D., Dawson, D.E., Bilbo, T.R., Salice, C.J., 2017. Transgenerational endpoints provide increased sensitivity and insight into multigenerational responses of *Lymnaea stagnalis* exposed to cadmium. Environ. Pollut. 224, 572–580.
Rohr, J.R., Sesterhenn, T.M., Stieha, C., 2011. Will climate change reduce the effects of a pesticide on amphibians?: partitioning effects on exposure and susceptibility to contaminants. Global Change Biol. 17 (2), 657–666.
Salinas, S., Brown, S.C., Mangel, M., Munch, S.B., 2013. Non-genetic inheritance and changing environments. Non-Genetic Inheritance 1, 38–50.
Salinas, S., Munch, S.B., 2012. Thermal legacies: transgenerational effects of temperature on growth in a vertebrate. Ecol. Lett. 15 (2), 159–163.
Sánchez, M., Ferrando, M.D., Sancho, E., Andreu, E., 2000. Physiological perturbations in several generations of *Daphnia magna* straus exposed to diazinon. Ecotoxicol. Environ. Saf. 46 (1), 87–94.
Schäfer, R.B., Piggott, J.J., 2018. Advancing understanding and prediction in multiple stressor research through a mechanistic basis for null models. Global Change Biol. 24 (5), 1817–1826.
Schulz, R., Bub, S., Petschick, L.L., Stehle, S., Wolfram, J., 2021. Applied pesticide toxicity shifts toward plants and invertebrates, even in GM crops. Science 372 (6537), 81–84.
Shama, L.N., Strobel, A., Mark, F.C., Wegner, K.M., 2014. Transgenerational plasticity in marine sticklebacks: maternal effects mediate impacts of a warming ocean. Funct. Ecol. 28 (6), 1482–1493.
Shaw, J.L., Judy, J.D., Kumar, A., Bertsch, P., Wang, M.B., Kirby, J.K., 2017. Incorporating transgenerational epigenetic inheritance into ecological risk assessment frameworks. Environ. Sci. Technol. 51 (17), 9433–9445.
Singmann, H., Bolker, B., Westfall, J., Aust, F., 2017. Afex: Analysis of Factorial Experiments. R Package Version.
Stehle, S., Schulz, R., 2015. Pesticide authorization in the EU—environment unprotected? Environ. Sci. Pollut. Control Ser. 22 (24), 19632–19647.
Stillman, J.H., 2019. Heat waves, the new normal: summertime temperature extremes will impact animals, ecosystems, and human communities. Physiology 34 (2), 86–100.
Topping, C.J., Aldrich, A., Berny, P., 2020. Overhaul environmental risk assessment for pesticides. Science 367 (6476), 360–363.
Tran, T.T., Janssens, L., Dinh, K.V., Op de Beeck, L., Stoks, R., 2016. Evolution determines how global warming and pesticide exposure will shape predator–prey interactions with vector mosquitoes. Evolutionary applications 9 (6), 818–830.
Tran, T.T., Janssens, L., Dinh, K.V., Stoks, R., 2018. Transgenerational interactions between pesticide exposure and warming in a vector mosquito. Evol. Appl. 11 (6), 906–917.
Uller, T., 2008. Developmental plasticity and the evolution of parental effects. Trends Ecol. Evol. 23 (8), 432–438.
Van Drooge, H.L., Groeneveld, C.N., Schipper, H.J., 2001. Data on application frequency of pesticide for risk assessment purposes. Ann. Occup. Hyg. 45, S95–S101.
Vasseur, D.A., DeLong, J.P., Gilbert, B., Greig, H.S., Harley, C.D., McCann, K.S., et al., 2014. Increased temperature variation poses a greater risk to species than climate warming. Proc. Biol. Sci. 281 (1779), 20132612.
Verberk, W.C., Bilton, D.T., 2013. Respiratory control in aquatic insects dictates their vulnerability to global warming. Biol. Lett. 9 (5), 20130473.
Verberk, W.C., Overgaard, J., Ern, R., Bayley, M., Wang, T., Boardman, L., Terblanche, J. S., 2016. Does oxygen limit thermal tolerance in arthropods? A critical review of current evidence. Comp. Biochem. Physiol. Mol. Integr. Physiol. 192, 64–78.
Verheyen, J., Delnat, V., Stoks, R., 2019. Increased daily temperature fluctuations overrule the ability of gradual thermal evolution to offset the increased pesticide toxicity under global warming. Environ. Sci. Technol. 53 (8), 4600–4608.
WHO, 2005. Guidelines for Laboratory and Field Testing of Mosquito Larvicides. World Health Organization, Geneve, Switzerland, pp. 1–41.