Environmental effects on the biology of the cestode *Schistocephalus solidus* and its interactions with aquatic hosts

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Environmental effects on host-parasite interactions in the aquatic cestode *Schistocephalus solidus*

Abstract

The broad aim of the work undertaken in this thesis was to increase understanding of how environmental changes in aquatic environments impact host-parasite interactions in the experimentally amenable copepod - three spined stickleback - *Schistocephalus solidus* model using controlled, laboratory experiments. In natural environments, animals are being exposed to a suite of anthropogenic stressors including temperature and heavy metal pollution. In this thesis the effects of temperature and heavy metals pollution are studied to determine the effect of these stressor on the growth development, survival and life cycle of both hosts and parasites.

This thesis first investigated the effect of the temperature on the survival of infective free-swimming stages (coracidia) of *Schistocephalus solidus*, and their subsequent development as procercoid larvae in copepods (first intermediate hosts). The survival of coracidia was temperature-dependent, with the longest survival times at 10°C, and being reduced at 15°C and 20°C. The growth of procercoids in copepod hosts was faster at 20°C than at 10°C or 15°C. The second part of this thesis investigated the effect of water hardness and the influence of heavy metals on the survival of *S. solidus* coracidia, and on the development of procercoids in copepods. The survival of coracidia was shown to be sensitive to low water hardness (i.e. soft water), but was unaffected by heavy metals at the concentrations used. Procercoids developing in copepod hosts attained a larger size when reared under heavy metal treatments in hard water. The third part of this thesis investigated the effect of elevated zinc concentration (0.2 μg/L, 2 μg/L, 20 μg/L and 200 μg/L) on the viability of developing *S. solidus* eggs, on the survival of coracidia and on the development and growth of procercoids in copepod hosts. Under elevated zinc concentrations, *S. solidus* eggs were able to develop and hatch, and coracidia were shown to survive longer at higher zinc concentrations. After being exposed to zinc, procercoids grew more quickly in copepod hosts. The last part of this thesis investigated how the growth of early larval stages of the parasite in copepod hosts exposed to zinc affected the subsequent performance of the second larval (plerocercoid) stage in the second intermediate host, the three-spined stickleback *Gasterosteus aculeatus*. *Schistocephalus solidus* procercoids, which grew larger in copepods exposed to zinc, developed into larger plerocercoids when transmitted to the three-spined stickleback hosts, suggesting carry-over effects in this complex parasite life cycle.

The overall findings of the thesis are that environmental stressors in aquatic environments, such as temperature and heavy metal pollutants, can have complex and divergent implications for different parasite life cycle stages, with potentially complex implications for the dynamics of parasite life cycles in ecosystems subject to environmental changes.

Acknowledgements

emptiness from our life.

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1.1 Parasitism

Parasitism is a relationship between individuals of two different species in which one (the parasite) uses the other (the host) as its environment from which it derives nourishment and can implies harm to the host (Esch and Fernandez, 2013). Parasites are a highly diverse group of organisms that have evolved different strategies for infecting and exploiting their hosts. Parasites have adapted to survive, feed, develop and reproduce on or in their hosts, and in doing so, exploit them (Clayton and Moore, 1997; Poulin, 2011). Parasites can be broadly classified as either endoparasites or ectoparasites. Endoparasites live inside the body of their hosts, and include a wide variety of protozoans, digeneans, cestodes, nematodes, and acanthocephalans (Bush, 2001). Endoparasites can be found in the gut, body cavity, lungs or tissues of their hosts (Smyth, 1994). In contrast, ectoparasites, including parasitic arthropods and monogeneans, inhabit the outer layer or live outside of the body of their host in areas such as the skin, gills, feathers, and hair (Smyth, 1994). Many parasites have quantifiably negative effects on their hosts, including influencing patterns of mortality, morbidity and lowering fecundity (Clayton and Moore, 1997).

Parasites can impact the health and fitness of humans, livestock, and wild animals in natural populations. For example, zoonotic parasites such as *Toxoplasma*, *Trichinella*, and *Opisthorchis* cause human illnesses, and are acquired through dietary preferences and food preparation practices such as the ingestion of raw or undercooked meat, poultry or seafood (Roberts et al., 1994). The outcome of these parasitic zoonotic diseases can influence the worldwide economy and costs billions of dollars annually in medical costs and productivity losses due to worker illness or death (Roberts et al., 1994). Studies also have shown that parasitic diseases can cause enormous costs to the livestock industry. For example, the widespread incidence of parasitic roundworms (*Haemonchus contortus* and *Trichostrongylus* spp.) cost the Australian sheep farming industry millions of dollars annually in production losses (McLeod, 1995).

Changing in the environment condition that inhabited by parasites and their host can influence the disease outbreak as the relationship of the host-parasite also affected by the changes (Poulin, 1992). As a consequences, these changes may affect the evolutionary of parasite and host. For example, environmental changes could increase the transmission

rate of the parasite which can cause to the selection for higher virulence (Anderson and May, 1982; Poulin, 1992).

1.2 Parasite life cycles

Life cycles are generally classified as direct or indirect. Direct life cycles do not require an intermediate host (Figure 1.1). For direct life cycles, only a single definitive host species is required – the species in which the parasite reaches sexual maturity and produces progeny (Friend and Franson, 1999). For example, many ectoparasites, including the fleas of mammals and birds (Bitam et al., 2010) have direct life cycles. Other examples include gregarine parasites, such as Apicomplexans which are single-celled eukaryotes that are transmitted by oocysts ingested by a host (David et al., 2012). Hosts ingest oocysts, containing infective sporozoites, which attach to or penetrate hosts' intestinal epithelium cells (Figure 1.1). Also, some nematodes are directly transmitted, for example *Syphacia obvelata*, the roundworm of pigs when the eggs do not hatch, pre-parasitic larvae develop inside their eggs and hatching will take place after these eggs are eaten by another host and the infective larva escapes (Marjorie, 1950).

In contrast, indirect parasite life cycles involve one or more intermediate hosts in addition to the definitive host (Figure 1.2). Intermediate hosts are required by the parasite for completion of its life cycle because of the morphological, developmental and physiological changes that usually take place in the parasite within those hosts (Friend and Franson, 1999). Top level predators, including piscivorous birds, often serve as the definitive hosts for the parasites such as nematodes, cestodes, acanthocephalans, and trematodes (Bråten, 1966; Chappell et al., 1994; Smyth, 1994; Barber and Scharsack, 2010).
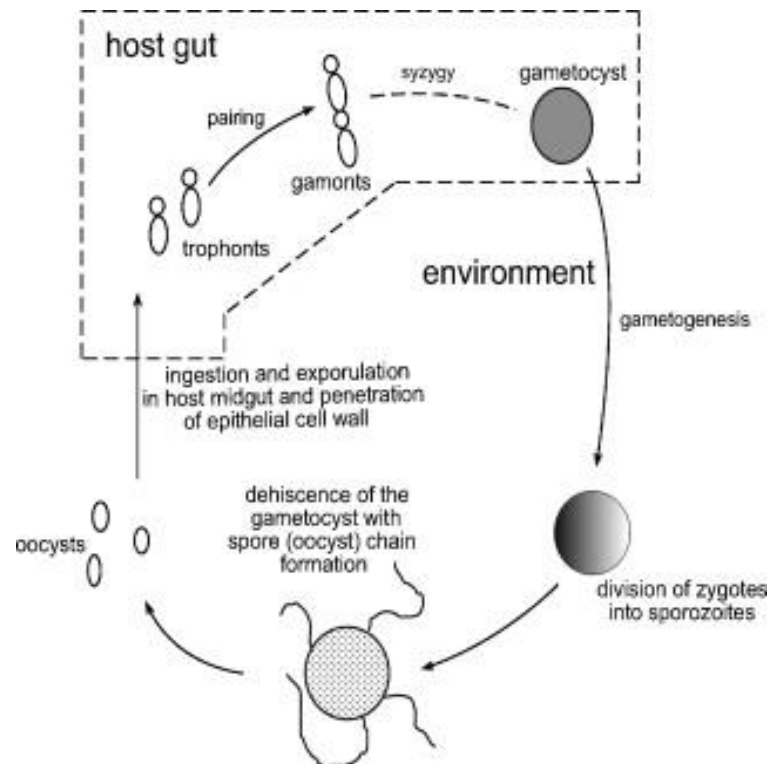
Figure 1.1 The direct life cycle of a gregarine parasite showing the transmission of the infection and the trophont production rate of infective agents (oocysts). Adapted from David et al., (2012).
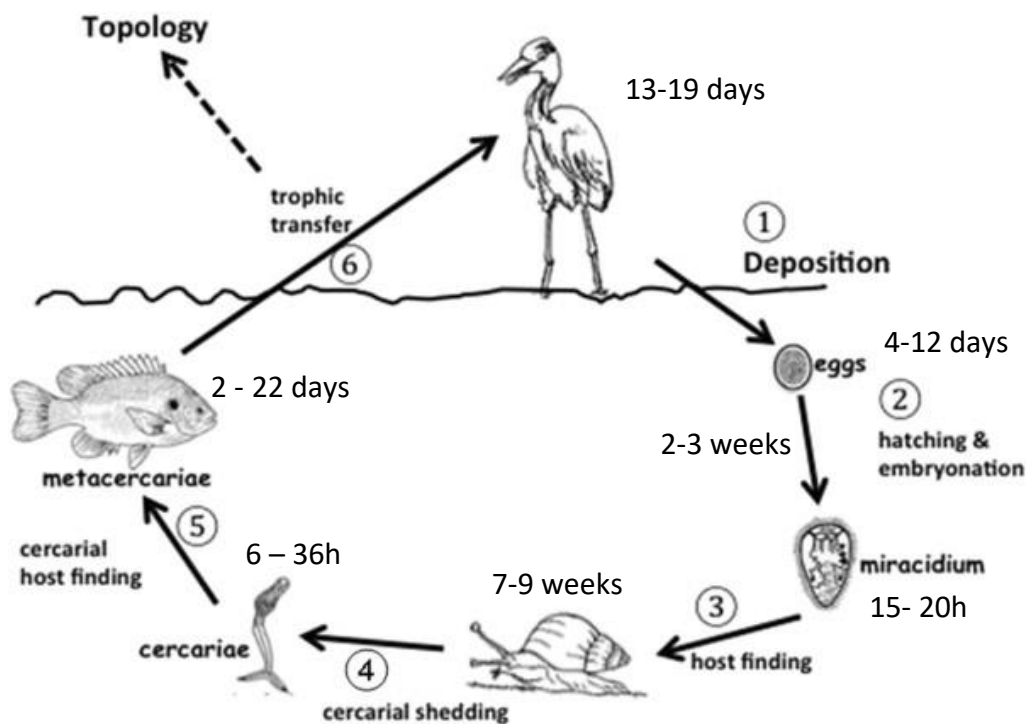
Figure 1.2 Example of an indirect parasite life cycles of a typical, unnamed trematode. Adapted from Sukhdeo (2012).

1.3 Host-parasite interactions in a changing world

Parasite infections and their effects on hosts might vary depending on the environmental context in which hosts and parasites interact. Understanding not only the typical outcome but also how these outcomes differ depending on factors such as environmental conditions (e.g. temperature and pollution) is important because these factors have implications for host populations and the strength of parasite-mediated selection over evolutionary time (Poulin, 2011; Goater et al., 2013). A number of reviews have highlighted the potential impact of climate change and pollution on parasitism such as disease outbreaks, transmission rates of parasites and pathogens and virulence of pathogens and parasites (Marcogliese, 2001; Sures, 2008). However, understanding how environmental changes affect parasites that have complex, multi-stage life cycles is challenging (Figure 1.3) (Morley et al., 2003; Barber et al., 2016).

In indirect life cycles, different life stages of a parasite occupy different hosts that often live in a range of habitats, with each parasite life stage adapted to ensure transmission efficiency and survival (Poulin, 2011). The complex life cycles of parasites, such as those exhibited by trematodes, nematodes and cestodes, often include free-living stages that experience external aquatic conditions directly without being buffered by host physiology, and so are directly vulnerable to environment perturbations such as chemical pollutants or altered temperature regimes. Such life cycle stages might therefore be expected to be highly sensitive to environmental change, with predicted consequences for the subsequent prevalence of infection in susceptible hosts (Evans, 1985; Morley et al., 2001).

Environmental changes may also affect potential host organisms by altering their survival, reproductive success or behaviour, or by otherwise influencing their susceptibility to parasite infection. Following infection, parasites that have established in a host could also be affected by external environmental factors indirectly, through effects on the host organism. Such influences (Figure 1.3) may affect the development growth or infectivity of the parasite, or the growth and survival of the host (Macnab and Barber, 2012).
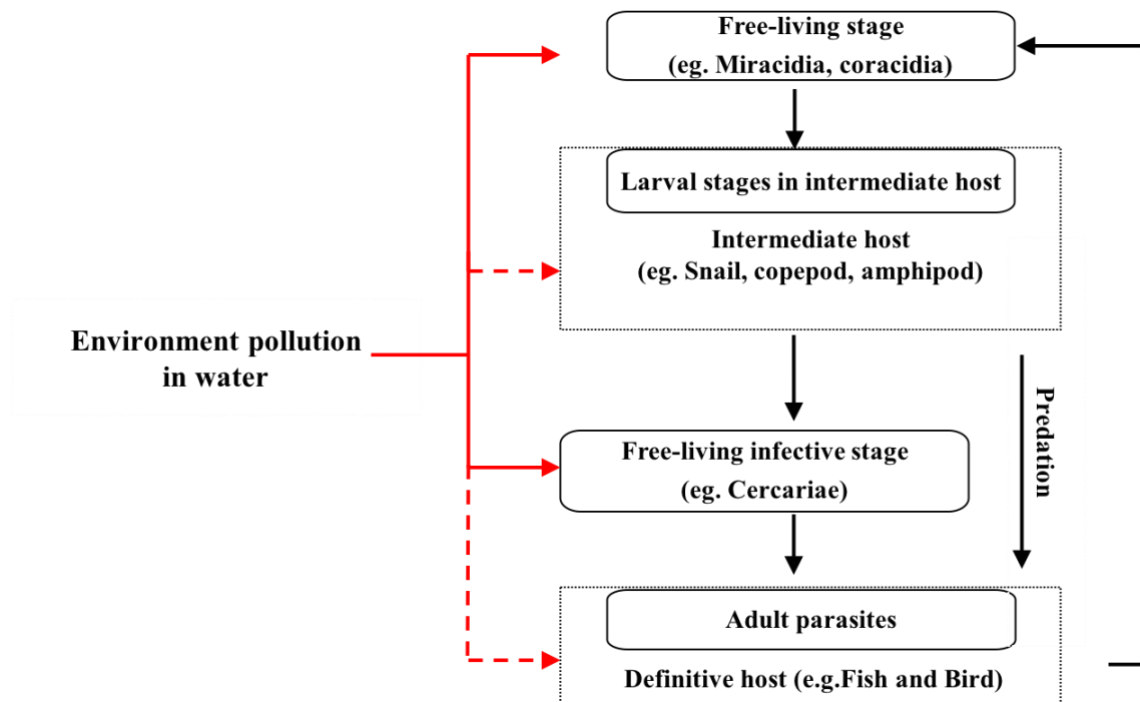
Figure 1.3 Possible effects of a water-borne environmental pollutant on a host-parasite system involving an indirectly-transmitted parasite. Solid red lines leading to a direct environment effect on the free-living stage of the parasite. Broken red lines represent an indirect effect of environmental pollution on the parasite stage via the intermediate or definitive host.

Environmental pollution can affect the host-parasite interaction and identifying the effect would be challenging especially for a parasite that have a complex life cycle. It is important to determine the environmental parameter involved and one way to do it is by generate a data from the controlled laboratory work. Using a *Schistocephalus solidus* experimental host-parasite model, the information on the multi- life cycle and the host-parasite interaction can be develop (Barber and Scharsack, 2010).

1.4 Effects of environmental change on emerging parasitic diseases

Changes in environmental condition can alter the growth and reproduction of parasite and host at once influence the existence of the zoonotic parasitic disease (Graczyk and Fried, 1997; Poulin, 2011). The host-parasite relationship can also be influence by other environmental perturbation such as deforestation and changes in patterns of land use and human settlement (Patz et al., 2000). For examples, to provide area for agricultural and

houses (human settlements), more forests area are cleared and this situation can affecting parasitic vector populations (Patz et al., 2000).

Host-parasite interactions also can be affected by habitat disturbances (Lafferty, 1993). For example, a small urban population of the horn snail *Cerithidea californica* that was separated from a marsh by the construction of a parking lot and bordered by a busy highway intersection harboured no digenean parasites, compared with a prevalence of infection of 25% in the adjacent marsh. This change was attributed to the loss of avian definitive hosts, which were conspicuously absent from the separated site (Lafferty, 1997). Toxic chemical pollutants such as petroleum hydrocarbons can also influence host-parasite interactions by suppressing immune responses, causing hosts to become more susceptible to infections (Khan, 1990). For example, infections with the trematode *Ribeiroia* sp. and pesticide exposure have synergistic effects in wood frogs *Rana sylvatica* by decreased the tadpoles host ability to resist infection, resulting in higher parasite loads and a higher risk of limb deformities (Kiesecker, 2002).

Host-parasite interaction including free-living, intermediate, or vector stages of pathogens infecting terrestrial animal also can be altered by climate change (Harvell et al., 2002). In this thesis, the effects of heavy metal pollutions and temperature change on a host and parasite life cycle have been studied.

1.5 Effects of temperature change on host-parasite interactions

The effect of temperature change on species will depend on their thermal tolerance and how close they are present to their physiological limit (Deutsch et al., 2008). For example, ectotherms are particularly at risk from climate change, as their physiological processes are directly influenced by the environmental temperature that they are experiencing (Harvell et al., 2002). In contrast, endotherms, which possess thermoregulatory mechanisms that allow them to maintain their body at a constant temperature (Deutsch et al., 2008), are expected to be less severely affected. While the majority of parasitic taxa are ectothermic, hosts may be endo- or ectotherms. In ectothermic hosts, parasites experience the same thermal conditions as the host and may be expected to be adapted to historical local environmental temperature regimes. Elevated temperatures therefore may be expected to have implications for both parasites and hosts in ectotherm host-parasite systems.

Altered temperature associated with climate change can potentially influence host-parasite interactions at many different points in the life cycles of multi-host parasites. For example, warmer temperatures may drive faster metabolic rates and speed up development rates, reducing the length of the embryonation period, meaning that eggs hatch quicker (Sakanari and Moser, 1985; Paull and Johnson, 2011). However, increases in metabolism may also deplete the limited energy reserves of motile free-living – but non-feeding – stages that emerge from eggs (Barber et al., 2016). Consequently, warmer temperatures have the potential to decrease the survival probability and hence persistence of infective stages after hatching (Nollen et al., 1979). As a result, temperature effects on the production of infective stages are predicted to be nonlinear, with intermediate temperatures expected to generate the highest overall hatching success. The temporal consistency of future thermal regimes will also likely have a major influence on egg development times (Barber et al., 2016).

However, most species appear to have the capacity to tolerate temperature ranges within those commonly encountered under natural conditions (Evans, 1985). For example, the activity and infectivity of cercariae *Echinostoma liei* increased at higher temperatures to encounter for their lower survival time (Evans, 1985).

Temperature can also directly influence the multi-host life stage of parasites by controlling the development and growth of parasitic stages. For example, under low temperatures, the development of larvae of *Anguillicola crassus* parasite in eels is reduced (Knopf et al., 1998). In trematode parasites system, the development of metacercariae *Maritrema novaezealandensis* in the amphipod *Paracalliope novizealandiae* also reduced at lower temperatures of 15ºC and 20ºC - non-infective immature and early cyst stages, compared at 25ºC almost a third had completed their development and were infective to the next host (Studer et al., 2010). Therefore, increasing temperatures can enhance parasite development, as long as this temperature can be tolerated by the host (Studer et al., 2010). Macnab and Barber (2012) also showed that plerocercoids growing in fish held at 15 ºC grew less quickly, with plerocercoids growing in fish held at 20ºC having a larger mass at the end of the study and exceeding the 50 mg threshold for infectivity to the final host, a fish-eating bird. Elevated temperatures, therefore, have the potential to increase the rate at which infective parasite stages are transmitted to definitive hosts. Temperature is likely to have a significant effect on parasites with multi-host life cycles, with free-living stages and ectothermic hosts most likely to be affected (Harvell et al., 2002) but

there is also the potential for opposing effects of temperature change on different parts of the life cycle.

1.6 Effects of heavy metal pollutants on host-parasite interactions

1.6.1 General effects of heavy metal pollutants on aquatic organisms

Toxic pollutants are becoming ubiquitous in freshwater ecosystems. Substances such as heavy metals, cyanides, ammonia and pesticides from various chemical industrial and agricultural practices and domestic waste can impact the biology of aquatic organisms (Poulin, 1992; Saeed and Shaker, 2008; Nhi et al., 2013). Pollutants present in the water column may accumulate in the sediment of aquatic biotopes where they can be dissolved again and enter the food chain (Sures, 2006). The uptake and accumulation of pollutants by aquatic organisms may cause harm, depending on the specific substance (Sures, 2006). The concentration at which a compound has lethal effects can depend upon many contributing factors, including species and water quality (Scott and Sloman, 2004), but sub-lethal concentrations can also have ecologically significant effects.

The effects of pollutants may be immediate (i.e. acute toxicity) or may only arise after prolonged exposure (i.e. chronic toxicity) (Sures, 2008). For example, in fish, pollutants are taken up through the gills or the intestine and are accumulated until they reach a steady-state concentration (Sures, 2008). After reaching this steady-state concentration, some pollutants are excreted by the organism (Figure 1.4a). The uptake and accumulation of pollutants are also normally connected with a general physiological response (Figure 1.4b) mainly comprising adverse effects, which can be either specific (e.g. induction of certain proteins, or DNA damage) or more general (e.g. changes in hormone levels or effects on the general metabolism), any of which can cause death (Sures, 2008).

Exposure to heavy metal pollutants can affect animals by changing their physiology or behaviour, including the rate of feeding, digestion, respiration, or excretion, with consequences for their energy budget, growth and reproduction (Luoma and Rainbow, 2008). For example, the hydroid *Campanularia flexuosa* showed a reduced colony growth rate with increasing dissolved metal concentrations (Stebbing, 1976), whereas the freshwater amphipod *Gammarus pulex* had a reduced feeding rate at 101 µg/L copper

compared to 8.3 µg/L copper (Taylor et al., 1993).
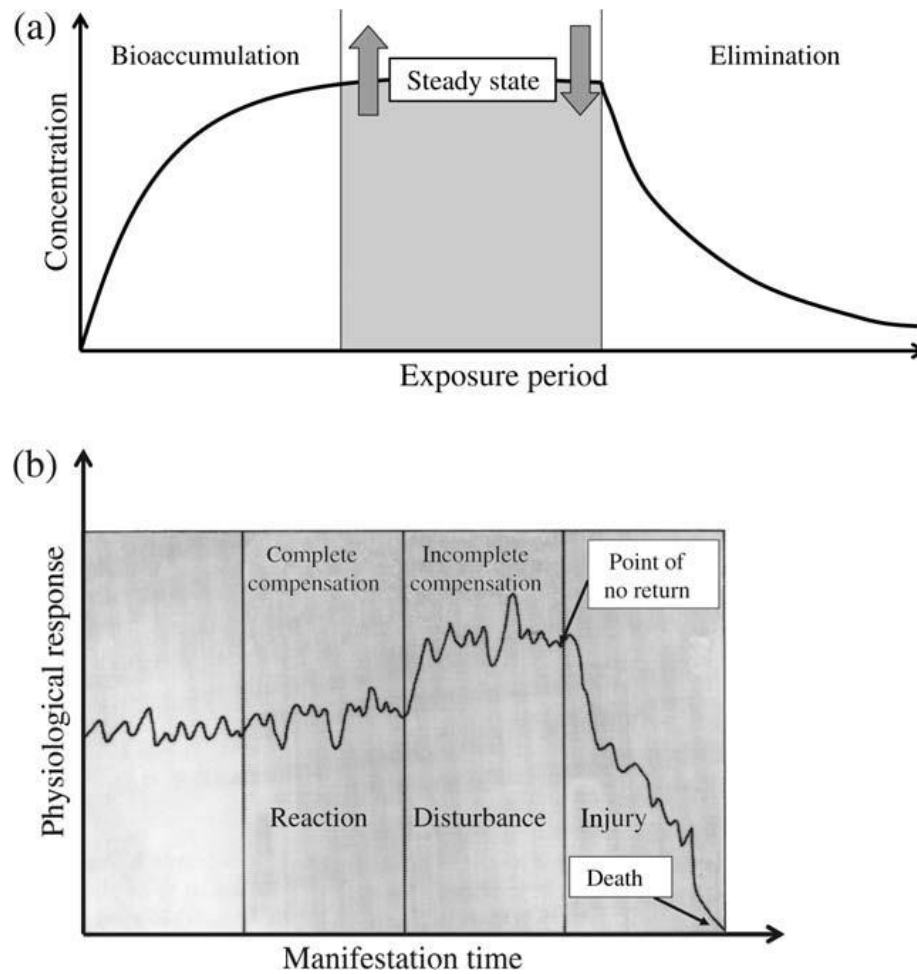
Figure 1.4 Example of (a) Uptake and accumulation of pollutants in organisms and (b) associated intensity of physiological response Sures (2008).

Heavy metal pollution can also affect the behaviour of aquatic animals. For example, mussels *Dreissena polymorpha* exposed to cadmium pollution exhibited altered shell-opening frequency (Sures, 2004) whereas the burrowing behaviour of the marine bivalve, *M. liliana* was significantly decreased at a sediment concentration of 25 µg/g copper, and at 80 µg/g zinc (Roper et al., 1995).

In this thesis the effects of three different heavy metals are studied; cadmium, copper and zinc as these heavy metals are naturally occurring metals commonly present in surface waters and can reach high concentrations near mining areas (Martins et al., 2017). They have been widely used in industry and are released into the environment as a byproduct of ore smelting, mining activity, domestic waste emission or application of fertilizers and pesticides (Morley et al 2002; Sanchez et al., 2017). In the UK, concentrations

chronically polluting the aquatic environment have been recorded as high as 160 μgL^{-1} for cadmium and 8800 μgL^{-1} for zinc (Morley et al 2002). Meanwhile, copper is a widespread pollutant found in surface waters at concentrations up to 100 gL^{-1} (Sanchez et al., 2017).

1.6.2 Effects of cadmium in aquatic environments

Cadmium is a biologically non-essential heavy metal that is typically found naturally at low (i.e. parts per billion) concentrations in rivers, lakes, and ponds. Cadmium can enter ecosystems through the application of phosphate fertilizers and other industrial sources (Williams and David, 1973). However, cadmium can become problematic in aquatic environments when its concentration rises above natural background levels. The most toxic form of cadmium is the divalent ion (Cd^{2+}) (Solomon, 2008). Exposure to this form induces the synthesis of low molecular weight metallothionein proteins, which bind with cadmium and decrease its toxicity (Wright and Welbourn, 2002). This normally takes place in the liver of fish and mammals. However, if cadmium concentrations are elevated, the metallothionein detoxification system can become overwhelmed and the excess cadmium will be available to produce toxic effects (Wright and Welbourn, 2002).

Cadmium occurs in low concentrations within the water column of rivers and estuaries and accumulates in the sediments (Bennet-Chambers et al., 1999). Cadmium has been reported to reach concentrations of 0.11–200 $\mu g.kg^{-1}$ and up to 500 $\mu g.kg^{-1}$ dry weight in the sediment of a sump adjacent to Lake Leschenaultia, Western Australia (Bennet-Chambers et al., 1999). The concentration of cadmium is generally dependent on whether the sediments were humic (high cadmium) or siliceous (low cadmium).

1.6.3 Effects of copper in aquatic environments

The main process leading to the mobilization of copper into the environment is extraction from its mining, milling and smelting, and also from agriculture and waste disposal (Wright and Welbourn, 2002). In aquatic ecosystems, copper generally enters from agriculture runoff, though the deliberate use of copper sulphate to control algal blooms and also from direct discharges from industrial processes (Wright and Welbourn, 2002). In uncontaminated freshwater, copper concentrations generally range from 0.001 to 0.1 $\mu g/L^{-1}$ and from 0.03 to 0.6 $\mu g/L^{-1}$ in uncontaminated ocean water. In contaminated water, copper usually leaves the water column and accumulate in sediment. Copper

concentrations ranging from 50 to 100 µg/L^{-1} can occur in aquatic ecosystems that receive mine tailings or where copper sulphate has been added to control algal blooms (Wright and Welbourn, 2002).

Copper is an essential trace metal, required in small doses by organisms for metabolic functions, but it is potentially highly toxic if the internal available concentration exceeds the capacity of physiological detoxification processes (Sunda and Hanson, 1987). Fish and crustacean generally are sensitive to copper (Wright and Welbourn, 2002). For example, the cyprinid fish *Rutilus kutum* had a lower body weight and survival rate when exposed to 0.23 mg/L^{-1} of copper sulphate compared to non-exposed fish (Gharedaashi et al., 2013). The effect of copper on osmoregulation in the freshwater amphipod *G. pulex* exposed to 100 µg/L^{-1}, caused a significant reduction in haemolymph sodium concentration and sodium influx within 4h. Copper concentrations as low as 10 µg/L $^{-1}$ also significantly reduced gill Na$^+$/K$^+$ ATPase activity (Brooks and Mills, 2003).

1.6.4 Effects of zinc in aquatic environments

Zinc can enter water naturally by the erosion of minerals from rocks and soils, and also as a by-product of steel production, the operation of coal-fired power stations, or the burning of waste materials (Irwin et al., 1997). Zinc is also used in some fertilizers that may leach into groundwater. High natural levels of zinc in water are usually associated with higher concentrations of other heavy metals, such as lead and cadmium (Irwin et al., 1997).

In water, zinc settles onto the sediment and a small amount remains dissolved in the water or is present as suspended particles (Irwin et al., 1997). Zinc is usually more concentrated in the sediments of streams and rivers compared to the water column. In non-polluted areas, zinc concentrations can be as low as 0.1µg/L. In rivers, the concentration of zinc is higher (20 µg/L) whereas, in streams affected by mine drainage, the zinc concentration can exceed 100 µg/L (Irwin et al., 1997). In aquatic ecosystems, the concentration of zinc is not only influenced by sediment type but also by other abiotic parameters such water hardness (Irwin et al., 1997).

Zinc is an essential metal for most aquatic organisms, due to its involvement in somatic cell functions including protein synthesis and enzymatic regulation (Bury et al., 2003).

For example, in teleost fish, zinc is taken up through transporters on the apical membrane of chloride cells in the gill epithelium. However, under pollution, excess of zinc may compete with Ca^{2+} at the apical membrane of chloride cells via nonspecific Ca^{2+} channels, which may, in turn, disrupt Ca^{2+} homeostasis (Bury et al., 2003). Another example of the acute effect of the zinc was documented in *Branchiura sowerbyi* and *Tubifex tubifex* where this two organism have shown to be more sensitive to zinc compare to the arsenic (Lobo et al., 2016). In nematode, zinc has shown to decreases the growth of the *Caenorhabditis elegans* population in a 500 mM of zinc solution (Dietrich et al., 2016).

1.6.5 Heavy metal pollution and host-parasite interactions

In aquatic environments, organisms are not only confronted with pollutants, but often also with parasites. As environmental pollutants may affect the physiological homeostasis of organisms that act as hosts, it can be expected that host-parasite interactions will also be altered (Sures, 2008). The effects of pollutants on parasitism are variable and may be positive or negative. For example, pollution may increase levels of parasitism by interfering with host defence mechanisms (Sures, 2004). Parasites may also be more resistant than their hosts to pollutants and thus tend to increase in number in polluted condition (Mackenzie, 1999). On the other hand, pollution could also decrease parasitism, if parasitized animals suffer increased mortality under polluted conditions, or if parasites are more susceptible to the pollutant than their hosts.

The complexity of the parasite life cycle may play an important role in determining the patterns of infection that emerge in polluted environments. Levels of infection with endoparasitic helminths with complex, indirect life cycles tend to decrease, while infections with ectoparasites with direct single-host life cycles tend to increase (Mackenzie, 1999). One explanation is that endoparasites with indirect life cycles can be affected directly (as free-living transmission stages or adult forms in the alimentary tract come into direct contact with the pollutant) and indirectly, through adverse effects of the pollutant on other hosts in the parasite life cycle (Mackenzie, 1999). Ectoparasites with direct life cycles, on the other hand, have a constant direct contact with the external environment and in the course of their evolution, they have developed flexibility and resistance to certain natural changes (Mackenzie, 1999). Consequently, many ectoparasites show higher tolerance of certain types of environmental change than their hosts (Mackenzie, 1999). However, there is variation in parasite responses toward

pollution depending on the parasite taxa and the pollutants involved (Mackenzie, 1999, Sures, 2008). For example, nematode parasites show an increase in abundance among hosts exposed to crude oil, whereas digenean parasite abundance in crude oil-affected host populations was decreased (Lafferty, 1997).

Pollution also influences parasitism by affecting the swimming behaviour and longevity of free-living infective parasite stages. For example, in *Cryptocotyle lingua*, acute exposure to Iron, zinc, and copper in artificial seawater can affect both the swimming rate and the longevity of cercariae released from snail hosts. The swimming behaviour and longevity of cercariae are relevant because both can influence the probability of transmission to the second intermediate hosts (Rea and Irwin, 1992).

Pollution could influence susceptibility to parasite infections by impairing host immune responses (Khan, 1990). Heavy metal pollutants appear to have a consistently negative effect on parasite numbers, but the magnitude of the effects can be dependent on concentration (Lafferty, 1997). For example, guppies (*Poecilia reticulata*) exposed to intermediate levels of zinc ($15 - 60$ µg/L^{-1}) were less likely to develop ectoparasite *Gyrodactylus turnbulli* infections (Gheorgiu et al., 2006) than when exposed to a concentration of 240 µg/L^{-1}. This is due to the proliferation of mucus on the fish epidermis as a result of high zinc exposure, which made it difficult for the parasite to attach.

Parasites can also modify the effect that heavy metal pollutants have on their hosts. For example, sticklebacks infected with *S. solidus* were affected more severely when exposed to cadmium than non-infected fish (Pascoe and Cram, 1977). However, some endoparasites, such as acanthocephalans, appear to be capable of accumulating heavy metals (Sures, 2008), and hence reducing their toxic effects on hosts. For example, the acanthocephalan *Pomphorhynchus laevis* accumulates heavy metals (Lead) in much higher concentrations than those found in the organs of its fish host, chub (*Leuciscus cephalus*) (Sures and Siddall, 1999).

At a population level, while the effect of anthropogenic stressors such as heavy metal pollutants may increase the susceptibility of individuals to parasites, if this stress also leads to a reduction in host density (through mortality of parasitized individuals) then disease levels may fall (Lafferty and Holt, 2003).

1.7 The *Schistocephalus solidus* parasite model

1.7.1 *Schistocephalus solidus*

Schistocephalus solidus is a diphyllobothriidean cestode whose definitive host is usually a fish-eating bird (Barber and Scharsack, 2010). It has a complex lifecycle with two intermediate hosts: freshwater copepods and fish, especially three-spined sticklebacks (Bråten, 1966; Barber and Scharsack, 2010). *Schistocephalus solidus* belongs to the Phylum Platyhelminthes, Class Cestoda, Order Diphyllobothriidea, Family Diphyllobothriidae. *Schistocephalus solidus* is a simultaneous hermaphrodite species because the plerocercoid stage in the definitive host can generate functional male and female reproductive organs at the same time (Schärer et al., 2001).

In nature, the life cycle of *S. solidus* begins within the intestine of a bird where the immature plerocercoid stage undergoes sexual maturation to the adult tapeworm form (Hopkins and Smyth, 1951). In the intestine, *S. solidus* reproduces sexually by cross-fertilization or self-fertilization (in the case of a single infection) (Barber and Scharsack, 2010). The eggs are then released into the water through the faeces of the bird. The hatching eggs produce coracidia − ciliated, free-swimming larval stages − which then infect cyclopoid copepod hosts (Hopkins and Smyth, 1951) following ingestion. Within 24-48 zinc of ingestion, the coracidia penetrate the copepod gut wall (Tierney and Crompton, 1992). In the haemocoel of the copepod, the coracidia develop into the procercoid stage (in approximately 3 days). When the copepod is eaten and digested by a three-spined stickleback fish (*Gasterosteus aculeatus*), the procercoid is released into the gut of the fish before burrowing through the gut wall to enter the abdominal cavity, where it develops into a plerocercoid (Bråten, 1966, 1992). Three-spined sticklebacks must then be eaten by a suitable definitive host for the life cycle of *S. solidus* to be completed (Figure 1.5). The life cycle of *S. solidus* in nature can be mimicked in the laboratory by *in vitro* cultivation (Smyth, 1946), with the bird intestine being replaced with semi-permeable dialysis tubing. This mimics a narrow 'small intestine' where fertilization can take place (Barber and Scharsack, 2010). Coracidia, copepods, and sticklebacks can also be easily maintained in the lab, so it is possible to study and manipulate all stages of the lifecycle (Smyth, 1946; Barber and Scharsack, 2010).
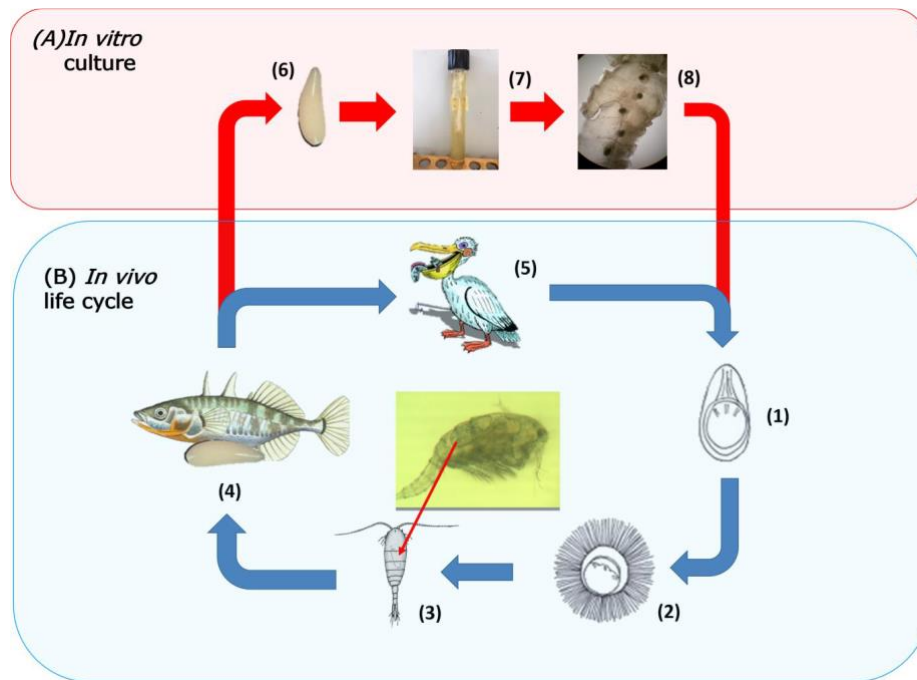
Figure 1.5 Life cycle of *Schistocephalus solidus* in nature and in an *in vitro* lab culture. (1) egg stage, (2) free-living stage (coracidia), (3) procercoid stage in copepod first intermediate host, (4) plerocercoid stage in stickleback second intermediate host, (5) avian definitive host, (6) mature plerocercoid, (7) *in vitro* culture, (8) dead plerocercoid after culturing to successfully produce eggs.

1.7.2 Copepods as first intermediate hosts

Copepods belong to a subphylum of crustaceans (Boxshall and Defaye, 2008) which inhabit almost every salinity range, from freshwater to saltwater (Boxshall and Defaye, 2008). There are approximately 13,000 known species of copepod, and 2,814 of these are found in freshwaters. Some copepods are parasitic to fish and molluscs. Those such as *Lernaea cyprinacea* and *Ergasilus sieboldin* are considered a threat in freshwater aquaculture where they can cause extensive damage (Boxshall and Defaye, 2008). Non-parasitic copepods may themselves be a host for other parasites. In the present study, the copepod species used as a host to the *S. solidus* parasite is *Cyclops strenuus* Fischer, 1851 (Figure 1.6).

Cyclops strenuus is generally found in small freshwater habitats and sometimes in low salinity water. This species belongs to the Family Cyclopidae which is native to Europe,

Alaska, the Yukon and the Northwest territories (Reed and McIntyre, 1995; Hudson et al., 1998; Tackx et al., 2004). *Cyclops strenuus* is a common host to many different parasites including cestodes, nematodes, tapeworms, acanthocephalid worms and helminths (George and Nadakal, 1973; Moravec et al., 1994; Barber and Svensson, 2003). The species commonly serves as a host to *S. solidus* parasites and has been used in a number of experimental *S. solidus* studies (Macnab and Barber, 2012; Simmonds, 2015). In a laboratory experiment, individual *C. strenuus* are usually exposed to a single or two *S. solidus* coracidia to develop a procercoid infection before been fed to fish.
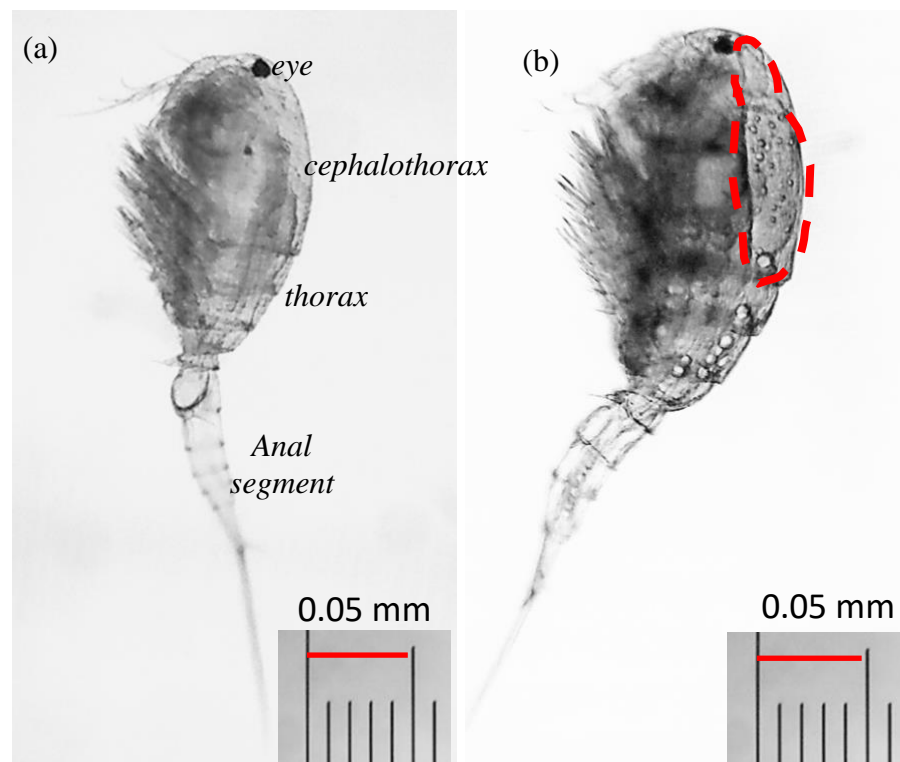
Figure 1.6 Copepod *Cyclops strenuus* in side view. (a) Non-infected copepod (b) copepod infected with the infective procercoid stage of *Schistocephalus solidus* (outlined by red dashed line).

1.7.3 Three-spined sticklebacks as second intermediate hosts

Three-spined sticklebacks (*Gasterosteus aculeatus*) have a wide geographic distribution, occupy a variety of habitat types spanning the temperate and sub-polar zones of the northern hemisphere (Wootton, 1976), and have a central position in aquatic food webs (Barber, 2013). These fish belong to the Class Actinopterygii, Order Gasterosteiformes, Family Gasterosteidae. Three-spined sticklebacks are a small fish with a maximum adult length of ca. 10cm (Wootton, 1976). They have a fusiform body shape with dorsal and anal fins. They get their common name from three prominent spines that develop in front of the dorsal fin. Their preferred habitat is slow-moving water in, for example, side branches of rivers drain and among rooted vegetation in lakes or ponds (Wootton, 1976). This species displays paternal care, with males guarding the eggs until they hatch and become active swimming young fish (Wootton, 1976). The female usually lays her colourless to light orange, spherical eggs in the male-built nest. Three-spined sticklebacks are largely carnivorous (Wootton, 1976) with a diet that includes tubificid worms, zooplankton (*Daphnia* and copepods) and other benthic invertebrates. Even though the diet of three-spined sticklebacks consists mostly of animals, they have also been recorded as having a plant material such as algae and diatoms in their diet (Wootton, 1976).

Three-spined sticklebacks have become a model species in evolutionary biology, as they have undergone substantial adaptive evolution in skeletal and feeding morphology following their widespread colonization of freshwaters from the sea (Peichel et al., 2001). With a small body size, the fish can be reared relatively easily in laboratory aquaria (Wootton, 1976) and can be induced to spawn under lab conditions, as well as being ideal for *in vitro* fertilisation (Barber and Arnott, 2000). There is extensive documentation of the biology, ecology, and evolution of this species as reviewed by Barber (2013). This species has, in particular, been documented as a suitable species in which to examine host-parasite interactions (Lafferty and Kuris, 2009; Barber and Scharsack, 2010). Sticklebacks are also relatively resilient to environmental change. This important feature allows analyses of host-parasite interactions under a range of altered environment conditions (Barber, 2013).

In experimental controlled temperature, stickleback and *Schistocephalus* was documented to respond differently in changing temperature (Macnab and Barber, 2012). For example, the growth of stickleback fish was increasing in cold temperatures. Contrast to their *Schistocephalus solidus* parasites, where the growth was higher at warm temperatures which might help the parasite to reach infective stage in shorted time and at once benefit the parasite to complete the life cycle. Furthermore, this experimental work also documented that there are potential adaptive manipulation of behaviour happen when infected fish show a behaviour of likely to choose warmer temperatures compared to cold temperature (Macnab and Barber, 2012).

1.8 Aims and objectives of the thesis

The way in which altered environments change the growth, energetics and reproductive development of parasites and their hosts is a major area of scientific and applied research interest (Macnab and Barber, 2012; Mackenzie, 1999). A wide range of anthropogenic environmental changes can alter both the survival and reproductive success of host fish and their parasites, but very little is known about how changing environments increase the susceptibility of fish to acquiring parasitic infections, how degraded environments alter the life cycles of parasites, and how the effects that parasites have on their host fish are affected by degraded environments. In this thesis, I will assess the effects of environmental stressors (elevated temperature and heavy metal pollutants) on the growth, energetic status, reproductive development, survival and life cycle of both hosts (the copepod *C. strenuus*, and three spined sticklebacks *G. aculeatus*) and their parasite *S. solidus*.

It is had been predicted that global climate change will influence disease dynamics of pathogens with complex life cycles. Chapter 2 examines temperature effects on the survival of free-living infective stages of parasites and the development of the parasite in the first intermediate hosts (copepods). Chapter 3 examines the effects of degraded environments on the parasite and host interaction. Environmental contamination with heavy metals including zinc, copper, and cadmium, was used to determine the effect of pollution on the *S. solidus* free-living stage and the progression of *S. solidus* to the next (copepod) host. In Chapter 4, this pollution effect is investigated in more detailed using a single metal (zinc) as a contaminant to determine how this pollutant affects *S. solidus* parasites at free-living and in the intermediate host stages. In Chapter 5 I determined how

the growth and size of the parasite inside the first intermediate host affects its subsequent performance and growth in the second intermediate host, the three-spined stickleback.

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Chapter 2

Effects of temperature on the survival, infectivity and development of an aquatic parasite
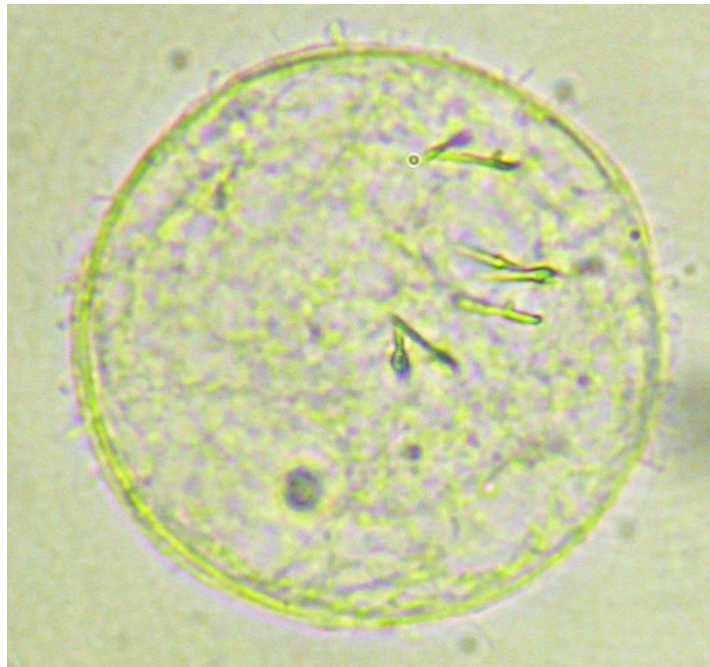
© Z. Ismail

2.1 Abstract

Global climate changes have considerable potential to impact the interactions between aquatic parasites and their hosts, both directly (for example, by acting on free-swimming infective stages) and indirectly (by affecting their interactions with intermediate host), altering transmission success, parasite development time and ultimately life cycle completion rates. Here, experimental studies examining how temperature affects the survival, infectivity, growth and development of larval stages of an ecologically-important parasite of stickleback fish, the diphyllobothriidean cestode *Schistocephalus solidus*, were investigated. The results indicated that the survival of free-swimming infective first-stage larvae (coracidia) is temperature-dependent, with the longest survival times at 10°C, reducing at 15°C and 20°C. When the first intermediate hosts – copepods *Cyclops strenuus* – were exposed to controlled doses of infective coracidia, the growth and development of second stage larvae (procercoids) at 10°C, 15°C or 20°C were tracked *in vivo* for 6 weeks post-infection. While there was no effect of temperature on the infectivity of coracidia to copepods, the growth of procercoids in copepod hosts was faster at 20°C than at 10°C or 15°C, and the appearance of a hooked cercomer – a developmental stage signalling infectivity to fish hosts – occurred earlier at the highest temperature. The results suggest that changes in thermal regimes in aquatic environments can have divergent implications for different parasite life cycle stages, with potentially complex implications for life cycle dynamics.

2.2 Introduction

Changes in climate or temperature can alter the relationships between aquatic organisms such as parasites and their hosts, particularly for parasites with complex life cycles (Harvell et al., 2002). These changes can affect the interactions between aquatic parasites and their hosts both indirectly (e.g., by affecting their interactions with the intermediate host) (Hakalahti et al., 2006) and directly (e.g., by acting on free-swimming infective stages (Koprivnikar et al., 2010). Parasites with a complex life cycle that have a free-living infective stage – such as the coracidia of cestodes, or the miracidia and cercariae of trematodes – may be especially sensitive to environmental stressors, since they have direct contact with aquatic environments (Poulin, 1992; Mackenzie, 1999). Any effect on the survival or transmission success of a free-living infective stage could also affect patterns of infection, host susceptibility, parasite development time and, ultimately, life cycle completion rates (Pietrock et al., 2002).

2.2.1 Effect of temperature on the free-living stage of parasites

A number of studies have documented the effect of temperature on the longevity or survival of free-swimming stages of parasites, but most of them have focussed on the cercariae of trematodes (Rea and Irwin, 1992; Pechenik and Fried, 1995; Ford et al., 1998; Koprivnikar and Poulin, 2009; Koprivnikar et al., 2010) and larvae of nematodes (Andersen et al., 1966; Pandey, 1972; Measures, 1996). For example, higher temperatures increase the activity level of the free-living cercariae of *Euhaplorchis* and *Acanthoparyphium* species (Koprivnikar et al., 2010) and cause the free-living infective stage of *Echinostoma trivolvis* cercariae to consume energy resources more rapidly and to have a shorter life-span (Pechenik and Fried, 1995). In cestode parasites, decreased survival of free-living parasite stages at higher temperatures could be associated with several factors affecting mortality, such as increased metabolic rate (Dubinina, 1980) due to a high level of activity, which might deplete stored carbohydrates and energy reserves present in the form of fat and glycogen droplets located in the ciliated embryonal shell (Dubinina, 1980). The diphyllobothriidean cestode *Schistocephalus solidus* is a parasite with a life cycle that spans various habitat types and includes a free-swimming stage, so it can be hypothesised that it may be particularly susceptible to changes in environmental conditions. The first free-living stage of the cestode *S. solidus* emerges from eggs that have been released from the faeces of birds (the definitive host) into bodies of fresh water

(Dubinina, 1980). This free-swimming larval stage ('coracidium') then infects a cyclopoid copepod (the first intermediate host) by penetrating the gut wall following ingestion (Tierney and Crompton, 1992a). The copepod may then be eaten by a three-spined stickleback (the second intermediate host), which in turn becomes infected by the parasite (Bråten, 1966). The coracidia of cestodes swim in random directions in the water until they collide with a copepod host when they may be detected as a food and eaten (Smyth and McManus, 1989). Coracidia are reported to survive for 4-5 days (Dubinina, 1980) during which they will be exposed to prevailing environmental conditions (including e.g., temperature and pollution) that may influence survival or longevity and affect their ability to infect the copepod host.

2.2.2   Effect of temperature on the parasite in first intermediate hosts

The relationship between parasites and their host is important in making sure that the life-cycle completion rates can be accomplished but this interaction can be affected by changes in temperature of the surrounding environment (Pietrock et al., 2002). In a complex life cycle, temperature dependent rates of larval development in the bodies of their hosts is a potentially important factor determining the dynamics of life cycles (Barber et al., 2016). To date, a number of studies have documented the effects of elevated temperatures on the rate of cercarial output of trematode-parasitised snails (Evans, 1985; Shostak and Esch, 1990; Paull et al., 2015), as temperatures are known to accelerate the production of cercariae and trigger the emergence of cercariae (Ataev, 1991). However, the effects of elevated temperatures may not always increase cercarial output (Koprivnikar and Poulin, 2009) and this response might vary between genotypes of a single parasite species (Berkhout et al., 2014). In another study, the development of metacercariae *Maritrema novaezealandensis* in amphipod *Paracalliope novizealandiae* was effected by the temperature, suggested that increased in temperatures in a range that hosts could resist can enhance parasite development (Studer et al., 2010).

The rate of development or growth of a parasite could have the potential to influence or effect the life cycle dynamics (Barber et al., 2016) and this process could be altered under elevated temperature. Several studies have shown decreased developmental times or transformation of parasite larvae into the next stage under warmer temperatures (Tokeson and Holmes, 1982; Lv et al., 2006; Macnab and Barber, 2012). There is also studied have look at the effects of temperature on the time of development of cestode procercoids

Ligulidae in crustacean host (copepod) where the parasite reach invasive state quickly in higher temperature (Dubinina, 1980). In *S. solidus* parasite, less is known about the effects of elevated temperatures on the time of maturation (infective stage) and development of the procercoid particularly in intermediate hosts, copepods. Even though the growth and development of cestode procercoids has been reported to be both density- (Nie and Kennedy, 1993) and host size-dependent (Wedekind, 1997) due to the constraining fixed size of the rigid copepod exoskeleton (e.g. compared to the fish host body cavity, which can expand), environmental temperature is still considered to be an important factor influencing the rate of growth and development of procercoids in copepods (Granath Jr and Esch, 1938*a*).

2.2.3 Aims of the study

Here, the effect of altered environment temperature on the *S. solidus* parasite at the free-swimming stage (coracidium) and the parasite (procercoid) progression in the copepod host was studied. Temperatures that represent those normally encountered in the UK during autumn and summer (10 and 15°C) were contrasted to a temperature of 20°C, which was chosen to reflect recent predictions for climate change in temperate regions (EEA, 2010), and the thermal tolerance range of stickleback fish (Heuts, 1947, Macnab and Barber, 2012). In this study, the following questions were addressed: (1) How is the life-span of the free-living, infective stage of *S. solidus* affected under different temperatures? (2) How does the exposure of copepod hosts to temperatures affect the growth and development of the procercoid stage of *S. solidus* parasites in copepod hosts?

2.3 Materials and Methods

2.3.1 Parasite culture

Three-spined sticklebacks *Gasterosteus aculeatus* from naturally-infected populations or experimentally-infected laboratory stocks were dissected to recover plerocercoids of the pseudophyllidean cestode *S. solidus* present in the body cavity. Infected fish can be readily identified outside the breeding season, since the abdomen of infected fish is distended compared to that of non-infected fish. Plerocercoids recovered from infected fish were cultured immediately, or were kept at 4°C overnight in a RPMI (Roswell Park Memorial Institute) -1640 culture medium (Sigma Aldrich, UK), in a covered, sterile Petri

dish. Plerocercoids of *S. solidus* from infected fish were weighed using an analytical balance (to 0.001g) and only those with mass >50mg were selected for culture. Parasites were cultured in specially-designed culture tubes, using published *in vitro* protocols (Smyth, 1946; Wedekind, 1997). Screw-top boiling tubes (volume: 70 ml) were filled with a 50:50 mixture of RPMI-1640 medium and heat-inactivated horse serum (H1138, Sigma Aldrich, UK) with 500 µg of Penicillin. Horse serum acts as buffer against the toxic effect of acidic metabolic products that are produced during the cultivation process (Smyth, 1946) and Penicillin acts as an antibiotic to reduce fungal infection of the culture (Barber and Scharsack, 2010). The RPMI-1640 medium was prepared by adding 4.16g of RPMI medium powder to 400ml of double deionised water (ddH$_2$O). To ensure the culture was sterile, the RPMI medium was autoclaved before being used.

In the tubes, worms were suspended in narrow dialysis tubing (6.3 mm in diameter) (Medicell Membranes Ltd) to compress the worm, to promote insemination and fertilization and stimulate egg production (Smyth, 1946). The procedure to insert the plerocercoid into the dialysis tubing was done carefully to avoid damaging the parasite. Surplus moisture from the parasite body was blotted away using absorbent tissue, and it was found to be easier to guide the plerocercoid into the tubing by pressing gently at the end of the plerocercoid body once in the tube and moving it forward into the tubing. In this study, two plerocercoids were placed together in the dialysis tube, to permit cross-fertilisation (Wedekind, 1997). The culture vessel containing the parasites was kept in a water bath at 40°C for 7d and was shaken gently to help the distribution of metabolic products from worms (Barber and Scharsack, 2010). The plerocercoids were monitored daily to check for worm survival and medium condition. Any dead plerocercoids were removed immediately from the dialysis tubing, to avoid a build-up of waste products that otherwise made it hard to clean and collect the eggs and which could have affected their development and hatching. The *in vitro* plerocercoid culturing period typically lasted 7d.

2.3.2 *Schistocephalus solidus* egg collection

Most parasite eggs were collected after 7d of the culturing period. At this time, the plerocercoids had mostly died or were senescent. If plerocercoids died before 7d, eggs were collected immediately. At the end of the culturing period, the dialysis tubing was carefully cut with scissors to remove the dead plerocercoids. The dialysis tubing was then

rinsed by continuously pipetting deionised water into it until all the eggs released from the plerocercoids had been washed into a sterile Petri dish. Debris, including cestode tegument, was removed to leave a clean egg culture, which was then aliquoted into replicate Petri dishes, to achieve approximately 800-1000 eggs per dish. Each Petri dish was then sealed using Parafilm™ and covered with aluminium foil to keep it dark and resistant to evaporation, and then incubated at 20°C in the dark for 3 weeks.

2.3.3 Free-living stage survival experimental design

On the day before an experiment was conducted, the Petri dishes containing *S. solidus* eggs ('egg plates') were taken out from the incubator and exposed to natural daylight to trigger hatching of the free-living coracidia. The egg plates were left at room temperature overnight and hatching success was assessed visually the next day using a dissecting microscope (Leica S6E, USA). Hatching was confirmed by the presence of swimming coracidia. Only egg plates that provided >500 hatched coracidia were used for the experiment. Once hatched, each coracidium was individually placed into one well of a 96-well flat bottom microtitre plate containing 100µl of autoclaved aquarium fresh water using a glass pipette. A total of 360 newly hatched coracidia from six worm-families were tested for their survival time. The experimental plates were distributed into one of three constant temperature treatments (10°C, 15°C or 20°C) in separate incubators (LMS, UK). Temperature was monitored hourly to the nearest 0.1°C throughout an experiment using a digital thermometer placed in each incubator. Coracidia were individually monitored in their 96-well microtitre plates at 1h intervals under the dissecting microscope. They were considered to be dead when they showed no swimming movements or rotation around their longitudinal axis. Dead coracidia also changed from clear and transparent to white and opaque, and expanded in size due to swelling of the embryonal shell.

2.3.4   Parasite development in first intermediate host experimental design

2.3.4.1 Copepod infection experiment

Cultures of *Cyclops strenuus abyssorum* were obtained from a commercial supplier (Sciento, Manchester, UK) and maintained in a series of glass conical flasks containing deionised water at 15°C. Copepod were fed weekly on *Alfalfa* infusion, prepared in the lab. For this experiment, copepodite stages were exposed to the infective, free-swimming coracidium stages of *S. solidus*. Three days before the infection, copepod cultures were

transferred in the conical flasks (500 ml) to three temperatures (10°C, 15°C or 20°C) and held without food in order to maximise the chance that copepodites would ingest the coracidia. One day before the experiment, copepodites were separated from the adults and nauplii using a 200μm mesh sieve and housed individually in the well of 24-well plates containing 2 ml of autoclaved freshwater before exposure to coracidia.

2.3.4.2 Copepod exposure

A single hatched coracidium generated from the *in vitro* culture of *S. solidus* plerocercoids from the River Soar stickleback population was placed in each well of the 24-well plate that contained a copepod. A total of 120 copepodites were fed with a single infective coracidia (40 per temperature treatment). The exposed copepod plates were then kept in three incubators under controlled temperature of 10°C, 15°C and 20°C with fifteen sham-exposed copepods (which experienced identical treatment but were not fed infective coracidia) as controls. Three days after the exposure / sham exposure, copepods (exposed and sham-exposed) were fed with protozoan (*Colpidium striatum*) culture.

2.3.4.3 Copepod screening

The development of infectivity of the procercoids under different temperatures was quantified by screening exposed copepods after 7d, and thereafter at 7d intervals, for 6 weeks. The development of procercoids inside the copepod hosts was tracked by microscopic examination and image analysis. Copepods were viewed under a compound microscope (LEICA CME, USA) by placing the copepod on a cavity slide (Agar scientific, UK) using a glass pipette. A drop of carbonated water (Sparkling mineral water) was placed onto the copepod on the cavity slide to slow down the copepod's movement during the screening procedure. The posterior half of the cephalothorax, the thorax, the abdomen, the tail, stomach and midgut were checked for infection. The infection status of the copepod was recorded along with the presence or absence of a cercomer on the procercoids. Photographs of copepods and procercoids were taken using a Moticam 3.0MP camera which was attached to the eyepiece of the compound microscope. Copepod size (cephalothorax length) and procercoid body area were measured in mm using the ImageJ 1.49V software packages. For the non-exposed

copepod (control), the cephalothorax length was measured to determine the growth during the experiment period.

2.3.5   Statistical analysis

For the free-living stage survival experiment Linear Mixed Models (LMM, IBM SPSS Statistics v22) were used to determine the significance of differences in survival time among temperature treatments, among parental pairs and in the potential interaction between these two factors. Mean survival time (h) was used as the dependent variable, while temperature and worm-pair identity were used as fixed categorical factors. Non-parametric Kaplan-Meier analysis with log-rank (Mantel-Cox) tests were used to detect overall survival distributions between the groups. Any significant differences result from the test then were followed by the Kaplan-Meier analysis with Wilcoxon (Gehan) tests (pairwise comparisons) to compare survival distribution among groups, with the test statistic based on differences in group mean. These pairwise comparisons show which group are significantly different in survival curves. Statistical significance was set at a value of $P < 0.05$.

Chi-square analysis were used to determine the infective status of procercoids in copepods. Procercoid growth in area, procercoids specific growth rate (SGR - data was log - transformed) and copepod growth were analysed by a repeated-measures ANOVA. A Bonferroni correction were applied to determine the significance of the F ratios, with basic $\alpha = 0.05$.   ANCOVA was used to determine the effect of copepod size on procercoids body area (square root - transformed). All statistical analyses were performed using SPSS v22 software packages.

2.4   Results

2.4.1 Effects of temperature and parasite family on survival of the free-living stage

Survival of *S. solidus* coracidia exposed to three temperatures (10°C, 15°C and 20°C) was affected by parasite family (Figure 2.1; Table 2.1), and temperature (Figure 2.1; Table 2.1), and there was a significant interaction (Table 2.1) between parasite family and temperature indicating that the thermal reaction norms significantly differed between the families. On average, coracidia from all families showed shorter mean survival times with

increasing temperatures, with coracidia surviving twice as long at 10°C than they did at 20°C (Figure 2.1).

Table 2.1 Results of a linear mixed model used to determine the effects of experimental treatment on survival of *Schistocephalus solidus* coracidia. Significant P value (<0.05) are shown in bold.

Variable	D.F.	F	P
Temperature	2	5.294	**0.016**
Parasite family	5	3.131	**0.033**
Temperature*parasite family	18	4.469	**0.045**
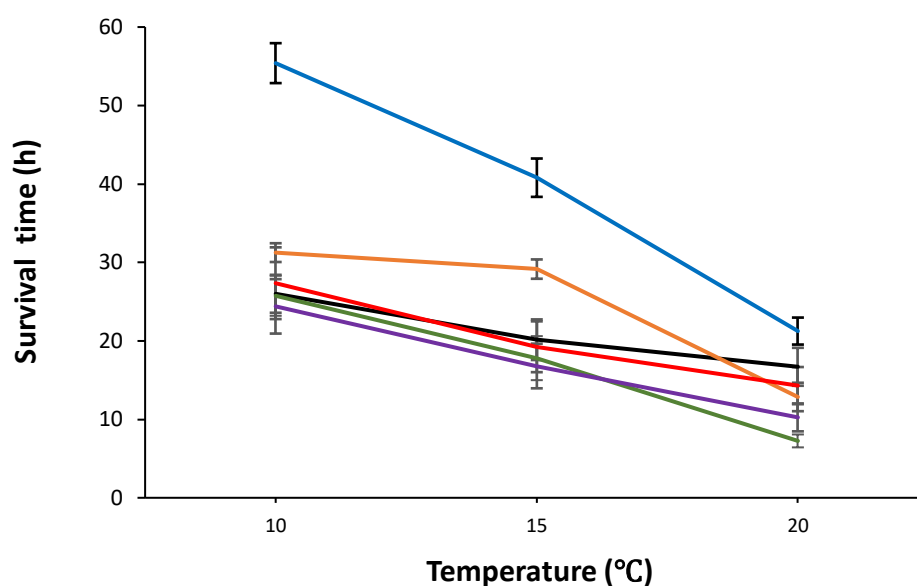
Figure 2.1 Survival time (mean ± 1 S.E.) of *Schistocephalus solidus* coracidia arising from six different worm-pair cultures (Family 1 (black line), Family 2 (green line), Family 3 (blue line), Family 4 (purple line), Family 5 (orange line); Family 6 (red line) exposed to three experimental temperatures 10°C, 15°C and 20°C. Linear Mixed Models statistic.

Wilcoxon (Gehan) tests (pairwise comparisons) were then used to compare survival distribution among the families, with the test statistic based on differences in families mean. At 10°C (Figure 2.2a), the coracidia survival distribution of Family 3 was significantly different from all other families: (Family 1, $\chi^2_2 = 28.193$, $P < 0.0005$, Families 2, $\chi^2_2 = 29.57$, $P < 0.0005$, Family 4, $\chi^2_2 = 25.53$, $P < 0.0005$, Family 5, $\chi^2_2 = 29.57$, $P < 0.0005$; and Family 6, $\chi^2_2 = 15.18$, $P < 0.0005$), with mean survival time of 55 h followed by; Family 5 (31 h) > Family 6 (27 h) > Family 1 (26 h) >Family 2 (25 h) and Family 4 (24 h). The survival distribution of coracidia from Family 5 was also differently

from those of Family 1 ($\chi^2_2 = 13.22$, $P < 0.0005$), Family 2 ($\chi^2_2 = 14.81$, $P < 0.0005$) and Family 4 ($\chi^2_2 = 25.53$, $P < 0.0005$).

Again at 15°C (Figure 2.2b), the coracidia survival distribution of Family 3 was differently from all other families: (Family 1, $\chi^2_2 = 13.100$, $P < 0.0005$, Family 2, $\chi^2_2 = 17.34$, $P < 0.0005$, Family 4, $\chi^2_2 = 16.54$, $P < 0.0005$. Family 5, $\chi^2_2 = 5.87$, $P = 0.005$ and Family 6, $\chi^2_2 = 11.47$, $P = 0.001$). The survival distribution of coracidia from Family 5 was also differently from those Family 1, $\chi^2_2 = 8.02$, $P = 0.005$, Family 2, $\chi^2_2 = 13.06$, $P < 0.0005$, Family 4, $\chi^2_2 = 11.74$, $P = 0.001$ and Family 6, $\chi^2_2 = 7.70$, $P = 0.006$. The mean survival time of Family 3 was longer, at 40 h, compared to Family 1 (20 h), Family 2 (18 h), Family 4 (16 h), Family 5 (29 h) and Family 6 (19 h).

At 20°C (Figure 2.2c), the survival distribution pattern of coracidia from Family 3 slightly changed since differences were only shown between Family 4 ($\chi^2_2 = 10.04$, $P = 0.002$), Family 5 ($\chi^2_2 = 4.69$, $P = 0.030$) and Family 2 ($\chi^2_2 = 25.12$, $P < 0.0005$). The differences in survival distribution were also documented between Family 4 with those from the families: Family1 ($\chi^2_2 = 5.20$, $P = 0.023$) and Family 2 ($\chi^2_2 = 6.83$, $P = 0.009$). In addition, the survival curve of Family 2 was also differed from those Family 1 ($\chi^2_2 = 10.06$, $P = 0.002$) and Family 5 ($\chi^2_2 = 18.29$, $P < 0.0005$). Overall, at 20°C, Family 2 had the lowest mean survival time 7 h, followed by Family 4 (10 h), Family 5 (12 h), Family 6 (14 h) and Family 3 (21 h).

Overall, coracidia of Family 3 survived 2.3 − 3 times longer in all three temperatures (10°C, 15°C and 20°C) than the coracidia of the shortest-lived families.
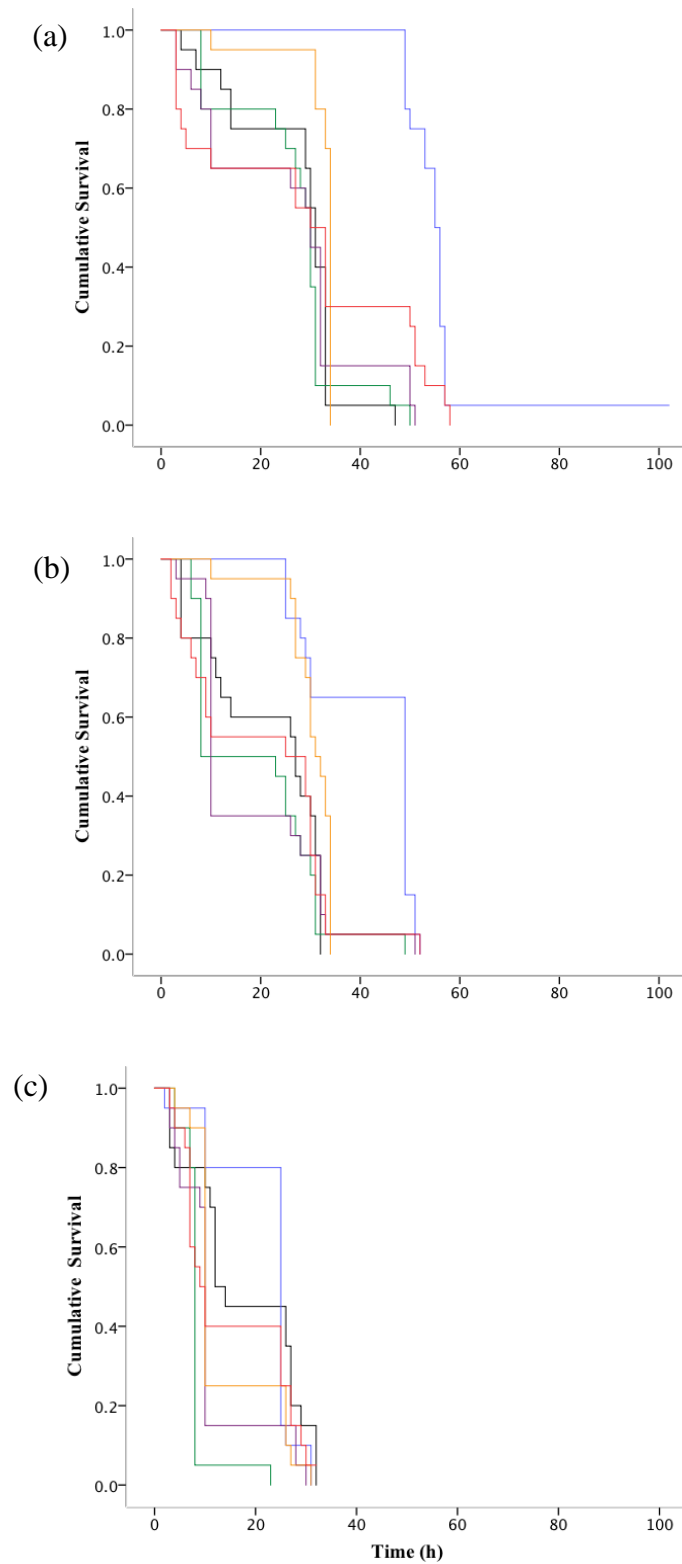
Figure 2.2 Comparisons of the cumulative survival of six families (Family 1(black line), Family 2 (green line), Family 3 (blue line), Family 4 (purple line), Family 5 (orange line), Family 6 (red line)) of coracidia at (a)10°C, (b) 15°C and (c) 20°C. Kaplan-Meier statistic.

2.4.2 Effects of temperature and infection status on copepod survival

Survival of copepods was significantly affected by infection status (Table 2.2) but not by temperature (Table 2.2). There were also interactions between infection status and temperature (Table 2.2, Figure 2.3).

Table 2.2 Two Way ANOVA of copepod (*Cyclops strenuus*) survival, testing the effect of temperature, and infection status. Significant P value (<0.05) are shown in bold.

Variable	D.F	F	P
(Intercept)	1	380.750	**.000**
Infection status	1	12.101	**.001**
Temperature	2	0.290	.749
Infection status * temperature	2	4.296	**.017**
Error	85		

A simple effect analysis was then used to test the effects of infection status on copepod survival in each temperature. At 10°C, there was a significant effect of infection status on copepod survival ($F_{1,85}$ = 5.308, P =0.024; Figure 2.3), with non-infected copepods surviving longer (mean survival time 5 weeks) than infected copepods (3 weeks). At 15°C, copepod survival was significantly affected by infection status ($F_{1,85}$ = 15.384, $P <$ 0.0005; Figure 2.3), with non-infected copepods living longer (mean survival time 5 weeks) than infected copepods (3 weeks). At 20°C, there was no significant effect of infection status on copepod survival ($F_{1,85}$ = 0.038, P =0.845; Figure 2.3), with both non-infected and infected copepods survived up to 3 weeks (mean survival time).

2.4.3 Parasite infection level

Infections established in copepod hosts under all temperature (10°C: 16/40, 15°C: 15/40 and 20°C: 16/40) and there was no statistically significant effect of temperature on the proportion of copepods that become infected across temperatures, X^2 (2) = 0.070, $P =$ 0.966. Tem*p*erature therefore did not affect the probability of coracidia infecting copepod hosts.
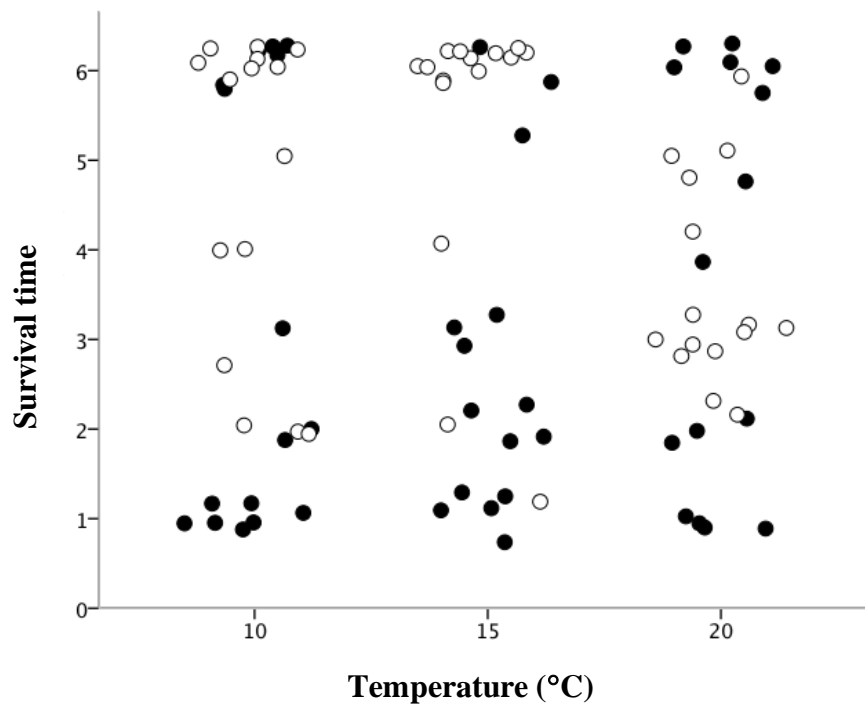
Figure 2.3 Scatter plot showing the effect of *Schistocephalus solidus* infection status (Infected copepod (black symbols) and Non-infected copepod (open symbols) and temperature (10°C, 15°C and 20°C) on the survival of copepods. Two-way ANOVA statistic.

2.4.4 Procercoid growth in the first intermediate host

Over the 42 days post-exposure, the mean size of procercoids (measured as body area) increased week-on-week throughout the experiment period and the rate of growth in body area size differed between the three temperature treatments. There was a significant procercoids weekly size x temperature treatment interaction indicating that there were some differences in the trajectories of the increase in body area between temperature treatments (Table 2.3; Figure 2.4).

A simple effect analysis was used to investigate the interaction between the temperature and the weekly size of procercoids. At week 1, after just 7d of growth, there already was a significant difference in body area of procercoids in the three temperatures ($F_{2,11} = 52.569$, $P < 0.0005$), with procercoids at 20°C temperature increased more rapidly in size, and attaining an area 2.5 times bigger, compared to 10°C and 15°C (Figure 2.4). The

Table 2.3 Result of repeated-measures ANOVA to determine the effects of temperature on the growth (body area size) of the *Schistocephalus solidus* procercoids. Significant P value (<0.05) are shown in bold.

Variable	D.F	F	P	Greenhouse-Geisser	Huynh-Feldt
Procercoids size	5	121.829	**< 0.0005**	**< 0.0005**	**< 0.0005**
Temperature	2	11.588	**0.002**		
Procercoids size * Temperature group	10	13.525	**< 0.0005**	**< 0.0005**	**< 0.0005**
Error	55				

difference in body area between temperature treatments continued into week 2 ($F_{2,11} = 26.156$, $P < 0.0005$), with procercoids at high temperature reaching a body area 1.7 times bigger than procercoids at 10°C. At week 3, significant differences in body area were recorded between 10°C and 20°C with procercoids at 20°C 1.4 times bigger than those at 10°C ($F_{2,11} = 14.324$, $P = 0.001$). After week 4 (Figure 2.4), the growth pattern changed dramatically due to the growth trajectories of body area of procercoids kept at 15°C only increasing 0.05% from week 3 and at 20°C procercoids increased 0.32%. But at 10°C, the body area of procercoids continued to grow (1.25%) and they reached approximately same and not significantly different body area as procercoids kept at 15°C or 20°C. For specific growth rate (SGR), there was a significant difference ($F_{4,28} = 4.846$, $P = 0.004$) in weekly SGR of procercoids body area for each temperature over 5 weeks of the experiment period. The SGR of body area also differed between the three temperature treatments ($F_{2,7} = 14.758$, $P = 0.003$), with procercoids at 10°C having a higher SGR of 0.935±0.105 followed by procercoids at 15°C with SGR of 0.381±0.258 and 20°C with SGR of -0.045±0.149. However, there was no significant interaction of SGR for weekly size * temperature treatment (Figure 2.5; $F_{8,28} = 1.250$, $P = 0.308$). Thus, the differences in the body area of procercoids only appeared at week 1 to week 3 but temperature does influence the amount of time taken by parasites (procercoids) to develop a cercomer which is important to infect fish. Development of a cercomer (a caudal appendage associated with infectivity to fish hosts) appeared early (at first week) at higher temperature (20°C) but took two weeks at 15°C and five weeks at 10°C (Figure 2.6).
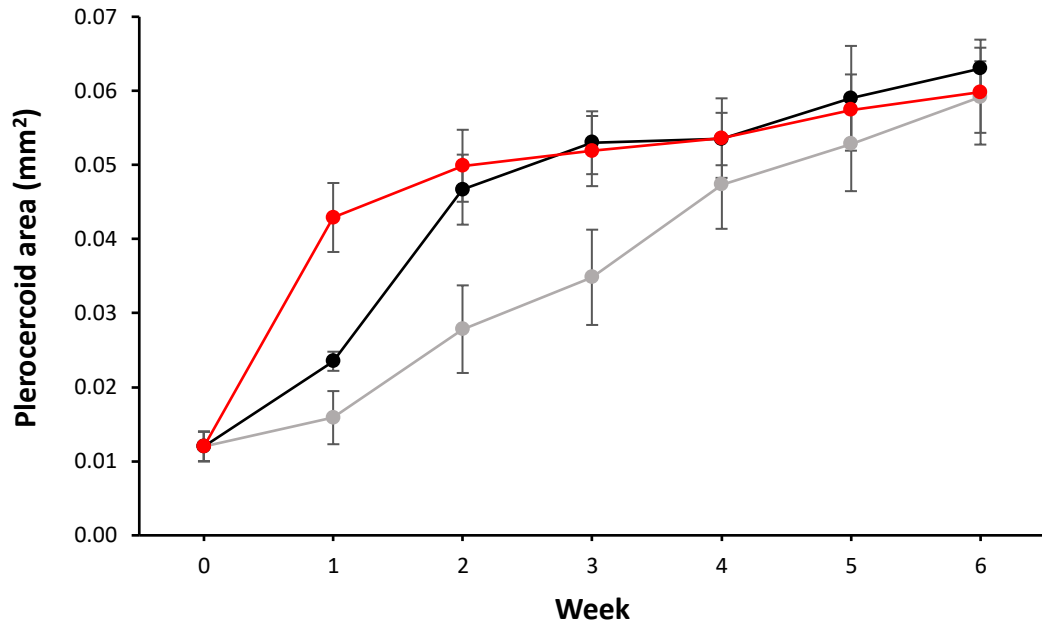
Figure 2.4 Growth trajectories (body area, mm^2) of the *Schistocephalus solidus* procercoids in three temperatures (10°C, 15°C and 20°C), recorded over 6 weeks post-infection. Error bars represent ± 1 standard deviation. Grey line represent 10°C; black line represent 15°C; red line represent 20°C. Repeated-measures ANOVA statistic.
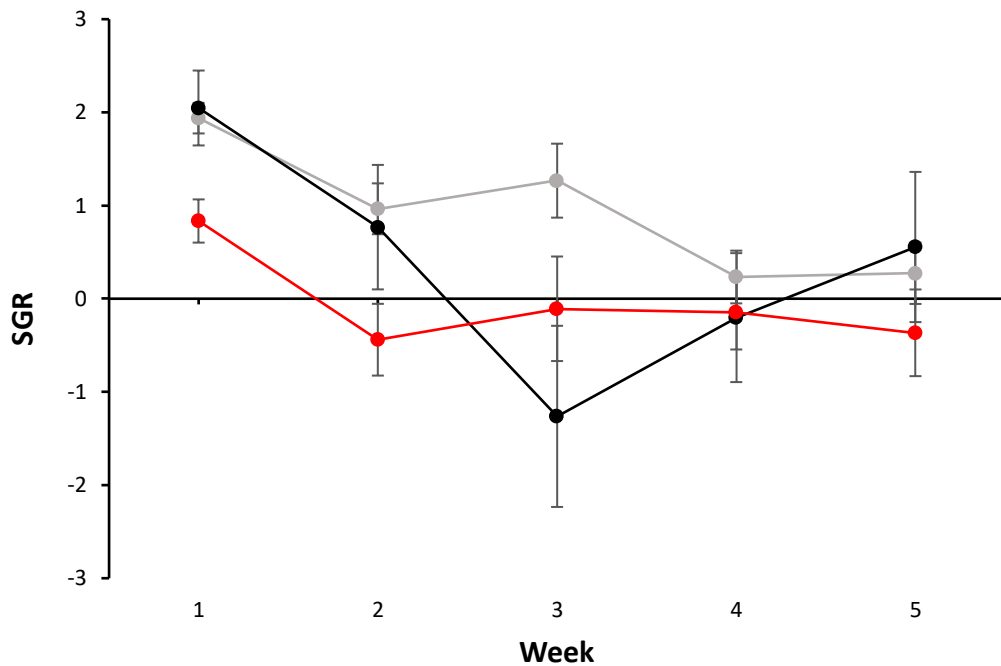
Figure 2.5 Specific growth rate (SGR) for body area of the *Schistocephalus solidus* procercoids in three temperatures (10°C, 15°C and 20°C), recorded over 5 weeks post-infection. Error bars represent ± 1 standard error. Grey line represent 10°C; black line represent 15°C; red line represent 20°C. Repeated-measures ANOVA statistic.
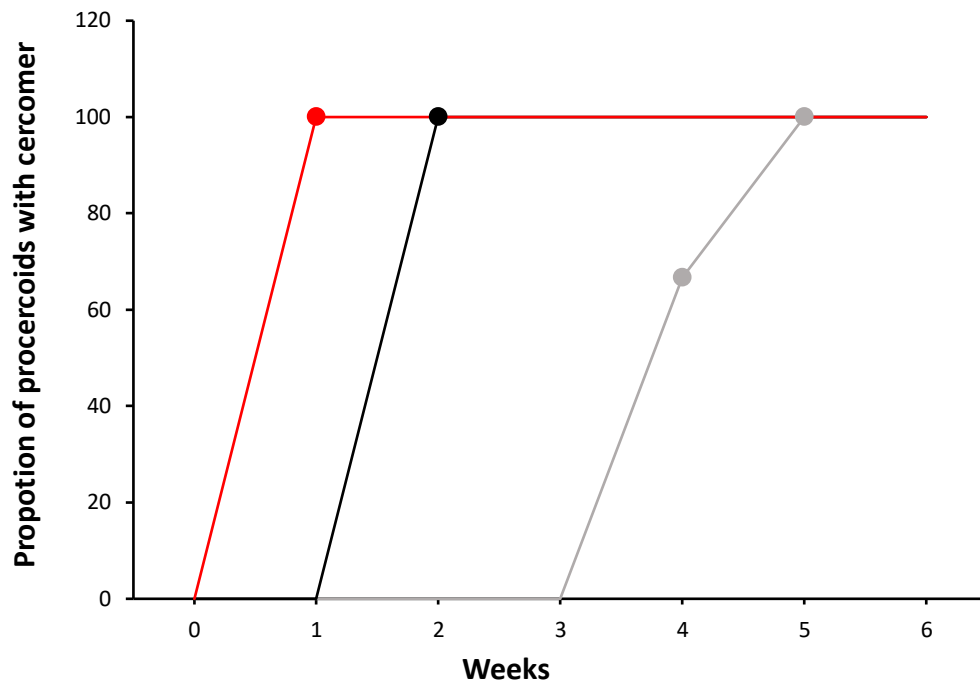
Figure 2.6 Proportion of the procercoids possessing a cercomer during the experiment kept in 10°C, 15°C and 20°C. Grey line represent 10°C; black line represent 15°C; red line represent 20°C. Data was plotted using excel.

2.4.5 Effects of temperature and infection status on the size of copepods

Throughout the 6-week period of the experiment, both infected and non-infected copepods kept at the three temperatures increased in size (measured as cephalothorax length) (Table 2.4; Figure 2.7). There was a significant effect of infection status on the size of copepods (Table 2.4; Figure 2.7) where the non-infected copepods have a larger size (measured as cephalothorax length) compared to the infected copepods. However, there was no significant effect of temperatures on copepod size (Table 2.4). There was an interaction between: (a) Copepod length and temperature, and (b) Copepod length and infection status but there was no significant interaction between (c) temperature and infection status (Table 2.4). There was no statistically significant three-way interaction between Copepod length, temperature and infection status (Table 2.4).

Table 2.4 Result of repeated-measures ANOVA to determine the effects of temperature and infection status on the cephalothorax length of *Cyclops strenuus* copepods. Significant P value (<0.05) are shown in bold.

Variable	D.F.	*F*	*P*	Greenhouse-Geisser	Huynh-Feldt
Copepod length	5	81.327	**< 0.0005**	**< 0.0005**	**< 0.0005**
Copepod length*temperature	10	3.010	**0.001**	**0.012**	**0.007**
Copepod length *infection status	5	7.917	**<0.0005**	**<0.0005**	**<0.0005**
Copepod length *temperature* infection status	10	1.613	0.105	0.158	0.142
Temperature*infection status	2	0.905	0.413		
Temperature	2	1.661	0.203		
Infection status	1	17.475	**<0.0005**		
Error	195				

2.4.6 Effect of copepod size and temperature on procercoid size

Copepod size was significantly related to procercoid size (Table 2.5; Figure 2.8). There was also a significant effect of temperature on procercoid size (Table 2.5). There was a significant interaction between treatment group and copepod size (homogeneity of regression slope) (Table 2.5).
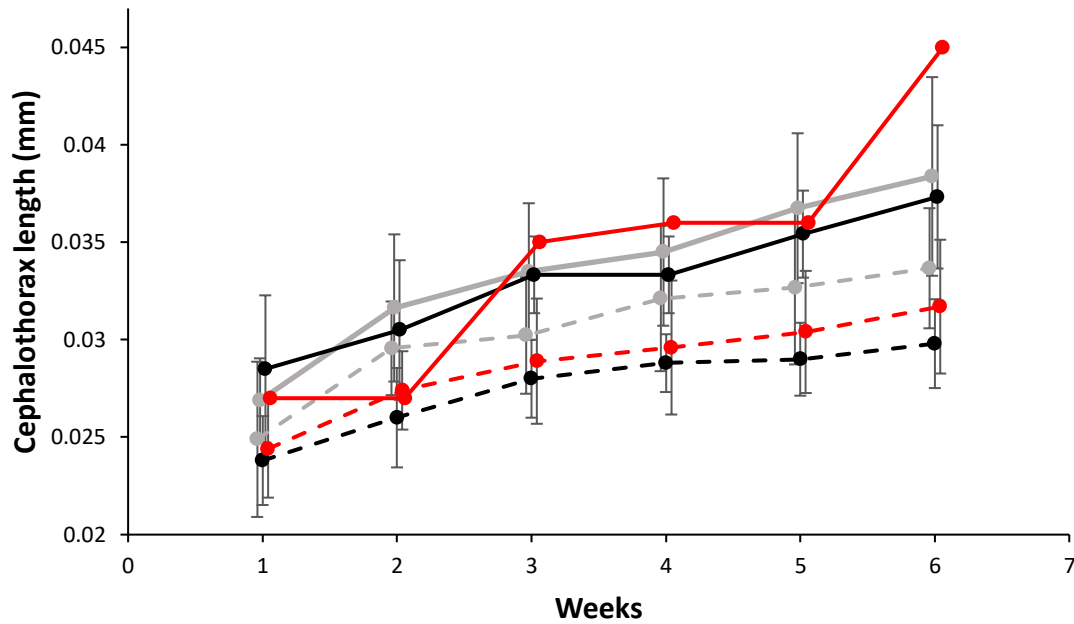
Figure 2.7 Growth in cephalothorax length for infected (broken lines) and non-infected (solid lines) *Cyclops strenuus* kept at 10°C (grey lines), 15°C (black lines) and 20°C (red lines). Data points show mean values, error bars represent ± 1 standard deviation. Repeated-measures ANOVA statistic.

Table 2.5 ANCOVA examining the effect of temperature and copepod size (cephalothorax length) on the body area of *Schistocephalus solidus* procercoids at the end of the study. Significant P value (<0.05) are shown in bold.

Variable	D.F	*F*	*P*
Intercept	1	0.030	.863
Temperature	2	7.663	**.001**
Copepods	1	12.459	**.001**
Temperature * Copepods	2	5.653	**.005**
Error	78		
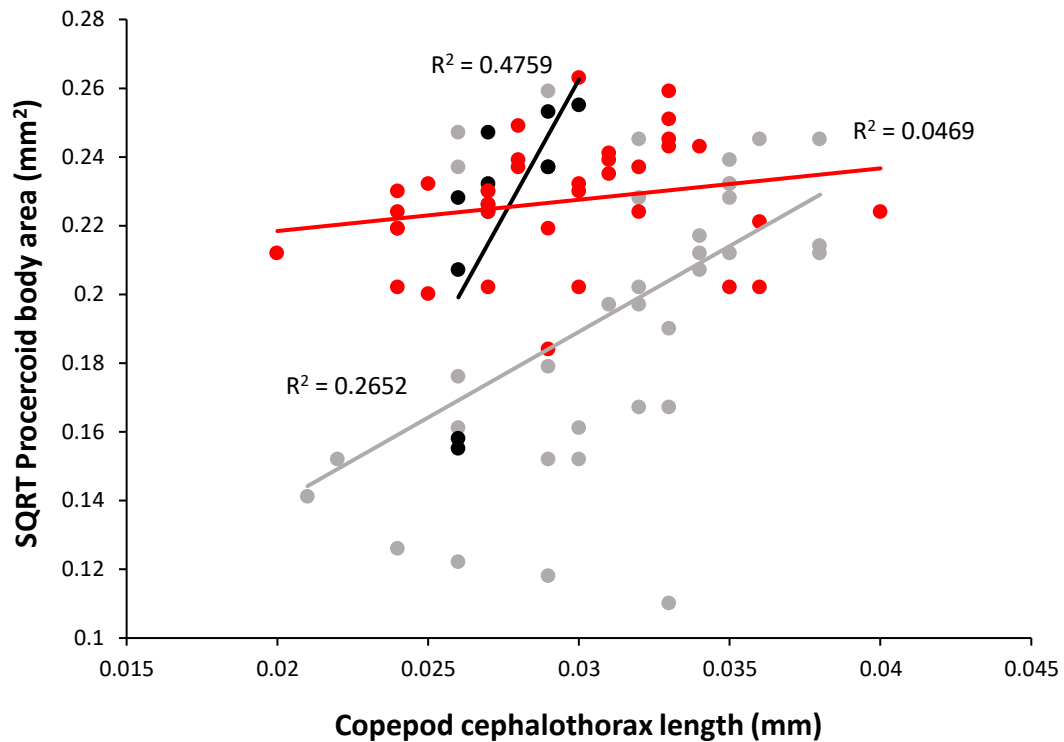
Figure 2.8 The effects of final copepod size and rearing temperature on procercoid body area after 6 weeks post-infection (10°C, grey symbols, grey line; 15°C, black symbols, black line; 20°C, red symbols, red line). ANCOVA statistic.

2.5   Discussion

2.5.1   Main findings of the study

In this study, the effect of environmental temperature on the survival of the free-living coracidium stage of the parasite *S. solidus* was studied, as was the development and growth of the procercoid stage of the parasite in the first intermediate host, the copepod *Cyclops strenuus*. These are potentially important regulating steps in determining the success and rate of life cycle completion. This results demonstrated that environmental temperature is an important factor affecting the survival of coracidia, the free-living stage. The mean survival time of all coracidia or free-living stage generated from six families decreased at higher temperature (20°C) compared to those coracidia exposed to lower temperature of 10°C and 15°C (Figure 2.1). However, the study also demonstrated significant differences in survival between parasite families (Figure 2.2).

This study also demonstrated an effect of the temperature on parasite development and growth, measured as body area size, in the copepod first intermediate host. At higher

temperature (20°C), the parasite tends to establish in host body cavity and increase in size rapidly after just one week, compared to those kept at lower temperatures of 10°C and 15°C (Figure 2.4). The development of a cercomer (a caudal appendage that is associated with infectivity to fish hosts) by the parasites was also affected by temperature, with the cercomer appearing at week-1 at the higher temperature (20°C) but developing more slowly at cooler temperatures, taking two weeks at 15°C and five weeks at 10°C (Figure 2.5).

2.5.2 Effect of temperature on the survival of *S. solidus* coracidia

In the present study, *S. solidus* coracidia were shown to be sensitive to temperature, as the mean coracidia survival time decreased with increased temperatures. The decrease in survival of coracidia stages at the highest temperature (20°C) might be related to the increased activity level in coracidia, in terms of their swimming and movements (Koprivnikar et al., 2010) which would lead to them consuming energy resources more rapidly (Pechenik and Fried, 1995), eventually causing death. Free-swimming stages of parasites are lecithotrophic and known to contain a limited amount of energy to sustain life and fuel locomotion (Barber et al., 2016). In cestodes, that energy exists in the form of fat and glycogen droplets located in the ciliated embryonal shell of free-living stage (Dubinina, 1980). Based on personal observations, the coracidia swam actively and moved randomly at the bottom and surface at high temperature compared to low temperatures where the coracidia generally moved slowly at the edge of the well. Increased activity and shortened lifespans at higher temperatures have also been observed in different free-swimming stage of other aquatic parasite species, particularly in trematode cercariae (Rea and Irwin, 1992; Pechenik and Fried, 1995; Ford et al., 1998; Koprivnikar and Poulin, 2009; Koprivnikar et al., 2010; Berkhout et al., 2014), which typically also survive longer at lower temperatures (Studer et al., 2010). For example, cercariae of *Cryptocotyle lingua* survived longer (up to 128 h) at 10°C compared only 44 h at 20°C (Rea and Irwin, 1992). But not all cercariae species showed worse survival performance under higher temperature. For example, *Centrocestus formosanus* and *Haplorchis pumilio* cercariae survived longer at higher temperatures of 15°C and 20°C compared to a lower temperature (Lo and Lee, 1996). This is might be because, most of free-living stage species could have the ability to tolerate the range of temperature that commonly appear in natural environmental conditions and therefore, the infectivity and

activity of this free-living stage could have the potential to increase under high temperature to recoup for their lower survival time (Evans, 1985).

The longevity of infective free-swimming stages of parasites is likely to be critical, as this stage needs to locate (or be located by) and invade the susceptible hosts either by penetrating the gut wall after ingestion, such as in cestodes and some nematodes (Dubinina, 1980) or by penetrating the external host tegument from the environment (as in the case of trematode cercariae) to proceed the life cycles. Therefore, effects on the longevity and activity levels of infective free-living parasite stages by temperature are is likely to influence the probability of host encounters as their habitat selection and ability to use effective host-finding behaviours were also affected (Barber et al., 2016). As the temperature can affect the free-living stage, it would also effect the intermediate host availability and suitability to act as a host to the parasite in a way that it can affect the probability that the infective parasites and susceptible hosts coexist spatiotemporally or by effect the ability of hosts to resist infection after exposed to infectious agents (Barber et al., 2016). Therefore, it would also be interesting to work on the other components of the *S. solidus* life cycle regarding the temperature effect, including their infectivity and growth of procercoids in copepod hosts in altered temperatures. This is because the effect of environmental temperature on the rate at which larval stages of multi-host parasites develop within the bodies of their hosts is a potentially important factor in determining the dynamics of life cycles (Barber et al., 2016).

2.5.3 Copepod survival

In this study, there was no difference in the survival of copepods between the different temperatures (10°C, 15°C and 20°C) over the 6 weeks of experimental period. Similar findings were documented by Ban (1994), who showed no significant differences in mortality of the copepodite stage of the copepod *Eurytemora affinis* kept at 10°C, 15°C and 20°C. This contrasts with the findings of some other studies, which have reported a significant mortality in the first copepodite stage (Uye, 1988) with increased temperatures due to large morphological and physiological changes (Epp and Lewis, 1980). However, in the present study, the survival of copepods was influenced by infection status. Infected copepods had a shorter survival time (3 weeks) when compared to non-infected copepods (5 weeks). Infections are expected to have a wide range of impacts on host copepods – including tissue damage arising from gut penetration and energetic drain as a consequence

of sequestration of host - derived nutrients (Rosen and Dick, 1983).

2.5.4 Parasite infection rate in first intermediate host

In this study, *C. strenuus* exposed to *S. solidus* parasite showed no significant differences in infection rate between three temperatures (10°C, 15°C and 20°C). These findings contrast with some other studies that have shown that temperature can affect the infectivity of parasites in first intermediate hosts. For example, in the cestode *Bothriocephalus acheilognathi* (Smyth and McManus, 1989) and cercariae of the trematode *Echinostoma trivolvis* (Pechenik and Fried, 1995), infectivity reduced as temperature increased. There are several factors that might have led to the apparent lack of effect of temperature in the present study such as susceptibility of host (copepod). It is known that changing temperatures can affect the susceptibility of invertebrate hosts to infection by affecting their ability to resist infections (Barber et al., 2016). Several studies have documented lower resistance to parasite infections with increasing temperatures (Seppälä and Jokela, 2011; Leicht et al., 2013; Paull et al., 2015). Another possibility is that, hosts that being exposed to parasites could have altered their behaviour to cope with the changing temperature environment (Barber et al., 2016). Organisms with temperature-dependent behaviour may also have the ability to alter the spatiotemporal relationships of hosts and parasites and affect the likelihood of encounter (Barber et al., 2016). For example, in ectothermic organisms, increases in temperature can affect metabolic rate and influence the rate of food consumption (Roessig et al., 2004), potentially leading to a greater exposure to trophically-transmitted parasites, either because prospective hosts eat more prey items, or because they become less selective in prey choice with increasing appetite. This could explain the similar infection level between high temperature and low temperature in the present study. In addition, the success of infection also probably depends on the number of coracidia that are ingested and subsequently penetrate the copepod intestine (Nie and Kennedy, 1993; Wedekind, 1997). In this study, a single infective coracidium was fed to each copepod, so this might be one of the reasons that contribute to the same infection level occurred between temperatures.

2.5.5 Procercoid growth and development in first intermediate host

Water temperature had a significant effect on *S. solidus* procercoid growth and development in the copepod first intermediate host. A few studies have previously documented the effect of temperature on the parasite growth with increases in parasite growth and/or development rates in hosts held at higher temperatures (Tokeson and Holmes, 1982; Lv et al., 2006; Studer et al., 2010; Macnab and Barber, 2012; Franke et al., 2017).

It is known that temperature can affect a wide range of physiological mechanisms, including development time, maturation, egg production, or reproduction, longevity, ammonia excretion, food intake, oxygen consumption and respiration (Gophen, 1976; Marcogliese, 2001; Pörtner, 2002).

A number of possible mechanisms may be involved to generate increased parasite size at higher temperatures in this host-parasite system, including elevated ingestion rate of food by host copepods (Gophen, 1976) giving parasites access to more host-derived nutrients. This is because cestode parasites are capable of utilizing carbohydrate (particularly glucose) and energy substrate from the host by the active mediated transport mechanism which transport the food materials (salts, glucose, amino acids and lipids) for synthesis purposes (Smyth and McManus, 1989). In the present study, the amount of food given to copepods at all three temperatures was not restricted; this might benefit the parasites that established in hosts, since higher temperatures increase food intake of host (Gophen, 1976). Feeding activity of the copepods may be inhibited at lower temperatures and thus lower levels of host-derived nutrients will be available to the procercoids at low temperature (Uye, 1988). In addition, the significant difference in procercoid growth between 20°C and 10°C was only observed until week 3 post-infection. After this point, the increase in procercoid body area in copepods held at 20°C slowed, and did not differ from 10°C, presumably because of the procercoids development rate reached its thermal maximum. If this thermal maximum is exceeded, enzyme systems are affected and death

occurs (Gophen, 1976). Another possibility is that the increased in procercoid body area size might be limited by the size of the copepod host (Wedekind, 1997). In present study, procercoid body area size was significantly related to the size of the host copepod, supporting previous studies (Wedekind, 1997). This could be because the rigid exoskeleton of the copepods constrains the maximum size that can be achieved.

In the present study, temperature also affected the timing of parasite development, with earlier maturation (i.e. a shorter development time) being documented at higher temperatures. This might result from a higher amount of assimilated energy flowing to the growth at higher temperatures (Gophen, 1976). All procercoids from copepods kept at the highest temperature (20°C) had reached infectivity (i.e. had developed hooked cercomers) after 7d, whereas none of procercoids kept at the lower temperatures (10°C and 15°C) had reached this stage of development after 7d. Procercoids that have faster cercomer development will be able to infect fish earlier than those that develop later. Although, maturation appeared early (at week 1) at high temperature, the body area size of procercoids was smaller compared to the procercoids kept at low temperature (10°C), which had a larger body size when they reached maturation. This is might be because of in cold temperatures, the animals are able to have large body size at maturation stage by sustaining the growth and restrain the reproduction (Angilletta Jr et al., 2004). This is known as an adaptive plasticity of body size (Angilletta Jr et al., 2004). Adaption also can be considered as the fecundity and survival increased and maturation process became slowed or delayed in cold temperatures (Stearns, 1992). Faster development of larvae under warmer temperatures has been demonstrated experimentally in several studies such as in *Angiostrongylus cantonensis*, a nematode parasite of humans and the causative agent of eosinophilic meningitis. This parasite uses larvae of the freshwater snail *Pomacea canaliculata* as its first intermediate host. Under higher temperatures (28°C), the larval nematodes complete their transformation from first stage larvae to human-infective third stage larvae in a shorter time (ca. 20 days) compared to 19°C, at which completion takes more than two months (Lv et al., 2006). In another study, by Tokeson and Holmes (1982), the development rate of the acanthocephalan *Polymorphus marilis* in laboratory-infected *Gammarus lacustris* increased linearly with temperatures between 10°C and 25°C. With faster larval development rates, the number of life cycles that could be completed per year could be altered, and also the number of hosts that are likely to be exposed to the parasite was also affected (Barber et al., 2016). This faster development also shortens the period

of time that parasites are at risk of pre-transmission host mortality through predation, as well as promoting the onset of parasite-induced manipulation of host behaviour (Poulin, 2010) either of which could increase the efficiency of transmission.

2.5.6 Family-specific differences in response to temperature

In this study, different parasite families responded differently in terms of their coracidia survival when reared at different temperatures. This study appears to be the first to look for differences among parasite families in survival and performance of free-living stages response to temperature in *S. solidus*. Mean coracidia survival time and distribution were found to vary between families. In the present study, the difference between families in their response to temperature was clear since there was a significant interaction between the families of coracidia and temperature were found. The data for six families of *S. solidus* coracidia reveal significantly different survivorships at each temperature, and significantly different reaction norms, for temperatures spanning 10-20°C which suggested that there is might be a genetic differences involved. This was shown in the trematode *M. novozealandensis* by Berkhout et al., (2014), who reported that temperature effects on cercarial activity vary within species. Some of the cercariae genotypes showed differences in activity after 8h between treatments and some of them appears to be similar in activity between temperatures. This indicated the existences of genetic differences in cercariae activity among conspecific parasites in their responses to rising temperature (Berkhout et al., 2014).

Temperatures have been shown to affect the free-swimming stage among the same species of different population. For example, two genetic clusters or batches ('northern' and 'southern') of the ectoparasitic copepod *Tracheliastes polycolpus* differed in terms of the survival the motile stages at different temperatures (Mazé-Guilmo et al., 2016). Populations can respond to climate change by either shifting their geographical distribution to track their favourable habitat (Hoffmann and Sgrò, 2011) or by adapting *in situ* (via phenotypic plasticity or microevolution) (Merilä and Hendry, 2014). In present study, the plate that kept the *S. solidus* eggs were incubated at higher temperature of 20 °C and the coracidia that hatched at this temperature then tested at the temperature of 10,

15 and 20°C. The higher survival from the coracidia exposed to 10 and 15°C indicates that the coracidia produced from 20°C when they shifted to lower temperature they were adapting better and suggested there is might be *in situ* adaptation. This adaptation can have varied even within the same population. In contrast to another parasite systems study, Berkhout et al., (2014) showed that cercariae produced in snails that experienced 20°C for several weeks coped better with more extreme temperatures of 25°C than cercariae that emerged from snails that experienced 15°C for several weeks. In the present study, the free-living coracidia were generated from different families drawn from a single population by *in vitro* culture in the laboratory, and no detailed genotype investigation was undertaken to determine the differences between the families. Therefore, future work needs to take this element into account as the genetic drift effects are likely to cause population variation in thermal tolerance (Mazé-Guilmo et al., 2016).

2.5.7 Conclusion and future directions

In summary, this study demonstrates the impact of temperature on the survival or life-span of the free-swimming infective stages (coracidia) of the parasite *S. solidus* and identifies an effect of temperatures on the development and growth of *S. solidus* procercoids in a first intermediate host, the cyclopoid copepod *C. strenuus*. The study has shown that the survival time of free-living stage of *S. solidus* coracidia was temperature-dependent, with higher temperatures shortening the life-span at which this survival element could affected the completion of the life cycle. But to continue to next stage, the free-living stage needs to infect the next host, the copepod. Here, factors such as age of free-living stage and speed or swimming behaviour at different temperatures could affect the transmission and infectivity. Therefore, it would be interesting to look at this element at different temperature and determine the successfulness of the transmission.

This present study also showed that development and maturation were affected by temperatures, with high temperature reducing the time taken for parasites to develop characteristics that are indicative of infectivity to the next host in the lifecycle – the three-spined stickleback *Gasterosteus aculeatus*. The study also shows that parasites kept at higher temperatures (20°C) have a larger body area size in the first three weeks at high temperature. These results indicate that increases in growth at higher temperatures could potentially affect the rate with at which parasites complete their life cycles in aquatic habitats subjected to elevated temperatures. Further investigations on the age and size at

infective stage of parasites in intermediate hosts at elevated temperature would be required to determine the probability of higher infection or establishment rate in the next intermediate host (stickleback fish). In addition, the effects observed occur independently of food intake, and further study is required where infected copepods are given fixed amount of food and held at different temperatures to determine the effects of food on the parasites growth and development.

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Chapter 3

Effects of water hardness and heavy metals on survival, growth and development of *Schistocephalus* coracidia and procercoids

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3.1   Abstract

Heavy metals enter the aquatic environment from a variety of natural and anthropogenic sources. Among the heavy metals, cadmium, copper and zinc are the most harmful pollutants of aquatic ecosystems. In natural environments, the toxicity of metals or other pollutants can be altered by environmental factors such as water hardness. In the present study, the effects of heavy metals on a cestode parasite, *Schistocephalus solidus* and its copepod host, *Cyclops strenuus,* were tested at two different levels of water hardness (hard water: 342 mg/L and soft water: 34.2 mg/L $CaCO_3$). Water hardness had a significant effect on the survival, development and growth of the parasite and host. Free-swimming, infective stages of the parasite (coracidia) exposed to copper, zinc and cadmium (10 µg/L) survived longer in hard water than in soft water. The survival of the host copepods was also affected by water hardness, with both infected and non-infected

copepods surviving for longer in hard water than in soft water. The size of the procercoid stage of the parasite within infected copepod hosts differed in hard and soft water. After one week of infection, procercoids within hosts kept in hard water solutions containing either copper or zinc were larger than those kept in soft water solutions containing the same amounts of metals. Infected copepods themselves were also larger when kept in hard water compared to soft water. In contrast, non-infected copepods were smaller in hard water than in soft water.

3.2   Introduction

3.2.1   General biological effects of heavy metals

Industrialization and urbanization processes have led to the introduction of pollutants including heavy metals into natural resources like soil, water and air, thus degrading the quality of the environment and affecting both plants and animals (Sethy and Ghosh, 2013). Heavy metals are generally defined as metals having relatively high densities, atomic weights, or atomic numbers (Luoma and Rainbow, 2008). Heavy metals which enter plants, animals and microorganisms have the ability to alter their physiological function (Heath, 1995; Stebbing, 1976; Seregin and Kozhevnikova, 2006; Luoma and Rainbow, 2008). In living plant cells, accumulation of toxic heavy metals can lead to

various deficiencies, reduction of cell activities, and inhibition of plant growth (Farooqi et al., 2009). For example, the germination indices and growth inhibition indices for the halophile seepweed *Suaeda salsa* decreased significantly at a $CdCl_2$ concentration of 0.1mg/L, and the inhibition was increased with increasing concentration of $CdCl_2$ (Liu et al., 2012). In another study, the growth of cork oak *Quercus suber* was affected by cadmium exposure via the nutrient solution. Even a low concentration of cadmium (10 μM Cd(II)-EDTA) resulted in major decreases in shoot growth (64% when compared with control values) and also significant decreases in the leaf concentrations of photosynthetic pigments (leaf chlorosis) (Gogorcena et al., 2011).

In aquatic invertebrates, heavy metals such as copper, zinc, Mercury and cadmium change both morphological (Martinez et al., 2002) and physiological parameters such as growth rate, swimming speed, food consumption, intensity of breathing, productivity, survival and life cycles (Gerhardt, 1993; Barata et al., 2002; LaBreche et al., 2002). Maltby and Naylor (1990) showed that a freshwater amphipod crustacean *Gammarus pulex* had reduced growth when exposed to 0.3 mg/L zinc. The reduced growth was associated with a decrease in the size of offspring released in the subsequent brood (Maltby and Naylor, 1990). Green crabs (*Carcinus maenas*) and rock crabs (*Cancer irroratus*) exposed to various concentrations of copper for 48 zinc showed a loss of osmoregulatory function with increasing copper concentration, so their normally hyperosmotic haemolymph tended to become isosmotic. When green crabs were exposed to cadmium for 48 zinc, however, serum osmolarity was elevated above its normally hyperosmotic state. Exposure to cadmium reduced the rate of gill oxygen consumption in both species whereas exposure to copper had no effect (Thurberg et al., 1973).

3.2.2   Heavy metals in aquatic environments

Heavy metals, including cadmium, copper and zinc, are among the most harmful pollutants of aquatic ecosystems (Evans, 1982b; Sullivan et al., 1983; Depledge et al., 1994). copper (Gutierrez et al., 2010), cadmium and zinc (Morley et al., 2001b) are particularly important because they are widely used in industry and are major pollutants in aquatic ecosystems. Heavy metals continuously enter the aquatic environment from a variety of sources including natural processes such as biogeochemical cycles, where metals are released from rocks by weathering processes. They are then cycled through various environmental compartments by biotic and abiotic processes, and passed into

soils and transported either with soil particles or in dissolved form, into rivers, estuaries and eventually to the deep oceans (Luoma and Rainbow, 2008). Although heavy metals are naturally occurring elements that are found throughout the crust of the earth, most environmental contamination results from anthropogenic sources such as industry, agriculture and domestic waste run-off (Lima et al., 2008; Nhi et al., 2013). Heavy metals are redistributed throughout the water column, accumulated in sediments or consumed by biota due to their incomplete biological degradation (Forstner and Prosi, 2013; Frémion et al., 2016). The sediments constitute a long-term source of contamination to the food chain due to desorption and remobilization of metals. Heavy metal residues in contaminated habitats have the ability to bio-accumulate in aquatic flora and fauna (Hasan et al., 2016), which, in turn, may enter the human food chain and generate health problems (Varol and Şen, 2012).

Copper is widely used in antifouling paint, treatment for fish diseases and as an algaecide (Gutierrez et al., 2010). Copper is an essential element in metabolic processes for both plants and animals (Irwin et al., 1997a), but high concentrations can be toxic to aquatic species such as fish, algae and crustaceans (Sullivan et al., 1983). The sensitivity of aquatic invertebrates to copper varies. For example, crustaceans have an LC50 at 48h ranging from 5 to 86 ug/L but for annelids (*Tubifex* sp.), the 48h LC50 ranges from 10 to 890 ug/L (Hodson et al., 1979). This variation in sensitivity depends in part on the surface area and respiration rates of the animals, which can influence the copper uptake (Hodson et al., 1979).

cadmium and zinc have similar physicochemical properties (Morley et al., 2002) and often occur together in pollution (Depledge et al., 1994). These two metals have been widely used in industry as a by-product of ore smelting (Morley et al., 2001b). In comparison with other heavy metals, cadmium is relatively water soluble and more mobile, especially in soil; and hence is more bioavailable and more likely to bio-accumulate. In animals such as fish and invertebrates, cadmium concentrates in internal organs such as the kidney and accumulates as the animal ages (Irwin et al., 1997b). Zinc is used in industry to make white paints, in the production of rubber, in preserving wood and in dyeing fabrics (Irwin et al., 1997c). It can be found contaminating the air, soil and water (Irwin et al., 1997c). Both cadmium and zinc can affect behaviour, growth and physiological processes in aquatic organisms if the concentration level is elevated. The acute toxicity of cadmium to aquatic organisms is varied and related to the free ionic

concentration of the metal. In fish, cadmium can inhibit the uptake of calcium from water, with long term exposure causing hypocalcaemia and affecting larval growth and mortality (Irwin et al., 1997b). For aquatic invertebrates, the toxicity of cadmium is increased by the presence of zinc which affects growth and reproduction (Irwin et al., 1997b).

3.2.3 Effect of water hardness on the concentration of heavy metals

In the natural environment, the toxicity of chemicals or pollutants depends on a variety of environment variables such as the water hardness, pH, and the concentration of humic substances (Pietrock and Marcogliese, 2003). These variables may alter the rate of metabolism of organisms, and the toxicity of the chemical (Rathore and Khangarot, 2003). Thus, it is important to look not only at the general toxicity of the chemical but also to study its interactions with other variables including different environmental conditions. Among all the environmental variables affecting toxicity, the effects of water hardness and temperature are perhaps best studied (Cairns et al., 1975; Calamari et al., 1980). Most heavy metals become less toxic in hard water (Mount, 1966; Evans, 1982a; Morley et al., 2001a; Rathore and Khangarot, 2003). For example, the toxicity of zinc and copper to *T. tubifex* decreases with increasing water hardness (Rathore and Khangarot, 2003). In some cases however – for example, in the parasitic digenean trematode *Notocotylus attenuatus* – the toxicity of copper and zinc can be less influenced by water hardness (Evans, 1982b). Increased toxicity in soft water might arise because heavy metals are more soluble in soft water. The toxicity of a metal is more related to the prevalent concentration of certain metal species than to the total concentration of metal, so the observed patterns of copper and zinc toxicity in relation to water hardness may reflect differences in the concentrations of soluble, dissolved forms of the metals present in the test solutions (Evans, 1982a; Rathore and Khangarot, 2003). To date, several digenean parasite species have been used as test organisms to investigate the effects of water hardness on the toxicity of heavy metals, but so far, there are few data concerning such effects on cestode parasites, including *Schistocephalus solidus*.

3.2.4 Implications of pollution for host-parasite interactions in aquatic environments

Toxic pollutants, including heavy metals, can directly influence the physiology and survival of aquatic organisms through toxic effects (Pietrock and Marcogliese, 2003) and can influence the prevalence, intensity and pathogenicity of parasites (Khan and Thulin,

1991). Many diseases in aquatic populations are dependent on complex interactions between hosts, pathogens and environmental factors (Möller, 1987). Aquatic pollution may affect the incidence and nature of parasitism in many ways, for example, by altering host susceptibility through alteration of host defence mechanisms, by altering the densities of suitable intermediate or final host populations (Lafferty and Kuris, 1999), or by reducing the performance or survival of infected hosts (Sures, 2004). The effects of pollutants on parasitism can differ between species and even between developmental stages. The latter effects can be particularly pronounced in parasites where different life stages live in very different environments including both external and internal environments (i.e. within hosts).

3.2.5 Effects of pollution on the survival of free-living parasite stages

During their off-host, free-living phases, parasites are directly exposed to the environmental conditions in their habitat (Poulin, 1992). Any natural environmental factor or pollutant caused by anthropogenic activities can therefore directly influence the survival and performance of free-living parasite stages in their transition from one host to the next (Pietrock and Marcogliese, 2003). Among taxa and species, the free-living infective stages of parasites display an enormous variety of morphological and physiological properties (Pietrock and Marcogliese, 2003). Regardless of morphological variability, these free-living stages must rely on their own energy reserves and physiological systems to survive in external habitats that provide very different conditions from those inside their hosts. During this time they must either passively wait for host contact or actively seek out a host in a possibly polluted environment (Morley et al., 2003). A number of studies have investigated the effects of chemical substances on the cercarial stage of digenean trematodes (Evans, 1982a; Cross et al., 2001; Pietrock et al., 2002), probably because of their large size and the ease with which cercariae can be produced by infected snails in the laboratory, and also because some (such as *Schistosoma mansoni*) can infect humans and affect their health (Crompton, 1999). Most studies of the effects of pollution on parasites have been performed using miracidia or cercariae. Thus, there is a need for information on how stages of other endohelminth taxa respond to pollutants (Pietrock and Marcogliese, 2003).

It is often assumed that the free-living stages, particularly of endohelminth parasites, are highly sensitive to pollutants (Mecham and Holliman, 1975; Wanas et al., 1998;

MacKenzie et al., 1995), but sometimes pollutants can even prolong survival, especially at low concentrations of metals (Morley et al., 2001b; Morley et al., 2002; Pietrock and Marcogliese, 2003). Reduced survival of digenean cercariae in the presence of a toxicant may arise because of binding of the toxicant to active sites of enzymatic molecules and de-activation of enzyme systems during encystment. Conversely, an increase in life-span could arise because of binding of metal ions to specific enzymes involved in glycogen utilization, reducing metabolic activity (Pietrock and Marcogliese, 2003). There is thus a diversity in the sensitivity of parasites to toxicants according to their species and stages (Pietrock and Marcogliese, 2003).

3.2.6   Effects of pollutants on survival and susceptibility of intermediate hosts

Copepods are dominant grazers as well as an important food source in aquatic food webs, and have become important study organisms for understanding the role of pollutants in toxicity tests (Hook and Fisher, 2001). Copepods accumulate metals by assimilating them from their food or by absorbing them from water (Reinfelder et al., 1998), but the uptake pathway varies considerably for different metals. Copepods also act as intermediate hosts for many fish parasites, including cestodes and nematodes (Torres et al., 2007; Barber and Scharsack, 2010).

Aquatic crustaceans (including copepods) infected with parasites might be more susceptible to pollution than uninfected ones (Sures and Radszuweit, 2007). For example, *Cyclops strenuus* infected with *Bothriocephalus acheilognathi* and exposed to cadmium died at a significantly earlier day of exposure compared to non-infected controls. This suggests that when both stressors, cadmium and parasite infection, are applied together, they have adverse synergistic and/or additive effects on the copepods compared to when the copepods are exposed to a single stressor (Khalil et al., 2014). In *Gammarus pulex* infected with the acanthocephalan *Pomphorhynchus laevis*, two weeks of exposure to cadmium (2.1 μg/l) was found to decrease the survival rate of *P. laevis* in an infected individual (Brown and Pascoe, 1989). In addition, cadmium levels were lower in the cystacanth stage of the parasite than in the gammarid host. Similarly, cadmium and Lead levels are lower in the cystacanth of *Acanthocephalus lucii* than in the intermediate host *Asellus aquaticus* (Sures and Taraschewski, 1995). This suggests that parasites infecting crustacean hosts do not accumulate high quantities of heavy metals. This might be because inside the haemocoel of crustacean hosts, parasites are surrounded by liquid

of host origin (Sures and Taraschewski, 1995) and the location of the parasite inside the host determines the availability of metals to the parasite (Sures and Siddall, 1999). For example, young adult *P. laevis* bio-concentrated lead above host tissue levels if they were located within the intestine (Sures and Siddall, 2001). In contrast, conspecifics which had passed through the intestinal wall and were located in the body cavity of the fish host showed little accumulation of lead (Sures and Siddall, 2001). Nevertheless, parasites within fish might sequester toxins from the fish, protecting the fish from their deleterious effects (Sures, 2008). Parasitic infection not only reduced the amounts of heavy metals absorbed by fish but also reduced the amounts accumulated in fish organs (Sures et al., 1994; Sures and Siddall, 1999). Environment pollutants not only affect the physiology of the hosts, but can also alter the interactions between hosts and parasites, and this effect might vary according to the development stage, specific pollutant and parasites involved (Sures, 2008).

3.2.7 Aims of the study

The current study aimed to examine whether water hardness can alter the effects of heavy metal toxins (copper, zinc and cadmium) on the survival, infectivity, parasite and host development of *Schistocephalus solidus* parasites and their copepod hosts, *Cyclops strenuus*. The following questions were addressed: (1) How do differences in heavy metals (copper, zinc and cadmium) with water hardness as a modulating factor affect the free-living stage of *S. solidus*? (2) How do differences in heavy metals with water hardness as a modulating factor affect the susceptibility to parasitic infection of hosts kept in different heavy metals? (3) How do differences in heavy metal with water hardness as a modulating factor affect the growth of parasites?

3.3   Materials and Methods
3.3.1   Preparation of hard and soft water for heavy metal exposure experiments

Hard water was prepared by dissolving 0.304 g of anhydrous Calcium chloride ($CaCl_2$) and 0.139 g of Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_20$) in deionised water type II provided by an Elgastat Option 2 water purifying system itself fed with chemically deionised and filtered water and making up to 1 L (WHO, 1989). This provided water with a hardness of 342 mg/L calculated as Calcium carbonate. Meanwhile, soft water was prepared by diluting one part (100 ml) of the WHO standard hard water, with nine parts (900 ml) of deionised water to give 34.2 mg/L hardness (WHO, 1989).

Stock solutions of heavy metals (zinc, copper and cadmium) were prepared by dissolving 40 mg of either copper sulphate ($CuSO_4 \cdot 5H_2O$), zinc chloride ($ZnCl_2$) or cadmium chloride ($CdCl_2 \cdot 2\frac{1}{2}H_2O$) (Sigma) in deionised water (200 ml) to give a heavy metal concentration of 200 mg/L. The test solution of 10 μg/L was obtained by diluting stock solution in 200 ml soft or hard water (ZINC.M.S.O., 1969). All stock solutions were prepared fresh daily.

3.3.2 Free-living stage experiment

Schistocephalus solidus plerocercoids were recovered from three-spined sticklebacks (*Gasterosteus aculeatus*) from the Llyn Frongoch population that had been bred and experimentally infected in the laboratory. Two worms were used as parents in paired culture and were matured in an *in vitro* culture system (Smyth, 1946) (see Chapter 2.3.1 for detailed parasite culture protocol). On day 7, the eggs of the plerocercoids were collected and kept in sealed Petri dishes in the dark in an incubator at 20°C for 3 weeks to embryonate.

After 3 weeks, eggs were removed from the incubator and exposed to natural daylight to stimulate hatching (Figure 3.1). For coracidia survival experiments, the wells of 96-well flat bottom microtitre plates were each filled with 100 μL of either soft, hard or test solution (copper, zinc and cadmium) at 10 μg/L. A single, newly-hatched coracidium was taken from the pool of hatching eggs using a glass Pasteur pipette, and added to one of the wells. Two replicates of each treatment were conducted, with ten individual coracidia being used in each treatment (control – soft and hard water, copper – soft and hard water, cadmium – soft and hard water, and zinc – soft and hard water). The 96-well microtitre plate was covered with a lid and kept in a 12:12 zinc light:dark incubator at 15°C until examination for survival. Each coracidium was examined at 1h intervals under a

dissecting microscope (Leica S6E, USA) and the survival of coracidia was recorded. Death was confirmed when no swimming movement was visible during 2 min observation (repeated 3 times), and there was a change from transparent to opaque white colour.
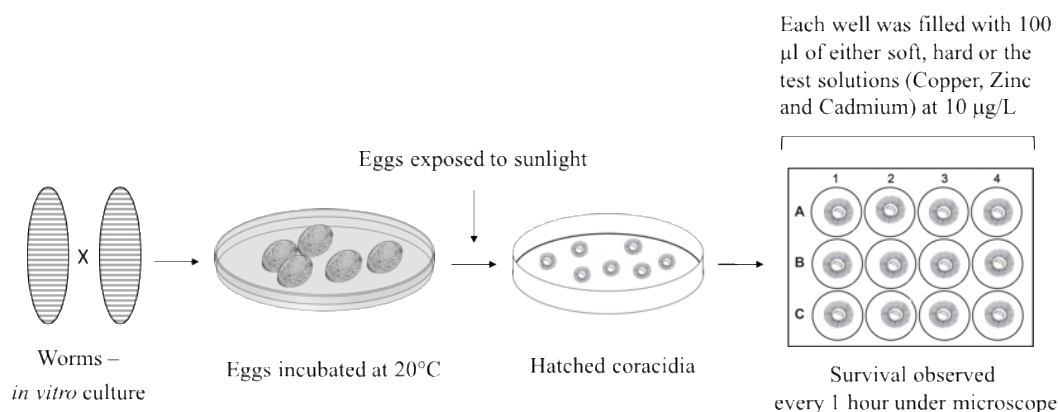
Figure 3.1 Experimental design schematic illustrating the procedure used to assess survival of *Schistocephalus solidus* coracidia exposed to either soft, hard or the test solutions (copper, zinc or cadmium) at 10 μg/L. Eggs and coracidia drawing are not to scale.

3.3.3   Copepod infection (Batch infection)

'Batch' infections were used to generate a large number of infected copepods for experiments in which the infection level (i.e. the number of procercoids per copepod host) was not precisely controlled. Batch infections were chosen for this kind of procedure because of the typically high rates of infection that could be achieved, compared to more controlled, individually generated single infections. Copepodites were acclimatized either in copper, zinc or cadmium solutions (10μg/L) made up in hard (342 mg/L) or soft water (34.2 mg/L) in 500 ml conical flasks. The acclimated copepodites were left unfed for 7d prior to parasite exposure to promote ingestion of coracidia and thus maximise the rate of infection. At this point, cadmium solutions were removed from the experiment because all of the copepods that been acclimatized in this solution had died by day 3.

A day before the remaining acclimatized copepods were exposed to parasites, the copepods were screening for mortality and only live individuals were transferred into a new conical flask containing 300 ml of heavy metal solution (copper or zinc) in either hard or soft water. A total of 240 copepodites were used in the experiment, divided into

12 conical flasks (20 copepods per conical flask, with 2 replicates of each heavy metal solution).

The next day, the eggs were observed under a dissecting microscope to confirm that the coracidia had hatched. Twenty newly hatched coracidia were transferred (Figure 3.2) into each of the 300 ml conical flasks (12 flasks) that contained acclimatized copepodites and were kept in the 15°C temperature incubator (LMS, UK). The copepodites were left unfed for three days before starting to feed them again with protozoan culture. The infection status of copepodites in the batch infection culture was checked after a week (see Section 2.3.4.3 for details of the screening method), and all infected copepods were then transferred individually into single wells of 24-well plates (Costar®), each containing one combination of metal solution (copper and zinc) and water hardness (hard and soft water), covered with a lid and kept for 3 weeks in an incubator at 15°C. Copepods and procercoids were photographed weekly for over 3 weeks to follow the progression and development of both hosts and parasites throughout the screening process. The body size of procercoids was measured in mm^2 as the sectional area of a photograph taken at 100x magnification. Body size of copepods was measured as the length of the cephalothorax.
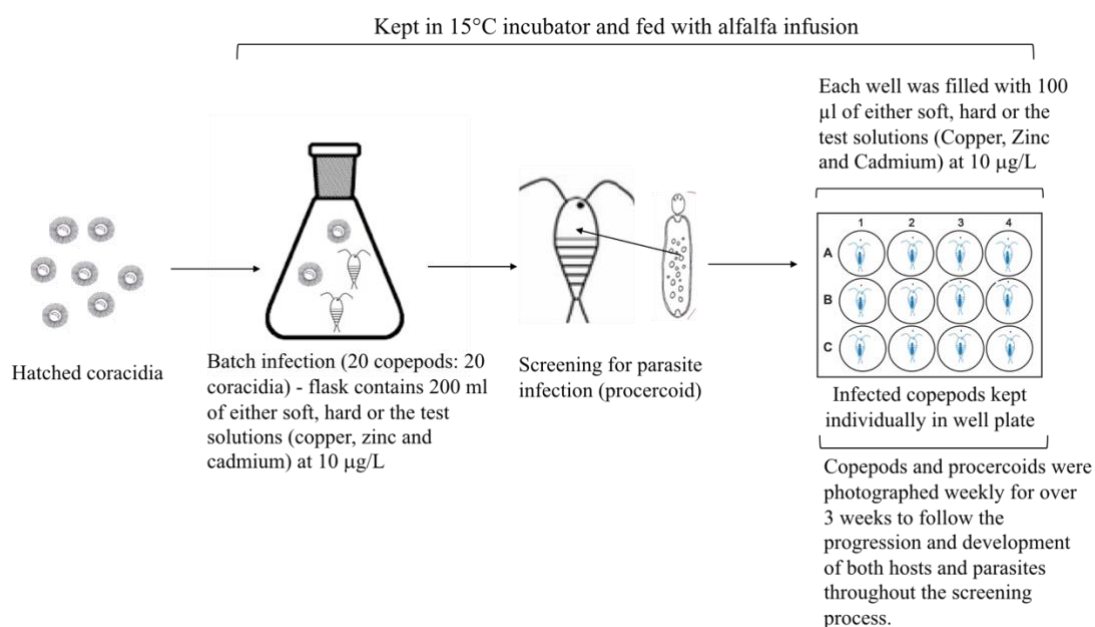
Figure 3.2 Experimental design schematic illustrating the batched infection procedure of *Schistocephalus solidus* exposed to copepods, *Cyclops strenuus*.

3.3.4 Statistical analysis

For the free-living stage survival experiment Linear Mixed Models (LMM) were used to determine the significance of differences in mean survival time (MST) among heavy metal treatments, among water hardness and in the potential interaction between these two factors. Mean survival time (zinc) was used as the dependent variable, while heavy metal and water hardness were used as fixed categorical factors. Non-parametric Kaplan-Meier

analysis with log-rank (Mantel-Cox) tests were used to detect overall survival distributions between the groups. Any significant differences resulting from the tests were then followed by a Kaplan-Meier analysis with Wilcoxon (Gehan) tests (pairwise comparisons) to compare survival distributions among groups, with the test statistic based on differences in group means. These pairwise comparisons show which groups are significantly different in survival curves. Statistical significance was set at a value of $P < 0.05$.

To examine the infective status of procercoids in copepods, defined as the presence of a cercomer, a chi-squared analysis was used. A Fisher's exact test was applied due to the low number of infections (less than 5) in some treatment groups. A Factorial ANOVA was used to determine the effects of heavy metals, water hardness and *Schistocephalus solidus* infection status on copepod survival, on procercoid growth in body area, and on copepod cephalothorax length. All statistical analyses were performed using IBM SPSS Statistics v22 (IBM Corp).

3.4 Results

3.4.1 Effects of heavy metal treatment and water hardness on survival of the free-living stage

Survival of *S. solidus* coracidia exposed to control, copper, zinc and cadmium conditions was affected by water hardness ($F_{1,8} = 21.748$, $P = 0.002$), but not by heavy metal treatment ($F_{3,8} = 0.217$, $P = 0.653$), and there was no significant interaction ($F_{1,8} = 0.021$, $P = 0.888$) between heavy metal type and water hardness. Coracidia had higher survival levels in hard water than in soft water in both control (i.e. with no heavy pollutant) and also in all three heavy metal treatments (Figure 3.3).

The survival distributions of *S. solidus* coracidia in uncontaminated hard and soft water (control groups) were significantly different (Log Rank Test: $\chi^2_2 = 21.159$, $P < 0.0005$) with a higher mean survival time (23 h) in hard water compared to soft water (8 h) (Figure 3.3a). Coracidia exposed to heavy metals in soft water had shorter life-spans (mean survival time 5-6 h) than those in corresponding hard water treatments (mean survival time 22-30 h).

In copper (Figure 3.3b), the survival distributions of coracidia were significantly different between hard and soft water (Log Rank Test: $\chi^2_2 = 21.16$, $P < 0.0005$), with coracidia exposed to hard water surviving significantly longer (mean survival time 25 h) than those in soft water (mean survival time 5 h). In zinc, the survival distributions were also different between hard (mean survival time 22 h) and soft (mean survival time 5 h) water (Log Rank Test: $\chi^2_2 = 26.55$, $P < 0.0005$) (Figure 3.3c). In cadmium, also (Figure 3.3d), coracidia survival distributions differed between hard (mean survival time 30 h) and soft (mean survival time 6 h) water (Log Rank Test: $\chi^2_2 = 33.21$, $P < 0.0005$).
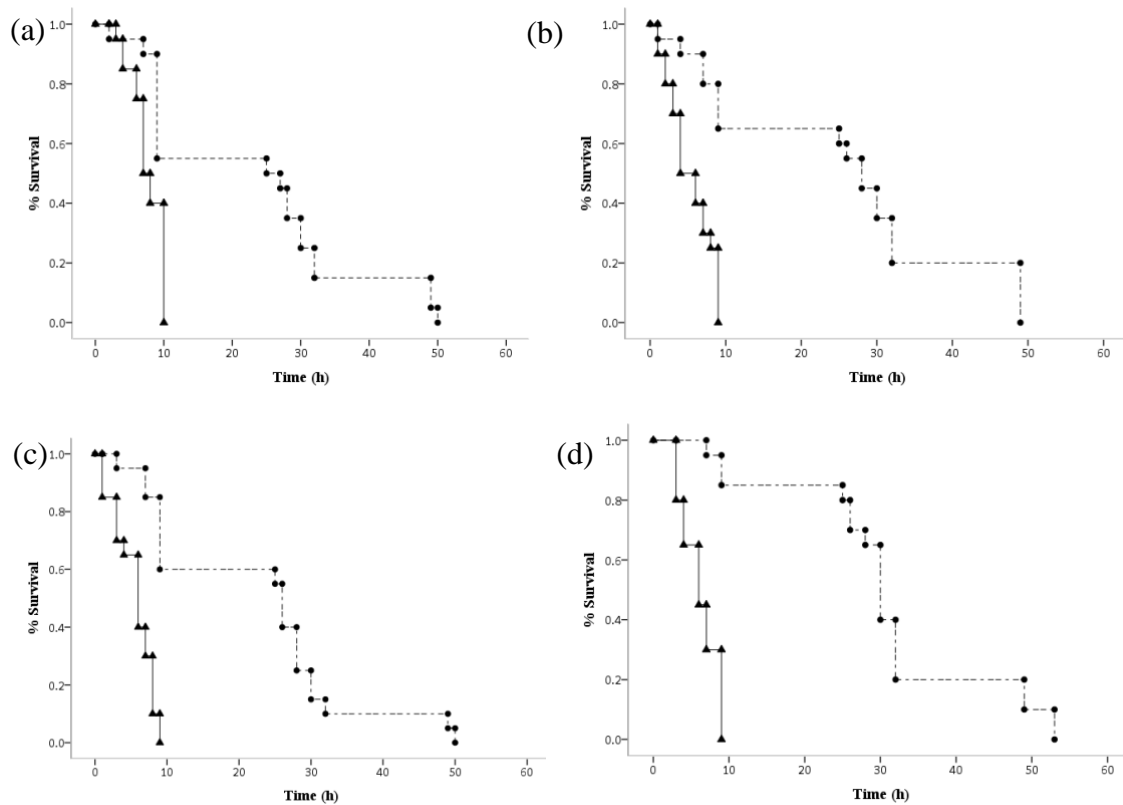
Figure 3.3 Survival of *Schistocephalus solidus* coracidia in heavy metals at concentration of 10 µg/l kept at 15°C. (a) control (n = 40) (b) copper (n = 40) (c) zinc (n = 40) (d) cadmium (n = 40). Hard water (dashed line); soft water (solid line). Kaplan-Meier statistic.

3.4.2 Effects of heavy metal treatment, water hardness and infection status on copepod survival

Survival of copepods was significantly affected by all predictor variables. There were also interactions between: (a) treatment group (control, copper, and zinc) and water hardness, (b) infection status and treatment group; but not (c) the interaction between water hardness and infection status. There was a statistically significant three-way interaction between water hardness, treatment group and infection status (Table 3.1, Figure 3.4).

A multifactorial ANOVA was then used to test the effects of water hardness and infection status on copepod survival in each heavy metal treatment separately. In control conditions (no heavy metal) there was a significant effect of infection on copepod survival ($F_{1,35}$ = 20.879, $P < 0.0005$), with non-infected copepods surviving longer (mean survival time 6 weeks) than infected copepods (mean survival time 4 weeks). There was a weaker significant effect of water hardness on survival of copepods ($F_{1,35}$ = 4.958, P =0.032) and there was an interaction between infection status and water hardness ($F_{1,35}$ = 11.266, P

=0.002; Figure 3.4).

Table 3.1 Factorial ANOVA of copepod (*Cyclops strenuus*) survival, testing the effect of zinc Concentrations, and infection status. Bold indicates significance at $\alpha < 0.05$.

Variable	D.F	f	p
(Intercept)	1	1601.477	**.000**
Hardness	1	26.577	**.000**
Infection status	1	23.288	**.000**
Metal	2	27.086	**.000**
Hardness * infection status	1	1.113	.293
Hardness * Metal	2	37.490	**.000**
Infection status * Metal	2	3.378	**.037**
Hardness * infection status * Metal	2	11.313	**.000**
Error	118		

Copepod survival in copper was significantly affected by water hardness ($F_{1,40} = 89.905$, $P < 0.0005$), with copepods in hard water living longer (mean survival time 5 weeks) than those in soft water (mean survival time 2 weeks). There was no effect of infection status on copepod survival ($F_{1,40} = 0.581$, $P = 0.450$) and there was little or no interaction between infection status and water hardness ($F_{1,40} = 40.049$, $P = 0.051$; Figure 3.4). Copepod survival in zinc was not affected by water hardness ($F_{1,43} = 1.787$, $P = 0.188$) but was affected by infection status ($F_{1,43} = 10.216$, $P = 0.003$) with non-infected copepods living longer (mean survival time 4 weeks) than infected copepods (mean survival time 3 weeks). There was an interaction between water hardness and infection status ($F_{1,43} = 10.216$, $P = 0.003$; Figure 3.4).
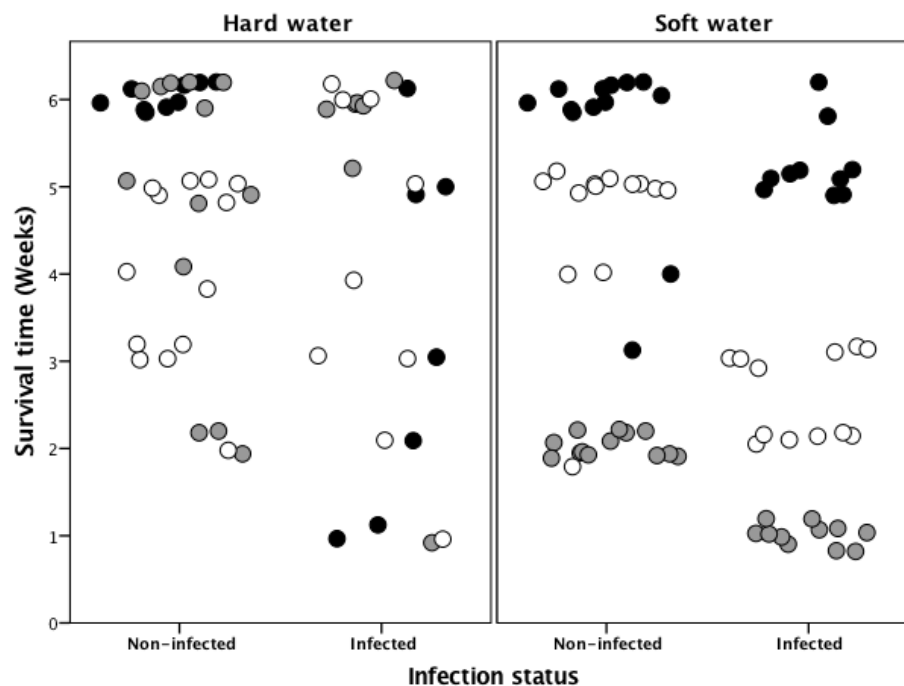
Figure 3.4 Scatter plot showing the effect of *S. solidus* infection status, water hardness and heavy metal treatment group (control (black symbols), copper (grey symbols) and zinc (open symbols)) on the survival of copepods. Factorial ANOVA statistic.

3.4.3   Effects of heavy metal treatment and water hardness on parasite infection levels in copepod hosts

There was no difference in the number of infected copepods in hard water ($\chi^2_2 = 561$, $P = 0.307$) (Figure 3.5) or soft water ($\chi^2_2 = 3.766$, $P = 0.876$) (Figure 3.5) between the treatment groups (control, copper and zinc). The total number of infected copepods (either single or multiple infection) in each treatment were: control (hard water, 7/40; soft water: 12/40); copper (hard water, 7/40; soft water, 11/40); and zinc (hard water, 9/40; soft water: 12/40).
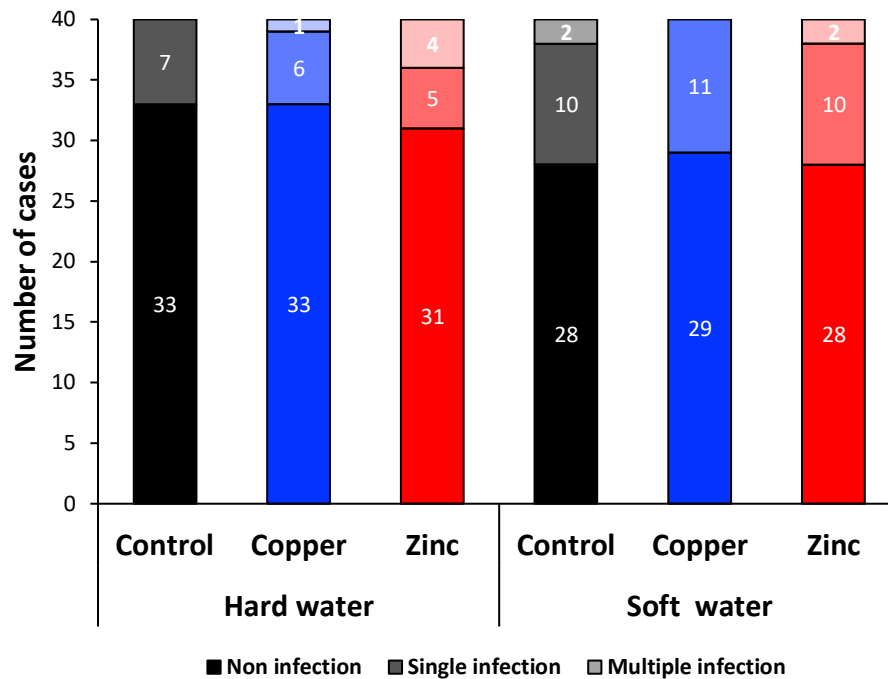
Figure 3.5 Number of copepod exposures that led to infections in hard water and soft water for each different heavy metal (at 10 µg/L). Chi-squared - A Fisher's exact test statistic.

3.4.5 Effects of heavy metal treatment, water hardness and copepod size on procercoid growth in the first intermediate host

Due to the shortened survival of infected copepod hosts exposed to copper and zinc, especially in soft water, the development of the parasitic procercoids could not be followed to 6 weeks. Instead, body area of procercoids was measured in all experimental treatments one week after infection. The analysis was restricted to single-parasite infections. The mean body area of procercoids in singly-infected hosts was significantly affected by water hardness but did not differ between heavy metal treatments and copepod size (Table 3.2, Figure 3.6). There was no significant interaction between water hardness and heavy metal treatment (Table 3.2). One week after infection no procercoids had developed a cercomer, so their infectivity and maturity could not be measured.

Table 3.2 Factorial ANOVA of procercoid size (body area) relative to water hardness and copepod size (cephalothorax length) after one week of infection. Bold indicates significant at α < 0.05.

Variable	D.F.	F	P
(Intercept)	1	18.833	**.000**
Hardness	1	32.447	**.000**
Copepod size	1	0.292	.592
Metal	2	0.936	.400
Hardness * Metal	2	2.827	.071
Error	42		
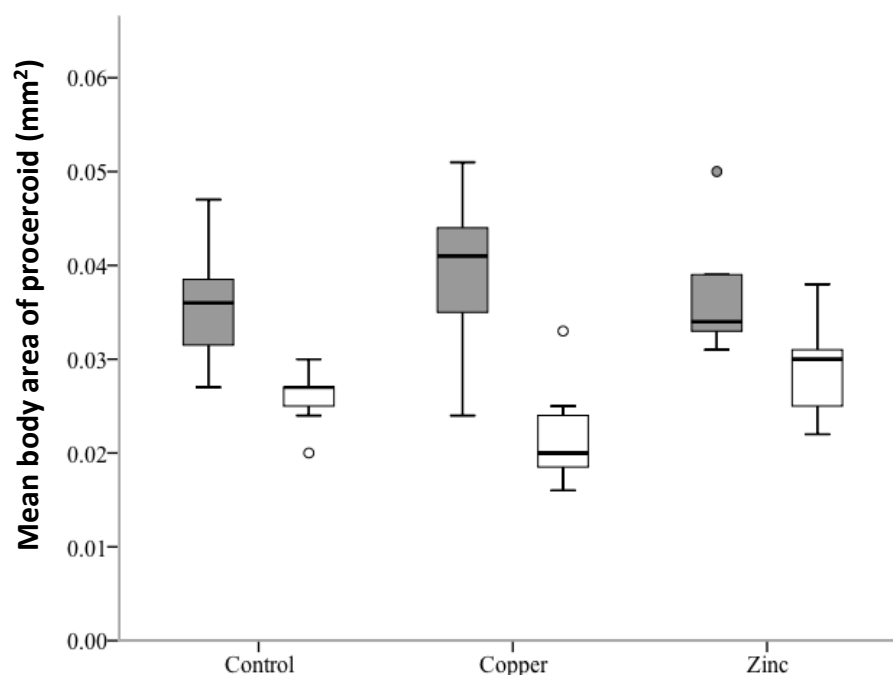
Figure 3.6 Boxplot showing the effect of hard (grey) and soft water (white) on the body area of procercoids that established in singly infected *Cyclops strenuus* experimentally exposed to different water treatments (control; hard water (N= 7), Soft water (N=10), copper; hard water (N= 6), Soft water (N=11), and zinc; hard water (N= 5), Soft water (N=10)) after one week of infection. The dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR) and the whiskers represent the largest and smallest value that are not outliers. Circles represent outliers. Factorial ANOVA statistic.

3.4.6 Effects of heavy metal treatment, water hardness and infection status on the size of copepods

There was no significant effect of water hardness or heavy metal treatment on the size of copepods at the end of week 1 (Table 3.3; Figure 3.7). However, irrespective of the heavy metal treatment, there was a significant effect of infection on copepod size, where infected copepods kept in hard water exhibited larger body size (measured as cephalothorax length) than non-infected copepods (Table 3.3; Figure 3.7). There were no interactions between: (a) water hardness and heavy metal treatment; or (b) infection status and heavy metal treatment; but there was a significant interaction between (c) water hardness and infection status (Table 3.3). There was no statistically significant three-way interaction between water hardness, metal group and infection status (Table 3.3).

Table 3.3 Factorial ANOVA of copepods (*Cyclops strenuus*) size, relative to heavy metals, water hardness and infection status. Bold indicates significance at $\alpha < 0.05$.

Variable	D.F.	F	P
(Intercept)	1	7317.989	**.000**
Hardness	1	0.993	.321
Infection status	1	35.742	**.000**
Metal	2	0.263	.769
Hardness * infection status	1	57.932	**.000**
Hardness * Metal	2	0.541	.583
Infection status * Metal	2	2.263	.109
Hardness * infection status * Metal	2	0.972	.382
Error	110		
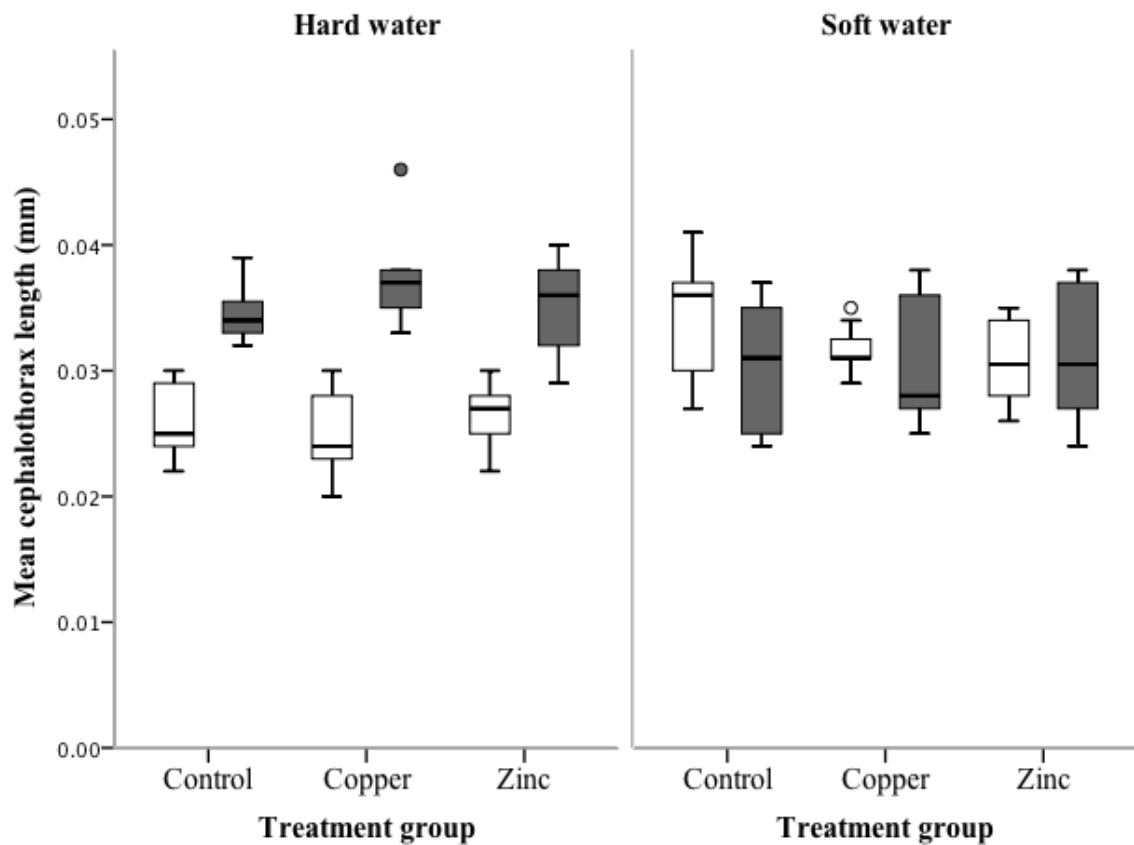
Figure 3.7 Boxplot showing the mean cephalothorax length of infected (grey) and non-infected (white) copepods in each treatment group (control, copper and zinc) in hard and soft water. The dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR) and the whiskers represent 1.5 times the IQR. Circle represents outliers. Factorial ANOVA statistic.

3.5 Discussion

3.5.1 Main findings

This study has demonstrated that water hardness has a profound effect on the survival of *S. solidus* coracidia. Coracidia exposed to copper, zinc and cadmium survived longer in hard water than in soft water (Figure 3.3). The survival of the first intermediate hosts of the parasite (copepods, *C. strenuus*) was influenced by water hardness, the presence of heavy metals and by infection status (Figure 3.4). The copepod exposed to parasite in hard water and soft water in all heavy metal treatments (control, copper and zinc) showed no difference in the number of infections (Figure 3.5). Water hardness also influenced the

size of the parasite developing inside copepod hosts, but not the size of the host (Figure 3.6). Procercoids growing in infected copepod hosts exposed to hard water were larger than those in hosts exposed to soft water, regardless of heavy metal treatment.

3.5.2   Survival of coracidia in hard and soft water

A number of studies of heavy metal toxicity have concentrated on the free-living stages of a number of common or medically important digeneans, such as the cercariae of *Schistosoma mansoni* (Holliman and Esham, 1977, Allah et al., 1996). However, these previous studies have typically neglected the influence of different environmental parameters such as water hardness on the toxic action of metals on the free-living stage. So far, only Evans (1982a, 1982b) studied the effects of different water hardness on copper and zinc toxicity to cercariae of *Echinoparyphium recurvatum* and *Notocotylus attenuatus.* The present study appears to be the first to look at differences among cestodes *S. solidus* coracidia survival when exposed to heavy metals (cadmium, copper and zinc) in water of differing hardness.

Water hardness was an important determinant of the survival of the free-living coracidia stage of *S. solidus* exposed to heavy metal (cadmium, copper and zinc). Specifically, soft water reduced survival by 5-6 h. The mean survival time of *S. solidus* coracidia in hard (342 mg/L $CaCO_3$) and soft water (34.2 mg/L $CaCO_3$) at $15°C$ were 23 and 8 h respectively. The reduction of survival time by soft water also documented by Evans, (1982a) where in 25 mg/l $CaCO_3$, the mean survival time for infective *Echinoparyphium recurvatum* kept at $18°C$ was 28.5 h compared to the survival time at 250 mg/l $CaCO_3$ where the *E. recurvatum* cercariae managed to survived up to 30.5 h.

In the present study, there was no main effects of metals across hard and soft water on *S. solidus* coracidia survival. The survival of coracidia was unaffected by exposure to metal solutions, which might suggest that the concentrations of soluble forms of the metals fell to broadly similar levels in test solutions (Evans, 1982b). In general, metal toxicity can be influenced by water hardness (WHO, 1992) with increasing water hardness causing an increased prevalence of less toxic forms of the metals (Evans, 1982a, Rathore and Khangarot, 2003).

Another factor that might also influence the survival or kill of the *S. solidus* coracidia survival might be the usage of the deionised water as a medium based when the metals solution was prepared. Deionised water is a water that had contain no mineral ions which has been removed during the chemical process and not suitable for the living organism. The effect of deionised water on the living organism such as plant have been document by Uulke et al., (2001), who showed that the leaf of the bouvardia flowers kept in deionized water has wilting on day 6 of vase life compared to the flowers that kept in tap water the leaf did not show any wilting during the experiment. In addition, the deionized water also reduced the fresh weight of chrysanthemum flowers after three days of the experiment (Uulke et al., 2001).

3.5.3 Effects of water hardness, heavy metal treatment and infection on first intermediate host survival

Water hardness, heavy metal treatment and parasite infection all affected copepod survival, and there were interactions between heavy metal treatment and both water hardness and infection. Water hardness was an important modulator of copper effects on the first intermediate hosts, but not of the effects of zinc. Infected and non-infected copepods in control conditions and under the copper treatment performed better in hard water (mean survival time 5-6 weeks) compared to soft water, where they only survived until week 2. As the hardness of freshwater increased, the heavy metal effect decreased, which might be due to competition between the heavy metals and Ca^{2+} and Mg^{2+} ions during the uptake by organisms (Yim et al., 2006). The uptake and accumulation of heavy metal are normally connected with a general physiological response mainly comprising adverse effects which can either be specific, for example induction of certain proteins, or DNA damage; or general such as effects on the general metabolism (Sures, 2008). In the environment, Calcium and Magnesium are present at much higher concentrations than heavy metals. Therefore, Calcium and Magnesium might be competing with heavy metals and blocking their access to aquatic organisms. In addition, Calcium and Magnesium are very important for successful moulting in crustaceans (Fieber and Lutz, 1982). Impaired moulting might lead to mortality and this might explain low survival in soft water in the present study as the water hardness decreased. Moulting frequency was not documented but personal observations suggested that some copepods moulted during the experiment. Therefore, future studies should take this point into consideration to investigate if the

water hardness effects the moulting process and at thus influences the survival rate of copepods.

Meanwhile, the severe effect of copper in soft water in the present study may have been caused by a greater availability and more rapid uptake of toxic forms of this metal by the organism (Evans, 1982a). In general, copper is considered to be more toxic than zinc to living organisms, probably because of the greater stability of the complexes which copper forms with organic molecules (Irving and Williams, 1953). Hagopian-Schlekat et al., (2001) showed that copper was more toxic to the estuarine copepod *Amphiascus tenuiremis* compared to zinc either in a sediment or in a water. Madhupratap et al., (1981) also showed that copper was more toxic to estuarine copepods *Acartia spinicauda* and *Tortanus forcipatus* when compared to zinc. Another study by Wong and Pak (2004), also showed that the survival of young *Mesocyclops pehpeiensis* copepods was reduced to a greater extent by copper than by zinc. The toxic effects of copper were more evident on young stage copepods than adults, perhaps because younger copepods have thinner exoskeletons than adults. A thick exoskeleton diminishes the entrance of metals and increases the resistance of the adult (Vesela and Vijverberg, 2007; Gutierrez et al., 2010). This is because copepods can potentially accumulate metals by taking up dissolved pore-water metals across their thin exoskeletons and soft tissues, especially during moulting, or by ingesting sediment-associated metals (Hagopian-Schlekat et al., 2001). In addition, the detoxification mechanisms are probably less effective in young individuals than in adults (Bryan and Hummerstone, 1971). The metabolic rates of smaller organisms are higher than those of larger ones and as a consequence, the influx rate of metals increases with decreasing body size (Gutierrez et al., 2010). The smaller size of copepods (young copepodites) used in the present study might explain the higher mortality, especially in copper solution at the soft water.

3.5.4   Effects of water hardness on procercoid growth

Water hardness had a significant effect on the size of *S. solidus* procercoids in the copepod host at week 1. Procercoids have a larger body area in hard water than in soft water, and this might be related to lack of Calcium and magnesium in soft water when compared to hard water. In hard water, the access of heavy metals during uptake are blocked by the presence of Calcium and Magnesium in the water and thus decrease the effect of heavy metal on the organism (Yim et al., 2006). For example, copepods are able to accumulate

heavy metals, as has been shown by Khalil et al., (2014) who reported that infected and non-infected copepods, *C. strenuus* accumulate cadmium content of 10 µg/L to 100 µg/L after 24 h exposure. Other studies also have suggested the ability of free-living crustaceans to accumulate cadmium in their body tissues and organs more than fish, for example, *Asellus aquaticus* accumulate more cadmium in their muscle, liver and intestine when compared to fish (chub *Leuciscus cephalus* and perch *Perca fluviatilis*) (Sures and Taraschewski, 1995). In the cladoceran *Daphnia magna*, cadmium body concentrations increased significantly from 13±3 mg Cd/g dry weight in acclimation concentrations of 0.23 nM Cd^{2+} to 236±30 mg Cd/g dry weight of 4.60 nM Cd^{2+} (25-250 mg Cd/l) (Muyssen and Janssen, 2004). Brown and Pascoe (1989) recorded the whole body cadmium concentration in the amphipod *Gammarus pulex* to be higher than in their acanthocephalan parasite *Pomphorhynchus laevis* (0.43-0.74µg Cd g^{-1} dry wt).

Parasites may or may not accumulate heavy metals, but they might alter the uptake and accumulation of chemicals by their hosts through their influence on physiological mechanisms associated with respective pollutant uptake mechanisms (Sures, 2008), which can influence or alter the metabolism and thus affect host feeding rates (Khalil et al., 2014). The significant decline of the feeding rate not only could increase the mortality but also could influence the growth of parasites in which this cestode is known as a parasite that utilize carbohydrate (particularly glucose) and energy substrates from the host. In the present study, the feeding rate of copepods was not measured and thus the effect of feeding rate on the procercoid growth cannot be determined.

3.5.5 Conclusion and future directions

This study shows that water hardness does influence the survival of the free-living stage and the growth of the parasite in the first intermediate host. The results show that soft water reduced the survival and the body area of the free-living stage. In future work it would be valuable to assess the accumulation of heavy metals in various organs and tissues of both infected and non-infected copepods to seek mechanisms underlying mortality of the copepods. It would be particularly valuable to determine how the copepod immune system responds to polluted environments and parasites.

The patterns of accumulation of metals in parasites and their hosts exposed to different water hardness were not measured, therefore, further study is needed to determine the

metal accumulation ability of *S. solidus* compared to their host *C. strenuus* in hard and soft water. This finding could contribute to the monitoring of increasing heavy metal pollution in the aquatic environment. Due to this accumulation capacity and to their abundance in different aquatic ecosystems, cestodes may serve as a useful indicator of heavy metal contamination in the aquatic environment in addition to other invertebrates.

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Chapter 4

Effects of zinc on the *Schistocephalus solidus* life cycle
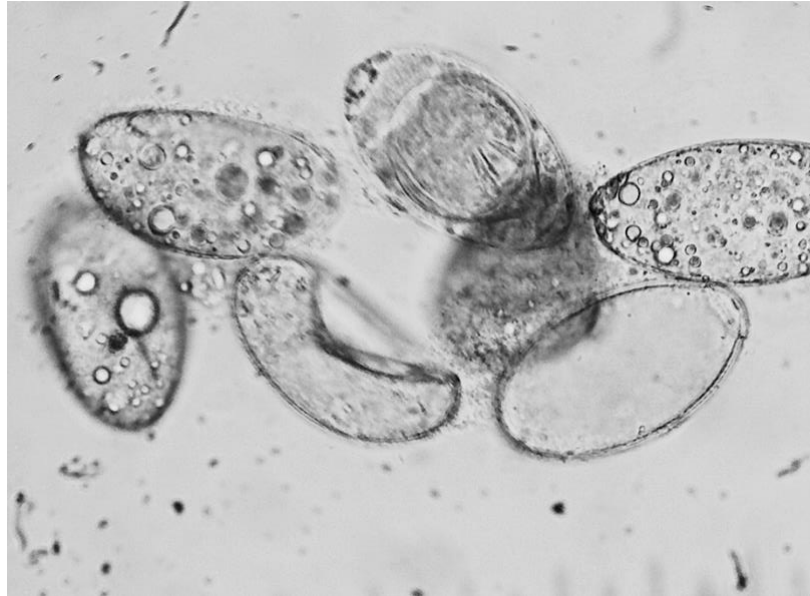
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4.1 Abstract

Water quality plays a major role in influencing the health of aquatic organisms and their interactions with parasites and disease. Pollutants that enter the aquatic environment, such as heavy metals, can potentially alter the relationships between hosts and their parasites and as a consequence influence the completion of parasites life cycles. Here, the influence of the heavy metal pollutant zinc on a range of life cycles stages of the cestode *Schistocephalus solidus* is investigated. *In vitro* culture of the parasite allowed the production of eggs, which were then incubated in a range of zinc concentrations (0.2 μg/L, 2 μg/L, 20 μg/L and 200 μg/L). The effect of zinc on *S. solidus* egg viability, on

the survival of emerging free-living, motile infective stages (coracidia) and on the subsequent development or growth of the parasite in copepod hosts was then quantified. The development and hatching success of *S. solidus* eggs developed normally in elevated zinc concentrations up to 0.2 μg/L but above this egg viability dropped. This high tolerance toward elevated zinc concentrations was also shown by the free-living stages, which exhibited an extended survival time with increasing zinc concentration. zinc concentration had no effect on the infectivity of coracidia to copepods, nor on the growth rate of *S. solidus* parasites in copepod hosts for 5 weeks post-infection. The implications of these findings for host-parasite interactions in polluted waters are discussed.

4.2 Introduction

4.2.1 Impact of pollutants on host-parasite interactions

Many parasites are extremely sensitive to environmental change, others are more resistant than their hosts and tend to increase in numbers in polluted conditions (Mackenzie, 1999). Pollution is expected to alter the interactions between hosts and their parasites in a way that may increase levels of parasitism by increasing host susceptibility or by increasing the abundance of intermediate hosts and vectors (Sures, 2008). For example, chemical pollutants such as endocrine disrupting chemicals (EDCs), which enter aquatic

environments through domestic and industrial effluents, and through agricultural runoff, represent one of the biggest threats to animal health in natural ecosystems (Martin et al., 2010), and particularly threaten aquatic habitats (Sumpter, 2009). Chemical pollutants can be a contributing factor in disease outbreaks and can influence host–parasite interactions directly, for example, through toxic effects on intermediate hosts or parasites, or indirectly, through suppressive effects on host immune systems (Poulin, 1992; King et al., 2010; Martin et al., 2010).

Another example of the potential impact of environmental degradation on host parasite interactions is provided by atrazine pollution. Atrazine is a herbicide that is used to prevent weeds growing in corn and sugar cane crops, and it can run off into wetland, where it has been shown to have negative effects on anuran tadpoles *Rana sylvatica*. Higher concentrations of atrazine can lead to a higher intensity of trematode cercaria and also can diminish the antiparasite behaviour of tadpoles (Koprivnikar et al., 2007). In another study, cercarial mortality was increased in a higher concentration (200 µg/L) of atrazine solution in two trematodes (*Echinostoma trivolvis* and *Haematoloechus sp.*) (Koprivnikar et al., 2006). Rohr et al. (2008) found that pesticide (Atrazine, Glyphosate, Carbaryl and Malathion) exposure increased the susceptibility of tadpoles *Rana clamitans* to *E. trivolvis* infection to a greater degree than it impeded cercarial transmission, resulting in net increases in infection.

4.2.2 Zinc in aquatic environments

Zinc, the twenty-fifth most abundant element, is a bluish-white shiny metal in its pure form, and is naturally found in rocks and soils, and is present in the soil, water, and as well as in foods (Vallee, 1959). Zinc is widely used in industry, for example as a constituent of paint, in the manufacture of rubber and in food preservation (Vallee, 1959), and its widespread use means that it is listed by the Environmental Protection Agency (EPA) as one of 129 priority pollutants (Irwin et al., 1997). From an ecological perspective, taking the example from the environmental quality standard (EQS), in an average of 8-500 µg/L of zinc as trace metal were recorded annually in river district in UK and Ireland (Peters et al., 2012).

In aquatic ecosystems, most zinc enters water bodies either through industrial effluents, domestic waste water or through run-off from contaminated soil (Irwin et al., 1997). In water, most zinc settles on the sediment with a small amount remaining dissolved in the

water or as suspended particles (Irwin et al., 1997). In aquatic ecosystems, the concentration of zinc is influenced by a range of parameters, including sediment type and water hardness (Irwin et al., 1997, Morley et al., 2001a) and can also be related to other abiotic characteristics of water, especially pH which can affect its solubility, polarity, volatility, stability and speciation, thereby affecting its bioavailability as well as its toxicity (Cooney, 1995). In non-polluted waterbodies, zinc concentrations have been recorded as low as 0.1µg/L and in the rivers, the concentration of zinc is higher (20 µg/L). In regions affected by mine drainage, the zinc concentrations in streams (water column) can be as high as 100 µg/L or above (Irwin et al., 1997).

4.2.3 Zinc uptake by aquatic animals

Zinc is an essential nutrient for life and is important for normal physiological functioning, growth, reproduction and development in animal life (Bury et al., 2003). Zinc is also often used as a dietary additive, for example, it is added to algal cultures for copepod diets (Bielmyer et al., 2006). Increases in zinc intake can provide protection against cadmium exposure (Irwin et al., 1997). If zinc concentration in an animal's diet is too low, it can cause reproductive problems and lower resistance to disease, but at levels exceeding the limits required by the body, zinc may harm health (Irwin et al., 1997). In freshwater fish, the uptake of zinc and other metals can be regulated over a wide range of ambient concentrations and is usually taken up through the gills or intestine (Sures, 2008). In invertebrates, zinc and other metals are usually bound to specific metal-binding proteins such as metallothioneins or in the case of crustaceans, incorporated into crystals by isomorphic substitution in the carapace (Sures, 2008). These metallothioneins are believed to play an important role in the regulation of tissue concentrations of essential metals, including zinc and copper, and are involved in the detoxification of non-essential metal, such as cadmium (Sures, 2008).

4.2.4 Implications of zinc toxicity for host-parasite interactions in aquatic environments

In aquatic environments, increases in zinc concentration are markedly toxic to aquatic organisms such as algae, crustaceans and fish (Irwin et al., 1997). Metal toxicity occurs when the total rate of uptake of metal ions exceeds the combined rates of detoxification and excretion, causing the metal to accumulate in its metabolically-available form at a concentration that exceeds a toxicity threshold, with ensuing toxic effects (Rainbow and

Luoma, 2011). Although zinc is essential for the physiological functioning of animals, several studies have documented reduced growth, development or survival associated with zinc toxicity, especially in copepods (Wong and Pak, 2004, Bielmyer et al., 2006).

In aquatic environments, organisms are not only faced with environmental pollution, but they are also confronted with parasites (Sures, 2008), with a high percentage of organisms in natural systems harbouring parasitic infections (Guth et al., 1977). Many parasites have complex life cycles that include motile, free-living infective stages and developmental life stages that infect a number of different intermediate hosts successively, with transmission between hosts being necessary to complete the life cycle. These life cycles typically begin with eggs that are released with the faeces of definitive hosts (often predatory fish or birds) into the external environment. These eggs hatch, releasing a free-living, often motile infective stage that locates, penetrates and develops inside a first intermediate host (typically an invertebrate), before being tropically transmitted to subsequent intermediate hosts following a period of development (Dubinina, 1980). Therefore, any environmental factor or pollutant impacting aquatic environments may influence the success of parasite transmission, progression and / or the development processes in intermediate or definitive hosts (Poulin, 1992, Poulin, 2006).

As well as directly affecting the parasites themselves, pollutants also potentially influence the capacity of hosts to resist infection, or to constrain the growth and development of invading parasites, through their physiological impact on hosts, further altering host-parasite interactions (Sures, 2008). For example, studies examining the effect of zinc on the life cycle of the digenean parasites *Notocotylus attenuates*, have shown that the effect of zinc on cercariae and metacercarial cysts of the parasite is low, with the infectivity of parasites being unaffected even at high zinc concentrations (Evans, 1982b). In other studies, the effect of zinc on cercariae was more prominent and reduced the life-span (Evans, 1982a, Morley et al., 2001a) but was influenced by other environmental variables, including water hardness and temperature. However, the effects of metals, particularly zinc, on the life cycle of other aquatic parasites with complex life cycles, such as cestodes, is not well documented. There is, therefore, a need for more information on the effects of metal pollutants on the life cycle stages of at each stage because larval and adults might be affected and respond differently.

4.2.5 Aims of the study

The current study aimed to examine whether differences in zinc concentration can

influence the survival, infectivity, parasite and host development of *Schistocephalus solidus* parasites and their copepod hosts, *Cyclops strenuus*. The following questions were addressed: (1) Do elevated concentrations of zinc effect the normal development of *S. solidus* eggs? (2) How is the life-span of the free-living, infective stage of *S. solidus* affected under elevated zinc concentrations? (3) How does the exposure of copepod hosts to zinc affect the growth and development of the procercoid stage of *S. solidus* parasites?

4.3 Materials and Methods

4.3.1 Solution preparation

A 200 mg/L stock solution of zinc was prepared by dissolving 40 mg zinc chloride ($ZnCl_2$) (Sigma chemicals, UK) into 200 ml deionised water type II provided by an Elgastat Option 2 water purifying system itself fed with chemically deionised and filtered water. A dilution series of 0.2 μg/L, 2 μg/L, 20 μg/L and 200 μg/L was generated by diluting the stock solution with deionised water (ZINC.M.S.O., 1969).

4.3.2 Parasite culture

Schistocephalus solidus plerocercoids were dissected from three-spined sticklebacks *Gasterosteus aculeatus* that had been bred and experimentally-infected in the laboratory. Host sticklebacks were the offspring of parents collected from Llyn Frongoch, mid-Wales (52°21'39''N, 3°52'46''W). The parents of the *S. solidus* parasite were collected from the infected fish from the River Soar in Leicestershire (52°37'43.5''N 1°08'33.5''ZINC). Three pairs of *S. solidus* plerocercoids were cultured using *in vitro* techniques, previously described in Chapter 2 (see section 2.1 for detailed parasite culture protocol) to generate three different parasite families. After 7 days, eggs were collected by transferring the content of the dialysis membrane tube into a 9 cm diameter Petri dish and examined visually under a binocular dissection microscope (Leica CME, USA). Any remnants of the adult parasite's outer tegument were removed. Eggs were then either used immediately in the egg viability experiment or were incubated at 20°C for use in later experiments.

4.3.3 Egg viability experiment

4.3.3.1 Experimental conditions

A pipette was used to transfer 13 ml of the zinc solution at a concentration of, 0.2 μg/L, 2 μg/L, 20 μg/L, 200 μg/L or deionised water into 5 cm diameter Petri dishes, with two replicates of each. Clean *S. solidus* eggs generated from the *in vitro* culture 7d before the experiment were transferred and distributed equally (approximately 100 eggs) between the 10 Petri dishes, using a micropipette by viewed under a dissection microscope (Leica S6E, USA). This procedure was applied to the three-pair worm eggs. Each of the worm pair eggs was incubated at each of the zinc concentrations and in double deionised water, with two replicates of each treatment. The Petri dishes were then sealed with Parafilm™ and covered with aluminium foil to prevent evaporation and kept in a dark incubator at 20°C for three weeks.

4.3.3.2 Egg count images and data analysis

After three weeks, all 30 Petri dishes containing egg cultures were removed from the incubator, exposed to natural light and left overnight. The next day the Petri dishes were agitated before removing approximately 30 eggs which placed on a glass slide with a cover slip. The slides were then observed under a compound microscope at 400x magnification, and a digital photograph was then taken of the field of view. The field of view was then moved and the process repeated until 10 unique images of the eggs in each Petri dish were captured. Images were then analysed in ImageJ (Schneider et al., 2012) using the 'cell counter' plugin (Simmonds and Barber, 2016). For this experiment, eggs were categorised according to their stage of development (hatched, embryonated, non-embryonated or damaged). Hatched eggs are characterised by a transparent appearance, sometimes with the operculum of the egg still visible (Figure 4.1a). Embryonated eggs were identified as containing a spherical hexacanth, which is a fully-developed embryo characterised by six hooks and is yet to emerge (Figure 4.1b). Non-embryonated eggs were uniform in appearance, giving a mottled pattern with no hexacanth visible (Figure 4.1c). Damaged eggs had a 'puckered' appearance, with no hexacanth visible (Figure 4.1d) (Simmonds and Barber, 2016).

4.3.4 Free-living stage survival experiment

In this experiment, each well of a 96-well flat bottom microtitre plate was filled with 100 µl of the either deionised water or zinc solution at concentration of 0.2 µg/L, 2 µg/L, 20 µg/L or 200 µg/L, with two replicates of each for each worm pair. Coracidia of *S. solidus* were hatched from eggs arising from the three worm-pairs, to provide three different families (see Section 2.1 for detailed parasite culture protocol). Hatched coracidia were transferred individually into a well of a 96-well microtitre plate containing either deionised water or a zinc solution. The coracidium in each well was examined at 1h intervals under a dissecting microscope (Leica S6E, USA) and its survival status (alive / dead) was recorded. The survival of 300 coracidia from the three worm-pair families (100 coracidia per family) was studied, with coracidia being divided equally between treatments, with 10 coracidia per worm pair * zinc treatment replicate used.

4.3.5 Parasite development and growth in first intermediate host experiment

In this experiment, three days before the exposure to the coracidium of *S. solidus*, the *Cyclops strenuus* copepodites were sieved from the lab stock culture using a 200µm mesh, to separate them from the adults and nauplii and placed individually in 24 well plate (Costar®) containing 2 ml of either dH$_2$O or zinc solution. A total of 500 copepodites were held without food for three days either in deionised water or zinc solution in order to maximise the chance that they would ingest the coracidia following exposure.
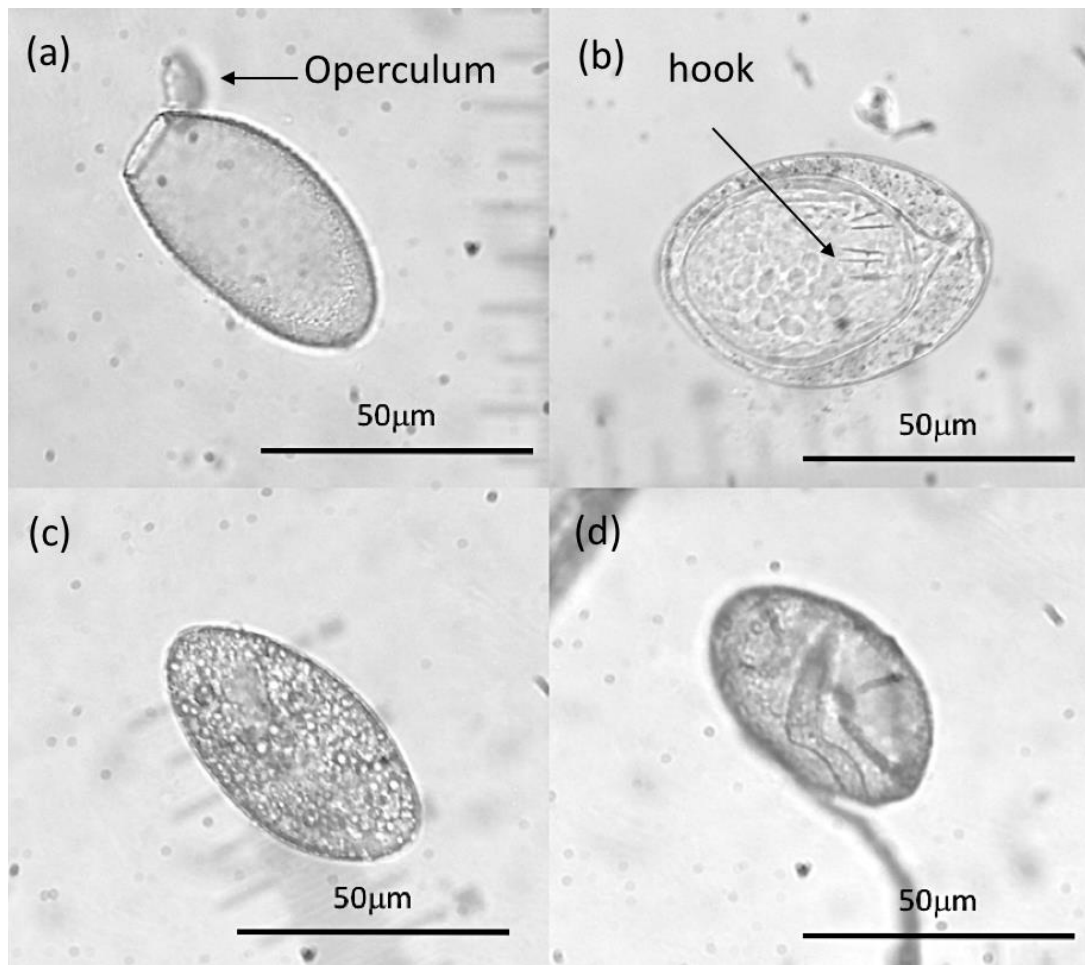
Figure 4.1 Reference plate of microscope images illustrating the categories used to differentiate between hatched, embryonated, non-embryonated and damaged *Schistocephalus solidus* eggs. Microscope image of control eggs showing: (a) hatched egg with a visible opening with the operculum absent where the coracidia emerged; (b) embryonated egg; (c) non-embryonated egg; (d) physical damaged egg.

After three days, a single hatched coracidium from one of the three parasite families generated by *in vitro* culture of *S. solidus* worm pairs was placed in each well of the 24-well plates that contained a copepodite. The plates containing parasite-exposed copepods were then transferred to incubators and held at a controlled temperature of 15°C, with 10 sham-exposed copepods (which experienced identical treatment but were not fed infective coracidia) as controls for each treatment. Exposed and sham-exposed copepods were held for 7d before quantifying infection status. At this point, copepods were left without food for three days before being fed with cultured protozoans (*Colpidium striatum*).

The development of infectivity of the procercoids under different treatments was quantified by screening exposed copepods after 7d and thereafter at 7d intervals for a total of 6 weeks. The development of procercoids inside the copepod hosts was tracked by microscopic examination using a Moticam 3.0MP camera which was attached to the eyepiece of a compound microscope (Leica CME, USA). Photographs were taken and ImageJ 1.49V was used to measure the body size (length and area) of both copepods and procercoids. Details of the copepod screening process was explained in Chapter 2 Section 2.2.4. Copepod size (measured as cephalothorax length) and procercoid body area were measured in mm^2 to determine the growth during the experimental period.

4.3.6 Statistical analysis

A multinomial logistic regression was completed in IBM SPSS Statistics v22 using a generalised linear model with a logit link, to examine the effects of increasing zinc concentration on the viability of *S. solidus* eggs. Eggs were then reclassified as being either potentially 'viable' (hatched or embryonated) or 'non-viable' (including both non-embryonated and damaged eggs). For the free-living stage survival experiment, the mortality of coracidia represented by the survival function (the percentage of surviving individuals) and mean survival time was estimated by non-parametric Kaplan-Meier analysis with log-rank (Mantel-Cox) tests (IBM SPSS Statistics v22). Linear mixed models (LMM, IBM SPSS Statistics v22) were used to determine the effect of zinc treatment and parasite family on survival, and any potential interaction between these two factors. Mean survival time (h) was used as the dependent variable, while treatment and parasite family were used as fixed categorical factors. Statistical significance was set at a value of $P < 0.05$. A repeated-measures ANOVA (with a Bonferroni correction applied to determine the significance of the F ratios, with basic $\alpha = 0.05$) was used to determine the effects of zinc concentration on the growth (body area) of the *Schistocephalus solidus* procercoids. A Chi-square analysis was used to test for association between zinc concentration and the infective status of procercoids in copepods. The specific growth rate of the parasite was analysed by a one-way ANOVA. ANCOVA was used to determine the effect of copepod size on the procercoids body area (into square root transformed). A Factorial ANOVA was used to determine the effects of zinc concentration and *Schistocephalus solidus* infection status on copepod survival. All statistical analyses were performed using IBM SPSS Statistics v22 (IBM Corp).

4.4 Results

4.4.1 The effect of zinc on egg viability

The development of a total of 601 *Schistocephalus solidus* eggs was quantified. All three worm-pairs produced viable eggs. Hatched eggs were present in samples incubated in zinc concentrations up to 200 µg/L, and damaged eggs were observed in zinc concentrations of 2 µg/L and above (Figure 4.2). There was some qualitative variability between eggs from different worm-pairs in their ability to withstand higher zinc concentrations; for example, eggs from worm pair 2 showed a higher proportion of damaged eggs between 2 µg/L and 200 µg/L than those from pairs 1 and 3, while eggs from pair 1 and 3 did not show any damage at 2 µg/L (Figure 4.2). At higher zinc concentrations (200 µg/L), eggs from pair 2 did not show any hatching compared with pair 1 and 3, which routinely hatched at these higher concentrations (Figure 4.2).

zinc is a significant predictor for egg viability (Logistic regression $P < 0.0005$, $F_{1,13} = 48.280$, Figure 4.3). The viability of all eggs declined with increasing zinc concentration. Although the viability of eggs varied slightly between individual parasites, there was no significant effect of individual parasites ($F_{1,26} = 30.183$, $P = 0.260$). Therefore, the variation between parasites is not significant and all parasites showed a decrease in egg viability with increasing zinc concentration.

4.4.2 Effects of zinc on the survival of coracidia

The mean survival time of coracidia was influenced both by the particular worm pair that generated the eggs ($F_{2,540} = 7.124$ $P = 0.001$), and the concentration of zinc to which the coracidia were exposed ($F_{5,540} = 8.554$, $P < 0.0005$). There was no significant interaction between parasite family and zinc concentration ($F_{8,540} = 0.821$ $P = 0.585$) (Figure 4.4). To determine the differences in coracidia survival time between the zinc concentration in each family, Kaplan-Meier log rank tests were used. In worm pair 1, the effect of zinc treatment was a marginally non-significant (Figure 4.4; Log rank test: $\chi^2_2 = 9.400$, $P = 0.052$). In worm pair 2, the survival of coracidia showed a significant difference in mean survival time between zinc concentrations (Figure 4.4; Log rank test: $\chi^2_2 = 12.042$, $P = 0.017$) with the highest mean survival time (18 h) being recorded in the highest zinc

concentration (200 μg/L) and the lowest mean survival time (10 h) observed at 0.2 μg/L zinc.
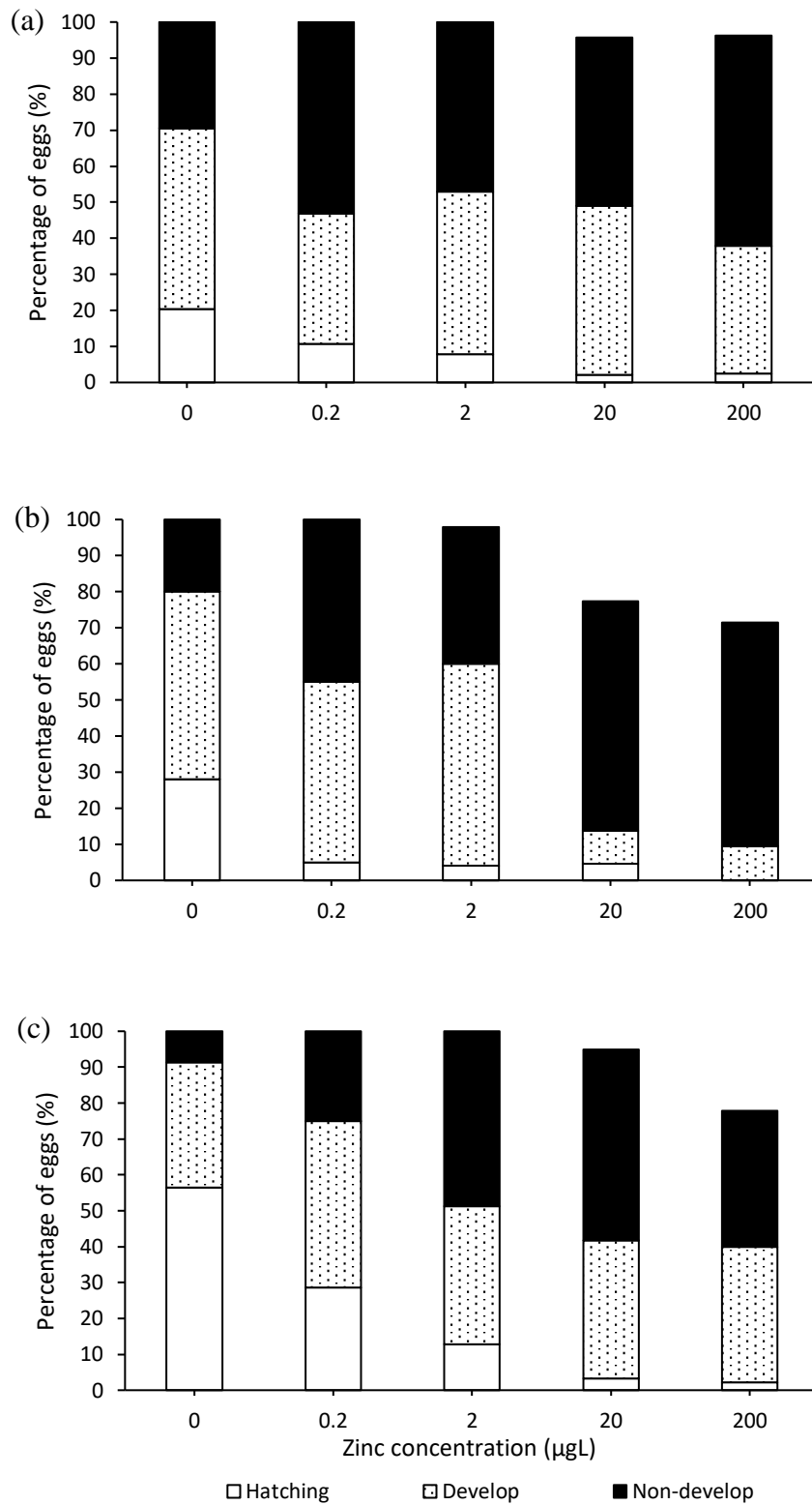
Figure 4.2 The proportion of *Schistocephalus solidus* eggs hatched, embryonated, non-embryonated and physical damaged from zinc concentration conditions in the study

ranging from 0.2 µg/L to 200 µg/L. (a) worm pair one; (b) worm pair two; (c) worm pair three. Data was plotted using excel.
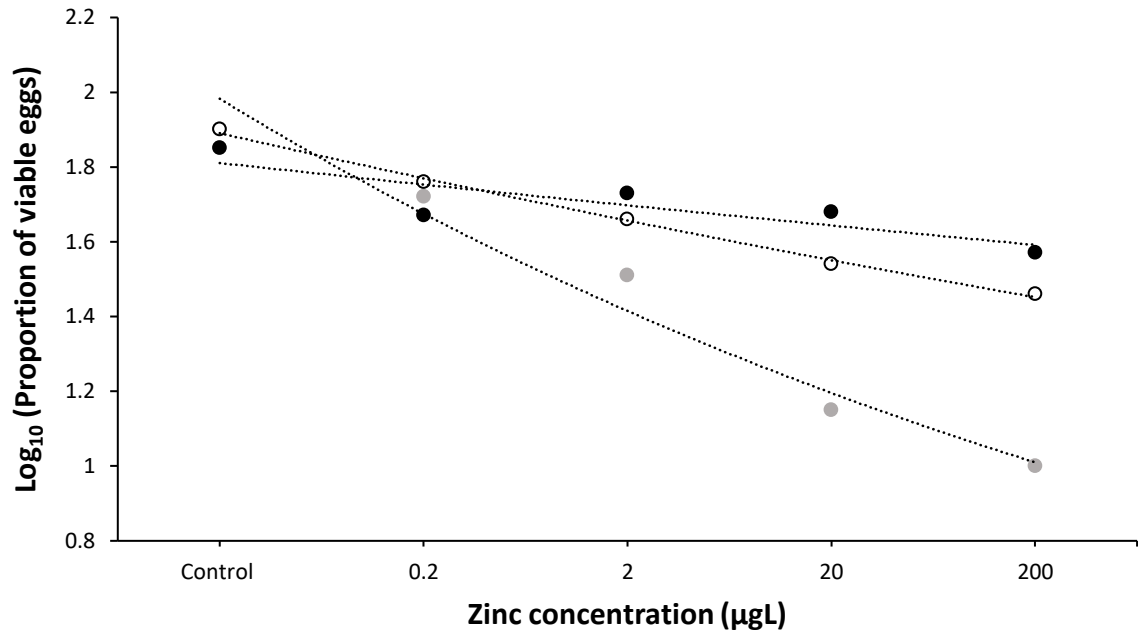
Figure 4.3 The proportion of viable eggs from *Schistocephalus solidus* worm pair 1 (black symbols), worm pair 2 (grey symbols) and worm pair 3 (white symbols) decreases with increasing zinc concentration. Curve is fitted using logarithmic. A multinomial logistic regression statistic.

Pairwise comparisons using the Kaplan-Meier, Wilcoxon Gehan statistic were then undertaken to determine where significant differences occurred. The mean survival time differences among zinc concentrations in worm pair 2 were; (1) 200 µg/L with 0.2 µg/L (Figure 4.4; $\chi^2_2 = 4.94$, $P = 0.026$) (2) 200 µg/L with 2 µg/L (Figure 4.4; $\chi^2_2 = 8.73$, $P = 0.003$) (3) 0.2 µg/L with 20 µg/L (Figure 4.4; $\chi^2_2 = 3.88$, $P = 0.049$). For worm pair 3, the mean survival time of coracidia among the zinc concentrations was significantly different (Figure 4.4; Log rank test: $\chi^2_2 = 13.06$, $P = 0.011$). The differences were between the concentrations; (1) 200 µg/L with 2 µg/L (Figure 4.4; Wilcoxon Gehan: $\chi^2_2 = 5.99$, $P = 0.014$) (2) 200 µg/L with 0.2 µg/L (Figure 4.4; Wilcoxon Gehan: $\chi^2_2 = 10.49$, $P = 0.001$) (3) 200 µg/L with control (deionised water) (Figure 4.4; Wilcoxon Gehan: $\chi^2_2 = 5.20$, $P = 0.023$). The highest mean survival time was recorded at 200 µg/L (14 h) and the lowest mean survival time (8 h) was recorded at 0.2 µg/L of zinc.
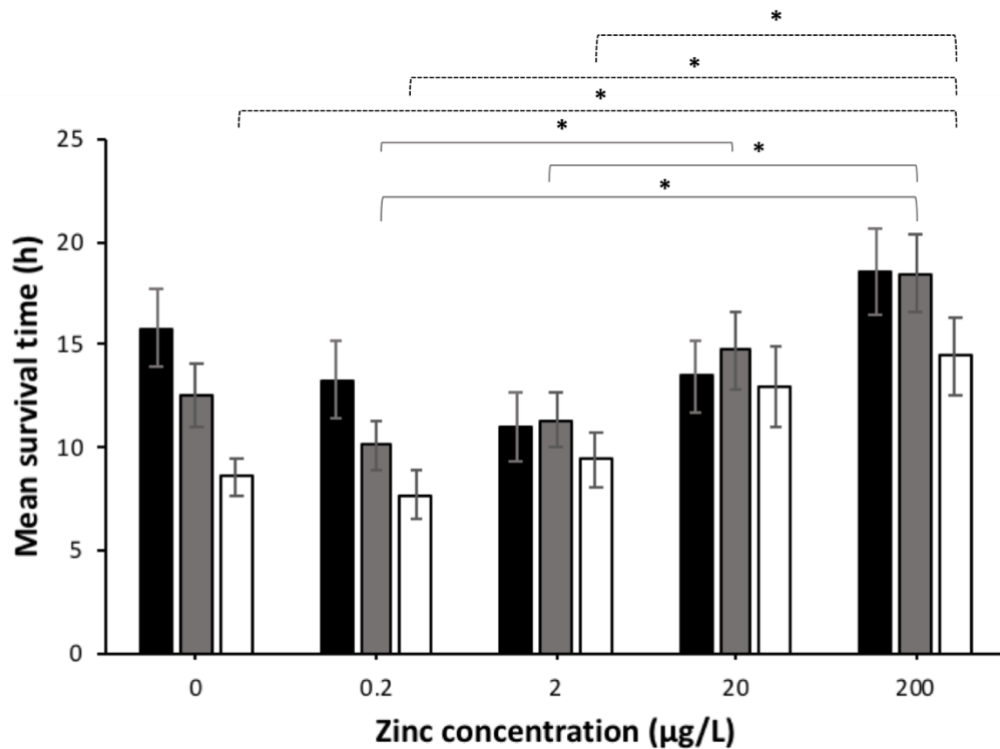
Figure 4.4 Mean survival time of *Schistocephalus solidus* coracidia arising from worm pair 1 (black bars), worm pair 2 (grey bars) and worm pair 3 (white bars) exposed to 0.2 µg/L, 2 µg/L, 20 µg/L and 200 µg/L of zinc. Brackets and asterisks illustrate significant differences between conditions at either end of the bracket. Straight line bracket represents significant difference for worm pair 2 and dotted line bracket represents significant different of worm pair 3. Error bars show standard error. Linear Mixed Model statistic.

4.4.3 Effects of zinc concentrations and infection status on copepod survival

Survival of copepods was significantly affected by zinc concentrations but not by infection status (Table 4.1). Pairwise comparison has revealed that the survival time of infected copepods in control conditions was longer and was statistically different to 2 µg/L ($P = 0.004$), 20 µg/L ($P = 0.013$) and 200 µg/L ($P = 0.008$) zinc (Figure 4.5). The survival time of infected copepods at 0.2 µg/L zinc also was significantly longer than at 2 µg/L ($P = 0.045$). However, the survival time of non-infected copepod at 200 µg/L was significantly shorter than in control ($P = 0.002$), 2 µg/L ($P = 0.004$) and 20 µg/L ($P = 0.002$) zinc (Figure 4.5). There was also an interaction between zinc concentration and infection status (Table 4.1, Figure 4.5).

Table 4.1 Two Way ANOVA of copepod (*Cyclops strenuus*) survival, testing the effect of zinc concentrations and infection status. Bold indicates significance at $\alpha < 0.05$.

Variable	D.F	F	P
(Intercept)	1	746.738	**.000**
Infection status	1	3.443	.068
zinc concentrations	4	4.305	**.004**
zinc concentrations * infection status	4	2.869	**.029**
Error	72		
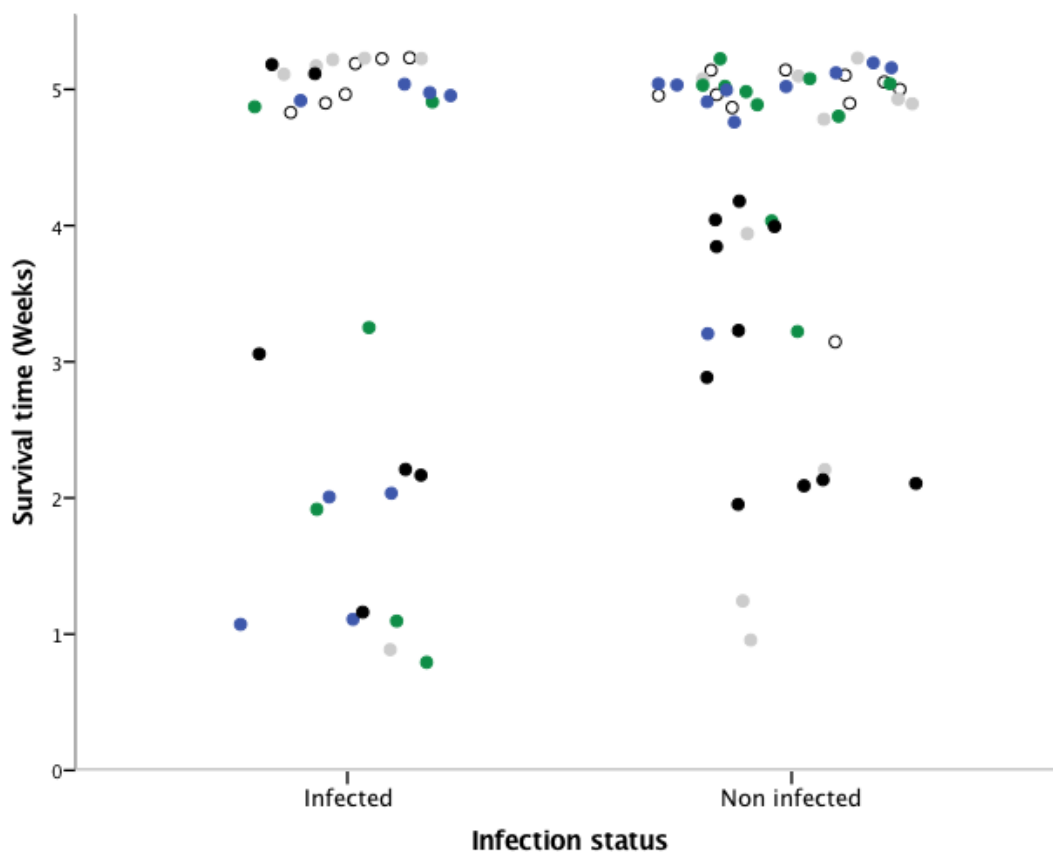
Figure 4.5 Scatter plot showing the effect of *Schistocephalus solidus* infection status and zinc concentration treatment group (control (open symbols), 0.2 µg/L (grey symbols), 2 µg/L (green symbols), 20 µg/L (blue symbols) and 200 µg/L (black symbols)) on the survival of copepods. Two Way ANOVA statistic.

4.4.4 Parasite infection level

Infections became established in copepod hosts under all concentration of zinc (0.2 µg/L: 6/40, 2 µg/L: 6/40, 20 µg/L: 8/40 and 200 µg/L: 6/40) and in deionised water (6/40). Statistically, there was no significant effect of zinc concentration on the proportion of copepods that become infected across concentrations (X^2_2= 0.595, P = 0.964).

4.4.5 Procercoid growth in the first intermediate host

Over 5 weeks of the experiment, the procercoids kept in control and all zinc concentration showed significant increases in mean size (measured as body area) weekly throughout the experimental period (Table 4.2; Figure 4.6). Procercoid size also differed between the control and 20 µg/L zinc (Pairwise comparison; P = 0.026) (Figure 4.6). However, there was no significant difference on weekly size * treatment group interaction (Table 4.2). There is no significant difference in procercoid specific growth rate (SGR) between the control and zinc concentrations) suggesting that SGR was not dependent on the treatment group (Figure 4.7; ANOVA: $F_{4,14}$ = 0.911 P = 0.484).

Table 4.2 Result of repeated-measures ANOVA to determine the effects of zinc concentration on the growth (body area) of the *Schistocephalus solidus* procercoids after 5 weeks of infection. Bold indicates significance at α < 0.05.

Variable	MS	D.F	*F*	*P*	Greenhouse-Geisser	Huynh-Feldt
Weekly size	0.003	4	85.448	**< 0.0005**	**< 0.0005**	**< 0.0005**
Weekly size* Treatment group	0.000065	16	1.808	0.053	0.097	0.055
Treatment group	0.000131	4	4.132	**0.020**		
Error	0.000036	56				

4.4.6 Effect of zinc concentration and infection status on the size of copepods

Due to the non-survival of non-infected copepods in the 200 µg/L zinc concentration, the growth (in cephalothorax length) of both infected and non-infected copepods were analysed only up to week 4. There was a significant effect of zinc concentration and infection status on the size of copepods at the end of week 4 (Table 4.3; Figure 4.8). There

were also interactions between: (a) weekly size and infection status and (b) weekly size and group (Table 4.3; Figure 4.8). There was a statistically significant three-way interaction between weekly size, infection status and treatment (Table 4.3).
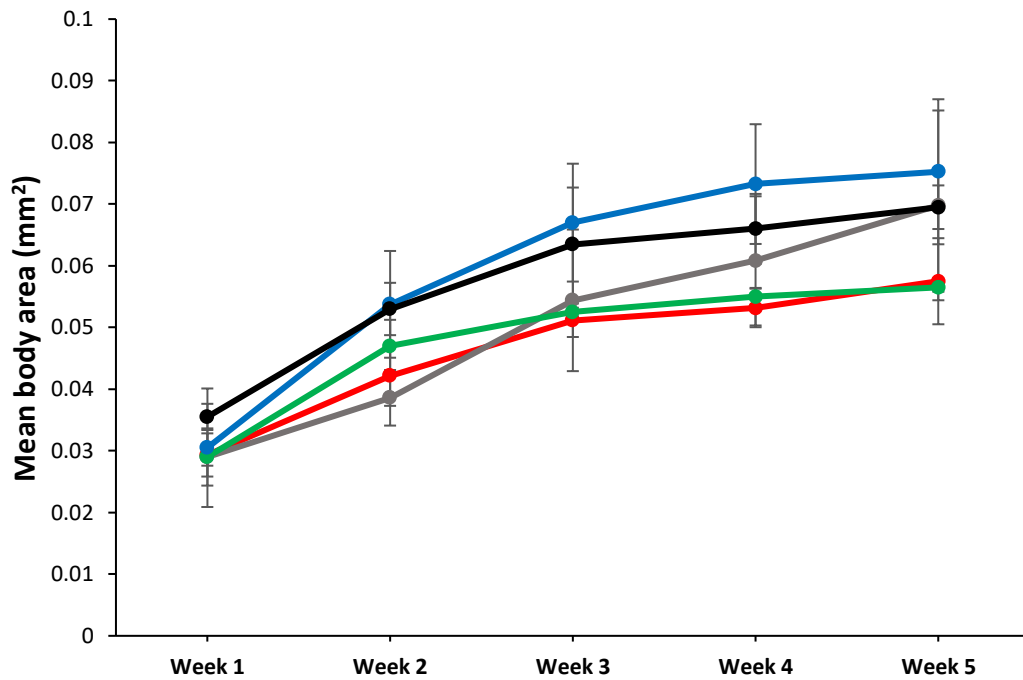
Figure 4.6 Growth trajectories (body area, mm^2) of the *Schistocephalus solidus* procercoids in zinc concentration group (control (red line), 0.2 µg/L (grey line), 2 µg/L (green line), 20 µg/L (blue line) and 200 µg/L (black line) recorded over 5 weeks' post-infection. Error bars represent ± 1 standard deviation. Repeated-measures ANOVA statistic.
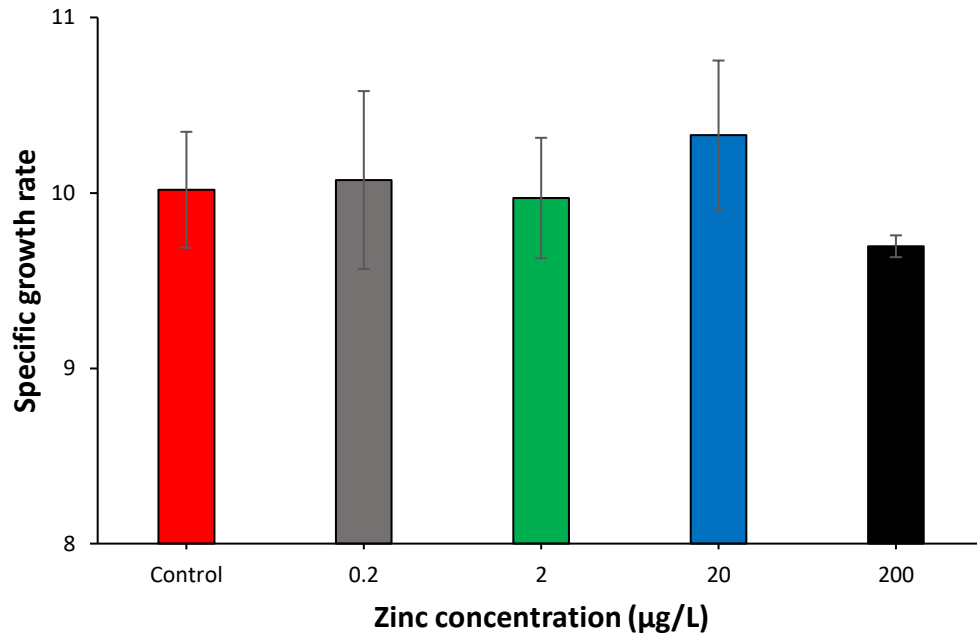
Figure 4.7 Specific growth rate (SGR) of procercoids in control (red line), 0.2 μg/L (grey line), 2 μg/L (green line), 20 μg/L (blue line) and 200 μg/L (black line) achieved over the 5 weeks. Error bars represent ± 1 standard deviation. Factorial ANOVA statistic.

Table 4.3 Result of repeated-measures ANOVA to determine the effects of zinc concentration on the cephalothorax length of *Cyclops strenuus*, copepods. Bold indicates significance at $\alpha < 0.05$.

Variable	D.F	f	p	Greenhouse-Geisser	Huynh-Feldt
Weekly size	3	144.329	**< 0.0005**	**< 0.0005**	**< 0.0005**
Weekly size*infection status	3	2.161	0.096	0.116	0.100
Weekly size*group	12	2.066	**0.023**	**0.041**	**0.027**
Weekly size*infection status*group	12	0.894	0.554	0.532	0.549
Infection status	1	30.793	**<0.0005**		
Group	4	2.617	**0.048**		
Error	132				
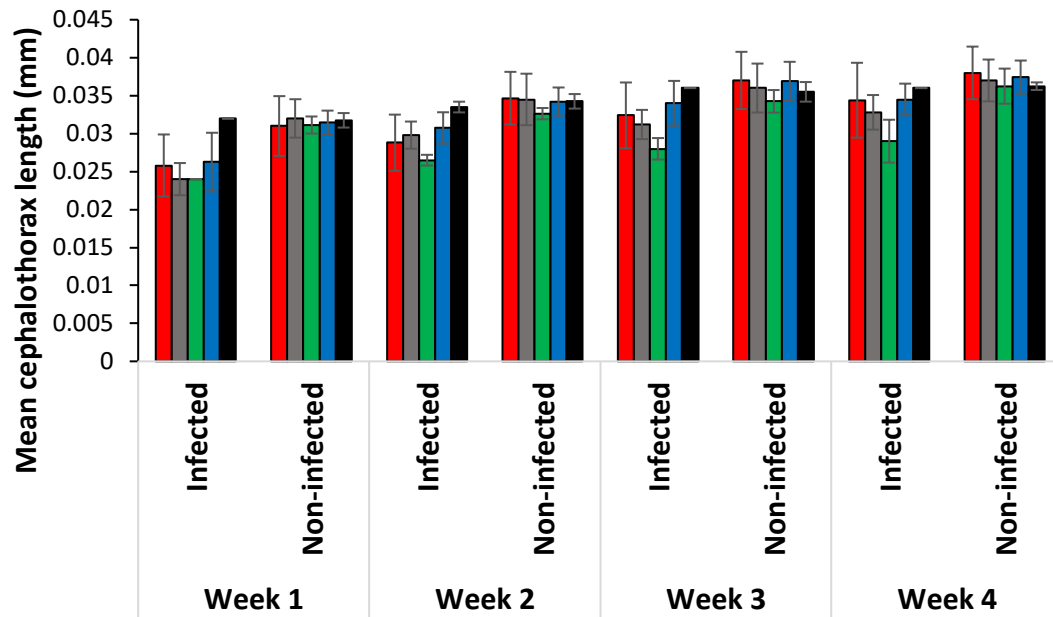
Figure 4.8 Mean cephalothorax length of non-infected and experimentally infected *Cyclops strenuus* copepods exposed to control (red bars) or zinc treatments of different concentration (0.2 μg/L (grey bars), 2 μg/L (green bars), 20 μg/L (blue bars), 200 μg/L (black bars), recorded over 4 weeks post-infection. Bar heights are means, error bars represent ± 1 standard deviation. Repeated-measures ANOVA statistic.

4.4.7 Effect of copepod size and treatment group on procercoid size

Copepod length was significantly correlated with procercoid size (Table 4.4; Figure 4.9). There was also a significant effect of zinc treatment group on procercoid size (Table 4.4). There was a significant interaction between treatment group and copepod size (homogeneity of regression slope) (Table 4.4).

Table 4.4 ANCOVA of procercoid size (body area) relative to treatment group and copepod size (cephalothorax length). Bold indicate significant at $\alpha = 0.05$.

Variable	D.F	*F*	*P*
Copepod size	1	57.263	**0.0005**
Treatment group	4	5.253	**0.001**
Treatment group * Copepod size	4	6.158	**0.0005**
Error	62		
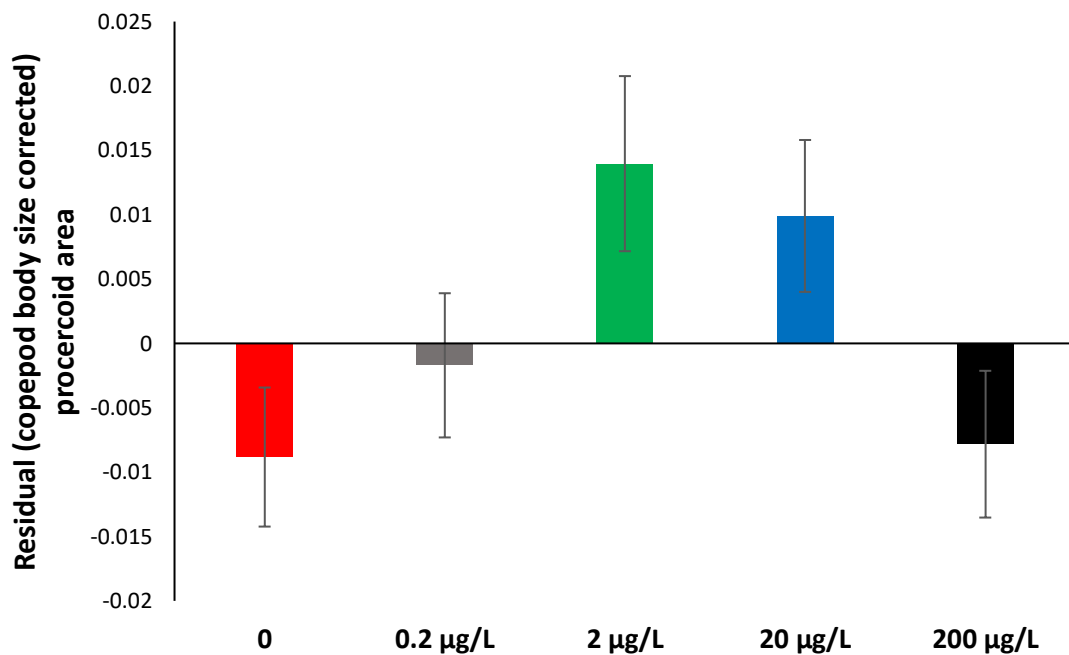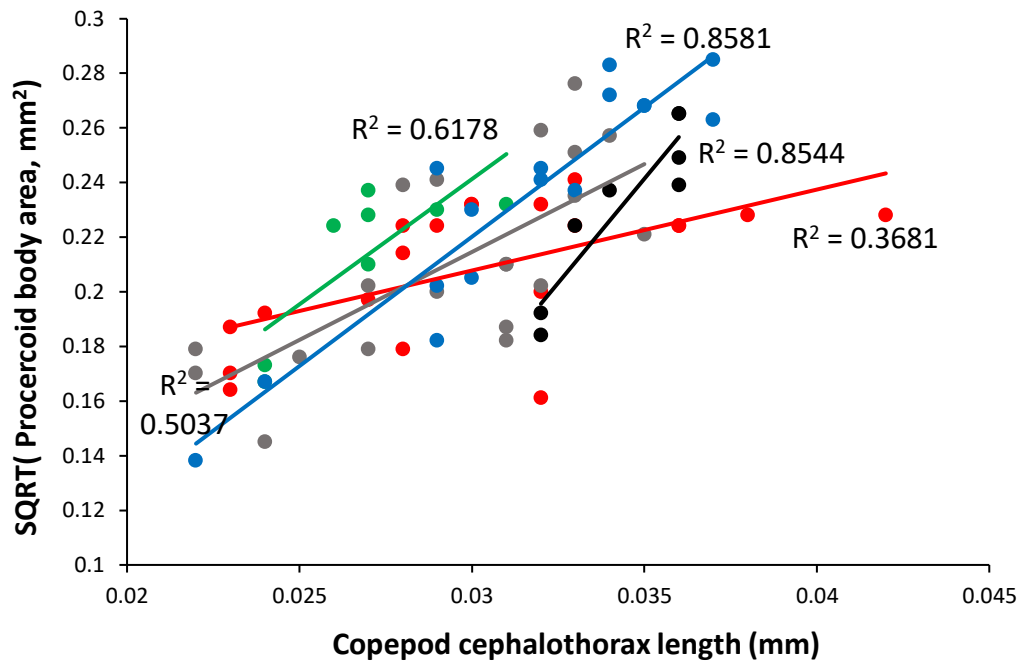
Figure 4.9 Effect of copepod size and zinc treatment on *Schistocephalus solidus* procercoid body area 4 weeks post infection. (a) the relationship between copepod cephalothorax length with the procercoid body area in zinc treatment separately (grey symbols and line, 0.2 µg/L; green symbols and line, 2 µg/L; blue symbols and line, 20 µg/L; black symbols and line, 200 µg/L; red symbols and line, control) (b) residual from the relationship between copepod cephalothorax length with procercoid area in zinc treatment. ANCOVA statistic.

4.5 Discussion

4.5.1 Main findings

In this study, elevated concentrations of zinc have been demonstrated to influence the viability of *Schistocephalus solidus* eggs. All eggs from three parasites used in this study could develop and hatch even at the highest zinc concentration (Figure 4.2), but the viability of the eggs declined as the concentration of zinc increased (Figure 4.3). Different effects of elevated zinc concentration on the free-living, motile coracidia of *S. solidus* were observed. Counterintuitively, it appeared that the survival of *S. solidus* coracidia was prolonged in high zinc concentrations, compared to deionised water (Figure 4.4). The longer survival time of the free-living stage in higher zinc concentrations does not necessarily indicate high rates of infection in the copepod host. The present study showed that parasites can infect copepod hosts in all treatment groups and did not influence the probability of *S. solidus* coracidia infecting copepod hosts. The survival of copepods was significantly and negatively affected by zinc concentrations, but not by *S. solidus* infection (Figure 4.5). The findings of the study also demonstrated the ability of *S. solidus* procercoids to continue to grow in copepod hosts exposed to zinc concentrations up to 200 μg/L (Figure 4.6). Elevated concentrations of zinc did not affect the time that *S. solidus* parasites took to attain infectivity, with all procercoids developing a cercomer by week 2.

4.5.2 Effect of zinc on the development and viability of eggs

The transmission of parasites in degraded aquatic habitats could be affected in several ways. It is important to understand at which conditions makes the transmission become possible. In a recent study by Simmonds and Barber (2016), the viability of *S. solidus* eggs in response to increasing salinity was investigated; however, the impacts of metal pollutants such as zinc on the development of *S. solidus* or other cestode eggs in freshwater is not well documented. This study has demonstrated effects of elevated zinc concentration on the development and hatching of *S. solidus* eggs. These findings may provide an information regarding the limitation on the environmental range on the ability of *S. solidus* to infect their intermediate hosts (both copepods and fish). The zinc concentration tested in this study are ecologically relevant to a water inhabited by aquatic organisms including fish three-spined stickleback which is host to *S. solidus* in the UK (Bervoets et al., 2001; Peters et al., 2012).

In comparison to other parasites species that have been investigated, it is likely that environments polluted by zinc at the concentrations investigated here would not prevent the parasite's eggs from developing and hatching. In this study, even though the *S. solidus* eggs viability was reduced in increasing zinc concentration, it did not inhibit the eggs in terms of their development and hatching rate in zinc concentration up to 200 μg/L. Similar findings are also documented by Morley et al., (2001b) who showed that hatching of *Schistosoma mansoni* eggs occurred at zinc concentrations as high as 10000 μg/L, although the percentage of eggs hatching was reduced under the highest concentration compared to lower concentration of 100 μg/L. In the present study, the percentage of *S. solidus* eggs hatching was low at 200 μg/L (less than 10%) when compared to *S. mansoni* parasites eggs hatching at 100 μg/L and 1000 μg/L (where 60% and 20% hatched respectively). This suggested that different parasites species respond differently toward the same metal pollutant.

This study has shown that whereas *S. solidus* eggs were capable of developing and hatching in zinc-polluted waters, this capacity reduces and eggs become damaged as zinc concentration increases. This suggests that the eggs of *S. solidus* are vulnerable to certain effects of zinc pollution. In common with other diphyllobothriidean cestodes (Smyth, 1954), the eggs of *S. solidus* are surrounded by a thick protective capsule that can prevent the toxicity of chemical pollutants (Smyth, 1954); however, in order hatch the eggs need to take up both oxygen and water. When a large amount of water been taken up, metals contained in the water might affect the probability of eggs hatching. Disruption by zinc of the eggs' enzymatic hatching reaction that is required for the operculum to open might also reduce the hatching success of eggs (Simmonds and Barber, 2016). However, in the present study, the inhibition mechanism was not investigated. Thus, further work is needed to determine the potential mechanism involved in *S. solidus* eggs development in zinc or other metal pollutants.

Another possible limitation of this study is that the plerocercoids used for culture were from the infected fish that had been bred in an aquarium with a good water quality and not from the wild or in an area that has a high level of pollutants. It might be *S. solidus* populations from the polluted area could have better tolerance to high zinc or metal concentration. Therefore, more work is needed to look in detailed and compared the tolerance or ability of *S. solidus* eggs from a range of polluted and non-polluted sites, to test for any local adaptation.

4.5.3 Effect of zinc on the free-living stage life-span

All coracidia generated from three different worm families exhibited their longest survival times (14-18 h) in the highest concentration of zinc studied (200 µg/L). These findings were unexpected and contrast with another study (Evans, 1982a), in which the survival time of *Echinoparyphium recurvatum* cercariae from the trematode parasite in 100 µg/L of zinc was 35 h. Morley et al., (2001b), demonstrated that the survival of *Schistosoma mansoni* miracidia in 100 µg/L of zinc was not different from the survival of miracidia in deionised water and a low zinc concentration of 10 µg/L (11 h). Even though the miracidia and coracidia were not from the same parasite species, miracidia are a motile, free-living stage that hatched from the eggs of trematode parasite. This suggested that even the parasites in equivalent, free-living stage respond differently toward the same metal pollutant (Sures, 2008).

Higher mean survival time of *S. solidus* coracidia at higher zinc concentrations in this study might arise through a number of different mechanisms. First, it may be the result of a reduction in coracidia activity at higher zinc concentrations, which leads to a decrease in the rate of utilization of finite glycogen reserves. Higher depletion of stored carbohydrates and energy reserves present in the form of fat and glycogen droplets located in the ciliate embryonal shell of cestode parasites was observed in coracidia when metabolic activities increased (Dubinina, 1980). As relatively little is known about the factors contributing to the free-living stage of *S. solidus* activity or metabolic mechanisms this hypothesis would require further study.

Secondly, prolonged coracidia survival at high zinc concentrations might arise because of the activation process of the oncosphere. In nature, the activation of the oncosphere within the embryophore has been shown to interact with the surrounding chemical environment through the ciliated epithelial coat (Jakobsen et al., 2012). This activation process helps the oncosphere to escape from their embryophore when the copepod rupture the coracidia shell (embryophore outer surface) (Dubinina, 1980). But in this study, copepods were not introduced to coracidia during the survival time experiment, therefore, the time and energy needed for the escape process and uptake of extra-nutrients from the medium (higher zinc solution) might, however, support the oncosphere with enough nutrients. Compared to other group, the level of zinc may be lower and this could lead to

more reduction of nutrient storage and result in death as zinc also known as one of the important nutrients for growth or development of organisms (Bielmyer et al., 2006, Bury et al., 2003).

4.5.4 Infected and non-infected copepod survival

Parasitism is known to increase the susceptibility of copepod hosts to a variety of toxicants (Guth et al., 1977, Khalil et al., 2014). In contrast to previous studies, the present findings have clearly demonstrated that the presence of *S. solidus* (at any level of zinc concentration) does not modify the toxicity of zinc to the copepod hosts, *Cyclops strenuus*. These findings were unexpected, since previous work suggested that infected *C. strenuus* were more susceptible to heavy metal pollutants than uninfected conspecifics (Khalil et al., 2014). Therefore, it might be expected that infected *C. strenuus* would be more susceptible to zinc. The possibility for this to happen might be due to an alteration of zinc uptake or elimination of zinc from the copepod host. It is known that parasites can alter the uptake and accumulation of chemicals by their hosts through their influence on the physiological mechanisms associated with pollutant uptake (Sures, 2008). The alteration of the chemical uptake and accumulation were observed in many fish-parasite studies, especially in acanthocephalans species (Sures and Siddall, 1999). It appeared that this alteration not only benefits the host, but eventually the parasites mainly need the chemicals for their development (Sures and Siddall, 1999). Reduced pollutant concentrations in organisms due to parasitism has also been observed in non-fish hosts such as clams (Heinonen et al., 1999), gastropods (Evans et al., 2001) and shrimps (Bergey et al., 2002). In the present study, the level of uptake or accumulation of zinc in the body of infected copepods was not measured. Therefore, more work is needed to determine the accumulation of toxin in copepod/parasites species. If this reduction of toxins in infected copepod is demonstrated, then it could help to clarify the health effect of parasites on their host. Therefore, it would be worthwhile studying the interaction between the effects of parasites on the physiological mechanisms and the pollutant uptake mechanisms.

In general, the uptake and accumulation of pollutant by organisms are usually directly connected with biological effects which mainly comprise detoxification and excretion mechanisms (Sures, 2008). It is assumed that the intensity of the biological effects is higher with increased pollutant levels in the body (Sures, 2008). If the general physiological response is too severe, the host will reach the point of critical damage and

die (Sures, 2008). Copepods have a defence mechanism that helps them regulate internal metal concentrations and bioreactivity (Stringer et al., 2012), namely the metallothionein system. In copepods, as in other animals, the production of metallothioneins is induced as a response to metal exposure (Poynton et al., 2007) and metallothionein induction is known to promote survival (Poynton et al., 2007).

4.5.5 Infectivity of parasite in first intermediate host

Most toxicants, including heavy metals, pesticides and sewage effluents, are known to reduce the level of infectivity of parasites, especially in digeneans (Morley et al., 2003). In contrast to previous work, the present study has demonstrated that there is no significant effect of zinc concentration on *S. solidus* infection of copepod hosts. In the present study, elevated zinc concentrations did not influence the infectivity of the parasite, or the number of copepods that became infected by *S. solidus*. A similar finding was documented by (Evans, 1982b), who showed no differences in infectivity of metacercariae of *Notocotylus attenuates* exposed to control, zinc or copper. The present study and (Evans, 1982b) findings contrast with other work, since the previous study suggested that the heavy metal not only stimulated the infectivity of the parasites (Morley et al., 2003) but also could increase the prevalence of parasites within the host population (Dreyfuss et al., 2000). Therefore, it might be expected that increased zinc concentration would increase the infection rate of infective *S. solidus* coracidia in *C. strenuus* copepods.

The mechanism that might relate to the non-significant infection could be the differences in target host species that may have different susceptibility to toxicant-exposed parasites (Morley et al., 2002). Differences in a strain of the host or parasites could also influence the infectivity. Evans, (1982a) and Morley et al., (2002) have showed that, within the same species in the same experimental condition (water medium), the susceptibility of parasites *Echinoparyphium recurvatum* differed in which suggested that it will be an impact on predicting and interpreting the effects of pollution on host/parasites systems (Morley et al., 2003).

4.5.6 Effect of copepod size on the procercoid size

In this study, elevated zinc concentration seems not to have restricted *S. solidus* parasites in terms of their development and increase in body area. In this study, the procercoids' size was related to the size of the host. Similar findings were documented by Wedekind, (1997), who showed the size of the copepods *Macrocyclops albidus* at the time of

infection correlated with the size of the *S. solidus* procercoids that developed after 14 days of infection. One reason that parasite size depends on the host size in crustacean hosts could be due to the fixed size of the rigid exoskeleton that constrains the maximum size that can be attained (Wedekind, 1997).

4.5.7 Conclusions and future directions

This study identifies an effect of elevated zinc concentration on the egg viability, survival and development of *S. solidus* parasites and host copepods *Cyclops strenuus*. The results show that under an elevated concentration of zinc, *S. solidus* egg development and hatching was negatively impacted. With the same range of zinc concentration, free-living infective stages of *S. solidus* were shown to survive even at higher zinc concentration. The prolonged survival time at a higher concentration did not influence the infection probability to *S. solidus*. I also showed that parasites exposed to zinc solution can increase in size (body area). In nature, heavy metal pollutants such as zinc enter freshwater ecosystems through a variety of routes. Aquatic organisms, including both hosts and parasites, are continuously exposed to those environmental stressors and this might impact the evolution of the organism and could also alter the relationship between parasites and host. With the information concerning the potential toxicity of heavy metal on the parasites from the earlier stage such as eggs viability, it can help to predict any disruption of the parasite life cycle.

4.6 References

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Chapter 5

The relationship between larval size and fitness in the tapeworm *Schistocephalus solidus* exposed to zinc

Z. Ismail ©

5.1 Abstract

The transition from the larval stage to the adult stage of species with complex life cycles, such as insects and amphibians, is defined as metamorphosis. For such animals, the environments experienced by larval stages can have profound implications for the performance of the adult form. For example, factors that influence the larval body size at metamorphosis potentially affect adult survival, body size and age at first reproduction, and ultimately their fecundity. Such phenomena are known as 'carryover effects'. For heteroxenous parasites, the transitional size and physiological status of larval stages as they move into their next host is potentially an important factor in determining the growth, fecundity or survival of the next stage. However, the size and age at the transition could also be influenced by environmental conditions, including thermal regimes and the presence of anthropogenic pollutants. This study was designed to first investigate how

exposure to zinc, an important heavy metal pollutant in freshwater ecosystems, impacts the growth of the first larval stage of a cestode parasite (i.e. the procercoid stage) in the copepod host, and then to determine whether these effects alter the performance of the second larval stage (plerocercoid) in the fish host. In this study, sticklebacks were exposed to infective stages of parasites that had established in experimentally-infected copepods that had been developing under two different environmental treatments (deionised water and 20 μg/L zinc solution). Procercoid growth rate correlated with the size of the host copepod, but procercoids also grew faster in copepods kept in 20 μg/L zinc solution, than those growing in copepods kept in deionised water. The size attained by the procercoid in the copepod host also affected plerocercoid growth in the stickleback host, with fish fed larger procercoids developing larger plerocercoids. The plerocercoid size in fish was found to be correlated with the size attained by the procercoid in copepods at the time of infection. This suggests that the environment conditions (in this case, zinc pollution) experienced by the larva can have long-lasting carryover effects on the next life cycle stage, with consequences of parasite fitness.

5.2 Introduction

5.2.1 Metamorphosis and ontogeny in organisms with complex life cycles

Many organisms exhibit complex, multi-stage life cycles characterised by developmental changes. Examples include tadpoles developing into frogs, caterpillars metamorphosing into butterflies, planktonic larvae settling to become sea stars and vegetative to flowering transition of the mustard plant *Arabidopsis thaliana*. Many parasites exhibit life cycles with multiple stages existing within different host species (Benesh, 2016). There are different opinions on the precise definition of a complex life cycle, with some opinions considering that metamorphosis a necessary part of a complex life cycle (Wilbur, 1980, Semlitsch et al., 1988), and others suggesting that ontogenetic niche shifts alone are sufficient to call a cycle complex (Werner, 1988).

In organisms such as insects and amphibians, the transition between larval stages, or from larval to adult stages is critical, and the success of the transition may be influenced by the size, development or physiological condition of the life stage at transmission and can impact the performance in the next stage (Benesh and Hafer, 2012). For example, large size of larval stages at transmission stage is often associated with a higher transmission success, infectivity and fecundity of the next stage (Benesh and Hafer, 2012).

In nature, phenotypic plasticity induced by environmental quality are common and the responses are usually adaptive, in a way that developing the health of the organism (Miner et al., 2005), especially the organisms that have a multi life cycles (Pechenik, 2006). The environment condition that inhabit by the larval stage can have effect on the next life cycle stage (Atkinson, 1994). For examples, the development time of Iberian painted tadpoles (*Discoglossus galganoi*) are longer at low temperatures. To cover up the lost, they accumulate more lipids that can be used by the adults tadpoles (Álvarez and Nicieza, 2002). In damselfly *Lestes viridis*, the mating success usually depend on the size and age of the adults. But, certain condition (nutritional condition and photoperiod treatment) exposed by the final instar larval also can have long lasting effect when transfer to adults stage and influence the mating success rate (Stoks et al., 2006). Such effects are known as carryover effects (Benesh, 2016). There are, several studies documented the environmental conditions that experienced during the larvae stage was carry over when they transfer into the juvenile stage, especially in amphibians (Altwegg and Reyer, 2003; Relyea, 2007; Gomez-Mestre et al., 2010; Tejedo et al., 2010).

5.2.2 The relationship between larval size and fitness in complex life cycles

In complex life cycles, separate life stages typically live in distinct habitats; in free-living organisms, they may occupy different habitat types, whereas in parasites, they may occupy different host environments (Benesh et al., 2012). In a diversity of taxa, larval stages that have larger body size at the transition between habitats have a better transmission probability, growth, survival and fecundity as a adults (Semlitsch et al., 1988). The correlation between larval size and the fitness of subsequent life stages may arise if larger size is itself beneficial, or if size is reflective of some other characteristic

related to fitness, such as physiological condition (Blanckenhorn, 2000). Larger larvae might confer lower mortality in the adult habitat, because of a shorter growth period, or increased reproductive success (Blanckenhorn, 2000). On the other hand, larger larvae may be in a better condition, due to an ability to accumulate more resources, that promotes survival and reproduction in the adult habitat (Van Noordwijk and de Jong, 1986).

5.2.3 Environmental factors affecting larval parasites, and their effects on future stages

Carry over effects are assumed to be predicated by size and age at metamorphosis (Chubb et al., 2010). For examples, individuals that transition at small sizes or old ages are likely to have been in poorer larval conditions and are thus expected to experience negative carryover effects as adults. However, how well size or age reflect larval stress is unclear, given that different types of environmental stressors can affect growth and development in different ways (Van Allen et al., 2010). It is known that the environment can also affect complex life cycle organisms (Benesh, 2016). For example, *L. viridis* growth rates were found to be higher in the delayed photoperiod than in the actual photoperiod, except for larvae that starved during the starvation period and for all larvae during the emergence period (Stoks et al., 2006). Temperature also can affect the performance of larval stages of parasites with complex life cycles, with potential consequences for their performance in the next host, by stimulating or slowing down metabolic processes (Pechenik and Fried, 1995) and also by affecting size and age at transmission (Atkinson, 1994). For example, larval organisms experiencing higher temperatures may reach metamorphosis at a younger age and/or smaller size (Benesh, 2016).

In Chapter 2 of this thesis, *S. solidus* procercoids were found to develop more slowly in intermediate copepod hosts reared under colder temperatures, but attained infectivity (as indicated by the development of a cercomer) at a larger size than those reared at higher temperatures. However, the carryover consequences for the next parasite stage (i.e. the pleroceroid in the fish hosts) could not be studied, as the experiment failed to produce infection in fish hosts. Other environmental factors experienced by one life stage of such parasites, such as the presence of pollutants, also potentially affect the performance of future life stages even after they move into their new habitat, or – for parasites – after transmission to a new host (Benesh, 2016). In parasites, pollutants such as heavy metals are known to accumulate in the tissues of parasite larvae (Sures et al., 1994) where they are apparently not always harmful to the larval parasites, and may even benefit them

(Sures, 2008). However, most of the effects of pollutants on parasites have focused on a single life stage, and little is known about the potential for carry over effects in parasite life cycles and the consequences of the growth variation in this pollution on parasite performance in the next host.

5.2.4 Aims of the study

Previous results reported in this thesis showed that exposure to zinc affected the growth of the procercoid stage of the tapeworm *Schistocephalus solidus* in host copepods (Chapter 4). In this Chapter, the effects of zinc exposure on the infectivity of the parasite to the next intermediate host in the parasite life cycle, the three-spined stickleback, is studied, as is the subsequent growth of the parasite in host fish. The experimental study was designed to examine how that growth of the larval stage (procercoid) can affect the infective stage of the parasite (plerocercoid) performance in its stickleback second intermediate host that experiences the same environmental conditions. The following hypotheses were tested: (1) that the larger body size of individual *Schistocephalus solidus* procercoids growing in copepod hosts exposed to zinc have a higher probability of successfully infecting sticklebacks; and (2) that the larger body size of individual *Schistocephalus solidus* procercoids growing in copepod hosts exposed to zinc had developed larger body size in the stickleback hosts than those exposed in freshwater.

5.3 Materials and Methods

5.3.1 Production of fish using IVF

Gravid female and sexually mature male three-spined sticklebacks were selected from lab-bred stock (second generations) from wild caught parents originally caught at Llyn Frongoch Reservoir in mid-Wales (52°21'39''N, 3°52'46''W). Seven families of sticklebacks were generated using standard *in vitro* fertilization (IVF) techniques during

August 2016. The reproductive status of females and males was checked regularly during the breeding season to ensure they were ready for IVF. The cloaca of gravid female fish was inspected for distension, and males were assessed based on nuptial colouration and nesting behaviours. The ratio of female and male fish used in each IVF procedure was 3 females : 1 male. Male sticklebacks were euthanized using a Home Office Schedule 1 method (overdose of Benzocaine anaesthetic (stock solution: 10g prepared in 1L of 70% EtOH) before the testes were removed.

Testes of male sticklebacks were then placed in a watch glass and macerated using clean micro-scissors to release sperm. The egg clutches of three fully gravid female fish were stripped into a clean watch glass by gently pressing each female's abdomen. The macerated testes were then mixed gently with eggs and were left for 20 – 30 min for fertilization to occur, before being transferred into a breeding tank (ca. 1L volume). The fertilization was observed under a microscope. Once the eggs started to become more transparent and the vitelline membrane separated from the egg proper, the perivitelline space filled with fluid. These fertilised eggs were transferred into approximately 1L plastic aquarium provided with gentle aeration. To make sure the eggs were free from fungal infections, 2ml of Methylene Blue solution (2 mg/L concentration) was added into the tank. Six days post-fertilization, 75% of the water was changed and the next day 50% was changed, to reduce levels of Methylene Blue, which can affect the development of newly-hatched fish. The eggs were left to hatch which usually took 8 to 10 days. The newly hatched fry were fed with Liquifry (Interpet, UK) for 8 days and thereafter with freshly-hatched *Artemia* nauplii. After a month, the fry that had been generated either from natural spawnings or through IVF procedures were transferred into clean 30 L glass aquarium tanks (40x25x30cm), and placed into the recirculating aquarium system. Fish were fed daily, *ad libitum* with frozen bloodworm. Fry were kept until they reached 6 to 8 months in age before being used for infection experiments.

5.3.2 Copepod culture and maintenance

Copepodite stages of the cyclopoid copepod *Cyclops strenuus abyssorum* were selected from lab-bred stock culture originally purchased from a commercial supplier (Sciento, Manchester, UK). The culture system consisting of a 1 L conical flask, containing

approximately 400-500 ml of deionised water, was maintained by monthly sieving through mesh sizes ranging from 45µm to 390µm, to separate the nauplii, copepodites, and adult copepod stages, which were then transferred to separate 1 L conical flasks. Copepod cultures were fed once per week with a protozoan culture (*Colpidium striatum*), until one week prior to experimental parasite exposure. At that point, the copepod cultures were again sieved into the three stages (nauplii, copepodites and adult copepods), and the copepodite stages were used for experimental *Schistocephalus solidus* infections.

5.3.3 Copepod exposure to infective *S. solidus* coracidia (free-living stage)

Five hundred copepodites were collected and distributed equally among flasks containing either 200 ml of deionised water or 200 ml of 20 µg/L zinc solution, freshly prepared in laboratory for acclimation process. A 200 mg/L zinc stock solution was prepared by dissolving 40 mg zinc chloride ($ZnCl_2$) (Sigma) into 200 ml deionised water type II provided by an Elgastat Option 2 water purifying system itself fed with chemically deionised and filtered water, and this was diluted with deionised water to generate a final copepod exposure solution of 20 µg/L (H.M.S.O., 1969). Copepods were held in in 200 ml of this solution for 7d, after which 420 (84%) had survived. Surviving copepods were distributed into individual wells of replicated 24-well cell culture plates (Costar, UK), each of which contained 2 ml of either deionised water or the 20 µg/L of zinc solution. Individually-housed copepods were then exposed to a single, freshly-hatched *S. solidus* coracidium. Coracidia emerged from eggs that had been produced following the *in vitro* culture of adult worms (Smyth, 1954) (see section 2.3.1 for detailed parasite culture protocol), and incubated in the dark at 20°C for three weeks. Hatching of coracidia was induced by exposure to light. During the post-exposure period, the zinc solution and deionised water in the cell culture plate wells were changed every two days to maintained the zinc concentration. This solution changing also helped remove left-over food on the bottom of the well. After three weeks of exposure, copepods held under both treatments were screened for infection status, and any infected copepods were transferred into a new 24-well plate containing the new solution of deionised water or zinc.

5.3.4 Fish selection and exposure to infected copepod

Sixty juvenile three-spined sticklebacks (mean standard length: 32.7±0.42 mm) were

selected from IVF families bred in the lab in August 2016, as described above. Prior to exposure, fish were kept in freshwater and left without food for 48 zinc. Fish were then exposed to infected copepods individually in small plastic aquaria (15 cm x 14 cm x 11 cm, 1.25 L) held under static freshwater conditions. Each fish was fed two singly-infected copepods, which had either been reared under the deionised water (n=30) or the 20 µg/L zinc treatment (n=30). Exposures to infective parasite stages were carried out under the authorisation of a UK Home Office licence (project license: 70/8148; personal licence: I0203BEDF). Two days after parasite exposure, experimental fish were transferred individually to small plastic aquaria (15 cm x 14 cm x 11cm, 1.25 L) held on a temperature-controlled recirculating water system and fed *ad libitum* with frozen bloodworm for a further 70 d. Individual rearing aquaria contained a plastic plant for shelter.

5.3.5 Fish dissection and tissue sampling

At the end of the experiment, fish were sacrificed by exposure to an overdose of Benzocaine anaesthetic (Stock solution: 10g prepared in 1L of 70% EtOH) according to UK Home Office Schedule 1. Fish were blotted dry to remove surface moisture before being weighed on an electronic balance to record the total mass (M, to 0.001 g) and standard length (SL, to 0.1mm) was measured manually using a dial calliper. Any *S. solidus* plerocercoids present were counted, blotted and weighed individually to find the total plerocercoid mass (M_p, to 0.001g). The parasite index (I_p) was then calculated (I_p, = [total *S. solidus* mass / M] * 100) (Simmonds, 2015). During the experiment, two fish died: one coming from fish that had been fed infected copepods reared in deionised water (died at 5 weeks post exposure), and 1 from the fish that had been fed an infected copepod kept in zinc solution (died at 7 weeks post exposure), leaving a total of 58 fish in the experiment. Dead fish were dissected, to determine the infection status.

Fish mass, i.e. excluding plerocercoid mass, (M_f) was then calculated ($M_f = M - M_p$). Body condition factor was calculated as [(M_f / (SL)^3) * 100000]. Liver mass (M_L) was recorded (to 0.001g) and the hepatosomatic index [(HSI=M_L / M_f) * 100)] was calculated. Spleen mass (M_S) was also recorded (to 0.001g) and the spleen somatic index [(HSI=M_S/M_f) * 100)] was calculated (Simmonds, 2015).

5.3.6   Data transformation and statistical analysis

All statistical analyses were carried out in SPSS v22. Data were tested for normality and homogeneity of variance using Explore and normal Q-Q plots. BCF, SSI and Fish mass were log-transformed to achieve normality. Chi-squared tests were used to compare the success of parasite exposures between treatment (fish exposed to infected copepods kept in deionised water and zinc solution). Treatment (H_2O vs zinc) and infection status (infected vs non-infected) were compared simultaneously, using two-way ANOVA tests to show their effect on fish growth and condition indices. The relationship between procercoid body area and terminal copepod cephalothorax length for copepods maintained under deionised water and 20 µg/L of zinc solution were determined using Spearman's correlation coefficient test. Mean comparison ANOVA test was also used to compare the M_p and I_p recovered from fish exposed to infected copepods that had been reared in deionised water and zinc solution.

5.4 Results

5.4.1 Effects of zinc exposure on procercoid and copepod growth

Three weeks after being exposed to parasites, copepods held in the 20 µg/L zinc solution had a larger mean cephalothorax length than copepods kept in deionised water (ANOVA, $F_{1,119} = 40.810$, $P < 0.0005$; Figure 5.1a). The mean body area of procercoids infecting copepods held in the 20 µg/L zinc solution was also larger than that of procercoids infecting copepods kept in deionised water (ANOVA, $F_{1,119} = 581.96$, $P < 0.0005$; Figure 5.1b).
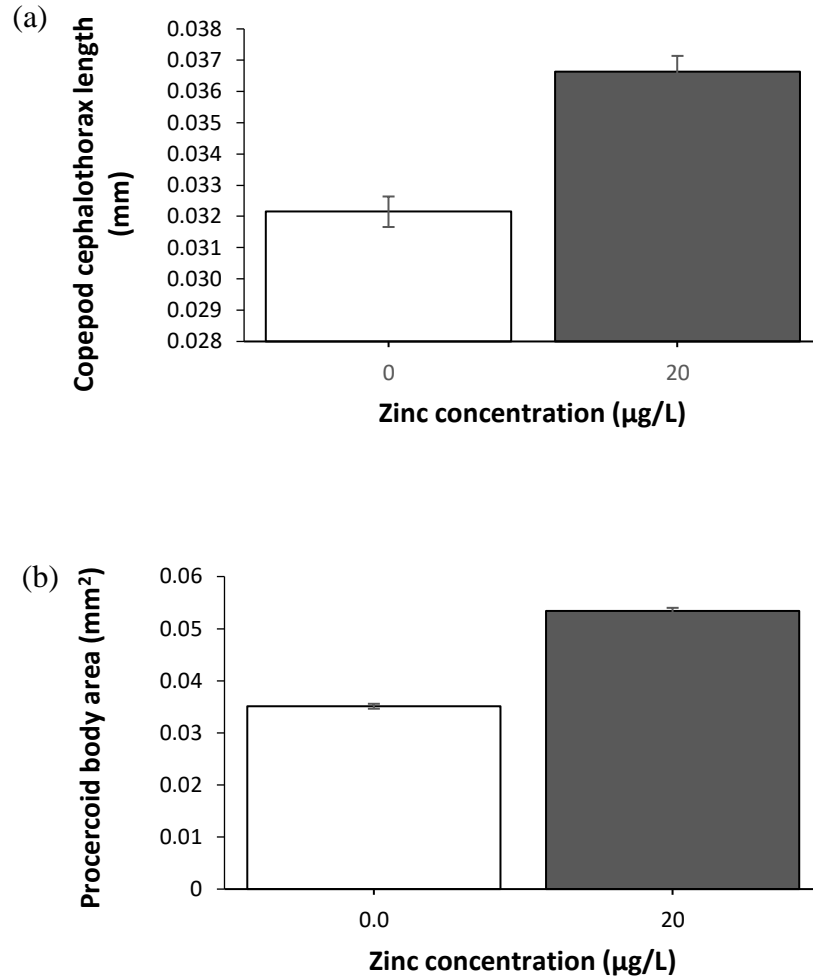
Figure 5.1 Differences in (a) copepod cephalothorax length at week 3 post-exposure to the parasite and (b) body size, shown as area of *Schistocephalus solidus* procercoids that established in copepods at week 3. Error bars represent ± standard error. Two-way ANOVA statistic.

5.4.2 Host growth rates and relationship with procercoid size

The body area of procercoids infecting copepods after 3 weeks of infection was strongly affected by the copepod size, measured as cephalothorax length (ANCOVA, $F_{1,116} = 69.311$, $P < 0.0005$), and also by zinc treatment (distilled vs. 20 µg/L zinc solution) (ANCOVA, $F_{1,116} = 28.116$, $P < 0.0005$) (Figure 5.2).
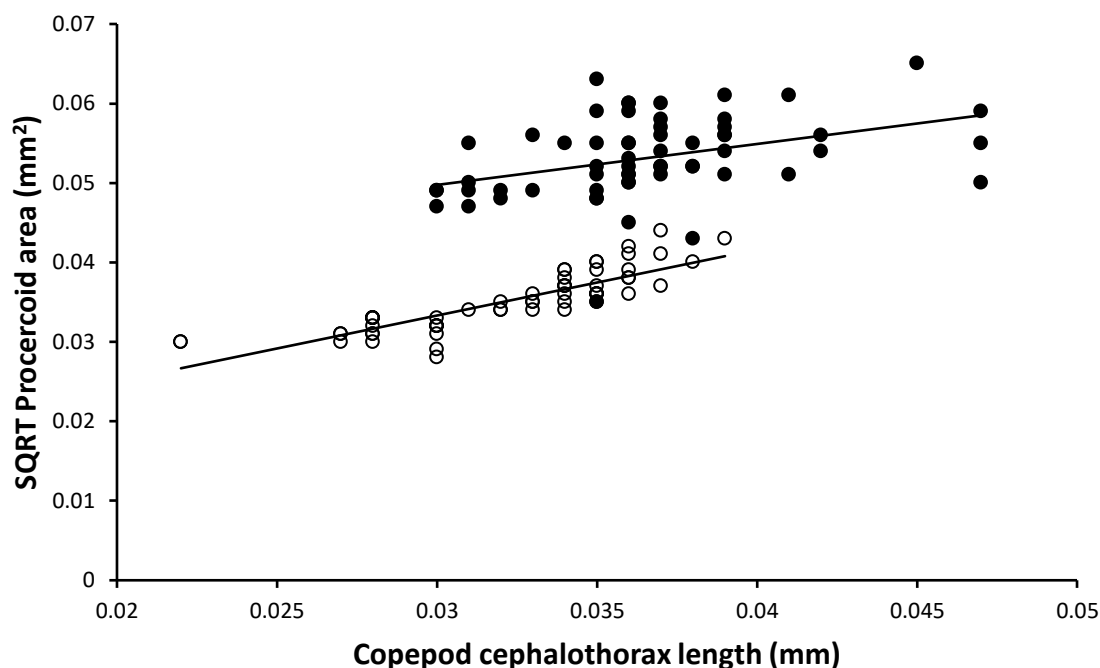
Figure 5.2 The relationship between procercoid body area and terminal copepod cephalothorax length after 3 weeks of exposure for copepods maintained under deionised water (open symbols) and 20 μg/L of zinc solution (filled symbols). ANCOVA statistic.

5.4.3 Probability of infection

The probability of infections establishing in stickleback hosts following exposure to infective *S. solidus* parasites depended on the rearing conditions experienced by procercoids in infected copepods. Fish fed with infective parasites that had developed in copepods held under 20 μg/L were more likely to harbour at least one plerocercoid (12/30) than those fed with parasites reared in copepod hosts kept in deionised water (3/30) ($X^2_2 = 7.200$, $P < 0.007$; Figure 5.3).

All fish were exposed to two infective procercoids, meaning that fish could potentially develop 0, 1 or 2 plerocercoids. The three infected fish exposed to deionised water-reared parasites harboured only a single plerocercoid, whereas of the fish exposed to zinc-reared parasites, eight harboured one plerocercoids and four harboured two plerocercoids.
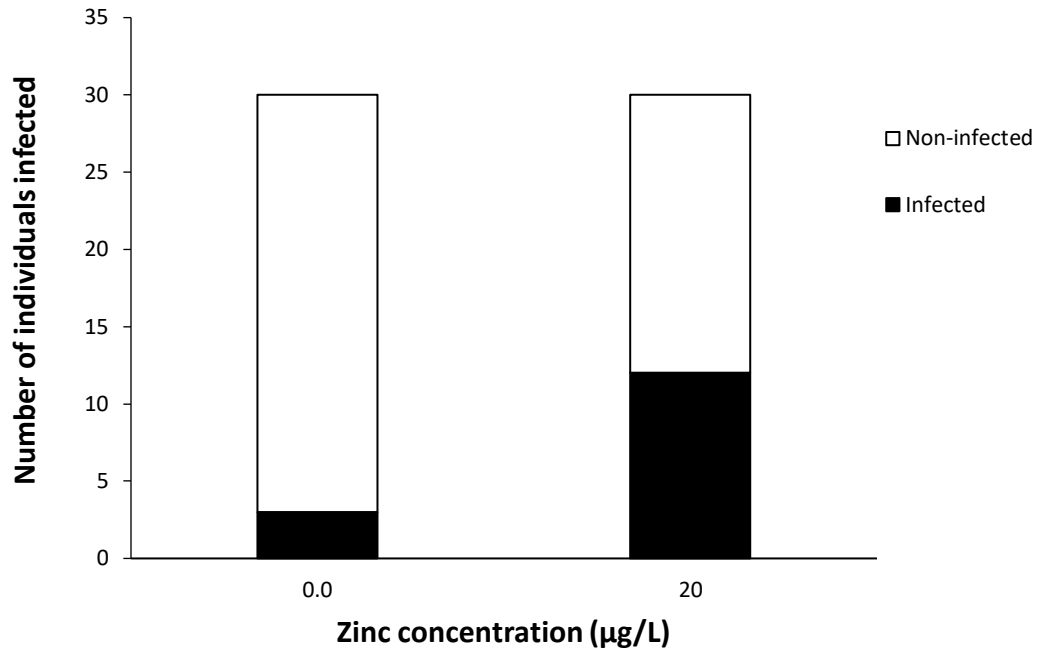
Figure 5.3 The number of infected and non-infected three-spined sticklebacks that had been experimentally exposed to infective *Schistocephalus solidus* procercoids reared in copepods held either in deionised water or 20 μg/L zinc solution. Chi-squared test statistic.

5.4.4 Host growth

There was no significant effect of infection status ($F_{1,54} = 1.134$, $P = 0.292$) or treatment ($F_{1,54} = 0.682$; $P = 0.412$) on specific growth rate (SGR), and also there was no significant interaction between infection status and treatment ($F_{1,54} = 0.065$, $P = 0.799$; Figure 5.4). It therefore seems that stickleback growth rates are not significantly affected by treatment or *S. solidus* infection.
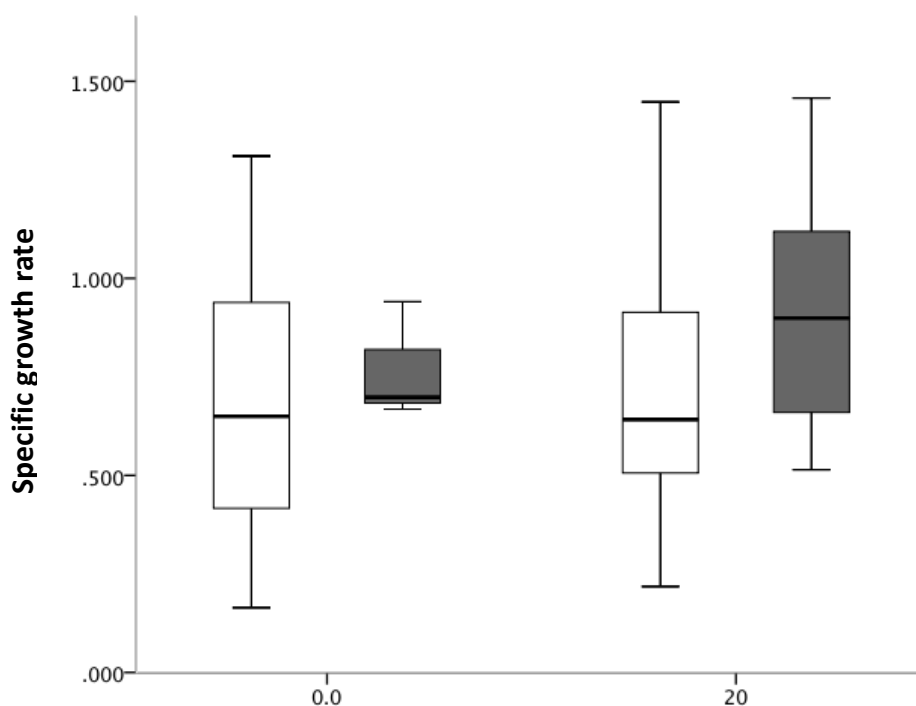
Figure 5.4 Boxplots showing specific growth rate (*SGR*) of Llyn Frongoch three-spined sticklebacks for non-infected and infected fish achieved over the 70d in the study fed either copepod-reared in deionised water or copepod-reared in zinc treatment (20 μg/L). The dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR) and the whiskers represent 1.5 times the IQR. Two-way ANOVA statistic.

5.4.5 Host body condition

Infected fish fed with infected copepod that kept in deionised water having relatively higher live mass than a fish fed with infected copepod that kept in 20 μg/L zinc solution (Table 5.2, Figure 5.5a). However, the was no significant effect of infection status on the HSI (Table 5.1, Figure 5.5a) and there was no interaction between infection status and zinc treatment (Table 5.1, Figure 5.5a).

The body condition factor (BCF) of fish was unaffected by zinc treatment (Table 5.2, Figure 5.5b), and infection status had only a marginal non-significant effect (Table 5.2, Figure 5.5b). There was no interaction between zinc treatment and infection status (Table 5.2, Figure 5.5b). Splenosomatic index (SSI) was not affected by infection status (Table 5.3, Figure 5.6) or by zinc treatment (Table 5.3, Figure 5.6) and there was no interaction between these two factors (Table 5.3, Figure 5.6).

Table 5.1 ANOVA table for growth of Llyn Frongoch three-spined sticklebacks over the course of the study using HSI as the response variable. *Schistocephalus solidus* infection status and copepod kept in two treatments were used as predictor variable. Significant values (*P*<0.05) are shown in bold.

Variables	DF	*f*	*p*
zinc treatment	1	6.777	**0.012**
Infection status	1	1.306	0.258
zinc treatment*infection status	1	3.612	0.063
Error	54		

Table 5.2 ANOVA table for growth of Llyn Frongoch three-spined sticklebacks over the course of the study using BCF as the response variable. *Schistocephalus solidus* infection status and copepod kept in two treatments were used as predictor variable.

Variables	DF	*f*	*p*
zinc treatment	1	0.083	0.775
Infection status	1	3.682	0.060
zinc treatment*infection status	1	1.476	0.230
Error	54		
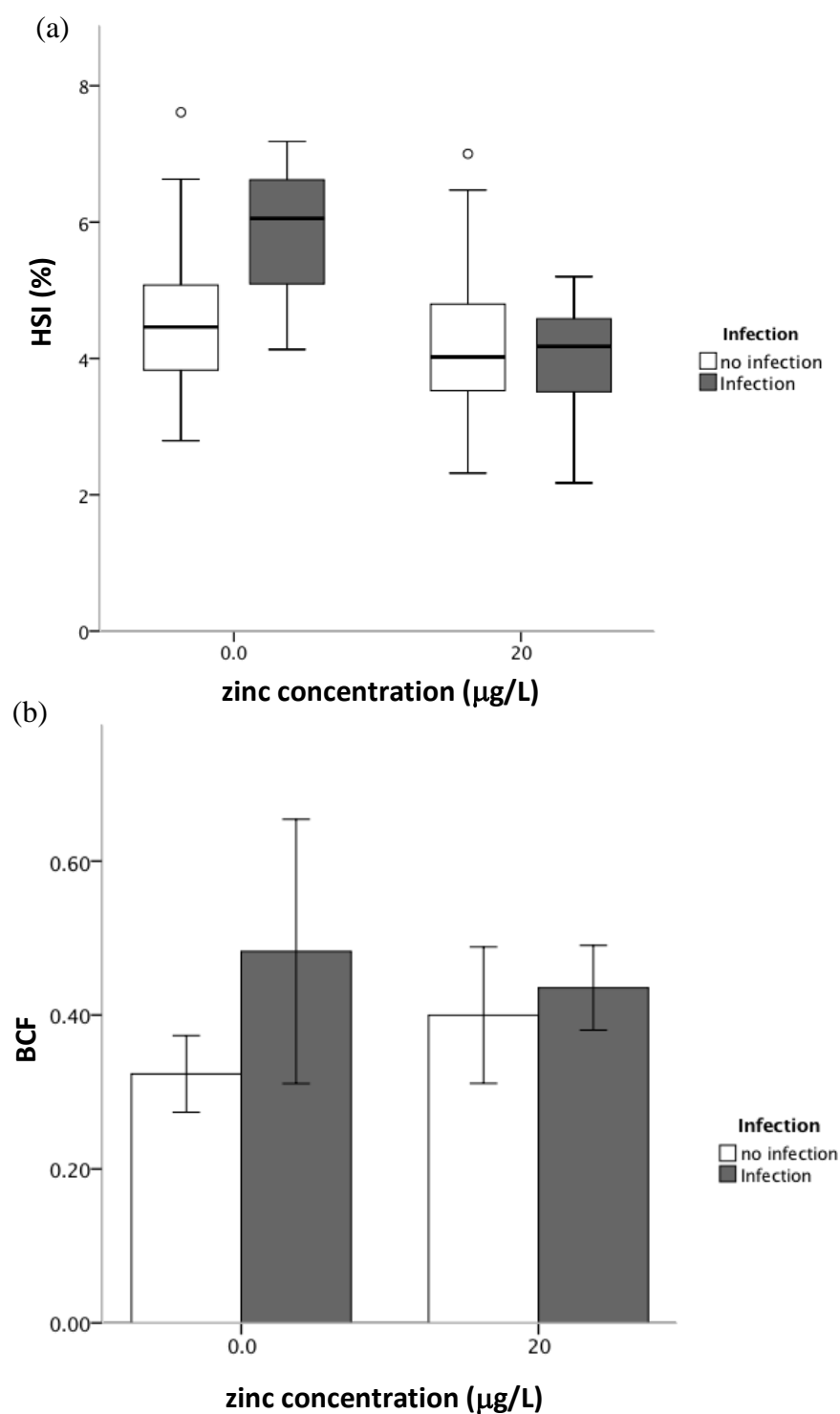
Figure 5.5 Three-spined sticklebacks condition graph showing (a) hepatosomatic index (HSI) and (b) body condition factor (BCF). Error bars represent $\pm$ 1 standard error. The dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR) and the whiskers represent 1.5 times the IQR. $\circ$ represents outliers. Two-way ANOVA

statistic.

Table 5.3 ANOVA table for growth of Llyn Frongoch three-spined sticklebacks over the course of the study using SSI as the response variable. *Schistocephalus solidus* infection status and the zinc treatment experienced by host copepods were used as predictor variables.

	DF	f	p
zinc treatment	1	0.736	0.395
Infection status	1	2.111	0.152
zinc treatment x infection status	1	0.017	0.895
Error	54		
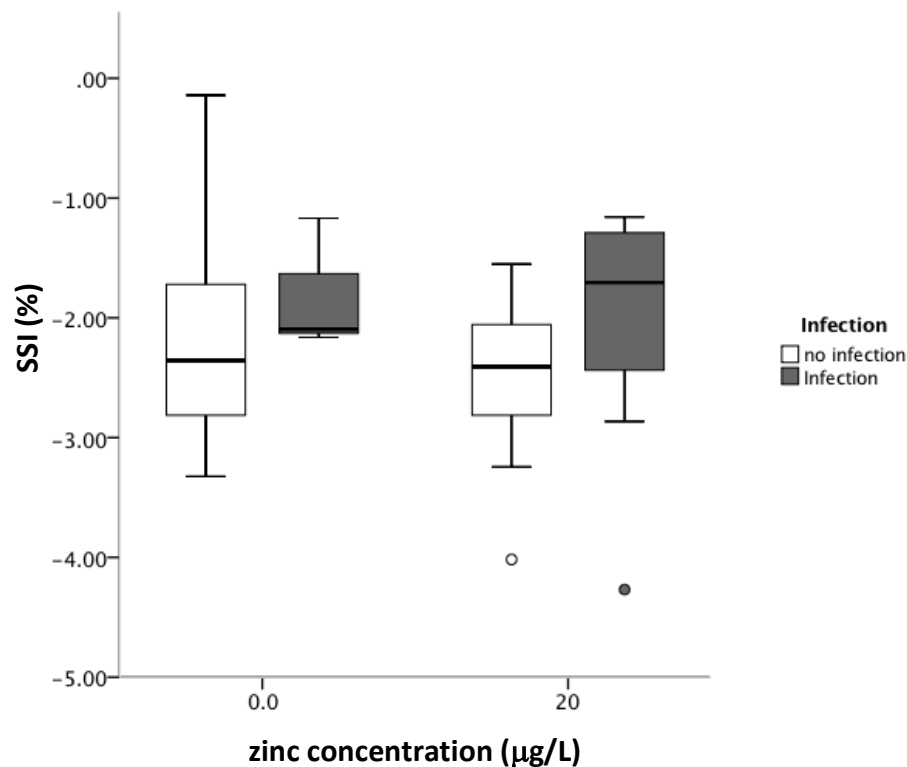
Figure 5.6 Boxplot showing spleen somatic index (SSI) of non-infected and *Schistocephalus solidus* infected Llyn Frongoch three-spined sticklebacks in the study fed either with copepod that kept in deionised water or 20 μg/L zinc solution. The dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR) and the whiskers represent 1.5 times the IQR. ○ represents outliers. Two-way ANOVA statistic.

5.4.6 Parasite growth in fish

Mean total mass of plerocercoids from infected fish fed with copepods reared in 20 μg/L zinc solution was considerably higher than that of plerocercoids from infected fish that were fed with copepods reared in deionised water (ANOVA, $F_{1,10} = 16.552$, $P = 0.003$, Figure 5.7a). However, none of the plerocercoids recovered from fish fed with copepods reared in deionised water or in 20 μg/L zinc solution had reached infective size, as all were < 50 mg (Figure 5.7a). When the mass of the parasite was controlled for the mass of the fish, by calculating parasite index (I_p), there was a significant difference between the two treatments, with fish fed with copepods reared in deionised water having significantly lower I_p values than those fed with copepods reared in 20 μg/L zinc solution (ANOVA; $F_{1,10} = 24.897$, $P = 0.001$; Figure 5.7b).

(a)

(b)
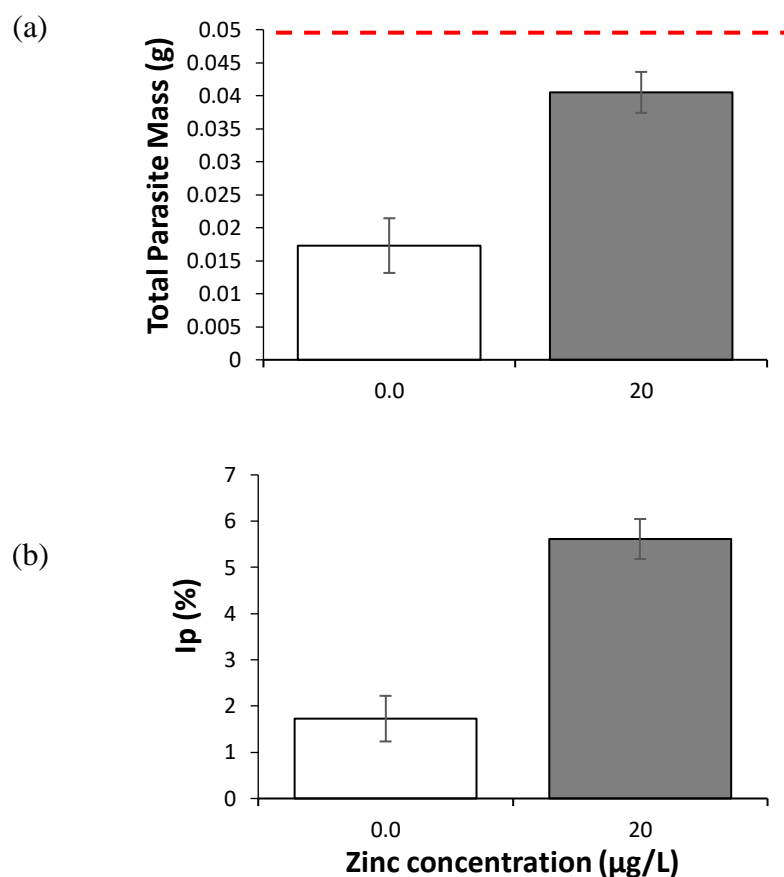
Figure 5.7 The size of *Schistocephalus solidus* plerocercoids from infected fish fed with copepods reared in deionised water or 20 μg/L recovered from sticklebacks kept in freshwater for 70d. (a) Total parasite mass, giving absolute parasite size. (b) Parasite index (I_p), showing parasite mass relative to host mass. Red line shows the size at which

S. solidus becomes infective. Error bars represent ± 1 standard error. Mean Comparison ANOVA test statistic.

5.4.7 Determinants of plerocercoid size

Among all parasites, i.e. combining infecting copepods kept in deionised water and kept in the zinc treatment, there was a positive correlation between the size (measured as area) that the procercoid achieved in the copepod host, and the mass of the plerocercoid recovered from the three-spined stickleback host (Spearman's correlation coefficient r_s = 0.0624, n =23, *P*= 0.040; Figure 5.8).
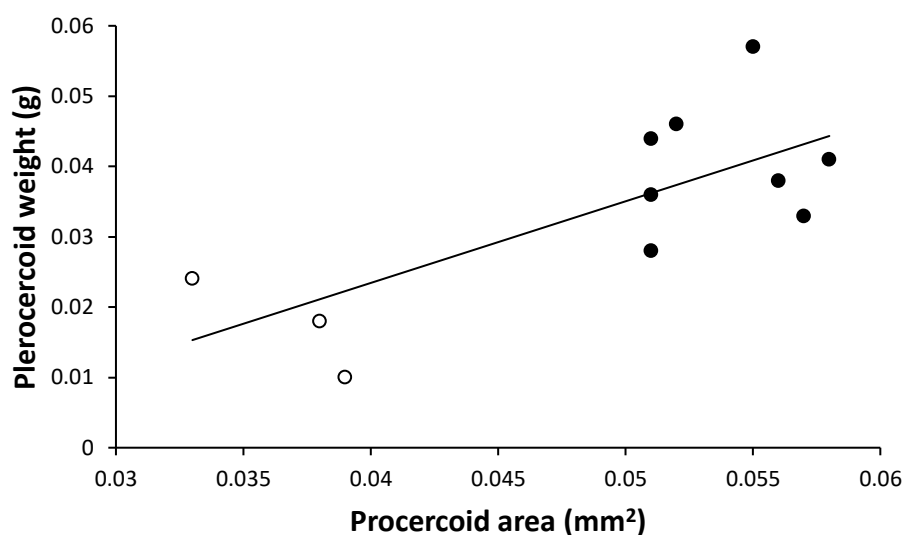
Figure 5.8 The correlation between procercoid area in copepods kept in zinc treatment and plerocercoid weight in fish. Data pooled for all groups with single regression line. Deionised water (open circles) and 20 μg/L (filled circles). Spearman's correlation coefficient statistic.

5.5 Discussion

5.5.1 Main findings

This study has demonstrated that aspects of the aquatic environment experienced by procercoid larvae of *S. solidus* infecting copepod hosts can have profound implications for the transmission success and growth of the parasite in the next host, the three-spined

stickleback fish. Procercoids grew faster in copepod hosts that had been exposed to the zinc treatment (Figure 5.1), and these larger procercoids were both more successful in infecting sticklebacks, and developed into larger plerocercoids following establishment, than those emerging from copepods that had been raised in deionised water (Figure 5.7).

5.5.2 What affects plerocercoid growth?

In this study, *Schistocephalus solidus* tapeworms that grew larger in copepods grew to a bigger size in fish and had a higher infectivity in fish. In contrast to the present study, Benesh et al. (2012), documented that the large worm of *S. solidus* that grew in large copepods did not have a higher infection success or growth in fish. A few studies suggest that the larval size-fitness correlation may depend on environmental factors like the intensity of infection in the intermediate host (Fredensborg and Poulin, 2005, Dorücü et al., 2007) or the size of the intermediate host (Benesh et al., 2012). In the present study, the size of procercoids was related to the size of the copepod host, and also treatment. The significant effect of copepod size on the procercoid size might arise because copepods – in common with all crustaceans – have a fixed size of the exoskeleton that can constrain the maximum size that can be achieved (Wedekind, 1997). Meanwhile, copepods kept in zinc solution developed to a larger size (measured as cephalothorax length) compared to those infecting copepods kept in deionised water. Martins et al., (2017), showed that a crustacean (*Daphnia longispina*) can tolerate and continue to grow with zinc concentrations up to 4000 µg/L. Cladocerans exposed to the two lowest concentrations (500 and 1000 µg/L) of zinc showed significant effects on growth rate at 0 - 7d, whereas higher concentrations of zinc (200 and 4000 µg/L) significantly affect the growth rate of older daphnids (Martins et al., 2017). Muyssen et al., (2006), have shown that *Daphnia magna* exposed to 80 µg/L of zinc seemed to perform as well as or better than conspecifics exposed to control conditions (without added zinc) in terms of filtration rate, energy reserves and individual weight. They also suggested this is more likely associated with the fact that organisms cultured without added zinc suffer from zinc deficiency. In crustaceans such as copepods, zinc is noticeable as a nutrient requirement (Bielmyer et al., 2006) and has been recorded as one of the higher metal contained in copepod body compared to rotifer and *Artemia* (Karlsen et al., 2015). Also, zinc is known as an important nutrient for growth, reproduction and development in animal life (Bury et al., 2003). In this study, the zinc concentrations tested were of ecological relevance, in

an average of 8-500 μg/L of zinc) to a water inhabited by aquatic organisms including fish three-spined stickleback and copepod which is host to *S. solidus* in the UK (Peters et al., 2012) and lower (20 μg/L) from the previous study. Therefore, the above explanation could explain the bigger size of copepod kept in zinc solution recorded in the present study which influence the growth of procercoid.

In this study, the procercoid that have larger body size in copepod host (at the time of exposure into fish) have a higher success in growth when transform in plerocercoid stage. This finding agree with the hypothesis that body size of larval at the transition (carryover effect) between habitats can influence the transmission probability, survival, fecundity and growth as adults (Semlitsch et al., 1988; Steinauer and Nickol, 2003; Benesh et al., 2012; Benesh and Hafer, 2012). In free-living organisms, the quality of the larval usually was related to the larval size during the transition. For example, larval that have big size often grew in better conditions (Hentschel and Emlet 2000, Morey and Reznick 2000, Phillips 2002, Altwegg and Reyer 2003, De Block and Stoks 2005). This is because, large larval have more resource and energy which can be used for survival and reproduction in the adult habitat (Van Noordwijk and de Jong 1986, Reznick et al. 2000). Dias and Dustin, (2010), documented that during the post-metamorphic, larger bryozoan *Celleporaria* sp offspring produce a larger colonies which have higher fecundity and reproductive output.

5.5.3 Conclusion

The performance of larval parasites can have significant and profound implications for their transmission and growth in subsequent hosts. Infection of the next host is typically an energetically-demanding process. The transitional size and fitness of larval stages into the next host is an important factor shaping the life history strategies of parasites with complex life cycle. The environment can also affect complex life cycle organisms. zinc as an environment stressor has been shown to affect growth and development in different ways. It affects procercoid growth in copepod hosts with consequences for performance in the next host (three spined sticklebacks) by stimulating the size of plerocercoid. For *S. solidus* procercoids, transmission to three spined sticklebacks as a small larva has clear costs in terms of lower infection probability and stunted, inefficient growth. In further studies, it would be interesting to look at the combined effects of simultaneously occurring parasitism and pollution on the health of the respective hosts such metal uptake

by the *S. solidus* parasite in three spined stickleback host and to determine if *S. solidus* can reduce metal levels in the tissues and organs of their hosts.

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Chapter 6

General Discussion

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6.0 General Discussion

6.1 Main Findings of the Thesis

In natural aquatic environment, parasite infections is one of the natural stressor that can intervene the reproduction and biology of their host by many mechanisms. But in natural aquatic environment, host organism also have to experience with the environmental changes including anthropogenic stressors such as climate change and pollution (Sumpter, 2009). Understanding how host-parasite interactions especially with complex life cycle affected with this changes are important in order to determine how far it will affect the disease outbreak. In this thesis the first and second intermediate host (copepod and stickleback) of *Schistocephalus* parasite, was used to investigate how environmental changes such as temperature and heavy metal pollution influences the interaction between host-parasite. In this discussion the key finding of the environmental effect on the host-parasite interaction will be summarised and suggestions for future work will be given.

There is potential for the balance of host-parasite interactions to be shifted in degraded ecosystems because the behaviour and physiology of both parasite and host species can change depending on environment conditions (Poulin, 2011). Thus, the effect of environmental changes is likely to vary between parasites species, and even between developmental stages because larval and adult parasites are affected in different ways. Parasites are ubiquitous components of the ecosystem, and it is thought that more than half of all animal species are parasitic either temporarily or permanently (Pietrock and Marcogliese, 2003).

Because of their complex life cycles, parasites inhabit at least two very different parts of the environment: the external environment, which is experienced by the free-living stages in their life cycles; and the internal environment(s) provided by the host(s). Changes in either of these environments can affect a parasite's ability to complete its life cycle. In this thesis the impacts of changes in the external and internal environments caused by anthropogenic changes on a parasite life cycle were investigated using controlled laboratory experiments. All of the work in the thesis has utilised an established experimental host-parasite model comprising the *Schistocephalus solidus* parasite free-living stage, the copepod *Cyclops strenuus* intermediate host*,* and the stickleback *Schistocephalus solidus* definitive host. In this discussion, I will summarise the

environmental factors identified as important for host-parasite interactions in this system and give suggestions for potentially interesting and fruitful future research directions.

6.1.1 External environmental effects

In the complex life cycle of a parasite, some parasites achieve transmission through passive mechanisms, with static eggs being consumed by intermediate hosts such as acanthocephalans (Cheng, 1973). In many cases eggs hatch to release free-swimming infective stages, which typically exhibit behavioural adaptations to facilitate active transmission (e.g., cestode coracida, trematode miracidia; (Cheng, 1973, Dubinina, 1980). For eggs that hatch and release free-swimming infective stages, at this point they are independent and directly exposed to the external environment (i.e. outside of any host). This independent stage of parasites is not shielded by a host from the external environment and therefore any changes or fluctuations in the environment can directly affected the egg (Pietrock and Marcogliese, 2003).

6.1.1.1 The effects of temperature on the host-parasite interaction

Chapter 2 showed that elevated temperature has the potential to decrease the survival of a free-living stage of a parasite. Chapter 2 showed that the survival of a free-living stage of a parasite decreased as temperature increased, suggesting that environmental temperature is an important factor affecting the biology of the parasite and might also influence their habitat selection and ability to use effective host-finding behaviours which then is likely to influence the probability of host encounters (Barber et al., 2016). To my knowledge, there are no empirical studies demonstrating changes in *S. solidus* parasite survival in a response to increasing temperature. Therefore, it is quite difficult to compare these findings with another study using a same parasite system. Shorter life-spans at higher temperatures might be related to an increased metabolic rate (Dubinina, 1980, Koprivnikar et al., 2010) and rapid depletion of stored carbohydrates and energy reserves. The free-living stage does not feed but instead depends on energy reserves in the form of fat and glycogen droplets located in the ciliate embryonal shell (Dubinina, 1980, Pechenik and Fried, 1995, Pietrock and Marcogliese, 2003). In a different parasite study-system, increase in temperature not only reduce (Rea and Irwin, 1992) the survival of a parasite but also can prolong (Lo and Lee, 1996) the survival. This might reflect the ability of the parasite species to tolerate the range of temperatures and therefore, the survival can be

increased. Therefore, under altered temperature environment, it is still not clear on how far this free-living stage might be affected (Measures, 1996). The longevity of infective free-living stages of parasites is important as this stage would need to invade susceptible hosts from the environment to complete the life cycle.

6.1.1.2 The effects of water hardness on the host-parasite interaction

Chapter 3 showed that water hardness (represented as $CaCO_3$) modulated the effect of cadmium, copper and zinc on the survival of the free-living stage of *S. solidus*. Hard water has been shown to have significant effects on the free-living stage of *S. solidus* by prolonging parasite survival. The results presented in Chapter 3 support other studies using different concentrations of water hardness such as in *Echinoparyphium recurvatum*. Differences in water hardness and parasite species make it difficult to compare, but still hard water was recorded to increase the survival (Evans, 1982ab). This is because toxicity of metals is generally reduced in hard water due to the differences in the concentrations of soluble, toxic forms of the metals. Increased water hardness can increase the prevalence of less toxic forms of the metals (Evans, 1982ab). In general, toxicity of metals in the environment can be affected by other environmental variables including water hardness, temperature, pH and salinity (Cooney, 1995, Evans, 1982ab). This study is, to my knowledge, the first to look at the water hardness as a factor that can influence the effect of heavy metals on *S. solidus* survival. The lack of knowledge in the life-span of the free-living stage make it impossible to determine the mechanism involved in contributing the survival/mortality of the parasite. Therefore, the results presented in Chapter 3 might have major implications for future research investigating the pollution effect on the parasite free-living stage.

6.1.1.3 The effects of zinc on the host-parasite interaction

Chapter 4 investigated the impacts of zinc acting as a pollutant, and its effects on the development of embryonic parasites and survival of free-living stage. The effect of zinc on the hatchability of *S. solidus* eggs has not been investigated in previous studies. The results of this study have revealed that eggs hatching of *S. solidus* is resistant to the different zinc concentrations (0.2–200 μg /L) studied. Chapter 4 illustrated that hatchability of *S. solidus* eggs occur in all zinc concentration after 21d of exposure. However, hatchability of *S. solidus* eggs decreased as zinc concentration increased. The

results presented in Chapter 4 support other parasite study who showed that *Bothriocephalus acheilognathi* eggs is highly resistant to cadmium and hatching occurs even at higher concentration of cadmium (Khalil et al., 2009). The previous study also revealed that cadmium accumulates on the surface of the egg and does not penetrate into the enclosed coracidium. This means that the parasite eggs may be able to withstand a heavy metal pollutant incident. Previous study suggested that the egg shell protects the developing and mature coracidium. The results of this chapter support this hypothesis, at least with respect to zinc. This is because, in cestodes, the parasite eggs also consists a thick sclerotin capsule surrounding the eggs (Smyth, 1954). However, once the operculum has opened and the coracidium has been exposed to the external environment then this free-living stage of the tapeworm life cycle appears to be very vulnerable to heavy metal pollution. This may result in a decrease in transmission to the intermediate host.

Chapter 4 further analysed the impact of elevated zinc concentrations on the free-swimming infective stage of *S. solidus*, the coracidium. The life-span of *S. solidus* coracidia was shortened with decreasing zinc concentration and in deionised water. All coracidia used in this study had a longer survival time (14-18 zinc) in higher concentrations of zinc (200 μg/L) than in lower concentrations (8-10h in 0.2 μg/L). This result contrasts with another study, which found that that the survival of *Echinoparyphium recurvatum* cercaria in zinc was shortened compared to the survival time of cercariae in deionised water (Evans, 1982a). The present study thus provides experimental evidence that the free-swimming infective stages of cestodes – coracidia - may be particularly resistant to zinc pollution, and provides further evidence of the range of responses of taxonomically diverse parasites to stressors. Free-living larval stages of parasites respond to aquatic environmental variables such as toxicants similarly to free-living animals in general, whilst adult parasites are often sheltered from the outer environment by living inside their hosts (Pietrock et al., 2002). In another study, *Schistosoma mansoni* miracidia survival in zinc was not different from the survival of miracidia in deionised water (Morley et al., 2001). This shows that the tolerances to pollutants of different stages in parasites' life cycles depend on the parasite species (Sures, 2008). The results of the present study can fill the gap in current knowledge about the effects of heavy metals on free-living cestode stages exemplified by the stickleback cestode *S. solidus*.

6.1.2 Internal environmental effects

Parasites that have become established in hosts may still be affected indirectly by external environmental factors such as temperature and pollution. Chapters 2, 3 and 4 examine the effects of environmental conditions on the development of *S. solidus* parasites inside the crustacean intermediate host. Chapter 2, showed that, elevated temperatures affected procercoids inside the first intermediate host differently to the free-living stage. Chapter 2, illustrated that at higher temperatures, procercoids developed more rapidly (in body area) and reached the next infective stage earlier than at low temperatures. A consequence of this rapid development, however, was that infective procercoids were smaller than those that developed at lower temperatures. These results support other study at which using a different parasite system, for example in *Angiostrongylus cantonensis* (nematode parasite of humans and the causative agent of eosinophilic meningitis), higher temperature has shown to reduce the transformation time of larval nematode into infective third stage larvae compared to a lower temperature at which takes more than two months for the transformation to complete (Lv et al., 2006). The development rate of the acanthocephalan *Polymorphus marilis* in laboratory-infected *Gammarus lacustris* increased linearly with increased temperatures (Tokeson and Holmes, 1982). In cooler temperatures, parasites grew slower. Therefore, less nutrients will be used in supporting the growth compared to those in warmer temperatures. This condition will help the parasite to growth into a larger size without interfere with the host reproduction process. Further laboratory studies are required to determine what are the mechanism that lead to increase in parasite growth at high temperatures.

Chapter 3 also assessed the impact of water hardness (hard water: 342 mg/L and soft water: 34.2 mg/L $CaCO_3$) and heavy metals (copper and zinc) on *S. solidus* procercoids in their copepod host, *C. strenuus*. To my knowledge, there are no studies measuring *S. solidus* parasite growth in the presence of a heavy metal at different levels of water hardness. Therefore, it is quite difficult to compare my new findings with other study using the same parasite system. previous studies that used water hardness as factor to influence the toxicity of heavy metal only looked at the free-living stage of parasites (Evans, 1982a, 1982b; Morley et al., 2001). Chapter 3 illustrated that at day-7, copper and zinc had no effects on procercoid growth, but it was affected by the water hardness. Procercoids grew to a larger size in hard water than in soft water. Future studies over

longer time periods (6 weeks post parasite exposure) are now needed, as these will probably show the parasite development progress under water hardness exposure.

Chapter 4 assessed the effect of elevated zinc concentration on *S. solidus* procercoid growth. This chapter investigated whether parasite growth or development might be affected by the elevated zinc concentration. *Schistocephalus solidus* procercoid was found developed and increased in size (body area) even in higher zinc concentration. This may be due to the levels of zinc (0-200 μg/L) used in my study, which are ecologically relevant levels of 8-500 μg/L occur in UK and Irish rivers (Peters et al., 2012) which are inhabited by the parasite's host. Parasite growth (body size area) differed between control and 20 μg/L of zinc . Further analysis revealed that the copepod (first intermediate host) size correlated with the parasite's growth. The results presented in Chapter 4 appear to support other studies which showed that the size of copepods *Macrocyclops albidus* at the time of infection correlated with the size of *S. solidus* procercoids after 14 days of infection (Wedekind, 1997). As the host is a crustacean, the fixed size of the exoskeleton constrains the maximum size that can be achieved by the parasite (Wedekind, 1997).

Studies that track the zinc pollution in natural environments and relate these to the laboratory work will be extremely useful in identifying if zinc pollution could create population differences in infectivity, growth and development of the parasite. This is because in natural environment, there would be other mechanism that involved and influence the parasite growth such as food ability and other water parameter.

6.1.3 Size at transmission affects performance in the next host

Chapter 5 investigated the relationship between procercoid size in the first intermediate host (which was kept in two different environmental conditions (zinc treatment and deionised water)) and performance in the second intermediate host (stickleback). The environmental conditions experienced by larvae can have consequences on post-metamorphic success not only through indirect effects derived from changes in metamorphic traits (Smith, 1987), but through their direct effects on traits influencing survival and growth in juvenile stages (Scott, 1994). In general, the size and fitness of parasite larvae at the point of transmission into the next host may affect adult survival, body size at first reproduction, age at first reproduction, and fecundity (Benesh and Hafer,

2012). However, there is very little information on the mechanistic relationship between size at transmission and survival to the next stage or reproductive/growth success. Only a few studies have examined the effects of factors operating during the larval period on measures of performance (Semlitsch et al. 1999; Álvarez and Nicieza, 2002; Benesh et al., 2012; Benesh and Hafer, 2012) and to my knowledge none of these evaluated the effects of developmental exposure to zinc or pollution on larval performance. Chapter 5 illustrated that individual *S. solidus* procercoids that grew larger in copepods (kept at 20 µg/L of zinc) when transferred into the next host, the three-spined stickleback fish, continued grow to a bigger size than those raised in deionised water. Put simply, parasites that were larger when entering a fish were also larger when recovered. The results presented in Chapter 5 appear to agree with other studies which showed that individual *S. solidus* tapeworms that grew larger in copepods they tended to grow to a slightly bigger size in fish (Semlitsch et al., 1988; Steinauer and Nickol, 2003; Benesh et al., 2012; Benesh and Hafer, 2012). In addition, the results presented in Chapter 5 appear to support the idea that, larvae that grow to a large size in the intermediate host generally have higher infection success in the next host (Rosen and Dick, 1983; Tierney and Crompton, 1992). For *S. solidus* procercoids, transmission to sticklebacks as a small larva has clear costs in terms of lower infection probability and stunted, inefficient growth. Given the seemingly strong selection for rapid growth and development in copepods, more work is needed to identify what prevents change in the ontogenetic schedule of *S. solidus*. In addition, if larvae prefer zinc that maximize growth and survival to the next stage (plerocercoid), approaches considering only the performance in the larval phase may undervalue adaptive behaviours. Developing the relationships between population-specific and environmental variables in the aquatic habitats will allow more mechanistic understanding of the impact.

6.2 Suggestions for future work

In Figure 6.1, I have highlighted the stages and variables that I have studied in this thesis. I have demonstrated that the survival of the free-living coracidium stage of *Schistocephalus solidus* is reduced with increasing temperatures, more work is required to determine how temperature affects metabolic activity of this stage. Free-living stage swimming activity is likely to be affected by the rate of metabolic activity and this can be altered by temperatures. Swimming speed or movement is important as it might influence transmission into next host.

This thesis demonstrates that the life-span of the free-living stage of *S. solidus* can differ markedly between lineages. It would be extremely interesting to investigate whether the environment affects differently different lineages or populations of the parasite. In this future work, molecular methods would be required to investigate the genotype for each worm cultured *in vitro* to determine how interactions between genotype and environment influence free-living stage survival.
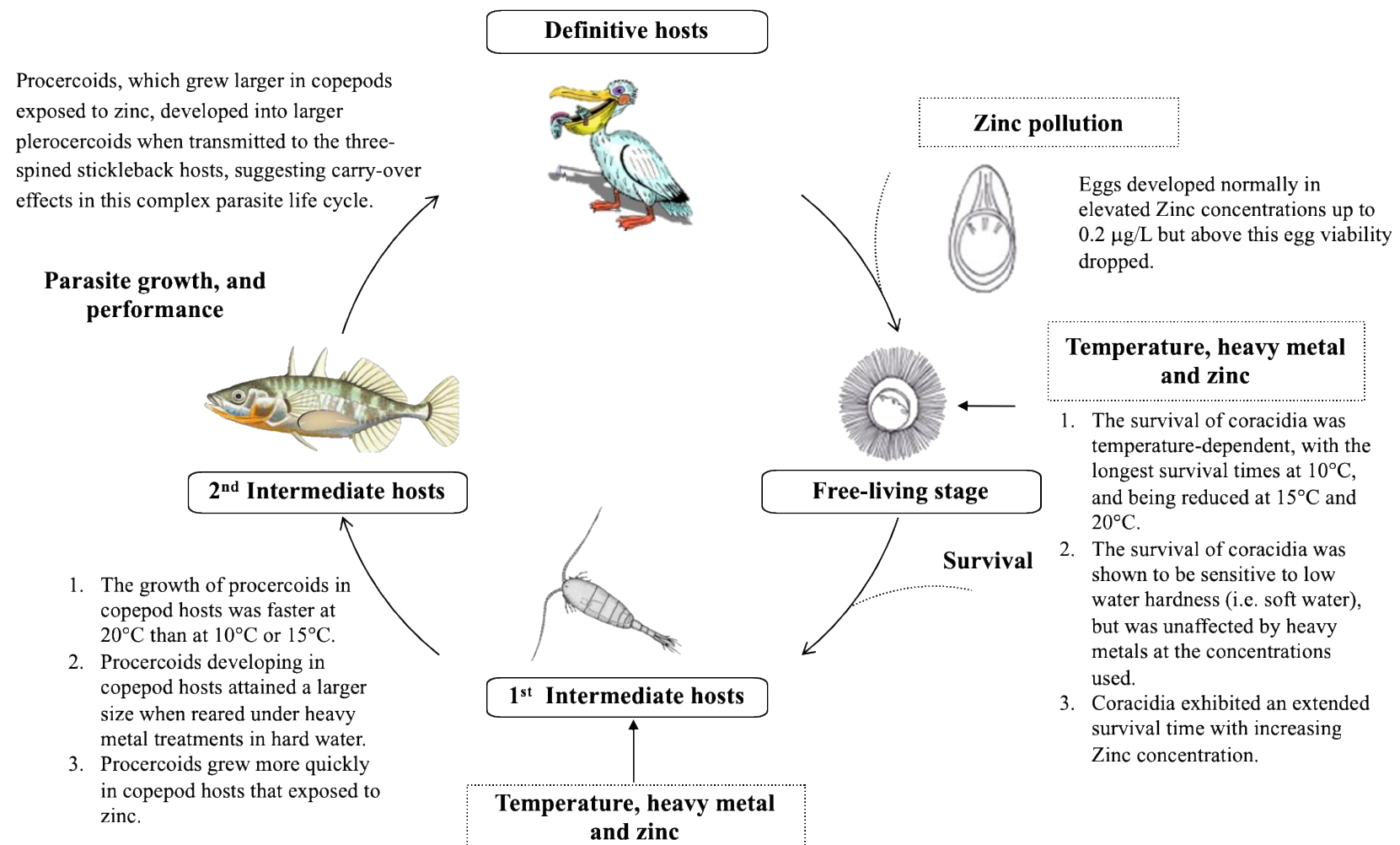
I have shown that temperature affects the development and infectivity of *S. solidus* procercoids in the first intermediate host. It would be interesting to investigate the effects of temperatures and age or stage (early or late) on the infective capacity. For example, *S. solidus* procercoids of different ages could be co-cultured with the first intermediate host to determine if age might affect the infection rate. This has been shown in a different parasite by Pechenik and Fried (1995) where temperature reduced the duration of infective period of young *Echinostoma trivolvis* cercariae by decreasing the infection in snails at higher temperatures.

An understanding of host-parasite interactions within polluted environments can contribute to ecotoxicological studies because parasites respond to the environment in different ways (Sures, 2004). I did not assess the amount of metal that accumulated in the free-living stage, in copepods (infected and non-infected), in infective stage of parasites or in fish. Future work should address this element to better understand how metals affect the host-parasite relationship in the *S. solidus* system. Determination of heavy metal accumulation in cestode parasites might serve as a useful indicator of contamination in the aquatic environment in addition to other invertebrates such as intestinal parasites of fishes, especially acanthocephalans (Sures, 2008) that are known to accumulate heavy metals from within their hosts.

6.3 Concluding comments

The conclusion of this thesis is that environmental pollution caused by anthropogenic activities has the potential to alter host-parasite interactions. These changes in host-parasite relationships could affect whether or not parasite life cycles are completed, or could affect the completion rate, through effects on intermediate, paratenic and final hosts.

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Definitive hosts

Zinc pollution

Procercoids, which grew larger in copepods exposed to zinc, developed into larger plerocercoids when transmitted to the three-spined stickleback hosts, suggesting carry-over effects in this complex parasite life cycle.

Eggs developed normally in elevated Zinc concentrations up to 0.2 µg/L but above this egg viability dropped.

Parasite growth, and performance

Temperature, heavy metal and zinc

2nd Intermediate hosts

Free-living stage

Survival

1. The growth of procercoids in copepod hosts was faster at 20°C than at 10°C or 15°C.
2. Procercoids developing in copepod hosts attained a larger size when reared under heavy metal treatments in hard water.
3. Procercoids grew more quickly in copepod hosts that exposed to zinc.

1st Intermediate hosts

1. The survival of coracidia was temperature-dependent, with the longest survival times at 10°C, and being reduced at 15°C and 20°C.
2. The survival of coracidia was shown to be sensitive to low water hardness (i.e. soft water), but was unaffected by heavy metals at the concentrations used.
3. Coracidia exhibited an extended survival time with increasing Zinc concentration.

Temperature, heavy metal and zinc

Figure 6.1 Schematic illustrating the life cycle of *S. solidus*, with environmental factors examined in the thesis.

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