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Exposure to a widespread non-pathogenic bacterium magnifies sublethal pesticide effects in the damselfly *Enallagma cyathigerum*: from the suborganismal level to fitness-related traits

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ABSTRACT

While there is increasing concern that pesticide stress can interact with stress imposed by antagonistic species including pathogens, it is unknown whether this also holds for non-pathogenic bacteria. We exposed *Enallagma cyathigerum* damselfly larvae to the pesticide chlorpyrifos and a non-pathogenic *Escherichia coli* strain. Both exposure to chlorpyrifos and *E. coli* reduced growth rate and fat storage, probably due to the observed energetically costly increases in physiological defence (glutathione-S-transferase and Hsp70) and, for *E. coli*, immune defence (phenoloxidase). Moreover, these stressors interacted for both fitness-related traits. Most importantly, another fitness-related trait, bacterial load, increased drastically with chlorpyrifos concentration. A possible explanation is that the upregulation of phenoloxidase in the presence of *E. coli* changed into a downregulation when combined with chlorpyrifos. We argue that the observed interactive, partly synergistic effects between pesticides and widespread non-pathogenic bacteria may be common and deserves further attention to improve ecological risk assessment of pesticides.

Key words: fitness; non-pathogenic bacteria; pesticide; physiological defence; synergistic interactions

Non-pathogenic bacteria reduce fitness-related traits and can synergistically interact with sublethal pesticide effects for physiological and fitness-related traits.

INTRODUCTION

Identifying and understanding the cumulative effects of multiple and interacting stressors is becoming a key issue to understand threats to biodiversity (Darling & Côté 2008). One specific concern is that the intensification of agriculture (Matson et al. 1997) and the associated declines in biodiversity in nearby ponds (Williams et al. 2004) is modulated by interactions between the increased use of pesticides (Schwarzenbach et al. 2006) and stress imposed by antagonistic species (Sih et al. 2004). Indeed, an increasing number of studies reported interactive effects between pesticides and stress imposed by competitors (e.g. Foit et al. 2012), predators (e.g. Schulz & Dabrowski 2001), and parasites such as pathogenic bacteria (e.g. Coors & De Meester 2008). Despite their importance for risk assessment, we still do not know whether interactions with pesticide exposure also occur with other types of organisms. Furthermore, to be able to generalize and make predictions about the occurrence of interactions between pesticides and stress imposed by other species, we need more insight in their mechanistic underpinnings (Campero et al. 2007).

Because of the general belief that widespread non-antagonistic organisms, such as non-pathogenic bacteria, do not impair fitness-related traits, their potential to modulate pesticide effects has been ignored. Yet, recently it has been documented that the non-pathogenic bacteria *Escherichia coli*, can negatively affect fitness-related traits in butterflies (Freitak et al. 2007, 2009). This generates the question whether non-pathogenic bacteria may also interact with pesticides. This could have major consequences for the impact of pesticides in natural systems where non-pathogenic bacteria can be very abundant (Leff & Lemke 1998). The impairment of life history traits under exposure to non-pathogenic bacteria has been attributed to energetic costs associated with the upregulation of the immune defence (Freitak et al. 2007, 2009). Therefore, information of the energy status and the immune defence may

be critical to start exploring the mechanistic base of potential interactions between non-pathogenic bacteria and pesticides.

We here test whether the non-pathogenic bacterium *E. coli* can negatively affect fitness-related traits, potentially in an interactive way with a pesticide, in a pond-inhabiting insect. As study organisms we chose damselfly larvae, important intermediate predators in ponds (Corbet 1999). As a pesticide we will look at effects of chlorpyrifos, an organophosphate insecticide that functions as an inhibitor of acetylcholinesterase (Stenersen 2004). Chlorpyrifos is used in agriculture and pest management all over the world. As a consequence it is often found in ponds through runoff or direct application. Several studies demonstrated negative effects of chlorpyrifos on non-target pond organisms (e.g. Widder & Bidwell 2006, Huynh & Nugegoda 2012) including aquatic insects (Rubach et al. 2012).

To get a multifaceted mechanistic view of the combined impact of this pesticide and the non-pathogenic bacterium we studied three types of response variables. As a first type we scored three fitness-related variables: growth rate, energy storage and bacterial load. Growth rate is an often used proxy for fitness (Arendt 1997). As component of energy storage we studied fat content, important for starvation resistance and linked to mating success and survival in damselflies (Stoks & Cordoba-Aguilar 2012). Given that organisms try to clear challenges with non-pathogenic *E. coli* (Willer et al. 2012), the number of internally accumulated *E. coli* ('bacterial load') after uptake from the water reflects their ability to fight the *E. coli* challenge. Furthermore, given that injection of lipopolysaccharides, components of the outer membrane of gram-negative bacteria such as *E. coli*, are known to reduce fitness in insects (e.g. Ahmed et al. 2002, Kelly 2011), a higher bacterial load reflects lower fitness. As a second type, we focused on two variables that likely mediate the effects of the pesticide: food intake and the inhibition of acetylcholinesterase (AChE). The latter has an important function in nerve transmission and is a target of many pesticides, including chlorpyrifos

(Stenersen 2004). Moreover, studies indicated a role for AChE in explaining synergisms with natural enemies (Campero et al. 2007). As a third type, we quantified three key physiological defence mechanisms: the expression of stress proteins, the activity of glutathione S-transferase (GST) and immune function. Specifically, we studied the stress protein Hsp70, that is upregulated in insects by chlorpyrifos (Nazir et al. 2001) and by bacteria (Sörensen et al. 2003). GST is an important detoxification enzyme that is upregulated in the presence of chlorpyrifos (e.g. Cinzia et al. 2006). GST is also an important defence mechanism against bacteria (e.g. Dubovskiy et al. 2008). As key component of the immune system we studied the enzyme phenoloxidase (PO) (Gonzalez-Santoyo & Cordoba-Aguilar 2012) which is involved in clearance of many different organisms, including bacteria (Pauwels et al. 2010).

MATERIALS AND METHODS

Collecting and housing

Twenty mated females of the damselfly *Enallagma cyathigerum* were collected in “De Ruiterskuilen”, a nature reserve without a known history of pesticide application in Opglabbeek (Belgium). Females were transferred to the laboratory for egg laying. Ten days after hatching, larvae were placed individually in 200 ml cups. Throughout their life, larvae were reared in a room with a constant temperature of 20 °C and a photoperiod of L:D 14:10 hours. Damselfly larvae were fed ad libitum with *Artemia* nauplii five days a week (average daily dose = 1347, SE = 102, n = 15).

Pesticide concentration

To select the pesticide concentrations, we first ran a range finding experiment where we exposed larvae individually in glass vials (100 ml) for seven days to several chlorpyrifos

concentrations. The pesticide was dissolved in synthetic water (pH 6.4) which guarantees constant rearing conditions. In order to make a stock solution of the synthetic water we dissolved 2.97 ml $\text{Na}_2\text{Si}_3\text{O}_7$, 250 mg $\text{Ca}(\text{NO}_3)_2$, 404 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 72 mg KCl in 1 liter milli-Q water. For the working solution we diluted the stock 200 times and added 25 ml soda-water. Pre-trials showed growth rates across seven days in synthetic water to be equal to those observed in natural pond water (Lizanne Janssens, unpublished data).

We tested 0.1 $\mu\text{g/l}$, 0.25 $\mu\text{g/l}$, 0.5 $\mu\text{g/l}$, 1 $\mu\text{g/l}$, 2.5 $\mu\text{g/l}$ and 5 $\mu\text{g/l}$ chlorpyrifos and used the synthetic water as control. We calculated growth rate over the exposure period of seven days for ten final instar larvae per pesticide concentration and selected the lowest (LOEC) and second lowest concentration with a negative effect on growth rate, but no effect on mortality, being 0.25 $\mu\text{g/l}$ and 0.5 $\mu\text{g/l}$ chlorpyrifos, respectively. Ten samples per concentration were analysed by the independent research laboratory Lovap NV using gas chromatography in combination with mass spectrometry. Initial concentrations were 0.263 $\mu\text{g/l}$ and 0.489 $\mu\text{g/l}$ chlorpyrifos, and after 24 h chlorpyrifos concentrations were 0.227 $\mu\text{g/l}$ and 0.455 $\mu\text{g/l}$, respectively. These concentrations fall within the range of chlorpyrifos concentrations reported in nature (Schulz 2004, Tierney et al. 2007).

We daily prepared the chlorpyrifos solution starting from a stock solution with a concentration of 10 $\mu\text{g/ml}$ chlorpyrifos (kept in the dark at 4°C). This stock solution was a 100 times dilution of another stock solution containing 1 mg/ml chlorpyrifos dissolved in ethanol. The chlorpyrifos concentration of the stock solution at the start and at the end (3 months later) of the experiment was 1.000 mg/ml and 0.975 mg/ml, respectively. We used synthetic water in the control treatment instead of a solvent control, since the amount of ethanol was only 0.25 or 0.5 $\mu\text{l/l}$ exposure medium. Previous research showed that there was no difference in growth and behaviour of the study species at ethanol concentrations up to 5 $\mu\text{l/l}$ (Lizanne Janssens, unpublished data).

153 *Bacterial exposure*

154 As bacterium we used *Escherichia coli*, which is typically non-pathogenic and widespread in
155 aquatic invertebrates (McEwen & Leff 2001). More specifically we used the non-pathogenic
156 strain ATCC 11775 (Guerrero-Beltran & Borbosa-Canovas 2005). This strain was genetically
157 modified by insertion of a plasmid coding for *dsRed* fluorochrome and kanamycin resistance,
158 allowing it to grow on a culture medium containing kanamycin. This enables us to directly
159 quantify the bacterial load inside the damselfly larvae resulting from the uptake of
160 experimentally provided *E. coli* bacteria (pink colonies) without confounding with the
161 background bacterial load.

162 In pilot experiments we determined a suitable way to administer the bacterium and the
163 associated sublethal concentration that reduced larval growth rate. We decided not to inject
164 larvae with a bacterial solution because this is particularly stressful. Instead, we chose one of
165 the main routes of natural exposure to bacteria in aquatic organisms: we administered the
166 bacterium by adding it to the synthetic water (cf. Freitag et al. 2007). We tested, besides a
167 control without *E. coli*, two sublethal concentrations of *E. coli*, namely 2×10^7 and 1×10^8 CFU
168 (colony forming units), by exposing ten final instar larvae to each treatment for seven days.
169 These concentrations are chosen based on field data showing that bacterial concentrations can
170 vary between $<1 \times 10^4$ and $>1 \times 10^9$ CFU/ml in natural ponds depending on the water
171 characteristics and nutrients present (Kirschner & Velimirov 1997, Palijan 2012). We selected
172 the lowest concentration that caused a negative effect on growth, namely 2×10^7 CFU.

173 Throughout the experiment, we daily prepared an *E. coli* solution with a concentration
174 of 1×10^9 CFU in mili-Q water using a spectrophotometer. We added 1 ml of this solution to
175 the vials of the bacterial condition resulting in a concentration of 2×10^7 CFU. To the control
176 condition we added 1 ml of mili-Q water. Because the pesticide treatment may affect

damselfly feeding rates (Janssens & Stoks 2012) and bacterial uptake may be associated with feeding we only exposed the larvae to the bacterium solution during periods without food. Therefore, the rearing medium was renewed twice a day. After the first renewal, the larvae were given two hours to feed in the absence of the bacterium. After 2h, the medium was renewed again and now the bacterium was added in the absence of food until the next renewal, 22h later. Note that also during the feeding period, the larvae allocated to the treatment with pesticide were exposed to chlorpyrifos.

Experimental setup

To test for the effects of exposure to the pesticide and exposure to the bacterium and their potential interactions on growth rate, food intake and biochemical/physiological variables we set up a full factorial experiment with all combinations of three pesticide treatments (control, 0.25 and 0.5 µg/l chlorpyrifos) and two bacterial treatments (*E.coli* absent and present). We set up a static renewal experiment with medium renewal during seven days. The number of larvae tested at each treatment combination was 50 (total of 300 larvae).

One day after larvae moulted into the final instar they were randomly attributed to one of the six treatment combinations for seven days. During the exposure period, larvae were placed individually in glass vials (100 ml) filled with 50 ml of the medium. Throughout the exposure period, larvae were daily fed ad libitum with *Artemia* nauplii. in the absence of the bacterium.

Response variables

We daily checked survival during the 7-day exposure period. To quantify growth rate, we weighed each larva to the nearest 0.01 mg at the start and at the end of this period. Growth rate was calculated as $[\ln(\text{final mass}) - \ln(\text{initial mass})] / 7 \text{ days}$ (Stoks et al. 2005).

On day four of the 7-day exposure period, we quantified food intake by counting the number of *Artemia* nauplii that each larva consumed during two hours. Per day that we measured foraging activity we stored the number of *Artemia nauplii* of two initial food ratios in 70% ethanol to afterwards estimate the average amount of nauplii given to each larva that day. At the end of each 2h-foraging period, we collected the remaining *Artemia* nauplii per vial and stored them in 70% ethanol. Afterwards, fixated nauplii were counted at magnification 10× using a stereomicroscope. The number of nauplii eaten by a larva on day four was calculated as the difference between the mean initial number given at that feeding day and the number of remaining nauplii in the vial of that larva.

At the end of the 7-day exposure period, ten larvae per treatment were used to quantify internal load of the administered *E. coli* strain ('bacterial load'). These larvae were washed in ethanol, homogenized and the supernatant was used for inoculation on a culture medium (LB-agar) containing kanamycin. Only the administered kanamycin-resistant *E.coli* strain is able to grow on this culture medium. Each larval homogenate was inoculated in duplicate on separate agar plates at each of three serially diluted assay concentrations: (1) 100 µl of a ten times diluted supernatant, (2) 50 µl of a ten times diluted supernatant, (3) 100 µl of a hundred times diluted supernatant. After inoculation, the agar plates were incubated at 28°C for 24 hours. Then, the number of colonies was counted at the assay concentration that had between 10 and 200 colonies. We quantified bacterial load as the mean number of CFU of both replicates expressed as CFU per larva.

The remaining 40 larvae per treatment combination were stored at -80°C for quantification of biochemical/physiological variables. We used the heads of the larvae to quantify the concentration of Hsp70 and the activity of acetylcholinesterase (AChE). On the body of the animals we measured energy reserves (total fat), immune function (activity of phenoloxidase [PO]) and activity of glutathione S-transferase (GST). Additionally, we

measured protein content in head and body homogenates using the Bradford method (Bradford, 1976). For all physiological variables we used 40 replicates (= larvae) per treatment combination, only for Hsp70 we used 20 replicates (= heads) per treatment combination.

Fat content was measured based on the protocol of Bligh and Dyer (1959). Fat concentrations were calculated using a standard curve of glyceryl tripalmitate and expressed as μg fat per mg wet mass. To measure the activity of AChE we used a modified version of the protocol of Ellman (Jensen et al. 1997). We expressed AChE activity in units with 1 unit representing 1 μmol hydrolyzed acetylcholine per minute per mg protein. We quantified the levels of the stress protein Hsp70 using the western blot assay described in Slos and Stoks (2008) (figure in Appendix). Levels of Hsp70 were expressed as mOD per μg protein. To correct for variation between blots, we ran on every blot a control sample of 1 μl Hela Cell Lysate (Heat shocked, Stressgen). The activity of GST was measured based on the protocol of McLoughlin et al. (2000). We expressed GST activity in units with 1 unit representing 1 μmol GS-DNB formed per minute per mg protein. The activity of total PO was quantified using a modified version of the protocol by Stoks et al. (2006). PO activity was determined as the slope of the linear part of the reaction curve (time lapse 500-1500s) and expressed in units with 1 unit representing 1 mmol dopachrome formed per minute per mg protein.

Statistical analyses

We used separate two-way ANOVAs to test for the effects of pesticide exposure and bacterial exposure on the different response variables. Interactions were further explored using Duncan posthoc tests.

To specifically explore the contributions of the physiological mode-of-action of the pesticide (through the activity of AChE) and of the physiological defence mechanisms against

the pesticide (Hsp70 levels and GST activity) and the bacterium (PO activity) to changes in the fitness-related variables under pesticide and bacterial exposure we tested for their covariation patterns at the individual level using ANCOVAs. Note that this was only possible where we had scored fitness-related variables and physiological variables on the same individual, which was the case for growth rate and fat content but not for bacterial load. In these ANCOVAs we included pesticide exposure and bacterial exposure as categorical independent variables, one of the physiological variables (AChE activity, Hsp70 level, GST activity or PO activity) as continuous covariate and tested for effects on one of the two fitness-related variables (growth rate and fat content).

RESULTS

Fitness-related variables

There was no mortality during the exposure period reflecting the sublethal pesticide and bacterium concentrations. Overall, exposure to the bacterium ($F_{1, 294} = 12.17$, $P < 0.001$) and to chlorpyrifos ($F_{2, 294} = 11.40$, $P < 0.001$) had a negative effect on larval growth rate (Fig. 1A). There was, however, a trend for an interaction between pesticide and bacterial exposure ($F_{2, 294} = 2.93$, $P = 0.055$; Fig. 1A), which indicated that the bacterium-induced growth reduction was present in the control larvae (Duncan post hoc test, $P = 0.011$) and the high-chlorpyrifos larvae ($P = 0.0011$) but not in the low-chlorpyrifos larvae ($P = 0.95$). Similarly, both exposure to the bacterium ($F_{1, 234} = 12.60$, $P < 0.001$) and to chlorpyrifos ($F_{2, 234} = 36.45$, $P < 0.001$) decreased fat levels (Fig. 1B) and this in interactive way ($F_{2, 234} = 5.65$, $P = 0.0040$; Fig. 1B). This interaction indicated that the bacterium-induced fat reduction was present in the control larvae ($P < 0.001$) and the high-chlorpyrifos larvae ($P = 0.013$) but not in the low-chlorpyrifos larvae ($P = 0.61$).

The number of *E. coli* RFP colonies present in the bacterium-exposed larvae at the end of the exposure period was strongly affected by the pesticide ($F_{2, 27} = 114.94$, $P < 0.001$): bacterial load more than tripled when larvae were exposed to the low chlorpyrifos concentration ($P = 0.0052$), and increased more than 10 times when exposed to the high chlorpyrifos concentration ($P < 0.001$) (Fig. 1C). In the control condition no *E. coli* RFP colonies were detected.

Food intake and AChE activity

Both exposure to the bacterium ($F_{1, 294} = 22.40$, $P < 0.001$) and to the pesticide ($F_{2, 294} = 66.08$, $P < 0.001$) caused a higher foraging activity (Fig. 2A). These effects did not interact ($F_{2, 296} = 1.49$, $P = 0.23$). Chlorpyrifos had an inhibiting effect on the activity of the enzyme AChE ($F_{2, 234} = 22.05$, $P < 0.001$), especially at the high chlorpyrifos concentration in the presence of the bacterium (Pesticide $\times$ Bacterium, $F_{2, 234} = 4.22$, $P = 0.016$) (Fig. 2B). From the point of view of the bacterium, *E. coli* had no negative effect on the AChE activity in the control ($P = 0.70$) and at the low pesticide concentration ($P = 0.70$), but a strong inhibiting effect at the high pesticide concentration ($P = 0.0015$).

Physiological defence mechanisms

Pesticide and bacterium interacted for the level of the stress protein Hsp70 ($F_{2, 114} = 18.34$, $P < 0.001$): larvae increased Hsp70 levels when exposed to chlorpyrifos (low pesticide concentration: $P = 0.045$, high pesticide concentration: $P = 0.00051$), except larvae jointly exposed to the high chlorpyrifos concentration and the bacterium which reduced Hsp70 levels ($P = 0.00024$) (Fig. 3A). From the point of view of the bacterium, larvae increased Hsp70 levels when exposed to the bacterium, except larvae that were also exposed to the high chlorpyrifos concentration which reduced Hsp70 levels. Both exposure to the pesticide ($F_{2, 234} = 14.64$, $P < 0.001$) and to the bacterium ($F_{1, 234} = 10.59$, $P = 0.0013$) increased GST levels

and this in an additive way (Pesticide $\times$ Bacterium, $F_{2, 236} = 1.036$, $P = 0.36$) (Fig. 3B). Pesticide and bacterial exposure interacted for the activity of PO ($F_{2, 234} = 8.70$, $P < 0.001$; Fig. 3C): while control larvae increased PO activity in the presence of the bacterium ($P = 0.019$), larvae exposed to chlorpyrifos decreased PO activity (low pesticide concentration: $P = 0.044$, high pesticide concentration: $P = 0.0036$). From the point of view of the pesticide, PO activity decreased with increasing chlorpyrifos concentration but only in the presence of the bacterium.

Covariation patterns between physiology and fitness-related variables

The AChE activity covaried positively with growth rate (ANCOVA $F_{1, 233} = 3.89$, $P = 0.005$; slope ± 1 SE = 0.18 ± 0.05) and fat content ($F_{1, 233} = 28.83$, $P < 0.0001$; slope ± 1 SE = 0.25 ± 0.08). Of the physiological defence mechanisms against chlorpyrifos, the Hsp70 concentration covaried negatively with growth rate ($F_{1, 113} = 9.44$, $P = 0.0027$; slope ± 1 SE = -0.073 ± 0.024), but not with fat content ($F_{1, 113} = 0.95$, $P = 0.33$). The GST activity tended to covary positively with growth rate ($F_{1, 233} = 3.71$, $P = 0.055$; slope ± 1 SE = 0.00039 ± 0.00020), but not with fat content ($F_{1, 233} = 1.55$, $P = 0.21$). The PO activity covaried negatively with growth rate ($F_{1, 233} = 4.83$, $P = 0.029$; slope ± 1 SE = -0.00011 ± 0.000048) and fat content ($F_{1, 233} = 98.59$, $P < 0.001$; slope ± 1 SE = -0.11 ± 0.011).

DISCUSSION

Both exposure to the non-pathogenic bacterium and the pesticide had an impact on all studied life history, behavioural and physiological response variables. A particularly important finding was that both stressors interacted for several of these variables. We first discuss the effects of each single stressor separately and then consider their interactions.

320 *Pesticide effects*

321 In the absence of the bacterium, chlorpyrifos reduced two of the fitness-related variables:
322 growth rate and fat content. Lower growth rates in the presence of chlorpyrifos have been
323 shown before in other taxa (e.g. fish: Huynh & Nugegoda 2012; tadpoles: Widder & Bidwell
324 2006). In contrast with the study of Widder & Bidwell (2006), the observed growth reduction
325 cannot be explained behaviourally since the foraging activity increased with increasing
326 pesticide concentration and therefore asks for a physiological explanation.

327 Most likely, exposure to the pesticide caused a re-allocation of energy away from
328 growth and energy storage. It has been shown that in the presence of other pesticides (atrazin
329 and endosulfan) damselfly larvae indeed showed a lower efficiency to convert assimilated
330 food into biomass (Campero et al. 2007). One reason for this may be that in the presence of
331 chlorpyrifos energy was shunted toward the observed upregulations of Hsp70 and GST. These
332 two physiological responses are well-known defence mechanisms when coping with
333 pesticides, including chlorpyrifos (Hsp70: e.g. Scheil et al. 2010; GST: e.g. Cinzia et al.
334 2006). Increased levels of Hsp70 have been linked to lower growth rates in damselfly larvae
335 (e.g. Slos & Stoks 2008, Stoks & De Block 2011) and other insects (e.g. Feder et al. 1992). In
336 line with this, we here documented also at the individual level a negative covariation between
337 Hsp70 levels and growth rate. Besides energy re-allocation also the mode-of-action of the
338 pesticide, namely the observed inhibition of AChE may have contributed to the growth
339 reduction as suggested in the study of Huynh & Nugegoda (2012). In line with this, our data
340 show a positive covariation at the individual level between growth rate and AChE activity.

341 *Bacterial effects*

342 Exposure to non-pathogenic *E. coli* RFP bacteria impaired the three studied fitness-related
343 traits in the studied damselfly larvae: it caused lower growth rates, reduced fat storage and the

internal accumulation of the bacterium. This extends the finding of fitness-impairing sublethal effects of naturally ingested non-pathogenic bacteria as observed in butterflies (Freitak et al. 2007, 2009). Also the bacterium-induced reductions in growth and energy storage need a physiological explanation as larvae experimentally exposed to *E. coli* increased food intake.

The *E. coli*-induced growth reduction in butterflies has been attributed to a costly upregulation of immune defence, which is considered a priming of the innate immune system against real pathogens (Freitak et al. 2007, 2009). Alternatively, non-pathogenic bacteria may also elicit a strong immune response as animals may not be able to discriminate between pathogenic and non-pathogenic bacteria (Willer et al. 2012). Whatever the reason, we also observed an upregulation of immune defence in the form of an increase in PO in bacteria-exposed larvae (see also Masova et al. 2010). Note that PO may not only have directly contributed to the defence against the foreign bacteria, but also indirectly through producing signalling molecules that target immune tissues in the fat body and midgut (Moreno-García et al 2012). We have shown before in damselfly larvae that high PO levels are costly to maintain (e.g. De Block & Stoks 2008, Slos et al. 2009). In line with this, we here documented a negative covariation between PO activity and growth (and fat storage) at the individual level. In addition, also the observed higher investments in Hsp70 and GST may have shunted energy away from growth and energy storage (see above). The upregulation of Hsp70 (e.g. Reilly et al. 2007) and GST (e.g. Al-Wabel et al. 2007) in response to non-pathogenic bacteria has been reported before and together with the upregulated immune system likely constitute a general mechanism underlying the reduction in growth and energy storage imposed by non-pathogenic bacteria. Additionally, competition between the natural midgut microbiota and the bacteria admitted to the damselfly larvae may have contributed to the observed growth reduction in the presence of the bacterium.

Pesticide x bacterium interactions

Pesticide and bacterium interacted with each other for two fitness-related variables (growth rate and fat content) and several physiological variables (AChE, Hsp70 and PO). Somewhat intriguingly, the bacterium caused a reduction of growth rate and fat storage in the control and high pesticide concentration, but not in the intermediate pesticide concentration. Our data seem to suggest that when dealing with only one stressor the larvae reduced their growth rate and energy storage while trying to maintain an “admissible level”(growth rate: ca. 0.0075 d⁻¹, fat storage: ca. 5 µg fat × mg wet mass⁻¹). Larvae seemed successful in maintaining this admissible level when both stressors were combined at the low pesticide concentration. Yet, at the high concentration growth rate and energy storage further dropped.

We hypothesize that when larvae were exposed to the bacterium at the low pesticide concentration, the reductions in PO levels and especially the considerable increase in food intake compared to the single stressor situations made it possible to keep growth rate and fat storage at the admissible levels and this despite the increased investment in two defence mechanisms (Hsp70 and GST) and the increased bacterial load. Yet, when exposed to the bacterium at the high pesticide concentration food intake did not further increase and the bacterial load considerably increased compared to the bacterial exposure at the low pesticide concentration. The associated considerable decrease in AChE activity further indicates bacterial-exposed animals were suffering heavily in the high pesticide concentration. This is further supported by the decrease in the levels of Hsp70 and PO: larvae did invest much less in these defence mechanisms at the high chlorpyrifos concentration.

A key finding of our study is that, in analogy with the few studies on pathogenic bacteria (e.g. Kerby & Storfer 2009, Alaux et al. 2010), pesticide exposure increased the internal load of the non-pathogenic bacterium by >10 times. Such synergistic effect between a pollutant and a non-antagonistic organism has not been reported before. Yet, we hypothesize it may be general as non-pathogenic bacteria (Freitak et al. 2007, 2009) as well as the non-

pathogenic cell wall compounds of gram-negative bacteria (Ahmed et al. 2002, Kelly 2011) have been shown to reduce fitness components (including increased mortality). Our data went one step further by indicating a potential mechanism for the synergism: the upregulation of PO activity in the presence of only the bacterium changed into a downregulation when the bacterium was combined with the pesticide. Higher PO levels have been associated with a higher resistance against bacteria (Pauwels et al. 2010). Noteworthy, the immunosuppressive effect of the pesticide chlorpyrifos, as described in previous studies (Holmstrup et al. 2010), was only apparent in the presence of the bacterium. Intriguingly, this would indicate that the presence of non-pathogenic bacteria would make damselfly larvae under pesticide exposure also more vulnerable to pathogens.

Conclusions

A growing number of studies documented that pollutant effects can be magnified, or only become apparent by the stress imposed by antagonistic species: predators (e.g. Schulz & Dabrowski 2001), competitors (e.g. Foit et al. 2012) and parasites (e.g. Coors & De Meester 2008). Such interactive effects were not expected for non-pathogenic bacteria as these were typically considered not to reduce fitness-related traits (but see Freitak et al. 2007). Yet, we here demonstrated that also non-pathogenic bacteria may reduce growth rate and fat storage, probably through energy allocation toward defence mechanisms and make animals more vulnerable to pesticides as immunosuppression by the pesticide was only present in bacterial-challenged larvae. Vice versa the ability of the damselfly larvae to withstand internal accumulation of the non-pathogenic bacteria was seriously impaired with increasing pesticide levels. Since non-pathogenic bacteria can be very abundant in natural systems (Leff & Lemke 1998) further testing of their interactions with pesticides at a wide range of bacterial concentrations will be crucial to fully understand and predict the effects of pesticides in natural systems.

419

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576

577 FIGURE LEGENDS

578 Figure 1. Mean (+ 1 SE) of the three fitness-related variables of *E. cyathigerum* damselfly
579 larvae in function of exposure to the pesticide chlorpyrifos and the bacterium *E. coli*: (A)
580 growth rate, (B) total fat content, and (C) number of colony forming units of the bacterium.

581

582 Figure 2. Mean (+ 1 SE) food intake (B) and activity of acetylcholinesterase (B) of *E.*
583 *cyathigerum* damselfly larvae in function of exposure to the pesticide chlorpyrifos and the
584 bacterium *E. coli*.

585

586 Figure 3. Mean (+ 1 SE) levels of three physiological defence mechanisms of *E. cyathigerum*
587 damselfly larvae in function of exposure to the pesticide chlorpyrifos and the bacterium *E.*
588 *coli*: (A) the stress protein Hsp70, (B) activity of glutathione-S-transferase, and (C) activity of
589 phenoloxidase.

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