Contaminant mixtures interact to impair predator-avoidance behaviours and survival in a larval amphibian

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ABSTRACT

Global declines in amphibian populations are a significant conservation concern, and environmental contamination is likely a contributing driver. Although direct toxicity may be partly responsible, contaminants are often present at sub-lethal concentrations in the wild. Behavioural end-points are becoming an increasingly useful method to estimate the impact of contaminants, particularly if the behavioural responses manifest to affect individual fitness (i.e. survival, growth, or reproduction). In the wild, most animals are affected by multiple stressors, and determining how these interact to affect behaviour is critical for understanding the ecological implications of contaminant exposure. Here, we examined the individual and interactive effect of the heavy metal copper and the insecticide imidacloprid on mortality rates and anti-predator behaviours of spotted marsh frog (*Limnodynastes tasmaniensis*) tadpoles. This common species frequently occupies and breeds in contaminated stormwater and agricultural wetlands, where copper and imidacloprid are often present. These contaminants may alter behaviour via physiological and neurological pathways, as well as affecting how tadpoles respond to chemical cues. Tadpoles suffered unexpectedly high mortality rates when exposed to imidacloprid concentrations well below published LC50 concentrations. Only unexposed tadpoles significantly avoided predator cues. Copper and imidacloprid reduced swimming speed and distance, and escape responses, while increasing erratic swimming. We observed an interactive effect of imidacloprid and copper on erratic swimming, but in general imidacloprid and copper did not act synergistically. Our results suggest that as contaminants enter waterbodies, tadpoles will suffer considerable direct mortality, reduced foraging capacity, and increased susceptibility to predation. Our results provide the first evidence of imidacloprid affecting amphibian behaviour, and highlight both the adverse effects of copper and imidacloprid, and the importance of exploring the effect of multiple contaminants simultaneously.

Keywords: *amphibian, behaviour, copper, HIREC, imidacloprid, multiple stressors, predator avoidance, urbanisation*

1. Introduction

Humans have caused unprecedented rates and scales of environmental change, with considerable impacts on wildlife (Pereira et al., 2010; Vitousek et al., 1997). These impacts are often assessed via changes in abundance or species richness (Sievers et al., 2018a). Although these population- and community-level metrics indicate that animals are present, they offer little information regarding functional responses to environmental change (Palmer and Febria, 2012), and may provide misleading information on the value of habitats since these metrics do not incorporate survival and reproductive measures (Sievers et al., 2018a).

When the environment changes, animals often respond initially by altering their behavior, and this can be pivotal in determining how successful animals are in these new conditions (Tuomainen and Candolin, 2011; Wong and Candolin, 2015). One reason this is important is that behavioural responses are closely linked to fitness (i.e. survival, growth and reproduction), particularly when considering contaminant-induced behavioural change (Montiglio and Royauté, 2014; Zala and Penn, 2004). The sensitivity of many behaviours may also allow sub-lethal effects to be detected at lower levels of contamination than those typically needed to observe direct reductions in fitness (Zala and Penn, 2004). Consequently, behavioural end-points are becoming increasingly useful for estimating the impact of contaminants within natural systems.

Amphibians are one of the most imperilled taxa (Monastersky, 2014), and often live and breed in locations that are designed to receive contaminated water (e.g. stormwater wetlands receiving urban runoff; Brand and Snodgrass, 2010) or located in areas inherently prone to intentional contamination (e.g. agricultural wetlands receiving pesticides and fertilisers; Hazell et al., 2001). Amphibians in general, and their aquatic larval stages in particular, are thus often exposed to a variety of contaminants that affect fitness (Egea-Serrano et al., 2012) and behaviour (Shuman-Goodier and Propper, 2016). For example, heavy metals can affect tadpole swimming by reducing sprint speeds and distances, and causing erratic swimming, behavioural alterations which may affect fitness by increasing predation risk (Brunelli et al., 2009; Hayden et al., 2015).

Amphibians use chemical cues to locate resources, detect conspecifics and avoid predators (Lürling and Scheffer, 2007). Exposure to contaminants can affect responses to cues in other taxa by compromising sensory acuity (e.g. copper can damage the olfactory bulb; Tierney et al., 2010) or by changing the characteristics of the cues (e.g. humic acid can bind to conspecific cues; Fisher et al., 2006). Contaminant exposure may also cause amphibians to fail to perceive or respond to important cues. For example, tadpoles exposed to a sub-lethal concentration of the herbicide glyphosate exhibited impaired responses to olfactory cues from injured conspecifics – an indicator of predation risk – due to the loss of function of alarm cues (Moore et al., 2015). Coined ‘info disruptors’ (Lürling and Scheffer, 2007), these contaminants – which typically act at concentrations well below those examined in standard ecotoxicological tests – may represent a significant threat to amphibians. Therefore, contaminants can impact growth, development and survival directly through toxicity and the resulting morphological or physiological changes (Egea-Serrano et al., 2012), or indirectly by impairing behaviours which can increase vulnerability to predators and reduce foraging efficiency (Bridges, 1999).

Most animals are affected by multiple stressors (Jackson et al., 2016; Ormerod et al., 2010), such as different classes of chemical contaminants (e.g. heavy metals and pesticides). Determining how these interact to impact behaviour is critical for understanding the ecological implications of contaminant exposure under natural conditions (Halfwerk and Slabbekoorn, 2015), yet it is more common to study the effect of individual, rather than combined, stressors. Multiple stressors can act: synergistically (outcome is stronger than predicted based on the sum of the individual effects); antagonistically (outcome is weaker than predicted based on the sum of the individual effects); additively (equal to the sum of the individual effects, i.e. no interaction); or even produce an ‘ecological surprise’, such as when two stressors exhibit no effects in isolation, but do together (Crain et al., 2008; Hale et al., 2017). Given that the various interaction types could have very different ecological consequences, we need to investigate responses to combinations of stressors that occur in nature.

Here, we examined the individual and interactive effects of copper and the insecticide imidacloprid on anti-predator behaviours of spotted marsh frog (*Limnodynastes tasmaniensis*) tadpoles. This common species frequently occupies and breeds in stormwater and agricultural wetlands throughout much of Australia (Hamer and Parris, 2011; Hazell et al., 2001). We previously showed that it responds to olfactory cues from predatory dragonfly larvae, and that this response can be affected by chronic exposure to contaminated natal environments (Sievers et al., 2018b). Copper and imidacloprid often contaminate wetlands in Australia (Allinson et al., 2015; Allinson et al., 2017) and around the world (Unrine et al., 2007; Weston et al., 2009). Imidacloprid acts on the central nervous system (Gervais et al., 2010), and other neurotoxic insecticides are known to affect normal swimming behaviours of amphibians (e.g. Brunelli et al., 2009). To our knowledge, no previous study has focused on behavioural changes caused by exposure to imidacloprid or the interaction between this insecticide and copper.

We conducted short-term laboratory exposures at environmentally relevant concentrations followed by choice experiments to document behavioural responses to predator olfactory cues and tail prodding (i.e. a simulated ‘attack’; Pauli et al., 1999; Van Buskirk and McCollum, 2000). We predicted that tadpoles exposed to contaminants, especially copper and imidacloprid, would be less able to recognise and avoid predators than unexposed tadpoles. We also predicted that pollutant exposure would compromise the swimming behaviour of tadpoles, which could enhance predation risk as sprint speed and distance can be correlated with the ability of a tadpole to escape predators (Bridges, 1999).

2. Materials and Methods

2.1 Study species

The spotted marsh frog *Limnodynastes tasmaniensis* (Günther, 1858) is a ground-dwelling frog native to Australia. We collected a portion of nine egg masses from an isolated wetland (37°37’08.06” S, 145°00’10.67” E) created for the endangered growling grass frog *Litoria raniformis* (Keferstein, 1867). We hatched eggs and reared tadpoles in RO/DI water with artificial salt medium within individual 2L, acid-washed containers, and fed tadpoles crushed flake food and lettuce until experimentation at 23 days old (mean length ± SE: 9.02 ± 0.21mm, n = 20).

2.2 Creation of predator cues

We created predator cues using locally collected dragonfly larvae (Suborder: Epiprocta); a ubiquitous and voracious tadpole predator commonly used in predator detection and avoidance experiments (Hanlon and Relyea, 2013; Sievers et al., 2018b). To harvest predator odours, we kept 36 dragonfly larvae in 4 L of aged tap water for 18 h, which was subsequently diluted to 20 L to achieve a final concentration of 1 larva per 0.56 L of odour water (similar to Carlson and Langkilde, 2013; Ehrsam et al., 2016). All cue water was made fresh daily and used within 12 h.

2.3 Contaminant preparation and exposure

Much of the copper within urban stormwater wetlands comes from vehicles, especially brake pads and tyres (Sansalone and Buchberger, 1997). The neonicotinoid insecticide imidacloprid is primarily registered in Australia for use against general household insects, insect pests of fruits and vegetables, and termites (Allinson et al., 2015), and has a high potential to enter urban wetlands due to its high solubility and persistence in water. Although widely studied for its unintended impact on bees (Cresswell, 2011), very few studies have investigated its impact on amphibians.

We conducted exposures using a fully crossed, 3 x 3 experimental design. We dosed copper at 0, 10 and 20 μg/L, matching levels found within urban wetlands (Allinson et al., 2017) and below lethal concentrations for other amphibians (Barry, 2011; Chen et al., 2007). We dosed imidacloprid at 0, 0.25 and 0.50 μg/L, again matching levels occurring within urban wetlands (Allinson et al., 2015; Weston et al., 2009), and below lethal concentrations for other amphibians (Feng et al., 2004; Quan et al., 2006).

We made up stock solutions of copper (from $CuNO_3$ salt; nominal 5 mg/L) and imidacloprid (as the commercial formulation Confidor 200SC (Bayer), 50 g/kg active ingredient; nominal 10 μg/L). During the trial period, we diluted stock solutions daily with RO/DI water, and samples from each treatment were analysed by SymBio Laboratories (Eight Miles Plains, QLD, Australia).

We exposed tadpoles for approximately 24 h within 2 L acid-washed containers. This time period was chosen following Hayden et al. (2015), who observed behavioural modifications following 24-h exposures to copper in wood frogs (*Lithobates* (*Rana) sylvatica*). Morning and afternoon batches of tadpoles received their treatments 6 hours apart to keep exposure time consistent.

2.4 Experimental trials

We conducted trials within plastic choice tanks (38 × 17 × 13 cm; L × W × H), with two 10 L carboys above each providing olfactory cues, as for previous work (Sievers et al., 2018b). We provided cues by dripping water into the tanks at a flow rate of ~50 ml/min, and switched the side that the predator cues were applied to the choice tanks every 15 trials. Each trial started with an initial 2-min acclimation period (and to allow the cues to reach the centre of the tank), followed by a 5-min examination period consisting of a 4-min observation period and 1-min manipulation period. We lifted the perforated cylinder (50 mm diameter) within the choice tank that held the tadpoles during the initial 2 min at the beginning of the examination period. Preliminary tests with dye revealed these times were sufficient for some cue to reach the middle of the choice tank by the beginning of the examination period without much mixing of cues by the end of the observation period. The manipulation period involved gently prodding the tail of each tadpole three times with a glass probe, to initiate a scare response or simulated predatory attack (Pauli et al., 1999; Van Buskirk and McCollum, 2000).

We recorded each trial using a GoPro Hero 3+ above each tank. Tanks were illuminated from underneath by a single fluorescent tube to avoid reflections from the water surface that can interfere with video analysis. Due to unexpected mortality and some videos cutting off part-way through a trial, the total number of replicates per treatment varied from 13 to 20 (Supplementary Table 1), and an additional two buckets with five tadpoles in each were added to the highest concentration treatment which suffered the greatest mortality (i.e. 20 µg/L copper and 0.50 µg/L imidacloprid).

2.5 Behavioural responses

Videos of the 4-min observation period were analysed using the tracking software SWISTrack 4.0 (Lochmatter et al., 2008) which provides an output of x and y coordinates of the centre of the tadpole at every frame of the video (see Supplementary Appendix 1 for details on the software). From the outputs, we calculated predator avoidance (the proportion of time spent at the predator side minus the proportion of time spent at the non-predator side, with the middle 50 mm excluded), the proportion of time spent inactive (the proportion of frames in which the tadpole had not moved since the previous frame), and the total distance travelled (the sum of the Euclidean distance travelled between frames based on x and y pixel coordinates, converted to mm). We used the plugin MTrackJ (Meijering, 2006) in ImageJ32 (Abràmoff et al., 2004) to analyse responses to a simulated attack (i.e. prodding). We calculated distance travelled per prod (mm), swimming speed per prod (mm/s), escape response (time taken to swim 25mm following the first prod; Van Buskirk and McCollum, 2000), and erratic swimming (the number of turns made greater than 90 degrees).

2.6 Statistical analysis

To examine the effect of contaminant exposure on mortality rates, we fitted a generalised linear model (family: binomial) with copper and imidacloprid concentrations fitted as fixed effects, and analysed the model using chi-squared analysis of deviance. For this analysis, the treatment container was considered the replicate, while for behavioural responses, the individual tadpole was considered the replicate. To examine behavioural responses, we created generalised linear mixed-effects models and conducted analysis of variance (ANOVAs) with copper and imidacloprid concentrations fitted as fixed effects, and treatment container fitted as a random effect. For the predator avoidance variable, we also obtained model estimates and their 95% CI. We assessed normality and homogeneity of variances before all analyses using Q-Q and residual plots, respectively. Variables violating these assumptions (predator avoidance, distance travelled during the 4 min, escape response, and erratic swimming) were log-transformed. We performed all analyses in R 3.2.2 (R Development Core Team, 2015).

3. Results

Contaminants were not detected in the 0 µg/L treatments. Actual concentrations for the 0.25 and 0.50 µg/L nominal imidacloprid treatments were (mean ± SE) 0.22 ± 0.02 and 0.46 ± 0.02, respectively. For copper, actual concentrations for the 10 and 20 µg/L nominal treatments were 10.1 ± 2.4 and 19.5 ± 3.5, respectively.

Imidacloprid and copper increased tadpole mortality (Table 1), although all tadpoles survived the copper treatments when imidacloprid was absent (Figure 1). In general, when imidacloprid was present, mortality rates increased as copper concentration increased (Figure 1). At 0.50 µg/L, imidacloprid caused 17% (95% CI: 1.4 to 31.9%) mortality when copper was absent, reaching 33% (95% CI: 16.2 to 50.5%) when copper was present at 20 µg/L (Figure 1).

Only tadpoles that were not exposed to any contaminant (i.e. the controls) avoided predator cues (95% CI: -0.62 to -0.21). All other treatment groups showed no substantial avoidance or attraction (Table 2; Figure 2a). During this 4-min observation period, there was little effect of contaminant exposure on the proportion of time tadpoles spent inactive (Table 2; Figure 2b). Tadpoles tended to swim shorter distances as copper concentrations increased (Table 2; Figure 2c), but the distance moved by tadpoles following prodding was little affected by exposure (Table 2; Figure 3a). After prodding, however, average swimming speed decreased with increasing copper concentration (Figure 3b).

Copper and imidacloprid also affected escape response (Table 2). In particular, the tadpoles exposed to the higher concentrations of both contaminants took more than twice as long to move 25 mm following a simulated attack than in any other treatment combination (Figure 3c). Copper and imidacloprid interacted to increase erratic swimming (Table 2); copper had little effect when imidacloprid was absent or at 0.50 µg/L, but at 0.25 µg/L, swimming became more erratic as copper concentration increased (Figure 3d).

4. Discussion

4.1 Direct mortality from contaminants

Mortality of exposed tadpoles during our study was unexpectedly high. This result contrasts strongly with the (limited) data published for imidacloprid LC50 concentrations: 52.6 mg/L (Pérez-Iglesias et al., 2014) and 165–219 mg/L (Feng et al., 2004), values thousands of times higher than the concentrations (up to 0.50 µg/L) we tested here. There may be several reasons for this discrepancy. Previous studies were conducted in different countries on different species which may be naturally hardier, or may have been exposed to imidacloprid for longer, and are thus, less susceptible (e.g. Boyd et al., 1963). In addition, there were considerable differences in tadpole age and size between our study and these toxicology studies. Our tadpoles were exposed well before limb-bud development at 9 mm SVL and approximately Gosner Stage 22, while those from Pérez-Iglesias et al. (2014) were at Stage 35–37, and the two species from Feng et al. (2004) were 33 and 37 mm SVL.

Regardless, the considerable mortality caused by short-term exposure to imidacloprid is concerning given similar levels have been recorded in wetlands within the study region and elsewhere (Allinson et al., 2017; Weston et al., 2009). Furthermore, when imidacloprid was present, there was some evidence that mortality increased as copper increased. Further research on how these contaminants may interact to affect mortality are needed, and work on exposing tadpoles across a range of size and age classes (not just at fixed life-history stages – i.e. embryo, tadpole, metamorph) is required to inform how susceptibility changes throughout development.

4.2 Avoidance of predator cues

Tadpoles exposed to environmentally relevant concentrations of copper and imidacloprid did not avoid predator odours. This is consistent with our previous work on spotted marsh frog tadpoles raised in low-quality natal environments containing a suite of contaminants which similarly did not avoid predator cues (Sievers et al., 2018b). Conversely, at a copper concentration (5 µg/L) lower than those tested here (10 and 20 µg/L), the ability of wood frog tadpoles to detect predator cues was not obviously affected (Reeves et al., 2011). Since copper concentrations within urban wetlands across Melbourne reached 15 µg/L (Allinson et al., 2017), some tadpoles in our study area likely experience reduced capacity to detect or avoid predators, particularly given the presence of numerous other contaminants within most urban wetlands.

4.3 Changes in swimming behaviour

Swimming speed and distance can govern an individual's ability to escape a predator (Bridges, 1997) or efficiently locate resources (Bridges, 1999). Swimming distance was affected by exposure to imidacloprid, while copper exposure reduced swimming speed following a simulated attack. Swimming speed was also reduced in Northern leopard frog (*Rana pipiens*) tadpoles following exposure to similar levels of copper (Chen et al., 2007). Escape response was affected by both contaminants, most notably a large increase in the time to move 25mm in the highest combined treatment. This metric approximates how quickly a tadpole can move to evade a strike from a sit-and-wait predator, and is related to velocity and acceleration, but also differentiates between tadpoles that swim in one direction and those that swim erratically (Van Buskirk and McCollum, 2000).

Imidacloprid exposure increased erratic swimming, and like escape response, this metric was most affected in the highest combined treatment. Erratic swimming has been observed in common toad (*Bufo bufo*) tadpoles following exposure to endosulfan, and was considered a sign of neurotoxic stress (Brunelli et al., 2009).

Like endosulfan, imidacloprid is a neurotoxin, acting on the central nervous system (Gervais et al., 2010). Similar neurotoxic effects (e.g. abnormal swimming and spasmodic movements) were observed in South American toad (*Rhinella arenarum*) tadpoles following exposure to low levels of copper (3.75–7.5 µg/L; Aronzon et al., 2011) and the authors posited this was due to acetylcholinesterase inhibition, which was also observed in wild frogs exposed to insecticides (Sparling et al., 2001).

Although low levels of copper (5 µg/L) reduced activity levels of wood frog tadpoles (Reeves et al., 2011), we observed little difference in activity levels amongst treatment groups. Increased activity levels can increase foraging rates but also increase predator detection (Azevedo-Ramos et al., 1992). Decreased activity can thus reduce predator detection, but also competitive ability and feeding rates, ultimately reducing energy intake and development (Dayton and Fitzgerald, 2001; Horat and Semlitsch, 1994). Therefore, the fitness consequences of activity levels can be variable and will likely be highly context-dependent.

4.4 Interactive effects

Understanding how contaminants may interact to affect tadpole behaviour was one of the key aims of this study. Multiple contaminants can interact in different ways to more or less strongly affect responses than would be predicted based on the sum of the two independent stressors (Piggott et al., 2015). We observed an interactive effect of imidacloprid and copper on erratic swimming, whereby copper increased erratic swimming at the low concentration of imidacloprid, but at the high concentration or when imidacloprid was absent, copper had little effect. In general, however, imidacloprid and copper did not interact to affect most of the behavioural responses measured here, potentially due to them having similar neurotoxic modes of action.

Still, copper affected several responses, while imidacloprid affected others, meaning that although these two contaminants may not often interact, the presence of both still affects more behavioural responses then either one in isolation.

This is the first work looking at the interactive effects of copper and imidacloprid on amphibian behaviour. Our results show that only tadpoles that were not exposed to either pollutant avoided cues from predators. Our results show that only tadpoles that were not exposed to either pollutant avoided cues from predators. In the future, it will be important to examine responses across a greater range of concentrations than we examined, particularly between 0 and 10 ug/L copper in order to identify the threshold copper concentration at which the detection of predator cues and subsequent behavioural responses are impaired.

4.5 Significance for wild amphibian populations

The tendency for exposed amphibians to not avoid predator odours in combination with impaired swimming can manifest to reduce survival, growth and development by decreasing foraging rates and efficiency, and increasing vulnerablility to predation. Tadpoles exposed to pesticides can be less likely to feed (Krishnamurthy and Smith, 2011) and be more susceptible to being eaten (Verrell, 2000). These outcomes may have considerable implications for amphibian populations living in contaminated habitats, and may help explain population declines within habitats containing contaminants at concentrations well below known toxic levels (Davidson et al., 2001; Davidson et al., 2002).

Although our results suggest that as contaminants enter waterbodies, young tadpoles will suffer considerable mortality, have reduced foraging capacity, and be more susceptible to predation, we echo the sentiments of Ehrsam et al. (2016) in cautioning against assuming that the behavioural modifications observed will mean that population-level responses occur. Exposed predators may, for example, be less able detect and capture prey (Weis et al., 2001)**,** partly offsetting impaired anti-predator behaviours. In addition, the ubiquity and diversity of potential contaminants (both natural and anthropic) may render accurate detection of predator olfactory cues impossible in most aquatic habitats, and the presence of visual cues in addition to olfactory cues may compensate for any olfactory disruption (e.g. Sievers et al., 2018b), allowing even exposed tadpoles to successfully avoid being eaten. Therefore, the impact of exposure to contaminants in the wild is likely to be a complex outcome of species-specific susceptibilities to particular contaminants, species interactions and various abiotic factors (Hayden et al., 2015; Sih et al., 2004).

Nonetheless, documenting behavioural end-points is becoming an increasingly useful method to estimate the impact of contaminants within natural systems. Our results add to this growing body of research, highlighting both the adverse effect of copper and imidacloprid, and the importance of researching multiple contaminants simultaneously. Future work should aim to investigate how these behavioural alterations manifest to affect individual fitness and population dynamics under more natural conditions.

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Tables

Table 1. Output from analyses of deviance (family: binomial) on tadpole mortality ($^{\#}$ p = 0.0458). Rdf = residual degrees freedom, Dev = deviance, and RDev = residual deviance. Boldface values indicate statistical significance.

	Mortality				
Treatment	df	Rdf	Dev	RDev	p
Copper	**2**	**33**	**6.2**	**10.8**	**$^{\#}$0.05**
Imidacloprid	**2**	**35**	**23.0**	**16.9**	**0.00**
Cu × Imid	4	29	2.6	8.2	0.63
Null		37		40.0	

Table 2. Output from analyses of variance (linear mixed effects models) on tadpole predator avoidance and swimming behaviour during the initial 4-min predator cue trial, and on tadpole behaviour following tail prodding. For detailed description of behavioural responses see Methods. Asterisks indicate variables that were log-transformed. P-values are calculated based on Satterthwaite's approximations ($^{\#}$ p = 0.0496). Ndf = numerator degrees freedom, and Ddf = denominator degrees of freedom. Boldface values indicate statistical significance.

Factor	**Ndf**	**MS**	**Ddf**	***F***	**p**
4-min observation period					
Predator avoidance*					
Copper	2	1.43	144	1.1	0.33
Imidacloprid	2	**4.61**	**144**	**3.6**	**0.03**
Cu × Imid	4	1.00	144	0.8	0.55
Distance moved*					
Copper	2	0.90	140	1.2	0.30
Imidacloprid	2	**2.28**	**140**	**3.1**	$^{\#}$**0.05**
Cu × Imid	4	1.02	140	1.4	0.25
Proportion inactive					
Copper	2	0.06	140	2.2	0.11
Imidacloprid	2	0.05	140	2.0	0.13
Cu × Imid	4	0.03	139	1.0	0.41
Tail-prodding					
Distance moved					
Copper	2	0.06	140	2.2	0.11
Imidacloprid	2	0.05	140	2	0.13
Cu × Imid	4	0.03	139	1	0.41
Swimming speed					
Copper	2	**1545**	**149**	**5**	**0.01**
Imidacloprid	2	195	149	0.6	0.54
Cu × Imid	4	381	149	1.2	0.3
Escape response*					
Copper	2	**2.74**	**145**	**6.3**	**0**
Imidacloprid	2	**10.97**	**145**	**25.3**	**0**
Cu × Imid	4	0.9	145	2.1	0.09
Erratic swimming*					
Copper	2	0.97	149	2.4	0.09
Imidacloprid	2	**10.63**	**149**	**26.7**	**0**
Cu × Imid	4	**1.36**	**149**	**3.4**	**0.01**

Figures

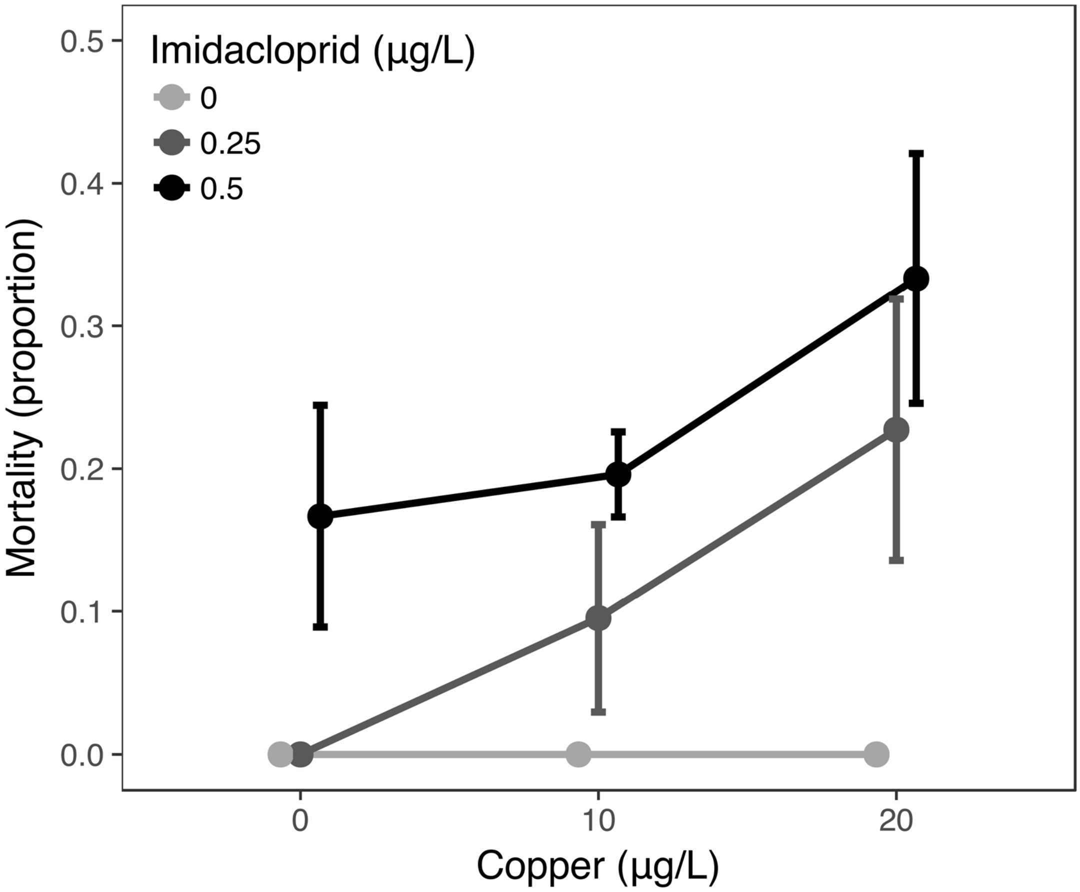

Figure 1. The proportion of tadpoles dying following 24-hour exposure to copper, imidacloprid and the combination of both. Mean ± SE are shown, with points intentionally jittered.

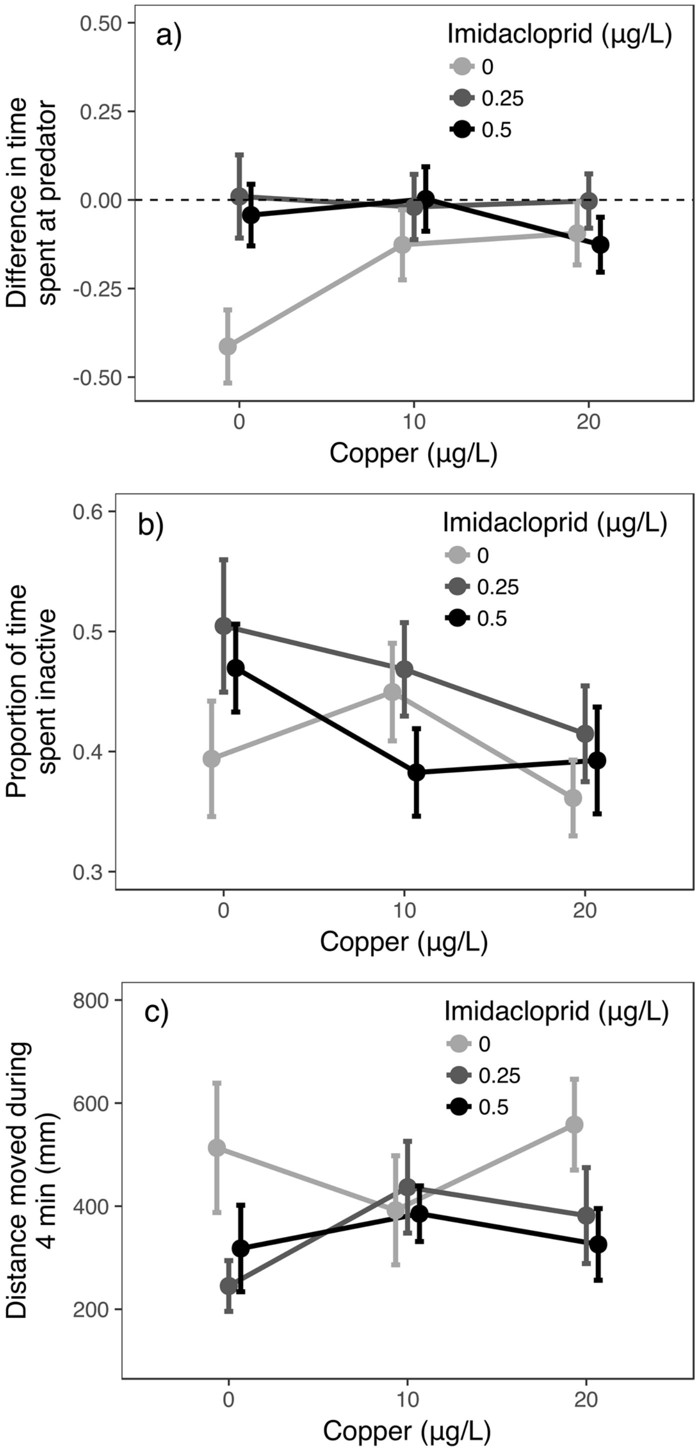

Figure 2. Behavioural responses recorded during the initial 4-min observation period following 24-hour exposure to copper, imidacloprid and the combination of both.. Predator avoidance (a) was calculated as the proportion of time spent at the predator side minus the proportion of time spent at the non-predator side, with the centre 50mm excluded. The proportion of time spent inactive (b) was calculated as the

proportion of frames in which the tadpole had not moved since the previous frame. Finally, the total distance travelled (c) was calculated as the sum of the Euclidean distance travelled (based on x, y pixel coordinates) between frames. Mean ± SE are shown, with points intentionally jittered.

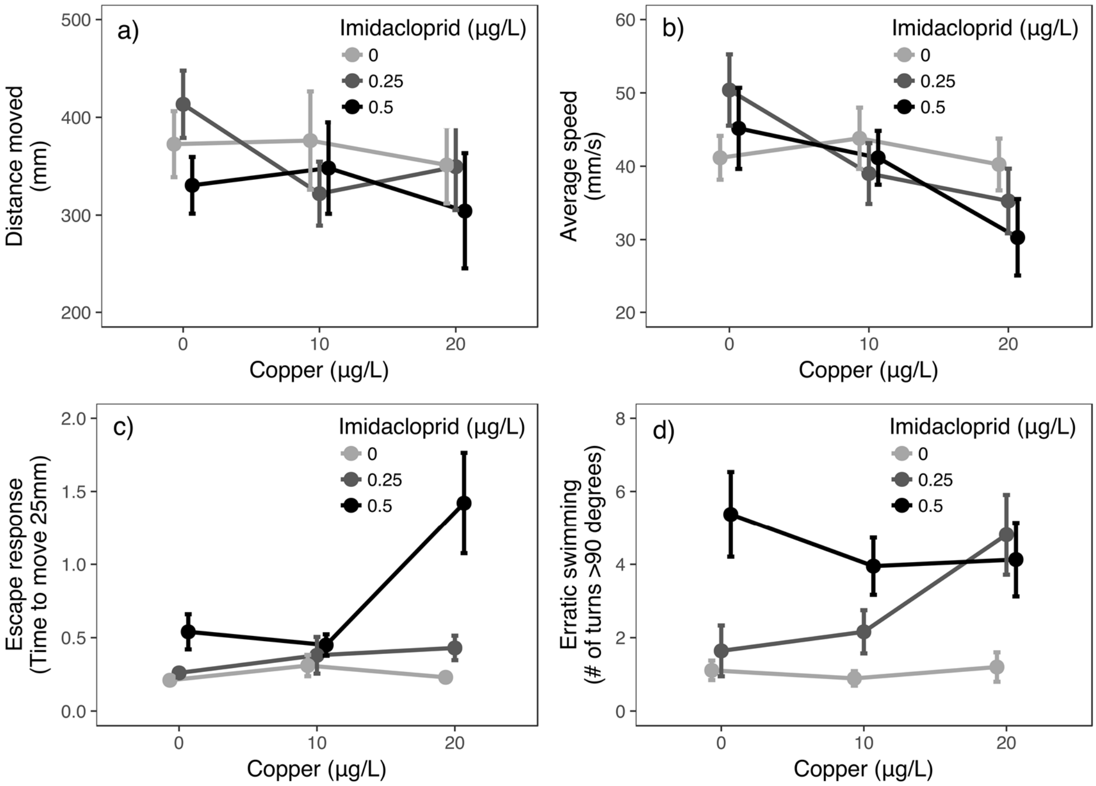

Figure 3. Behavioural responses recorded during the prodding period following 24-hour exposure to copper, imidacloprid and the combination of both.. Total distance travelled (a), average swimming speed (b), escape response (c; time taken to swim 25mm following the first prod), and erratic swimming (d; the number of turns made greater than 90 degrees). Mean ± SE are shown, with points intentionally jittered.

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