1 A widespread morphological antipredator mechanism reduces the sensitivity to pesticides and
2 increases the susceptibility to warming

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Abstract

Pollution and predation are two omnipresent stressors in aquatic systems that can interact in multiple ways, thereby challenging accurate assessment of the effects of pollutants in natural systems. Despite the widespread occurrence of morphological antipredator mechanisms, no studies have tested how these can affect the sensitivity of prey to pesticides. Sensitivity to pesticides is typically measured via reductions in growth rates and survival, but also reductions in heat tolerance are to be expected and are becoming increasingly important in a warming world. We investigated how autotomy, a widespread morphological antipredator mechanism where animals sacrifice a body part (here the caudal lamellae) to escape when attacked by a predator, modified the sensitivity to the insecticide chlorpyrifos in larvae of the damselfly *Coenagrion puella*. Exposure to chlorpyrifos reduced the growth rate and heat tolerance (measured as CT_{max}). A key finding was that the pesticide had a greater impact on growth rates of intact animals, i.e. those that retained their lamellae. This reduced sensitivity to chlorpyrifos in animals without lamellae can be explained by the reduced outer surface area which is expected to result in a lower uptake of the pesticide. Larvae that underwent autotomy exhibited a lower heat tolerance, which may also be explained by the reduced surface area and the associated reduction in oxygen uptake. There is a wide diversity of morphological antipredator mechanisms, suggesting that there will be more examples where these mechanisms affect the vulnerability to pollutants. Given the importance of pollution and predation as structuring forces in aquatic food webs, exploring the potential interactions between morphological antipredator mechanisms and sensitivity to pollutants will be crucial for risk assessment of pollutants in aquatic systems.

Keywords: autotomy, chlorpyrifos, damselfly, climate change, predation, thermal tolerance

Introduction

Pesticides (Liess et al. 2008) and predation (Kerfoot & Sih 1987) are omnipresent stressors in aquatic ecosystems that may interact in multiple ways. Recent studies show that exposure to predation risk makes pesticides more toxic (e.g. Relyea & Mills 2001; Campero et al. 2007), likely through energy limitation (Sokolova 2013; Liess et al. 2016), and that pesticide exposure impairs antipredator mechanisms (e.g. Janssens & Stoks 2012; Shuman-Goodier & Propper 2016) and predator recognition (e.g. Polo-Cavia et al. 2016). Yet, such interactions are not found consistently (e.g. Jansen et al. 2011) and a recent study showed that exposure to the herbicide glyphosate actually increased the chemical antipredator defence of toad larvae (*Bufo bufo*) (Bokony et al. 2017). Morphological antipredator mechanisms are widespread (Kerfoot & Sih 1987; Barbosa & Castellanos 2005) and are therefore likely to also be displayed in polluted habitats. However, so far no studies directly tested how morphological antipredator mechanisms can affect the sensitivity to pesticides.

An important morphological antipredator mechanism in both vertebrates and invertebrates (McVean 1975; Fleming et al. 2007) is autotomy, the amputation of a body part at a breakage plane to escape when caught by a predator (Fleming et al. 2007). There are many delayed costs associated with autotomy; for example, a lower escape speed (e.g. Gyssels & Stoks 2005), a reduced balance during locomotion (Hessel et al. 2017) and reduced energy stores because of re-growing a body part (Dias-Guisado et al. 2006), resulting in an increased sensitivity to predation in the future (e.g. Stoks 1998a). In damselfly larvae, autotomy has been shown to not affect resting metabolic rate (Sesterhenn et al. 2013), but to lower antioxidant defense and immune function and (Slos et al. 2009). Whether autotomy may influence the sensitivity to pesticides is an unexplored, yet exciting pathway in the interaction between

predation and pesticides. If autotomy reduces the outer surface area, then the impact of pollutants may be reduced. Indeed, in many aquatic organisms the majority of the uptake of pollutants occurs through the surface and it has been shown that aquatic arthropods with a larger outer surface area and external gills are more sensitive to pesticides due to a higher uptake, hence higher internal accumulation of the toxicant (e.g. Rubach et al. 2012; Wiberg-Larsen et al. 2016).

Sensitivity to pesticides is typically measured via reductions in growth rates and survival, but also reductions in heat tolerance are to be expected and are becoming increasingly important in a warming world. Heat tolerance is set by oxygen limitation, particularly a mismatch between the capacity for oxygen supply to body tissues and the rate at which oxygen is required (Ern et al. 2015; Verberk et al. 2016a). In ectotherms, the demand for oxygen increases with temperature, potentially outpacing the rate at which oxygen can be supplied (Pörtner 2010; Verberk et al. 2011). Pesticide exposure is expected to reduce heat tolerance (see e.g. Op de Beeck et al. 2017) as pesticide exposure increases oxygen demand, making a mismatch between the capacity for oxygen supply and the rate at which oxygen is required more likely (Sokolova & Lanning 2008; Noyes & Lema 2015). This general phenomenon of contaminants reducing an organism's ability to cope with increased temperatures has been named the "contaminant-induced climate sensitivity concept" (Sokolova & Lanning 2008; Noyes & Lema 2015). How antipredator mechanisms may shape the ability to cope with warming is unexplored. If heat tolerance is governed by a mismatch between the capacity for oxygen supply and the rate at which oxygen is required, then gill autotomy will decrease the heat tolerance because of a reduction in the capacity for oxygen uptake. This would be an important overlooked mechanism how predators may shape the ability of prey populations to deal with warming. Given the

potential of pesticide exposure to increase oxygen demand and of gill autotomy to reduce oxygen uptake, their combination may especially reduce heat tolerance.

Pesticides and heat waves are important stressors in ponds as the inhabitants cannot escape exposure (Stoks et al. 2015; Hassall & Thompson 2008). Moreover, most aquatic insects employ under-water gas exchange, which also involves a diffusive step. This makes them especially relevant to study in the context of heat tolerance because the low solubility and diffusivity of oxygen challenges diffusive uptake of oxygen (Verberk & Atkinson 2013). Indeed, aquatic insects that relied on under-water gas exchange, including damselfly larvae (Verberk & Calosi 2012), were more prone to oxygen-limited heat tolerance (Verberk & Bilton 2013), and will likely show reduced heat tolerance when also exposed to pesticides.

In this study, we tested the single and combined effects of pesticide exposure and autotomy on survival, growth rate and heat tolerance (quantified as CTmax) on larval damselflies. We chose the pesticide chlorpyrifos. This model pesticide for the class of organophosphates is one of the most frequently used insecticides worldwide (Eaton et al. 2008) and a priority pollutant in the European Water Framework Directive (2000/60/EC). As study species we use *Coenagrion puella* a common damselfly species in Europe for which we have shown negative effects of chlorpyrifos on food intake (Tüzün et al. 2015) and growth rate (Dinh Van et al. 2016). Growth rate is an indicator for the adult mass, hence reproductive potential in damselflies (Stoks & Cordoba-Aguilar 2012). CTmax is considered a useful proxy for the capacity of individuals to withstand extreme heat (Huey et al. 2012). The ability of CTmax to predict field patterns in the distribution of species and how these are affected by warming indicates CTmax is informative for how organisms can cope with climate change (e.g. Calosi et al. 2010; Verberk et al. 2016b; Birkett et al. 2018). We chose to study damselfly larvae as these

often autotomize their caudal lamellae, with frequencies of autotomy in natural populations going from 30% to 90% (Stoks 1998a; Witt et al. 2013). Furthermore, the benefits and costs of autotomy have been well studied in these animals (e.g. Stoks 1998a; Slos et al. 2009). We expect the pesticide to reduce both the growth rate (e.g. Janssens & Stoks 2013) and heat tolerance (e.g. Op de Beeck et al. 2017), because of energy limitations and a disturbance of the oxygen metabolism. Due to the reduction of the surface area, autotomy is expected to lower the oxygen uptake and therefore reduce the heat tolerance (Verberk & Bilton 2011), but at the same time the lower pesticide uptake and accumulation will reduce the sensitivity to the pesticide. We therefore expect the effects of the pesticide and autotomy to interact with autotomy conferring the benefit of reduced pesticide sensitivity.

Methods

We collected larvae of the damselfly *Coenagrion puella* in a fishless pond in Heverlee (Belgium). Large dragonfly larvae (*Anax* and *Aeshna* species) are top predators in these water bodies (Stoks et al. 2003). We only collected damselfly larvae with three intact, not regenerated lamellae. This pond is not surrounded by agricultural fields making it less likely larvae experienced pesticide exposure (Coors et al. 2009). Because damselfly larvae are cannibalistic (De Block & Stoks 2004), individuals were kept in the laboratory in separate 100 mL plastic vials under standard conditions of light (14:10 L:D), temperature (22°C) and food (*ad libitum* *Artemia nauplii*) for at least four weeks.

Experimental setup

To test for the effects of autotomy and pesticide exposure and their interaction on growth and heat tolerance, we set up a full factorial 2 x 4 experiment combining two autotomy treatments

(control or autotomized larvae) with four pesticide treatments (0, 0.75, 1.0, 1.5 $\mu\text{g/l}$ chlorpyrifos), giving a total of eight treatment combinations. We used penultimate instar (F_1) larvae for the experiment and exposed the animals to the treatments for seven days. We quantified mortality and growth rate across the 7-day exposure period and CT_{max} at the end of the 7-day exposure period. The mass of the damselfly larvae at the beginning of the exposure experiment varied between 15.65 and 33.28 mg with a mean (± 1 S.E.) of 24.04 ± 6.92 mg. We started 24 replicates per treatment combination (total $N = 192$).

One day after moulting into the penultimate instar, the larvae were transferred to 100 ml glass vials filled with 50 ml of medium (solvent control or chlorpyrifos) for the exposure treatment. During a chronic 7 day exposure period, larvae were fed *Artemia* nauplii *ad libitum* daily. The chlorpyrifos concentrations (0.75, 1.0 and 1.5 $\mu\text{g/l}$) were selected based on previous experiments on damselfly larvae, where they induced sublethal effects on growth, behaviour and physiology without causing mortality (Dinh Van et al. 2014a; Janssens et al. 2017). The chosen chlorpyrifos concentrations are high, yet within the range reported in edge-to-field water bodies (Schulz 2004). To prepare the chlorpyrifos solutions for the exposure we first prepared a stock solution containing 1 mg/ml of chlorpyrifos dissolved in ethanol which was kept in the dark at 4°C . In a second step, we diluted this stock solution 100 times to obtain a concentration of 10 $\mu\text{g/ml}$ chlorpyrifos. The latter solution was prepared daily to prepare the actual chlorpyrifos exposure solutions (in aerated tap water) used in the experiment. In the control condition we added the same amount of ethanol to the aerated tap water as in the condition with the highest chlorpyrifos concentration (1.5 $\mu\text{l/l}$). Previous studies on damselfly larvae showed this ethanol concentration not to affect behaviour and growth (e.g. Janssens et al. 2017). We performed a static renewal experiment whereby the medium was completely renewed every day. A mixed

sample of ten experimental cups per concentration was analyzed by the laboratory of
Phytopharmacy (University of Ghent) using liquid chromatography combined with tandem mass
spectrometry. The measured initial concentrations were 0.64 (nominal 0.75), 0.94 (nominal 1.0)
and 1.49 (nominal 1.5) $\mu\text{g/l}$ chlorpyrifos; after 24h (just before the renewal of the medium) the
concentrations dropped to 0.41, 0.61 and 0.91 $\mu\text{g/l}$.

For the autotomy treatment, we haphazardly divided larvae into two groups and removed
all three lamellae from one group of larvae per pesticide exposure combination by gently pulling
them with tweezers until the animals autotomized these appendages at the specialized breaking
joints (as in Stoks 1999). Damselfly larvae often lose all three lamellae simultaneously in the
field (Stoks 1998b). The other half of the larvae underwent a sham operation without removing
the lamellae.

Response variables

We monitored survival daily during the exposure period. We quantified growth rate as the
increase in body mass per day; due to some mortality (see results) we were able to do so in 19-23
replicates per treatment combination (total $N = 166$). Animals were blotted dry using tissue paper
and weighed at the beginning and at the end of the 7-day exposure period using an electronic
balance (0.01 mg precision) and growth rate was calculated as relative growth rate using the
formula $(\ln_{\text{end mass}} - \ln_{\text{begin mass}})/7$. This measure of growth rate is preferred as it is independent of
the initial mass of the damselfly larvae (e.g. Stoks et al. 2003).

Immediately after the exposure period (on day 7), we quantified the critical thermal
maximum (CT_{max}) as a proxy for the heat tolerance following the methodology described in Op
de Beeck et al. (2017). For logistic reasons we tested CT_{max} for a randomly chosen subset of the
larvae (13-17 per treatment combination, total $N = 120$). Shortly, the larvae were placed

individually in plastic vials filled with aged tap water. Those vials were placed in a temperature controlled water bath filled with water with an initial temperature of 22°C. An aquarium heater heated up the water with a constant rate of 0.30°C per minute. This heating rate falls within the range used to study CTmax in freshwater insects (e.g. Verberk & Bilton 2011; Dallas & Rivers-Moore 2012). The temperature at which the larvae entered a heat coma, i.e. became moribund and no longer showed any body movements, was defined as the CTmax (Verberk & Calosi 2012; Verberk & Bilton 2013). After the CTmax test, larvae were placed again at 22°C; all larvae recovered. CTmax trials were done on different, consecutive days, reflecting the fact that not all larvae reached the penultimate instar at the same day and hence the period during which they were exposed to pesticides also ended at different, consecutive days. We included the trial day (the day at which the CTmax was measured) in the statistical analyses. Per trial day we tested 17-18 larvae (2-3 larvae per treatment combination).

Statistical analyses

Survival was analyzed using a general linear model (GLM) with a binomial error structure with autotomy, the pesticide treatment and their interaction as fixed effects. We tested for an effect of autotomy, the pesticide treatment and their interactions on growth rate and CTmax using separate two-way GLMs with a normal error structure. For growth rate, we initially included initial body mass as a covariate but as it was not significant ($p = 0.21$) it was removed from the final model. Note this also confirmed our estimate of growth rate was size-independent. For CTmax we initially included trial day (day of measurement) as a random effect and body mass as covariate, but since these had no effect (both $p > 0.25$), we removed them from the final model. None of the covariates interacted with the autotomy or pesticide treatment. In case of an interaction between the pesticide and autotomy treatments, we tested the main effect of pesticide in additional GLMs

separately for damselfly larvae with and without lamellae. When the main effect of pesticide was significant in these additional GLMs, we used follow-up Tukey post hoc tests to investigate which of the different pesticide concentrations differed significantly from one another. We confirmed normality using Shapiro Wilk tests (growth: $p = 0.15$; CTmax: $p = 0.37$) and homogeneity of variances using Levene tests (growth: $p = 0.18$; CTmax: $p = 0.49$). All tests were done in Statistica v13 (Statsoft Inc, OK, USA).

Results

Survival was high overall (on average 86.1 %) and did not differ between the treatments (all $p > 0.35$).

There was a significant interaction between the pesticide and autotomy treatment for growth rate ($F_{3, 158} = 2.70$; $p = 0.048$; Figure 1). The additional GLMs per autotomy treatment indicated that the pesticide significantly reduced the growth rate both in larvae with lamellae (main effect pesticide: $F_{3, 82} = 7.23$; $p = 0.0023$) and in larvae without lamellae (main effect pesticide: $F_{3, 76} = 5.24$; $p = 0.0024$). However, the follow-up Tukey tests showed that the concentration at which the growth reduction occurred depended on the autotomy treatment: damselfly larvae with lamellae showed considerably reduced growth rates at the intermediate (1 $\mu\text{g/l}$: Tukey $p = 0.012$) and high (1.5 $\mu\text{g/l}$: Tukey $p < 0.001$) chlorpyrifos concentrations compared to the control condition. Moreover, in damselfly larvae with lamellae, there was also a significant difference in growth rate between the two highest chlorpyrifos concentrations, with the growth being further reduced at 1.5 $\mu\text{g/l}$ (Tukey $p = 0.029$). In contrast, in larvae that autotomized their lamellae, growth was reduced only at the highest pesticide concentration (1.5 $\mu\text{g/l}$: Tukey $p = 0.018$).

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222 Figure 1: Mean growth rate (with 95 % confidence intervals) of *Coenagrion puella* damselfly larvae as a
 223 function of pesticide exposure and autotomy. Different letter codes indicate significant differences
 224 between the treatments based on Tukey post hoc tests.

225

226 There was no significant interaction between the pesticide and the autotomy treatment for
 227 CTmax ($F_{3, 106} = 1.00$; $p = 0.39$), although there was a trend that in the larvae with lamellae
 228 chlorpyrifos reduced CTmax already at a lower concentration compared to the animals without
 229 lamellae (Figure 2). In general, CTmax was lower in autotomized larvae (main effect autotomy:

230 $F_{1, 106} = 21.60$; $p < 0.001$) and in animals exposed to chlorpyrifos (main effect pesticide: $F_{3, 106} =$
 231 17.70 ; $p < 0.001$).

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233

234 Figure 2: Mean upper thermal tolerance (measured as critical thermal maximum, CTmax) (with 95 %
 235 confidence intervals) of *Coenagrion puella* damselfly larvae as a function of pesticide exposure and
 236 autotomy. Different letter codes indicate significant differences between the treatments based on Tukey
 237 post hoc tests with lower case letter codes illustrating the effect of the pesticide for larvae with lamellae
 238 and capital letters illustrating the effect of the pesticide for larvae without lamellae.

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Discussion

We tested whether a widespread morphological antipredator mechanism such as autotomy may shape the sensitivity of prey organisms to pesticides and to heat. As expected, gill autotomy resulted in damselfly larvae being less sensitive to chlorpyrifos (for growth rate), but more sensitive to warming (reduced CT_{max}). Furthermore, our results support the “contaminant-induced climate sensitivity concept” (Sokolova & Lanning 2008; Noyes & Lema 2015) by showing that larvae exposed to chlorpyrifos had a reduced heat tolerance.

The pesticide caused no mortality but resulted in a reduced growth rate (on average -54 %) and a decrease in heat tolerance (CT_{max}) (on average -0.95 °C). A lower growth rate during chlorpyrifos exposure has been shown in many aquatic species (e.g. tadpoles [Widder & Bidwell 2006], fish [Huynh & Nugegoda 2012]) including damselfly larvae (e.g. Dinh Van et al. 2014a; Janssens et al. 2017). This chlorpyrifos-induced growth reduction can be explained by a combination of a decrease in food consumption (e.g. Widder & Bidwell 2006; Dinh Van et al. 2014b) and an increased investment in costly defence processes against the pesticide. Examples of the latter are increased levels of the stress protein Hsp70 (Scheil et al. 2010; Janssens & Stoks 2013), and increased activity of the detoxification enzyme Glutathione-S-transferase, GST (Cinzia et al. 2006; Janssens & Stoks 2013).

The observed pesticide-induced reduction in heat tolerance matches the general pattern in pesticides (Noyes & Lema 2015), including chlorpyrifos (e.g. Patra et al. 2007; Op de Beeck et al. 2017). Stressors such as pesticides may result in a reduced heat tolerance by causing oxygen demand to be higher than oxygen supply (Verberk & Bilton 2011, 2013). Individuals exposed to pesticides indeed often increase their aerobic metabolism, and thus oxygen consumption. For example, Bednarska & Kaszowska (2014) showed in ground beetles exposed to chlorpyrifos an

increase in respiration rate. Increases in respiration rate in response to moderate stress are assumed to be general and to be driven, amongst others, by the increased energy demand to activate mechanisms for protection and damage repair (Sokolova 2013). Whether pesticide exposure also reduces the internal supply of oxygen to body tissues is unknown. Other processes that reduce the available energy for the organism and thereby affect the aerobic performance may also contribute to the pesticide-induced lower CT_{max} (reviewed in Noyes & Lema 2015). For example, exposure to chlorpyrifos has been shown to lower food intake in the study species (Tüzün et al 2015), and to lower food intake and energy stores in other damselfly species (e.g. Dinh Van et al. 2014a, b; Arambourou & Stoks 2015; Janssens et al. 2017).

As expected, the heat tolerance was lower in animals without lamellae. The caudal lamellae of damselfly larvae function as gills for the uptake of oxygen, and this function gets more important with increasing temperature (Eriksen 1986). Given the importance of oxygen supply for the heat tolerance in damselfly larvae (Verberk & Calosi 2012), this may explain the lower CT_{max} in autotomized animals. Similarly, Eriksen (1986) showed in another damselfly species, that larvae with lamellae could better survive long-term exposure to higher water temperatures than larvae without lamellae. A reduction in heat tolerance has been a largely ignored cost of autotomy (for example, not included in the overview given by Fleming et al. 2007) that likely will become more important in a warming world. Note that autotomy did not reduce the growth rate, which may be explained by the presence of ad libitum food (Starostova et al. 2017).

A key finding of our study was that the negative impact of the pesticide for growth was greater in intact, non-autotomized animals. Indeed, the lowest observed effect concentration (LOEC) for growth rate was higher for autotomized larvae (1.5 µg/l) compared to larvae without

lamellae (1 µg/l). Since chlorpyrifos is a hydrophobic compound it is expected to sorb onto the outer surface of the larvae, making surface area to volume relationships important determinants of chlorpyrifos accumulation (Buchwalter et al 2004; Rubach et al. 2012). Given the surface of lamellae can make up a considerable amount relative to the total outer surface area of damselfly larvae (e.g. up to 68% in the damselfly *Lestes disjunctus*, Eriksen 1986), lamellae are not only important for oxygen uptake (Eriksen 1986), but likely also for the uptake of pollutants. Hence, animals with lamellae likely had a higher internal chlorpyrifos accumulation. Similarly, Phillips et al. (2002) showed an increased sensitivity to chlorpyrifos in juvenile walleyes at the moment they developed gill filaments and more sophisticated respiratory systems and also hypothesized this to be the result of increased uptake of the pesticide. As damselflies cannot actively remove their lamellae they cannot exploit autotomy to reduce their sensitivity. Moreover, lamellae autotomy comes with costs such as increased susceptibility to predation (see introduction). Nevertheless, exposure to chlorpyrifos has been shown to increase the degree of autotomy in larvae from northern populations in another coenagrionid damselfly when tested under mild warming (Op de Beeck et al. 2018). Noteworthy, in *Tubifex* worms the self-amputation of the contaminated, posterior body regions has been described as an active strategy to excrete contaminants (Paris-Palacios et al. 2010).

Implications for risk assessment of pesticides

Our findings are important to improve risk assessment of pesticides and illustrate the relevance of integrating freshwater ecology and ecotoxicology (Gessner & Tlili 2016). Coping with extreme temperatures will become increasingly important under climate change, since heat waves will become more intense, more frequent and of longer duration (Christidis et al. 2015). Our data demonstrate a clear interaction between a pollutant and an antipredator mechanism in

terms of growth rate and show that heat tolerance can be a more sensitive biomarker than the more typically used sublethal biomarker growth rate. Indeed, the non-autotomized larvae already showed a decrease in CT_{max} at a chlorpyrifos concentration of 0.75 µg/l, while the growth reduction was only observed at a concentration of 1 µg/l.

An increasing number of studies demonstrated that predation risk can make pesticides more toxic (e.g. Relyea & Mills 2001; Campero et al. 2007) and that pesticide exposure may impair antipredator mechanisms, such as predator escape responses (Janssens & Stoks 2012). To our knowledge this study is the first to show an antipredator mechanism reducing the sensitivity to a pollutant. The beneficial effect of autotomy by reducing pesticide sensitivity is expected to be general as it is most likely operating through the reduction of uptake of the pesticide because of a reduced surface area. This would mean that in natural damselfly populations where autotomy frequently occurs (Stoks 1998a; Witt et al. 2013), animals could be less sensitive to pesticides than predicted based on laboratory studies. While the mechanism is likely common amongst taxonomic groups, the predicted effects can vary. For example, tadpoles typically increase their tail surface area when exposed to predatory dragonfly larvae (e.g. Van Buskirk & McCollum 2000), which by increasing the outer surface area may make them more vulnerable to pollutants such as chlorpyrifos. Other examples of morphological antipredator responses that will increase the outer surface area are the change to a deep-bodied morph in carps in response to gape-limited predators (e.g. Domenici et al. 2007) and the formation of neck teeth in water fleas in response to predator kairomones (e.g. Hanazato & Dodson 1995). Given the importance of pollution, predation and warming as structuring forces in aquatic food webs (Kerfoot & Sih 1987; Thomas et al. 2004; Halstead et al. 2014) exploring these potential interactions between antipredator

331 mechanisms, their associated costs, and sensitivity to pollutants will be crucial to improve the
332 risk assessment of pollutants in aquatic systems under climate change.

333 **Conflict of interest**

334 The authors have no conflict of interest to declare.

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