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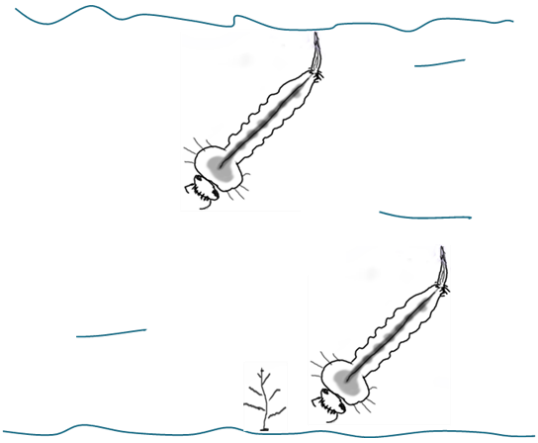
Combined effects of global warming and pesticide exposure on mosquitoes: integrating temporal aspects within and across generations in ecotoxicology



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Dissertation presented in partial fulfilment of the requirements for the degree of Doctor of Science (PhD): Biology

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Summary

Pesticides and warming are two major environmental stressors in aquatic ecosystems that can cause biodiversity loss and can interact with each other. Therefore, investigating the combined effects of warming and pesticide exposure is important for assessing the net impact of pesticides on non-target aquatic species. Despite the growing awareness of the roles of temporal aspects in stressor interactions and of the effects of warming in ecotoxicological studies, current knowledge about the interactive effects between warming and pesticides is largely based on empirical studies of simultaneous exposure to both stressors. The purpose of this thesis was to evaluate the importance of considering realistic temporal exposure scenarios to warming for the risk assessment of pesticides. I thereby studied the within- and across-generation combined effects of warming-related factors (increase in mean temperature and heat extremes) and pesticide exposure on the mosquito species *Culex pipiens*.

In chapters 1 and 2, I tested whether and how the toxicity of the pesticide chlorpyrifos was modulated by the sequential exposure to a heat spike within a generation. I found that the heat spike applied in the first larval stage caused considerable direct and delayed mortality, and the survivors were less sensitive to the subsequent exposure to chlorpyrifos in the final larval stage which probably was caused by survival selection and cross-tolerance (chapter 1). In contrast, the heat spike strongly magnified the toxicity of chlorpyrifos when the heat spike was applied in the final larval stage and directly followed by the chlorpyrifos exposure, and this synergism disappeared or was weakened when the exposure order was reserved (chapter 2).

In chapters 3 and 4, I investigated whether and how the duration of exposure to warming can predictably modify pesticide toxicity. I thereby contrasted the effects of acute, developmental and transgenerational warming on the toxicity of chlorpyrifos. The net impact of chlorpyrifos on life history and physiology (chapter 3) and on heat tolerance and antipredator behaviour (chapter 4) was weakened under warming, caused by the warming-induced increase in the degradation rate of chlorpyrifos. In addition, the impact of chlorpyrifos under warming was affected by the exposure duration to warming, where acute warming increased the toxicity of chlorpyrifos more compared to developmental and transgenerational warming.

In chapter 5, I tested for the sequential exposure of a heat spike followed by exposure to chlorpyrifos and this in a full factorial way in the parental and offspring generations. Parental exposure to chlorpyrifos caused adaptive transgenerational effects in terms of increased

tolerance to chlorpyrifos in the offspring, suggesting an underestimation of the risk of a toxicant in field-collected animals in case that their parents already experienced the toxicant. Nevertheless, the synergism between the heat spike and exposure to chlorpyrifos did not change in the offspring when parents had been exposed to chlorpyrifos.

In general, my thesis provides considerable evidence from life history, heat tolerance, physiology and antipredator behaviour that global warming-related factors can strongly modulate the impact of exposure to a pesticide, and importantly that this can be strongly dependent on the temporal exposure scenario. Hence, my results emphasize the importance of integrating ecologically relevant temporal exposure scenarios in ecotoxicological studies and risk assessment of pollutants.

Samenvatting

Pesticiden en opwarming zijn twee belangrijke omgevingsstressoren in aquatische ecosystemen die biodiversiteitsverlies kunnen veroorzaken en met elkaar kunnen interageren, waardoor het onderzoeken van de gecombineerde effecten van opwarming en blootstelling aan pesticiden belangrijk is voor het beoordelen van de netto-impact van pesticiden op niet-doelsoorten. Ondanks het groeiende bewustzijn van de rol van temporele aspecten in stressorinteracties en de effecten van opwarming in ecotoxicologische studies, is de huidige kennis over de interactieve effecten tussen opwarming en pesticiden grotendeels gebaseerd op empirische studies van gelijktijdige blootstelling aan beide stressoren. Het doel van dit proefschrift was om het belang te evalueren van realistische temporele blootstellingsscenario's aan opwarming en een pesticide voor de risicobeoordeling van pesticiden. Ik bestudeerde daarbij de gecombineerde effecten binnen en tussen generaties van ‘global warming’-gerelateerde factoren (stijging van gemiddelde temperatuur en extreme hitte) en blootstelling aan pesticiden op de muggensoort *Culex pipiens*.

In hoofdstukken 1 en 2 heb ik getest of en hoe de toxiciteit van pesticide chloorpyrifos werd gemoduleerd door opeenvolgende blootstelling aan hittepieken binnen en tussen generaties. Ik ontdekte dat de hittepiek in het eerste larvale stadium aanzienlijke directe en vertraagde sterfte veroorzaakte, en de overlevenden waren minder gevoelig voor de daaropvolgende blootstelling aan chloorpyrifos in het laatste larvale stadium, wat waarschijnlijk werd veroorzaakt door overlevingsselectie en kruistolerantie (Hoofdstuk 1). Daarentegen verhoogde de hittepiek de toxiciteit van chloorpyrifos sterk wanneer de hittepiek werd toegepast in het laatste larvale stadium en direct gevolgd door de blootstelling aan chloorpyrifos, en de synergismen tussen hen verdwenen of werden verzwakt toen de belichtingsvolgorde werd gereserveerd (hoofdstuk 2) .

In hoofdstukken 3 en 4 heb ik onderzocht of en hoe de duur van blootstelling aan opwarming de toxiciteit van pesticiden voorspelbaar kan veranderen. Ik heb daarbij de effecten van acute, ontwikkelings- en transgenerationele opwarming op de toxiciteit van chloorpyrifos vergeleken. De netto impact van chloorpyrifos op levensgeschiedenis en fysiologie (hoofdstuk 3) en op hittetolerantie en antipredatorgedrag (hoofdstuk 4) werd verzwakt door opwarming; dit kon worden verklaard door de door opwarming veroorzaakte toename van de afbraaksnelheid van chloorpyrifos. Bovendien werd de impact van chloorpyrifos onder opwarming beïnvloed door de blootstellingsduur aan opwarming, waarbij acute opwarming de

toxiciteit van chloorpyrifos meer verhoogde in vergelijking met ontwikkelings- en transgenerationele opwarming.

In hoofdstuk 5 heb ik dieren sequentieel blootgesteld aan hitte en daarna een het pesticide en dit in een volledig gekruiste proefopzet in zowel de parentale als in de nakomelingen-generatie. De blootstelling van de ouders aan chloorpyrifos veroorzaakte adaptieve transgenerationele effecten in termen van verhoogde tolerantie voor chloorpyrifos bij de nakomelingen, wat een onderschatting suggereert van het risico van een pesticide bij in het veld verzamelde dieren in het geval dat hun ouders het pesticide al hadden ervaren. Nochtans veranderde het synergisme tussen de blootstelling aan hitte en het pesticide niet in de nakomelingen van wie de ouders waren blootgesteld aan het pesticide.

In het algemeen leverde mijn proefschrift aanzienlijk bewijs op basis levensgeschiedenisvariabelen, hittetolerantie, fysiologie en antipredatorgedrag dat opwarmingsgerelateerde factoren de impact van blootstelling aan pesticiden sterk kunnen moduleren, en vooral dat dit sterk afhankelijk was van de temporele blootstellingsscenario's. Mijn resultaten tonen dus het belang van het integreren van ecologisch relevante temporele blootstellingsscenario's in ecotoxicologische studies en risicobeoordeling van toxische stoffen.

List of abbreviations

Anova	analysis of variance
ATP	adenosine triphosphate
AChE	acetylcholinesterase
CEA	cellular energy allocation
CITS	climate-induced toxicant sensitivity
CPF	chlorpyrifos
CTmax	critical thermal maximum
Ea	energy available
Ec	energy consumed
ETS	electron transport system
FDR	false discovery rate
GLM	General Linear Model
IA	independent action
IPCC	Intergovernmental Panel on Climate Change
MDA	malondialdehyde
MDR	model deviation ratio
ROS	reactive oxygen species
SE	standard error
TICS	toxicant-induced climate change sensitivity
TPC	thermal performance curve
UPLC MS/MS	ultra performance liquid chromatography – tandem mass spectrometer
WHO	World Health Organization

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General introduction

Despite the important ecosystem services and rich biodiversity of freshwater ecosystems, they are facing increasing threats due to human activities (Dudgeon et al., 2006; Vörösmarty et al., 2010). Among the multiple stressors that threaten freshwater organisms, exposure to pesticides is of serious concern as driver of biodiversity loss (Beketov et al., 2013). The pesticides applied in agriculture can enter surface water bodies, especially edge-to-field water bodies, through run-off or drift, resulting in adverse acute and/or chronic effects on non-target organisms (Schulz et al., 2021; Topping et al., 2020). Global warming is another anthropogenic stressor that can cause considerable effects in aquatic ecosystems (Heino et al., 2009), and moreover can interact with pesticides. Warming has been shown both to magnify the toxicity of many pesticides at constant concentrations (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015), and to accelerate their rate of degradation (overview in Hooper et al., 2013; Op de Beeck et al., 2017a). Since the latter mechanism may buffer or even overrule the increased toxicity of pesticides under warming, it is necessary to consider both aspects to understand the net impact of pesticides under warming. In addition, pesticides can also negatively affect the ability of ectotherms to tolerate thermal stress (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013).

There is growing awareness to consider ecologically relevant temporal exposure scenarios in multistressor ecology (overviews in Gunderson et al., 2016; Orr et al., 2020; Jackson et al., 2021; Ashauer et al., 2010). Several temporal aspects are relevant to study in a multistressor context. A first one is about co-exposure versus sequential exposure. Currently, the impact of pesticides under warming is mainly studied by co-exposing organisms to both stressors (overview in Holmstrup et al., 2010). However, under sequential exposure to both stressors the mechanisms underlying the higher toxicity of pesticides under warming may differ from those under co-exposure, moreover the degradation of pesticides is then unlikely to be altered by warming. Hence, the combined effects of both stressors under sequential exposure can differ considerably compared to simultaneous exposure. Sequential exposure is especially relevant to consider for heat extremes and pesticide exposure, as both are transient stressors, and heat extremes are expected to occur more frequent under warming (IPCC 2021; Hansen et al., 2012; Rahmstorf and Coumou, 2011). Notably, heat extremes may have more adverse effects on population persistence than mild warming (Thompson et al., 2013; Vasseur et al., 2014; Ma et al., 2015 and 2021). A second temporal aspect is the exposure duration to a stressor. While pesticides are typically applied as pulses, warming can go from an acute to a continuous

stressor, thereby organisms may have already experienced warming before a pesticide application starts, and different exposure durations to warming may induce different plastic responses (Sgro et al., 2016), likely causing variation in the interaction with the pesticide exposure. The exposure duration may also occur across generations, the stressors experienced by parents may thereby affect the overall fitness of offspring and/or their ability to deal with these stressors (Bonduriansky et al., 2012; Salinas et al., 2013). This phenomenon that parents can modulate the phenotype of the offspring has been referred to by various names, such as transgenerational effects, carry-over effects and multigenerational effects (Bonduriansky et al., 2012; Salinas et al., 2013). I here use the term transgenerational effects in its large sense where it broadly describes all non-sequence-based effects that can be transmitted from one generation to the next (Heard and Martienssen, 2014; Bell and Hellmann, 2019). In its strict sense, transgenerational effects only apply when parental exposure to a stressor affects a later generation whose germ cells have not been exposed to that stressor, which is the F2 or F3 generation depending whether the animals in the parental generation were exposed before maturity. (Donelson et al., 2018). It is important to consider transgenerational effects in ecotoxicological (Shaw et al., 2017; Head, 2019) and global warming studies (Salinas and Munch, 2012; Donelson et al., 2018). For example, maladaptive transgenerational effects can lead to an underestimation of the total impact of stressors on populations when they are ignored. Despite the frequent interplay between the effects of warming and pesticide pulses, and the fact that maladaptive transgenerational effects have been shown both for warming (e.g. Tran et al., 2018) and for pesticides exposure (e.g. Sánchez et al., 2000), combined transgenerational effects between these two stressors have been rarely studied (but see e.g. Tran et al., 2018 and 2019) and never for heat extremes.

In this introductory part, I will discuss the environmental stressors studied and their interactions, introduce the study organism and the key endpoints investigated. I will end this general introduction with an overview of the research chapters and their aims.

1. Stressors and their effects

1.1 Stress and stressor

Organisms are seldom living at optimal conditions in their natural habitats, this is especially true for ectotherms (Hofmann and Todgham, 2010). When the external environmental factors

(see Figure 1; Van Straalen, 2003), in this case stressors, are out of the normal range of their ecological niche or amplitude, organisms start suffering from stress (an internal state). However, through various stress responses, like dispersal, plasticity and evolution, organisms can stay alive and relieve the stress by returning to their niche, or by shifting the edge of the niche which will make a previous stressor no longer be a stressor (Van Straalen, 2003; Calow, 1989).

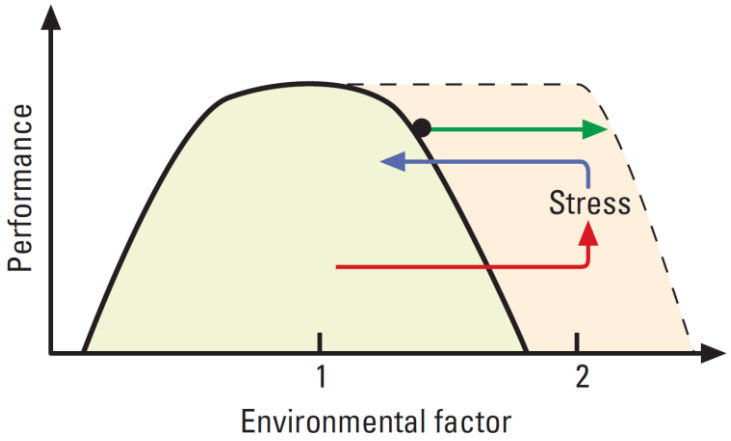


Figure 1: Schematic illustration of the niche-based definition of stress (from Van Straalen, 2003). The ecological niche of a species was reflected by the light green area under the black line. When the intensity of a stressor increases from level 1 to 2 where it is out of the ecological niche of a species, stress arises (red line). Stress responses then take place which help the species go back to the niche (blue line) or broaden the borders of the niche making the previous stressor no longer be a stressor (green line).

1.2 Global warming

Global warming is the process of long-term increase in average temperature of the near-surface air and oceans (Ghommem et al., 2012; Guilyardi et al., 2018). According to The Intergovernmental Panel on Climate Change (IPCC, 2021), warming induced by human activities has reached about 1 ℃ by 2017 relative to pre-industrial levels, with an increase of ca. 0.2 ℃ per decade. It is necessary or even vital to maintain the temperature increase below 1.5 ℃ to limit the negative effects of extreme events on ecosystems, biodiversity and human well-being (IPCC, 2021). However, according to the SSP (Shared Socio-economic Pathway) 5-8.5, an increase of 4.4 ℃ can be expected by 2100 (IPCC, 2021). The SSP5-8.5 scenario represents the scenario with very high greenhouse gas emissions and CO_2 emissions that

roughly double from current levels by 2050 (Figure 2; IPCC, 2021).

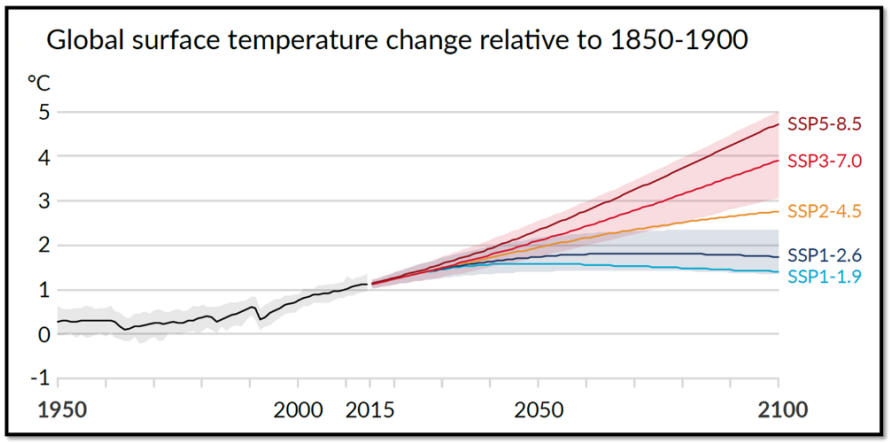


Figure 2: Global surface temperature changes in ºC relative to 1850-1900 (based on IPCC,2021). Changes relative to 1850–1900 based on 20-year averaging periods are calculated by adding 0.85°C (the observed global surface temperature increase from 1850–1900 to 1995–2014) to simulated changes relative to 1995–2014. *Very likely* ranges are shown for the different scenarios.

Temperature plays an important role in the performance of ectotherms (Paaijmans et al., 2013; Sinclair et al., 2016). Temperature can affect the rate of physiological and biochemical processes by influencing bio-macromolecules, and thereby, amongst others the structure and functioning of proteins, and membrane fluidity (Grigaltchik et al. 2012; Sinclair et al., 2016). Related to this, metabolism, growth, development and reproduction, and all other cellular and physiological functions are dependent on temperature, and the effects of temperature can also cascade to higher organization levels (Grigaltchik et al. 2012; Sinclair et al., 2016). At the organismal level, the effect of temperature can be graphically summarized in the "Thermal Performance Curve (TPC)", which illustrates how temperature affects the performance and fitness of ectotherms (Huey and Berrigan, 2001). When temperatures are below the optimal temperature (T_{opt}), higher temperatures will increase performance, up to a maximum at T_{opt} (Figure 3; Noyes and Lema, 2015; Stoks et al., 2017). However, when temperatures go above T_{opt}, temperature increases will decline performance strongly, down to zero at the critical thermal maximum (CTmax). Therefore, global warming may cause either positive or negative effects on ectotherms, depending on where the temperature is located at the TPC.

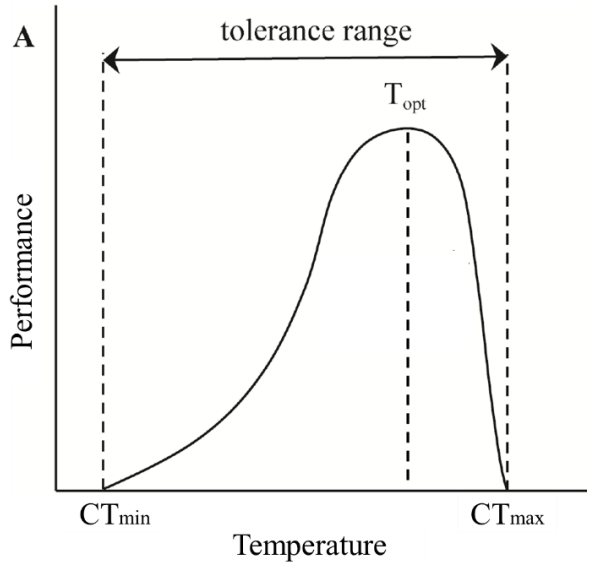


Figure 3: Conceptual visualization of a thermal performance curve of a typical ectotherm (based on Noyes and Lema, 2015). The temperature between the critical thermal minimum (CTmin) and maximum (CTmax) reflects the tolerance range, ectotherms reach their highest performance at the optimal temperature (T_{opt}).

Besides increases in mean temperature, temperature variability and the occurrence of heat extremes is also expected to rise under warming (IPCC, 2021; Stillman, 2019; Woolway et al., 2021; Buckley and Huey, 2016). Different from mild warming, heat extremes are typically a transient stressor and often lethal (Stillman, 2019; Ma et al., 2021), and may therefore act as a selective factor (Buckley and Huey, 2016). While there is still no unified definition of heat extremes from a biological perspective, they can be described as the phenomenon where temperature triggers the threshold of a biological response (Ma et al., 2021).

Despite that heat extremes may only occur periodically, they can cause stronger adverse effects on population persistence than gradual temperature shifts (Harley, 2011; Wethey et al., 2011). The lethal and sublethal effects caused by heat extremes (overview in Stillman, 2019; Ma et al., 2021) can be explained by several reasons. Firstly, heat extremes can damage the structure and functions of bio-macromolecules (e.g. the denaturation of proteins, and changes in the fluidity of membranes), causing heat injury at several biological (e.g. molecular, biochemical and physiological) levels (Chown and Nicolson, 2004; Ma et al., 2021). Secondly, they can affect neurophysiological functions by disrupting the balance of cellular ions (hyperkalemia), and impair mitochondria, and the latter can further affect the ATP synthesis

and the energy supply (Harada et al., 2019; Stillman, 2019). Heat extremes can also cause desiccation by accelerating the body water loss, resulting in death (Chown et a., 2011). In addition, at extreme high temperatures, the limited ability of oxygen supply relative to the increased oxygen demand is another reason constraining the performance, as described in the oxygen and capacity limitation of the thermal tolerance (OCLTT) theory (Pörtner, 2001; Verberk et al., 2016). Despite their potential stronger impact, the effects of heat extremes on organisms are still less studied compared to mean temperature increases.

1.3 Pesticides

Pesticides are substances used to control pests, such as vectors of diseases, and insects that harm crops. The use of pesticides originates about 4500 years ago, but only till 1940s when synthetic pesticides were largely produced, the amount of pesticides use started surging (Kroma and Flora, 2003). After the book *Silent Spring* by the marine biologist Rachel Carson was published, scientists started becoming more aware of the adverse effects of pesticides on non-target species and their bioaccumulation through the food chain. Pesticides applied in agriculture can enter surface water bodies (especially edge-to-field water bodies) via spray drift and run-off, and are one of the major stressors that threaten biodiversity in aquatic ecosystems (Beketov et al., 2013; Topping et al., 2020; Schulz et al., 2021).

Depending on the applied dose, pesticides can also cause positive effects on organisms. It is well known that high doses of pesticides when exceeding the threshold can induce negative effects on the focal and non-target species, acting as a strong environmental selective factor. However, as shown in the hormetic dose-response model (see Figure 4), they can also increase the performance of organisms at low doses, a phenomenon known as the hormetic effect (Agathokleous et al., 2018; Brevik et al., 2018).

In this thesis, I chose chlorpyrifos as model pesticide. Chlorpyrifos is a member of the organophosphate pesticides (a pesticide class accounting for more than half of the total use of insecticide worldwide), and has been widely used in agriculture, domestic applications and mosquito control (Sumon et al., 2016). The IUPAC (International Union of Pure and Applied Chemistry) name of chlorpyrifos is *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate, with a chemical formula of $C_9H_{11}Cl_3NO_2PS$. Chlorpyrifos is soluble in most organic solvents (Rahman et al., 2021). The persistence of chlorpyrifos has been shown to vary

from days to years depending on the environmental factors, like temperature and light. Its metabolites (e.g. chlorpyrifos-oxon, 3,5,6-trichloro-2-pyridinol) are more persistent in the body, and can induce equal or stronger toxic effects (Rahman et al., 2021). Chlorpyrifos has recently been banned in Europe and the USA because of its higher toxicity for the human nervous system. However, it is still widely used in many developing countries due to its high effectiveness and low price (Hites, 2021; Rahman et al., 2021). It was classified as a priority substance in Europe by the Water Framework Directive of Europe (2000/60/EC: Ojec, 2000), and listed among the top ten chemicals that pose high threat to freshwater ecosystems (Johnson et al., 2017). The measured concentrations of chlorpyrifos in European surface water range between 0.07 and 0.69 µg/L (Stehle and Schulz, 2015), and the peak concentrations in edge-to-field water bodies can reach between 1 to 100 µg/L (Bernabò et al., 2011).

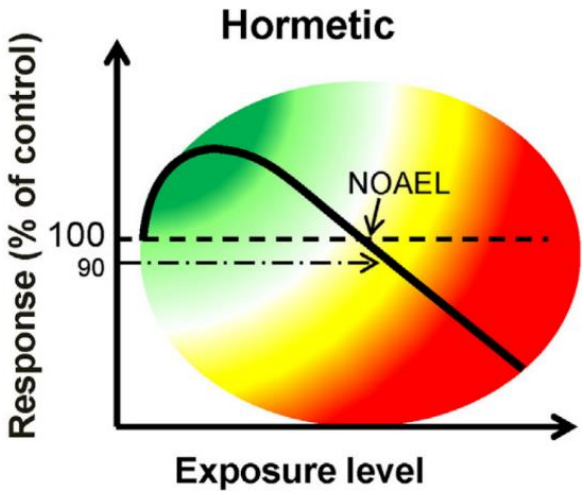


Figure 4: The hormetic dose-response model with no-observed-adverse-effects-level (NOAEL, also known as NOEC) indicating the toxicological threshold above which adverse effects occur (from Agathokleous et al., 2018). Stimulatory effects occur when the dose is lower than NOAEL (left region), while adverse effects occur when doses are higher than NOAEL (right region).

The toxicity of chlorpyrifos is attributed to the ability to block the active sites of its target enzyme acetylcholinesterase (AChE), which regulates the signal transmission at the neuromuscular junction and nerve synapses (Fukuto, 1990). When phosphorylated by chlorpyrifos, AChE is no longer able to hydrolyze acetylcholine (ACh), a member of

neurotransmitting agents responsible for the transmission of nerve pulses, which can cause a continuous stimulation of muscles or nerve fibers, leading to spasms or death (Fukuto, 1990; Domingues et al., 2010).

1.4 Interactions between stressors

In nature, organisms are always facing multiple biotic (e.g. predation) and abiotic (e.g. pesticides) stressors that can further interact with each other (Côté et al., 2016; Birk et al., 2020), which may contribute to the observation that the current risk assessment of pesticides fails to protect biodiversity (Liess et al., 2016). Therefore, it is important to consider the combined effects between stressors in multi-stressor studies.

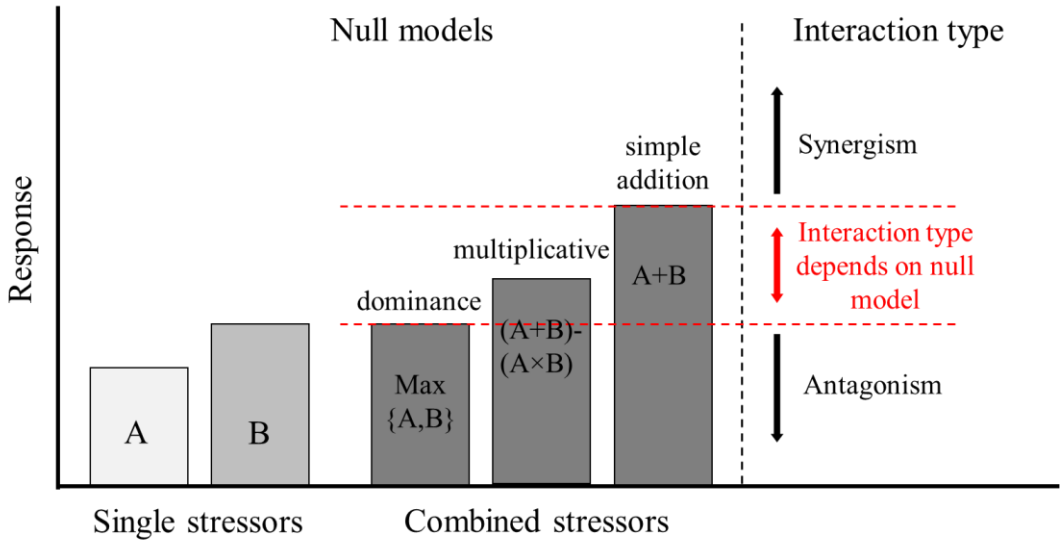


Figure 5: Schematic illustration about how null models can (mis)identify the type of interaction between stressor A and B (based on Côté et al., 2016; Delnat et al., 2019a). Three types of interaction between two stressors can be identified: (1) addition (no interaction); (2) synergism (larger observed combined effects than predicted by a null model); (3) antagonism (smaller observed combined effects than predicted by a null model). When the observed combined effects are within the range between the two red dashed lines, the interaction type will depend on the choice of null model. For example, the multiplicative null model (e.g. independent action model, IA model) would identify a combined effect as a synergism, while the simple addition null model (e.g. general linear models, GLM) will determine it as an addition or antagonism.

Three types of interactions can be identified based on the net combined effects of two stressors (Schäfer and Piggott, 2018; Todgham and Stillman, 2013): (1) addition, where the two stressors are independent and do not influence each other; (2) synergism, where the combined effects are larger than the additive effects; (3) antagonism, where the combined effects are less than the additive effects. As indicated in figure 5, there are three major null models which are models used to predict the interaction type based on the effects of individual stressors following the assumption of additivity (Schäfer and Piggott, 2018): (1) simple addition, which simply sums up the effects of both individual stressors and compares the combined effects of them (Schäfer and Piggott, 2018); (2) multiplicative, which accounts for the sum of both stressors but subtracts their product, thereby taking into account that once killed by one stressor one cannot be killed again by the other stressor (Bliss, 1939); (3) dominance, which applies to the situation where the combined effects are dominated by one of the two stressors (the dominant stressor). Selecting an appropriate null model is important for correctly determining the interaction type, since in some situations different null models can result in the identification of different interaction types (see figure 5). For example, when two stressors with different modes of action (e.g. warming and pesticides), or when both stressors are lethal, the simple addition null model (e.g. general linear model when response variable is not log-transformed) may identify an actual synergism as an addition or even an antagonism. In this case, the multiplicative null model (e.g. the independent action model) is recommended (Bliss, 1939; Schäfer and Piggott, 2018). Despite the important role of null models in determining interaction types, this is still less considered in multi-stressor ecotoxicological studies (but see e.g. Delnat et al., 2019a; Pestana et al., 2009).

1.5 Magnitude and timing of stressors

The intensity of stressors matters in ecotoxicology, since only when exceeding the threshold level (thereby moving out of the ecological niche of an organism, Figure 1), an environmental factor induces stress. A stressor can cause sublethal and/or lethal effects according to its intensity. Survival selection caused by lethal effects can erode genetic diversity, thereby reducing the tolerance of organisms to deal with other stressors in the future. Yet, the survivors may be those of the highest quality and instead be more tolerant to future stressors compared to a control group that did not undergo survival selection.

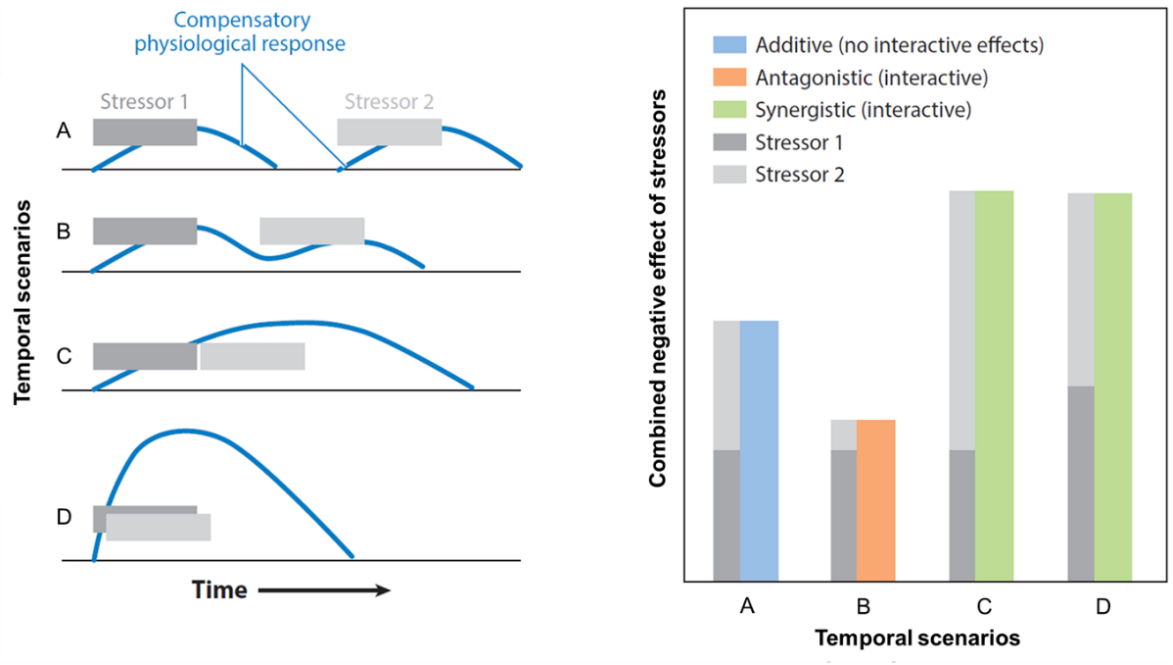


Figure 6: A temporal model to predict when additive, antagonistic and synergistic interactions may occur between two stressors (based on Gunderson et al., 2016). The left part lists four ecologically relevant temporal exposure scenarios: scenarios A, B, C and D. The curved blue lines reflect the temporal pattern in the compensatory physiological response to the stressors. The right part shows the expected consequence for each temporal exposure scenario. The dark gray and light gray bars represent the expected magnitude of the response to each stressor in the presence of the other stressor based on a specific temporal model, and the coloured bars represent the sums of those responses, hence the observed combined effects.

Also sublethal effects caused by one stressor can either positively or negatively affect the ability to deal with other stressors; this may strongly depend on the temporal exposure scenarios (Gunderson et al., 2016; Jackson et al., 2021; Orr et al., 2020). When transient stressors are involved (e.g. heat extremes and pesticide pulses), sequential exposure will be common. Based on the time lag between two stressors, three types of sequential exposure scenarios can be identified (Figure 6; Gunderson et al., 2016): (1) when there is a long time interval between two stressors (scenario A), individuals may have enough time to repair the damage caused by the first stressor and recover to their homeostasis before the second stressor starts, in this case there is likely no interaction; (2) when the time interval is relatively short, they may have enough time to finish the damage repair, but the compensatory physiological responses to the first stressor may be still ongoing at the start of exposure to the second stressor

(scenario B), in this case the exposure to the first stressor may prime the defense system and lead to a better handling of the subsequent second stressor if both stressors share the same defense mechanisms, a phenomenon known as cross-protection or cross-tolerance; thereby an antagonism is likely to be detected; (3) when two stressors are in close succession (scenario C), the individuals weakened by the first stressor may be more vulnerable to the second stressor, thereby a synergism can be expected. (4) In addition, stressors may occur simultaneously (scenario D, Figure 6), whereby the second stressor may act to extend the intensity of the first stressor in magnitude, and synergisms are also likely to be detected (Gunderson et al., 2016).

When sequential exposure is the case, the exposure order of two transient stressors can alter their combined effects. Stressors may differ in the rate of inducing negative effects and in the modes of action which further determine the rate of recovery to homeostasis (toxicodynamic recovery; Ashauer et al., 2010). When a stressor has a slower rate of inducing the negative effects and recovery ("slow stressor"), it is more likely to induce delayed effects, magnifying the noxious effects of the subsequent second stressor. Therefore, reversing the exposure order between a "slow" (e.g. a heat spike) and a "fast" (e.g. some pesticides) stressor is likely also to modulate the type and strength of interaction between two stressors, especially when they are in close succession.

There is increasing evidence that transgenerational effects (in large sense in this thesis), whereby the parental exposure to stressors can affect the overall fitness of offspring and/or their ability to deal with the same or different stressors (Bonduriansky et al., 2012; Salinas et al., 2013), may play an important role in ecotoxicological (Head, 2019; Shaw et al., 2017) and warming-related studies (Donelson et al., 2018; Salinas and Munch, 2012). Transgenerational effects can either be positive, whereby the legacy of parental exposure overall increases the fitness of offspring (Kopp and Matuszewski, 2014; Oppold et al., 2015) and/or their tolerance to the stressor (for warming: e.g. Shama et al., 2014; for pesticides: e.g. Oppold et al., 2015), or negative, whereby parental exposure makes their offspring perform worse. Such maladaptive transgenerational effects have been well-documented (e.g. for warming: Tran et al., 2018; for pesticide: Sánchez et al., 2000), and can lead to an underestimation of the total impact of stressors on populations when ignored. However, the combined transgenerational effects of multiple stressors are rarely studied despite the ubiquitous interaction between stressors (but see Tran et al., 2018 and 2019).

1.6 Combined effects of warming and pesticides

As two major stressors in aquatic ecosystems, warming and pesticide exposure have been well-documented to interact, which may be one reason why pesticides applied at protective concentrations according to the current risk assessment, are still causing declines in aquatic biodiversity (Beketov et al., 2013; Liess et al., 2016). Studying the combined effects of pesticides and warming-related climate change factors is of paramount importance for evaluating the actual risk posed by pesticides to the non-target organisms in a warming planet, and is becoming one of the focuses in ecotoxicological studies (Van den Brink et al., 2018). Notably, warming may modulate the impact of pesticides in two opposing ways. Firstly, the toxicity of many pesticides (at a constant concentration) has been shown to be magnified at higher temperatures (e.g. Lydy et al., 1999; Delnat et al., 2019a), due to a higher uptake and metabolic rate, or a quicker internal conversion to more toxic metabolites (Harwood et al., 2009; Noyes et al., 2009; Hallman and Brooks, 2015). This general pattern is encapsulated in the "climate change induced toxicant sensitivity" (CITS) concept (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). Secondly, pesticides can also break down faster at higher temperatures, probably offsetting or even overruling the warming-induced higher toxicity. While the latter mechanism has been predicted (Hooper et al., 2013), it is still rarely explicitly quantified in experimental studies (but see Op de Beeck et al., 2017a). In addition, pesticides can decrease the heat tolerance of organisms (e.g. Verheyen et al., 2019; Delnat et al., 2019b), which is encapsulated in the "toxicant induced climate change sensitivity" (TICS) concept (Hooper et al., 2013; Moe et al., 2013; Noyes et al., 2009).

Currently, the combined effects of warming and pesticide exposure are mainly studied by co-exposing organisms to both stressors (overview in Holmstrup et al., 2010). Given that under sequential exposure, the above-mentioned mechanisms underlying the higher toxicity of pesticides under warming may not play a role, and also the degradation of pesticides will not be modulated by temperature, the combined effects between the two stressors can differ considerably relative to simultaneous exposure. Sequential exposure is especially relevant to consider for heat extremes and pesticide exposure, as both are transient stressors, thereby are likely to be encountered in a sequential way in nature. While simultaneous exposure to mild warming and pesticide may typically result in a synergism (overview in Holmstrup et al., 2010), heat extremes can have a different interaction type with pesticides as this may be highly dependent on the temporal exposure scenarios (e.g. the time interval between the two stressors, and the exposure order). For example, heat-induced cross-tolerance has been shown before with

cadmium and zinc (e.g. Pestana et al., 2016). However, it has also been recorded that a pesticide can be more toxic when it directly followed a heat wave (Dinh et al., 2016).

2 Study species

Mosquitoes (Diptera: Culicidae) are semi-aquatic insects and are important vectors for many diseases, such as West Nile, malaria, Zika, filariasis and yellow fever (Becker et al., 2010). Their life cycle consists of four stages: egg, larva, pupa and adult. Eggs are laid at the water surface, larvae feed on algae and other organic material and live in the water as well as pupae that metamorphose into terrestrial adults. Adult males feed on carbohydrate food sources, like floral nectar, plant sugar and honeydew, to get energy, thereby serving as important pollinators. Besides sugar-heavy food sources, adult females typically need blood meals from humans or other vertebrates (e.g. birds) for the maturation of their eggs. During a bite of female mosquitoes, they can transfer not only saliva but also pathogens to their host, acting as vectors. As such they are studied as target species for pest control. In contrast, mosquitoes can occur at high biomass and therefore play an important role in food webs (Figure 7), hence can also be studied as important non-target species in ecological risk assessment of pesticides. For example, the aquatic larvae are an important food source in freshwater ecosystems, such as for many fishes, damselfly larvae and ducks, and the terrestrial adults are food of dragonflies and bats (Becker et al., 2010).

Acting as vectors of diseases, mosquitoes are widely used in epidemiological studies. Meanwhile, they are also increasingly studied as model species in stress ecology and ecotoxicology because of their short life cycle and the easy maintenance in lab conditions (Prud'homme et al., 2017). In this thesis, the mosquito *Culex pipiens* biotype *molestus* (Forskål 1775) was chosen as the study species, which is referred to as common house mosquito (Becker et al., 2010; Farajollahi et al., 2011). This species is widely distributed in North America and Europe, and is an important vector for West Nile virus (Farajollahi et al., 2011). The advantage of choosing this biotype for maintaining a lab culture is that even without a blood meal the adult females can still reproduce one batch of eggs.

The duration of the life cycle of *C. pipiens* is approximately one month at 20 °C. Eggs start hatching around three days after oviposition. The aquatic larval stage consists of four instars, and it takes ca. ten days at 20 °C before entering the final larval stage, which is the

recommended stage by WHO (2005) for toxicological studies in mosquitoes. Larvae can stay in the final instar for around one week before pupation. About four days after metamorphosis, females start laying eggs. This short life cycle makes it a good model for testing both the within and trans-generational effects of multiple stressors. Despite the semi-aquatic lifestyle, I mainly applied stressors (temperature and pesticides) during the larval stage, since the aquatic larvae have relatively limited mobility to escape from stressors compared to the terrestrial adults, making the larval stage a more vulnerable period. In addition, in this thesis I regarded mosquitoes as a component of food webs, thus focusing on how stressors impact them as a non-target species in freshwater ecosystems.

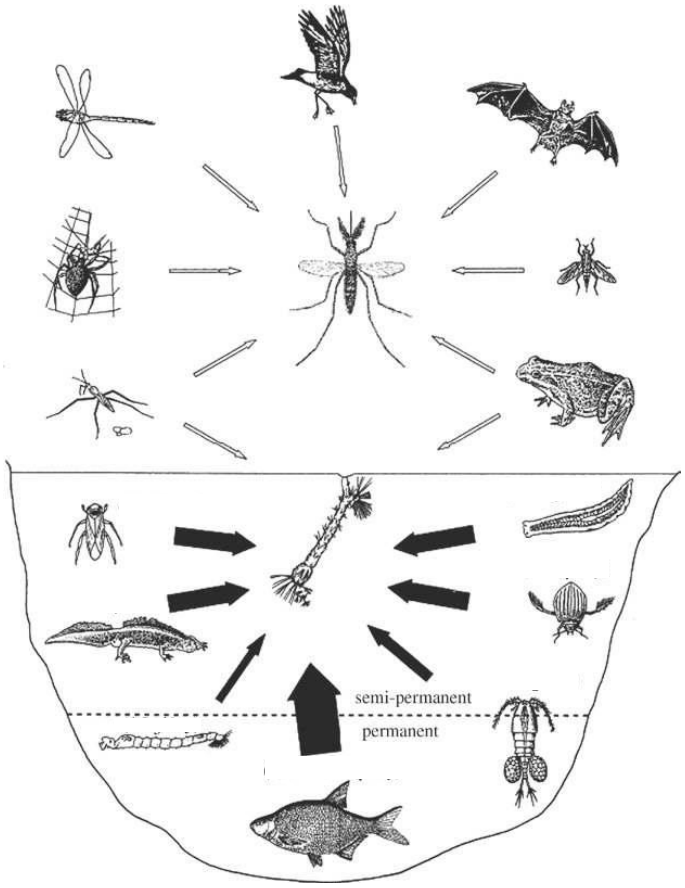


Figure 7: The role of mosquitoes as food source in aquatic and terrestrial food webs (based on Becker et al., 2010)

All experiments started by collecting egg clutches from a lab culture kept in a temperature-controlled room at 20 °C. This culture originated from three natural German pond

populations and the rearing temperature of 20 °C matches the mean summer temperature in German shallow water bodies (Tran et al., 2016). Given that a 4.4 °C increase in mean temperature is predicted by 2100 according to scenario SSP5-8.5 of IPCC (2021), I chose the temperature of 24 °C for treatments of mild warming. For the lab culture, the larvae were reared in white trays (length × width × height: 28 × 40 × 10 cm) containing 5 L dechlorinated tap water at a density of ca. 400 larvae per tray. Before the emergence of adults, trays were covered by white netting to avoid escape of adults. The emerged adults were then collected by an aspirator and transferred to small insectaries (length × width × height: 25 × 25 × 40 cm) covered by white netting, and fed with a 6% glucose solution. The aquatic larvae were fed with a powder mixture of Supradyn® vitamins (3%), wheat germs (51%) and Olvarit® 7 cereal flakes (46%).

3 Endpoints

To obtain a comprehensive understanding about the combined effects of warming and pesticide exposure, I quantified besides lethal effects, sublethal effects on multiple variables: life history, heat tolerance, physiology and antipredator behaviours.

3.1 Life history

The life history variables studied in this thesis are mortality, growth rate, development time, female wing length and population growth rate. For mortality, I quantified both the direct mortality during the exposure to a stressor and the delayed mortality after the exposure ended. Delayed effects are relevant to record since they can modulate the impact of the subsequent stressor in sequential exposure. Both growth rate (increase of body mass) and development time may affect the mass of adults at emergence, further impacting the reproductive success (Costanzo et al., 2011). Female wing length was measured as it is a good proxy for fecundity in mosquitoes (Costanzo et al., 2011). Based on the above-measured variables, I estimated the effects on population growth rate by calculating the composite index of population performance (*r'*, based on Livdahl and Sugihara, 1984).

Both pesticides and warming (including heat extremes) have been shown to affect these life history traits. For example, exposure to pesticides including chlorpyrifos (Baker et al., 2013; Dinh Van et al., 2014; Delnat et al., 2019a) and higher temperatures (for heat extremes: overview in Ma et al., 2021; Stillman, 2019) can induce higher mortality. Warming has been

shown to accelerate growth and development, resulting in smaller adults (temperature-size rule; Atkinson, 1994). Despite the deleterious effects of pesticides, growth and development have been recorded several times to be accelerated by pesticides in the study species (e.g. Delnat et al., 2019a) and other semi-aquatic insects (e.g. for damselfly larvae: Janssens and Stoks, 2014).

3.2 Heat tolerance

Heat tolerance is critical to quantify when assessing the biological impact of pesticides under warming, since it is a good proxy of the ability of organisms to tolerate mild warming (Åsheim et al., 2020) as well as heat extremes (Jørgensen et al., 2019; Kaspari et al., 2015). In this thesis, heat tolerance was estimated as CTmax (critical thermal maximum), which is the temperature when performance declines to zero (Noyes and Lema, 2015; Huey et al., 2012). All CTmax measurements were conducted in the larval stage just after the exposure to stressors ended. Specifically, randomly selected mosquito larvae were heated up till the temperature (CTmax) when they were floating motionlessly at the water surface. The heating rate applied (0.3 ℃ per minute) is commonly used for assessing the CTmax of aquatic invertebrates (e.g., Cambronero et al., 2018; Verberk and Bilton, 2013), and allows them to closely track the change of water temperature while avoiding acclimation (Dallas and Rivers-Moore, 2012).

CTmax occurs when oxygen demand cannot be met by oxygen supply (Verberk et al., 2016). Since aquatic organisms are more likely to suffer from hypoxia than terrestrial organisms (Verberk and Bilton, 2016), both pesticides and warming are likely to negatively affect the heat tolerance of aquatic larvae by increasing the demand and/or reducing the supply of oxygen. Indeed, by both increasing oxygen demand for detoxification (Sokolova, 2013; for chlorpyrifos: Narváez et al., 2016) and decreasing oxygen supply because of damaged respiratory system (for chlorpyrifos: Negro and Collins, 2017; Marigoudar et al., 2018), exposure to pesticides can shift the mismatch to a lower temperature, thereby reducing CTmax. Similarly, heat extremes may also increase oxygen demand when upregulating processes to cope with thermal stress and decrease oxygen supply by impacting the oxygen transport cascade, causing a decline in CTmax (Verberk et al., 2016). However, warming (including heat extremes) may also increase heat tolerance via acclimation, thereby shifting CTmax to higher values (Dallas and Rivers-Moore, 2012; Gunderson and Stillman, 2015), which has been recorded in some semi-aquatic insects (e.g. damselfly larvae: Verheyen et al., 2019).

3.3 Physiological variables

Physiological variables can not only provide mechanistic understanding for the observed patterns in other fitness-related traits, but may also capture effects that are not (yet) detectable at the organismal level (Janssens and Stoks, 2013a; Karl et al., 2011). Three types of physiological variables were measured in this thesis: the target enzyme of the pesticide chlorpyrifos, oxidative damage and bioenergetic response variables.

1) Target enzyme

The activity of acetylcholinesterase (AChE) was measured as this is the target enzyme of organophosphates and carbamates (Fukuto,1990), and the inhibition of its activity can cause neurotoxic effects, leading to spasms and death (Domingues et al., 2010). Chlorpyrifos has been shown to reduce the AChE activity of the study species (Delnat et al., 2019a), and other semi-aquatic insects (for damselfly larvae: Verheyen et al., 2019), which may contribute to the chlorpyrifos-induced lethal effects. In addition, warming-related factors have also been shown to influence AChE activity. For example, mild warming and temperature variation have been recorded to cause increases in AChE activity (Dinh Van et al., 2014; Willming et al., 2013).

2) Oxidative stress

Organisms can generate reactive oxygen species (ROS), like the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), as a result of normal biological activities (Valavanidis et al., 2006; Monaghan et al., 2009). Low levels of ROS can play important roles in key functions, such as the regulation of blood flow and smooth muscle relaxation, cell signaling and transformation, and immune defense (Droge, 2002; Monaghan et al., 2009). However, high levels of ROS can strongly affect key biological macromolecules, (e.g. DNA, proteins and lipids). When the produced ROS cannot be neutralized by antioxidants (e.g. superoxide dismutase, glutathione peroxidase and catalase (CAT), oxidative stress occurs (Monaghan et al., 2009), suggesting oxidative stress cannot be simply estimated by only quantifying one side of the balance (either ROS production or the capacity of antioxidant system). Therefore, it is important to quantify the net consequence in terms of oxidative damage to these macromolecules (Monaghan et al., 2009).

In this thesis, the oxidative damage to lipids was quantified as an estimate of oxidative stress by measuring the level of malondialdehyde (MDA) (Miyamoto et al., 2011). Exposure to chlorpyrifos has been shown to increase the generation of ROS (Itziou et al., 2011; Patetsini et

al., 2013). Related to this, chlorpyrifos has been recorded to increase the MDA level in the study species (e.g. Delnat et al., 2019a). While more ROS can be generated under warming because of a higher metabolic rate (Lushchak, 2011), this may not necessarily result in higher MDA level (e.g. Janssens and Stoks, 2017; Tu et al., 2018), since the antioxidant enzymes may also be upregulated under warming (e.g. increase in CAT activity: Op de Beeck et al., 2017a).

3) Bioenergetic variables at the cellular level

Although stressors may widely differ in their mode of action, they all tend to reduce the overall energy budget and increase the allocation of energy from other biological activities (such as development, growth and reproduction) to stress defense and damage repair mechanisms (Calow and Sibly, 1990; Sokolova, 2013; Verberk et al., 2020), as described by the dynamic energy budget theory (Nisbet et al., 2000; Sousa et al., 2008). Thus, how stressors individually and in combination influence the energy budget at the cellular level can provide important mechanistic insights of their impact on organismal fitness. De Coen and Janssen (1997) proposed a valid biomarker, the cellular energy allocation (CEA), to estimate the net energy budget that can be used for fitness-related traits like development and reproduction. CEA is determined by integrating the energy available to use and the energy consumed (De Coen and Janssen, 2003). Specifically, the energy stored in reserve molecules (energy availability, Ea) is being divided by the energy consumed (energy consumption, Ec).

In a given period, the energy consumed by an organism is determined by its metabolic rate, and can be estimated by the consumption of O_2, the production of CO_2, or the heat produced (De Coen and Janssen, 1997). The activity of the electron transport system (ETS) based on the consumption of O_2 has been shown to be a reliable estimation of organismal metabolic rate (De Coen and Janssen, 1997). Generally, metabolic rate will increase when a stress response is activated (Hawlena and Schmitz, 2010). Metabolic rate and the associated energy consumption have been shown to be modulated by higher temperatures, where mild warming normally increases metabolic rate (e.g. Kühnhold et al., 2017; Van Dievel et al., 2017), however, extreme high temperatures can cause thermal stress and lead to metabolic depressions (Dinh et al., 2016; Storey, 2015). Exposure to pesticides has been shown to increase metabolic rate and energy consumption since pesticide-exposed organisms need more energy for detoxification (Van Dievel et al., 2019; Verheyen and Stoks, 2020).

The energy available can be estimated by integrating the energy stored in the three main energy reserve molecules: lipids, carbohydrates and proteins (De Coen and Janssen, 1997).

Lipids stored in organisms are used for long-term energy demand, they have higher caloric content per unit of mass and are considered to be the most important energy reserve in invertebrates (Arrese and Soulages, 2010; Azeez et al., 2014). Carbohydrates are used by organisms for short-term energy storage, they are mainly stored in the form of glycogen and can be rapidly converted into glucose serving as a vital compound for all biological activities (Steele, 1982; Sterner and Elser, 2002). When the above two reserves are not enough to support the energy demand, proteins can also be broken down to generate more glucose (Hawlena and Schmitz, 2010). Both pesticides and warming have been shown to decrease energy reserves (for pesticides: e.g. Verheyen et al., 2019; for warming: e.g. Janssens et al., 2014a).

3.4 Antipredator behaviours

The aquatic mosquito larvae are prey for many species at higher trophic levels, such as larval damselflies and backswimmers, and have evolved several antipredator behaviours to avoid being detected by their predators (Becker et al., 2010). Larvae of *Culex* mosquitoes spend most of their time at the water surface to obtain oxygen via an air tube located at the end of their body (Corbet et al., 2000). They respond to a sudden light interruption by diving to the bottom, which has been regarded as an alarm escape response to avoid being attacked by predators, like the backswimmer *Notonecta glauca* (Futami et al., 2008; Reynaldi et al., 2011). A strong ability of predator avoidance has been associated with a higher proportion of larvae showing the diving response (responsiveness) and a longer diving time before resurfacing (Futami et al., 2008; Reynaldi et al., 2011).

Both the responsiveness and diving time have been shown to be modulated by pesticides and warming. For example, it has been shown that the pyrethroid pesticide fenvalerate can decrease the diving responsiveness (Reynaldi et al., 2011), and both chlorpyrifos and warming can reduce the diving time (Tran et al., 2019) in the study species.

4. Research aims and outline

This thesis focuses on the combined effects of global warming and pesticide exposure within and across generations on the mosquito species *Culex pipiens*, with special attention to temporal exposure scenarios. Specifically, I studied the roles of sequential exposure to transient stressors (e.g. heat extremes and pesticide exposure), and of the exposure duration to a stressor (within

and across generations). In this thesis, four ecologically relevant temporal exposure scenarios were investigated with following research questions (Figure 8): (1) when mosquito larvae are exposed to a heat spike followed by a pesticide exposure with a time lag between them, whether the delayed effects of the preceding heat spike affect the sensitivity of survivors to the pesticide; (2) when the heat spike and pesticide exposure are in close succession, how they interact and whether the type and strength of the interaction will change when the exposure order is reversed; (3) whether differences in the exposure duration to warming will differently modulate the impact of pesticides under warming; (4) based on research questions 1 and 2, whether the parental exposure to a heat spike and pesticide exposure can influence the overall fitness of offspring, and/or their ability to deal with these stressors.

Based on the number of mosquito generations involved in each research question, this thesis was divided into two parts: part 1, which focused on the combined effects of successive exposure to a heat spike and pesticide within a single generation, mainly addressing research questions 1 and 2; part 2, which focused on the combined effects of warming (including heat spikes) and pesticide exposure across generations, addressed research questions 3 and 4. I here studied transgenerational effects in the broad sense, also called multigenerational effects, where I tested for exposure of the parental generation on effects in the offspring (F1) generation.

Part 1: The combined effects of a heat spike and pesticide exposure within a single generation

Part 1 consists of two chapters, both of which are about the sequential exposure to a heat spike and the pesticide chlorpyrifos within a single generation. The difference is that in chapter 1 there was a long time lag between the two stressors, while in chapter 2 they were in close succession with a special focus on the exposure order.

In chapter1, I tested how the delayed effects of a heat spike may affect the sensitivity of mosquito larvae to the subsequent exposure to chlorpyrifos. Both the lethal and sublethal effects were quantified on life history variables (mortality and growth rate), heat tolerance and physiology. This allowed not only testing both the CITS and the TICS concepts but also their potential interdependence. Specifically, I tested the effects of exposure to a lethal heat spike in the first larval (L1) stage, and subsequently to an ecologically relevant lethal pulse exposure to the pesticide chlorpyrifos in the final larval (L4) stage. The delayed mortality (after the heat spike finished) was also recorded, as this might help explain the interactive effects with the

subsequent chlorpyrifos exposure.

In chapter 2, I tested how a heat spike interacts with the exposure to chlorpyrifos when they are in close succession, and whether the type and strength of the interaction will change when the exposure order is reversed. Specifically, the mosquito larvae were exposed to a heat spike in the L4 stage directly followed by an exposure to chlorpyrifos, or they were first exposed to chlorpyrifos followed by the heat spike. For both the heat spike and chlorpyrifos exposure, three levels were included: control, low (causing no or minor mortality), and high (causing higher mortality) concentrations, allowing to compare the effects in the absence and presence of any survival selection. Moreover, both lethal and sublethal effects on growth, heat tolerance and physiology were studied, which allowed to assess order effects on both the CITS and TICS concepts, as well as their potential interdependence, and to provide insights in the underlying mechanisms.

Part 2: The combined effects of warming and pesticide exposure across generations

This part consists of three chapters, all of which involve transgenerational effects. The main topics of this part are about how the impact of pesticide under warming may be modulated by the exposure duration to warming (chapters 3 and 4), and about how the exposure history of parents with stressors may transmit to their offspring (chapter 5).

In chapters 3 and 4, I investigated whether and how the duration of exposure to warming can predictably modify pesticide toxicity in a two-generation experiment. I thereby contrasted the effects of acute, developmental and transgenerational warming on the toxicity of chlorpyrifos under warming in mosquito larvae. In chapter 3, I quantified the effects on life history variables (mortality and growth rate) and physiology, and provided mechanistic understanding for the observed pattern using a bioenergetic perspective. In chapter 4, I quantified the effects on two other fitness-related variables: heat tolerance and antipredator behaviour.

In chapter 5, I conducted a two-generation experiment in mosquitoes to test the within- and transgenerational single and combined effects of a heat spike and chlorpyrifos exposure. Specifically, I 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 (F1) generations with a full factorial design. I thereby tested the legacy from exposure to a stressor in the parents

on the fitness of the offspring in general and their tolerance to these stressors with special attention for the interactive effect between both stressors. Effects were quantified on life history variables (mortality, growth rate, developmental time, female wing length, composite index of population performance *r'* as an estimate of population growth), heat tolerance and physiology.

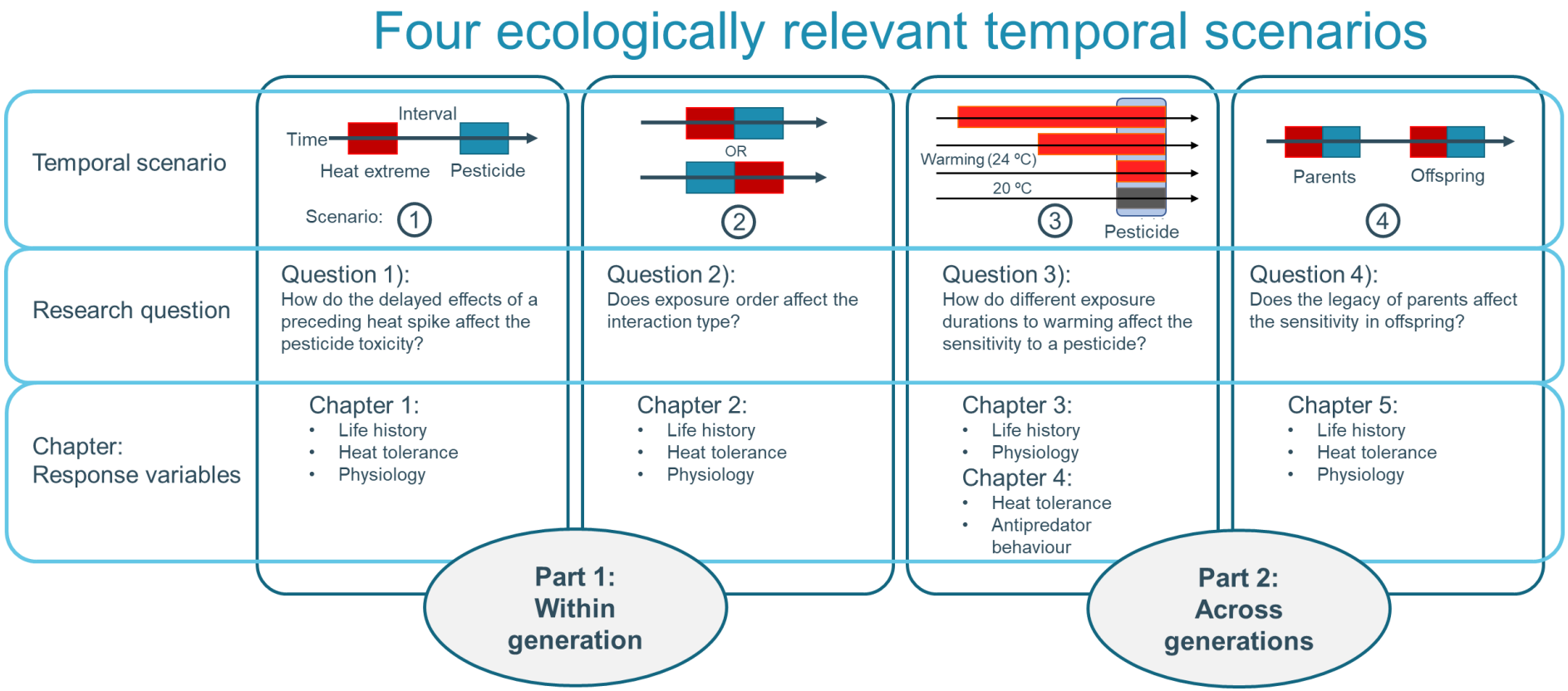
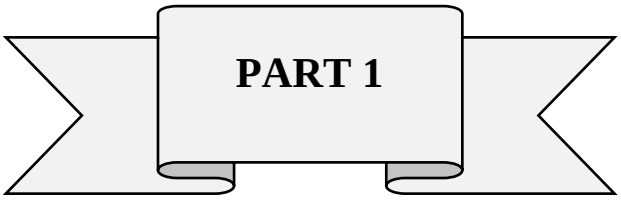


Figure 8. Overview of the five data-based chapters of my thesis with the associated temporal exposure scenarios and research questions. The stressors involved are mild warming, heat extremes and the pesticide chlorpyrifos. The thesis consists of two parts: part 1 only considers within-generation effects while part 2 also considers across-generation effects of warming and pesticide exposure. The single and combined effects of both stressors were estimated in terms of life history, heat tolerance, physiology and antipredator behaviour.



THE COMBINED EFFECTS OF A HEAT SPIKE AND PESTICIDE EXPOSURE WITHIN A SINGLE GENERATION

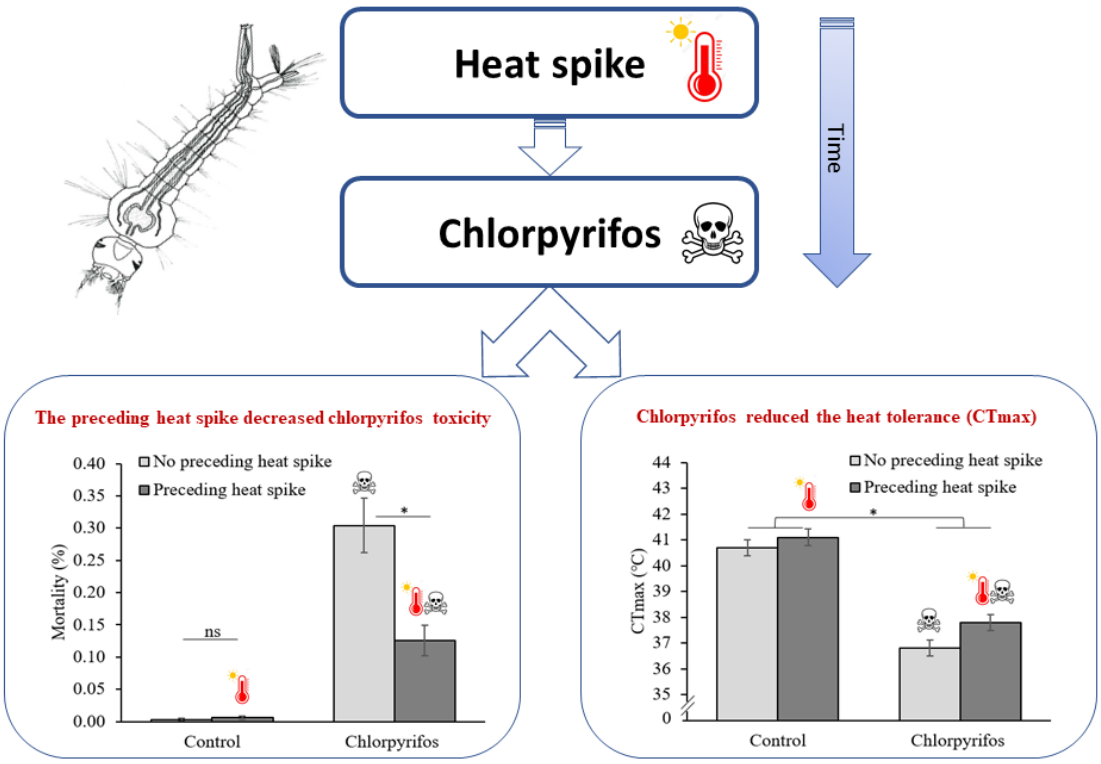
Chapter 1

Mosquito larvae that survive a heat spike are less sensitive to subsequent exposure to the pesticide chlorpyrifos

Shandong Meng, Vienna Delnat and Robby Stoks

Environmental Pollution (2020), 265: 114824

Slightly adapted version



Abstract

While extreme high temperatures are an important aspect of global warming, their effects on organisms are relatively understudied, especially in ecotoxicology. Sequential exposure to heat spikes and pesticides is a realistic scenario as both are typically transient stressors and are expected to further increase in frequency under global warming. We tested the effects of exposure to a lethal heat spike and subsequently to an ecologically relevant lethal pulse exposure of the pesticide chlorpyrifos in the larvae of mosquito *Culex pipiens*. The heat spike caused direct and delayed mortality, and resulted in a higher heat tolerance and activity of acetylcholinesterase, and a lower fat content in the survivors. The chlorpyrifos exposure caused mortality, accelerated growth rate, and decreased the heat tolerance and the activity of acetylcholinesterase. The preceding heat spike did not change how chlorpyrifos reduced the heat tolerance. Notably, the preceding heat spike did lower the lethal effect of the pesticide, which makes an important novel finding at the interface of ecotoxicology and global change biology, and adds a new dimension to the "climate-induced toxicant sensitivity" (CITS) concept. This may be due to both survival selection and cross-tolerance, and therefore a widespread phenomenon. Our results emphasize the importance of including extreme high temperatures as an important transient global change stressor in ecotoxicology.

Keywords: Climate change, cross-tolerance, multiple stressors, "climate-induced toxicant sensitivity" (CITS) concept, "toxicant-induced climate change sensitivity" (TICS) concept

Introduction

A recent key focus of ecotoxicology is how to study and integrate interactive effects of toxicants with natural stressors (Liess et al., 2019; Van den Brink et al., 2019). A specific challenge for risk assessment of toxicants in a warming world is to evaluate the combined impact of toxicants and warming-related stressors (Moe et al., 2013; Landis et al., 2013). The increasing number of studies at the interface of global warming biology and ecotoxicology resulted in the identification of two widely supported concepts. Firstly, the vulnerability of organisms to toxicants can be altered by climate change factors, named the "climate-induced toxicant sensitivity" (CITS) concept (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015). The general pattern is that increases in mean temperature can make many pesticides more toxic (De Silva et al., 2009; Dinh Van et al., 2014; Verheyen et al., 2019, Tran et al., 2018). Secondly, exposure to toxicants can also change the sensitivity of organisms to climate change, named the "toxicant-induced climate change sensitivity" (TICS) concept (Noyes et al., 2009; Moe et al.,2013; Hooper et al., 2013). In line with this, toxicant exposure generally lowers the heat tolerance (e.g. Patra et al., 2007; Janssens et al., 2018; Op de Beeck et al., 2018a; Delnat et al., 2019b; Quiroga et al., 2019). Moreover, both concepts may be interrelated as warming may modify the strength of the TICS patterns (Op de Beeck et al., 2017b).

Compared to increases in mean temperature, there has been much less studies that explicitly looked at the effects of extreme high temperatures, both in global warming biology and in ecotoxicology. Nevertheless, temperature extremes are expected to have an increased frequency and intensity under global warming (Hansen et al., 2012; IPCC, 2021; Rahmstorf and Coumou, 2011; Arambourou et al., 2017). In addition, dealing with temperature extremes may be more important for survival than dealing with mean temperature increases under global warming (Bauerfeind and Fischer, 2014; Dinh et al., 2016; Thompson et al., 2013; Vasseur et al., 2014; Ma et al., 2015). An important difference with increases in mean temperature is that extreme temperatures are often lethal (Stillman 2019) and are transient. As such, they resemble pesticide pulses, and may affect the toxicity of pesticides differently than continuous exposure to warming.

Studying the impact of multiple transient stressors asks for explicitly considering the temporal component of exposure (Gunderson et al. 2016; Ashauer et al., 2010). The relatively few studies on the combined impact of extreme temperatures and pesticide exposure co-exposed animals to both transient stressors simultaneously (Jacquin et al., 2019; overview in

Holmstrup et al., 2010, but see e.g. Dinh et al., 2016). Yet, given the transient nature of both exposure to heat spikes and to pesticides, and given agricultural practices (Defra et al., 2006), successive exposure is much more likely in nature. When transient stressors are imposed successively, it might increase the negative effect of the second stressor because animals might not yet have recovered from the first stressor (Ashauer et al., 2017). However, this may not always be the outcome for two reasons. Firstly, survival selection imposed by the first applied stressor may remove the weakest animals and the survivors may therefore be more tolerant to the second applied stressor compared to animals that did not experience the first stressor (Heckwolf et al., 2018). Secondly, cross-tolerance may occur whereby activation of the defensive system to the first stressor may lead to a better handling of the subsequent stressor (Gunderson et al., 2016; Kaunisto et al., 2016; Todgham and Stillman, 2013).

In this study, we tested the effects of a heat spike and subsequent pesticide exposure on the aquatic larvae of a mosquito. We quantified both lethal effects and sublethal effects on life history, heat tolerance and physiology. This allowed not only testing both the CITS and the TICS concepts but also their potential interdependence. As study organism, we chose the mosquito *Culex pipiens* (Linaeus, 1758) form *molestus*, which is an ubiquitous domestic mosquito species in the urban areas of northern Europe and the United States (Paz, 2015; Fonseca et al., 2004). This mosquito lives in shallow water bodies where temperature extremes occur frequently (Jacobs et al., 2008). Mosquito larvae play a key role as food in aquatic food webs (Becker et al., 2010). As pesticide we used chlorpyrifos, a globally used pesticide (although it has recently been banned or its use restricted in several countries), which is a priority substance in the European Water Framework Directive (2000/60/EC) (Johnson et al., 2017). Chlorpyrifos is one of the model pesticides for the group of the organophosphates and has a stronger impact on aquatic insects at a higher temperature (e.g., Dinh Van et al., 2014; Dinh et al., 2016; Tran et al., 2018). We expected exposure to chlorpyrifos to reduce heat tolerance (TICS concept, Noyes et al., 2009; Moe et al.,2013; Hooper et al., 2013). In case of survival selection and/or cross-tolerance (Gunderson et al., 2016; Kaunisto et al. 2016; Todgham and Stillman, 2013) we expected a lethal heat spike to reduce the toxicity of chlorpyrifos.

We quantified three important fitness-related physiological variables that respond to chlorpyrifos exposure. We estimated the activity of the enzyme acetylcholinesterase (AChE), as this is the target enzyme that is inhibited by organophosphate pesticides such as chlorpyrifos. AChE plays a vital role in maintaining the normal nerve functions (Domingues et al., 2010).

The inhibition of AChE results in an accumulation of the neurotransmitter acetylcholine, which can cause the continuous stimulation of the muscles and eventually lead to paralysis and death (Fukuto, 1990). We measured the total fat content, the major energy storage in insects (Azeez et al., 2014) that is important for adult fitness (Arrese and Soulages, 2010). Chlorpyrifos is known to reduce the fat content in insects (e.g. Dinh et al., 2016). Finally, we quantified the malondialdehyde (MDA) level as an estimate of oxidative damage to lipids. Pesticides such as chlorpyrifos can cause oxidative damage (e.g. lipid peroxidation) (Kavitha and Rao, 2008), which in its turn may reduce fitness (Monaghan et al., 2009; Valavanidis et al., 2006).

Materials and methods

For the experiment we used *C. pipiens* from a continuous culture that has been kept in the laboratory for ca. 3 years before starting the experiment. The mosquito culture was housed at 20 ℃, the mean temperature in summer in the shallow ponds of origin of the lab culture in Germany (see Tran et al., 2016).

Experimental setup

A full factorial experiment was set up with two levels of a heat spike treatment (presence and absence) crossed with two levels of a pesticide treatment (presence and absence). We first exposed 24 h old L1 larvae for two days to the heat spike treatment. Afterwards, when they moulted into the final L4 stage (ca. 6-7 days later), the two-day pesticide exposure was started (see figure 1). The L4 stage was chosen for the pesticide exposure based on the guidelines by WHO (2005) as this is typically the most tolerant larval stage. Any effects in this stage would therefore also be present, and likely stronger in the younger larval instars. For chlorpyrifos, this has been shown in another dipteran (Buchwalter et al., 2004). We started 50 replicate jars (each with 40 larvae) at each of the two heat spike treatments (total of 100 jars and 4,000 larvae). To impose the heat spike, larvae were exposed to 30 ℃ for 48 h. The jars containing larvae were first heated to 26 °C in ~2.5 h, and kept at this temperature for another 1 h, then heated to 30 °C in ~1.5 h. This matches a ramping rate of ~2 °C per h, which reflects a rapid increase in temperature associated with heat spikes in shallow lakes and ponds (Cambronero et al., 2018). Temperature in shallow ponds in Germany inhabited by the study species may rise up to 30 ℃ for several days in summer (German Climate Data Centre, https://www.dwd.de/EN/climate_environment/cdc/cdc_node.html).

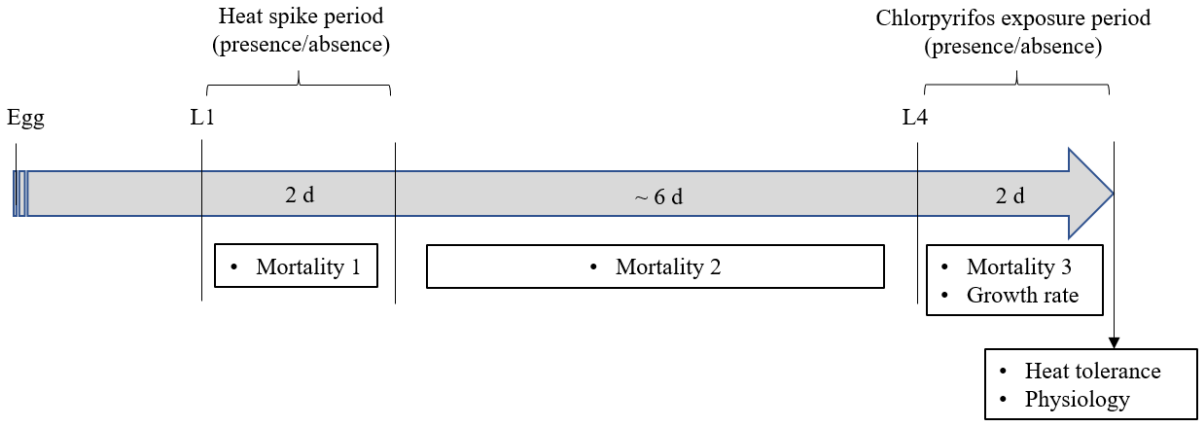


Figure 1. Scheme of the full factorial experimental design with the timing of the quantification of the response variables. During the heat spike period L1 (= first instar) larvae were exposed to a 30 °C heat spike or not. During the chlorpyrifos exposure period L4 (= fourth instar) larvae were exposed to 0.35 µg/L chlorpyrifos or a solvent control. Mortality was separately quantified for three different periods. Mortality 1 refers to the mortality during the 2-d heat spike period. Mortality 2 refers to the delayed mortality between the end of the heat spike and the start of the chlorpyrifos exposure period. Mortality 3 refers to the mortality during the 2-d chlorpyrifos exposure period.

For selecting the suitable concentration of chlorpyrifos, a range finder was conducted at 20 °C with following concentrations tested: 0 (ethanol solvent control), 0.10, 0.20, 0.25, 0.30, 0.32, 0.35, 0.37, 0.40, 0.42, 0.45, 0.50, 0.60, 0.70, 0.80 and 0.90 µg/L. Two pesticide pulses were given during the 2-day chlorpyrifos exposure period: at the start and 24 h later when the medium was refreshed. We chose a nominal concentration of 0.35 µg/L as this concentration caused a mild ~18% mortality (see results in Appendix A). This allowed to detect the lethal effect of the combination of the two stressors and guaranteed enough survivors to test heat tolerance and physiology. This concentration of chlorpyrifos is ecologically relevant since it is within the range measured in European surface waters: 95% CI = [0.07 µg/L, 0.69 µg/L] (Stehle and Schulz); and the pulse concentrations can reach maximum 100 µg/L in edge-to-field water bodies (Bernabò et al. 2011).

Chlorpyrifos (purity grade > 99%) was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). By dissolving chlorpyrifos powder in absolute ethanol, a stock solution of 0.50 mg/mL was made and stored in a cold room (4 °C) in the dark. From this solution, a secondary stock solution of 10 µg/mL was prepared in MilliQ water. All pesticide treatments, as well as

the solvent control, contained 0.70 µL/L ethanol. We took three pooled samples of 5 experimental jars both at the start and after 24 h to determine the chlorpyrifos concentrations using LC-MS/MS. The measured initial chlorpyrifos concentration in those experimental jars was 0.181 ± 0.008 µg/L (mean ± SE, N = 3 pooled samples), and the concentration after 24 h was 0.041 ± 0.008 µg/L (N = 3).

Experimental procedure

The experiment was started with 80 egg clutches collected in a single day. Egg clutches were placed in 100 mL plastic cups with 80 mL water in a climate-controlled room at 20 °C. Within 24 h after they hatched, sets of 40 first instar larvae were pooled and placed in 210 mL jars with 125 mL dechlorinated tap water (total of 100 jars). To impose the heat spike treatment, 50 jars were randomly selected and moved to the water bath at 30 °C for 48 h, the other 50 jars were kept at 20 °C. Thereafter, the medium was refreshed and all jars were placed in the climate-controlled room at 20 °C and kept there till the end of the experiment. From then onwards, the medium was refreshed every three days until the larvae entered the L4 stage.

When the larvae entered L4, the 2-day chlorpyrifos-exposure period started. Not all larvae of the same jar moulted into L4 at the same day. The newly moulted L4 larvae (within 24 h) from all jars of the same heat spike treatment were daily collected and transferred to a white tray filled with 5 L dechlorinated tap water. From this daily pooled set of L4 larvae, we randomly composed sets of 30 in the same kind of glass jars containing 125 mL of chlorpyrifos or solvent medium. This approach ensured that all larvae had the same age in each jar when the chlorpyrifos exposure period started. For the larvae that had not experienced a heat spike, we ran 21 replicate jars per pesticide treatment. For the larvae that had experienced a heat spike, we ran 19 replicate jars per pesticide treatment (because of mortality caused by the heat spike). As a result, the total number of jars during this step of the experiment was 80 (total of 2,400 larvae). Throughout the experiment, the larvae were given per day and per larva 0.313 mg of a mixture of Supradyn® vitamins (3%), wheat germs (51%) and Olvarit® 7 cereal flakes (46%). This ensures food was not limiting (Beketov and Liess, 2007).

After the chlorpyrifos exposure, two sets of five pooled larvae per jar were weighed, and stored in a -80 °C freezer for physiological measurements. After blotting the larvae dry, we took the wet masses to the nearest 0.01 mg (electronic balance, AB135 S, Mettler Toledo, Zaventem, Belgium). Another four larvae per jar were directly used for individual measurement of the heat tolerance (in a few jars more than four larvae were measured).

Life history

After the heat spike period, the number of larvae that died per jar was recorded to measure the mortality during the heat spike (mortality 1 in figure 1) . When the first L4 larva was observed, the number of larvae that died in each jar was recorded again to assess possible delayed effects of the heat spike (mortality 2 in figure 1) . Finally, we determined mortality during the 2-day chlorpyrifos-exposure period (mortality 3 in figure 1). We expressed mortality per jar by the total number of larvae that died divided by the initial number of larvae for a given period.

The growth rate was measured during the chlorpyrifos exposure period. To determine the start mass (M1), we took the average wet mass of five pooled larvae that freshly moulted into the L4 stage. As end mass (M2), we used per jar the mean mass of two sets of five pooled larvae; these sets were later used for physiology. This resulted in two growth rate estimates per jar. Growth rates were estimated by the following formula: (ln(M2) – ln(M1)) / 2 days.

Heat tolerance

The heat tolerance was estimated as the critical thermal maximum (CTmax) following the protocol of Op de Beeck et al. (2017). CTmax is considered a good parameter to evaluate the ability of organisms to deal with warming (Huey et al., 2012). To measure the heat tolerance, larvae were individually heated up till the temperature (CTmax) when they floated at the water surface motionlessly and did not respond to a gentle tap by a plastic pipette (based on Lutterschmidt and Hutchison, 1997; Verberk and Bilton, 2011; Delnat et al., 2019b). For this, we moved the larvae individually to the same type of transparent plastic jars containing 50 mL dechlorinated tap water. Jars were kept floating in an aquarium that contained distilled water. The initial water temperature in these plastic jars was 20 °C. A heating rate of 0.3 °C per minute was generated by a heater (TC120 optima immersion thermostat, Cambridgeshire, UK). This rate is within the used range for quantifying the CTmax of aquatic invertebrates (e.g. Verberk et al., 2011; Dallas et al., 2012), and suitable for larvae to track the temperature change but avoids acclimation during the trials (Verheyen et al., 2019).

When the CTmax was reached, the larva was immediately removed to 20 °C to recover. Only five (= 1.52%) larvae did not recover within 20 minutes; these were considered dead and were not used in the analyses. The larvae that recovered after the measurement were weighed to the nearest 0.01 mg to mass-correct the CTmax values.

Physiology

We measured three physiological traits based on the two pooled sets of five larvae per jar: the acetylcholinesterase (AChE) activity which is the target enzyme of chlorpyrifos (Fukuto, 1990; Domingues et al., 2010), the total fat content as measure of long-term energy storage in insects (Azeez et al., 2014), and lipid peroxidation as estimate of oxidative damage to lipids (Monaghan et al., 2009).

Physiological traits were measured using spectrophotometry following established protocols for mosquito larvae (Delnat et al., 2019a). Each set of five larvae was first homogenized in phosphate buffer saline (PBS-buffer, 50 mM, pH 7.4) with a body-mass adjusted volume (wet mass × 10 µL/mg) and then centrifuged to obtain the supernatant which was used for all physiological measurements. The AChE activity was determined based on a modified version of the Ellman method (Jensen et al., 1997). The total fat content was quantified based on a modified protocol of Marsh and Weinstein (1966). The level of malondialdehyde (MDA) was measured as an estimate of lipid peroxidation using a modified protocol of the thiobarbituric acid reactive substance (TBARS) assay following Miyamoto et al. (2011). Detailed protocols can be found in Appendix B.

Statistical analyses

We used R v3.6.1 (Core Team R, 2019) to analyse the data. The packages used were drc v3.0-1 (Ritz et al., 2015), lsmeans v2.30-0 (Lenth, 2016), afex v0.25-1 (Singmann et al., 2017), lme4 v1.1-21 (Bates et al., 2015), and car v3.0-3 (Fox and Weisberg, 2018).

The effect of heat spike exposure on mortality (binary response variable: dead vs alive) during the simulated heat spike period in the L1 stage and during the next period (the post-heat spike period until the first L4 larvae appeared in the jars) was tested using generalized linear mixed models (GLMM) with a binomial error distribution and the logit-link function. Similarly, the effects of a previous heat spike (present vs absent) and pesticide exposure (present vs absent) on mortality (binary response variable) during the 2-day pesticide exposure period in the L4 stage were tested using a generalized linear mixed model (GLMM) with a binomial error distribution and the logit-link function. Rearing jar and the start date of pesticide exposure were included in the model as random factors.

The effects of a previous heat spike (present vs absent) and pesticide exposure (present vs absent) on growth rate during the pesticide exposure period and on heat tolerance (CTmax)

were tested using general linear mixed models. Because we had several estimates of these variables per jar, we added jar as a random factor to avoid pseudoreplication. For CTmax, the mass of larva was added as a continuous covariate. The effects of a previous heat spike and the pesticide exposure on the three physiological response variables were tested using general linear models.

Results

Life history

Mortality during the 2-day heat spike period in the L1 stage was >3 times higher in the presence of the heat spike (mean ± SE: 7.1 ± 0.9%) than in its absence (2.1 ± 0.4%) (χ_1^2 = 32.0, P < 0.001). In addition, mortality during the post-heat spike period (until the first L4 was detected) was >5 times higher in larvae experiencing a previous heat spike (21.2 ± 1.1%) compared to those without a heat spike treatment (4.1 ± 0.5%) (χ_1^2 = 187, P < 0.001).

During the 2-day chlorpyrifos-exposure period, mortality was higher in larvae exposed to chlorpyrifos (main effect CPF, Table 1, Figure 2A). Moreover, chlorpyrifos-induced mortality was ~2.5 times higher in larvae not pre-exposed to a heat spike (30.4 ± 4.2%), compared to those pre-exposed to the heat spike (12.6 ± 2.4%), as also indicated by the marginally non-significant CPF exposure × Heat spike interaction (P = 0.051, Table 1, Figure 2A). Previous heat spike exposure did not show a main effect on mortality during the chlorpyrifos-exposure period (Table 1, Figure 2A).

The larvae exposed to chlorpyrifos grew ~16% faster than those of the solvent control (Table 1, Figure 1B). Previous heat spike exposure had no effect on the growth rate, neither did it interact with the chlorpyrifos effect (Table 1, Figure 2B).

Heat tolerance (CTmax)

Chlorpyrifos exposure decreased the CTmax with ~9% from 40.9 °C to 37.3 °C (main effect CPF: Table 1, Figure 3). Larvae that had experienced the heat spike had a ~2% higher CTmax (main effect Heat spike: Table 1, Figure 3). No significant interaction was detected between the heat spike and chlorpyrifos treatments (Table 1, Figure 3).

Physiology

Chlorpyrifos exposure decreased the activity of AChE with ~60% (Table 1, Figure 4A). Previous heat spike exposure caused an increase with ~43% in the activity of AChE (Table 1, Figure 4A). No significant interaction was observed between the heat spike and chlorpyrifos treatments for AChE (Table 1, Figure 4A). Previous heat spike exposure decreased the total fat content with ~12% (Table 1, Figure 4B). No effect of chlorpyrifos exposure or an interactive effect with the heat spike treatment was detected for total fat content (Table 1, Figure 4B). Previous heat spike exposure and chlorpyrifos did not affect the MDA levels (Table 1, Figure 4C).

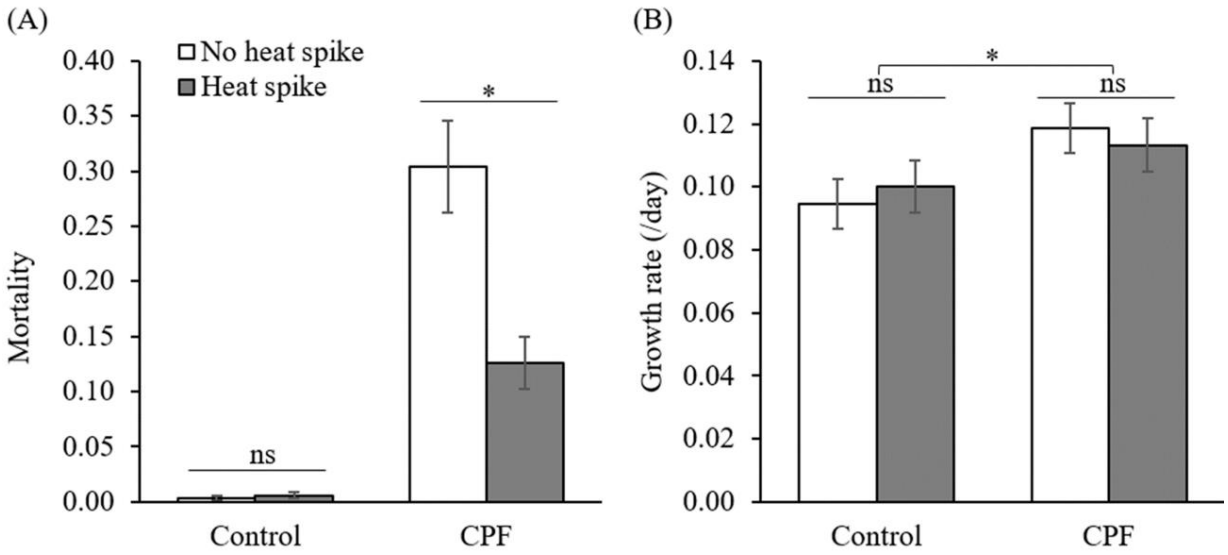


Figure 2. Effects of preceding heat spike exposure and exposure to the pesticide chlorpyrifos (CPF) on the two life history variables during the 2-day pesticide exposure period: (A) mortality, and (B) growth rate. Means are given with standard error. P-values associated with contrast analyses are coded as follows: * $P < 0.05$, ns $P > 0.05$.

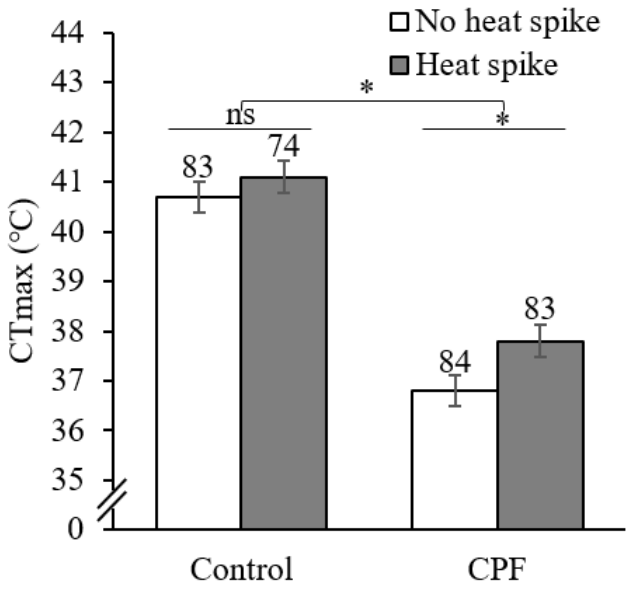


Figure 3. Effects of preceding heat spike exposure and exposure to the pesticide chlorpyrifos (CPF) on heat tolerance (CTmax). Means are given with standard error. The numbers of larvae measured are shown above the bars. P-values associated with contrast analyses are coded as follows: * $P < 0.05$, ns $P > 0.05$.

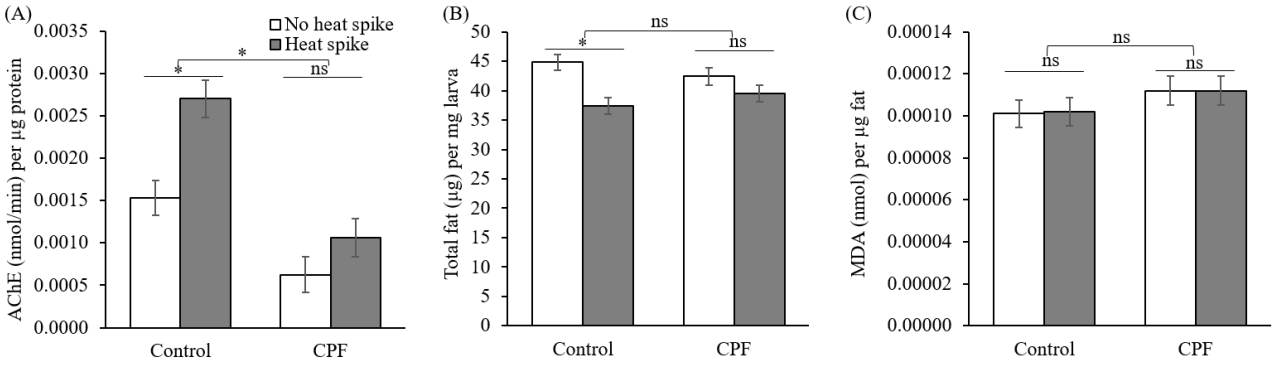


Figure 4. Effects of preceding heat spike exposure and exposure to the pesticide chlorpyrifos (CPF) on the three physiological variables: (A) AChE (acetylcholinesterase) activity, (B) total fat content, and (C) MDA (malondialdehyde) level. Means are given with standard error. P-values associated with contrast analyses are coded as follows: * $P < 0.05$, ns $P > 0.05$.

Table 1. The effects of previous heat spike exposure and exposure to the pesticide chlorpyrifos (CPF) on two life history traits, heat tolerance (CTmax) and three physiological traits. Bold *P*-values represent significant effects ($P < 0.05$).

Effect	Mortality			Growth rate			CTmax		
	χ^2	Df	P	χ^2	Df	P	χ^2	Df	P
Heat spike	0.225	1	0.635	0.000	1	0.998	5.43	1	**0.020**
CPF	82.5	1	**< 0.001**	5.19	1	**0.023**	122	1	**< 0.001**
Heat spike × CPF	3.81	1	0.051	0.443	1	0.506	0.942	1	0.332
Mass							0.625	1	0.429
	AChE activity			Total fat			MDA content		
	χ^2	Df	P	χ^2	Df	P	χ^2	Df	P
Heat spike	13.8	1	**< 0.001**	14.0	1	**< 0.001**	0.014	1	0.871
CPF	34.8	1	**< 0.001**	0.011	1	0.831	2.71	1	0.066
Heat spike × CPF	2.92	1	0.070	2.73	1	0.107	0.014	1	0.964

Discussion

While both the heat spike and chlorpyrifos exposure were lethal, they also caused sublethal effects on growth rate, heat tolerance and physiological traits that were partly in opposite directions. Notably, the preceding heat spike caused less chlorpyrifos-induced mortality, making a link to the "climate-induced toxicant sensitivity" (CITS) concept (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015). Chlorpyrifos reduced the ability of dealing with extreme high temperatures as indicated by the lower CTmax, thereby supporting the "toxicity-induced climate change sensitivity" (TICS) concept (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013).

Effects of single exposure to the heat spike

Exposure to the simulated heat spike of two days at 30 °C was lethal, which is also documented in other studies (reviewed by Stillman, 2019). Too high temperatures may cause damage to macromolecules, such as proteins, and collapse of ATP synthesis capacity, resulting in deleterious effects on organisms (Harada et al., 2019; Somero et al., 2017; Stillman, 2019). The larvae exposed to the heat spike moulted to the L4 stage ~1-2 days earlier compared to those without a heat spike. This may partly buffer the negative effect of the heat spike on mortality for the resulting population growth rate. There was also delayed mortality after the exposure to the heat spike ended, which was probably associated with an energy deficit. The heat spike decreased the total fat content, which may have resulted in less energy to repair the damage caused by the heat spike. The heat spike did, however, no longer cause delayed mortality during the chlorpyrifos exposure period. A first reason for this may be that when the larvae entered the chlorpyrifos exposure period the time interval was long enough to recover from the heat spike. A second reason may be that all larvae that suffered most from the heat spike had died before the pesticide exposure period started.

The direct and delayed mortality caused by the heat spike may have imposed survival selection by removing the weakest larvae, those with the lowest body condition. Hence, the heat spike may have removed the larvae with the lowest growth rate and lowest baseline AChE activity levels. In support of this, the larvae surviving the heat spike were larger in size at the end of the heat spike exposure and entered the L4 stage ~1-2 days earlier than the larvae that did not undergo the heat spike (S. Meng, personal observation). Furthermore, animals with a lower energy content, for example reared at a low food level, indeed typically grow slower (Dinh et al., 2016) and have lower AChE activity levels (Pedersen et al., 2002). Survival

selection may therefore explain why previous heat spike exposure (i) did not reduce growth during the chlorpyrifos exposure period, and (ii) even resulted in a higher activity of AChE in the larvae that survived the heat spike. Previous studies documented a lower growth after exposure to extreme high temperatures (e.g. Dinh et al., 2016). Yet, these studies did not allow animals to recover from the extreme high temperature. Note that survival selection against the weakest larvae with the lowest body condition does not have to conflict with the observation that the survivors of the heat spike showed a lower fat content. Non-lethal extreme high temperatures are known to reduce energy storage (e.g. butterflies: Karl et al., 2011; crickets: Adamo et al., 2012; damselflies: Dinh et al., 2016), and the here observed decrease in fat content (~12%) might have been even stronger if there would have been no survival selection. In addition, there may have been a reallocation of energy storage toward investment in growth and AChE activity, and other 'defense' mechanisms (such as upregulation of heat shock proteins, see below).

As expected, the preceding heat spike increased the heat tolerance as reflected by the higher CTmax. This resembles the general pattern that higher acclimation temperatures can lead to higher upper thermal limits (Gunderson and Stillman, 2015). Yet, our results differ from most acclimation studies (e.g. Boher et al., 2012; Hu et al., 2014) as we used a transient, short-duration heat spike and there was a long time interval between the exposure to the higher temperature and the actual measurement of the heat tolerance. Higher levels of heat shock proteins (Hsp) caused by the heat spike may have contributed to the enhanced heat tolerance. Increased Hsp levels have been observed after heat stress to repair the damage to proteins (Roberts et al., 1997; Soren et al., 2018), and have been associated with enhanced heat tolerance (Dahlhoff and Rank, 2000; King and MacRae, 2015; Somero et al., 2017).

Effects of single exposure to chlorpyrifos

As expected, the used chlorpyrifos concentration increased mortality. This may be partly explained by the reduced activity of AChE, the target enzyme of organophosphates like chlorpyrifos (Domingues et al., 2010). When AChE is inhibited, it is no longer able to hydrolyze the neurotransmitter acetylcholine leading to a continuous activation of muscles and nerve cells, eventually resulting in exhaustion and spasms (Fukuto, 1990). In addition, chlorpyrifos may cause mortality by generating oxidative damage (Kumar et al., 2011; Tripathi and Shasmal, 2010). Nevertheless, we did not detect a chlopyrifos-induced rise in oxidative damage to lipids (measured as the level of MDA). This contrasts with other studies (e.g. Singh & Prasad 2018;

Hatami et al., 2019; for the study species: Delnat et al., 2019a), where chlorpyrifos did cause increased MDA levels. This may not have occurred in our study because of rapid heat hardening (Zhao and Jones, 2012), where pre-treating insects with a heat stressor can induce expression of Hsp and antioxidant enzymes (Kang et al., 2017; Zhu et al., 2017) that protect against subsequent stress (oxidative stress in our study). Furthermore, larvae may have offset the costs of detoxification processes by increasing food acquisition (Jeon et al., 2010), reducing the potential oxidative damage caused by chlorpyrifos. In apparent contrast with this lethal effect, chlorpyrifos-exposed larvae that survived had a faster growth rate. This has been shown before in *C. pipiens* (Delnat et al., 2019a) and other aquatic organisms (e.g. damselfly larvae: Janssens and Stoks, 2013b). This may be an adaptive response for semi-aquatic animals to escape from the toxic aquatic environment (Rohr et al., 2011), yet may also reflect survival selection where the weakest and slowest growing larvae were killed by chlorpyrifos.

As expected by the TICS concept (Noyes et al., 2009; Moe et al.,2013; Hooper et al., 2013), pesticide exposure reduced the heat tolerance. This has been shown before after chlorpyrifos exposure in *C. pipiens* (Delnat et al., 2019b) and in other aquatic insects (e.g. damselfly larvae: Verheyen et al., 2019). This phenomenon can be caused by the mismatch between the supply and demand of oxygen (Verberk et al., 2016) occurring at lower temperatures under chlorpyrifos exposure. This can be expected because of the combination of an increasing oxygen demand for detoxification and damage repair mechanisms (Sokolova, 2013; for chlorpyrifos: Narváez et al., 2016), and a decreasing oxygen supply caused by the impairment of respiratory functioning (for chlorpyrifos: Negro and Collins, 2017; Marigoudar et al., 2018).

Effects of chlorpyrifos after heat spike exposure

Our results suggest that a preceding heat spike made the pesticide less lethal. This contrasts with the general empirical support for the CITS concept (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015) that climate change would magnify the toxicity of many pesticides, including organophosphates. There is indeed wide support that at higher temperatures, pesticides such as chlorpyrifos get more lethal (e.g. De Silva et al., 2009; Dinh Van et al., 2014; Verheyen et al., 2019, for *C. pipiens*: Tran et al., 2018). In contrast with most previous studies, we did not co-expose the larvae simultaneously to the heat spike and the pesticide, but pre-exposed the larvae to a heat spike and then exposed all larvae to chlorpyrifos at the same mild temperature of 20 °C. As a result, the supposed mechanisms that make chlorpyrifos more toxic

under warming, such as a higher uptake and a faster conversion into the more toxic chlorpyrifos-oxon (Lydy et al., 1999; Buchwalter et al., 2003) are not expected to occur. Two non-exhaustive mechanisms may explain our finding. First, survival selection caused by the heat spike may have removed the weaker larvae, hence only relatively stronger ones remained (see above). These relatively high-quality larvae that had a higher baseline activity of AChE may have been more tolerant to chlorpyrifos. For example, a damselfly species with a higher baseline of AChE activity was less sensitive to chlorpyrifos compared to its congeneric damselfly species with a lower baseline activity of AChE (Op de Beeck et al., 2018b). Second, cross-tolerance can be another reason. When organisms deal with different stressors by using the same physiological mechanisms, the defensive system to the first stressor may lead to a better handling of the subsequent stressor (Gunderson et al., 2016; Kaunisto et al., 2016; Todgham and Stillman, 2013). Cross-tolerance to other stressors induced by heat stress has been reported (e.g. Chen and Stillman, 2012; Kellett et al., 2005; Todgham et al., 2005), yet rarely in ecotoxicology. One exception is the study by Pestana et al. (2016) reporting an increased acute tolerance to cadmium and zinc in the brine shrimp after a sub-lethal heat shock that was driven by the up-regulation of heat shock proteins. Both survival selection and cross-tolerance may have contributed to the absence of sub-lethal interactive effects between the heat spike and chlorpyrifos exposure for the traits measured in this study (growth rate, heat tolerance and the three physiological traits). Both mechanisms may have different evolutionary implications. Indeed, only survival selection is expected to erode genetic diversity, hence may reduce the ability to deal with other stressors in the future. In contrast, cross-tolerance will not affect genetic diversity, but is likely energetically costly (Feder and Hofmann, 1999) and therefore may have delayed costs, possibly even after metamorphosis.

We simulated the net numerical effect of the single and combined exposure to the heat spike and the pesticide at the population level based on the observed mortalities at the individual level. Based on the observed mortality values in the three different steps of the experiment, there would be ~94 larvae surviving at the end of the experiment in the control treatment (without heat spike and without chlorpyrifos exposure). The number of the survivors at the end of the experiment would be ~73 for the single exposure to the lethal heat spike, ~65 for the single exposure to chlorpyrifos, and ~64 larvae for the combined exposure to the lethal heat spike and chlorpyrifos. This suggests that the preceding heat spike may also reduce the effect of the pesticide pulse at the population level.

Conclusions and future directions

While extreme high temperatures are an important aspect of global warming (Hansen et al., 2012; Ma et al., 2015; Rahmstorf and Coumou, 2011), they are relatively understudied in ecotoxicology. Sequential exposure to heat extremes and pesticides is a realistic scenario as both are typically transient stressors and are predicted to further increase in frequency in a warming world (heat extremes: IPCC, 2021; pesticide exposure: Kattwinkel et al., 2011). By exposing animals to a lethal heat spike and subsequently to a lethal pesticide concentration, our results suggest an important novel pattern at the interface of ecotoxicology and global change biology: a preceding heat spike may reduce the lethal impact of a pesticide pulse. This effect may be widespread, given the two assumed underlying reasons for this interactive effect, survival selection and cross-tolerance, are considered to be general. This adds a new dimension to the CITS concept (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015). Notably, our results also suggest that preceding heat spikes may bias the outcome of ecotox tests on field-collected animals. Indeed, we may underestimate the impact of pesticides when testing animals that have experienced an extreme high temperature in nature. Consistent with the TICS concept (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013), pesticide exposure caused a lower heat tolerance. Interestingly, this was partly buffered by the preceding heat spike that caused a slightly higher heat tolerance. Despite support for both the TICS and CITS concepts, we did not find evidence both concepts were interrelated: previous exposure to the heat spike did not change how chlorpyrifos reduced the heat tolerance. More general, our findings provide further support to the importance of the temporal component of exposure to multiple transient stressors (Gunderson et al. 2016; Ashauer et al., 2010), which is still understudied in ecotoxicology (but see e.g. Ashauer et al. 2017). Our results particularly emphasize the importance of including extreme high temperatures as an important transient global change stressor in ecotoxicology. To improve insights at the interplay of heat spikes and toxicants, our current results suggest several important topics for future research: the underlying physiological mechanisms of the cross-tolerance and whether these mechanisms are transient and cause any long-term costs of cross-tolerance, the impact of the magnitude and the order of exposure, and of the time lag between exposure to a heat spike and toxicants.

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Appendix A: Range finder for Chlorpyrifos (CPF)

To select the CPF concentration used in the experiment, we first did a range finding experiment at 20 ℃ in the same climate-controlled room as in the real experiment. The following concentrations were tested to obtain the $LC_{20,48h}$: 0 (ethanol solvent control), 0.1, 0.2, 0.25, 0.3, 0.32, 0.35, 0.37, 0.40, 0.42, 0.45, 0.50, 0.60, 0.70, 0.80 and 0.90 µg/L. The stock solution of CPF was prepared in absolute ethanol at a concentration of 500 µg/mL and was stored in a cold room (4 ℃) in the dark. Then a secondary stock solution of 10 µg/mL was prepared in milliQ water. From this secondary stock solution, the final medium of different concentrations was obtained in dechlorinated tap water. The same procedure as in the real experiment was followed in the CPF exposure. Briefly, 30 larvae were exposed in each jar for 48 h. Two pesticide pulses were given in total with the first one at the start of the CPF exposure period and the second one 24 h later, respectively.

We ran 4 to 8 replicate jars for each CPF concentration. The number of dead larvae was recorded 48h later. Dose-response curve was fitted using a log-logistic function and were used to obtain the $LC_{20,48h}$ values with the 95% confidence interval using the “drm function” in the package drc v3.0-1 (Ritz et al., 2015).

The $LC_{20,48h}$ for CPF was 0.353 µg/L (SE: 0.007; 95% CI [0.339, 0.367], Figure A). Based on the results, a concentration of 0.35 µg/L was chosen for the real experiment which caused ca. 18% mortality.

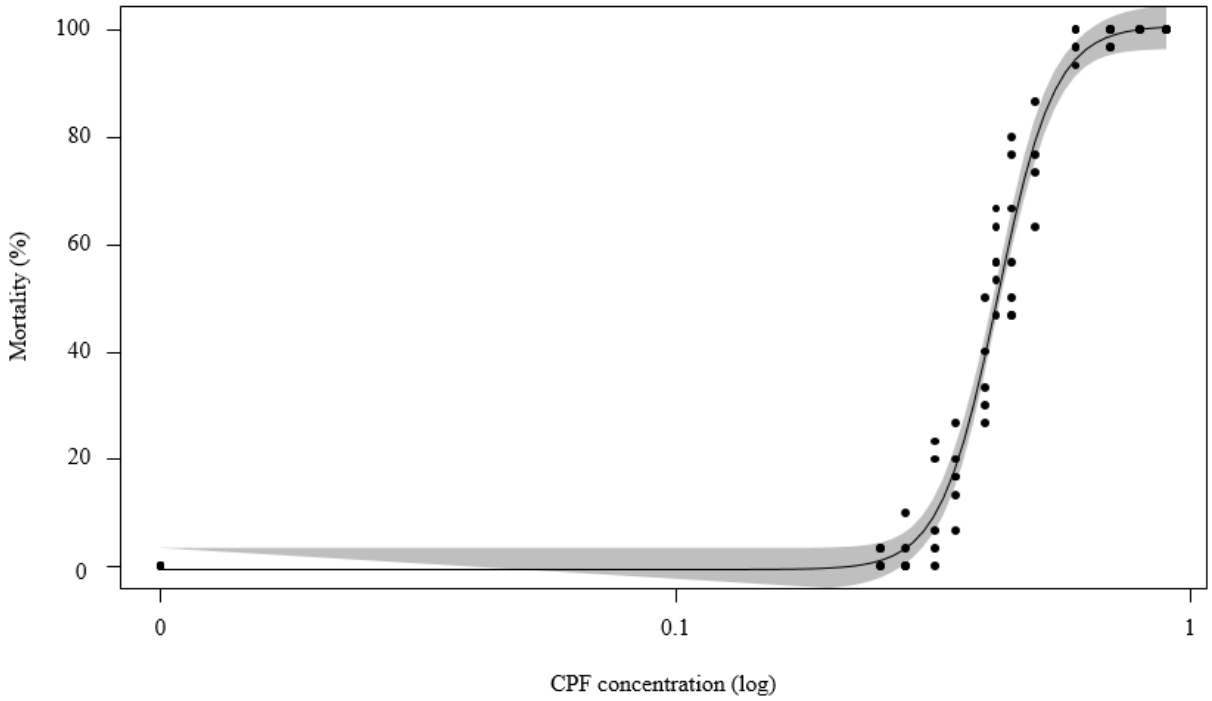

Figure A. Dose-response curve for the effect of chlorpyrifos (CPF) on mortality after 48 h in the mosquito *Culex pipiens*. The grey area gives the 95% confidence interval and the dots visualize the observed mortality percentage for a given jar (note that some dots overlap).

Appendix B: Detailed protocols of the physiological assays

Three physiological variables were measured based on the two pooled sets of five larvae per jar following the protocols of Delnat et al. (2019): the activity of acetylcholinesterase (AChE) which is the target enzyme inhibited by chlorpyrifos (Fukuto, 1990; Domingues et al., 2010), the total fat content as measure of long-term energy storage in insects (Arrese and Soulages, 2010), and lipid peroxidation as estimate of oxidative damage to lipids (Monaghan et al., 2009). We first homogenized each set of five larvae in PBS-buffer (phosphate buffer saline, 50 mM, pH 7.4) with a body-mass adjusted volume (wet mass × 10 µL/mg). To obtain the supernatants used for all physiological measurements, the homogenate was centrifuged for 7 minutes at 13,000 g (4 °C). All physiological measurements were based on spectrophotometric methods with an Infinite M200 (TECAN) plate reader.

The activity of AChE was measured in duplicate based on a modified version of the Ellman method (Jensen et al., 1997). We added 170 µL of acetylcholine iodide (mM) and 330 µL of 5,5-dithiobis-2-nitro-benzoic acid (DTNB, 3 mM) to 8.8 mL PBS-buffer. Then, we added 5 µL supernatant to 25 µL of this mixture in a 384 well plate. Next, we recorded the change in absorbance at 412 nm for 20 minutes. Following the formula of Lambert-Beer, the absorbance was converted to the concentration of AChE using an extinction coefficient of 13.6/mM × cm. The activity of AChE was expressed in nmol/min per µg protein, and both estimates per pooled sample were averaged for the statistical analyses. The protein content was measured in quadruplicate using the Bradford (1976) method.

The total fat content was measured in triplicate based on a modified protocol of Marsh and Weinstein (1966). We pipetted 56 µL of sulfuric acid (100%, H_2SO_4) to a glass tube together with 8 µL of the supernatant. We heated the tubes to 150 °C for 20 min, and then cooled them down to room temperature. Next, we added 64 µL of Milli-Q water to each tube. We added 30 µL of this mixture to a transparent 384-well plate with flat bottom and measured the absorbance at 340 nm. A standard curve was made in chloroform with glyceryl tripalmitin. Total fat content was expressed as µg per mg larva (wet mass). The mean of the three estimates per pooled sample was averaged for the statistical analyses.

The level of malondialdehyde (MDA) was measured in triplicate as an estimate of lipid peroxidation using a modified protocol of the thiobarbituric acid reactive substance (TBARS)

assay following Miyamoto et al. (2011). We heated a mixture of 40 µL supernatant with 40 µL TBA-buffer (0.4%, in 0.1 M HCl) for 1 h at 100 ℃ and cooled it down immediately on ice afterwards. Then, we added 132 µL of 1-butanol and centrifuged the mixture for 4 minutes at 3,000 g at room temperature. Next, we pipetted 30 µL of the upper phase solution from the final mixture to a 384 well microtiter plate and measured the absorbance at excitation-emission wave lengths of 535-570 nm. To calculate MDA concentrations, a standard curve was made using 1,1,3,3-tetramethoxypropan (99%) malonaldehyde bis (dimethyl acetal) (99%). The MDA level was expressed in nmol per µg fat. The mean of the three estimates per pooled sample was averaged for the statistical analyses.

Chapter 2

The exposure order strongly modifies how a heat spike increases pesticide toxicity

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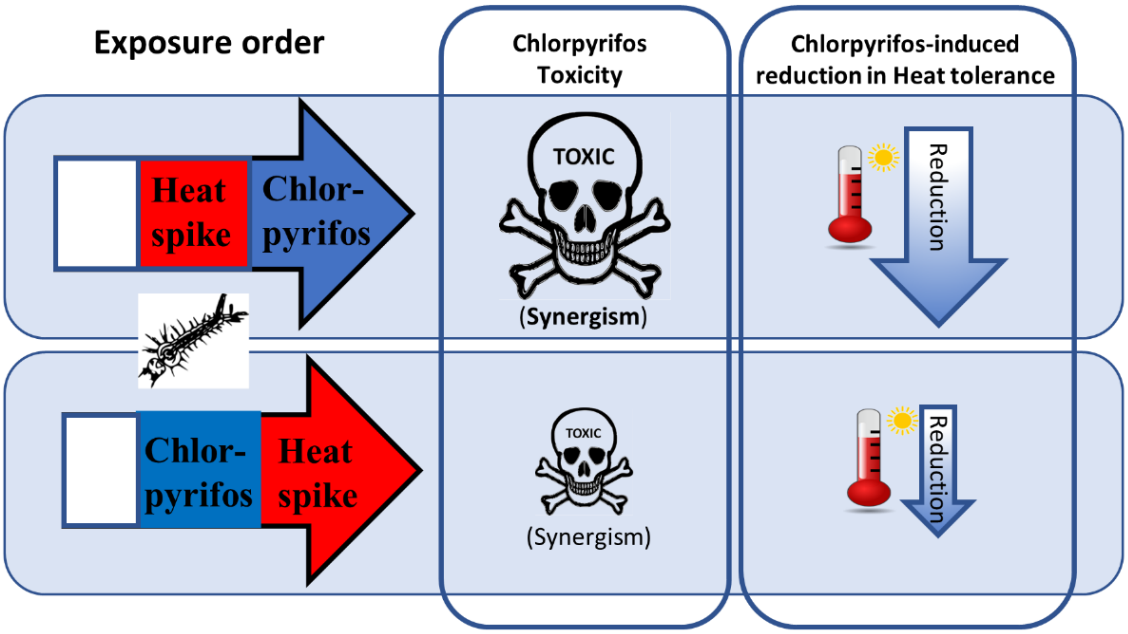
Slightly adapted version



Abstract

The exposure order may strongly affect the impact of stressors, yet is largely ignored for the frequently occurring combinations of toxicants with natural stressors. We tested how exposure order shaped the interactive effects of serial exposure to the pesticide chlorpyrifos and to a heat spike in the larvae of the mosquito *Culex pipiens*. Notably, the chlorpyrifos-induced mortality was much more magnified by the heat spike and a synergism was already detected at the low concentration when exposure to chlorpyrifos followed the heat spike. This suggests that the preceding heat spike weakened the larvae as reflected in their lower net energy budget, moreover the chlorpyrifos-induced inhibition of its target enzyme (acetylcholinesterase) was only magnified by the heat spike when it was the first stressor. Also the chlorpyrifos-induced reduction in heat tolerance was stronger when the pesticide pulse followed the heat spike, and was buffered by the heat spike when this was the second stressor. Our results provide the first evidence that the exposure order can strongly change the magnifying effect of an important climate change factor on the toxicity of a pesticide. This highlights the importance of exposure order in ecological risk assessment of toxicants under realistic combinations with natural stressors.

Keywords: Cellular energy allocation, exposure sequence, independent action model, synergisms between toxicants and natural stressors, survival selection, toxicodynamic recovery

Introduction

There is growing concern that organisms are exposed to multiple, often interacting stressors in their natural habitats (Côté et al., 2016, Gunderson et al., 2016; Orr et al., 2020). This may be an important reason why current risk assessment of pesticides fails to protect biodiversity (Beketov et al., 2013; Liess et al., 2016). Most focus is on the combined effects of simultaneous exposure to stressors. Yet, many stressors (such as pesticides) are transient, hence organisms will be sequentially exposed to them whereby the exposure order may have a strong effect on their combined impact at different levels of biological organization (Vinebrooke et al., 2004; Ashauer et al., 2017). An exposure order effect at the organismal level can be expected because after exposure to a stressor, organisms will try to re-establish homeostasis through toxicodynamic recovery (Ashauer et al., 2010), and the speed of this recovery process can differ strongly among stressors (Ashauer et al., 2017). In case of close sequential exposure, slow toxicodynamic recovery from the first stressor is likely to generate delayed effects, which may magnify the toxicity of the second stressor. Hence, the combined effects of stressors with a different toxicodynamic recovery rate may differ when the exposure order is reversed. In a milestone study in ecotoxicology, Ashauer et al. (2017) demonstrated that reversing the order of exposure to chemical toxicants with a different mode-of-action strongly changed their toxicity in the freshwater crustacean *Gammarus pulex*.

Despite the increasing interest in the temporal component of exposure to stressors (Gunderson et al., 2016, Orr et al., 2020), and the alarming insight that natural stressors may strongly magnify the toxicity of chemicals (Holmpstrup et al., 2010; Liess et al., 2016), the order of exposure has been largely ignored in the studies combining toxicants with natural stressors. Given that toxicants and natural stressors have a different mode-of-action and are often transient, their rate of induction of the negative effects and their toxicodynamic recovery rates may be different, hence exposure order effects can be expected. Just as for order effects between exposure to toxicants (Ashauer et al., 2017), order effects on how natural stressors magnify the toxicity of chemicals may be of paramount importance for ecological risk assessment.

One particularly important natural stressor in ecotoxicology is temperature. Toxicants and warming are indeed two widespread stressors known to interact with each other (Noyes et al., 2009; Moe et al., 2013; Wang et al., 2019). On the one hand, the toxicity of many toxicants increases under warming, the so-called “climate change induced toxicant sensitivity” (CITS)

concept (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015). On the other hand, the heat tolerance of organisms may be reduced by toxicants, the so-called "toxicant induced climate change sensitivity" (TICS) concept (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013). Our insights in the effects of toxicants under warming are mostly limited to studies that looked at simultaneous exposure to toxicants at a higher mean temperature (but see e.g. Dinh et al., 2016; Russo et al., 2018). Yet, heat extremes, another key aspect of global warming that may more negatively impact organisms (Ma et al., 2015), typically occur as pulses (Thompson et al., 2013; Stillman, 2019), implying that sequential exposure to toxicants (such as pesticides) and heat extremes may often be the case (Dinh et al., 2016).

In this study, we tested to what degree the exposure order shaped how a heat spike magnified the toxicity of a pesticide pulse in a mosquito. We studied both lethal and sublethal effects on growth, heat tolerance and physiology. This allowed us to assess order effects on both the CITS and TICS concepts (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015), as well as their potential interdependence (Op de Beeck et al., 2017), and to provide insights in the underlying mechanisms. We studied this in the mosquito *Culex pipiens* form *molestus* (Forskal, 1775), a common mosquito species in Europe and the United States (Fonseca et al., 2004; Paz, 2015). The aquatic larvae of this mosquito inhabit shallow ponds and lakes where heat extremes can occur frequently in summer (Jacobs et al., 2008). We tested effects of the pesticide chlorpyrifos that is listed in the top ten chemicals that have highest risk to aquatic animals (Johnson et al., 2017). This organophosphate pesticide is used worldwide (although has recently been banned or restricted to use in some countries), and has a higher lethal effect on aquatic animals at higher temperatures (Dinh Van et al., 2014; Tran et al., 2018; Verheyen et al., 2019). We expected the heat spike to magnify the toxicity of the pesticide (CITS, Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015), and the pesticide to reduce the heat tolerance (TICS, Noyes et al., 2009; Moe et al.,2013; Hooper et al., 2013). Recent studies showed strong delayed mortality in mosquito larvae after exposure to heat extremes (Meng et al., 2020a), but much less after exposure to the pesticide chlorpyrifos (Delnat et al., 2019c), suggesting a more rapid action of chlorpyrifos, and/or a longer toxicodynamic recovery after a heat spike. We therefore expected the toxicity of the pesticide to be more magnified when preceded than when followed by the heat spike.

Materials and methods

Experimental setup

We set up a full factorial experiment to test whether the exposure order alters the effects of a heat spike and a pesticide. For both stressors, we included a low level that caused no or slight mortality, and a high level that caused considerable mortality. The latter allowed to evaluate the potential effects of survival selection induced by the first stressor on the effect of the second one. Thus, the experiment consisted of 18 treatment combinations (see Figure S1 in Appendix S1 for a schematic design): 3 heat spike treatments (no, low and high level) × 3 pesticide treatments (solvent control, low and high concentration) × 2 exposure orders (exposure to heat spike first, exposure to chlorpyrifos first). Each treatment combination was replicated in 19-22 jars, each jar containing 30 larvae (total of 363 jars and 10,890 larvae). We applied a sequential exposure procedure where larvae in the fourth, final larval stage (L4) were first exposed to one stressor for 48 h, and then to the second stressor for the next 48 h. This reflects realistic exposure durations for both pulse-like stressors.

For the heat spike treatments, 20 °C was chosen as control temperature as it is the rearing temperature of the mosquito culture and represents the mean summer water temperature in the shallow German ponds where the mosquito culture originated (Tran et al., 2016). The low heat spike level was set at 30 °C. Exposure of L4 larvae to 30 °C for 48 h did not cause mortality in pre-trials. This temperature can occur for several days in shallow German ponds in summer (German Climate Data Centre, https://www.dwd.de/EN/climate_environment/cdc/cdc_node.html). As high heat spike level we used 34 °C, which caused ~16% mortality in pre-trials. Heat spikes of 34 °C are currently very rare in the region of origin of the culture, but an overall increase in the frequency and intensity of heat spikes is expected under global warming (IPCC 2021; Stillman, 2019). Both simulated 48 h heat spikes consisted of three parts: an increasing part where the temperature gradually increased from 20 °C to 30 °C (or 34 °C) in 6 h; a constant period at 30 °C (or 34 °C) for 36 h; and a decreasing part where the temperature gradually decreased to 20 °C in 6 h. The imposed temperature changes are rapid, yet realistic in shallow ponds inhabited by mosquito larvae (Cambronero et al., 2018).

Based on a range finder where L4 larvae were exposed to chlorpyrifos for 48 h at 20 °C, we chose for the low level a nominal concentration of 0.25 µg/L causing minor mortality (0.67%), and for the high level a nominal concentration of 0.35 µg/L causing ~27% mortality.

Note that in the study species, the dose-response curve for chlorpyrifos is steep (Delnat et al., 2019a; Meng et al., 2020a), hence low- and high-effect concentrations are relatively close to each other. Peak concentrations of chlorpyrifos can range between 1 µg/L and 100 µg/L in edge-to-field shallow water bodies (Bernabò et al., 2011). The solvent control contained the same amount of ethanol (3.5 µL/L) as in the high chlorpyrifos treatment. This ethanol concentration is unlikely to affect the mosquito larvae as concentrations of up to 500 µL/L did not cause effects on the larval survival and growth to the study species (Delnat et al., 2019a). The medium in the chlorpyrifos treatments was refreshed after 24 h. To avoid biases, the medium in the heat spike treatments was also refreshed after 24 h. In addition, the medium was renewed and the larvae were moved to clean jars after 48 h when switching the stressor treatments to avoid any pesticide in the medium after the pesticide treatments ended. To prepare the chlorpyrifos concentrations, we first made a stock solution of 0.1 mg/mL chlorpyrifos (purity grade > 99%) in absolute ethanol. Then, a secondary stock solution of 1.0 µg/mL was made in milliQ water. The chlorpyrifos concentrations were measured in three random sets of five pooled experimental jars per concentration using UPLC-MS/MS. The measured start concentrations were 0.191 ± 0.018 (mean ± 1 SE) µg/L for the low level, and 0.280 ± 0.026 µg/L for the high level. After 24 h (just before renewal of the medium), the measured concentrations were 0.060 ± 0.004 µg/L for the low level, and 0.073 ± 0.007 µg/L for the high level.

To obtain L4 mosquito larvae for the experiment, egg clutches were collected from the continuous lab culture and put into white trays containing aerated tap water till the larvae hatched. Both in the culture and in the experiments the photoperiod was set at 14:10 h light:dark. Sets of 100 recently (within 24 h) hatched L1 (= first larval stage) larvae from different egg clutches were pooled and placed into 2 L containers with 1 L aerated tap water at 20 °C till the start of the exposure period. When the larvae entered L4, the experiment started. The recently (within 24 h) moulted L4 larvae were collected from all 2 L containers and placed together in a white tray containing aerated tap water. Then, sets of 30 larvae were randomly grouped and placed in 210 mL experimental jars made of glass that contained 125 mL aerated tap water (for heat spike treatments) or 125 mL chlorpyrifos solution (for chlorpyrifos treatments). During the experiment, the larvae were provided with a high amount of food consisting of a mixture of Supradyn® vitamins (3%), wheat germs (51%) and Olvarit® 7 cereal flakes (46%) (0.313 mg per larva per day, Op de Beeck et al., 2016).

Life history variables

During the 4-d experiment, the number of dead larvae in each jar was recorded daily. We reported the total mortality across the 4 days in the manuscript, the mortality after exposure to the first stressor, hence after the first 2 days is presented in Appendix S2. Directly after the 4-d experiment, two sets of five larvae were collected from each experimental jar and weighed to the nearest 0.01 mg, one set was used for physiological measurements (stored at -80 °C). For growth rate, the mean wet mass of the two sets of five pooled larvae was used as the end mass (M2). At the start of the 4-d experiment, extra sets of five pooled larvae that were recently molted to L4 were weighed to the nearest 0.01 mg, and their average wet mass was used as the start mass (M1). Based on this, we calculated a single value for growth rate per jar across the 4-d experiment as [ln(M2) – ln(M1)]/4 d.

Heat tolerance

We measured CT_{max} (critical thermal maximum) as an estimate of heat tolerance following the method of Meng et al. (2020a). At the end of the 4-d exposure period, larvae were randomly selected from each experimental jar for the CT_{max} measurement. We heated the larvae in individual vials, and the temperature at which the larvae started floating motionlessly and did not react to a gentle disturbance was recorded as the CT_{max} (details in Appendix S3). Where possible we tested 3 larvae per jar, but because of mortality the number per jar was sometimes lower (only in ca. 10% of the total amount of jars; see details Appendix S3). The total number of tested larvae was 925 (exact sample sizes per treatment combination are given in Figure 1).

Physiological variables

We assayed six physiological variables: the activity of AChE (acetylcholinesterase) as this is the target enzyme inhibited by chlorpyrifos (Fukuto, 1990), oxidative damage to lipids (measured as the level of MDA, malondialdehyde, Monaghan et al., 2009), the activity of ETS (the electron transport system) as a measure of metabolic rate (De Coen and Janssen, 2003), and the contents of the three major storage molecules (total proteins, total fat and total carbohydrates). Assays were done on one pooled set of 5 larvae per jar, and the jar means of the 3-4 technical replicates were used in the statistical analyses (exact sample sizes per treatment combination are given in Figure 2A&B and Figure S4 in Appendix S6). All assays were based on spectrophotometry as described in Delnat et al. (2019a) and Verheyen and Stoks (2020). The detailed assay protocols can be found in Appendix S4.

Based on ETS and the combined contents of the three storage molecules, the cellular energy allocation (CEA) was calculated. CEA is a biomarker reflecting the total net energy budget (De Coen and Janssen, 2003). It was calculated as the total energy available (Ea) divided by the energy consumed (Ec) (De Coen and Janssen, 2003; Pestana et al., 2019; Verheyen and Stoks, 2020). The total energy available was estimated by integrating the energy in the three different storage molecules using energetic equivalents with the following enthalpy of combustion: 24,000 mJ/mg for proteins, 39,500 mJ/mg for lipids, and 17,500 mJ/mg for glycogen. To calculate the energy consumed, the total amount of O_2 consumed per larva (based on ETS activity) was transformed into energetic equivalents following the oxyenthalpic equivalents of 484 kJ/mol O_2 for a mixture of the average lipid, protein and carbohydrate molecules. Both Ea and Ec were expressed in mJ per mg larval wet mass.

Data analyses

The main effects of the heat spike, exposure to chlorpyrifos and the exposure order, and all their interactions on mortality (0 = alive and 1 = dead) were analyzed by a generalized linear mixed model with a binomial error distribution and the logit-link function. We added jar as a random factor to take into account that sets of 30 larvae shared the same jar. The main effects of the heat spike, exposure to chlorpyrifos and the exposure order, and all their interactions on CT_{max} were analyzed using a general linear mixed model, with the mass of the larva as covariate and rearing jar as a random factor (we individually measured several larvae per jar). The main effects and their interactions on all other variables for which we had a single value per jar (growth rate, AChE activity, MDA level, Ea, Ec and CEA) were analyzed using general linear models. For CEA, the mass of larva was added as covariate. To meet the model assumptions of normality, the AChE activity and MDA level were log10-transformed. When exposure order had effects, a separate model was run per exposure order to analyze the main and interactive effects of chlorpyrifos and the heat spike.

All statistical analyses were conducted using R v3.6.1 (R Development Core Team, 2019). Packages ‘lme4’ (v1.1-21, Bates et al., 2015) was used to fit the models, ‘afex’ (v0.25-1, Singmann et al., 2017) was used to set effect coding, package ‘car’ (v3.0-3, Fox and Weisberg, 2018) was used to calculate the wald chi-square and F statistics and the p-values, package ‘emmeans’ (v2.30-0, Lenth, 2019) was used to obtain the contrasts to further analyze the interactions between treatment combinations, the associated p-values were false discovery rate (FDR) corrected.

To explicitly determine the interaction type for mortality between chlorpyrifos and a heat spike, we used the independent action (IA) model (see Appendix S5). To estimate the strength of the interaction effect, the model deviation ratio (MDR) was calculated as the observed combined mortality divided by the predicted combined survival by the IA model (based on Belden et al., 2007; Shahid et al., 2019). Defined this way, MDR values are higher than one for synergistic interactions. Note that the identification of the synergism is based on the IA model and that the MDR values only indicate the strength of the synergism.

Results

For none of the response variables, the treatment without any stressor (being the solvent control at 20 °C) differed between the exposure orders (Contrasts: all *P*-values > 0.20), indicating no order effect under stressor-free conditions. To keep the results section focused we present results on growth rate, malondialdehyde, and the two components of the net energy budget (Ea and Ec) in Appendix S6.

Mortality

Exposure to chlorpyrifos, and especially to its high concentration increased the mortality during the 4-d experiment (main effect CPF, Table 1, Figure 1A-B). In the absence of a heat spike, the low chlorpyrifos concentration was slightly lethal (~5%) while the high concentration caused ~36% mortality. The chlorpyrifos-induced increase in mortality was differently magnified by the heat spike in both exposure orders with an overall higher mortality when exposure to chlorpyrifos followed the heat spike (CPF × Heat × Order, Table 1, Figure 1). When first exposed to chlorpyrifos and then to the heat spike, the chlorpyrifos-induced mortality was only magnified by the high heat spike level at the high concentration reaching a mortality of ~50% (CPF × Heat when chlorpyrifos first: χ_4^2 =9.73, P = 0.045; Figure 1A Contrasts at low concentration 'low CPF-20 vs low CPF-30' and 'low CPF-20 vs low CPF-34': both P > 0.67, Contrasts at high concentration 'high CPF-20 vs high CPF-30': P = 0.130, 'high CPF-20 vs high CPF-34': P = 0.001). This was confirmed by the IA model where only between the high heat spike level and the high chlorpyrifos concentration a synergism was detected, all other interaction types were additive (see Table S1A, Appendix S5). When first exposed to the heat spike and then to chlorpyrifos, the heat spike tended to magnify the chlorpyrifos-induced mortality already at the low concentration with mortality going up to ~61% after the 34 °C heat

spike, and mortality even going up to ~91% at the high concentration after the 34 °C heat spike (CPF × Heat when heat spike first: χ^2_4 =8.35, *P* = 0.079; Figure 1B all contrasts 'low CPF-20 vs low CPF-30', 'low CPF-20 vs low CPF-34', 'high CPF-20 vs high CPF-30' and 'high CPF-20 vs high CPF-34': all *P* < 0.001). This was confirmed by the IA model that indicated a synergistic interaction at each combination of the two chlorpyrifos concentrations and the two heat spike levels, with the interaction strength consistently stronger than in the other order (Table S1B, Appendix S5).

Table 1. The effects of exposure to chlorpyrifos (CPF), a heat spike (Heat), and exposure order (Order) on mortality, heat tolerance (CT_{max}), acetylcholinesterase (AChE) activity and the net energy allocation (CEA) of the mosquito *Culex pipiens*. Bold *P*-values indicate significant effects.

	Mortality			CT_{max}		
	χ^2	Df	P	χ^2	Df	P
CPF	1476.8	2	**<0.001**	199.34	2	**<0.001**
Heat	204.47	2	**<0.001**	77.07	2	**<0.001**
Order	90.09	1	**<0.001**	257.57	1	**<0.001**
Heat × Order	124.49	2	**<0.001**	10.82	2	**0.004**
CPF × Order	3.25	2	0.197	160.25	2	**<0.001**
CPF × Heat	3.51	4	0.476	44.75	4	**<0.001**
CPF × Heat × Order	19.46	4	**<0.001**	24.51	4	**<0.001**
Mass				0.42	1	0.518
	AChE			CEA		
	F	Df	P	F	Df	P
CPF	201.40	2,331	**<0.001**	0.92	2,329	0.399
Heat	3.82	2,331	**0.023**	3.09	2,329	**0.046**
Order	68.51	1,331	**<0.001**	7.42	1,329	**0.007**
Heat × Order	4.58	2,331	**0.011**	0.12	2,329	0.886
CPF × Order	12.31	2,331	**<0.001**	0.00	2,329	0.997
CPF × Heat	0.97	4,331	0.425	2.68	4,329	**0.032**
CPF × Heat × Order	2.46	4,331	**0.045**	1.16	4,329	0.329
Mass				2.71	1,329	0.100

The heat spike itself increased mortality, but mainly when it was the first stressor the larvae experienced and much less so in the other order, indicating delayed mortality after the 2-day heat spike (Heat × Order, Table 1, Figure 1; main effect Heat: when the heat spike first, χ_2^2 =483.85, P < 0.001, when chlorpyrifos first, χ_2^2 =5.80, P = 0.055). In the absence of the pesticide and when the first stressor, the low heat spike caused ~3% mortality and the high heat spike caused ~15% mortality (Figure 1B, Contrasts 'solvent control-20 vs solvent control-30' and 'solvent control-20 vs solvent control-34': both $P \leq 0.001$).

Heat tolerance (CT_{max})

In general, chlorpyrifos decreased the CT_{max} (main effect CPF, Table 1, Figure 1C-D), yet this strongly depended on the heat spike and especially the order of exposure (CPF × Heat × Order, Table 1, Figure 1C-D). When chlorpyrifos was the first stressor, chlorpyrifos only reduced CT_{max} at 20 °C by ~1% (Figure 1C; contrasts: both $P \leq 0.026$). When chlorpyrifos was the second stressor, it decreased CT_{max} more strongly by ~5%, and this reduction was further altered by the preceding heat spike (CPF × Heat, when heat spike first: P < 0.001). At the low concentration, the chlorpyrifos-induced reduction of CT_{max} was more pronounced when preceded by a heat spike; at the high concentration, however, this was most pronounced when not preceded by a heat spike (Figure 1D). The heat spike in general increased CT_{max} (main effect Heat, Table 1, Figure 1C-D) and this more consistently so when the heat spike was the second stressor (Heat × Order).

Physiology

Overall, chlorpyrifos decreased the AChE activity, especially at the high concentration (main effect CPF, Table 1, Figure 2A-B). This inhibition was stronger when chlorpyrifos was the second stressor (~54%) than when it was the first stressor (~48%) (CPF × Order). When chlorpyrifos was the second stressor, the heat spike magnified the AChE inhibition at the low concentration (contrasts: P = 0.087 for low heat spike level, P < 0.001 for high level), in all other combinations the heat spike did not change the inhibitory effect of chlorpyrifos (CPF × Heat × Order; contrasts: all P > 0.488). The heat spike had no effect on itself (in the solvent control) (contrasts: all $P \geq 0.754$).

Chlorpyrifos decreased the net energy budget (measured as the cellar energy allocation, CEA), but only in the absence of the heat spike (CPF × Heat, Table 1, Figure 2C-D). The heat spike reduced the CEA in the solvent control (main effect Heat, contrasts: low heat spike level:

P = 0.078; high level: P = 0.001), and the high heat spike level also in the low chlorpyrifos concentration (CPF × Heat, contrast: P = 0.166 for low level, P = 0.048 for high level). The main effect of Order indicated that the CEA was generally lower when chlorpyrifos was the first stressor (Table 1, Figure 2C-D).

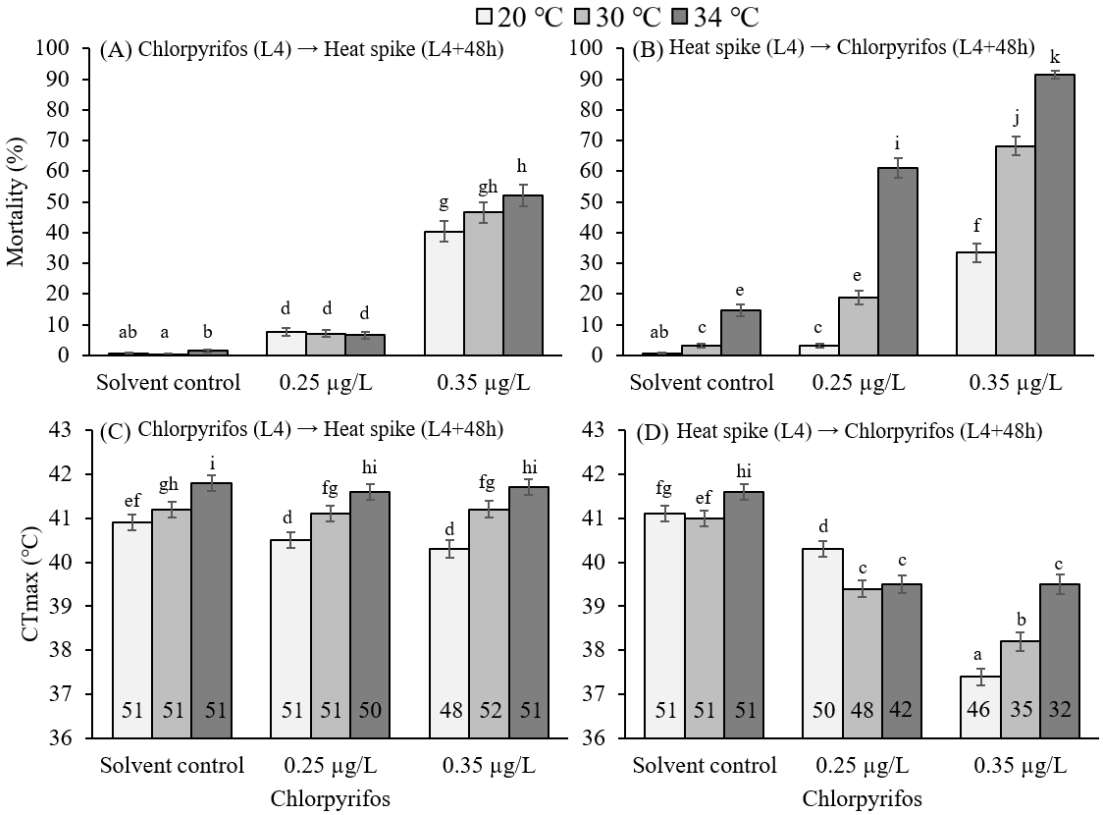


Figure 1. Effects of exposure to chlorpyrifos, a heat spike and their exposure order on mortality (A-B) and the heat tolerance (CT_{max}, C-D) of L4 larvae of the mosquito *Culex pipiens*. Means are shown with standard error. Means that differed significantly (false discovery rate corrected P < 0.05) between treatments are indicated by different letters. Numbers in bars represent sample sizes.

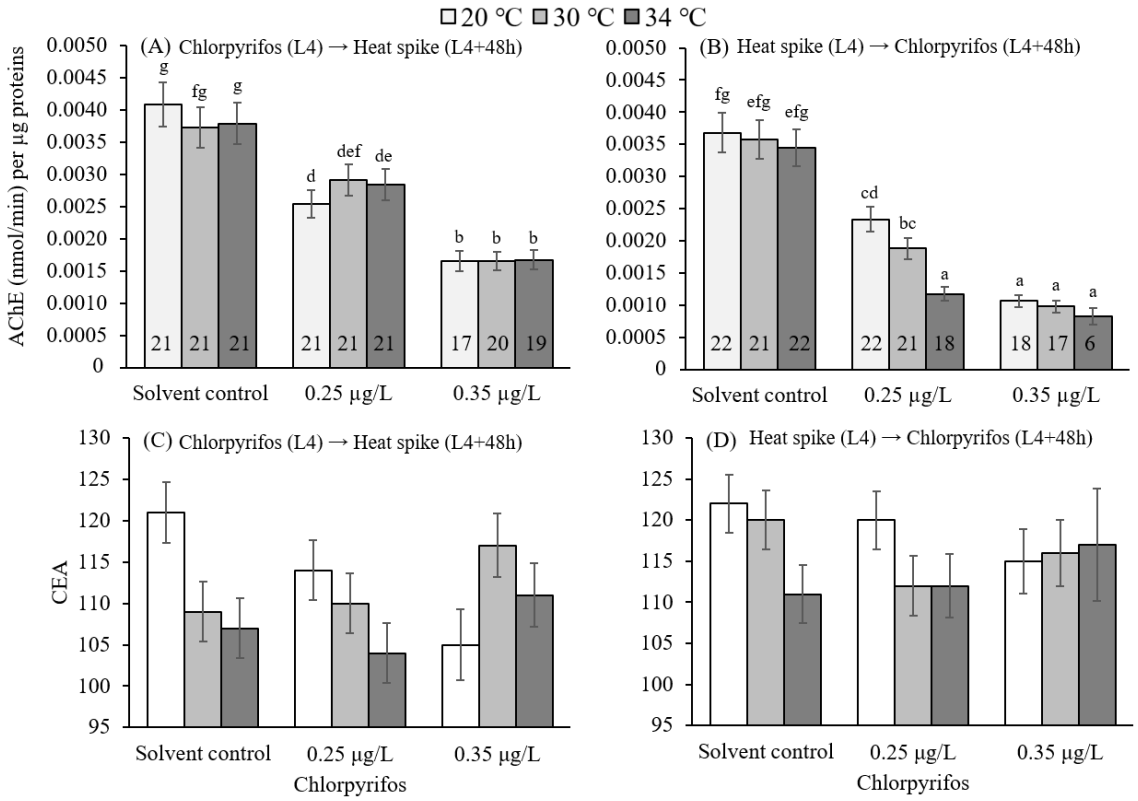


Figure 2. Effects of exposure to chlorpyrifos, a heat spike and their exposure order on acetylcholinesterase (AChE, A-B) activity and the net energy allocation (CEA, C-D) of L4 larvae of the mosquito *Culex pipiens*. Means are shown with standard error. Means that differed significantly (false discovery rate corrected $P < 0.05$) between treatments are indicated by different letters. Numbers in bars represent sample sizes.

Discussion

We here focus on the effects of chlorpyrifos, and how these were modulated by the heat spike, the effects of single exposure to the heat spike are discussed in Appendix S7.

Effects of chlorpyrifos in the absence of the heat spike

As expected, chlorpyrifos caused mortality, especially at the high concentration. This can be explained by the reduced activity of AChE, leading to lethal neurotoxic effects (Fukuto, 1990), and the increased oxidative damage (manifested by the increased MDA level, Appendix S6) (Kumar et al., 2011; Tripathi and Shasmal, 2010). The chlorpyrifos-induced oxidative damage on its turn may be explained by the increase in energy consumption (Ec, Appendix S6) generating more reactive oxygen species (Milatovic et al., 2006), and a lower investment in

energetically costly antioxidant defense because of the lower total net energy budget (CEA). A chlorpyrifos-induced lowered CEA has been associated with a higher mortality in aquatic damselfly larvae (Verheyen and Stoks, 2020).

In line with the "Toxicant-induced climate sensitivity" (TICS) concept (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013), chlorpyrifos reduced the heat tolerance (measured as CT_{max}). This matches the pattern observed for chlorpyrifos in the study species (Delnat et al., 2019b) and aquatic damselfly larvae (Verheyen et al., 2019). A reduction of heat tolerance has been explained by a mismatch between the demand and supply of oxygen under extreme high temperatures (Verberk et al., 2016). Exposure to chlorpyrifos likely both increased the oxygen demand to generate more energy for detoxification and repair of damage (Sokolova, 2013; for chlorpyrifos: Narváez et al., 2016), and reduced the oxygen supply due to the impairment of the respiratory system (Negro and Collins, 2017; Marigoudar et al., 2018). Therefore, under chlorpyrifos exposure this mismatch likely already occurred at lower temperatures, hence the lower CT_{max}.

Some effects of single exposure to chlorpyrifos were dependent on the timing of application. When applied during the final two days of the 4-day experiment, the pesticide had a stronger effect in terms of the reduction of heat tolerance and the inhibition of AChE. This suggests that when applied during the first two days, counteracting mechanisms may have been operating during the subsequent two days in the absence of the pesticide, thereby reducing its effects. For example, chlorpyrifos-exposed animals have been shown to upregulate AChE levels to counteract the inhibition by this pesticide (Suarez-Lopez et al., 2018). Besides, as single exposure to chlorpyrifos when applied during the first two days caused a somewhat higher mortality across the 4-day experiment than when applied during the last two day (Appendix S2), survival selection by removing the weakest larvae may also have contributed to this phenomenon.

Exposure order shapes interactive effects between chlorpyrifos and the heat spike

A key finding was that the expected pattern whereby a heat spike magnifies the pesticide toxicity (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015), was predictably and strongly dependent on the exposure order. Indeed, when first exposed to the heat spike, both chlorpyrifos concentrations interacted with both heat spike levels in a synergistic way as formally identified based on the IA model. Yet, when first exposed to chlorpyrifos, there was only a synergistic interaction effect between the high chlorpyrifos concentration and the heat

spike level of 34 °C. Moreover, the synergistic interaction between the high chlorpyrifos concentration and the heat spike of 34 °C was stronger when first exposed to the heat spike (resulting in a mortality of ~91%) than when first exposed to chlorpyrifos (with a mortality of ~50%). Note, this order effect was not just an age effect because the 2-day mortality caused by exposure to the heat spike was very low and did not differ between larvae that were exposed in days 1-2 of the L4 stage (Figure S2) and larvae that were exposed in days 3-4 of the L4 stage (Figure 1A) (χ_2^2 = 5.92, P = 0.052). This higher toxicity of the pesticide when preceded than when followed by the heat spike suggests that the preceding heat spike weakened the larvae, making them less able to cope with the pesticide. Indeed, the heat spike negatively affected the net energy budget, and may have caused malfunctioning of biomolecules and key physiological processes involved in detoxification and ATP synthesis (Somero et al., 2017; Harada et al., 2019; Stillman, 2019). Moreover, the chlorpyrifos-induced inhibition of AChE was only magnified by the heat spike when the heat spike was the first stressor. In general, this order effect on the interaction between both stressors is consistent with a more rapid action of chlorpyrifos and/or a longer toxicodynamic recovery time after the heat spike than after exposure to the pesticide (Ashauer et al., 2017). This is supported by the observation that the mortality in the next two days without stressor is higher after 2-day exposure to the heat spike than to the pesticide (see Appendix S2). Furthermore, single exposures to a high heat spike level and to a high chlorpyrifos concentration during the first two days caused ~14% and ~6% more mortality across the 4-day experiment, respectively, than their single exposures during the last two days.

This order-dependence of the synergism between the pesticide and the heat spike for mortality implies that also support for the "climate change induced toxicant sensitivity" (CITS) concept is order-dependent (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015). The few studies that looked at sequential exposure to a toxicant and a heat wave, only exposed the animals first to the heat wave and did show that extreme heat may magnify the sensitivity to toxicants (for cadmium: Kimberly and Salice, 2014; for chlorpyrifos: Dinh et al., 2016, but see Meng et al., 2020a). Our results contrast with those of Meng et al. (2020a) where the mosquito larvae that survived a lethal heat spike in the first larval (L1) stage were more tolerant to the exposure to chlorpyrifos in the final larval (L4) stage. There are two non-exclusive reasons for this: (i) the two stressors in previous study were not in close succession, allowing ample time to recover from the heat spike, and (ii) the heat spike imposed in the L1 stage in previous study was highly lethal thereby imposing stronger survival selection, making the high-quality larvae

that survived the heat spike better in coping with the subsequent exposure to chlorpyrifos.

The order of exposure to the heat spike and the pesticide also strongly affected how the heat tolerance was reduced. Indeed, the chlorpyrifos-induced reduction of CT_{max} was small when chlorpyrifos was the first stressor and only occurred in the absence of a heat spike, suggesting that in this order the chlorpyrifos-induced reduction of heat tolerance was balanced by the heat spike-induced increase of heat tolerance. In contrast, the chlorpyrifos-induced reduction of CT_{max} was larger when chlorpyrifos was the second stressor, and was further magnified by the heat spike for the low chlorpyrifos concentration. This order effect on the interaction between both stressors is likely also partly the result of the above discussed differential toxicodynamic recovery after exposure to each stressor (Ashauer et al., 2010).

Our results also indicate the importance of contrasting low- and high-effect levels when combining stressors because this may unravel dose-dependent interaction effects driven by survival selection. Some interactive effects of exposure to chlorpyrifos and the heat spike indeed depended on the chlorpyrifos concentration. For example, when the heat spike was the first stressor, the chlorpyrifos-induced reduction of AChE activity was further exacerbated by the heat spike but only at the low chlorpyrifos concentration; furthermore, when combined with the heat spike, chlorpyrifos increased the energy availability (see appendix S6) but also only at the high concentration. Because the low chlorpyrifos concentration was already able to generate at 20 °C negative effects on endpoints such as mortality, CT_{max} and AChE activity, these concentration-dependent interactive effects may be explained by survival selection at the high concentration. When combined with the heat spike, the high chlorpyrifos concentration indeed caused very high mortalities (when the heat spike first: ~ 68% at the low heat spike level, and ~ 91% at the high level; when chlorpyrifos first: ~47% at the low level, ~52% at the high level). This may have removed the larvae with the lowest body condition, that probably also had the lowest baseline AChE activity level and lowest amount of energy available, resulting in the different interactive pattern with the heat spike at the low versus the high chlorpyrifos concentration. In support of this, animals reared at low food level (hence, lower body condition) have been shown to have lower AChE activity levels (Pedersen et al., 2002) and a lower lipid content (Dinh et al., 2016). Moreover, at the high chlorpyrifos concentration, the maximum AChE inhibition velocity may already have been reached at 20 °C since AChE inhibition exhibits a first-order kinetic (Zhao et al., 2019). Survival selection may also explain the increased energy availability at the high chlorpyrifos concentration when combined with the heat spike (see appendix S6), and why the heat spike decreased CEA both in the solvent control

and at the low chlorpyrifos concentration, but not at the high chlorpyrifos concentration.

Implications for risk assessment under global warming

Despite increasing indications that the exposure order may play an important role in ecotoxicology, this has been mainly limited to studies combining toxicants (e.g. Ashauer et al., 2017, but see Dhakal et al., 2020), and never for the studies combining toxicants with the many and widespread abiotic natural stressors. We here provide the first evidence at the interplay of ecotoxicology and global change biology that the exposure order can strongly change how an important climate change factor magnifies the toxicity of a pesticide, and how the pesticide reduces the heat tolerance. This adds an important order effect to both the CITS and TICS concepts (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015). Notably, the chlorpyrifos-induced reduction in heat tolerance was buffered by the heat spike when the heat spike was the second stressor. This provides evidence that the exposure order also determines the degree to which both concepts interrelate with each other. Our results highlight the importance of integrating exposure order when estimating and predicting the general effects of chemical toxicants under climate change. More general, we provided important proof-of-principle of the importance of exposure order in ecological risk assessment of toxicants under realistic combinations with natural abiotic stressors.

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Abbreviations

AChE acetylcholinesterase; CEA cellular energy allocation; CITS climate change induced toxicant sensitivity concept; CPF chlorpyrifos; CT_{max} critical thermal maximum; Ea energy available; Ec energy consumed; ETS electron transport system; IA independent action model; FDR false discovery rate; L4 fourth and final larval stage; M1 start mass; M2 end mass; MDA

malondialdehyde; TICS toxicant induced climate change sensitivity concept; UPLC-MS/MS ultra performance liquid chromatography - tandem mass spectrometer.

Appendix S1: Scheme of the experimental setup

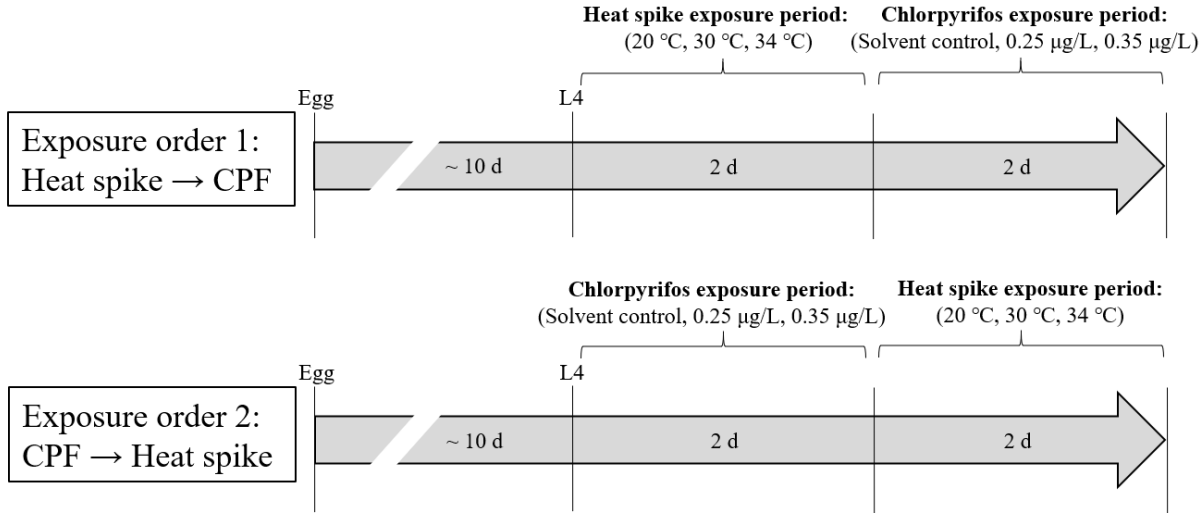


Figure S1. Scheme of the bifactorial, fully crossed design to test sequential effects of a heat spike and pesticide exposure (CPF = chlorpyrifos). Both stressors had three levels: a control level, a low level that caused no or slight mortality, and a high level that caused considerable mortality. For the heat spike these levels were 20 °C, 30 °C and 34 °C. For the chlorpyrifos exposure these levels were solvent control, 0.25 µg/L and 0.35 µg/L. Both exposure periods occurred in the final (L4) larval stage. Exposure order 1: larvae were first given a heat spike treatment for 2 days, then a chlorpyrifos treatment for the next 2 days. Exposure order 2: larvae were first given a chlorpyrifos treatment for 2 days, then a heat spike treatment for the next 2 days. Note that the control treatments for both stressors (the no heat spike treatment and the solvent control treatment) were both run at 20 °C, yet differed in the medium. In the control treatment for the heat spike the medium was water, while in the solvent control treatment, the same amount of solvent (ethanol, 3.5 µL/L) as in the high chlorpyrifos concentration was added to the water.

Appendix S2: The mortality during the first 2 days of the exposure period

Methods:

During the first 2 days of the experiment, the animals were either undergoing one of the three chlorpyrifos treatments (solvent control, 0.25 μg/L and 0.35 μg/L) or one the of the three heat spike treatments (20 ℃, 30 ℃ and 34 ℃). We separately evaluated per stressor the magnitude of delayed mortality of single exposure to a given stressor, by comparing the mortality during the first 2 days (2-d mortality) with the total mortality across the four days (4-d mortality) in larvae that in the last two days were kept at the control (no pesticide, 20 ℃). We did so by using generalized linear mixed models with the stressor treatment as between group factor, and time (2 d, 4 d) as the within group factor. In addition, jar was added as a random factor. A significantly higher 4-d mortality than 2-d mortality, would be indicative of delayed mortality after exposure to a single stressor. This was tested by using contrast analyses.

Results:

The mortality patterns after exposure to a single stressor during the first 2 days and across the 4 days are shown in Figure S2.

Both the low chlorpyrifos concentration and the low heat spike level caused minor mortality (contrasts: for chlorpyrifos, $P < 0.001$, for the heat spike, $P = 0.025$). The high chlorpyrifos concentration caused considerable immediate mortality (~39%) during the first two days (contrast: $P < 0.001$), but the high heat spike level only caused minor immediate mortality (~5%) (contrast: $P < 0.001$). No significant difference was detected between the 2-d mortality and the 4-d mortality after the chlorpyrifos exposure for all three chlorpyrifos treatments (solvent control, the low and the high concentration) (contrasts: all $P > 0.095$), meaning the chlorpyrifos exposure did not cause obvious delayed mortality (+3%) in the next 2 days. However, for the high heat spike exposure, the 4-d mortality was significantly higher (+10%) than the 2-d mortality (contrasts: for the high heat spike level, $P < 0.001$; for control and the low heat spike level, both $P > 0.230$), indicating that 2-day exposure to the high heat spike level caused considerable delayed mortality in the next two days when at conditions control (20 °C, no pesticide).

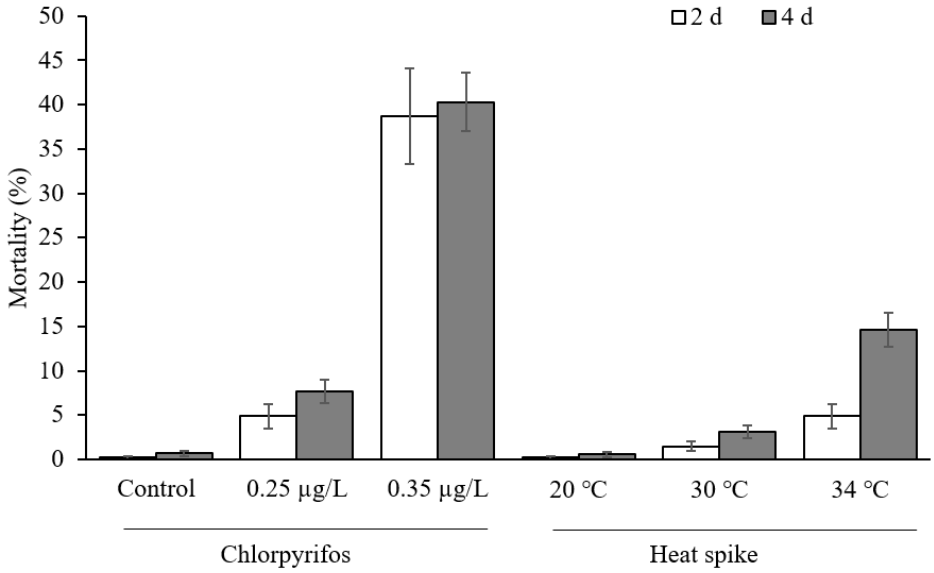


Figure S2. The temporal pattern of mortality when exposing mosquito larvae to chlorpyrifos or the heat spike during the first two days and then keeping them another two days in the absence of the stressor. Given are the mortality percentages occurring during the first 2 days and across the 4 days of the experiment. Given are means with standard error.

Appendix S3: Detailed protocol for CT_{max} measurement

CT_{max} (critical thermal maximum) was measured as an estimate of heat tolerance following the method of Meng et al. (2020b). At the end of the 4-d exposure period, larvae were randomly selected from each experimental jar for the CT_{max} measurement. Where possible we tested 3 larvae per jar, but because of mortality the number per jar was sometimes lower. We could only collect 2 larvae instead of 3 per jar for CTmax in 6 jars, and 1 larva in 1 jar of the 'Heat spike → low chlorpyrifos' group; and 2 larvae in 9 jars and 1 larva in 1 jar of the 'Heat spike → high chlorpyrifos' group.

To start the measurement, larvae were individually placed into transparent plastic cups (50 mL) which were floating at the water surface of a temperature-controlled water bath. The water temperature was increased by a heater (TC120 optima immersion thermostat, Cambridgeshire, UK) at a constant rate of 0.3 °C/min. This rate is within the commonly applied range when measuring the CT_{max} of aquatic invertebrates (Cambronero et al., 2018; Verberk and Bilton, 2011; Verheyen and Stoks, 2019). The temperature at which the larvae started floating motionlessly at the water surface and did not react to a gentle disturbance using a plastic pipette was recorded as the CT_{max}. Afterwards, the larvae were moved immediately to 20 °C to recover. The recovered larvae were weighed; those that did not recover (only 9 larvae, 1.02%) were excluded from the analysis.

Appendix S4: Detailed protocols for the physiological measurements

All physiological endpoints were quantified using spectrophotometry with an Infinite M200 (TECAN) plate reader following the established protocols of Delnat et al. (2019a) and Verheyen and Stoks (2020). The sets of five larvae were first homogenized in PBS-buffer (phosphate buffer saline, 50 mM, pH 7.4) with a body-mass adjusted volume (wet mass × 10 µL/mg) and centrifuged at 13,000 g for 7 minutes (4 °C) to obtain the supernatants used for all physiological measurements.

The activity of AChE was measured in triplicate based on a modification of the Ellman method (Jensen et al., 1997). 170 µL of acetylcholine iodide (mM) and 330 µL of 5,5-dithiobis-2-nitro-benzoic acid (DTNB, 3 mM) were added to 8.8 mL PBS-buffer and mixed. 25 µL of this reaction mixture was added to 384 well plates filled with 5 µL supernatant. Then, the change in absorbance was recorded at 490 nm for 30 minutes with readings every 60 seconds. The AChE concentration was calculated based on the absorbance following the formula of Lambert-Beer, with an extinction coefficient of 13.6/mM × cm. The activity of AChE was expressed in nmol/min per µg protein.

The malondialdehyde (MDA) level was measured in triplicate to assess the lipid peroxidation using a modified protocol of the thiobarbituric acid reactive substance (TBARS) assay following Miyamoto et al. (2011). The cryotubes filled with a mixture of 50 µL supernatant and 50 µL TBA-buffer (0.4%, in 0.1 M HCl) were incubated at 100 °C for 60 minutes, and cooled down immediately on ice afterwards. Then, 165 µL 1-butanol was added and the mixture was centrifuged for 4 minutes at 3,000 g at room temperature. Next, 30 µL upper phase solution of the final mixture was added to 384 well microtiter plates and the absorbance was measured at excitation-emission wave lengths of 535-570 nm. A standard curve was made by using 1,1,3,3-tetramethoxypropan (99%) malonaldehyde bis (dimethyl acetal) (99%) for calculating the MDA concentration, The MDA level was expressed in nmol per µg fat.

The protein content was measured in quadruplicate using the Bradford (1976) method. Specifically, 1 µL supernatant and 40 µL BioRad reagents were added to 160 µL Milli-Q water and mixed thoroughly. After incubation for 5 minutes at 25 °C, the absorbance was measured at 595 nm. The protein concentrations were calculated based on a standard curve of Bovine

Serum Albumine. The sugar content (glucose and glycogen, Hahn and Denlinger, 2007) was measured in triplicate following a protocol based on the glucose kit of Sigma-Aldrich USA (Stoks et al., 2006). A mixcure of 13 µL PBS-buffer, 5 µL supernatant and 2 µL amyloglucosidase was added to a 384 well microtiter plate and mixed. After an incubation at 37 °C for 30 minutes, all glycogen was transformed to glucose. Then, 40 µL glucose reagent was added to each well and mixed. After a final incubation at 30 °C for 20 minutes, the absorbance was measured at 340 nm. The total sugar content was calculated according to a standard curve based on the known glucose concentrations and absorbances. The total fat content was quantified in triplicate using a modified protocol of Marsh and Weinstein (1966). 8 µL supernatant and 56 µL sulphuric acid (98%, H_2SO_4) were added to fat free glass tubes. After incubated at 150 °C for 20 minutes, all tubes were cooled down to room temperature and filled with 64 µL Milli-Q water. 30 µL of the final mixture was added to 384 well plates and absorbance was measured at 340 nm. A standard curve of glyceryl tripalmitate was created for calculating the total fat content. All energy reserve biomolecules (total protein, total sugar, total fat content) were expressed in µg per mg wet mass.

The activity of the electron transport system (ETS) was measured in triplicate to assess metabolic rate using a modified protocol of De Coen and Janssen (2003). 15 µL buffered substrate solution (0.13 M Tris-HCl, 0.3% Triton X-100, 1.7 mM NADH, 250 µM NADPH, pH 8.5) was added to 384 well plates filled with 5 µL supernatant. Then, 10 µL INT (8 mM p-iodonitrotetrazolium) was added to replace O_2 as electron acceptor and to receive electrons from NADPH via NADH-cytochrome oxidoreductase, which caused the formation of formazan. The increase in formazan absorbance was quantified at 20 °C based on a kinetic run of 30 minutes at 490 nm with readings every 30 seconds. The formazan concentration was calculated using the formula of Lambert-Beer with an extinction coefficient of 15.9/mM × cm. The final cellular oxygen consumption rate was calculated based on the theoretical stoichiometric relationship that 1 µmol O_2 was needed for forming 2 µmol formazan in the ETS system. ETS activity was expressed as nmol per minute per mg larva.

Appendix S5: Independent action model

Methods

We applied the Independent Action (IA) model separately for each combination of a given concentration of pesticide (low or high) and heat spike level (30 °C or 34 °C), and this for both exposure orders. We thereby followed the procedure detailed in Coors and De Meester (2008).

We calculated the predicted combined effect of chlorpyrifos and the heat spike following the equation $E_{combined} = 1 - \prod^{i}(1 - E_i)$, with E_i the proportional mortality imposed by the single effects of chlorpyrifos or the heat spike. We calculated E_i based on the equation $E_i = \frac{(e_i - e_{control})}{(e_{max} - e_{control})}$. Hereby, e_i is the observed mortality in absolute units caused by a single stressor (a given concentration of the pesticide or the heat spike level), $e_{control}$ is the observed mortality in absolute units in the absence of a stressor (solvent control at 20 °C), and e_{max} is the maximum possible effect of a single stressor (100% for mortality). Finally, we back-transformed the predicted combined effect $E_{combined}$ to absolute units to allow comparison with the 95% confidence interval of the observed combined effects using the equation $e_{combined} = E_{combined} \times (e_{max} - e_{control}) + e_{control}$.

The predicted combined additive effect by the model is significantly different from an observed combined effect if the predicted effect in absolute units is not within the 95% confidence interval of the observed effect. Hereby, a synergistic interaction is defined when the observed mortality for the combined effect is significantly higher than the predicted mortality for the combined effect.

Results

The results of the independent action model mortality are shown in Table S1 (see next page).

Table S1. Identification of the interaction types between chlorpyrifos (CPF) and a heat spike based on the independent action model (A) when first exposed to chlorpyrifos and (B) when first exposed to the heat spike. A synergism was identified when the observed 95% confidence interval (CI) of the combined effect was larger than the predicted mean of the combined effect. The strength of the interaction is determined using the model deviation ratio (MDR) whereby a synergism has a MDR-value > 1.

		Predicted mean combined stressor effect	Observed 95%CI combined stressor effect	Interaction type	Interaction strength (MDR)
(A) Exposure Order: CPF → Heat spike					
low CPF	30 °C	8.37	[5.27, 11.07]	Additive	0.98
low CPF	34 °C	9.60	[4.29, 10.71]	Additive	0.78
high CPF	30 °C	42.52	[39.04, 55.56]	Additive	1.11
high CPF	34 °C	43.29	[43.89, 62.78]	Synergism	1.23
(B) Exposure Order: Heat spike → CPF					
30 °C	low CPF	6.58	[14.85, 26.48]	Synergism	3.14
34 °C	low CPF	18.86	[51.97, 71.70]	Synergism	3.28
30 °C	high CPF	37.45	[57.90, 77.97]	Synergism	1.81
34 °C	high CPF	45.67	[87.58, 93.63]	Synergism	1.98

Appendix S6: Extended results (growth rate, malondialdehyde level, energy availability and energy consumption)

Growth rate

Chlorpyrifos exposure increased the growth rate, especially when this was the first stressor (main effect CPF, CPF × Order, Table S2, Figure S3A-B). When exposed to the pesticide first, this growth acceleration was ~56% at the high concentration (contrast: $P < 0.001$), and when first exposed to the heat spike it was ~17% at the high concentration ($P < 0.001$). The heat spike itself slightly increased the growth rate but only at the low heat spike level (+6%, contrasts: low level, $P = 0.027$; high level, $P = 0.130$; main effect Heat, Table S2).

The accelerated growth rate caused by chlorpyrifos exposure has been shown in other studies involving the study species (Delnat et al., 2019a) and other semi-aquatic insects (e.g. damselfly larvae: Janssens and Stoks, 2013b). This phenomenon can be an adaptive strategy for semi-aquatic animals to escape the pesticide in the aquatic environment (Rohr et al., 2011), and may have contributed to the increased energy consumption (see below) under chlorpyrifos exposure (Downs et al., 2016). Furthermore, the chlorpyrifos-induced increase in growth rate was stronger when the pesticide was applied during the first two days of the 4-day experiment. Likely, any upregulation of growth rate may take some time and therefore was more easily detectable when the pesticide was applied early in the experiment.

MDA level

Chlorpyrifos exposure increased the MDA level but only in the absence of a heat spike and at the high concentration (CPF × Heat, Table S2, Figure S4A-B). This increase at 20 °C was ~40% at the high chlorpyrifos concentration (contrasts: for the high concentration $P = 0.004$; for the low concentration $P = 0.473$). The heat spike on itself (at the solvent control) tended to increase the MDA level at the low level (30 °C, contrast: $P = 0.067$).

Energy availability (Ea)

Chlorpyrifos exposure increased the energy availability (Ea) in the survivors but only at the high concentration and when combined with a heat spike (Table S2, Figure S5A-B, Contrasts for CPF effect, no heat spike: $P = 0.174$, low heat spike level: $P = 0.053$, high heat spike: $P = 0.019$). The high heat spike level decreased the Ea in the absence of chlorpyrifos (contrast: $P =$

0.022), while both heat spike levels instead tended to increase the Ea at the high chlorpyrifos concentration (contrasts: both *P*-values = 0.059). There was a main effect of order indicating that Ea was generally lower when chlorpyrifos was the first stressor (Table S2, Figure S5A-B).

Energy consumption (Ec)

Chlorpyrifos exposure in general increased the energy consumption (Ec) at the high concentration (contrast: $P < 0.001$), but not at the low concentration ($P = 0.762$) (main effect CPF, Table S2, Figure S5C-D). The heat spike increased the energy consumption but only when it was the first stressor and at the high level (contrast, high level vs 20 °C as first stressor, $P < 0.001$, other contrasts: $P > 0.114$) (Heat × Order, Table S2).

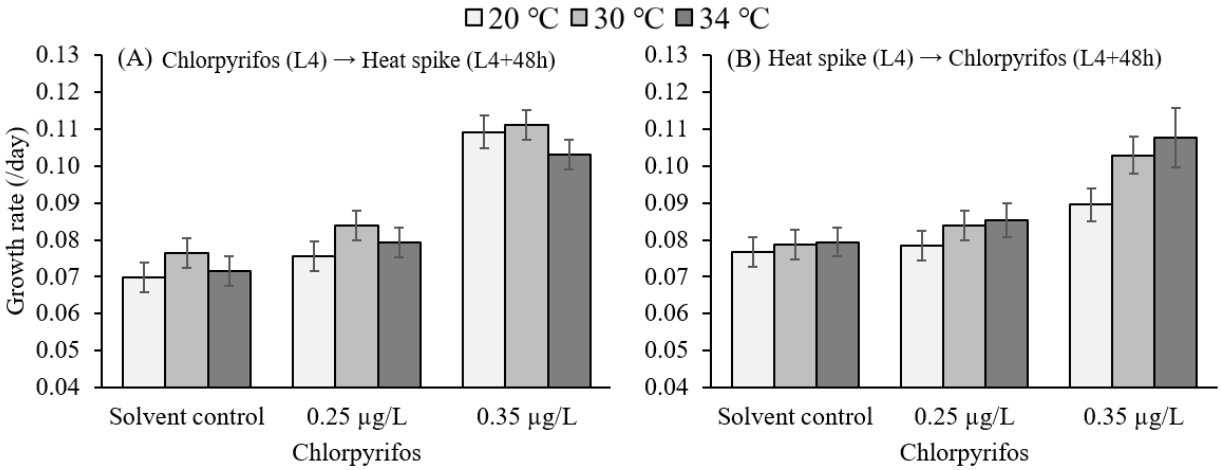


Figure S3. Effects of exposure to chlorpyrifos, a heat spike and their exposure order on growth rate of L4 larvae of the mosquito *Culex pipiens*. Means are shown with standard error.

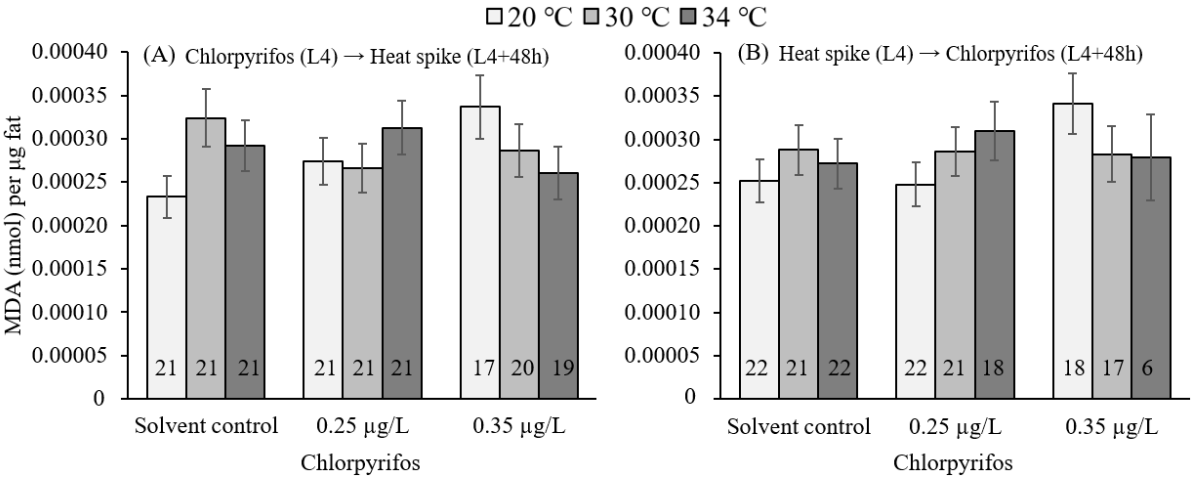


Figure S4. Effects of exposure to chlorpyrifos, a heat spike and their exposure order on malondialdehyde (MDA) level of L4 larvae of the mosquito *Culex pipiens*. Means are shown with standard error. Numbers in bars represent sample sizes.

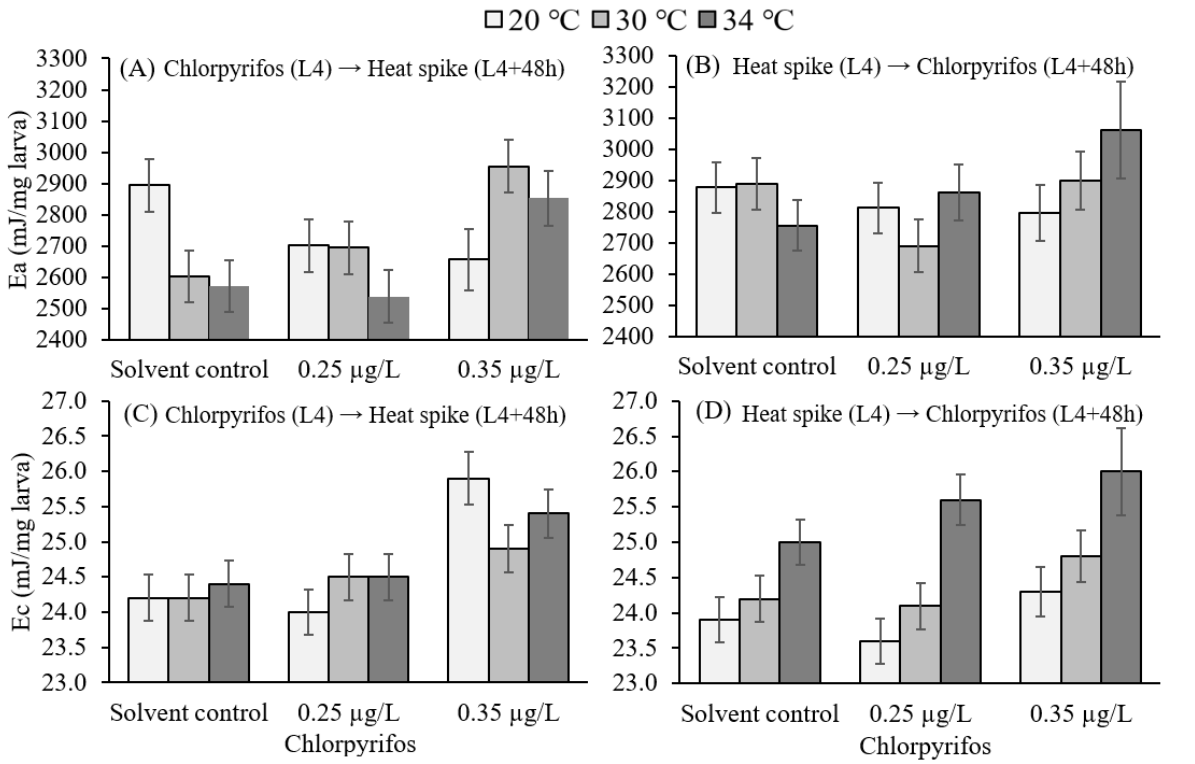


Figure S5. Effects of exposure to chlorpyrifos, a heat spike and their exposure order on the energy availability (Ea) and the energy consumption (Ec) of L4 larvae of the mosquito *Culex pipiens*: (A-B) Ea, and (C-D) Ec. Means are shown with standard error.

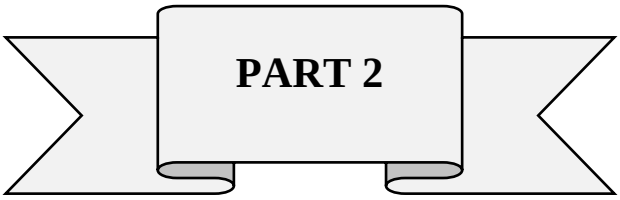
Table S2. The effects of exposure to chlorpyrifos (CPF), a heat spike (Heat), and exposure order (Order) on growth rate, malondialdehyde (MDA) level, the energy availability (Ea) and the energy consumption (Ec) of the mosquito *Culex pipiens*. Bold *P*-values indicate significant effects.

	Growth rate			MDA		
	F	Df	P	F	Df	P
CPF	59.81	2,308	**<0.001**	0.66	2,286	0.518
Heat	3.65	2,308	**0.027**	0.25	2,286	0.783
Order	0.01	1,308	0.913	0.03	1,286	0.867
Heat × Order	1.77	2,308	0.172	0.01	2,286	0.988
CPF × Order	3.32	2,308	**0.037**	0.10	2,286	0.905
CPF × Heat	0.14	4,308	0.969	2.87	4,286	**0.023**
CPF × Heat × Order	0.94	4,308	0.441	0.45	4,286	0.770
	Ea			Ec		
	F	Df	P	F	Df	P
CPF	3.97	2,329	**0.020**	9.76	2,330	**<0.001**
Heat	0.05	2,329	0.951	8.27	2,330	**<0.001**
Order	9.35	1,329	**0.002**	0.13	1,330	0.721
Heat × Order	1.43	2,329	0.241	6.26	2,330	**0.002**
CPF × Order	0.13	2,329	0.875	0.82	2,330	0.443
CPF × Heat	3.35	4,329	**0.010**	0.82	4,330	0.512
CPF × Heat × Order	1.52	4,329	0.197	0.93	4,330	0.448

Appendix S7: Extended discussion about the single effects of the heat spike in the solvent control

When applied during the first two days of the experiment, the heat spike was lethal and increased oxygen consumption (at 34 °C). This suggests delayed effects of the heat spike, as observed before in this species (Meng et al., 2020a). Too high temperatures can be deadly since they can affect the structure and function of proteins, and impair ATP synthesis (Somero et al., 2017; Stillman, 2019; Harada et al., 2019). In response to the damage caused to proteins, additional energy will be allocated to the molecular chaperones (such as heat shock proteins) to repair the damaged proteins (Chen et al., 2018). This may explain the increased energy consumption (Ec) after the heat spike when the heat spike occurred during the first two days. Besides, the heat spike-exposed larvae had a lower energy availability (Ea) and a lower total net energy budget (CEA), thus less energy could be allocated to recover from the heat stress. The lower energy availability may also have important sublethal fitness consequences in *Culex* mosquitoes. For example, a lowered total fat content may negatively affect reproduction (Shin et al., 2012) and female survival (Vrzal et al., 2010). The growth rate was increased at the low heat spike level which was also observed in other studies involving heat extremes (e.g. Van Dievel et al., 2017). However, the high heat spike level did no longer accelerate growth possibly because of the impairments of biomolecules as mentioned above and the lowered amount of energy allocated to growth.

In line with the general pattern that higher acclimation temperatures increase the upper thermal limits (Gunderson and Stillman, 2015), the heat spike increased CTmax. This can be explained by the induction of heat shock proteins under heat stress, resulting in an enhanced heat tolerance (King and MacRae, 2015).



THE COMBINED EFFECTS OF WARMING AND PESTICIDE EXPOSURE ACROSS GENERATIONS

Chapter 3

Acute warming increases pesticide toxicity more than transgenerational warming by reducing the energy budget

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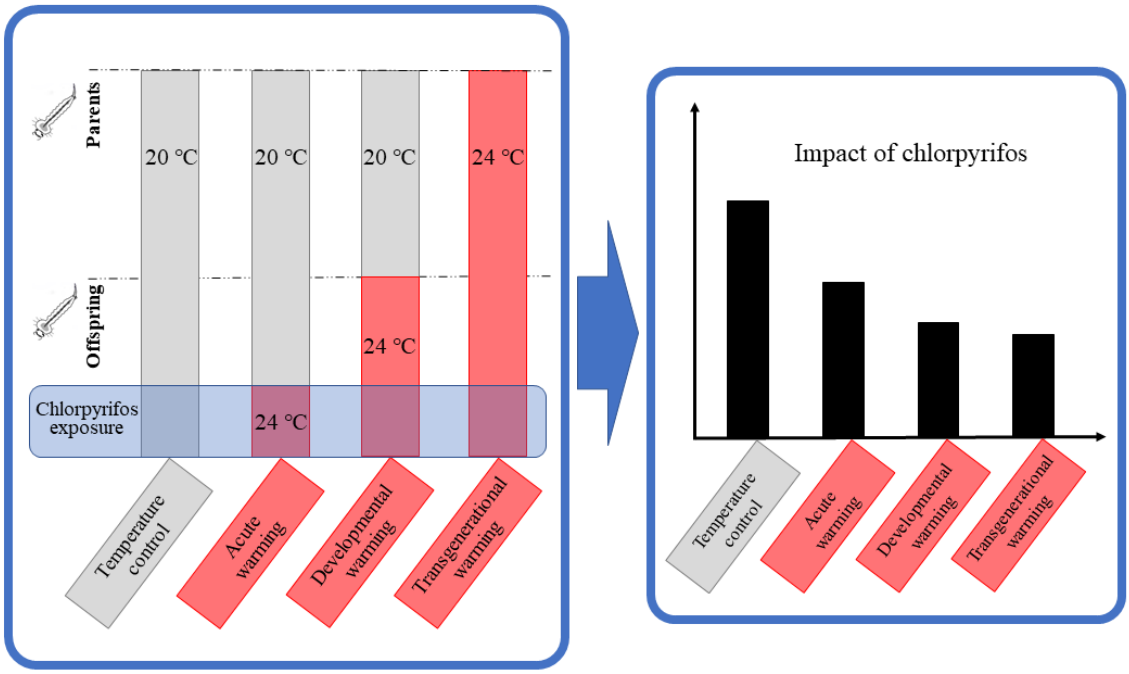
Slightly adapted version



Abstract

There is increasing awareness that the toxicity of pesticides can to a large extent be modulated by warming, and that temporal exposure scenarios may strongly affect the impact of two stressors. Nevertheless, we lack information on how the exposure duration to warming may shape pesticide toxicity under warming. Furthermore, despite that bioenergetic responses have the potential to generate mechanistic insights in how toxicants interact with warming, this has been understudied in ecotoxicology. To investigate whether warming duration modifies pesticide toxicity, mosquito larvae were exposed to a control temperature at 20 ºC or three warming treatments at 24 ºC (acute, developmental and transgenerational warming), and to four pesticide treatments (solvent control, and three chlorpyrifos concentrations) in a full factorial design. Chlorpyrifos increased mortality, growth rate and the energy consumed, and reduced the AChE (acetylcholinesterase) activity, the energy available, and the net energy budget (estimated as cellular energy allocation). The warming treatments did not affect mortality, AChE activity, and the energy consumed. However, acute warming increased the growth rate and decreased the energy available, while both acute and developmental warming decreased the cellular energy allocation. A first key finding was that the lethal and sublethal effects of chlorpyrifos were less strong under warming because of a higher degradation in the medium under warming. A second key finding was that, among the warming treatments, the pesticide toxicity was more increased under acute warming than under transgenerational warming. This could be explained by the negative impact of acute warming but not transgenerational warming on the net energy budget. The results in this study provide mechanistic insights that the exposure duration to warming can play an important role in modulating the impact of pesticides under warming. Therefore, including ecologically relevant temporal scenarios of exposure to warming is important in ecotoxicological studies.

Key words: Degradation rate, energy budget, global warming, temporal scenario, transgenerational effect

Introduction

Freshwater ecosystems are increasingly exposed to multiple stressors that moreover may interact with each other (Birk et al., 2020). Pesticides applied in agriculture can enter freshwater ecosystems through runoff and cause adverse ecological effects on non-target communities (Beketov et al., 2013; Schulz et al., 2021). Another anthropogenic stressor that can cause biodiversity loss is global warming (Heino et al., 2009). The net impact of pesticides under warming is typically not additive. On the one hand, warming magnifies the toxicity of many pesticides (Moe et al., 2013; Hooper et al., 2013; Noyes and Lema, 2015) due to a higher metabolic rate, a higher uptake, or a faster internal conversion of pesticides to a more toxic metabolite (Harwood et al., 2009; Noyes et al., 2009; Hallman and Brooks, 2015). On the other hand, warming can speed up the external degradation rate of pesticides and may buffer or even overrule the warming-induced higher toxicity. While the latter mechanism has been predicted (Hooper et al., 2013) and is important in assessing the net combined effects, it has still rarely been documented (but see Op de Beeck et al., 2017). Note that under both scenarios, warming on its own may not be necessarily stressful to the organism. In other words, these scenarios may operate without warming itself reducing fitness and performance.

The temporal exposure scenarios to two stressors are of importance in driving the direction and strength of their combined impact, and therefore are getting increasing attention in multistressor studies (overviews in Gunderson et al., 2016, Orr et al. 2020, Jackson et al., 2021). Despite the fact that warming can strongly affect the toxicity of pesticides (e.g. Delnat et al., 2019; Meng et al., 2020a&b; overview in Noyes and Lema, 2015), no study so far tested whether different exposure durations to warming differ in modulating the toxicity of pesticides. While pesticides are typically applied as acute pulse-like stressors, warming may go from an acute to a chronic stressor. Different exposure durations to warming may generate different plastic responses (Sgro et al., 2016). In general, three types of exposure scenarios to warming are identified: acute warming (typically hours to days), developmental warming (typically weeks), and transgenerational warming (where warming already occurs in the previous generation). Transgenerational warming is especially relevant for organisms with a short life cycle (Sgro et al., 2016). Since under developmental and transgenerational warming, organisms may already be acclimated to warming, these warming scenarios may be experienced as less energetically demanding compared to acute warming (Leung et al., 2021; Veilleux et al., 2015; Sørensen et al., 2003). Synergistic interactions with pollutants are more likely to occur when the second stressor is energetically costly (Liess et al., 2016). One could therefore hypothesize

that acute warming may be more likely to magnify the toxicity of pesticides than developmental and transgenerational warming.

Despite the widely different modes of action about how stressors operate, they all tend to reduce the overall energy budget and to increase the allocation of energy to defense and repair mechanisms away from other functions such as growth and development (Calow and Sibly 1990; Sokolova, 2013; Verberk et al. 2020), as described in the dynamic energy budget theory (Nisbet et al., 2000; Sousa et al., 2008). Therefore, information on how stressors singly and in combination affect the energy budget is important to understand the multistressor impact on organismal fitness (Kaunisto et al., 2016; Liess et al., 2016; Verberk et al., 2020). Nevertheless, the potential of bioenergetic responses to advance mechanistic insights in multistressor effects is still underexplored in ecotoxicology (but see e.g. Verheyen and Stoks, 2020; Meng et al., 2020a; Wu et al., 2021).

This study investigated whether and how the duration of exposure to warming can predictably modify pesticide toxicity, and thereby contrasted the effects of acute, developmental, and transgenerational warming on the toxicity of chlorpyrifos in larvae of the mosquito *Culex pipiens* form *molestus* (Forskal, 1775). The aquatic larvae of mosquitoes can serve as an important food source in aquatic food webs since they can reach a high biomass (Becker et al., 2010). The organophosphate chlorpyrifos was chosen since it lists in the top ten of hazardous chemicals that pose threats to aquatic organisms (Johnson et al., 2017). While recently banned in some countries, it is still widely used (Rahman et al., 2021). Chlorpyrifos has been shown to be more toxic at higher temperatures (Buchwalter et al., 2003; Tran et al., 2018; Meng et al., 2020a). In a companion study (Meng et al., 2021), it has been shown that the degradation rate of chlorpyrifos was faster under warming, and that transgenerational warming made the larvae less sensitive to chlorpyrifos compared to acute warming in terms of heat tolerance and antipredator behaviour. Here, this study was extended to pesticide toxicity: effects on survival, growth, and the inhibition of acetylcholinesterase (the target enzyme of chlorpyrifos). Moreover, the effects on bio-energetic variables were quantified to obtain mechanistic insights. We have two key predictions. First, it is expected that chlorpyrifos will have less impact under warming because of a faster external degradation. Second, assuming higher energetic costs of acute warming (Veilleux et al., 2015; Sørensen et al., 2003), it is expected that of the three warming treatments the toxicity of the pesticide will be highest under acute warming and lowest under transgenerational warming.

Materials and methods

Experimental setup

To examine how the sensitivity of mosquito larvae to the pesticide chlorpyrifos under warming is regulated by different types of thermal plasticity, a two-generation experiment was conducted where mosquito larvae were exposed to one of the 16 temperature-by-chlorpyrifos treatment combinations (four temperature treatments crossed by four chlorpyrifos treatments) as shown in Figure 1. The chlorpyrifos exposure was only applied for four days in L4 (final instar) larvae in the offspring generation. Exposure of L4 larvae is recommended by WHO (2005) as this is the most tolerant mosquito stage. The four temperature treatments consisted of a temperature control at 20 °C and three warming treatments at 24 °C (acute, developmental and transgenerational warming). In the temperature control, both the parents and their offspring never experienced warming (temperature parents – offspring until L4 – offspring during L4: 20-20-20). Under acute warming (20-20-24), the offspring were only exposed to warming in L4 during the pesticide exposure period. Under developmental warming (20-24-24), exposure to warming started from the egg stage. Finally, under transgenerational warming (24-24-24) both the parents and their offspring were continuously exposed to warming. The four-day pesticide treatments started in the offspring generation once the larvae entered the L4 stage. Mosquito larvae then were exposed to the solvent control or a chlorpyrifos concentration either at 20 °C in the temperature control (20-20-20), or at 24 °C in each of the three warming treatments. The temperature of 20 °C reflects the current mean summer water temperature of ponds in Germany where the mosquito culture originated from (Tran et al., 2016), while 24 ºC represents the temperature expected by 2100 under a 4.4 °C increase in mean temperature based on IPCC (2021) scenario SSP5-8.5.

To start the parental generation, 144 egg rafts were obtained from a lab culture that was housed at 20 °C. The freshly hatched larvae (less than 24h old) were pooled and transferred in sets of 100 to 2-L containers where they were kept till the pupal stage. The 2-L containers were put at 20 or 24 °C in temperature-controlled rooms based on the temperature treatment. When the pupation started, groups of three 2-L containers at each temperature were randomly selected and combined into white trays containing 5 L dechlorinated tap water which were covered by netting to avoid the escape of adults. The emerged adults from three white trays were moved to one insectary and fed with a 6% glucose solution. For both temperatures (20 and 24 °C) in the parental generation, we installed 27 2-L containers resulting in nine white trays, hence three

insectaries.

To start the offspring generation, egg rafts were collected from all three insectaries at each temperature. The hatched larvae were reared in 2-L containers as in the parental generation and placed at 20 or 24 °C based on the temperature treatment. The egg rafts or larvae from different parental temperature treatments were never mixed. When the larvae entered L4, the acute warming treatment and the pesticide exposure were started. The L4 larvae were exposed in groups of 25 in 210 mL glass vials containing 125 mL solvent control or pesticide medium for 96 h, with a refreshment of the medium after 48 h. Each treatment combination was replicated in 18-31 vials (in total 352 vials, 8800 larvae).

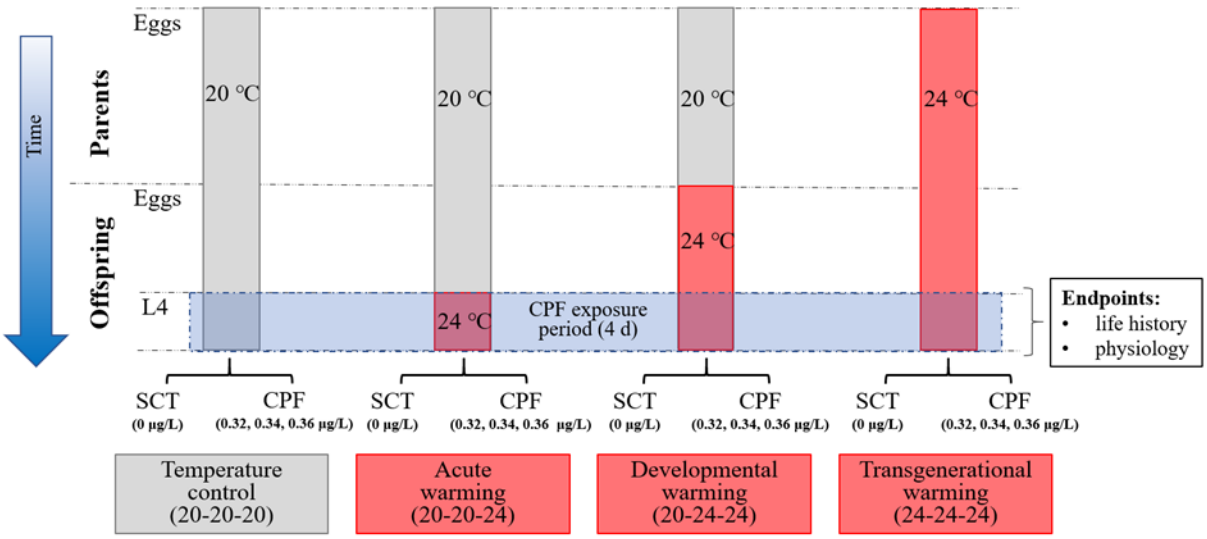


Figure 1. Scheme of the experimental design to test the effects of different exposure durations to warming on the sensitivity to chlorpyrifos. In the offspring generation, freshly moulted L4 (final instar) larvae were exposed to the solvent control (SCT) or one of the three chlorpyrifos (CPF) concentrations (0.32, 0.34 or 0.36 µg/L). In the temperature control (20-20-20), both the parents and their offspring always stayed at 20 °C. In the three warming treatments, exposure to 24 °C started from the L4 stage in the offspring generation for acute warming (20-20-24), from the egg stage in the offspring generation for developmental warming (20-24-24), or already from the egg stage of the parental generation for transgenerational warming (24-24-24).

Throughout the experiment, larvae were daily fed with a food mixture consisting of Olvarit® 7 cereal flakes, Supradyn® vitamins and wheat germs at a high food level (0.313 mg

per larva per day) (Beketov and Liess, 2007).

Pesticide exposure concentrations

To determine nominal concentrations that caused low, moderate and high mortality, a pilot experiment was conducted where freshly moulted L4 larvae were exposed to a range of chlorpyrifos concentrations for 96 h at 20 ℃ with the medium refreshed after 48 h. Based on the results of the pilot experiment, three (0.32, 0.34, and 0.36 µg/L) nominal concentrations were chosen which induced ~15%, ~30%, and ~40% mortality, respectively. The three concentrations cover a narrow range but induced considerable differences in mortality because of the steep dose-response curve of chlorpyrifos in the study species (see in the companion study Meng et al., 2021; see also Delnat et al., 2021). The chosen concentrations are environmentally realistic as they are within the range of measured chlorpyrifos concentrations in European surface waters (Stehle and Schulz, 2015). The measured chlorpyrifos concentrations in the experimental vials for the three nominal concentrations were 0.262 ± 0.021 (mean ± SE), 0.297 ± 0.037, and 0.345 ± 0.023 µg/L at the start of the exposure. Concentrations were quantified using UPLC-MS/MS with Triple Quadrupole Mass Spectrometry based on two pooled samples of 10 vials per temperature. The chlorpyrifos medium was prepared from a stock solution of 100 µg/mL which was made by dissolving chlorpyrifos powder (purity > 99%) in absolute ethanol and was stored in the dark at 4 °C. Ethanol (3.6 µL/L) was added to the solvent control at the same concentration as in the high pesticide concentration (0.36 µg/L).

Life history variables

The number of dead larvae was recorded daily per vial during the 4-day pesticide exposure period and used to quantify the total mortality across the exposure period. Growth rate was quantified based on the increase in wet body mass (weighed to the nearest 0.01 mg) during the 4-day exposure period. At the start, the average body mass of 20 sets of five freshly molted L4 larvae was used as the start mass (SM). After the 4-day exposure period, the average mass of five pooled larvae from each experimental vial was used as end mass (EM). Growth rate was calculated per vial using the formula [ln(EM) − ln(SM)]/4. After weighing, the set of five larvae per vial was stored at −80 °C for physiological measurements.

Physiological variables

The activity of acetylcholinesterase (AChE) was quantified as this is the target enzyme inhibited

by chlorpyrifos (Domingues et al., 2010), and a set of bio-energetic variables was determined to estimate the net energy budget. For the latter, the activity of the electron transport system (ETS) as an indicator of metabolic rate, and the body contents of the three major storage molecules (total proteins, total fat, and total carbohydrates) were measured. The cellular energy allocation (CEA) was calculated as an estimate of the total net energy budget (De Coen and Janssen, 1997), which was obtained as the energy availability (Ea) divided by the energy consumption (Ec) (Pestana et al., 2009). To quantify the energy availability (Ea), the total energy stored in the three major storage molecules was integrated following the energetic equivalents with the enthalpy of combustion of 39,500 $mJ.mg^{-1}$ for lipids, 24,000 $mJ.mg^{-1}$ for proteins, and 17,500 $mJ.mg^{-1}$ for glycogen (De Coen and Janssen, 2003). The energy consumption (Ec) was determined based on ETS activity by transforming the total amount of O2 consumed per larva to energetic equivalents with the oxyenthalpic equivalent of 484 $kJ.mol^{-1}$ for a mixture of the average lipid, protein and carbohydrate molecules (De Coen and Janssen, 2003). Both Ea and Ec were reported in mJ/mg larval mass.

All variables were quantified using spectrophotometry based on protocols adapted for mosquito larvae (Delnat et al., 2019). Physiological variables were quantified using the collected sets of five larvae per vial. The detailed physiological assays can be found in Appendix A.

Statistical analyses

All statistical analyses were done using R v4.0.2 (R Core Team, 2020) with the following packages: “lme4” (v1.1-23, Bates et al., 2015), “afex” (v0.28-0, Singmann et al., 2017), “car” (v3.0-9, Fox and Weisberg, 2018), and “emmeans” (v1.5.1, Lenth et al., 2019).

The effect of temperature during the pesticide exposure period on the measured concentration 48h later was analyzed using a general linear model, including temperature (20 and 24 °C), nominal concentration, and their interaction as factors. The effects of the temperature and chlorpyrifos treatments, and their interaction on mortality (binary data) were analyzed using a generalized linear mixed model with a binomial error distribution and the logit-link function. The number of larvae that died and were alive in each vial was binary coded, and the experimental vial was included as a random factor to take into account that each vial started with 25 larvae. The main effects of the temperature and chlorpyrifos treatments, and their interaction on the other response variables (growth rate, AChE activity, Ea, Ec, and CEA) were analyzed separately using general linear models. Significant interactions between

temperature and chlorpyrifos treatments were further analyzed using contrasts between treatment combinations, and the associated *p*-values were false discovery rate (FDR) corrected.

Results

Pesticide concentrations

The measured concentrations of chlorpyrifos 48 h after the start (just before refreshing the medium) were ~3× lower under warming at 24 °C than in the temperature control at 20 °C (main effect Temperature: $F_{1,6} = 14.96$, $P = 0.008$; Figure 2), and this was so for all the three nominal concentrations (contrasts: all $P < 0.001$). There was no interaction between temperature and the nominal concentration ($F_{2,6} = 2.51$, $P = 0.161$).

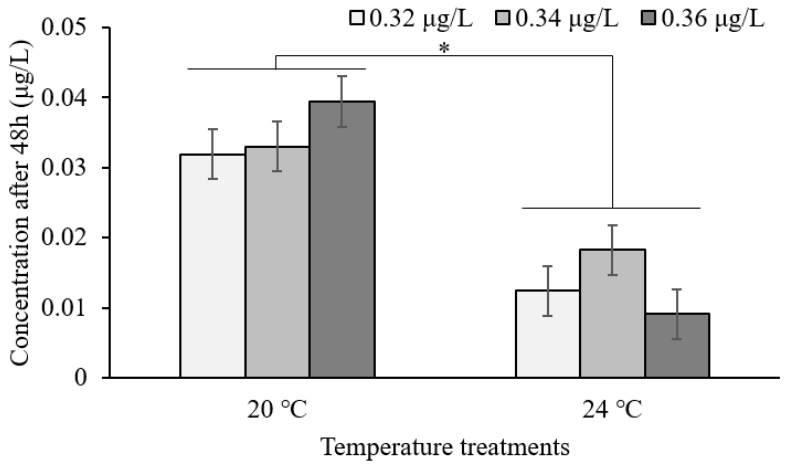


Figure 2. The effect of temperature during the chlorpyrifos exposure period on the measured concentrations of chlorpyrifos in the experimental vials for the three nominal start concentrations (0.32, 0.34 and 0.36 µg/L) 48 h after the start of the exposure. Means were based on two pooled samples of 10 vials and are shown with standard error. Significant differences (*P*-value < 0.05) are coded as *.

Life history variables

In the absence of chlorpyrifos, mortality was very low (0.2%) and was not affected by warming. Chlorpyrifos exposure caused mortality and this strongly depended on the temperature treatment (Chlorpyrifos × Temperature, Table 1, Figure 3A). At 20 °C, chlorpyrifos caused

mortality gradually going up to 52.6% at the highest concentration of 0.36 µg/L. In the three warming treatments (at 24 °C), the chlorpyrifos-induced mortality also increased with increasing concentrations, but remained considerably lower than at 20 °C (Chlorpyrifos × Temperature). Across the three warming treatments, chlorpyrifos-induced mortality was consistently higher under acute warming (up to 17.4% at 0.36 µg/L), lower under developmental warming (up to 11.4% at 0.36 µg/L) and lowest under transgenerational warming (up to 10.1% at 0.36 µg/L) (Figure 3A).

In the absence of chlorpyrifos, the growth rate was increased by acute warming, but not by developmental and transgenerational warming. Chlorpyrifos exposure increased the growth rate but this strongly depended on the temperature treatment (Chlorpyrifos × Temperature, Table 1, Figure 3B). At 20 °C, chlorpyrifos exposure already increased the growth rate at the low concentration going up to an increase of 58.4% at the highest concentration. In the three warming treatments, chlorpyrifos only increased the growth rate at the highest concentration (0.36 µg/L) under acute warming (+23.1%), but never under developmental and transgenerational warming.

Physiological variables

In the absence of chlorpyrifos, warming did not affect the AChE activity. Chlorpyrifos exposure inhibited the AChE activity, especially at 20 °C (Chlorpyrifos × Temperature, Table 1, Figure 4A). At 20 °C, chlorpyrifos decreased the AChE activity, especially at the two high concentrations. At 24 °C, the chlorpyrifos-induced reduction in AChE activity at 0.34 µg/L was less under acute (-42.4%) and developmental (-24.8%) warming than at 20 °C (-61.6%), and at 0.36 µg/L less under acute warming (-43.2%) than at 20 °C (-64.1%).

In the absence of chlorpyrifos, the energy consumed (Ec) was not significantly affected by warming (Figure 4C), while the available energy (Ea) was decreased by acute warming, but not by developmental and transgenerational warming (Table 1, Figure 4B). This resulted in the net energy budget (CEA) being reduced by acute warming and slightly by developmental warming, but not by transgenerational warming (Figure 4D). Chlorpyrifos exposure generally increased the energy consumed (Ec) (main effect Chlorpyrifos, Table 1, Figure 4C), which was not affected by the temperature treatments. Instead, chlorpyrifos reduced Ea at 20 °C but not in the warming treatments (Chlorpyrifos × Warming, Table 1, Figure 4B). This resulted in chlorpyrifos reducing CEA at 20 °C, but not under the three warming treatments (Chlorpyrifos × Warming, Figure 4D).

Table 1. The effects of chlorpyrifos exposure and the temperature treatments on life history and physiological variables in larvae of the mosquito *Culex pipiens*. Significant *P*-values ($P < 0.05$) are indicated in bold. Ea = energy available, Ec = energy consumed, CEA = cellular energy allocation.

	Mortality			Growth rate			AChE activity		
	χ^2	Df	P	F	Df	P	F	Df	P
Chlorpyrifos	186.06	3	**< 0.001**	8.06	3,331	**< 0.001**	59.13	3,320	**< 0.001**
Temperature	22.73	3	**< 0.001**	18.33	3,331	**< 0.001**	2.88	3,320	**0.036**
Chlorpyrifos × Temperature	17.07	9	**< 0.001**	2.52	9,331	**0.008**	2.37	9,320	**0.013**
	Ea			Ec			CEA		
	F	Df	P	F	Df	P	F	Df	P
Chlorpyrifos	4.91	3,288	**0.002**	6.68	3,298	**< 0.001**	4.45	3,266	**0.005**
Temperature	19.35	3,288	**< 0.001**	1.77	3,298	0.152	22.20	3,266	**< 0.001**
Chlorpyrifos × Temperature	1.94	9,288	**0.046**	0.66	9,298	0.741	2.18	9,266	**0.023**

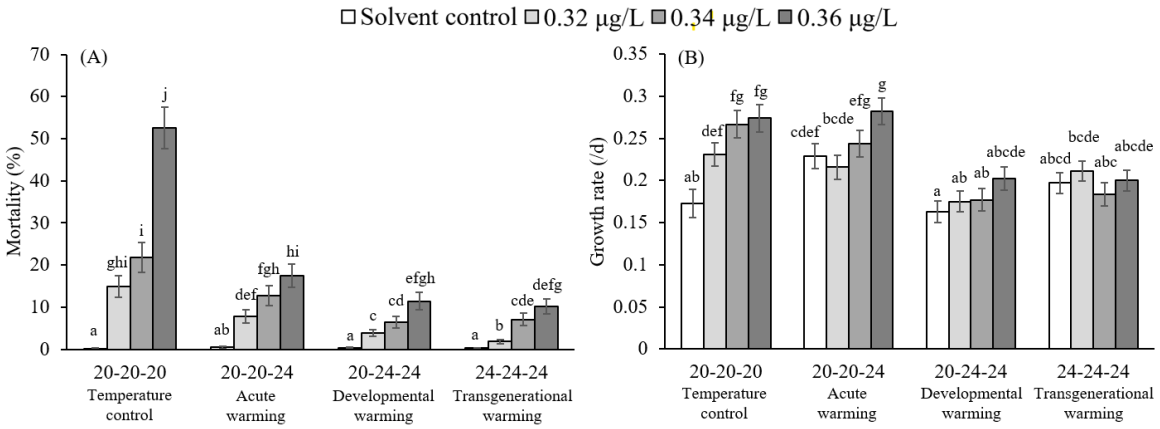


Figure 3. The effects of chlorpyrifos exposure and the temperature treatments on life history variables of L4 larvae of the mosquito *Culex pipiens*: (A) mortality and (B) growth rate. Means are shown with standard error. Different letters above the bars indicate significantly different means (false discovery rate corrected $P < 0.05$).

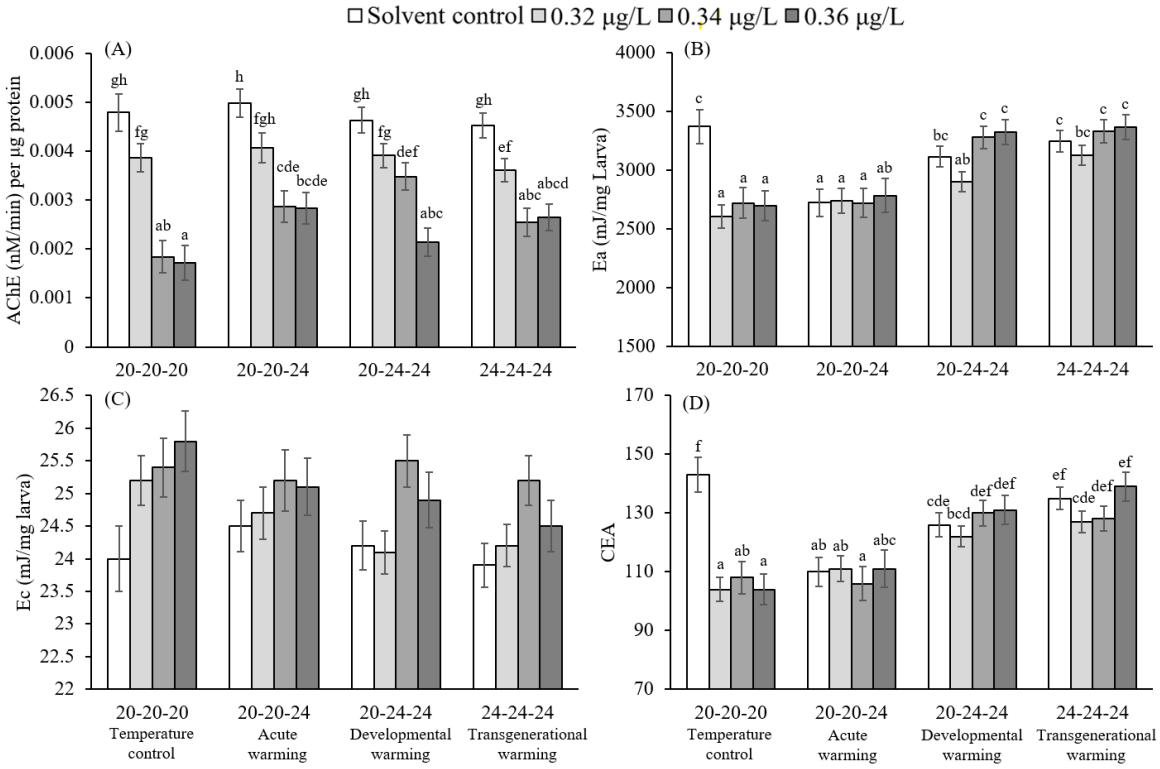


Figure 4. The effects of chlorpyrifos exposure and the temperature treatments on physiological variables of L4 larvae of the mosquito *Culex pipiens*: (A) AChE (acetylcholinesterase) activity, (B) Ea (the energy available), (C) Ec (the energy consumed) and (D) CEA (cellular energy allocation). Means are shown with standard error. Different letters above the bars indicate significantly different means (false discovery rate corrected $P < 0.05$).

Discussion

As expected, the used chlorpyrifos concentrations caused mortality and affected all studied sublethal variables. A first key finding was that warming, by inducing a faster external degradation of the pesticide, reduced the impact of chlorpyrifos. Indeed, the chlorpyrifos-induced lethal and sublethal effects were only present or more pronounced in the absence of warming. A second key finding was that the chlorpyrifos-induced toxicity predictably depended on the type of warming. Indeed, the pesticide-induced mortality and the sublethal effect on growth were less strong under transgenerational warming than under acute warming. Related to this, while acute warming decreased the net energy budget, this was not the case for transgenerational warming. In next parts, we first discuss the effects of single exposure to either warming or chlorpyrifos, then focus on the overall combined effects of warming and chlorpyrifos exposure (first key finding), and finally discuss how the exposure duration to warming shaped the pesticide toxicity (second key finding).

The effects of warming in the absence of chlorpyrifos

In the absence of the pesticide, the 4 °C warming did not induce mortality, and acute warming even increased the growth rate. This confirms previous work on the study species (Delnat et al., 2019a) and other semi-aquatic insects (e.g. damselflies: Dinh Van et al., 2014) that 4 °C warming is not lethal and may even be beneficial for growth. This matches the general pattern in terrestrial ectotherms that ambient temperatures in temperate regions are lower than the optimal temperatures for fitness (Deutsch et al., 2008). In line with this, the AChE activity and the energy consumed were also not negatively affected by warming. The higher growth under acute warming may explain the reduced energy available and the net energy allocation under acute warming. Indeed, a faster growth is often associated with less energy storage (e.g. Janssens and Stoks, 2020). In addition, the energy budget was measured only four days after larvae of the acute warming treatment were switched to 24 °C, hence their lower budget may reflect the ongoing investment to adjust at the molecular/physiological level to the 4 °C higher temperature. The longer exposure duration to warming may have resulted in the disappearance of the effects of transgenerational warming on the energy-related variables (except for a smaller reduction in energy budget under developmental compared to acute warming). Related to this, the energetically costly upregulation of heat shock proteins has been shown only under acute warming but not under developmental and transgenerational warming (overview in Chen et al., 2018; Sørensen et al., 2003; Veilleux et al., 2015).

In contrast with acute warming, the larvae did not increase their growth under developmental and transgenerational warming. This suggests these larvae had enough time to acclimate to the higher temperature, resulting in the disappearance of the potential beneficial effect on growth compared to acute warming (Schulte et al., 2011; Rohr et al., 2018). This may further explain the absence of (under transgenerational warming) or a smaller (under developmental warming) decrease in the net energy budget.

The effects of chlorpyrifos in the absence of warming

As expected, chlorpyrifos exposure at 20 ℃, especially at high concentrations, increased mortality, growth rate and the energy consumed, and decreased AChE activity, the energy available and the cellular energy allocation. The chlorpyrifos-induced mortality may partly be explained by the chlorpyrifos-induced inhibition of AChE activity (Domingues et al., 2010). In addition, chlorpyrifos increased the energy consumed and reduced the energy availability and the net energy budget as also recorded before (damselfly larvae: Verheyen and Stoks, 2020; for the study species: Meng et al., 2020b), resulting in less energy available for detoxification. Furthermore, the increased energy consumption by chlorpyrifos can also have generated more reactive oxygen species (Milatovic et al., 2006), hence increased oxidative stress, which may also have contributed to the lethal effect (Tripathi and Shasmal, 2010; Kumar et al., 2011). Despite the concentration-dependence of the increase in mortality, this was less so for the energy availability and the cellular energy allocation, which might be caused by survival selection removing the weakest larvae with the lowest energy available and the lowest cellular energy allocation.

Despite the chlorpyrifos-induced mortality and the negative effects on physiological variables at the cellular level, chlorpyrifos caused an adaptive increase in growth rate at 20 ℃. Accelerated growth caused by chlorpyrifos has been recorded several times in the study species (e.g. Meng et al., 2020a; Delnat et al., 2019a) and other semi-aquatic insects (for damselfly larvae: Janssens and Stoks, 2013b), and regarded as an adaptive response of semi-aquatic organisms to escape exposure to toxicants in the aquatic environment (Rohr et al., 2011). In addition, survival selection induced by chlorpyrifos may also have contributed to the increased growth by removing the weakest larvae with the slowest growth rate.

The overall effects of warming on chlorpyrifos toxicity

A first key finding was that the chlorpyrifos-induced lethal and sublethal effects were less

strong under warming, and this is irrespective of the exposure duration to warming. Indeed, chlorpyrifos caused considerably less mortality at 24 ºC than at 20 ºC. This seems in contrast with the "climate change-induced toxicant sensitivity" (CITS) concept stating that many pesticides (including organophosphates) become more toxic at higher temperatures (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). This apparent deviation can, however, be explained by the here observed warming-induced higher external degradation rate overruling the warming-induced higher toxicity of chlorpyrifos. A similar pattern of lower toxicity of chlorpyrifos under warming because of faster degradation was recorded by Op de Beeck et al. (2017) in a study on damselfly larvae. Related to this, under warming chlorpyrifos inhibited the AChE activity less and increased the growth rate less. Also the chlorpyrifos-induced reduction in energy availability and the net energy budget did not occur under warming, allowing more energy to be allocated to detoxification.

The effects of the exposure duration to warming on chlorpyrifos toxicity

A second key finding was that, while in each of the three warming treatments the animals were exposed to chlorpyrifos at 24 °C for the same four-day period, acute warming caused a higher toxicity than developmental and transgenerational warming. In the three warming treatments, chlorpyrifos-induced mortality was indeed highest under acute warming, intermediate under developmental warming and lowest under transgenerational warming. Similarly, when combined with warming, the chlorpyrifos-induced adaptive increase in growth only occurred under acute warming but not under developmental and transgenerational warming. The same pattern was also observed for chlorpyrifos-induced reductions in heat tolerance and in antipredator behaviours in the companion study (Meng et al., 2021). These different effects of the three warming treatments on chlorpyrifos sensitivity cannot be explained by a different degradation rate of chlorpyrifos, because the chlorpyrifos solution in all the three warming treatments was exposed to 24 °C for the same duration (two days in between renewals). Our bio-energetic results suggest that under developmental and transgenerational warming, the larvae had already acclimatized to the higher temperature, hence experienced less combined energetic stress during the chlorpyrifos exposure period at 24 °C. Indeed, long-term warming did not (under transgenerational warming) or did less (under developmental warming) reduce the energy budget compared to acute warming as explained above. Instead, under acute warming, the larvae suffered from both chlorpyrifos exposure and a switch to a higher temperature (and associated lower energy budget) when they entered the L4 stage, which may explain that acute warming caused a higher toxicity.

Conclusions

While warming can magnify the toxicity of pesticides (overview in Noyes et al., 2009 and Moe et al., 2013), we here provide rare evidence that warming, by increasing the external degradation, can lower the lethal and sublethal impact of a pesticide. Moreover, while studies are increasingly highlighting the importance of temporal exposure scenarios (Orr et al., 2020; Jackson et al., 2021), no studies so far tested the role of exposure duration to warming in modulating the toxicity of pesticides under warming. By exposing mosquito larvae for three durations (acute, developmental and transgenerational) to warming and the pesticide chlorpyrifos, it was found that under acute warming the larvae suffered more from chlorpyrifos compared to under transgenerational warming. This could be explained by the lowered energy budgets available for detoxification under acute warming. The results in this study provide evidence and mechanistic insights that the exposure duration to warming can play an important role in modulating the toxicity of pesticides under warming. This study thereby further highlights the importance of integrating ecologically relevant temporal scenarios of exposure to warming, and of the added value of quantifying bio-energetic variables in ecotoxicological studies.

Acknowledgements

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Appendix A: Detailed assays for the physiological measurements

We quantified the activity of acetylcholinesterase (AChE), and the four bio-energetic variables: the activity of the electron transport system (ETS), and the body contents of total proteins, total fat, and total carbohydrates, using the collected sets of five larvae per vial. All physiological variables were quantified based on the protocols adapted for mosquito larvae (Delnat et al., 2019a) using spectrophotometry with an Infinite M200 (TECAN) plate reader. The sets of five larvae were first homogenized in PBS-buffer (phosphate buffer saline, 50 mM, pH 7.4) with a body-mass adjusted volume (wet mass × 10 µL/mg) and centrifuged at 13,000 g for 7 minutes (4 °C) to obtain the supernatants. These supernatants were used for all physiological measurements.

The AChE activity was measured in triplicate based on a modified Ellman method (Jensen et al., 1997). We added 170 µL of acetylcholine iodide (mM) and 330 µL of 5,5-dithiobis-2-nitro-benzoic acid (DTNB, 3 mM) to 8.8 mL PBS-buffer and mixed them. We added 25 µL of this reaction mixture and 5 µL supernatant to 384 well plates. Then, the change in absorbance was recorded at 490 nm for 30 minutes with readings every 60 seconds. Following the formula of Lambert-Beer, we calculated the AChE concentration based on the absorbance with an extinction coefficient of 13.6/mM × cm. The activity of AChE was expressed in nmol/min per µg protein.

The activity of the electron transport system (ETS) was measured in triplicate to assess metabolic rate based on a modified protocol of De Coen and Janssen (2003). We added 15 µL buffered substrate solution (0.13 M Tris-HCl, 0.3% Triton X-100, 1.7 mM NADH, 250 µM NADPH, pH 8.5) and 5 µL supernatant to 384 well plates. Then, 10 µL INT (8 mM p-iodonitrotetrazolium) was added to replace O_2 as electron acceptor and to receive electrons from NADPH via NADH-cytochrome oxidoreductase, which caused the formation of formazan. We quantified the increase in formazan absorbance at 20 °C based on a kinetic run of 15 minutes at 490 nm with readings every 30 seconds. The formazan concentration was calculated using the formula of Lambert-Beer with an extinction coefficient of 15.9 /mM × cm. We calculated the final cellular oxygen consumption rate based on the theoretical stoichiometric relationship that 1 µmol O_2 was needed for forming 2 µmol formazan in the ETS system. ETS activity was expressed as nmol per minute per mg larva.

The protein content was measured in quadruplicate using the Bradford (1976) method. Specifically, we added 1 µL supernatant and 40 µL BioRad reagents to 160 µL Milli-Q water and mixed them thoroughly. After an incubation for 5 minutes at 25 °C, the absorbance was measured at 595 nm. We calculated the protein concentrations based on a standard curve of Bovine Serum Albumine. The sugar content (glucose and glycogen: Hahn and Delinger, 2007) was measured in triplicate following a protocol based on the glucose kit of Sigma-Aldrich USA (Stoks et al., 2006). We added 13 µL PBS-buffer, 5 µL supernatant and 2 µL amyloglucosidase to 384 well microtiter plates and mixed them well. All glycogen was transformed to glucose after an incubation at 37 °C for 30 minutes. Then, 40 µL glucose reagent was added to each well and mixed. After a final incubation at 30 °C for 20 minutes, the absorbance was measured at 340 nm. We calculated the total sugar content using a standard curve based on the known glucose concentrations and absorbances. The total fat content was quantified in triplicate based on a modified protocol of Marsh and Weinstein (1966). We added 8 µL supernatant and 56 µL sulphuric acid (98%, H_2SO_4) to fat free glass tubes. After an incubation at 150 °C for 20 minutes, all tubes were cooled down to room temperature and filled with 64 µL Milli-Q water. We added 30 µL of the final mixture to 384 well plates and the absorbance was measured at 340 nm. A standard curve of glyceryl tripalmitate was created for calculating the total fat content. All energy reserve biomolecules (total protein, total sugar, total fat content) were expressed in µg per mg wet mass.

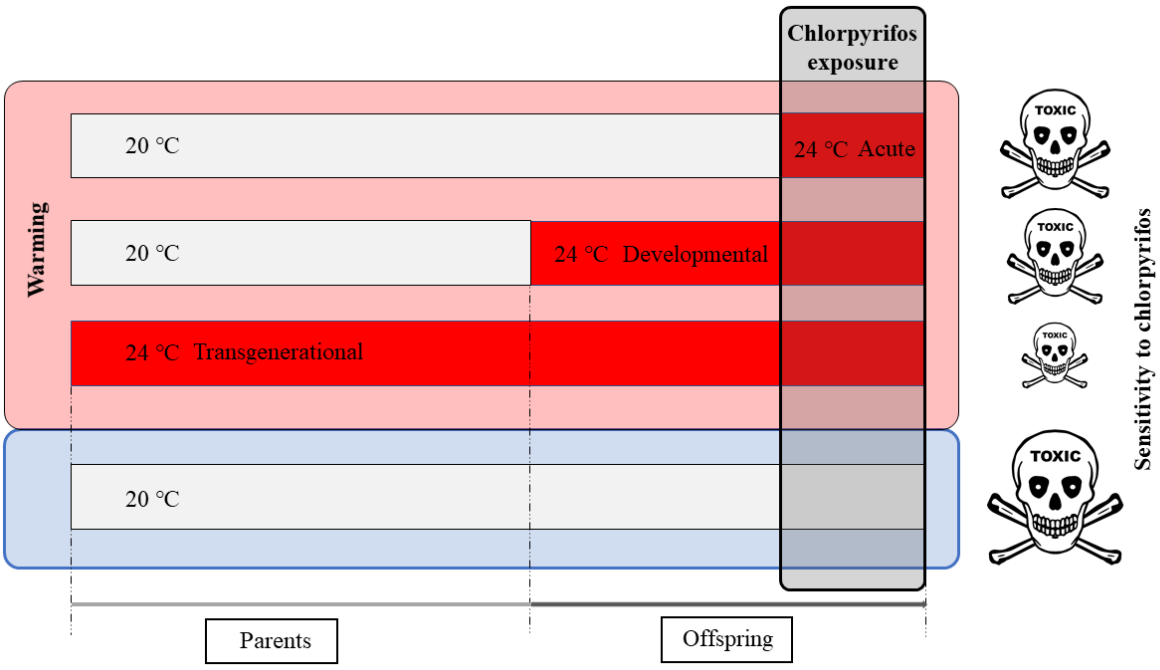
Chapter 4

Transgenerational exposure to warming reduces the sensitivity to a pesticide under warming

Shandong Meng, Tam T. Tran, Vienna Delnat, Robby Stoks

Environmental Pollution (2021), 284: 117217

Slightly adapted version



Abstract

Despite the increased attention for temporal aspects of stressor interactions and for effects of warming in ecotoxicological studies, we lack knowledge on how different exposure durations to warming may affect pesticide sensitivity. We tested how three types of exposure duration to 4 ℃ warming (acute, developmental and transgenerational exposure to 24 ℃ vs 20 ℃) shape the effect of the pesticide chlorpyrifos on two ecologically relevant fitness-related traits of mosquito larvae: heat tolerance and antipredator behaviour. Transgenerational (from the parental generation) and developmental (from the egg stage) warming appeared energetically more stressful than acute warming (from the final instar), because (i) only the latter resulted in an adaptive increase of heat tolerance, and (ii) especially developmental and transgenerational warming reduced the diving responsiveness and diving time. Exposure to chlorpyrifos decreased the heat tolerance, diving responsiveness and diving time. The impact of chlorpyrifos was lower at 24 °C than at 20 °C indicating that the expected increase in toxicity at 24 °C was overruled by the observed increase in pesticide degradation. Notably, although our results suggest that transgenerational warming was energetically more stressful, it did reduce the chlorpyrifos-induced negative effects at 24 °C on heat tolerance and the alarm escape response compared to acute warming. Our results provide important evidence that the exposure duration to warming may determine the impact of a pesticide under warming, thereby identifying a novel temporal aspect of stressor interactions in risk assessment.

Key words: Antipredator behaviour, global warming, heat tolerance, transgenerational effects, thermal plasticity

Introduction

Warming and pesticides are two stressors of high concern as they can cause biodiversity loss, especially in aquatic organisms (warming: Heino et al., 2009; pesticides: Beketov et al., 2013). Both stressors are increasingly studied jointly as they may co-occur and can interact with each other. Notably, warming may change the net impact of pesticides in opposing ways. On the one hand, the toxicity of a given concentration of many pesticides such as carbamates and organophosphates is higher under warming (Noyes et al., 2009; Holmstrup et al., 2010; Moe et al., 2013). On the other hand, and much less considered in experiments, warming is expected to increase the degradation of pesticides thereby potentially buffering or even overruling the warming-induced increase in toxicity (Hooper et al., 2013; Op de Beeck et al., 2017a).

Another largely neglected aspect in studies on the impact of pesticides under warming is the exposure duration to warming. In general, temporal aspects of stressor interactions need more attention as these may strongly determine their net impact (Orr et al., 2020; Meng et al., 2020a&b). Most ecotoxicological studies co-exposed organisms for a couple of days to warming and to a pesticide (reviewed by Noyes et al., 2009; Holmstrup et al., 2010; Wang et al., 2019). Nevertheless, as warming is typically a chronic stressor (except for heat extremes) and pesticides are pulse stressors, organisms may often already experience warming before being exposed to a pesticide. Three types of exposure duration to warming, and associated thermal plasticity, can be distinguished and may determine how organisms respond to warming (Sgrò et al., 2016), and therefore also how warming may shape the toxicity of a pesticide. Organisms may respond differentially to temperature when they are exposed for a short period (typically hours-days, acute plasticity), for a longer period (typically weeks, developmental plasticity), and when also their parents have already been exposed (transgenerational plasticity) (Sgrò et al., 2016). While animals may show adaptive acute plasticity to warming, long-term developmental (Rohr and Palmer, 2013) and transgenerational (Veilleux et al., 2015; Tran et al., 2018) exposure to warming may cause negative effects (Kingsolver and Woods, 2016), thereby likely further increasing the sensitivity to pesticides at higher temperatures. Notably, studies of transgenerational exposure to warming followed by pesticide exposure are rare (but see Tran et al., 2018; Pham et al., 2020) but highly relevant. Indeed, in organisms with a short life cycle both parents and their offspring can be expected to experience the same level of warming, and how offspring deal with warming can thereby be modulated by the thermal exposure history of their parents, which is defined as transgenerational plasticity (Bell and Hellmann, 2019; Donelson et al., 2018). Thus, how the exposure duration to warming and the

associated type of thermal plasticity will interact with pesticide exposure is highly relevant for risk assessment. Nevertheless, no studies so far compared how the three types of exposure duration to warming (and associated thermal plasticity) affect pesticide sensitivity.

Two important fitness-related traits that are much less quantified in multi-stressor studies compared to the traditional life history traits are heat tolerance and antipredator behaviour. The heat tolerance of organisms is important to quantify as it correlates with the tolerance to mild warming (Åsheim et al., 2020) and reflects the ability to deal with heat extremes (Kaspari et al., 2015; Jørgensen et al., 2019). Heat extremes have increased during last decade and are expected to further increase in frequency and intensity under global warming (Stillman, 2019; IPCC, 2021). Antipredator behaviour is another crucial trait, especially in freshwater systems where predation is an important structuring force (Wellborn et al., 1996). When stressors impair antipredator behaviour this will increase mortality due to predation, which eventually may affect the persistence of populations (Relyea and Hoverman, 2006). Pesticides have been shown to reduce the heat tolerance (Hooper et al., 2013; Moe et al., 2013; Noyes et al., 2009), and to negatively affect antipredator behaviour (e.g. Pestana et al., 2010; Reynaldi et al., 2011). Acclimation to higher temperatures can increase the heat tolerance and thereby partly buffer the negative effect of the pesticide on heat tolerance (Meng et al., 2020b; Op de Beeck et al., 2017b), and modulate the negative effect of the pesticide under warming on antipredator behaviour (Janssens et al., 2014b; Tran et al., 2019). However, it has not been tested whether the three types of exposure duration to warming (acute, developmental and transgenerational) differentially affect the heat tolerance and antipredator behaviour, and more importantly how these types interact with exposure to pesticides.

In this study, we tested how acute, developmental and transgenerational 4 °C warming determine the sensitivity of mosquito larvae to a pesticide. We thereby focused on effects on two ecologically relevant fitness-related traits: heat tolerance and antipredator behaviour. Aquatic stages of insects are considered especially sensitive to warming and pesticides as they cannot escape exposure (Woodward et al., 2010, Brönmark and Hansson, 2002). Mosquitoes may reach a high biomass and therefore play an important role as food in aquatic and terrestrial food webs (Becker et al., 2010). We studied the mosquito *Culex pipiens* form *molestus* (Forskal, 1775). This is a commonly distributed species in Europe and North America (Fonseca et al., 2004). As pesticide, we used the organophosphate chlorpyrifos which is listed among the top ten chemicals threatening aquatic organisms (Johnson et al., 2017). It is commonly used worldwide (although in some countries, its use was banned or restricted recently), and shows

an increased toxicity at higher temperatures in aquatic insects (e.g. Lydy et al., 1999; Dinh et al., 2016; Verheyen et al., 2019). Compared to other aquatic insects, *C. pipiens* is especially sensitive to chlorpyrifos (Rubach et al. 2012). Based on previous work in the study species and other aquatic insects (Meng et al., 2020a; Verheyen et al., 2019; Tran et al., 2019), we expected chlorpyrifos to reduce the heat tolerance, warming to increase the heat tolerance, and each stressor to reduce the antipredator behaviour. Chlorpyrifos is known to degrade faster at higher temperatures (Buchwalter et al., 2003; Op de Beeck et al., 2017a). We allowed pesticide degradation during the exposure period, and thereby tested whether the net impact of the pesticide under warming is determined mainly by the increased toxicity under warming or by the higher degradation under warming. Given that long-term exposure to warming is stressful to *C. pipiens* (Tran et al., 2018), we expected a longer exposure duration to generate more negative effects, including a higher sensitivity to the pesticide.

Materials and methods

Experimental setup

To test how the three types of exposure duration to 4 °C warming influence the sensitivity to the pesticide chlorpyrifos under warming, we set up a 2-generation experiment with four temperature treatments crossed with four pesticide treatments (solvent control vs three nominal chlorpyrifos concentrations: 0.32, 0.34 and 0.36 µg/L) (Figure 1). The chlorpyrifos treatments were imposed in the offspring generation for four days when the larvae entered the final, fourth (L4) instar. This instar is recommended to be exposed by WHO (2005), as it is typically the most robust larval stage in mosquitoes. The four temperature treatments differed in the duration of exposure to warming (24 °C compared to the 20 °C control): never (parental temperature – offspring egg-to-L4 temperature – offspring L4 temperature: 20-20-20), only during the pesticide exposure period in L4 (acute exposure: 20-20-24), already from the egg stage (developmental exposure: 20-24-24), or already from the parental generation (transgenerational exposure: 24-24-24). Note that mosquito larvae in the offspring generation were exposed to the solvent control or to the pesticide at 20 °C in the thermal control treatment, yet at 24 °C in the three warming treatments. Note also that in all three warming treatments the animals were exposed to the pesticide for 4 days, so no differential degradation of the pesticide is to be expected among the three warming treatments. To keep the experiment feasible and because we were interested in the effect of exposure duration within and across generations, we did not

include treatments where the warming treatment shifted back to the control temperature in the next stage. We measured the performance traits, heat tolerance and antipredator escape response (responsiveness and diving time), directly after the 4-day pesticide exposure period, hence in the absence of chlorpyrifos. During the entire experiment, larvae were fed daily 0.313 mg/per larva of a food mixture consisting of Supradyn® vitamins (3%), wheat germs (51%) and Olvarit® 7 cereal flakes (46%). This equals a high food amount (Op de Beeck et al., 2016) and avoids an effect of food scarcity.

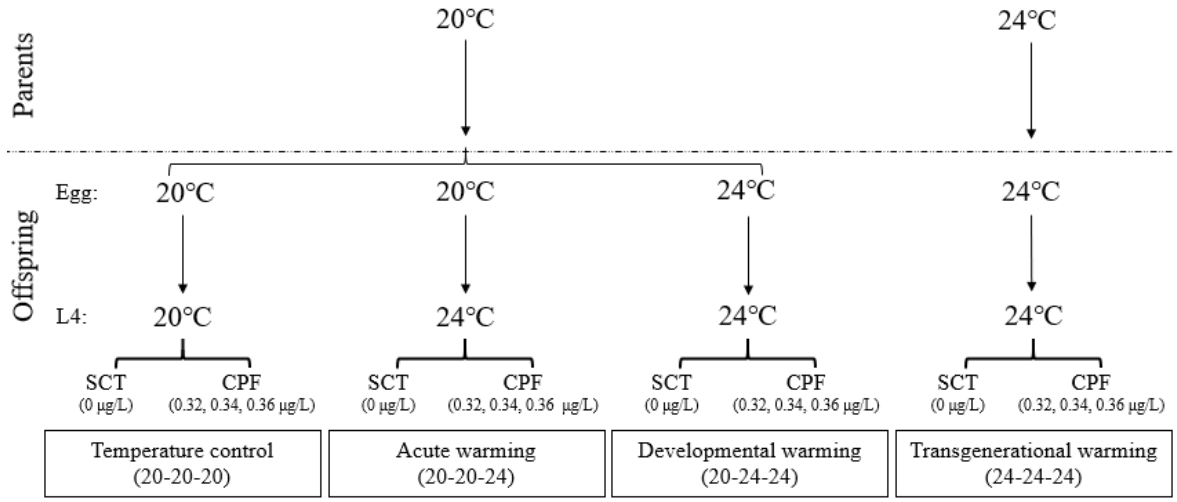


Figure 1. Scheme of the experimental design to test how exposure to 4 °C warming and its duration affects the sensitivity of mosquito larvae to chlorpyrifos. In the control temperature treatment both parents and offspring were continuously kept at 20 °C (parental temperature – offspring egg-to-L4 temperature – offspring L4 temperature: 20-20-20). In the three warming treatments, warming started in the L4 stage of the offspring generation (acute warming, 20-20-24), in the egg stage of the offspring generation (developmental warming, 20-24-24), or already in the parental generation (transgenerational warming, 24-24-24). When larvae had recently (< 24h) moulted into the L4 (= final instar) stage, they were exposed to the solvent control (SCT) or chlorpyrifos solution (CPF).

The mosquito egg clutches were obtained from a lab culture which was housed at 20 °C. This rearing temperature represents the current mean summer temperature in the German source populations that inhabit shallow water bodies (Tran et al., 2016). Because by 2100 a 4.4 °C increase in mean temperature is expected according to IPCC (2021) scenario SSP5-8.5, larvae

were exposed to 24 °C in the warming treatments. Currently, larvae may already experience temperatures of 24 °C during warm periods in summer.

In both the parental and the offspring generations, all mosquitoes were reared from the egg stage in white 2 L containers filled with 1 L aged tap water. To start the parental generation, newly hatched L1 (= first instar) larvae from 144 egg clutches were combined in a white tray with 5 L aged tap water, and sets of 100 larvae were transferred to white 2 L containers. The containers were placed in temperature-controlled rooms at 20 °C or 24 °C depending on the temperature treatment. When the first pupa was detected, groups of three randomly selected containers at each temperature were combined into a white tray containing 5 L aged tap water from which adults could emerge. The trays were covered with white netting. After metamorphosis, the adults from three trays per temperature were transferred with an aspirator to one cage (25 cm × 25 cm × 40 cm) covered with netting and fed a 6% glucose solution. We set up three cages with adults (ca. 400 in each cage) at 20 °C and three cages at 24 °C.

To start the offspring generation, we collected egg clutches from all cages at the same temperature and pooled the newly hatched L1 larvae into 2 L containers (as in the parental generation) which were then kept at 20 °C or 24 °C depending on the temperature treatment. When the larvae of the offspring generation entered L4, the acute warming treatment and the chlorpyrifos treatments started. At that moment, sets of 25 recently (< 24 h) moulted L4 larvae that were pooled from different 2 L containers at the same temperature were placed in 210 mL glass jars containing 125 mL medium (solvent control or chlorpyrifos solution) and exposed for 96 h. The medium during the chlorpyrifos exposure period was refreshed after 48 h. We ran 22-23 replicated jars for each treatment combination (total of 361 jars and 9,025 mosquito larvae).

Based on a range finding experiment where we exposed L4 larvae to chlorpyrifos for 96 h at 20 °C (Appendix A), we chose a nominal concentration of 0.32 µg/L as this caused low (~16%) mortality. To compare the effects of different chlorpyrifos concentrations, we included two higher concentrations: 0.34 µg/L and 0.36 µg/L, which caused ~27% and ~38% mortality in the range finder, respectively. These chlorpyrifos concentrations are ecologically relevant as these are within the range measured in European surface waters: 95% CI = [0.07 µg/L, 0.69 µ/L] (Stehle and Schulz, 2015; personal communication Sebastian Stehle). Note that these three concentrations are close, yet generate considerably different mortality due to the steep dose-response curve of chlorpyrifos in the study species (see the results of the range finder in

Appendix A).

Actual chlorpyrifos concentrations in the experiment were quantified by UPLC-MS/MS. Per concentration, two pooled samples (each sample pooled the medium of 10 jars) were taken at the start of the experiment, and per combination of concentration and temperature two pooled samples were taken after 48 h (just before renewal of the medium). To prepare the chlorpyrifos medium, a stock solution of 0.1 mg/mL was made by dissolving chlorpyrifos (purity grade > 99%) in absolute ethanol, from which a secondary stock solution of 1 µg/mL was made in milliQ water. The same amount of ethanol (3.6 µL/L) as in the high chlorpyrifos concentration (0.36 µg/L) was included in the solvent control. This amount of ethanol is not expected to affect the larvae since ethanol concentrations of up to 500 µL/L do not affect survival and growth of the larvae of the study species (Tam Tran, unpublished data).

Heat tolerance

Heat tolerance was assessed by measuring the critical thermal maximum (CT_{max}) following the method of Meng et al. (2020b) immediately after the 4-day chlorpyrifos exposure period. Specifically, the larvae were heated separately in cups filled with 50 mL aged tap water and the CT_{max} was recorded as the temperature when the larvae started floating motionlessly at the water surface and did not respond to a slight disturbance. The cups containing the larvae were fixed at the water surface of a temperature-controlled tank which was heated by a heater (TC120 optima immersion thermostat, Cambridgeshire, UK) at a rate of 0.3 °C/min. This ramping rate is within the commonly applied range when measuring the CT_{max} of aquatic organisms (e.g. Cambronero et al., 2018; Verberk and Bilton, 2013). The starting temperature for the measurements was 20 °C or 24 °C, matching the rearing temperature during the chlorpyrifos exposure period. When the CT_{max} was reached, the larva was moved immediately to 20 °C or 24 °C (matching the rearing temperature during the chlorpyrifos exposure period) to recover. The few larvae that died (10 out of 738) were excluded from the analyses. The recovered larvae were weighed to the nearest 0.01 mg using an electronic balance (AV135-S, Mettler Toledo, Columbus, OH, USA) to mass-correct the CT_{max} value. CT_{max} was in most cases measured on two randomly selected larvae per jar, yet in 14 out of 361 jars three larvae per jar were tested (to make the number of larva measured as equal as possible across treatments). The exact sample sizes for each treatment combination are shown in Figure 3.

Alarm escape response

Culex mosquito larvae stay most of the time at the water surface to obtain oxygen (Corbet et al., 2000). They react to a sudden change of light intensity by diving to the bottom; this is regarded as an alarm escape response to avoid predation (Reynaldi et al., 2011; Futami et al., 2008). A stronger escape response, being a higher percentage of mosquitoes that show the diving response (= responsiveness) and a longer diving time, is considered more effective against predators such as the water boatman *Notonecta glauca* (Reynaldi et al., 2011; Futami et al., 2008).

The alarm escape response was measured following the protocol of Tran et al. (2019) at the end of the 4-d chlorpyrifos exposure period. Specifically, five larvae were randomly selected from each experimental jar and transferred to a new jar containing aged tap water (irrespective of the chlorpyrifos treatment). Jars with larvae were first kept in the dark for 5 minutes and afterwards placed below a light source with an intensity of 1500 lux. When all five larvae were located at the water surface (within 5 minutes), the light was turned off and we recorded the number of larvae per jar that showed the diving response. Five seconds later we turned the light on again and recorded the time (diving time) needed for each larva to return to the water surface to the nearest 0.01 second using a chronometer. The temperature during the measurements matched the temperature the larvae experienced during the chlorpyrifos exposure period. All measurements were conducted by the same observer (Meng Shandong) between 11:00 to 14:00. One mean value for diving time was obtained per jar for the statistical analyses.

Statistical analyses

We analyzed all variables using R v3.6.1 (R Core Team, 2019) with the following packages: ‘lme4’ (v1.1-21, Bates et al., 2015), ‘afex’ (v0.25-1, Singmann et al., 2017), ‘car’ (v3.0-3, Fox and Weisberg, 2018), ‘emmeans’ (v2.30-0, Lenth et al., 2019) and ‘drc’ (v3.0.1, Ritz et al., 2015).

The effect of temperature on the measured chlorpyrifos concentrations after 48 hours was analyzed using a general linear model with temperature, nominal chlorpyrifos concentration and their interaction as factors. The main effects of the warming treatments and chlorpyrifos exposure, and their interaction on CT_{max} were analyzed by a general linear mixed model with the body mass of the larva as a covariate and experimental jar as a random factor.

The main effects of the warming treatments and chlorpyrifos exposure, and their interaction on the diving response were analyzed using a generalized linear mixed model with a binomial error structure and the logit link function. We categorized the number of larvae showing and not showing the diving response in each jar. We added the date of testing as a random factor. The main effects of the warming treatments and chlorpyrifos exposure, and their interaction on diving time were analyzed using a general linear model. When there was an interaction between warming and exposure to chlorpyrifos we compared the estimated marginal means of the different treatment combinations with the function contrasts in the package 'emmeans'; the obtained p-values were false discovery rate (FDR) corrected and coded on the figures. Based on these posthoc tests, we first described the effects of single exposure to warming (in the absence of chlorpyrifos), single exposure to chlorpyrifos (in the absence of warming), and then how the warming treatments (exposure duration) shaped the effect of chlorpyrifos.

Table 1. The main and interactive effects of the warming treatments and chlorpyrifos exposure on heat tolerance (CT_{max}), and the two alarm escape response variables (responsiveness and diving time). Significant *P*-values ($P < 0.05$) are indicated in bold.

	CT_{max}			Responsiveness			Diving time		
	χ^2	Df	P	χ^2	Df	P	F	Df	P
Chlorpyrifos	177.93	3	**< 0.001**	30.67	3	**< 0.001**	40.52	3,288	**< 0.001**
Warming	51.14	3	**< 0.001**	57.37	3	**< 0.001**	6.75	3,288	**< 0.001**
Chlorpyrifos × Warming	25.48	9	**0.002**	17.14	9	**0.046**	3.01	9,288	**0.002**
Mass	3.06	1	0.080						

Results

Chlorpyrifos concentrations

The measured chlorpyrifos concentrations at the start of the exposure period for the three nominal concentrations of 0.32, 0.34 and 0.36 µg/L were (mean ± SE) 0.262 ± 0.021, 0.297 ±

0.037, and 0.345 ± 0.023 µg/L (N = 2 pooled samples per concentration), respectively. For all three nominal concentrations, the measured concentrations in the experimental jars after 48 h were ~ 61.8% lower at 24 °C than that at 20 °C (main effect Temperature: $F_{1,6} = 14.96$, $P = 0.008$, Figure 2, contrasts between 20 °C and 24 °C for each concentration: all $P \leq 0.001$). No significant interaction between temperature and nominal concentration was observed (Temperature × Nominal concentration: $F_{2,6} = 2.51$, $P = 0.161$).

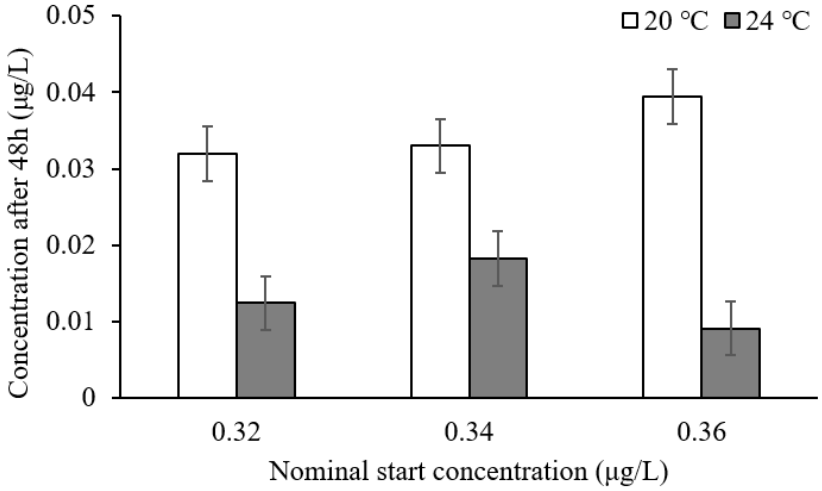


Figure 2. The measured chlorpyrifos concentrations 48 h after the exposure started in the experimental jars for the three nominal start concentrations at the two temperatures. Mean concentrations (± 1 standard error) are based on two pooled samples each of 10 experimental jars.

Heat tolerance (CT_{max})

Warming, exposure to chlorpyrifos and their interaction significantly shaped CT_{max} (Table 1, Figure 3). Warming in general increased CT_{max} (main effect Warming, Table 1). In the absence of chlorpyrifos, CT_{max} increased under acute warming (20-20-24) but no longer under developmental (20-24-24) and transgenerational warming (24-24-24). Exposure to chlorpyrifos in general decreased CT_{max} (main effect Chlorpyrifos, Table 1). This chlorpyrifos-induced reduction of CT_{max} was stronger (up to -2.0 °C) at 20 °C (20-20-20) than under the three warming treatments at 24 °C (up to -1.6 °C) (Chlorpyrifos × Warming, Table 1). At 20 °C, chlorpyrifos reduced CT_{max} more at 0.34 and 0.36 µg/L than at 0.32 µg/L. At 24 °C, CT_{max} was more sensitive to chlorpyrifos under acute warming than under transgenerational warming at

0.32 µg/L: the chlorpyrifos-induced reduction of CT_{max} was 2.8 times larger under acute warming than under transgenerational warming. Yet, at 0.34 and 0.36 µg/L there was no longer a difference among warming treatments in the extent the heat tolerance was reduced by chlorpyrifos.

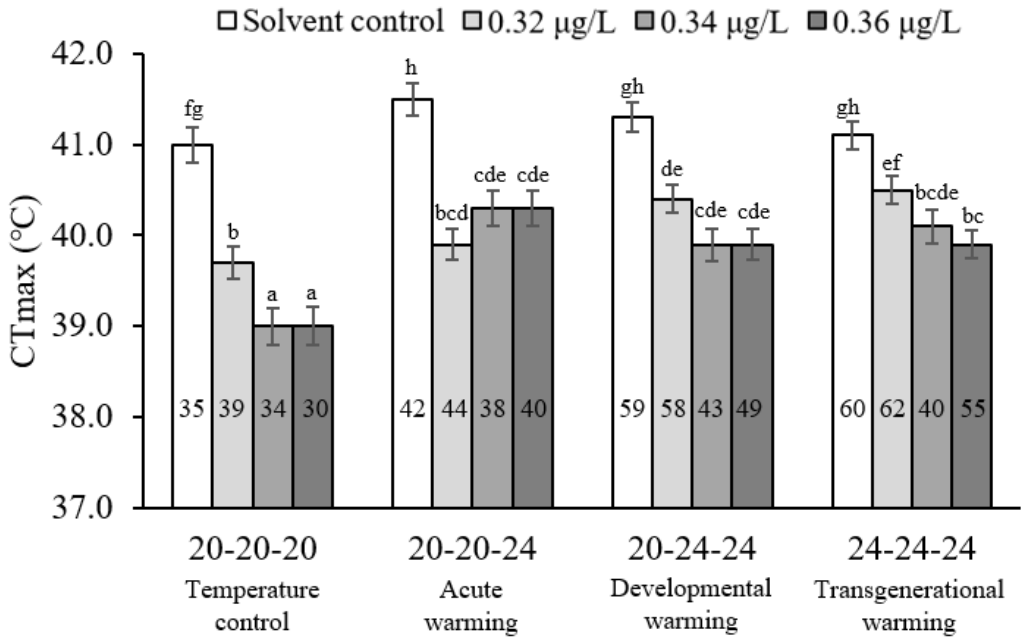


Figure 3. Effects of exposure to chlorpyrifos, warming and their interactions on heat tolerance (CT_{max}) of L4 larvae of the mosquito *Culex pipiens*. Means are shown ± 1 standard error. Means that differ significantly (false discovery rate corrected $P < 0.05$) are indicated by different letters. Numbers in bars represent sample sizes (number of larvae measured).

Alarm escape response

Warming, exposure to chlorpyrifos and their interaction significantly affected the percentage of mosquitoes that showed an escape diving response (= responsiveness) (Table 1, Figure 4a). Warming in general reduced the responsiveness (main effect Warming, Table 1). In the absence of chlorpyrifos, the responsiveness was higher under acute warming (20-20-24) than under developmental (20-24-24) and transgenerational warming (24-24-24). Exposure to chlorpyrifos in general decreased the responsiveness, which further depended on the warming treatment (main effect Chlorpyrifos, Chlorpyrifos x Warming, Table 1). At 20 °C, exposure to chlorpyrifos reduced the responsiveness, and this reduction was stronger at 0.34 and 0.36 µg/L

(-23.2%) than at 0.32 µg/L (-9.0%). At 24 °C, the responsiveness was more sensitive to chlorpyrifos under acute warming than under long-term warming. Under acute warming (20-20-24), all chlorpyrifos concentrations reduced the responsiveness to the same degree (-22.5%); however, under developmental warming (20-24-24) only the highest chlorpyrifos concentration (0.36 µg/L) significantly reduced the responsiveness (-31.3%), and under transgenerational warming (24-24-24) none of the three concentrations reduced the responsiveness.

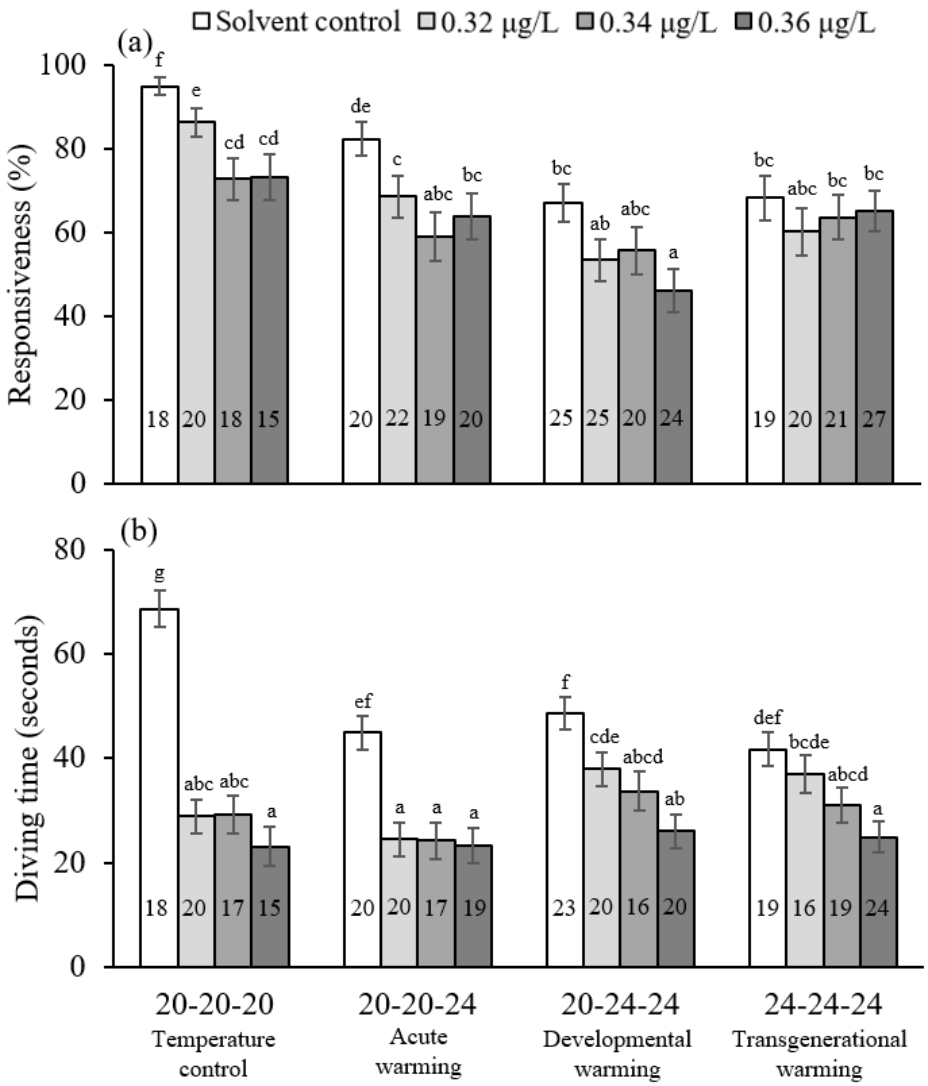


Figure 4. Effects of exposure to chlorpyrifos, warming and their interactions on the alarm escape diving response of L4 larvae of the mosquito *Culex pipiens*: (a) responsiveness and (b) diving time. Means are shown ± 1 standard error. Means that differ significantly (false discovery rate corrected $P < 0.05$) are indicated by different letters. Numbers in bars represent sample sizes.

Warming, exposure to chlorpyrifos and their interaction significantly shaped the escape diving time (Table 1, Figure 4b). In the absence of chlorpyrifos, warming shortened the diving time by 34.3%, and this to the same extent across warming treatments. At 20 °C, exposure to chlorpyrifos shortened the diving time by 60.6%, and this did not depend on the concentration (Figure 4b). The chlorpyrifos-induced reduction of diving time was smaller at 24 °C than at 20 °C (Chlorpyrifos × Warming, Table 1). At 24 °C, the diving time was more sensitive to chlorpyrifos under acute warming than under transgenerational warming. Under acute warming the chlorpyrifos-induced reduction was -46.6% and this already at 0.32 µg/L and did not further reduce at 0.34 and 0.36 µ/L. Instead, under developmental warming the chlorpyrifos-induced reduction in diving time went from -22.0% at 0.32 µg/L to -46.5% at 0.36 µg/L, and under transgenerational warming the diving time was only significantly reduced at the highest concentration (0.36 µg/L) by -40.3%.

Discussion

Effects of warming in the absence of chlorpyrifos

Our results support the hypothesis that developmental and transgenerational warming were energetically more costly than acute warming. Indeed, only acute warming resulted in an adaptive increase of CT_{max}, and the maladaptive reduction in diving responsiveness was smaller under acute compared to long-term warming. These patterns cannot be explained by differential effects of the three warming treatments on body mass (see Appendix C).

Acute 4 °C warming increased the heat tolerance measured as CT_{max}, which is in line with the general adaptive pattern that the upper thermal limit increases after acclimation to higher temperatures (Gunderson and Stillman, 2015). Note that the observed increase in CT_{max} under warming is conservative and not a result of the higher start temperature during the CT_{max} test (see Appendix B). This higher heat tolerance may be caused by increased levels of heat shock proteins (King and MacRae, 2015; Dahlhoff and Rank, 2000). Notably, the warming-induced increase in heat tolerance did no longer exist when the duration of warming was prolonged under developmental and transgenerational warming. This can be explained by observations that heat shock genes no longer are upregulated and can even be downregulated under long-term developmental and transgenerational exposure to warming because of associated energetic costs (Chen et al., 2018; Veilleux et al., 2015; Dixon et al., 2015; Meyer

et al., 2011). In general, the increase in metabolic rate can be much higher than the increase in feeding rate at evaluated temperatures reducing the energetic efficiency under warming (Vucic-Pestic et al., 2011). This mechanism may indeed make long-term exposure to warming energetically more costly compared to short-term exposure (see also Meyer et al., 2011; Cloyed et al., 2019). Notably, this may occur also at abundant food levels (e.g. Veilleux et al., 2015) as in the current study.

Under warming, less larvae showed the alarm escape by diving away from the water surface, and those that did had a shorter diving time. This reduction in diving time under developmental warming was shown before in mosquito larvae (e.g. Tran et al., 2019). Similarly, diving beetles show at higher temperatures a decreased antipredator behaviour associated with a higher surfacing frequency (e.g. Calosi et al., 2007). An increased demand of oxygen under warming due to a higher metabolic rate (Verberk et al., 2011) may contribute to these patterns since mosquito larvae mainly obtain oxygen from the air (Silberbush et al., 2015). Furthermore, as diving is energetically costly (Olsson and Klowden, 1998), the shortened diving times under warming may also be associated with energy deficiency, as the total energy budget is decreased by warming in the study species (Meng et al., 2020b). Interestingly, the diving responsiveness was further reduced under developmental and transgenerational warming compared to acute warming. This further suggests that more energy was utilized, thus more oxygen was needed, to offset the negative effects of prolonged warming.

Effects of chlorpyrifos in the absence of warming

At 20 °C, chlorpyrifos negatively affected CT_{max} and the two diving response variables. For CT_{max} and diving responsiveness this negative impact was stronger at 0.34 and 0.36 µg/L than at 0.32 µg/L.

As expected, exposure to chlorpyrifos decreased the heat tolerance, which supports the "toxicant induced climate change sensitivity" (TICS) concept (Hooper et al., 2013; Moe et al., 2013; Noyes et al., 2009). A chlorpyrifos-induced reduction in heat tolerance supports previous findings in the study species (e.g. Meng et al., 2020b, b) and other freshwater organisms (e.g. damselfly larvae: Verheyen et al., 2019; fish: Patra et al., 2007). The heat tolerance of an organism is thought to be determined by a mismatch between the oxygen demand and supply (Verberk et al., 2013). This mismatch is likely to occur at lower temperatures under exposure to chlorpyrifos since the damage repair and detoxification mechanisms may increase the oxygen demand (Sokolova, 2013; for chlorpyrifos; Narváez et al., 2016), while an impaired respiratory

functioning may decrease the oxygen supply (for chlorpyrifos: Marigoudar et al., 2018; Negro and Collins, 2017). CTmax values were much higher than the experienced mean rearing temperatures, which is a general pattern (e.g. Jørgensen et al., 2019). Yet, CTmax values are highly correlated with maximum environmental temperatures thereby being a good proxy of the relative ability to deal with thermal extremes (e.g. Huey et al., 2012; Jørgensen et al., 2019). Moreover, the heat tolerance of organisms correlates with the tolerance to mild warming (Åsheim et al., 2020), hence may inform about the ability of organisms to deal with mild warming. The chlorpyrifos-induced decrease in CTmax (ca. 2 °C) observed in the current study is likely ecologically relevant as such absolute changes match those observed among thermally adapted populations from different thermal environments differing in 4 °C (e.g. for damselfly larvae: Carbonell and Stoks, 2020; Janssens et al., 2021).

The increased oxygen demand and possibly reduced oxygen supply under chlorpyrifos exposure together with the assumed energetic cost of diving may also explain why exposure to chlorpyrifos reduced the proportion of larvae showing escape diving and shortened the diving times. These reductions in escape responses match the previously recorded reduced responsiveness when exposed to the pesticide fenvalerate (Reynaldi et al., 2011), and the shortened diving time when exposed to chlorpyrifos (Tran et al., 2019) in the study species. Both a decreased escape responsiveness and a shortened diving time have been associated with increased risk of being killed by predators (Reynaldi et al., 2011; Tran et al., 2016), suggesting chlorpyrifos exposure increased the vulnerability of larvae to their predators.

General effects of chlorpyrifos under warming

Chlorpyrifos reduced the heat tolerance and the diving time less for the three warming treatments at 24 °C than for the control temperature of 20 °C. This may be explained by the higher degradation rate, as evidenced by the observed lower chlorpyrifos concentrations after 48 h at 24 °C than at 20 °C. This higher degradation at 24 °C hence overruled the documented stronger toxicity of chlorpyrifos at 24 °C in the study species (Delnat et al., 2019a; Tran et al., 2018). Such pattern has been predicted in general (Moe et al., 2013), but has surprisingly only been rarely described (but see for the damselfly *Ischnura elegans*, Op de Beeck et al., 2017a). In line with this explanation, lower chlorpyrifos concentrations indeed reduced the heat tolerance less as shown at 20 °C (see also Meng et al., 2020b). In addition, the warming-induced increase in heat tolerance may also partly have counteracted the chlorpyrifos-induced reduction in heat tolerance (for the study species: Meng et al., 2020b; for damselfly larvae: Verheyen et

al., 2019). Warming may thereby have increased the CTmax to an upper limit value in the solvent controls, and as a result made the difference in CTmax between the solvent control and the three chlorpyrifos treatments smaller in each warming treatment. For diving time which was already strongly reduced by warming, the species may have tried to maintain a minimum diving time to guarantee a minimum effect of this antipredator behaviour. An alternative mechanism whereby warming initiated an overall stress response that helped the larvae to better deal with the pesticide seems less likely. Indeed, the general pattern is that warming magnifies the toxicity of organophosphate pesticides (Noyes et al., 2009; Holmstrup et al., 2010; Moe et al., 2013). More general, co-exposure to stressors is expected to cause synergistic interactions (Gunderson et al. 2016).

Differential effects of chlorpyrifos under the three warming types

Contrary to our expectation, a key finding was that the negative effects of chlorpyrifos at 24 °C on CT_{max} and the two escape response variables were less pronounced under transgenerational, and to some extent also under developmental warming, than under acute warming. Notably, the chlorpyrifos-induced reduction in CT_{max} at the low concentration (0.32 µg/L) was nearly 3-fold smaller under transgenerational warming than under acute warming. Hence, transgenerational warming weakened the chlorpyrifos-induced negative effects on heat tolerance at 24 °C compared to acute warming. Similarly, the chlorpyrifos-induced reduction of escape diving at 24 °C was smaller under transgenerational warming since none of the three chlorpyrifos concentrations significantly reduced the responsiveness, and no significant reductions in diving time were detected at 0.32 and 0.34 µg/L. A similar pattern also tended to occur under developmental warming where chlorpyrifos concentrations of 0.32 and 0.34 µg/L did not statistically reduce the diving responsiveness, and 0.32 µg/L chlorpyrifos resulted in a longer diving time than under acute warming.

Intriguingly, while long-term exposure to warming was energetically more stressful than acute warming, particularly transgenerational warming reduced the sensitivity of mosquito larvae to the pesticide compared to acute warming (especially at the low concentration of 0.32 µg/L). We can exclude several potential reasons for this apparently counterintuitive pattern. First, any differential effects of the pesticide among the three warming treatments cannot be due to differential degradation of the pesticide as in all three warming treatments the pesticide was kept at 24 °C for the same duration. Moreover, this pattern is unlikely to be fully mediated by warming-induced effects on larval body mass. Indeed, the body mass in the solvent control

(likely reflecting the body mass patterns at the start of the exposure period) was largest under acute warming, intermediate under transgenerational warming and smallest under developmental warming (Appendix C). Given that smaller larvae of aquatic insects are more vulnerable to chlorpyrifos (chironomid larvae: Buchwalter et al., 2002; damselfly larvae: Verheyen and Stoks, 2019), differences in body mass among the three warming treatments can therefore not explain our key finding that the negative effects of chlorpyrifos were strongest under acute warming and least strong under transgenerational warming (for more details see Appendix C). Furthermore, any limit values for CTmax or diving time may also not explain differential effects on these response variables among the three warming treatments.

Instead, this pattern might be explained because under developmental and transgenerational warming larvae were already acclimated to 24 °C before being exposed to chlorpyrifos, while under acute warming larvae experienced both a switch to 24 °C and exposure to the pesticide when they entered the final L4 stage. The latter may have resulted in a stronger acute stress response. Related to this, as the energetically costly heat shock proteins have commonly been shown to be upregulated under acute thermal stress (reviewed by Sørensen et al., 2003; Chen et al., 2018) but no longer under developmental and transgenerational warming (Chen et al., 2018; Veilleux et al., 2015), larvae under the two long-term warming treatments may have had more energy available for detoxification. An alternative reason why transgenerational warming reduced the sensitivity may be that under transgenerational warming the parents were reared at 24 °C, hence likely had a smaller body mass (given that this was the case for the larvae reared from the egg stage at 24 °C, appendix C), compared to the parents of the three other warming treatments (that all developed at 20 °C). Bagni et al. (2020) showed in the moth *Spodoptera littoralis* that offspring from parents with a smaller maternal body mass were less sensitive to chlorpyrifos, which was unrelated to the offspring body mass. Unfortunately, they could not identify the underlying mechanism for this transgenerational effect. Nevertheless, even if partly contributing to our pattern, this mechanism cannot explain why larvae also suffered less from the pesticide under developmental warming than under acute warming, as the parents of both groups were reared under identical conditions (hence likely did not differ in mass).

Conclusions

Despite the increased attention for temporal aspects of stressor interactions (Orr et al., 2020) and for effects of warming in ecotoxicological studies (Moe et al., 2013, Holmstrup et al., 2010), no study so far compared how different exposure durations to warming may affect pesticide sensitivity. Our results suggest that warming generally reduced the impact of chlorpyrifos on heat tolerance and antipredator behaviour, probably caused by an increased degradation of chlorpyrifos. Notably, while long-term exposure to warming seemed energetically more stressful than acute warming, transgenerational warming did make mosquito larvae better in coping with the exposure to pesticide chlorpyrifos at 24 °C than acute warming. While our experiment simulated an increase of 4.4 °C by 2100 under IPCC (2021) warming scenario SSP5-8.5, mosquito larvae currently already may experience water temperatures of 24 °C making the observed effects also relevant for current populations during warm summers. Our results provide important evidence that the duration of warming may modulate the interactive effects with pesticides, thereby identifying a novel temporal aspect in ecotoxicological studies.

Acknowledgments

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Appendix A: Range finding experiment for chlorpyrifos

We did a separate trial to select the suitable concentration used for the experiment. Mosquito larvae were reared in 2 L containers at a density of 100. When they entered final larval instar, sets of pooled newly molted L4 larvae (within 24 h) were transferred to 210 mL jars with 125 mL medium (solvent control or relevant chlorpyrifos solution). The jars were kept in a temperature-controlled room at 20 ℃. Two pulses were given during the 96-h exposure with the first one at the start of the chlorpyrifos exposure and the second one 48 h later.

The following concentrations were tested: 0 (ethanal solvent control), 0.15, 0.20, 0.25, 0.28, 0.30, 0.33, 0.35, 0.38, 0.40, 0.50, 0.60, 0.80 μg/L. We ran 5 to 6 replicate jars for each treatment. The mortality was recorded at the end of the 96h exposure. Dose-response curve was fitted by using a log-logistic function and were used to obtain the $LC_{20,96h}$ values with the 95% confidence interval using the "drm function" in the package drc v3.0-1 (Ritz et al., 2015).

The $LC_{20,96h}$ for chlorpyrifos was 0.325 μg/L (SE: 0.005; 95% CI [0.316, 0.335], Figure A). We chose 0.32 μg/L as the concentration used in the real experiment which caused ca. 16% mortality. To compare the effects of different chlorpyrifos concentrations, we included two higher concentrations, 0.34 μg/L and 0.36 μg/L, which caused ca. 27% and ca. 38% mortality, respectively.

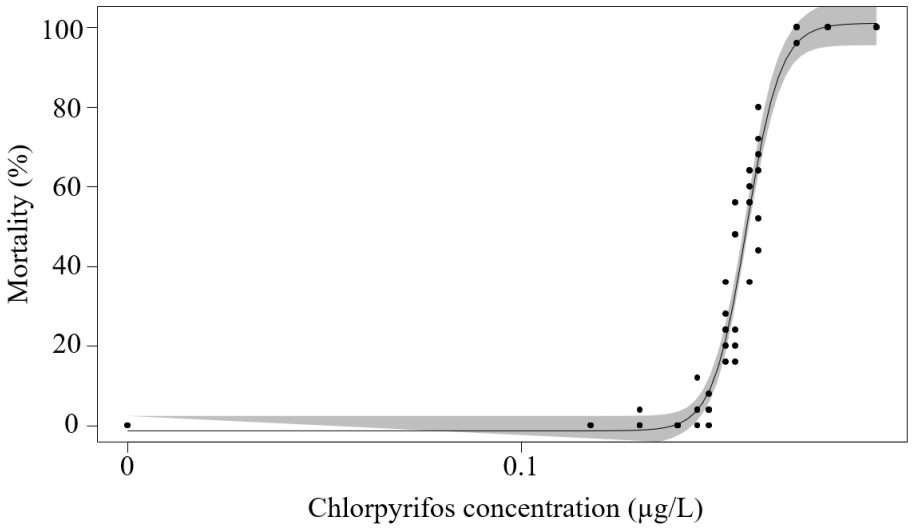


Figure A. The dose-response curve for the lethal effect of chlorpyrifos after 96 h in the larvae of mosquito *Culex pipiens*. The grey area represents the 95% confidence interval, and the dots visualize the observed mortality for a given jar (note that dots may overlap)

Appendix B: The effects of starting temperature on CTmax measurements

Methods

We did separate trials to test if the starting temperature affects the outcomes of CTmax measurements for the three warming treatments. Larvae were reared at 20 ℃ in white 2 L containers with a density of 100. When they entered L4 (= final larval stage), sets of 25 newly molted L4 larvae (within 24 h) were pooled to 210 mL glass jars with 125 mL aged tap water (in total we got 7 jars) which were then kept in a temperature-controlled room at 24 ℃. Larvae were daily fed with the same amount of food as in the formal experiment. 96 h later when the CTmax measurements started, two sets of five larvae were randomly selected from each jar. One set was measured with the starting temperature of 20 ℃ and the other set was measured from 24 ℃. For each starting temperature we measured 35 larvae. The procedure of the CTmax measurements was exactly same as in the formal experiment. We used general linear model to analyze the effect of starting temperature on CTmax.

Results

The starting temperature had effects on the outcomes of CTmax measurements ($F_{1,66} = 6.03$, $P = 0.017$). For larvae reared at 24 ℃, the CTmax was 41.4 ± 0.141 ℃ (mean ± 1SE) when the starting temperature was 20 ℃, and was 40.9 ± 0.141 ℃ when the starting temperature was 24 ℃.

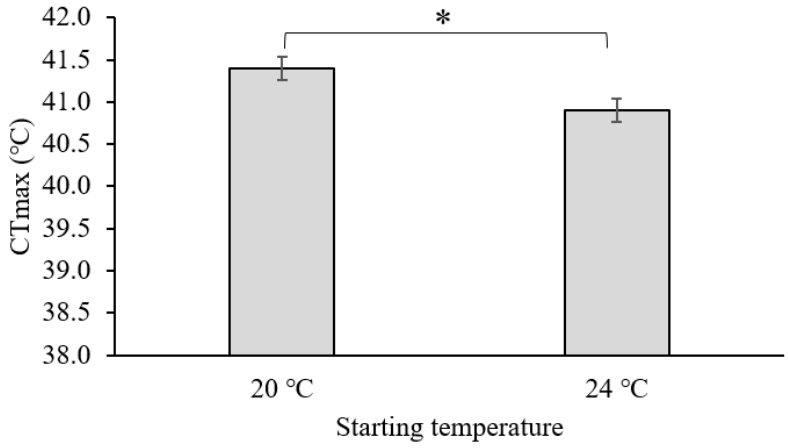


Figure B. The effects of starting temperature on CTmax measurements

Appendix C: Response patterns in larval body mass to the warming and chlorpyrifos treatments, and its potential role in affecting treatment effects on the three endpoints

Methods

At the end of the 4-d chlorpyrifos exposure, two to three larvae were randomly selected per jar for CTmax measurements, after which the living larvae were weighed to the nearest 0.01 mg (see details in Materials and methods). From each jar one mean value of body mass was obtained.

The main and interactive effects of warming and chlorpyrifos exposure on larval body mass were analyzed by using general linear models. The main and interactive effects of warming, chlorpyrifos exposure and larval body mass on CTmax were analyzed by a general linear mixed model with experimental jar as a random factor. The main and interactive effects of warming, chlorpyrifos exposure and larval body mass on the diving response were analyzed using a generalized linear mixed model with a binomial error structure and the logit link function, and the date of testing was added as a random factor. The main and interactive effects of warming, chlorpyrifos exposure and larval body mass on diving time were analyzed using a general linear model.

Results and discussion

Effects of warming and chlorpyrifos exposure on larval body mass

The larval body mass was generally smaller at 24 ℃ than at 20 °C at the end of the 4-d chlorpyrifos exposure period (Warming: $F_{3,316} = 37.64$, $P < 0.001$), yet this reduction only was significant under developmental and under transgenerational warming (contrasts: both $P < 0.001$), but not under acute warming (contrast: $P = 0.909$) (Warming × Chlorpyrifos: $F_{9,316} = 3.17$, $P = 0.001$, Table C, Figure C). This difference among warming treatments is expected as under acute warming the larvae have only been exposed to 24 °C for 4 days in the final L4 stage, while both under developmental and transgenerational warming this was from the egg stage onwards. This fits the idea that the temperature-size rule is driven by higher temperatures increasing development rates more than growth rates, leading to smaller sizes in later instars (Forster and Hirst, 2012). In addition, the larval body mass was smaller under developmental

warming than under transgenerational warming (contrast: P = 0.009). This is in line with the phenomenon that offspring from mothers that experienced warmer conditions are bigger, compared to offspring from mothers that experienced cooler conditions (Verberk et al., 2021). Chlorpyrifos exposure at the two highest concentrations increased larval body mass but only at 20 °C (main effect Chlorpyrifos: $F_{3,316}$ = 6.20, P < 0.001; Warming × Chlorpyrifos: $F_{9,316}$ = 3.17, P = 0.001, Table C, Figure C). This might be caused by survival selection at the two highest concentrations removing the weakest larvae, probably those with the smallest body mass.

Effects of larval body mass on CTmax, responsiveness and diving time

When including besides larval body mass (as in the original manuscript), also the interactions Warming × Body mass, Chlorpyrifos × Body mass and Warming × Chlorpyrifos × Body mass, these interactions were not significant for the response variables CTmax (all P > 0.06), and diving time (all P > 0.55). Hence, this indicates that the significant effects of Warming, Chlorpyrifos and their interaction on CTmax and diving time could not be explained through effects on body mass.

For diving responsiveness, however, the Warming × Chlorpyrifos × Body mass interaction was significant (χ_9^2 = 17.16, P = 0.045). This indicates that there is no general mass correction possible for the different warming treatments. We therefore analyzed the Chlorpyrifos effect separately in each warming treatment. Note that within each warming treatment no correction for body mass is needed as all larvae of the same warming treatment have been affected the same for body mass. This set of separate analyses showed that chlorpyrifos had a significant effect on responsiveness in the temperature control (χ_3^2 = 19.96, P < 0.001), acute warming (χ_3^2 = 13.08, P = 0.004) and developmental warming (χ_3^2 = 13.95, P = 0.003) groups, but chlorpyrifos had no effect in the transgenerational warming group (χ_3^2 = 1.95, P = 0.583). This further supports that, if anything, chlorpyrifos reduced the diving responsiveness less (even not) under transgenerational warming, while it did so under acute warming and to a lesser extent under developmental warming. Given the above-mentioned patterns in body mass, this pattern cannot be driven by larval body mass differences among the warming treatments.

To specifically check whether developmental and transgenerational warming differentially affected the response variables compared to acute warming by affecting body mass, we further analyzed the Warming × Body mass interaction in the solvent control. For all three endpoints (CTmax, diving responsiveness and diving time), the effects of Body mass and

the Warming x Body mass interaction were not significant (all *P*-values > 0.27). Thus, the different patterns under developmental and transgenerational warming compared to under acute warming cannot be explained by the warming effects on body mass.

Table C. The main and interactive effects of the warming treatments and chlorpyrifos exposure on the larval body mass at the end of the 4-d chlorpyrifos exposure. Significant *P*-values ($P < 0.05$) are indicated in bold.

	Larval body mass		
	F	Df	P
Chlorpyrifos	6.20	3,316	< **0.001**
Warming	37.64	3,316	< **0.001**
Chlorpyrifos × Warming	3.17	9,316	**0.001**

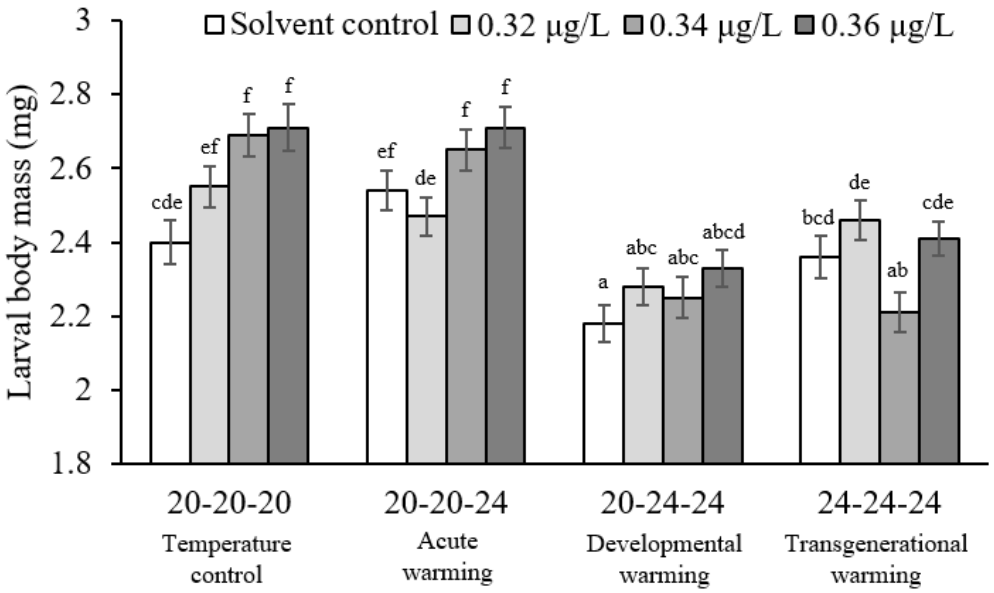


Figure C. Effects of exposure to chlorpyrifos, warming and their interactions on the larval body mass of the mosquito *Culex pipiens* at the end of the 4-d chlorpyrifos exposure. Means are shown ± 1 standard error. Means that differ significantly (false discovery rate corrected $P < 0.05$) are indicated by different letters.

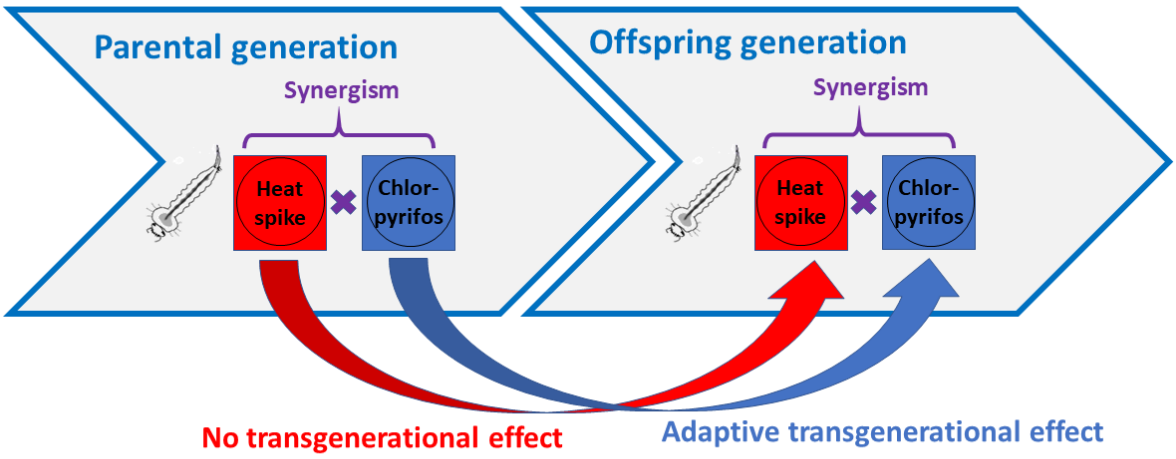
Chapter 5

Transgenerational 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

Environmental Pollution, accepted

Slightly adapted version



Abstract

While interactions with global warming and transgenerational effects are considered crucial to improve risk assessment of pesticides, these have rarely been studied in an integrated way. While heat extremes can strongly 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 transgenerational effect for the heat spike. Despite the adaptive transgenerational 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 transgenerational effects in risk assessment.

Key words: Global change, multiple stressors, synergistic interaction, temperature extremes, transgenerational effects

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., 2020b; 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, 2021; 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 transgenerational 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). Transgenerational effects can cause a negative 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, transgenerational effects of a parental stressor may reduce the tolerance of the offspring to the same stressor (Uller, 2008). Such maladaptive transgenerational 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. When ignored, maladaptive transgenerational effects may underestimate the total impact of stressors on populations. Yet, transgenerational 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 transgenerational 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 transgenerational 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., 2020b), 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 transgenerational effects of warming and pesticide exposure being (mal)adaptive we expected these to reduce or increase the synergism in the offspring generation.

Materials and methods

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 (Figure 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., 2020b). 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; 2,880 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 ℃ 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 ℃ 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 ℃. When larvae entered L4, we pooled the newly (< 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 ℃, the other half experienced a heat spike. To impose the heat spike, the jars were first heated from 20 to 30 ℃ in 5 h, and kept at 30 ℃ for 38 h, then cooled from 30 to 20 ℃ 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 48h 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.

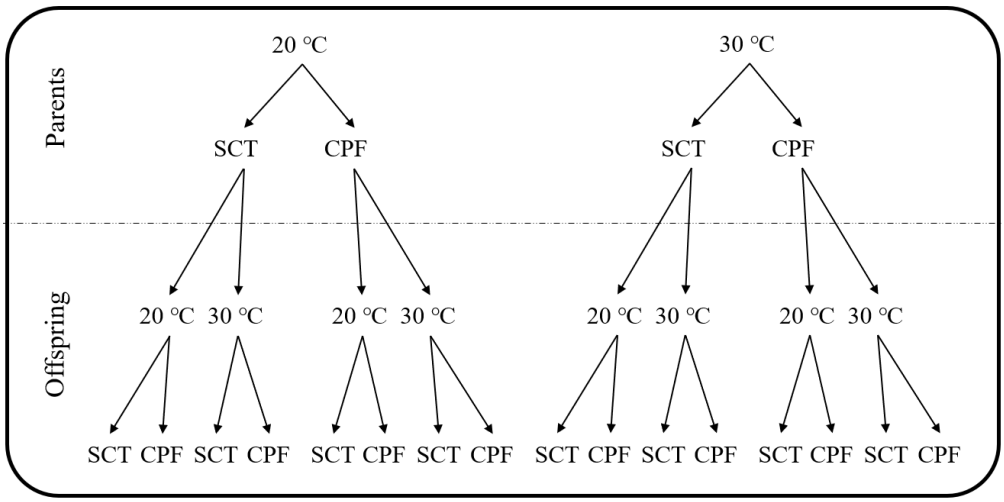


Figure 1. Scheme of the bifactorial transgenerational experimental design to test how legacies of the heat spike and/or the exposure to chlorpyrifos in parents affect the sensitivity of offspring when dealing with the heat spike and chlorpyrifos. In both generations, L4 larvae were first exposed to 20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

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 µ/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 24h 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 ℃ 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%).

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 ℃ for physiological measurements. 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).

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 ℃ 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 ℃ for 20 minutes, 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.

Physiology

We measured the activity of acetylcholinesterase (AChE), the target enzyme of chlorpyrifos (Domingues et al., 2010), following the protocol 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).

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.

Results

Mortality

In the parental generation, chlorpyrifos caused mortality, especially in larvae pre-exposed to the heat spike (10.8% vs 0.6%) (Table 1, Figure 2A). This synergism was confirmed by the IA model (Appendix E). The heat spike itself (in the solvent control) did not cause mortality. In the offspring generation, both the heat spike (1.0%) and chlorpyrifos exposure (1.2%) in isolation slightly increased mortality (Table 2, Figure 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%).

CTmax

In the parental generation, chlorpyrifos reduced CTmax, especially in larvae pre-exposed to the heat spike (-3.4% vs -1.5%) (Table 1, Figure 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, Figure 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%).

Growth rate

In the parental generation, chlorpyrifos generally increased the larval growth rate (+10.3%), while the heat spike itself did not (Table 1, Figure 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, Figure 3C).

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, Figure 2D). The heat spike itself (in the solvent control) did not affect the AChE activity. In the offspring generation, chlorpyrifos also reduced the AChE activity, and again especially after the heat spike (–56.6% vs -37.1%) (Table 2, Figure 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.

Wing length

In the parental generation, chlorpyrifos reduced female wing length but only in larvae pre-exposed to the heat spike (Table 1, Figure 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, Figure 3E).

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, Figure 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, Figure 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.

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, Figure 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

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 transgenerational effects in response to chlorpyrifos exposure. This adaptive transgenerational effect was not found for parental heat spike exposure. Despite the adaptive transgenerational 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 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

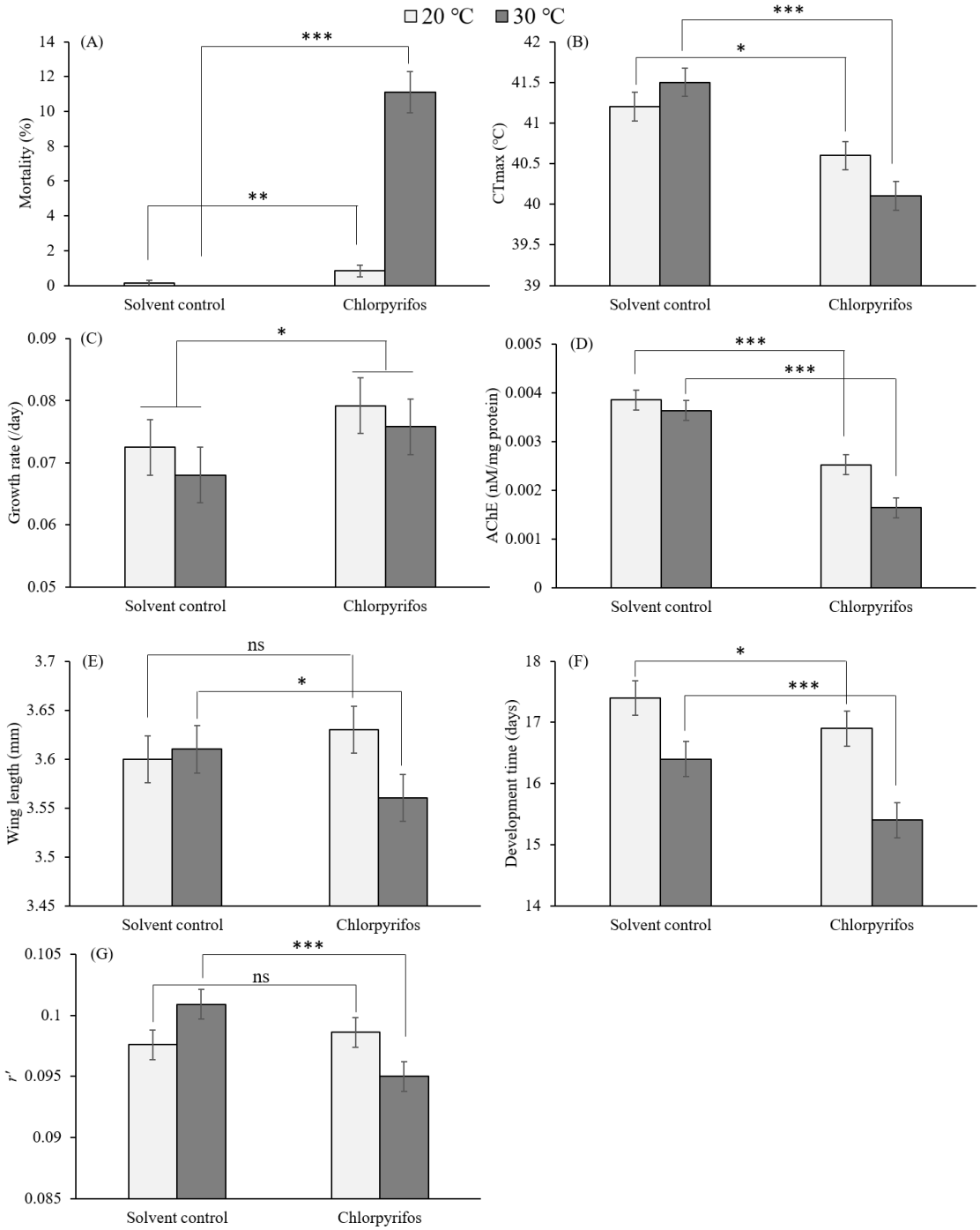


Figure 2. The single and combined effects of the heat spike and chlorpyrifos exposure on life history, heat tolerance and physiological variables in the parental generation of the mosquito *Culex pipiens*. (A) mortality (mortality is zero in the solvent control at 30 ºC), (B) heat tolerance (CTmax), (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': $P > 0.05$; '*': $P < 0.05$; '**': $P < 0.01$; '***': $P < 0.001$.

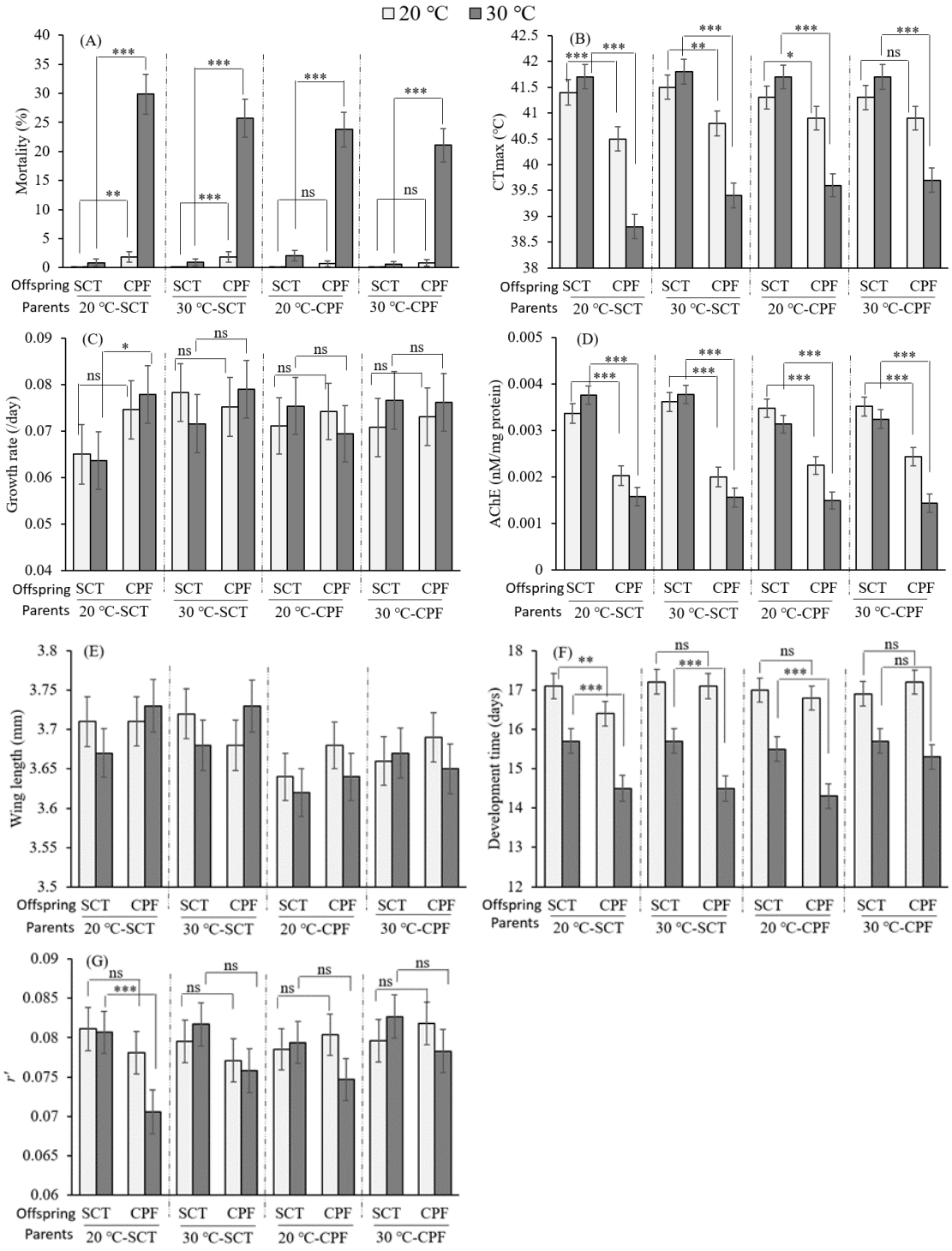


Figure 3. The single and combined effects of the heat spike and chlorpyrifos exposure in the parental and the offspring generations on life history, heat tolerance and physiological variables in the offspring generation of the mosquito *Culex pipiens*. (A) mortality, (B) heat tolerance (CTmax), (C) growth 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 the absence of any stressor. Note, this would not bias the patterns in the transgenerational 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, transgenerational effects caused by larval densities might be an important avenue for future research.

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., 2020b) 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., 2020b). 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., 2011), 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 ºC) 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 whereby 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).

In contrast with the negative effects on survival and heat tolerance, chlorpyrifos exposure accelerated the growth rate 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., 2019a) and other aquatic insects (in damselfly larvae: Janssens and Stoks, 2013b), 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.

Transgenerational 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 transgenerational 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 a., 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 transgenerational 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 transgenerational pattern in mortality. In addition, parental exposure to chlorpyrifos reduced the accelerated growth rate 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 transgenerational 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 transgenerational 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 transgenerational effects have been reported before, and associated with non-genetic and epigenetic processes (e.g. DNA methylation, histone modifications and chromatin remodeling) which can be influenced by environmental conditions (reviewed by Donelson et al., 2018; Brevik et al., 2018); these include 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 transgenerational 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 transgenerational effects after exposure to a heat spike for more than two generations, and 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 transgenerational 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 transgenerational 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%).

Conclusions

While transgenerational effects of stressors are receiving increased attention in ecotoxicology (Shaw et al., 2017; Brevik et al, 2018), transgenerational 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 transgenerational 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 transgenerational 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 transgenerational effect to the pesticide. Despite the importance of temporal aspects in multi-stressor studies (Orr et al., 2020), transgenerational 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 transgenerational effects in risk assessment.

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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**

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						

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 ($P < 0.05$).

	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									

Appendix A: Range finding experiment for chlorpyrifos

To select the chlorpyrifos concentration that is suitable for this study, we did a separate range finding experiment at 20 ºC under the same conditions as in the real experiment. Specifically, larvae were reared in white 2 L containers at a density of 120 larvae per container. When they entered the final larval (L4) stage, sets of 30 newly (< 24 h) moulted L4 larvae were placed in 210 mL glass jars containing 125 mL dechlorinated tap water in water baths at 20 ºC for 48 h (same duration as the heat spike treatments). Then, these larvae were exposed to a series of chlorpyrifos concentrations: 0 (solvent control), 0.10, 0.20, 0.25, 0.27, 0.30, 0.32, 0.35, 0.37, 0.40, 0.45, 0.5, 0.60, 0.70 and 0.80 µg/L.

Based on this, a nominal concentration of 0.32 µg/L was chosen as it caused minor (2.4%) mortality. This concentration 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 ºC in dark. The same amount of ethanol (3.2 µL/L) as in the chlorpyrifos treatment was included in the solvent control.

Appendix B: Measurements of water quality parameters

We measured the following physio-chemical parameters: pH, dissolved oxygen (mg/L), conductivity (µS/cm) and hardness (mg/L $CaCO_3$). Specifically, we measured these parameters at different key moments of the experiment: (i) before the heat spike treatment started (at 0 h when all jars were at 20 °C), (ii) and 24 h later just before renewal of the medium (separately for jars at 20 °C and vials at 30 °C), (iii) at the start of the chlorpyrifos exposure period (0 h) and 24 h later before the medium was refreshed (separately for both the solvent control and pesticide treatment). Each time three jars per condition were measured and their mean with SE were quantified.

There was a narrow range of pH values (between 8.41 and 8.49) across all conditions (Table B1). The dissolved oxygen levels ranged between 7.09 and 8.66 mg/L. Oxygen levels were after 24 h ~11% lower at 30 °C than at 20 °C, and after 24 h also slightly lower in the presence of chlorpyrifos than in the solvent control. This lowered dissolved oxygen is not likely to cause effects as *C. pipiens* larvae obtain oxygen mainly from the air via the air tube located at the end of their body (Silberbush et al., 2015). Conductivity values ranged between 652.67 and 720.33 µS/cm. Conductivity values were after 24 h slightly (~7%) higher at 30 °C than at 20 °C, and very similar after 24 h in the presence of chlorpyrifos and in the solvent control. There was a narrow range of hardness values (151.67-158.33) across all conditions. Note that any differential changes in physio-chemical parameters among the treatments were offset each 24 h by renewing the medium.

Table B1. Values of the physio-chemical parameters at several key moments of the experiment. Means and standard errors (SE) are given.

Treatment	Time	pH		Dissolved oxygen (mg/L)		Conductivity (µS/cm)		Hardness (mg/L $CaCO_3$)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Heat spike	0 h	8.44	0.00	8.63	0.01	652.67	0.33	151.67	1.67
Heat spike (20 ºC)	24 h	8.41	0.01	7.99	0.02	672.00	0.00	158.33	1.67
Heat spike (30 ºC)	24 h	8.49	0.01	7.09	0.02	720.33	0.33	157.67	2.67
Chlorpyrifos (Solvent control)	0 h	8.43	0.00	8.65	0.01	654.67	0.33	151.67	1.67
Chlorpyrifos (0.32 µg/L)	0 h	8.42	0.01	8.66	0.02	655.33	0.33	151.67	1.67
Chlorpyrifos (Solvent control)	24 h	8.41	0.00	8.00	0.02	671.67	0.33	157.33	1.45
Chlorpyrifos (0.32 µg/L)	24 h	8.41	0.01	7.75	0.06	668.00	1.53	153.33	1.67

Appendix C: The calculation of the composite index of population performance (*r'*)

We estimated how the single and combined effects of the heat spike and chlorpyrifos exposure affected the population growth rate within and across generations by calculating the composite index of population performance (*r'*) following the method in Op De Beeck et al. (2016) based on Livdahl and Sugihara (1984). *r'* is an estimate of the realized per capita rate of population change, and was calculated per jar following the formula:

$$r' = \frac{\ln\left[\left(\frac{1}{N_0}\right)\sum_x A_x f(w_x)\right]}{D + \left[\sum_x x A_x f(w_x) \,/\, \sum_x A_x f(w_x)\right]}$$

N_0 is the initial number of females in each jar, and was estimated as the total number of females that emerged plus half of the number that died before metamorphosis. We thereby assumed that half of the dead animals were females since the sex cannot be determined before metamorphosis. A_x is the number of females that emerged on day *x* (*x* was counted from the day of egg hatching). w_x is the mean size of the adult females. estimated as the mean wing length (in mm) of the three collected females per jar. $f(w_x)$ is a function relating fecundity to female size, and *D* (expressed in days) is the time between metamorphosis and reproduction. For the study species, *D* is approximately eight days (Vinogradova, 2020), and the size-fecundity relationship is $f(w_x) = 32.88w_x - 89.72$ ($r^2 = 0.99$) (Costanzo et al., 2011; calculated from Vinogradova, 2000).

Appendix D: Detailed protocol for the measurements of acetylcholinesterase (AChE) activity

We measured the activity of AChE, following the protocol of Delnat et al. (2019). Specifically, we first homogenized the pooled set of five larvae per jar in PBS buffer (phosphate buffer saline, 50 mM, pH 7.4) with a body-mass adjusted volume (wet mass × 10 mL/mg). We centrifuged the homogenate for 7 min at 13,000 g (4 °C), and measured the AChE activity on the supernatant using an Infinite M200 (TECAN) plate reader. We measured the activity of AChE in triplicate following a modified version of the Ellman method (Jensen et al., 1997). First, 330 mL of 5,5-dithiobis-2-nitro-benzoic acid (DTNB, 3 mM) and 170 mL of acetylcholine iodide (mM) were added to 8.8 mL PBS-buffer. Then, 5 mL supernatant and 25 mL of the mixture were added in a 384 well plate. The change in absorbance was recorded at 412 nm for 30 min. Following the Lambert-Beer formula, we converted the absorbance to the concentration of AChE using an extinction coefficient of 13.6/mM × cm. We expressed the activity of AChE in nmol/min per mg protein, and one mean value was obtained per jar for analyses. The protein content was measured in quadruplicate using the Bradford (1976) method.

Appendix E: Independent action model

Methods

We applied the independent action (IA) model for both the parental and the offspring generations. In the offspring generation, this was done separately for the four parental treatment combinations of heat spike x chlorpyrifos exposure, and across all four treatments. We thereby followed the procedure detailed in Coors and De Meester (2008).

We calculated the predicted combined effect of the heat spike and chlorpyrifos in each generation following the equation $E_{combined} = 1 - \prod^{I}(1 - E_i)$, with E_i the proportional mortality imposed by the single effects of the heat spike or chlorpyrifos. E_i was calculated based on the equation $E_i = \frac{(e_i - e_{control})}{(e_{max} - e_{control})}$. Hereby, e_i is the observed mortality in absolute units caused by a single stressor (the heat spike or chlorpyrifos), $e_{control}$ is the observed mortality in absolute units in the absence of any stressor (in the solvent control at 20 °C), and e_{max} is the maximum possible effect of a single stressor (100% mortality). Finally, we back-transformed the predicted combined effect $E_{combined}$ to absolute units to allow comparison with the 95% confidence interval of the observed combined effects using the equation $e_{combined} = E_{combined} \times (e_{max} - e_{control}) + e_{control}$.

The predicted combined additive effect by the model is significantly different from an observed combined effect if the predicted effect in absolute units is not within the 95% confidence interval of the observed effect. Hereby, a synergistic interaction is defined when the observed mortality for the combined effect is significantly higher than the predicted mortality for the combined effect.

Results

The results of the independent action model on mortality are shown in Table E1 for the parental generation, and in Table E2 for the offspring generation.

Table E1. Identification of the interaction type between the heat spike and chlorpyrifos based on the IA model in the parental generation. A synergism was identified when the observed 95% confidence interval (CI) of the combined effect was larger than the predicted mean of the combined effect. The strength of the interaction is determined using the model deviation ratio (MDR) whereby a synergism has a MDR-value > 1.

Predicted mean combined stressor effect	Observed 95%CI combined stressor effect	Interaction type	Interaction strength (MDR)
1.39	[9.62, 15.94]	Synergism	9.19

Table E2. Identification of the interaction types between the heat spike and chlorpyrifos based on the IA model in the offspring generation when the parents were exposed to: (A) neither the heat spike nor chlorpyrifos, (B) only the heat spike, (C) only chlorpyrifos, (D) both the heat spike and chlorpyrifos. A synergism was identified when the observed 95% confidence interval (CI) of the combined effect was larger than the predicted mean of the combined effect. The strength of the interaction is determined using the model deviation ratio (MDR) whereby a synergism has a MDR-value > 1.

Parental treatment	Predicted mean combined stressor effect	Observed 95%CI combined stressor effect	Interaction type	Interaction strength (MDR)
(A) No stressor	7.14	[25.48, 45.19]	Synergism	4.95
(B) Heat spike	6.19	[20.46, 39.26]	Synergism	4.82
(C) Chlorpyrifos	5.47	[19.53, 32.24]	Synergism	4.73
(D) Heat spike and Chlorpyrifos	3.77	[16.81, 30.37]	Synergism	6.26
(E) Across all treatments	5.60	[24.42, 32.54]	Synergism	5.09

General discussion

In this thesis, I studied the within- and across-generation single and combined effects of warming-related factors (increase in mean temperature and heat extremes) and pesticide exposure on the mosquito species *Culex pipiens*. Specifically, I investigated the roles of temporal exposure scenarios in determining the interactive effects between warming and chlorpyrifos exposure. In this chapter, I will first discuss the effects of exposure to each of these stressors separately, hence the effects of individual stressors (chlorpyrifos exposure, heat spike and increase in mean temperature) on life history variables, heat tolerance, physiology and antipredator behaviour. Next, I will discuss the interactive effects between warming and pesticide exposure, hence how the impact of chlorpyrifos exposure was modulated by mild warming or the heat spike within and across generations. Following this, I will discuss the role of temporal exposure scenarios in risk assessment of pesticides, followed by a discussion about the limitations of this thesis and future directions.

1. Effects of individual stressors

All stressors (chlorpyrifos, the heat spike and the 4 ℃ increase in mean temperature) were found to generate lethal effects and/or sublethal effects on life history variables, heat tolerance, physiology and/or antipredator behaviour within a single generation. Besides, chlorpyrifos exposure and the 4 ℃ increase in mean temperature also induced adaptive transgenerational effects.

1.1 Within-generation effects of chlorpyrifos exposure

As expected, chlorpyrifos exposure caused considerable lethal and sublethal effects when applied at the ecologically relevant concentrations (Chapters 1, 2, 3, 4 and 5). The concentrations used in this thesis were 0.30, 0.32, 0.34, 0.36 μg/L, all of which are within the measured concentrations of chlorpyrifos in surface waters in Europe (Stehle and Schulz, 2015). These concentrations only covered a narrow range, but the induced mortality varied from minor to ca. 50% because of a steep dose-response curve of chlorpyrifos in the study species (see Appendix A in chapter 1 and 4).

The chlorpyrifos-induced lethal effect can partly be explained by the chlorpyrifos-

induced inhibition in AChE activity (as quantified in Chapters 1, 2, 3 and 5) causing neurotoxic effects (Domingues et al., 2010). Chlorpyrifos has been shown to reduce AChE activity in the study species before (e.g. Delnat et al., 2019a) and in other organisms (for damselfly larvae: e.g. Dinh et al., 2016). Besides, a chlorpyrifos-induced increase in oxidative damage (seen as an increase of the MDA level, Chapter 2) may also contribute to the lethal effect by affecting key bio-molecules (e.g. lipids, proteins and DNA; Tripathi et al., 2010). Increased oxidative damage can be expected since organisms may increase their metabolic rate to detoxify pesticides and prevent damage and repair to tissues (estimated as ETS, Chapter 2; Hawlena and Schmitz, 2010; De Coen and Janssen, 2003; Van Dievel et al., 2019; Verslycke et al., 2004), thereby more reactive oxygen species will be generated as a by-product (Monaghan et al., 2009). However, chlorpyrifos did not change the oxidative damage in Chapter 1, which might be caused by cross-tolerance where the exposure to the heat spike in the first larval (L1) stage may prime the defense system to better cope with the chlorpyrifos pulses in the final larval (L4) stage. In addition, chlorpyrifos-induced mortality may also be explained by the lowered energy availability and net energy budget by chlorpyrifos (as quantified in Chapters 2 and 3); the latter has been related to the chlorpyrifos-induced higher mortality in damselfly larvae (Verheyen and Stoks, 2020).

Chlorpyrifos exposure consistently reduced the heat tolerance (estimated as CTmax, Chapters 1, 2, 4 and 5), which is in line with the “toxicant induced climate change toxicity” (TICS) concept (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013). CTmax occurs when the oxygen supply can no longer match the oxygen demand (Verberk et al., 2016). Chlorpyrifos can shift the mismatch to a lower temperature by increasing the oxygen demand for detoxification and damage repair (e.g. Narváez et al., 2016) and by decreasing the oxygen supply through impairing the respiratory functioning (e.g., Negro and Collins, 2017; Marigoudar et al., 2018). Chlorpyrifos-induced reductions in heat tolerance have been recorded before in the study species (e.g. Delnat et al., 2019b) and in aquatic damselfly larvae (e.g. Verheyen et al., 2019). CTmax values are highly correlated with heat extremes and mild warming, thus are a good proxy of the ability of organisms to tolerate heat extremes (Jørgensen et al., 2019; Kaspari et al., 2015) as well as mild warming (Åsheim et al., 2020).

Exposure to chlorpyrifos reduced two important antipredator responses of mosquito larvae, the diving response and the diving time, likely making them more vulnerable to predation (Chapter 4). The reduced diving responses may be caused by the chlorpyrifos-induced increase in oxygen demand as mosquito larvae mainly obtain oxygen from the air. The

shortened diving time may be related to energy deficiency since the diving response is energetically costly and chlorpyrifos has been shown to reduce the energy budget (see above).

Despite the negative effects, chlorpyrifos caused adaptive increases in growth rate and decreases in development time (Chapters 1, 2, 3 and 5). Similar accelerated growth and development responses to chlorpyrifos have been documented several times in the study species (e.g. Delnat et al., 2019a; Tran et al., 2019), and in aquatic damselfly larvae (e.g. Janssens and Stoks, 2013b), and regarded as an adaptive response for semi-aquatic organisms to escape the toxic aquatic environment (Rohr et al., 2011). When the applied chlorpyrifos concentration was lethal (Chapters 1, 2 and 3), survival selection by removing the weakest larvae with the lowest growth rate may also contribute to this phenomenon. Moreover, a sublethal concentration (Chapter 5) may cause hormetic effects that positively influence life history traits (Brevik et al., 2018; Margus et al., 2019).

Chlorpyrifos exposure did not show effects on female wing length, a good proxy of fecundity in mosquitoes (Costanzo et al., 2011), and on the estimate of population growth rate (Chapter 5). The applied chlorpyrifos concentration which caused only minor mortality in Chapter 5, may have not been severe enough to cause negative effects on adults. Besides, the chlorpyrifos-induced reduction in development time likely counteracted other negative effects on the population growth rate which can be increased by faster development due to a reduced time to reproductive maturity (Porter et al., 1991). Similarly, it has been recorded before that chlorpyrifos exposure in the larval stage did not influence the female wing length (Tran et al., 2018) in the study species.

The detection of chlorpyrifos-induced sublethal effects may strongly depend on the applied dose. When chlorpyrifos caused high mortality, survival selection may offset the negative sublethal effects by removing the larvae with the lowest condition. For example, when multiple chlorpyrifos concentrations were applied (Chapters 2, 3 and 4), the negative effects induced by chlorpyrifos were not further exacerbated at higher concentrations for some endpoints, such as CTmax in Chapter 2, the energy budget in Chapter 3, and the alarm diving responses in Chapter 4.

1.2 Within-generation effects of an increase in mean temperature

The 4 °C increase in mean temperature (mild warming) was not lethal. The sublethal effects

caused by 4 °C warming on life history variables, heat tolerance, energy budget and antipredator behaviour were either positive or negative, and strongly differed in terms of the duration of exposure to warming. Generally, acute warming had stronger negative/positive effects than developmental and transgenerational warming.

Mild warming itself did not cause mortality (Chapter 3), consistent with previous studies in the study species (Delnat et al., 2019a) and in aquatic damselfly larvae (e.g. Dinh Van et al., 2014). This matches the general pattern in terrestrial ectotherms that ambient temperatures in temperate areas are lower than the optimal temperatures for fitness (Deutsch et al., 2008), thereby mild warming may be even beneficial. Related to this, mild warming did not affect AChE activity and the energy consumed, and the growth rate was even accelerated under acute warming. The faster growth may explain the decrease in energy availability and net energy budget under acute warming (e.g. Janssens and Stoks, 2020). Since the energy budget was measured during the 4-d exposure period to acute warming, the lower budget may also reflect the ongoing demand of energy to adjust to the switch of 4 °C temperature increase. In contrast, the effects on energy-related variables disappeared under transgenerational warming, and were weaker under developmental than acute warming. This may be caused by larvae having compensated this energy investment before the exposure to the pesticide started, for example, through increasing feeding. Related to this, the energetically costly upregulation of heat shock proteins has been observed only under acute warming, but not under developmental and transgenerational warming (overview in Veilleux et al., 2015; Chen et al., 2018; Sørensen et al., 2003).

The growth rate was not affected under developmental and transgenerational warming, suggesting the beneficial effects of the 4 °C temperature increase disappeared after acclimation. This may also explain why the energy budget was less (developmental warming) or not (transgenerational warming) reduced when the exposure duration to warming was prolonged. Acclimation may also explain why the heat tolerance was increased by acute warming, but not by the two long-term warming treatments. An increased heat tolerance after acclimation to higher temperatures has been well-recorded (Gunderson and Stillman, 2015; Verheyen et al., 2019; Delnat et al., 2019b), and associated with the heat stress-induced increase in heat shock proteins (Somero et al., 2017; King and MacRae, 2015; Dahlhoff and Rank, 2000). The disappearance of the upregulation of heat shock proteins under developmental and transgenerational warming compared to acute warming (overview in Veilleux et al., 2015; Chen et al., 2018; Sørensen et al., 2003) may explain why the heat tolerance was not increased in the

two prolonged warming treatments.

Warming reduced the number of larvae showing the alarm diving response, and shortened the diving time, suggesting an increased vulnerability to predation under warming. A reduced diving time had been recorded before in the study species under developmental and transgenerational warming (Tran et al., 2019). Similarly, diving beetles have also been shown to be more vulnerable to predation at higher temperatures associated with a higher surfacing frequency (e,g. Calosi et al., 2007). The reduced diving responses may be caused by warming increasing the metabolic rate, hence increasing the oxygen demand. In addition, the diving responsiveness was further reduced under developmental and transgenerational warming compared to acute warming, suggesting that long-term exposure to warming was energetically more costly than acute warming. However, this shows no necessary conflict with the observations that the energy budget was lower under acute warming, since this may be driven by the higher growth.

1.3 Within-generation effects of the heat spike

The heat spike caused both lethal effects and sublethal effects on life history variables, heat tolerance and physiology (Chapters 1, 2 and 5), that moreover differed between ontogenetic stages. The chosen heat spike temperatures of 30 ℃ (Chapters 1, 2 and 5) and 34 ℃ (Chapter 2) are ecologically relevant (Tran et al., 2016). The temperature of 34 ℃ (Chapter 2) is currently still rare, however, may become common in the future since the intensity of heat extremes is expected to increase under warming (IPCC, 2021).

The effects caused by the heat spike were dependent on its intensity and the exposed ontogenetic stage. Exposure to the heat spike of 30 ℃ for 2 d caused considerably direct and delayed mortality in the L1 stage (Chapter 1). Too high temperatures can be lethal (overview in Stillman, 2019; Ma et al., 2021) by damaging key bio-macromolecules (e.g. proteins), and collapsing the capacity of ATP synthesis (Somero et al., 2017; Harada et al., 2019; Stillman, 2019; Ma et al., 2021). Survival selection caused by the heat spike may alter the fitness-related sublethal effects. For example, the increased AChE activity (Chapter 1) after the heat spike was likely to be caused by survival selection removing the weakest larvae with the lowest baseline AChE activity. In addition, the heat spike-induced delayed mortality may be related to the energy deficiency, since the heat spike reduced the total fat content (an estimate of long-term

energy storage, Chapter 1), and the energy availability and the net energy budget (Chapter 2). Similarly, a previous heat wave was also recorded to reduce total fat content in damselfly larvae (Dinh et al., 2016). However, the heat spike of 30 ℃ did not cause lethal and sublethal effects when applied in the L4 stage (Chapters 2 and 5), suggesting the L4 stage is more heat tolerant. A similar phenomenon was also recorded in other insects showing that older larval instars are more tolerant than younger ones (e.g. for the moth *Plutella xylostella*: see Zhang et al., 2015; for another mosquito: Lyons et al., 2012). When the intensity of the heat spike was, however, increased to 34 ℃, it also increased mortality and the energy consumption, and decreased both the energy available and the net energy budget in the L4 stage (Chapter 2).

1.4 Transgenerational effects of warming and/or chlorpyrifos exposure

Exposure to either the heat spike or chlorpyrifos in the parental generation on themselves did not affect the overall fitness of the offspring (Chapter 5). This absence of "main" transgenerational effects has also been documented before for parental exposure to tolfenpyrad in ladybird beetle *Coccinella septempunctata* (Chi et al., 2021), and to coumarin 47 in zebrafish (Blanc et al., 2020). However, positive (e.g. Lim et al., 2021) and negative (e.g. Tran et al., 2018) main transgenerational effects on offspring survival have also been documented. The timing and exposure duration to stressors in parents are critical for the expression of transgenerational effects. Two critical periods are early development (e.g. embryogenesis) and the period prior to and during reproduction (Feil and Fraga, 2012; Burton and Metcalfe, 2014). The absence of main transgenerational effects (Chapter 5) may be explained by the fact that the larvae in the parental generation were only exposed to a stressor for a short term in the relatively less sensitive final larval stage. In addition, the magnitude of stressors is an important factor for inducing transgenerational effects (Donelson et al., 2018). The applied heat spike and chlorpyrifos caused no or only minor mortality in the parental generation, thereby may not be severe enough to exceed the threshold for inducing transgenerational main effects.

However, parental exposure to chlorpyrifos did weaken the chlorpyrifos-induced lethal and sublethal effects in offspring on life history variables (growth rate, development time and population growth rate), heat tolerance and physiology, suggesting parental exposure to chlorpyrifos caused adaptive transgenerational effects to chlorpyrifos. The adaptive transgenerational effect of chlorpyrifos on CTmax adds a new transgenerational dimension to the TICS concept (Noyes et al., 2009; Moe et al., 2013; Hooper et al., 2013). Adaptive

transgenerational effects have been associated with non-genetic and epigenetic processes (e.g. DNA methylation, histone modifications and chromatin remodeling) which can be modulated by environmental conditions (overview in Donelson et al., 2018; Brevik et al., 2018); these include in utero exposure to stressors of the developing embryo (Heard and Martienssen, 2014; Lim and Brunet, 2013). Different from previous studies that mainly exposed the parents to a stressor across the entire life cycle (e.g. Shama et al., 2014; for toxicants: e.g. Marshall, 2008), or during the most sensitive ontogenetic stages (Lim et al., 2021; overview in Donelson et al., 2018; for toxicants: e.g. Reátegui-Zirena et al., 2017; Oppold et al., 2015), my results indicate that even a short-term exposure to a pesticide in the relatively less sensitive final larval stage can induce transgenerational effects and partly offset the negative effects of the pesticide in the offspring. This may be important at the population level as the estimate of population growth rate in the offspring generation was not affected by the pesticide when also the parents had been exposed. In contrast, parental exposure to the heat spike did not alter the sensitivity of offspring to the heat spike, which may be explained by the fact that the heat spike itself caused fewer within-generation effects as discussed above.

2. Interactive effects of warming and chlorpyrifos exposure

Generally, the impact of chlorpyrifos was strongly modulated by warming-related factors, and this was strongly dependent on the temporal exposure scenario. Moreover, the results in this thesis provided support for some key aspects of the temporal model proposed by Gunderson et al. (2016) as shown in Figure 6 in the General introduction. I here adjusted their conceptual figure to the temporal aspects I studied in this thesis (see Figure 1, below).

Firstly, when there was a long time interval (~ one week, with exposure to stressors in L1 and L4) between the heat spike and chlorpyrifos exposure, the larvae surviving the preceding heat spike were more tolerant to the subsequent exposure to chlorpyrifos, indicating an antagonistic interaction (Chapter 1). This may be explained by cross-tolerance, where the preceding heat spike primes the defense system, making the survivors better in coping with the chlorpyrifos exposure. Cross-tolerance can indeed be expected since both the exposure to heat stress (overview in King and MacRae, 2015) and to chlorpyrifos (e.g. in common carp: Xing et al., 2013; in Chironomidae: Lee et al., 2006) have been shown to upregulate the level of heat shock proteins. This suggests that organisms share some of the physiological defense systems against both a heat spike and chlorpyrifos exposure. Indeed, irrespective of the modes of action,

warming and contaminants have been shown to share some general stress defense responses, including antioxidant processes, detoxification, protection of macromolecules and energy metabolism/allocation (Sulmon et al., 2015), which was shown explicitly for the warming and the pesticide chlorpyrifos in my study species (Delnat et al., 2020). In line with the scenario B in the temporal model of Gunderson et al. (2016) (see Figure 1), these results suggest that the time interval was long enough for the survivors to recover from the heat spike, while the compensatory physiological responses (e.g. the upregulated levels of heat shock proteins) are still ongoing when chlorpyrifos exposure started. A similar pattern where a preceding non-lethal heat shock make the brine shrimp more tolerant to the subsequent exposure to cadmium and zinc was recorded by Pestana et al. (2016). It should, however, be noted that this antagonism may also be caused by survival selection removing the weakest larvae with lowest body condition, given considerable mortality was caused by the heat spike, thereby the survivors with relatively higher body condition were more tolerant to a future stressor (here chlorpyrifos exposure). When the time interval between both stressors is long enough, scenario A (Figure 6, general introduction) may be expected where there is no interaction between two subsequent stressors. While this was not observed in my thesis, it may be expected if the applied preceding heat spike would be less intense, whereby the mosquitoes would need less time for damage repair and recovery to homeostasis.

Secondly, supporting scenario C (see Figure 1), synergisms were observed between the heat spike and chlorpyrifos exposure when they were in close temporal succession (without time gap), and this consistently occurred in different studies with similar design (Chapters 2 and 5). For example, the chlorpyrifos-induced mortality was magnified when combined with the heat spike, while the heat spike itself caused negligible mortality (Chapters 2 and 5); and only in the presence of the heat spike, chlorpyrifos affected CTmax, AChE activity, female wing length and the population growth rate (Chapter 5). This is in line with the "climate change induced toxicant sensitivity" (CITS) concept (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). Notably, the synergism was further modulated by the exposure order. When the heat spike was applied first and followed by the chlorpyrifos exposure, there was already a synergistic interaction in the combination of the low heat spike level (30 °C) and the low chlorpyrifos concentration; however, a synergism was only detected between the high chlorpyrifos concentration and the high heat spike level (34 °C) when the chlorpyrifos exposure was followed by the heat spike, and the strength of the synergism was also weaker (Chapter 2). The stronger combined effects when the heat spike was followed by chlorpyrifos exposure

compared to the reserved order is consistent with a faster action of chlorpyrifos and/or a longer toxicodynamic recovery time after the heat spike than after chlorpyrifos exposure. Although the effect of exposure order was recorded in chemical toxicants with a different mode-of-action (Ashauer et al., 2017), this had never been shown before when combining chemicals with a natural stressor. Furthermore, despite the adaptive transgenerational effects of parental exposure to chlorpyrifos, the synergistic interaction between the heat spike and chlorpyrifos exposure did not alter in both generations (Chapter 5). Given that generating the adaptive transgenerational effects for chlorpyrifos can be energetically costly, thereby less energy can be used to tolerate the heat spike which has a different mode of action from chlorpyrifos, probably resulting in a higher sensitivity to the heat spike in the offspring. These results suggest that for risk assessment considering the offspring generation may be important for pesticides but not for the heat spike and their combined effect.

Thirdly, when the larvae were co-exposed to mild warming and chlorpyrifos, the net impact of chlorpyrifos was less strong under warming, which seems to contrast with the CITS concept (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015) and deviate from the scenario D in Gunderson et al. (2016). This deviation can be explained by the warming-induced higher external degradation rate overruling the warming-induced higher toxicity of chlorpyrifos, as also recorded in damselfly larvae (Op de Beeck et al., 2017a). The degradation of chlorpyrifos occurs via several processes in aquatic environment, like photolysis, hydrolysis and microbial degradation, and can be affected by temperature (Kamrin, 1997; Simon et al., 1998). Related to this, lower chlorpyrifos concentrations indeed caused less lethal and sublethal effects (Chapters 2, 3 and 4). Furthermore, the net impact of chlorpyrifos under warming was also influenced by the exposure duration to warming. When larvae were exposed to warming from the early life (egg) stage onwards (developmental warming), and especially from the parental generation (transgenerational warming), the chlorpyrifos-induced lethal and sublethal effects were less strong than those under acute warming. Indeed, chlorpyrifos-induced mortality was highest under acute warming, intermediate under developmental warming and lowest under transgenerational warming, which cannot be explained by a different degradation rate since the chlorpyrifos solution in the three warming treatments was placed at 24 ℃ for the same duration. This may be caused by the fact that under the two long-term warming treatments, the larvae may have already acclimatized to the evaluated temperature, hence experienced less combined energetic stress during the chlorpyrifos exposure period, while the larvae under acute warming suffered from both chlorpyrifos and a switch to a higher temperature when they entered the L4

stage.

Besides supporting the temporal model of Gunderson et al. (2016), especially when two stressors were in sequential exposure, this thesis highlighted several additional dimensions of temporal aspects in ecotoxicology (see Figure 1). Firstly, the exposure order should be considered when two transient stressors are applied sequentially with a short time gap in between. Secondly, the exposure duration to warming can influence how warming modulate pesticides toxicity. Thirdly, the effects of a stressor or the combination of multiple stressors may be influenced by the legacy from previous generations. Moreover, this thesis also highlights the importance of considering the net impact (e.g. also the degradation) in risk assessment of pesticides in a warmer world.

3. Limitations and future perspectives

The primary goal of this thesis was to evaluate the net impact of pesticides under warming on aquatic non-target species. This was done by investigating the within- and transgenerational combined effects of exposure to pesticide and warming-related factors (increases in mean temperature and heat extremes) in different ecologically relevant temporal exposure scenarios. However, there are several aspects that can be further developed.

Firstly, besides increases in mean temperature and heat extremes, temperature fluctuations are another important aspect of global warming (IPCC, 2021). Temperature fluctuations are ecologically relevant to consider as ambient temperatures are not constant in nature (e.g. daily and seasonal fluctuations), and important to integrate in risk assessment of pesticides as they can increase the toxicity of pesticides (e.g. Verheyen et al., 2019; Willming and Maul, 2016). For example, coping with temperature fluctuations has been shown to be energetically costly (Colinet et al., 2015), leading, for example, to a higher toxicity of chlorpyrifos in aquatic damselfly larvae (Verheyen and Stoks, 2020). Daily temperature fluctuations have also been shown to make mosquito larvae more vulnerable to a mixture of a chemical pesticide and a biopesticide (Delnat et al., 2019d). Meanwhile, temperature fluctuations have also been shown to lower the impact of chlorpyrifos in the study species by increasing degradation rate (Delnat et al., 2021).

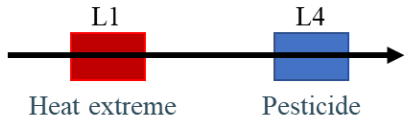
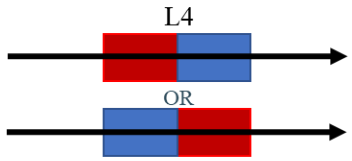
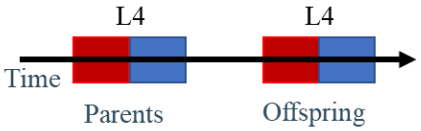
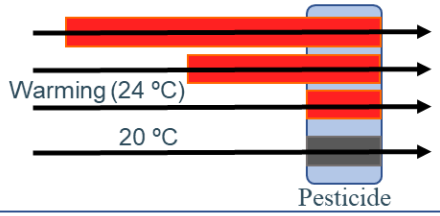
Four temporal scenarios in this thesis	Research chapter	Interaction type	Hypothesized underlying mechanism	Comparison with the four scenarios in Gunderson et al. (2016)
	1	Antagonism	Cross-tolerance Survival selection	• Matching scenario B • Extra mechanism: survival selection
	2	Synergism	Larvae weakened by the first stressor were less able to deal with the second stressor	• Matching scenario C • Extra dimension: strong effects of exposure order
	5	Synergism (within generation)	Larvae weakened by the first stressor were less able to deal with the second stressor	• Matching scenario C (within generation) • Extra dimension: consistent synergism across generations
	3 & 4	———	Degradation of the pesticide	• Deviating from scenario D • Extra dimension: effects of exposure duration to the first stressor

Figure 1. An overview of the main findings in each of the four temporal exposure scenarios studied in this thesis, and a comparison with the hypothesized combined effects of two stressors based on the temporal scenarios in Gunderson et al. (2016).

Secondly, aquatic organisms are still facing other natural stressors (e.g. food scarcity and predation) besides warming, and these stressors can also influence the impact of pesticides and/or the combined effects of pesticides and warming. Indeed, organisms may always be suffering from food scarcity, and low food availability has been shown to reduce survival in the study species (Beketov and Liess, 2007) and increase the toxicity of chlorpyrifos under exposure to a heat wave in damselfly larvae (Dinh et al., 2016). Predation risk has also been shown to increase the mortality of a mosquito species to a biopesticide Bti (Delnat et al., 2020). Therefore, it is important to include other natural stressors and field studies to make the risk assessment of pesticides more realistic.

Thirdly, pesticides and heat extremes can be strong selective factors, and how the animals selected by one stressor across multiple generations will deal with another stressor and the combination of both stressors are highly relevant but largely unstudied. According to the cost of tolerance concept (Moe et al., 2013), it can be expected that animals that evolved tolerance to one stressor will be more sensitive to another stressor. Besides, in the absence of selection, transgenerational effects may shape the sensitivity to the same stressor in the offspring, as shown in current PhD thesis. Yet, I only studied effects of parental exposure on the performance of their offspring. It would be very interesting to study more generations, thereby also explore how exposure of parents to a pesticide still would have effects on the third generation whose germ cells have not been exposed to the stressor, to identify transgenerational effects in the strict sense (Donelson et al., 2018; Salinas et al., 2013).

Next, given that one of the main focuses of this thesis was on the temporal exposure scenario of multiple stressors, it would be interesting to use a more stable toxicant (e.g. metals) to avoid the problem of degradation in detecting the combined effects. Moreover, it may be easier to work with a study species that shows a relatively flat dose-response curve to the chosen pesticide, which can offset the potential effects of subtle changes in pesticides concentration (e.g. caused by degradation). One of the frequently used organisms in the biomonitoring of aquatic environmental pollution is the freshwater isopod *Asellus aquaticus* (O'Callaghan et al., 2019), a species that is widely distributed in North America and Europe. Since this species is tolerant to many toxicants but sensitive to high temperatures (overview in O'Callaghan et al., 2019), it would be a good model species to study the combined effects of warming and pesticides in aquatic ecosystems. One other important difference between freshwater isopods and semi-aquatic insects (e.g. mosquitoes and damselflies) is that isopods cannot avoid the toxic

aquatic environment by growing and developing faster. However, given isopods have a longer life cycle (O'Callaghan et al., 2019), it may not be suitable for studying transgenerational effects of stressors compared to mosquitoes.

Finally, building on the many endpoints that were quantified in this thesis, it would also be very interesting to apply toxicokinetic-toxicodynamic (TKTD) models and individual-based model (IBM) in these studies. TKTD models can predict the effects for any time-variable exposure profile (Ashauer and Escher, 2010), while IBM can be used to extrapolate the impacts at lower biological levels (e.g. sub-organismal level) to the impacts on higher levels (e.g. population and ecosystem process) (Galic et al., 2018). By applying these models, one could make generalizations and predictions on the effects of multiple stressors.

4. Conclusions and implications for risk assessment of pesticides

Pesticides and warming are two major environmental factors in aquatic ecosystems that can cause biodiversity loss and can interact with each other (Beketov et al., 2013; Holmstrup et al., 2010; Liess et al., 2016), thereby investigating the combined effects of warming and pesticide exposure is important for assessing the net impact of pesticides on non-target aquatic species. The purpose of this thesis was to evaluate the importance of considering realistic temporal exposure scenarios to warming for the risk assessment of pesticides. The key findings of this thesis can inform a critical evaluation of safety (uncertainty) factors used in risk assessment. Indeed, some of these have been little updated since they were originally introduced in 1945, and the current applications of safety factors are mainly based on policy rather than on empirical science (Chapman et al., 1998). My results thereby can serve as important background information to improve the current risk assessment of pesticides in a context of global warming.

Despite the well-documented higher toxicity of many pesticides at higher temperatures, the net impact of the pesticide chlorpyrifos on life history, heat tolerance, physiology and antipredator behaviour was weakened under mild warming, which was caused by warming increasing the degradation rate of chlorpyrifos. Warming-induced higher degradation rates therefore should not be neglected when co-exposing organisms to both stressors, as this can offset or even overrule the warming-induced higher toxicity of pesticides. For example, when pesticides co-occur with mild warming, the net impact of a pesticide is likely to be magnified under warming when the degradation of the pesticide is less affected by temperature; while it

can either be magnified or weakened when the degradation rate is faster at higher temperatures, depending on whether the degradation effect will be larger than the increased toxicity effect. This suggests that it is highly relevant to assess the net impact of pesticides under warming and not only how warming may alter the toxicity of pesticides. When focusing only on the temporal scenarios, it may be important to choose a more stable pesticide to avoid the effect of warming on degradation. In addition, the impact of chlorpyrifos under warming was also affected by the exposure duration to warming. Acute warming increased the toxicity of chlorpyrifos more compared to developmental and transgenerational warming, which suggests an underestimation of the impact of pesticides under warming when the organisms have already acclimatized to the elevated temperatures.

Sequential exposure is ecologically relevant to study for transient stressors, and can generate different interactive effects compared to simultaneous exposure. When first exposing animals to a heat spike followed by a chlorpyrifos exposure, antagonism in lethal effect and no interaction in sublethal effects were observed when there was a time interval between them, and synergisms in lethal and sublethal effects were detected when they were in close temporal succession. Notably, the synergisms between the two stressors when in close succession were weakened or disappeared when the exposure order was reserved. In addition, the parental exposure to chlorpyrifos caused adaptive transgenerational effects in terms of increased tolerance to chlorpyrifos in the offspring. Above all, when an individual is sequentially exposed to heat extremes and a pesticide, the toxicity of the pesticide is likely to be magnified when both stressors are in close succession, especially when the pesticide exposure follows the heat extreme. The pesticide toxicity may not be influenced by the preceding heat extreme when the time lag between them is long enough for the individual to recover from the heat extreme; or the toxicity is likely to be weakened by the preceding heat extreme when the time lag is long enough for the individual to repair the damage caused by the heat extreme while the defense systems are still ongoing when the pesticide exposure starts. In addition, the risk of a pesticide can be underestimated in field-collected animals in case that their parents already experienced the toxicant. Moreover, given *Culex* mosquitoes are important vectors for some diseases, this thesis also suggests that a more efficient mosquito control practice in an ongoing warmer world could be spraying directly after a heat event.

In general, this thesis provided considerable evidence that warming-related factors can modulate the impact of pesticide exposure, which further depends on the actual exposure

scenarios. My thesis thereby emphasizes the importance of integrating ecologically relevant temporal exposure scenarios in ecotoxicological studies and risk assessment of pollutants.

References:

Adamo, S. A., Baker, J. L., Lovett, M. M., & Wilson, G. (2012). Climate change and temperate zone insects: the tyranny of thermodynamics meets the world of limited resources. *Environmental Entomology*, 41(6), 1644-1652.

Agathokleous, E., Kitao, M., & Calabrese, E. J. (2018). Environmental hormesis and its fundamental biological basis: rewriting the history of toxicology. *Environmental Research*, 165, 274-278.

Arambourou, H., Sanmartín-Villar, I., & Stoks, R. (2017). Wing shape-mediated carry-over effects of a heat wave during the larval stage on post-metamorphic locomotor ability. *Oecologia*, 184(1), 279-291.

Arrese, E. L., & Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. *Annual Review of Entomology*, 55, 207-225.

Ashauer, R., & Escher, B. I. (2010). Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. *Journal of Environmental Monitoring*, 12(11), 2056-2061.

Ashauer, R., Hintermeister, A., Caravatti, I., Kretschmann, A., & Escher, B. I. (2010). Toxicokinetic and toxicodynamic modeling explains carry-over toxicity from exposure to diazinon by slow organism recovery. *Environmental Science & Technology*, 44(10), 3963-3971.

Ashauer, R., O'Connor, I., & Escher, B. I. (2017). Toxic Mixtures in Time - The Sequence Makes the Poison. *Environmental Science & Technology*, 51(5), 3084-3092.

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. *Journal of Experimental Biology*, 223(23).

Atkinson, D. (1994). Temperature and organism size: a biological law for ectotherms?. *Advances in Ecological Research*, 25, 1-58.

Azeez, O. I., Meintjes, R., & Chamunorwa, J. P. (2014). Fat body, fat pad and adipose tissues in invertebrates and vertebrates: the nexus. *Lipids in Health and Disease*, 13(1), 1-13.

Bagni, T., Siaussat, D., Maria, A., Couzi, P., Maïbèche, M., & Massot, M. (2020). A maternal

effect influences sensitivity to chlorpyrifos pesticide in the pest moth *Spodoptera littoralis*. *Ecotoxicology and Environmental Safety*, 204, 111052.

Baker, N. J., Bancroft, B. A., & Garcia, T. S. (2013). A meta-analysis of the effects of pesticides and fertilizers on survival and growth of amphibians. *Science of the Total Environment*, 449, 150-156.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using Lme4. *Journal of Statistical Software*, 67, 1-48.

Bauerfeind, S. S., & Fischer, K. (2014). Simulating climate change: temperature extremes but not means diminish performance in a widespread butterfly. *Population Ecology*, 56(1), 239-250.

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., Kefford, B. J., Schäfer, R. B., & Liess, M. (2013). Pesticides reduce regional biodiversity of stream invertebrates. *Proceedings of the National Academy of Sciences*, 110(27), 11039-11043.

Beketov, M. A., & Liess, M. (2007). Predation risk perception and food scarcity induce alterations of life-cycle traits of the mosquito *Culex pipiens*. *Ecological Entomology*, 32(4), 405-410.

Bell, A. M., & Hellmann, J. K. (2019). An integrative framework for understanding the mechanisms and multigenerational consequences of transgenerational plasticity. *Annual Review of Ecology, Evolution, and Systematics*, 50, 97-118.

Belden, J. B., Gilliom, R. J., & Lydy, M. J. (2007). How well can we predict the toxicity of pesticide mixtures to aquatic life?. *Integrated Environmental Assessment and Management*, 3(3), 364-372.

Bernabò, I., Sperone, E., Tripepi, S., & Brunelli, E. (2011). Toxicity of chlorpyrifos to larval *Rana dalmatina*: acute and chronic effects on survival, development, growth and gill apparatus. *Archives of Environmental Contamination and Toxicology*, 61(4), 704-718.

Birk, S., Chapman, D., Carvalho, L., Spears, B. M., Andersen, H. E., Argillier, C., ... & Hering, D. (2020). Impacts of multiple stressors on freshwater biota across spatial scales and

ecosystems. *Nature Ecology & Evolution*, 4(8), 1060-1068.

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. *Ecotoxicology and Environmental Safety*, 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*). *Science of the Total Environment*, 779, 146404.

Bliss, C. I. (1939). The toxicity of poisons applied jointly 1. *Annals of Applied Biology*, 26(3), 585-615.

Boher, F., Trefault, N., Piulachs, M. D., Bellés, X., Godoy-Herrera, R., & Bozinovic, F. (2012). Biogeographic origin and thermal acclimation interact to determine survival and hsp90 expression in *Drosophila* species submitted to thermal stress. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 162(4), 391-396.

Bonduriansky, R., Crean, A. J., & Day, T. (2012). The implications of nongenetic inheritance for evolution in changing environments. *Evolutionary Applications*, 5(2), 192-201.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254.

Brevik, K., Lindström, L., McKay, S. D., & Chen, Y. H. (2018). Transgenerational effects of insecticides - implications for rapid pest evolution in agroecosystems. *Current Opinion in Insect Science*, 26, 34-40.

Brönmark, C., & Hansson, L. A. (2002). Environmental issues in lakes and ponds: current state and perspectives. *Environmental Conservation*, 290-307.

Buchwalter, D. B., Jenkins, J. J., & Curtis, L. R. (2002). Respiratory strategy is a major determinant of [^{3}H] water and [^{14}C] chlorpyrifos uptake in aquatic insects. *Canadian Journal of Fisheries and Aquatic Sciences*, 59(8), 1315-1322.

Buchwalter, D. B., Jenkins, J. J., & Curtis, L. R. (2003). Temperature influences on water permeability and chlorpyrifos uptake in aquatic insects with differing respiratory strategies. *Environmental Toxicology and Chemistry*, 22(11), 2806-2812.

Buchwalter, D. B., Sandahl, J. F., Jenkins, J. J., & Curtis, L. R. (2004). Roles of uptake, biotransformation, and target site sensitivity in determining the differential toxicity of chlorpyrifos to second to fourth instar *Chironomous riparius* (Meigen). *Aquatic Toxicology*, 66(2), 149-157.

Buckley, L. B., & Huey, R. B. (2016). Temperature extremes: geographic patterns, recent changes, and implications for organismal vulnerabilities. *Global Change Biology*, 22(12), 3829-3842.

Burton, T., & Metcalfe, N. B. (2014). Can environmental conditions experienced in early life influence future generations? *Proceedings of the Royal Society B: Biological Sciences*, 281(1785), 20140311.

Calosi, P., Bilton, D. T., & Spicer, J. I. (2007). The diving response of a diving beetle: effects of temperature and acidification. *Journal of Zoology*, 273(3), 289-297.

Calow, P. (1989). Proximate and ultimate responses to stress in biological systems. *Biological Journal of the Linnean Society*, 37(1-2), 173-181.

Calow, P., & Sibly, R. M. (1990). A physiological basis of population processes: ecotoxicological implications. *Functional Ecology*, 283-288.

Cambronero, M.C., Beasley, J., Kissane, S., & Orsini, L. (2018). Evolution of thermal tolerance in multifarious environments. *Molecular Ecology*, 27(22), 4529-4541.

Carbonell, J. A., & Stoks, R. (2020). Thermal evolution of life history and heat tolerance during range expansions toward warmer and cooler regions. *Ecology*, 101(10), e03134.

Chapman, P. M., Fairbrother, A., & Brown, D. (1998). A critical evaluation of safety (uncertainty) factors for ecological risk assessment. *Environmental Toxicology and Chemistry*, 17(1), 99-108.

Chen, B., Feder, M. E., & Kang, L. (2018). Evolution of heat-shock protein expression underlying adaptive responses to environmental stress. *Molecular Ecology*, 27(15), 3040-3054.

Chen, X., & Stillman, J. H. (2012). Multigenerational analysis of temperature and salinity variability affects on metabolic rate, generation time, and acute thermal and salinity tolerance in *Daphnia pulex*. *Journal of Thermal Biology*, 37(3), 185-194.

Chi, W., Mingyuan, H., Fengshou, D., Jun, X., Xiaohu, W., Bing, C., ... & Xingang, L. (2021). The influence of tolfenpyrad on fitness, development, and reproduction in parents and offspring of *Coccinella septempunctata*. *Ecotoxicology and Environmental Safety*, 210, 111875.

Chown SL, Nicolson SW. 2004. *Insect Physiological Ecology: Mechanisms and Patterns*. Oxford, UK: Oxford Univ. Press.

Chown, S. L., Sørensen, J. G., & Terblanche, J. S. (2011). Water loss in insects: an environmental change perspective. *Journal of Insect Physiology*, 57(8), 1070-1084.

Cloyed, C. S., Dell, A. I., Hayes, T., Kordas, R. L., & O'Gorman, E. J. (2019). Long-term exposure to higher temperature increases the thermal sensitivity of grazer metabolism and movement. *Journal of Animal Ecology*, 88(6), 833-844.

Colinet, H., Sinclair, B. J., Vernon, P., & Renault, D. (2015). Insects in fluctuating thermal environments. *Annual Review of Entomology*, 60, 123-140.

Coors, A., & De Meester, L. (2008). Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in *Daphnia magna*. *Journal of Applied Ecology*, 45(6), 1820-1828.

Corbet, S. A., Tiley, C., Moorhouse, T., Giam, C., Pursglove, S., Raby, J., & Rich, M. (2000). Surface films as mosquito larvicides: partitioning the mode of action. *Entomologia Experimentalis et Applicata*, 94(3), 295-307.

Costanzo, K. S., Muturi, E. J., Lampman, R. L., & Alto, B. W. (2011). The effects of resource type and ratio on competition with *Aedes albopictus* and *Culex pipiens* (Diptera: Culicidae). *Journal of Medical Entomology*, 48(1), 29-38.

Côté, I. M., Darling, E. S., & Brown, C. J. (2016). Interactions among ecosystem stressors and their importance in conservation. *Proceedings of the Royal Society B: Biological Sciences*, 283(1824), 20152592.

Dahlhoff, E. P., & Rank, N. E. (2000). Functional and physiological consequences of genetic

variation at phosphoglucose isomerase: heat shock protein expression is related to enzyme genotype in a montane beetle. *Proceedings of the National Academy of Sciences*, 97(18), 10056-10061.

Dallas, H. F., & Rivers-Moore, N. A. (2012). Critical thermal maxima of aquatic macroinvertebrates: towards identifying bioindicators of thermal alteration. *Hydrobiologia*, 679(1), 61-76.

De Coen, W. M., & Janssen, C. R. (1997). The use of biomarkers in Daphnia magna toxicity testing. IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. *Journal of Aquatic Ecosystem Stress and Recovery*, 6(1), 43-55.

De Coen, W. M., & Janssen, C. R. (2003). The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environmental Toxicology and Chemistry*, 22(7), 1632-1641.

De Silva, P. M. C., Pathiratne, A., & van Gestel, C. A. (2009). Influence of temperature and soil type on the toxicity of three pesticides to *Eisenia andrei*. *Chemosphere*, 76(10), 1410-1415.

Defra, H.S.E., WAG, 2006. Pesticides: Code of Practice for Using Plant Protection Products. Defra Publications, London, UK. http://www.pesticides.gov.uk/farmers_growers_home.asp#Codes_of_Practice.

Delnat, V., Janssens, L., & Stoks, R. (2019a). Whether warming magnifies the toxicity of a pesticide is strongly dependent on the concentration and the null model. *Aquatic Toxicology*, 211, 38-45.

Delnat, V., Verborgt, J., Janssens, L., & Stoks, R. (2021). Daily temperature variation lowers the lethal and sublethal impact of a pesticide pulse due to a higher degradation rate. *Chemosphere*, 263, 128114.

Delnat, V., Swaegers, J., Asselman, J., & Stoks, R. (2020). Reduced stress defence responses contribute to the higher toxicity of a pesticide under warming. *Molecular Ecology*, 29(23), 4735-4748.

Delnat, V., Tran, T. T., Verheyen, J., Van Dinh, K., Janssens, L., & Stoks, R. (2019b).

Temperature variation magnifies chlorpyrifos toxicity differently between larval and adult mosquitoes. *Science of The Total Environment*, 690, 1237-1244.

Delnat, V., Tran, T. T., Janssens, L., & Stoks, R. (2019c). Resistance to a chemical pesticide increases vulnerability to a biopesticide: Effects on direct mortality and mortality by predation. *Aquatic Toxicology*, 216, 105310.

Delnat, V., Tran, T. T., Janssens, L., & Stoks, R. (2019d). Daily temperature variation magnifies the toxicity of a mixture consisting of a chemical pesticide and a biopesticide in a vector mosquito. *Science of the Total Environment*, 659, 33-40.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C., & Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences*, 105(18), 6668-6672.

Dhakal, S., Meyling, N. V., Pedersen, K. E., Cedergreen, N., & Fredensborg, B. L. (2020). Timing of sub-lethal insecticide exposure determines parasite establishment success in an insect-helminth model. *Parasitology*, 147(1), 120-125.

Dillon, M. E., Wang, G., & Huey, R. B. (2010). Global metabolic impacts of recent climate warming. *Nature*, 467(7316), 704-706.

Dinh Van, K., Janssens, L., Debecker, S., & Stoks, R. (2014). Temperature-and latitude-specific individual growth rates shape the vulnerability of damselfly larvae to a widespread pesticide. *Journal of Applied Ecology*, 51(4), 919-928.

Dinh, K. V., Janssens, L., & Stoks, R. (2016). Exposure to a heat wave under food limitation makes an agricultural insecticide lethal: a mechanistic laboratory experiment. *Global Change Biology*, 22(10), 3361-3372.

Dixon, G. B., Davies, S. W., Aglyamova, G. V., Meyer, E., Bay, L. K., & Matz, M. V. (2015). Genomic determinants of coral heat tolerance across latitudes. *Science*, 348(6242), 1460-1462.

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. *Environmental Toxicology and Chemistry*, 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 Biology*, 24(1), 13-34.

Downs, C. J., Brown, J. L., Wone, B. W., Donovan, E. R., & Hayes, J. P. (2016). Speeding up growth: selection for mass-independent maximal metabolic rate alters growth rates. *The American Naturalist*, 187(3), 295-307.

Droge, W. (2002). Free radicals in the physiological control of cell function. *Physiological Reviews*, 82(1), 47-95.

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z. I., Knowler, D. J., Lévêque, C., ... & Sullivan, C. A. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological Reviews*, 81(2), 163-182.

Farajollahi, A., Fonseca, D. M., Kramer, L. D., & Kilpatrick, A. M. (2011). "Bird biting" mosquitoes and human disease: a review of the role of Culex pipiens complex mosquitoes in epidemiology. *Infection, Genetics and Evolution*, 11(7), 1577-1585.

Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*, 61(1), 243-282.

Feil, R., & Fraga, M. F. (2012). Epigenetics and the environment: emerging patterns and implications. *Nature Reviews Genetics*, 13(2), 97-109.

Fonseca, D. M., Keyghobadi, N., Malcolm, C. A., Mehmet, C., Schaffner, F., Mogi, M., ... & Wilkerson, R. C. (2004). Emerging vectors in the *Culex pipiens* complex. *Science*, 303(5663), 1535-1538.

Forster, J., & Hirst, A. G. (2012). The temperature-size rule emerges from ontogenetic differences between growth and development rates. *Functional Ecology*, 26(2), 483-492.

Fox, J., & Weisberg, S. (2018). An R companion to applied regression. Sage publications.

Fukuto, T. R. (1990). Mechanism of action of organophosphorus and carbamate insecticides. *Environmental Health Perspectives*, 87, 245-254.

Futami, K., Sonye, G., Akweywa, P., Kaneko, S., & Minakawa, N. (2008). Diving behavior in *Anopheles gambiae* (Diptera: Culicidae): avoidance of a predacious wolf spider

(*Araneae: Lycosidae*) in relation to life stage and water depth. *Journal of Medical Entomology*, 45(6), 1050-1056.

Galic, N., Sullivan, L. L., Grimm, V., & Forbes, V. E. (2018). When things don't add up: quantifying impacts of multiple stressors from individual metabolism to ecosystem processing. *Ecology Letters*, 21(4), 568-577.

Ghommem, M., Hajj, M. R., & Puri, I. K. (2012). Influence of natural and anthropogenic carbon dioxide sequestration on global warming. *Ecological Modelling*, 235, 1-7.

Grigaltchik, V. S., Ward, A. J., & Seebacher, F. (2012). Thermal acclimation of interactions: differential responses to temperature change alter predator–prey relationship. *Proceedings of the Royal Society B: Biological Sciences*, 279(1744), 4058-4064.

Guillaume, A. S., Monro, K., & Marshall, D. J. (2016). Transgenerational plasticity and environmental stress: do paternal effects act as a conduit or a buffer?. *Functional Ecology*, 30(7), 1175-1184.

Guilyardi, E., Lescarmontier, L., Matthews, R., Point, S.P., Rumjaun, A.B., Schlüpmann, J., Wilgenbus, D. (2018). IPCC special report global warming of 1.5 °C: Summary for teachers coordinator. IPCC.

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. *Annual Review of Marine Science*, 8, 357-378.

Gunderson, A. R., & Stillman, J. H. (2015). Plasticity in thermal tolerance has limited potential to buffer ectotherms from global warming. *Proceedings of the Royal Society B: Biological Sciences*, 282(1808), 20150401.

Hahn, D. A., & Denlinger, D. L. (2007). Meeting the energetic demands of insect diapause: nutrient storage and utilization. *Journal of Insect Physiology*, 53(8), 760-773.

Hallman, T. A., & Brooks, M. L. (2015). The deal with diel: temperature fluctuations, asymmetrical warming, and ubiquitous metals contaminants. *Environmental Pollution*, 206, 88-94.

Hansen, J., Sato, M., & Ruedy, R. (2012). Perception of climate change. *Proceedings of the National Academy of Sciences*, 109(37), E2415-E2423.

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*. *Frontiers in Physiology*, 10, 213.

Harley, C. D. (2011). Climate change, keystone predation, and biodiversity loss. *Science*, 334(6059), 1124-1127.

Harwood, A. D., You, J., & Lydy, M. J. (2009). Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: toxicokinetic confirmation. *Environmental Toxicology and Chemistry*, 28(5), 1051-1058.

Hatami, M., Banaee, M., & Haghi, B. N. (2019). Sub-lethal toxicity of chlorpyrifos alone and in combination with polyethylene glycol to common carp (*Cyprinus carpio*). Chemosphere, 219, 981-988.

Hawlena, D., & Schmitz, O. J. (2010). Physiological stress as a fundamental mechanism linking predation to ecosystem functioning. *The American Naturalist*, 176(5), 537-556.

Head, J. (2019). An ecotoxicological perspective on transgenerational epigenetic inheritance. *Environmental Toxicology and Chemistry*, 38(6), 1149-1151.

Heard, E., & Martienssen, R. A. (2014). Transgenerational epigenetic inheritance: myths and mechanisms. *Cell*, 157(1), 95-109.

Heckwolf, M. J., Meyer, B. S., Döring, T., Eizaguirre, C., & Reusch, T. B. (2018). Transgenerational plasticity and selection shape the adaptive potential of sticklebacks to salinity change. *Evolutionary Applications*, 11(10), 1873-1885.

Heino, J., Virkkala, R., & Toivonen, H. (2009). Climate change and freshwater biodiversity: detected patterns, future trends and adaptations in northern regions. *Biological Reviews*, 84(1), 39-54.

Hites, R. A. (2021). The Rise and Fall of Chlorpyrifos in the United States. *Environmental Science & Technology*, 55(3), 1354-1358.

Hofmann, G. E., & Todgham, A. E. (2010). Living in the now: physiological mechanisms to tolerate a rapidly changing environment. *Annual Review of Physiology*, 72, 127-145.

Holmstrup, M., Bindesbøl, A. M., Oostingh, G. J., Duschl, A., Scheil, V., Köhler, H. R., ... & Gerhardt, A. (2010). Interactions between effects of environmental chemicals and

natural stressors: a review. *Science of the Total Environment*, 408(18), 3746-3762.

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. *Environmental Toxicology and Chemistry*, 32(1), 32-48.

Hu, J. T., Chen, B., & Li, Z. H. (2014). Thermal plasticity is related to the hardening response of heat shock protein expression in two *Bactrocera* fruit flies. *Journal of Insect Physiology*, 67, 105-113.

Huey, R. B., & Berrigan, D. (2001). Temperature, demography, and ectotherm fitness. *The American Naturalist*, 158(2), 204-210.

Huey, R. B., Kearney, M. R., Krockenberger, A., Holtum, J. A., Jess, M., & Williams, S. E. (2012). Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 367(1596), 1665-1679.

IPCC. (2021). *Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press.

Itziou, A., Kaloyianni, M., & Dimitriadis, V. K. (2011). Effects of organic contaminants in reactive oxygen species, protein carbonylation and DNA damage on digestive gland and haemolymph of land snails. *Chemosphere*, 85(6), 1101-1107.

Jacobs, A. F., Heusinkveld, B. G., Kraai, A., & Paaijmans, K. P. (2008). Diurnal temperature fluctuations in an artificial small shallow water body. *International Journal of Biometeorology*, 52(4), 271-280.

Jackson, M. C., Pawar, S., & Woodward, G. (2021). The temporal dynamics of multiple stressor effects: From individuals to ecosystems. *Trends in Ecology & Evolution*, 36(5), 402-410.

Jacquin, L., Gandar, A., Aguirre-Smith, M., Perrault, A., Le Hénaff, M., De Jong, L., ... & Jean, S. (2019). High temperature aggravates the effects of pesticides in goldfish. *Ecotoxicology and Environmental Safety*, 172, 255-264.

Janssens, L., Dinh Van, K., Debecker, S., Bervoets, L., & Stoks, R. (2014b). Local adaptation and the potential effects of a contaminant on predator avoidance and antipredator responses under global warming: a space-for-time substitution approach. *Evolutionary Applications*, 7(3), 421-430.

Janssens, L., & Stoks, R. (2013a). Synergistic effects between pesticide stress and predator cues: conflicting results from life history and physiology in the damselfly *Enallagma cyathigerum*. *Aquatic Toxicology*, 132, 92-99.

Janssens, L., & Stoks, R. (2013b). 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., & Stoks, R. (2017). Chlorpyrifos-induced oxidative damage is reduced under warming and predation risk: Explaining antagonistic interactions with a pesticide. *Environmental Pollution*, 226, 79-88.

Janssens, L., & Stoks, R. (2020). Oxidative stress mediates rapid compensatory growth and its costs. *Functional Ecology*, 34(10), 2087-2097.

Janssens, L., Van, K. D., & Stoks, R. (2014a). Extreme temperatures in the adult stage shape delayed effects of larval pesticide stress: a comparison between latitudes. *Aquatic Toxicology*, 148, 74-82.

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*, 117019.

Jensen, C. S., Garsdal, L., & Baatrup, E. (1997). Acetylcholinesterase inhibition and altered locomotor behavior in the carabid beetle *Pterostichus cupreus*. A linkage between biomarkers at two levels of biological complexity. *Environmental Toxicology and Chemistry*, 16(8), 1727-1732.

Jeon, J., Ra, J. S., Lee, S. H., Lee, M. J., Seung, H. Y., & Kim, S. D. (2010). Role of food and clay particles in toxicity of copper and diazinon using *Daphnia magna*. *Ecotoxicology and Environmental Safety*, 73(3), 400-406.

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. *Science of the Total Environment*, 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. *Functional Ecology*, 33(4), 629-642.

Kamrin, M. A. *Pesticide Profiles: Toxicity, Environmental Impact, and Fate*, 1997. CRC/Lewis Publishers, Boca Raton, FL.

Kang, Z. W., Liu, F. H., Liu, X., Yu, W. B., Tan, X. L., Zhang, S. Z., ... & Liu, T. X. (2017). The potential coordination of the heat-shock proteins and antioxidant enzyme genes of *Aphidius gifuensis* in response to thermal stress. *Frontiers in Physiology*, 8, 976.

Karl, I., Stoks, R., De Block, M., Janowitz, S. A., & Fischer, K. (2011). Temperature extremes and butterfly fitness: conflicting evidence from life history and immune function. *Global Change Biology*, 17(2), 676-687.

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 Biology*, 21(3), 1092-1102.

Kattwinkel, M., Kühne, J. V., Foit, K., & Liess, M. (2011). Climate change, agricultural insecticide exposure, and risk for freshwater communities. *Ecological Applications*, 21(6), 2068-2081.

Kaunisto, S., Ferguson, L. V., & Sinclair, B. J. (2016). Can we predict the effects of multiple stressors on insects in a changing climate?. *Current Opinion in Insect Science*, 17, 55-61.

Kavitha, P., & Rao, J. V. (2008). Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in mosquito fish, *Gambusia affinis*. *Environmental Toxicology and Pharmacology*, 26(2), 192-198.

Kellett, M., Hoffmann, A. A., & McKechnie, S. W. (2005). Hardening capacity in the *Drosophila melanogaster* species group is constrained by basal thermotolerance. *Functional Ecology*, 19(5), 853-858.

King, A. M., & MacRae, T. H. (2015). Insect heat shock proteins during stress and diapause. *Annual Review of Entomology*, 60, 59-75.

Kimberly, D. A., & Salice, C. J. (2014). Complex interactions between climate change and toxicants: evidence that temperature variability increases sensitivity to cadmium. *Ecotoxicology*, 23(5), 809-817.

Kingsolver, J. G., & Woods, H. A. (2016). Beyond thermal performance curves: modeling time-dependent effects of thermal stress on ectotherm growth rates. *The American Naturalist*, 187(3), 283-294.

Kopp, M., & Matuszewski, S. (2014). Rapid evolution of quantitative traits: theoretical perspectives. *Evolutionary Applications*, 7(1), 169-191.

Kroma, M. M., & Flora, C. B. (2003). Greening pesticides: A historical analysis of the social construction of farm chemical advertisements. *Agriculture and Human Values*, 20(1), 21-35.

Kühnhold, H., Kamyab, E., Novais, S., Indriana, L., Kunzmann, A., Slater, M., & Lemos, M. (2017). Thermal stress effects on energy resource allocation and oxygen consumption rate in the juvenile sea cucumber, *Holothuria scabra* (Jaeger, 1833). *Aquaculture*, 467, 109-117.

Kumar, N., Prabhu, P. A. J., Pal, A. K., Remya, S., Aklakur, M., Rana, R. S., ... & Jadhao, S. B. (2011). Anti-oxidative and immuno-hematological status of Tilapia (*Oreochromis mossambicus*) during acute toxicity test of endosulfan. *Pesticide Biochemistry and Physiology*, 99(1), 45-52.

Landis, W. G., Durda, J. L., Brooks, M. L., Chapman, P. M., Menzie, C. A., Stahl Jr, R. G., & Stauber, J. L. (2013). Ecological risk assessment in the context of global climate change. Environmental Toxicology and Chemistry, 32(1), 79-92.

Lee, S. M., Lee, S. B., Park, C. H., & Choi, J. (2006). Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, chironomidae) larvae exposed to various environmental pollutants: a potential biomarker of freshwater monitoring. *Chemosphere*, 65(6), 1074-1081.

Lenth, R. V. (2016). Least-squares means: the R package lsmeans. Journal of statistical software, 69(1), 1-33.

Lenth, R., Singmann, H., Love, J., Buerkner, P., & Herve, M. (2019). emmeans: Estimated Marginal Means, aka Least-Squares Means (Version 1.3. 4).

Leung, J. Y., Russell, B. D., Coleman, M. A., Kelaher, B. P., & Connell, S. D. (2021). Long-term thermal acclimation drives adaptive physiological adjustments of a marine gastropod to reduce sensitivity to climate change. *Science of The Total Environment*, 771, 145208.

Liess, M., Foit, K., Knillmann, S., Schäfer, R. B., & Liess, H. D. (2016). Predicting the synergy of multiple stress effects. *Scientific Reports*, 6(1), 1-8.

Liess, M., Ratte, T., Ebke, P., & Hollert, H. (2019). 20 years SETAC GLB: increasing realism of pesticide risk assessment. p.13

Lim, J. P., & Brunet, A. (2013). Bridging the transgenerational gap with epigenetic memory. *Trends in Genetics*, 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*, 146704.

Livdahl, T. P., & Sugihara, G. (1984). Non-linear interactions of populations and the importance of estimating per capita rates of change. *Journal of animal ecology*, 573-580.

Lushchak, V. I. (2011). Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology*, 101(1), 13-30.

Lutterschmidt, W. I., & Hutchison, V. H. (1997). The critical thermal maximum: data to support the onset of spasms as the definitive end point. *Canadian Journal of Zoology*, 75(10), 1553-1560.

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*. *Archives of Environmental Contamination and Toxicology*, 37(4), 542-547.

Lyons, C. L., Coetzee, M., Terblanche, J. S., & Chown, S. L. (2012). Thermal limits of wild and laboratory strains of two African malaria vector species, *Anopheles arabiensis* and *Anopheles funestus*. *Malaria Journal*, 11(1), 1-14.

Ma, C. S., Ma, G., & Pincebourde, S. (2021). Survive a warming climate: insect responses to extreme high temperatures. *Annual Review of Entomology*, 66, 163-184.

Ma, G., Rudolf, V. H., & Ma, C. S. (2015). Extreme temperature events alter demographic rates, relative fitness, and community structure. *Global Change Biology*, 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. *Scientific Reports*, 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. *Ecotoxicology and Environmental Safety*, 148, 327-335.

Marsh, J. B., & Weinstein, D. B. (1966). Simple charring method for determination of lipids. *Journal of Lipid Research*, 7(4), 574-576.

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). Mosquito larvae that survive a heat spike are less sensitive to subsequent exposure to the pesticide chlorpyrifos. *Environmental Pollution*, 265, 114824.

Meng, S., Delnat, V., & Stoks, R. (2020b). The exposure order strongly modifies how a heat spike increases pesticide toxicity. *Environmental Science & Technology*, 54(18), 11476-11484.

Meng, S., Tran, T. T., Delnat, V., & Stoks, R. (2021). Transgenerational exposure to warming reduces the sensitivity to a pesticide under warming. *Environmental Pollution*, 284, 117217.

Meng, S., Tran, T. T., Dinh, K. V., Delnat, V., & Stoks, R. (2022). Acute warming increases pesticide toxicity more than transgenerational warming by reducing the energy budget. *Science of The Total Environment*, 805, 150373.

Meyer, E., Aglyamova, G. V., & Matz, M. V. (2011). Profiling gene expression responses of coral larvae (*Acropora millepora*) to elevated temperature and settlement inducers using a novel RNA-Seq procedure. *Molecular Ecology*, 20(17), 3599-3616.

Milatovic, D., Gupta, R. C., & Aschner, M. (2006). Anticholinesterase toxicity and oxidative stress. *The Scientific World Journal*, 6, 295-310.

Miyamoto, S., de Almeida, E. A., Nogueira, L., Gennari de Medeiros, M. H., & Di Mascio, P. (2011). Evaluation of malondialdehyde levels. *Oxidative stress in aquatic ecosystems*, 440-447. John Wiley & Sons, Chichester, UK.

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. *Environmental Toxicology and Chemistry*, 32(1), 49-61.

Monaghan, P., Metcalfe, N. B., & Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology Letters*, 12(1), 75-92.

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. *Ecotoxicology and Environmental Safety*, 140, 116-122.

Nisbet, R. M., Muller, E. B., Lika, K., & Kooijman, S. A. L. M. (2000). From molecules to ecosystems through dynamic energy budget models. *Journal of Animal Ecology*, 913-926.

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., ... & Levin, E. D. (2009). The toxicology of climate change: environmental contaminants in a warming world. *Environment International*, 35(6), 971-986.

O'Callaghan, I., Harrison, S., Fitzpatrick, D., & Sullivan, T. (2019). The freshwater isopod *Asellus aquaticus* as a model biomonitor of environmental pollution: A review. *Chemosphere*, 235, 498-509.

Olsson, B., & Klowden, M. J. (1998). Larval diet affects the alarm response of *Aedes aegypti* (L.) mosquitoes (Diptera: Culicidae). *Journal of Insect Behavior*, 11(4), 593-596.

Op de Beeck, L., Janssens, L., & Stoks, R. (2016). Synthetic predator cues impair immune function and make the biological pesticide *Bti* more lethal for vector mosquitoes. *Ecological Applications*, 26(2), 355-366.

Op de Beeck, L., Verheyen, J., Olsen, K., & Stoks, R. (2017a). Negative effects of pesticides under global warming can be counteracted by a higher degradation rate and thermal adaptation. *Journal of Applied Ecology*, 54(6), 1847-1855.

Op de Beeck, L., Verheyen, J., & Stoks, R. (2017b). Integrating both interaction pathways between warming and pesticide exposure on upper thermal tolerance in high-and low-latitude populations of an aquatic insect. *Environmental Pollution*, 224, 714-721.

Op de Beeck, L., Verheyen, J., & Stoks, R. (2018a). Competition magnifies the impact of a pesticide in a warming world by reducing heat tolerance and increasing autotomy. *Environmental Pollution*, 233, 226-234.

Op de Beeck, L., Verheyen, J., & Stoks, R. (2018b). Strong differences between two congeneric species in sensitivity to pesticides in a warming world. *Science of The Total Environment*, 618, 60-69.

Oppold, A., Kreß, A., Bussche, J. V., Diogo, J. B., Kuch, U., Oehlmann, J., ... & Müller, R. (2015). Epigenetic alterations and decreasing insecticide sensitivity of the Asian tiger mosquito *Aedes albopictus*. *Ecotoxicology and Environmental Safety*, 122, 45-53.

Orr, J. A., Vinebrooke, R. D., Jackson, M. C., Kroeker, K. J., Kordas, R. L., Mantyka-Pringle, C., ... & Piggott, J. J. (2020). Towards a unified study of multiple stressors: divisions and common goals across research disciplines. *Proceedings of the Royal Society B*, 287(1926), 20200421.

Paaijmans, K. P., Heinig, R. L., Seliga, R. A., Blanford, J. I., Blanford, S., Murdock, C. C., & Thomas, M. B. (2013). Temperature variation makes ectotherms more sensitive to climate change. *Global Change Biology*, 19(8), 2373-2380.

Patetsini, E., Dimitriadis, V. K., & Kaloyianni, M. (2013). Biomarkers in marine mussels, *Mytilus galloprovincialis*, exposed to environmentally relevant levels of the pesticides, chlorpyrifos and penoxsulam. *Aquatic Toxicology*, 126, 338-345.

Patra, R. W., Chapman, J. C., Lim, R. P., & Gehrke, P. C. (2007). The effects of three organic chemicals on the upper thermal tolerances of four freshwater fishes. *Environmental*

Toxicology and Chemistry, 26(7), 1454-1459.

Paz, S. (2015). Climate change impacts on West Nile virus transmission in a global context. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370(1665), 20130561.

Pedersen, L. F., Dall, L. G., Sørensen, B. C., Mayntz, D., & Toft, S. (2002). Effects of hunger level and nutrient balance on survival and acetylcholinesterase activity of dimethoate exposed wolf spiders. *Entomologia Experimentalis et Applicata*, 103(3), 197-204.

Pestana, J. L., Loureiro, S., Baird, D. J., & Soares, A. M. (2009). Fear and loathing in the benthos: responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. *Aquatic Toxicology*, 93(2-3), 138-149.

Pestana, J. L., Loureiro, S., Baird, D. J., & Soares, A. M. (2010). Pesticide exposure and inducible antipredator responses in the zooplankton grazer, *Daphnia magna* Straus. Chemosphere, 78(3), 241-248.

Pestana, J. L., Novais, S. C., Norouzitallab, P., Vandegehuchte, M. B., Bossier, P., & De Schamphelaere, K. A. (2016). Non-lethal heat shock increases tolerance to metal exposure in brine shrimp. *Environmental Research*, 151, 663-670.

Pham, H. T., Dinh, K. V., Nguyen, C. C., & Quoc, L. B. (2020). Changes in the magnitude of the individual and combined effects of contaminants, warming, and predators on tropical cladocerans across 11 generations. *Environmental Science & Technology*, 54(23), 15287-15295.

Pörtner, H. (2001). Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften*, 88(4), 137-146.

Porter, J. H., Parry, M. L., & Carter, T. R. (1991). The potential effects of climatic change on agricultural insect pests. *Agricultural and Forest Meteorology*, 57(1-3), 221-240.

Prud'homme, S. M., Chaumot, A., Cassar, E., David, J. P., & Reynaud, S. (2017). Impact of micropollutants on the life-history traits of the mosquito *Aedes aegypti*: On the relevance of transgenerational studies. *Environmental Pollution*, 220, 242-254.

Quiroga, L. B., Sanabria, E. A., Fornés, M. W., Bustos, D. A., & Tejedo, M. (2019). Sublethal concentrations of chlorpyrifos induce changes in the thermal sensitivity and tolerance

of anuran tadpoles in the toad *Rhinella arenarum*?. *Chemosphere*, 219, 671-677.

R Development Core Team. (2017). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing.

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*, 142649.

Rahmstorf, S., & Coumou, D. (2011). Increase of extreme events in a warming world. *Proceedings of the National Academy of Sciences*, 108(44), 17905-17909.

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. *Environmental Pollution*, 224, 572-580.

Relyea, R., & Hoverman, J. (2006). Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecology Letters*, 9(10), 1157-1171.

Reynaldi, S., Meiser, M., & Liess, M. (2011). Effects of the pyrethroid fenvalerate on the alarm response and on the vulnerability of the mosquito larva *Culex pipiens molestus* to the predator *Notonecta glauca*. *Aquatic Toxicology*, 104(1-2), 56-60.

Ritz, C., Baty, F., Streibig, J. C., & Gerhard, D. (2015). Dose-response analysis using R. *PloS One*, 10(12), e0146021.

Roberts, D. A., Hofmann, G. E., & Somero, G. N. (1997). Heat-shock protein expression in *Mytilus californianus*: acclimatization (seasonal and tidal-height comparisons) and acclimation effects. *The Biological Bulletin*, 192(2), 309-320.

Rohr, J. R., Civitello, D. J., Cohen, J. M., Roznik, E. A., Sinervo, B., & Dell, A. I. (2018). The complex drivers of thermal acclimation and breadth in ectotherms. *Ecology Letters*, 21(9), 1425-1439.

Rohr, J. R., & Palmer, B. D. (2013). Climate change, multiple stressors, and the decline of ectotherms. *Conservation Biology*, 27(4), 741-751.

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 Biology*, 17(2), 657-666.

Rubach, M. N., Baird, D. J., Boerwinkel, M. C., Maund, S. J., Roessink, I., & Van den Brink, P. J. (2012). Species traits as predictors for intrinsic sensitivity of aquatic invertebrates to the insecticide chlorpyrifos. *Ecotoxicology*, 21(7), 2088-2101.

Russo, R., Becker, J. M., & Liess, M. (2018). Sequential exposure to low levels of pesticides and temperature stress increase toxicological sensitivity of crustaceans. *Science of the Total Environment*, 610, 563-569.

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. *Ecology Letters*, 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. *Ecotoxicology and Environmental Safety*, 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 Biology*, 24(5), 1817-1826.

Schulte, P. M., Healy, T. M., & Fangue, N. A. (2011). Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integrative and Comparative Biology*, 51(5), 691-702.

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.

Sgro, C. M., Terblanche, J. S., & Hoffmann, A. A. (2016). What can plasticity contribute to insect responses to climate change?. *Annual Review of Entomology*, 61, 433-451.

Shahid, N., Liess, M., & Knillmann, S. (2019). Environmental stress increases synergistic effects of pesticide mixtures on *Daphnia magna*. *Environmental Science & Technology*, 53(21), 12586-12593.

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. *Functional Ecology*, 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. *Environmental Science & Technology*, 51(17), 9433-9445.

Shin, S. M., Akram, W., & Lee, J. J. (2012). Effect of body size on energy reserves in *Culex pipiens pallens* females (Diptera: Culicidae). Entomological Research, 42(3), 163-167.

Sheldon, K. S., Yang, S., & Tewksbury, J. J. (2011). Climate change and community disassembly: impacts of warming on tropical and temperate montane community structure. *Ecology Letters*, 14(12), 1191-1200.

Silberbush, A., Abramsky, Z., & Tsurim, I. (2015). Dissolved oxygen levels affect the survival and developmental period of the mosquito *Culex pipiens*. *Journal of Vector Ecology*, 40(2), 425-427.

Simon, D., Helliwell, S., & Robards, K. (1998). Analytical chemistry of chlorpyrifos and diuron in aquatic ecosystems. *Analytica Chimica Acta*, 360(1-3), 1-16.

Sinclair, B. J., Marshall, K. E., Sewell, M. A., Levesque, D. L., Willett, C. S., Slotsbo, S., ... & Huey, R. B. (2016). Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures?. *Ecology Letters*, 19(11), 1372-1385.

Singh, P., & Prasad, S. M. (2018). Antioxidant enzyme responses to the oxidative stress due to chlorpyrifos, dimethoate and dieldrin stress in palak (*Spinacia oleracea* L.) and their toxicity alleviation by soil amendments in tropical croplands. *Science of The Total Environment*, 630, 839-848.

Singmann, H., Bolker, B., Westfall, J., & Aust, F. (2017). afex: Analysis of factorial experiments. R package version.

Sokolova, I. M. (2013). Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integrative and Comparative Biology*, 53(4), 597-608.

Somero, G. N., Lockwood, B. L., & Tomanek, L. (2017). *Biochemical adaptation: response to environmental challenges, from life's origins to the Anthropocene*. Sinauer Associates, Incorporated Publishers.

Soren, S., Vir Singh, S., & Singh, P. (2018). Seasonal variation of mitochondria activity related and heat shock protein genes in spermatozoa of Karan Fries bulls in tropical climate. *Biological Rhythm Research*, 49(3), 366-381.

Sørensen, J. G., Kristensen, T. N., & Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, 6(11), 1025-1037.

Sousa, T., Domingos, T., & Kooijman, S. A. L. M. (2008). From empirical patterns to theory: a formal metabolic theory of life. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1502), 2453-2464.

Steele, J. E. (1982). Glycogen phosphorylase in insects. *Insect Biochemistry*, 12(2), 131-147.

Stehle, S., & Schulz, R. (2015). Pesticide authorization in the EU - environment unprotected? *Environmental Science and Pollution Research*, 22(24), 19632-19647.

Sterner, R. W., & Elser, J. J. (2002). Ecological stoichiometry: *The biology of elements from molecules to the biosphere*. Princeton university press, Princeton, NJ.

Stillman, J. H. (2019). Heat waves, the new normal: summertime temperature extremes will impact animals, ecosystems, and human communities. *Physiology*, 34(2), 86-100.

Stoks, R., Block, M. D., & McPeek, M. A. (2006). Physiological costs of compensatory growth in a damselfly. *Ecology*, 87(6), 1566-1574.

Stoks, R., & Cordoba-Aguilar, A. (2012). Evolutionary ecology of Odonata: a complex life cycle perspective. *Annual Review of Entomology*, 57, 249-265.

Stoks, R., Verheyen, J., Van Dievel, M., & Tüzün, N. (2017). Daily temperature variation and extreme high temperatures drive performance and biotic interactions in a warming world. *Current Opinion in Insect Science*, 23, 35-42.

Storey, K. B. (2015). Regulation of hypometabolism: insights into epigenetic controls. *Journal of Experimental Biology*, 218(1), 150-159.

Suarez-Lopez, J. R., Butcher, C. R., Gahagan, S., Checkoway, H., Alexander, B. H., & Al-Delaimy, W. K. (2018). Acetylcholinesterase activity and time after a peak pesticide-

use period among Ecuadorian children. *International Archives of Occupational and Environmental Health*, 91(2), 175-184.

Sulmon, C., Van Baaren, J., Cabello-Hurtado, F., Gouesbet, G., Hennion, F., Mony, C., ... & Gérard, C. (2015). Abiotic stressors and stress responses: What commonalities appear between species across biological organization levels?. *Environmental Pollution*, 202, 66-77.

Sumon, K. A., Rico, A., Ter Horst, M. M., Van den Brink, P. J., Haque, M. M., & Rashid, H. (2016). Risk assessment of pesticides used in rice-prawn concurrent systems in Bangladesh. *Science of the Total Environment*, 568, 498-506.

Thompson, R. M., Beardall, J., Beringer, J., Grace, M., & Sardina, P. (2013). Means and extremes: building variability into community-level climate change experiments. *Ecology Letters*, 16(6), 799-806.

Todgham, A. E., Schulte, P. M., & Iwama, G. K. (2005). Cross-tolerance in the tidepool sculpin: the role of heat shock proteins. *Physiological and Biochemical Zoology*, 78(2), 133-144.

Todgham, A. E., & Stillman, J. H. (2013). Physiological responses to shifts in multiple environmental stressors: relevance in a changing world. *Integrative and Comparative Biology*, 53(4), 539-544.

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. *Evolutionary Applications*, 11(6), 906-917.

Tran, T. T., Janssens, L., Dinh, K. V., & Stoks, R. (2019). An adaptive transgenerational effect of warming but not of pesticide exposure determines how a pesticide and warming interact for antipredator behaviour. *Environmental Pollution*, 245, 307-315.

Tripathi, G., & Shasmal, J. (2010). Reparation of chlorpyrifos-induced impairment by thyroxine

and vitamin C in fish. *Ecotoxicology and Environmental Safety*, 73(6), 1397-1401.

Tu, X. H., Zhuo, Y. L., Jiang, X. Y., Liu, H. L., Cao, Y., & Liu, S. (2018). Molecular characterization of a mitochondrial manganese superoxide dismutase from *Chilo suppressalis* (Lepidoptera: Crambidae). *Journal of Economic Entomology*, 111(5), 2391-2400.

Uller, T. (2008). Developmental plasticity and the evolution of parental effects. *Trends in Ecology & Evolution*, 23(8), 432-438.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., & Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*, 64(2), 178-189.

Van den Brink, P. J., Boxall, A. B., Maltby, L., Brooks, B. W., Rudd, M. A., Backhaus, T., ... & van Wensem, J. (2018). Toward sustainable environmental quality: Priority research questions for Europe. *Environmental Toxicology and Chemistry*, 37(9), 2281-2295.

Van den Brink, P. J., Bracewell, S. A., Bush, A., Chariton, A., Choung, C. B., Compson, Z. G., ... & Baird, D. J. (2019). Towards a general framework for the assessment of interactive effects of multiple stressors on aquatic ecosystems: Results from the Making Aquatic Ecosystems Great Again (MAEGA) workshop. *Science of The Total Environment*, 684, 722-726.

Van Dievel, M., Janssens, L., & Stoks, R. (2019). Additive bioenergetic responses to a pesticide and predation risk in an aquatic insect. *Aquatic Toxicology*, 212, 205-213.

Van Dievel, M., Stoks, R., & Janssens, L. (2017). Beneficial effects of a heat wave: higher growth and immune components driven by a higher food intake. *Journal of Experimental Biology*, 220(21), 3908-3915.

Van Drooge, H. L., Groeneveld, C. N., & Schipper, H. J. (2001). Data on application frequency of pesticide for risk assessment purposes. *The Annals of Occupational Hygiene*, 45, S95-S101.

Van Straalen, N. M. (2003). Ecotoxicology becomes stress ecology. *Environmental Science & Technology*, 37(17), 324A-330A.

Vasseur, D. A., DeLong, J. P., Gilbert, B., Greig, H. S., Harley, C. D., McCann, K. S., ... &

O'Connor, M. I. (2014). Increased temperature variation poses a greater risk to species than climate warming. *Proceedings of the Royal Society B: Biological Sciences*, 281(1779), 20132612.

Veilleux, H. D., Ryu, T., Donelson, J. M., Van Herwerden, L., Seridi, L., Ghosheh, Y., ... & Munday, P. L. (2015). Molecular processes of transgenerational acclimation to a warming ocean. *Nature Climate Change*, 5(12), 1074-1078.

Verberk, W. C., Atkinson, D., Hoefnagel, K. N., Hirst, A. G., Horne, C. R., & Siepel, H. (2021). Shrinking body sizes in response to warming: explanations for the temperature–size rule with special emphasis on the role of oxygen. *Biological Reviews*, 96(1), 247-268.

Verberk, W. C., & Bilton, D. T. (2011). Can oxygen set thermal limits in an insect and drive gigantism?. *Plos One*, 6(7), e22610.

Verberk, W. C., & Bilton, D. T. (2013). Respiratory control in aquatic insects dictates their vulnerability to global warming. *Biology Letters*, 9(5), 20130473.

Verberk, W. C., Bilton, D. T., Calosi, P., & Spicer, J. I. (2011). Oxygen supply in aquatic ectotherms: partial pressure and solubility together explain biodiversity and size patterns. *Ecology*, 92(8), 1565-1572.

Verberk, W. C., Buchwalter, D. B., & Kefford, B. J. (2020). Energetics as a lens to understanding aquatic insect's responses to changing temperature, dissolved oxygen and salinity regimes. *Current Opinion in Insect Science*, 41, 46-53.

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. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 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. *Environmental Science & Technology*, 53(8), 4600-4608.

Verheyen, J., & Stoks, R. (2019). Shrinking body size and physiology contribute to geographic variation and the higher toxicity of pesticides in a warming world. *Environmental Science & Technology*, 53, 11515-11523.

Verheyen, J., & Stoks, R. (2020). Negative bioenergetic responses to pesticides in damselfly larvae are more likely when it is hotter and when temperatures fluctuate. *Chemosphere*, 243, 125369.

Verslycke, T., Roast, S. D., Widdows, J., Jones, M. B., & Janssen, C. R. (2004). Cellular energy allocation and scope for growth in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following chlorpyrifos exposure: a method comparison. *Journal of Experimental Marine Biology and Ecology*, 306(1), 1-16.

Vinebrooke, R., L. Cottingham, K., Norberg, Marten Scheffer, J., I. Dodson, S., C. Maberly, S., & Sommer, U. (2004). Impacts of multiple stressors on biodiversity and ecosystem functioning: The role of species co-tolerance. *Oikos*, 104(3), 451-457.

Vinogradova, E. B. (2000). *Culex pipiens pipiens* mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control (No. 2). Pensoft Publishers.

Vörösmarty, C. J., McIntyre, P. B., Gessner, M. O., Dudgeon, D., Prusevich, A., Green, P., ... & Davies, P. M. (2010). Global threats to human water security and river biodiversity. *Nature*, 467(7315), 555-561.

Vrzal, E. M., Allan, S. A., & Hahn, D. A. (2010). Amino acids in nectar enhance longevity of female *Culex quinquefasciatus* mosquitoes. *Journal of Insect Physiology*, 56(11), 1659-1664.

Vucic-Pestic, O. L. I. V. E. R. A., Ehnes, R. B., Rall, B. C., & Brose, U. (2011). Warming up the system: higher predator feeding rates but lower energetic efficiencies. *Global Change Biology*, 17(3), 1301-1310.

Wang, Z., Lui, G. C., Burton Jr, G. A., & Leung, K. M. (2019). Thermal extremes can intensify chemical toxicity to freshwater organisms and hence exacerbate their impact to the biological community. *Chemosphere*, 224, 256-264.

Wellborn, G. A., Skelly, D. K., & Werner, E. E. (1996). Mechanisms creating community structure across a freshwater habitat gradient. *Annual Review of Ecology and Systematics*, 27(1), 337-363.

Wethey, D. S., Woodin, S. A., Hilbish, T. J., Jones, S. J., Lima, F. P., & Brannock, P. M. (2011). Response of intertidal populations to climate: effects of extreme events versus long term change. *Journal of Experimental Marine Biology and Ecology*, 400(1-2), 132-144.

WHO, (2005). Guidelines for Laboratory and Field Testing of Mosquito Larvicides. *World Health Organization*, Geneve, Switzerland, pp. 1-41.

Willming, M. M., Qin, G., & Maul, J. D. (2013). Effects of environmentally realistic daily temperature variation on pesticide toxicity to aquatic invertebrates. *Environmental Toxicology and Chemistry*, 32(12), 2738-2745.

Willming, M. M., & Maul, J. D. (2016). Direct and indirect toxicity of the fungicide pyraclostrobin to Hyalella azteca and effects on leaf processing under realistic daily temperature regimes. *Environmental Pollution*, 211, 435-442.

Woodward, G., Perkins, D. M., & Brown, L. E. (2010). Climate change and freshwater ecosystems: impacts across multiple levels of organization. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365(1549), 2093-2106.

Woolway, R. I., Jennings, E., Shatwell, T., Golub, M., Pierson, D. C., & Maberly, S. C. (2021). Lake heatwaves under climate change. *Nature*, 589(7842), 402-407.

Wu, F., Sokolov, E. P., Dellwig, O., & Sokolova, I. M. (2021). Season-dependent effects of ZnO nanoparticles and elevated temperature on bioenergetics of the blue mussel *Mytilus edulis*. *Chemosphere*, 263, 127780.

Xing, H., Li, S., Wang, X., Gao, X., Xu, S., & Wang, X. (2013). Effects of atrazine and chlorpyrifos on the mRNA levels of HSP70 and HSC70 in the liver, brain, kidney and gill of common carp (*Cyprinus carpio* L.). *Chemosphere*, 90(3), 910-916.

Zhang, W., Chang, X. Q., Hoffmann, A., Zhang, S., & Ma, C. S. (2015). Impact of hot events at different developmental stages of a moth: the closer to adult stage, the less reproductive output. *Scientific Reports*, 5(1), 1-9.

Zhao, L., & Jones, W. A. (2012). Expression of heat shock protein genes in insect stress responses. *Invertebrate Survival Journal*, 9(1), 93-101.

Zhao, S., Kamelia, L., Boonpawa, R., Wesseling, S., Spenkelink, B., & Rietjens, I. M. (2019). Physiologically based kinetic modeling-facilitated reverse dosimetry to predict *in vivo* red blood cell acetylcholinesterase inhibition following exposure to chlorpyrifos in the Caucasian and Chinese population. Toxicological sciences, 171(1), 69-83.

Zhu, G., Xue, M., Luo, Y., Ji, G., Liu, F., Zhao, H., & Sun, X. (2017). Effects of short-term

heat shock and physiological responses to heat stress in two *Bradysia* adults, *Bradysia odoriphaga* and *Bradysia difformis*. *Scientific Reports*, 7(1), 1-11.

Publications

Meng, S., Delnat, V., & Stoks, R. (2020). The exposure order strongly modifies how a heat spike increases pesticide toxicity. *Environmental Science & Technology*, 54(18), 11476-11484.

Meng, S., Delnat, V., & Stoks, R. (2020). Mosquito larvae that survive a heat spike are less sensitive to subsequent exposure to the pesticide chlorpyrifos. *Environmental Pollution*, 265, 114824.

Meng, S., Tran, T. T., Delnat, V., & Stoks, R. (2021). Transgenerational exposure to warming reduces the sensitivity to a pesticide under warming. *Environmental Pollution*, 284, 117217.

Meng, S., Tran, T. T., Dinh, K. V., Delnat, V., & Stoks, R. (2022). Acute warming increases pesticide toxicity more than transgenerational warming by reducing the energy budget. *Science of The Total Environment*, 805, 150373.

Meng, S., Delnat, V., & Stoks, R. (2021). Multigenerational effects modify the tolerance of mosquito larvae to chlorpyrifos but not to a heat spike and do not change their synergism. *Environmental Pollution*, accepted.

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