Effects of host variation and environmental conditions on *Schistocephalus solidus* infections in sticklebacks

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Abstract

The outcomes of host-parasite interactions are potentially affected by both the genotype and phenotype of the hosts and parasites involved, and modulated by the environmental conditions under which they interact. Anthropogenic environmental changes therefore have the potential to shift the balance in host-parasite interactions, with consequences for disease processes. This thesis examines how host factors and environmental conditions influence the outcome of host-parasite interactions in the experimentally amenable three-spined stickleback-Schistocephalus solidus model.

When sticklebacks invade freshwaters, their lateral plate count typically reduces and becomes more variable. In a freshwater population with unusually high diversity in plate morphology, fish with fewer numbers of lateral plates were found to show increased susceptibility to experimental S. solidus challenge. Hypoxic conditions often arise in degraded environments and have the potential to interact with infection status. Schistocephalus solidus infected sticklebacks showed significantly reduced expression of genes (including GADD45 and LDHA) usually associated with the normal cellular hypoxic response of fish. These results suggest that S. solidus infections impair the normal cellular response to hypoxia, and may contribute to observed behavioural changes observed in infected individuals under hypoxic conditions. The results of a study investigating the effects of salinity on the development of S. solidus showed embryonic development was prevented at salinity greater than 20 ppt, indicating that the threat of infection is confined to brackish and freshwaters. Schistocephalus solidus therefore represents a novel selection pressure for sticklebacks colonising freshwaters from ancestral marine populations. Both the level of host food intake and host body size significantly affected the outcome of experimental parasite challenge. Experimentally manipulated host ration generated faster plerocercoid growth under greater levels of alimentation. The establishment of S. solidus plerocercoids was related to host size, with infections developing more frequently in smaller than larger sticklebacks. However, plerocercoids grew more quickly in larger hosts. Hence, environmental changes that affect host size, the timing of infections or the availability of food have the potential to influence parasite growth and life cycle completion rates.

The overarching conclusion of the thesis is that the biology of host-parasite interactions is highly susceptible to environmental changes, which can exert their effects through direct impacts on hosts, on parasites or on the interaction between them.
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Chapter 1

Introduction

Image courtesy of I. Barber ©
1.1 Host-parasite interactions

Parasites have evolved morphological or physiological adaptations to living in or on another organism, have a metabolic commitment to the host and the potential to decrease host fitness (Goater et al., 2013a). Some parasites exert extremely detrimental effects on their hosts, including mortality, morbidity and lowering fecundity (Clayton and Moore, 1997, Ebert, 2005). Because of their potential to negatively impact the health of humans and livestock, and the fitness of animals in natural populations, it is perhaps justifiable that these negative impacts of parasites have received the greatest attention (Minchella, 1985). However, some parasite infections are benign (Bollatti and Ceballos, 2014), and the outcomes of infection might vary considerably depending on a range of factors, including the specific genotype of host and parasite and the environmental context in which they interact. Understanding not only the typical, average or ‘normal’ outcome of a particular host-parasite interaction – but also how these outcomes differ depending on modulatory factors such as environmental conditions – are therefore of particular interest, as they have implications for host populations and the strength of parasite-mediated selection over evolutionary time (Poulin, 2007, Goater et al., 2013a). Disease causing agents – pathogens and parasites – are found in almost every population of free-living organisms. For hosts, a consequence of harbouring a parasite infection can be an alteration of host physiology or behaviour, which is not beneficial to the needs of the host. At the population level, parasites can reduce overall population size (Møller, 2005) and even change the course of evolution via parasite-mediated selection (Clayton and Moore, 1997, Mitchell, 2005). Increasingly the ecosystem consequences of infections are appreciated as a major driver of community structure (Lafferty, 2008). How severe these consequences are varies between host-parasite systems. For example bacterial coldwater disease of fish caused by pathogenic Flavobacterium psychrophilus causes rapid mortality (Starliper, 2010) while infection with gill lice Salmincola edwardsii in brook trout (Salvelinus fontinalis) results in a relatively benign infection with mortality occurring only in individuals with the heaviest parasite loads (Vaughan and Coble, 1975).

Significant research effort has been dedicated to predicting the impacts of climate change. However, despite parasites being ubiquitous they have received
relatively little attention compared to other aspects (Marcogliese, 2001). While studies examining solely climate change are invaluable for determining the relative importance of individual factors they do not represent an accurate model of the environmental heterogeneity encountered by natural populations. Indeed empirical studies examining interacting environmental effects have shown an exacerbation of pathology and mortality when infections and environmental change coincide (Tucker et al., 2000, Sirois and Dodson, 2000).

In parasites with multi-stage life cycles predicting how changing environments is likely to alter disease outcome is particularly complex. Free-living life cycle stages are directly vulnerable to physiological perturbations and therefore might be expected to be sensitive to environmental changes, with predicted consequences for their encounter rate with susceptible hosts. Equally, environmental effects that alter the survival, reproductive success or behaviour of intermediate host taxa may have repercussions both for their availability as hosts, and for the rate of exposure of subsequent hosts of the parasite. Once individuals have been exposed to parasites, external environmental factors could affect host parasite resistance or the infectivity of the parasite; for example, hosts in high quality environments (e.g. with high food availability) might be expected to have increased immune function (Kolluru et al., 2006). Even after the parasite has become established in a host, the growth and survival of host and parasite are potentially impacted by local environmental conditions (Macnab and Barber, 2012).

Building on this conceptual overview, I have considered the theoretical implications of environment in each of these infection stages in greater detail whilst reviewing the empirical evidence available (Figure 1.1).
1.2 Parasite life stages and effects of environment

1.2.1 Environmental effects on parasite encounter rate

The likelihood that a parasite will become established in a host is determined by the encounter rate in the environment, and it can be reasonably predicted any increase in encounter rate is expected to increase infection levels in susceptible hosts.

1.2.1.1 Release of infective stages

Environmental conditions can exert control over encounter rate, for example via the release of infective stages increasing parasite density and therefore encounter rate. Such a scenario was proposed by Thuy et al. (2010) who suggested temperature-dependent release of trematode cercariae from snails may explain increased prevalence of metacercariae in the Sutchi catfish (*Pangasianodon hypophthalmus*) in the rainy season. Höglund and Thulin (1990) showed that the release of thermal effluent from a power plant can cause short term increases in *Diplostomum* sp. infection levels in one year old perch (*Perca*
through an increase in the relative density of metacercariae, although fish from the unheated and heated treatments had similar parasite densities after two years indicating a possible role of host mortality in this system. In a study by Studer et al. (2010) the release of cercariae of the trematode *Maritrema novaezealandensis* from the snail host *Zeacumans subcarinatus*, to subsequently infect amphipod (*Paracalliope novizealandiae*) hosts, was also temperature dependent. Here, the release of cercariae was greatest at intermediate temperatures of 20-25°C rather than at extreme temperatures, although cercariae activity increased with temperature suggesting that encounter rates might be highest at the intermediate temperatures due to an interaction between density and activity effects (Studer et al., 2010). While the authors established that host susceptibility to infection was unaffected by temperature, the overall effect of high cercariae density on infection prevalence was not evaluated, and although it can be assumed that increased number would lead to increased infection evidence to confirm this is still absent.

The spatial distribution of infective stages can also have a profound effect on parasite transmission. In aquatic ecosystems, flooding can aid dispersal of free-living stages of parasites, enabling wider exposure. For example Thuy et al. (2010) suggested that seasonal rises in temperature coincide with flooding, distributing infected snails and accounting for the increased prevalence of trematode metacercariae (*Haplorchis pumilio, H. taichui, Centrocestus formosanus* and *Procerovum* sp.). Flood aided transmission has also been shown in the digenean parasite, *Posthodiplostomum cuticola*, in a complex snail-fish-bird life cycle (Ondrackova et al., 2004).

Johnson et al. (2007) reported eutrophication can increase the release of trematode (*Ribeiroia ondatrae*) cercariae from snails. High parasite load and nutrient levels in the water produced the greatest number of infected snails with a higher rate of cercariae release per snail leading to a higher incidence of infected amphibian definitive hosts. This is in part due to high nutrient loads increasing availability of algae food for snail hosts which increases the number of snail intermediate hosts. In addition higher food availability increases snail growth, and larger snails can release more cercariae at a faster rate. The case of eutrophication and *R. ondatrae* is interesting because it exemplifies the
complexity of environmental variables on multi-host life cycles. Eutrophication is expected to attract the definitive host bird due to abundant prey which will lead to further disease transmission both locally and to other populations, resulting in higher rates of infection and associated malformations in the amphibian intermediate hosts (McKenzie, 2005, McKenzie and Townsend, 2007).

1.2.1.2 Host density

Encounter rates between hosts can be increased with host density for parasites where horizontal transmission is a means of infection (Rohde, 1991). In coral reef fish (Haemulon flavolineatum) the establishment of monogenean parasites (Haliotrema sp.) in the population was higher at increased stocking density (Sasal, 2003), although it is not clear if this was due to increased transmission or crowding stress of the host. High stocking density is of particular concern in aquaculture, and due to the potential economic and ecological consequences of infection has received much attention (Johnson et al., 2004). Infection of sea lice (Lepeophtheirus salmonis) on salmonids increases with stocking density, and has been shown to increase in fish farms which are surrounded by a high density of farmed salmonids (Jansen et al., 2012). Although these commercial settings are artificial they do effectively mimic high host abundance in the wild. Similar results were found for natural populations of piranhas (Pygocentrus nattereri, Serrasalmus spilopleura, Serrasalmus marginatus, Osteichthyes: Serrasalminae) where branchiuran parasite density is higher in the dry season, and water levels are lower fish are brought into close proximity, facilitating transmission and increasing stress which increases susceptibility to infection (Carvalho et al., 2003).

One mechanism which could increase host density in changing environments is the stratification of lakes (Ficke et al., 2007). The uppermost layer of a lake, the epilimnion, is directly heated by sunlight and is oxygenated by diffusion and turbulence at the water surface. The lower hypolimnion has low light exposure and lower oxygen levels. Differential density prevents the layers from mixing, and is exacerbated by higher temperatures at the surface which are predicted to occur more frequently and for long with changing climates. For some species of fish which have limited heat and oxygen tolerances, we might also predict that as temperatures rise and the concentration of dissolved oxygen lowers fish may be
forced into higher densities (Figure 1.2), facilitating exposure to parasites (Marcogliese, 2001). Experimental evidence for this comes from a study tracking the movements of striped bass (*Morone saxatilis*) in a reservoir, Cheek et al. (1985) reported that fish had wider distribution during winter compared to summer due to temperature and dissolved oxygen limits.

![Figure 1.2](image-url) Illustration of predicted climate changes effects on lake stratification. Higher temperatures widen the epilimnion, and higher metabolic rates of fish consume more oxygen, leading to a narrowing of pelagic habitat and increased fish density. Adapted from Ficke (2007).

For parasites with complex multi-host life cycles, their survival and reproductive fitness is not only determined by their ability to encounter and invade one host, but several in succession. Environments favourable to the intermediate hosts, serving to increase host abundance would also aid transmission both by increasing host density for parasite infective stages and encounter rate for the next host. Conversely, a decline in intermediate host populations may also cause a reduction in parasite infections in definitive hosts. By examining data over a 30 year period, Khan et al. (2008) identified a decrease in temperature coincident with a decline in the population size of capelin (*Mallotus villosus*), a primary food
source for Atlantic cod and a host in the life cycle of *Echinorhynchus gadi* (Acanthocephala). Infection prevalence in the cod increased when capelin abundance recovered, thus indicating climatic variables were closely linked to the acquisition of parasites via predation. Elevated production caused by nutrient input from sewage effluent was found by Marcogliese and Cone (2001) to have increased the prevalence and mean number of myxozoan parasite species per host, the spottail shiner (*Notropis hudsonius*). In a further study, Marcogliese et al. (2009) suggested that high densities of the intermediate host oligochaete *Limnodrilus hoffmeistereri* downstream may have increased the exposure of spottail shiners (*Notropis hudsonius*) to myxozoan parasite species (inc. *Myxobolus bartai*) and caused local increases in parasite prevalence. Therefore effects of environment may indirectly affect parasite infection by acting on a different stage of the life cycle.

High nutrient availability benefiting the parasite rather than the host, which can lead to higher encounter rates of the disease or of the intermediate host, have been shown in a number of systems. McKenzie and Townsend (2007) surveyed examples of anthropogenic nutrient pollution, across parasite species including helminths, protozoans, bacteria, viruses, fungi and myxozoans. Increasing the nutrient input into aquatic systems was considered likely to increase disease risk; of 55 different parasite infection studies they surveyed, 51 exhibited a positive response to higher nutrient levels. McKenzie and Townsend (2007) explained the relationship by suggesting higher nutrient levels either gives more resources to the parasite directly, as in bacterial diseases such as *Streptococcus agalactiae* of Wild Mullet (*Liza klunzingeri*) (Glibert et al., 2002), or increases the resources available to intermediate hosts and thereby increasing exposure to infective stages, such as *Schistosoma* sp. infections of snails (Lindblade, 1998).

### 1.2.1.3 Timing of exposure

The life cycles of multiple host parasites, and the timing of key life events, have evolved to take advantage of the natural ecology of their hosts, and as such transmission events are often timed to ensure that parasite infective stages are released during periods when hosts are most abundant and / or susceptible (Kearn, 1998). This evolutionary ‘fine tuning’ of parasites to their host’s biology can lead to potential problems for parasites when environmental change alter the
life history of the hosts and parasites differentially, such that they do not overlap. By using the parasite model *Ribeiroia ondatrae* infecting snails and amphibians in a mesocosm experiment, Paull and Johnson (2014) demonstrated that warming by 3°C caused the intermediate host snail to release cercariae 9 months earlier. Early release of parasite infective stages leads to a mismatch in timing of the parasite and host amphibian abundance and consequently a reduction in parasite loads and associated pathology (Paull and Johnson, 2014). Mismatches can also occur spatially as environmental change causes differential distribution of the parasite and host (Lafferty, 2009). For example by modelling distributional shifts in the larval stages of the nematode *Parelaphostrongylus tenuis* compared to the intermediate and definitive hosts, Pickles et al. (2013) predicted a mismatch between host and parasite. The range of the definitive host white-tailed deer (*Odocoileus virginianus*) was predicted to expand, while the parasite range was shifted away from drier climates such as the Great Plains, indicating that infection would be predicted to decrease in some areas of the hosts range while in others the parasite might be able to invade with its host (Pickles et al., 2013).

A shift in climate may lengthen the exposure period, as is the case in a nematode-snail system. Kutz et al. (2005) demonstrated a rise in temperature of 1°C caused the development of the parasite to shift from a two to a one year cycle, therefore removing the high overwinter mortality and causing the definitive host musk-ox (*Ovibos moschatus*) to be exposed to increased infective stages. Exposure periods may also be shifted to earlier in the year. For example for the much studied *Ribeiroia ondatrae* parasite, increased temperatures caused parasite eggs to develop more rapidly which is predicted to cause intermediate host snails (*Planorbellada trivolvis*) to be exposed to the parasite earlier while they are smaller leading to higher susceptibility (Paull and Johnson, 2011). Therefore in multistage lifecycles the effects of environment can be complex, and only be understood by examining all stages in the life cycle.

1.2.2 Environmental effects on host susceptibility and parasite infectivity
The ability of hosts to resist infection, and conversely the ability of parasites to overcome mechanisms of host resistance, will determine if an infection
establishes and persists. Therefore, any perturbation of host immunocompetence and/or parasite infectivity potentially causes a shift in parasite prevalence. Environmental perturbations have been demonstrated to affect host defences in a wide range of taxa. In the mesograzer *Idotea baltica*, Roth et al. (2010) demonstrated that phagocytosis – a key innate immune response of invertebrates – was reduced by 50% under simulated extreme heat conditions. Over the long term, parasite mediated selection can cause the host population to adapt to increased parasite pressure. In Atlantic salmon (*Salmo salar*), variation in the Major Histocompatibility Complex (MHC) region of the genome, which is associated with immunocompetence and encodes cell surface proteins in vertebrates, was found to increase in diversity with higher temperatures. This may reflect the greater diversity and virulence of pathogens in these regions, exerting selection pressure on their hosts by requiring a broader immune response (Dionne et al., 2007).

Susceptibility to infection may also be dependent on the level of host nutrition, where energy intake provides resources for immune responses. In *Daphnia magna* exposed to the bacterium *Pasteuria ramosa*, feeding hosts on a diet with a lower phosphorus content reduced the prevalence of infection (Frost et al., 2008), indicating that poor diet hindered the growth of the parasite. Bize et al. (2008) showed the ectoparasitic louse fly *Crataerina melbae*, took smaller blood meals from food deprived, and consequently immunosuppressed, nestling hosts (Alpine swift, *Apus melba*). Although susceptibility was higher in food deprived individuals, parasites were sensitive to host nutrition and moderated their own energy intake. When the physical nature of environments change, the infectivity of parasites can be altered, as is illustrated by the ciliate *Ichthyophthirius multifiliis* – the causative agent of white spot disease in fish. Price and Clayton (1999) took common carp (*Cyprinus carpio*) reared at 16°C and reared them separately at 16°C, 19°C and 24°C, followed by exposure to different strains of *I. multifiliis*. Infection levels increased with temperature in the tropical strain of the parasite, but decreased with temperature in the temperate strain showing local environmental adaptation by the parasite. This result demonstrates the principle that coevolution of hosts and parasites can result in differential infection outcomes in different environments.
1.2.3 Environmental effects on parasite life histories

1.2.3.1 Temperature

Parasites can be envisioned as organisms living in one environment (provided by the host) embedded within a wider environment (provided by the ecosystem that the host lives in) (Smith and Holt, 1996). Whether or not the parasite experience the same external environment as its host depends on a range of factors, including whether or not the parasite is ecto- or endoparasitic, and for endoparasites, on the buffering capacity offered by the host. If we consider temperature, in ectothermic hosts the parasite experiences the same environmental conditions as the host and may therefore be expected to be adapted to local temperature regimes. However, in the stickleback-\textit{Schistocephalus} system, recent experimental work has demonstrated that hosts and parasites benefit differentially from an elevation in temperature (Macnab and Barber, 2012). Whereas hosts grew better at lower temperatures, parasites grew better at higher temperatures, and in fish held at 20°C plerocercoids attained a fourfold increase in growth compared to over those reared in fish held at 15°C. Furthermore, infected fish exhibited different temperature preferences; hosts harbouring infective parasites preferred warmer temperatures, which benefitted parasites, suggesting adaptive manipulation of behaviour (Macnab and Barber, 2012). Faster growth rates would mean parasites reach infective size in less time, increasing the likelihood of completing the life cycle. In addition, as adult parasite fecundity is proportional to plerocercoid size in this system (Dörcü et al., 2007), increases in temperature are predicted to increase the input of infective stages into the environment.

Differences in the optimum temperature for the host and parasite have been observed in other host-parasite systems. This can be evident in early development of the parasite, for example in a study on infections by the protist \textit{Perkinsus marinus} of oysters (\textit{Crassostrea virginica}) which can cause high mortality of the host, La Peyre et al. (2010) manipulated temperature and salinity in order to test the conditions which would end an epizootic and control the spread of the disease. At combined low temperature and salinity, cell viability was reduced, indicating that proliferation of the parasite is limited by combined effects of salinity and temperature, benefitting the host (La Peyre et al., 2010).
Conversely, at high temperature and salinity Atlantic salmon infected with salmon lice *Lepeophtheirus salmonis*, copepodids are able to benefit from increased settlement and development rates (Tucker et al., 2000). For the monogenean parasite *Dendromonocotyle pipinna* infecting the blotched fantail ray (*Taeniurus meyeni*), increased temperatures increase the speed of egg hatching, although this does not affect hatching success (Chen et al., 2010). For the eye fluke *Diplostomum spathaceum* infecting rainbow trout (*Oncorhynchus mykiss*) cercarial shedding increased from 10000 cercariae / snail / d at 10°C to 58000 cercariae / snail / d at 20°C, showing a strong temperature dependence (Lyholt and Buchmann, 1996). The ability of the bacterial parasite *Pasteuria ramose* to infect its *Daphnia* host is increased at high temperatures followed by an increased growth rate (Mitchell, 2005). Ectoparasites such as *Neobenedenia girellae* infecting amberjack fish (*Seriola dumerili*) also exhibit temperature sensitivity by achieving a greater size at 30°C than both 25°C and 20°C at 16 days post-exposure (Hirazawa et al., 2010). In a novel experiment, Bates et al. (2010) tested the response of the ciliated protozoan *Orchitophrya stellarum* to temperature, both in isolation and in association with host starfish species (*Asterina miniata* and *Pisaster ochraceus*). The protozoan parasite feeds on sperm from the testes of infected starfish and is associated with reduced reproductive output. In this study, ciliate densities of the free-living parasite in isolation were considerably higher in the warmer treatment. In tests on infected hosts, infections were more prevalent and more advanced in the warmer treatment as testis mass was shown to be reduced in size in the 15°C treatment compared to the 10°C, showing that higher temperatures not only facilitate increased parasite density, but also increase reproductive costs for the host (Bates et al., 2010).

Some parasites only develop over a threshold temperature, for example *I. multifiliis* only develops in fish at temperatures over 16°C, which opens the possibility that rising temperatures might increase disease risk for some pathogens (Marcos-Lopez et al., 2010). Increased temperatures have also been shown to increase metacercariae accumulation rates of *Diplostomum baeri* in Perch (*Perca fluviatilis*) (see Höglund and Thulin, 1990). Therefore changing thermal regimes have the potential to dramatically widen both a parasites
geographical range and its temporal infection period, allowing exposure to novel populations and develop new host-parasite relationships. This idea was tested by King et al. (2009) who transferred *Gyrodactylus bullatarudis* from the guppy (*Poecilia reticulata*) to the three-spined stickleback at both 15°C and 25°C. *G. bullatarudis* was able to transmit and survive on both hosts at both temperatures, but only reproduced under the higher temperature conditions. As the guppy has expanded in its range as environmental temperatures increase (King et al., 2009), the implication is that novel host-parasite associations may become more common.

The link between temperature and parasite life history may not always be so straightforward. Studer et al. (2010) reported maximum metacercariae development rates of the trematode parasite *Maritrema novaezealandensis* and associated host mortality of the amphipod host *Paracalliope novizealandiae* at the intermediate temperatures of 20°C and 25°C than at 16°C and 30°C. By speeding up the parasite life cycle and increasing the numbers of infective stages in the system, the overall effect of parasite infections in the ecosystem could be further exacerbated.

When temperature effects on parasites are tested in the field, the results are more complex, depending on the species. When examining the effect of thermal discharge on winter flounder flatfish (*Pleuronectes americanus*), Khan and Hooper (2007) found the prevalence of ectoparasites differed between sites further away from the power station and the site where the warm water was discharged. Infection with the trematode *Cryptocotyle lingua*, which utilises the periwinkle (*Littorina littorea*) as its intermediate host, was higher in and abundance at the site affected by warm water effluent from the power station. The abundance of the parasite is known to increase in warmer waters (Sekhar and Threlfall, 1970), therefore – perhaps unsurprisingly – the mean abundance of *C. lingua* was greater at the warmer site than those further from it. As predicted, ectoparasites (such as *Gyrodactylus* and *Steringophorus*) which in this region favour cooler temperatures were lower in abundance. Lafferty (1997) surveyed the effects of environments on parasite infections, including warm water effluent. The effects of warm water effluent varied dramatically, in part due to the diversity of parasite responses. Eutrophication was found to have a positive effect on most
groups parasites overall, including cestoda, nematode and acanthocephala (Lafferty, 1997).

From the evidence available to date, it seems a plausible prediction is that changing environments have considerable potential to affect the timing and speed of key events in parasite life cycles, and often this will be of advantage of the parasite. Potentially a shift towards warmer temperatures, particularly warmer winters, may mean that the risk of acquiring some infections will no longer be restricted to the summer months, but may occur all year round (Marcogliese, 2001).

The increased development and growth of parasites at higher temperatures may create the need for hosts to make a behavioural trade-off; while being in warmer temperatures may exacerbates infection, being at lower temperatures could limit the host’s other essential physiological functions. This principle has been experimentally tested in *Daphnia magna* infected with *Pasteuria ramosa*. Here, the hosts suffered the costs of increased parasite growth at high temperatures, but reduced fecundity at low temperatures (Allen and Little, 2010). Host sensitivity to temperature may also produce a cost to the parasite, for example ambient temperature can have a direct influence on host body size, and for endoparasites this could restrict growth (Randhawa and Poulin, 2009).

**1.2.3.2 Host nutrition**

The availability of host nutrition can be a critical factor in determining parasite growth rates and reproduction. The outcome of the competition for host resources between hosts and parasites will influence both the costs of parasitism for the host and the benefits gained by the parasite. Equally, hosts in favourable nutritional condition potentially offer an abundance of resources to parasites, allowing parasites to grow and reproduce at elevated levels without the risk of killing the host. In *Aedes aegypti* mosquitos infected with the microsporidian *Vavraia culicis*, increased host nutrition allows parasites to elevate spore production (Bedhomme et al., 2004). Within the copepod host *Schistocephalus solidus* grows larger under higher feeding regimes, however the developmental time scale is unhindered (Benesh, 2010). This may be reflective of the need for
the parasite to reach infectivity as quickly as possible, although smaller body size may carry a cost in the next (stickleback) host.

In a study investigating the effect of feeding regime on the immune system and parasite infection of Sea Bream, Sitjà-Bobadilla et al. (2003) found that restricted feeding regimes did not negatively impact immunity and respiratory burst activity was actually higher in the restricted feeding group compared to those fed to satiation. The overall prevalence of parasites increased when sea bream were fed to satiation rather than on a restricted feeding regime, particularly for the endoparasites *Ceratomyxa sparusaurati* and *Leptotheca sparidarum*. Although conversely the opposite effect was observed on coccidian *Cryptosporidium molnari* which infects gut epithelium (Sitjà-Bobadilla et al., 2003). A similar pattern was observed in guppies with a delayed increase in parasite growth in the high food ration group leading to an eventual elevated parasite prevalence in this group compared to their low ration counterparts (Kolluru et al., 2006).

1.2.4 Environmental effects on free-living parasite stages

Intuitively we might predict that without a host to shield from the immediate environment, parasites would be more sensitive to changes in environmental conditions. Additionally, parasite free-living stages are without a host to provide resources and therefore must survive on their own reserves until a susceptible host becomes available, meaning harsh environmental conditions could diminish the chances of an individual parasite surviving to make this transition. Indeed, Blanar et al. (2009) were able to show that free-living parasite stages are in general more sensitive than parasitic life cycle stages to pollutants. Although in general parasite growth rates are higher at higher temperatures the limits of this are variable between and within taxa (Kutz et al., 2005, Hudson et al., 2006). In *Sphaerothecum destruens*, the causative agent of rosette disease in fish, lower temperatures reduce the development rates of the parasite sufficiently to delay the production of zoospores, and these temperatures also lengthen host lifespan (Andreou et al., 2009). The cercariae of trematodes exhibit differing responses to temperature, with philophthalmid cercariae exhibiting increased emergence with increasing temperature, and microphallid cercariae showing the opposite pattern (Koprivnikar et al., 2010).
Overall it can be observed that although the optimal temperatures for free-living stages are variable and species-specific, environmental perturbations away from the optimum typically have negative effects on survival and infectivity. There appears to be a trade-off for free-living stages between increased developmental and activity rates experienced at high temperatures, which typically reduce longevity and survival. It is also true that environmental sensitivity varies between taxa due to diversity in morphological structures in parasite free-living stages, for example the coracidia produced by pseudophyllidean cestodes appear to be more vulnerable to temperature changes than trematode cysts (Pietrock and Marcogliese, 2003).

1.2.5 Summary
The outcome of a host-parasite interaction is clearly affected by host factors such as nutrition and body size, parasite factors such as genotype and free-living stages, and the environment(s) in which the interactions between the two organisms are being played out. To what extent individual environmental factors are involved in modulating host-parasite interactions is, however, typically poorly understood, and there is a general lack of empirical data from controlled lab experiments. Developing a better understanding of how disease processes and host-parasite interactions are likely to be affected by changing environments can be facilitated by identifying which environmental variables are important. The value of these studies can be maximised by using an established host-parasite system.

1.3 The model host: the three-spined stickleback
1.3.1 Stickleback distribution
The three-spined stickleback is a teleost fish that is commonly found in a wide range of aquatic habitats, including reservoirs, rivers, lakes and ponds. Although the stickleback is ancestrally a marine species, and indeed there still extant populations of three-spined sticklebacks in marine environments, since the last Pleistocene glaciers retreated repeated colonisations of freshwater habitats have occurred across the northern hemisphere (Bell and Foster, 1994b, Barber, 2013). There are several species in the stickleback family (Gasterosteidae, Teleostei) spread over the northern hemisphere above 35°N. These include the brook
stickleback (*Culea inconstans*) and the four-spined stickleback (*Apeltes quadracus*) which are only found in North America, and the marine fifteen-spined stickleback (*Spinachia spinachia*), which is limited to the coastal waters of Europe. The most widespread genera are *Pungitus* and *Gasterosteus*, which are found on all three northern continents. Both the black spotted stickleback (*Gasterosteus wheatlandi*) and Ukrainian stickleback (*Pungitus platygaster*) are limited in range to the Atlantic seashore of North America and the European rivers systems of the Black Sea area respectively. However, the nine-spined stickleback (*Pungitus pungitius*) is more widespread, occurring on all three northern continents and is commonly sympatric with *Gasterosteus*, although has a more northerly distribution. *Gasterosteus aculeatus* is found on all three northern continents, where it is confined to relatively coastal areas. In Europe *G. aculeatus* occurs as far south as Spain, Italy and the Balkan Peninsula and stretches north to Iceland and the White Sea. While in Asia *G. aculeatus* is found in Japan and Korea, and in North America is found on the east coast from Alaska down to California and on the west coast from Hudson Bay down and inland to Lake Ontario (Wootton, 1984).

The three-spined stickleback has been has risen to prominence as a biological model, and this fish will be the focus of this thesis. The three-spined stickleback has a long history as a model for behaviour, ecology and evolution. It is this rich background which makes the three-spined stickleback particularly useful as a parasitological model, as many aspects of the host biology are well established (Barber, 2013). The three-spined stickleback is also ecologically relevant as species found in a wide range of habitats, making it an excellent model of environmental change (Katsiadaki, 2007). In addition the vast genetic and phenotypic variation found naturally in the wild make a fascinating subject for evolutionary studies.

### 1.3.2 Stickleback biology

Firstly, I will describe briefly relevant aspects of the biology of the three-spined stickleback, given that the literature on this subject forms the basis of research contained in the following chapters. Populations of three-spined stickleback are described as marine, anadromous or freshwater. The reproductive biology of
sticklebacks requires that the male builds a nest on the substratum in shallow water; therefore while some populations may spend some of their life in marine open waters they must return to the coast or freshwater to breed (Wootton, 1976). While body size varies between populations, the average size is approximately 5cm standard length (SL) (Bell and Foster, 1994a). The stickleback does not have scales unlike many teleost fish, and instead has a skin covered by a thin cuticle layer. Colouration is variable between populations, but varies in tones between black and silver of the pigment cells in the skin. Males typically develop red nuptial colouration along the ventral surface of the head and trunk as they approach the breeding season. Males also exhibit a series of complex behaviours to encourage the female to lay eggs in the nest, after which the male is responsible for parental care. These courtship behaviours which can be used as a measure of reproductive condition include zig-zag movements, dorsal pricking, leading to the nest and biting. Females in reproductive condition can be identified by abdominal distension towards the vent (Wootton, 1976).

1.3.3 Three-spined stickleback and optimal foraging

The prey of the three-spined stickleback is mainly zooplankton (e.g. Crustacea, Copepoda and Cladocera) and benthic invertebrates, including the larvae of chironomids, and although the specific prey species may vary between populations and season, they still fall into these broad categories as the stickleback is restricted in the prey it can take by its small size. The energy gained from each of these prey items in terms of dry weight is approximately the same, although the profitability of prey items (measured as energy gain per unit time) will vary depending on ease of detection and capture and water content (Hart and Gill, 1994). Therefore, energy is limited by both food availability in the environment and physical limitations such as handling time and stomach capacity (Hart and Gill, 1994). The energy gained from prey must also be balanced with the energy requirements of maintenance such as metabolism and swimming activity, and tissue growth including somatic growth, energy storage and reproduction (Wootton, 1994). The relative balance of investment in these energy allocations should maximise survival and production of offspring, and therefore are reflective of the environment of the individual both in the short and long term. For example in the short term fish which are well fed and do not have high energy
requirements would be expected to have higher liver mass and body condition (Tierney et al., 1996), while fish with high energy demands such as high metabolic rates or parasite burdens would have less energy to invest in growth, and instead invest in maintenance to ensure survival (Barber et al., 2008). In the longer term, fish which are consistently under pressure from size-selective predators might be expected to invest in somatic growth to avoid mortality (Reimchen, 1992, Reimchen, 1994). Therefore, in the three-spined stickleback measuring energetic investment can yield interesting information about the overall energy budget and the extent of pressure on the individual.

1.3.4 Morphological variation in the three-spined stickleback

The three-spined stickleback has a complex series of polymorphisms (Wootton, 1984) across its distribution making it a species of research interest from an evolutionary perspective. This phenotypic diversity is generated by a wide geographic distribution, inhabiting marine, brackish and freshwater followed by isolation or semi-isolation of populations (Bell and Foster, 1994a). There are a series of morphological structures which sets the three-spined stickleback apart from most other teleosts, commonly referred to as ‘bony armour’. These include the three dorsal spines, two of which are prominent and serrated, one pelvic spine on both side, and the lateral plates. The pelvic and dorsal spines lock in place (Hoogland, 1951), offering protection against predation by increasing handling time offering opportunities for escape (Reimchen, 1983). At their maximum extent, typically observed in marine populations, the lateral plates form a continuous row along either side of the fish, running from the pectoral girdle, also referred to as the cleithrum, along the abdomen to the caudal peduncle. There is a well-developed pelvic girdle, which – in addition to the dorsal spines supported by the lateral plates – forms the armour that protects the stickleback from puncturing injuries from toothed predators (Hoogland et al., 1956, Reimchen, 1983, Reimchen, 1994). These lateral plates begin to develop when the fish is around 13 mm long, although this is variable between populations, and they are not fully developed until the fish is approximately 30 mm (Wootton, 1976). The number of lateral plates and other armour morphologies is highly variable between populations, and is subject to continued research interest (Bell et al., 1993, Marchinko and Schluter, 2007, Bell et al., 2010, Spence et al., 2013,
Recently, the genetic basis of plate phenotypes has been elucidated, and is determined by variation in the Ectodysplasin gene (Peichel et al., 2001, Colosimo et al., 2005).

### 1.4 Parasitism and the three-spined stickleback

#### 1.4.1 Parasite diversity

The three-spined stickleback typically harbours a diverse range of parasites, revealed by surveys of fish parasite (Barber, 2007). In one such survey, Zander et al. (2002) examined fish of the Baltic Sea including nine-spined sticklebacks (*Pungitius pungitius*), common goby (*Pomatoschistus microps*) and European flounder (*Pleuronectes flesus*), and found that three-spined sticklebacks harboured both a broader range of parasites and a greater intensity of infection.

Parasite diversity is in part due to the widespread distribution of the three-spined stickleback across a range of environments meaning it frequently encounters generalist parasites. There are also a number of stickleback specialists which require the three-spined stickleback as a host in at least one stage of the life cycle. Co-infections of multiple parasites are common, and reflect the habitat and foraging preferences of the individual (Poulin et al., 2011). As there are such a diverse range of parasites recorded, I will concentrate on those which are commonly encountered in the populations studied in the following chapters.

#### 1.4.1.1 *Diplostomum sp.*

Infections by *Diplostomum* sp. (Platyhelminthes, Digenea, Echinostomata, Strigeata) metacercariae are often found in the eye of three-spined sticklebacks, leading to the common name of ‘eye fluke’. *Diplostomum* sp. infections are widespread among freshwater fish, infecting over 100 species worldwide (Karvonen, 2011). In three-spined sticklebacks, two of the most commonly recorded species are *Diplostomum gasterosteii*, which is found in the retina, and *D. spathaceum*, which uses the lens of the eye as the site of infection (Kalbe et al., 2002). Infections by *Diplostomum* sp. have been shown to impair foraging behaviours as the parasites reduce the visual acuity of the host fish (Owen et al., 1993), and cause cataracts in the lens (Seppälä et al., 2011, Goater et al., 2013b). The parasite life cycle is typical of a trematode, with the definitive host a
piscivorous bird, eggs passed in the faeces where the miracidia hatch in water. The miracidia actively seek the intermediate host, a freshwater snail (commonly *Lymnaea* sp.). Sporocysts are formed by asexual reproduction containing cercariae which are released from the snail and penetrate the epithelium of the host fish whereupon the parasite migrates to the eye (Karvonen, 2011). Given the manner of the transmission of *Diplostomum*, infections in fish reflect habitat use of the host. In the benthic-limnetic sympatric pairs of three-spined sticklebacks found in British Columbia, limnetic sticklebacks were found to have a higher intensity of *D. scudderii* infection than benthics (MacColl, 2009).

1.4.1.2 *Gyrodictyulus* sp.
The gyrodactylids are ectoparasites of marine and freshwater fish, and can cause severe host mortality, such as the epidemic of *Gyrodictyulus salaris* in Norway during the 1970s (Bakke et al., 2007). *Gyrodictyulus* (Platyhelminthes, Monogenea) is found on all external surfaces of the host stickleback including the skin, gills and fins (Özer et al., 2004). While *Gyrodictyulus* is usually transferred by close contact of hosts, the parasite is capable of surviving independently for short periods (Cable et al., 2002). After transmission, *Gyrodictyulus* is capable of rapid increases in number owing to a viviparous reproduction system, most notable during the fish breeding season when opportunities for transmission are high. Once established on the host gills, the parasite anchors itself using the characteristic hooks on the opisthaptor (Goater et al., 2013b).

1.4.1.3 *Diphyllobothrium* sp.
Diphyllobothriid cestodes infect birds, mammals and humans (Goater et al., 2013b). *Diphyllobothrium* sp. (Platyhelminthes, Cestoda, Pseudophyllidea) are found in the body cavity and viscera of three-spined sticklebacks in the populations under study, indicating the species is likely to be either *Diphyllobothrium dendriticum* or *D. ditremum*, which are commonly considered to have a reduced impact on host biology than *Schistocephalus solidus* (see Barber, 2007). As with many indirectly transmitted endoparasites, frequency of *Diphyllobothrium* in three-spined sticklebacks reflects feeding activity (Morozinska-Gogol, 2002). Co-infections between *S. solidus* and *Diphyllobothrium* sp. are common as both infections are acquired by eating
infected copepods and require a definitive host bird for reproduction (Zander et al., 2002).

1.4.2 Schistocephalus solidus

1.4.2.1 Life cycle

Plerocercoids of *Schistocephalus solidus* (Platyhelminthes, Cestoda, Pseudophyllidea) are a common parasite of three-spined stickleback, *Gasterosteus aculeatus*, occupying still or slow flowing freshwaters throughout the range of the host fish (Wootton, 1976, Barber, 2007, Barber et al., 2008). *S. solidus* is an indirect trophically transmitted parasite, which requires multiple hosts to complete its life cycle. The parasite infects sticklebacks after the ingestion of an infected cyclopoid copepod. Infections are timed such that the stage that procercoids are fully developed in the copepod, and ready to move to the next host, coincides with when the three-spined stickleback is at optimal size for predation on copepods. The exact timing of exposure varies between populations, with some researchers finding an increase in prevalence in May (Pennycuick, 1971a, Morozinska-Gogol, 2002), but in local populations infections appear to be acquired in young of the year fish in late summer, leading to externally evident plerocercoids later in the year (unpublished data. Simmonds, N., Figure 1.3).

![Prevalence of Schistocephalus solidus infection among young of the year three-spined sticklebacks in the River Welland, Leicestershire (52°48 N.,-0°92 W.), May to December 2012](image-url)
After being ingested by the three-spined stickleback, the parasite then penetrates the gastro-intestinal tract to develop into a plerocercoid within the fish body cavity, acquiring nutrients through the fish gut. The parasite can grow up to 50% of the total infected fish weight over approximately three months while it develops to infectivity for the definitive host (Arme and Owen, 1967, Pennycuick, 1971b, McPhail and Peacock, 1983, Barber, 2007), by which time the parasite is often detectable by abdominal distension (Aeschlimann et al., 2000, Barber and Svensson, 2003). Plerocercoids are infective and capable of producing eggs when above 50mg in mass (Tierney and Crompton, 1992). The nutrients acquired from the host to reach this size are absorbed through the parasite’s body surface, as is characteristic of the Cestoda (Smyth, 1994), with glycogen thought to be one of the major energy sources (Körting and Barrett, 1977). S. solidus only reaches sexual maturity when the stickleback is ingested by the endothermic definitive host, usually a piscivorous bird (Tierney and Crompton, 1992). Parasite development into the adult form is triggered by increases in ambient temperature from the ectothermic fish to that of the endothermic bird. Once established the adult parasite produces eggs, and as total fecundity is proportional to plerocercoid size (Dörücü et al., 2007), maximising growth whilst in the intermediate host stickleback is pivotal to the parasite’s fitness. The eggs, which are released with the bird’s faeces, hatch in water into free swimming coracidia. These coracidia are then ingested by copepods which develop into procercoids in the haemocoel completing the parasite life cycle (Figure 1.4) (Hammerschmidt and Kurtz, 2007, Jakobsen et al., 2012). S. solidus is specific to G. aculeatus in the larval plerocercoid stage, while Schistocephalus pungitii infect a close relative, the nine-spined stickleback (Pungitius pungitius) (see Chubb et al., 2006, Barber and Scharsack, 2010, Nishimura et al., 2011). In contrast, the adult parasite is non-specific in the definitive host stage, which can be any piscivorous bird or mammal (Smyth, 1954, Smyth, 1994).
1.4.2.2 Effects on the three-spined stickleback host

Three-spined stickleback infected with *S. solidus* that have reached an infective size are readily detectable externally by abdominal distension (Arme and Owen, 1967, Barber, 1997) and in some individuals a reduction in skin pigmentation by demelanisation (LoBue and Bell, 1993, Ness and Foster, 1999).

As *S. solidus* transfers all its energy to reproduction in the definitive host, the parasite is reliant on the intermediate host three-spined stickleback to provide all nutrients required for growth, and as such is expected to exert a considerable energetic cost. As a result of this energetic drain, resting metabolic rates of infected sticklebacks are higher (Meakins and Walkey, 1975) and swimming is more energetically costly than for non-infected conspecifics (Lester, 1971). Infected individuals also have lower energy reserves stored in the liver (Pascoe and Mattey, 1977), reduced body condition (Tierney et al., 1996, Bagamian et al., 2004), reduced reproductive investment in males (Tierney et al., 1996, Rushbrook and Barber, 2006, Macnab et al., 2009, Macnab et al., 2011) and females (Schultz et al., 2006, Heins and Brown-Peterson, 2010, Heins, 2012). If
the host is unable to compensate for the energy needs of the parasite, infections may also result in mortality (Pennycuick, 1971a, Heins et al., 2010).

In addition to the energetic drain of the parasite, the presence of the plerocercoid is likely to reduce space for the expansion of the stomach whilst foraging and as a result infected three-spined stickleback consume fewer prey items in one feeding bout (Milinski, 1985, Cunningham et al., 1994). Infected sticklebacks also experience longer prey handling times (Cunningham et al., 1994). As a result infected sticklebacks switch prey type (Hynes, 1950, Jakobsen et al., 1988, Tierney, 1994) and size to smaller prey items when starved (Milinski, 1984) or larger prey items when ad libitum (Ranta, 1995) to avoid competition with non-infected conspecifics with greater foraging efficiency than their own.

Another aspect of *S. solidus* infections which has received considerable attention is the parasite induced behavioural changes in host three-spined sticklebacks. Sticklebacks infected with heavy *S. solidus* burdens show impaired anti-predator behaviours, such as spending more time at the water surface (Giles, 1987), reduced escape responses from avian attack (Barber et al., 2004), emerging from shelter and returning to foraging quicker after being startled (Giles, 1983, Milinski, 1985, Barber et al., 2004) and a reduced tendency to shoal (Barber and Huntingford, 1995). It is thought that these behaviours increase the chance of avian predation, and as this would constitute a beneficial response for the parasite by aiding transmission, it may in fact be an example of behavioural manipulation (Moore, 2002, Barber and Dingemanse, 2010). There is a possible role for neuroendocrine disruption in these observed behavioural changes, with 5-hydroxytryptamine (5-HT) and norepinephrine (NE) being significantly reduced in infected three-spined sticklebacks (Øverli et al., 2001).

### 1.5 Aims of the thesis

By definition parasites cause harm to the host by using resources, but controlled experiments examining how the severity of parasite effects on host biology are modulated by environmental factors are scarce. In this thesis I will focus on variation in host-parasite interactions within a single host species. A major aim of the research in this thesis is to investigate the effect of both host and environmental biologically relevant factors, on the interactions between a
naturally prevalent parasite and its wide-ranging and ecologically well-understood host. One objective was to determine if the genetic background and phenotype of the host influenced the host-parasite interaction. The three-spined stickleback has repeatedly colonised freshwater environments from marine waters. After becoming established the three-spined stickleback is known to adapt to the new environment with changes to its morphology and associated genotype, and therefore the effects of potentially novel parasite infections on the host are of interest as a possible source of parasite mediated selection. A naturally variable three-spined stickleback population with unusually high variation in plate morphology was used to examine the effect of host genotype and phenotype on the susceptibility and subsequent progression of experimentally-induced parasite infections (Chapter 2). In Chapter 3, the effect of environmental disturbance on molecular responses of infected and non-infected hosts was examined, to study how the response of sticklebacks to short term changes in oxygen availability may vary with parasite burden. Direct environmental effects on S. solidus were then examined in Chapter 4, in which the viability of S. solidus eggs was tested across a salinity gradient, from marine to brackish and freshwater. It was from this data that it was determined if the parasite S. solidus, would in fact represent a novel infection because it is limited to freshwater environments or if its tolerance may be wider. In Chapters 5 and 6, variation in host food availability and host body size on subsequent parasite infections was studied. The objective of these studies was to use controlled experiments to determine the relative importance of host factors that can vary substantially both within and between host populations, on the development of parasite infections.

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Chapter 2

Consequences of stickleback morphology and *Eda* genotype for *Schistocephalus* infection: field and laboratory studies

N. Simmonds ©
2.1 Abstract

The three-spined stickleback has risen to prominence as an evolutionary model, partly due to the vast range of morphologies and associated genetic variation they display. Upon invading freshwaters, the lateral plate count of three-spined sticklebacks typically reduces and becomes more variable, although the cause of such rapid evolution has not been conclusively determined. The *Eda* gene has been found to be associated with the development of lateral plates and can be determined easily by PCR. In the present study, a freshwater population with unusually high variation in plate morphology was used to examine the link between parasite infections and lateral plate number as a possible contributory factor of stickleback evolution. Among wild fish, parasite prevalence did not differ significantly with genotype. In an experimental *Schistocephalus solidus* challenge, fish with fewer numbers of lateral plates were found to show increased susceptibility to infection. The implications of these results for stickleback evolution are discussed.
2.2 Introduction

2.2.1 Host ecological immunity

The ability of a host to resist infection and tolerate the presence of parasitic organisms can be thought of as a host trait that is governed by the same rules of natural selection as other traits that increase host fitness (Schulenburg et al., 2009). As resources are expected to be limited in the environment, trade-offs are predicted between immunity to parasites and other traits that also impact fitness. One example of this is variation in parasite burdens between individuals linked to diversity in sexual ornamentation and other sex-based morphological differences. Although developing energetically-costly sexual ornamentation can increase host fitness by increasing reproductive rate, it can also place individuals under greater susceptibility to parasites and lower survival rates (Zuk and McKean, 1996, Sheldon and Verhulst, 1996). For example, more elaborate sexual ornamentation was generated in chickens (Gallus domesticus) that were chosen for their lower immune function, indicating a trade-off between immune function and sexual ornamentation (Verhulst et al., 1999). Higher reproductive effort has also been associated with reduced immunity (Nordling et al., 1998) and high parasite burdens (Richner et al., 1995) in birds (Norris and Evans, 2000). This ‘immunocompetence handicap hypothesis’ has also been examined in fish. Male chub (Leuciscus cephalus) that invest in higher sperm quality also have higher levels of infection with digenean trematodes (Rohlenova and Simkova, 2010). However, the converse situation can also arise, where individuals with genetic predisposition for higher immunocompetence have a lower fecundity, such as in fruit flies (Drosophila melanogaster), indicating an evolutionary cost of maintaining elevated immune defences (McKean et al., 2008).

As well as host morphology acting as a factor influencing the susceptibility to parasites, infections can also influence host morphology. Such effects may then impact host fitness. For example, intertidal mud snails infected with the digenean trematodes Coitocaecum parvum (see Lagrue et al., 2007) and Maritrema novaezealandensis (see Hay et al., 2005) exhibit differences in size and shell shape associated with reproductive castration and loss of host fitness. It is possible that the host gigantism and shape changes induced by trematode infection may be a side effect of reproductive castration, leading to more
resources being available for host growth, or be a symptom of the specific parasite infection. By examining both *Acanthoparyphium* sp. and *Maritrema novaezealandensis* infections in the same host, Hay et al. (2005) were able to determine that shape varied with parasite identity, meaning that host gigantism and shape are species specific changes in host phenotype and may even be adaptations of the parasite to aid transmission. A similar response is shown by the freshwater snail *Elimia livescens* in response to infection with the digenean *Proterometra macrostoma*; these changes are caused by parasite induced changes in growth rather than differential mortality of differently shaped hosts (Krist, 2000).

Parasite infections have also been shown to affect the morphology of fish. Killifish (*Fundulus heteroclitus*) infected with endoparasitic metacercariae digenean of the digeneans *Ascocotyle diminuta* and *Echinochasmus schwartzi* showed increased gill branching compared to non-infected conspecifics (Bass et al., 2007). *Ascocotyle pachycysti* metacercariae cause morphological changes in the heart of minnows (*Cyprinodon variegatus*), specifically enlarging the heart to compensate for mechanical blockage by the parasite, decreasing swimming performance particularly at low temperature and oxygen levels (Coleman, 1993). Infections by *Diplostomum* sp. trematodes also cause morphological changes to the eye of bullheads (*Ictalurus melas* and *I. nebulosus*) by encysting in the lens (Larson, 1965), and due to disruption of vision are associated with reduced foraging efficiency in Artic charr (*Salvelinus alpinus*) (see Voutilainen et al., 2008). Experimental infection with *Ornithodiplostomum ptychocheilus* metacercariae induces an enlargement of the cranium in minnows (*Pimephales promelas*) as a result of the encystment of the parasite in the brain, interfering with normal development (Sandland and Goater, 2001); this is more likely to be a side effect of infection than an adaptation by the parasite. In sticklebacks infected with *Schistocephalus solidus*, fish exhibited smaller and deeper head morphology than non-infected conspecifics, which was more pronounced among fish with higher parasite burdens (Dingemanse et al., 2009). This change in morphology has the potential to impact host feeding ecology and fitness, although it is unclear whether the changes observed by Dingemanse et al. (2009)
arise as a by-product of infection, or an adaptation by the host or parasite to increase food intake.

### 2.2.2 Genetic aspects of parasite infections

The genetic background of the host may also interact with the environment to affect host fitness, with some genotypes performing better in one environment than others (Lazzaro and Little, 2009). These genotype x environment effects have been shown for temperature in *Daphnia* (see Mitchell, 2005) and food availability in mosquitos (Bedhomme et al., 2004).

Parasite-mediated selection has the potential to drive high genetic diversity among host populations (Poulin and Morand, 2004), with host populations exhibiting low genetic heterozygosity typically being most susceptible to infection. In inbred Soay sheep (*Ovis aries*), susceptibility to infection by nematode parasites was higher in inbred than in outbred individuals, when parasite burden was determined by faecal egg counts and inbreeding by heterozygosity at selectively neutral microsatellite loci. Among sheep that were experimentally cleared of nematodes with an anthelmintic treatment, overwinter survival was random with respect to heterozygosity, but inbred and untreated sheep were significantly less likely to survive compared to untreated sheep with high heterzygosity. This illustrates how high genetic diversity may be maintained in the population (Coltman et al., 1999). Among unmanaged (feral) populations of Soay sheep, individuals with high allelic diversity in the major histocompatibility complex (MHC) – a region associated with immunity in vertebrates (Goater et al., 2013, Apanius et al., 1997) – have lower nematode parasite burdens (Paterson et al., 1998).

Variation in MHC has also been observed in house finches (*Carpodacus mexicanus*) which show a down-regulation of MHC genes in the spleen of birds infected with *Mycoplasma gallisepticum* (see Hess et al., 2007). Successive cohorts of great reed warblers (*Acrocephalus arundinaceus*) were shown to vary in their MHC alleles more than neutral microsatellite markers, and two MHC alleles varied more between cohorts than would be expected from random (Westerdahl et al., 2004). In a second study on great reed warblers and malaria-causing parasites, the number of MHC class I alleles was found to be positively
associated with prevalence of the milder malaria GRW2 lineage (*Plasmodium ashfordi*), which was not explained by inbreeding as there was no association between microsatellite heterozygosity and an increased number of MHC alleles (Westerdahl et al., 2005). The authors also found the MHC allele B4b to be positively correlated with GRW2 infection, suggesting immunity was shifted to offer protection against the lethal effects of malaria (Atkinson et al., 2001, Sol et al., 2003) and towards the milder GRW2 infection. Malaria infections have also been found to be associated with MHC variation in house sparrows (*Passer domesticus*), where specific MHC alleles were found to be associated with higher or lower infections of *Plasmodium relictum* depending on population, indicating that diversity in parasites may cause increased genetic diversity in the host (Loiseau et al., 2011). This spatial pattern of MHC diversity indicating adaptation to infections experiences in local environments has also been shown in other birds including the great snipe (*Gallinago media*) (Ekblom et al., 2007) and the lesser kestrel (*Falco naumanni*) (Alcaide et al., 2008).

One of few examples in which variation in parasite diversity has been shown to affect the genetic diversity of host populations is the three-spined stickleback (Bernatchez and Landry, 2003), where the diversity of MHC in sticklebacks has also been shown to have reached an optimal level in response to multiple parasite infections (Wegner et al., 2003). Environments have also been shown to influence the genetic background of sticklebacks. In a study on Icelandic populations the most common number of alleles at the MHC class IIB was associated with the lowest levels of infection, suggesting that parasites had shifted the evolution of their hosts. Furthermore in sympatric populations parasite load was associated with habitat type with fish caught from lava substrates showing the highest prevalence of infection (Natsopoulou et al., 2012). In a separate study using lake and river ecotypes in Germany, the lower parasite species richness found in riverine populations (Kalbe et al., 2002) was suggested to have lowered the genetically-determined immunocompetence of lab-bred fish from riverine habitats compared to lakes (Scharsack et al., 2007). In a mesocosm study using second generation lab-bred hybrid sticklebacks, fish with lake ecotypes showed higher divergence in MHC genotypes which would confer advantages against a range of parasite infections, while river ecotypes would
have selective advantage against a narrow range of parasites (Eizaguirre et al., 2012a). This study illustrates that parasite mediated selection can change genetic background of fish, and differs between the specific parasites found in the environment. Under experimental conditions exposure to two nematode species (Anguillicoloides crassus and Camallanus lacustris), only the MHC alleles which conferred resistance to the specific parasite infection exposed to that population increased in the next generation, demonstrating rapid parasite mediated selection (Eizaguirre et al., 2012b).

2.2.3 The three-spined stickleback as a model for studying morphological evolution

The three-spined stickleback, Gasterosteus aculeatus, is a teleost fish found commonly in freshwater and marine habitats. Due to its ubiquity and ease of rearing under laboratory conditions the stickleback has become a common model in a range of fields (Bell and Foster, 1994, Ostlund-Nilsson et al., 2010, Barber and Nettleship, 2010, Barber, 2013). The three-spined stickleback has become a classic example of evolution in the wild, with numerous field studies examining the extensive variation in anti-predator morphology (Reimchen, 1983) and feeding morphology (Nosil and Reimchen, 2005) across different aquatic habitats. This has led to many studies that have sought to understand the source of this variation, and have considered the role of predator abundance (Reimchen, 1992, Reimchen, 1994, Reimchen, 2000), salinity (Hagen and Gilbertson, 1972), and calcium (Spence et al., 2012). Ancestrally a marine species, the three-spined stickleback has repeatedly invaded brackish and freshwater environments from extant marine populations (Bell and Foster, 1994). Populations of sticklebacks invading freshwaters have therefore evolved repeatedly in response to the new environment. In freshwaters, three-spined sticklebacks typically become more streamlined in appearance with reduced body depth and reduced size and number of lateral plates. In the marine form, three-spined sticklebacks have a row of lateral plates forming a strong bony exterior, but these are dramatically reduced in most freshwater populations forming either a partially plated morph with a gap in plates after the pelvic girdle, or a low plated morph with no posterior plates (Figure 2.1). These forms of the three-spined stickleback are sometimes referred to as trachurus, semiarmatus and leiurus respectively (Morozińska-
Gogol, 2011, Bell and Foster, 1994). More recently the molecular basis of the evolutionary change in morphology has been explored in detail.

![Diagram of fish morphs](image)

**Figure 2.1** Lateral plate morphs of the three-spined stickleback, *Gasterosteus aculeatus*, adapted from Bell and Foster (1994). A: low plated morph. B: partial plated morph. C: complete plated morph.

### 2.2.4 Stickleback plate morphology and the *Eda* gene

The ectodysplasin (*Eda*) gene is present across animal taxa and is involved in development of dermal structures including bone (Mikkola and Thesleff, 2003). In the three-spined stickleback, *Eda* is linked to the number of lateral plates found in the bone structure of the fish (Colosimo et al., 2005), and was located in this landmark paper. By using two genetic markers found in most of the study populations, STN380 and STN381, Colosimo et al. (2005) found the low plated allele was present at low frequencies even in completely plated populations, indicating that standing genetic variation in extant marine populations allows
them to repeatedly adapt to freshwater environments when they are encountered, typically during post-glacial invasions. Furthermore, by comparing single nucleotide polymorphisms (SNPs) Colosimo et al. (2005) were able to show that genetic similarities were grouped geographically rather than by morph, demonstrating that the low plate morph was unlikely to have evolved from one low plated population that had then colonised new habitats. Rather, their results support the hypothesis of a repeated transition from marine to freshwater.

One question is, why have these alleles become repeatedly fixed in so many derived freshwater populations? What is the selective agent? There are currently two major perspectives on this; firstly the Eda allele encoding the low-plated form could confer a direct advantage if it allows individuals greater survival and/or reproduction success in freshwaters, thereby increasing fitness. On the other hand there could be co-selection of a gene that is closely linked to Eda.

A possible candidate as a selective force on three-spined stickleback is predation (Hagen and Gilbertson, 1973). If the completely plated morph were less susceptible to predation by piscivorous fish, this could provide a selective advantage particularly in marine environments and freshwater with high visibility. In freshwaters, which often offer low visibility and where the risk of predation by grappling invertebrates – which potentially use plates to gain a hold on stickleback prey – may pose a greater threat, it has been argued that plates offer less protection (Reimchen et al., 2013). Sticklebacks have dorsal and pelvic spines that have been shown to lower predator success rates when mouth diameter is a limiting factor such as in piscivorous fishes (Reimchen, 1994). In an experimental study, in which the predation success of cutthroat trout (Oncorhynchus clarki) was measured, the number of handling failures by the predator was highest for completely-plated morph sticklebacks, and the presence of posterior plates was associated with increased handling time and escape rates (Reimchen, 2000). This effect was most pronounced at the highest ratios of prey diameter to predator mouth diameter, and therefore was attributed to the additional posterior plates interfering with swallowing, by disrupting pharyngeal jaw retraction and facilitating escape.
By increasing the number of lateral plates it is proposed that sticklebacks increase their likelihood of escape and reduce puncture damage, particularly in the anterior region from plates 4 to 8 as these support the dorsal and pelvic spines from retracting under pressure (Reimchen, 1983). Such a mechanism is most likely where toothed predators are abundant in the limnetic and pelagic zones. However the converse of the theory states that the low plate morph should be favoured where there is a lower predation intensity, as developing extra bony structures is energetically costly and should only be beneficial where predation risk is high (Gross, 1978, Bourgeois et al., 1994). Developing the low plate morph should also occur where there is a high frequency of invertebrate predators using grappling in their feeding technique making long spines and armour a disadvantage as they would impinge attempts at escape (Reimchen, 1992, Reimchen, 2000). Plate number has also been associated with swimming performance, as plate reduction improves the fast-start response with increased velocity and displacement, which might be advantageous in predation regimes where predators have a low pursuit efficiency such as diving birds rather than piscivorous fishes (Bergstrom, 2002).

Reimchen’s perspective is further supported by a reported case of reversed evolution in an urban lake, where the frequency of completely plated morphs increased over a 40 year period (Kitano et al., 2008). This was attributed to mitigation of eutrophication in the lake, leading to increased visibility and predation by piscivorous fishes.

In contrast, the proposition that low plate morph should be favoured where there is a high frequency of invertebrate predators has recently been questioned. Zeller et al. (2012) illustrated in a polymorphic population that there was no significant difference in the survival of the three plate morph genotypes when placed in experimental pond with invertebrate predators. When individually tested with an invertebrate predator, predation success did not depend on size of either prey or predator.

Historically, it has been suggested that the number of lateral plates is associated with salinity, as completely plated morphs are commonly found in marine environments (Heuts, 1947). However this explanation was later contradicted as
the number of plates was found not to correlate with relative salinity (Hagen and Gilbertson, 1972). The reduction in bone structures associated with the invasion of freshwaters may alternatively result from the lack of calcium availability, as freshwater has a much lower concentration than sea water (3-60 mg/L and 420 mg/L respectively) (see Wootton, 1998, Spence et al., 2012). As calcium is important for many aspects of development, reducing the level of demand imposed by bony structures may reduce energetic expenditure, allowing for faster growth through juvenile stages where mortality is typically high. This is supported by experimental studies, which have found a reduced growth rate of complete morph fish when reared under low calcium conditions (Spence et al., 2012). The authors also found evidence of local adaptation to salinity, but this was independent of plate morph. This result is further supported by field studies on the island of North Uist in Scotland. Here there are a series of naturally occurring lakes, which vary in calcium content and predation regime. In the lakes where calcium concentration is very low (2.5 mg/L) unusual populations of spine-deficient morph sticklebacks occur, indicating calcium is a selective agent in these populations (Giles, 1983). Spence et al. (2013) also studied the wild populations on North Uist and suggest that the calcium concentration is a significant predictor of plate morph, with low plated fish being found more frequently in low calcium lakes. Interestingly in this study there was no evidence of predator type being an important factor in selection of plate morph (Spence et al., 2013). However, these results have been disputed by MacColl and Aucott (2014) who have also studied the stickleback populations on North Uist and find that lateral plate variation is strongly correlated with trout abundance. Bell at al. (1993) did a comprehensive survey of 179 lakes around the Cook inlet, Alaska and found correlational evidence that reduction in pelvic structures was associated with low calcium concentrations, but only where this also occurred in the absence of predatory fishes suggesting that selection for defensive structures is relaxed in the absence of predators. Bell et al. (1993) suggest that their results illustrate how environmental variables interact and influence morphological traits and there may yet be other environmental variables which are important.
2.2.5 Stickleback plate morphology and parasite infections

Another possible explanation for the change in plate morphology could be that parasites encountered only in freshwaters (and not in marine populations) impose novel selection pressures on the morphology of fish that invade freshwater environments. This might occur, for example, if morphology either affects the susceptibility to infection, or the subsequent survival or reproductive success of infected fish.

As a population disperses to a new environment, the success of this new colonisation can depend on changes in parasite pressure (Møller, 2005). Theoretically parasites of the host may be left behind leading to ‘missing’ parasites, allowing the host to dramatically increase in population size – a hypothesis commonly referred to ‘enemy release’ (Prenter et al., 2004, Dunn, 2009). In a survey of colonisations by animal hosts, introduced populations had an average of 40% less parasites than in their native range (Torchin et al., 2003). However, Torchin et al. (2003) also found native parasites that invaded the introduced host population had as high a prevalence as parasite which were introduced with their host.

It is also possible that the individuals which colonise new environments are have stronger immune responses, as heavily parasitized individuals with chronic infections would not be expected to survive the stressful transition to a new environment. This is supported by a study by Møller and Cassey (2004) which examined the success of introduced birds to New Zealand and showed nestling T-cell mediated immune responses was a reliable predictor of establishment success.

However, examples of enemy release typically involve introduced species entering an environment for the first time rather than those with resident populations of that species already established. In this instance, we might expect the colonisers to be at a disadvantage compared to the resident populations, as they could lack local adaptation to the new environment. Hendry (2004) examined reproductive isolation which arises from fitness being higher for individuals breeding in environments they are adapted to rather than those in which they are not. Using single-locus and quantitative-genetic models Hendry
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(2004) determined that selection against migrants can contribute to reproductive isolation in less than 30 generations.

As parasites can contribute to reproductive fitness they may constitute one way in which migrants are selected against, as novel parasites may have more severe consequences for hosts than infections in their native range. In a meta-analysis study of local adaptation across host-parasite interactions, Hoeksema and Forde (2008) found that when gene flow between populations is low, hosts can show a higher susceptibility to novel parasite infections than those from their native host range. This may be especially true when host gene flow is higher than parasite gene flow (Gandon et al., 1996). However this pattern is not universal for all host-parasite systems, and the degree of local adaptation by parasites may also contribute. In a study on freshwater snails (Potamopyrgus antipodarum) the trematode Microphallus sp. was found to infect sympatric hosts with common genotypes from the same geographic area significantly more often than rare host genotypes (Lively and Dybdahl, 2000). Therefore, the novel parasites experienced by hosts when colonising new environments have the potential to either provide either an advantage or disadvantage to the migrants, affecting fitness and selection.

There is some recent evidence to suggest that there may be a link between plate morph and the level of parasite infection in three-spined sticklebacks. In Gdynia Marina on the Baltic coast, a survey of parasite species of three-spined sticklebacks found that cestode parasites were more frequent among individuals with fewer lateral plates (Morozińska-Gogol, 2011). Infections of three-spined sticklebacks have been reported in anadromous sticklebacks in Alaska, suggesting that S. solidus is capable of surviving in oceanic stickleback for several years once infecting fish in freshwater (Confer et al., 2012). MacColl and Chapman (2010) used a freshwater mesocosm infection study to examine the effect of marine or freshwater ancestry on parasite susceptibility, and found fish from marine populations suffer from higher susceptibility to cestode parasites and Diplostomum sp. infections than those from freshwater environments, when reared in freshwater enclosures and naturally exposed to parasites. Parasite infection was also associated with reduced growth, but was alleviated when in groups given an antihelminthic treatment. MacColl and Chapman (2010) suggest
that as reduced growth is associated with reproductive success, their results indicate parasites would reduce the fitness of marine fish when they migrate to freshwater environments. This contrasts with the enemy release hypothesis and instead suggests that parasites select against migrants (MacColl and Chapman, 2010). Parasites have the ability to affect the mortality of fish (Threlfall, 1968), which potentially could influence overall fitness of a particular phenotype altering its frequency in the population over time. While MacColl and Chapman’s (2010) study did not look at plate number directly, as marine fish are commonly completely plated it suggests there may be a parasite induced fitness cost to having more lateral plates in freshwater habitat.

2.2.6 Aims
The main aim of this study was to test whether variation in lateral plate morphology in Gasterosteus aculeatus, and/or the underlying Eda genotype, is associated with either parasite load, or susceptibility to infection. I took advantage of the discovery of a landlocked freshwater population that – highly unusually – exhibited the full variation of lateral plate morphs. This provided a unique opportunity to examine the how variation in plate phenotype and Eda genotype affected parasite load. This was examined in two ways. First, a parasite survey of wild caught fish was undertaken, to examine the hypothesis that plate morph was related to parasite load, both in terms of the type and intensity of infections. Second, an experimental laboratory infection experiment was carried out to examine the susceptibility of different plate morphs / Eda genotypes to experimental Schistocephalus solidus challenge, and the subsequent severity of infection.
2.3 Methods

2.3.1 Study Site

Carsington Water (53° 3'32"N, 1°37'42"W) is a reservoir created in 1991 in North Derbyshire, UK. Although water is fed into the reservoir intermittently by a series of small streams, the main input is via a 10 km pipe connected to the River Derwent at the village of Ambergate. This pipe is only opened to facilitate the refilling of the reservoir after water is drained from it for public water use (Young, 2012). The River Derwent eventually reaches a confluence with the River Trent at Derwent Mouth before reaching the North Sea at the Humber estuary. Therefore Carsington Water lies approximately 260 km upstream from the sea, once river meandering is accounted for (Figure 2.2). As such, recent and repeated migration of marine sticklebacks into the population seems unlikely (Jones et al., 2006).

![Map of central England showing the 260km linear distance of Carsington Water from the sea, accounting for river meandering. (Google Earth v7.1.2.2041, 2013).](image)
2.3.2 Molecular techniques

2.3.2.1 DNA extraction from swab samples
Fish in individual tanks were then swabbed for DNA extraction following an procedure adapted from Smalley & Campanella (2005) and Livia et al. (2006). In brief, fish were blotted dry before a cotton sterile swab (Transport swab plastic applicator rayon tipped white cap, Thermo Scientific Sterilin, UK) was applied to the skin from the operculum to the caudal peduncle under slight pressure twenty times. The swab was then returned to the sterile container and immediately taken through the DNA extraction procedure.

DNA was extracted from swabs using the Isolate Genomic DNA minikit (Bioline, UK) according to kit protocol for buccal swabs. The applicator of the swab was cut approximately 1 cm above the cotton and placed in a 1.5 ml Eppendorf, and digested in 400 µl of Lysis Buffer D and 25 µl Proteinase K at 50°C for 15 min. Swabs were removed from the solution, transferred to spin column D and centrifuged at 12,000 rpm for 2 min. Two cycles of washes with wash buffer D were applied to the spin column before centrifuging at 12,000 rpm for 1 min. After centrifuging at 16,000 rpm for 2 min the spin column was transferred to the elution tube and 200 µl elution buffer was applied to the membrane, left at room temperature for 1 min before a final centrifuge at 8000 rpm for 1 min.

2.3.2.2 DNA extraction from fin clips
Protocols followed a standard Isopropanol DNA extraction method without organic solvents (Sambrook and Russel, 2001). Tissue samples were transferred from the 100% ethanol storage tubes to a fresh Eppendorf tube containing 1 ml of ddH20, and left to rehydrate for 30 min. Tissue was digested overnight at 55°C in a 400 µl buffer solution of 1M TRIS pH 7.5, 0.5 M EDTA, 2 M NaCl, 10% SDS and 7 µl of 20 mg/ml proteinase K. Samples were then heated to 92°C for 10 min, vortexed, and centrifuged at 16,000 rpm for 2 min. 300 µl of supernatant was removed and placed in to a fresh Eppendorf tube, and 300 µl of Isopropanol was pipetted into the solution. The Eppendorf tubes were tilted to mix the contents before being incubated at -80°C for 10 min. Samples were then defrosted and centrifuged at 16,000 rpm for 10 min. The pellet was dried using a pipette before adding 198 µl of 70% Ethanol and centrifuging at 16,000 rpm for 2 min. The pellet
was once again dried before the tube was covered with micropore tape and allowed to dry completely overnight. The pellet was rehydrated in 100 µl ddH2O and stored at 4°C overnight, after which the concentration of DNA in the samples was quantified using a Nanodrop™ 1000 (Thermo Fisher Scientific, UK).

2.3.2.3 Optimisation of PCR protocols

Primers for STN381 and STN382 were used following Colosimo et al. (2005). Primer sequences were for STN381 F: CACGGACCTACACCACAACG and R: ATTGAGGTTCCAGCTCTGG which due to an indel in the sequence on intron 6 should amplify a 175 and/or a 165 bp allele in completely plated fish and a 193 bp allele in low plated fish. For STN382 the primer sequences were F: CCCTTAGAGAATTGCCTAGCAG and R: CTTGTCCGGATCATACGC, amplifying a 150 bp allele in low plated fish and a 218 bp allele in completely plated fish due to an indel polymorphism at intron 1. After testing the PCR protocol used by Colosimo et al. (2005), Bell et al. (2010) and standard PCR conditions, the STN381 primer set failed to produce any bands on the electrophoresis gel. This could be due to divergence in the stickleback genome between the North American populations used by Colosimo et al. (2005) and Bell et al. (2010) and the population in the United Kingdom used in this study. For the STN382 primers the PCR conditions described by Colosimo et al. (2005) did not reliably produce a product and were adjusted accordingly.

The STN382 primer failed to produce bands with the original primers and after further inspection of the stickleback genome using Ensembl genome browser (Flicek et al., 2012, Jones et al., 2012) a discrepancy was found in the forward primer sequence showing the beginning of the sequence was the same but changes after TTT. Therefore new forward primers were designed with a sequence of CCCTTAGAGAATTGCCTAGCAG, which subsequently produced the characteristic bands as described by Colosimo et al. (2005).

PCR reactions were in a 10 µl volume containing 5 µl Red Taq (Sigma, U.K.), 0.5 µl forward primer, 0.5 µl reverse primer, 3 µl ddH2O and 1 µl DNA. The PCR conditions were found to be optimum using 1 cycle at 94°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds and a final extension at 72°C for 10 minutes before returning to 4°C.
Due to the variability of fin clip size, the concentration of DNA extracted varied from between 10 ng/µl and 600 ng/µl. After optimising the PCR protocol it was found to increase reliability of the gel electrophoresis if samples were between 10 ng/µl and 20 ng/µl; any samples which were above 20 ng/µl were therefore diluted to within this optimum range. For samples below 10 ng/µl, the amount of DNA in the PCR was increased to 3 µl per 10 µl reaction.

As the product sizes expected for STN382 are 150 bp for the low plated allele and 218 bp for the completely plated allele, a high percentage gel of 4% was used to differentiate the 68 bp difference between the bands. A volume of 50 ml 1XTAE was chilled to 4°C before adding 2 g high definition agarose (Sigma, UK) and heating to dissolve. Agarose was incubated at 50°C for 1 h before adding 0.5 µl ethidium bromide and pouring in to the gel former. PCR products were run on a two rowed gel at 60 volts for 60 min. A high resolution ladder (Hyperladder V, Sigma, U.K.) was used for which produced bands every 25 bp in the 100 bp to 200 bp range allowing the distinction between low and complete alleles. Samples from fish with the low plated genotype only produce one strong band at 150 bp, the completely plated genotype only produces one strong band at 218 bp while the heterozygous EdaC/EdaL partial genotype produces two weaker bands at both 150 bp and 218 bp (Figure 2.3).

**Figure 2.3** Gel image illustrating the bands produced by PCR using primers STN382 for Eda from three-spined stickleback DNA. LL: low plated genotype. CL: partial plated genotype. CC: completely plated genotype.

### 2.3.3 Clearing and staining for morphological analysis

Protocols were adapted from Dingerkus and Uhler (1977). After specimens had been fixed in 10% NBF for a minimum of 14 d, they were rinsed in dH₂O for 10 minutes before being allowed to rehydrate in dH₂O overnight. Specimens were immersed in Alician blue stain (20 mg Alcian blue 8GX, 70 ml 100% Ethanol, 30 ml glacial acetic acid) for 2 hours before being rehydrated through an
ethanol:dH$_2$O series of 3:1→1:1→1:3 and a final dH$_2$O step, each lasting 1 h. Two 5 minute washes of 30% saturated sodium borate solution (stock: saturated di-sodium tetraborate (Na$_2$B$_4$O$_7$.10H$_2$O) solution) was used before immersing the specimens in 1% trypsin solution (Sigma, U.K.), made with 30% saturated sodium borate solution, and incubating at 30°C for 48 hours. Two 5 minutes washes of 2% potassium hydroxide were used before immersing in Alizarin red solution (0.002g Alizarin complexone (C$_{19}$H$_{15}$NO$_8$), 100 ml 2% potassium hydroxide (KOH)) overnight to allow the identification of plates (Peichel et al., 2001). Specimens were cleared by immersing in bleaching solution (500 µl 60% hydrogen peroxide (H$_2$O$_2$), 150 ml 0.5% potassium hydroxide (KOH), 50 ml glycerol) for 48 hours, then rebalanced in a 0.5% potassium hydroxide: glycerol series of 1:1, 1:3 and finally 100% glycerol for 2 hours each step. Specimens were stored in fresh 100% glycerol.

Specimens were viewed under a dissection microscope and the presence or absence of each of the possible 36 lateral plates was recorded following a method adapted from Reimchen (1994). Firstly, plate 6 was identified as the plate that descends from the first two prominent spines from the anterior, flanked on either side by plates 5 and 7 descending from the two dorsal spine plates. Most stickleback have a cleithrum, also referred to as a pectoral girdle, which is counted as plate number 1, and indeed in all the fish examined in this study the cleithrum was present. Immediately adjacent to the cleithrum there is the small plate 2, and plates 3 and 4 descending from the first dorsal spine is present. Plate numbers 8 onwards can be counted from anterior to posterior. In the fish examined in this study, low plated fish commonly possessed only the cleithrum and plates 5, 6, and 7 (Figure 2.4A). The plate number of partially plated fish varied considerably, but often appeared similar to low plated fish with additional plates at the posterior end (Figure 2.4B). Completely plated fish had a full row of pectoral plates, with no gaps but individual plates were sometimes reduced in size (Figure 2.4C).
Figure 2.4 Cleared dissected three-spined stickleback specimens, stained with Alizarin red, with pectoral plates visible. A: completely plated fish. B: partially plated fish. C: low plated fish.
2.3.4 Parasite survey of wild fish

2.3.4.1 Stickleback collection and husbandry
Fish were collected in March and April 2012 from accessible sites around Carsington Reservoir, Derbyshire, U.K. (53°3’30"N 1°37’50"W) (Figure 2.5), using Gee’s minnow traps (n = 150).

After returning to the laboratory aquaria young of the year fish were euthanized by overdose of Benzocaine anaesthetic (stock solution: 10 mg L-1) according to Schedule 1 methods. Fin clips were then taken by removing the right-hand pectoral fin and placing them into 100 µl absolute ethanol contained in an Eppendorf tube. The remaining body was immediately placed into an individual 30 ml universal tube and filled with 10% neutral buffered formalin (Stock:4 g Sodium dihydrogen orthophosphate dihydrate (NaH$_2$PO$_4$·H$_2$O), 6.5 g di-Sodium hydrogen orthophosphate anhydrous (Na$_2$HPO$_4$), 100ml 37% Formaldehyde, 900ml dH$_2$O) ensuring the entire body was covered.
2.3.4.2 Dissection and parasite identification

Fish fixed in 10% NBF were blotted, measured using a dial calliper (standard length, SL, to 0.1 mm) and weighed (mass, M, to 0.001 g). First, the skin surface of the fish was scanned (under x30 magnification) for ectoparasites such as *Gyrodactylus* sp., and if present the total number of each species was recorded. Eyeballs were then removed and placed into a watch glass for dissection and covered in ddH$_2$O. The location and number of any eye parasites, including *Diplostomum gasterostei* and *D. spathaceum*, was recorded. An incision was then made along the ventral surface of the fish to the operculum, and the sides of the fish removed. The body cavity and all internal organs were screened for parasites, and the location and number of any parasites recovered, including *Schistocephalus solidus*, *Diphyllobothrium* sp. and nematodes, was recorded.
2.3.5 Experimental infection study

2.3.5.1 Stickleback collection and husbandry
Adult fish were housed in 100 L glass aquaria (41 cm x 60 cm x 40 cm large tanks) and fed daily with bloodworms (Chironomus sp. larvae) and fish pellets (Medium Premium Granular, ZM systems, UK) ad libitum. The feeding regime ensured a supply of carotenoids to allow males to develop nuptial colouration, and combined with a day length regime of L14:D10, induced sexual maturation. Males developing nuptial colouration and females developing clutches of eggs were identified and separated into individual tanks with gravel and aeration, and arranged as alternate male and females. Males were provided with nesting material in the form of black polyester thread, and nesting behaviour was used as a measure of reproductive activity.

Fish in individual tanks were then swabbed for DNA and PCR to identify the Eda genotype of each fish by gel electrophoresis. Parents were then crossed using in vitro fertilisation techniques (Barber and Arnott, 2000), to create families that exhibited the full range of Eda genotypes. Testes were dissected from male fish and placed in a watch glass over ice. Females were stripped of eggs into a watch glass, before being covered in the macerated testes solution in autoclaved aquaria water, and left for 30 minutes. Eggs were checked under dissection microscope for the development of the outer membrane, indicating successful fertilisation. Developing eggs were reared in 1 L plastic aquaria with constant aeration and methyl blue solution as an anti-fungal agent. Fry were kept in their plastic aquaria until able to accept live Artemia sp. nauplii. Juvenile fish were then transferred and reared in family groups in 30 L glass aquaria (40 cm x 25 cm x 30 cm) in a temperature controlled, filtered, recirculating water system, and fed daily ad libitum with Artemia sp. nauplii.

2.3.5.2 Experimental parasite infection procedure
Schistocephalus solidus plerocercoids recovered from the Carsington Water population of Gasterosteus aculeatus were cultured using techniques adapted from Smyth (1954). Plerocercoids were removed from the body cavity of infected sticklebacks, by making an incision along the ventral side of the fish from the vent to the operculum. Parasites were placed into a 6.3 mm diameter dialysis tubing
(Visking, U.K.) suspended in a 70 ml screw-top glass tube (PYREX™ Screw Cap Culture Tubes, Fisher, U.K.) containing 50% RPMI media and 50% horse serum (Sigma, U.K.). The glass tubes were placed in a shaking water bath at 40°C for 5 days, which has previously been shown to be the optimal time for egg production (Arnott et al., 2000, Barber and Svensson, 2003, Macnab et al., 2011). The eggs were flushed from the dialysis membrane using a wash bottle filled with ddH2O, in to a 9 cm diameter Petri dish. The culture liquid was viewed under a dissection microscope and excess liquid and remains of the parasite outer tegument removed. Eggs were incubated in dark conditions at 20°C for 21 days to allow them to develop before exposing them to natural light to induce hatching.

A lab population of copepods (*Cyclops strenuus abyssorum*) were sorted by sieving into three groups; adults at 250 µm, copepodites at 150 µm and nauplii at 45 µm. Copepodites and adults were placed in 250 ml conical flasks and exposed to a selection of *S. solidus* eggs observed to have swimming coracidia or hatched eggs after 30 minutes. These exposure flasks were left in natural daylight for 3 hours before being returned to the aquaria. After 2 days the copepodites and copepods were filtered to remove parasite eggs and coracidia (to ensure no further infections could be acquired), and placed into fresh autoclaved filtered aquarium water. Copepods were screened for infection 14 days following the initial exposure to *S. solidus* coracidia. By viewing each copepod on a glass slide, procercoids were visible in the body cavity and copepods were classified as harbouring infective procercoids when the cercomere was observed.

Juvenile fish (n = 142) were starved the day before parasite exposure, then selected from families in a manner determined by a random number generator. Exposures were carried out in plastic 1 L aquaria filled with 400 ml of filtered, recirculating system water, and surrounded by black plastic on all sides with illumination provided from above. Fish were presented with two copepods, estimated to harbour a total of three or four procercoids. Copepods were introduced to the aquaria via a glass pipette, and a drop of dilute *Artemia* sp. nauplii culture was added immediately afterwards to stimulate feeding. Exposure tanks were left undisturbed for 3h before fish were transferred to individual 1.25 L plastic aquaria (15 cm x 14 cm x 11cm), which were held on a recirculating
water system. Exposed fish were then fed live *Artemia* sp. nauplii *ad libitum* for
70 d. Water temperature was 17°C ±1.4 and day length regimes were 10L:14D. Exposures
to parasite infective stages were carried out under home office licence (Project licence: 80/2327, Personal Licence: 40/9978).

2.3.5.3 Dorsal profile image analysis
In order to facilitate the subsequent estimation of parasite growth rates, digital photographs of the dorsal profile of each fish were taken immediately prior to parasite exposure at 0 d and at the termination of the study at 70 d. The images were analysed using the ImageJ software package (Schneider et al., 2012) for accurate measurements of standard length (*SL*) and area (Barber and Svensson, 2003). The change in length over the course of the study (*dSL*) was calculated as the standard length on day 0 (*SL0*) subtracted from standard length on day 70 (*SL70*). The residual dorsal profile area (*rDPA*) was then calculated from the relationship between the length and dorsal profile area for non-infected fish, to give a baseline measurement of the expected swelling for a fish of given size. The difference in *rDPA* from the start to the end of the study (*drDPA*) thus indicates how the extent of swelling changes for each individual fish, and so indicates parasite growth. The *rSwelling* was then calculated from the residuals of the relationship between total parasite mass and *rDPA* for infected fish, which would then give an indication of whether each fish was exhibiting more or less abdominal distension than would be expect for an infected fish with their total parasite mass (*Mp*).

2.3.5.4 Dissection
After 70 d, fish were euthanized using an overdose of Benzocaine anaesthetic (stock solution: 10mg L-1) according to UK Home Office Schedule 1 methods. Fish were blotted dry, measured using a dial calliper (standard length, *SL*, to 0.1 mm), weighed (*M*, to 0.001 g) and dissected. Any plerocercoids recovered were blotted dry and weighed (to 0.001 g); in the case of multiply infected fish, the mass of each individual plerocercoid was recorded and the total parasite mass (*Mp*) of plerocercoids recovered from each fish calculated.

The fish mass (*Mf*) was then calculated as (*Mf* = *M* − *Mp*). Body condition factor (*BCF*) was calculated as [(*Mf*/(*SL*)³) * 100000]. Liver mass (*Ml*) was recorded (to
0.001 g) and the hepatosomatic index (HSI=$M_t/M_f$*100) was calculated. Specific growth rate ($SGR$) was calculated as (100*(ln($M_{56}$ - $M_p$)-ln($M_0$))/d), where $M_0$ is the wet mass of fish at the start of the study and $M_{56}$ is the wet mass of the fish at the end of the 56 day study.

2.3.6 Statistical Analysis
All statistical analysis was carried out in $R$ 2.15.3 (R Core Team, 2012). Data were tested for normality and homogeneity of variance using box and normal Q-Q plots. Chi-squared tests were used to compare prevalence of parasites between each genotype in wild fish, and also to test if procercoid exposure increased likelihood of infection. A principle components analysis was used to test for clustering of parasite species in wild fish, and ANOVA used to examine parasite intensity against genotype and plate number. In order to determine susceptibility to experimental exposure to $S. solidus$, a binary logistic regression was completed using a general linear model with a binomial link function. A series of ANOVA models were then used to test for relationships between genotype, plate morph, infection status, host growth and condition.
2.4 Results

2.4.1 Survey of parasite loads in relation to plate morph

2.4.1.1 Genotypes present
All *Eda* genotypes were found among sticklebacks collected from the Carsington Reservoir population (n = 150). Although all sampled fish were young-of-the-year (i.e. 0+), all were of sufficient size to have had the potential to develop a full set of plates (Figure 2.6). There was considerable variation in plate numbers within genotypes with the partial (CL) genotype showing the greatest range (Figure 2.7).

![Figure 2.6](image)

**Figure 2.6** Total number of plates for each three-spined stickleback examined for parasite fauna. Data is shown with variation in standard length and genotype.
Figure 2.7 Probability density plot for the total number of plates in wild three-spined sticklebacks from Carsington Water examined for parasite fauna, separated by genotype.

2.4.1.2 Parasite fauna

A number of common freshwater parasite species were found in juvenile sticklebacks from Carsington Water, including *Diplostomum gasterosteii*, *D. spathaceum*, *Gyrodactylus* sp., *Schistocephalus solidus*, *Diphyllobothrium* sp. and an unidentified nematode (Figure 2.8).
Figure 2.8 Plate of microscope images illustrating the diversity of the parasite fauna of wild three-spined stickleback from Carsington Water. A: Diplostomum gasterostei. B: Diplostomum spathaceum, C: Gyrodactylus sp. D: Schistocephalus solidus. E: Diphyllobothrium sp. F: encysted nematode sp.
Most parasites were found to be evenly distributed between *Eda* genotypes (Figure 2.9), although CC genotype sticklebacks appeared to lack cestode (*S. solidus* and *Diphyllobothrium* sp.) and nematode infections (Figure 2.9). There was no association between any of the genotypes and any individual parasite infection (*S. solidus*: $\chi^2=1.31$, d.f. = 2, $P = 0.52$), (*D. gasterostei* $\chi^2 = 3.20$, d.f. = 2, $P = 0.20$), (*D. spathaceum* $\chi^2 = 0.27$, df = 2, $P = 0.88$), (*Gyrodactylus* sp. $\chi^2 = 0.60$, df = 2, $P = 0.74$), (*Diphyllobothrium* sp. $\chi^2 = 0.92$, df = 2, $P = 0.63$). Although it should be noted that the assumptions of the Chi-squared test were not met in the case of *Gyrodactylus* sp. and *Diphyllobothrium* sp. due to insufficient numbers of non-infected individuals, it is unlikely there was a significant effect of genotype on this effect.

**Figure 2.9** Prevalence of parasites recovered from three-spined sticklebacks at Carsington Water. Data is shown for each *Eda* genotype separately.

Principle components analysis (PCA) did not show any obvious clustering of parasite species, and four principle components were required to explain 75% of the data (PC1 = 0.236, PC2 = 0.417, PC3 = 0.586, PC4 = 0.746). Parasite intensity was not significantly associated with genotype in any of the infections (*S. solidus*: ANOVA, $F_{1,144} = 0.56$, $P = 0.5678$, *D. gasterosteii* ANOVA, $F_{1,144} = 1.71$, $P = 0.1853$, *D. spathaceum* ANOVA, $F_{1,144} = 0.34$, $P = 0.7135$, *Gyrodactylus* sp. ANOVA, $F_{1,144} = 0.17$, $P = 0.8448$, *Diphyllobothrium* sp. ANOVA, $F_{1,144} = 0.66$, $P = 0.5197$; Figure 2.10). There was also no association between plate number
and parasite intensity for *Gyrodactylus* sp. (ANOVA, $F_{1,144}= 0.09$, $P = 0.7698$; Figure 2.11), or any other parasite infection (*S. solidus*: ANOVA, $F_{1,144}= 0.27$, $P = 0.6032$, *D. gasterosteii* ANOVA, $F_{1,144}= 1.05$, $P = 0.3082$, *D. spathaceum* ANOVA, $F_{1,144}= 0.01$, $P = 0.9193$, *Diphyllobothrium* sp. ANOVA, $F_{1,144}= 1.35$, $P = 0.2477$).

**Figure 2.10** Intensity of *Gyrodactylus* sp. infection recovered from three-spined sticklebacks at Carsington Water. A, *Eda* genotype. B, number of lateral plates. Error bars represent standard error.
2.4.2 Experimental Infection study

2.4.2.1 Eda, Plate number and probability of infection

Among experimentally infected fish, the probability of infection following exposure to *S. solidus* was not associated with any of the *Eda* genotypes ($X^2 = 2.39$, df = 2, p-value = 0.303; Figure 2.12), but was significantly related to the number of lateral plates exhibited by the host fish (Binary logistic regression: $Z = -2.153$; $P=0.031$; Figure 2.13). Fish with lower numbers of plates were more likely to become infected than those with more plates.

**Figure 2.11** Intensity of parasite infections recovered from three-spined sticklebacks at Carsington Water. A, *Eda* genotype. Error bars represent standard error.

```
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1

Intensity of Infection

Genotype

LL
CL
CC

Diplostomum gasterostei
Diplostomum spathaceum
Schistocephalus solidus
Diphyllobothrium sp.
```

$X^2 = 2.39$, df = 2, p-value = 0.303; Figure 2.12, but was significantly related to the number of lateral plates exhibited by the host fish (Binary logistic regression: $Z = -2.153$; $P=0.031$; Figure 2.13). Fish with lower numbers of plates were more likely to become infected than those with more plates.
Figure 2.12 Frequency of *Schistocephalus solidus* infected and non-infected three-spined sticklebacks of *Eda* genotypes LL, CL and CC.

Figure 2.13 Histogram and logistic regression combination plot. Red line indicates probability of three-spined stickleback infection with *Schistocephalus solidus* according to the logistic regression model. Data are presented as a histogram and logistic regression curve combination plot as recommended by de la Cruz Rot (2005).
To test whether individuals that deviated furthest from the mean number of plates for their genotype were more or less susceptible to infection, the mean number of plates was calculated for each genotype, and the residual number of plates (rPLATE) was calculated for each fish. There was no significant difference in the residual number of plates of fish that did and did not develop infections (Student's t-test: \( t = -0.98, \text{d.f.} = 88, \ P = 0.33 \); Figure 2.14), showing that fish developing more or fewer plates than expected for their genotype were no more (or less) susceptible to infection.

Figure 2.14. Residuals of the expected and observed number of plates for each three-spined stickleback depending on genotype, for infected and non-infected fish. 0, non-infected. 1, infected. 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.
2.4.2.2 Exposure level and parasite establishment

Among exposed fish, there was no significant association between the estimated number of procercoids to which the fish were exposed and whether or not an infection established ($\chi^2 = 2.59$, d.f. = 2, $P = 0.27$; Figure 2.15). Exposing fish to larger numbers of procercoids did increase the variation in the number of worms establishing, but did not increase the numbers of infected individuals overall. A non-parametric Kruskal-Wallis test examining only infected fish showed a significant effect of procercoid dose on the number of plerocercoids that developed (Kruskal-Wallis $\chi^2 = 8.97$, d.f. = 2, $P = 0.011$). This shows that, among individuals that were susceptible to infections, higher levels of exposure did increase parasite intensity (Figure 2.16).

![Figure 2.15](image)

**Figure 2.15** Infection frequency of three-spined stickleback experimentally exposed to procercoids of *Schistosomephalus solidus*. 
Figure 2.16 Boxplot showing the number of plerocercoids that established in threespined sticklebacks experimentally exposed to an estimated 2, 3 or 4 *Schistocephalus solidus* procercoids. 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.

2.4.2.3 Doral profile area as a measure of parasite mass

Swelling, as measured by the distension of the dorsal profile, could be used as an accurate measure of parasite mass in infected fish. There was a significant relationship between the residual dorsal profile area and the total mass of plerocercoids recovered from experimentally infected fish ($F_{1,89} = 34.97, P < 0.0001$; Figure 2.17a). The relationship between residual dorsal profile area and total plerocercoid mass was not affected by host genotype ($F_{1,83} = 0.92, P = 0.454$; Figure 2.17b) nor was there any significant interaction ($F_{1,83} = 1.45, P =$...
It seems clear that the *Eda* genotype of host fish did not affect the extent of abdomen distension among infected fish.

**Figure 2.17** The relationship between total *Schistocephalus solidus* parasite mass and rDPA. A: data pooled for all genotypes with single regression line. B: Each genotype shown separately, with a regression line for each genotype.

In order to determine if having more lateral plates constricted the swelling associated with heavy parasite burden, the residual swelling was used as a measure of whether infected fish showed more swelling than expected for an infected fish with a given total parasite mass (*M_p*). The number of lateral plates did significantly predict the rSwelling value for each fish, fish with greater numbers of plates showed more swelling than expected, indicating that the body
armour does not constrict the parasite from swelling within the abdominal cavity ($t_{1,58} = 2.060, P = 0.0439$, Figure 2.18). When genotype was added as a factor into the linear model, NPLATE was still a significant factor ($F_{1,90} = 4.2782, P = 0.0435$) but genotype was not a significant predictor of rSwelling ($F_{1,84} = 0.9570, P = 0.4199$) and there was no interaction between them ($F_{1,84} = 1.2922, P = 0.2832$), indicating that the observed increased swelling for a given total parasite mass with increased number of plates was not an effect of the underlying $Eda$ genes and related was instead to phenotype.

![Figure 2.18](image.png)

**Figure 2.18** The residual swelling of each *Schistoscephalus solidus* infected three-spined stickleback against the number of lateral plates.

### 2.4.2.4 Fish growth and plate morph

The difference in residual dorsal profile area ($drDPA$) (i.e. the increase in swelling) was significantly affected by infection status, with infected fish showing a greater increase in area over the course of the study, reflecting the parasite-induced swelling ($F_{1,84} = 37.84, P < 0.001$). There was no significant effect of $Eda$ genotype on $drDPA$ ($F_{1,84} = 0.26, P = 0.90$) and there was no interaction between $Eda$ genotype and infection status ($F_{1,84} = 1.45, P = 0.24$; Figure 2.19), meaning that the degree of swelling exhibited among infected fish did not differ between genotypes.
The increase in the length of fish (dSL) experimentally exposed to *S. solidus* was not significantly affected by *Eda* genotype (*P* = 0.43, *F*<sub>1,90</sub> = 0.85; Figure 2.20), or by infection status (*P* = 0.29, *F*<sub>1,1</sub> = 1.12; Figure 2.20). There was also no interaction between *Eda* genotype and *S. solidus* infection, suggesting that the effects of infection on growth did not differ between *Eda* genotypes (*F*<sub>1,90</sub> = 0.60, *P* = 0.55). There was also no significant effect of genotype (*F*<sub>1,84</sub> = 1.13, *P* = 0.33) or infection status (*F*<sub>1,84</sub> = 1.99; *P* = 0.16) on specific growth rate (SGR), and no interaction between genotype and SGR (*F*<sub>1,84</sub> = 1.50, *P* = 0.23; Figure 2.21). It therefore seems that, under standard laboratory conditions at least, stickleback growth rates are not significantly affected by *Eda* genotype, and *S. solidus* infection has a uniform effect on host growth across *Eda* genotypes.

![Figure 2.19](image.png) Difference in the swelling of three-spined sticklebacks from the beginning to the end of the study, with the *Schistoscephalus solidus* infection status of the fish at dissection shown as separate bars. Error bars represent +/- standard error.
Figure 2.20 The mean increase in the standard length of three-spined sticklebacks over the course of the study for each genotype, with the *Schistocephalus solidus* infection status of the fish at dissection shown as separate bars. Error bars represent +/- standard error.

Figure 2.21 The mean specific growth rate (SGR) of three-spined sticklebacks during the study for each genotype, with the *Schistocephalus solidus* infection status of the fish at dissection shown as separate bars. Error bars represent +/- standard error.
In order to determine whether faster growing fish develop fewer plates than expected, the effect of specific growth rate (SGR) and genotype on the residual number of plates was tested. Analysis of covariance (ANCOVA) found no significant effect of SGR on rPLATE ($F_{1,90} = 1.28, P=0.26$) or Genotype ($F_{1,90} = 0.02, P=0.98$) or infection status ($F_{1,90} = 0.68, P=0.41$). Therefore fish with faster growth rates did not develop fewer plates than expected for their genotype, and this was not affected by infection status (Figure 2.22).

**Figure 2.22** The specific growth rate (SGR) of three-spined sticklebacks grouped by genotype, showing the regression of residual number of plates (rPLATE) for each fish and SGR.

However on closer visual inspection of the data using a lattice plot of the regression for SGR and rPLATE for each genotype separately indicated an interesting relationship for Eda heterozygotes (CL) (Figure 2.23). The regression was significant for fish with the genotype CL ($F_{1, 34} = 7.0518, P=0.0120$), but not for the CC genotype ($F_{1, 12} = 0.1485, P = 0.7067$) or LL genotype ($F_{1, 38} = 1.1193, P= 0.2968$). Therefore among fish with the highly phenotypically variable CL genotype, individuals with fastest growth rates developed more plates than expected for their genotype, whereas slow growing heterozygous fish developed
fewer plates than expected. One possible explanation could be that slow growing fish had not fully finished development and therefore had not had enough time to fully develop all their plates.

![Figure 2.23](image)

**Figure 2.23** Scatter plots for the regression of rPLATE and SGR for all three-spined stickleback genotypes and each given genotype separately. A dashed line at 0 is included for reference.

### 2.4.2.5 Effect of infection on body condition

The hepatosomatic index (HSI) was not affected by infection status, *Eda* genotype or the number of plates (Table 2.1, Figure 2.24). Body condition factor (BCF) significantly related to infection status (Table 2.2, Figure 2.25), with infected fish having lower body condition factors than non-infected conspecifics in the genotypes with the low plated allele. There was no effect of genotype or NPLATE on BCF (Table 2.2, Figure 2.25), and no interaction between these
factors, indicating that lateral plate development did not affect the condition of fish.

**Table 2.1** ANOVA table with three-spined stickleback heptato-somatic index (HSI) as the response variable

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>Infection status</td>
<td>1</td>
<td>0.94</td>
<td>0.336</td>
</tr>
<tr>
<td>Genotype</td>
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<td>2.25</td>
<td>0.112</td>
</tr>
<tr>
<td>NPLATE</td>
<td>1</td>
<td>1.20</td>
<td>0.276</td>
</tr>
<tr>
<td>Infection status x Genotype</td>
<td>2</td>
<td>0.07</td>
<td>0.929</td>
</tr>
<tr>
<td>Infection status x NPLATE</td>
<td>1</td>
<td>1.76</td>
<td>0.189</td>
</tr>
<tr>
<td>Genotype x NPLATE</td>
<td>2</td>
<td>0.20</td>
<td>0.817</td>
</tr>
<tr>
<td>Infection status:Genotype x NPLATE</td>
<td>2</td>
<td>2.72</td>
<td>0.072</td>
</tr>
<tr>
<td>Residuals</td>
<td>80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.2** ANOVA table with three-spined stickleback body condition factor (BCF) as the response variable. Significant P values (<0.05) are shown in bold

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection status</td>
<td>1</td>
<td>8.77</td>
<td>0.004</td>
</tr>
<tr>
<td>Genotype</td>
<td>2</td>
<td>0.59</td>
<td>0.558</td>
</tr>
<tr>
<td>NPLATE</td>
<td>1</td>
<td>3.17</td>
<td>0.079</td>
</tr>
<tr>
<td>Infection status x Genotype</td>
<td>2</td>
<td>0.50</td>
<td>0.609</td>
</tr>
<tr>
<td>Infection status x NPLATE</td>
<td>1</td>
<td>0.79</td>
<td>0.376</td>
</tr>
<tr>
<td>Genotype x NPLATE</td>
<td>2</td>
<td>1.21</td>
<td>0.305</td>
</tr>
<tr>
<td>Infection status x Genotype x NPLATE</td>
<td>2</td>
<td>2.17</td>
<td>0.121</td>
</tr>
<tr>
<td>Residuals</td>
<td>80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.24 Hepatosomatic index (HSI) for three-spined sticklebacks from the *Schistocephalus solidus* experimental infection study. A: HSI shown for the three *Eda* genotypes. Error bars represent ± standard error. B: HSI shown against the total number of lateral plates. Regression lines are shown for non-infected and infected fish separately.
Figure 2.25 Body condition factor (BCF) for three-spined sticklebacks from the *Schistocephalus solidus* experimental infection study. A: BCF shown for the three *Eda* genotypes. Error bars represent ± standard error. B: BCF shown against the total number of lateral plates. Regression lines are shown for non-infected and infected fish separately.
2.5 Discussion

2.5.1 Parasite survey

The population of three-spined sticklebacks at Carsington Water showed a wide range of lateral plate numbers, which is highly unusual for a landlocked, freshwater population (Bell and Foster, 1994). Furthermore, there was considerable variation in plate numbers within *Eda* genotypes. Substantial variation was found in the number of plates developed by heterozygous CL fish and homozygous CC fish, indicating that there is substantial phenotypic variation even within a single genotype. This has been reported before in European populations and hypothesised to be associated with gene modifiers or environmental constraints (Lucek et al., 2012).

The results of the field survey showed that many of the common parasites of freshwater three-spined sticklebacks (Kennedy, 1974, Barber, 2007) were represented in the population resident in Carsington Water. However, parasites were found to be evenly distributed between plate morphs, with no significant difference in parasite prevalence between *Eda* genotypes. This may indicate that parasites do not play a strong role in the evolution of plate morphology in three-spined sticklebacks at this site. Sticklebacks with the low plated (LL) genotype had the highest prevalence of *Schistocephalus* infections, although this relationship was non-significant, possibly due to the small numbers of CC fish in the sample. Overall, in the present study there was a lack of clear association between *Eda* genotype or lateral plate morphology and any individual parasite infection. Such relationships might have been expected if the susceptibility to, or detrimental effects of, parasite infections varied across genotypes.

Inhabiting different environments is commonly associated with the development of distinct parasite communities, which are influenced in part by patterns of foraging on intermediate hosts of trophically-transmitted parasites and associating with infected conspecifics, which in this survey might explain why there was no significant difference between genotypes. For example, a study comparing the natural parasite fauna of three-spined stickleback in brackish and freshwater habitats in Poland as part of an international parasite survey of sticklebacks, showed some parasites were present in freshwaters which were
not observed at the Baltic coast such as *Proteocephalus sp.* and *Argulus foliaceus* (see Morozińska-Gogol, 2006).

The results presented here are in some ways surprising, as previous work has indicated morphology is associated with differences in feeding ecology and habitat use in the three-spined stickleback, and therefore we might expect a difference in parasite fauna. MacColl (2009) found evidence that parasite species composition differed between sympatric benthic and limnetic pairs, with limnetic characteristics being associated with *Diplostomum* sp. and *S. solidus* infections and glochidia (*Anodonta* sp.) more common in benthic fish. While differences in parasites communities of single host species are often observed, it can sometimes be difficult to determine the cause of such variation. In a survey of twelve populations of sticklebacks in Scottish lochs in close geographic proximity, loch surface area, pH, calcium concentration, chlorophyll A concentration and dissolved organic carbon (DOC) were measured, yet none of these variables explained the differences in parasite communities, despite distinct differences being observed (de Roij and MacColl, 2012).

The parasite fauna may not simply be a function of the environment but may also represent a selective pressure influencing the phenotypes and genotypes of the organisms that inhabit it. While the parasite survey could potentially give an indication of which parasites are influential in driving evolutionary processes at Carsington Water, there are a number of problems with this approach. First, the correlational nature of the data precludes an analysis of whether the parasite fauna are influencing host genotypes or *vice versa*. Second, it is difficult to determine if the fish caught truly represent the full range of parasite fauna, or simply the most common while missing the most virulent parasites, as it is impossible to know if some parasites may have caused mortality before the sample was taken (Poulin and Morand, 2004). For example relatively few fish with the CC genotype were caught; whilst this could be indicative of the true frequency of the genotype in the population, it might also indicate selective (possibly parasite-mediated) mortality of CC genotypes at an earlier life history stage. Therefore it was necessary to undertake an experimentally study, to give a better indication of the potential for parasite-mediated selection on *Eda* genotypes and / or plate morphology.
2.5.2 Experimental study

The experimental infection study was undertaken to test the hypothesis that susceptibility to *Schistocephalus solidus* infection, or the subsequent growth rates of the parasite and development of the disease phenotype, was related to the *Eda* genotype and/or plate morphology of sticklebacks. Fish bred from parents from the same population showed substantial variation in plate number of *Eda* genotype. This permitted a test of the hypothesis that susceptibility to infection was related to plate morph, without the usual confounding effect of having to use fish from different ecosystem types. Significant differences were found in the susceptibility to infection, with fish with fewer numbers of plates exhibiting an increased likelihood of acquiring infections. A similar result was found recently by Morozińska-Gogol et al. (2011) in a wild sample of sticklebacks in the Baltic Sea. Sticklebacks infected with *S. solidus* had significantly fewer plates, and were identified as either *leiurus* or *semiarmatus*. They hypothesised that this may be due to differences in spawning preferences leading to diet changes, as *leiurus* migrated to freshwater to spawn and *semiarmatus* stayed in shallow water whereas *trachurus* spawned in shallow water but then migrated to open sea. Our results contradict this explanation, since all fish were fed the same diet and were exposed in a controlled manner to infections, meaning that differences in exposure is unlikely to be the best explanation for their result. Therefore differences in susceptibility must be due to an intrinsic factor that is linked to the development of lateral plates. The results presented here also indicate that individuals with infections had lower body condition factors in low plated and heterozygous genotypes, but higher body condition in the completely plated *Eda* genotype. This may indicate that fish genetically predisposed to develop more plates had better body condition. As developing resistance to infection can be energetically costly and is only expected to evolve where there is sufficient selective advantage (Rigby et al., 2002), one explanation for this could be that individuals with sufficient resources to develop protection against predators also develop defences against parasites. However, this would need further testing on greater numbers of fish with the completely plated *Eda* to confirm.
It is possible that the formation of lateral plates is also linked to other phenotypic changes, by pleiotropic effects independent of environment, linking immune factors to plate morphology. Local parasite communities have been shown to have consequences for stickleback immune function in experimental settings (Scharsack and Kalbe, 2014), with fish from lake ecotypes commonly exposed to *Diplostomum pseudospathaceum* showing lower susceptibility and higher respiratory burst (immune) activity in head kidney samples than fish from river ecotypes. However, repeated experimental exposures caused reduced susceptibility in both ecotypes.

The results of the experimental study also demonstrate variation in the number of plates that develop in fish carrying the high plated *Eda* allele, with a genotype of either CL or CC, which is in agreement with previous research (Lucek et al., 2012). The authors suggested in their study population the number of lateral plates developed may be reflective of environmental constraints. However, in our experimental study the fish were bred from parents from the same population and kept in the same laboratory aquaria, therefore the number of plates that are developed by fish with the same genotype cannot simply reflect environmental variation. Given the apparent advantage of high plate number in terms of resisting infection, lateral plates may linked to defence from hostile environments. As the fully plated phenotype is closer to the ancestral marine form, there may be responses linked to plate number which are associated with managing the transition between marine and freshwater. For example, introduced individuals have been shown to have stronger immune responses because they are able to survive the transition to a new environment while infected (Møller and Cassey, 2004). Once in the new environment local adaptation can result in evolved diminished immunity. As marine and anadromous stickleback move between different environments to breed, they might be more resistant to stress. While low plated stickleback in the wild can exploit faster growth rates in freshwater (Marchinko and Schluter, 2007, Barrett et al., 2008, Barrett et al., 2009) by not diverting energy to defence, an advantage as faster growth rates have been associated with response to insect predation (Marchinko, 2009) and to increase survival over the winter (Curry et al., 2005). This would need further testing, with microcosm studies to validate. In one such study where microcosms with
freshwater and marine populations were used, selection against migrants was found, as marine fish artificially migrated to freshwater were found to be more susceptible to freshwater parasites than native freshwater fish (MacColl and Chapman, 2010). The authors do not explicitly state what the plate morph of their fish were, but presumably the marine ancestry fish they used were fully plated, meaning their results contradict those presented here as they found marine fish to be more susceptible to infection than freshwater fish. Therefore studies examining the stress response of fish, such as cortisol levels, and the immune response may be beneficial to separate the effects of genetic background and environment. It is possible that the selection pressures influencing stickleback evolution differ between populations, meaning parasites have a high degree of influence in some populations whereas in others, which may show less diversity in parasite communities, other environmental factors have a greater influence.

Studies on the island of North Uist have shown that calcium may be a particularly important factor influencing the number of plates shown in populations (Spence et al., 2012). North Uist has a relatively low parasite diversity compared to other stickleback populations (de Roij and MacColl, 2012), but populations differ considerably in salinity and calcium levels in close geographic proximity (Giles, 1983). Spence et al. (2012) reared complete and low morph sticklebacks in high and low calcium concentrations, some of which from Scottish populations on Uist. Fish grew more slowly in low calcium and low salinity conditions, but the effect was much more pronounced in complete morphs thereby giving a growth advantage to low plated fish. The predation regime has also been hypothesised to have an effect on plate evolution in stickleback (Reimchen, 1992), but experimental tests of this have not given support to this theory (Zeller et al., 2012). Therefore in some populations the abiotic environment may have a greater influence on stickleback evolution than the biotic.

The results of the experimental study showed a significant positive correlation between specific growth rate (SGR) and the residual plate number of fish, but only among heterozygous CL fish. This could be because heterozygous fish are genetically predisposed to develop more plates if they are able, and faster growing fish are more successful in the environment meaning they are able to grow more quickly and obtain the resources needed to develop energetically
costly bone structures. The water at Carsington Reservoir is relatively high in dissolved calcium (83.9 mg/L), particularly compared with those considered to be high by Spence et al. (2012) in experimental treatments (calcium chloride (CaCl₂.2H₂O) 1.470 g/L⁻¹) and in wild populations (dissolved calcium 13.72 mg/L) (Spence et al., 2013). The high levels of limestone rock surrounding Carsington Water contribute to the high levels of calcium found in the water (Appendix 2; Carsington water geological map), and also in the water supply for the Severn Trent area, including the aquaria at the University of Leicester (calcium hardness 160 mg/L, total hardness 222 mg/L, tested with LaMotte calcium hardness kit, LaMotte Company, Chestertown, MD). Therefore we would not expect calcium availability to be a factor limiting the development of plates in this population in the wild, or in our experimental study. Differences in growth rates between complete and low morph (Marchinko and Schluter, 2007), and highly plated compared to low plated genotypes (Barrett et al., 2008) have been previously reported, with fish exhibiting reduced lateral plates having the advantage. In our study we found no such difference, possibly due to the high water hardness (calcium concentration) of the aquarium used, which might lower the growth costs associated with developing plates.

2.5.3 Conclusions and future directions
This study identifies an effect of plate morphology on susceptibility to S. solidus infections. We have shown that in a population with a range of plate morphs and a relatively high availability of calcium for a freshwater environment, sticklebacks are capable of growing more plates than expected for their Eda genotype, and this may be related to their overall growth rate. We have also shown that the number of plates developed may influence infection susceptibility to S. solidus. Further investigation on the selective agents influencing plate number in threespined sticklebacks from a wide range of environments with differing parasite communities would determine the circumstances where each of these factors becomes important.
2.6 References


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Chapter 3

Consequences of limited dissolved oxygen on gene expression in infected sticklebacks
3.1 Abstract

The three-spined stickleback is a species tolerant of temperature and oxygen concentration fluctuations. A recent study on three-spined sticklebacks identified genes which show differential expression under hypoxia, demonstrating a genetic basis for hypoxia tolerance in this species. Three-spined sticklebacks infected with the cestode *Schistocephalus solidus* spend longer performing aquatic surface respiration and greater periods of time at the surface, where levels of dissolved oxygen (DO) are higher, indicating higher oxygen requirement. In the present study, wild three-spined sticklebacks naturally exposed to *S. solidus* were held under low (21%) or high (98%) DO saturation treatments. Expression of genes previously identified as being upregulated under hypoxia were compared between treatment groups and infection status. Expression of *LDHA* was did not differ between control and treatment groups, although was more in fish with heavier plerocercoids. *JMJD6* was unaffected by treatment or infection status. Infected fish showed a reduced expression of *GADD* and *DDIT4* compared to non-infected fish. The ecological consequences of these changes in hypoxia response are discussed.
3.2 Introduction

3.2.1 Dissolved oxygen in aquatic environments

Dissolved oxygen (DO or \( \text{dO}_2 \)) is the biologically available oxygen source for aquatic organisms, which enters the water column as a result of aquatic photosynthetic organisms releasing oxygen, and by diffusion from the air (Davis, 1975). Aquatic photosynthetic organisms, including macrophytes and phytoplankton, also respire aerobically, especially during the hours of darkness, and hence require DO. In addition, other animals and microorganisms have DO demands that remove oxygen from the water. If DO concentration falls below 2–3 mg/L, the water is considered hypoxic, while 5 mg/l is generally sufficient for the survival of most aquatic organisms (Ficke et al., 2007).

The natural levels of DO found in aquatic habitats vary between the storage reservoirs in the hydrological cycle. Estuaries are highly variable, with rapid increases in DO due to high rates of photosynthesis, and decomposition of organic matter decreasing oxygen levels all changing with tidal conditions. The dissolved oxygen concentration of water is inversely correlated with temperature. Therefore headwaters of streams tend to have high oxygen concentrations due to cooler temperatures and thorough mixing of the water due to higher gradients. Large rivers are calmer, have higher temperatures and consequently lower DO concentrations. However most organisms inhabiting rivers are not usually limited by oxygen due to the constant movement of the water ensuring diffusion of oxygen from the air is high. In lakes, changes in DO concentration have a number of causes. In oligotrophic lakes, which tend to be deeper and therefore maintain lower water temperatures in summer, the DO is high and they support cool water fishes that have high DO demands such as trout (\( \text{Salmo} \) sp.) and whitefish (\( \text{Coregonus} \) sp.) accompanied by a rich benthic invertebrate fauna commonly including caddis and mayfly larvae. In oligotrophic lakes there are low levels of production due to limited availability of phosphorus and nitrogen leading to low densities of photosynthetic organisms. By contrast, eutrophic lakes have high levels of nutrients and are more shallow increasing water temperature in summer. This leads to lakes regularly diurnally depleted of their oxygen overnight as photosynthesis stops but respiration continues (Richards et al., 2009), and also seasonally due to thermal stratification in summer causing high decomposition of
organic matter and oxygen consumption below the thermocline. These lakes tend to be inhabited by fish that are capable of tolerating temperature and DO fluctuations, such as common carp (*Cyprinus carpio*) and catfish (*Ictalurus* sp.), and benthic invertebrates such as midge larvae and tubificid worms (Molles, 2005). It would take thousands of years for an oligotrophic lake to become eutrophic without human intervention (Khan and Ansari, 2005).

The surface waters of the aquatic environment commonly contain high DO levels with nitrogen present mainly as nitrate (Camargo and Alonso, 2006). However in eutrophic waters, where the addition of nutrients results in the excessive growth of phytoplanktons, the DO is restricted in the water due to limited diffusion of oxygen from the air. The green layer of phytoplankton also reduces light penetration resulting in the death of photosynthetic plants below the surface and further reduction of DO (Khan and Ansari, 2005). Rivers and streams, which normally have relatively high DO levels, can receive waste from industry with high biochemical oxygen demand (BOD) therefore limiting aquatic life to those tolerant of low oxygen concentrations (Molles, 2005).

### 3.2.2 Climate change and DO in freshwater ecosystems

Climate change as a whole is expected to have consequences for aquatic organisms, as the atmospheric CO$_2$ levels increase the climate becomes warmer. Warmer climate reduce the overall water supply of the basin due to evaporation, dryer catchments leads to less dissolved organic carbon input and lowering of dissolved oxygen below the thermocline having impacts on the phytoplankton, zooplankton, benthos and fishes. The effect of climate change and associated lowering of DO concentration may also have more severe effects on shallow lakes and streams, as suggested by Magnuson et al. (1997) when considering the lakes, streams and wetlands of the Laurentian Great Lakes region of North America.

Climate change is predicted to have a pronounced effect on populations of fish that are sensitive to water temperatures, and have evolved to live in particular habitat niches. Therefore fish will be forced to adapt to the new environment or alter their species range. Rising temperatures also has the effect of increasing metabolic rates of fish, which in turn increases aerobic respiration and oxygen
demands. DO is inversely related to temperature, temperature increase has a two-fold effect on fish by increasing their demands while limiting their oxygen supply. As higher temperatures increase biochemical oxygen demand, some areas are predicted to experience a decrease in DO (Ficke et al., 2007).

3.2.3 Effects of reduced DO on aquatic organisms in freshwater ecosystems

There are repeated examples of limited DO or hypoxia affecting the aquatic organisms, although much of the research focuses on fish. In common carp (Cyprinus carpio) prolonged hypoxia has been shown to disrupt reproduction by decreasing levels of testosterone, estradiol, and triiodothyronine resulting in underdeveloped gonads and lower fertilisation success (Wu et al., 2003). Hypoxia also impairs the subsequent embryonic development in fertilised fish eggs (Shang and Wu, 2004). There are species differences in responses to hypoxia, for example the fathead minnow (Pimephales promelas) has a greater tolerance for reduced oxygen concentration than their major predator, the yellow perch (Perca flavescens) even when matched for size (Robb and Abrahams, 2003). Metabolic scope models based on laboratory and field data have shown that activity in Atlantic cod (Gadus morhua) is inversely proportional to DO, and in water with a DO of less than 20% air saturation, swimming and feeding activity becomes close to zero risking mortality (Chabot and Claireaux, 2008). Hypoxia can also affect anti-predator behaviours in fish, while escape responses and schooling are disrupted the frequency of aquatic surface respiration (ASR) is increased therefore exposing fish to aerial predation (Domenici et al., 2007). The susceptibility of tadpoles (Rana clamitans) to predation by the fishing spider (Dolomedes triton) is also increased under hypoxia due to the increased amount of time spent at the surface (Moore and Townsend, 1998). Predation can also be increased during hypoxia as a result of increased ventilation rate, as startled common sole (Solea solea) show a ventilation rate of 2 beats per min in normoxia and 58.8 beats per minute in hypoxia (Cannas et al., 2012). However, in some predator-prey systems, lowering DO can actually reduce predation due to the greater sensitivity of predators to hypoxia. In laboratory behaviour trials, reduction in DO significantly reduced predation rates of guppies (Poecilia reticulata) by oscars (Astronotus ocellatus) (see Poulin et al., 1987).
The response of aquatic organisms to hypoxia has also been investigated in gene expression studies. Since aquatic organisms are frequently exposed to oxygen-limited environments they may be expected to have evolved mechanisms to tolerate such conditions (Gracey et al., 2001). Studies on organisms adapted to hypoxic conditions have shown that extreme hypoxia resistance is achieved by the up-regulation of some genes and down-regulation of others to reduce ATP demand (Hochachka and Lutz, 2001). Therefore, gene expression studies provide an opportunity to study the physiological response of an animal and judge its ability to cope with anoxia (Nikinmaa, 2002).

3.2.4 Effects on dissolved oxygen concentration on parasitized fish
Climate change and its effects on DO levels may also have an impact on the parasite infections of animals. For example, thermal stratification makes water below the thermocline hypoxic, forcing fish into higher densities (Ficke et al., 2007), and increasing parasite transmission (Marcogliese, 2001). Parasites may also exert more severe effects when combined with a stressor in the environment, meaning infections which are not lethal in optimum conditions may induce mortality when the host is challenged (Marcogliese, 2008). For example in common carp fry infected with varying levels of Dactylogyrus vastator and exposed to decreasing oxygen concentrations, heavily infected fish died first and non-infected fish had the longest survival (Molnar, 1994). When exposed to sub-lethal levels of hypoxia, European eels (Anguilla anguilla) infected with the swimbladder nematode parasite Anguillicoloides crassus (Dracunculoidea) have shorter survival times than non-infected conspecifics. Effects are more pronounced with heavier infections, potentially explaining summer mortality of infected fish (Molnár, 1993). Eel mortality under decreasing oxygen availability is inversely correlated with pathology of the parasite in the swimbladder rather than number of parasites (Lefebvre et al., 2007). Infected eels also show elevated cortisol in blood plasma, indicating stress, compared to non-infected conspecifics when exposed to 4h of hypoxia (Gollock et al., 2005). Gene expression studies have shown a negative relationship between parasite abundance and several genes involved in coping with environmental stress (Fazio et al., 2008), but not fish growth (Fazio et al., 2009), giving a possible mechanism for the mortality associated with A. crassus under hypoxia.
3.2.5 Effects of reduced DO on three-spined sticklebacks

The three-spined stickleback is a teleost fish that has repeatedly colonised brackish and freshwater habitats in the Northern hemisphere from ancestral marine populations. This adaptability, together with its ease of housing in lab aquaria, have made it a prominent model in ethology, genetics and parasitology. The stickleback has a high tolerance to warming and hypoxia making it an excellent model for effects of global change and environmental quality (Katsiadaki et al., 2002, Katsiadaki, 2007). In a mesocosm experiment using increased temperature and nutrient enriched conditions, Moran et al. (2010) showed that combined treatments resulted in extinction of experimental stickleback populations, which was attributed to hypoxia as lower DO concentrations were recorded in these treatments. 

Early studies by Jones (1952) into the reactions of fish to hypoxia, have shown sticklebacks react to hypoxia below 3 mg/L or 28% saturation at 13°C by increasing their swimming and ventilation rate. Oxygen concentrations above 4 mg/L or 38% saturation at 13°C were not sufficient to induce the fish to move to the aerated zone of an experimental chamber. The avoidance behaviour shown to low DO water was faster at 20°C than at 13°C. Similar responses were shown by common minnows (*Phoxinus phoxinus*), with less of temperature dependence. However, trout responded with higher ventilation rates more than high swimming speeds, although this may be due to the source of the sample population (Jones, 1952). This behavioural response is common in fishes exposed to low dissolved oxygen concentrations (Kramer, 1987). The level of oxygen saturation which induced behavioural changes in three-spined sticklebacks may be variable between populations, as more recent studies have reported normal behaviour at as low as 20% oxygen saturation (Sneddon and Yerbury, 2004).

Utilising the fully sequenced genome of the three-spined stickleback (Jones et al., 2012), a gene expression study has also been completed on three-spined sticklebacks exposed to hypoxia of 24% to 28% oxygen saturation. Several transcripts were found to be differentially expressed under hypoxia including those associated with regulation of energy metabolism (Leveelahti et al., 2011).
3.2.6 *Schistocephalus solidus* infections and environmental change

*Schistocephalus solidus* plerocercoids occur naturally in three-spined sticklebacks, and are known to alter host growth, reproduction and behaviour (Barber and Svensson, 2003, Barber, 2013). The parasite is commonly found in still waters and slow flowing rivers, where fluctuation in DO is likely to be commonplace.

Temperature can have consequences for *Schistocephalus solidus* growth, with elevated temperatures resulting in increased parasites size and a shift in host preferences towards warmer water (Macnab and Barber, 2012). Sticklebacks infected with *S. solidus* are also sensitive to other environmental perturbations, such as feeding regime and cadmium exposure (Pascoe and Woodworth, 1980).

The effect of *Schistocephalus solidus* on stickleback physiological response to hypoxia has not been confirmed. Early studies examining the effect of reducing DO have shown that sticklebacks parasitized with *S. solidus* show behavioural changes sooner – at higher DO concentrations – indicating that they may have a reduced ability to respond to changing conditions. Infected fish showed aquatic surface respiration (ASR) for longer than 10 sec at 2.6 to 3.0 mg/L or 3.6 to 4.0 mg/L, and fish with higher parasite index (PI) reacted sooner. This is compared to non-infected conspecifics, which performed ASR for longer than 10 sec at 1.6 to 2.0 mg/L (Giles, 1987). These results were found to concur with an earlier study on *S. solidus* infected sticklebacks where parasitized fish were found to consume more oxygen than non-parasitized fish at maximum activity levels (Meakins and Walkey, 1975).

In studies on nine-spined stickleback infected with *Schistocephalus* sp., infected fish spent more time performing aquatic surface respiration (ASR) than non-infected fish in hypoxic conditions at 0.75–2.0 mg/L oxygen concentration. Parasitized fish were also quicker to return to the surface after being exposed to a simulated bird predator attack. Parasitized fish were concluded to have a higher oxygen demand as parasitized fish also showed a higher lethal oxygen level (Smith and Kramer, 1987). However, nine-spined stickleback and three-spined stickleback show divergent preferences for oxygen concentration in the wild, with three-spined sticklebacks typically being found in water with higher oxygen
concentrations (Copp and Kováč, 2003), meaning the result found by Smith and Kramer (1987) may be difficult to generalise. It has been suggested that stickleback infected with *S. solidus* may spend longer periods close to the water surface, due to higher DO levels (Lester, 1971), and more recently behavioural manipulation to increase predation by birds and consequently transmission of the parasite to the definitive host has been implicated (Barber et al., 2004).

### 3.2.7 Aims

In a previous study by Leveelahti et al. (2011) three-spined sticklebacks responded to hypoxia by upregulating selected genes, and the authors cite parasitism as one factor likely to alter hypoxia tolerance. There is already a wealth of knowledge on the detrimental effects of parasitism. From my own observations and past studies, infected sticklebacks show aquatic surface respiration earlier than non-infected conspecifics, which may indicate that parasitized individuals are less able to cope with short-term fluctuations in oxygen availability. Therefore, the current study aims to examine if three-spined sticklebacks infected with *S. solidus* show the same changes in gene expression in response to hypoxia as non-infected stickleback. Naturally infected and non-infected stickleback were exposed to hypoxia for a 24 h period, and their expression of candidate genes, already identified as being upregulated in response to hypoxia in non-infected three-spined sticklebacks, was quantified using qPCR. Two possible outcomes were considered; firstly, since parasitism may increase the oxygen demand of the host, we might expect hypoxia associated genes to be further upregulated compared to non-infected individuals in order to compensate for the parasite burden. On the other hand, it is possible that there would be no increase in expression of hypoxia genes in infected fish, or even a decrease, as energetic drain or other detrimental factors associated with parasite infection may prevent the host from mounting a sufficient response to hypoxia, which may in turn lead to the increased aquatic surface respiration behaviour observed.
3.3 Methods

3.3.1 Fish collection and husbandry
Forty adult three-spined sticklebacks were caught in June 2012 from the River Welland, Leicestershire, UK (N 52°28'33, W 0°55'29). Fish were held in stock tanks (40cm x 60cm x 40cm) on a recirculating system maintained at 16 ± 1°C under a 16L:8D photoperiod to match natural conditions. Experimental treatments took place in July 2012.

3.3.2 Experimental treatments
Trials were completed to find the oxygen concentration where fish from this population showed some behavioural responses, such as aquatic surface respiration, but were not distressed. Oxygen concentration was decreased incrementally from 100% to 30% and held for fish to acclimatise but behavioural responses did not start until 20% saturation, the level used for the hypoxia treatment in the study.

Fish were held in two experimental tanks (40cm x 60cm x 30cm) each containing 20 fish. Tanks receiving the hypoxia treatment were maintained at 20% oxygen saturation using nitrogen gas bubbled through an air stone until stable to ±1%. DO levels were monitored throughout by an oxygen meter (PreSens Precision Sensing GmbH, Regensburg, Germany) and visualised using OxyView software (PST3-V6.02). Measurements of oxygen saturation were automatically temperature compensated in the software. Fish were then placed in an acclimatisation tank for 1 h as oxygen levels were gradually reduced to 20% before entering the experimental hypoxia tank for a further 23 h, to give a total exposure time of 24 h.

Data from the oxygen meter showed the experimental hypoxia tank had a mean oxygen saturation of 21.32% ±2.53 S.D. and a temperature of 16.33°C ±0.38 S.D. In the control tank oxygen saturation was maintained, by bubbling compressed air through an airstone, at 97.84% ± 0.32 S.D. and temperature was 15.94°C ±0.08 S.D.
3.3.3 Fish dissection and tissue sampling

Fish were removed from their experimental tanks and euthanized using an overdose of Benzocaine anaesthetic according to UK Home Office Schedule 1 methods. Fish were rinsed with ddH$_2$O, blotted dry, measured ($SL$, to 0.1 mm) and weighed ($Mass$, $M$, to 0.001 g). Any *Schistocephalus solidus* plerocercoids were removed following an incision along the ventral surface, from the vent to the operculum. If present, plerocercoids were blotted dry and weighed (total plerocercoid mass, $M_p$, to 0.001 g). Not many other parasite infections were noted in this sample. The liver, kidney, spleen and brain tissue were removed and snap frozen in liquid nitrogen before being stored at -80°C prior to RNA extraction. In total 21 out of 40 three-spined stickleback were infected with *Schistocephalus solidus*.

3.3.4 RNA isolation

RNA was extracted using Sigma Mammalian Total RNA extraction kit following manufacturer instructions. Briefly, tissue was homogenised in 500 µl of lysis buffer containing β-mercaptoethanol (stock 1 ml lysis solution, 10 µl ME). Samples were transferred to a filtration column and centrifuged at 12000 rpm for 2 min. 500 µl 70% ethanol was added to the sample, then transferred to a binding column. Two washes were applied, followed by centrifugation for 15 seconds, with a final wash step followed by centrifuging for 1 min. Finally the sample was eluted in 55 µl and centrifuged for 1 min.

Genomic DNA was removed from the RNA samples by DNasel (Sigma, UK) following protocols provided. 50 µl per samples was incubated with 5 µl reaction buffer and 5 µl DNasel enzyme for 15 min at 70°C, before adding 5 µl stop solution.

RNA quantity and quality was determined by Nanodrop 1000 spectrophotometer (LabTech International, Lewes, UK). Gel electrophoresis was used to check for RNA degradation, using 1µl RNA sample with 2 µl loading dye and 7 µl ddH$_2$O on a 1% agarose gel.
3.3.5 Reverse Transcription Quantitative PCR analysis

RT-qPCR was undertaken to examine the expression of genes that are known to be upregulated in sticklebacks exposed to hypoxia. These genes of interest were selected on the basis of their known function, and differential expression in sticklebacks, following Leveelahti et al. (2011). The genes chosen were lactate dehydrogenase (*LDHA*), growth and DNA damage-induced protein 45 gamma (*GADD45G*), Jumonji domain containing 6 (*JMJD6*) and DNA damage-inducible transcript 4 (*DDIT4*). Primers were designed based on Ensemble IDs to yield a product size of 70 to 120 bp. The housekeeping gene used for reference was ribosomal protein L8 (*rpL8*), as this gene has been used on local stickleback populations previously (Geoghegan et al., 2008, Seear et al., 2014) and showed the lowest variation in Ct in all samples compared to ribosomal protein L13 (*rpL13*) and ubiquitin (*UBQ*). *LDHA*, *DDIT4* and *JMJD6* are associated closely with hypoxia responses and the *GADD* family attributed to cell differentiation.

First strand cDNA was reverse transcribed using 0.5µg RNA with Moloney Murine Leukemia Virus Reverse Transcriptase (mmLV-RT) (Promega, UK), and then diluted to 2µg concentration. RT-qPCR reactions used a master mix of 10µl SYBR Green JumpStart Taq ReadyMix (Sigma, UK), 1 µl of 5µM forward and reverse primer (Table 3.1), 7 µl water and 1 µl diluted cDNA to a total volume of 20µl.

qPCR was carried out on a Chromo4 qPCR thermocycler (BioRad Laboratories, Hercules, CA). PCR condition were as follows: 40 cycles with an initial incubation step of 95°C for 3 min followed by 95°C for 30 sec (denaturing steps), 60°C for 30 sec (annealing) and 72°C for 30 sec (extension). A melting curve step from 50°C to 95.1°C, with a reading every second, was then used to ensure only one product was amplified and confirmed by a single peak in the dissociation curve. A “no reverse transcriptase” control was performed for each primer and cDNA combination. Ct values were determined by finding the baseline where no reaction occurred then manually setting the threshold on a log plot of fluorescence where exponential amplification occurred. Gene expression data was normalised using ribosomal protein L8 as a reference gene.
Table 3.1 Primers used for qPCR on three-spined stickleback cDNA for hypoxia associated genes, after Leveelahti et al. (2011) and Geoghegan et al. (2008)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene</th>
<th>Primers 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSGACG00000011270</td>
<td>LDHA</td>
<td>F: TAATGGAGTTGTCTGTCCCCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAAATTGCCTTTGTGCTCAACTGA</td>
</tr>
<tr>
<td>ENSGACG0000006793</td>
<td>GADD45G</td>
<td>F: CTGAGAAGGATTGGGAATATGCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CCATTTCGTGCAAAGTCTGAAAC</td>
</tr>
<tr>
<td>ENSGACG0000015017</td>
<td>JMJD6</td>
<td>F: GCCTTCAGTCCACCTCTGTGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GAACAGGTTTAAGGGGTCCAGT</td>
</tr>
<tr>
<td>ENSGACG0000008596</td>
<td>DDIT4</td>
<td>F: GACTGTGGCCAAAAGTTCAGAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTCTTGTGGCCAATGTTCAAGAAG</td>
</tr>
<tr>
<td>ENSGACG0000002035</td>
<td>L8</td>
<td>F: CGACCCTACCGCTTTCAAGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGACATTGCCAATGTTCAAGCTGA</td>
</tr>
</tbody>
</table>

3.3.6 Data analysis

Gene expression was analysed by calculating the delta Ct value for each sample, by subtracting the average reference Ct value from the average target Ct value, as used by (Seear et al., 2014). All data analysis was completed in R (R Core Team, 2012). Linear models were used, after checking for assumptions of normality, with delta Ct values as the response variable. Multifactorial analysis of variance (MANOVA) was used to examine the effects of infection status, hypoxia treatment and gene on expression. Each gene was then analysed individually, again using ANOVA, to determine the effects of infection status and hypoxia treatment on relative expression.
3.4 Results

3.4.1 Dissection data

Fish used in the study had a mean standard length (SL) of 33.1 ± 3.5 mm. At dissection it was possible to confirm infection status; the length of infected fish (32.4 ± 3.7 mm) did not differ significantly from that of non-infected fish (33.7 ± 3.3 mm), and fish selected for the hypoxia treatment did not differ in length (33.5 ± 3.8 mm) from those selected for the control treatment (32.8 ± 3.3 mm). There was no significant difference in SL with infection status and treatment and there was no interaction between the two variables (ANOVA; Infection status, $F_{1,37} = 1.40$, $P = 0.24$; Treatment, $F_{1,37} = 0.92$, $P = 0.34$; Interaction, $F_{1,37} = 1.12$, $P = 0.30$, Figure 3.1).

At dissection, fish mass (i.e. excluding the mass of plerocercoids removed from infected fish) was found to differ significantly between infected (0.370 ± 0.156 g) and non-infected fish (0.473 ± 0.137 g) in the study. Fish mass varied significantly with infection status but not with experimental treatment and there was no interaction between the two variables (ANOVA; Infection status, $F_{1,37} = 4.95$, $P = 0.03$; Treatment, $F_{1,37} = 0.71$, $P = 0.40$; Interaction, $F_{1,37} = 0.54$, $P = 0.47$, Figure 3.1).

The mean total parasite mass ($M_p$) among infected fish in the study was 0.130 ± 0.041 g. Mean $M_p$ did not differ between infected fish maintained under the hypoxia treatment (0.134 ± 0.042 g) and the control treatment (0.125 ± 0.043 g; ANOVA, $F_{1,17} = 0.196$, $P = 0.664$, Figure 3.2). When fish mass was taken used to calculate parasite index ($I_p$) there remained no significant difference between treatments (hypoxia 36.6 ± 14.3, control 43.4 ± 17.3; ANOVA, $F_{1,17} = 0.8524$, $P = 0.369$, Figure 3.2).
Figure 3.1 Post-mortem data from River Welland three-spined sticklebacks, including standard length (SL) and the mass of the fish excluding *Schistocephalus solidus* parasite mass (Fish Mass). \( n = 40; \) Control non-infected \( n = 11, \) Control infected \( n = 9, \) Hypoxia treatment non-infected \( n = 8, \) Hypoxia treatment infected \( n = 12. \) 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.
Figure 3.2 *Schistocephalus solidus* data for three-spined sticklebacks in the control and hypoxia treatment. Data presented is for infected fish only. A; Total parasite mass (g). B; Parasite index ($I_p$). 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.

### 3.4.2 Gene expression, hypoxia and infection status

Expression of hypoxia-associated genes from liver tissue was significantly affected by all predictor variables. There was also an interaction between treatment and gene, showing that the change in expression pattern under hypoxia differed between genes (Table 3.2, Figure 3.3).
Table 3.2 Factorial ANOVA of three-spined stickleback gene expression level relative to L8, testing the effect of hypoxia treatment, Schistocephalus solidus infection status and gene.

<table>
<thead>
<tr>
<th>Variable</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection status</td>
<td>1</td>
<td>15.01</td>
<td>0.00016</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>7.20</td>
<td>0.0081</td>
</tr>
<tr>
<td>Gene</td>
<td>3</td>
<td>116.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Infection status x Treatment</td>
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<td>0.60</td>
<td>0.438</td>
</tr>
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<td>0.229</td>
</tr>
<tr>
<td>Treatment x Gene</td>
<td>3</td>
<td>8.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Infection status x Treatment x</td>
<td>3</td>
<td>0.12</td>
<td>0.948</td>
</tr>
<tr>
<td>Gene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>144</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The delta Ct values were then analysed using a multifactorial ANOVA (MANOVA) to test the effect of hypoxia treatment and infection status on each gene separately. There was a marginally non-significant effect of infection on LDHA expression (F_{1,36} = 2.99, P = 0.093), with infected fish showing a trend towards lower levels of gene expression than non-infected fish. There was no significant effect of hypoxia treatment on delta Ct values for LDHA (F_{1,36} = 0.65, P = 0.427) and no interaction between infection status and treatment (F_{1,36} = 0.020, P = 0.888), meaning that hypoxia did not alter expression of LDHA in this study (Figure 3.3).

Expression of GADD was significantly affected by infection status, with infected fish showing reduced expression compared to non-infected fish (F_{1,36} = 6.05, P = 0.019). There was a marginally non-significant effect of hypoxia treatment (F_{1,36} = 2.98, P = 0.093), with GADD being upregulated under hypoxia. There was no interaction between infection status and hypoxia treatment (F_{1,36} = 0.009, P = 0.927, Figure 3.3).

The expression JMJD6 gene was not affected by infection status (F_{1,36} = 0.13, P = 0.721) or hypoxia treatment (F_{1,36} = 2.37, P = 0.133) with no interaction between the two variables (F_{1,36} = 2.08, P = 0.158, Figure 3.3).
There was a significant effect of infection status on the expression of DDIT4 \( (F_{1,36} = 7.38, P = 0.0101) \), with gene expression being upregulated further in non-infected fish than infected fish. There was also a highly significant effect of hypoxia treatment \( (F_{1,36} = 23.84, P < 0.0001) \), as fish held under the hypoxia treatment showed reduced DDIT4 expression than control fish held under control conditions. There was no significant interaction between infection status and hypoxia treatment \( (F_{1,36} = 0.16, P = 0.694, \text{Figure } 3.3) \).

**Figure 3.3** Boxplots showing the effect of *Schistocephalus solidus* infection status and hypoxia treatment on the expression of the genes *LDHA, GADD, JMJD6* and *DDIT4* relative to that of the reference gene, ribosomal protein L8, by three-spined sticklebacks in the study. Note different y-axis variation between plots. Sample sizes: control, non-infected \( n = 11 \); control, infected \( n = 10 \); hypoxia treatment, non-infected \( n = 7 \); hypoxia treatment, infected \( n = 12 \). 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.
3.4.3 Parasite burden and gene expression

The parasite index, which takes into account the relative size of the parasite to the fish, was not a significant predictor for expression of any of the genes studied (Table 3.3, Figure 3.4).

Among infected fish, the expression of *LDHA* showed a significant positive relationship with largest worm mass (Table 3.4, Figure 3.5), meaning that fish with larger plerocercoids showed the highest *LDHA* expression. This relationship was not found for any other genes in the study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>df</th>
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<tbody>
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<td>LDHA</td>
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<table>
<thead>
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<th>Gene</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>0.03214</td>
</tr>
<tr>
<td>GADD45</td>
<td>17</td>
<td>0.7044</td>
<td>0.413</td>
</tr>
<tr>
<td>JMJD6</td>
<td>17</td>
<td>0.0163</td>
<td>0.8998</td>
</tr>
<tr>
<td>DDIT4</td>
<td>17</td>
<td>0.0285</td>
<td>0.8679</td>
</tr>
</tbody>
</table>
Figure 3.4 Gene expression of *Schistocephalus solidus* infected River Welland threespined sticklebacks for each gene of interest with parasite index ($I_p$). The dashed line represents 0 normalised expression of the gene. Solid lines show the regression between PI and delta Ct.
Figure 3.5 Gene expression of *Schistocephalus solidus* infected River Welland threespined sticklebacks for each gene of interest, shown against the mass of the largest plerocercoid present. The reference line dashed line represents 0 normalised expression of the gene. Solid lines show the regression between PI and delta Ct. The dotted line represents the size at which the parasite become infective, at 50mg.
3.5 Discussion

3.5.1 General findings
Relatively few studies have studied hypoxia and gene expression in sticklebacks, and none to our knowledge have studied this in combination with the added burden of parasite infection. This is despite sticklebacks having a wide range of naturally occurring parasites, and hypoxia being known to interact with parasite infection in other fish species. The results of this chapter indicate that both S. solidus infection status and the availability of DO influences the pattern of expression of genes that are known to normally be upregulated in the hypoxic response of fish. However, the effect was not consistent across all genes studied. Fish in experimental groups were of similar size and parasite burden, therefore it seems unlikely that individual variation in body size was a significant factor in the analysis of gene expression.

3.5.2 Gene expression and potential physiological function in sticklebacks
In contrast with previous studies on hypoxia in fish (Leveelahti et al., 2011), we found no evidence of upregulation of LDHA compared with controls. As LDHA is associated with increasing ATP production by glycolysis, in other words anaerobic respiration, upregulation in LDHA may help in maintaining activity under low oxygen conditions as has been previously shown in mammals. LDHA was found to be upregulated in the liver tissue of longjaw mudsucker gobies (Gillichthys mirabilis) which are tolerant to hypoxia (Gracey et al., 2001). However, high expression of LDHA in this study began after 24 h and therefore may differ between species and populations, perhaps as a response which is activated after the initial metabolic suppression response immediately after the onset of hypoxia has occurred (Hochachka et al., 1996). It is possible that in the current study, the duration or intensity of hypoxia induced was not sufficient to cause a shift to anaerobic respiration. However, LDHA expression was significantly related to the mass of the largest plerocercoid found in the fish, indicating LDHA is expressed more in fish with larger worms. Given the role of LDHA in anaerobic respiration, expression may signify hypoxic stress on the host when carrying infective parasites.
Both LDHA and GADD are regulated by hypoxia-inducible factor 1 alpha (HIF-1α), which has been well studied in mammals as a being associated with hypoxia tolerance (Shams et al., 2005). HIF is transcription factor with two subunits, the alpha subunit being stabilised by oxygen. In the absence of oxygen HIF-1α is no longer degraded by enzyme activity, and accumulates in cells triggering the expression of genes. This function which seems to have been preserved across taxa makes it of interest to medical science due to its role in retinal disease (Arjamaa and Nikinmaa, 2006), and in particular in fish due to their tendency to be exposed to oxygen fluctuations (Gracey et al., 2001).

The cell cycle is slowed in tissues experiencing hypoxia by pathways involving HIF-1α, GADD and DDIT (Brugarolas et al., 2004). Leveelahti et al. (2011) found GADD45 is upregulated after 3 h and 48 h exposure to hypoxia, and DDIT4 showed increased transcripts after hypoxia. In the present study, GADD was shown to slightly upregulated in response to hypoxia, but significantly less in infected fish. A much greater response was seen in DDIT4 where downregulation was seen under hypoxia, with significantly less expression seen in infected fish. This is unusual as DDIT4 has been previously reported to be upregulated in response to hypoxia (Hu et al., 2006), as an inhibitor to mTOR which prevents accumulation of HIF-1α.

In contrast to Leveelahti et al. (2011) there was no differential expression of JMJD6 with hypoxia found in this population of sticklebacks. Recent studies have suggested JMJD6 is a target for HIF-1α (Yang et al., 2009, Wellmann et al., 2008), although this role is not always clear as hypoxia does not always induce JMJD6 activity and it may instead have a greater role in blood vessel growth (Boeckel et al., 2011). Hypoxia has even been shown to upregulate transcription of some enzymes containing jumonji (JMJ) domains (Pollard et al., 2008).

3.5.3 Ecological consequences for host-parasite interactions

The behavioural changes induced by S. solidus infections are likely to increase the likelihood of predation by susceptible predators (Barber et al., 2004), therefore if infection reduces the ability to cope with lower DO then it may also increase predation or reduce anti-predator behaviour further as seen in other non-infected fish species, for example spending greater periods of time at the
surface and aquatic surface respiration. Increased time at the surface is more likely to increase predation by birds which would complete the parasite life cycle, giving a benefit to the parasite of manipulating the host. Expression of LDHA was found to increase in fish harbouring larger parasites. This may be beneficial to the parasite as larger plerocercoid size is associated with reproductive output, therefore causing fish to spend greater amounts of time at the surface may increase parasite fitness. However the expression of the other hypoxia associated genes examined in this study showed no change in expression with parasite index, therefore further study with a greater range of parasite burdens would be needed to clarify if gene expression changes are consistent.

It is also possible that the effects of infection on DO response could also be consequence of energetic drain of parasite. To try to determine this more data would be needed on fish harbouring parasites around the 50 mg infective size. We would expect that parasites under 50 mg would not cause their hosts to spend time at the surface as transmission at this stage would not facilitate reproduction. While fish with worms over 50 mg would be expected to induce a behavioural response coupled with higher gene expression. We do know that infected fish suffer more under hypoxia from previous studies by Giles (1987) and Meakins and Walkey (1975) but this will need further study to understand the physiological basis.

The hypoxia response in sticklebacks also might have consequences for reproduction because the embryos depend on male fanning the nest (Moran et al., 2010), therefore infected males who are expressing hypoxia genes might spend more time at the surface with higher DO rather than performing parental care behaviours. Therefore if infected males do manage to successfully build a nest, offspring may suffer higher mortality due to lack of oxygen.

### 3.5.4 Future directions

To summarise I have shown that the capacity of sticklebacks to respond adaptively to reduced DO levels in changing environments appears to be affected by parasite infections. However, one of the problems with this kind of study, which is based on naturally infected fish, is that we cannot know at what stage the responses change. In addition, the data is correlational meaning it is difficult to
determine if the expression patterns of fish differ because they are infected or if they are infected because they differ in gene expression profiles, such as being more susceptible to *S. solidus* infection. In order to clarify these effects of infection on gene expression the experimental infection system of *Schistocephalus solidus* could be used to look at how responses change as the infection progresses. By using an experimental infection study the effect of infection and change in dissolved oxygen could also be tested. It is possible that early infections cause hypoxia sensitivity in young of the year fish therefore increasing mortality, which could not be tested in this study due to difficulties in diagnosing early *S. solidus* infections. Alternatively hypoxia at a later stage when the plerocercoid is at infective size may cause greater transmission to the definitive host as fish rise to the surface, which has been previously been identified as a behaviour known to increase predation of sticklebacks (Barber et al., 2000).

From my own observations, not all fish showed aquatic surface respiration at the same time in this study as the fish were held in groups. This could be a result of differing parasite indexes for the wild fish, as reported by Giles (1987). This may present an issue because not all fish may have been under the same level of oxygen stress, meaning a highly variable gene expression profile. To solve this, fish could be housed individually and oxygen saturation sequentially lowered until behavioural responses were observed. Alternatively an experimental infection study could be used to control the time between exposure and treatment, in an effort to produce plerocercoids of similar sizes. This would also allow for a study to examine the effect of *S. solidus* on gene expression at pre-infective and infective parasite sizes. If the parasite is manipulating the behaviour of the host by altering gene expression, forcing the fish to spend greater periods of time at the surface, we would expect the response to only be produced over the 50 mg infectivity limit when the parasite could successfully transmit to the definitive host and reproduce. Unfortunately this was not possible to do in the current study because naturally infective wild fish were used, which had already attained infective size.
3.6 References


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Chapter 4

The effect of salinity on the development and viability of *Schistocephalus solidus* eggs

N. Simmonds ©
4.1 Abstract

Schistocephalus solidus plerocercoids commonly infect three-spined stickleback Gasterosteus aculeatus populations in brackish and freshwaters but infections are typically absent from marine populations. Here, we test the salinity tolerance of S. solidus eggs, to determine the role of salinity in limiting the distribution of infection in coastal zones. We find that S. solidus eggs, derived from the in vitro culture of three different plerocercoids, developed normally in salinities of up to 12.5‰, but above this egg viability dropped rapidly, and no egg hatching was observed at salinities above 20‰. Our results are consistent with the observed distribution of infection in natural stickleback populations, and demonstrate that S. solidus presents a novel disease challenge to marine populations of three-spined sticklebacks entering brackish and freshwater environments.
4.2 Introduction

4.2.1 Parasite distribution and environmental constraints

Parasites vary considerably in their sensitivity to their environment, both in their ability to survive and also transmit successfully between hosts. While some endoparasites are shielded from external environmental fluctuations by their hosts, others are either ectoparasitic (and therefore permanently exposed to the external environment), or have life cycles that include free-living infective stages that are in direct contact with the external environment. As such, all parasites that are, at some point in their life cycle, exposed to the external environment are expected to have evolved mechanisms to allow them to cope with changes in external stimuli. For example, some infective stages of parasites enclose themselves within protective cysts that are passed with the faeces and in which they can lie dormant until ingested by the next host. Examples of this include *Giardia duodenalis*, a unicellular zooflagellate protist which commonly infects humans from contaminated water supplies (Goater et al., 2013) and the acanthocephalan *Plagiorhynchus cylindraceus* (Moore, 1983). Another solution employed by parasites to avoid encountering unfavourable external environments is to arrest development altogether at the larval stage within the host until outside conditions are more favourable. This requires parasites to be capable of sensing the external environment either directly, or through the responses of the hosts, and is a strategy adopted by *Trichostrongylus* nematodes (Anderson, 2000). However, despite these adaptations, for many parasites surviving sub-optimal external environments outside of the host represents a considerable challenge, and parasites are often vulnerable to changes in conditions. External environments may place strict limitations on the geographic range of parasites, and potentially allow hosts to widen their distribution beyond the tolerance of the parasite.

Arguably one of the most important environmental elements affecting the distribution of parasites is the availability of other obligate hosts in the parasite’s life cycle, or vectors that may be required for transmission. Environmental factors that affect the distribution of these hosts or vectors also play a critically important role in determining the distribution of parasites. For the malaria-causing protozoan *Plasmodium falciparum*, geographic distribution is limited by the
mosquito vector. Consequently, temperature increases related to climate change are predicted to shift the distribution of mosquito vectors (Patz et al., 1996, Tanser et al., 2003). Similarly, digenean parasites – whose distribution is restricted to areas where molluscs are present - are limited to high-calcium water bodies since this mineral is required for mollusc shell development. In this instance the parasite is not directly affected by the calcium levels but is still dependent on them on to find a suitable host and complete its life cycle (Bush et al., 2001).

Other effects of the environment influence parasite survival in a more direct manner, influencing the parasite themselves rather than the host. Many ectoparasitic nematodes, which typically have a free-living terrestrial larval stage in their life cycle, are sensitive to temperature and humidity, as these affect survival and infectivity. For example, *Elaphostrongylus rangiferi*, adults of which infect reindeer, utilises a terrestrial snail as an intermediate host but requires moisture in the soil or a water source for the parasites to successfully transmit to the snail (Skorping, 1982). Without these requirements being met the parasite larva are in danger of desiccation and mortality before moving on to the next stage in the life cycle. Therefore if an environment is not favourable to the free-living stages of the parasite it will not survive to reproduce and the parasite will be unable to expand its geographic range in to that area (Bush et al., 2001).

A similar process applies to ectoparasites of hosts that move between the extremes of environments. For example, Pacific salmonids of the genus *Oncorhynchus*, are anadromous, hatching in freshwater and spending their adult life stage feeding in marine waters before returning to freshwater to breed. Freshwater ectoparasites, such as *Gyrodactylus salaris*, are lost in the transition to marine waters (Soleng and Bakke, 1997); sea lice (Copepoda: Caligidae), on the other hand, are marine specialists that routinely infect salmonids in seawater but drop off when fish return to freshwaters to spawn.

4.2.2 Effects of novel parasites on non-coevolved hosts

Although hosts invading new habitats may lose their parasite in this transition, the converse scenario may also occur, where a host moving to a new area may acquire novel parasites. While release from native parasites has received
considerably more attention in the literature (Torchin and Mitchell, 2004, Mack et al., 2000), other studies have considered the implications of parasites specialised to a particular environment on colonisation by new hosts. Over time an invasive species may accumulate pathogens, potentially limiting its success, as illustrated in invasive grasses (Flory et al., 2011). Alternatively, species introduced to an area for the first time may serve as additional hosts for resident parasites, such as the increased species richness observed in the parasite community of an introduced goby Neogobius kessleri (Ondrackova et al., 2009). A common hypothesis suggests parasites which have coevolved with their hosts over long periods will be less virulent than novel parasites, because this ensures the parasites is able to develop to infectivity to transmit to the next stage of the life cycle without inducing host mortality (Lenski and May, 1994, Frank, 1996). However, this is not always the case and can vary between host-parasite interactions (Ebert, 1994). Therefore the results of hosts moving to new environments can result in both the loss of parasites and novel parasite challenges.

4.2.3 Schistocephalus solidus development, life cycle and distribution

The tapeworm Schistocephalus solidus is a parasite known to infect three-spined sticklebacks, Gasterosteus aculeatus, in both freshwater and brackish water environments (Barber, 2006). As for all pseudophyllidian cestodes, the eggs of S. solidus are released from adult worms into aquatic environments (Smyth, 1976). The egg is surrounded by a thick capsule which is thought to provide a water-proof barrier and is formed from the quinone protein sclerotin, giving the egg a tanned appearance as they pass through the uterus and harden before being released from adult worms (Smyth and McManus, 2007). Within the eggs, provided they are laid into oxygenated water, embryonic parasites undergo a process of development to form the hexacanth (‘6-hooked’) stage (Smyth, 1976), which emerges from the eggs when the operculum ruptures following exposure to daylight (Chappell, 1980). The hexacanth develops into a coracidium and becomes enclosed in a ciliated embryophore membrane to permit free-swimming, which it achieves with a characteristic swirling motion. The coracidia are then ingested by the first intermediate host copepods, making this is a passive process for the parasite (Rohde, 2005). A light stimulus triggers hatching,
although other stimuli may be involved there is little known about the precise timing of hatching in the wild. It is likely that the mechanism of hatching is similar to that of the trematodes, with an enzymatic reaction breaking open the operculum (Smyth, 1976).

*Schistocephalus solidus* exhibits variable levels of host specificity through its life cycle. Whereas the plerocercoid stage appears to be highly specific to *Gasterosteus aculeatus* (Orr et al., 1969, Bråten, 1966), a wide range of vertebrates can serve as suitable definitive hosts of the adult stage due to the progenetic plerocercoid carrying enough energy reserves to mature in the definitive host without a food source (Smyth, 1976). Experimental infections of rats, hamsters, ducks, chicken and pigeon have all been successful (McCaig and Hopkins, 1963, Tierney and Crompton, 1992). In the wild, infections of piscivorous birds are more common with gulls (Fam. Laridae), grebes (Fam. Podicipedidae) and ducks (Fam. Anatidae) all being reported as harbouring infections of adult worms (Rolbiecki et al., 1999). The first intermediate hosts of the parasites are copepods, and a number of species have been recorded in the wild as harbouring *S. solidus* procercoids (Dubinina, 1959, Bråten, 1966, Sysoev, 1985, Marcogliese, 1995b). In the laboratory * Cyclops* sp. are commonly used in the culture of *S. solidus* to ingest the coracidia and become visibly infected (Orr and Hopkins, 1969, Jakobsen et al., 2012). It therefore appears that *S. solidus* can potentially utilise a range of copepod hosts. Since copepods are ubiquitous components of aquatic ecosystems – including brackish and marine systems – it is therefore possible that potentially suitable hosts for the *S. solidus* life cycle exist in a wide range of aquatic habitats. It would seem that transmission could potentially extend to estuaries and coastal areas.

There are reports of three-spined sticklebacks infected with *S. solidus* at the coast particularly in the Baltic Sea (Morozińska-Gogol, 2011) and in anadromous stickleback which experience both freshwater and marine environments (Confer et al., 2012) although these are relatively rare. This is generally assumed to be because *S. solidus* is a freshwater specialist relying on the abundance of copepod and bird hosts found commonly in slow flowing waters such as lakes. However, *S. solidus* is specific to *Gasterosteus aculeatus* which are found in a wide variety of habitat including coastal areas, but non-specific for the first
intermediate host copepod and definitive host (Wootton, 1976). It is therefore unclear whether *S. solidus* is in fact restricted to freshwater, and if so whether this is due to a complex life cycle relying on the presence of certain freshwater-specific host species, or a limitation of the parasite itself to tolerate marine conditions. The evolutionary origins of the Pseudophyllidea are thought to have been in the marine environment (Rohde, 2005), and *S. solidus* has close extant relatives inhabiting marine hosts. Given this, and the fact that sticklebacks in a range of salinities have been shown to harbour *S. solidus* infections, we might expect *S. solidus* to be tolerant to a range of salinities.

### 4.2.4 Aims

If *S. solidus* shows sensitivity to salinity and is a freshwater specialist then infection would present a novel challenge to marine sticklebacks attempting to colonise freshwater environments, as the parasite would not have co-evolved with marine hosts. However, if developmental stages of *S. solidus* can tolerate brackish water salinities, then it is possible that anadromous populations will have been exposed to *S. solidus* infection, facilitating the flow of resistance or tolerance genes. In this study, I tested the hypothesis that *S. solidus* is a parasite that is largely restricted to fresh and brackish waters due to an inability of eggs to develop hatch in marine environments.

### 4.3 Methods

#### 4.3.1 Parasite culture and egg production

*Schistoscephalus solidus* plerocercoids were dissected from sticklebacks that had been sampled from the River Welland (52°28'33.02"N, 0°55'29.10"W) in June 2012, and kept in the laboratory for ten weeks to allow the plerocercoids to develop sufficiently to be easily detectable, by visually inspecting the fish for abdominal distension. Plerocercoids were cultured using *in vitro* techniques, previously described in Chapter 2 (Barber and Svensson, 2003). Contents of the dialysis membrane were transferred to a 9 cm petri dish and inspected visually for eggs. Any remnants of the adult parasite’s outer tegument were removed.
4.3.2 Experimental conditions

Saline solutions were made up to the required concentrations by adding the appropriate quantity of aquarium salt (Tropic Marin® Synthetic Sea Salt, Dr. Biener GmbH, Wartenberg, Germany; Table 4.1) to 100 ml filtered distilled water in a 250 ml conical flask, then mixed using a magnetic stirrer to ensure the salt was fully dissolved. Solutions were then measured using a conductivity meter to give precise measurements of salinity (Table 4.1). Ten salinity conditions were tested (Table 4.1), with two replicates of each. A Gilson Macroman™ pipette was used to pipette 15 ml of the appropriate solution into each 5 cm Petri dish. Eggs were then distributed equally between the 20 Petri dishes by viewing under a dissection microscope, agitating the solution and using a glass pipette to remove the eggs in a minimal volume of culture liquid.

It was decided that 0‰ would not be tested as this would be difficult to control for pH and not representative of natural conditions. Instead 0.05‰ was used as a control and pH was tested for all solutions prior to use.

Table 4.1 Experimental conditions used to culture Schistocephalus solidus eggs, recovered from River Welland three-spined sticklebacks and brought to maturity in vitro

<table>
<thead>
<tr>
<th>Condition Number</th>
<th>Sea salt added per 100 ml (g)</th>
<th>Nominal Salinity (‰)</th>
<th>Conductivity measured (mS.cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.005</td>
<td>0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.5</td>
<td>0.911</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>5</td>
<td>7.69</td>
</tr>
<tr>
<td>3</td>
<td>0.8</td>
<td>8</td>
<td>12.73</td>
</tr>
<tr>
<td>4</td>
<td>1.1</td>
<td>11</td>
<td>18.27</td>
</tr>
<tr>
<td>5</td>
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<td>14</td>
<td>21.3</td>
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<td>6</td>
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<tr>
<td>8</td>
<td>2.3</td>
<td>23</td>
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</tr>
<tr>
<td>10</td>
<td>3.5</td>
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</tr>
</tbody>
</table>
The Petri dishes were then sealed with Parafilm to prevent evaporation and transferred to an incubator at 20°C for three weeks. This has proved to be sufficient time for successful embryonation in the laboratory previously and is the timing described by Smyth (1976).

4.3.3 Egg count images and data analysis
Petri dishes were removed from culture and agitated before removing a drop of culture solution and placing on a glass slide with cover slip. A digital photograph was then taken of the field of view at x400 magnification. 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 (De Vos, 2010).

Eggs were categorised according to their stage of development or non-development, as being ‘hatched’, embryonated ‘non-embryonated or ‘damaged’. Damaged eggs appear sickle shaped with no hexacanth visible (Figure 4.1.A). Non-embryonated eggs are uniform in appearance giving a mottled pattern with no hexacanth visible (Figure 4.1.B). Embryonated eggs have a spherical hexacanth, which is a fully developed embryo characterised by six hooks, and is yet to emerge (Figure 4.1.B). Hatched eggs are characterised by a clear appearance, sometimes with the operculum of the egg still visible (Figure 4.1B & C).
The data was then reclassified as eggs being either ‘viable’ (hatched or embryonated) and ‘non-viable’ (including both non-embryonated and damaged eggs). A multinomial logistic regression was then completed in R (R Core Team, 2012) using a generalised linear model with a logit link, to examine the effects of increasing salinity on the viability of eggs.

4.3.4 Statistical Analysis
Viability of eggs was modelled using a general linear model with a quasibinomial link function including *Schistocephalus solidus* individual as a factor to account for individual variation.
4.4 Results
In total, the development of 6519 eggs was quantified in this study. All of the three Schistocephalus solidus worms that were cultured produced eggs, and of these all produced some eggs that hatched successfully. Hatched eggs were present in samples incubated in salinities up to and including 20‰ salinity and damaged eggs were present at 23‰ and above (Figure 4.2). There is some variability between S. solidus individuals in their ability to withstand higher salinities, for example the eggs from plerocercoid one show a greater proportion of damaged eggs between 23‰ and 35‰ than those from plerocercoid two, whereas eggs from plerocercoid three do not show damage at 23‰ (Figure 4.2). However with only three S. solidus individuals cultured it is difficult to test these differences statistically.

Salinity is a significant predictor for egg viability (Logistic regression p= 0.0005, F1, 9=27.358, Figure 4.3). Although the viability of eggs varies slightly between individual parasites, the viability of all eggs declined with increasing salinity. The salinity condition was a significant predictor for egg viability (t= -4.707, P<0.0001) but there was no significant effect of individual parasites (t= -0.075, P=0.941) and no interaction between the parasite identity and salinity condition (t= -0.170, P=0.866). Therefore the variation between parasites is not significant and all parasites show a decrease in egg viability with increasing salinity.
Figure 4.2 Bar chart showing the proportion of *Schistoccephalus solidus* eggs damaged, non-embryonated, embryonated and hatched from salinity culture conditions in the study ranging from 0.5‰ to 35‰. A, plerocercoid one data. B, plerocercoid two data. C, plerocercoid three data.
Figure 4.3 The proportion of viable eggs from each individual *Schistocephalus solidus* decreases with increasing salinity. Curve is predicted from the logistic regression model using the collated data set as no significant differences between worms were found.
4.5 Discussion

Given the wide use of the three-spined stickleback as a model for behavioural and evolutionary studies and the close association of the tapeworm parasite *Schistocephalus Solidus* (Barber and Scharsack, 2010), understanding the conditions in which transmission of the parasite is possible is particularly important. Significant attention has been given to infectivity of the procercoid and the subsequent effects on the stickleback host (Wedekind, 1997, Christen and Milinski, 2003, Parker et al., 2003), but little is known about the conditions which give rise to infective coracidia. Although *S. solidus* infected sticklebacks have been found in marine conditions, it is commonly assumed that freshwater is a more favourable environment (Morozinska-Gogol, 2011). This study has demonstrated an inhibitory effect of salinity on the development and hatching of *S. solidus* eggs. This will effectively provide an environmental and geographical limitation on the range over which *S. solidus* is able to infect three-spined stickleback hosts.

To put the results from this chapter in an ecological context taking the example of a typical river estuary, a study measuring the salinity along the Humber estuary, UK in relation to fish species including sticklebacks, the salinity was found to vary from 32.2 psu at the widest point to 0.3 psu at the tributary (Marshall and Elliott, 1998). As psu and ‰ are almost equivalent, the salinities tested in the present study are ecologically relevant to a river inhabited by three-spined stickleback. Given the inhibitory effect of salinity demonstrated in this chapter, it is likely hatching of *S. solidus* would be limited to the upper estuary in this example location. Anadromous stickleback are known to migrate along the Humber estuary to breed (Jones et al., 2006), indicating that there is overlap between the theoretical hatching range of *S. solidus* and the presence of *Gasterosteus aculeatus* which then return to the coast. This seems the most likely explanation for reports of infected stickleback at higher salinities in other locations (ref barber 2006 stickleback book).

In a parasite survey on nine-spined stickleback (*Pungitius pungitius*) inhabiting brackish and freshwater ponds, the sister parasite species *Schistocephalus pungitius* was found only in freshwater ponds (Marcogliese, 1995a), a finding
consistent with our own results. Given the isolation of Sable island, Nova Scotia on which Marcogliese et al. (1995a) conducted their study, and the close proximity of the freshwater and brackish ponds to each other it seems reasonable that the bird hosts which allowed *Schistocephalus* to colonise this island dispersed parasite eggs into both ponds. The brackish ponds’ salinity varied from 10‰ to 25‰ depending on seasonal variation, which would be consistent with our results showing egg viability being limited over 20‰.

In some marine areas, such as in the Baltic Sea, salinities are particularly low. A study by Zander et al. (2007) found that in Mecklenberg in the Baltic, with a salinity of around 10 to 18 psu, three-spined stickleback infection with *S. solidus* can be as high as 14%. In the Gulf of Gdańsk on the Polish Baltic coast, the psu is around 7 and *S. solidus* infection rates have been reported between 6.3% and 94.4% (Morozinska-Gogol, 2011, Rolbiecki et al., 1999). Given our results showing that *S. solidus* eggs remain viable up to 20‰ salinity or approximately 20 psu, it is possible that there is some proportion of *S. solidus* eggs hatching in the Baltic Sea, leading to these higher than usual levels of infection. As the low surface salinities of less than 10 psu in the Baltic sea are maintained by freshwater run-off (Ehlin, 1981) some infections may be caused by the influx of parasite infective stages from rivers.

Smyth (1954, 1976) investigated the structure of the cestode egg in great detail, using histological examinations and noted the presence of a thick capsule in most pseudophyllidean species and oxygen and water being necessary for hatching, although the influence of water salinity has not been tested experimentally. In this study there was visible folding of the capsule, indicating a physical barrier to hatching. It is possible the elevated salinity disrupts the enzymatic reaction required for the operculum to open at the mid-range of salinities tested preventing hatching. While at high salinities close to marine conditions of 35‰ embryogenesis is prevented by changing the surrounding water potential and removing the trigger for development. The lowering of the water potential surrounding the eggs also seems to result in dehydration, causing the inner membrane to withdraw from the outer shell. However this would require further study to confirm the mechanism of inhibition.
A possible limitation of the study is that the plerocercoids used for culture were only from one freshwater population. It is conceivable that *S. solidus* populations from areas closer to estuaries have a greater tolerance for higher salinity. However, given the wide range of dispersal of *S. solidus* by the definitive host, a piscivorous bird, it seems unlikely the populations of *S. solidus* would segregate to an extent to cause local adaptation. Rather, gene flow is likely to be high due to migration and sexual reproduction between these hermaphroditic parasites in the avian gut.

*S. solidus* is a cestode with many close relatives in the family pseudophyllidean which inhabit marine teleosts (Rohde, 2005, Olson et al., 2001). It is possible that the ability of *S. solidus* to tolerate brackish waters could be due to ancestral forms infecting marine stickleback. These marine sticklebacks would then have colonised freshwater with infections, leading to co-evolution of extant freshwater populations of three-spined sticklebacks and *S. solidus*. However, molecular evidence suggests that the diphyllobothriids including *S. solidus* which inhabit birds, mammals and reptiles as their definitive hosts must have diverged from the rest of the order pseudophyllidean early on in its evolution, as most of the other families inhabit teleost fish as their definitive hosts (Olson et al., 2001, Kodedova et al., 2000). Given this gap in evolutionary time between *S. solidus* and marine ancestors it is likely marine three-spined stickleback populations encounter diphyllobothriids infrequently.

The inhibitory effect of higher salinity on hatching of *S. solidus* has interesting implications for evolution of the three-spined stickleback. It has been demonstrated by genetic analysis that *Gasterosteus aculeatus* has repeatedly invaded freshwater from marine habitats, the adaptive changes observed facilitated by standing genetic variation rather than on freshwater population invading other freshwater sites (Colosimo et al., 2005, Jones et al., 2012). As parasites are known to exert significant pressure on host populations by disrupting reproduction and causing mortality they are capable of influencing the evolution of a species. If each population separately invades freshwater then encountering *S. solidus* and potentially other freshwater parasite would be a novel challenge for the stickleback. Potentially the harmful effects of infection may be more serious in some individuals, with those better able to resist or
tolerate the infection able to survive long enough to reproduce thereby increasing fitness and increasing their frequency in the population over successive generations. As relatively little is known about the factors contributing to stickleback evolution this hypothesis would require further study.

4.6 References


ROHDE, K. 2005. Marine Parasitology, CSIRO PUBLISHING.


Chapter 5

Effects of food intake on plerocercoid growth in Schistoscephalus solidus infected sticklebacks

N. Simmonds ©
5.1 Abstract

A number of studies have demonstrated increased appetite of *S. solidus* infected sticklebacks, but the consequences of increased food intake are unknown, and may potentially benefit host fish or parasites. Here, the ration of infected sticklebacks was experimentally manipulated to investigate the relationship between food intake and plerocercoid. Lab-bred and reared juvenile sticklebacks that had been experimentally infected with *S. solidus*, or sham-exposed, were subsequently held under 8% or 16% body mass per day ration treatments for 56d. Image analysis of dorsal photographs taken at the beginning and end of the experimental feeding study, allowing the change in infection-induced swelling to be quantified and permitting host and parasite growth to be quantified in relation to host ration. At the end of the study, plerocercoids recovered from infected fish were weighed. Parasites gained more weight during the feeding trials in hosts fed the higher, compared to the lower, ration. All fish held under higher rations developed larger livers for their size compared to the lower ration, regardless of infection status. The implications of these findings for host-parasite interactions are discussed.
5.2 Introduction

5.2.1 Energetic costs of parasites

Parasites represent a diverse group of organisms, with one common characteristic; they interact with another organism in a way which causes detrimental effects. The negative impact on the hosts are a result of parasites relying on host organisms for resources necessary to fuel their growth, development and reproduction (Goater et al., 2013) and therefore impose energetic costs on their hosts, which often exhibit reduced growth as a consequence. For example, in fish a wide range of both ecto- and endoparasitic infections are associated with reduced host growth rates. In whitefish (*Coregonus lavaretus*) infections with the eye fluke *Diplostomum spathaceum* has been shown to reduce weight of the fish, but only under high parasite burden (Karvonen and Seppälä, 2008), presumably because infection of the lens hindered the ability to forage efficiently. Cardinalfish (*Cheilodipterus quinque lineatus*) infected with parasitic isopods (*Anilocra apogonae*) are shorter and have a lower body mass than non-infected fish (Fogelman et al., 2009), indicating significantly reduced growth rates. Similarly in Perch (*Perca fluviatilis*) with liver infections of *Triaenophorus nodulosus* plerocercoids, fish growth both in terms of length and mass for a given age, was slower in infected individuals. Brinker and Hamers (2007) found negative effects on mass were more pronounced in heavy infections (reduced by 16%) compared with fish with an average parasite load (reduced by 9%). Suppression of growth rate in fish infected with *Loma salmonae* has also been observed in Rainbow trout (*Oncorhynchus mykiss*) (see Speare et al., 1998) and Chinook salmon (*Oncorhynchus tshawytscha*) (see Ramsay et al., 2004). Growth suppression has also been found in other taxa, such as water striders (*Gerris buenoi*) infection with gut parasites (trypanosomatid flagellates, *Blastocrithidia* sp. and *Leptomonas* sp.) cause lower growth rates and longer development time (Klingenberg et al., 1997), a pattern seen across parasite infections (Crompton, 1984). Such effects may have effects on the health, welfare and reproductive potential of infected animals, and have ecological or economic consequences (Longshaw et al., 2010).
5.2.2 General effects of nutrition on organisms

The nutritional status of an individual has important implications, as the energy gained must be used for both maintenance and growth (Wootton, 1994). Studies which have examined the effect of reduced food availability in invertebrates have shown negative impacts on reproduction (Antunes et al., 2003) and growth (Antunes et al., 2003). In fish, the nutritional content of food can also have implications for innate immune function (Landolt, 1989, Duncan and Klesius, 1996, Jeney et al., 1997, Magnadottir, 2006). In a study examining the role of vitamin C and E on the Gilthead seabream (Sparus aurata), fish fed a diet with vitamin C and E supplements showed enhanced respiratory burst activity indicating improved innate immune function compared to controls with no supplements (Ortuño et al., 2001). In a study on male guppies (Poecilia reticulata) exposed to Gyrodactylus turnbulli, the availability of food and carotenoid was manipulated in order to distinguish between these two variables (Kolluru et al., 2006). Carotenoids are used in males as a pigment for attracting mates and also confer immunological benefits. Kolluru et al. (2006) found that intermediate levels of carotenoid supplementation was most beneficial as this resulted in the lowest parasite load, indicating a beneficial effect of carotenoids to hosts at low levels but benefit to parasites at higher levels. High food availability initially resulted in lower parasite loads at 3 days, suggesting an increased immune response compared to fish with low food availability, but 6 days later in the experiment high food availability resulted in greater parasite loads indicating a benefit of well-fed hosts for the parasite. Therefore fish raised under high food availability, with consequently better condition, may then facilitate higher parasite growth rates in the long term.

5.2.3 Effects of host nutrition on parasites

The consequences of the energetic reliance of parasites on their hosts can also be studied from a parasite-centred perspective, since parasite growth and development may be sensitive to the nutritional conditions experienced within the host, and thus can be affected by changes in the quantity or quality of food available to the host. For example in the cestode Hymenolepis diminuta growth is limited by host glucose levels (Dunkley and Mettrick, 1969).
In some cases, infection may cause a temporary increase in host food intake or hyperphagy, possibly in an attempt to compensate for the energy demand of the parasite. The European eel (Anguilla anguilla) infected by the nematode Anguillicoloides crassus shows a positive correlation between relative gut mass and parasite mass, indicating an increase in food intake in proportion with parasite burden (Lefebvre et al., 2013). Parasite associated growth enhancement has also been reported in cestode (Ligula intestinalis) infections of Roach (Rutilus rutilus) (Loot et al., 2002b), which may be caused by early increases in host foraging activity in response to infection. Increased early growth in the first two years of parasitized whitefish (Coregonus lavaretus) infected with Triaenophorus crassus plerocercoids has also been observed, and attributed to high initial feeding rates (Pulkkinen and Valtonen, 1999). However, it is difficult to determine if this increase in feeding rate is an attempt by the host to mitigate the effects of parasite burden, or a manipulation strategy of the host to increase the availability of nutrients for its own growth.

5.2.4 Food availability and host-parasite interactions in context

Resource allocation between the host and the parasite in a heterogeneous environment is particularly interesting in indirectly transmitted parasites as the need for growth must be balanced with transmission success. Models examining resource ecology of parasitic infections have suggested parasites will continue to use host resources for their own growth until the parasite’s minimum energy requirements are met (Hall 2009). However, from an evolutionary perspective selection will only favour traits that increase parasite fitness, and the survival of hosts until the parasite is transmitted is a crucial factor in determining parasite fitness in complex lifecycles (Poulin, 2007). Understanding the balance between the energetic needs of the host and parasite in variable environments is particularly important, since alterations in the quantity and quality of food available to individual animals are often predicted as a result of anthropogenic environmental change (De Stasio et al., 1996, Micheli, 1999, Woodward et al., 2010). Increased food availability and changes to nutritional quality are the norm in intensive agriculture and aquaculture (Descroix et al., 2010) as a method of improving production, therefore a detailed understanding of how host ration affects the growth and development of parasites is desirable.
5.2.5 The Three-spined stickleback – *Schistcephalus solidus* system

Plerocercoids of the cestode *Schistcephalus solidus* are common parasites of three-spined sticklebacks, *Gasterosteus aculeatus*, throughout the range of the host (Barber, 2007, Wootton, 1976). Sticklebacks become infected after ingesting infected cyclopoid copepods, which are the first intermediate hosts of the parasite. Infective procercoid larvae then penetrate the gastro-intestinal tract and develop into plerocercoids within the body cavity of the host. Plerocercoids achieve infectivity to the definitive host at a mass of approximately 50 mg (Tierney and Crompton, 1992), by which time the parasite is detectable by abdominal distension (Barber, 1997, Aeschlimann et al., 2000, Barber and Svensson, 2003). *Schistcephalus solidus* only reaches sexual maturity in the avian gut following the ingestion of host sticklebacks by piscivorous birds, which serve as definitive hosts of the parasite.

Sticklebacks infected with *S. solidus* typically exhibit changes in behaviour and time budgets that lead to a greater foraging effort post-infection, in an attempt to balance their energy intake with the parasitic burden (Walkey and Meakins, 1970). Infected fish have been shown to increase their access to food through a variety of mechanisms, including foraging under threat of predation (Milinski, 1985, Barber et al., 1995) and becoming more selective in prey choice (Milinski, 1984, Jakobsen et al., 1988, Cunningham et al., 1994, Tierney, 1994, Ranta, 1995). The net result is that infected fish potentially increase their energetic intake beyond that of non-infected conspecifics, yet it is unclear in this and in other systems in which infection-associated hyperphagy has been demonstrated (Lefebvre et al., 2013) whether the consequent increase in food intake ultimately benefits the host (for example, in allowing it to ‘outgrow’ the infection) or the parasite. In a threat-sensitive feeding study, Aeschlimann et al. (2000) experimentally exposed three-spined sticklebacks to *S. solidus* infection and monitored fish foraging decisions and growth rates in the presence or absence of a pike (*Esox lucius*). There was no significant difference in the growth rate of infected and non-infected fish, and infected fish consumed less in the presence of a predator than infected fish without predation threat. Therefore these results indicate that when survival risk is high, infected fish do not use the opportunity to maximise their growth (Aeschlimann et al., 2000). Previous studies have
demonstrated that, among experimentally-infected group housed fish, plerocercoid growth rates and host growth rates co-varied positively (Barber 2005); however, the specific effect of host ration has not yet been investigated.

5.2.6 Aims

The aim of this study was therefore to examine the consequences of experimentally manipulated host ration for parasite growth, specifically testing the hypothesis that an increase in food intake by host sticklebacks led to an increase in the growth rate of plerocercoids. The growth rate of plerocercoids in experimentally infected fish held under different ration regimes was compared with their hosts by a non-invasive morphometric technique (Barber, 1997).

5.3 Methods

5.3.1 Sampling and fish breeding

Juvenile three-spined sticklebacks were selected from lab-bred stock bred in June 2010 from wild caught parents originally captured at Carsington Reservoir, Derbyshire (53°06' N, 1°64' W). Fish were reared in family groups in 30 L tanks (40x25x30cm), fed ad libitum to excess on frozen bloodworms and were naïve to infection prior to parasite exposure. The mean standard length of the fish was 26.56 mm at week 0 (n = 90).

5.3.2 Exposure to parasite infective stages

*Schistocephalus solidus* plerocercoids were dissected from two UK populations of three-spined stickleback, Carsington Reservoir and the River Welland, Leicestershire (52°48' N, 0°92' W). These worms were cultured separately in vitro to produce eggs (Smyth, 1954) which were then incubated in the dark at 21°C for 3 weeks. Hatching was induced by exposure to light (Scharsack et al., 2007). Individual lab bred and reared copepods (*Cyclops strenuus abyssorum*, Sciento, UK) were exposed to two emergent coracidia and screened after 14 d to confirm infection status. Three-spined sticklebacks were exposed to infective stages of *S. solidus* by ingestion of a single infected copepod, or sham-exposed by feeding a non-infected unexposed copepod (infective exposed n = 60, sham exposed n = 30). Exposures to parasite infective stages were carried out under home office licence (Project licence: 80/2327, Personal Licence: 40/9978).
5.3.3 Animal husbandry
Post exposure / sham-exposure, fish were housed individually in 2.5 L (22.7 cm x 14.7 cm x 14 cm) tanks containing a gravel substratum, plastic plant and supplied with compressed air via an air stone. Water temperature was maintained at 16°C throughout the study and a photoperiod of 14L:10D was implemented, to mimic natural environmental conditions. Fish were randomly assigned to either the 8% body weight per day (bw.d⁻¹) food ration or the 16% bw.d⁻¹ food ration treatment and were fed for 14 d. A total of 90 sticklebacks were used in the study, with 45 assigned to each treatment. Feeding manipulation was for 56 d duration, with ration recalculation occurring every 14d.

5.3.4 Non-invasive measurement of parasite growth
Fish were photographed digitally, backlit in dorsal profile (Barber 1997), blotted gently and weighed in water (wet mass, $M_w$, to 0.001 g) at the beginning (day 0) and the end of the experimental feeding period (day 56). An additional weighing on day 7 allowed the recalculation of bloodworm ration to compensate for any change in mass. Dorsal profile images were analysed using ImageJ software (Schneider et al., 2012), to record standard length ($SL_w$) and area ($DPA$) as described in Chapter 2. Standard length on day 0 ($SL_0$) was subtracted from standard length on day 56 ($SL_{56}$) to derive the change in length over the course of the study ($dSL$). The $DPA$ values were square root transformed ($sqrtDPA$) and corrected for body size by calculating the residuals ($rDPA$) from the relationship between $sqrtA$ and $SL_w$ for non-infected fish to give a baseline measurement of swelling for a stickleback of a given size. $rDPA$ was therefore a measure of the signed deviance in profile area from the expected value of a non-infected fish of the same length, and has previously been shown to relate to the magnitude of the plerocercoid burden (Barber, 1997, Barber and Svensson, 2003). The change in $rDPA$ from day 0 to day 56 was then calculated for each fish ($drDPA$).

5.3.5 Post mortem analysis
At the end of each study, fish were sacrificed by exposure to an overdose of Benzocaine anaesthetic. Fish were blotted to remove surface moisture before being weighed on an electronic top pan balance to record the total Fish Mass ($M$, to 0.001 g) and Standard Length ($SL$) was measured manually to an accuracy of
0.1mm using a dial calliper. Any *S. solidus* plerocercoids present were counted, blotted and weighed individually to find the Total Parasite Mass (*M*<sub>p</sub>, to 0.001g), allowing the parasite index (*l*<sub>p</sub>) to be calculated as the total parasite mass divided by the fish mass and multiplied by 100 (*l*<sub>p</sub> = [total *S. solidus* mass / *M*] * 100). Fish were then assigned to either a non-infected or infected status. During the course of the study 18 fish died: 1 control and 10 exposed from the 8% bw.d<sup>-1</sup>; 1 control and 6 exposed from 16% bw.d<sup>-1</sup> treatment leaving a total of 72 fish.

The fish mass (*M*<sub>f</sub>) was then calculated as (*M*<sub>f</sub> = *M* – *M*<sub>p</sub>). Body condition factor was calculated as ([(*M*<sub>f</sub> / (SL<sup>3</sup>)] * 100000]. Liver mass (*M*<sub>L</sub>) was recorded (to 0.001g) and the hepatosomatic index (*HSI* = *M*<sub>L</sub> / *M*<sub>f</sub> * 100) was calculated. Spleen mass (*M*<sub>S</sub>) was also recorded (to 0.001g) and the spleen somatic index (*HSI* = *M*<sub>S</sub> / *M*<sub>f</sub> * 100) was calculated. Specific growth rate (*SGR*) was calculated as (100*(ln(*M*<sub>70</sub> – *M*<sub>p</sub>)-(ln(*M*<sub>0</sub>))/d), where *M*<sub>0</sub> is the wet mass of fish at the start of the study and *M*<sub>70</sub> is the wet mass of the fish at the end of the 70 day study.

### 5.3.6 Statistical analysis

All statistical analysis was carried out in *R 2.15.3* (R Core Team, 2012). Ration and infection status were compared simultaneously, using two-way ANOVA tests to show their effect on: change in *SL*, *SGR*, *HSI*, *BCF*, *SSI*, *drDPA*. ANOVA was also used to compare the *M*<sub>p</sub> and *l*<sub>p</sub> recovered from fish held on the 8% and 16% bw.d<sup>-1</sup> ration. A mixed effects model was then used to show the changes in *rDPA* of fish in each week of the study, in order to compare the growth of parasites in each treatment over time.

### 5.4 Results

#### 5.4.1 Host growth

All fish surviving to 56 d increased in length. There was a significant effect of ration on *dSL*, but no discernible effect of infection status and no interaction between ration and infection status (ANOVA: ration: *F*<sub>1,68</sub> = 31.82 *P*<0.0001; infection: *F*<sub>1,68</sub> = 0.96 *P* = 0.331; interaction: *F*<sub>1,68</sub> = 0.21 *P* = 0.650; Table 5.1, Figure 5.1.A). When examining changes in mass controlled for size at the start of the study by calculating the specific growth rate (*SGR*), *SGR* in all treatment groups
was positive, indicating all fish grew over the course of the study. Fish fed the higher ration achieved significantly higher SGR than conspecifics fed the lower ration, and growth was significantly affected by infection status (ANOVA: ration: $F_{1,68} = 59.92 \ P < 0.0001$; infection: $F_{1,68} = 6.62 \ P = 0.012$; Table 5.2, Figure 5.1.B). There was no interaction between ration and infection status on SGR indicating the effect of ration on SGR was not dependent on infection status (ANOVA: interaction: $F_{1,68} = 0.08 \ P = 0.778$).

Table 5.1 ANOVA table for growth of Carsington Reservoir three-spined sticklebacks over the course of the study using dSL as the response variable. *Schistoscephalus solidus* infection status and feeding ration were used as predictor variables. Significant values ($p < 0.05$) are shown in bold.

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Table 5.2 ANOVA table for growth of Carsington Reservoir three-spined sticklebacks over the course of the study using specific growth rate (SGR) as the response variable. *Schistoscephalus solidus* infection status and feeding ration were used as predictor variables. Significant values ($p < 0.05$) are shown in bold.

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Figure 5.1 Boxplots showing growth of Carsington Reservoir three-spined sticklebacks in the study fed either low (8% body weight per day) or high (16% body weight per day) ration. A; Difference in length from the start of the study to the end (dSL) achieved over the 56d for non-infected and *Schistocephalus solidus* infected fish. B; Specific growth rate (SGR) achieved over the 56d for non-infected and infected fish. 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.

5.4.2 Host body condition

At the termination of the study, there was a highly significant effect of host ration on hepatosomatic index (*HSI*), with fish held under higher rations having relatively higher liver mass (ANOVA: $F_{1,68}=24.32 \ P<0.0001$; Table 5.3, Figure 5.2A). There was a non-significant trend for experimentally infected fish to have
higher HSI (ANOVA: \( F_{1,68}= 3.79 \ P=0.056; \) Table 5.3, Figure 5.1A), but no interaction between infection status and ration (ANOVA: \( F_{1,68}= 0.85 \ P=0.359; \) Table 5.3, Figure 5.1A). The body condition factor (BCF) of the fish also showed a highly significant effect of ration (ANOVA: \( F_{1,68}= 12.42 \ P<0.001; \) Table 5.4, Figure 5.2B), again there was a non-significant trend for experimentally infected fish to have higher BCF (ANOVA: \( F_{1,68}= 3.00 \ P=0.088; \) Table 5.4, Figure 5.2B) but no interaction between ration and infection (ANOVA: \( F_{1,68}= 0.28 \ P=0.5958; \)Table 5.4, Figure 5.2B). Spleen somatic index was significantly affected by infection status, but not feeding ration and there was no interaction between them (ANOVA: ration: \( F_{1,68}= 0.18 \ P=0.671; \) infection: \( F_{1,68}= 5.50 \ P=0.022; \) interaction: \( F_{1,68}= 0.16 \ P=0.688; \)Table 5.6, Figure 5.3), indicating infected fish had larger spleens.

**Table 5.3** ANOVA table for the condition of Carsington Reservoir three-spined sticklebacks at the end of the study using hepatosomatic index (HSI) as the response variable. *Schistocephalus solidus* infection status and feeding ration were used as predictor variables. Significant values (\( p < 0.005 \)) are shown in bold.

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**Table 5.4** ANOVA table for the condition of Carsington Reservoir three-spined sticklebacks at the end of the study using body condition factor (BCF) as the response variable. *Schistocephalus solidus* infection status and feeding ration were used as predictor variables. Significant values (\( p < 0.005 \)) are shown in bold.

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Figure 5.2 Boxplots showing body condition of Carsington Reservoir three-spined sticklebacks in the study fed either low (8% body weight per day) or high (16% body weight per day) ration. A; hepatosomatic index (HSI) for non-infected and *Schistosomephalus solidus* infected fish. B; Body condition factor (BCF) for non-infected and infected fish. 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.
Table 5.5 ANOVA table for the Spleen Somatic Index (SSI) of Carsington Reservoir three-spined sticklebacks at the end of the study with ration and Schistocephalus solidus infection status as predictors. Significant values ($p < 0.005$) are shown in bold.

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<td>Ration x infection status</td>
<td>1</td>
<td>0.1623</td>
<td>0.68831</td>
</tr>
<tr>
<td>Residuals</td>
<td>68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.3 Boxplot showing Spleen Somatic Index (SSI) of non-infected and Schistocephalus solidus infected Carsington Reservoir three-spined sticklebacks in the study fed either low (8% body weight per day) or high (16% body weight per day) ration. 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.
5.4.3 Analysis of parasite growth

The change in $rDPA$ (i.e. $drDPA$, the change in the size of abdominal swelling) over the experimental feeding period was strongly affected by both host ration and infection status (2-way ANOVA: ration: $F_{1,68} = 14.75$ $P<0.001$; infection: $F_{1,68} = 18.00$ $P<0.001$; Table 5.6, Figure 5.4), with fish on higher ration, and infected fish increasing swelling. Importantly, there was also a significant interaction between ration and infection status ($F_{1,68} = 4.62$ $P = 0.035$, Table 5.6, Figure 5.4), with infected fish surviving to 56d held under the high ration exhibiting substantially higher $drDPA$ compared to other groups.

Table 5.6 ANOVA table for the growth of parasites from Carsington Reservoir three-spined sticklebacks measured as the difference in swelling from the start of the study to the end, using $drDPA$ as the response variable. *Schistoccephalus solidus* infection status and feeding ration were used as predictor variables. Significant values ($p < 0.05$) are shown in bold.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>$F$ value</th>
<th>$P$ value</th>
</tr>
</thead>
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<tr>
<td>Ration</td>
<td>1</td>
<td>14.7481</td>
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</tr>
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<td>Infection status</td>
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<td>0.0351</td>
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<tr>
<td>Residuals</td>
<td>68</td>
<td></td>
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</tr>
</tbody>
</table>
Figure 5.4 Boxplot showing the change in the size of the swelling of *Schistocephalus solidus* non-infected and infected Carsington Reservoir three-spined sticklebacks from the start to the end of the study fed either low (8% body weight per day) or high (16% body weight per day) ration for non-infected and infected fish. 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.

The mean mass of plerocercoids extracted from fish which survived to 56 d held under the higher ration treatment was considerably higher than those on lower rations; however, due to limited sample sizes, statistical testing showed only a marginally non-significant effect ($F_{1,10} = 3.6139, P = 0.0865$; Figure 5.5A). When the mass of the parasite was controlled for the mass of the fish, by calculating parasite index ($I_p$), there was no significant difference between the two ration treatments ($F_{1,10} = 0.6733, P = 0.431$; Figure 5.5B).
5.4.4 Changes in parasite growth during the study

When examining the mean rDPA over the course of the experiment, including fish with early mortality, the growth trajectories are steeper for parasites on high ration compared to their low ration counterparts and unexposed controls (Mixed effects model: Week x Ration: $F = 13.08$ $P<0.001$; Table 5.7, Figure 5.6A and
Figure 5.6B). In addition, when examining the mean rDPA over the course of the experiment the parasites within fish in the high ration treatment only show increased size at week 6 (Figure 5.6B).

When individual growth trajectories are considered, the majority of parasites follow the pattern shown in Figure 5.6. However there is an outlier on the 16% ration which showed an initial growth phase followed by a reduction in fish swelling followed by mortality (Figure 5.7B). This individual did not eat the entire food ration provided to it for 1 week prior to mortality although appeared otherwise healthy. Examining the feeding conditions separately reveals that, aside from the anomaly mentioned previously, the majority of individuals go through an initial lag phase where growth is minimal followed by a rapid increase. In the low feeding condition some individual fish showed a decrease in swelling in the initial 2 week period (Figure 5.7A).

When parasite mass was examined post mortem, the parasites from early mortalities had not reached a large size and were not all on the low ration treatment (Figure 5.8), indicating that mortality may not be simply due to fast growing parasites or a side effect of low food availability.

Table 5.7 Linear mixed effects model for the growth of *Schistocephalus solidus* from Carsington Reservoir three-spined sticklebacks measured as abdominal swelling, using rDPA as the response variable each week of the study. *Schistocephalus solidus* infection status, feeding ration and week measured were used as predictor variables. Significant values (*p* < 0.05) are shown in bold.

<table>
<thead>
<tr>
<th>Predictor Variable</th>
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<th>denDF</th>
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<td>135.55</td>
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</tr>
<tr>
<td>Week</td>
<td>1</td>
<td>308</td>
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<tr>
<td>Ration</td>
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<td>0.005</td>
</tr>
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<td>Infection Status</td>
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<tr>
<td>Week x Ration</td>
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<td>308</td>
<td>13.08</td>
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</tr>
<tr>
<td>Week x Infection Status</td>
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<td>308</td>
<td>11.05</td>
<td>0.001</td>
</tr>
<tr>
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<td>0.3265</td>
</tr>
<tr>
<td>Week x Ration x Infection Status</td>
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<td>308</td>
<td>4.90</td>
<td>0.0275</td>
</tr>
</tbody>
</table>
Figure 5.6 The mean \( r_{DPA} \) of Carsington Reservoir three-spined sticklebacks over the course of the study showing the difference in swelling between control fish that were never exposed to infective *Schistosomeles solidus* stages and experimentally infected fish. A; Fish fed on low ration (8% body weight per day) treatment. B; Fish fed on high ration treatment (16% body weight per day). Error bars represent ± 1 standard deviation.
Figure 5.7 Swelling of individual Schistocephalus solidus infected fish in feeding ration treatment, indicating estimated growth trajectories of parasites recovered from Carsington Reservoir three-spined sticklebacks. A: Fish fed on low ration (8% body weight per day) treatment (n = 7). B: Fish fed on high ration treatment (16% body weight per day) (n = 8). Red lines indicate means of the controls for respective feeding condition.

Figure 5.8 The total Schistocephalus solidus parasite mass recovered from Carsington Reservoir three-spined sticklebacks post mortem from fish held under the low ration (8% body weight per day) treatment and high ration treatment (16% body weight per day).
5.5 Discussion

5.5.1 Parasite growth and food availability

The level of host feeding can have important implications for the growth of *Schistoscephalus solidus*. In a study of experimentally infected fish, infected fish fed a regulated ration of 16% showed a greater increase in the level of parasite-induced swelling than infected fish held under a ration of 8% bw.d\(^{-1}\), because the extent of swelling correlates closely with the proportion of host mass contributed by plerocercoids in stickleback-*S. solidus* and other fish-pseudophyllidean systems (Barber, 1997, Loot et al., 2002a, Loot et al., 2002b, Barber and Svensson, 2003), these results strongly suggest that feeding ration causes increased parasite growth. The relative size of the swelling did not change among infected fish fed at 8% bw.d\(^{-1}\) over the experimental feeding period, indicating plerocercoid growth that was proportional to fish growth. These results suggest that the growth rates of parasites is responsive to short term changes in nutrients available to the host. Previous work examining the growth of *S. solidus* plerocercoids in experimentally infected sticklebacks reared in mixed infection groups – in which the food intake could not be controlled – showed that the fastest-growing fish developed the largest plerocercoids (Barber, 2005). One explanation given for this result was that plerocercoids might benefit from high levels of alimentation experienced by the most competitively able fish. The results of the present study are consistent with this interpretation (Barber, 2005), and provide further evidence that parasites can benefit through accelerated growth when host food intake increases.

Sticklebacks infected with *S. solidus* face a major energetic problem; as well as having higher metabolic rate than non-infected fish as a result of sustaining the growth and development of the parasites (Lester, 1971, Meakins and Walkey, 1975), they are competitively disabled by infection (Barber and Ruxton, 1998). In addition, infected sticklebacks have a reduced voluntary meal size (Cunningham et al., 1994, Wright et al., 2006), possibly because growing plerocercoids restrict the capacity of the stomach to expand. A range of studies have demonstrated that infection with *S. solidus* is associated with altered foraging behaviour in host sticklebacks. For example, infected fish switch prey preferences to avoid highly
contested prey (Milinski, 1984, Ranta, 1995, Tierney, 1994) and take greater risks to access available food, including foraging close to predators (Milinski, 1985, Giles, 1987, Tierney et al., 1993) and choosing individual foraging opportunities over shoal membership (Barber et al., 1995). In natural environments, with typically high levels of competition for food resources, such behaviours are likely to compensate for other foraging deficiencies of infected fish, such as slower foraging on spatially aggregated prey (Barber and Ruxton, 1998). However, the results presented here suggest that if behavioural changes cause infected fish to increase food intake sufficiently, there are likely benefits to plerocercoid growth.

The initial lag phase observed in parasite growth could be attributed to (1) The parasite has a natural growth lag immediately post-establishment, (2) Exposure to the parasite caused an initial body condition loss for the fish host whilst the immune response was mounted which offset any parasite-induced swelling, (3) Parasite size has not yet increased significantly enough for swelling to be detectable.

### 5.5.2 Host body condition and feeding ration

In this study, *S. solidus* only exhibited disproportionate growth in stickleback hosts held under the 16% bw.d\(^{-1}\) ration. Under this level of alimentation, both infected and non-infected sticklebacks developed large livers relative to their body size. The liver acts as a medium term energy storage organ in fish (Chellappa et al., 1995), and in nature *S. solidus* infection is often associated with reduced liver size, suggesting infection-induced energetic stress (Tierney et al., 1996). The fact that liver size was elevated under the higher ration, even among infected fish, combined with the increased growth of plerocercoids, suggests that the 16% ration was sufficient to supply both hosts and parasites with sufficient energy to allow close to maximal growth and still permit fish to deposit energy reserves. Body condition factor showed a similar pattern, as higher body conditions were found under high ration, lending further suggesting infected fish were able to allocate energy to both host and parasite growth. The results support the suggested energetic model presented by Walkey & Meakins (1970), in which parasites are able to transform energy from food more efficiently than their hosts.
Previous studies have also demonstrated that experimentally infected fish can outgrow non-infected conspecifics when there are no constraints on food intake, possibly as a result of the behavioural changes associated with infection (Arnott et al., 2000, Barber et al., 2008).

The spleen mass of infected fish was elevated in under both feeding regimes. Spleen mass has been previously shown to be higher in parasitized sticklebacks (Kalbe and Kurtz, 2006), and has been examined as a marker of fisher health (Handy et al., 2002). In teleosts, the spleen is a lymphoid organ and related to the immunocompetence of the fish (Zapata et al., 2006). Therefore the data presented here show higher spleen mass in infected fish regardless of feeding condition. One possibility is that infection did cause an immune response, but this was unaffected by energy intake.

5.5.3 Mechanisms and implications

Under the 8% ration parasites grew in proportion to the fish host, resulting in no net change in the relative size of the swelling. This could simply be because the levels of nutrients available within the fish are too low for the parasite to attain higher growth rates, therefore parasite growth rates reflect a by-product of resource availability. Alternatively, the parasite could be operating ‘restraint’ in its exploitation of the host. Schultz et al. (2006) tested the by-product and restraint hypotheses by examining reproductive investment in S. solidus infected female sticklebacks. By differentiating between the absolute amounts of energy that three-spined sticklebacks allocated to reproduction (reproductive investment) and the proportion of energy they allocated to reproduction (reproductive effort), they determined if reduced reproductive ability was due to parasite strategy or simply a side effect of infection. Although reproductive investment was lower in infected female fish, reproductive effort was unaltered. This was considered to be consistent with the by-product hypothesis; parasites did not appear to be selectively reducing host reproduction for their own benefit. Consequently, the authors suggested that the reduced reproductive development of individual three-spined sticklebacks was explained as a case of nutrient theft by the parasite rather than ‘parasitic castration’. The study by Schultz et al. (2006) demonstrated that under food-limited conditions, parasites did not maximise their
own growth rates despite having evolved adaptations to compete with their hosts for nutrients (Halton, 1997). One implication of these results is that anthropogenic alterations to aquatic habitats – for example increased nutrient input, climate change or alteration of food webs following species introductions – may create conditions in which food availability is altered, and thus potentially made more available to parasites infecting fish. Such changes may shift the balance of host-parasite interactions. The results in this study also highlight the importance of the environmental conditions experienced by hosts during periods of parasite establishment and growth for ultimate infection phenotype.

5.6 References


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Chapter 6

Host body size effects on parasite susceptibility and growth

N. Simmonds ©
6.1 Abstract

Responses to infection changes with age and body size in many host-parasite systems, although most studies do not examine the relative importance of each factor and simply use one as a proxy for the other. In trophically transmitted parasites, the timing of infection can affect prevalence and has the potential to be shifted by anthropogenic change, making the characterising the influence of age or body size as a determining factor important. Lab-bred three-spined sticklebacks were experimentally exposed to *S. solidus* in two batches in order to generate variation in age and body size. Body size was found to be a strong predictor of infection susceptibility and subsequent parasite growth rates, while age was relatively unimportant. These finding have implications for host-parasite interactions in the wild.
6.2 Introduction

6.2.1 How does host body size affect parasite infections?

Host body size can play an important role in determining the infection susceptibility of hosts and the growth and development of parasites, and consequently have implications for dynamics of infection and the ecology of host-parasite interactions (Poulin, 2000, Poulin, 2007). Body size, for example, may be important if the effectiveness of immune responses changes as individuals grow. In feral pigeons (Columba livia), intensity of the blood parasite Haemoproteus columbae decreases dramatically in sub-adults over time compared to adults, with the parasite only causing mortality among juveniles until they develop an effective immune response (Sol et al., 2003). Changes in immune response with age could be due to acquired immunity related to previous experience of pathogens, or increases of innate immune responses with age. In humans, acquired immunity to Schistosoma haematobium increases with age leading to lower infection intensity among older individuals (Woolhouse et al., 1991). Age has been observed to be important in other systems such as red-spotted newts (Notophthalmus viridescens); by examining host age and the intensity of infection intensity with 12 different parasites, Raffel et al. (2009) showed that acquired immune response can influence the infection dynamics, including the protist Amphibiocystidium viridescens, by shifting infection rates to younger individuals. In the garter snake (Thamnophis elegans), the innate immune response increases with age and body size until maturity, when innate immune responses decreased (Sparkman and Palacios, 2009). Increases in immune response over time have also been shown in fish. In several salmonid species, the immunity of fry was found to be function of their size rather than age (Johnson et al., 1982). Sea bass (Dicentrarchus labrax) have also exhibited increased immunoglobulin levels with age and size (Scapigliati et al., 1999).

Body size could also be a crucial factor if ontogenetic niche shifts and / or changes in behaviour alter the exposure level of host organisms to infective parasite stages (Rohde, 1994). For example in slow worms (Anguis fragilis) exposure to the nematode Neoxysomatium brevicaudatum increases with age, but age-related acquired immunity causes parasite prevalence to show a convex pattern, with intermediate age classes exhibiting the highest infection rates.
In the case of parasite infections that are acquired throughout the host’s lifetime, infection intensity may also be expected to correlate positively with host age (and hence, potentially, size). This pattern of parasite accumulation has been shown in Arctic Charr (*Salvelinus alpinus*), where prevalence of the ectoparasitic copepod *Salminctola edwardsii* increased with host age and size (Amundsen et al., 1997). In a survey of fish parasite communities, Poulin and Valtonen (2001) found that larger fish tended to have richer parasite communities as they ingested more prey and therefore increased their level of exposure, and they also tended to be older and therefore had accumulated more parasites. Larger individuals are therefore likely to have higher parasite burdens, and harbour the less common parasites, whereas smaller individuals are much more likely to harbour common parasites (Poulin and Valtonen, 2001). By studying the accumulation of *Triaenophorus crassus* plerocercoids in whitefish (*Coregonus lavaretus*) Pulkkinen and Valtonen (1999) were able to demonstrate that while parasites did accumulate with age, larger young fish had increased parasite burdens compared to smaller fish of the same age. In older fish the opposite pattern was observed, as smaller fish had harboured more parasites than larger conspecifics. The authors attributed this pattern to increased feeding in the first two years leading to faster growth rates and higher exposure to infected copepods, but after two years a slower rate of growth in infected fish due to parasite infection (Pulkkinen and Valtonen, 1999).

Host body size may also be important in determining patterns of infection, for example if larger individuals present a more readily-located or easily-colonised target for infective parasites (Haas, 2003), or if they allow greater opportunities for the growth, reproduction or accumulation of parasites (Guégan et al., 1992, Grutter and Poulin, 1998, Lo et al., 1998). In a host choice experiment, significantly more *Schistosoma mansoni* miracidia were attracted to sub-adult snail hosts (*Biomphalaria glabrata*) than juveniles and adults (Theron et al., 1998). Theron et al. (1998) also found that when exposed individually the smallest juvenile size class had greater prevalence and abundance than sub-adults and adults. The authors suggest that while the smallest individuals had the highest susceptibility when presented in isolation, parasites may choose the host size with the optimal resources such as space for parasite growth, longevity,
energy availability and higher parasite reproduction (Theron et al., 1998). Anderson and Crombie (1984) studied the effect of snail size, as a proxy for age, on infection by *S. mansoni* and found intermediate sizes to show the highest prevalence. Again in these studies, the effects of age and size were not separated. Furthermore, increased levels of food intake by larger individuals may mean that hosts encounter more infective stages of trophically acquired parasites (des Clers, 1991, Poulin, 2000, Poulin and Valtonen, 2001) and hence develop higher infection intensities. However, because age and body size typically co-vary, the great majority of field and lab studies that examine relationships between parasite load and host size confound the two factors. Distinguishing the effects of age from those of body size is therefore challenging, and the importance of these factors cannot readily be separated by studying naturally infected hosts in field surveys, especially in species that exhibit indeterminate growth such as fish (Wootton, 1998).

### 6.2.2 Consequences of altered host body size for parasite infections in the context of environmental change

Understanding the consequences of host body size for the dynamics of parasite infection may become even more important in the context of changing environmental conditions. Many parasites have evolved seasonal life histories that ensure infective stages encounter hosts during temporal windows that coincide with the optimal availability of hosts for invasion or exploitation. Environmental perturbations, which include (but are not limited to) climate change, can drive changes in the timing of emergence of infective parasites (Sures, 2008, Studer and Poulin, 2014, Paaijmans et al., 2009), as well as the reproduction (Hance et al., 2007) and / or the early growth rates of hosts (Altizer et al., 2006, Marcogliese, 2001), potentially generating ecological mismatches between hosts and parasites. In extreme cases, altered host body size may lead to complete temporal separation and breakdown of infection (e.g. Paull and Johnson, 2014); however, in less extreme cases it may lead to seasonally-acquired parasites being faced with larger or smaller (or older or younger) hosts than in historical populations. Developing a detailed understanding of how host body size affects interactions with ecologically-important parasites is therefore highly relevant and timely.
6.2.3 Size dependent effects of parasites and host evolution

It has been predicted from life-history models that if conditions for the host worsen over time, then selection should favour earlier reproduction (Poulin and Morand, 2004). This is because an organism should begin reproduction at an age that maximises lifetime fitness (Roff, 1992, Klingenberg and Spence, 1997); therefore if parasites limit host reproduction then their presence should select for limited reproduction earlier rather than risk no offspring being produced (Agnew et al., 2000). There is empirical evidence in support of this from studies on molluscs. Marine snails (*Cerithidea californica* and *C. mazatlanica*) reached maturity at smaller sizes where larval trematodes were present (Lafferty, 1993). In the freshwater snail *Biomphalaria glabrata*, individuals infected with *Schistosoma mansoni* showed higher levels of egg production immediately after infection (Minchella and Loverde, 1981) but subsequently exhibit reduced egg production below that of non-infected conspecific, and lower fecundity over their lifetime (Thornhill et al., 1986), indicating a shift towards earlier reproductive effort.

Parasite infections of fish are particularly interesting, as fecundity in female fish is usually directly proportional to female body size (Roff, 1984, Trippel et al., 1997), therefore delaying sexual maturation until an optimal body size is reached should increase fitness. However, as macroparasites may limit host reproduction while growing (Macnab et al., 2009) and may cause host mortality in order to complete their life cycle, we might predict a shift towards earlier host reproduction. In a survey of 33 fish species, larval helminth diversity was correlated with early host reproductive maturity (Morand, 2003). Therefore if the costs of parasitism are higher at larger body sizes (or greater age) then this may represent an important selective pressure on fish, and have implications for fisheries by lowering productivity in much the same way as the selective removal of larger individuals from populations (De Roos et al., 2006).

6.2.4 The three-spined stickleback-*Schistocephalus solidus* system

Plerocercoids of the pseudophyllidean cestode *Schistocephalus solidus* commonly infect three-spined sticklebacks (*Gasterosteus aculeatus*) across the geographic range of the host fish (Wootton, 1976). Once established in the body cavity of the host, this parasite is known to affect the behaviour (Giles, 1987,
Barber et al., 2004), growth (Barber et al., 2008), physiology (Arnott et al., 2000, Scharsack et al., 2004) and reproductive development (Rushbrook et al., 2007, Macnab et al., 2009, Heins and Brown-Peterson, 2010) of host sticklebacks, with fish harbouring larger worms typically being more severely affected by infection. In turn, the mass attained by the plerocercoid directly affects transmission success, with larger plerocercoids being capable of adaptively manipulating host behaviour (Tierney et al., 1993, Barber et al., 2004), having a greater likelihood of successfully establishing in definitive avian hosts (Tierney and Crompton, 1992), and producing more eggs as adults (Dörücü et al., 2007) than smaller worms. The mass attained by plerocercoids is therefore a major factor determining both parasite fitness and the fitness consequences of infection for stickleback hosts, so understanding the consequences of host body size per se for infection susceptibility and subsequent plerocercoid growth in this system is particularly relevant.

6.2.5 Aims

Here the results of an experimental infection study designed to examine the role of stickleback body size in determining the susceptibility to S. solidus infection and subsequent plerocercoid growth rate are reported. Sticklebacks were bred and reared in the lab to generate variation in both age and body size before exposing these fish to infective stages of S. solidus and allowing worms to establish and grow for 70d. By using this well-characterized, experimentally amenable host-parasite system (Barber and Scharsack, 2010) allowed the following questions to be addressed: (1) How do differences in host body size at the time of exposure affect infection susceptibility? (2) How do differences in host body size at the time of exposure affect subsequent plerocercoid growth? (3) Do faster growing host sticklebacks develop heavier infections?

6.3 Methods

6.3.1 Fish collection and husbandry

Adult fish collected in May, 2011 from Carsington Reservoir, Derbyshire, U.K. (53°3’30”N 1°37’50”W) were transferred to laboratory aquaria, fed daily with frozen bloodworms (Chironomus sp. larvae) and exposed to environmental conditions that induced the development of sexual maturation. Nuptial coloured
males and gravid females were selected as parents, and nine families of juvenile fish were generated using in vitro fertilisation techniques as described in Chapter 2 (Barber and Arnott, 2000). In brief, male three-spined sticklebacks were dissected under a dissection microscope, the testis removed and placed on ice. Eggs stripped from female three-spined sticklebacks were placed into a watch glass and covered in macerated testis in autoclaved aquaria water and left for 30 minutes for fertilisation to take place. Fertilised eggs were transferred to 1 L plastic aquaria containing methyl blue solution and constant aeration. Juvenile fish were reared in family groups (n = 20) in glass aquaria (40 cm x 25 cm x 30cm, 30 L) placed within a temperature controlled, filtered, recirculating water system and fed daily ad libitum with Artemia sp. nauplii.

6.3.2 Experimental parasite infections

*Schistocephalus solidus* plerocercoids recovered from adult three-spined sticklebacks from Carsington Reservoir were cultured individually to produce eggs, using techniques adapted from Smyth (1954). In brief, plerocercoids were inserted into 6 mm diameter dialysis tubing (Visking, U.K.) suspended in a boiling tube containing 50% RPMI media and 50% horse serum (Sigma, U.K.). Boiling tubes were placed into a darkened shaking water bath for 5 days before eggs were collected (Arnott et al., 2000, Barber and Svensson, 2003, Macnab and Barber, 2012). Eggs were incubated in the dark at 20°C for 21 d before being hatched by exposure to natural daylight. To limit any potential effect of inter-individual differences in worm virulence, eggs from a single culture were used in the experiment; the selection of the worm chosen was based on high levels of hatchability of a sample of eggs. Copepodites from a lab population of copepods (*Cyclops strenuus abyssorum*) were exposed individually to two newly hatched coracidia, and exposed copepods were screened for infection status after 14 d. Exposures to parasite infective stages were carried out under home office licence (Project licence: 80/2327, Personal Licence: 40/9978).

To generate size and age variation among prospective hosts, fish were exposed in two batches, at mean age 46d and 87d. In the first batch of exposures, 50 juvenile fish were fed a single copepod containing 1 or 2 procercoids, with 10 control fish being fed with a non-infected copepod (i.e. sham-exposed). In the
second batch, 72 juveniles were exposed, with 11 fish being sham-exposed. All fish were measured for Standard Length ($SL_0$, to 0.1 mm), blotted dry, and weighed ($M_0$, to 0.001 g).

After parasite exposure / sham-exposure, experimental fish were transferred individually to small plastic aquaria (15 cm x 14 cm x 11 cm, 1.25 L) held on a temperature-controlled recirculating water system, and fed live *Artemia* sp. nauplii under an *ad libitum* regime for a further 70 d. Individual rearing aquaria contained a gravel substratum and a plastic plant for shelter. Water temperature was maintained at 19°C throughout the study, and a L:D photoperiod of 11:13 was implemented until the end of the experiment. Experimental design is summarised in Figure 6.1.

![Figure 6.1](image)

**Figure 6.1** Experimental design schematic illustrating the two staggered batches of *Schistocephalus solidus* exposure from the same set of three-spined stickleback families.

### 6.3.3 Post mortem analysis

Mortality among experimental fish was low, with only 6 of 143 fish (ca. 4%) dying before the end of the study. After 70 d post-exposure (dpe), all surviving experimental fish were euthanized using an overdose of Benzocaine anaesthetic (stock solution: 10 mg L$^{-1}$). Fish were Standard Length ($SL_{70}$, to 0.1 mm) was measured using a dial calliper, blotted dry, and weighed ($M_{70}$, to 0.001 g) and dissected. Any plerocercoids recovered were blotted and weighed (to 0.001 g); in the case of multiply infected fish, the mass of each individual plerocercoids was recorded and the total parasite mass of plerocercoids ($M_p$) recovered from each fish calculated.
Digital photographs of the dorsal profile of each fish were taken immediately prior to parasite exposure / sham exposure (i.e. at 0 dpe) and at the termination of the study (at 70 dpe), facilitating the accurate measurement of changes in body size by image analysis using the ImageJ software package (Schneider et al., 2012).

This permitted the calculation of the specific growth rates of host fish ($SGR = 100\times (\ln(M_{70})-\ln(M_0)) / 70$) over the post-exposure period, which allowed a consideration of how post-exposure fish growth rates related to plerocercoid growth. Prior to further analysis, host $SGR$ was corrected for the expected negative correlation with initial host size, generating residual ($rSGR$) values, which were subsequently used in all analyses.

### 6.3.4 Statistical analysis

Statistical analysis was carried out in R (R Development Core Team, 2012). Data were tested for normality and homogeneity of variance using box and normal Q-Q plots, and subject to transformation if necessary prior to using parametric analyses. A general linear model was used to examine the effect of size and age on infection susceptibility. As the numbers of infected individuals were low overall the model was constructed by comparing $SL_0$ and age of infected and non-infected fish. The effect of initial body size ($SL_0$), age at exposure (days) and exposure batch (early / late) on $M_p$ was analysed using factorial ANOVA. A series of logistic regression analysis was then used to determine the effect of $SL_0$ on the probability of an infection developing following parasite exposure. There were three different versions of the model. In the first version, only fish that survived were included in the analysis, i.e. omitting data from exposed fish that died during the study. However, because infection status could not be ascertained in the fish that died, and also because they were smaller than average (and so could potentially affect the logistic regression model) the model was run two more times to represent the most extreme possible situations. In the second iteration of the model it was assumed that all of the fish that died had been infected, and in the third iteration it was assumed they were non-infected.

Multi-factorial ANOVA was used to examine the influence of (a) host size-at-exposure ($SL_0$), (b) host age-at-exposure, (c) whether the fish were exposed in the early (‘young’) or late (‘old’) batch and (d) whether the fish developed a one
or two plerocercoids on the total plerocercoid mass ($M_p$) recovered from experimentally infected fish. In the first model the interaction terms were included in order to determine if there might be an interaction between age and $SL_0$ (Table 6.2). None of the interaction terms were significant, therefore the non-significant terms were sequentially deleted leaving only those factors showing a significant effect on total parasite mass ($M_p$) or deemed to be of interest to the experimental design (Table 6.3).

In order to estimate what the potential repercussions would be for parasite fitness if infection were shifted to higher or lower host body sizes, equations taken from Dörücü et al. (2007) were used to predict the egg output of parasites from a fish of a given size. A series of egg output estimates were made based on parasite data. There are two equations given for single and multiple infections (eqn.1 and eqn.2 respectively, Dörücü et al. (2007)), therefore predictions were made based on the single infection equation alone (Eqn.1) and both equations (Eqn.1 and 2).

\[ \log_{10} \text{egg output} = 7.33 + 2.11 \times \log_{10}(\text{parasite mass}) \quad \text{Eqn.1} \]

\[ \log_{10} \text{egg output} = 4.66 - 0.472 \times \log_{10}(\text{parasite mass}) \quad \text{Eqn.2} \]
6.3.5 Size variation and parasite exposure success

Body size ($SL_0$) variation was generated successfully, with the ‘early’ and ‘late’ batches differing by an average of 8.1 mm (Table 6.1).

Plerocercoids established in 34 (29%) of the 117 parasite-exposed fish that survived to the end of the study. Whether or not plerocercoids established was influenced by the size of the host at exposure ($SL_0$) and the level of exposure (1 vs. 2 procercoids).

Table 6.1 The mean (± sd) age and body size of sham-exposed and *Schistocephalus solidus*-exposed three-spined sticklebacks in the two batches of exposures carried out in the study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Batch 1 ('early')</th>
<th>Batch 2 ('late')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at exposure</strong> ($A_0$, days)</td>
<td>Sham-exposed</td>
<td>47 (± 5)</td>
<td>86 (± 6)</td>
</tr>
<tr>
<td></td>
<td>Exposed</td>
<td>46 (± 5)</td>
<td>87 (± 6)</td>
</tr>
<tr>
<td><strong>Length at exposure</strong> ($SL_0$, mm)</td>
<td>Sham-exposed</td>
<td>14.4 (± 2.1)</td>
<td>22.7 (± 4.1)</td>
</tr>
<tr>
<td></td>
<td>Exposed</td>
<td>14.5 (± 2.0)</td>
<td>22.4 (± 3.6)</td>
</tr>
<tr>
<td><strong>Mass at exposure</strong> ($M_0$, g)</td>
<td>Sham-exposed</td>
<td>0.026 (± 0.011)</td>
<td>0.104 (± 0.055)</td>
</tr>
<tr>
<td></td>
<td>Exposed</td>
<td>0.027 (± 0.013)</td>
<td>0.099 (± 0.044)</td>
</tr>
</tbody>
</table>
6.4 Results

6.4.1 Host body size and exposure level as determinants of infection susceptibility

There was no significant difference in the $SL_0$ of fish receiving one or two procercoids ($t = 0.12, df = 1, P = 0.73$). Being exposed to two procercoids, however, significantly increased the likelihood of fish becoming infected, with 13 of 27 fish developing a plerocercoid when exposed to two procercoids, compared with 21 of 69 when exposed to one ($\chi^2 = 6.20, df = 1, P = 0.013$). There were more parasites established from doubly infected copepods than singly infected copepods ($\chi^2 = 8.58, df = 2, P = 0.014$; Figure 6.2).

Figure 6.2 Frequency of *Schistocephalus solidus* infected three-spined sticklebacks exposed to copepods infected with one or two procercoids.

Fish developing infections were smaller at exposure than those that did not become infected ($F_{1,115}=8.54 P=0.004$, Figure 6.3). In each version of the logistic regression analysis, $SL_0$ was found to have a highly significant effect on the probability of fish becoming infected (using data from surviving fish only: $G = 7.11, df = 1, P = 0.008$; assuming all dead exposed fish were infected: $G = 11.44, df = 1, P = 0.001$; assuming all exposed dead fish were non-infected: $G = 6.58, df = 1, P = 0.010$; Figure 6.4).
Figure 6.3 The initial standard length ($SL_0$) of three-spined sticklebacks that developed *Schistocephalus solidus* infections (‘infected’: black) and those that did not develop infections (‘uninfected’, white) after being fed copepods harbouring infective procercoids. Main figure: the fate of each individual fish in the two experimental trials. Inset figure: mean +/- sd $SL_0$ of fish that developed infections and those that did not after being exposed. *** denotes statistical significance at P<0.005. Non-infected, n = 88; Infected, n = 34.
Figure 6.4 The probability of *Schistocephalus solidus* infection for the range of $SL_0$ generated in the study, as determined by the logistic regression model. 0, non-infected. 1, infected. Actual data points are shown (○) as well as predicted probability values (●) from the logistic regression. The bold regression line represents the model including only data from fish which survived the full course of the study. The lower and upper dashed regression line denotes the predicted probability of infection, using data from the model assuming all of the three-spined sticklebacks which did not survive the course of the study were non-infected and infected respectively.
6.4.2 Effects of initial host body size on parasite growth potential

When all factors were included in the multi-factorial ANOVA only $SL_0$ was shown be a significant determining factor of $M_p$ (Table 6.2).

Table 6.2 ANOVA table showing the influence of different host factors and the interaction terms between them on the total mass of *Schistcephalus solidus* plerocercoids recovered from experimentally-infected three-spined sticklebacks, 70d post-exposure. P values shown in bold indicate a significance level of <0.005

<table>
<thead>
<tr>
<th>Term</th>
<th>df</th>
<th>$F$ value</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\log_{10} SL_0$</td>
<td>1</td>
<td>11.48</td>
<td>0.0195</td>
</tr>
<tr>
<td>Batch (early / late)</td>
<td>1</td>
<td>0.81</td>
<td>0.4091</td>
</tr>
<tr>
<td>Age at exposure</td>
<td>11</td>
<td>0.47</td>
<td>0.8612</td>
</tr>
<tr>
<td>Infection type (single / multiple)</td>
<td>1</td>
<td>6.26</td>
<td>0.0543</td>
</tr>
<tr>
<td>$\log_{10} SL_0$ : Batch</td>
<td>1</td>
<td>0.47</td>
<td>0.5215</td>
</tr>
<tr>
<td>$\log_{10} SL_0$ : Age</td>
<td>8</td>
<td>0.09</td>
<td>0.9982</td>
</tr>
<tr>
<td>$\log_{10} SL_0$ : Infection type</td>
<td>1</td>
<td>0.69</td>
<td>0.4438</td>
</tr>
<tr>
<td>Batch: Infection type</td>
<td>1</td>
<td>0.0004</td>
<td>0.9857</td>
</tr>
<tr>
<td>Age: Infection type</td>
<td>2</td>
<td>0.06</td>
<td>0.9402</td>
</tr>
<tr>
<td>$\log_{10} SL_0$ : Batch: Infection type</td>
<td>1</td>
<td>1.68</td>
<td>0.2513</td>
</tr>
<tr>
<td>Residuals</td>
<td>5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After the sequential deletion of non-significant interaction terms from the model, $SL_0$ had a highly significant, positive effect on $M_p$, with fish that were larger at exposure developing larger plerocercoid loads (Table 6.3, Figure 6.5). After accounting for this highly significant effect of host body size in the model, neither individual host age nor exposure batch had a significant effect on $M_p$, either as a main effect or in interaction with other factors (Table 6.3). Whether fish developed one or two plerocercoids also had a significant effect on $M_p$ (Table 6.3); when controlling statistically for the strong effect of size at exposure, fish developing two plerocercoids exhibited significantly higher $M_p$'s than those developing a single plerocercoid (ANCOVA; $F_{1, 31} = 10.23$ $P = 0.003$ Figure 6.5a); however,
although the total plerocercoid mass in doubly-infected fish exceeded the parasite mass of singly-infected fish, the mass of the largest worm present was significantly lower in doubly-infected fish compared to those with single worms (ANCOVA; $F_{1,31} = 8.03 \ P = 0.008$ Figure 6.5b).

**Table 6.3** ANOVA table showing the influence of different host factors on the total mass of *Schistocephalus solidus* plerocercoids recovered from experimentally-infected three-spined sticklebacks, 70d post-exposure. *P* values shown in bold indicate a significance level of <0.005

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log$<em>{10}$ SL$</em>{0}$</td>
<td>1</td>
<td>25.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Infection batch ('early' / 'late')</td>
<td>1</td>
<td>1.78</td>
<td>0.199</td>
</tr>
<tr>
<td>Age at exposure</td>
<td>11</td>
<td>1.03</td>
<td>0.458</td>
</tr>
<tr>
<td>Infection type (single / double)</td>
<td>1</td>
<td>13.71</td>
<td>0.0015</td>
</tr>
<tr>
<td>Residuals</td>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 6.5 Effect of three-spined stickleback SL₀ on Schistocephalus solidus growth. A: SL₀ and the total parasite mass at the end of the study. B: SL₀ and the largest worm mass recovered. Single infections (*) and double infections (●) are shown separately. The dashed regression line refers to single infection data, whilst the solid line refers to double infections.
6.4.3 Host growth rates and relationship with plerocercoid mass

The total plerocercoid mass recovered from experimentally infected fish at the end of the study was strongly affected by the growth rate ($rSGR$) of host sticklebacks during the post-exposure period (ANCOVA, $F_{1,33} = 8.59$, $P = 0.006$). There was no difference in the slope ($F_{1,33} = 0.63$, $P = 0.434$) or elevation ($F_{1,33} = 2.45$, $P = 0.128$) of the function linking plerocercoid mass to host $rSGR$ in singly- and doubly-infected fish (Figure 6.6).

![Figure 6.6](image)

**Figure 6.6** Effect of three-spined stickleback $rSGR$ on the total parasite mass of *Schistocephalus solidus* at the end of the study. Single infections (*) and double infections (●) are shown separately. The regression line is for the significant relationship between $rSGR$ and total parasite mass for all data with single and double infections combined.

6.4.4 Estimated parasite fecundity

Predictions of the egg output of parasites taken from fish of a given size made based on equations given by Dörück et al. (2007) (see Section 6.3.3). Only when Eqn.1, based on single infections, was used did $SL_0$ affect predicted egg output (Table 6.4). Despite variation in the estimated egg output of parasites from each fish of a given size, $SL_0$ showed a significant positive association with predicted
egg output, meaning that parasite fecundity is predicted to increase with fish body size at exposure (Figure 6.7).

**Table 6.4** ANOVA table for egg output predictions for *Schistocephalus solidus* based on the initial length of the three-spined stickleback at exposure (*SL₀*). Prediction Eo.1 used eqn.1 and based prediction on total parasite mass. Prediction Eo.2 used both eqn.1 and eqn.2 and used total parasite mass. Total.eo.3 used both eqn.1 and eqn.2 but calculated predicted egg output separately for each parasite and totalled for parasites from the same host. Total.eo.4 used only eqn.1 but calculated predicted egg output separately for each parasite and totalled for parasites from the same host.

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>F value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>eo.1</td>
<td>1</td>
<td>152.04</td>
<td>1.068e-13</td>
</tr>
<tr>
<td>eo.2</td>
<td>1</td>
<td>1.5923</td>
<td>0.2161</td>
</tr>
<tr>
<td>total.eo.3</td>
<td>1</td>
<td>1.518</td>
<td>0.2269</td>
</tr>
<tr>
<td>total.eo.4</td>
<td>1</td>
<td>80.984</td>
<td>2.802e-10</td>
</tr>
<tr>
<td>Residuals</td>
<td>32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6.7** Predicted egg output from the *Schistocephalus solidus* parasites of each three-spined stickleback infected in the study, plotted against fish length at parasite exposure (*SL₀*). Predicted values are based on Eqn.1 using the total parasite mass.
6.5 Discussion

6.5.1 Main findings

Among lab-bred fish exposed to a controlled dose of infective parasites and reared under controlled laboratory conditions, host body size at exposure was an important predictor of both susceptibility to infection and the growth rate attained by the parasites. Whereas smaller fish were more readily invaded by infective *S. solidus* parasites, plerocercoids exhibited a faster growth rate in initially larger hosts, and hence attained a larger size. Furthermore, although age co-varied with body size in this study, the experimental design allowed body size and age to be analysed separately, factors which are commonly confounded. In contrast to the strong effects of body size, the age at which fish were exposed to parasites appeared relatively unimportant in determining susceptibility to infection and subsequent parasite growth rates. Among infected fish, those attaining the fastest body-size corrected growth rates ended the study with the heaviest plerocercoid burdens, which is consistent with other studies in fish (Arnott et al., 2000, Randhawa and Poulin, 2009) and molluscs (Taskinen and Valtonen, 1995). Finally, parasites are predicted have higher fecundities when infecting fish at a larger body size. The consequences for parasite success when faced with a host population composed of larger or smaller individuals therefore appear complex; smaller sticklebacks appear to be more readily invaded, but larger sticklebacks offer better prospects for rapid growth and development for those parasites that can infect them.

6.5.2 The problem of covariance between age and body size

Since age and body size are typically linked closely in fish and other animals that exhibit indeterminate growth, separating the importance of each factor is challenging, and experimental infection studies are required. *Schistocephalus* prevalence increases in smaller fish under one year old (Pennycuick, 1971), although being a natural infection study no attempt was made to disentangle the effects of body size and age. Some previous studies have used manipulations to decouple the age and size of prospective fish hosts. Jones et al. (2008) acknowledged that the age and body size were both significantly affected resistance when the two effects were decoupled by rearing fish under separate
temperatures to create different growth rates. Ryce et al. (2005) experimentally manipulated growth rate (and hence body size) by rearing rainbow trout *Onchorhynchus mykiss* at different temperatures before exposing them to *Myxobolus cerebralis*, the causative agent of whirling disease. Their study identified a significant effect of body size, but also an interaction with age, such that trout needed to be both larger and older to resist infection. In our study, fish showed a highly significantly increased susceptibility (+10%) to infection at smaller body sizes, without a significant effect of age. A significant effect of exposure age was found in both Chinook salmon and Rainbow trout exposed to *Myxobolus cerebralis* with younger fish having a higher prevalence of infection five months post-exposure (Sollid et al., 2003). Sutherland (2011) hypothesised that greater susceptibility in smaller size classes of pink salmon (*Oncorhynchus gorbuscha*) to lice infection may be caused by nutrient diversion at an early developmental stage, as cell stress and decreased proliferation were detected in gene expression analysis. It is possible similar processes occur in the early stages of stickleback infections with smaller individuals having nutrients diverted away from growth processes meaning they are less able to withstand infection. While food availability was standardised during the experiment it is possible stomach capacities differ across body sizes, with infected fish showing increased meal sizes (Wright et al., 2006).

In contrast to other studies, size-at-age was not manipulated experimentally, and instead natural variation in growth rates was utilised, generated by both within- and between-family variation. Although the experimental design allows the examination of the role of age and body size separately, one limitation of this approach is that body size is potentially confounded by pre-exposure growth rates. Therefore, we are unable to rule out the possibility that the increased growth rate of parasites following infection result from the rate at which fish were growing prior to exposure, rather than from body size *per se*. However, our approach does have the advantage that potentially highly stressful factors such as manipulating food availability or temperature can be excluded. Therefore it can be assumed individuals were not experiencing a side effect of delayed growth as all fish were kept under the same environmental conditions.
6.5.3 Importance of host internal environment on parasite success

The findings presented here demonstrate the importance of variation in the environment created by the host for parasite growth. The body size of sticklebacks at exposure has a significant impact on infection levels, even when there is no further accumulation of parasite individuals in an experimental setting. Similar findings were shown by Barber et al. (2005), who demonstrated that of fish held in competitive groups, those with greater growth rates due to high competitive ability also showed increased parasite burden. However, in that study fish were held in groups and so individual food intake was not controlled, and slow growing fish may have been simply unable to secure sufficient resources. In the present study, all fish were housed individually and fed ad libitum, indicating that food availability per se was not a factor limiting the growth rate of parasites and hosts. Our findings are supported by field studies, such as the ectoparasite *Tracheliastes polycopus* infecting European dace (*Leuciscus leuciscus*), the size of which has been shown to be positively correlated with parasite burden in a field study (Cardon et al., 2011). A host size-parasite size correlation was also shown for the isopod *Ichthyoxenus fushanensis* infecting the body cavity of *Varicorhinus bacbatulus* (see Tsai et al., 2001). Models examining invertebrate hosts have shown that the abundance of parasites is higher at larger host size classes (Hasu et al., 2007).

6.5.4 Conclusions

While the current study has highlighted the relationship between body size and cestode infection in sticklebacks, this raises the further question of what mechanisms are involved. It is possible that with greater size comes an immunological maturity (Sollid et al., 2003, Sutherland et al., 2011). However in this study age was not a significant factor in the susceptibility to infection; rather size per se appeared to be the determining factor. Interestingly in the study by Sutherland et al. (2011) using ectoparasites, size classes of host Atlantic salmon were found to have differing patterns of gene expression after exposure, but not all of these were immune related. Further studies utilising the fully sequenced genome of the stickleback might provide greater insight into the mechanisms that underpin the host factors that determine both susceptibility to infection and disease phenotype.
6.6 References


PENNYCUICK, L. 1971. Differences in the parasite infections in three-spined sticklebacks (Gasterosteus aculeatus L.) of different sex, age and size. Parazitologija, 63, 407-418.


Chapter 7

Discussion

Image adapted from Barber et al. (2008)
7.1 Main Findings
Parasites are ubiquitous components of all environments and exert varying degrees of pressure on their hosts, ranging from the benign to the fatal. In fact the consequences of a particular parasite species can change depending on conditions, and as environments change rapidly due to anthropogenic influences there is the potential for the balance of host-parasite interactions to be shifted. From the perspective of an endoparasite, there are two ‘layers’ to the environments they inhabit and experience. The external environment, which is experienced by ectoparasites and endoparasites that have free-living stages in their life cycles, and the internal environment created by the host. Variation in either of these environments has the potential to affect a multitude of infection parameters. This thesis investigates how variation in the internal environment generated by host variability, and how variation in external environments generated by anthropogenic changes, affect parasite infections. Most of the work in the thesis has utilised the three-spined stickleback – *Schistoscephalus solidus* experimental host-parasite model. In this discussion I will summarise the host and environmental factors identified as important for host-parasite interactions and give suggestions for interesting and fruitful future research directions.

7.1.1 Environmental effects on host-parasite interactions

7.1.1.1 Wider external environment
The impact of water quality and chemistry on *S. solidus* was investigated in Chapters 4, 5 and 6. Salinity, and its effects on the development of embryonic parasites, was shown to be a factor limiting the distribution of *S. solidus* to fresh- and brackish waters in Chapter 4. The concentration of oxygen was found to interact with *S. solidus* infections to determine patterns of gene expression in sticklebacks in Chapter 6.

Aquatic organisms are exposed to fluctuating oxygen availability to a much greater extent than most terrestrial organisms (Ficke et al., 2007, Marcogliese, 2001). The concentration of oxygen in the environment is of particular interest to fish biologists, as it directly impacts the health of wild fish populations (Magnuson et al., 1997, Diaz, 2001) and fisheries production (Breitburg, 2002, Breitburg et al., 2009). In sticklebacks hypoxia has been associated with increased time at
the surface (Giles, 1987a), greater predation risk (Meakins and Walkey, 1975, Giles, 1987b), lower glucose levels (O'Connor et al., 2011), loss of dominance hierarchies (Sneddon and Yerbury, 2004) and patterns of gene expression (Levelahti et al., 2011). Sticklebacks infected with S. solidus fish consume more oxygen at rest and whilst swimming (Lester, 1971) and spend more time at the surface in oxygenated water than non-infected conspecifics (Giles, 1987a).

The expression of genes previously linked to hypoxia was less consistent than in other biological systems commonly studied (Chapter 6). Not all genes showed significant upregulation in response to hypoxia, which could be due to a high resilience to hypoxia in the study population. Perhaps surprisingly, infected fish did not show the expected upregulation of hypoxia genes, and in general had lower expression levels than non-infected fish. However, expression of LDHA did increase with parasite size. As this gene is involved in the switch to anaerobic respiration by glycolysis and the subsequent fall in glucose levels, this may indicate that parasitism mimics physiological hypoxia responses in fish causing them to seek high oxygen at the surface. The behaviour of stickleback infected with S. solidus has been suggested to increase success by avian predators (Tierney et al., 1993, Barber et al., 2004), and may be an example of behavioural modification by the parasite to increase its own transmission to the definitive host (Poulin, 1994). The utilisation of an existing physiological response to hypoxia may represent one mechanism of how the parasite achieves this.

Salinity can have an impact on the distribution of aquatic organisms, including parasites, with narrow osmotic ranges. This is particularly true of ectoparasites and parasites with free-living stages in their life cycles, as they are not buffered by hosts from external fluctuations (Pietrock and Marcogliese, 2003). Some freshwater parasites can tolerate a slight increase in salinity, such as the eye flukes Philophthalmus megalurus and P. gralli hatching from freshwater snails in 18‰ and 24‰ salinity respectively (Nollen et al., 1979). However once outside of the protective cuticle, even short term exposure to salt solutions can cause mortality, such as in miracidia of Echinostoma caproni (Ford et al., 1998).

The hatchability of S. solidus eggs decreased with increasing salinity, becoming completely non-viable at 35‰ (Chapter 4). However, at salinities equivalent to
brackish water, at around 10‰ similar to that found in estuaries and the Baltic Sea, some eggs were able to develop and hatch. This may explain why sticklebacks infected with *S. solidus* are found in the Baltic Sea and are reported in coastal regions (Rolbiecki et al., 1999, Zander, 2007). In the wider context, sticklebacks are likely to be exposed to *S. solidus* only after entering freshwaters, which indicates that it provides a novel challenge for marine populations of three-spined sticklebacks when colonising freshwaters. A relatively recent freshwater reservoir population was investigated in Chapter 5 to further study the impact of genetic background on host-parasite interactions.

### 7.1.1.2 Internal environment of the host

Endoparasites are often reliant on their host for resources as they are unable to forage themselves, therefore the nutritional status of the host is expected to impact parasite growth. This has been given considerable attention in fish species as this energetic drain may also have an impact on fish growth as resources are diverted from host metabolic function to support the parasite burden (Kim and Lovell, 1995, Descroix et al., 2010). In tapeworm species, such as *S. solidus*, the parasite absorbs nutrients from the body cavity of intermediate hosts to rapidly increase in size before transmission to the definitive host. Once there, the parasite no longer allocates resources to somatic growth, but instead becomes reproductively active. As parasite size is related directly to both the probability of establishing in the avian gut (Tierney and Crompton, 1992) and the fecundity of adult parasites once established (Dörücü et al., 2007), attaining maximum size whilst at the plerocercoid stage is likely to maximise the fitness of parasites. However, as *S. solidus* is unable to reproduce in the fish host, early mortality of infected sticklebacks would mean the parasite would be unable to be transmitted and release eggs. Therefore a trade-off between attaining high growth rates without causing host death is predicted. It could alternatively be predicted that increasing food availability to hosts could benefit the fish, either because the additional nutrition allows the host to mount effective immune responses, such that they have a reduced susceptibility to infection, or because their higher growth rate allows them to outgrow parasites once they establish, particularly as infected fish have been previously shown to have higher meal sizes (Wright et al., 2006).
As expected fish held on higher rations were able to achieve faster growth rates, both in terms of increasing length and specific growth rate (Chapter 2). Evidence was found that infected fish held under low food rations show an increase in specific growth rate compared to non-infected fish provided with the same food availability. In a previous study, three-spined sticklebacks held individually and fed to satiation showed parasite associated growth enhancement, with growth of hosts increasing following infection (Arnott et al., 2000). The results presented here (Chapter 2) lend further support to this hypothesis. The infection associated faster growth rates in terms of mass may reflect a greater increase in liver and spleen size which were also shown to be higher in infected fish under both feeding regimes, and consistent with other studies (Arnott et al., 2000, Kalbe and Kurtz, 2006).

Interestingly, increased food ration to hosts was found to increase S. solidus plerocercoid size in experimentally infected stickleback (Chapter 2). The evidence presented here suggests that rather than taking advantage of the host to grow at maximum rates at any cost, S. solidus moderates its own growth rate to permit the hosts basic metabolic functions. This finding supports previous results from group housed stickleback showing that parasites grow faster in faster growing hosts (Barber, 2005), although in that study it was unclear if this was due to increased investment in competitive foraging by infected fish. As the study presented in this thesis (Chapter 2) was an experimental infection study of individually housed stickleback, we can be confident that the sizes attained by parasites must have been achieved within the study period by the specific food ration given.

### 7.1.2 Host factors and parasite infection

#### 7.1.2.1 Host body size

Host size at exposure may have important implications for parasite infections, as hosts may change their foraging behaviour through with size (Rohde, 1994) thereby altering exposure rates, or growth rates may have impacts on immune function (Sol et al., 2003). Growth and overall body size may be altered in some species as global environmental change associated with anthropogenic effects brings higher temperatures (Chen et al., 2009) and species range shifts
Environmental change may also impact parasites as timing of emergence is seasonally linked (Marcogliese, 2008). Therefore understanding the effect of the timing of parasite exposure as the host grows has an important ecological context.

In *S. solidus* infections, faster growing fish have been shown to have faster growing parasites (Arnott et al., 2000, Heins et al., 2002, Barber, 2005) and the studies presented in Chapters 3 and 5 provide further experimental demonstration of this. By using natural variation in growth rates, rather than potentially stressing fish with changes in temperature or food availability, body size was shown to significantly affect infection rates and parasite growth. Smaller fish were shown to be more susceptible to acquiring parasite infection following a controlled challenge, but parasites that managed to establish in larger fish were able to attain a greater mass than those establishing in smaller fish. Age did co-vary with body size, but was found to be less important to both susceptibility and parasite growth rate. This is in contrast with other studies on fish that have shown age to be an important factor in determining parasite susceptibility, such as larger and older rainbow trout showing higher resistance to whirling disease (Ryce et al., 2005). However, in the study by Ryce et al. (2005) temperature was used to control growth rates.

It is possible that separate mechanisms are involved in determining a host’s susceptibility to acquiring parasites and the growth rate achieved by parasites once established. For instance, while the greater body size causing higher parasite growth may be linked to stickleback meal size increasing with size (Wright et al., 2006), susceptibility to infection could be linked to the allocation of energy between growth and resistance to infection (Sutherland et al., 2011). Salmon size classes have been shown to induce different gene expression patterns when challenged with sea lice infection (Sutherland et al., 2011), and a similar pattern may be observed in sticklebacks infected with *S. solidus*.

The results presented here suggest that if environmental change were to cause *S. solidus* to change their timing of emergence to earlier in the year, when sticklebacks had achieved only small body size, or to reduce the availability of food, sticklebacks would be likely to be more susceptible to infection. Given that
S. solidus infections can interfere considerably with stickleback reproduction (Rushbrook and Barber, 2006, Rushbrook et al., 2007, Macnab et al., 2009, Heins et al., 2010, Heins and Brown-Peterson, 2010, Heins, 2012), greater susceptibility may put the longevity of certain populations into question through reproductive castration (Heins et al., 2010).

Given that smaller fish will are more likely to become infected, and infected fish have been shown to increase their food intake, the most advantageous of hosts are small fish with high food intake. This scenario may be made more likely by the parasite encouraging foraging even under predation threat (Milinski, 1985).

7.1.2.2 Host genotype and phenotype

In three-spined stickleback, the identity and diversity of parasite infections have been associated with variation in host phenotype, with (for example) benthic and limnetic morph fish being differentially infected with parasites in a manner that reflects habitat use and foraging preferences (Stutz et al., 2014). The authors also showed that, between populations, the relationship between phenotype and parasite infection was absent, indicating that other process may be involved at larger spatial scales. However, while correlations between morphology and infection might indicate phenotype dependent exposure due to foraging on infected prey items, this cannot be determined by wild fish sampling alone.

Here, in a controlled parasite exposure study, the host phenotype for lateral plates was found to be associated with infection susceptibility, with fish exhibiting a greater number of plates having a lower probability of acquiring infection following controlled experimental exposure to S. solidus (Chapter 5). The ectodysplasin (Eda) genotype and plate morph phenotype was also been associated with body condition, again greater number of plates was an advantage. While infected fish had lower body condition factors than non-infected conspecifics, greater numbers of plates meant infected fish suffered less from diminished body condition than fish with fewer plates and the low plated Eda allele. The unexpected disadvantage to three-spined sticklebacks with low lateral plate number may represent an example of parasite adaptation to the host most frequently encountered.
Fish infected with *S. solidus* were also found to have a greater abdominal distension, as measured by dorsal profile area, with more lateral plates. This is contrary to the prediction that have a body armour of lateral plates might constrict parasite induced swelling, and possibly increase the pressure on internal organs. It is possible that the low-plated phenotype only showed swelling after plate 8 below the second prominent dorsal spine, while the completely plated phenotypes show swelling along the whole of the lateral side pushing parasite toward the anterior of the fish. Studies allowing the infection to progress to greater parasite burdens while monitoring parasite growth would determine this.

There was no effect of plate morph phenotype or *Eda* genotype on the growth of fish in our study. This contradicts results by other researchers who have found enhanced growth in fish with the low plated *Eda* genotype (Barrett et al., 2008) and low plate phenotypes (Marchinko and Schluter, 2007). One explanation for this could be the relatively high levels of calcium in the laboratory aquaria, which may have negated the additional growth costs normally experienced by high-plated fish in freshwater environments.

### 7.2 Suggestions for future work

As the factors influencing parasitic infections with a multi-host life cycle are complex, predicting parasite responses to environmental change requires an understanding of each stage. In Figure 7.1 I have illustrated the life cycle of *S. solidus* and highlighted the variables I have examined in this thesis. There still remains many unanswered questions regarding the effects of the wider environment on infections by this parasite. For example, I have demonstrated that eggs of *S. solidus* are sensitive to salinity conditions, but the subsequent effect on hatched coracidia is unknown and these stages may yet be sensitive to perturbations in other environmental variables, such as temperature, which has already been shown to affect the plerocercoid stage (Macnab and Barber, 2012). By culturing *S. solidus* eggs in temperatures corresponding to those experienced in the wild through the year, the potential impact of timing and annual temperature variation would be clarified. As smaller fish were shown to be more susceptible to parasite infection in Chapter 6, one such scenario could be high parasite hatching success earlier in the year increasing infection rate, due to the temporal
overlap of infective stages and susceptible hosts. There were a limited number of *S. solidus* individuals tested for salinity tolerance and all from the same population, meaning local adaptation could not be tested. In order to test for geographic variation in salinity tolerance, populations from marine, brackish and freshwater areas in addition to low salinity marine waters, such as the Baltic, should be examined.

**Figure 7.1** Schematic illustrating the life cycle of *S. solidus*, with environmental and host factors examined in the thesis

While we know from the studies presented here that increased nutrition post-exposure increases parasite growth rather than host growth, the effects of differential nutrition prior to exposure is still unknown. As nutrition is linked to immune function in many systems (Trichet, 2010) it is possible that increased food availability before parasite exposure might offer an advantage to the host in terms of lower susceptibility to infection or higher body condition (Descroix et al., 2010). The quality of nutrition (food type, or diet composition) before exposure may also have an effect as prey type affects body condition (dos Santos et al., 1993) and immune function in fish (Amar et al., 2004, Kolluru et al., 2006). For example, as carotenoids are an important precursor to vitamin A (Torrissen and
Christiansen, 1995), which plays an important role in the immune response, their absence in the diet may lead to higher parasite susceptibility, although this has not been studied in three-spined sticklebacks. Studies providing a diet supplemented with carotenoids to three-spined sticklebacks then exposed and non-exposed to *S. solidus*, would be one way of simulating variation in prey quality. Immune function could then be quantified as has been done previously in sticklebacks (see Scharsack et al., 2004, Scharsack and Kalbe, 2014), to further clarify the effect of host condition on susceptibility to *S. solidus*.

The results presented here give an interesting indication that the expression of hypoxia genes may differ between populations and infection phenotypes. Studies on hypoxia in sticklebacks have found behavioural changes start at different oxygen saturation levels depending on the study population used (Sneddon and Yerbury, 2004, O’Connor et al., 2011, Leveelahti et al., 2011). Examining the gene expression of fish from a range of populations would determine if the observed variation in hypoxia behaviour is a local adaptation or simply a result of rearing environment. There may be changes in expression of *LDHA* and other genes associated with hypoxia responses with increasing parasite burden, although there would need to be further studies to validate these results with a greater sample size. If gene expression patterns did alter with parasite burden, this may indicate a possible mechanism for parasite behavioural modification in the stickleback-*Schistocephalus* system. To test this, an experimental exposure study could be used to monitor behavioural changes under hypoxia as infection progresses.

I have shown that body size rather than age is the most important factor for three-spined stickleback susceptibility to *S. solidus*. The mechanism of this change is unclear, and could be due to growth rates prior to infection or perhaps development of immune function. In a study on juvenile pink salmon (*Oncorhynchus gorbuscha*), Sutherland et al. (2011) were able to characterise the development of innate immunity by comparing transcriptional profiles of different size classes. Using this approach would further clarify if the effect of body size on infection susceptibility in three-spined sticklebacks is due to immune function development.
I have also found evidence of a link between morphological phenotype and infection susceptibility, as fully-plated three-spined sticklebacks were less susceptible to *S. solidus* infections following controlled challenge. However, the mechanism behind this difference is unknown. As variation at the MHC has been implicated in resistance to infection this provides an interesting avenue for determining genetic predisposition for immune responses. By examining relative MHC and actual immune responses of fully plated and low plated three-spined sticklebacks, we would be able to determine if the difference in infection susceptibility is due to immune function. There is evidence from previous studies that migrant marine fish are selected against by being more susceptible to freshwater parasites (MacColl and Chapman, 2010), but this could not be determined in the study presented here as all fish were from the freshwater population. It would also be interesting to compare fully plated fish from freshwater and marine populations to see if ‘migrant’ highly plated fish have weaker immune responses as shown previously by MacColl and Chapman (2010) or if high numbers of plates is associated with higher immune function as predicted by my results.

### 7.3 Concluding comments

The overarching conclusion of this thesis is that host-parasite interactions are influenced by both host factors and the environmental context in which they interact. The outcomes of host-parasite interactions have the potential to be shifted by anthropogenic environmental change, and have consequences for host responses and disease progression. Given the pathogenic effects of *S. solidus* infection, shifts in the host-parasite interaction have wider population level implications for the host.
7.4 References


Appendix 1

Carsington Water, (53°3'30"N 1°37'50"W), OS Map 1:40000 Digitmap® Ordinance Survey®. Sampling sites shown in red.
Appendix 2

Carsington Water geological map, (53°3’30"N 1°37’50"W), OS Map 1:40000 Digitmap® Ordinance Survey®. Grey colouration denotes limestone formation.