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15 Synergistic effects between pesticide stress and predator cues: conflicting results from life

16 history and physiology in the damselfly *Enallagma cyathigerum*

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25 Running headline: synergistic stressor effects

26 Abstract

27 There is increasing awareness that the negative effects of anthropogenic stressors may be
28 magnified in the presence of natural stressors. Very few of these studies included physiology,
29 yet this may learn about the mechanistic base of such synergisms at the life history level and
30 identify synergistic interactions not translated in life history traits. We studied in *Enallagma*
31 *cyathigerum* damselfly larvae potential synergistic effects between exposure to the pesticide
32 glyphosate and predator cues on a key life history trait, growth rate, its associated behavioral
33 trait, food intake, and three types of physiological traits known to be affected by both stressors
34 in isolation: the stress protein Hsp70, energy storage and variables related to oxidative stress
35 and damage. The pesticide and predator cues reduced growth rate in an additive way. Food
36 intake increased under pesticide exposure and was not affected by the predator cues,
37 indicating physiological mediation of the growth reduction. One potential physiological
38 mechanism was that both stressors additively increased Hsp70 levels, this may also have
39 contributed to the reduced levels of total carbohydrates when exposed to predator cues.
40 Chronic exposure to predator cues reduced oxygen consumption, possibly to avoid too high
41 costs of an increased metabolic rate. This reduction did not occur in the presence of the
42 pesticide, reflecting the need for energetic expensive defense mechanisms (such as Hsp70
43 upregulation). When both stressors were combined, there was a reduction of the antioxidant
44 enzyme superoxide dismutase (SOD) and an associated increase of oxidative damage in
45 lipids. While synergistic interactions were not present for growth rate and food intake, they
46 were identified for antioxidant defense and oxidative damage. This novel type of “hidden”
47 synergistic interactions may have profound fitness implications, and when ignored will lead to
48 underestimations of the impact of pollutants in natural populations where predators are
49 omnipresent.

Key-words. Antioxidant defense, damselfly larvae, multiple stressors, oxidative damage, pesticide, synergistic interactions

1. INTRODUCTION

One important threat to biodiversity is the presence of synergisms between stressors (Darling & Côté, 2008; Bancroft et al., 2008; Lindenmayer et al. 2010). While most studies focused on interactions between anthropogenic stressors, the effects of these stressors may also be magnified by natural stressors such as the presence of natural enemies (e.g., Relyea and Mills, 2001; Alton et al., 2010). The occurrence of such synergisms is still poorly understood (Sih et al., 2004; Relyea and Hoverman, 2006). Traditionally, synergisms have been studied at the level of life history traits with a strong bias towards effects on mortality (Darling & Côté, 2008, Bancroft et al., 2008). Yet, stressors may also negatively affect physiological traits. Evaluating the presence of synergistic interactions at the level of physiology may be important for two reasons. Firstly, one may learn about the mechanistic base of synergisms at the life history level (Campero et al., 2007; Alton et al., 2012). Secondly, one may identify synergistic effects that are not detected when only focusing on life history traits because several physiological traits (such as energy reserves), although tightly linked to fitness, may be affected independently from traditional life history traits such as mortality and age and mass at maturity (Rolff et al., 2004; Karl et al., 2011). Ignoring effects on physiology may therefore lead to underestimates of the impact of stressors and their synergistic interactions in nature.

Several studies demonstrated synergistic effects between pesticide exposure and predator cues for life history traits such as mortality and growth rate (e.g., Releya and Mills, 2001; Campero et al., 2007; Trekels et al., 2011). Yet, such synergistic effects on life history are not always detected (e.g., Coors and De Meester, 2008; Jansen et al., 2011) and

75 physiological traits have been largely ignored (but see Campero et al., 2007; Trekels et al.,
76 2012). Three types of neglected physiological traits are especially relevant to consider in the
77 context of synergistic interactions between exposure to pesticides and predator cues as these
78 types are related to fitness and are known to be influenced by both stressors separately.
79 A first type of physiological traits is the expression of stress proteins, key cellular
80 defense mechanisms (Korsloot et al., 2004). Specifically, the stress protein Hsp70 can be
81 induced both under pesticide exposure (Lee et al., 2006) and exposure to predator cues
82 (Pauwels et al., 2005; Slos and Stoks, 2008). Moreover, high Hsp70 levels have been
83 associated with reduced growth rates (Stoks and De Block, 2011). Secondly, energy reserves
84 are very relevant for fitness and both pesticide exposure (Frontera et al., 2011) and exposure
85 to predator cues (Stoks et al., 2005c, 2006) can reduce them, and this independently from life
86 history traits (Stoks et al., 2006). Thirdly, a particular type of physiological traits that are
87 increasingly gaining attention are those related to oxidative stress (McGraw et al., 2010).
88 Oxidative stress occurs when reactive oxygen species (ROS) are not fully neutralized by
89 antioxidant defenses, thereby generating oxidative damage (Monaghan et al., 2009). A recent
90 paradigm shift recognizes oxidative stress as a key mediator of trade-offs between life history
91 traits (Dowling and Simmons, 2009; Monaghan et al., 2009; Metcalfe and Alonso-Alvarez,
92 2010). Oxidative damage may have profound fitness consequences as it can, amongst other,
93 reduce reproductive output and accelerate ageing (Monaghan et al., 2009). Although both
94 pesticides (Lushchak, 2011) and predator cues (Slos and Stoks, 2008) have been shown to
95 interfere with antioxidant defense, so far no studies directly looked at potential synergistic
96 interactions on oxidative damage.
97 In this study, we investigated potential synergistic effects between pesticide exposure
98 and predator cues on a key life history trait, growth rate, its associated behavioral trait, food
99 intake, and the neglected three types of physiological traits mentioned above. Specifically, we

100 studied effects on the stress protein Hsp70, energy reserves (fat, total carbohydrates) and
101 physiological traits related to oxidative stress: the activity of the electron transport system
102 (ETS), the activity of the two key antioxidant enzymes in insects (superoxide dismutase SOD
103 and catalase CAT, Korsloot et al., 2004) and oxidative damage to lipids (lipid peroxidation), a
104 key marker of oxidative stress (Monaghan et al. 2009). We measured ETS activity because
105 ROS are generated as a by-product of normal metabolic processes and energy is produced in
106 the form of ATP, generated in the mitochondria via the electron transport chain (Balaban et
107 al., 2005). As study animals, we used damselfly larvae: important intermediate predators in
108 aquatic food webs, being predators of small invertebrates (e.g. mosquito larvae) and prey for
109 larger organisms (e.g. fish and dragonfly larvae). Effects of predator cues and pesticide
110 exposure that negatively affect damselfly larvae therefore have the potential to disturb the
111 whole ecosystem (Stoks and Cordoba-Aguilar, 2012). As pesticide we used glyphosate, the
112 active compound in many herbicides (e.g. Roundup). The use of glyphosate is increasing
113 since several broad spectrum herbicides are recently forbidden in Europe (e.g. atrazin)
114 (Donaldson et al., 2002). Glyphosate functions as an enzyme inhibitor in plants by inhibiting
115 the enzyme enolpyruvylshikimate phosphate synthase resulting in an inhibition of the
116 shikimate pathway of biosynthesis of aromatic amino acids, thereby disturbing growth
117 (Stenersen, 2004). There is evidence that glyphosate-based herbicides can also negatively
118 affect animals (Tsui and Chu, 2003; Relyea, 2005). In animals, glyphosate has been shown to
119 cause mortality and to reduce growth rates (e.g., Frontera et al., 2011; Paetow et al., 2012).
120 These effects can be expected given that exposure to glyphosate in animals has been linked to
121 increased oxidative stress (e.g., Glusczak et al., 2011; Lushchak 2011), disturbed energy
122 metabolism (e.g., Peixoto, 2005; Hanana et al., 2012) and upregulation of energetically costly
123 defense mechanisms (Costa et al., 2008).
124

125 2. MATERIALS AND METHODS

126 *2.1 Collecting and housing*

127 Twenty-five females in copula of the damselfly *Enallagma cyathigerum* were collected in
128 "De Ruiterskuilen", a protected nature area without a history of pesticide application in
129 Opglabbeek (Belgium). This is a fishless pond with larvae of the large dragonfly *Anax*
130 *imperator* as the most important predator. Females were transferred to the laboratory for egg
131 laying. Ten days after hatching, larvae were placed individually in 200 ml cups. Throughout
132 their life, larvae were reared in a room with a constant temperature of 20 °C and a
133 photoperiod of L:D 16:8 hours. Damselfly larvae were daily fed ad libitum with *Artemia*
134 nauplii five days a week (average daily dose = 604, SE = 36, N = 10). When larvae moulted
135 into the final instar, they were used for the experimental trials. Starting from that point the
136 larvae were fed seven days a week with *Artemia* nauplii (average daily dose = 1347, SE =
137 102, N = 15). The wet mass of the larvae when entering the final instar varied between 24.32
138 and 43.52 mg.

139 *2.2 Pesticide concentration*

140 To select the pesticide concentration, we first ran a range finding experiment where we
141 exposed larvae individually in glass vials (100 ml) for seven days to several concentrations of
142 glyphosate. The pesticide was dissolved in synthetic water which guarantees constant rearing
143 conditions. In order to make a stock solution of the synthetic water we dissolved 2.97 ml
144 $Na_2Si_3O_7$, 250 mg $Ca(NO_3)_2$, 404 mg $MgSO_47H_2O$ and 72 mg KCl in 1 liter milliQ water. For
145 the working solution we diluted the stock 200 times and added 25 ml soda-water. Pre-trials
146 showed growth rates across 7 days in synthetic water (mean ± 1 SE: 0.017 d^{-1} ± 0.0019, n =
147 10 larvae) to be equal to those observed in natural pond water (0.017 d^{-1} ± 0.0019, n = 10
148 larvae) (t-test, t = -0.043, df = 18, p = 0.97). Also the frequency of three general behaviors of
149 damselfly larvae during a 7-min observation period did not differ between both media:

150 walking activity (synthetic water: 17.4 ± 2.11, pond water: 17.4 ± 2.10, $t = 0.00$, $df = 18$, $p =$
151 1.00), head orientations toward prey (synthetic water: 9.2 ± 1.07, pond water: 9.7 ± 1.49, $t = -$
152 0.43, $df = 18$, $p = 0.67$) and feeding strikes toward prey (synthetic water: 27.8 ± 2.13, pond
153 water: 28.7 ± 2.56, $t = -0.43$, $df = 18$, $p = 0.67$).

154 We tested 0.5 mg/l, 1 mg/l, 2 mg/l, 3 mg/l and 4 mg/l glyphosate (based on Relyea,
155 2005) and used the synthetic water as control. We calculated growth rate over the exposure
156 period of seven days for ten final instars per pesticide concentration and selected the lowest
157 concentration that generated an observable negative effect on growth rate (LOEC). The
158 selected nominal concentration for the experiment was 2 mg/l glyphosate, this is a sublethal
159 concentration which falls within the range observed in natural water bodies in Flanders (Giesy
160 et al., 2000).

161 *2.3 Experimental setup*

162 To test for effects of pesticide exposure and predator cues and their potential interactions on
163 growth rate, food intake and the physiological traits we set up a full factorial design with all
164 four combinations of two pesticide treatments (control and 2 mg/l glyphosate) and two
165 predator cues treatments (predator cues absent and present). To be able to evaluate effects on
166 growth rate exposure we exposed the larvae for seven days to this pesticide concentration
167 with daily refreshment of the medium. We exposed half of the larvae to a combination of
168 visual and chemical predator cues, reflecting the cocktail of predator cues damselfly larvae
169 encounter in nature. *Enallagma* larvae are responsive to both types of predator cues (Stoks et
170 al., 2003, 2005a; Mortensen and Richardson, 2008). The number of larvae tested at each
171 treatment combination was 40 (total of 160 larvae).

172 One day after larvae moulted into the final instar they were randomly allocated to one
173 of the four treatment combinations for seven days. During the exposure period, larvae were
174 placed individually in glass vials (100 ml) filled with 50 ml of the medium. Glass vials were

175 placed in groups of four in larger containers (750 ml). Each container was allocated to one
176 predator cue treatment. To avoid any bias due to a specific predator or container, we randomly
177 re-distributed vials among containers of the same predator cue treatment on a daily basis.
178 Throughout the exposure period, larvae were daily fed ad libitum with *Artemia* nauplii.

179 To ensure visual predation cues, a large *Anax* dragonfly larva, important predators of
180 *Enallagma* larvae (Stoks et al., 2005b), was placed in the containers of the treatment with
181 predator cues. Additionally, larvae could see the conspecific larvae in the other vials in the
182 container (damselfly larvae are cannibalistic; De Block and Stoks, 2004). To avoid visual
183 predator cues in the treatment without predator cues, these vials were made non-transparent
184 using dark tape. For the chemical predator cues, we homogenized one *E. cyathigerum* larva in
185 20 ml of water from an aquarium filled with 300 ml aged tap water (i.e. tap water that was
186 aerated and filtered with a carbon filter for at least 24h) in which a large *Anax* dragonfly larva
187 had eaten a larva of *E. cyathigerum*. We daily added 1 ml of this predator medium to each vial
188 of the treatment combinations with predator cues. To the vials of the treatment combinations
189 without predator cues we daily added 1 ml of water. Previous work on damselfly larvae
190 (including the study species) has shown that the chosen combination of visual and chemical
191 predator cues elicits responses on growth, behavior and physiological parameters (McPeek et
192 al., 2001; Stoks and McPeek, 2003; Stoks et al., 2005a; Slos and Stoks, 2008; Slos et al.,
193 2009).

194 *2.4 Response variables*

195 We daily checked survival during the exposure period. To quantify growth rate across the 7-
196 day exposure period, we weighed each larva to the nearest 0.01 mg at the start and at the end
197 of this period. Growth rate was calculated as [ln(final mass) – ln(initial mass)] / 7 days.

198 On day four of the 7-day exposure period, we quantified foraging activity of 30 per
199 treatment combination (total of 120 larvae) by calculating the number of *Artemia* nauplii each

200 individual larva consumed during two hours. Per day that we measured foraging activity we
201 stored the number of Artemia *nauplii* of two food rations in 40 % ethane diol to afterwards
202 count the amount of nauplii given to each larva. At the end of each 2h-foraging period, we
203 collected the uneaten *Artemia* nauplii per vial and also stored them in ethane diol. Afterwards,
204 fixated nauplii were coloured using lugol and counted at magnification 10× using a
205 stereomicroscope. The number of nauplii eaten by a larva was calculated as the difference
206 between the mean initial number of a food ration at that feeding day and the number of
207 remaining uneaten nauplii in the vial of that larva. Given that most *Artemia* die in fresh water
208 during the first two hours and damselfly larvae only feed on living prey items, food intake
209 during this 2h-period is a good measure of food intake of the larva per daily food ration given.
210 There was no difference in mortality between glyphosate-exposed nauplii (mean number of
211 living nauplii ± 1 SE, start: 1289 ± 7, after 1 h: 853 ± 8, after 2 h: 395 ± 4 [N = 10]) and
212 control nauplii (start: 1308 ± 7, after 1 h: 864 ± 8, after 2 h: 386 ± 4 [N = 10]) (repeated
213 measures ANOVA, pestide effect: $F_{1, 36} = 1.6$, $p = 0.23$). Therefore, we are confident that the
214 survival of the *Artemia* nauplii was not affected by the glyphosate and did not interfere with
215 our measure of food intake.
216 At the end of the 7-day exposure period, larvae were frozen individually in eppendorf
217 tubes and stored at -80°C. Afterwards, we prepared head and body homogenates to quantify
218 the physiological traits. To measure the expression of the stress protein Hsp70, individual
219 heads (18 per treatment combination) were homogenized using a pestle in 250 µl phosphate
220 buffer saline (pH 7.4, 100 mM PBS) and centrifuged for 5 minutes (16,100 g, 4 °C); the
221 resulting head supernatant was used in the assays. To measure energy reserves, antioxidant
222 defense and oxidative damage in the bodies, 30 bodies were pooled per two and homogenized
223 using a pestle and diluted 15 times in PBS (pH 7.4, 100 mM), afterwards they were
224 centrifuged for 5 minutes (16,100 g, 4 °C). This resulted in 15 replicated body supernatants

225 per treatment combination for the assays. To measure ETS the remaining bodies were
226 individually homogenized and diluted 15 times in a Tris buffer (pH 8.5), resulting in 10 ETS
227 replicates per treatment combination. Additionally, we measured protein content in head and
228 body homogenates using the Bradford method (Bradford, 1976). We converted whole animal
229 mass into head mass and into body mass based on the fact that head mass made up 10 % of
230 the total mass (based on a subset of larvae whose total mass and body mass were weighted
231 separately).
232 We used the western blot assay described in Slos and Stoks (2008) for quantification of
233 the levels of the stress protein Hsp70. Shortly, we first diluted 50 µl of the head supernatant
234 with 50 µl of Laemmli sample buffer, from this mixture we used 18 µl for the analysis. The
235 proteins were separated using SDS-polyacrylamide gel electrophoresis (PAGE). Afterwards,
236 we used two antibodies to detect Hsp70, a monoclonal primary antibody (dilution 1:1500,
237 anti-Hsp70 SPC-103D, Gentaur Europe, Kampenhout, Belgium) and a alkaline phosphatase-
238 conjugated secondary antibody (dilution 1:1000, D0486, DakoCytomation, Glostrup,
239 Denmark). We scanned the blotting membrane using the HP Scanjet 8270 and quantified the
240 optical density of the stress protein bands using Image Pro Plus. Levels of Hsp70 were
241 expressed as mean optical density (mOD) per µg protein. To correct for variation between
242 blots, we ran on every blot a control sample of 1 µl Hela Cell Lysate (Heat shocked,
243 Stressgen).
244 Fat content was measured based on the protocol of Bligh and Dyer (1959). We mixed
245 75 µl of the body supernatant and 250 µl sulfuric acid (100%) in a glass tube. The tubes were
246 heated for 15 minutes at 200 °C. Afterwards we added 350 µl of miliQ water. We filled a 96
247 well microtiter plate with 100 µl of the sample and measured absorbance at 340 nm (in
248 duplicate, intra-assay coefficient of variation 2.46 %). Fat concentrations were calculated
249 using a standard curve of glyceryl tripalmitate. For carbohydrates (glucose + glycogen), we

250 used the protocol described in Stoks et al. (2006) based on the glucose kit of Sigma Aldrich
251 USA. In a first step, all glycogen was transformed to glucose. Therefore, we mixed 65 µl
252 miliQ water, 25 µl body supernatant and 10 µl amyloglucosidase (Sigma A7420) in a 96 well
253 microtiter plate. After 30 minutes of incubation at 37 °C glycogen was transformed to
254 glucose. We measured the glucose levels by adding 200 µl of glucose assay reagent (Sigma
255 G3293) to each well (in duplicate, intra-assay coefficient of variation 1.85 %). After another
256 incubation period of 20 minutes at 30 °C we measured absorbance at 340 nm. We calculated
257 sugar concentration based on a standard curve of known concentrations of glucose and their
258 absorbance. Fat content and total sugar concentration were expressed as µg per mg wet mass.
259 Measurement of the electron transport system (ETS) activity was based on the protocol
260 of De Coen and Janssen (2003). In the mitochondria electron transfers between electron
261 donors (e.g. NADH) and electron acceptors (e.g. O_2) occur during the transfer of H^+ ions
262 across the inner membrane. This results in an electrochemical proton gradient which is
263 necessary for the formation of adenosine triphosphate (ATP) out of adenosine diphosphate
264 (ADP). In the protocol iodonitrotetrazolium (INT) replaces O_2 as electron acceptor. The body
265 of the larvae was diluted 15 times in an homogenizing buffer (0.1 M Tris-HCl, pH 8.5, 15%
266 polyvinyl pyrrolidone, 153 µM $MgSO_4$ and 0.2% Triton X-100) and centrifuged at 4 °C
267 during 5 minutes (15,600 g). Afterwards, a 96 well microtiter plate was filled with 150 µl
268 buffered substrate solution (0.13 M Tris HCl, 0.3% Triton X-100, 1.7 mM NADH, 250 µM
269 NADPH, pH 8.5) and 50 µl of the supernatant (in duplicate, intra-assay coefficient of
270 variation 2.31 %). We started the reaction by adding 100 µl INT (8 mM p-
271 iodonitrotetrazolium), what receives electrons from NADPH via the NADH-cytochrome
272 oxidoreductase and causes the formation of formazan. We followed the increase in
273 absorbance at 490 nm and 20 °C during 5 minutes with measurements every 30 seconds.
274 Using the formula of Lambert-Beer we calculated the concentration of formazan (extinction

275 coefficient 15,900 $M^{-1}cm^{-1}$) and afterwards converted this to cellular oxygen consumption
276 based on the theoretical stoichiometric relationship that for each 2 µmol of formazan formed,
277 1 µmol of O_2 was consumed in the ETS system. We expressed ETS activity as nmol O_2/s per
278 mg wet mass.

279 We measured the activity of two key antioxidant enzymes in insects,
280 superoxidedismutase (SOD) and catalase (CAT) (Korsloot et al., 2004) in the body
281 supernatant. For the SOD activity we used the protocol of De Block and Stoks (2008) based
282 on the SOD assay kit WST (Fluka, Buchs, Austria). This measures the formation of a
283 formazan dye upon reduction of the tetrazolium salt WST-1 with superoxide anion. In a 96
284 well microtiter plate we mixed 200 µl of WST working solution, 20 µl of the body
285 supernatant and 20 µl enzyme working solution. After an acclimatization period of 20 minutes
286 at 37 °C, absorbance at 450 nm was measured. The more SOD activity, the less formazan
287 production. One SOD unit is the amount of enzyme needed to cause 50 % inhibition of the
288 rate of the colorimetric reaction per µg protein. To measure CAT activity we used the
289 protocol of De Block and Stoks (2008) based on Aebi (1984). The body supernatant was
290 further diluted 16 times with PBS. We filled a 96 well microtiter plate (suited for the UV-
291 spectrum) with 80 µl of PBS, 20 µl of the diluted supernatant and 100 µl of 20 mM hydrogen
292 peroxide (in duplicate, intra-assay coefficient of variation 1.43 %). CAT activity was
293 measured as the degradation of H_2O_2 with absorbance measurements at 240 nm every 30
294 seconds during 2 minutes. CAT activity was calculated based on the slope of the linear part of
295 the reaction plot. One CAT unit is the amount of enzyme needed to decompose 1 µmol
296 H_2O_2/min per µg protein.

297 We measured oxidative damage to lipids using the thiobarbituric acid reactive substance
298 assay (TBARS assay). In this assay, measurement of MDA is based on its reaction with
299 thiobarbituric acid (TBA) (Ohkawa et al., 1979). We closely followed the protocol of Barata

300 et al. (2005). Lipids were extracted by mixing 150 µl of the body supernatant and 650 µl of
301 chloroform/methanol butylated hydroxytoluene. After centrifugation, the chloroform (bottom
302 layer) and methanol (upper layer) fraction were separated. To quantify the level of TBARS
303 we used 200 µl of the chloroform fraction. We added 32 µl of SDS (81 %), 280 µl of acetic
304 acid (20 %) and 280 µl of TBA-buffer. The solution was mixed and heated for 60 minutes at
305 100 °C. Afterwards, we added 200 µl of a mixture of butanol and pyridine (15:1). After
306 mixture, the solution was centrifuged for 5 minutes (4 °C, 16,100 g). The supernatant (organic
307 layer) was used to fill a black 384 well microtiter plate (in duplicate, intra-assay coefficient of
308 variation 3.51 %) and fluorescence was measured at an excitation/emission wavelength of
309 530/550 nm. We calculated concentration of TBARS based on a standard curve of 1,1,3,3-
310 tetramethoxypropan 99% malonaldehyde bis (dimethyl acetol) 99%. We corrected the level of
311 TBARS for the total fat content by dividing by this variable. TBARS levels were expressed as
312 nmol MDA per µg fat.
313 *2.5 Statistical analyses*
314 We used AN(C)OVAs to test for the effects of pesticide exposure and exposure to predator
315 cues on the different response variables. When a test indicated a significant interaction
316 between the two stressors, we performed contrasts to investigate the effect of pesticide
317 exposure in the absence and presence of predator cues, as well as the effect of predator cues in
318 the absence and presence of pesticide exposure. For all variables we tested the assumptions of
319 ANOVA (normal distribution with Shapiro Wilk tests; homogenenity of variances with
320 Levene tests). For food intake we included the mass of the animals as a covariate. To correct
321 for variation between the different blots of Hsp70 measurements, the optical density of the
322 Hela band was included as a covariate. In an additional analysis, we re-ran the model testing
323 for effects of pesticide exposure and predator cues on TBARS levels with the activity of the
324 antioxidant enzymes SOD and CAT as covariates. This analysis allows to evaluate the

325 covariation of both antioxidant enzymes with the level of oxidative damage in lipids. We
326 tested for interaction between the pesticide or predator cues exposure and the covariates, yet
327 these interactions were never significant. All tests were done in STATISTICA 10. Values of
328 $0.05 < p < 0.10$ were considered as trends, values of $p < 0.05$ were considered significant.
329

330 ## 3. RESULTS

331 Survival was high overall, reflecting the use of a sublethal glyphosate concentration (control
332 97.5 %, control with predator cues 100 %, 2 mg/l glyphosate 97.5 %, 2 mg/l glyphosate with
333 predator cues 95.2 %). Growth rates tended to be lower in larvae exposed to the pesticide ($p =$
334 0.065), and were significantly lower in larvae exposed to predator cues (Table 1, Figure 1A).
335 There was no interaction between the two stressors for growth rate. Food intake was higher in
336 larvae exposed to the pesticide (Table 1, Figure 1B). Predator cues nor the interaction
337 between the two stressors affected food intake. Heavier animals had a higher food intake.

338 Levels of the stress protein Hsp70 were higher in the heads of the larvae exposed to the
339 pesticide, and there was a trend ($p = 0.083$) for higher Hsp70 levels in larvae exposed to
340 predator cues (Table 1, Figure 2A). The two stressors did not interact for Hsp70. Energy
341 reserves, total fat and total carbohydrates, were not affected by the pesticide. Only total sugar
342 levels were lower in the presence of predator cues (Table 1, Figures 2B-C). The two stressors
343 did not interact for energy reserves.

344 Electron transport system (ETS) activity in larvae exposed to the pesticide was only
345 higher when they were also exposed to predator cues as indicated by the significant
346 interaction between pesticide exposure and predator cues (Table 1, Figure 3A; contrast
347 analyses for pesticide exposure, without predator cues: $p = 0.32$, with predator cues: $p =$
348 0.043). This was mainly due to the fact that larvae exposed to predator cues had a lower ETS

349 activity in the absence of the pesticide (contrast analyses for predator cues, without pesticide:
350 $p = 0.021$, with pesticide: $p = 0.51$).
351 Of the two antioxidant enzymes measured, only SOD activity was affected by the
352 treatments (Table 1, Figures 3B-C). There was a significant interaction between pesticide
353 exposure and predator cues: the pesticide inhibited SOD activity, but only in the presence of
354 predator cues (contrast analyses for pesticide exposure, without predator cues: $p = 0.72$, with
355 predator cues: $p = 0.0089$). Seen from the point of view of predator cues: predator cues
356 resulted in reduced SOD activity, but only when the pesticide was present (contrast analyses
357 for predator cues, without pesticide: $p = 0.63$, with pesticide: $p = 0.012$).
358 There was a trend ($p = 0.055$) for an interaction between pesticide exposure and
359 predator cues for TBARS levels (Table 1, Figure 3D). This trend was confirmed in a
360 statistical model where also the activity levels of both antioxidant enzymes, SOD and CAT,
361 were included (ANCOVA, pesticide x predator cues: $F_{1, 46} = 6.94$, $p = 0.011$). TBARS levels
362 nearly doubled in larvae exposed to the pesticide, but only in the presence of predator cues
363 (contrast analyses for pesticide exposure, without predator cues: $p = 0.16$, with predator cues:
364 $P = 0.025$). From the point of view of predator cues, this interaction indicated that TBARS
365 levels increased under predator cues but only in the presence of the pesticide (contrast
366 analyses for predator cues, without pesticide: $p = 0.52$, with pesticide: $p = 0.0061$). The SOD
367 activity covaried positively with the TBARS level (SOD activity: $F_{1, 50} = 4.70$, $p = 0.035$;
368 slope ± 1 SE = 0.29 ± 0.13) while this was not the case for CAT activity ($F_{1, 51} = 1.56$, p =
369 0.22; slope ± 1 SE = 0.17 ± 0.14). In the larvae jointly exposed to the pesticide and predator
370 cues SOD activity levels were lowest, while TBAR levels were highest.
371
372 # 4. DISCUSSION
373 ## *4.1 Growth rate, energy storage and Hsp70*

374 Several studies reported a decreased growth rate when animals were exposed to glyphosate
375 (e.g., Frontera et al., 2011; Paetow et al. 2012) and predator cues (reviewed in Benard et al.,
376 2004). Since the larvae did not reduce their foraging activity in the presence of predator cues
377 and even increased food intake in the presence of the pesticide, the observed decreased
378 growth rates cannot be explained behaviorally and ask for a physiological explanation. In line
379 with this, a reduced efficiency to convert food into biomass has been documented in animals
380 in the presence of predator cues (e.g. McPeek et al., 2001; Stoks et al., 2005c; Trussell et al.,
381 2006) and in the presence of pesticides (e.g., Ribeiro et al., 2001; Campero et al., 2007). Such
382 reduced allocation of energy to growth in the presence of glyphosate may be explained by
383 several non-exclusive mechanisms including increased oxidative stress (e.g., Glusczak et al.,
384 2011; Lushchak, 2011), disturbed energy metabolism (e.g., Peixoto, 2005; Hanana et al.,
385 2012) and upregulation of energetically costly defense mechanisms (Costa et al., 2008). Our
386 data tentatively suggest that the upregulation of Hsp70 levels is one such defense mechanism
387 that may have contributed to this growth reduction. Previous studies also have shown that
388 exposure to predator cues (e.g., Pauwels et al., 2005; Slos and Stoks, 2008) may increase
389 Hsp70 levels. The above-mentioned mechanisms may as well have caused the observed
390 reduction in energy storage in terms of total carbohydrates in the presence of predator cues as
391 documented before (e.g., Stoks et al. 2005c; Thaler et al., 2012).
392 In contrast with previous studies (e.g., Relyea and Mills, 2001; Campero et al., 2007;
393 Trekels et al., 2011; but see e.g., Pestana et al., 2009) we did not find an interaction between
394 pesticide exposure and exposure to predator cues for growth rate. This could be due to the fact
395 that the larvae tried to cope with the higher energy demands when exposed to the pesticide by
396 increasing their foraging activity. In contrast, a decreased food uptake and activity when
397 pesticide and predator cues were combined, was observed by studies that did report the
398 synergism (Relyea and Mills, 2001; Campero et al., 2007). If general, this may indicate that

399 the presence of synergistic interactions on growth rate may depend on the energy level of the
400 animals and generates the testable prediction that such interactions are more likely to occur at
401 low food levels (see also Campero et al., 2007).
402 *4.2 Oxidative stress and damage*
403 A key novel finding of our study is that oxidative damage in terms of lipid peroxidation
404 (measured as TBARS levels) increased when both stressors were combined, indicating a
405 synergism. Oxidative damage occurs when ROS production is not balanced by a sufficient
406 increase in antioxidant defense (Monaghan et al., 2009). Such imbalance may be due to an
407 increased ROS production and/or a decreased antioxidant defense, and both mechanisms may
408 have played a role in our study. First, although we have no data on this, ROS levels may have
409 been increased under glyphosate exposure as it disrupts normal physiological processes in
410 cells and induces excessive leaking of ROS from the electron transport pathway (Wang et al.,
411 2012). Second, our data support a reduced antixodant defense, as measured by a reduced SOD
412 activity, one of the two most important antioxidant enzymes in insects (Korsloot et al., 2004),
413 when both stressors were combined. Reductions in antioxidant defense (including SOD) have
414 been reported under isolated exposure to predator cues (Slos and Stoks, 2008; Travers et al.,
415 2010) and glyphosate (Lushchak, 2011). Antioxidant enzymes are costly to maintain at
416 constant levels and are typically reduced at low food level (De Block and Stoks, 2008; Slos et
417 al., 2009). We therefore hypothesize that the energetic content of the larvae was too low to
418 maintain constant SOD levels when they were facing both stressors due to a disturbed energy
419 metabolism and an increased investment in costly defense mechanisms (see above).
420 Our data indicate that the observed synergism for oxidative damage cannot be explained
421 by increased ROS production associated with increased ETS activity at the mitochondrial
422 level (Balaban et al., 2005). Indeed, ETS activity was not higher when both stressors were
423 combined. Noteworthy, ETS activity was reduced in the presence of predator cues when the

424 pesticide was absent, suggesting under these conditions larvae had lowered cellular
425 respiration rates (see De Coen & Janssen 2003). Previous studies showed an increased ETS
426 activity during exposure to pesticides (De Coen and Janssen, 2003), yet no previous studies
427 looked at the combined effect of exposure to pesticides and predator cues on ETS activity.

428 *4.3 Conclusions*

429 Starting with the study by Relyea and Mills (2001), there is an increasing interest for
430 synergistic interactions between pesticides and predator cues. Predators are abundant in nature
431 and effects imposed by predator cues are widespread (e.g. Luttbeg and Kerby, 2005; Preisser
432 et al., 2005), indicating that animals often will face both types of stressors (Sih et al., 2004).
433 Ignoring their synergistic interactions will lead to underestimations of the impact of pesticides
434 in natural systems and therefore are crucial to consider for ecological risk assessment (Relyea
435 and Hoverman, 2006). Studies looking for synergisms mainly considered life history (survival
436 and growth), yet several of these studies did not detect synergisms (e.g., Coors and De
437 Meester, 2008; Pestana et al., 2009; Jansen et al., 2011). By also focusing on oxidative stress
438 and damage we could unravel the presence of “hidden” synergisms on these endpoints which
439 were not observably associated with synergisms at the level of life history (growth rate) and
440 behavior (food intake). Given that the proposed energetic constraints underlying reduced
441 antioxidant defense under combined exposure to predator cues and pesticides are likely
442 general, we hypothesize that this novel type of synergistic interaction at the level of oxidative
443 damage may be widespread. Increased levels of oxidative damage are thought to reduce
444 fitness through several mechanisms, including reduced reproductive output and acceleration
445 of ageing (Monaghan et al., 2009). Given their likely wide occurrence and their fitness impact
446 synergisms associated with oxidative damage are an important novel type of synergisms that
447 ask our attention to understand the impact of pollutants in natural populations where predators
448 are omnipresent.

449

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454

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622 FIGURE LEGENDS

623 Figure 1. Mean growth rate (A) and food intake (B) of *E. cyathigerum* larvae in function of
624 exposure to the pesticide glyphosate and predator cues. Given are least-squares means + 1 SE.
625 The replicate number per treatment combination was 40 (A) or 30 (B).

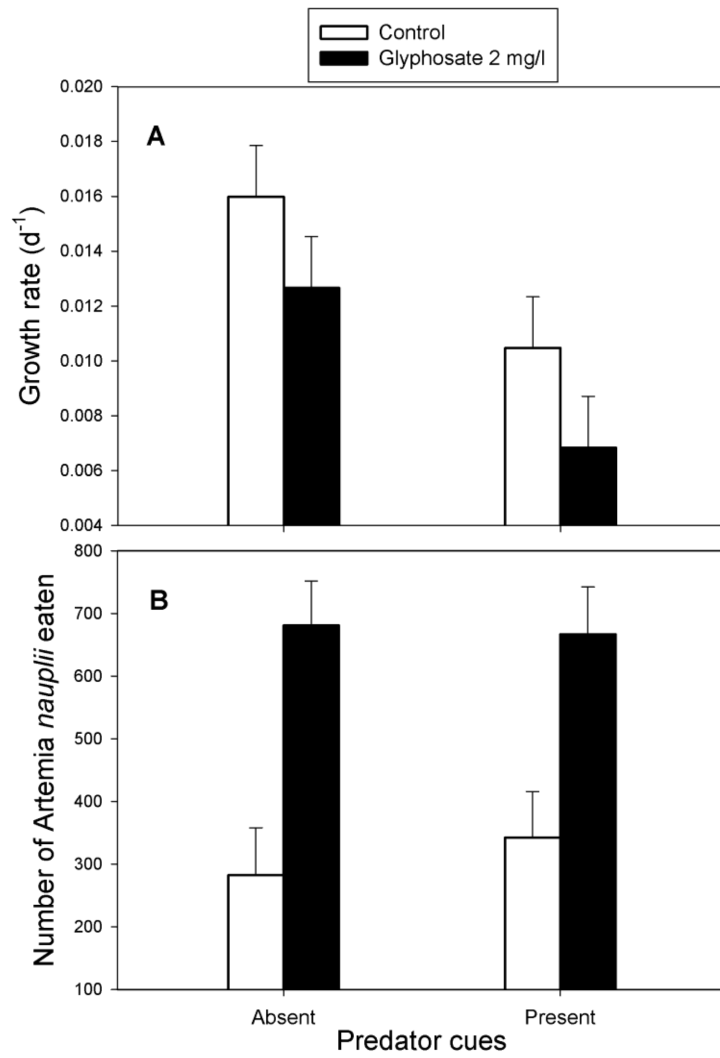
626

627 Figure 2. Mean levels of the stress protein Hsp 70 (A) and energy storage molecules, total fat
628 content (B) and total carbohydrates (C), of *E. cyathigerum* larvae in function of exposure to
629 the pesticide glyphosate and predator cues. Given are least-squares means + 1 SE. The
630 replicate number per treatment combination was 18 (A) or 15 (B, C).

631

632 Figure 3. Mean levels of oxygen consumption (activity of the Electron Transport System,
633 ETS) (A), activity of two antioxidant enzymes, SOD (B) and CAT (C), and oxidative damage
634 to lipids (TBARS levels) (D) of *E. cyathigerum* larvae in function of exposure to the pesticide
635 glyphosate and predator cues. Given are least-squares means + 1 SE. The number of replicates
636 per treatment combination was 10 (A) or 15 (B, C).

637



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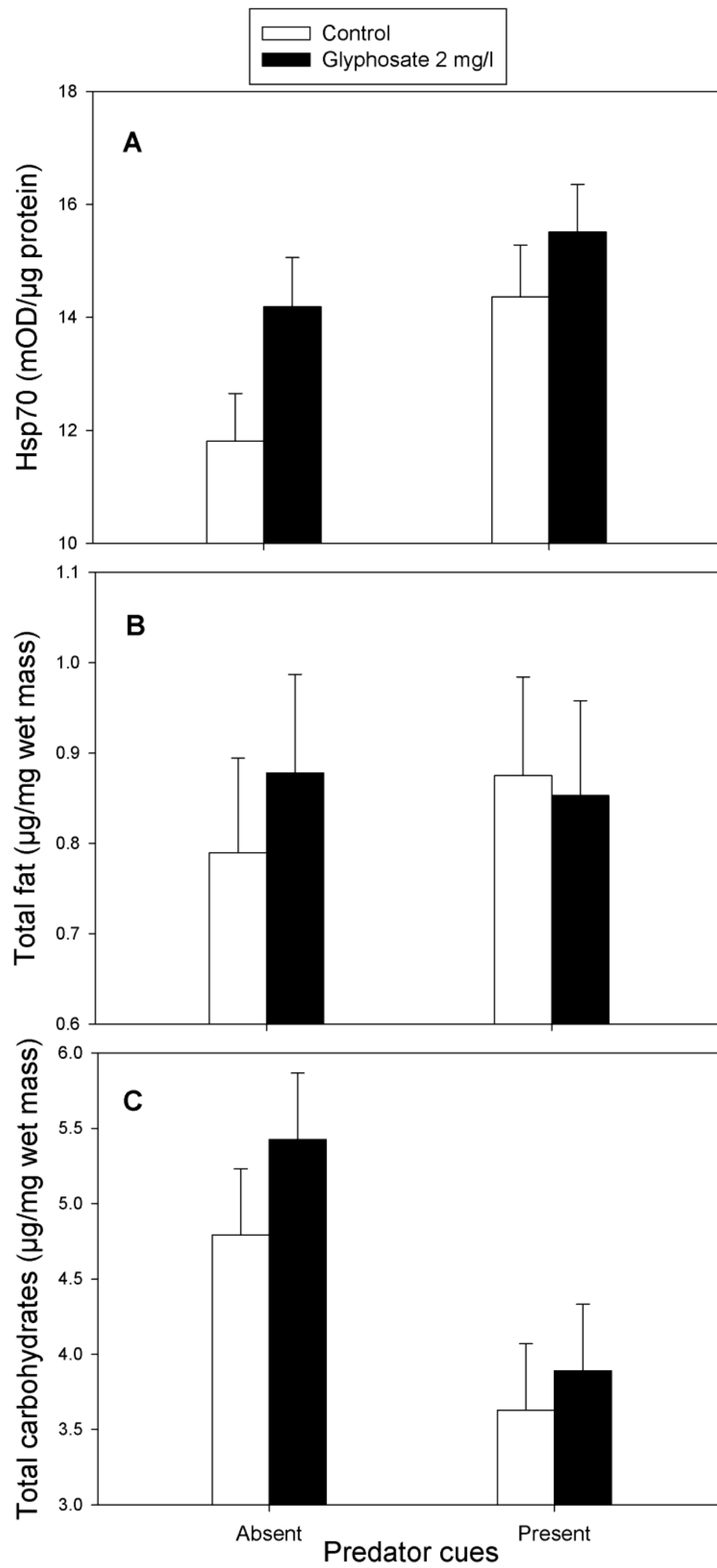

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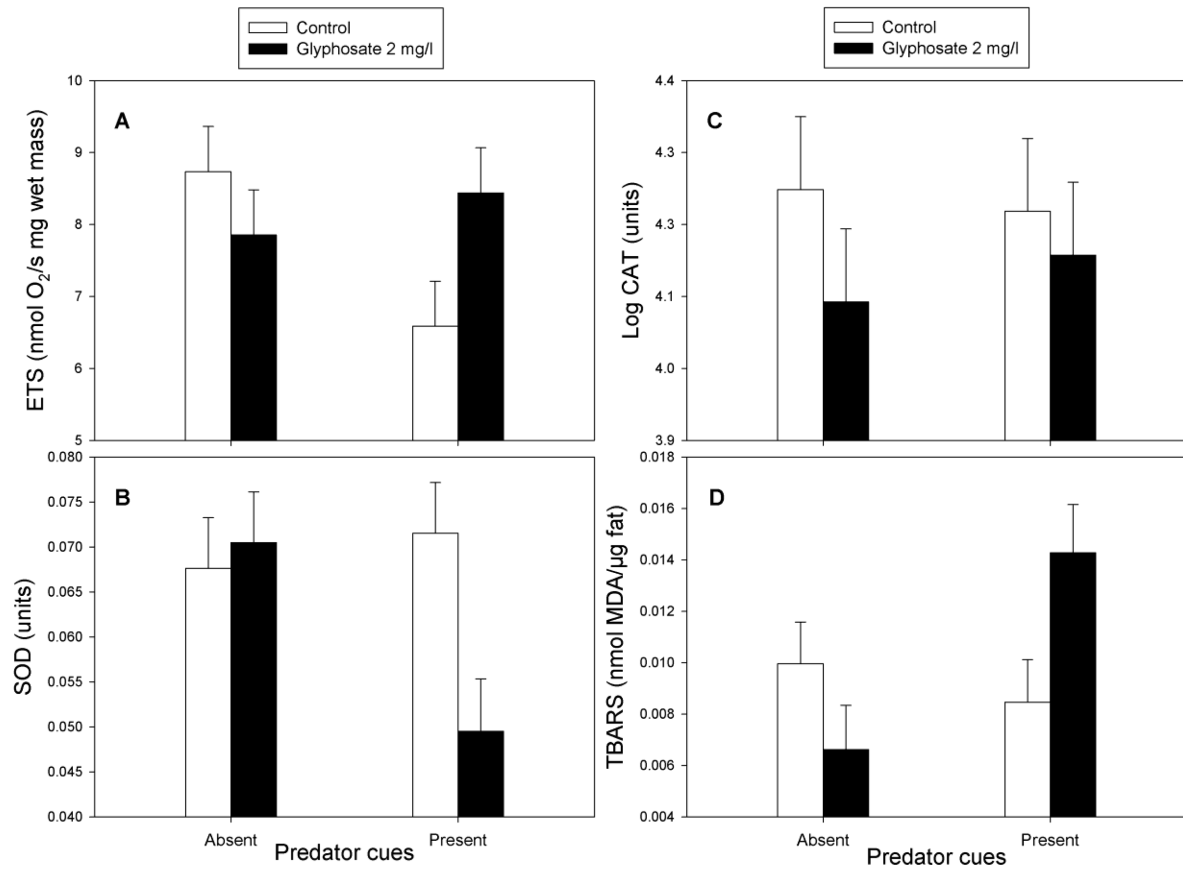

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