1 Testing the time-scale dependence of delayed interactions: a heat wave during the egg stage
2 shapes how a pesticide interacts with a successive heat wave in the larval stage

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ABSTRACT

Under global change organisms are exposed to multiple, potentially interacting stressors. Especially interactions between successive stressors are poorly understood and recently suggested to depend on their timing of exposure. We particularly need studies assessing the impact of exposure to relevant stressors at various life stages and how these interact. We investigated the single and combined impacts of a heat wave (mild [25°C] and extreme [30°C]) during the egg stage, followed by successive exposure to esfenvalerate (ESF) and a heat wave during the larval stage in damselflies. Each stressor caused mortality. The egg heat wave and larval ESF exposure had delayed effects on survival, growth and lipid peroxidation (MDA). This resulted in deviations from the prediction that stressors separated by a long time interval would not interact: the egg heat wave modulated the interaction between the stressors in the larval stage. Firstly, ESF caused delayed mortality only in larvae that had been exposed to the extreme egg heat wave and this strongly depended upon the larval heat wave treatment. Secondly, ESF only increased MDA in larvae not exposed to the egg heat wave. We found little support for the prediction that when there is limited time between stressors, synergistic interactions should occur. The intermediate ESF concentration only caused delayed mortality when combined with the larval heat wave, and the lowest ESF concentrations only increased oxidative damage when followed by the mild larval heat wave. Survival selection mitigated the interaction patterns between successive stressors that are individually lethal, and therefore should be included in a predictive framework for the time-scale dependence of the outcome of multistressor studies with pollutants. The egg heat wave shaping the interaction pattern between successive pesticide exposure and a larval heat wave highlights the connectivity between the concepts of ‘heat-induced pesticide sensitivity’ and ‘pesticide-induced heat sensitivity’.

Capture: An egg heat wave shapes the interaction between successive pesticide exposure and a heat wave on mortality during the larval stage.

Keywords: Delayed pesticide effects; global warming; multistressor studies; pyrethroid; stressor interactions; survival selection

INTRODUCTION

The growing concern that animals will not only have to cope with warming, but also with the changed impact of pollutants in a warming world boosted research at the interface of global change biology and ecotoxicology (Moe et al. 2013; Noyes & Lema 2015). In the field of global change biology there is a strong bias toward research on mild warming, while effects of heat waves have been understudied (Thompson et al. 2013). Recent insights indicate, however, that the effects of heat waves may be stronger and may overrule the effects of mild warming (Jentsch et al. 2007; Williams et al. 2016). This bias towards mild warming studies is also present in ecotoxicology. Nevertheless, the number of combined studies on pollutants and extreme temperatures as experienced during heat waves is increasing (e.g. Chang et al. 2007; Holmstrup et al. 2010; Arambourou et al. 2015). This follows the general insight that we need more studies that consider interactions between stressors to improve the risk assessment of pollutants and conservation management in general (Côté et al. 2016; Liess et al. 2016).

Especially interaction patterns between successive stressors are poorly understood and suggested to depend on their timing of exposure (Ashauer et al. 2010; Gunderson et al. 2016). The majority of studies exposing animals to heat waves and pollutants did so simultaneously with the general outcome of a stronger, synergistic impact of pollutants under extreme temperatures (Holmstrup et al. 2010). Simultaneous exposure to two stressors is indeed more likely to generate synergistic interactions since the physiological response to the first stressor

is still being mounted when the second stressor occurs, resulting in no toxicodynamic recovery (Ashauer et al. 2010). Yet, as animals typically encounter heat waves (Meehl & Tebaldi 2004) and pesticides (Van Drooge et al. 2001) only for limited periods of time, animals will be more likely exposed successively to both stressors. When two stressors are in close succession, the physiological response to the first stressor may still be ongoing, and if most of the damage is repaired or removed before the second stressor occurs, this may generate cross-resistance and antagonistic interactions (Gunderson et al. 2016). When stressors are instead spread over a long timescale, no interactive effects are expected since the organisms are thought to have returned to their homeostatic phase before the second stressor occurs (Gunderson et al. 2016). Alternatively, exposure to one stressor can lead to a permanent increase of an organism's baseline defence levels, making it easier for it to cope with future stressors (Monaghan et al. 2009).

Crucial to advance our insights into how heat waves will interact with pollutants, are studies that expose animals successively to these stressors. We particularly need studies assessing the impact of exposure to relevant stressors at various life stages and how these interact. These will be essential to test the conceptual framework of Gunderson et al. (2016) and to address other questions related to the timing of exposure to stressors. Many organisms will be exposed to more complex stressor combinations and one outstanding question is whether exposure to one stressor early in life may change the way two other stressors later in life interact with each other. Given the rarity of three-stressor studies in ecotoxicology in general (but see e.g. Przeslawski et al. 2005; Coors & De Meester 2008; Matthaei et al. 2010; Dinh Van et al. 2016a), this has been unexplored. Nevertheless, such studies will be crucial to advance our insights in the still poorly understood occurrence of interactions between pollutants and natural stressors (Crain et al. 2008; Liess et al. 2016).

While there is increasing appreciation that stressors may interact in a delayed way (Segner 2011; Gunderson et al. 2016), the studies integrating stress exposure in two life stages typically consider the larval stage and the adult stage (e.g. Rohr & Palmer 2005; Janssens et al. 2014). In contrast, delayed effects of stressors experienced in the egg stage on traits later in the development, and especially their interaction with stressors occurring later in life have been rarely studied (but see Broomhall 2004; Zhang et al. 2014; Sniegula et al. 2017). Such studies are, however, highly relevant as in the egg stage organisms are less mobile and therefore do not have the possibility to avoid exposure to stressors (Potter et al. 2011).

In this study we investigated the single and combined effects of a heat wave during the egg stage, followed by successive exposure to a pyrethroid pesticide and a heat wave during the larval stage of a damselfly. The study species, *Lestes viridis*, deposits eggs in summer, which will hatch the following spring; making a heat treatment for the eggs and pesticide exposure and a heat treatment for the larvae, relevant stressors to study in these different life stages. Special focus was on the delayed effects of the stressors and how a stressor in the egg stage interacted with stressors encountered later in life. Based on Gunderson et al. (2016) we may expect few effects of the egg heat wave on the impact of the larval pesticide exposure and the larval heat wave, while the latter two are expected to interact in a synergistic way because they are in close succession. We studied effects on survival and growth, and an important physiological variable linked with life history and performance, oxidative damage to lipids (Monaghan et al. 2009). Oxidative stress is thought to be a key molecular mechanism involved in pyrethroid-induced toxicity (e.g. Rodrigues et al. 2015). As pyrethroid pesticide we chose the insecticide esfenvalerate (ESF), one of the most widely applied pyrethroids (Beketov & Liess 2005; Spurlock & Lee 2008). Pyrethroids function by damaging the nervous system through interference with ion channels in the nerve axons (Tomlin 2001). We chose damselflies as study organisms since they are particularly vulnerable in the aquatic larval

stage to both global warming (Hassall & Thompson 2008) and to ESF exposure (Beketov 2004).

METHODS

Collection and housing

Lestes viridis oviposits in tree twigs overhanging ponds. Eggs of this species are laid in summer, enter a winter diapause and hatch from April to half June in the study region (De Block et al. 2005), with a hatching peak at the end of April (De Block & Stoks 2004). The duration of the larval period is ca. 65 days (De Block & Stoks 2003). Twigs containing eggs of *L. viridis* were collected from a population in Edegem (51°09'19.1"N, 4°23'55.6"E), Belgium on 27 January 2015. Twigs with eggs can be easily recognized by the gall-like structures where the eggs have been deposited, and the oviposition marks are easily visible (each mark typically is associated with 4 eggs). The population was chosen in an area without agriculture and therefore is unlikely to be exposed to pesticides (Coors et al. 2009). The twigs with eggs were placed in incubators in trays filled with dechlorinated tap water. The eggs were gradually warmed (10°C on 28 January, 15°C on 1 February, 20°C on 5 February). After hatching, groups of 10 larvae were placed together in 200 ml plastic cups filled with dechlorinated tap water in water baths (20°C, 14:10 L:D) to enhance survival (De Block & Stoks 2003). Two weeks after hatching, larvae were placed individually in the same cups. During their life, the larvae were fed *ad libitum* with *Artemia* nauplii.

Experimental setup

To test for the single and combined effects of subsequent exposure to a heat wave in the egg stage ('egg heat wave'), pesticide exposure in the larval stage and a heat wave in the larval stage ('larval heat wave'), we set up a full factorial experiment (Figure 1). To test how one stressor early in life may change the way two other stressors later in life interact with each

other, the egg heat wave was separated by 30-36 days from the larval pesticide exposure (depending on when the larvae hatched), while the larval heat wave was directly succeeding the pesticide exposure. The full setup consisted of the 36 combinations of three egg heat treatments (20°C, 25°C, 30°C) × four larval pesticide treatments (0, 0.05, 0.1 and 0.4 µg/l ESF) × three larval heat treatments (20°C, 25°C, 30°C). Each treatment combination started with 28 replicates (total of 1,008 animals).

For the simulation of the heat wave in the egg stage, the eggs were divided in three equal groups based on the number of oviposition marks on the twigs. The first group was kept at an air temperature of 20°C, the second group was warmed to an air temperature of 25°C for two days (6 and 7 February), and the third group was gradually warmed to an air temperature of 30°C for two days (25°C at 6 February, 30°C at 7 and 8 February, and 25°C at 9 February). After the heat exposure, all eggs returned to 20°C. No larvae hatched before the heat exposure during the egg stage was completed. Heat waves with air temperatures up to 30°C have been recorded as early as May in Belgium (www.meteo.be) and therefore exposure of eggs to the here applied temperature of 25°C (i.e. ‘mild heat wave’) and 30°C (i.e. ‘extreme heat wave’) is ecologically relevant, with 25°C being experienced more often.

Four weeks after hatching, the larvae entered the ESF exposure period. At this age they may experience spring pesticide applications in ponds in agricultural areas. Per egg heat wave treatment, animals were divided over four pesticide treatment groups: a control, 0.05 µg/l, 0.1 µg/l and 0.4 µg/l ESF. The concentrations were chosen as they induced a growth reduction in larvae of other damselfly species (Dinh Van et al. 2016b; Sniegula et al. 2017) and are ecologically relevant in water bodies nearby agricultural fields (Cooper et al. 2003; Forbes & Cold 2005; Rodrigues et al. 2015). A stock solution was prepared by dissolving ESF powder in absolute ethanol obtaining a concentration of 0.4 mg/ml. The stock solution was then diluted with Milli-Q water to obtain the target concentrations in the cups; a separate

solution was made for each ESF concentration (0.05, 0.1 and 0.4 µg/ml). The water in the cups of the ESF treatment groups was spiked with the diluted solution and the control group received the highest concentration of ethanol used in the ESF exposed animals (0.1µl/l). Previous research showed that ethanol concentrations up to 5µl/l did not affect growth and behaviour of damselfly larvae (Lizanne Janssens, unpublished data). A mixed sample of ten experimental cups per concentration was analyzed by the research laboratory Lovap NV (Geel, Belgium) using gas chromatography combined with mass spectrometry. The measured initial concentrations were 0.024, 0.056 and 0.25 µg/l ESF; after 48h (before the second pulse) all ESF concentrations were below the detection level of 0.020 µg/l. The exposure period lasted for four days during which we applied two pulses of the pesticide (on days 1 and 3). This is a realistic scenario of repeated exposure to pesticides through run-off in agricultural ponds during spring (Van Drooge et al. 2001). At the end of the exposure period, all larvae were transferred to clean water for the subsequent larval heat wave period.

Immediately after the pesticide exposure period we simulated the heat wave treatment in the larval stage. This was done similar as in the egg stage. Per egg heat wave × pesticide treatment combination, larvae were exposed to one of three heat wave treatments, being 20°C, 25°C and 30°C for four days. The longer duration of the heat wave treatment in the larval stage (4 days) compared to the egg stage (2 days) was chosen given the longer periods with high temperatures when the season progresses.

Response variables

We counted the number of larvae that hatched at each egg temperature. Survival was recorded daily during the larval pesticide exposure period and during the larval heat wave period. We quantified larval growth rate during the larval heat wave period (see Appendix 1).

At the end of the larval heat wave, all larvae ($n = 844$) were frozen for the quantification of oxidative damage to lipids. This was measured as the concentration of malondialdehyde (MDA) based on the protocol described in Janssens & Stoks (2017).

Statistical analyses

As we were unable to accurately count the eggs in the twigs, we assumed all three treatments started with the same number of eggs. This is motivated by the fact that we initially divided the twigs containing the eggs equally over the three egg temperature treatments based on the number of oviposition marks. Egg survival was compared between the three egg heat treatments using Fisher's exact tests where we set the number of larvae that hatched at the temperature with the highest number of hatchlings as 100% and calculated the percentage of hatching at the other temperatures as a proportion of this maximum hatching success. Larval survival was analyzed using logistic regression models with a binomial error structure. When analyzing larval survival during the pesticide exposure period, we added the egg heat wave treatment, the pesticide treatment and their interaction as independent variables. When analyzing larval survival during the larval heat wave period, we also added the larval heat wave treatment and its interactions. Significant interactions were further explored using Fisher's exact tests. We tested for an effect of the egg heat wave treatment, the pesticide treatment, the larval heat wave treatment and their interactions (three-way ANOVA) on the MDA content at the end of the larval heat wave period. Significant interactions were further explored using contrast analyses. The ANOVA assumptions were met without the need of transformations. All tests were done in Statistica v13 (Statsoft Inc, OK, USA).

RESULTS

Survival

Egg hatching success strongly depended on the egg heat wave treatment, with the number of hatchlings being highest at 25 °C ($\chi^2 = 739.39$; $p < 0.001$), intermediate at 20°C and lowest at 30°C (Fisher's exact tests, all $p < 0.001$; Figure 2a).

During the larval pesticide exposure period, the pesticide induced significant mortality (Table 1; Figure 2b), but only at the highest concentration (Fisher's exact test, control vs 0.4 µg/l: $p = 0.0019$; all other comparisons $p > 0.37$). The egg heat wave treatment did not affect the mortality during the pesticide exposure period, nor did it interact with the pesticide treatment (both $p > 0.34$).

During the larval heat wave period, there was a negative effect on survival of the previous egg heat wave treatment and the current larval heat wave treatment (Table 1; Figure 3). There was a significant three-way interaction between the egg heat wave treatment, the pesticide treatment and the larval heat wave treatment (Table 1; Figure 3) indicating that the initial egg heat wave modulated how the larval pesticide exposure and larval heat wave interacted in shaping mortality. In larvae originating from eggs kept at 20°C, there was no effect of the pesticide, unless the larvae were exposed to the extreme heat wave; in this case the highest pesticide concentration resulted in a higher survival ($p = 0.023$). In larvae originating from eggs kept at 30°C, previous exposure to the highest pesticide concentration reduced the survival when the larvae were kept at 20°C ($p = 0.026$). This survival reduction already occurred at the intermediate concentration when the larvae were kept at 25°C ($p = 0.017$).

Oxidative damage (MDA levels)

Overall, both the egg heat wave and the larval heat wave) resulted in higher levels of MDA. Although there was no main effect of the pesticide, it strongly interacted with both the egg heat wave and the larval heat wave (Table 1; Figure 4).

We observed a significant interaction between the pesticide and the egg heat wave treatment indicating that the stress experienced early in life affected the response to a stressor in a later life stage. In animals originating from eggs kept at 20°C, the two highest ESF concentrations resulted in higher levels of MDA (contrast analyses, 0.1 µg/l: $p = 0.030$; 0.4 µg/l: $p = 0.025$). In animals originating from eggs kept at 30°C, MDA levels were lower when the animals were exposed to the highest pesticide concentration ($p = 0.001$) (Figure 4a).

There was a significant interaction between the pesticide exposure and the successive larval heat wave treatment. Larvae that were kept at 20°C increased MDA levels when exposed to the two highest ESF concentrations (contrast analyses, 0.1 µg/l: $p = 0.0044$; 0.4 µg/l: $p = 0.0069$). When the larvae were exposed to a mild larval heat treatment, MDA levels were higher at the two lowest ESF concentrations (0.05 µg/l: $p = 0.012$; 0.1 µg/l: $p = 0.042$) (Figure 4b).

DISCUSSION

We detected delayed effects of both the egg heat wave treatment and the larval pesticide exposure on survival and oxidative damage during the subsequent larval heat wave period. The effects of the heat wave during the egg stage thereby bridged the egg-larva boundary. The pesticide only reduced survival at the highest concentration during the exposure period. Yet, pesticide exposure at intermediate and high concentrations affected mortality and oxidative damage after the exposure period. Notably, these delayed lethal pesticide effects were dependent on the heat wave treatments in the egg and larval stages, indicating that stressors separated in time interacted with each other. A key finding thereby was that the initial egg heat wave treatment modulated how the two larval stressors (the pesticide and the larval heat wave) interacted in shaping mortality.

Heat wave effects in the egg and larval stage

The mild heat wave (25°C) during the egg stage resulted in an increased number of hatchlings (compared to 20°C) which confirms that mild warming may increase embryonic survival (e.g. in birds [Nord & Nilsson 2011], copepods [Holste & Peck 2006] and newts [Smith et al. 2015]). The mild heat waves in both the egg and larval stages resulted in a higher growth rate (see Appendix 1), which was associated with higher levels of oxidative damage as measured by MDA. This is consistent with the general belief that higher growth rates are associated with a higher metabolic rate and ROS production (Dmitriew 2011). Increased ROS production and damage under mild warming have been shown for example in clams (Bocchetti et al. 2008).

In contrast, the extreme heat wave (30°C) in the egg stage caused a reduced hatching success and this lethal effect also carried over to the larval stage. In fact, a lower survival of all larvae originating from eggs exposed to 30°C was observed during the larval heat wave period. These findings confirm a study in ladybeetles where not only the hatching success, but also the subsequent larval survival was reduced when the eggs had been exposed to a heat shock (Zhang et al. 2014). The authors hypothesized this to be caused by a slow accumulation of damage caused by the heat shock with detrimental effects being displayed later in the development. Also the larval heat wave of 30°C reduced survival. Similarly as for the mild heat wave, also the extreme heat wave was associated with increased oxidative damage (see e.g. also Zhang et al. 2015).

Pesticide effects and their interactions with the heat wave treatments

The highest ESF concentration (nominal 0.4 µg/l, effective 0.25 µg/l) imposed ca. 7% mortality during the exposure period. This concentration falls within the range of concentrations encountered in nature during the spring spraying season in agricultural ponds (Stampfli et al. 2013) and also has been shown to cause minor (2%) mortality in *Chironomus riparius* larvae (Rodrigues et al. 2015). We exposed the larvae to the pesticide when they

were three weeks old, corresponding to May-June (based on De Block & Stoks 2004, 2005), suggesting that young damselfly larvae may be at risk in agricultural ponds during spring application scenarios. Our results thereby further confirm the vulnerability of *Lestes* damselfly larvae to ESF (Beketov 2004).

When the pesticide exposure had ended, ESF still affected survival, but this critically depended upon the heat wave treatments in the egg and larval stages. Delayed mortality caused by ESF has been demonstrated before in other aquatic insects: the caddisflies *Limnephilus lunatus* (Liess & Schulz 1996) and *Anabolia nervosa* (Rasmussen et al. 2017). We extend this insight by showing its dependence on extreme temperatures. In larvae originating from eggs kept at 20°C, there was no effect of the pesticide unless the larvae were exposed to the extreme heat wave. In this case previous exposure to the highest pesticide concentration resulted in an increased survival during the larval heat wave. This seemingly counterintuitive effect suggests survival selection by the highest ESF concentration, where the weakest animals already died during the exposure period and only the strongest survived, combined with possible ‘cross-tolerance’ between the pesticide stress and the larval heat stress. Hereby, the exposure to one stressor (here the pesticide) prepares the animal to cope with a future stressor (here the extreme larval heat wave) (Vinebrooke et al. 2004, Kaunisto et al. 2016). Cross-tolerance can be expected because of the activation of shared defence mechanisms such as the expression of stress proteins, which are upregulated in response to ESF exposure (e.g. Eder et al. 2009) and to heat waves (e.g. Janssens et al. 2014).

When the larvae originated from eggs that had been exposed to the extreme heat wave (30°C), the interaction pattern with ESF differently depended on the larval heat wave treatment. When the larvae were exposed as egg to 30°C and as larva to 20°C, we observed delayed mortality of previous exposure to the highest 0.40 µg/l ESF concentration. This may be because after the extreme egg heat wave, the larvae were already pushed to their energetic

limits making it no longer possible to defend against the highest pesticide concentration. Moreover, ESF has been shown to reduce the energy budget and increase energy consumption in aquatic insects (Rodrigues et al. 2015; Rasmussen et al 2017). When the eggs were exposed to the extreme heat wave (30°C) and the larvae to the mild heat wave (25°), the intermediate 0.10 µg/l ESF concentration already induced delayed mortality. Most likely, because the mild larval heat wave caused stress as indicated by the increase in MDA, the energy budget was already too low at this intermediate ESF concentration.

The pesticide effect on oxidative damage was highly dependent on the egg and larval heat wave treatments. When larvae originated from eggs not exposed to a heat wave (eggs kept at 20°C), the pesticide resulted in increased lipid peroxidation, which confirms a study in the midge *Chironomus riparius* and can be explained by a decrease in the antioxidant defence (Rodrigues et al. 2015). When larvae originated from eggs that did undergo a heat wave, the pattern changed. After the mild egg heat wave (25°C), the pesticide did not cause increased oxidative damage. This might be because the animals increased their antioxidant defence during the egg heat wave to balance the increased ROS production at the higher temperature (Lushchak 2011). After the heat wave had ended, they kept the baseline levels of the antioxidant defence high, resulting in no increase in oxidative damage when confronted with the second stressor. Such cross-resistance for oxidative stress between successive stressors separated in time has been suggested in general by Monaghan et al. (2009). An alternative explanation would be that hormesis played a role, where intermediate stress levels (here being the mild heat wave) result in a better performance (Calabrese & Baldwin 2002). After the extreme egg heat wave (30°C), the MDA concentration even decreased at the highest ESF concentration. This may be due to a combination of cross-resistance and survival selection. Indeed, in the treatment combining the extreme egg heat wave and the high pesticide concentration, the total mortality was the highest (23%), making it possible that the weaker

animals, most susceptible to oxidative damage, already died, while the strongest animals with the most pronounced antioxidant defence survived.

The impact of the heat wave on the pesticide toxicity was slightly different when imposed in the larval stage than in the egg stage. Again, ESF caused increased oxidative damage when the larvae were not exposed to the larval heat wave. When exposed to the mild larval heat wave also the lowest ESF concentration resulted in increased lipid peroxidation. That the mild egg heat wave did not result in higher pesticide-induced oxidative damage, but the larval heat wave did, could be the result of the time lapse between the stressors. In the first case, there was plenty of time between the stressors, therefore it might be that the encountered damage due to the heat wave was repaired or removed before the second stressor (i.e. the pesticide) was encountered (i.e. toxicodynamic recovery, Ashauer et al. 2010). In the second case, there was no time lag between the two stressors. Therefore, it is possible that the animals were still fighting the increased ROS production by the pesticide, when the increased ROS production due to the higher temperature was added. This might have made it impossible for the animals to neutralize both stressors, resulting in increased oxidative damage.

Conclusions

Organisms are exposed to combinations of stressors that can interact with each other and thereby challenge risk assessment of pesticides (Holmstrup et al. 2010; Vighi 2013; Liess et al. 2016). Despite the growing number of multistressor studies, little is known when and how successive stressors interact with each other (Gunderson et al. 2016). The here documented delayed impact of stressors experienced in the egg stage, and their interaction with stressors occurring later in life are especially poorly studied (but see Broomhall 2004; Sniegula et al. 2017).

Our findings only partly match the predictions made by Gunderson et al. (2016) about the interactive effects of successive stressors. First, and in contrast to our results, no

interactive effects were predicted when the stressors are spread over a longer timescale, such as the egg heat wave and the larval pesticide exposure in our study. Second, when there is only limited time between the two stressors, such as the pesticide exposure and the larval heat wave in our study, synergistic interactions were predicted (Gunderson et al. 2016). We found only limited support for the latter prediction. More mechanistic studies are needed to explicitly consider the physiological stress responses and their time frame in determining the interaction type between successive stressors. Our results point to the potential strong interference of survival selection mitigating the interaction patterns between lethal stressors. This highlights that survival selection should be included in a general predictive framework for the time-scale dependence of the outcome of multistressor studies with pollutants. We hypothesize that the predictions by Gunderson et al (2016) are more likely to hold when stressors that are individually sublethal are combined. As recently indicated by Ashauer et al. (2017) an extra complicating factor that may cause deviations from the framework of Gunderson et al (2016) is that the sequence of exposure to the stressors may also play a role in shaping the interactive effects between stressors.

Whatever the underlying mechanisms, our key finding that previous exposure to a heat wave during the egg stage modulates the interaction pattern between successive pesticide exposure and a heat wave during the larval stage is directly relevant for multistressor studies in ecotoxicology. Indeed, while ESF only caused direct mortality at the highest concentration tested, delayed effects on survival caused by exposure to low and intermediate ESF concentrations depended on the combination of the egg and larval heat wave treatments. This may hamper extrapolation of safe concentrations based on lab ecotox tests to field situations where heat waves are becoming increasingly frequent (Fischer & Schär 2010). One should consider these delayed interactions when designing regulatory acceptable concentrations for pyrethroids. As far as we know, this is the first demonstration that a preceding stressor may

shape the interaction between two stressors later in life, and thereby may explain differences between studies focusing on the same two-stressor combinations. Furthermore, this delayed interaction pattern underscores the connectivity between the concepts of ‘heat-induced pesticide sensitivity’ and ‘pesticide-induced heat sensitivity’ (Hooper et al. 2013; Noyes & Lema 2015) that are typically considered in isolation (but see Op de Beeck et al. 2017).

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FIGURE LEGENDS

Figure 1: Experimental design with indication of the timing of exposure to the three successive stressors: the egg heat wave, the larval pesticide exposure and the larval heat wave. Day zero was defined as the day when the first stressor treatment (the egg heat wave) started.

Figure 2: (a) Number of hatchlings of *Lestes viridis* larvae. Effect of the egg heat wave. (b) Percentage survival during the pesticide exposure period of *Lestes viridis* larvae. Effect of the pesticide. Sample sizes per treatment group range from 80 to 81 larvae.

Figure 3: Percentage survival during the larval heat wave period of *Lestes viridis* larvae.

Effects of the egg heat wave, previous pesticide exposure and the larval heat wave. Sample sizes per treatment group range from 21 to 27 larvae.

Figure 4: Mean MDA levels (with 1 SE) at the end of the larval heat wave period of *Lestes viridis* larvae. Effects of the egg heat wave, previous pesticide exposure and the larval heat wave. Sample sizes per treatment group range from 21 to 27 larvae.

Figure 1.

Figure 2.

580

581 Figure 3.

Figure 4.