Synergistic effects of glyphosate formulation and parasite infection on fish malformations and survival

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Summary

1. Anthropogenic pollution and disease can cause both lethal and sub-lethal effects in aquatic species but our understanding of how these stressors interact is often not known. Contaminants can reduce host resistance to disease, but whether hosts are impacted at environmentally relevant concentrations is poorly understood.

2. We investigated the independent and combined effects of exposure to the common herbicide glyphosate and the trematode parasite *Telogaster opisthorchis* on survival and the development of spinal malformations in juvenile *Galaxias anomalus*, a New Zealand freshwater fish. We then investigated how exposure to a glyphosate concentration gradient (0·36, 3·6, 36 mg active ingredient (a.i.) L^{-1}) affected the production and release of the infective cercarial stage of the parasite by its snail intermediate host *Potamopyrgus antipodarum*.

3. Survival of juvenile fish was unaffected by exposure to glyphosate alone (at an environmentally relevant concentration; 0·36 mg a.i. L^{-1}) or by *T. opisthorchis* infection alone. However, simultaneous exposure to infection and glyphosate significantly reduced fish survival.

4. Juvenile fish developed spinal malformations when exposed either to infections alone or to infections and glyphosate, with a trend towards greater severity of spinal malformation after exposure to both stressors.

5. All snails exposed to the highest glyphosate concentration (36 mg a.i. L^{-1}) died within 24 h. Snails exposed to a moderate concentration (3·6 mg a.i. L^{-1}) produced significantly more *T. opisthorchis* cercariae than snails in the control group or the low concentration group (0·36 mg a.i. L^{-1}; the same concentration as in the fish experiment).

6. *Synthesis and applications.* This is the first study to show that parasites and glyphosate can act synergistically on aquatic vertebrates at environmentally relevant concentrations, and that glyphosate might increase the risk of disease in fish. Our results have important implications when identifying risks to aquatic communities and suggest that threshold levels of glyphosate currently set by regulatory authorities do not adequately protect freshwater systems.

Key-words: disease, fish populations, glyphosate, malformation, multiple stressors, parasite, Roundup, toxicity tests

Introduction

Anthropogenic stressors are a pervasive problem in freshwaters because these ecosystems can act as conduits or sinks that accumulate and concentrate contaminants (Rohr *et al.* 2008a; Relyea 2009). Understanding how aquatic systems respond to stressors, which is fundamental to their management, is far from straightforward because often multiple stressors are in

operation (Allan 2004; Culp & Baird 2006). Stressors can have both lethal and non-lethal effects that impact on individuals and populations, and these may be additive, antagonistic or synergistic (Relyea 2005a; Budischak, Belden & Hopkins 2008; Townsend, Uhlmann & Matthaei 2008; Hu *et al.* 2009). Regulatory bodies tend to impose maximum allowable concentrations for aquatic pollutants based on standard short-term (96-h) toxicity tests, but by failing to account for interactions among stressors managers may underestimate or overestimate risk (Relyea 2005a; Budischak *et al.* 2008; Townsend *et al.* 2008).

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In contrast to anthropogenic pollutants, parasitism and disease are natural stressors that can influence hosts by affecting survival, reproduction and/or development (Kiesecker 2002; Tompkins *et al.* 2002; Johnson & Chase 2004). Despite increasing awareness that anthropogenic stressors and disease may interact (Lafferty & Kuris 2005; Johnson *et al.* 2007), their combined effects have received attention only in recent studies (e.g. Budischak *et al.* 2008; Sures 2008). For example, the host immune response may be weakened by other forms of environmental stress (Hoole *et al.* 2003; Rohr *et al.* 2008b). Chemically induced immunosuppression can increase host susceptibility to existing infections, or render the host more susceptible to future infection (Daszak, Cunningham & Hyatt 2001; Forson & Storfer 2006; Rohr *et al.* 2008a). Infection can also impair reproduction when anthropogenic contaminants are also present (Morley, Lewis & Hoole 2006). Thus, in aquatic systems, chronic exposure to sub-lethal levels of pollutants may change the impact of diseases on host species (Poulin 1992) by, for example, increasing host morbidity at a given infection level or by increasing infection probability (e.g. Rohr *et al.* 2008b).

Freshwater fish are common hosts for trematode worms whose infective cercariae are released by aquatic snails, the first intermediate host, before encysting as metacercariae in the body or organs of fish, the second intermediate host (Woo 2006; Kelly *et al.* 2009). Infection can cause fish skeletal malformations and mortality, with effects particularly acute in juvenile stages (Kelly *et al.* in press; Cunningham *et al.* 2005). In a previous study, we showed that *Telogaster opisthorchis* infection caused spinal malformations and increased mortality in juvenile *Galaxias anomalus*, affecting population dynamics in the field (Kelly *et al.* in press). *G. anomalus*, a threatened non-migratory freshwater fish native to New Zealand (New Zealand's Department of Conservation 2005), is a common second intermediate host for metacercariae of *T. opisthorchis*; infection prevalence and intensity range 33–100 and 2·6–370, respectively; Kelly *et al.* 2009). *G. anomalus* become infected after the release of cercariae by the snail *Potomopyrgus antipodarum*. Completion of the parasite life-cycle requires transmission to the definitive host (a freshwater eel) by predation on infected *G. anomalus*. For parasites with complex life-cycles, toxicants may modulate such disease impacts in several ways: (1) by direct immunosuppression of the host, (2) by changing transmission probabilities of infective stages from alternative hosts, (3) by changing the survival probability of alternative hosts and (4) by directly influencing survival or infectivity of the disease-causing agent (e.g. Poulin 1992; Rohr *et al.* 2008b). Conversely, infection may weaken host susceptibility to toxins and metal contaminants (Poulin 1992; Hoole *et al.* 2003; Morley, Irwin & Lewis 2003). Here we test whether a common herbicide causes such effects in the *T. opisthorchis/G. anomalus* system.

Glyphosate (*N*-phosphonomethyl-glycine), often applied as the commercial formulation Roundup®, is the most widely used herbicide in the world and, because it inhibits amino-acid synthesis in plants, is of broad spectrum use (Conners & Black 2004; Relyea 2005b; Kolpin *et al.* 2006). The use of glyphosate has expanded rapidly in concert with growth in the agricultural sector and with the production of genetically modified glyphosate-resistant crops such as soybean and corn (Perez *et al.* 2007; Struger *et al.* 2008). While glyphosate as the active ingredient is toxic to aquatic vertebrates only at high concentrations (reported 96h LC50 for fish and amphibians range 97–149 mg L^{-1} and 108–9729 mg L^{-1}, respectively), the surfactant polyethoxylated tallowamine (POEA), which is included in the commercial formulation, is primarily responsible for toxicity (Folmar, Sanders & Julin 1979; Relyea 2005a). Although glyphosate adsorbs to soil and has been considered of low risk to aquatic ecosystems, it may reach freshwaters via spray drift, surface run-off and/or soil leachate, its release being enhanced in the presence of inorganic phosphorus fertilizer (Perez *et al.* 2007; Siemering, Hayworth & Greenfield 2008; Contardo-Jara, Klingelmann & Wiegand 2009).

The current study comprises two laboratory experiments; the first designed to test the independent and combined effects of exposure to *T. opisthorchis* infection and glyphosate on the development of spinal malformations and survival in juvenile *G. anomalus*. The second experiment was designed to determine the influence of a glyphosate concentration gradient on the production of infective cercariae by *P. antipodarum* and on the survival of *P. antipodarum*.

Materials and methods

LABORATORY TRIALS WITH JUVENILE *GALAXIAS ANOMALUS*

To provide infective trematodes, the intermediate host of *T. opisthorchis*, *Potamopyrgus antipodarum* snails, were collected from a small (unnamed) tributary of the Upper Taieri River in New Zealand's South Island (45°06′45″S, 170°15′18″E) in December 2008 and housed in laboratory aquaria at 12 °C. Infected snails were identified by placing individuals in plastic 12-well trays (12 per well), incubating them at 16 °C under intense light, and screening for cercariae after 24 h. Snails not shedding cercariae were repeatedly screened and considered uninfected if no cercariae were shed after five screening cycles.

The fish experiment was conducted during the austral summer (January–February 2009). Juvenile *G. anomalus* were collected from a site of low infection prevalence (3·3%; *n* = 119) in the Upper Taieri catchment (45°08′50″S 170°17′35″E) and maintained in large holding aquaria for 5 weeks. Fish were fed on fine commercial pellet food prior to experimental infections, at which stage they were 7–8 weeks old (length 27·2 mm ± SE 0·2; *n* = 94). Four fish were randomly allocated to each of 32 2 L experimental aquaria and allowed to acclimatise for 2 days. The experiment was conducted in ambient temperature (12–14 °C) and lighting conditions (8 h:16 h L:D cycle) to simulate natural conditions as closely as possible. There were four experimental groups, each with eight replicates: (1) Controls – addition of two uninfected snails plus standard filtered water, (2) *T. opisthorchis* infection alone – addition of two infected snails plus standard filtered water, (3) Glyphosate formulation alone – addition of two uninfected snails plus filtered water with commercial formulation Glyphosate 360 (360 mg L^{-1} plus 10–20% POEA as the surfactant; supplier: Ravensdown New Zealand), diluted to 0·36 mg active ingredient (a.i.) L^{-1} and (4) Combined *T. opisthorchis* and glyphosate

formulation—addition of two infected snails plus glyphosate (0·36 mg a.i. L^{-1}).The choice of the glyphosate formulation exposure concentration was environmentally realistic and within the environmental exposure limit (EEL) of 0·37 mg a.i. L^{-1} set for New Zealand freshwaters by New Zealand's Environmental Risk Management Authority (ERMA NZ, 2005). This is an order of magnitude below toxicity values of glyphosate-based formulations for fish or manufacturer's recommended application rates (e.g. see Folmar *et al.* 1979; Relyea 2005a). The glyphosate concentration used in the formulation was also within or below the range recorded in natural freshwaters (e.g. 2·3–2·6 mg a.i. L^{-1} by Giesy, Dobson & Solomon 2000 and Perez *et al.* 2007; ; 0·33 mg L^{-1} by Battaglin *et al.* 2009). Although glyphosate can adsorb to soil, which we did not include in experimental aquaria, studies have shown that soil presence has no effect on the toxicity of glyphosate formulation (see Relyea 2005a).

Fish were fed fine pellets (twice daily) and snails were fed algal pellets (every 3 days). Snails were allowed to shed cercariae naturally, with the experiment running for 26 days to allow any potential effects of the treatments on fish development and survival to occur. Each replicate was monitored twice daily and at the end of the experiment all fish (including those that died prematurely) were assessed for spinal malformations and numbers of metacercariae were counted. The methods used to prepare fish for fish spinal malformation and infection intensity assessment are reported elsewhere (see Kelly *et al.* in press), but briefly involved specimen fixation in formalin, dehydration in ethanol and tissue clearing in clove oil. The extent of spinal malformation was assessed by counting the total number of spinal deviation points in both scoliotic (lateral) and lordotic (dorsoventral) planes (see Kelly *et al.* in press). Mean infection intensity of *T. opisthorchis*, the severity of spinal malformations, and percentage fish surviving, were compared among treatments using ANOVA. All data were log$_{10}(x + 1)$ or arcsine square root transformed prior to analysis.

CERCARIAL SHEDDING AND SURVIVAL OF *P. ANTIPODARUM*

Potamopyrgus antipodarum infected with *T. opisthorchis* were identified using the methods described in the fish experiment. Infected snails were individually allocated to plastic 2 L aquaria. There were four treatment groups covering a concentration gradient of glyphosate, each with 12 replicates: (1) Control group—standard filtered water, (2) low glyphosate formulation exposure (0·36 mg a.i. L^{-1}), (3) moderate glyphosate formulation exposure (3·6 mg a.i. L^{-1}) and (4) high glyphosate formulation exposure (36 mg a.i. L^{-1}). The treatments were within the glyphosate formulation range used in toxicity experiments (Folmar *et al.* 1979; Relyea 2005b; Rohr *et al.* 2008b) and encompassed levels previously recorded in freshwaters (Battaglin *et al.* 2009). The manufacturer's recommended maximum application rate for Roundup in North America of 3·8 mg a.i. L^{-1} (see Relyea 2005b) is similar to the moderate formulation concentration used here. Each aquarium was supplied with a 5 cm stem of the aquatic macrophyte *Rorrippa nasturtium aquaticum* as a periphyton substratum for snail grazing.

All treatments were run for 10 days at 8° C to allow any responses in snail survival or cercarial production to manifest. The choice of temperature was a compromise between a low temperature that would reduce the metabolic rate of snails and a higher temperature that might induce premature cercarial shedding by snails (see below). Treatments were examined daily and fresh macrophyte was supplied at the start of the second week. After two weeks, snails were individually transferred to 12-well plates and exposed to intense lighting at a temperature of 14 °C for 24 h to stimulate cercarial shedding.

T. opisthorchis cercariae were counted and the snails were allowed to rest for 24 h. This shedding cycle was repeated 6 times to account for possible heterogeneity in cercarial production cycles among snails. At the end of the experiment snail lengths were measured; analysis of covariance (ANCOVA), controlling for snail size (length), was used to test for differences across treatments in mean *per capita* cercarial production per 24 h.

Results

GALAXIAS ANOMALUS SURVIVAL AND MALFORMATIONS

There was a significant treatment effect on mean intensity of *T. opisthorchis* infection ($F_{3,28}$ = 861·6, P < 0·0001) with Fisher's protected least significant difference (FPLSD) *post hoc* analyses showing that fish exposed to *T. opisthorchis* alone or in combination with the glyphosate treatment had significantly higher infection intensities than fish in controls or those exposed to glyphosate alone (FPLSD, all P < 0·0001; Fig. 1a). A single fish in the control group was infected by one metacercaria, whereas two fish in the glyphosate formulation only group had one metacercaria each; these infections probably occurred naturally prior to the collection of juvenile fish from the site of low overall infection abundance (see above). However, the aforementioned infections were rare and insignificant when compared to infection levels of fish in the exposure groups that averaged between 80 and 100 cercariae per fish (Fig. 1a). There was no significant difference in the infection intensity of fish exposed to *T. opisthorchis* only or *T. opisthorchis* in combination with the glyphosate formulation. A *t*-test was conducted to determine whether this lack of difference in infection intensity was confounded by the high mortality of fish in the combined stressor group (see below). For example, because of a shorter exposure time, fish dying prematurely would be expected to have fewer metacercariae than those surviving until the end of the experiment. However, there were no significant difference in mean infection intensity between fish surviving to the end of the experiment in the group exposed to *T. opisthorchis* alone, and those in the combined exposure group (df = 11, *t* = 0·66, NS).

The extent of spinal malformation differed significantly among treatments ($F_{3,28}$ = 9·74, P < 0·0001), being higher in fish exposed either to *T. opisthorchis* infection alone or a combination of *T. opisthorchis* and glyphosate formulation exposure, than in control fish or those exposed to the glyphosate formulation alone. No developmental malformations occurred in the latter two groups (FPLSD, all P < 0·01; Fig. 1b). Although there was a trend toward greater severity of spinal malformation in fish exposed to the combined stressors, as compared to *T. opisthorchis* alone, this was not significant. Fish survival differed significantly among treatments ($F_{3,28}$ = 6·75, P < 0·001; Fig. 1c), but only fish exposed to a combination of *T. opisthorchis* infections and the glyphosate formulation had significantly reduced survival than controls (P < 0·01); exposure to glyphosate formulation or *T. opisthorchis* infection alone had no effect on fish survival as compared to controls.
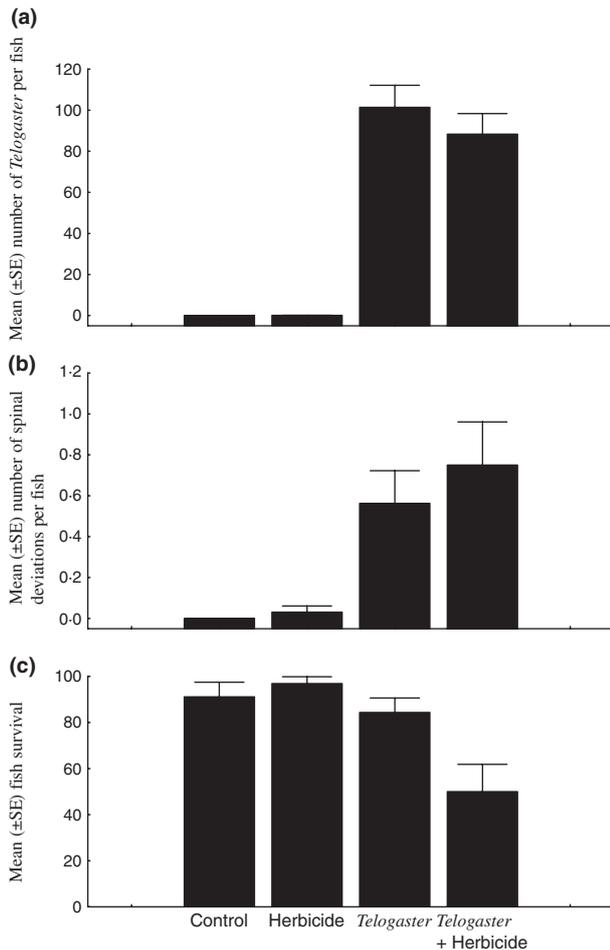
Fig. 1. Differences among treatments in (a) *Telogaster opisthorchis* infection intensities in juvenile *Galaxias anomalus*; (b) the extent of spinal malformation in *G. anomalus*; (c) Percentage survival of *G. anomalus*.

Fig. 2. Differences among treatment groups in *per-capita* shedding rates of *Telogaster opisthorchis* cercariae by *Potamopyrgus antipodarum*.

CERCARIAL SHEDDING AND SURVIVAL OF *P. ANTIPODARUM*

Snail survival did not differ among controls and the low and medium glyphosate concentration groups (66%, 100% and 91·6%, respectively). However, all snails in the high glyphosate formulation exposure group (36mg a.i. L^{-1}) died within 24 h; this group could thus not be included in the cercarial shedding experiment. After controlling for snail size, which showed a trend for increasing shedding rates with shell length (NS at $P = 0·056$), there was a significant effect of glyphosate concentration on *per capita* production of *T. opisthorchis* (ANCOVA; $F_{2,25} = 4·85$, $P = 0·017$). *Per-capita* production in snails in the moderate glyphosate formulation concentration group (3·6 mg a.i. L^{-1}) was higher than that in the control or low concentration groups (both $P < 0·05$, FPLSD; Fig. 2).

Discussion

Despite increasing recognition of interactions between disease and aquatic pollution, few studies have explored how these stressors act tog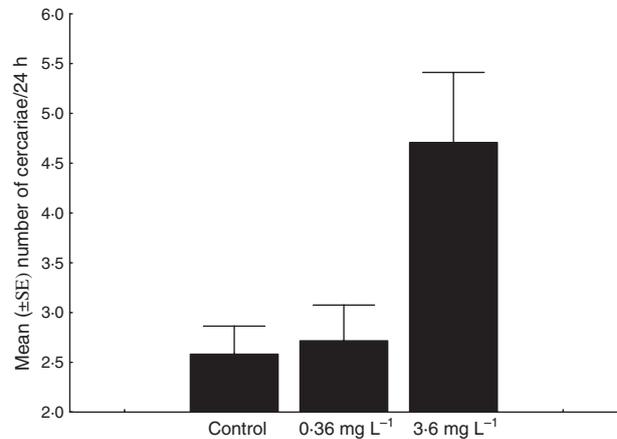ether on aquatic vertebrates at environmen- tally relevant contaminant concentrations. In the current study, the combined effects of trematode infection, and glyphosate formulation exposure at an environmentally rele- vant sub-lethal concentration, were not predictable from their individual effects because we observed a synergistic effect on fish survival but no effect of glyphosate formulation alone. We are aware of a single study reporting increased virulence of micro-parasitic infections in aquatic micro-crustaceans after simultaneous exposure to the pesticide carbaryl (Coors *et al.* 2008). Studies on vertebrates have shown synergistic increases in infection rates in the presence of contaminants, but impacts on host survival were either insignificant or not reported (e.g. Kiesecker 2002; Forson & Storfer 2006; Rohr *et al.* 2008a,b). On the other hand, simultaneous exposure to contaminants does not always lead to increases in host infection levels (e.g. Griggs & Belden 2008), paralleling our results from the first experiment here. Similarly, in our second experiment, infected snails exposed to the same concentration of glyphosate formu- lation as fish were in the first experiment (0·36 mg a.i. L^{-1}), did not differ from controls in their production of *T. opisthorchis* cercariae.

Hosts may become more susceptible to the morbidity effects of disease without any change in *per capita* infection because contaminants can induce cortisol production and suppress blood leukocytes (Forson & Storfer 2006). For example, sup- pressed immune responses and stress have been reported in fish and invertebrates after exposure to sub-lethal concentrations of glyphosate and its formulation (El-Gendy, Aly & El-Sebae 1998; Contardo-Jara *et al.* 2009). In the current study, it is pos- sible that parasite-induced host immunosuppression could have increased susceptibility to toxic effects of the glyphosate formulation. In a similar way, predator chemical cues enhance the lethality of Roundup to tadpoles (Relyea 2005b) while tapeworm infections in salmon and sticklebacks reduce fish survival during exposure to heavy metals (see Poulin 1992). Whether the synergistic effect observed in the current study reflects a direct effect of the herbicide or the parasite on host immunosuppression, requires further investigation.

Simultaneous exposure to parasites and toxicants can also cause sub-lethal effects on host morphological development (e.g. Kiesecker 2002; Budischak *et al.* 2008). We have previously shown that *T. opisthorchis* infection causes spinal malformations and reduced survival in 4- to 5-week-old *G. anomalus*, and that both responses were heightened by increasing infection intensity (Kelly *et al.* in press). Although infection alone did not influence fish survival in the current study, it did cause malformations. The glyphosate formulation alone had no effect on malformations but there was a trend for more severe malformations in fish exposed to both stressors. The difference in effect of *T. opisthorchis* here as compared to our previous study, and the lack of significant effects of the glyphosate formulation on development, is most probably due to timing. Fish age, and thus size, at the end of the current experiment were greater than in the previous study (mean length 27 mm ± SE 0·4 and 25·1 mm ± SE 0·6, respectively), and larger fish may be more resistant to the effects of infection and the glyphosate formulation on development (e.g. Ryce *et al.* 2005). Thus, field patterns and laboratory experiments indicate that trematode-induced malformation and mortality occur during a critical window of development corresponding to larval-juvenile metamorphosis (Kelly *et al.* in press). Since herbicide formulations of glyphosate are applied in spring at the start of the freshwater fish growing season (see Forson & Storfer 2006), impacts in the field may be extensive.

For parasites with complex life-cycles, the impact of toxins and infections on vertebrate hosts will also be influenced by direct toxic effects on the parasite or its intermediate hosts (see Poulin 1992; Morley *et al.* 2003; Rohr *et al.* 2008b). In our second experiment, however, we did not observe a change in survival of infected *P. antipodarum* at the glyphosate formulation concentration (0·36 mg a.i. L^{-1}) to which juvenile *G. anomalus* were exposed in the first experiment. While all snails died within 24 h at the high glyphosate formulation concentration (36 mg a.i. L^{-1}), levels in freshwaters rarely reach this high (e.g. Battaglin *et al.* 2009). Notably, though, we observed the unusual effect of increased cercarial production by *P. antipodarum* at an environmentally realistic concentration (i.e. 3·6 mg ai L^{-1}) that is within the manufacturer's recommended maximum application rate in North America (3·8 mg a.i. L^{-1}; see Relyea 2005a) and less than the maximum predicted in wetlands (3·7 mg a.i. L^{-1}; Giesy *et al.* 2000) or worst-case scenario levels expected for freshwaters (e.g. Perez *et al.* 2007). It is also within the range reported to have no observable effects on fish in North America (Siemering *et al.* 2008).

Increased shedding in the presence of the glyphosate formulation is not straightforward to explain because cercarial emergence from mollusc intermediate hosts is usually curtailed in the presence of metals, pesticides and low pH (e.g. Morley *et al.* 2003). One possibility is that the glyphosate formulation debilitates snails in ways that facilitate the use of host resources by the parasites and their conversion into cercariae, or that a cue for the parasite of impending snail mortality induces increased cercarial production. Alternatively, the observed pattern could be due to an increase in periphyton production in the presence of glyphosate, leading to enhanced availability of snail resources for cercarial production. In outdoor mesocosms, Perez *et al.* (2007) showed that periphytic cyanobacteria and picoplankton abundance increased 6–10-fold after exposure to 6 mg glyphosate a.i. L^{-1}, attributing the effect to greater phosphorus availability associated with the glyphosate formulation. Increased periphyton availability can translate into increased *per capita* shedding rates of cercariae by snails, and increased intensities of infection by metacercariae in amphibians (Johnson *et al.* 2007). Whatever the mechanism, the increase in *per capita* shedding in the presence of the glyphosate formulation, coupled with the synergistic effects on fish survival, can be expected to increase the risk to fish populations of malformation and mortality.

RECOMMENDATIONS FOR MANAGEMENT

The effects we observed on *G. anomalus* survival were caused by a glyphosate formulation at concentrations within the environmental exposure limit for glyphosate of 0·37 mg a.i. L^{-1} set for New Zealand freshwaters (ERMA, NZ 2005), and lower than levels normally used in toxicity tests for fish. This is of particular concern because many species of freshwater fish, including those in New Zealand, are already threatened by invasive fish, habitat modification and pollution (Duncan & Lockwood 2001; Department of Conservation New Zealand, 2005; Leprieur *et al.* 2006; Olden, Hogan & Vander Zanden 2007); such stressors could interact in complex ways with parasite infection and glyphosate formulation levels. Indeed, the use of glyphosate formulations have increased dramatically in New Zealand and worldwide as a consequence of expansion and intensification in the agricultural sector (Manktelow *et al.* 2005; Perez *et al.* 2007).

By overlooking naturally occurring stressors that can affect morbidity and survival, risk assessments of toxicants based on standard tests may underestimate ecological risks (e.g. Rohr *et al.* 2008a; Sures 2008). Glyphosate is the most commonly used herbicide in the world and numerous studies have reported effects of its commercial formulations on aquatic communities (e.g. Folmar *et al.* 1979; Mitchell, Chapman & Long 1987; Wan, Watts & Moul 1989; Relyea 2005a; Rohr *et al.* 2008b), but most impacts have been observed at unrealistic concentrations. We used glyphosate in a formulation at concentrations that were similar to levels recorded in freshwaters, or within acceptable limits designated by regulatory authorities. A number of observations indicate that the synergistic effects observed could have deleterious consequences for fish populations at a broad scale: (1) Glyphosate is commonly recorded at concentrations at which we observed synergistic effects with parasite infection on fish mortality; (2) Metacercarial infection are very common in freshwater fish; (3) The timing of the application of glyphosate formulations is often in spring, coinciding with the period of larval fish emergence and early susceptibility to parasite infection; and (4) early fish life stages are highly susceptible to environmental stressors, and factors that affect their survival are key determinants of population recruitment (e.g. Cushing 1996). Our results thus have important implications for the validity of setting thresholds for

contaminants, such as glyphosate formulations, based on standard toxicity testing methods.

Acknowledgements

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