



Predator cues magnify effects of the pesticide endosulfan in water bugs in a multi-species test in outdoor containers



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ARTICLE INFO

Article history:

Received 31 December 2012
Received in revised form 18 April 2013
Accepted 20 April 2013

Keywords:

Competition
Insecticide
Outdoor container experiment
Predation risk
Sublethal effects
Survival

ABSTRACT

Pesticides have become major stressors in many aquatic communities. Laboratory studies suggest their impact may be further magnified in the presence of cues from predators. Despite their importance for ecological risk assessment, synergisms between pesticides and predator cues have not been confirmed under semi-natural outdoor conditions. We evaluated how the presence of predator cues and the presence of a non-corixid community affect the pesticide sensitivity of five water bug (Corixidae) species in an outdoor, multi-species container experiment. The experiment employed a full factorial design with two pesticide treatments, two predator cue treatments and two (non-corixid) community treatments (absence versus presence of *Cloeon dipterum* mayfly larvae, *Ischnura elegans* damselfly larvae and *Physa acuta* snails). The pesticide treatment negatively affected survival in *Cymatia coleoptrata*, and to a lesser extent, *Sigara lateralis*, but not in the other three Corixidae species (*Hesperocorixa linnaei*, *Sigara iactans* and *Sigara striata*). The addition of pesticides did not significantly affect body mass in the latter four species, unless combined with predator cues. To our knowledge this is the first report of this synergism under semi-natural, outdoor conditions. Neither lethal nor sublethal pesticide effects in the Corixidae depended on the community context, yet the presence of the non-corixid community when combined with predator cues reduced survival and body mass. Our results suggest that the here documented synergism between pesticides and predator cues may occur in nature.

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1. Introduction

Freshwater habitats worldwide are facing a biodiversity crisis due to increasing human impact (Dudgeon et al., 2006; Voerlesmarty et al., 2010). Moreover, aquatic organisms also face natural stressors such as suboptimal abiotic conditions and natural enemies that potentially magnify the effects of anthropogenic stressors (Holmstrup et al., 2010; Laskowski et al., 2010). There is increasing concern that the prevalence and magnitude of interactions among stressors may result in accelerated biodiversity loss (Sala et al., 2000), yet such synergisms remain one of the largest uncertainties when predicting future ecological change (Darling and Côté, 2008; Lindenmayer et al., 2010). Therefore, the study of interactions between anthropogenic and natural stressors is crucial

to our understanding and ability to project how natural aquatic communities will change with increasing human impact.

Anthropogenic stressors that have received much attention in this context are pesticides. These pollutants are increasingly used worldwide (Tilman et al., 2001) and present a major threat to many natural aquatic ecosystems (Butchart et al., 2010; Relyea and Hoverman, 2006; Schwarzenbach et al., 2006). A natural stressor that is especially important in aquatic ecosystems is the stress imposed by predator cues (Preisser et al., 2005). The non-consumptive effects imposed by predators can be as important or even more important for prey population dynamics than the consumptive effects (Preisser et al., 2005). There is a growing literature indicating that the effects of pesticide exposure and predator cues may not always add up, and that predator cues may magnify the impact of pesticide stress (e.g. Campero et al., 2007; Relyea, 2004, 2003; Relyea and Mills, 2001). Such synergistic interactions between pesticides and predator cues are, however, not always detected (see e.g., Pestana et al., 2009; Qin et al., 2011), and both stressors can sometimes also interact antagonistically (Relyea, 2012). Currently, it is poorly understood under which conditions the presence of synergisms is most likely. Moreover, nearly all studies tested for the presence of these synergisms using laboratory experiments whereas we need to know whether they also

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occur under more natural conditions (Relyea, 2012; Relyea and Hoverman, 2006). Of the three studies that tested for the presence of a synergism between pesticides and predator cues under semi-natural outdoor conditions, none could demonstrate its presence (Relyea, 2012, 2006; Rohr and Crumrine, 2005).

The increasing number of studies showing interactions between pesticides and natural stressors identified the need for a community-level approach when evaluating the impact of pesticides (Relyea et al., 2005; Relyea and Hoverman, 2006; Rohr et al., 2006). Beside predation, other biotic interactions within the community also may play a role, yet have been much less considered in ecotoxicology (Clements and Newman, 2002, but see e.g. Buck et al., 2012; Jones et al., 2011; Rohr and Crumrine, 2005). For example, competitive interactions and food shortage may directly affect the response to a pesticide (Boone and Semlitsch, 2002; Rohr and Crumrine, 2005), but also may modulate the expression of interactions between pesticides and predator cues. Yet, only very few studies jointly manipulated pesticide stress, predator cues and interactions with a community (but see Rohr and Crumrine, 2005). Documenting interactions with pesticide stress and to what extent they themselves depend on other stressors is an important step to arrive at a predictive community ecotoxicology (Clements and Rohr, 2009; Rohr et al., 2006).

In the current study, we investigated the combined effects of pesticide stress, predator cues and the presence of a non-corixid community on five Corixidae (Hemiptera) species. By applying a full factorial design in an outdoor container experiment, we obtained a gradient from a more artificial (only Corixidae) to a more natural setting (including predator cues and a non-corixid community). As predator cues, pesticides and competition may be important for prey population dynamics (Preisner et al., 2005), we quantified effects on survival and per capita dry mass, key traits linked to fitness. Our main objective was to test the independent and interacting effects of pesticide effects, predator cues and a non-corixid community in a full factorial design.

2. Materials and methods

2.1. Corixidae species

Corixidae are common inhabitants of all types of lentic waters, and feed on small invertebrates, detritus and algae (Savage, 1989). The species of Corixidae used in this study were *Cymatia coleoptrata* (Fabricius, 1777), *Hesperocorixa linnaei* (Fieber, 1848), *Sigara iactans* (Jansson, 1983), *Sigara lateralis* (Leach, 1817) and *Sigara striata* (Linnaeus, 1758), all of which commonly co-occur in ponds in Belgium. Corixidae were caught in August 2008 from ponds located in Flanders that have no known pesticide application history, and that were surrounded by natural land use.

2.2. Experimental setup

We set up an outdoor container experiment to evaluate how the presence of predator cues and the presence of a non-corixid community affect the pesticide sensitivity of the five water bug (Corixidae) species under semi-natural conditions. The experiment employed a full factorial design with two pesticide treatments \times two predator cue treatments \times two (non-corixid) community treatments. Each treatment combination contained all five Corixidae species, and was replicated in three containers (total of 24 containers).

For the pesticide treatment, we chose to use the pesticide endosulfan, which is a neurotoxic chlorinated cyclodiene insecticide that stimulates the central nervous system through the GABA-receptor (Stenersen, 2004). We chose this pesticide because it is a global

insecticide (Weber et al., 2010), shown to interact under laboratory conditions with predator cues in aquatic insects (Campero et al., 2007) including Corixidae (Trekels et al., 2012). Some studies also showed increased mortality by predation in the presence of endosulfan (Broomhall, 2002; Carlson et al., 1998). In Belgium, concentrations of up to 54.5 $\mu\text{g/L}$ have been measured recently (VMM Flemish Environment Agency, 2006, unpublished data), but elsewhere peak concentrations might be as high as 700 $\mu\text{g/L}$ (Ernst et al., 1991). Before endosulfan was banned in Belgium in 2007, concentrations exceeded the water quality standards of 10 ng/L in ca. 30% (2005–2006) of all sample locations of the Flemish government, including several observations of concentrations over 10 $\mu\text{g/L}$ annually (Van Steertegem, 2012). Five years later, and after endosulfan had been banned, concentrations of this persistent organic pollutant and its metabolites still exceeded these limits in 10% of the sample locations (Van Steertegem, 2012). According to the species sensitivity distribution of endosulfan, ca. 50% of all freshwater species would be affected at the chosen concentration of 8 $\mu\text{g/L}$ (Wan et al., 2005). The toxicity values of some often used aquatic test organisms are the following: LC50 48 h = 1.0 $\mu\text{g/L}$ for the salmonid fish *Oncorhynchus kisutch*, LC50 48 h = 840 $\mu\text{g/L}$ for the cladoceran *Daphnia magna* and LC50 96 h = 5.7 $\mu\text{g/L}$ for the freshwater amphipod *Hyalella azteca* (Wan et al., 2005).

We used endosulfan $\alpha:\beta$ 1:2 from Sigma Aldrich® (Pestanal class, purity ≥ 98.9). At the start of the exposure period, endosulfan from a 1000 mg/L stock solution (solvent: acetone) was added once to install the nominal pesticide concentrations of 8 $\mu\text{g/L}$. This mimicked a realistic pulse pesticide application. This nominal concentration (8 $\mu\text{g/L}$) was chosen based on the results of previous experiments where it had caused species-specific lethal and sub-lethal (i.e. growth rate) effects (Trekels et al., 2012, 2011). All containers, including the pesticide-free ones, received the same amount (800 μL) of acetone. Water samples for pesticide analyses were taken by pooling samples across all containers of the pesticide treatment at the start of the experiment and after ten days. Concentrations of endosulfan (α -, β - and -sulphate) were analyzed by gas chromatography with electron capture detection (column: cp-Sil 8CB 50 m 0.39 μm ; 1.38 bar; carrier: He; injection: 250 °C; detection: 275 °C) after liquid extraction in dichloromethane. The initial endosulfan concentration was 6.68 $\mu\text{g/L}$ in the treatment with pesticide presence. In line with the rapid dissipation of endosulfan (Stenersen, 2004), the endosulfan level was reduced to 0.078 $\mu\text{g/L}$ after ten days. This residual fraction is comparable with previous container experiments (Trekels et al., 2011).

The two predator cue treatments were absence and presence of a caged pumpkinseed sunfish, *Lepomis gibbosus* (Linnaeus, 1758). This species has become widespread in Flanders after the first introduction in 1885 (Verreycken et al., 2007), and is a major predator of Corixidae (F. Van de Meutter, unpublished data). All fish were collected in a pesticide-free lake in a nature reserve in Genk (Belgium). In the treatment with predator cues, one pumpkinseed sunfish (standard length ca. 6 cm) was placed in a transparent cylindrical cage (diameter: 20 cm) with two 5 cm \times 5 cm holes on opposite sides covered by 200 μm mesh to provide both visual and chemical predator cues while excluding consumptive predation. Fish were fed 20 frozen chironomid larvae every two days. The containers of the treatment without predator cues had similar but empty cages. If a fish died, it was replaced within 24 h.

For the community treatment, a set of non-corixid macroinvertebrates was added to the containers. We used mayfly larvae (*Cloeon dipterum* Linnaeus, 1761), damselfly larvae (*Ischnura elegans* Vander Linden, 1820), and snails (*Physa acuta* Draparnaud, 1805). This simplified community was composed of the three most abundant species that commonly co-occurred in the ponds sampled (H. Trekels and F. Van de Meutter, unpublished data), and that share food resources with the five Corixidae species. Size-matched

organisms were collected and added to half of the containers in the following numbers: 40 mayfly larvae (8–10 mm, corresponding to instars F-2 and F-1; total number 480), 10 damselfly larvae (12–16 mm, corresponding to instars F-3 and F-2, total number 120) and 16 snails (7–8 mm, total number 192). All non-corixid macroinvertebrates were collected from pesticide-free ponds near Leuven (Belgium) and kept in 10 L buckets for 2–4 days before the start of the experiment. The chosen densities reflect the natural densities in the sampled ponds (H. Trekels and F. Van de Meutter, unpublished data).

We used black plastic 230 L containers placed at the outdoor experimental area of the Laboratory of Aquatic Ecology, Evolution and Conservation in Heverlee (Belgium) as experimental units. Each container was filled with 100 L of synthetic pond water (Weber, 1993) that had aged outside for two months and had been colonized by algae. A layer of small pebbles was added as a bottom substrate. All containers were covered throughout the experiment with a 1 mm mesh to prevent emigration and immigration of Corixidae and other insects.

At the start of the experiment, each container was inoculated with 16 individuals (eight males and eight females) of each of the five Corixidae species (total number 1920). This mimicked natural densities in ponds in Belgium (H. Trekels and F. Van de Meutter, personal observations). We took several precautions to avoid differences in initial body mass between individuals of a given Corixidae species among containers. First, for each species, we only used adults that were collected in the same pond. Second, prior to the experiment, all Corixidae were kept for two weeks in identical 230 L containers filled with 100 L of a 1:10 mixture of pond water and synthetic pond water and small pebbles as a bottom substrate to alleviate historical background differences. Additionally, Corixidae were daily fed *ad libitum* with frozen chironomid larvae during this period. Third, we randomly assigned Corixidae individuals of each species to the different containers. At the start of the experiment, we added 600 frozen chironomid larvae to each container as food. This amount provided food for only three to four days. This was done to cause food competition during the 10-day exposure period.

2.3. Response variables

After ten days of exposure, we quantified two response variables for each macroinvertebrate species per container: the number of survivors and the average per capita dry mass (from hereon called dry mass). In order to do this, all surviving Corixidae (sexes separated), damselfly larvae, mayfly larvae and snails were counted and weighed, pooled per container, after being dried for 48 h at 60 °C.

To help with the interpretation of the treatment effects on the experimental animals, we monitored dry mass of periphyton (on 50 cm² ceramic tiles), the concentration of chlorophyll *a* in the water body as a proxy for phytoplankton densities (method by Talling and Driver, 1963), and the physicochemical parameters total N, total P, Secchi-depth, pH, dissolved oxygen and conductivity in each container at the end of the experiment. None of the treatments had a significant effect (all $P > 0.15$) on final periphyton dry mass (mean \pm SE: 312 ± 25 mg/m²), chlorophyll *a* concentration (21.3 ± 1.8 µg/L), or on physicochemical parameters, all of which showed limited variability between containers (total N: 795 ± 24 µg/L; total P: 156 ± 15 µg/L; Secchi-depth: 48 ± 2 cm; pH: 8.94 ± 0.06 ; O₂: 11.37 ± 0.20 mg/L; conductivity: 249 ± 1 µS/cm).

2.4. Statistical analyses

We tested for effects of the pesticide treatment, predator cues and species identity on the survival and dry mass of the three “non-corixid community” species using separate ANOVAs per response

variable. Similarly, effects of the pesticide treatment, predator cues, the presence of the non-corixid community, and the Corixidae species identity on survival and dry mass of the five Corixidae were analyzed using separate ANOVAs per response variable. Because of high mortality of *C. coleoptrata* in the pesticide treatment, dry mass data are lacking for several treatment combinations. Therefore, the model for dry mass only included the other four Corixidae species. To take into account that the five (or four) Corixidae species were present in the same container (i.e. that we had sets of non-independent species means per container), we included container as a factor in the models. Because each container belonged to a certain combination of pesticide treatment, predator cue treatment and non-corixid community treatment, the factor container was nested in their 3-way interaction (see also Millar and Anderson, 2004). Initially, we also included sex in the statistical models. The effect of sex (and its interactions) on survival was not significant, and therefore not retained in the final model for survival. The species-specific sex effect on dry mass (females were heavier than males) was independent of all experimental treatments and will therefore not be discussed. Assumptions were met in all ANOVAs. Container was the unit of replication in all analyses and the average per capita dry mass of a given species per container was used for analysis.

3. Results

3.1. Survival and per capita dry mass of the non-corixid community

The number of survivors and the per capita dry mass of the three non-corixid species (*I. elegans* damselfly larvae, *C. dipterum* mayfly larvae and *P. acuta* snails) were independent of the pesticide and the predator cue treatments (all $P > 0.14$; Table A.1 and Fig. B.1 in Appendices). The per capita dry mass differed among the non-corixid community species, but survival did not ($P < 0.001$ and $P = 0.19$, respectively; Table A.1).

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2013.04.008>.

3.2. Survival of Corixidae

The ANOVA of all five Corixidae species indicated a species-specific pesticide effect on survival, which was independent of predator cues and community context (Fig. 1, Table 1). Separate ANOVAs per species showed that these species-specific survival patterns were driven by a high (80%) pesticide-induced mortality of *C. coleoptrata* ($F_{1,16} = 115$; $P < 0.001$), and to a lesser extent (18%), of *S. lateralis* ($F_{1,16} = 6.28$; $P = 0.023$), while the other three species did not have lower survival in the presence of the pesticide (*H. linnaei*: $F_{1,16} = 0.45$; $P = 0.51$, *S. iactans*: $F_{1,16} = 0.44$; $P = 0.52$ and *S. striata*: $F_{1,16} = 0.41$; $P = 0.53$; Fig. 1). Independent of this species-specific pesticide effect, there was a predator cue \times community context interaction on survival (Table 1). In the presence of predator cues, the non-corixid community induced 11% mortality in the Corixidae (Tukey posthoc test: $P = 0.033$), yet the non-corixid community had no effect on Corixidae survival when predator cues were absent ($P = 0.99$) (Fig. 2A).

3.3. Per capita dry mass of Corixidae

Low survival in several treatment combinations of *C. coleoptrata* precluded an analysis on dry mass for this species. The ANOVA of dry mass for the set of the other four Corixidae species (*H. linnaei*, *S. iactans*, *S. lateralis* and *S. striata*) indicated a pesticide \times predator cues interaction (Table 1). The pesticide had a negative effect on per

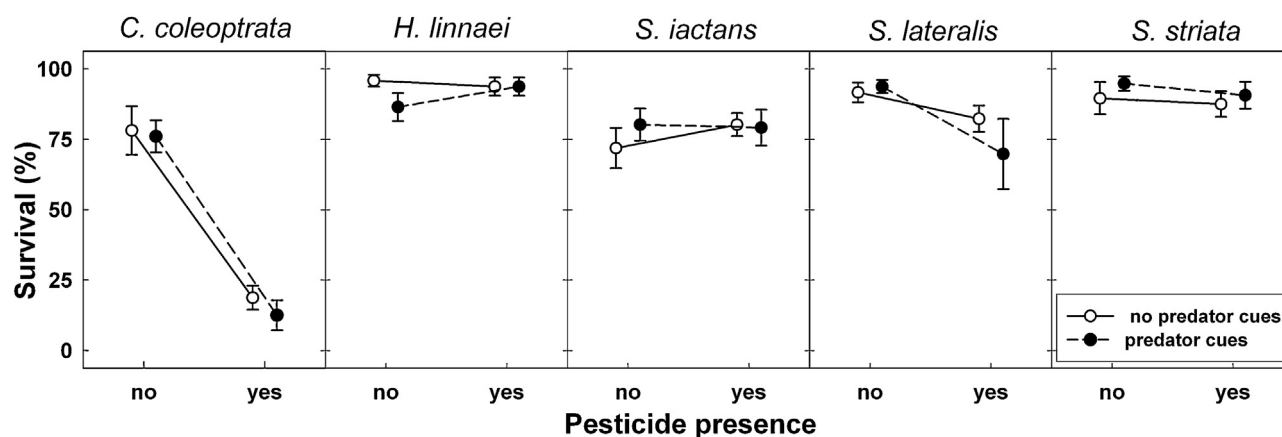


Fig. 1. Average (percentage) survival after ten days of exposure to the pesticide (absence or presence of endosulfan) and predator cue treatments (absence or presence of a caged fish predator) for each of the five studied Corixidae species. Initial numbers were 16 individuals per container for each species. Means are given with 1 SE.

Table 1

Results of the ANOVAs testing for the effects of pesticide, predator cues, the non-corixid community, Corixidae species identity, and sex on survival (all five Corixidae) and per capita dry mass (four Corixidae species, without *C. coleoptrata*). As there were no sex effects on survival, sex was removed from the analysis of survival.

Factor	Survival (all five Corixidae)			Per capita dry mass (without <i>C. coleoptrata</i>)		
	df	F	P	df	F	P
Pesticide (1)	1	41.4	<0.001	1	14.9	<0.001
Predator cues (2)	1	0.29	0.593	1	1.79	0.183
(non-corixid) Community (3)	1	3.20	0.077	1	7.78	0.006
Species (4)	4	51.9	<0.001	3	1058	<0.001
Sex (5)	–	–	–	1	226	<0.001
Pesticide × species	4	27.2	<0.001	3	0.60	0.613
Pesticide × predator cues	1	0.85	0.360	1	3.92	0.049
Pesticide × community	1	2.04	0.156	1	0.78	0.379
Predator cues × species	4	0.84	0.504	3	0.15	0.928
Predator cues × community	1	4.60	0.035	1	2.76	0.098
Species × community	4	1.67	0.164	3	2.32	0.078
Species × sex	–	–	–	3	33.6	<0.001
1 × 2 × 3	1	0.00	1.000	1	2.31	0.130
1 × 2 × 4	4	0.78	0.539	3	0.06	0.979
1 × 3 × 4	4	1.11	0.358	3	0.83	0.477
2 × 3 × 4	4	1.75	0.148	3	0.47	0.705
1 × 2 × 3 × 4	4	0.32	0.865	3	1.51	0.216
Container (1 × 2 × 3)	19	1.35	0.172	19	4.57	<0.001
Error	88			154		

Bold values are statistically significant if $P \leq 0.05$.

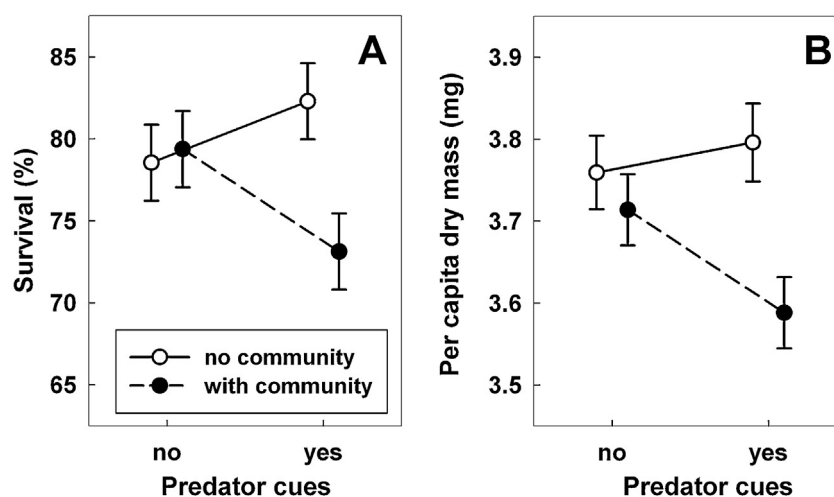


Fig. 2. Average (percentage) survival of the Corixidae (averaged across all five Corixidae species, A) and average per capita dry mass of Corixidae (averaged across four Corixidae species without *C. coleoptrata*, B) after ten days of exposure to predator cue treatment (absence or presence of a caged fish predator) in the absence and presence of the non-corixid community. Initial numbers were 16 individuals for all species in all containers. Means are given with 1 SE.

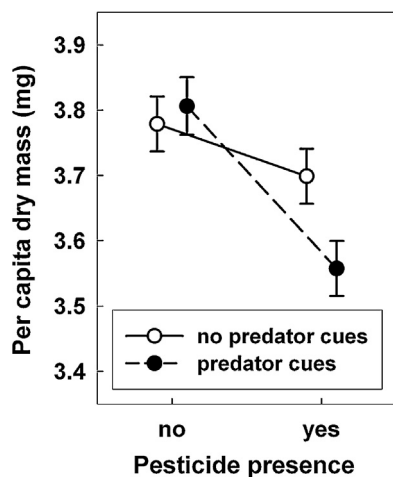


Fig. 3. Average per capita dry mass of Corixidae (averaged across four Corixidae species without *C. coleoptrata*) after ten days of exposure to predator cues (absence or presence of a caged fish predator) and pesticide treatments (absence or presence of endosulfan). Means are given with 1 SE.

capita dry mass, but only significantly so (7% decrease in mass) in the presence of predator cues (pesticide effect; in the presence of predator cues: $P=0.041$, without predator cues: $P=0.11$; Fig. 3). Per capita dry mass of the Corixidae also decreased in the presence of the non-corixid community (Table 1). Although there was no significant predator cues \times community interaction ($P=0.10$, Table 1), this mass reduction tended to be present only in the presence of predator cues (Fig. 2B). In the presence of predator cues, the non-corixid community reduced dry mass of the Corixidae by 5% (Tukey post hoc test: $P=0.020$), yet the non-corixid community had no effect on dry mass when predator cues were absent ($P=0.53$).

4. Discussion

The endosulfan concentration used in this study only strongly negatively affected survival of *C. coleoptrata*. This is in line with the much lower LC_{50} of this species compared to the four other Corixid species tested (*H. linnaei*, *S. iactans*, *S. lateralis* and *S. striata*) (Trekels et al., 2011). For the latter four species, we could demonstrate a pesticide-induced reduction of per capita dry mass, but only in the presence of predator cues, reflecting a synergism between both stressors under semi-natural conditions. Despite negative effects of the non-corixid community on corixid survival and per capita dry mass, no synergistic effects with pesticide exposure were detected. Given that neither pesticide exposure nor predator cues affected survival and dry mass of the species of the non-corixid community, any treatment effects on the Corixidae were likely not mediated through treatment effects on the non-corixid community.

4.1. Effects of the pesticide in the presence of predator cues

Stress imposed by predation is an important driver of prey population dynamics in aquatic ecosystems (Luttbeg and Kerby, 2005; Preisser et al., 2005). Aquatic ecosystems are increasingly suffering from pesticide pollution (Butchart et al., 2010) making the study of joint effects of predator cues and pesticide exposure particularly relevant. While many studies reported synergistic interactions between pesticide exposure and predator cues for survival (e.g. Maul et al., 2006; Relyea and Mills, 2001), growth rate (e.g. Campero et al., 2007; Relyea and Mills, 2001), and other end points such as genotoxicity (Giri et al., 2012), synergistic effects between predator cues and pesticides are not general (e.g., Pestana et al., 2009;

Qin et al., 2011; Relyea, 2005). Moreover, all studies showing this synergism were based on laboratory studies.

Our findings are important, as the few previous semi-natural, outdoor studies were not able to confirm synergisms between pesticides and predator cues (Relyea, 2012, 2006; Rohr and Crumrine, 2005), despite the fact that in two of these studies, a synergism was previously documented for the same combination of species and pesticides in the laboratory (*Rana catesbeiana* bullfrogs and *Rana clamitans* green frogs exposed to carbaryl in the study by Relyea (2006) and *Rana sylvatica* wood frogs and glyphosate in the study by Relyea (2012)). In a previous laboratory study where we investigated the combined effects of exposure to the same endosulfan concentration we used in this study and fish predator cues in four of these species (*C. coleoptrata*, *H. linnaei*, *S. lateralis* and *S. striata*), we found no support for synergistic interactions on survival or on per capita dry mass increase (Trekels et al., 2012). In line with this, no synergism for survival between predator cues and the pesticide could be detected in the current study, despite the fact that the community treatment became lethal in the presence of predator cues. We did, however, observe synergistic effects of pesticides and predator cues on per capita dry mass, and this was consistent across species. This further illustrates that the extrapolation of synergisms from laboratory to semi-natural settings is not straightforward and together with previous work (Relyea, 2006) indicates that synergisms between pesticides and predator cues may either disappear or become apparent when moving from laboratory to semi-natural field conditions.

Several mechanisms may explain the discrepancy between the occurrence of synergisms between pesticides and predator cues in indoor laboratory and outdoor container experiments (Relyea, 2006). It seems unlikely that differences in the imposed levels of pesticide and of predator cues played a role in our study system. With regard to pesticide exposure, pesticide levels on average were likely lower in the current semi-natural, outdoor experiment than in previous laboratory experiments. In both experiments, a pulse application was applied at the start of the experiment, yet endosulfan breakdown may have been more rapid under outdoor conditions due to photolysis (but see Penuela and Barcelo, 1998). Other abiotic conditions likely played a minor role as both experiments were run in reconstituted soft water of the same composition and pH. That endosulfan concentrations remained at higher levels for a longer time in the laboratory study is supported by the observation that three out of four species had lower survival in the presence of endosulfan in the laboratory study compared to two out of five species in current study. The assumed lower actual pesticide concentrations throughout parts of the exposure period in the outdoor containers than in the laboratory is unlikely to explain the appearance of the synergism in the present study (Relyea, 2006).

With regard to predator cues, the imposed stress levels by the predator were also likely lower in the current experiment than in previous laboratory studies. In the laboratory study, we used a stickleback of a similar size as the pumpkinseed sunfish used here. While the kairomones released by pumpkinseeds may have imposed a stronger predator stress than those released by sticklebacks (Stoks and De Block, 2000), the total level of predator cues (including alarm cues by the prey) was likely stronger in the laboratory, because sticklebacks were fed daily with ground Corixidae while pumpkinseed sunfish were fed chironomid larvae in the current container study. Prey species are known to respond much stronger to conspecific alarm cues than to predator kairomones alone (Ferrari et al., 2010; Schoeppner and Relyea, 2009). The assumed lower level of predator-induced stress in the containers is supported by the observation that the predator cue treatment slightly reduced survival and growth rate in some Corixidae species in the laboratory experiment while it did not in the current container study. Taken together, this suggests that the

levels of pesticide stress and of predator cues were likely lower in the outdoor container experiment, and therefore, cannot explain that a synergism was only detected then (see also Relyea, 2012, 2006).

More likely, the synergism between pesticides and predator cues on dry mass was detected in the outdoor container experiment but not in the laboratory experiment because the outdoor container conditions, and more specifically the presence of conspecifics and heterospecifics (i.e. a community), were perceived as a more stressful environment. Synergisms between two stressors may indeed be more pronounced or only detectable in the presence of a third stressor (Crain et al., 2008). General conditions in the outdoor containers were likely more stressful because animals were confronted with both conspecific and heterospecific Corixidae at a suboptimal food level, likely generating some degree of food stress during the 10-day exposure period, while in the laboratory, animals were exposed individually and given food ad libitum. Synergisms between pesticides and predator cues are more likely to occur under food-limited conditions as animals are less able to defend themselves and compensate for both stressors (Campero et al., 2007). Analogously, previous studies also showed prey animals suffered more from predator cues in terms of life history response under low food conditions (e.g. Beketov and Liess, 2006).

4.2. Effects of the non-corixid community

In the absence of other stressors, the presence of the non-corixid community had no significant effects on survival and per capita dry mass of the Corixidae species. Yet, when combined with predator cues, both life history traits were negatively impacted. Corixidae are generalist feeders (Candolin, 2005; Popham et al., 1984). Therefore, they likely competed for food with all members of the non-corixid community: with damselfly larvae (that also feed on chironomids), and with mayfly larvae and snails (that also graze on periphyton). In the presence of predator cues, the energy reserves of the Corixidae may already have been lowered due to reduced food intake and/or shunting of energy to costly defense mechanisms such as upregulation of stress proteins (Slos and Stoks, 2008; Stoks et al., 2005a,b). Adding the non-corixid community and likely food competition may have further reduced energy reserves, resulting in reduced dry mass and ultimately, mortality. Additionally, the reduced activity levels of the water bugs in the presence of fish predator cues (F. Van de Meutter, unpublished results) may have made them more vulnerable to predation by damselfly larvae and less strong competitors against the mayflies and snails.

Despite the synergistic interaction between the non-corixid community and predator cues, the presence of the non-corixid community did not magnify the pesticide effect. Studies that manipulated interspecific competition to investigate its effects on the sensitivity to pollutants are relatively rare and, like our study, do not seem to indicate that any negative pesticide effects are magnified under interspecific competition. Distel and Boone (2010) found no effect of different densities of Northern leopard frogs (*Rana pipiens*) and American toads (*Bufo americanus*) on the sensitivity to carbaryl. Distel and Boone (2010) even found that *B. americanus* toads exposed to carbaryl tended to survive better in the presence than in the absence of competing *R. pipiens* frogs possibly due to competitive release.

In our study, it may also be that the assumed negative effects of the non-corixid community on food levels in the containers were offset by reduced densities (conspecifics and heterospecifics) in the presence of the pesticide (see also Chang et al., 2005). The only other study that manipulated interspecific competition, endosulfan and predator cues, also showed no synergistic interactions (Rohr and Crumrine, 2005). Interspecific competition did, however, modulate the endosulfan effect, as endosulfan's indirect benefit on tadpole

mass was greater in the absence of snails (Rohr and Crumrine, 2005). This is not to say that interspecific competition may not negatively impact communities exposed to pesticides. For example, two recent studies showed that interspecific competition delayed recovery of communities from pesticide pulses (Foit et al., 2012; Knillmann et al., 2012). This indicates that combined effects of pesticides and competition may be subtle and not apparent unless when tested in the recovery phase.

5. Conclusions

Our study demonstrates for the first time a synergistic interaction between pesticide exposure and predator cues under semi-natural, outdoor conditions. This urges caution when extrapolating synergisms from laboratory tests to semi-natural conditions. Our study further highlights the need for ecological realism when testing effects of pollutants (Relyea and Hoverman, 2006; Relyea et al., 2005; Rohr et al., 2006). A better understanding of whether and how pesticide effects depend on interactions with species at the same (competition) or higher (predation) trophic levels is a major challenge to arrive at a predictive community ecotoxicology (Clements and Rohr, 2009; Rohr et al., 2006). While the here documented treatment-related responses (including the synergistic effect) are likely better mimicking the actual responses occurring in real pond ecosystems compared to responses observed in indoor aquarium experiments, a future challenge will be to study these synergisms in real pond ecosystems with treatments replicated at the pond level. Such experiments have been carried out to separately study effects of predators (e.g., Werner and McPeck, 1994) and of pollutants (see e.g. Brodman et al., 2010), but so far have not studied the combined impact of pollutants under the stress of predation. Combining similarly focused multi-species studies as the present study with food web modeling may provide mechanistic insights into the underlying ecological mechanisms, and a fruitful avenue for further research.

Conflict of interest

We declare no conflict of interest.

Acknowledgements

During this study HT was a PhD fellow of the IWT-Flanders and FVDM was a postdoctoral fellow of FWO-Flanders. This research was financially supported by research projects of the KU Leuven Research Fund and FWO-Flanders to FVDM and RS. We acknowledge the constructive comments of two reviewers that greatly improved the paper. We would like to thank Dr. Lisa Shama for her final contributions on English grammar and spelling.

References

- Beketov, M.A., Liess, M., 2006. Predation risk perception and food scarcity induce alterations of life-cycle traits of the mosquito *Culex pipiens*. *Ecological Entomology* 32, 405–410.
- Boone, M.D., Semlitsch, R.D., 2002. Interactions of an insecticide with competition and pond drying in amphibian communities. *Ecological Applications* 12, 307–316.
- Brodman, R., Newman, W.D., Laurie, K., Ostereld, S., Lenzo, N., 2010. Interaction of an aquatic herbicide and predatory salamander density on wetland communities. *Journal of Herpetology* 44, 68–82.
- Broomhall, S., 2002. The effects of endosulfan and variable water temperature on survivorship and subsequent vulnerability to predation in *Litoria citropa* tadpoles. *Aquatic Toxicology* 61, 243–250.
- Buck, J.C., Scheessele, E.A., Relyea, R.A., Blaustein, A.R., 2012. The effects of multiple stressors on wetland communities: pesticides, pathogens and competing amphibians. *Freshwater Biology* 57, 61–73.
- Butchart, S.H.M., Walpole, M., Collen, B., van Strien, A., Scharlemann, J.P.W., et al., 2010. Global biodiversity: indicators of recent declines. *Science* 328, 1164–1168.

- Campero, M., Slos, S., Ollevier, F., Stoks, R., 2007. Sublethal pesticide concentrations and predation jointly shape life history: behavioral and physiological mechanisms. *Ecological Applications* 17, 2111–2122.
- Candolin, U., 2005. Why do multiple traits determine mating success? Differential use in female choice and male composition in a water boatman. *Proceedings of the Royal Society B: Biological Sciences* 272, 47–52.
- Carlson, R.W., Bradbury, S.P., Drummond, R.A., Hammermeister, D.E., 1998. Neurological effect on startle response and escape from predation by medaka exposed to organic chemicals. *Aquatic Toxicology* 43, 51–68.
- Chang, K.H., Sakamoto, M., Hanazato, T., 2005. Impact of pesticide application on zooplankton communities with different densities of invertebrate predators: an experimental analysis using small-scale mesocosms. *Aquatic Toxicology* 72, 373–382.
- Clements, W.H., Newman, M.C., 2002. *Community Ecotoxicology*. John Wiley and Sons, Chichester.
- Clements, W.H., Rohr, J.R., 2009. Community responses to contaminants: using basic ecological principles to predict ecotoxicological effects. *Environmental Toxicology and Chemistry* 28, 1789–1800.
- Crain, C.M., Kroeker, K., Halpern, B.S., 2008. Interactive and cumulative effects of multiple human stressors in marine systems. *Ecology Letters* 11, 1304–1315.
- Darling, E.S., Côté, I.M., 2008. Quantifying the evidence for ecological synergies. *Ecology Letters* 11, 1278–1286.
- Distel, C.A., Boone, M.D., 2010. Effects of aquatic exposure to the insecticide carbaryl are species-specific across life stages and mediated by heterospecific competitors in anurans. *Functional Ecology* 24, 1342–1352.
- Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z.I., Knowle, D.J., Leveque, C., Naiman, R.J., Prieur-Richard, A.H., Soto, D., Stiassny, M.L.J., Sullivan, C.A., 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological Reviews* 81, 163–182.
- Ernst, W.R., Jonah, P., Doe, K., Julien, G., Hennigar, P., 1991. Toxicity to aquatic organisms of off-target deposition of endosulfan applied by aircraft. *Environmental Toxicology and Chemistry* 10, 103–114.
- Ferrari, M.C.O., Wisenden, B.D., Chivers, D.P., 2010. Chemical ecology of predator–prey interactions in aquatic ecosystems: a review and prospectus. *Canadian Journal of Zoology* 88, 698–724.
- Foitt, K., Kaske, O., Liess, M., 2012. Competition increases toxicant sensitivity and delays the recovery of two interacting populations. *Aquatic Toxicology* 106, 25–31.
- Giri, A., Yadav, S.S., Giri, S., Sharma, G.D., 2012. Effect of predator stress and malathion on tadpoles of Indian skittering frog. *Aquatic Toxicology* 106, 157–163.
- Holmstrup, M., Bindesbol, A.-M., Oostingh, G.J., Duschl, A., Scheil, V., Kohler, H.R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: a review. *Science of the Total Environment* 408, 3746–3762.
- Jones, D.K., Hamond, J.I., Relyea, R.A., 2011. Competitive stress can make the herbicide Roundup more deadly to larval amphibians. *Environmental Toxicology and Chemistry* 30, 446–454.
- Knillmann, S., Stampfli, N.C., Noskov, Y.A., Bketov, M.A., Liess, M., 2012. Interspecific competition delays recovery of *Daphnia* spp. populations from pesticide stress. *Ecotoxicology* 21, 1039–1049.
- Laskowski, R., Bednarska, A.J., Kramarz, P.E., Loureiro, S., Scheil, V., Kudlek, J., Holmstrup, M., 2010. Interactions between toxic chemicals and natural environmental factors – a meta-analysis and case studies. *Science of the Total Environment* 408, 3763–3774.
- Lindenmayer, D., Likens, G.E., Krebs, C.J., Hobbs, R.J., 2010. Improved probability of detecting ecological surprises. *Proceedings of the National Academy of Sciences of the United States of America* 107, 21957–21962.
- Luttbeg, B., Kerby, J.L., 2005. Are scared prey as good as dead? *Trends in Ecology & Evolution* 20, 416–418.
- Maul, J.D., Farris, J.L., Lydy, M.J., 2006. Interaction of chemical cues from fish tissues and organophosphorous pesticides on *Ceriodaphnia dubia* survival. *Environmental Pollution* 141, 90–97.
- Millar, R.B., Anderson, M.J., 2004. Remedies for pseudoreplication. *Fisheries Research* 70, 397–407.
- Penuela, G.A., Barcelo, D., 1998. Photosensitized degradation of organic pollutants in water: processes and analytical applications. *Trends in Analytical Chemistry* 17, 605–612.
- Pestana, J.L.T., Loureiro, S., Baird, D.J., Soares, A.M.V.M., 2009. Fear and loathing in the benthos: responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. *Aquatic Toxicology* 93, 138–149.
- Popham, E.J., Bryant, M.T., Savage, A., 1984. The role of front legs of British corixid bugs in feeding and mating. *Journal of Natural History* 18, 445–464.
- Preisser, E.L., Bolnick, D.I., Benard, M.F., 2005. Scared to death? The effects of intimidation and consumption in predator–prey interactions. *Ecology* 86, 501–509.
- Qin, G., Presley, S.M., Anderson, T.A., Gao, W.M., Maul, J.D., 2011. Effects of predator cues on pesticide toxicity: toward an understanding of the mechanism of the interaction. *Environmental Toxicology and Chemistry* 30, 1926–1934.
- Relyea, R.A., 2003. Predator cues and pesticides: a double dose of danger for amphibians. *Ecological Applications* 13, 1515–1521.
- Relyea, R.A., 2004. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. *Environmental Toxicology and Chemistry* 23, 1080–1084.
- Relyea, R.A., 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications* 15, 618–627.
- Relyea, R.A., 2006. The effects of pesticides, pH, and predatory stress on amphibians under mesocosm conditions. *Ecotoxicology* 15, 503–511.
- Relyea, R.A., 2012. New effects of Roundup on amphibians: predators reduce herbicide mortality; herbicides induce antipredator morphology. *Ecological Applications* 22, 634–647.
- Relyea, R.A., Mills, N., 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). *Proceedings of the National Academy of Sciences of the United States of America* 98, 2491–2496.
- Relyea, R., Hoverman, J., 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecology Letters* 9, 1157–1171.
- Relyea, R.A., Schoepner, N.M., Hoverman, J.T., 2005. Pesticides and amphibians: the importance of community context. *Ecological Applications* 15, 1125–1134.
- Rohr, J.R., Crumrine, P.W., 2005. Effects of an herbicide and an insecticide on pond community structure and processes. *Ecological Applications* 15, 1135–1147.
- Rohr, J.R., Kerby, J.L., Sih, A., 2006. Community ecology as a framework for predicting contaminant effects. *Trends in Ecology & Evolution* 21, 606–613.
- Sala, O.E., Chapin, F.S., Armesto, J.J., Berlow, E., Bloomfield, J., Dirzo, R., et al., 2000. Biodiversity – global biodiversity scenarios for the year 2100. *Science* 287, 1770–1774.
- Schoepner, N.M., Relyea, R.A., 2009. Interpreting the smell of predation: how alarm cues and kairomones induce different prey defences. *Functional Ecology* 23, 1114–1121.
- Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. *Science* 313, 1072–1077.
- Slos, S., Stoks, R., 2008. Predation risk induces stress proteins and reduces antioxidant defense. *Functional Ecology* 22, 637–642.
- Stenersen, J., 2004. *Chemical Pesticides: Mode of Action and Toxicology*. CRC Press, Florida.
- Stoks, R., De Block, M., 2000. The influence of predator species and prey age on the immediate survival value of antipredator behaviours in a damselfly. *Archiv für Hydrobiologie* 147, 417–430.
- Stoks, R., De Block, M., McPeck, M.A., 2005a. Alternative growth and energy storage responses to mortality threats in damselflies. *Ecology Letters* 8, 1307–1316.
- Stoks, R., De Block, M., Van de Meutter, F., Johansson, F., 2005b. Predation cost of rapid growth: behavioural coupling and physiological decoupling. *Journal of Animal Ecology* 74, 708–715.
- Talling, J.F., Driver, D., 1963. Some problems in the estimation of chlorophyll-A in phytoplankton. In: Doty, M. (Ed.), *Proceedings on Primary Productivity Measurement, Marine Freshwater*. U.S. Atomic Energy Engineering Commission, Honolulu, pp. 142–146.
- Tilman, D., Fargione, J., Wolff, B., D'Antonio, C., Dobson, A., Howarth, R., Schindler, D., Schlesinger, W.H., Simberloff, D., Swackhamer, D., 2001. Forecasting agriculturally driven global environmental change. *Science* 292, 281–284.
- Trekels, H., Van de Meutter, F., Stoks, R., 2011. Effects of species-specific interactions with predation risk on the relative species sensitivities to a pesticide in water boatmen (Corixidae). *Oikos* 120, 897–905.
- Trekels, H., Van de Meutter, F., Bervoets, L., Stoks, R., 2012. Species-specific responsiveness of four enzymes to endosulfan and predation risk questions their usefulness as general biomarkers. *Ecotoxicology* 21, 268–279.
- Van Steertegem, M., 2012. MIRA indicator rapport 2011. Milieuraapport Vlaanderen, Vlaamse Milieumaatschappij.
- Verreycken, H., Anseeuw, D., Van Thuyne, G., Quataert, P., Belpaire, C., 2007. The non-indigenous freshwater fishes of Flanders (Belgium): review, status and trends over the last decade. *Journal of Fish Biology* 71, 160–172.
- Voerlesmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan, C.A., Liermann, C.R., Davies, P.M., 2010. Global threats to human water security and river biodiversity. *Nature* 467, 55–561.
- Wan, M.T., Kuo, J.-L., Buday, C., Schroeder, G., Van Aggelen, G., Pasternak, J., 2005. Toxicity of α -, β -, ($\alpha + \beta$)-endosulfan and their formulated and degradation products to *Daphnia magna*, *Hyalella azteca*, *Oncorhynchus mykiss*, *Onchorhynchus kisutch*, and biological implications in streams. *Environmental Toxicology and Chemistry* 24, 1146–1154.
- Weber, C., 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. In: *Environmental Monitoring Systems Laboratory*, fourth ed. Environmental Protection Agency, Cincinnati.
- Weber, J., Halsall, C.J., Muir, D., Teixeira, C., Small, J., Solomon, K., Hermanson, M., Bidleman, T., 2010. Endosulfan, a global pesticide: a review of its fate in the environment and occurrence in the arctic. *Science of the Total Environment* 408, 2966–2984.
- Werner, E.E., McPeck, M.A., 1994. Direct and indirect effects of predators on 2 anuran species along an environmental gradient. *Ecology* 75, 1368–1382.