Indirect effects of herbicide on trematode proliferation in the freshwater snail host *Potamopyrgus antipodarum*

Sabrina Desireé Hock

A thesis submitted for the degree of
Master of Science Ecology
University of Otago
Dunedin, New Zealand
June 2012
I would like to dedicate this thesis

to my grandfather, Clair Clark Hock and grandmother, Nancy Hock
I would first like to thank Robert, for bringing me into this lab! I have become a better thinker, researcher and writer because of you. Whenever there was an issue, your door was always open for advice.

And to my lab/office mates..you guys became more than just that, thank you for the friendship, support, motivation, laughs, beach trips, field assistance, and editing comments. In particular, I would like to thank Bronwen Presswell, Melanie Lloyd, Katie O’dwyer, Haseeb Randhawa, Fatima Jorge, Amanda Valois, Isa Blasco-Costa, and Anja Studer for all your help, one way or another, over the years.

For technical support, thank you to the members of the Zoology/Ecology Department Staff: Ken Miller, Phil Bishop, Murray McKenzie, Kim Garrett, Nicky McHugh, Stephen Wing, and Ronda Peacock.

To my friends and family back home: mom and dad you have always been there for me, through thick and thin. TJ and Kristin (and lil fig), thank you for keeping me cool when I thought the worse was going to happen, and lighting up my face with a first time niece on the way 😊 To my friends: Nira, Stef, Vishal, Britt, Tessa, Drea, Paul, Anna, LJ, Matt..you guys are awesome and even with a world apart things never change between good friendship.

To the Doc (R.I.P) and Christine..for being the best employers I could of ever had during my B.Sc., and being my inspiration to advance my education and research with something I love studying. Dr. Jerry Davidoff, you are missed but not forgotten.

To my (flat) mates in NZ: Ellis Schriefer, James Shelley and Scott Flemming...my family away from home, ta...for the memories, dinners, trekking adventures, road trips, FIFA games on wii, music, movies. WU-TANG CLAN!

Kim Garrett-my former flat mate from when I first arrived to Dunedin, words can not express how inspirational you and your family have been to my life. Thank you for making me a stronger/wiser person inside and out during good times and bad. Christchurch will always have a place in my heart.
And to everyone else who has made an impression on my life, during this adventure...

:::Namaste :::
ABSTRACT

Freshwater ecosystems are often exposed to intense agricultural pollution, which can impact species interactions such as those between parasites and their hosts. I studied the effect of glyphosate (the active ingredient of a widely-used agricultural herbicide, Roundup®) on the proliferation and transmission of trematode parasites in the New Zealand mud snail, *Potamopyrgus antipodarum*. This ubiquitous and highly abundant snail serves as the first intermediate host to a wide diversity of trematodes. Trematode larval stages multiply within the snail to form free-living infective stages known as cercariae which then go on to infect native invertebrates, fish and birds. Earlier evidence suggested that herbicides from agricultural run-off might weaken the immune system of the snail and promote the within-snail multiplication of the trematode *Telogaster opisthorchis*. I tested the effect of long-term exposure to different levels of glyphosate on snail behaviour, cercarial production by three trematode species parasitic in *P. antipodarum*, and cercariae survival. Glyphosate had an effect on snail behaviour, however infection by an undescribed renicolid trematode did not. Snails exposed to the pollutant were hidden more than their conspecifics in the control treatment. Exposure of snails to glyphosate doubled, and in some cases tripled, cercarial output in three trematode species, i.e. the previously-mentioned renicolid, *Coitocaecum parvum*, and *Apatemon* sp.. In addition, survival time of renicolid cercariae was 1.57% greater when glyphosate was present at moderate concentrations. The more a parasite’s quality and quantity increase, the more likely we will see cascading effects on other hosts (fish, amphibians and molluscs). My results provided evidence that there are indirect effects from agricultural run-off on freshwater systems, and add weight to the pressure on the agricultural sector to limit the large-scale use of herbicide.
Table of contents

Acknowledgments...........................................................................................................i
Abstract........................................................................................................................iii
Table of contents ..........................................................................................................iv
List of figures................................................................................................................vi
List of tables.................................................................................................................vii

CHAPTER ONE General introduction..........................................................................1
  1.1 Background ........................................................................................................2
  1.2 Trematode parasitism........................................................................................5
  1.3 Potamopyrgus antipodarum host........................................................................6
  1.4 Species studied...................................................................................................8
  1.5 Herbicides and parasitism................................................................................11
  1.6 Synergistic effects from pollution and parasitism............................................13
  1.7 Aims and objectives...........................................................................................15

CHAPTER TWO Potamopyrgus antipodarum behaviour in response to trematode
infection and glyphosate exposure.............................................................................16
  2.1 Introduction.......................................................................................................17
  2.2 Methods...........................................................................................................20
  2.3 Results..............................................................................................................24
  2.4 Discussion.........................................................................................................26

CHAPTER THREE Effect of glyphosate exposure on cercariae production in the snail
host, Potamopyrgus antipodarum.................................................................................29
  3.1 Introduction.......................................................................................................30
  3.2 Methods...........................................................................................................32
  3.3 Results..............................................................................................................36
  3.4 Discussion.........................................................................................................46
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOUR</td>
<td>Effect of glyphosate exposure on survival of cercariae of a renicolid trematode</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>4.1 Introduction</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>4.2 Methods</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>4.3 Results</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>4.4 Discussion</td>
<td>56</td>
</tr>
<tr>
<td>FIVE</td>
<td>General conclusion</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>5.1 Introduction</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>5.2 General results</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>5.3 Prevention</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>5.4 Future Studies</td>
<td>62</td>
</tr>
</tbody>
</table>

References ...................................................................................... 64

Appendix ............................................................................................ 82
List of figures

Figure 1.1 Generic trematode life cycle.................................................................6
Figure 1.2 *Potamopyrgus antipodarum* snail..........................................................8
Figure 1.3 renicolid cercaria..................................................................................9
Figure 1.4 Sporocysts of renicolid........................................................................9
Figure 1.5 *Coitocaecum parvum* cercaria............................................................10
Figure 1.6 *Apatemon* sp. cercaria........................................................................11
Figure 1.7 Metabolic pathway inhibited by glyphosate.........................................13
Figure 2.1 Tomahawk Lagoon map.......................................................................21
Figure 2.2 Diagram of experimental design..........................................................23
Figure 2.3 Proportion of snail’s hidden during day...............................................25
Figure 2.4 Proportion of snail’s hidden during afternoon.......................................25
Figure 3.1 Lake Waiholo map..................................................................................33
Figure 3.2 Kaka Point map....................................................................................34
Figure 3.3 Daily cercariae production among all species used...............................37
Figure 3.4 Daily renicolid (TM) production............................................................39
Figure 3.5 Overall renicolid (TM) production.........................................................39
Figure 3.6 Daily renicolid (LW) production............................................................41
Figure 3.7 Overall renicolid (LW) production.........................................................41
Figure 3.8 Daily *Coitocaecum parvum* production..........................................43
Figure 3.9 Overall *Coitocaecum parvum* production...........................................43
Figure 3.10 Daily *Apatemon* sp. production..........................................................45
Figure 3.11 Overall *Apatemon* sp. production.....................................................45
Figure 4.1 Number of renicolid cercariae used for each treatment........................54
Figure 4.2 Frequency distribution of renicolid.......................................................55
Figure 4.3 Overall mean renicolid cercariae survival.............................................55
Appendix Figure 1.8 Phylogenetic tree of renicolid..............................................84
List of Tables

Table 2.1 Snail behaviour............................................................................................24
Table 3.1 Prevalence....................................................................................................37
Table 3.2 Renicolid (TM) production results...............................................................38
Table 3.3 Renicolid (LW) production results...............................................................40
Table 3.4 Coitocaecum parvum production results.....................................................42
Table 3.5 Apatemon sp. production results.................................................................44
Table 4.1 Renicolid cercariae survival results.............................................................54
CHAPTER ONE

General introduction
1.1 Background

Anthropogenic and natural stressors, such as pollution (e.g. herbicides), and their interactions with parasitism, have been shown to cause widespread problems in freshwater environments (Holden, 1972; Morley et al., 2006; Relyea, 2005a). It is essential to understand how stressors can potentially interact with each other to alter ecosystem function and processes, through changes in foraging behaviour and predator avoidance strategies of individual animals, and in the prevalence/effects of parasites. Parasites are known to impose energetic demands on their host, alter foraging behaviour and increase risk of predation (Levri and Lively, 1996). Likewise, pollutants are associated with the weakening of immune responses in many species, and subsequently increasing their susceptibility to infection from parasites (Arkoosh et al., 1998; Rohr et al., 2008b).

Ecotoxicology (‘environmental toxicology’) is concerned with the maintenance of biodiversity, in response to effects from pollutants on the constituents of an ecosystem in an integrated context (Truhaut, 1977; Van Straalen, 1993). It deals with the nature, effects and interactions of substances that are harmful to the environment. Previous ecotoxicological research has revealed the detrimental effects from pollutants such as DDT, heavy metals and pesticides/herbicides on aquatic organisms (i.e. fish and snails) (Koprivnikar and Walker, 2011; McKim et al., 1974). These pollutants have a negative impact on different species in a number of ways, which have cascading effects that apply to all organisms in the food chain, including parasites. For instance, in terms of trematode parasites, the combination of cadmium/zinc mixtures has been found to increase cercarial (refer to glossary) survival, causing a cascading effect on the parasite’s host which could lead to mortality (Morley et al., 2002a). Because of these observations, recent attention has been focused on impacts from the common pesticides/herbicides atrazine and Roundup® (Kelly et al., 2010a; Koprivnikar and Walker, 2011). A few studies have investigated the effects of the pesticide atrazine in freshwater ecosystems.

One study found *Stagnicola elodes* snails infected with trematodes had higher mortality in the presence of atrazine, compared to uninfected snails (Koprivnikar and Walker, 2011). However, there is a paucity of research evaluating the synergy
between one of the most widely-used commercial herbicide, Roundup®, and trematodes in the environment, let alone the impact of both on freshwater hosts. Roundup®’s active ingredient and surfactant are known to cause environmental damage (Altieri, 2009; Diamand and Barron, 2001). Therefore understanding how these pollutants and trematodes affect a common freshwater host is needed for monitoring and maintaining the health of freshwater ecosystems.

Roundup® (focal herbicide for this study) is a systemic post-emergence herbicide which acts on the synthesis of amino acids and other endogenous chemicals important in plant metabolism. It is commonly used in agriculture, forestry and nurseries for the control, or elimination, of most herbaceous plants (Peluso et al., 1996). However it has been found to harm the health of fish and amphibians, such as *Salmo gairdneri* (rainbow trout) and *Rana sylvatica* (North American frog) (Folmar et al., 1979; Relyea, 2005b, 2011). Other impacts, such as circulatory failure in dogs, eye and skin irritation, pneumonia and erosion to the gastrointestinal lining in humans are areas of concern when Roundup® residues are left on vegetation or in drinking water (Cox, 1995a, 1995b; Hued et al., 2011). After rainfall, through agricultural run-off, traces of Roundup® have been found in watersheds, flowing into rivers and lakes (Kramer et al., 1980; Wang, 1991). Since Roundup® is used on farms and home gardens, it is becoming of increasing concern to ecosystems affected by run-off.

In addition to the stress associated with pollutant run-off into freshwater, natural stresses from trematode parasites affect the behaviour, survival, reproduction, and/or development, of their freshwater hosts. For instance, trematode infections in snails induce changes in behaviour and morphology (Lagrue and Poulin, 2007; Levri, 1999; Levri and Lively, 1996). In addition, metacercarial cysts in fish hosts cause abnormal spinal column development (Kelly et al., 2010b) and cysts in amphibians cause improper limb growth (Rohr et al., 2008a; Rohr et al., 2008b). Also, most rediae/sporocysts of trematodes castrate snail intermediate hosts (Poulin, 2007). However, few studies have focused on the combined effect of pollutants and parasitism on the hosts of trematodes.
Box 1.1 Glossary

**Cercaria:** A juvenile larval stage of a trematode, produced by asexual reproduction within a sporocyst or redia inside the first intermediate host (plural = cercariae).

**Definitive host:** The host (usually a vertebrate) in which a parasite achieves sexual maturity.

**First intermediate host:** The host (usually a mollusc) in which trematode larvae (sporocysts or rediae) multiply asexually, producing cercariae.

**Immunosuppression:** The partial or complete suppression of an individual’s immune response.

**Metacercaria:** The larval stage between the cercaria and adult in the life cycle of most trematodes, occurring in the second intermediate host (plural = metacercariae).

**Miracidium:** The first larval stage of a trematode hatched from the egg, usually free-swimming and ciliated, and responsible for infecting the first intermediate host (plural = miracidia).

**Pollution:** Harmful substances of anthropogenic origin (e.g., agricultural run-off).

**Prevalence:** Number of individuals of a host species infected with a particular parasite species divided by the number of hosts examined (usually written as a percentage).

**Redia:** One of two types of larval stage in trematodes, produced by asexual reproduction inside the first intermediate host, originating from a miracidium (plural = rediae).

**Second intermediate host:** The host (either an invertebrate or a small vertebrate) infected by cercariae and in which the metacercariae develop.

**Sporocyst:** One of two types of larval stage in trematodes, produced by asexual reproduction inside the first intermediate host, originating from a miracidium.
1.2 Trematode parasitism

Trematodes, commonly called ‘flukes’, are ubiquitous parasitic worms requiring up to four different vertebrate and invertebrate host species in their life cycle (Morley, 2008). The name fluke refers to the worm’s suckers, organs used for attachment, usually located both around the mouth and on the ventral surface of the body.

Trematodes are simultaneous hermaphrodites, having both male and female reproductive systems. Eggs are released after mating by adult worms in the definitive host, usually exiting in host faeces. During the first larval stage, the miracidium hatches from the egg and infects an aquatic snail, or the egg is inadvertently ingested by the snail while it is feeding (Morley, 2008). Within the snail, the miracidium takes on a sac-like appearance (sporocyst or redia depending on species) in the central body of the snail’s digestive gland and gonads, extending a brood sac into the snail’s head. Within the sporocyst or redia, asexual reproduction produces many embryos which develop into cercariae, damaging the host’s gonads and causing castration to the snail (Zbikowska and Nowak, 2009). The cercariae subsequently emerge from the body of the snail host when mature (Haas, 1994). An increase in temperature has been shown to trigger the emergence of cercariae and increase their production within a snail, which may be due to an increase in the trematode’s metabolic activity (Poulin, 2006).

After leaving the snail, free-swimming cercariae penetrate their next host, being either a second intermediate host or the definitive host. If the cercariae go to an intermediate host, they will then encyst (metacercariae) and await ingestion by the definitive host. The final host is a vertebrate and provides a habitat for the parasite, where the adult fluke will reproduce sexually, passing out its eggs in the host’s faeces or urine. Once the eggs hatch in the water, miracidia are released, repeating the trematode life cycle once a mollusc ingests them (Poulin, 2006) (Figure 1.1). Because of the multiple obligate hosts needed for a trematode to survive, they are said to have “complex life cycles” (Park et al., 2007).

Parasites exert stress on their hosts, but this normal situation can be exacerbated by anthropogenic impacts that change the parasite-host relationship. For example, in one study, molluscan hosts exposed to heavy metal pollutants (cadmium
and zinc) have been observed to exhibit physiological changes and a higher susceptibility to trematode infection (Morley, 2008). However, there is a dearth of information on indirect effects of herbicides on trematode biology.

Figure 1.1 Generic trematode life cycle (A) Free-swimming miracidium before entering snail; (B) Sporocyst with rediae; (C) Redia with daughter rediae; (D) Cercaria produced by daughter redia; (E) Cercaria, having escaped from snail; (F) Metacercaria in flesh of fish; (G) Adult in bird (Meyer and Olsen, 1971).

1.3 *Potamopyrgus antipodarum* host

New Zealand is home to the native freshwater snail, *Potamopyrgus antipodarum* (Gray), which has recently invaded the USA, Australia, UK and other parts of Europe (Kerans et al., 2005). Grazing gastropods like *P. antipodarum* are an essential link between primary producers (algae) and higher consumers (Gérard et al., 2008), making them a useful invertebrate model system for monitoring toxicological impacts and environmental changes. As adults, these snails range from four to five millimetres in shell length, and have five or six whorls on their narrow shells. Their diet includes diatoms, plant and animal detritus and attached periphyton (Alonso and
Castro-Diez, 2008). Shell colours vary from gray to dark brown (Holomuzki and Biggs, 2006) (Figure 1.2).

*Potamopyrgus antipodarum* are born live and females are ovoviviparous (i.e. they possess a brood pouch and give birth to live juveniles). The female snails are born with 20-120 developing embryos in their reproductive system, reaching maturity at three to six months of age. Males are born with fully functional sexual organs and every year the snails mate around spring and summer, producing approximately 230 young per brood (Benson, 2006).

Dawkins (1982) argued that an infected host is an ‘extended phenotype’ of its parasites. The parasite modifies the host to ensure its own survival and development. Overall, those effects of parasitism are detrimental to the host and advantageous for the parasite, yet determining the net outcome for the host is often neglected. From an evolutionary perspective, an ‘arms race’ develops, as both parasite and host genomes respond to reciprocal selective pressures exerted by the host on the parasite, and vice versa (Anderson and May, 1982). Although the outcome of the snail-trematode arms race almost invariably benefits the parasite, the influences of external factors like pollutants on the net outcome are still poorly understood.

These mud snails exist in a wide range of habitats, tolerating anything from brackish eutrophic mud bottoms to clear fresh running waters. Typical sites include lakes, ponds, streams, rivers, lagoons and estuaries (Hine, 1978). In addition, the snails have a wide temperature tolerance, ranging from an upper thermal limit of 28°C to a lower thermal limit near freezing (Holomuzki and Biggs, 2006). Because of these flexible traits, *P. antipodarum* snails have high survival and successful colonization rates, though both of these key traits are biologically controlled by trematodes via castration of infected snails (Morley, 2008).

Fourteen different trematode species are known to exploit the snail *P. antipodarum* as first intermediate host, although rates of infection differ between the different species (Hechinger, in prep), making this snail the main host of trematodes in New Zealand freshwaters.
Figure 1.2 *Potamopyrgus antipodarum* adult with algae growth on shell; shell length = 4.5mm.

### 1.4 Species studied

Three trematode species were chosen for this study: an undescribed renicolid species, *Coitocaecum parvum* (Crowcroft), and *Apatemon* sp.. They were chosen for their high prevalence, since these species are the ones most likely to have broad impacts on populations and communities. Their prevalence throughout New Zealand varies between location and season.

The first species belonged to the family Renicolidae (Dollfus), but the species is currently unnamed, and had a prevalence of about 10% in snails from the study area. Species-level identification using morphological features has not been possible; therefore molecular characterisation was required to confirm infections consisted of a single species (Appendix 1.8). Renicolids exhibit life cycle plasticity, usually starting with a snail as first intermediate host, and molluscs or fishes as second intermediate hosts. Adults are found in kidneys and ureters of aquatic birds (Gibson et al., 2008) (Figure 1.3 and 1.4).
Figure 1.3 Renicolid cercaria, ventral (mid section) and oral sucker (anterior end) present. Body length ~300 µm.

Figure 1.4 Sporocysts of renicolid extracted from mantle wall and visceral mass of a snail. They appear yellow, sausage-shaped and filled with germ balls and cercariae. Length ~1000 µm.
The second most common trematode in our study area is *Coitocaecum parvum*, with approximately 1% prevalence in snails. Its cercariae are non-oculate, with a short glandular sucker-like tail, with both ventral and oral suckers, and an oral stylet. Their body length is around 300µm; tail length is about 1/6 the body length (Hechinger, in prep). *C. parvum* cercariae have a leech-like mobility due to the stumpy tail, moving along the substratum using their oral and ventral suckers (Erasmus, 1972). Snails are once again the first intermediate host, followed by crustaceans (mainly amphipods), and fish are the definitive host (MacFarlane, 1939) (Fig 1.5).

![Figure 1.5 Coitocaecum parvum cercaria, with oral and ventral suckers visible. The posterior end has the short tail. Body length ~300µm.](image)

The final trematode species used in this study is a furcocercous cercaria, *Apatemon* sp. (to be described by Bronwen Presswell, in preparation), with a prevalence of about 0.5% in snails from our study site. The cercariae are colourless, translucent and have a protrusable anterior organ rather than a normal sucker. The tail is distinct from most other species, having a fork-shaped split dividing it into two furcae at about 3/4 of its length. The body length is 0.06 – 0.10mm, the width up to 0.04mm, and the tail stem length up to 0.15mm (Hechinger, in prep; Winterbourn, 1973). *Apatemon* sp. uses *Gobiomorphus cotidianus* (McDowall), the common bully,
as second intermediate host, and a duck as the definitive host (Bronwen Presswell, personal communication) (Fig 1.6).

Figure 1.6 *Apatemon* sp. cercaria, with tail stem divided into 2 furcae. Body length ~130μm (photo by Bronwen Presswell).

1.5 Herbicides and parasitism

Herbicides impact life-forms at many biological scales including molecules, tissues, organs, individuals, populations and communities (Pratt et al., 1997). Due to the irresponsible disposal of spray liquids, casual spillage, and herbicide discharge into streams and rivers, organisms that encounter the toxins are either directly or indirectly affected (Holden, 1972). For example, Roundup is not only applied to vegetation near waterways, but also directly to water bodies to control Raupo and other aquatic plants. More ecotoxicological studies are needed to further assess the effects of these toxins. However, natural ecosystems are diverse and these effects are complicated, which hinders the progress of any assessment (Hanazato, 2001). Pollution, whether as agricultural run-off or in other form, and parasitism may have
both separate and synergistic effects that need to be elucidated, so that their future impacts on ecosystems can be assessed (Rohr et al., 2008a).

Among animals exposed to agricultural pollutants within their ecosystem, aquatic species, especially semi-sessile invertebrates like snails, are one ‘at risk’ group that is easily monitored (Rosenberg and Resh, 1993). Trematodes can indirectly benefit from waters that are polluted, as the toxins can suppress the immune response of snails or other hosts, making the host more vulnerable to either infection by parasites or their subsequent proliferation within the host (Kelly et al., 2010a; Rohr et al., 2008b).

Furthermore, host vulnerability comes from the fact that herbicide contamination can induce cortisol production in vertebrates, a stress hormone secreted by the body to treat inflammation, and suppress blood leukocytes (Cericato et al., 2008; Dhabhar et al., 1996). These blood leukocytes are key components of the immune system, acting as the main cells to aid in defence against infectious diseases and foreign materials such as parasites. Because of this, host animals (e.g. fish, amphibians and potentially invertebrates) are vulnerable to immunosuppression after being exposed to sub-lethal concentrations of Roundup®’s main active ingredient, glyphosate, and its surfactant (i.e., a chemical that has prominent surface activity in aqueous solutions) polyoxyethyleneamine (referred to as POEA) (Kelly et al., 2010a). In natural habitats, concentrations of Roundup® have been found between 0.1 to 2.3mg a.i. (active ingredient)/L (Feng and Thompson, 1990; Newton et al., 1984) and these moderate concentrations have been lethal to aquatic invertebrates, fish and amphibians (Edginton et al., 2004; Giesy et al., 2000; Tsui and Chu, 2003)

1.5.1 Glyphosate

Glyphosate (N-phosphonomethylglycine), the active ingredient in Roundup®, inhibits the production of chorismate (an important biochemical intermediate in plants and microorganisms) via disruption of the aromatic amino acid biosynthesis in plants and several microorganisms, by inhibiting the enzyme 5-enolpyruvyl shikimic acid-3-phosphate (EPSP) (Amrhein et al., 1980) (see Fig 1.7). Glyphosate adsorbs to soil particles and consequently is easily dispersed from soil to surface water by wind and
surface run-off of suspended particulate matter (Pérez et al., 2007). Approximately 60% of the initial Roundup® volume applied to vegetation is washed away with soil and can end up in waterways (Burchett and Burchett, 2011).

Figure 1.7 The molecular process of glyphosate blocking the plant’s enzymes from the metabolic pathway (Rhodes, 2009).

1.5.2 Polyoxyethyleneamine

Polyoxyethyleneamine (POEA) is the active surfactant in glyphosate. The function of a surfactant is to increase the likelihood of an active ingredient penetrating plant cuticle. A review by (Diamand and Barron, 2001) showed POEA is actually more toxic than glyphosate to many aquatic species.

1.6 Synergistic effects from pollution and parasitism

Toxins may influence infection of hosts from parasites with complex life cycles by: (1) direct immuno-suppression of the host, (2) altering transmission probabilities of infective parasite stages between alternate hosts, (3) altering the survival probability of infected hosts (including indirectly via altered foraging behaviour and predator avoidance) or (4) directly influencing survival or infectivity of the disease-causing parasite (Kelly et al., 2010a).
A decade ago, (Kiesecker, 2002) published results linking amphibian deformities with both trematode infection and pesticide exposure. Kiesecker found that parasites infecting tadpoles caused the development of limb deformities observed in frogs, and that stress from the pesticides decreased the host’s ability to resist any infection (Kiesecker, 2002). A couple of other case studies (below) further illustrate the potential for parasites and pollutants to interact synergistically.

1.6.1 Case Studies

*Freshwater fish*

In a study involving toxic treatments of glyphosate, (Kelly et al., 2010a) looked at the roundhead galaxias, *Galaxias anomalus*, a freshwater fish that serves as an intermediate host for metacercariae of the trematode *Telogaster opisthorchis*. The cercaria forms a cyst (which turns into the metacercaria) inside the fish’s body and, depending on its location, can cause spinal malformations, if not mortality, when infecting the fish at the juvenile stage. The frequency of deformities was found to increase when fish were exposed to the combination of trematodes and glyphosate (Kelly et al., 2010b). Further, Kelly et al. (2010a) found that exposure of the snail first intermediate host to glyphosate lead to a vastly increased production and release of cercarial infection stages, further exacerbating the effects on fish. The snail in this case was *Potamopyrgus antipodarum*, which is also the first intermediate host of the three trematode species investigated in this thesis.

*Amphibians*

As of 2010, a consensus within the biological community estimated that 32% of amphibian species are currently threatened, 44% are in population decline, and 120 species have likely become extinct in the past 25 years (Shirk, 2010). (Budischak et al., 2008) performed research on amphibians inhabiting polluted areas near agricultural run-off, and their results showed weakened immune systems with frequent limb abnormalities in response to parasite (trematode) infections.

The development of limbs by amphibians depends on the spatial organization of cells in the tadpole limb bud, which are responsible for producing and releasing signalling molecules that set up the primary limb axes (Stopper et al., 2002).
Trematodes form cysts inside the amphibian host, including within limb buds, and disrupt the spatial organization of the cells, triggering intercalation in the limb buds during early stages of development (Sessions and Ruth, 1990; Stopper et al., 2002). Therefore, the compounding impact of pollutants on the risk of malformations caused by parasites has serious consequences on frog populations (Johnson et al., 2007).

1.7 Aims and objectives

The main objective of this research was to investigate the indirect effects of various levels of glyphosate and POEA on the common freshwater snail host, Potamopyrgus antipodarum, and three of its trematode species. The specific objectives were to:

1. Assess the survival and describe the behaviour of the host P. antipodarum when exposed to various concentrations of Roundup®.
2. Measure the impact of different Roundup® concentrations on the rates at which the different species of trematodes multiply within the snail host.
3. Measure the survival of trematode cercariae (infective stages) issued from the first intermediate host and exposed to various glyphosate concentrations.

I hypothesised that snail behaviour and survival of the trematode transmission stages (cercariae) would be influenced by the herbicide Roundup® (glyphosate and POEA) and predicted that higher concentrations of the herbicide would alter snail behaviour, reduce cercarial survival but augment cercarial production of the trematodes inside the snail host. For this thesis, Chapters Two, Three and Four have been written in manuscript style in order to fully report the results of different experiments as stand-alone pieces, and accordingly there is an element of repetition to them.
CHAPTER TWO

_Potamopyrgus antipodarum_ behaviour in response to
trematode infection and glyphosate exposure
2.1 Introduction

When an animal is hungry it will forage for food; if parched it will look for water; when threatened by a predator it will take measures to defend its life. However, what is an animal to do when it is simultaneously hungry, parched, and at risk of predation? In such an instance, natural selection will benefit individuals within a species that achieve optimal trade-offs between these demands in order to maximise fitness (i.e. advantageous survival and reproduction traits) (Barnard and Behnke, 1990). As a result, current foraging decisions can be an indication of past selection pressures, being a trade-off between predator-avoidance and the physical demands of nutrition. Foraging behaviour can also be influenced by a number of biotic (e.g. intra- and inter-specific competition, parasitism) and environmental (e.g. habitat structure, pollution) factors (Lefcort et al., 2000; Michel and Adams, 2009); parasitism and pollution being the focus of this chapter.

Firstly, parasites can induce rapid and dramatic changes in host behaviour. In the context of foraging, they can modify an organism’s optimal trade-off between feeding and avoiding predators by manipulating the behaviour of their animal host (Poulin, 1994). This manipulation manifests as altered host behaviour, benefiting the parasite (McCarthy et al., 2000). In particular, trophically-transmitted parasites with complex life cycles are able to induce changes to one or more of their hosts in order to favour their own survival or transmission. Manipulation is often through reduced predator avoidance and increased host growth (Parker et al., 2003).

Parasites can alter a host’s behaviour by reducing the host’s nutritional level and increasing foraging time (Barnard and Behnke, 1990). Parasites can do this via subtle biochemical manipulation, but more often they achieve it by simply consuming tissue and exacting an energetic cost upon the host. To compensate for the energy deficit, a host may increase foraging time or alter its behaviour, thus exposing itself to predation for longer periods in order to obtain more food (Hughes, 1993). For example, three-spine sticklebacks (*Gasterosteus aculeatus*) infected with the cestode *Schistocephalus solidus* were found to take risks to obtain food at any distance from their predator, showing no fear response. However, hungry uninfected sticklebacks only risked foraging at a far distance from the predator and partially satiated individuals did not risk foraging (Milinski, 1985).
Compared to trophically-transmitted parasites, parasites with a free-swimming infective stage may have a different approach to host manipulation. For instance, trematode cercariae exit from the first intermediate host and actively penetrate the next host. (Curtis, 1987) demonstrated how *Gynaecotyla adunca* benefit from altering the behaviour of their snail host, *Ilyanassa obsoleta*. Infected snails crawl towards the beach, whereas uninfected conspecifics remain at lower levels of the shore. *G. adunca* cercariae need to gain access to the next host, which is a semi-terrestrial crustacean, often the beach hopper amphipod *Talorchestia longicornis* (Curtis, 1987). For renicolid (focal parasite of this study) there may be no advantage in altering snail foraging behaviour. Instead, any change in snail behaviour may be due to pathological side effects (Bates et al., 2011). Because there is no advantage to the snail in this action, the parasite must be altering the host’s behaviour purely for its own advantage. Snail castration is one side effect renicolid trematodes inflict upon their host (Martorelli et al., 2008). Notwithstanding castration, renicolids may also induce changes in shell morphology of their snail host, which have been observed in *Zeacumantus subcarinatus* snails (Hay et al., 2005; Martorelli et al., 2008).

Secondly, pollution weakens the host’s immune system (immunosuppression) hence, endocrine and behavioural disruptions are considered useful bioindicators of anthropogenic impacts from pollutants (e.g. herbicides). Animals are exposed to these pollutants in the air, water and food (Clotfelter et al., 2004), creating a potential risk for natural communities (Relyea, 2005a). Their weakened immune system can also make them more susceptible to parasitism (Poulin, 1992).

Indirect effects from herbicides, such as the commonly used product Roundup®, have been detected in aquatic systems and are of importance for bio-monitoring (Jiraungkoorskul et al., 2002). Jiraungkoorskul et al. (2002) found juvenile *Oreochromis niloticus* fish exposed to Roundup® incurred histopathological changes to their gills, liver and kidneys. These effects are understudied, especially when it comes to an organism’s behavioural response when exposed to environmental stressors (Rohr and Crumrine, 2005). In ecotoxicology it is important to fully understand the interaction between pollution and parasitism. For instance, behavioural alterations that affect the intake and accumulation of pollution by different hosts, are also associated with parasitic infections (Sures, 2006).
Previous research has found that the pesticide atrazine, and other pollutants, inhibit anti-predator reactions to chemical cues in fish (Lürling and Scheffer, 2007; Moore and Lower, 2001). For instance, exposure to nominal concentrations (1.0µg/L) of the insecticide diazion significantly inhibited responses to anti-predator cues in *Oncorhynchus tshawytscha* salmon (Scholz et al., 2000). This could increase the risk of disease and predation by altering the host’s fear response (Rohr et al., 2009), and the same behaviour may be applicable in invertebrates. Snails exposed to atrazine are known to exhibit reduced reproduction and growth (Rohr and Crumrine, 2005). These indirect effects were a result of the depletion in the snail’s primary food source, periphyton algae, due to the pollutant decreasing photosynthesis and growth of the algae (Rohr and Crumrine, 2005).

Benthic invertebrates, such as snails, are ideal model organisms to study the effects of pollutants in aquatic environments due to their short lifespan and variability in sensitivity to pollutants (Clements and Kiffney, 1995). Invertebrates also live and feed in the sediments where pollutants accumulate, often accumulating these toxins before transferring them higher up the food web (Reynoldson, 1987). Additionally, snails are also important intermediate hosts to many trematode parasites (Jayawardena et al., 2010; Jokela and Lively, 1995). Infection levels can thus be used to monitor any effects from anthropogenic pollution (Sures, 2004).

Changes in snail behaviour may affect interactions between the free-swimming cercariae of trematodes and their next host (Rohr et al., 2008a). Accordingly, selection should favour parasites capable of modifying snail behaviour to their own advantage. This creates an evolutionary “arms race” between parasites (which influences a host to follow the parasite’s preferred foraging regime), and its host counterpart (Barnard and Behnke, 1990). Therefore, parasites and pollution might have synergistic impacts that are more harmful to the host than effects from either parasitism or pollution alone. If the pollution is capable of inhibiting certain anti-predator responses, then the snail host may become more susceptible to infections.

The effects of herbicides on snail behaviour have not been fully explored in terms of their interaction with parasites, and in this chapter I will explore those effects.
in the laboratory. The objective of this study was to quantify the separate and combined effects of renicolid parasitism and exposure to the herbicide Roundup® (using the active ingredient glyphosate and its surfactant polyoxyethylene amine, POEA) on the foraging behaviour of the freshwater snail Potamopyrgus antipodarum. This was achieved by comparing the behaviour of infected and uninfected snails exposed to different concentrations of glyphosate.

2.2 Methods

2.2.1 Collections

Potamopyrgus antipodarum snails were collected from Tomahawk Lagoon, Dunedin, South Island of New Zealand Latitude 46° S, 170° E (Figure 2.1). Tomahawk Lagoon has an upper and lower body of water; for this research, snails were collected only from the upper lagoon. They were collected during the summer months (December, January, February) of 2011-2012. Infection was expected to be more prevalent at this time due to increased parasite transmission promoted by higher temperatures (Barnard and Behnke, 1990). The trematode considered in this study was an undescribed species of the family Renicolidae, and was chosen due to its relatively high prevalence.

To obtain snails, a dip net was dragged through aquatic vegetation. Snails were then collected in containers. Additional lake water was collected for experimental and husbandry purposes, along with fresh Ruppia polycarpa (algae) to feed the snails. Larger snails (around 4-5.5mm) were preferred during collection, as they are more likely to be infected (Johnson et al., 2007).
2.2.2 Animal husbandry

In order to identify snails infected with trematode parasites, snails were placed individually into 12-well flat bottom culture plates (volume 3ml) filled with 2.1ml of freshwater and incubated for two hours at 20°C, stimulating the cercariae to emerge for observation under a dissecting microscope. Each snail was screened for infection once a day, over four consecutive days. Screening is the process of observing the well plates under the dissecting microscope, and focusing on each individual well, looking for recently shed cercariae. Snails experienced no mortality throughout the shedding process.

Once the parasitised snails were identified and those harbouring trematodes other than renicolids were discarded, the shell length of each snail was measured under a dissecting microscope from the tip of the spire to the base of the operculum and to the nearest 0.1mm. Snails were placed in a two litre aerated freshwater tank
Chapter 2: Snail behaviour

(LxWxH = 210 x 150 x 80mm) filled with one litre of freshwater with *Ruppia polycarpa* and kept at 12°C. Once all infected and uninfected snails were assembled in the tank (uninfected snails were identified by a bright pink nail polish marking on the shell’s apex), they were given 24 hours to acclimatise before conducting the experiment. This allowed simultaneous introduction of all infected and uninfected snails into experimental tanks. Once a week, water was changed and fresh macrophytes collected from the field were placed in the tank for feeding.

2.2.3 Experimental trials

Four experimental treatments and three replicates each were assembled for observing snail behaviour, with three of them made up of different concentrations of Roundup® and a control, consisting of freshwater only. Because the glyphosate and POEA came in a mixed concentration of 360mg/20litre, dilution was required to get the desired concentrations. The three treatments were made with both freshwater and the commercial formulation Glyphosate 360 (360mg L⁻¹ plus 10-20% POEA, as the surfactant; supplier: Ravensdown, New Zealand). Each of the three distinct glyphosate treatments was diluted to either: (1) low, 0.36 mg active ingredient (a.i.) L⁻¹, (2) medium, 3.6 mg a.i. L⁻¹, or (3) high, 36 mg a.i. L⁻¹ (Kelly et al., 2010a).

2.2.4 Laboratory procedures

Snail behaviour was assessed using separate two litre containers (LxWxH = 165 x 165 x 80mm) filled with one litre of treatment (control, low, medium or high concentrated glyphosate) and a rock (approx. 100mm diameter) collected from the site. Sediment was not included to prevent any confounding factors. All snails in the high glyphosate concentration (36mg/L) died within two days, and that treatment was therefore not considered in the analyses, leaving three treatments (control, low and medium glyphosate).

Each container had four infected snails and four uninfected snails, n=8 snails per container, for a total of 24 snails per replicate trial. Each replication used different snails, with a total of 72 snails used. Snails were observed twice a day (morning and afternoon) for 28 consecutive days, and the position where they settled relative to a
“shelter” rock was recorded. If a snail was under the rock or crawling around the bottom of the container, the snail was considered hidden (lower risk of predation). Likewise, if the snail was found on top of the rock or crawling up the side of the container, the snail was considered exposed (where algal food was present). Thus, for each observation period, each snail was recorded as either hidden or exposed (Figure 2.2).

Figure 2.2 Diagram showing an example of one experimental design container, containing both infected and uninfected (pink apex) snails and rock in either the control, low or medium concentration.

2.2.5 Data analysis

The snail behaviour data were analysed with a generalized linear model (GLM), with a normal error structure. The proportion of snails hidden (arcsine-transformed) in each container at each observation time was the response variable. The factors included in the model were treatment (control, low or medium glyphosate concentration), infection status (uninfected or infected), day of experiment (total of 28 days), time of observation (morning or afternoon) and snail container nested (levels of a factor are nested within the levels of another factor) within treatment. All tests were
performed using the statistical program JMP. Throughout the study, all values are given as the mean +/- SD. Results were considered significant at P<0.05.

2.3 Results

The analysis revealed that treatment and container nested within treatment had significant effects on the hiding rate of snails (Table 2.1). However, renicolid infection, time of day and day of observation did not have any significant effect on snail behaviour. Snails with no infection remained hidden around 75% of the time throughout the day, regardless of glyphosate level (Figure 2.3). The medium glyphosate concentration (3.6mg/L) appeared to increase the infected snail’s hiding time.

Table 2.1 Effect of treatment (control, low or medium concentrated glyphosate), infection status (uninfected or infected), day of experiment (total of 28 days), time of observation (morning or afternoon) and snail container nested within treatment on *Potamopyrgus antipodarum* behaviour. Results of a General Linear Model.

<table>
<thead>
<tr>
<th></th>
<th>Degrees of freedom</th>
<th>Chi-squared</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>14.86</td>
<td>0.0006*</td>
</tr>
<tr>
<td>Infection status</td>
<td>1</td>
<td>0.76</td>
<td>0.384</td>
</tr>
<tr>
<td>Day</td>
<td>1</td>
<td>0</td>
<td>0.9479</td>
</tr>
<tr>
<td>Time of observation</td>
<td>1</td>
<td>0.09</td>
<td>0.7647</td>
</tr>
<tr>
<td>Container [treatment]</td>
<td>15</td>
<td>157.76</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

* Denotes a statistically significant effect
Chapter 2: Snail behaviour

Figure 2.3 Proportion of *P. antipodarum* snails (Black = uninfected, Red = infected) hidden during the day (including morning and afternoon), by treatments (control, low, or medium concentrated glyphosate), across the whole exposure period.

The proportion of snails hidden during the afternoon, when the renicolid’s next host (fish) forages, was observed (Figure 2.4). However, there was no significant effect on the host’s behaviour during this time of day. Again, the medium glyphosate concentration appeared to increase the infected snail’s hiding time.

Figure 2.4 Proportion of *P. antipodarum* snails (Black = uninfected, Red = infected) hidden during the afternoon, by treatments (control, low, or medium glyphosate concentration), across the whole exposure period.
2.4 Discussion

This chapter focused on the effects of two factors, parasitism and pollution, on a host’s foraging behaviour. Infection by renicolid trematodes had no effect on snail behaviour, although parasitism has been shown in other studies to increase the snail’s exposure to predators through its foraging behaviour (Levri and Lively, 1996). The manipulation hypothesis explains how a parasite might have altered host behaviour to increase its chances of reaching the next appropriate host (Bethel and Holmes, 1977). For example, (Levri and Lively, 1996) found that uninfected *Potamopyrgus antipodarum* individuals and those infected by the trematode *Microphallus* sp. foraged at different times of the day, via manipulation. Similarly, the trematode *Microphallus piriformes* altered the dispersal of their snail host, *Littorina saxatilis* (McCarthy et al., 2000). This parasite-induced change might increase the chances of predation by the definitive host (herring gulls) and could be a survival strategy for *M. piriformes* (McCarthy et al., 2000). In this study, there was an absence of altered behaviour in infected snails, which suggested that renicolid did not influence host foraging behavior. Unlike the undescribed renicolid I studied here which is transmitted via a free-swimming cercariae, members of the genus *Microphallus* are trophically-transmitted to birds (Bethel and Holmes, 1977), thus host manipulation might benefit the *Microphallus* species more than renicolid trematodes.

While a behavioural manipulation was not detected here, perhaps renicolid trematodes have other pathological side effects on their host that may coincidentally improve transmission. Because renicolids reach their second intermediate host via free-swimming cercariae, and actively penetrate it, there might be no purpose to extending the host’s foraging time. It would be implausible for the renicolid to manipulate its host towards predators (Bernot, 2003). Instead, the parasite might benefit from re-allocating host energy, gained from foraging, to another function such as growth (Hall et al., 2007; Minchella, 1985). Previously, a study on trematodes of the family Opecoelidae were found to influence the movement and dispersal of their snail host, *Diloma subrostrata* (Miller and Poulin, 2001). Infected *D. subrostrata* snails were limited in their range of movement and direction, and had an increased shell growth compared to uninfected conspecifics. Since this parasite’s next host was a crustacean, reached by free-swimming cercariae, the altered behaviour could not
easily be reconciled with host manipulation (Miller and Poulin, 2001). Cercariae-
transmitted trematodes have been associated with a range of other behavioural shifts
in infected snails. For example, the foraging behaviour of uninfected *Littorina littorea*
snails resulted in 40% more ephemeral algae being consumed than by snails infected
with the trematode *Cryptocotyle lingua* (Wood et al., 2007).

Pollution did affect the *P. antipodarum* host. Exposure to Roundup® at
moderate glyphosate concentration (3.6mg/L) resulted in behaviour change, with
exposed snails remaining hidden more than their conspecifics in the control treatment.
Past studies have shown alterations in snail and fish feeding behaviour in response to
heavy metals (Lürling and Scheffer, 2007; Morley, 2010) and pesticides (Poulin,
1992). Behaviour has been suggested to be one of the most sensitive indicators of sub-
lethal effects of toxins (Gerhardt, 2007). However, the change in behaviour observed
here was unexpected and suggested that exposure to higher concentrations of
glyphosate reduced foraging and/or increased anti-predator behaviour. This is a
similar effect to that of heavy metal pollution on other snail species (Clements et al.,
2000; Lefcort et al., 2002; Lefcort et al., 1999).

Reduced foraging behaviour was associated with an overall reduction in
movement, and it is possible that the pollutant physiologically weakened the snail. A
review by (Morley et al., 2006) concluded that the combination of anthropogenic
pollutants and trematode infection led to a reduction in snail activity and movement.
Low cardiac activity might affect behaviour, feeding and respiration (Thompson,
1997). Similar effects on activity were demonstrated in this study by synergistic
effects from renicolid parasites and glyphosate exposure on the *P. antipodarum* host.
In addition to behaviour, the high glyphosate concentration (36mg/L) caused
relatively rapid mortality of all snails. Survival was similar to that in Kelly et al.’s
(2010a) experiment, which found *P. antipodarum* died within 24 hours of exposure to
high concentration. At the same time, there was no reduction in snail survival for the
medium concentration (3.6mg/L) in this study, which was also seen in previous
studies. Under certain conditions, it may be possible to find the medium concentration
in the field, before rain dilutes the pollutant towards a lower concentration (Rohr et
al., 2008a).
As mentioned, the renicolid trematode alone did not appear to have any effect on *P. antipodarum*’s behaviour, however moderate concentrations of glyphosate did. Together, parasitism and glyphosate could modify the host’s natural behaviour, but future studies should use a larger data set and multiple trematode species before reaching a definitive conclusion. In the case of renicolid, glyphosate may have weakened *P. antipodarum*’s immune response (Blakley et al., 1999), which not only altered foraging behaviour but increased host susceptibility to infection (Rohr et al., 2008a). Because the trematode castrated the snail host (see Chapter One), any energy gained from foraging went towards growth rather than reproduction, which would indirectly benefit the parasite (Hall et al., 2007). Although the phenomenon by which parasites encourage host gigantism is still puzzling researchers, we do know that parasites in larger hosts achieve higher fecundity (Ebert et al., 2004). Therefore, I suggest the renicolid trematode benefited from the weakened host, causing pathological side effects which lowered the risk of exposure to predation (enhanced anti-predatory behaviour) and re-allocated the host’s energy towards growth instead of reproduction. In general, it remains difficult to distinguish whether effects are caused through host manipulation or a pathological side effect from infection (Barber et al., 2000; Poulin, 1995).

Animals are subjected to multiple stressors, thus it is important to study potential interactions among a combination of stressors where possible. This study demonstrated that sub-lethal concentrations of Roundup® affected a snail host’s foraging behaviour, although caution should be advised before assuming causality. Any assessments should also take into consideration that trophically-transmitted parasites and those with cercarial stages have different requirements for transmission. In contrast, renicolid infection had no effect on snail behaviour, contrary to reports for other trematode taxa, possibly indicating differential effects of parasites based on differences in transmission modes.
CHAPTER THREE

Effect of glyphosate exposure on cercariae production in the snail host, Potamopyrgus antipodarum
3.1 Introduction

Anthropogenic stressors and climate change are a global concern, since they will affect ecosystems one way or another (Danz et al., 2007; Poulin and Mouritsen, 2006). The focus of this chapter is on anthropogenic stress caused from agricultural run-off, a less studied but equally concerned impact on the environment (Freedman, 1995). Pollutants found in run-off can accumulate and negatively affect aquatic organisms (Rosenberg and Resh, 1993). Among those aquatic organisms are trematode parasites, which are a ubiquitous part of biodiversity (Poulin and Mouritsen, 2006) and important players in food-web dynamics (Lafferty et al., 2008). It is therefore surprising that parasites have received relatively little attention with regards to anthropogenic pollution. We urgently need more information on how anthropogenic changes in the environment will alter the transmission and impact of parasites.

Trematodes are very common parasites in aquatic habitats, where their abundance is greatly influenced by abiotic factors like temperature, salinity and pollutants (Morley et al., 2003; Poulin, 2006). In order for a trematode parasite to complete its life cycle, it must first pass through the intermediate cercarial stage (Horák and Kolářová, 2011). Cercariae are the free-swimming stage of trematodes and the product of asexual reproduction within the first intermediate snail host; their role is to seek out and infect the next host in the trematode’s complex life cycle (Erasmus, 1972).

Environmental factors can influence both the rates at which trematodes multiply within a snail to produce new cercariae, and the rate at which stored cercariae are released from the snail (Pietrock and Marcogliese, 2003). Certain factors can serve as cues to the window of time during which the larvae should emerge to swimfreely, including response to light-dark cycles, shadows and chemical stimuli (Haas, 1994; Haas and Schmitt, 1982; Lewis et al., 1989). These signals may be altered by climate change and anthropogenic stressors, having an influence on mature cercariae within the molluscan host waiting to expel into the open water (i.e. shedding).
Importantly, the entire output of cercariae depends on the rate at which they are produced and released from the first intermediate host. Cercarial output is an important component of trematode transmission success, i.e. fitness (Erasmus, 1972). However, within an aquatic ecosystem certain external factors, including temperature change and exposure to pollutants, can alter the daily production of cercariae from their snail host (Marcogliese, 2001; Morley et al., 2002b; Studer et al., 2010).

Temperature is generally the most important external factor affecting cercarial output, with an increase in temperature having a positive effect on most trematode cercariae, increasing their emergence from snail hosts (Lo and Lee, 1996; Poulin and Mouritsen, 2006). However, this simultaneous increase only continues until the optimum temperature has been reached, after which threshold the cercarial output decreases (Koprivnikar and Poulin, 2009; Thielges and Rick, 2006). Additionally, snails living at higher salinity may shed more cercariae than snails in lower salinity, but only at higher temperatures (Mouritsen, 2002).

Anthropogenic stressors, such as pollutants (e.g. herbicides), have also been found to alter daily cercarial production (Morley, 2010). (Kelly et al., 2010a) investigated the relationship between glyphosate (the active ingredient in the herbicide Roundup®) and Telogaster opisthorchis cercarial output from the snail host *Potamopyrgus antipodarum*. These authors found that the number of cercariae shed from infected snails was highest when snails were kept in water with intermediate glyphosate concentration (3.6mg/L), compared to control snails. Moreover, the synergy between parasitism and glyphosate caused spinal malformations and decreased survival in the parasite’s next host, the New Zealand native freshwater fish, *Galaxias anomalus* (Kelly et al., 2010b).

Conversely, the pesticide atrazine was found to decrease daily cercarial production from the molluscan host, *Stagnicola elodes* (Koprivnikar and Walker, 2011). Furthermore, Rohr et al. (2008a) looked at four common pesticides (atrazine, glyphosate, carbaryl and malathion) and their effects on host-parasite interactions, focusing on cercariae of the trematode *Echinostoma trivolvis*. Exposure to those pesticides caused no significant enhancement to the virulence of *E. trivolvis* cercariae, however glyphosate’s accompanying surfactant was not included and is equally as
important as glyphosate for monitoring any affects to the host (Rohr et al., 2008a; Tsui and Chu, 2003).

Sub-lethal concentrations of pollutants have been known to affect the physiology, immunology and ecology of snail populations (Rittschof and McClellan-Green, 2005). These potential effects can be beneficial or detrimental to the cercariae produced within snails, and in particular alter the daily production of cercariae from the host. If pollution benefits the production of cercariae from their first intermediate host (e.g. mollusc) then there is increasing concern for the health of subsequent hosts, such as insects, crustaceans, amphibians and fish (see Kelly et al. 2010a).

Molluscs serving as first intermediate hosts to several trematode species provide ideal model systems to monitor these environmental impacts on parasite production. Measuring cercarial output in this single host allows assessment of the risk to several other species serving as second intermediate hosts for trematodes (Morley, 2010). This chapter focuses on the daily production (shedding) of a renicolid species (an unnamed trematode species from two different sites), Coitocaecum parvum and Apatemon sp. cercariae from the snail host, Potamopyrgus antipodarum. The effect of the exposure to the commercial herbicide Roundup® (using the active ingredient glyphosate and its surfactant polyoxyethylene amine, POEA) will be assessed. Comparisons will be made between the daily cercarial output of snails exposed to different glyphosate concentrations and that of control snails. In each experiment, the expectation was that glyphosate would alter the production of daily shedding, compared to the control.

3.2 Methods

The collection, experimental set up, and animal husbandry used for Chapter 3 followed the same protocol as in Chapter 2 (see methods sections 2.2.1-2.2.3).

3.2.1 Collection

Both Tomahawk Lagoon and Lake Waikola were used as field sites for collection of snails infected with renicolids (genetically similar 28S ribosomal DNA sequences confirm these cercariae are the same species; I. Blasco-Costa, unpublished data). As for the other two species, Coitocaecum parvum infected snails were
collected from a section of the Clutha River (near Kaka Point) and *Apatemon* sp. infected snails from Tomahawk Lagoon.

Lake Waihola is predominantly freshwater, receiving brackish water on regular tidal cycles from the Pacific Ocean coast (10km away). This lake is located 40km south from Dunedin, 46°01’S, 170°05’E (Figure 3.1).

![Figure 3.1 Lake Waihola: single collecting site along the publicly accessible shoreline (black arrow).](image)

A location near Kaka Point was used as the collection site for snails harbouring *C. parvum*. This section is part of the Clutha River’s Koau Branch, which drains into the Pacific Ocean about 12km east of the sampling site. This location is 85km south of Dunedin at 46°17’S, 169°45’E (Figure 3.2).
3.2.2 Laboratory procedures

Infection status and trematode species were identified by incubation (see Chapter Two). Afterwards, they were placed individually into wells of a 24-well plate (volume 3.3ml) with 4 individual snails in each row (n=16), except for the *Apatemon* sp. infected snails, which only had 3 snails per row (n=9).

Each snail was placed into a treatment containing either the control (freshwater) or various (low, medium, high) concentrations of glyphosate; see Chapter Two for details regarding the preparation of the glyphosate solutions. This was achieved by filling each well with 2.1ml of either water or the glyphosate solutions; which well received which treatment was determined at random. The high glyphosate concentration was only used for the renicolid, because sufficient snails were only available for this species. Cercariae in each well were counted on a daily basis at the same time every morning, using a dissecting microscope. After the daily count, snails were moved to a new plate with fresh solution (control or glyphosate
solutions, depending on the snail). This was repeated for 28 days, and macrophytes collected from the respective sites were given as food for snails once every week.

Two further replicate trials followed the initial experiment but with variations in snail sample size. This gave a total sample size of n=48 (Tomahawk Lagoon-renicolid), n=36 (Lake Waihola-renicolid), n=18 (Coitocaecum parvum) and n=9 (Apatemon sp.), dispersing the cercariae equally for each treatment used. The variation in snail numbers was due to the extremely low prevalence of some species. Thus, the high concentration (36mg/L) was not used for Apatemon sp. and C. parvum due to their low prevalence. Low concentrated glyphosate (0.36mg/L) is of most importance because it approximates the concentration found in run-off, whereas the medium concentration (3.6mg/L) is found in Roundup®, prior to being diluted from rain and wind (Giesy et al., 2000; Pérez et al., 2007).

When the experiment was completed after 28 consecutive days, snails were dissected for confirmation of their infection status. A snail was defined as uninfected when there was no evidence of sporocyst or rediae in the body. At the end of the experiment, snails initially deemed infected but containing no parasite tissue (sporocysts or rediae) upon dissection, were removed from the analysis. Additionally, snails with concurrent infection by other trematode species were also excluded. In total, only two snails had to be excluded for these reasons.

3.2.3 Data analysis

Each analysis was tailored to the data, and chosen to provide the most appropriate distribution of residuals. The factors included in the model were treatment (control, low glyphosate, medium glyphosate, high glyphosate), snail identity nested (levels of a factor are nested within the levels of another factor) within treatment and day of observation. As mentioned, Coitocaecum parvum and Apatemon sp. were an exception, as these trials had no high glyphosate exposure. All tests were performed using the statistical program JMP. Results were considered significant at P<0.05.

renicolid from Tomahawk Lagoon

Because the data had a large number of zeros (i.e. no cercarial shedding by a particular snail on a given day), the cercarial output data were analysed with a logistic
regression model. The numbers of cercariae released per snail per day was used as response variable, and treated as an ordinal variable with values of 0 (no cercariae), 1 (1 cercaria), 2 (2 cercariae), 3 (3 cercariae), 4 (4-5 cercariae), 5 (6-9 cercariae) or 6 (≥10 cercariae).

**rencolid from Lake Waihola**

The cercarial output data (log x+1 transformed) were analysed with a generalized linear model (GLM), with a Poisson error structure and a log link function. The numbers of cercariae released per snail per day was the response variable.

**Coitocaecum parvum**

The cercarial output data (log x+1 transformed) were analysed with a generalized linear model (GLM), with a normal error structure and an identity link function. The numbers of cercariae released per snail per day was the response variable.

**Apatemon sp.**

The cercarial output data (log x+1 transformed) were analysed with a generalized linear model (GLM), with a Poisson error structure and a log link function. The numbers of cercariae released per snail per day was the response variable.

**3.3 Results**

Prevalences (total number of infected snails divided by total number of snails examined) of infection among field-collected snails for all field sites are given in Table 3.1. Two snails were excluded from Tomahawk Lagoon, due to a double infection with both renicolid and Maritrema sp. during the experiment.
Table 3.1 Prevalence (percentage of infected snails) of infection by a renicolid trematode from two sites, *Coitocaecum parvum* from Kaka Point and *Apatemon* sp. from Tomahawk Lagoon, based on the total number of snails screened from each site.

<table>
<thead>
<tr>
<th>Species</th>
<th>Total snails</th>
<th>Infected snails</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>renicolid (Tomahawk)</td>
<td>934</td>
<td>122</td>
<td>13%</td>
</tr>
<tr>
<td>renicolid (Waihola)</td>
<td>708</td>
<td>70</td>
<td>9.8%</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>1500</td>
<td>18</td>
<td>1.2%</td>
</tr>
<tr>
<td><em>Apatemon</em> sp.</td>
<td>1920</td>
<td>10</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

On average, all three trematode species considered in the present study produced a higher quantity of cercariae on a daily basis when exposed to the low glyphosate concentration (0.36mg/L) than under the control and other concentrated glyphosate conditions (Figure 3.3).

![Figure 3.3](image)

Figure 3.3 Average number of cercariae produced per snail per day, for each trematode species, for either the control or (low, medium, high) glyphosate concentration, across the whole exposure period.

TL- Tomahawk Lagoon, LW-Lake Waihola, KP-Kaka Point

Each trematode species is analysed below.
3.3.1 renicolid (Tomahawk)

The production of renicolid cercariae from snails at Tomahawk Lagoon were significantly affected by the treatment, i.e. the presence and concentration of glyphosate, snail identity nested within the treatment, and the day of observation (Table 3.2). Snails exposed to low glyphosate produced cercariae daily over 25 days, compared to the control (doubled) and medium, high glyphosate (Figure 3.4), which is also seen when averaged over the entire experimental period (Figure 3.5). This experiment was intended to last 28 days, but due to unforeseen circumstances it ended after 25 days, and snails remaining in the high concentration died after 18 days.

Table 3.2 Effect of treatment (control and low, medium, high glyphosate concentration), snail identity nested within treatment, and the day of the experiment on the average number of renicolid cercariae produced from infected snail hosts collected at Tomahawk Lagoon over the entire period of the experiment (25 days). Results of the General Linear Model.

<table>
<thead>
<tr>
<th></th>
<th>Degrees of freedom</th>
<th>Chi-squared</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td>13.18</td>
<td>0.0043*</td>
</tr>
<tr>
<td>Snail [Treatment]</td>
<td>30</td>
<td>51.24</td>
<td>&lt;.0092*</td>
</tr>
<tr>
<td>Day</td>
<td>1</td>
<td>36.6</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

* Denotes a statistically significant effect
Figure 3.4 Average daily production of renicolid cercariae from infected *P. antipodarum* snail hosts from Tomahawk Lagoon for each treatment, over 25 days.

Figure 3.5 Overall mean (±standard error among replicates) number of renicolid cercariae produced per day by infected *P. antipodarum* snail hosts from Tomahawk Lagoon for each treatment, across the whole exposure period.
3.3.2 *renicolid* (Waihola)

Based on the model, treatment had little effect on cercarial production (Table 3.3). However, snail identity nested within each treatment and the day of observation did have a significant effect on the number of cercariae produced by the snails. Snails exposed to low glyphosate produced more cercariae daily over 28 days and nearly doubled compared to the control and medium, high glyphosate (Figure 3.6), which is also seen when averaged over the entire experimental period (Figure 3.7). Renicolid cercarial production was generally higher in Lake Waihola, than in Tomahawk Lagoon.

| Table 3.3 Effect of treatment (control and low, medium, high glyphosate concentration), snail nested within treatment, and the day of the experiment on the average number of renicolid cercariae produced from infected snail hosts collected at Lake Waihola over the entire period of the experiment (28 days). Results of the General Linear Model. |
|---------------------------------|------------------|------------------|
| Degrees of freedom | Chi-squared | Probability |
| Treatment             | 3      | 6.94            | 0.0738 |
| Snail [Treatment]   | 41     | 162.93          | 0.0001* |
| Day                  | 1      | 47.41           | <.0001* |

* Denotes a statistically significant effect
Figure 3.6 Average daily production of renicolid cercariae from infected *P. antipodarum* snail hosts from Lake Waihola for each treatment, over 28 days.

Figure 3.7 Overall mean number (±standard error among replicates) of renicolid cercariae produced per day by infected infected *P. antipodarum* snail hosts from Lake Waihola for each treatment, across the whole exposure period.
3.3.3 Coitocaecum parvum

Similar to the renicolid cercariae from Tomahawk Lagoon, Coitocaecum parvum cercariae production from infected snails was significantly affected by all three factors: treatment used, snail identity nested within treatment, and day of observation (Table 3.4). Snails exposed to low glyphosate produced more cercariae daily over 28 days and nearly doubled compared to the control and medium glyphosate (Figure 3.8), which is also seen when averaged over the entire experimental period (Figure 3.9).

Table 3.4 Effect of treatment (control and low, medium glyphosate concentration), snail nested within treatment, and the day of the experiment on the average number of C. parvum cercariae produced from infected snail hosts collected at Kaka Point over the entire period of the experiment (28 days). Results of the General Linear Model.

<table>
<thead>
<tr>
<th></th>
<th>Degrees of freedom</th>
<th>Chi-squared</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>12</td>
<td>112.56</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Snail [Treatment]</td>
<td>15</td>
<td>144.6</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Day</td>
<td>24</td>
<td>78.7</td>
<td>&lt;.0001*</td>
</tr>
</tbody>
</table>

* Denotes a statistically significant effect
Chapter 3: Cercariae production

Figure 3.8 Average daily production of *C. parvum* cercariae from infected *P. antipodarum* snail hosts from Kaka Point for each treatment, over 28 days.

Figure 3.9 Overall mean number (±standard error among replicates) of *C. parvum* cercariae produced per day by infected *P. antipodarum* snail hosts from Kaka Point for each treatment, across the whole exposure period.
3.3.4 *Apatemon* sp.

For *Apatemon* sp., both the treatment used and snail nested within treatment had a significant effect on the cercarial production. However, the day of the experiment had little effect on the snail’s cercarial production (Table 3.5). This particular trematode species was only tested in one trial; due to the low prevalence there were no repeated trials. Snails exposed to low glyphosate produced more cercariae daily over 28 days and tripled compared to the control and medium glyphosate (Figure 3.10), which is also seen when averaged over the entire experimental period (Figure 3.11).

<table>
<thead>
<tr>
<th></th>
<th>Degrees of freedom</th>
<th>Chi-squared</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>16.79</td>
<td>0.0002*</td>
</tr>
<tr>
<td>Snail [Treatment]</td>
<td>6</td>
<td>23.38</td>
<td>0.0007*</td>
</tr>
<tr>
<td>Day</td>
<td>1</td>
<td>0.58</td>
<td>0.4455</td>
</tr>
</tbody>
</table>

* Denotes a statistically significant effect
Figure 3.10 Average daily production of *Apatemon* sp. cercariae from infected *P. antipodarum* snail hosts from Tomahawk Lagoon for each treatment, over 28 days.

Figure 3.11 Overall mean number (±standard error among replicates) of *Apatemon* sp. cercariae produced per day by infected *P. antipodarum* snail hosts from Tomahawk Lagoon for each treatment, across the whole exposure period.
3.4 Discussion

Many environmental factors influence the production of cercariae within and their release from their snail host. Herbicide run-off was chosen as the key factor in this particular study, which aimed to quantify the effects of glyphosate exposure on the daily production of cercariae from the snail host, *Potamopyrgus antipodarum*. Infected snails collected from all sites had a diverse range of trematode species, all with a low prevalence. The low prevalence could be due to the timing of collection (seasonal, temperature, climate), or the sampling location.

In all experiments, the cercarial output from infected snails started with an initial rise in the first few days followed by a slow decline, producing a characteristic time-dependent curve (Anderson and Crombie, 1984). This could be due to the decreasing quality of the snail’s diet, which caused less energy to be passed on to the parasite for reproduction. (Rondelaud et al., 2004) demonstrated that a higher quality diet given to snail hosts led to increased cercarial shedding. At the same time, snails used in this experiment may have varied in age, which can affect the quantity or quality of resources available to multiplying trematodes (Gérard and Theron, 1997). The daily output plots illustrated a stronger negative impact of the day of observation on the daily production of renicolid cercariae than in the other two species. Control snails had an overall low daily production throughout the experiment, while exposure to low levels of glyphosate raised cercarial production. The results from this study were slightly different from those of Kelly et al. (2010a), who studied the trematode *Telogaster opisthorchis* and found that infected *P. antipodarum* snails exposed to medium glyphosate (3.6mg/L) produced the most cercariae. Other than pesticides, snail hosts have been exposed to heavy metals, which decreased overall cercarial production with increasing exposure (Cross et al., 2001; Morley, 2010).

Additionally, with the exception of *Coitocaecum parvum*, the infected snails produced as many cercariae, if not less, under the control conditions as when exposed to medium and high concentrations of glyphosate. Under low glyphosate concentration, snails showed a nearly two-fold greater production of cercariae, compared to control snails, for all the trematode species except *Apatemon* sp., in which cercarial output was nearly three-fold higher in the low concentration.
On average, *Coitocaecum parvum* infected snails produced more cercariae than the other trematodes. Renicolid trematodes from both sampling locations had the lowest average daily production, yet the highest prevalence compared to the other two trematode species. This interspecific variation in daily emergence is expected because of differences in trematode species, e.g. cercarial body sizes (Poulin, 2007). Values for the control snails show how the natural output profiles of the different trematodes would look in non-polluted environments. Also, individual snails naturally shed cercariae at a different rate, such that an infected snail could take days to release a few mature cercariae, whereas other snails shed cercariae at a high rate per day (Curtis and Hubbard, 1990). The generalized linear models used to analyse the data took individual differences among snails in the rate of cercarial output into consideration to uncover the true effect of glyphosate.

Cercariae often emerge from the snail in an environment that may also contain metals, pesticides and variable acidity (pH), making it difficult to assess which possible anthropogenic factor is causing the increased production (Morley et al., 2003). Firstly, one reason for increased shedding in the presence of glyphosate could involve the pollutant weakening the snail just enough to allow the trematodes to exploit stored host energy no longer used for the host’s reproduction (due to castration by the parasite). Chapter Two suggested parasites may benefit from this castration and use the host’s energy for growth, with a positive effect on parasite reproduction and daily cercarial output (Hall et al., 2007). Alternatively, glyphosate (in lower concentrations) may lead to increased biomass of periphyton algae, enhancing the snail’s resources for cercarial production under experimental conditions (Kelly et al., 2010a).

Secondly, with pollution weakening the host’s immune system, it was expected that cascading effects could alter the production of resident parasites within the host. Similar to glyphosate, heavy metals such as zinc and cadmium cause immunosuppression within the snail host (Morley et al., 2006). However, in contrast to glyphosate’s effect on cercarial production, heavy metals delay the development of trematode sporocysts within the mollusc and decrease cercarial production (Hira and Webbe, 1972; Yescott and Hansen, 1976). This difference may be due to glyphosate increasing glycogen levels in the snail host, as reported for the fish *Rhamdia quelen*.

Chapter 3: Cercariae production
(Glusczak et al., 2007), thus providing the energy parasites needed for increased cercarial production. Parasites impose a metabolic burden on the host, consuming the host’s glycogen and amino acids, therefore levels of intracellular aminotransferase (enzyme used to produce amino acids for protein) are increased in infected snails to replenish the lost metabolites (Manohar et al., 1972). (Christian et al., 1993) found that *Pseudosuccinea columella* infected snails increase their production of amino acids at lower concentrations of glyphosate (0.1mg/L), compared to higher ones (1.0mg/L), which may explain why the infected *P. antipodarum* snails exposed to the low concentration (0.36mg/L) in this study consistently produced a higher quantity of cercariae. Future studies should focus on the infected molluscan host’s aminotransferase levels in conjunction with consumption rates of metabolites of the parasites.

This chapter focused on parasite-snail relationships, and any alterations to the balance between these antagonists can have cascading effects on the rest of the ecosystem (Rohr and Crumrine, 2005). For instance, if parasites exiting from snails increase in quantity, then their next intermediate host, where the parasite encysts (e.g. the fish *Galaxias anomalus*), is at an increased risk of mortality (Kelly et al., 2010a; Kelly et al., 2010b). Fish are not the only second intermediate host that may be harmed from the increased release of cercariae. Depending on the trematode species, the next host can also be an insect, crustacean, mollusc or amphibian. As an example, the recent global concern over declining amphibian populations is now common knowledge (Kiesecker, 2002; Relyea, 2011; Rohr et al., 2008a; Stopper et al., 2002). Both herbicides and parasitism have synergistic effects on amphibians. For instance, the pesticide atrazine suppressed immune responses in the leopard frog, *Rana pipiens*, which prevented it from fighting off larval trematode encystment (Rohr et al., 2008b). This increased vulnerability has severe consequences for the host, because trematodes encysting within the amphibian host disrupt the normal development of limb buds (Stopper et al., 2002). This pollutant-parasitism synergy leads to amphibian mortality (Shirk, 2010). In the wild, amphibians located near agricultural run-off sites have more limb deformities than other conspecifics, suggesting this is a widespread phenomenon (Kiesecker, 2002).
There is a growing awareness of the impact environmental stressors have on host-parasite relationships (Koprivnikar et al., 2006). This study found trematode cercariae benefited from exposure to a low concentration of glyphosate, which may have subsequent detrimental effects on their second intermediate host (e.g. fish, amphibians). In freshwater ecosystems, we would mostly expect to see lower concentrations in run-off, as a result of irrigation practices and rain storms, and these results therefore suggest that the indirect effects of glyphosate on parasitism could be common and widespread (Giesy et al., 2000; Pérez et al., 2007; Richards and Baker, 1993). Therefore, the next step should be to quantify the effect of glyphosate on what cercariae do after leaving their snail host, i.e. their survival, infectivity to the next host and their impact on the latter; this would provide a more complete assessment of the impact of the parasitism-pollution synergy on freshwater systems.
CHAPTER FOUR

Effect of glyphosate exposure on survival of cercariae of a renicolid trematode
4.1 Introduction

Environmental assessment involves quantifying the impacts caused by any environmental changes, including pollution (Lafferty, 1997), e.g. agricultural run-off. These pollutants can alter parasite infectivity and host susceptibility because parasitic transmission is dependent on the surrounding environment (Morley et al., 2006). Trematode parasites with complex life cycles need successful transmission between intermediate and definitive hosts to succeed and complete life stages. Transmission refers to the ‘events occurring from the departure of an organism from one host until contact is made with the next host in the cycle’ (Crompton and Joyner, 1980). During this transition, they are vulnerable to effects of the environment, and for this reason are a good indicator for anthropogenic effects on the surrounding communities (Koprivnikar et al., 2006).

Within the molluscan intermediate host, asexual production of cercariae (trematode transmission stages) continues over time. The daily emergence of cercariae from an intermediate host is said to have a circadian rhythm (Galaktionov and Dobrovolskij, 2003). Cercariae often follow adaptive patterns of emergence, usually at the time of day when the probability of finding the next host is maximal. After exiting from a host, cercariae are in a vulnerable free-swimming stage (Pietrock and Marcogliese, 2003) and quickly expend their limited energy stores while swimming to the next host. Time is of the essence for these larvae, as their main objective is to race against predation (by an inappropriate host) and other external factors (e.g. pollution) to infect the next suitable host (Erasmus, 1972; Gibson et al., 2008).

During this vulnerable free-swimming stage, the parasite could be exposed to agricultural pollutants, e.g. herbicides, coming from surrounding fields into freshwater (Gibson et al., 2008; Pietrock and Marcogliese, 2003). The cercariae might come into contact and absorb those pollutants either directly, during their short free-swimming stage, or during their endoparasitic stage inside the host’s tissues (Cross et al., 2001). At the same time, a host’s lifespan can be shortened by both parasite infection (pathology) and pollution (weakened immune response) (Hechinger et al., 2009; Rohr et al., 2008b).
Survival of the free-swimming larvae is greatly affected by their environmental surroundings (Pietrock and Marcogliese, 2003; Thielges et al., 2008). Previously, researchers have mostly looked at the effects of temperature, molluscicide pesticides (Ghandour and Webbe, 1975; Viyanant et al., 1982) and heavy metals on cercarial survival (Morley et al., 2002b). For example, in terms of temperature, cercariae of *Transversotrema patialense* had a maximum life-span at 24°C of 44 hours, with 50% of the larvae surviving over 26 hours, with survival dropping sharply at lower or higher temperatures (Anderson and Whitfield, 1975). Also, unlike pesticides, which decreased survival, the combination of cadmium and zinc heavy metals was found to increase cercariae survival (Morley et al., 2002b).

Few studies have looked at the effects of common herbicides on cercariae longevity (Koprivnikar et al., 2006; Rohr et al., 2008a). Therefore, this chapter utilised the snail host, *Potamopyrgus antipodarum*, and an undescribed renicolid trematode to quantify cercarial survival after exposure to the common commercial herbicide Roundup® (using the active ingredient glyphosate and its surfactant polyoxyethylene amine, POEA). Survival was measured during the open-water swimming stage, in between hosts.

### 4.2 Methods

The general experimental set up used for this chapter followed the same protocol as in Chapter Two (see methods sections 2.2.1-2.2.3).

#### 4.2.1 Collections

Snails were collected from Tomahawk Lagoon, and the parasite of the study was the same unnamed and undescribed renicolid cercariae studied previously.

#### 4.2.2 Laboratory procedures

After snails were screened and sorted by infection status (see Chapter Two), a total of n=24 snails were confirmed to be infected by renicolid trematodes. The infected snails were divided into two replicate experiments, each involving a 12-well
plate (volume 3ml). In each replicate experiment, four snails were randomly assigned to each of the treatment (control, low and medium glyphosate). Individual snails were placed into each well, along with 2.1ml of either a control or (low, medium) concentrated glyphosate; see Chapter Two for details regarding the preparation of the glyphosate solutions. Snails were observed for cercarial shedding under a dissecting microscope, and snails were removed from the well after cercariae emerged.

Each hour, cercariae were monitored individually under a dissecting microscope to measure the duration of their survival (determined by swimming and/or twitching motion) in each well. Cercariae start to disintegrate immediately after they die, making their time of death evident (Croft, 1933). There was a total of n=61 cercariae, each snail producing one to five cercariae at a time. The 61 cercariae consisted of 21 in the control treatment, 19 in the low glyphosate treatment, and 21 in the medium glyphosate treatment (Figure 4.1). Each snail was used once after the first shedding was complete.

4.2.3 Data analysis

Because cercarial lifespans fell into two distinct time classes (Figure 4.2), the survival data were analysed with a logistic regression model. The lifespan of cercariae was used as a response variable, and treated as an ordinal variable with values of 1 (35 hours or less), or 2 (>40 hours). The factors included in the model were treatment (control, low and medium concentrated glyphosate), and snail identity nested within treatment. All tests were performed using the statistical program JMP. Results were considered significant at P<0.05.

4.3 Results

The renicolid cercariae survived from 27 to nearly 60 hours. Treatment, i.e. the presence and concentration of glyphosate, had a significant effect on the survival of renicolid individuals (Table 4.1). The control treatment, without glyphosate, produced lowest survival time while the medium concentration (3.6mg/L) had highest survival (Figure 4.3). These results suggest glyphosate increased the survival of renicolid cercariae from infected _P. antipodarum_ hosts. There was no significant
inter-individual differences in the survival rate of cercariae among snails, which were nested (levels of a factor are nested within the levels of another factor) within each treatment.

Table 4.1 Effect of treatment (control, low and medium concentrated glyphosate) and snail nested within a treatment on renicolid cercariae survival time; results of the General Linear Model.

<table>
<thead>
<tr>
<th></th>
<th>Degrees of Freedom</th>
<th>Chi-squared</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>42.26</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>Snail [Treatment]</td>
<td>21</td>
<td>16.91</td>
<td>0.7163</td>
</tr>
</tbody>
</table>

* Denotes a statistically significant effect

Figure 4.1 Number of renicolid cercariae used in each treatment (control, low and medium glyphosate concentration).
Chapter 4: Cercariae survival

Figure 4.2 Frequency distribution of renicolid cercariae survival, 34% of cercariae did not survive past 35 hours whereas 66% of cercariae exceeded 40 hours in survivorship.

Figure 4.3 Overall mean survival (± standard error among replicates) of renicolid cercariae for each treatment (control, low and medium glyphosate concentration).
4.4 Discussion

Free-swimming cercariae represent a brief transmission stage associated with limited energy supply (Gannicott and Tinsley, 1998). Cercaria survival, along with infectivity, is essential to the success of trematode transmission, and is strongly dependent on external factors (e.g., temperature, pH, salinity, pollution) (Ghandour and Webbe, 1975; Kelly et al., 2010a; Morley et al., 2002b; Viyanant et al., 1982). For example, (McCarthy, 1999) found a five-fold decrease in survival of the cercariae Echinoparyphium recurvatum when water temperatures approached 30°C. The presence of pollutants, e.g., herbicides, pesticides and heavy metals, have also been shown to affect cecarial life span (Koprivnikar and Walker, 2011; Morley et al., 2002b), however, the results were contradictory. Therefore, the study presented here quantified effects of glyphosate exposure on survival of renicolid cercariae, after shedding from the Potamopyrgus antipodarum snail host.

Pollutants were hypothesized to have a strong effect on parasite transmission as a result of direct toxicity to both the hosts and the free-living parasite stages as well as indirect effects on host densities and immune responses (Lafferty and Holt, 2003). In this study, cercarial longevity showed a gradual increase with exposure to higher glyphosate concentrations. More importantly, the expected environmental concentration (3.6mg/L) had a significantly higher survival rate (~52 hours) than the control (~33 hours). These results differ from previous studies, which have shown that atrazine, another common herbicide, significantly increased Echinostoma trivolvis cercarial mortality (living around 14-18 hours) at the highest expected environmental concentration (0.2mg/L) (Koprivnikar et al., 2006; Rohr et al., 2008a). Other agricultural pollutants (e.g., glyphosate, carbaryl, melathon) had no effect on cercariae (Rohr et al. 2008a). However, Rohr et al. (2008a) did not include glyphosate’s accompanying surfactant (POEA) in the formulation, which is known to be more deleterious to hosts than glyphosate (Rohr et al., 2008a; Tsui and Chu, 2003). Additionally, cercariae exposed to heavy metals were found to have slower swimming speeds during their free-living stage, along with lower longevity (Cross et al., 2001). Overall, it would appear that pollution effects on cercariae are strongly species and pollutant specific.
Although it is unknown why cercarial survival increased with exposure to glyphosate, longevity is generally mostly dependent upon the amount of glycogen reserves available for utilization (Lawson and Wilson, 1980). In Chapter Three’s discussion, I suggested lower concentrations of glyphosate may increase the production of certain proteins within the snail host (aminotransferase) (Manohar et al., 1972). Because the snail may have increased protein production at lower concentrations of glyphosate, perhaps the infected snail was able to provide a better habitat for cercariae, which increased their subsequent survival. (Yoon, 1964) found that increased aminotransferase activity benefited some parasites, by synthesising proteins required for fitness. There is a dearth of studies on enzyme systems in trematodes, in particular with relation to herbicides, but it is known that trematodes are able to absorb amino acids from diffusion of materials through the snail host’s gut (Min and Seo, 1966). Therefore, aminotransferase is potentially an important link with cercarial fitness (quality and/or quantity) and should be researched further.

Increased survival and glycogen reserves are not the only traits involved with cercarial transmission. In addition to longevity, cercariae need to successfully infect their next host (Combes, 1991; Pietrock and Marcogliese, 2003). Although increased survival and energy would increase the chances of transmission, the pollutants could have adverse effects on infection. For example, one study found atrazine and glyphosate had no effect on *Echinostoma trivolvis* infectivity (Rohr et al., 2008a), while another study using different methodology found similar concentrations of atrazine reduced cercarial infectivity (Koprivnikar et al., 2006). Once again, Rohr et al. (2008a) did not conduct cercarial infectivity with exposure to glyphosate along with its surfactant. More research, with more standardized methods, are necessary to make sense of these contradictory results and to further investigate the effects of exposure to pollutants on successful cercarial transmission.

The difference in results between this current study and others might be due to the different trematode species or type of pollution applied and its concentration. We are still far from a final assessment of the general impact of pollutants on the survival and infectivity of parasite free-living stages. In general, a parasite’s success depends upon the balance between the effects pollution have on a host’s behaviour and immunity, as well as a parasite’s infectivity (Koprivnikar et al., 2006). Further studies
will need to investigate the possible consequences of exposure to pollution for free-swimming cercariae as well as the relationship between pollutants and aminotransferase production by the molluscan host.
CHAPTER FIVE

General conclusion
5.1 Introduction

Pollution, after habitat loss, is the second greatest threat to aquatic and amphibious species (Wilcove et al., 1998), yet it is understudied in conservation biology (Lawler et al., 2006; Wiens et al., 2010). Pollution, in particular, may impair the immune response of a wide range of host species, increasing their susceptibility to parasites and pathogens (Sures, 2006). While the impact of disease is often considered less important, its role in conservation, via its effects on population dynamics, has been remarkably underestimated (Smith et al., 2009). Studies on the effects of parasites and pathogens on their hosts are often conducted in isolation of other potentially detrimental influences. However, it is the interactions between these stressors and other environmental factors, such as pollution or habitat loss, that may exacerbate their impact (Koprivnikar, 2010; Smith et al., 2009). Thus, studies overlooking parasitism and/or pollutants in an ecosystem, may considerably underestimate the actual ecological risks of host mortality (Rohr et al., 2008b).

Of the many sources of pollutants entering aquatic ecosystems, agricultural activities have been identified as major contributors, leading to substantial inputs of fertilisers and herbicides into freshwater systems (Tsui and Chu, 2003). There is growing concern regarding the use of herbicides on large agricultural farms, along with the risks of those pollutants entering watercourses. Herbicides bind to soil particles and are subsequently transported from their intended area of application to surface and ground waters (Vogel and Linard, 2011). Even if farms use no-till, and require only direct seed planting without disturbing soil, the soil still erodes via run-off (Burchett and Burchett, 2011; Richardson and Chichester, 1992). Run-off results from erosion and the amount of rain and wind affects how much run-off spreads pollutants into the water (Fargasova, 1994). Run-off exposes non-target organisms such as aquatic invertebrates and parasites to these potentially harmful substances (Tsui and Chu, 2003). Moreover, until two decades ago no research existed on the effects of pollutants on trematodes, specifically cercarial survival (Poulin, 1992).

Among the potential pollutants found in New Zealand run-off are chemicals from the common commercial herbicide Roundup®, such as glyphosate and its surfactant POEA. Large-scale farmers and small-scale gardeners use Roundup® to
control weeds (Hued et al., 2011). Because this product adsorbs to soil, low concentrations of the product (containing glyphosate and POEA) enter freshwater streams and lakes via run-off (Relyea, 2005b). Detrimental effects to exposed organisms such as molluscs, which typically serve as first hosts of trematodes can have cascading effects on transmission dynamics of the parasite to subsequent hosts, which include invertebrates, fish and amphibians (Johnson et al., 2007). As long as the molluscan host is able to persist in the environment and provide essential resources to the trematodes in the presence of glyphosate, the parasites can at least sustain or even increase their infectivity and/or quantity (Min and Seo, 1966).

5.2 General results

The research presented here found, firstly, that renicolid trematodes had no effect on the behaviour of their freshwater snail host *Potamopyrgus antipodarum*, other than leaving the host castrated. Glyphosate had an effect on the host, which left the snail weakened and less active (Chapter Two). Secondly, lower concentrations of glyphosate (0.36mg/L) resulted in the highest cercarial production of three trematodes species (taxonomically distinct) from within the host compared to the control and higher concentrations (3.6 and 36 mg/L) (Chapter Three). Finally, renicolid cercariae had increased fitness when exposed to glyphosate, i.e. surviving longer after leaving the snail host, while searching for the next host (Chapter Four).

My studies suggest the phenomenon of hormesis (Sanders et al., 1983), whereby the low toxic concentration of glyphosate appeared to benefit the overall quality and quantity of cercariae from different trematode species. Hormesis needs to be more widely recognised in ecotoxicological research, with a focus on host-parasite relationships and their cascading effects (Schreck, 2010). Thus, knowledge from these studies must be integrated into future management and conservation strategies (Rohr et al., 2008b).

5.3 Prevention

There are four major preventative measures that can be taken to reduce the risk of pollutants entering waterways via run-off. First, encouraging the development of organic farms and gardens (Parr et al., 1986). Second filter strips could be
established on riverbanks and lakeshores to filter out the pollutants from run-off. These filters are wide strips of vegetation that intercept overland sheet flows of run-off (Heede, 1990). This would prevent substantial quantities of pesticides/herbicides from entering waterways. Third, sediment basins (ponds excavated to retain run-off) and silt fences (vertical fences of filter fabric) might be suitable for farms. In the USA, organisations including The Environmental Protection Agency (EPA) and the National Oceanic and Atmospheric Administration (NOAA) have participated jointly in developing and implementing these filters in coastal zones (EPA, 1995). A fourth and relatively new method is hydroponics and/or aquaponics, which require no soil and maintain animals and their surrounding plants through a sustainable technique (Diver, 2006).

**5.4 Future studies**

Parasites tend to be overlooked in the ecological community, but in order to solve global biodiversity issues such as decreasing amphibian communities (Wake and Vredenburg, 2008) and endangered native freshwater fish (Helfman, 2011; Hunt et al., 2011) we must consider them as causal factors and identify what regulates their abundance (Van Allen et al., 2012). In addition to parasites, decreasing the usage of agricultural pollutants may reduce limb deformities in amphibians and spinal malformations in native fish (Kelly et al., 2010a; Rohr et al., 2008a; Rohr et al., 2008b). Future studies should investigate the impacts of pollutants on a greater number of host-parasite interactions and of pollution on food web modifications, which may result from the increased impact of parasitism on their hosts and reduced resilience of aquatic ecosystems. Additionally, the hypothesis that glyphosate causes increased aminotransferase within a snail host and subsequently provides the parasite with more glycogen reserves should be investigated. Enzyme activity is hardly looked at within parasite-host relationships, but there could be a relationship with the presence of pollutants. Another aspect of pollutant exposure that was not researched in this thesis, but should be explored, is the infectivity rate for free-swimming cercariae to their next host. Chapters Three and Four found an increased cercarial quantity and quality with exposure to the expected environmental concentrations of glyphosate (3.6mg/L). However, in order to fully assess the success rate of cercarial transmission, infectivity must be studied. Although these studies are performed on invertebrates, they can be used as a model for fish and amphibian conservation.


EPA, 1995. Erosion, sediment and runoff control for roads and highways. United States Environmental Protection Agency


Folmar, L., Sanders, H., Julin, A., 1979. Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates. Archives of Environmental Contamination and Toxicology 8, 269-278.


References


Hechinger, R.F., in prep. Faunal survey and identification key for the trematodes infecting Potamopyrgus antipodarum (Gastropoda: Hydrobiidae) as first intermediate host. in prep.


Hued, A.C., Oberhofer, S., de los Ángeles Bistoni, M., 2011. Exposure to a commercial glyphosate formulation (Roundup®) alters normal gill and liver histology and affects male sexual activity of *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes). Archives of Environmental Contamination and Toxicology **1**, 1-11.


Appendix
Appendix to Chapter One

In order to confirm the identity of the unidentified trematode, we amplified a partial sequence of the 28S ribosomal DNA region. Forward and reverse PCR primers for 28S were U178: 5’-GCACCCGCTGAAYTTAAG-3’ and L1642: 5’-CCAGCGCCATCCATTTTCA-3’, respectively (Lockyer et al., 2003). The PCR profile used for amplification of 28S was initial DNA denaturation (94°C for 2 min), 35 cycles of amplification (94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min 10 sec), and an extension hold at 72°C for 4 min.

PCR primers and an additional internal primer, LSU1200R: 5’-GCATAGTTCCACCATCTTTTCGG-3’ (Littlewood et al., 2000), were used for sequencing. PCR amplicon was cycle-sequenced from both strands using ABI BigDye™ Terminator v3.1 Ready Sequencing Kit, alcohol-precipitated, and run on an ABI 3730xl automated sequencer. Contiguous sequence was assembled and edited using Sequencher™ (GeneCodes Corp. 4.10.1). Sequences of closely related species available in Genbank were obtained by performing a search in BLAST® using the megablast algorithm. Newly obtained sequences were aligned with those sequences with high similarity (86%) using MUSCLE implemented in MEGA 4.0 with default parameter values. A Neighbour Joining (NJ) phylogenetic tree (Figure 1.8) was built using the GTR evolutionary model to cluster the sequence to a family. Divergence among the closest sequences was estimated as p-distance. The newly sequenced specimens clustered as sister to Renicola sp. (AY116871) and showed 15.6% genetic divergence suggesting that it represents a different genus of the Renicolidae not previously sequenced (I. Blasco-Costa, unpubl. data). Morphological features, e.g. suckers, tail and stylet of the cercariae, also agrees with the molecular classification of the specimens sequenced to Renicolidae.
Figure 1.8 Phylogenetic tree for unknown renicolid trematode species infecting *P. antipodarum* snails from both Lake Waihola (SabLwsx) and Tomahawk Lagoon (SabTmLSx) based on 28S ribosomal DNA sequences (Isa Blasco, 2011 unpublished data). Values on the nodes represent bootstrap support, and sequences in blue represent Renicolidae species available in Genbank.
Appendix

Poster presented at Australian Society for Parasitology Conference 2011

Herbicides affect proliferation of trematode in freshwater snail

Sabrina Hock, Robert Poulin

Department of Zoology, University of Guelph

Objective:

Does glyphosate affect daily production and survival of Renicolidae cercariae?

Methods

- Snails were kept individually in wells in culture plates, at different glyphosate concentrations for 28 days.
- Cercarial release was checked daily, and all cercariae died were counted.
- Survival of cercariae over time was also monitored at the different glyphosate concentrations.

Graph 1: Daily cercariae production

Central
Low
Medium
High

tu

Graph 2: Cercariae survival

Central
Low
Medium
High

Conclusions

- The lowest concentration has the highest impact on cercarial production.
- The highest concentration has a lethal effect on small hosts, consequently killing cercariae.
- Cercariae in medium concentrations have the longest survival time.
Appendix references

