Effects of host phenotypic and genotypic variations on parasitic infections in sticklebacks

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ABSTRACT

Host phenotype and genotype have a direct effect on the outcome of host-parasite interactions, and therefore have the potential to alter the consequences of the disease processes. This thesis examines how host-parasite interactions might be affected by host factors in both naturally and experimentally infected three-spined sticklebacks using the *Schistocephalus solidus* and *Diplostomum spathaceum* models.

In the River Soar, Leicestershire, 12 different parasite species belonging to various taxa have been recorded in the course of eight months survey. The prevalence and intensity of *D. spathaceum* infection was high throughout the year. *S. solidus* plerocercoid masses varied between the sexes, and infected fish had a poorer body condition than non-infected fish. In contrast to wild study results, fish sex was not found to be a strong predictor of fish susceptibility to experimental *S. solidus* infection. The establishment of *S. solidus* plerocercoids was not related to host size or mass. Neither host sex nor fish size or mass at exposure had a significant influence on subsequent parasitic growth rates. However, the parasite index was higher in female than male fish. Reproductive reduction often arise in infected males and it does not rely on gonad development alone, but also on the glue spiggin production for nest building as well. The effect of parental genotype and phenotypes on offspring susceptibility to *S. solidus* infections and spiggin gene expression have been investigated. I found that the prevalence of infected offspring sired by infected males that subsequently became infected following exposure was significantly higher than those sired by non-infected fathers. *S. solidus*-infected sticklebacks showed significantly increased *spiggin C1* and *spiggin C2* expression compared to non-infected fish, expression of all gene investigated did not affected by paternal infection treatment.

A significant effect of provenance (host population) and host sex on susceptibility to *D. spathaceum* infection was found. This variation reflected the genetic differences in the coevolutionary processes between the host and parasite. Sticklebacks invade freshwater, they typically evolved by reduce lateral plate count. Therefore, in the association between parasite infection and *Eda* genotype, plate number, age and sex were examined as possible factors determining fish susceptibility to *D. spathaceum* infection. Fish with a greater number of lateral plates were found to show increased parasitic loads, and older fish had higher loads than juveniles.

The overall conclusion of the thesis is that the outcome of host-parasite interactions is significantly affected by host phenotype and genotype factors. Pre-existing host variation effects have the potential to directly alter parasitic development and shift the consequences of the host-parasite interaction. In addition, host variation has helped to shed some light on the ecology and evolution of stickleback-parasite interactions.
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Chapter 1

Introduction

R. Shalal ©
1.1 Host-parasite interactions

1.1.1 The ecology and evolution of host-parasite interactions

Parasitism, as a mode of life, is taxonomically widespread and successful (Poulin and Morand, 2000) as measured by the large species and life cycle diversity (Smyth and Wakelin, 1994). Parasites are ubiquitous, with estimates made that for every host species, there is probably at least one parasite species that is unique to it (Windsor, 1998). Parasites live in a close association with other organisms (their hosts) in a symbiotic relationship (Bowman, 2014). Through the evolution of physiological and morphological adaptations to life in or on their hosts’ bodies, parasites have considerable potential to affect host fitness (Schmid-Hempel, 2011). Often, parasites have a strongly negative effect on their hosts, with effects including mortality, nutrient theft, morbidity and reproductive disruption (Perrin et al., 1996, Clayton and Moore, 1997, Knudsen et al., 2002). Nevertheless, the severity of parasite virulence can become reduced over evolutionary time in some infections, as a result of coevolutionary processes between parasites and hosts (Minchella, 1985, Poulin, 2007). Several empirical studies have had an attempt to understand the outcome of infection and how it can be linked considerably to a number of factors, such as genetic correlation across both host and parasites, and how they interact in an environmental context (Ebert and Herre, 1996, Wolinska and King, 2009).

Significant research effort has been investigated the impacts of environmental change on host-parasite interaction. However, little effort has been devoted to understanding the potential response of parasites of animal populations to climate changes, especially those in aquatic systems (Marcogliese, 2001). Parasites live in one environment (host body) which in a relative association with a wider environment (provided by the habitat that the host lives in) (Mackenzie, 1999). Therefore, parasite community structures may depend on a number of abiotic factors such as temperature, salinity, pH, etc. which could be directly or indirectly influenced their interaction with the host (Poulin, 2011).

While the ectoparasites with a single-host life cycle are directly sensitive to environmental change, consequently, many ectoparasites have shown that they are more tolerant than their hosts in varied types of environmental change (Mackenzie,
The endoparasites with indirect and multi-stage life cycles can be affected directly or indirectly by host external environment, and this likely to alter disease phenotype. Free-living stages of complex life cycle parasites are expected to be directly vulnerable to environmental changes that might have crucially affected their chance to be in a direct contact with the suitable intermediate/definitive host. Environmental changes effect can alter the intermediate/definitive host behaviour, reproduction and even their survival.

For example, climate change and the temperature increases, have considerable implications for host-parasite interactions, by increase pathogen development, survival rate and host susceptibility (Harvell et al., 2002). This is particularly in ectothermic hosts, as their body temperature matches their external environment, which a potential parasite must successfully adapt and develop under host habitat condition (Thomas and Blanford, 2003, Ward et al., 2010). Such a scenario was proposed by Macnab and Barber (2012) who suggested temperature regime had a dramatic effect on the growth of Schistocephalus solidus plerocercoids, when parasites had reached infectivity after eight weeks in high temperature regime than parasites taken from the experimentally exposed three-spined stickleback held at 15°C. Furthermore, the authors reported that stickleback’s growth and survival are potentially influenced by high temperature when control and exposed fish growing significantly slower individuals at lower temperatures.

It is becoming increasingly recognised that parasites are ubiquitous and important components and may have important consequences for ecosystems. Food webs are very incomplete without parasites (Lafferty et al., 2006). Indeed they influence a range of ecosystem functions and have a major effect on host manipulation to increase their transmission to their next host (Hudson et al., 2006, Lafferty, 2008). Parasites can have strong regulatory effects on host population dynamics (Tompkins et al., 2002, Phillips et al., 2010). It has been reported that host life history could be modified by parasites (Minchella, 1985, Agnew et al., 2000), and that many parasites are capable of altering host behaviour (Thomas et al., 2005, Libersat et al., 2009). It has been suggested that parasite-mediated selection plays a significant evolutionary role through maintaining host genetic variation (Coltman et al., 1999, Eizaguirre et
al., 2012). Increasingly, ecosystem stability has been driven by the consequences of parasitic infection via the regulation of host population structure (Lafferty, 2008). For example, avian malaria caused by the apicomplexan parasite *Plasmodium relictum* played a key role in annual mortality of the juvenile (hatch-year) ‘apapane (*Himatione sanguinea*) leading to the decline and ultimate extinction of this native Hawaiian forest bird, and is representative of one of the negative consequences of parasite infections (Atkinson and Samuel, 2010). Theoretical studies have been dedicated to clarifying the role of parasites on host evolution and explaining a range of evolutionary phenomena. For example, sexual selection (Hamilton and Zuk, 1982, Moore and Wilson, 2002), or natural selection and regulation of genetic polymorphism (Escalante et al., 1998, Wegner et al., 2003).

1.1.2 Factors affect host susceptibility to parasitic infection

The existing variations between particular host species might influence the susceptibility or resistance to parasitic infection. It is possible that several factors such as environmental conditions, genotype, age / size, sex etc. are involved in determining a host’s susceptibility to develop infection, recognition of this factors offers the possibility for understanding the reasons behind increase infection levels in susceptible hosts.

1.1.2.1 Environmental changes

One factor which could increase host susceptibility is the changing in environmental conditions. Environmental perturbations have been reported to affect host immune defences in a wide range of animals, and also it can exert control over host and parasite encounter rate. For example, the influence of temperature on shedding of *Diplostomum spathaceum* cercariae from infected snail (*Lymnaea stagnalis*) host is increased at high temperatures, up to 58000 cercariae per snail/day (Lyholt and Buchmann, 1996). Moreover, the environmental variables such as increase host habitat temperature might increase disease risk for some hosts (Höglund and Thulin, 1990). The severity of the amoebic pathogen *Entamoeba invadens* that infect eight different species of snakes was dramatically increased at high temperatures at 25°C, however, infected snakes of all species failed to show any gross pathology at 13-14°C (Barrow and Stockton, 1960).
1.1.2.2 Host nutrition

Susceptibility to parasitic infection may also be related to host nutrition. Parasites are often have a strong impacts in host food webs which has been apparent for over a hundred years (Lafferty et al., 2008). In trophically transmitted parasites, host foraging behaviour is likely to be associated directly with an increased host susceptibility to infection by consuming larval stage with the food. Host nutrition might have an indirect effect on increase parasites infection in potential hosts by providing energy resources for immune responses. In the ruminant animals that exposed to gastrointestinal nematode infections, feeding hosts on a diet with supplementation at the higher levels of protein has reduced faecal egg output and worm burdens and improve infected animals ability to cope with the consequences of parasites (Coop and Kyriazakis, 2001).

1.1.2.3 Genetic and phenotypic variations

High genetic diversity may differ in the population. In wild Daphnia magna infected with the bacterium Pasteuria ramosa, significant interactions were found between host and parasite. Host clones were differed in their susceptibility to parasite and showed resistance specific to certain parasite isolates, meaning that parasites are able to track specific host genotypes and some hosts are susceptible to the same parasite isolates (Carius et al., 2001). Parasite-mediated selection drives genetics in natural populations by change host infection susceptibility. For example, Duffy and Sivars-Becker (2007) found that genotypes of the cladoceran Daphnia dentifera from lakes that had been subjected to recent epidemics of the fungus Metschnikowia bicuspidate, showed more resistant to infection, and had less variance in susceptibility, than genotypes from lakes that had not seen recent epidemics. This result suggested that a parasitic epidemic could be rapidly halted by quick host genetic changes in natural populations and that parasites could drive natural selection among host directly by promoting the frequency of resistant genotypes. Age and body size can play an important role in determining host susceptibility to pathogen directly by change immunity response (acquired and innate) with age. For example, the development and severity of whirling disease Myxobolus cerebralis in rainbow trout (Oncorhynchus mykiss) was known to be generally age and size
dependent (Ryce et al., 2004). Higher disease severity found among younger host age and the parasite effect was gradually reduced in older fish group. In addition, fish that reared in parasite free water for nine weeks post-hatch or longer exhibited higher survival rate than fish that exposed to the parasite at younger ages as contributed to non-specific immune mechanisms that develop with age (Ryce et al., 2004).

There are a number of differences between males and females in terms of ecological, social and behaviour, immunity, physiology and ecology that might give rise to one sex becoming more susceptible to parasitic infection than the other (Barger, 1993, Zuk and McKean, 1996). The trade-offs between investment in sexually selected traits and the immune system might play the major role in increase host susceptibility to pathogen as immunity may decline.

1.1.3 Mechanisms of parasite avoidance and resistance
Parasite live in symbiosis with another organism (host), as a result of parasitic infection, host fitness is often negatively affected. The term “virulence” defines as the reduction in host fitness caused by pathogens (Levin and Pimentel, 1981, Read, 1994). As parasites basically are highly virulent to their host, however, evolution acts on parasites to exist a trade-off between their reproduction and host survival (Poulin, 2007, Lafferty and Kuris, 2009). Therefore resistance to parasites might be extensively mediated by host-parasite evolutionary outcome (Minchella, 1985).

As suggested by Minchella (1985), the impact of parasitism on potential host resistance is dependent on four principal factors: (1) the presence of resistance genes in the host population; (2) infection outcome severity and its potential effect on host reproductive success; (3) the probability of parasite-host encounters in the host habitat; and (4) the cost of resistance. For example, the evolutionary costs of resistance to the microsporidian Nosema whitei (obligately killing parasites) on eight distinct lines of flour beetles Tribolium castaneum was demonstrated by Bérénos et al. (2009). The authors used 11 generations of coevolved lines (which were subject to selection by coevolving the N. whitei infection) and control beetles as a host line with original and coevolved parasite as a source of experimental infection. The result of this study demonstrated that the coevolved host line had a higher survival rate.
and had developed higher resistance towards non-coevolved and coevolved parasites than the control line. In addition, parasites showed lower virulence in both coevolved and control hosts. The authors propose that this reduction in parasitic coevolution virulence may result from the trade-off between virulence and the potential for successful transmission.

1.1.3.1 Behavioural resistance

One mechanism to avoid exposure to a variety of pathogens and parasites is for hosts to behave in a manner that actively prevents or limits infection. Parasitic infection has provided a selective pressure on host behavioural resistance. Consequently, defence behavioural traits that have effectively been evolved by the potential host will be governed by natural selection (Schulenburg et al., 2009). There are various forms of behavioural resistance and parasitic avoidance mechanisms in animals. For example, Grooming, mate choice, social behaviour, hygiene behaviours, preening, habitat avoidance etc. (Hart, 1997).

To name a few examples of behavioural avoidance, there are noticeable parallel evolution solutions that have been created in host responses, as shown by social insects' behavioural defence (Cremer and Sixt, 2009). By developing a unique olfactory learning behaviour mediated by serotonin signalling in the nervous system, the nematode Caenorhabditis elegans successfully avoids odours from the pathogenic soil bacteria Pseudomonas aeruginosa and Serratia marcescens after sequential exposure to these pathogens (Zhang et al., 2005). A host might simply avoid parasitic infection by habitat choice. The great tit, Parus major, when given the choice of nesting in experimental hematophagous hen flea, Ceratophyllus gallinae, infected or non-infected nests, they will invariably chose the one without parasites (Oppliger et al., 1994). Behavioural experiments in the laboratory have shown that rainbow trout, Oncorhynchus mykiss, react physically to avoid Diplostomum spathaceum cercariae invasion (Karvonen et al., 2004). The authors thought that it was unlikely that the fish could distinguish the cercariae visually, and they could not use their odour response to avoid infection. However, they suggested that the mechanical disruption that some cercariae had caused by penetrating gills and skin allows fish to protect themselves from further exposure to parasites.
Through sexual selection for the avoidance of parasitic transmission, a host can avoid infection. By mating with brightly coloured males, female Rock Doves, *Columba livia*, may benefit from a reduced risk of disease transmission by choosing between "clean" males without lice than "lousy" males with experimentally increased parasite loads (Clayton, 1990).

### 1.1.3.2 Immunological mechanisms

One of the most important host defence mechanisms against parasitic infection is the immune system. Host immune defence is likely to reflect the evolution of the host-parasite interaction and, in particular, is host evolution dependent (Minchella, 1985). The host immune system is classically divided into innate and adaptive immunity (Vivier et al., 2011). The innate is a natural response to pathogens and is not related to prior antigen production, while the adaptive is an acquired immunity whereby systemic cells and processes or antibody production have worked to eliminate pathogens or prevent their growth (Iwasaki and Medzhitov, 2010, Buchmann, 2014). One example of innate host immunity is the snail immune system, which is relatively weak and unable to act against most trematode pathogens and which are often effectively established within the host body (Minchella, 1985).

However, in vertebrates, the coevolution of host pathogens, including parasites, are well relied on the genes of the major histocompatibility complex (MHC). MHC variability is believed to be maintained by pathogen-driven selection (Sommer, 2005). The MHC family is divided to three groups: class I, class II and class III. This gene is highly polymorphic, therefore a large alleles diversity has been recorded across a wide range of individuals within the population of vertebrates (Wegner et al., 2003, Spurgin and Richardson, 2010). Parasites have been reported as having an important role in maintaining the diversity of the large set of MHC alleles, which appears to persist over many host generations through host-pathogen coevolution (Reusch et al., 2001, Borghans et al., 2004). For example, in fish, immune investment as measured by variability of the MHC genes and parasitism has been found in wild populations among 14 species of cyprinid fish, and which has shown a high genetic diversity in exon 2 of the MHC in the spleen of fish infected with ectoparasites. This suggests that immunity plays a role in protecting each species
of fish from high parasitic pressure by maintaining immune gene diversity, leading to a decreased mortality rate (Šimková et al., 2006). (MHC) genes play a central role in the presentation of antigens to the adaptive immune system, and three-spined sticklebacks with low MHC allele diversity found more susceptible to S. solidus experimental exposure (Kurtz et al., 2004).

1.1.3.3 Host tolerance

In addition to host resistance evolution, a potential host can respond to the threat of parasitic infection through tolerance strategies which do not solely limit the infection itself, but by reduce or offset consequences to fitness (Roy and Kirchner, 2000). Roy and Kirchner (2000) defined resistance as the potential of a host to prevent pathogen development, while tolerance is used to describe a reduction or offset of fitness consequences due to infection, though both can improve host fitness. The terms "resistance" and "tolerance" have been used by different authors to describe different host-parasite effects, and they are often used in ways that confuse the two concepts (Clarke, 1986, Roy and Kirchner, 2000).

Recently, Rohr et al. (2010) illustrated that in the American toad (Bufo americanus) and the green frog (Rana clamitans) after exposing them experimentally to three trematode species, Echinostoma trivolvis, Ribeiroia ondatrae and plagiorchid trematode cercariae. Severity of infection varied between the two hosts as a result of differences in host tolerance, particularly between host age groups with older and larger-sized tadpoles having a higher tolerance than younger and smaller-sized tadpoles, who showed a higher likelihood of mortality than adults. While the concept of tolerance towards parasites has been well studied in plant-parasite systems, only a few studies have investigated tolerance in animal hosts (Schulenburg et al., 2009). The first study to demonstrate animal tolerance towards a parasite was reported by Råberg et al. (2007) in laboratory mice (Mus musculus) infected with the causative agent of rodent malaria Plasmodium chabaudi. The tolerance of a host genotype might vary between individuals that exhibit genetic variation for both resistance and tolerance. A tolerant genotype might exhibit high levels of fitness even with an increasing pathogen burden (Råberg et al., 2007). The authors demonstrated that host resistance and tolerance to infection were negatively genetically correlated and
that there was genetic variation for tolerance, meaning that, like plants, animals could develop the genetic selection for defence against a pathogen through increased tolerance towards infection as well as resistance against it.

1.1.4 Spatial variation in host-parasite interaction
Spatial variation in parasitism is generally observed in intermediate and definitive host populations. Host-parasite interactions can be complex and can sometimes show a local adaptation. In a natural habitat, the consequences of any interaction between species can differ across geographical distance, creating a geographical mosaic view of coevolution (Thompson, 1999). For example, Thieltges et al. (2009) found there was a spatial variation in parasite diversity across the same host species, particularly when the level of infection increased in variability over large (>100 km) spatial scales.

While several studies have investigated the host-parasite coevolution with regards to the temporal dynamic of parasite-mediated selection, others have considered variability in space as a force driving the evolution (Tack et al., 2012). Host-parasite interactions are known to vary geographically within the same host and parasite species. The freshwater snail (Bulinus globosus) as infected with the trematode Schistosoma haematobium, showed significant genetic variability between populations. Snails collected from different sites in the Zimbabwean highveld were more genetically structured than their parasite. S. haematobium showed significant genetic differences over larger distances (i.e., between two unconnected rivers), as represented by reduced population structure over larger areas (Davies et al., 1999).

Local adaptation in a host-parasite system is one of the fundamental principles of evolutionary theory to be driven by natural selection (Hereford, 2009). According to this theory, the host should become more susceptible to infection by local parasites; however, the parasites themselves become adapted to the local conditions of their hosts’ environments. Furthermore, parasites have to adapt to host evolution, leading to oscillatory dynamics in both host and parasite allele frequencies (Krist et al., 2000). Nonetheless, an allopatric host should be more susceptible to a parasite drawn from the local population than a parasite from a sympatric population (Lively, 1999). For example, the trematode parasite Microphallus sp. had shown high levels
of local adaptation to the snail host (*Potamopyrgus antipodarum*). The authors suggested that a sympatric parasite had significantly infected local common host genotypes more frequently than rare host genotypes (Lively and Dybdahl, 2000).

### 1.2 Effects of host variations on host-parasite interaction

The host population can be heterogeneous as it consist of different host type (Schmid-Hempel, 2011). Such a variations might be due to the host morph phenotype, genotypic, body condition, behaviour, sex, nutrition status or because they all associated with environmental condition (Schmid-Hempel, 2011). If hosts are sufficiently similar, then the parasites will adapted to exploit their host in a manner that causes severe harm, however, if individual variations between hosts do exist, then the cost of parasitism and the level of virulence in one host might be varied (Regoes et al., 2000). The authors suggested that as a result of host variations, parasites start to specialize on different host leading to a reduction in virulence and consequently have implications for host-parasite interaction. In fact, the environmental parameters such as temperature may be altered growth and overall host characters such as immune response, food intake etc. in some species and this could then influence how hosts interact with parasites in affected environments (Chen et al., 2011 and Schmid-Hempel, 2011).

#### 1.2.1 The effect of host body size on parasitic infection

Body size can play an important role in determining host susceptibility to parasitic infection, and it may influence parasites development (Poulin, 2011). Host body size is predicted to be one of the main factors influencing host encounter rates with parasites and the extent of parasitic species infecting individuals in wild populations (Vitone et al., 2004); typically, body mass has been observed to be important in a number of host-parasite systems. Larger host bodies are expected to be easier targets for parasitic invasion by provide larger surface areas and a larger number of available niches for colonization and also provide more cues like olfactory, chemical, visual, auditory, etc. that can be tracked by motile infective stages than small hosts. (Kuris et al., 1980, Poulin, 1995).
Body mass could be an essential factor when it also covaries with other host life histories such as diet (Lindenfors et al., 2007). For example, endoparasites intensity and richness have increased in larger-bodied hosts as they are likely to ingest a larger number of infectious stages purely due to their relatively increased food consumption (Lindenfors et al., 2007). Host life span may be crucially related to host body size, especially when some mammals show consistently faster development across their life-history stages than others (Harvey and Clutton-Brock, 1985).

Therefore, a host’s longevity and body size can be positively correlated with parasite diversity; in anthropoid primates, for instance, the diversity and intensity of both total helminths and nematode parasites have been positively related to host body size (Vitone et al., 2004). The authors suggested that as primates grow, they will naturally eat more leaves, leading to an increased probability of encountering the infective stages of gastrointestinal nematode parasites. Furthermore, larger-bodied carnivores that have slower life histories and lower mortality rates tended to live to be older and therefore accumulate more parasites (Lindenfors et al., 2007).

The size of a species is often correlated with parasite richness. The variability of monogenean gill ectoparasites species richness in 19 West African cyprinid fish species was significantly linked to host size when the maximal size of the fish species accounted for 77% of monogenean species variability (Guégan et al., 1992).

Age and body size are typically linked in many host-parasites studies to help in host growth assessment. Host body size and age may be essential in shaping parasitic patterns of infection. For example, among Canadian freshwater fish, their small size and short lifespans lead to them encountering fewer parasites (Bell and Burt, 1991). In another example of the relationship between a demographic parameter (prevalence, intensity, abundance, etc.) of parasites and the size of individual hosts, Theron et al. (1998) suggested that selective forces may be responsible for age-size preferences in parasitic infection. In their host-choice experiment, the authors used Schistosoma mansoni miracidia and three age groups (juvenile, subadult, and adult) of their snail hosts (Biomphalaria glabrata) where the juveniles showed a significantly higher prevalence than sub-adult and adults snails when exposed individually. The authors also found that S. mansoni miracidia were attracted more to sub-adult snail
hosts than juveniles and adults when exposed to the parasite larvae simultaneously. It has been suggested that *S. mansoni* miracidia showed a particular preference to a specific host size-age as they might provide higher energy resources for their development and reproduction, and also enough space for parasite growth (Theron et al., 1998).

1.2.2 The effect of host sex on parasitic infection

There are a number of differences between males and females in terms of behaviour, immunity, physiology and ecology that might give rise to one sex becoming more susceptible to parasitic infection than the other (Barger, 1993, Zuk and McKean, 1996). The abundance of parasites, including endoparasites (Poulin, 1996) and ectoparasites (Morand et al., 2004), often varies between the sexes of the same host species, leading to distinct biases in the infestation of various host-parasite systems (Poulin, 1996, Zuk and McKeen, 1996). Male hosts of higher vertebrates (birds and mammals) are frequently reported as consistently harbouring a greater number of parasites than females (Poulin, 1996, Krasnov et al., 2005).

There are a number of hypotheses that explain the male bias in parasitism. First is whether the higher mobility of males, as related to social activity such as looking for females for mating purposes and claiming a territory (Lang, 1996, Khokhlova et al., 2011), leads to their greater exposure to parasites than females. Second, immune responses increase/decrease as males reach maturity and hormonal changes take place, whilst mating competition leads to reproductive physiological trade-offs which can result in higher levels of stress and lower immunocompetence (Clutton-Brock and Parker, 1992, Folstad and Karter, 1992, Zuk and McKeen, 1996, Morales-Montor et al., 2004).

However, several studies have found female-biased parasitism (Sciutto et al., 1991, Larralde et al., 1995, Morales-Montor et al., 2002). For example, female wood ducks, (*Aix sponsa*) show a high prevalence of helminth infections during the egg-laying period, potentially a consequence of hyperphagia during the egg production period (Drobney et al. (1983)).
Therefore, there are other extrinsic factors that should be taken into account when attempting to explain sex-biased parasitism than intrinsic factors such as host sex, age, host body size and reproductive stage (Moura et al., 2003, Behnke et al., 2004, Krasnov et al., 2005). Guégan et al. (2005) suggested that some studies have excluded the likelihood of a host accidently avoiding parasites due to behavioural or ecological reasons which might have an important role in parasites-biased between sex. Additionally, as a result of certain morphological, physiological or immunological host factors, a parasite may not be able to develop properly or indeed even survive (Guégan et al., 2005, Krasnov et al., 2005).

Parasites may actively choose their hosts, and as a result of their active and adaptive choice for a more beneficial host, this could lead to a strong sex-linked contrast in infection (Christe et al., 2007). In the discrimination of host sex by a haematophagous ectoparasite experiment, Khokhlova et al. (2011) found that female fleas (*Xenopsylla ramesis*) chose a male rodent host significantly more often than a female rodent host, *Meriones crassus*. These experiments demonstrated that male behaviour and their low immunity status and also parasite sex was the main factors driving host choice.

### 1.3 The genetic basis of host-parasite interactions

Host susceptibility or resistance to parasite infection plays an essential role in shaping host-parasite coevolution models. Genetic variation may be seen in the host-parasite system as direct evidence for the potential of both rigorous host defence and parasite infectivity.

Genetic adaptation to a local environment can lead to the production of a unique genetic basis for host-parasite interactions (Sorci et al., 1997). Some parasites evolve the ability to maintain their reproduction via the production of new generations of life stages that can cope with new host adaptations (Minchella, 1985). The authors described genetic host-parasite interaction via two models: gene-for-gene (GFG) and matching-allele (MA). In the GFG genetic model of infection, it is assumed that for each host resistance allele there is a matching parasite allele (Frank, 1997). In other words, each parasite having a virulent allele that can equally infect resistant
and susceptible host genotypes (Flor, 1971, Lambrechts et al., 2006). By contrast, in the MA genetic model, infection occurs only when the virulence alleles of the parasite match the corresponding alleles of the host (Agrawal and Lively, 2002, Summers et al., 2003). As a result, in both genetic models there are specific genotype-genotype host-parasite responses as represented by host susceptibility/resistance cost variations (Schmid-Hempel and Ebert, 2003).

In natural populations, host fitness might be driven by two important factors, namely their genetic background and environmental conditions, so alternative host genotypes must perform better in distinct environmental states (Lazzaro and Little, 2009b). Parasites have provided quite specific evidence to measure genetic diversity among plant, animal and human hosts. Variation in MHC has also been noticed in Brandt's voles (Lasiopodomys brandtii), which show an association between the nematode burden and specific MHC alleles, this diversity also varying between geographical locations among wild populations (Zhang and He, 2013).

1.4 The model host: the three-spined stickleback, *Gasterosteus aculeatus*

The three-spined stickleback, *Gasterosteus aculeatus*, is a small fish belonging to the family Gasterosteidae, which is widely distributed across freshwater, marine and brackish aquatic environments throughout the Northern hemisphere (Wootton, 1976). The three-spined stickleback is ancestrally a marine species, and marine populations still exist in oceans of the Northern hemisphere. Freshwater populations of three-spined sticklebacks have evolved morphological, physiological and behavioural specialisations since colonising the newly-formed lakes and streams that around 8.000-10.000 years ago when ice sheets retreated at the end of the last period of glaciation (Bell and Foster, 1994, Peichel and Boughman, 2003). Since then, freshwater populations have adapted rapidly to their new habitat and have evolved a remarkable level of diversity across geographical and ecological scales. Similar phenotypic traits have been recorded among *G. aculeatus* freshwater populations that are geographically isolated, suggesting that repeated convergent evolution has occurred as a consequence of adaptation to alternative environments.
(Rundle et al., 2000). Three-spined sticklebacks are native to a range of different aquatic environments in Europe, Asia and North America, where they are typically abundant and readily collected (Katsiadaki et al., 2007, Barber and Nettleship, 2010).

As a result of high levels of morphological diversity between three-spined sticklebacks, they are ideal for use in evolutionary ecology studies (Barber and Nettleship, 2010). The species has also been used widely to investigate a wide range of questions in ethology, behavioural ecology and more recently in the study of animal personalities (FitzGerald and Wootton, 1986, Von Hippel, 2010, Dingemanse et al., 2012). They have been used to address evolutionary questions in a wide range of habitats (Leinonen et al., 2006, Kitano et al., 2008, Chan et al., 2010). More recently, with the rich background publication on the stickleback genome, offering a powerful system for studying the molecular basis of adaptive evolution in vertebrates (Jones et al., 2012), they have also been widely used in molecular genetic of morphological divergence (Peichel et al., 2001) parallel evolution of dramatic phenotypic change by repeated fixation of Ectodysplasin alleles (EDA) (Colosimo et al., 2005) and fish genome evolution (Roesti et al., 2013).

Since sticklebacks have been reported to be an intermediate and definitive host to various parasitic taxa, they have received considerable attention in host-parasite interaction studies (Barber, 2013). Sticklebacks are popular experimental models because they exhibit a number of advantageous features that makes them well suited for research. They are relatively easy to breed in the laboratory using in vitro fertilisation (IVF) techniques (Barber and Arnott, 2000), they can be readily housed in small laboratory aquaria and cope well under laboratory conditions.

1.4.1 Stickleback biology

Here I briefly outline the basic biology of the three-spined stickleback that is relevant to the research in this thesis. Marine sticklebacks must visit freshwater at least once in their life for breeding purposes (Wootton, 1976). Body size varies between populations, but generally they are small fish averaging approximately 4-8 cm in length, and a maximum 11 cm. The body is characterised by three strong spines in front of the dorsal fin (Maitland and Linsell, 2006).
Eggs laid by females are guarded by males in a nest, with embryo development rate depending on water temperature (Wootton, 1976). After hatching, the fry initially depend on their egg yolk for feeding, and subsequently infusoria; subsequent growth rates depend on variation in food availability, changes in the length of the day and water temperature (Wootton, 1976). In natural populations, genetic differences in growth rate and body size between three-spined stickleback populations has been observed. These variations are consistent with the hypothesis that adaptation to different migratory lifestyles has occurred (Snyder, 1991). However, under laboratory conditions of constant temperature and high food availability, wild fish can grow rapidly to maturity four months more quickly than in the wild; even in cases of forced starvation, stickleback can show low compensatory growth responses (Wootton, 1976, Zhu et al., 2001).

The life span of *G. aculeatus* varies considerably between populations, ranging between one to four years (Jones and Hynes, 1950, Mann, 1971, Pennycuick, 1971c), though with most UK freshwater populations living for just one year. During the breeding season, sticklebacks are highly sexually dimorphic, with males developing conspicuous red coloration on the throat and belly while females remain cryptically coloured (Wedekind et al., 1998). However, out of the breeding season, skin colouration is variable between populations as these fish have evolved major genetic changes in pigmentation as a result of their migration from ancestral environments into new ones (Miller et al., 2007). The stickleback skin does not have scales, unlike many teleost fish, and instead has a skin covered by a thin cuticle layer (Wootton, 1976).

1.4.2 Reproductive biology and sexual behaviour

In the breeding season, both males and females move to shallow, slow water for mating. Female fish have paired ovaries with different developed stages of oocytes. In the summer, the ovaries’ structure become clearer and forms a high proportion (between 8 to 30%) of the female body weight (Wootton, 1976). The number of eggs produced, and number spawned in the same breeding season, depend on female size and food abundance (Wootton, 1973). Males have paired testes that form about 1% of body weight; they consist of germ cells (spermatogonia) which develop to
spermatids then mature spermatozoa (Wootton, 1976). Males are usually responsible for finding and defending a territory in which to build a nest. Males typically show distinctive secondary sexual traits by developing nuptial colouration along the ventral surface of the head and trunk with bright blue eyes. To build a nest, males use nesting glue, which is called “spiggin” and is a multimeric glycoprotein and is synthesized in the male’s kidney and stored in the urinary bladder prior to secretion (Jakobsson et al., 1999). Males usually collect plant debris or algae filaments that is then joined with the aid of the spiggin as an adhesive agent (Wootton, 1976). How much an individual male fish invests in glue during nest building might provide clear signals of male quality, nest structure may also act as a quality-revealing ornament (Barber et al., 2001). For example, female fifteen-spined sticklebacks, Spinachia spinachia, have shown significant mate preference for males that had invested highly in nesting glue (“tångspiggin”) in their nests (Östlund-Nilsson, 2001).

The courtship and reproductive behaviour of male sticklebacks is complex, and males exhibit a series of ritualised behaviours. The courtship begins when females respond to male stimulation, including zig-zag dancing behaviour and biting the female’s abdomen to encourage her to lay eggs in his nest. After that, the male will enter the nest and fertilise the eggs, subsequently entering the paternal responsibility stage (Östlund-Nilsson, 2007). Paternal duties include providing eggs with water by the male fanning the nest with his pectoral fins, cleaning and oxygenating the eggs, and otherwise providing full protection to his nest against predators (Östlund-Nilsson, 2007).

### 1.4.3 Three-spined stickleback morphology

There is a remarkable variation in morphological characters in both freshwater and marine three-spined sticklebacks. These variations include the number of armoured plates and body shape (Bell and Foster, 1994). The evolutionary history of three-spine sticklebacks provides a fascinating subject for polymorphism studies. Complex polymorphism and speciation are obviously noticed within three-spined stickleback species (McKinnon and Rundle, 2002). Polymorphism in this species commonly occurs after geographical isolation, where freshwater populations have undergone
rapid adaptive radiation as they adapt to their new habitat (Bell and Foster, 1994). Six such pairs of three-spined sticklebacks have been discovered to have adapted morphologically to their particular habitats in six small lakes in British Columbia, (McPhail 1994). In each lake, there is one species with a strong body form that has shown a benthos living style and have a distinct trophic adaptation to forage on large invertebrates. However, other species have adapted to a limnetic habitat with a small slender body shape and are zooplankton consumers (McPhail 1994).

Despite this variation, three-spined stickleback typically have three dorsal spines and one pelvic spine on each side, and have lateral plates. The pelvic spine can be locked erect (Hoogland, 1951), where the pelvic girdle combines with other bones to offer a defence mechanism that increases the opportunity of escaping from predators (Reimchen, 1983, Reimchen, 1994). The number of spines and morphology are significantly affected by fish habitat, where these changes often lead to a reduced or even absent first dorsal spine in benthic fish as a result of a lower predation regime (Hagen and Gilbertson, 1972, Reimchen, 1980). Nevertheless, limnetic fish have shown the longer dorsal and pelvic spines that offer greater protection against predators. Interestingly, MacColl and Aucott (2014) found that spineless and/or plateless sticklebacks (fish are less armoured) are commonly available where the predator brown trout (Salmo trutta) are common too in the North Uist populations.

The number of skeletal armour plates is one of the traits that shows the most morphological variability between different stickleback populations (Wootton, 1976). In many populations, juvenile fish develop lateral plates which become fully developed when the fish reach 30 mm in length (Wootton, 1976). The typically observed number of plates found in marine populations is characterised by a complete row of 30-35 lateral plates (Mattern, 2007). Starting from the pectoral girdle and running continuously to the tail, these plates interact with a well-developed pelvic girdle. As a result of the high variation in number of plates between three-spined stickleback populations, recent research has investigated the gene responsible for the divergence in the number of plates.
The *Ectodysplasin* (*Eda*) gene appears to be play a fundamental role in the three-spined stickleback’s adaptation to a new environment by controlling polymorphism in their lateral ectodermal bony armours (Colosimo et al., 2005, Barrett et al., 2009b). Several studies have focused on the *Eda* gene as being associated with *G. aculeatus*’ behaviour (Barrett et al., 2009b), the pattern of expression of key immune system genes (Robertson et al., 2017), fish evolution (Rennison et al., 2014) and environmental factors (Barrett et al., 2009a). Recently, there have been a few reports in the literature investigating the association between parasitic infections and plate number. For example, the relationship between plates number and the cestode *Schistocephalus solidus* in wild fish from Gdynia Marina on the Baltic coast has been investigated by Morozińska-Gogol (2011). In their experimental study, Robertson et al. (2017) found that the *Eda* haplotypes in three-spined stickleback that infected with ectoparasites are associated with variation in immune gene expression.

### 1.5 Sticklebacks as hosts for parasites

The three-spined stickleback typically serves as host to a large number of parasitic taxa Barber (2007). Some of these parasites use sticklebacks as an intermediate host, while for others it represents the definitive host. Host specificity in parasites-sticklebacks is rare. However, due to the widespread distribution of this fish and its adaptation to a wide range of environments leading them to encounter parasites easily. Furthermore, an omnivorous diet exposes sticklebacks to a wide range of trophically transmitted parasites (Barber, 2007).

As sticklebacks often play a fundamental role in aquatic food webs, they often serve as an intermediate host to wide range of trophically transmitted parasites. For instance, Andersen and Valtonen (1992) found that *G. aculeatus* was the most heavily infected fish species, both in terms of parasite intensity and diversity, of the 13 species examined in the Baltic Sea. In this section, I will concentrate on some of the parasitic species that were most frequently recorded in this thesis.
1.5.1 Ectoparasites

1.5.1.1 Gyrodactylus sp.

*Gyrodactylus* is a genus of ectoparasitic monogenean flatworms, the number of described species of which numbers at over 400 (Bakke et al., 2007). These worms attach themselves to the scales on the bodies of marine and freshwater fish. They have direct life cycles; adult worms do not lay eggs, but rather give birth viviparously to live offspring (Turnbull, 1956). In heavily infected individuals, this parasite can cause high fish mortality, as recorded for Norwegian *G. salaris* during the 1970s (Bakke et al., 2007). A number of *Gyrodactylus* spp. are found on the skin, gills and fins of stickleback hosts (Raeymaekers et al., 2011). A free-living larval stage is absent in this parasite’s life cycle. However, its widespread appearance in most years and its short lifespan have been noted (Appleby, 1996). Furthermore, host behaviour might play the principal role of *Gyrodactylus* transmission (Cable et al., 2002). These parasites might cause severe tissue damage because of their hooks, and some parasitic species can cause skin discoloration, increase skin mucus secretion and reduce mucous cell density (Cone and Odense, 1984, Wells and Cone, 1990).

1.5.1.2 Argulus sp.

*Argulus* is a genus of ectoparasitic branchiuran crustaceans, commonly known as fish lice, and is one of the most common and widespread parasites found in a wide variety of fish worldwide (Walker et al., 2004). Infection with *Argulus* spp. can be lethal to the host, as it can lead to skin lesions as a result of skin damage tissue (Taulescu et al., 2010). Furthermore *Argulus* spp. lice are haematophagic, and cannot survive for any great length of time without their host (Mikheev et al., 1998).

*Argulus* spp. lice can be found on all external surfaces of the host stickleback, including the outside of the gills behind the pectoral fins and pelvic spines (Eaves et al., 2014). Three-spined stickleback acquire *Argulus* infections when they come into direct contact with the parasite, where the parasites can launch an attack particularly when water levels are low (Poulin and FitzGerald, 1988). After it has attached to the host, male and female *Argulus* can mate upon the host body, with
the females leaving the host body to lay eggs depending on water temperature and light levels (Hakalahti et al., 2006, Harrison et al., 2007).

1.5.2 Endoparasites

1.5.2.1 Acanthocephalus lucii and A. anguillae

Acanthocephalans, also known as thorny-headed or spiny-headed worms, are obligatory endoparasites found in the alimentary tract of vertebrates (Haustein et al., 2010). Teleost fish are usually well known as adult acanthocephalan hosts. _Acanthocephalus lucii_ and _A. anguillae_ are a common parasite of the digestive tract of many freshwater fish (Kennedy, 1974). Their life cycle is complex, starting with eggs and then moving to the acanthor, which leaves egg membranes in the gut of the intermediate host (usually crustaceans) to develop into the second larval stage (acanthella) that later encysts to become a cystacanth. As with many trophically transmitted parasites, infection with these two parasites reflects the feeding regime of the three-spined sticklebacks, which become infected when they consume infected isopods (Dezfuli et al., 1994).

1.5.2.2 Triaenophorus nodulosus

_Triaenophorus nodulosus_ is a parasitic cestode that infects predatory European pike _Esox lucius_ as its most common definitive host, although there are other fish species that have been reported to act as definitive hosts of this parasite in the United States and Canada (Chubb, 1963). As with other cestodes, its life cycle starts with eggs, then free-swimming ciliated coracidia that are eaten by the first intermediate host (crustacean) to develop into a procercoid. Fish that serve as a second intermediate host can develop infection after consuming infected copepods (Chubb, 1963). The plerocercoids of _T. nodulosus_ are often found in the livers and body cavities of three-spined sticklebacks (Poulin and Valtonen, 2001) and transmission to definitive hosts is via predation. _T. nodulosus_ infections in intermediate fish hosts can cause growth rate reduction and, significantly, serious pathological liver alterations (Brinker and Hamers, 2007).

1.5.2.3 Proteocephalus filicollis

This is a common parasite of three-spined sticklebacks, and is found as an adult worm in the intestine of infected fish. Infection with this parasite is a function of the
copepods intermediate host’s abundance and is also water temperature related. Hopkins (1959) reported that water temperature is the main factor in preventing fish from contracting *P. filicollis* infection by inhibiting eggs from hatching and due to copepods mortality during winter. However, Chappell (1969) found that growth and maturation of *P. filicollis* possibly occur during winter, leading to reduced appearance of the parasite in that season.

### 1.5.2.4 *Phyllodistomum folium*

Three-spined sticklebacks represent the main host for *P. folium*, although it has been reported in other species of fish (Kennedy, 1974, Stunžėnas et al., 2017). The metacercariae of *P. folium* are thought to be encysted within the sporocyst, and they are infective for the fish after 24 h when mussel or insects larvae (the intermediate hosts) are ingested by the fish to finally establish as a helminths in the urinary bladder (Goodchild, 1943). There may be two possible routes of three-spined sticklebacks infection with *P. folium* which is diet type dependent. In winter the fish diet consisted primarily of the mussel *Sphaerium* sp. while in the spring and summer fish are tend to consume variety of larval insects to develop *P. folium* infection (Chappell, 1969).

### 1.6 Parasite study species

#### 1.6.1 *Diplostomum* sp.

Parasites of the genus *Diplostomum* are common in European and North American freshwater fish. Kennedy (1974) recorded six species belonging to the genus *Diplostomum* in fish. These parasites harbour gulls as adult worms and the eyes or brain of freshwater fish (Paperna and Dzikowski, 2006). This parasite require to infect three host species in order for the life cycle to be completed, usually snails, fish and birds.

In host fish, infection due to many species of *Diplostomum* are often found in the eye, and cause partial or total blindness, leading to the common name ‘eye fluke’. However, *D. phoxini* metacercariae settle in the brain of the European minnow, *Phoxinus phoxinus*, which has significant effects on behaviour. Infected fish show higher levels of boldness and repetitive behaviour, and infection may influence the
sensory physiology or result in behavioural change in the personalities of their hosts (Kekäläinen et al., 2014). Infection with Diplostomum spp. parasites leads to considerable economic loss in fish farms because of their ocular pathology, which leads to increased mortality in fish. This disease is usually entitled diplostomiasis or diplostomaosis, parasitic cataract or eye fluke disease, is caused by the larval stages (metacercariae) settling and developing in the retina and humour of infected fish (Chappell et al., 1994). In three-spined sticklebacks, Diplostomum gasterosteii is one of the most common species found in the retina; similarly, D. spathaceum infects the lens of the eye (Paperna and Dzikowski, 2006).

1.6.1.1 *Diplostomum spathaceum* life cycle

*Diplostomum spathaceum* is one of the most common parasites of freshwater fish in the UK. Karvonen (2011b) recorded over 100 different species of fish serving as intermediate hosts for this parasite. There are six morphologically distinct stages in the life cycle (Figure 1.1), which include:

1. **Eggs to sporocyst**

Adult worms start to producing eggs which should contact water to start embryonic development with the aid of light, temperature and salinity; miracidia are then released into water to find a suitable host within 24-48 hours (Karvonen et al., 2006). Snails normally of the family Lymnaeidae, principally *Lymnaea pregera* and *L. stagnalis*, act as a suitable first intermediate host (Williams, 1966, Chappell, 1995). By using cilia and penetrating enzymes within a temperature range of 6-20°C, infection can be achieved eight weeks post-exposure. Within the snail’s body, asexual development takes place via a single mother and multiple daughter sporocysts, ending with the production of cecariae (Waadu and Chappell, 1991).

2. **Free-swimming cercariae and metacercariae**

Successfully infected snails can produce hundreds or thousands of active swimming cercariae which enter the water column using their tails (Figure 1.1). Cercariae swimming is affected by different factors such as temperature, direction and day light intensity, cercariae age, and dark and light stimulation (Haas, 1992). The best infective age of cercariae is between 0-5 hours after shedding (Whyte et al., 1991).
The mechanism of skin penetration employed by cercariae has been explained by (Höglund, 1991, Whyte et al., 1991) by supporting an incidental contact hypothesis when metacercariae settle in the lens of *Oncorhynchus mykiss* within 5-24 h of entry through the gills via fish ventilation hydrodynamics. Host specificity in *D. spathaceum* and the penetration process are stimulated by specific environmental conditions and host chemical signals (Haas, 1992). Establishment in the lens is completed at 12°C within 28 days in *P. phoxinus* and *Gobio gobio*; nevertheless, it takes 35-40 days in *Rutilus rutilus* and 120 days in *Perca fluviatilis* (Sweeting, 1974). The development of metacercariae is therefore dependent on both temperature and host species.

![Diagram of the life cycle of Diplostomum spathaceum](image)

**Figure 1.** The life cycle of *Diplostomum spathaceum*, after Dogiel (1961).

### 1.6.1.2 Diplostomum spathaceum pathology

The clearest sign of *D. spathaceum* infection is when the lens becomes grey and cloudy. Cataract severity depends on fish age, and the number and distribution of metacercariae inside the lens (Karvonen, 2011). Cercarial invasion and their
migration through the fish’s skin to the eye’s lens can result in acute effects to the epidermis, especially in small fish when they are exposed to 300-600 cercariae, as well penetrating blood vessels which results in internal haemorrhaging (Larsen et al., 2005, Karvonen, 2011). In addition, diplostomiasis basically increase fish stress, predation risk and heavily infection induced host mortality; in contrast, body condition and growth rate are decreased noticeably in heavy infected fish because of the consequent reduction in feeding (Karvonen, 2011).

1.6.2 *Schistocephalus solidus*

1.6.2.1 Life cycle and biology

*Schistocephalus solidus* (Platyhelminthes, Cestoda, Pseudophyllidea) is a common parasite of the three-spined stickleback in freshwater populations and is widely spread through the range of distribution of the host fish. The cestode *Schistocephalus solidus* has three hosts in its life cycle: cyclopoid copepods, stickleback fish and fish-eating birds (Wootton, 1976, Barber and Scharsack, 2010). About 40 different species of piscivorous birds have been recorded as suitable definitive hosts for this parasite, which is harboured in their intestines (Nishimura et al., 2011). Fertilized eggs are passed with the faeces of the definitive hosts to the water, where they develop and hatch, releasing ciliated, free-swimming first stage coracidia (Smyth and Wakelin, 1994). Copepods (the first intermediate hosts) develop infections after feeding on these larvae, which develop in their haemocoel into procercoids (Smyth and Wakelin, 1994). When the procercoid develops a cercomer, it becomes infective, and when three-spined sticklebacks ingest copepods harbouring infective procercoids the larvae shed the outer cercomer inside the stomach of the fish (Smyth, 1969). Within 12-24 hours, the parasite larvae then penetrate the intestines of the fish and reach the body cavity (Figure 1.2), by which time the parasite’s growth is often noticeable by abdominal distension (Aeschlimann et al., 2000).

Here, the plerocercoid grows until it attains an infective size to birds (> 50 mg) (Tierney and Crompton, 1992). Hence, the stickleback is the only specific host in the *S. solidus* life cycle (Hammerschmidt and Kurtz, 2007, Barber and Scharsack, 2010). This parasite has become a very useful model for studying ecological and
evolutionary aspects of host-parasite interactions (Barber, 2013). Serval wild studies have reported temporal variation in three-spined sticklebacks' exposure to waves of *S. solidus*. For example, Pennycuick (1971c) found a high parasitic prevalence in spring, while Tierney et al. (1996) found a major infective wave in autumn.

Figure 1.2 Lifecycle of *Schistocephalus solidus*.

1.6.2.2 The effect of *S. solidus* infection on three-spined sticklebacks

Three-spined sticklebacks infected with *S. solidus* that have grown to become infective to piscivorous definitive hosts are detectable by abdominal distension (Barber and Scharsack, 2010). As the *S. solidus* plerocercoid is large in size, this results in the expansion of the infected fish’s stomach, leading to reduced food consumption and longer prey handling times (Cunningham et al., 1994). Therefore, in some stickleback populations, heavily infected fish showed a loss in weight and slow growth rates (Pennycuick, 1971a).
In addition to the foraging mechanism and behaviour manipulation, infected fish exhibit delayed sexual maturation and reproductive disruption (Schultz et al., 2006), although in one Alaskan population female fish showed an ability to mature and develop egg clutches (Heins et al., 2000, Heins and Baker, 2008). *Schistocephalus solidus* infected fish show reduced reproductive capacity as a result of this energetic drain and nutrient theft (Heins and Baker, 2003). Several influential studies have found that infected fish were unable to engage in reproductive activities (McPhail and Peacock, 1983, Tierney et al., 1996). The effects of *S. solidus* infection on males have been investigated since reproduction in males does not rely on gonad development alone, but also on other behavioural traits such as the glue spiggin production for nest building and the courtship ritual (Wootton, 1976, Rushbrook et al., 2007, Macnab et al., 2009). Infected males have been shown rarely engaged in reproductive behaviours and reduce levels of spiggin in the kidney (Rushbrook et al., 2007, Macnab et al., 2009).

A larger parasitic mass might be a crucial factor for successful transmission to the definitive host by direct host behaviour manipulation (Barber et al., 2004). Sticklebacks infected with an *S. solidus* burden present different anti-predation behaviour such as spending more time at the water surface (Giles, 1987). It is thought that infection can alter the decision to join a shoal by swimming at a distance from it (Barber and Huntingford, 1995), and further by reducing escape responses from avian attack (Barber et al., 2004).
1.7 Structure of the thesis and main aims

*First I looked at parasite diversity and seasonality in a natural population.

Chapter 2: Parasite diversity of *Gasterosteus aculeatus* from River Soar and seasonal variations in two common parasites infection

I conducted a survey of macroparasite communities in River Soar, Leicestershire, population in one year to examine whether: (1) There is variation in parasite communities among population, (2) if this variation is consistent across year and (3) To study the effect of *S. solidus* and *D. spathaceum* infection on the body condition of infected fish in comparison to non-infected fish.

*Second I used experimental studies to investigate how a range of host factors affect host-parasite interactions.

Chapter 3: How do host sex and body size affect infection susceptibility and parasite growth?

I used lab-bred fish, varying in size and sex, were experimentally exposed to infective stages of *S. solidus* and worms to investigate:

(1) How do differences in host body size at the time of parasite exposure affect susceptibility to infection and fish health? (2) How do differences in host sex and body size affect subsequent plerocercoid growth and the development of heavier infection? (3) Does stickleback host sex affect the fecundity of adult parasites after transmission to the definitive host?

Chapter 4: Consequences of stickleback provenance, morphology and *Eda* genotype for *Diplostomum spathaceum* infection

(1) First experiment: I carried out an infection experiments with *Diplostomum spathaceum* in two genetically separated freshwater populations from two distinct habitats lab-reared stickleback to investigate: The relative importance of sex and population in determining susceptibility to *Diplostomum* infection. (2) The second experiment focussed on: The three-spined stickleback has repeatedly invaded freshwater habitats from marine ancestry; therefore, new adaptive changes,
including morphological and genetic traits, have evolved repeatedly in response to the new environment. Therefore, fish from the Carsington Reservoir population, which shows large variation in their plate number provide an ideal opportunity to examine experimentally whether variation in plate phenotype and Eda genotype affects parasitic load, and how this may be related to the effects of sex and age.

Chapter 5: Effect of paternal infection status on offspring susceptibility to *Schistocephalus solidus* infection

Here, three-spined stickleback and *S. solidus* were used as a model to examine: (1) Whether paternal infection status affects offspring susceptibility to *S. solidus* infection, and therefore how this may increase or decrease the resistance of new generations to infection. (2) By linking fish reproductive biology in this study, spiggin genes expression was used as a proxy for male reproductive development and whether sire infection status affected male offspring infection phenotype.

1.8 Aims of the thesis

Parasites are often small-sized organisms that exploit the food resources of hosts and influence their fitness. However, the severity of parasitic effects on host biology might be varied when individuals within host populations often show their own strong variation in genetic and phenotypic traits. By using the cestode parasite *S. solidus* and the trematode *D. spathaceum* as parasite models in this study, the overall aim of this research was to investigate the effect of both phenotypic and genetic biologically relevant factors on the interactions between a naturally prevalent parasite and its host.

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Chapter 2

Parasite diversity of three-spined sticklebacks from River Soar and seasonal variations in two common parasites infection
2.1 Abstract

In the River Soar, Leicestershire, the parasite community of three-spined sticklebacks (Gasterosteus aculeatus) has been investigated. From November 2014 to June 2015, a survey of 271 fish revealed 12 different parasite species belonging to varied taxa. Three species were present throughout the months: Argulus sp., Schistocephalus solidus and Diplostomum spathaceum. Only the prevalence and intensity of infection of D. spathaceum was high throughout the months. Fish standard length varied over the months and with S. solidus infection status. Plerocercoid masses varied between both sexes, and infected fish had a poorer body condition than non-infected fish. The number of D. spathaceum metacercariae was higher in June than in other months. The ecological consequences of these results are discussed.
2.2 Introduction

2.2.1 Parasite life stages and seasonal changes

Parasitism is the ecological interaction between host and parasite populations. The prevalence and intensity of infection varies depending on the frequency and distribution of the host (Crofton, 1971, Bush et al., 1997). Seasonal variations exerts a strong effect on parasite-host dynamics and plays a significant role in shaping host population structures (Altizer et al., 2006). Parasitic transmission is directly affected by seasonal changes in the host environment, therefore, directly transmitted parasites have to develop successful transmission mechanisms that allow them to cope with seasonal changes in the host environment (Feist and Longshaw, 2008). Other, complex life-cycle parasites that have free-living stages are additionally under directly environmental pressures such as temperature, pollution, intermediate host availability, etc.

A reduction/increase in the parasites prevalence of infection is driven by how the parasite responds to the conditions of their host habitat via an increased/decreased resistance to environmental change which consequently affect parasite abundance in host population (Mackenzie, 1999). Some parasite species have evolved effective strategies by going through a sequence of different host species to complete their life cycles (Lefebvre and Poulin, 2005). However, other parasites have developed a specific adaptation to one particular host. It has been shown that hosts exposure to very different environments is reflected in considerable variation in parasitic diversity (Overstreet, 1997). It has been suggested that parasitic diversity and successful transmission are often crucially affected by seasonal changes in intermediate and definitive host habitat conditions (Marcogliese, 2004).

Parasite community structures may depend on a number of abiotic factors, particularly for ectoparasites which are in constant direct contact with the external environment and they directly affected by the host environment conditions such as temperature, salinity, pH, etc. (Poulin et al., 2011). Consequently, many ectoparasites have shown that they are more tolerant than their hosts in varied types of environmental change (Mackenzie, 1999). However, endoparasites with indirect life cycles can be affected by seasonal changes by directly effect on free-living
transmission stages or adult forms when they indirectly affected through environmental effect on their host (Mackenzie, 1999).

In trophically transmitted parasites, host foraging behaviour is likely to be associated with an increased probability of infection. In addition, intermediate host abundance and its population density could be the main factors in determine parasite species richness and possibly introduction of another parasitic species (Takemoto et al., 2005, Locke et al., 2014). Furthermore, Thieltges et al. (2008) summarized six biotic factors that may interact with abiotic factors to achieve high rates of transmission by the free-living larval stages of endohelminths. These factors are: hyperparasites; predation risk; alternative hosts; decoy organisms; physical disturbance by organisms and toxic exudates from organisms. These factors are likely to interact with natural abiotic factors and anthropogenic pollutant which might help to anticipate the effect of climate change on helminth parasites and their host communities (Thieltges et al., 2008).

Seasonal changes are periodic, largely predictable, and exert strong pressures on human and other organisms, as seasonal variations can cause noticeable fluctuations in parasite abundance and host population structure (Altizer et al., 2006). The consequences of infectious diseases and host population dynamics under seasonal variation pressures present a considerable challenge to ecologists and parasitologists.

Parasites are often vulnerable to the seasonal and climate changes in the wild resulting in experiencing prominent variations in terms of the prevalence and intensity of infection. Moreover, seasonal changes in infectious diseases have been attributed to changes in the behaviour of the host. For instance, the influenza virus was recorded at high levels of infection in America during winter. Human susceptibility to this virus has increased perhaps due to the annual light/dark cycle; other human body physiological changes and human behaviour by spreading the virus from infected individuals to non-infected (Dowell, 2001). There is considerable evidence to suggest that parasite richness and their disease transmission in aquatic ecosystems will increase with global warming (Marcogliese, 2008). As suggested by Mackenzie (1999) that parasites have developed a flexibility and resistance to host
environmental changes through their course of evolution. For example, the distribution of parasites in aquatic hosts was directly affected by environmental changes, and indirectly through hosts’ ability to survive during winter when food is scarce and temperatures are low (Marcogliese, 2008).

Numerous studies have shown that parasites showing seasonal changes in appearance and distribution in freshwater and marine ecosystems fish host, particularly with the increased of global warming effect. To name just a few examples, in Finland two harmful parasitic species have found in fish culture, the directly transmitted ectoparasite, Argulus coregoni, and a complex life cycle endoparasite, Diplostomum spathaceum. The life histories of these two parasites have been dramatically affected by the increasing temperatures in Finnish waters, leading to a high abundance of these two parasites (Hakalahti et al., 2006). The crustacean A. coregoni typically appears to infect fish in one generation per year in Finland; however, increasing temperatures have allowed a potential shift to a two-generation cycle. Increased water temperatures possibly affect D. spathaceum life history dynamics via higher reproduction rates of the (intermediate host) snails which has subsequently enhanced parasite production. It seems therefore that the interaction between biotic and abiotic factors on parasites prevalence is complex and it need to be better understand; predict and more attention needs to be directed towards the effect of seasonal changes on parasite-disease systems.

2.2.2 Seasonal variations in parasite community of Gasterosteus aculeatus

Gasterosteus aculeatus are a common fish as they often present with high population densities with a global distribution, and are easy to catch (Wootton, 1976). Furthermore, they have a vital role in the ecosystem as a prey for many other fish, birds and otters, as well as acting as hosts for variety of parasites.

Many ecological studies have investigated parasite diversity in three-spined sticklebacks in both the UK and Europe. Eight species of parasite were recorded by Chappell (1969) in a Yorkshire pond, with only the ciliate Trichodina sp. being unique in its prevalence in infecting all 601 fish examined in this study. However, the author attributed the fluctuations in other parasite species’ abundance to the noticeable seasonal variations in the number of fish within a population and to the differences
in the stickleback's age, rather than any physical environmental parameters. From a pond in Somerset, Pennycuick (1971c) demonstrated that the infections of *Schistocephalus solidus*, *Diplostomum gasterostei* and *Echinorhynchus clavula* were changing between seasons as a result of seasonal changes in the feeding activities of the sticklebacks and in the abundance of the first intermediate host.

Sticklebacks are distributed across the entirety of the Northern hemisphere. In Europe, they inhabit freshwater, brackish and marine waters, and are therefore parasitized by a wide range of different parasite species. Many studies have investigated parasite diversity within three-spined stickleback populations in freshwater and marine ecosystem. In Norway, 19 parasite species have been recorded in freshwater stickleback populations (Levsen, 1992). A high species richness was recorded by Zander (2007), attaining 15 species of parasitic infection in four localities in Germany. In a review, Barber (2007) listed 122 parasitic species from worldwide three-spined sticklebacks in a variety of freshwater habitats. Three morphotypes of *G. aculeatus* in Gdynia Marina on the Baltic coast have been studied by Morozińska-Gogol (2011), showing a high prevalence of infection with the larval stage of the cestode *S. solidus*, at 94.4%. The parasites were more frequent among individuals with fewer lateral plates, with a maximum of six plerocercoids for each fish. A total of 12 taxa of parasites were recorded in two populations in Norway with quantitatively significant variations between parasite communities in the two populations, which might explained by differences in the density of sticklebacks and intermediate host’s abundance (Kuhn et al., 2015). Recently, Young and Maccoll (2017) used a large dataset of 12 species of macroparasites that infect three-spined stickleback from 14 locations on North Uist, Scotland. The authors found that some investigated parasites species have shown differences in relative abundance and prevalence across populations and the time of the year at which samples were collected, however, in other parasites they were consistent throughout the study.

Parasites can be used as natural biological indicators to evaluate and efficiently manage a fish population in terms of growth, migrations, diet and immunity status, etc. (Tierney et al., 1996, MacKenzie and Abaunza, 1998). Therefore, more information is still required about aquatic parasites’ biology to help with the
interpretation of infection phenotype. For example, *Schistocephalus solidus* and *Diplostomum* sp., two common parasites in freshwater stickleback populations, were widely used to provide information on various aspects of host biology. These two parasites are used to measure habitat types in parasite susceptibility as well as in immunological and host body condition parameters (Kalbe and Kurtz, 2006).

2.2.3 *Schistocephalus solidus* development, life cycle and distribution

The plerocercoid of the tapeworm *Schistocephalus solidus* is specific to three-spined sticklebacks, *Gasterosteus aculeatus*. Stickleback populations infected with *S. solidus* have been generally reported in the literature, but most are lake population studies (Confer et al., 2012). For lake stickleback examples, one might consider LoBue and Bell (1993), Heins and Baker (2008), MacColl (2009) and Morozińska-Gogol (2011); for brackish water populations, and Confer et al. (2012) in anadromous sticklebacks of Mud Lake, Alaska. In the UK, *S. solidus* infections are common among river and pond stickleback populations, as reported in several studies; for example, see (Arme and Owen, 1967, Pennycuick, 1971a).

2.2.3.1 How does infection with *S. solidus* affect host biology?

Parasitic infections can be associated with morphological, physiological and behavioural effects to their hosts. Quantification of the host phenotypic and disease phenotype changes by using long-term study reviews might help to understand some of the evolutionary consequences of parasite-induced alterations in host phenotypes (Poulin and Thomas, 1999, Baker et al., 2008). Host age and parasite number may potentially create complex changes in host morphology (Goodman and Johnson, 2011). In some instances, host phenotypic changes possibly occur due to the same parasite in a way that offers an advantage to the parasite only by increasing successful parasitic phenotypic growth and transmission (Goodman and Johnson, 2011).

Infection with *S. solidus* might modify more than one host character and create clear morphological variations in fish body shape (Barber and Svensson, 2003). The weight-length relationship indicates impaired body condition in *S. solidus* infected fish (Tierney et al., 1996). In addition to morphological changes, physiological and endocrine disruption by infection have been shown to have a substantial impact on
fish reproductive ability (Heins and Baker, 2008, Macnab, 2012). Reproductive disruption in three-spined sticklebacks was attributed to nutrient theft by the S. solidus, rather than parasite-induced castration (Schultz et al., 2006). The other important effect of this macroparasite is a behavioural change by reducing escape responses to attacking predators (Barber et al., 2004).

2.2.4 Diplostomum spathaceum development, life cycle and distribution
Parasites belonging to the genus Diplostomum sp. are common in European and North American freshwater fish. Kennedy (1974) recorded six species of the genus Diplostomum in fish. These parasites infect gulls as adult worms and the eyes or brains of freshwater fish (Paperna and Dzikowski, 2006). This parasite can exploit three animals (snails, fish and birds) over six distinct morphologically stages of their life cycle. In fish, many species of Diplostomum can cause partial or total blindness, or otherwise effect host sensory physiology or behaviour. Diplostomum spathaceum is distributed worldwide and one of the most common parasites of freshwater fish, it has been recorded in more than 125 different species of freshwater fish (Höglund, 1991b). In the UK, Kennedy (1974) recorded 21 different species of fish as intermediate hosts for this parasite.

2.2.4.1 Diplostomum spathaceum pathology
Infection with D. spathaceum leads to considerable economic loss in fish farms because of the ocular pathology which leads to an increased death rate in fish. This disease, usually referred to as diplostomiasis or diplostomaosis, parasitic cataract or eye fluke disease, is caused by the larval stage (metacercariae) that settles and develops in the lens and humour of infected fish (Chappell et al., 1994).

The clearest sign of heavy D. spathaceum infection is when the fish’s lens become grey and cloudy. Diplostomiasis cataract severity depends on fish age, and the number and distribution of metacercariae inside the lens (Karvonen, 2011). Cercarial invasion and their migration through fish skin to the eye lens can result in acute effects on the epidermis, especially in small fish. Diplostomiasis cataract severity was considered to be related to the number of metacercariae established in the eyes, specifically when fish were exposed to 300-600 cercariae, as well as penetrating blood vessels and causing internal haemorrhages (Larsen et al., 2005). In addition,
diplostomiasis increases fish stress and vulnerability to predation, and noticeably decreases fish body condition and growth rate in heavily infected fish because of feeding reduction (Karvonen, 2012).

**2.2.4.2 Diplostomiasis epidemiology and infection seasonal changes**

Seasonal changes in the abundance of *D. spathaceum* metacercariae in fish have been considered in several studies. In the UK, a positive relationship between parasite appearance and temperature were reported when higher parasite burdens were recorded between summer and autumn (Chappell, 1969, Pennycuick, 1971a). Successful *Diplostomum* sp. transmission to the definitive host might be achieved when a large number of metacercariae presenting in the lenses of a fish hosts leading to increase fish susceptibility to predation and (Pennycuick, 1971 a).

Pennycuick (1971b) and Burrough (1978) demonstrated that *Diplostomum* sp. exhibited an over-dispersed distribution (limited number of hosts with a high percentage of parasitic infection). *D. spathaceum* metacercariae accumulation in the eye of eight fish species from Hanningfield Reservoir, Essex, was positively associated with fish age and length (Wootten, 1974). However, from a long-term dataset obtained from Slapton Ley, Devon, Kennedy (1984) found that *D. spathaceum* metacercariae accumulation was decreased in older roach and perch hosts. This decline in mean rate of infection in older fish has attributed to parasite-induced host mortality in heavily infected fish and possibly to the parasite life span which is significantly shorter than that of the host (Kennedy, 1984).
2.2.5 Aims

The work in this chapter investigates the distribution and abundance of the parasitic fauna of three-spined sticklebacks in River Soar in Leicestershire. As I am interested in studying two common parasites for my PhD project. Therefore, I decided to investigate the prevalence and intensity of infection of two common parasites, *Schistocephalus solidus* and *Diplostomum spathaceum*. Infection data were compared over a period of eight months in order to collect information on the distribution and abundance of these parasites as linked to time and the sex of the fish. The most vital aims of this study are:

- To describe the parasite communities of the three-spined stickleback population in the River Soar.
- To study the effect of *S. solidus* and *D. spathaceum* infection on the body condition of infected fish in comparison to non-infected fish.
- To study seasonal changes in immunity function and fish energy reserves as indicated by HSI in relation to their infection status.
- To present a dataset regarding the pattern of infection for two parasites to aid future studies of *G. aculeatus* in the River Soar.
2.3 Methods

2.3.1 Study Area
The River Soar is one of the main tributaries to the River Trent in the East Midlands, UK, and a main body of water in Leicestershire (Smith et al., 2005). It passes through Loughborough and Leicester. It provides excellent environmental conditions for wildlife including several species of birds, plants, algae, fish and a wide range of invertebrates, etc. (personal observations). Fish were collected from a location between Mill Lane bridge and Newarke bridge (N 52°37’42.7", W 1°08’33.0"), as shown in Figure (2.1).

![Map of study area in Leicestershire, England, showing the River Soar. The fish were collected in the region of the red arrow between Mill Lane and Newarke bridges. (Google Earth, 2018).](image-url)
2.3.2 Stickleback collection and husbandry

A total of 271 three-spined sticklebacks were collected from the River Soar, Leicestershire (N 52°37'42.7", W 1°08'33.0") the time during November 2014 to June 2015 using Gee’s minnow traps. The number of individual samples measured in this survey was 103 fish in November 2014, 54 in January 2015, 31 in February, 53 in April and 30 in June. Fish were taken immediately to the university aquarium and maintained in a glass tank under aquarium conditions in a filtered, recirculating freshwater system. It was quite rare to catch fish between August and September 2015. I left the trap for more than two days, but no fish were caught due to high fish mortality after the breeding season (Shalal, personal observation).

2.3.3 Dissection and parasite screening

Dissection was started by euthanizing fish with an overdose of benzocaine anaesthetic (stock solution: 10 mg L\(^{-1}\)) according to Schedule 1 methods under home office licence (Project licence: 80/2327, Personal Licence: IAD9DF470). During the time out of breeding season, it was difficult to identify fish sex. Therefore, fin clips were then taken by removing one of the pectoral fins and placing them into 100 µl absolute ethanol in an Eppendorf tube.

Fish were blotted dry on a piece of tissue, and length was measured using a dial calliper (standard length, \(SL\), to 0.1 mm) and weighed (mass, \(M\), to 0.001 g). First, eyeballs were removed directly and placed into a watch glass and covered in ddH\(_2\)O. To inspect any \(Diplostomum\) sp. infection, eyes were dissected separately and the number and location of parasites were determined. Then the skin surface was examined under dissecting microscope for ectoparasites such as \(Glugea\) sp. and \(Gyrodactylus\) sp., the number of each parasite being recorded if it was present. Then, an incision was made along the ventral surface of the fish to the operculum, and all visceral organs and body cavity were examined for any possible parasitic infection.

Plerocercoids recovered from infected fish were quantified to total mass (\(M_p\)) to (0.001g). Parasite index calculations were performed: \(IP = \frac{M_p}{(M-M_p)} \times 100\), where \((M-M_p)\) is the mass of the infected fish minus the parasite mass (Pennycuick, 1971a,b). Body condition factor (BCF) was calculated using the equation \(BCF= \frac{M}{M_p}\).
\( -M_p) \times 10^5 \) (Pennycuick, 1971 c). Hepatosomatic (HSI) and spleensomatic (SSI) indices were calculated using the formulae Hepatosomatic Index (HSI) = \([\text{Liver mass}/(M-M_p)] \times 100\), SSI = \([\text{Spleen mass}/(M-M_p)] \times 100\). Data for the prevalence and intensity of each parasite infection were defined after Bush et al. (1997), except for the monogenean *Gyrodactylus* sp.

### 2.3.4 Molecular techniques

#### 2.3.4.1 DNA extraction from fin clips

To extract DNA from fish fins, a homemade chemical were used, by standard isopropanol DNA extraction method without using a kit following Sambrook and Russell (2001) protocol was used. In brief, tissue samples were transferred from the 100% ethanol storage tubes to a fresh Eppendorf tube containing 1 ml of ddH2O and left for 30 min. Fin samples were left overnight at 55°C in 400 µl of DNA extraction buffer (200 mM TRIS pH 7.5, 25 mM ethylenediaminetetraacetic acid pH 8.0, 250 mM NaCl, and 0.5% w/v sodium dodecyl sulphate) with 7 µl of 20 mg/ml proteinase K to digest the tissue.

On the second day, samples were heated to 92°C for 10 min, then vortexed, and centrifuged at 16,000 RPM for 2 min. Fresh Eppendorf tubes were prepared to transfer 300 µl of supernatant, then 300 µl of Isopropanol was added to the solution. Solution tubes were shaken to mix the contents before being frozen at -80°C for 10 min. Samples were then defrosted and centrifuged at 16,000 RPM for 10 min. Solutions were removed by pipette leaving the pellet to dry before adding 190 µl of 70% Ethanol and centrifuging at 16,000 RPM for 2 min. The pellet was allowed to dry completely overnight before being rehydrated in 100 µl ultrapure H2O and stored at 4°C overnight. The DNA concentration of the samples was quantified using a Nanodrop™ 1000 (Thermo Fisher Scientific, UK).

#### 2.3.4.2 Polymerase chain reaction (PCR)

The PCR protocol was undertaken using the marker isocitrate dehydrogenase IDH Reverse R primers TTATCGTTAGCCAGGAGATGG, forward F GGGACGAGCAAGATTATTGG following (Peichel et al., 2004), which successfully produced the characteristic bands amplifying at 302 bp from the X chromosome,
whilst the band at 271 bp was from the Y chromosome. Single bands of approximately 300 bp were linked to females, while males produce two products at 270 bp and 300 bp, as described by Peichel et al. (2004).

PCR reactions were in a 10 μl volume (9 μl of master mix plus 1 μl DNA sample), containing of 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 samples were briefly spun down before loading them into the PCR machine. The PCR conditions were found to be optimum using 1 cycle at 94°C for 5 minutes, 40 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds and a final one-cycle extension at 72°C for 10 minutes.

A high percentage gel, at 4% (high resolution for DNA/RNA fragments 10-500 bp; Sigma UK), was used to differentiate the 68 bp difference between the bands. A volume of 50 ml 1XTAE (Tris base, acetic acid and Ethylenediaminetetraacetic acid) was used, with 2 g high definition agarose added followed by heating to cause dissolution, after which 0.5 μl ethidium bromide was added followed by the gel former. The gel was placed in the gel tank and covered in 1XTAE. The samples were removed from the PCR machine and spun down briefly before being loaded into the gel, and 2 μl of high resolution DNA ladder (Hyperladder V, Sigma, U.K.) was added. PCR products were run at 60 volts for 75 min (Figure, 2.2)

![Figure 2. 2](image) Molecular sex determination gel showing female sticklebacks producing a single band at approximately 300 bp and males producing two products at 270 bp and 300 bp.
2.3.5 Data analysis

All statistical analysis were carried out in Minitab 17 statistical software. To analyse the effects of fish sex, months of the study, and both *S. solidus* and *D. spathaceum* infection status on the change in host standard length, the multivariable linear models (LM) were fitted and the significance of the independent variables and their interactions were determined. To determine the effects of fish sex, months and infection status of both parasites on each host body factor (HSI, BCF, and SSI), ANOVA were used and Tukey test within ANOVA was used then to confirm where the differences occurred between groups. Chi-squared tests were used to compare *D. spathaceum* infection susceptibility between male and female hosts. The *t*-test was used to investigate if there were any differences in body condition factors between non-infected and mixed infected fish (fish that had both *S. solidus* and *D. spathaceum* infection). For all boxplots in this chapter the dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR) and the whiskers represent variability outside the upper and lower quartiles, outliers are shown as asterisks in figures.
2.4 Results

2.4.1 Parasites community of three-spined sticklebacks in River Soar
The analysis of 271 three-spined stickleback specimens from the River Soar revealed a wide parasitic diversity (Table 1). A total of 12 different common internal and external freshwater parasite species belonging to different taxa were identified: *Acanthocephalus lucii*; *A. anguillae*; *Schistocephalus solidus*; *Triaenophorus nodulosus*; *Proteocephalus filicollis*; *Diplostomum spathaceum*; *D. gasterostei*; *Phyllodistomum folium*; Encysted Nematodes larvae sp.; *Argulus* sp.; *Glugea* sp. and *Gyrodactylus* sp. A high prevalence was found for *Triaenophorus nodulosus* and *Proteocephalus filicollis*. Nematodes and Acanthocephalans were less prevalent. The prevalence and intensity of infection for each parasite is reported in Table 2.1 except for *Gyrodactylus* sp.

2.4.2 The occurrence of *S. solidus* and *D. spathaceum* infection
Differences in the prevalence and intensity of infection over the months of the study could be clearly observed for *S. solidus* plerocercoids. A higher prevalence of infection was found in January at 31.5%; however, infection occurred with only a 3.8% prevalence in April (Figure 2.3, A).

The trematodes *D. spathaceum* showed a high prevalence of infection over most months of the study with a 93% higher appearance during February and lowest of 72.2% in January. No distinct pattern in intensity of infection between the months could be observed except in June with a higher rate at 28.73, although prevalence data showed a lower value in that month of 76.7% (Figure 2.3, B). Among all 271 fish collected over six months, only 34 fish were found to harbour both parasitic infection. Therefore, I decided to analyse the effect of each parasite on the fish separately and later compare the mixed infected fish with non-infected fish.
Table 2. 1 Parasites species found in three-spined sticklebacks sampled from the River Soar, Leicestershire. (P %): Prevalence of infection; (IF): intensity of infection average across the months and (S.E): standard errors.

<table>
<thead>
<tr>
<th>Endoparasites</th>
<th>P (%)</th>
<th>IF±S.E</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthocephala:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acanthocephalus lucii</em></td>
<td>2</td>
<td>1±0</td>
<td>Intestine</td>
</tr>
<tr>
<td><em>A. anguillae</em></td>
<td>0.7</td>
<td>1±0</td>
<td>Intestine</td>
</tr>
<tr>
<td>Cestoda:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Triaenophorus nodulosus</em></td>
<td>7</td>
<td>2.7±1.72</td>
<td>Liver, Body cavity</td>
</tr>
<tr>
<td><em>Proteocephalus filicollis</em></td>
<td>6</td>
<td>1.1±0.09</td>
<td>Intestine</td>
</tr>
<tr>
<td>Trematoda:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Diplostomum gasterostei</em></td>
<td>3.7</td>
<td>1.9±0.5</td>
<td>Eye Retina</td>
</tr>
<tr>
<td><em>Phyllodistomum folium</em></td>
<td>4</td>
<td>27.1±8.5</td>
<td>Urinary Bladder</td>
</tr>
<tr>
<td>Nematoda:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Encysted larvae sp.</td>
<td>1.3</td>
<td>1.5±0.5</td>
<td>Body cavity, Liver</td>
</tr>
<tr>
<td>Ectoparasites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maxillopoda:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Argulus</em> sp.</td>
<td>33.6</td>
<td>2±0.44</td>
<td>Skin</td>
</tr>
<tr>
<td>Haplophasea:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Glugea</em> sp.</td>
<td>2.3</td>
<td>1±0</td>
<td>Skin</td>
</tr>
<tr>
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<td>Gills</td>
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Figure 2. Variation in prevalence (%) (∆) and intensity of infection (O) of A: *Schistocephalus solidus* and B: *Diplostomum spathaceum* of infected three-spined sticklebacks from the River Soar over the course of the study.
2.4.3 Fish body condition, sex and S. solidus infection status

2.4.3.1 Fish length

Three-spined stickleback lengths were compared during months of the study and between the sexes. Fish length did not differ between the sexes regardless of infection status. However, significant differences in fish size were seen over the course of the study ($F_{4,268} = 10.87, P < 0.0001$, Table 2.2). To investigate whether sticklebacks infected with S. solidus were comparable in size, for the given mass and sex to non-infected specimens during months of the study, the multivariable linear models (LM) was used (Table 2.2). The analysis revealed that the effect of infection status on fish length was not significantly different between the sexes (Infection Status*Fish sex: $F_{1,268} = 2.47, P = 0.117$, Figure 2.4).

Figure 2.4 The effect of host sex and S. solidus infection on three-spined stickleback length, collected from River Soar during the eight months of the study.
The relationship between standard length and body mass was positively linear over the size range of the fish samples ($F_{1,268} = 65.24$, $P < 0.0001$). The relationship between fish length and mass was significantly affected by time of year and no effect of infection status was found on body mass for giving body length (Table 2.2; Figure 2.5).

**Figure 2.5** The relationship between length and mass of three-spined sticklebacks collected from the River Soar for each given month. Non-infected fish (○) are indicated with a solid line, infected (△) with a red dashed line.
Table 2. The multivariable linear models (LM) results for the standard length of *G. aculeatus* from the River Soar as a response variable for each month of the study using fish mass, *Schistocephalus solidus* infection status, months and sex as predictor variables. Significant values (P < 0.05) are shown in bold.

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<td>0.117</td>
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<td>4</td>
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2.4.3.2 Body Condition Factor (BCF)

The host body condition of three-spined sticklebacks were examined to identify whether there was any variation in BCF across *S. solidus* infected and non-infected fish. BCF varied significantly across the study months in relation to the infection status of the fish. Generally, infected fish had lower BCF values than non-infected fish (ANOVA Table 2.3; Figure 2.6). Infected males had a lower BCF than non-infected individuals, where the mean of BCF in infected males was 0.977± 0.123 S.D., and in non-infected males was 1.136 ± 0.152 S.D. (GLM, Tukey’s post-hoc: P
Moreover, infected males had a lower BCF than infected females (mean BCF of infected females was 1.04 ± 0.171 S.D. (GLM, Tukey's post-hoc: \( P = 0.041 \); Figure 2.7).

**Table 2. 3** ANOVA table showing the body condition factor (BCF) of three-spined sticklebacks from the River Soar as a response variable. *Schistcephalus solidus* infection status, months and sex as predictor variables. Significant values (\( P < 0.05 \)) are shown in bold.

<table>
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<td>8.65</td>
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<td>0.732</td>
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<td>2.97</td>
<td><strong>0.020</strong></td>
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<td>Fish Sex*Infection Status</td>
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<td>10.24</td>
<td><strong>0.002</strong></td>
</tr>
</tbody>
</table>

**Figure 2. 6** Body condition factor (BCF) of River Soar three-spined sticklebacks during the months of the study for (NI) non-infected and (I) *S. solidus* infected fish.
Figure 2.7 The effect of host sex and *S. solidus* infection on the body condition factor (BCF) of three-spined sticklebacks from the River Soar.

### 2.4.3.3 Hepatosomatic Index (HSI) and Spleensomatic Index (SSI)

The hepatosomatic index (HSI) was not affected by infection status, fish sex or the months of the study (Table 2.4, Figure 2.8 and 2.9). Spleensomatic index was significantly affected by infection status. SSI did not vary between fish sex either (Figure 2.10). However, SSI varied significantly between the months, with lower values in April 2015 and a higher rate was in November 2014 (Table 2.5, Figure 2.11).
Table 2.4 ANOVA table for the hepatosomatic Index (HSI) of three-spined sticklebacks from the River Soar as a response variable. *Schistocephalus solidus* infection status, months and sex as predictor variables.

<table>
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</table>

Table 2.5 ANOVA table for the spleensomatic index (SSI) of *G. aculeatus* from the River Soar as a response variable. *Schistocephalus solidus* infection status, months and sex were used as predictor variables. Significant values (p < 0.05) are shown in bold.

<table>
<thead>
<tr>
<th></th>
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<th>P value</th>
</tr>
</thead>
<tbody>
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<td>Fish Sex*Infection Status</td>
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<td>1.26</td>
<td>0.262</td>
</tr>
</tbody>
</table>
Figure 2.8 The effect of host sex and *S. solidus* infection on the hepatosomatic index (HSI) of three-spined sticklebacks from the River Soar.

Figure 2.9 Hepatosomatic index (HSI) of River Soar three-spined sticklebacks during the months of the study for (NI) non-infected and (I) *S. solidus* infected fish.
Figure 2.10 The effect of host sex and *S. solidus* infection on spleensomatic index (SSI) of three-spined sticklebacks from the River Soar.

Figure 2.11 Spleensomatic index (SSI) of River Soar three-spined sticklebacks during the months of the study for (NI) non-infected and (I) *S. solidus* infected fish.
2.4.3.4 Fish Sex and plerocercoids growth

Pooled across the months of the study, fish sex was not found to be significantly associated with *S. solidus* infection (Chi-squared: $X^2 = 1.016$, $P = 0.314$; Figure 2.12). Plerocercoid mass did differ significantly with the month the fish was collected ($F_{4,48} = 8.80$, $P < 0.0001$). There was a significant difference in the total parasitic mass between infected male and female sticklebacks, with a heavier plerocercoids mass being found in the males ($F_{1,48} = 19.38$, $P < 0.0001$; Figure 2.13). However, no interaction between sex and months was included in the model because the data did not allow analysis of sex differences per month, as only few infected fish/per month were recoded. When the mass of the parasite was controlled for the mass of the host by calculating the parasite index (PI), there was highly significant difference between the fish sex ($F_{1,48} = 8.39$, $P < 0.0001$; Figure 2.14) where males had a higher PI than females. Parasite indices were significantly increased across months ($F_{4,48} = 8.99$, $P < 0.0001$; Figure 2.14).

![Graph showing frequency of infected and non-infected fish by sex](image)

**Figure 2.12** Frequency of *Schistocephalus solidus* infected and non-infected three-spined sticklebacks of the sex given.
**Figure 2. 13** The relationship between total *Schistocephalus solidus* parasite mass data pooled over all months for each sex. Females (O) and males (Δ).

**Figure 2. 14** The relationship between *Schistocephalus solidus* parasite index data pooled over all months for each sex. Females (O) and males (Δ).
2.4.4 Fish sex, body condition and *Diplostomum spathaceum* infection status

2.4.4.1 Fish sex and *Diplostomum spathaceum* infection status

Among all the three-spined sticklebacks collected, the probability of having *D. spathaceum* infection was not associated with fish sex (Chi-Square: $X^2 = 1.484$, df =1, $P = 0.223$). Among infected fish, there were no significant differences between the males and females in terms of the number of metacercariae that developed in the fishes’ lenses ($F_{1, 228} = 1.86$, $P = 0.174$; Figure 2.15). The number of metacercariae was significantly varied across months of the study ($F_{4, 228} = 11.54$, $P < 0.0001$). There was a strong interaction between fish sex and months in terms of the intensity of the infection ($F_{4, 228} = 9.91$, $P < 0.0001$; Figure 2.15), infection intensity was roughly similar between sexes and also comparable between months.

![Figure 2.15](image)

**Figure 2.15** The effect of host sex on the intensity of *Diplostomum spathaceum* infection in three-spined sticklebacks from the River Soar during the six months of the study (F): females and (M): males.
2.4.4.2 Effect of infection on fish standard length

To examine whether sticklebacks infected with *D. spathaceum* varied in size compared to non-infected specimens as based on their sex during the months of the study, the multivariable linear model was used. *Diplostomum* infection and sex were not significant factors affecting fish length (Table 2.6). The average standard lengths of infected females was $37.31 \pm 4.88$ S.D., and males $40 \pm 4.45$ S.D.; and for non-infected females, $37.87 \pm 4.44$ S.D., and non-infected males, $37.52 \pm 4.72$ S.D. Fish length varied significantly during the months of the study; however, sex had no significant effect on fish size during the sampled months (Table 2.6; Figure 2.16).

Table 2. 6 The multivariable linear models (LM) results for the standard length (SL) of *G. aculeatus* from the River Soar as a response variable. *Diplostomum spathaceum* infection status, months and sex used as predictor variables. Significant values ($P < 0.05$) are shown in bold.

<table>
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<th>P value</th>
</tr>
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<td>0.332</td>
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<td>0.37</td>
<td>0.545</td>
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</table>
2.4.4.3 The effect of infection status on body factors

There was no significant effect of *Diplostomum* infection on BCF (ANOVA Table 2.7, Figure 2.17), SSI (ANOVA Table 2.8, Figure 2.18) and HSI (ANOVA Table 2.9, Figure 2.19) indices during the months of the study, and no significant variation with sex was found.

**Figure 2.16** Standard length of three-spined sticklebacks during the months of the study. (NI) non-infected and (I) for *Diplostomum spathaceum* infected fish.
Table 2. ANOVA table for the body condition of River Soar three-spined sticklebacks for the six months of the study using body condition factor (BCF) as the response variable. *Diplostomum spathaceum* infection status, months and sex were used as predictor variables.

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Figure 2. Body condition factor of River Soar three-spined sticklebacks over the months of the study for (NI) non-infected and (I) *D. spathaceum* infected fish.
Table 2. ANOVA table for the body condition of River Soar three-spined sticklebacks, using spleensomatic index (SSI) as the response variable. *Diplostomum spathaceum* infection status, months and sex were used as predictor variables.

<table>
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Figure 2. Spleensomatic index (SSI) of River Soar three-spined sticklebacks over the months of the study for (NI) non-infected and (I) *D. spathaceum* infected fish.
**Table 2.** ANOVA table for the body condition of River Soar three-spined sticklebacks, using hepatosomatic index (HSI) as the response variable. *Diplostomum spathaceum* infection status, months and sex were used as predictor variables.

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<td>0.958</td>
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**Figure 2.** Hepatosomatic index (HSI) of River Soar three-spined sticklebacks over the months of the study for (NI) non-infected and (I) *D. spathaceum* infected fish.
Body condition factors for the fish that harboured both *Schistocephalus* and *Diplostomum* infection were compared to non-infected fish, to investigate whether mixed infection had influenced fish health to a greater extent than fish with a single infection was presented previously. There were significant differences in BCF between mixed-infection fish which showed lower rate than non-infected fish ($t$-test: $t = 2.48$, df = 40, $P = 0.018$; Figure 2.20). No differences were found between fish in HSI ($t$-test: $t = -0.47$, df = 41, $P = 0.643$). Mixed-infection fish had a larger spleen size than non-infected SSI ($t$-test: $t = -5.61$, df = 49, $P < 0.0001$; Figure 2.21).

![Figure 2.20](image-url)  
*Figure 2.20* Body condition factor (BCF) of River Soar three-spined sticklebacks infected with both *Schistocephalus solidus* and *Diplostomum spathaceum* compared to non-infected fish.
Figure 2.21 Spleensomatic index (SSI) of River Soar three-spined sticklebacks infected with both *Schistocephalus solidus* and *Diplostomum spathaceum* compared to non-infected fish.
2.5 Discussion

2.5.1 General finding

Comparison of the macroparasite communities of three-spined sticklebacks collected from the River Soar population were examined over the course of the study. The manner in which the occurrence pattern of the two common parasites, *Schistocephalus solidus* and *Diplostomum spathaceum*, might affect fish health in this ecosystem was also considered. Nine endoparasites species and three ectoparasites were found in this survey, which belonged to various taxa. In general, infection levels were observed to be rather high for some species; however, in other species the level of infection was low or fluctuated.

Despite the fact that the three-spined stickleback is widely distributed across freshwater systems in the UK, only a few studies have presented parasites fauna information in various populations. To my knowledge, no previous study has considered the parasitic community in the River Soar. For example, Chappell and Owen (1969) have listed 20 stickleback parasites species in Britain. Three parasites species were recorded in the population of three-spined sticklebacks from Priddy Pool in Somerset: *S. solidus, D. gasterosteii* and *Echinorhynchus clavula* by Pennycuick (1971a). Kennedy (1974) has listed 51 species that have parasitized in sticklebacks in British and Irish freshwater bodies. Nine macroparasite species were recorded from 12 populations of three-spined stickleback, on North Uist, Scotland by De Roij and MacColl (2012).

The direct life cycle parasite *Argulus* sp. was the dominant species across all the months of the study. *Argulus* sp. life cycle is crucially related to water temperature and direct physical contact with the fish (Shafir and Van As, 1986). Therefore, the current study result suggests that this parasite has possibly evolved certain adaptations to cope with the host’s environment as represented by increased variability in hatching time (i.e. in winter), as suggested by Pasternak et al. (2000). The strikingly high infection rates throughout all seasons that I observed may be attributable to climate change and indicate that the parasite might benefits from the higher average winter temperatures. This is supported by a previous studies by Shafir and Van As (1986) and Gault et al. (2002) which found that *Argulus* infection
levels peaked as temperatures rose. In addition, Mackenzie (1999) suggested that ectoparasites are highly resistant to environmental change and will respond directly by increased levels of infection. In contrast, *Glugea* sp., which develops in up to 15°C (Woo et al., 2011) was recorded in only 7 fish in November 2014, and April and June 2015. This was possibly due to the water temperature, which might be unsuitable for its reproduction and transmission.

The seasonal cycle of certain parasite species that need an intermediate host to complete their transmission to the definitive host was reported in several wild studies. It is possible that the prevalence of infection was seasonally varied due to the short life cycle of their intermediate hosts, such as snails and crustaceans especially in winter when it is hard to survive with the food resource reduction.

For example, the isopod crustaceans are serving as intermediate host to the acanthocephalan *Acanthocephalus* sp. (Brattey, 1988). Fish food preference might explain why only a few fish were recorded as being infected with these parasites, it seems that isopods are not a preferred food item of stickleback. The three-spined stickleback presents as a definitive host for the cestode *Proteocephalus filicollis*, though this was found only in 18 fish. Infection with this parasite was found for all months, which might be explained by the variety of food being consumed by the fish, particularly copepods. Chappell (1969) found that *P. filicollis* infection occurred throughout the year in sticklebacks from a pond in Yorkshire, the author suggested that adult worms can mature over winter and would be gravid adults late summer and autumn.

### 2.5.2 Occurrence of *S. solidus* and *D. spathaceum* Infection

In this study, data were collected from 271 individuals, and seasonal changes of both *G. aculeatus* common parasites, *S. solidus* and *D. spathaceum*, have been investigated. *S. solidus* was present in over 14% of fish samples, where infection occurred at only a low percentage over all months except January (31.5%), after which the prevalence decreased dramatically in the following months, showing only a slight increase in June. In the survey of three-spined stickleback parasites in Yorkshire, only a few fish harboured *S. solidus* infection during the autumn, and no seasonal variations were recorded for the population studied by Chappell (1969).
Pennycuick (1971c) showed that *S. solidus* infection prevalence increased significantly in winter and spring during the two years of her study. It is possible that the fluctuation in prevalence of infection in the River Soar fish could be attributed to several reasons such as low survival rate of heavily infected fish which is in agreement with Pennycuick (1971c). In addition, definitive and intermediate host abundance might play a significant role in restricting the spread of infection, particularly after a considerable drop in crustacean numbers over the winter which lead to the significant dropped of infection level between February to April. Moreover, another possible explanation is monthly sample errors during fishing, where the number of fish caught in some months was possibly biased towards non-infected fish. In fact, in the last period between June and August 2015, it was hard to catch any fish because of their sexual behaviour, which involves emigrating to river bottom to find nesting areas and look for mates.

There was no difference between the sexes in infection prevalence, though males harboured the heaviest plerocercoids total mass. Differences in the levels of infection between the sexes have been previous investigated via wild studies. Ecological differences such as geographical differences between habitats (MacColl, 2009) and each sex feeding habits have been found to be the main reasons for sex-bias in *S. solidus* infection (Pennycuick, 1971a, Reimchen and Nosil, 2001).

The findings of the current study support the previous research results in the sense that the development of heavier plerocercoids mass in males is likely to be attributable to their feeding ecology, especially in April when food resources started to become more abundant. In addition, females’ requirement for food differs from that of males, especially at the start of breeding season, which might explain the higher parasite mass in June’s infected females. Reimchen and Nosil (2001) suggested that one sex probably exhibit more temporal changes in the relative amount of limnetic and benthic foraging that could mainly affect the levels of male vs. female infected fish. The results presented here also suggest that males’ requirement for food differs from that of females leading to *S. solidus* plerocercoids rapid growth and high food resources investments perhaps be available in male than female host.
Infection with *Diplostomum* sp. can occur when fish are exposed to free-swimming cercariae which penetrate the skin or pass directly from the gills to the blood stream. In contrast to *S. solidus*, *D. spathaceum*’s prevalence was over 85% in the fish sample, with a slight reduction observed in January only. The prevalence of infection decreased in winter and started to increase in early spring, followed by a large number of parasites were acquired in summer; when infection prevalence decreased slightly, and the infection intensity increased rapidly this was attributed to the abundance of a new generation of suitable intermediate hosts (snails). Another possible reason for this high level of infection is the accumulation of *Diplostomum* by the time, especially after different sizes of metacercariae were observed inside the lenses which suggests that a new infection has always occurred.

This result is consistent with that of an earlier study by Pennycuick (1971 c) and provide further evidence that the trematode *Diplostomum* has exhibited seasonal variations in infection occurrence of River Soar three-spined stickleback population.

Another possible explanation for *Diplostomum* prevalence increase in early spring, is that host immunity reduction that might be associated with sexual behaviour during the breeding season. This seemed apparent in June’s data when both sexes were found to have greater levels of infection (28.3%) that month. Mating competition leads to physiological reproductive trade-offs for both sexes, which can result in higher levels of stress and immunity reduction (Zuk and McKean, 1996, Morales-Montor et al., 2004).

### 2.5.3 Effects of *S. solidus* and *D. spathaceum* infection on the body condition of three-spined sticklebacks

A strong relationship between BCF and *S. solidus* infection was found in this study. Infected male fish had lower body condition factors than infected females and both non-infected males and females. Body condition factors are significant predictors of energy reserves (Chellappa et al., 1995). It is possible that infected male exhibit a negative response to *S. solidus* higher nutrient theft levels than infected female and non-infected fish, the mechanisms by how each sex response to nutrient theft and the factors responsible for variation in their body condition remains debatable.
A higher body condition factor was found in June 2015 with the adults of three-spined sticklebacks, as only four fish were harboured *S. solidus* infection. This suggested that non-infected adult fish are possibly able to store energy better *S. solidus* infection fish. One possible explanation of BCF reduction is that fish might have been exposed to *S. solidus* infection or other pathogens which transform the energy from food more efficiently than their host.

*Diplostomum* infection had no significant effect on BCF, this is unsurprising result as this may reflect the small body size of the parasite, so this parasite has low nutrient demands compared with *S. solidus*. Mixed infection fish, as infected by both parasites (*S. solidus* and *D. spathaceum*), showed lower BCFs than non-infected fish, possibly due to the behavioural changes associated with both infections, namely causing dietary stress and the manipulation of host swimming behaviour (LoBue and Bell, 1993, Barber et al., 2008).

The spleen is a lymphoid organ and related to fish immunity, variations in SSI was used as a proxy for fish immune status. The spleensomatic index, surprisingly, was not significantly affected by *D. spathaceum* infection status. As most fish samples were infected with a range of 1-60 metacercariae except one fish with 120 parasites, which might be considered to be a low or mild infection rate compared to the most heavily infected fish (443 parasites in one fish), as recorded by Pennycuick (1971c). *Diplostomum* intensity of infection in the River Soar is dependent upon visits of infected definitive host most probably gulls, which were seen occasionally at this river. The SSI of the mixed infection fish was higher than that for non-infected fish. Spleen mass has been previously shown to be higher in *D. pseudospathaseum* infected sticklebacks (Kalbe and Kurtz, 2006).

HSI was not affected by both parasites infection status. Arme and Owen (1967) found that the HSI was negatively correlated with *S. solidus* infection. However, Tierney et al. (1996) found that HSI showed a variable prevalence as it was more associated with *S. solidus* infection status and the season of the year.
2.5.4 Conclusions

In conclusion, this study provides a dataset regarding the parasites seasonal occurrence of *G. aculeatus* in the River Soar, Leicestershire over eight months. Differences in parasite occurrence could therefore be attributed to ecological factors and intermediate hosts availability. Generally *S. solidus* infected fish had lower body condition than non-infected fish. The survey could serve as a useful comparison with other studies of three-spined sticklebacks parasites both in the UK and elsewhere populations and monitoring future change that could affect sticklebacks-parasites ecosystem.

2.6 References


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Chapter 3

How do host sex and body size affect infection susceptibility and parasite growth?
3.1 Abstract

Parasites do not affect all of their hosts equally, and both the level of infection and the severity of the effects that parasites cause can be influenced by pre-existing variation among hosts. Host sex and body size are two potentially important factors influencing the interaction between parasites and their hosts, which have been shown to affect susceptibility to infection, as well as the subsequent growth and development of the infecting parasite. Plerocercoid larvae of the cestode *Schistoscephalus solidus* often affect the health, growth and development of three-spined sticklebacks, *Gasterosteus aculeatus*, in natural populations. In the investigations of this chapter, I used experimental infection techniques to examine how pre-existing variation among individual stickleback hosts influences the outcome of *S. solidus* infections. Sticklebacks that differed in body size and sex – determined non-invasively by PCR analysis of a sex-linked marker – were exposed to controlled doses of infective *S. solidus* parasites and reared under lab conditions for 70 d. Fish sex, mass and standard length were not found to be strong predictors of susceptibility to *S. solidus* infection. Furthermore, none of these host factors had a significant influence on subsequent parasite growth rates.
3.2 Introduction

Parasites do not affect all of their hosts equally, and both the level of infection and the severity of the effects that parasites cause can be influenced by pre-existing variation among hosts. Then the cost of parasitism and the level of virulence in one host might be varied (Regoes et al., 2000). Host sex and body size are two potentially important factors influencing the interaction of between parasites and their hosts, which have been shown to affect susceptibility to infection, as well as the subsequent growth and development of the infecting parasite. Larger host bodies are expected to be easier targets for parasitic invasion by provide larger surface areas and a larger number of available niches for colonization and also provide more cues like olfactory, chemical, visual, auditory, etc. that can be tracked by motile infective stages than small hosts (Kuris et al., 1980, Poulin, 1995). There are a number of differences between sexes in terms of behaviour, immunity, physiology and ecology that should be taken into account when attempting to explain sex-biased parasitism (Barger, 1993, Zuk and McKean, 1996). Meta-analysis studies showed nematode parasite levels are often unevenly distributed between host sexes in a range of mammal, bird and fish species. Evidence of a higher levels of parasitism may be biased towards males as a results of host behavioural and social interactions that might influence their exposure to infective parasite stages (Poulin, 1996). The levels of ectoparasite infections are often more male biased, but this observation was negatively related to male sexual size dimorphism in ten species of rodents infected with flea species (Morand et al., 2004).

3.2.1 How might host sex affect parasite infections?

3.2.1.1 Sex-related physiological factors

There are intrinsic biological differences between vertebrate animal sexes that might give rise to one sex being more susceptible to parasitic infections than the other. Alexander and Stimson (1988) summarized many reasons why parasite infections might differ as a consequence of physiological, morphological, or behavioural differences between male and female hosts, leading to sexual biases in infection levels. For example, host sex may be important if the effectiveness of immune
responses increases or decreases as individuals reach maturity and hormonal changes take place.

Mating competition leads to physiological reproductive trade-offs for both sexes, which can result in higher levels of stress and immunity reduction (Clutton-Brock and Parker, 1992, Zuk and McKean, 1996, Morales-Montor et al., 2004). Sex-biased parasitism might also be influenced by the trade-offs between investment in sexually selected traits and the immune system. The immunocompetence handicap hypothesis suggests that immune function in males may be negatively associated with their secondary sexual traits (Folstad and Karter, 1992). The authors reported that while testosterone stimulates the development of secondary sexual selection characters, it can also negatively affect immune function.

Furthermore, host sex may impact the level of parasites infection due to the direct effects of host hormones. A high parasite load in males was found in several studies in vertebrates as a result of higher levels of estrogens that stimulate female’s immunity, whereas androgens (testosterone) depress males immunity (Schuurs and Verheul, 1990). For example, Kamis and Ibrahim (1989) found that male mice (Mus musculus) are more susceptible to Plasmodium berghei and P. chabaudi experimentally infection than females. The authors suggested that testosterone may suppresses the production of leukocytes and that testosterone-treated mice become more susceptible to parasite infection. In humans, acquired immune responsiveness to Schistosoma haematobium infection has been found associated with patient gender, for instance adult Senegalese men were found to have higher immune responsiveness than women due to the effect of non-immunological factors such as severity of infection combined with sexual hormone levels (Remoue et al., 2001).

Host behaviour has been linked to sex differences in the presence, and diversity, of parasites in free-ranging hosts, especially when it has been correlated with physiological factors such as hormones and immune response changes (Poulin, 1996). In Cape ground squirrels (Xerus inauris) allogrooming social behaviour reduces ectoparasites load across the population (Hillegass et al., 2008). However, ectoparasites had an increased presence in males, which harboured three times more lice than females. It was suggested by Hillegass et al., (2008) that extensive
daily movements through bushes and trees could be associated with immunity suppression through high androgen levels. In contrast, surveys of short home range females showed they had higher levels of endoparasite infections than males (Hillegass et al., 2008). As a result of differences in sex-dependent, behaviour, and hormones level, a review of literature on mammalian host, showed that males with higher testosterone levels tend to have a larger home range than females, which might increase their risk of exposure to parasites (Morand et al., 2004).

3.2.1.2 Ecological, social and behavioural factors and parasite infection risk
Sex-biases in parasitism have been linked to differences in host ecological, social and behavioural factors, especially when it has been associated with other factors such as body size, age, habitat and genetic background (Poulin, 1996). Sex-dependent differences in behaviour may alter the exposure level of host to infective parasite stages (Robinson et al., 2008). For example, sex differences in territoriality, movement patterns and social activity can influence parasite transmission. Territoriality in male yellow-necked mice (Apodemus flavicollis) is associated with lower immunity response and higher nematode (Heligmosomoides polygyrus) transmission than non-territorial females in wild populations (Ferrari et al., 2004).

Differences in host behaviour could also reflect sex-dependent variation in the prevalence and intensity of parasite infections. In humans, sex-related differences in parasitic disease rates might attribute to racial, ethnic, cultural and social behaviour that may increase the chance that hosts encounter more infective stages (Bundy, 1988). For example, women may be more exposed to water-borne infections, including schistosomiasis, than males as a result of gender role-specific behaviours such as water collection and washing (Bundy, 1988).

Variation in diet between females and males, both in terms of quality and quantity of food ingested with the potential of physiological-immunity basis, might also affect their susceptibility to trophically transmitted parasites (Poulin, 1996). For example, in wood duck females (Aix sponsa), a high prevalence of helminth infection during the egg-laying period has been found by Drobney et al. (1983), potentially as a consequence of hyperphagia during the egg production period. Male ducks
harboured higher levels of infection during the breeding season, which was thought to be due to increased testosterone levels (Drobney et al., 1983).

In contrast to the pattern observed in other vertebrate, female bats have a higher ectoparasite prevalence than males. Mite infections were higher in intensity and showed significantly higher parasite survival in females than male Daubenton’s bats, \textit{(Myotis daubentonii)} (Christe et al., 2007). The authors suggested that the bats’ particular social life compared with other mammals is likely to imply different selective pressures that result in a parasitic bias between host sexes.

Parasites may actively choose their hosts, and as a result of their active and adaptive choice for more beneficial hosts, this could lead to a strong sex-linked contrast in infection (Christe et al., 2007). In the discrimination of host sex in a haematophagous ectoparasite experiment, Khokhlova et al. (2011) found that female fleas \textit{(Xenopsylla ramesis)} chose a male rodent host significantly more often than a female rodent host \textit{(Meriones crassus)}. These experiments demonstrated that parasite sex was the only factor driving host choice in this instance. Female fleas require multiple blood meals prior to successful reproduction, therefore, they may have developed a specific strategy of trade off the time required for host search against quality of a host by choosing low immunity males than females (Khokhlova et al., 2011).

3.2.2 The effect of host size differences in parasite infection

Host body size can play an important role in determining the infection susceptibility of hosts, parasite growth and potentially influence the host-parasite interactions (Poulin, 2011). Host body size may be important if it associated with an effective immune responses changes as individuals grow (Sol et al., 2003). Host immunity might have a direct effect on determining parasitic infection level; since age and body size are typically linked together in host growth and immunity assessment. The innate immunity can be correlated passively with host age, where older hosts might be expected to show a significant immune reduction against parasites and pathogens. High levels of innate immunity were found to be increased with age in the garter snake \textit{(Thamnophis elegans)} depending on their growth rate, size and age, after which the rate of immune development decreased up to maturity (Sparkman and Palacios, 2009).
In vertebrates, body size variation between the sexes is common, and such differences in body size between the sexes may play an important role in determining a pattern of infection by presenting an easier target for parasitic invasion (Haas, 2003). Host sex and age are important factors in determining the levels of parasitic infection in wood mice (Apodemus sylvaticus) in Southern England with the nematode Syphacia stroma (Behnke et al., 1999). Older males were more heavily infected than younger males and older females, and it was suggested that embryonated eggs were perhaps transferred more easily to the male's fur during mating competition. However, females showed a reduction in infection that was related to a lower number of encounters of infective parasites as they roamed less in the summer and autumn due to pregnancy and lactating periods (Behnke et al., 1999).

The level of parasite infection might be positively correlated with host age and size as a result of accumulated parasite infection encounters over the lifetime of the host. In a survey of the parasites of three-spined sticklebacks (Gasterosteus aculeatus) from Priddy Pool in Somerset, UK, the intensity of infection with both Diplostomum and Echinorhynchus was found to both increase with the age and size of the fish (Pennycuick, 1971a). Younger fish showed increase in Echinorhynchus infection than older fish due to their feeding habitat containing crustaceans and for higher exposure to Diplostomum cercariae, which probably leads to the accumulation of metacercariae over time. Larger whitefish (Coregonus lavaretus) accumulated more Triaenophorus crassus plerocercoids as they got older (Pulkkinen and Valtonen, 1999).

It remain unclear whether host slower growth rate is due to the consequences of parasitic infection or host size is the factor that increased their susceptibility to infection in several wild studies. Nevertheless, size differences among fish of the same age showed a growth rate variation which was associated with differences in parasite load negative effect of parasites on whitefish growth. Smaller-sized fish belonging to the older age class showed higher levels of parasites than larger fish of the same age, while in the younger age class, larger fish harboured more parasites
than smaller fish because of their relatively fast growth rate and their higher feeding rate that exposed them to increased risk of infection (Pulkkinen and Valtonen, 1999).

Host body size plays a crucial role in the survival of the infected host as individuals grow; across three different populations of guppies (Poecilia reticulate) it was found that smaller individuals tended to survive and shed more Gyrodactylus sp. infection than larger fish, who showed a higher intensity of infection and suffered a higher mortality rate (Cable and Van Oosterhout, 2007). Ryce et al. (2005) found that rainbow trout, (Oncorhynchus mykiss) that are less than 9 weeks post-hatching and are at least 40 mm in fork length were more susceptible to whirling disease, caused by the parasite Myxobolus cerebralis, and became more resistant to the disease when older and larger.

3.2.3 The effects of host body size and sex on Schistocephalus solidus infections in sticklebacks

Schistocephalus solidus is a common parasite of the three-spined stickleback in freshwater populations (Poulin et al., 2011). When three-spined sticklebacks ingest copepods harbouring infective parasite procercoids, the larvae shed the outer cercomer inside the stomach of the fish, and bore through the intestine to grow and develop to the plerocercoid stage in the body cavity (Clarke, 1954, Smyth, 1969).

Stickleback body size has the potential to play an important role in determining fish susceptibility to S. solidus infection, as the fish’s response to infection might change with age. Body size was found to be a strong predictor of stickleback susceptibility to S. solidus infection (Simmonds, 2015).

In some stickleback populations, heavily infected fish showed a significant loss in body mass for the same length of non-infected fish, and which also grow more slowly and exhibit delayed sexual maturation and reduced reproduction in both sexes (Pennycuick, 1971c), although in one Alaskan population, infected female fish showed an ability to mature and develop egg clutches faster (Heins et al., 2000, Heins and Baker, 2008). In contrast, several influential studies have suggested that infected fish are unable to engage in reproductive activities (McPhail and Peacock, 1983, Tierney et al., 1996).
With a large fish size, parasite might typically being able to achieve rapid growth. Then, the mass attained by the plerocercoid directly affects transmission success, with larger plerocercoids being able to alter fish behaviour which might increase the likelihood of successfully establishing in definitive hosts (Tierney and Crompton, 1992). Therefore three-spined sticklebacks- *Schistocephalus*, offer a well-studied system to test hypotheses concerning differential parasitism between the sexes (Zuk, 1990, Reimchen and Nosil, 2001). Therefore, understanding the consequences of host sex and body size for *Schistocephalus solidus* infection and plerocercoid growth in an experimental fish infection model is required.

Since, stickleback body size has the potential to play an important role in determining fish susceptibility to *S. solidus* infection and body size was found to be a strong predictor of stickleback susceptibility to *S. solidus* infection (Simmonds, 2015). Therefore, this current study designed to clarify the extent to which host sex and size are causative factors in determining stickleback susceptibility to *S. solidus* infection under laboratory conditions; and to determine the direct effect of host sex and size on parasite growth and development.

### 3.2.4 Aims

The aim of this chapter was to investigate how body size and sex differences among fish hosts influences the susceptibility of three-spined sticklebacks to experimental *Schistocephalus solidus* infection, and to examine how these host factors affect the subsequent growth and development of parasites among fish that become infected. Lab-bred fish, varying in size and sex, were exposed to infective stages of *S. solidus* and worms were allowed to establish and grow for 70 d post-infection. The study will enable the following questions to be addressed: (1) How do differences in host body size at the time of parasite exposure affect susceptibility to infection and fish health? (2) How do differences in host sex and body size affect subsequent plerocercoid growth and the development of heavier infection? (3) Does stickleback host sex affect the fecundity of adult parasites after transmission to the definitive host?
3.3 Methods

3.3.1 Fish breeding and husbandry

Adult lab-bred three-spined sticklebacks, originally bred from parents collected from Inverleith Park, Edinburgh, UK, were used as the parents of families generated by standard *in vitro* fertilisation techniques (IVF) following Barber and Arnott (2000).

Male three-spined sticklebacks were dissected under a stereomicroscope, testes were removed and placed in a watch glass over ice before being macerated using sterile forceps. Eggs were stripped from females into a watch glass before adding the macerated testes solution in aquarium water, and being left for 30 minutes. A stereomicroscope was used to check for fertilisation, which was confirmed by the appearance of the fertilisation membrane. Fertilised eggs were kept in 1 L plastic aquaria with constant aeration and 2 ml/L methylene blue solution (stock solution: 2 mg/L) as an anti-fungal agent.

Hatched fry were kept in the same 1 L aquaria and fed daily with Liquify No. 1™ for egg-laying fish until they were capable of consuming 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. They were fed daily *ad libitum* with *Artemia* sp. nauplii, which were supplemented with frozen bloodworms as the fish grew. Aquarium conditions tracked natural temperature and day light regimes.

3.3.2 Molecular sex determination

3.3.2.1 Fish skin swab samples

From a group size of 50, individual fish were netted from stock tanks and the skin was swabbed to collect DNA samples following the procedure by Breacker et al. (2017). In brief, fish were blotted and gently swabbed ten times from the operculum to the caudal fin with a sterile cotton swab stick (‘swab virus transport plastic stick’, VWR International Ltd, UK). Then, the swabbed fish were kept in 1 L plastic tanks individually until the DNA sex determination result was known. Sex-identified fish were housed together in sex-matched groups in a glass aquarium (41 cm x 60 cm x
40 cm large tanks) containing artificial plants and gravel substratum until the time of parasite exposure.

### 3.3.2.2 DNA extraction from swab samples

The sterile cotton swab stick was returned to its sterile container and immediately taken through the DNA extraction procedure. The swab sample was placed into a 1.5 ml microcentrifuge tube containing 400 µl DNA extraction buffer (200 mM TRIS pH 7.5, 25 mM ethylenediaminetetraacetic acid, pH 8.0, 250 mM NaCl, and 0.5% w/v sodium dodecyl sulphate), warmed to 55°C, and then incubated at room temperature for 15 min. The swab was then removed and 400 µl of chilled isopropanol was added to the DNA solution and mixed three times. The DNA solution was then chilled at -80°C for 10 min, the solution was centrifuged for 10 min at 13,000 RPM, and the remaining pellet washed with 190 µl of 70% EtOH. After a further centrifugation step of 2 min at 13,000 rpm, the DNA pellet was air dried and re-suspended in 30 µl of ddH$_2$O. DNA was quantified using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific, UK). The laboratory methods used for swabbing fish were those given by Breacker et al. (2017).

### 3.3.2.3 Polymerase chain reaction (PCR)

The PCR protocol was followed using the marker isocitrate dehydrogenase IDH Reverse R primers TTATCGTTAGCCAGGAGATGG, forward F GGGACGAGCAAGATTATG following (Peichel et al., 2004). These primers successfully produced the characteristic bands amplifying at 302 bp from the X chromosome and the band at 271 bp from the Y chromosome. A single PCR band of approximately 300 bp was linked to females, while males produce two products of 270 bp and 300 bp (See Figure 2.2 in Chapter 2), as described by Peichel et al. (2004) PCR reactions proceeded in a 10 µl volume (9 µl of master mix plus 1 µl DNA sample) containing 5 µl Red Taq (Sigma, U.K.), 0.5 µl forward primer, 0.5 µl reverse primer, 3 µl ddH$_2$O and 1 µl DNA. The samples were briefly spun down before loading them into the PCR machine. The PCR conditions were found to be optimum using 1 cycle at 94°C for 5 minutes, 40 cycles at 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds and a final 1 cycle extension at 72°C for 10 minutes.
A high percentage gel, at 4% (high resolution for DNA/RNA fragments 10-500 bp; Sigma UK), was used to differentiate the 68 bp difference between the PCR bands. A volume of 50 ml 1X TAE (Tris-acetate and Ethylenediaminetetraacetic acid) was used, adding 2 g high definition agarose and heating to dissolve, 0.5 μl ethidium bromide was added and then poured into the gel former. The gel was placed in the gel tank and covered in 1X TAE. The samples were removed from the PCR machine and spun down briefly before loading into gel, after which 2 μl high resolution DNA ladder (Hyperladder V, Sigma, U.K.) was added. PCR products were run on a two-rowed gel at 60 volts for 75 min.

### 3.3.3 Experimental parasite infection procedure

*Schistoscephalus solidus* plerocercoids were recovered from naturally-infected *Gasterosteus aculeatus* collected from the River Soar, Leicester, UK, by making an incision along the ventral side of the euthanized fish from the vent to the operculum. Infective plerocercoids, i.e. > 50 mg (Tierney and Crompton, 1992) were cultured in pairs using techniques adapted from (Smyth, 1954). Plerocercoids were placed into a loop of 6.3 mm diameter dialysis tubing (Visking, UK) and suspended in a 70 ml PYREX screw-top glass tube (Fisher, UK) filled with 1:1 mix of RPMI media and horse serum (Sigma, UK), using a total of 30 ml of each. To this solution, 0.5 ml of penicillin-streptomycin-glutamine solution was added (Thermo Scientific UK). Culture tubes were placed in a shaking water bath at 40°C for 6 days depending on the worm’s life span as previously recommended by (Arnott et al., 2000, Macnab, 2012). Eggs were collected from the dialysis tube; the culture liquid was removed, and eggs were washed with ddH$_2$O to remove any remaining parasite tegument. Collected eggs were stored in ddH$_2$O in a 9 cm diameter sealed Petri dish, covered in aluminium foil and incubated in the dark at 20 °C for 21 days.

Eggs were then taken from the incubator and exposed to daylight for 24 h to induce them to hatch. Laboratory-reared copepods (*Cyclops strenuus abyssorum*) were size-sorted by sieving into three groups; adults were retained by a 250 μm sieve, copepodites retained by a 150 μm sieve and nauplii passed through. Approximately 70 copepodites were placed in 100 ml conical flasks and exposed in batches to hatching coracidia (approximately 150) for approximately 24 h in natural daylight.
before being moved to a new flask and stored into fresh, filtered aquarium water under aquarium conditions. Three weeks after being exposed to the parasites, copepods were screened individually to determine the level of infection by observing each copepod in a drop of carbonated water on a glass slide under the compound microscope (300x magnification). The infection status and intensity of infection in each copepod was scored, as was the infective status of any procercoids, based on the presence or absence of a cercomer.

Copepods containing infective (i.e., cercomer bearing) procercoids were fed to lab-bred, previously sexed juvenile sticklebacks (see above). Fish were starved for 2 d prior to experimental infections to maximise the likelihood they ingested the infected copepod, and measured (Standard Length, SL$_0$, to 0.1 mm), blotted and weighed (M$_0$, to 0.001 g) before parasite exposure. Experimental fish were randomly selected from the male and female stock tanks. Each known-sex fish was either exposed to a known, controlled dose of procercoids by being fed an infected copepod, or was sham-exposed by being fed a non-infected copepod, via a glass pipette, in a small 1 L plastic aquarium (15.5 x 9.5 x 8.5 cm) filled with 500 ml of filtered system water.

A total of 80 fish (40 males and 40 females) were used in the experiment; 60 fish (30 males and 30 females) were exposed to a controlled dose of procercoid parasites whilst 20 (10 males and 10 females) were sham-exposed. Exposure tanks were left undisturbed for 24 h before fish were transferred to individual 1.25 L plastic aquaria (15 cm x 14 cm x 11 cm), which were held on a recirculating system and fed blood worms *ad libitum* to excess for 70 d. The water temperature was held at 19 ± 1.4°C and day length regimes were 12L: 12D. Exposure to parasite infective stages was carried out under the authority of a UK Home Office licence (Project licence: 80/2327, Personal Licence: IAD9DF470).

### 3.3.4 Post mortem analysis

At the end of the study, each fish was euthanized by an overdose of benzocaine anaesthetic (stock solution: 10g L$^{-1}$ in 70% EtOH) according to UK Home Office Schedule 1 methods. Fish were blotted dry, measured using a dial calliper (Standard Length, SL$_{70}$, to 0.1 mm) and weighed (wet mass, M$_{70}$, to 0.001 g) before dissection. Plerocercoids recovered from infected fish were weighed (to 0.001 g); in the case of
multiply infected fish, the mass of each individual plerocercoid was recorded and total parasite mass \((M_p)\) calculated. Parasite index was calculated as \(PI = \frac{M_p}{(M_{70}-M_p)} \times 100\), where \((M_{70}-M_p)\) is the mass of the infected fish following subtraction of parasite mass (Pennycuick, 1971a). Liver mass \((M_{liv})\), spleen mass \((M_{spl})\), kidney mass \((M_{kid})\) and gonad mass \((M_{gon})\) were weighed (all to 0·0001 g). Body condition factor (BCF) was calculated using the equation: \(BCF = \left[\frac{(M_{70} - M_p) \cdot Ls^3}{3}\right] \times 10^5\) (Pennycuick, 1971c). Specific growth rate (SGR) was calculated as \((100 \times (\ln(M_{70}-M_p)-\ln(M_0))/d)\), \(M_0\) is the initial wet mass of fish at the start of the study and \(M_{70}\) is the final wet mass of the fish at the end of the 70 day study. These data were used to calculate the following condition and health indices: Hepatosomatic Index (HSI) = \(((M_{liv} / (M_{70}-M_p)) \times 100)\); Splenosomatic Index (SSI) = \((M_{spl} / (M_{70}-M_p)) \times 100\); Gonadsomatic Index (GSI) = \(((M_{gon} / (M_{70}-M_p)) \times 100)\) and Kidney-somatic Index (IK) = \(((M_{kid} / (M_{70}-M_p)) \times 100)\).

### 3.3.5 Calculating adult parasite fecundity

The largest individual plerocercoid recovered from each infected host fish was cultured singly, in vitro, to quantify adult parasite fecundity and investigate whether stickleback host factors influenced adult egg production. Plerocercoids were cultured using in vitro techniques previously described above (Barber and Svensson, 2003). A procedure adapted from Dörücü et al. (2007) was also used to quantify the egg output of adult worms. Briefly, eggs were flushed from the dialysis membrane with distilled water and the suspension was centrifuged in 10 ml tubes at 1,500 RPM for 4 min. Extra fluids were removed via a glass pipette to adjust the volume to 2 ml, and the eggs were re-suspended by vigorous pipette action. A haemocytometer was then used to quantify egg density, and hence estimate the total egg output from each adult worm. The numbers of eggs in ten replicated 1.8 μl samples of re-suspended egg solution for each worm were counted under 40x magnification using a light microscope. For each worm, a trimmed mean was calculated, omitting the highest and lowest egg counts.
3.3.6 Statistical Analysis

All statistical analysis was carried out in Minitab 17 statistical software. Data were tested for normality and homogeneity of variance using Anderson-Darling tests and the test of equal variance. A Chi-squared test for independence was used to investigate if fish sex and exposure level influenced infection status (infected / non-infected) and infection level (single / multiple). Proportional data (BCF, HSI, SSI, GSI, KSI) were arcsine square root transformed and tested for normality using the Kolmogorov-Smirnov statistic, and non-normal data were transformed using the Box-Cox transformation or the Johnson transformation.

Binary logistic regression was used to test if initial length (SL₀) and mass (M₀) at the beginning of the study had an effect on the probability of fish becoming infected. For parametric data, ANOVAs were used to investigate whether fish sex and infection status had a significant impact on a given measured variable (M₇₀-Mₚ, SL₇₀, HSI, SSI, GSI and KSI in males only). Effects of host factors on plerocercoid growth were tested using multivariable linear model.

Among infected females and males, ANCOVA was used to compare the infection phenotypes in terms of M₇₀-Mₚ, SL₇₀ and condition indices (BCF, HSI) males and females, using PI as the covariate. Adult fecundity and plerocercoid mass were normalized using a log₁₀ transformation to meet the requirements of parametric testing to allow for statistical analysis. In order to estimate what the potential parasite's fecundity would be if infection developed in male or female hosts and if the worm developed as a single or multiple infection inside the host body, ANCOVA with plerocercoid mass Mₚ, as a covariate was perform. For all boxplots in this chapter the dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR) and the whiskers represent variability outside the upper and lower quartiles, outliers are shown as asterisks in figures.
3.4 Results

3.4.1 Exposure level, fish sex, and body size as determinants of infection susceptibility
Parasites became established in 36 (60%) of the 60 exposed fish. Among these fish, the probability of infection following exposure to *S. solidus* was not associated with fish sex ($X^2 = < 0.0001$, df = 1, $P = 1.000$; Figure 3.1). Being exposed to multiple procercoids, significantly increased the probability of fish becoming infected with at least one parasite ($X^2 = 15.901$, df = 1, $P = <0.0001$). This means that increasing the number of procercoids which the fish were exposed to positively increase the number of infected fish. Binary logistic regression tests examining only exposed fish showed the significance of procercoid exposure level on the probability of a stickleback developing at least one plerocercoid ($X^2 = 7.43$, df = 1, $P = 0.006$; Figure 3.2), meaning that higher levels of exposure positively increase the probability of fish to develop infection.

![Figure 3.1](image_url) **Figure 3.1** Frequency of experimental female and male three-spined sticklebacks exposed to copepods becoming infected with single and multiple procercoids.
Fish of both sexes were exposed to singly and multiply infected copepods. Seven fish of each sex were exposed to singly infected copepods, and the remaining 23 fish were exposed to multiply infected copepods. Among exposed fish, females harboured more parasites established from multiply infected copepods than males did ($X^2 = 6.415$, df = 2, $P = 0.040$; Figure 3.1).

![Figure 3.2](image)

**Figure 3.2** The probability of *Schistocephalus solidus* infection against initial number of procercoids, as determined by the logistic regression model. 0: Non-infected. 1: Infected. Data points are shown in (●) with the size of the circle representing the number of fish.

However, there were no differences found between males and females in terms of the number of parasites that were subsequently established compared to the estimated number of procercoids to which they had been exposed ($F_{1,35} = 0.31$, $P = 0.584$; Figure 3.3). In other words, once exposure level had been accounted for, among individuals that were susceptible to infections, sex was not found to have any effect on the number of plerocercoids that developed.
Fish pre-exposure length ($SL_0$) and mass ($M_0$) had no significant effect on the probability of fish becoming infected ($SL_0$: $X^2 = 0.77$, df = 1, $P = 0.379$; Figure 3.4 A; $M_0$: $X^2 = 0.16$, df = 1, $P = 0.688$; Figure 3.4 B).

**Figure 3.3** The relationship between the number of *Schistocephalus solidus* procercoids which fish were exposed and the number of plerocercoids that successfully established in three-spined stickleback hosts 70 d following exposure, separated by fish sex.
Figure 3.4 The probability of *Schistocephalus solidus* infection following exposure to infective parasites in relation to (A) the pre-exposure length ($SL_0$) and (B) the pre-exposure mass ($M_0$) of experimentally exposed three-spined sticklebacks, as determined by the logistic regression model. 0: Non-infected, 1: Infected fish.
Fish that went on to develop infections did not differ significantly in length or mass at the time of exposure from those that did not become infected (SL₀: F₁,₅⁹ = 0.100, P = 0.321; Figure 3.5 A; M₀: F₁,₅⁹ = 0.98, P = 0.328; Figure 3.5 B). There was also no significant differences between fish sex in length and mass at exposure (SL₀: F₁,₅⁹ = 1.13, P = 0.292; Figure 3.5 A), (M₀: F₁,₅⁹ = 0.32, P = 0.574; Figure 3.5 B) and no interaction between sex and infection status (SL₀: F₁,₅⁹ = 0.31, P = 0.582), (M₀: F₁,₅⁹ = 1.01, P = 0.320).

Figure 3.5 Mean chart of A: Pre-exposure standard length (SL₀), B: Pre-exposure mass (M₀) of three-spined sticklebacks infected with Schistocephalus solidus and those that were not, as separated by sex. Error bars represent +/- standard error.
3.4.2 Effect of host factors on plerocercoid growth

The effect of host factors on total plerocercoid mass recovered from experimentally infected fish at day 70 of experiment was tested using multivariable linear models (LM). The analysis showed that there was no significant relationship between $M_p$ and either $S L_0$ or $M_0$; when sex and infection level (single/multiple) were included in the model, neither these factors, nor the interaction terms between them, had a significant effect on the total mass of *Schistocephalus solidus* plerocercoids recovered from experimentally infected three-spined sticklebacks (Table 3.1).

Table 3.1 Results of multivariable linear models analysis investigating the influence of initial host body size, $S L_0$, initial mass, $M_0$, host sex and infection level and the interaction terms between them on the total mass of *Schistocephalus solidus* plerocercoids recovered from experimentally infected three-spined sticklebacks.

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<td>0.04</td>
<td>0.841</td>
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</table>
3.4.3 Growth of plerocercoids in singly and multiply infected hosts

Although the total plerocercoid mass in multiply infected fish exceeded the parasitic mass in singly infected fish ($F_{1,35} = 11.16, P = 0.002$; Figure 3.6), the mass of the largest individual plerocercoid in an infection was negatively correlated with the number of co-infecting parasites ($F_{1,35} = 19.9, P < 0.001$). The mass of the largest individual plerocercoid in an infection was significantly lower in multiply than in singly-infected fish ($F_{1,35} = 23.46, P < 0.0001$). The sex of the fish had no effect on the mass of the largest worms ($F_{1,35} = 1.53, P = 0.225$; Figure 3.6).

**Figure 3.6** The relationship ‘between the number of co-infecting parasites and the mass of the largest plerocercoid present’ female and male three-spined sticklebacks infected with *Schistocephalus solidus* after 70 d of exposure. (A) SL~0~ and the total parasite mass. (B) M~0~ and the total parasite mass. (*) represents female fish and (▲) male fish. The solid line refers to females and dashed regression line refers to males.
3.4.4 The effect of host sex and infection level on parasite index (PI)

The parasite index, which takes into account the relative mass of the parasite to the fish mass, differed significantly between male and female host fish ($t$-test: $t = 4.00$, $df = 30$, $P < 0.001$; Figure 3.7), with female fish having higher PIs than males. A significant effect of infection level (single or multiple) was also found, ($t$-test: $t = -5.37$, $df = 33$, $P <= 0.001$; Figure 3.7), with multiply infected fish having higher PIs than singly infected fish.

![Box plot showing the effect of host sex and infection level on parasite index (PI).](image)

**Figure 3.7** Post-mortem data from three-spined sticklebacks experimentally infected with *Schistocephalus solidus*, showing the effect of host sex and infection level (single/multiple) on parasite index.
3.4.5 Effects of infection on host biology

There was no effect of infection status on fish SL\textsubscript{70}, BCF and HSI (Table 3.2; Figure 3.8 A C D); however, \textit{S. solidus} infection had a strong effect on fish mass, with infected fish showing a lower body mass than non-infected fish after 70 d of infection (Figure 3.8 B). None of these variables differed significantly between males and females in the study (Table 3.2; Figure 3.8 A, B, C and D). When testing changes in mass controlled for initial size by calculating the specific growth rate (SGR) over the post-exposure period, which allowed to measure fish growth after exposure, no significant differences were found between infected and non-infected fish and no variation in SGR between fish sex (Table 3.2).

The spleensomatic index was significantly affected by infection status but not by host sex (2-way ANOVA: infection: $F_{1,59} = 39.97, P < 0.0001$; host sex $F_{1,59} = 0.03, P = 0.874$; Figure 3.9 A), with infected fish of both sexes having higher SSIs than non-infected fish. There was no interaction between infection status and host sex ($F_{1,59} = 1.05, P = 0.311$).

Among exposed fish, significant differences were found between infected and non-infected females with respect to GSI (ANOVA: $F_{1,29} = 5.82, P = 0.023$; Figure 3.9 B), infected females had significantly reduced GSI. When considering exposed males only, males had significantly reduced GSI values compared to non-infected fish (ANOVA: $F_{1,29} = 4.36, P = 0.046$), there was a significant difference in the KSI of infected and non-infected males (ANOVA: $F_{1,29} = 4.51, P = 0.043$; Figure 3.9 C), with infected males having lower KSI than non-infected males.
Table 3. ANOVA results showing the impact of infection status and fish sex on measurements of Final body length (SL$_{70}$); Fish mass (M$_{70}$-M$_p$), Body condition factor (BCF), Hepatosomatic index (HSI) and Specific growth rate (SGR). There were no significant interactions for any of the measured variables. P values that are significant are given in bold.

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Figure 3.8 The effect of *Schistocephalus solidus* infection and fish sex on measurements of body condition in experimentally exposed three-spined sticklebacks. The mean +/- standard error. (A) Final Length (SL 70); (B) Fish mass (M70*M0). (C) Body condition factor (BCF) and (D) Hepatosomatic index (HSI). Separated by fish sex (females: white bars; males: black bars).
Figure 3. 9 The effect of *Schistocephalus solidus* infection and fish sex on measurements of body condition in experimentally exposed sticklebacks. The mean +/- standard error. (A) Spleensomatic index (SSI) separated by fish sex (females: open bars; males: black bars); (B) Females gonadosomatic index factor (GSI) C- Kidneysomatic index (KSI) in males only.
3.4.6 Relationships between fish mass ($M_{70-M_p}$), length ($SL_{70}$) and condition indices (BCF, HSI) with parasite index (PI)

Analyses were therefore carried out in order to determine whether fish sex and parasitic infection level (single vs multiple infections) had an effect on fish mass, when controlling for parasite index. Two-way ANCOVA, with PI as a covariate, showed that fish mass was significantly affected by $S. solidus$ PI during the post-exposure period ($F_{1,35} = 4.92, P = 0.035$), since fish with higher PIs exhibited reduced body mass. However, there was no significant effect of sex ($F_{1,35} = 0.09, P = 0.77$; Figure 3.10) or parasite infection level ($F_{1,35} = 3.48, P = 0.073$; Figure 3.10) on fish mass. The gradient of the relationship between $M_{70}$ and PI was not found to differ significantly with fish sex ($F_{1,35} = 0.01, P = 0.921$; Figure 3.10) or infection level ($F_{1,35} = 2.99, P = 0.095$; Figure 3.10).

![Graph](image.png)

**Figure 3. 10** Relationship between parasite index (PI) and fish mass of three-spined sticklebacks experimentally infected with *Schistocephalus solidus*. Data was pooled for each sex separately regarding their infection level (single or multiple) with a regression line for each group.
Among all infected fish, host BCF was significantly affected by PI (ANCOVA: $F_{1,35} = 4.39, P = 0.045$; Figure 3.11), with fish having higher PIs exhibiting reduced BCF. There was no significant effect of either host sex or the level of infection (ANCOVA: host sex: $F_{1,35} = 1.13, P = 0.296$; infection level: $F_{1,35} = 0.22, P = 0.646$; Figure 3.11) on BCF. There were no differences in the gradient of the relationship between BCF and PI between the fish sexes ($F_{1,35} = 1.03, P = 0.319$; Figure 3.11) and between parasite infection level ($F_{1,35} = 0.38, P = 0.542$; Figure 3.11). The effect of host sex and infection level was not significant for all other variables for a given PI (Table 3.3).

Figure 3.11 Relationship between parasite index (PI) and body condition factor (BCF) of three-spined sticklebacks experimentally infected with *Schistocephalus solidus*. Data was pooled for each sex separately with regards to their infection level (single or multiple) with a regression line for each group.
Table 3. Results of two-way ANCOVA presenting the effect of three-spined sticklebacks sex and *S. solidus* infection level on SL$_{70}$ and HSI with PI as a covariate after 70 days of experimental exposure.

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<th>Sex</th>
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<td>P value</td>
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<td>0.740</td>
<td>0.559</td>
<td>0.714</td>
<td>0.849</td>
</tr>
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</table>

3.4.7 Effect of host factors on parasite eggs output

Among all the worms collected, there was a highly significant positive relationship between parasite mass and total egg output ($F_{1,33} = 18.85$, $P < 0.0001$; Figure 3.12). Heavier plerocercoids developed into adults that produced more eggs. After accounting for this highly significant effect of plerocercoid mass on egg output, neither host sex (ANOVA: $F_{1,33} = 0.03$, $P = 0.856$; Figure 3.13) nor infection level ($F_{1,33} = 0.013$, $P = 0.718$; Figure 3.13) were found to have a significant effect on parasitic egg production. This means that whether plerocercoids develop in female or male fish does not affect egg production and, also, that worms recovered from singly infected fish did not show higher egg output than worms from multiply infected fish for a given plerocercoid mass.
Figure 3. 12 The relationship between plerocercoid mass and egg output for \( n = 36 \) plerocercoids recovered from host three-spined sticklebacks infected with \( S. \) solidus.

Figure 3. 13 Predicted egg output from the \( Schistocephalus solidus \) parasites of infected three-spined sticklebacks after 70 d of exposure, as plotted against parasite mass and separated by fish sex and infection level.
3.5 Discussion

3.5.1 The effect of host size and sex on *S. solidus* infection

In this study, I tested the hypothesis that host sex and body size are two potentially important factors influencing the interaction between host and parasite. First, I tested whether there was a sex difference in the susceptibility of male and female sticklebacks to infection by *S. solidus* parasites. One potential outcome was that males might be expected to be more susceptible than females, as male-biased parasitism is often reported due to the cost of sexual selection in vertebrates (Zuk and McKeen, 1996). After male and female lab-bred fish were exposed to controlled doses of procercoids, they were kept under similar lab conditions corresponding to a breeding environment in terms of temperature and daylight in order to exclude the role of environmental factors.

In this current study, host sex was found to not be important as a factor in determining fish susceptibility to *S. solidus* infection. At the end of the experiments, both sexes showed a similar prevalence of infection at 60%, meaning that there was no sex-based bias in terms of susceptibility to *S. solidus* infection. Variations in parasitism between both sexes has been reported in the studies of a wide range of wild animal hosts including fish (Tierney et al., 1996), amphibians (Tinsley, 1989), birds (Poulin, 1996) and mammals (Folstad et al., 1989).

The previously reported sex-related differences in terms of parasite infection found in previous studies may therefore be more likely explained this can be by differences in ecological factors, differences in host behaviour, physiology and immune competence. High levels of testosterone in males might result in immunosuppression as a cost of sexual selection leading to a sex-based bias in parasitism between individuals. Recent studies have clearly investigated the role of 11-Ketotestosterone in stimulating secondary sexual characters in male three-spined sticklebacks, and it has been positively correlated with male ornamentation (Mayer et al., 2004, Mayer and Páll, 2006) but negatively with immunocompetence (Kurtz et al., 2007). Furthermore, a recent study by Macnab et al. (2011) used *S. solidus*-infected stickleback males from two UK populations, and despite being infected, some males were able to exhibit normal reproductive behaviour and were
able to breed normally. However, generally the infected males showed an 11-ketotestosterone titre that correlated negatively with infection severity. In the present study, despite these costs of reproduction associated with immunity reduction, and since the experiment was done in the summer during the fish breeding season, our findings still did not indicate any differences between the sexes. These results suggest that there are other factors that might influence fish susceptibility to *S. solidus* infection leading to a sex-based bias between individuals in natural populations, such as habitat use, diet and behavioural traits, which is consistent with other sex difference studies (Pennycuick, 1971a, Reimchen and Nosil, 2001).

There is evidence from previous studies that being in a group of same species or even the sex, it might increase host immunity, as being in social environment is a further important factor that can affect animal immune response strength (Schmid-Hempel, 2011). In fish host, sex-steroids and changes in immune parameters might together play an important role in increase fish susceptibility to pathogens (Milla et al., 2011). Mating competition leads to physiological reproductive trade-offs for both sexes, which can result in higher levels of stress and immunity reduction (Clutton-Brock and Parker, 1992, Zuk and McKeen, 1996, Morales-Montor et al., 2004). The immunocompetence handicap hypothesis suggests that immune function in males may be negatively associated with their secondary sexual traits (Folstad and Karter, 1992).

As fish in this current study showed no significant differences in their susceptibility to *S. solidus* infection between sexes, and the experimentally exposed fish were raised individually and have not experience mating or other competition such as space and food etc. Therefore it is possible that male fish had similar immune response level as females; and that potentially other factors such as social immunity, mating competition or other competition (space, food etc.) effects can be excluded as factors causing male immune reduction.

Differences in prevalence of *Schistocephalus* infection were also noticed in smaller wild fish caught when under one year old (Pennycuick, 1971a). In this current study, the experimental design allowed to investigate fish body size as a factor in determine their susceptibility to *S. solidus* and also if fish length at the end of the study was
affected already after acquired the infection. In contrast to Simmonds (2015) study, who found a significant effect of three-spined sticklebacks' body size as essential factor to increased fish susceptibility to \( S. \ solidus \) infection without interaction with age. The author found that smaller three-spined sticklebacks showed higher susceptibility to infection than larger fish after exposing them experimentally to a controlled dose of \( S. \ solidus \) procercoids. However, in our study, fish size was found to have no significant effect on fish susceptibility to \( S. \ solidus \) infection following experimental challenge to controlled levels of infection.

In fish, age and body size are usually linked; particularly in parasite-host growth rate studies, the establishment of age-dependent parasitic species should not be affected by host size, and they should therefore be present in most host age and size classes (Zelmer and Arai, 1998). Ryce et al. (2005) found that rainbow trout (\( Onchorhynchus \ mykiss \)) which were less than 9 weeks post-hatch and at least 40 mm in fork length were more susceptible to \( Myxobolus \ cerebralis \), whirling disease, and they became more disease resistant when older and larger. Adult sea bass (\( Dicentrarchus \ labrax \)) and sea bream (\( Sparus \ auratus \)) were on average significantly lighter and shorter when infected with the isopod \( Ceratothoa \ oestroides \) in Turkish aquaculture than non-infected fish (Horton and Okamura, 2001). Although the age of all fish in the same cage were known, the authors hypothesised that increased susceptibility to this parasitic infection might be attributed to keeping fish in high densities, which might have led to increased fish stress, thus inhibiting their growth.

### 3.5.2 Effects of \( S. \ solidus \) infection level on the body condition and reproductive development of sticklebacks

In the wild, infection with the cestode \( S. \ solidus \) is usually associated with noticeable variations in the body condition of infected fish compared with non-infected fish (Pennycuick, 1971a, Tierney et al., 1996). The results of the present lab study found no significant differences in BCF between infected and non-infected fish, and no sex-based differences were found either. This would suggest that fish in this study were able to acquire and ingest sufficient food for both the host and the parasite(s) they harboured, most likely due to the fact that food was provided in abundance, which is in contrast to the natural situation.
Several studies strongly suggested that infected fish had lower BCF values than non-infected fish in wild studies when seasonal changes were found to have a crucial effect on infected fish; this was especially so in winter they were in very poor physical condition (Pennycuick, 1971a, Tierney et al., 1996). A similar observation was made for lab studies (Macnab, 2012) when experimentally infected females showed a lower BCF than sham exposed fish. In another experimental study, infected fish likewise had a lower BCF than non-infected fish, especially within the Eda genotypes with low plated allele leading to lower body condition than other genotypes (Simmonds, 2015).

The medium-term energetic status of a host can be measured by hepatosomatic index (HSI) (Chellappa et al., 1995). The HSI in this study showed a similar pattern to BCF as no differences between sex or infection status were found. Since fish in the current study were fed blood worms, *Chironomus* sp., *ad libitum* there were no constraints on food intake, which suggests that infected fish were able to assimilate sufficient energy from their daily food intake to meet their needs. This result is similar to that of Barber et al. (2008), as found in their review of experimental stickleback–*Schistoscephalus* infection studies.

Infected fish exhibited larger spleens than non-infected individuals; therefore, spleensomatic index (SSI) was higher in infected fish, though there were no apparent differences between males and females in this regard. This was an expected result, because spleen enlargement is often associated with fish immune response against infection (Seppänen et al., 2009).

The results of this study showed that infected three-spined stickleback males had reduced gonadosomatic (GSI) and kidney-somatic (KSI) indices than non-infected males, which is consistent with data obtained from other studies that suggested that *S. solidus* infected males are unable to breed (Arme and Owen, 1967, Pennycuick, 1971c, Tierney et al., 1996). In contrast, other, more recent, studies have investigated the potential reproductive ability of infected wild males from two fish populations in the UK (Macnab et al., 2009, Macnab et al., 2011). The authors found that infected males were able to exhibit normal reproductive behaviour and they could engage in breeding whilst infected. Further support for this finding can be
found in the study of Geraudie et al. (2010), which found that the gonads of roach (*Rutilus rutilus*) infected with *Ligula intestinalis* could still develop and the affected fish were still able to breed. The reasons behind reproductive differences between stickleback populations in the UK remain unknown but it might possibly be due to the evolutionary interaction that has developed between *G. aculeatus* and the cestode *S. solidus* (Macnab et al., 2009), therefore, in some populations hosts have adapted better to the parasites and are able to maintain reproduction.

### 3.5.3 The effect of fish sex on infection level and parasite growth

In the present study, host sex, size and body mass were not found to have any effect on total plerocercoid mass; however, the parasite index was higher in females than males. These findings are consistent with a wild fish study by Pennycuick (1971a) where infected female three-spined sticklebacks had a higher PI than males, although there were fewer number of parasites established in female fish, but they had a higher mass than the worms developed in male host. The author noted the small parasite size in males generally, that there were feeding habit differences between the sexes, and the higher parasitic nutrient theft in order for them to grow rapidly in females who invest more energy in egg production (Pennycuick, 1971a). Since all the fish used in this current experiment were lab bred and well fed, there were no possible differences in feeding limitations between the sexes. There is some agreement with Pennycuick (1971a) opinion that females provide a better environment for rapid plerocercoid growth by consuming more food than males, as I found that female fish had a higher PI and lower body mass than males. In a survey of *G. aculeatus* parasite communities in a pond in Farnley, Leeds (UK), Arme and Owen (1967) found a negative relationship between fish mass and parasite index, and that heavier fish had a smaller PI. In the current results, there was a negative relationship between fish mass and PI. The reason for this finding is unclear as the fish were housed individually and no food competition with other fish existed. Possibly, this was due to an adaptive strategy of energy investment by female hosts that was not exhibited in males.

Infected females in this study had significantly reduced GSI compared to non-infected females. In contrast and support to these findings, Heins and Baker (2008)
observed some infected females in Alaskan populations were unable to produce eggs clutches and the other were retained the ability to produce eggs. Heins et al. (2010) found that clutch mass, egg numbers and ovum mass were negatively associated with severity of infection, with a higher parasite index host showing a greater reproductive performance decline. The authors suggested that infected females that had a PI of more than 10% were able to develop mature eggs, which strongly supports the “side effect” hypothesis that nutrient theft by parasites leads to reproductive disruption in three-spined sticklebacks (Heins and Baker, 2003, Schultz et al., 2006). Higher food demand will be exhibited whenever an increase in parasite size can be associated with decreasing reproduction metrics (Heins et al., 2010).

As the lab-bred fish in the current experiment were exposed within the breeding season and were housed individually for 70 d during this time, it was difficult to investigate if fish were able to produce eggs or not as it was difficult to distinguish between ovulation swelling in gravid females that were ready to spawn or if the appearance of a swollen body was actually due to the increasing size of the growing parasites. However, after fish were dissected it was clear that some of the infected females had developed small ovaries with immature eggs. One possible explanation for this result is that fish showed a lower energy investment in reproduction as a result of the nutrient theft cause by S. solidus infection. Barber and Svensson (2003) observed that sticklebacks experimentally infected with S. solidus developed significantly larger ovaries than controls, meaning that they showed successful gonad growth patterns despite the development of the infection. The authors suggested four reasons behind infected females’ gonad growth, which are that females exhibited an adaptive strategy in favourable environmental conditions, side effects of the infection, that the parasite could easily manipulate host energy resources, and finally early sexual maturation lead to increased susceptibility to infection.

3.5.4 Conclusions and Future work
The experimental infection study in this chapter yielded information which suggests that host sex and body size have no significant effects on increase the susceptibility of stickleback hosts to S. solidus infection. Observed sex differences in S. solidus
infections in three-spined sticklebacks might therefore be more likely to be due to ecological factors, immunity, or feeding habits in wild fish. However, the reasons for the published differences are not clear, and future experimental studies regarding these factors are still required. Infection had a strong effect on the GSI of both sexes, therefore more research is required to investigate if infected wild population fish are still able to spawn. However, the parasite index in female fish was higher than in male fish. Parasite growth is possibly affected by host nutrition (Barber, 2005), parental genetic effects, parental immunity and environmental factors, and so there is the potential for population differences in three-spined sticklebacks’ susceptibility to *S. solidus* infection. Future studies should focus on population variations and how genetic and environmental factors interact to create sex-based biases in the dynamics of stickleback host-parasite interactions.

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Chapter 4

Consequences of sticklebacks provenance, morphology and \textit{Eda} genotype for \textit{Diplostomum spathaceum} infection
4.1 Summary

Host provenance, age, sex and body size are important factors that potentially influence the interactions of hosts with their parasites, affecting their susceptibility to infection and the progression of disease. Experimental estimates of the effects of fish morphology on parasite infection might help in understanding the evolutionary relationship of host-parasite interaction. I used experimental exposure studies of three-spined stickleback, *Gasterosteus aculeatus*, to examine how host variation influences the outcome of parasite infections. Fish that varied in body size and sex were exposed individually to 100 infective *Diplostomum spathaceum* cercariae. I found a significant effects of provenance (host population) and host sex on infection susceptibility. Associations between infection levels and a range of host fitness indicators including energetic condition and immunity status were measured further. Three-spined stickleback populations that have invaded freshwaters showed a reduction in the number of lateral plates, although the reason behind these evolutionary variations is not well known. In the present study, therefore, a Carsington Reservoir population that exhibits a genetically and morphologically determined variation in plate number was used to examine the association between parasite infections and *Eda* genotype, plate number, age and sex as a possible factor of determine fish susceptibility to infection. Fish with more lateral plates were found to show increased parasite loads. The implications of these results for stickleback evolution are discussed.
4.2 Introduction

4.2.1 Host ecology and susceptibility to parasitic infection
Parasites are widely used as a biological indicators to understand host biology and evolution (Williams et al., 1992). Macroparasites have developed a range of adaptations to achieve the successful transmission between definitive hosts (Thomas et al., 2005). Parasitic infections can show remarkable variability between wild populations of the same host species, leading to differences in species diversity and an abundance of infection within populations (Bagge et al., 2004). In fish hosts, it is widely recognized that this phenomenon might be attributable to habitat type (ecosystem, lakes or rivers), host population size, parasite species (Kalbe et al., 2002, Sures, 2004, Eizaguirre et al., 2011) and to physical and chemical environmental effects (Hartvigsen and Halvorsen, 1994, Sures, 2004). For example, total fish population size was reported to be the main factor in determining the variations in the mean number and abundance of three species of monogeneans Dactylogyrus and one Gyrodactylus infection between individuals of the crucian carp (Carassius carassius) in nine isolated ponds in Finland that held no other fish species (Bagge et al., 2004).

Spatial variation in parasitism is generally observed in intermediate and definitive host populations. Host-parasite interaction can be complex and can sometimes show a spatial adaptation, as mediated by environmental conditions in different habitats. In a natural habitat, the consequences of any interaction between species can differ across geographical distance, creating a geographical mosaic view of coevolution (Thompson, 1999). In some parasite community studies, a distance decay of similarity (the similarity between two observations often decreases or decays as the distance between them increases (Tobler, 1970)) have been reported (Tobler, 1970, Poulin, 2003). Spatial variation in the diversity of parasite species has been found to be associated with variations in environmental factors (Krasnov et al., 2005). Such a negative relationship between distance and similarity in parasite load has been found, for example, to show a significant decay of similarity in the composition of parasite communities of three coastal marine fish species, as reported by Oliva and Teresa González (2005).
Local adaptation in a host-parasite system that is driven by natural selection is one of the fundamental principles of evolutionary theory (Hereford, 2009). According to this theory, the host should become more susceptible to infection by local parasites; however, the parasites themselves should become adapted to the local conditions of their hosts’ environments. Furthermore, parasites have to adapt to host evolution, leading to oscillatory dynamics in both host and parasite allele frequencies (Krist et al., 2000). Nonetheless, an allopatric host should be more susceptible to a parasite drawn from their local population than a parasite from a sympatric population (Lively, 1999).

4.2.2 The genetic basis of host susceptibility to parasite infections

In natural populations, host fitness can be modulated by two important factors: their genetic background, and environmental conditions. Efficiency of natural selection and maintenance of polymorphism might be negatively affected by environmental variations providing alternative host genotypes that perform better in distinct environmental states than in others (Lazzaro and Little, 2009). One example is resistance genetic variation in response to changes in local environmental conditions. Highly significant genotype-by-environment interactions have been found in variable temperature backgrounds in three different populations of fruit fly (Drosophila melanogaster) (Lazzaro et al., 2008). The authors suggested that local adaptation to geographically heterogeneous environments and genotype-by-environment interactions may explain the increase in resistance to bacterial infection for each population.

In natural populations, parasites have provided a measure of the genetic diversity among plant, animal and human hosts (Coltman et al., 1999b). This is clearly represented in an increased susceptibility to gastrointestinal nematode parasites in inbred Soay sheep (Ovis aries) than in outbred individuals as measured by increasing microsatellite heterozygosity leading to high genetic variations which might be driven by parasite-mediated selection (Coltman et al., 1999).

One well-understood example of the importance of genetics in determining susceptibility to parasite infection, and thus for the coevolution of hosts and parasites, are the genes of the vertebrate major histocompatibility complex (MHC).
MHC genes are responsible for initiating the vertebrate immune response to infection by pathogens, including parasites. The MHC gene is highly polymorphic and therefore a larger allele diversity has been recorded across a wide range of individuals within the population of vertebrates (Godot et al., 2000, Wegner et al., 2003, Spurgin and Richardson, 2010).

Brandt's voles (Lasiopodomys brandtii) showed an association between the nematode-mediated selection burden and specific rare MHC alleles which may increase/ decrease susceptibility and resistance to infection between individuals in a population. The lack of MHC genetic diversity and parasitic load varied between geographical locations among populations, which might give an advantage to the selective parasite species in terms of infecting a new host (Zhang and He, 2013).

In another study, a reduced infective load of the nematodes Aspiculuris tetraptera in the bank vole (Myodes Glareolus) was associated with an intermediate number of MHC alleles; however, negative relationship was reported between high parasitic loads and the number of MHC alleles (Kloch et al., 2010). Malaria infections have also been found to be associated with MHC variation in bird species; where there are between three and 23 functional MHC alleles in flycatchers, malaria infection was found to be decreased with increasing MHC diversity (Radwan et al., 2012). A similar/ comparable diversity in MHC alleles has been found across 13 populations of house sparrows (Passer domesticus). Under selection pressure, a new host’s allelic lineages emerge to create a specific MHC alleles-infection relationship with malarial parasites, Plasmodium relictum, indicating that allele diversity has been driven by population-specific infection pressures (Loiseau et al., 2011). Furthermore, MHC allele diversity was found to vary between individuals in the same population. In individual males of the common yellowthroat (Geothlypis trichas), mask size and long survival to Plasmodium spp. infection was positively correlated to the number of MHC class II alleles. Furthermore, MHC allele diversity has been linked to mating ornaments as males with a larger number of MHC alleles were actively been chosen by females for mating and showed more resistance to malaria infection, consistent with the good genes model of sexual selection (Dunn et al., 2013).
In fish, diversity in MHC gene has been found in wild populations among 14 species of cyprinid fish, which have shown high genetic diversity in the exon 2 of the MHC in the spleen of fish infected with ectoparasites. This suggests that immunity plays a role in protecting each fish species from high parasitic pressure by maintaining immune gene diversity, leading to a decreased mortality rate (Šimková et al., 2006).

Due to the wide distribution of sticklebacks across different habitats, environments have significantly affected their genetic background, and allelic richness is considerably varied between sympatric species pairs due to the contrasting parasite communities of varied habitats (Matthews et al., 2010). Sticklebacks provide an interesting model for the relationship between MHC diversity and parasite pressure in different environments, as they are widely distributed geographically, and have colonised a wide range of habitats across their geographic range. It has been shown that exposure to very different environments is reflected in considerable individual variation in MHC diversity. It has been suggested that this MHC diversity is optimised according to habitat-specific parasite pressures: *G. aculeatus* from a river in northern Germany showed lower MHC class II B diversity than isolates from a sympatric lake (Wegner et al., 2003).

In three-spined sticklebacks, MHC class II B has been found to vary between habitats with lowest allele diversity in river fish, *G. aculeatus*, from a river in northern Germany that showed lower MHC class II B diversity than a sympatric lake (Wegner et al., 2003). The authors found that female fish show highly mate sexual selection by aiming to have ten different MHC alleles to combine (from their own and from males), so that offspring fitness of progeny will be maximized, as five alleles on average match the optimal individual response to minimum level of parasites infections. Therefore, this natural and sexual selection could interact, resulting in the MHC polymorphism found in the sticklebacks’ wild populations, and which might thus be driven by parasitic infections (Wegner et al., 2003). Similarly, in three-spined sticklebacks fish collected from different Icelandic populations showed a high MHC class II B diversity that was related to a lower prevalence of parasitic infection and parasite load which differed between habitats (Natsopoulou et al., 2012). These
findings suggest that individual allele diversity in sticklebacks is optimised according to habitat-specific infection pressures.

4.2.3 Sticklebacks plate morphology, *Eda* gene and parasite infections

Ectodysplasin is a protein that encoded by the *Eda* gene which is responsible for ectodermal organ development throughout the animal kingdom (Mustonen et al., 2003). Genetic studies indicate that *Eda* plays a key role in lateral plate development evolution in natural populations of three-spined sticklebacks (Colosimo et al., 2005). These authors found that two genetic markers, STN 380 and STNS 381, can be used to identify *Eda* alleles in sticklebacks.

Marine sticklebacks have a continuous row of 32 to 36 armour plates extending from head to tail represented (completely plated alleles). After invasion of freshwater habitats, *G. aculeatus* have evolved repeatedly in response to their new environment. Freshwater three-spined stickleback populations exhibit a varying reduction in the number of plates, with a maximum of 9 plates in the low-plated morph (Bell and Foster, 1994). Several factors have been proposed as explanations for this variation in plate number between habitats: low calcium levels (Giles, 1983); enhanced swimming performance (Bergstrom, 2002) and avoidance of predators in freshwater populations (Bergstrom and Reimchen, 2000, Reimchen, 2000). Another explanation, however, is that of habitat differences in freshwater fish (but not in marine) parasite communities, creating a selection pressure on the fish plate morphology (Simmonds, 2015).

In three-spined sticklebacks, the association between parasite infections and plate number has been investigated, for example in fish infected with the cestode *Schistocephalus solidus*, where the prevalence of infections was markedly decreased in the completely plated morph (Morozińska-Gogol, 2011). A few studies have compared the parasitic fauna between freshwater and marine fish without considering the effects of the *Eda* gene and plate number as possible contributory factors to stickleback evolution (Simmonds, 2015). As marine fish are completely plated, they were also found to be more susceptible to exposure to freshwater parasites including the cestode parasites and *Diplostomum* sp. than freshwater fish (MacColl and Chapman, 2010). The authors suggested that immunological
resistance, changes in behaviour and host local adaptation to the native parasites are the reasons for increased marine fish susceptibility, and so supports the role of parasites in selection against migrants and population diversification. In three-spined stickleback, Eda locus haplotype is correlated with the expression of important immune genes (Robertson et al., 2017). The authors found that different Eda genotype is associated with differences in overall immune gene expression when F2 hybrid fish of two lake stickleback from North Uist, Scotland were experimentally exposed to ectoparasites.

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

The three-spined stickleback, *Gasterosteus aculeatus*, is a small teleost fish. They are native to a range of different aquatic environments, both marine and brackish aquatic environments. After freshwater invasion, three-spined sticklebacks developed evolved responses to new habitats through repeated reductions in body size and depth and number of armour plates. They are widely distributed across freshwater sources (Wootton, 1976, Peichel and Boughman, 2003). Marine sticklebacks typically have a continuous row of 32 to 36 armour plates extending from head to tail, in contrast with a gap in the middle of the row of plates in partial morph or a few plates less than nine in freshwater populations, as per Figure (4.1) (Bell and Foster, 1994, Deagle et al., 1996).

Due to its size and ease of rearing under laboratory conditions, the stickleback has become widely used as a model in behavioural, ecological, evolutionary, and molecular genetic studies. This makes sticklebacks a particularly useful model for studying host-parasite interactions (Barber, 2007, Katsiadaki et al., 2007, Barber and Nettleship, 2010), as well as a model organism for evolutionary and ecological research (Barber, 2013). In spite of its complex life cycle which consists of five stages, *Diplostomum spathaceum* is one of the most common parasites in both wild and aquacultural populations of freshwater fish (Kennedy, 1974), and sticklebacks are one of more commonly infected fish as intermediate hosts.
4.2.5 Aims
In the first set of experiments, I aimed to establish the relative importance of host body size, sex and population in determining susceptibility to Diplostomum infection. I addressed this aim through two laboratory experiments using controlled infections. This experiment took advantage of differences between two genetically separated freshwater populations from two distinct habitats (Carsington Reservoir and Llyn Frongoch). This was done because genetic differences between host populations might give rise to variation in susceptibility to parasitic infections. A previous study found considerable differences in the prevalence of infection with Diplostomum sp. across populations of naturally infected three-spined sticklebacks depending on the habitat type (Kalbe and Kurtz, 2006). Morphological variation in plate number is mediated by genetic variation in the Eda gene; to test if plate number and Eda genotype are potential factors in determining susceptibility to infection, the second experiment focussed on fish from the Carsington Reservoir population, which shows large variation in their plate number. Fish from this population provide an ideal opportunity to examine experimentally whether variation in plate phenotype and Eda genotype affects parasitic load, and how this may be related to the effects of sex and age.
Figure 4.1 Lateral plate morphs of the three-spined stickleback, *Gasterosteus aculeatus*, adapted from Taugbøl et al. (2014). I: complete plated morph; II: partial plated morph and III: low plated morph.

4.3 Methods

4.3.1 Fish stocks
Juvenile three-spined sticklebacks were used from lab-bred stock bred in June 2014 from wild caught parents originally captured at Carsington Reservoir (CAR), Derbyshire (53°3’30”N 1°37’50”W) and Llyn Frongoch (LF), Aberystwyth wales (52°21’37.1”N 3°52’40.5”W). Fish were reared in family groups in glass tanks (40 x 25 x 30 cm) and fed frozen blood-worms (*Chironomus* sp. larvae; 3F Fish food, The Netherlands) *ad libitum*. The water temperature was maintained at 16°C during experiments, and a photoperiod of 15 L: 9 D was used to mimic environmental conditions.
4.3.2 Experimental infection

4.3.2.1 Snails collection and cercariae identification

To obtain *Diplostomum* cercariae, freshwater snails were collected with a hand net from four locations in Leicestershire: River Soar (N 52°37’42.7”, W 1°08’33.0”), River Welland (N 52°28’33, W 0°55’29), Abbey park (52°38’43.6”N 1°08’01.6”W), Braunstone park (52°37’11.4”N 1°10’32.4”W). Snails were maintained in the lab in glass tanks under identical conditions in filtered freshwater and fed daily with washed lettuce *ad libitum*. To collect cercariae snails were placed individually in 15 ml universal sample tubes. Cercariae shedding was stimulated by exposing the tubes containing the snails to direct sunlight near a window or by using a bright bulb lamp. The appearance of cercariae in the water was observed first by the naked eye and then confirmed under a light microscope. For all experiments with *Diplostomum* during the two years period from October 2014 to September 2016, five species of snails (N=2655) were identified using the criteria of Beedham (1972) and Fitter and Manuel (1986) which are: *Potamopyrgus jenkinsi; Lymnaea stagnalis; Radix peregra; Planorbis corneus and P. vortex*. Parasite larval stages were identified according to Dawes (1946); Nasir and Erasmus (1964); Blair (1977); Niewiadomska (1986). Cercariae from seven species were found in this survey: *Echinostoma* sp.; *Apatemon* sp.; *Diplostomum spathaceum; D. phoxini; Notocotylus* sp.; *Cercaria ephemera* (Ephemera type after Dawes, 1946) and *Cyathocotyle* sp. (Figure 4.2).

4.3.2.2 *Diplostomum* species identification by DNA sequencing

The identification of many *Diplostomum* species is difficult due to their high morphological similarity. The molecular study used was based on cercariae samples to help with more reliable species identification. The molecular identification of the digenetic trematodes collected was based on DNA sequencing further, using mitochondrial cytochrome c oxidase subunit 1; *cox1* and nuclear (ribosomal internal transcribed spacer region ITS1-5.8S-ITS2) loci. The same batches of cercariae shed from the same snail were used for DNA extraction and as for experimental infection.
Figure 4. Cercariae recorded in the current study from the rivers Soar and Welland. A: *Notocotylus* sp. B: *Echinostoma* sp. C: *Cyathocotyle* sp. D: *Cercaria ephemera* (Ephemera type after Dawes, 1946). E: *Apatemon* sp. F: *Diplodostomum phoxini*. G: *D. spathaceum*.
To extract total genomic DNA, approximately 100 shed cercariae in 1 ml of ddH$_2$O were transferred to 1.5 ml microfuge tubes and put on ice to aid pelleting of the cercariae. Samples were then spun at 13,000 RPM at 4°C in a microcentrifuge for 10 minutes; the water was removed, leaving the pelleted cercariae behind. DNA was extracted using the same protocol as for fish samples following Breacker et al. (2017), except that 7 µl of 20 mg/ml proteinase K were added at the beginning to digest cercariae tissues. Extracted DNA was stored at −20°C until needed.

Sequences comprising the ribosomal ITS1-5.8S-ITS2 cluster of the rRNA gene was amplified by using the primers of Galazzo et al. (2002). Forward D1 (5’-AGG AAT TCC TGG TAA GTG CAA G-3’) and reverse: D2 (5’-CGT TAC TGA GGG AAT CCT GGT-3’); primers performed in a total volume of 25 µl (10 pmol of each primer) with 50 ng/µl of genomic DNA (gDNA). The following thermocycling profile was applied: denaturation at 94 °C for 2 min followed by 30 cycles (94°C for 60 s, 56°C for 60 s and 72°C for 2 min) and a final extension step at 72°C for 5 min). Partial fragments of the barcode region of the cox 1 mitochondrial gene Moszczynska et al. (2009) were amplified by polymerase chain reaction (PCR) by using the diplostomid-specific PCR primers Plat-diploCOX1F (5’-CGT TTR AAT TAT ACG GAT CC-3’) and Plat-diploCOX1R (5’-AGC ATA GTA ATM GCA GCA GC-3’) designed by Moszczynska et al. (2009). For PCR product analysis, 1% agarose gels (high resolution for DNA/RNA fragments 500-1000 bp) to which 0.5μl of ethidium bromide/ml was added were run.

PCR amplicons were purified using either a homemade procedure by using Isopropanole just in brief PCR fragments were transferred to new 1.5 ml tubes then 40 µl of 75% Isoproponol were added to each 10 µl sample of PCR product, samples were left at room temperature for at least 15 min then centrifuged for 30 minutes at 13°C. Samples were dried on a piece of tissue then 150 µl of 75% Isoproponol were added to each sample before being spun at 4,500 RPM for 2 min at 13°C. Samples were left to dry approximately 5-10 min at 65°C in heat block. Then, 20 µl of dH$_2$O were added after which the concentration of DNA in the samples was quantified using a Nanodrop™ 1000 (Thermo Fisher Scientific, UK). For PCR, 5 µl of the following primers were used (cox1: Plat-diploCOX1F and Plat-diploCOX1R) with 5
µl of PCR fragments samples. PCR Products were sent directly to GATC company for sequencing, contiguous sequences were compared with library database of sequences by using BLAST website.

4.3.3 Stickleback exposure to parasite infective stages

4.3.3.1 Experiment 1: Host susceptibility in relation to spatial variations

In this experiment (N= 118) fish were used. In the CAR population fish, 40 juvenile fish were exposed to parasites with 15 control fish being a sham-exposed and for LF, 40 juvenile fish were exposed to parasites with 23 control fish being a sham-exposed. After cercariae had emerged, 1 ml of cercariae suspension was measured using microcentrifuge tubes, and 100 cercariae per fish were counted for later exposure. Each fish was kept in a plastic tank with 1L of water, then cercariae were added by using glass pipette, fish were left for 24 h under regular aquarium light and temperature conditions.

Post exposure/sham-exposure, fish were housed in a glass aquaria (41 cm x 60 cm x 40 cm large tanks) containing artificial plants and gravel substratum. After 40 days of exposure, fish were euthanized using an overdose of Benzocaine anaesthetic (stock solution: 10 mg L\(^{-1}\)) according to UK Home Office Schedule 1 methods. Fish were blotted dry, measured using a dial calliper (standard length, SL\(_{40}\), to 0.1 mm), weighed (M\(_{40}\), to 0.001 g) and dissected. Eyes were removed and immediately dissected, the metacercariae were counted per eye using a stereo dissection microscope. Liver and spleen were weighed to calculate both hepatosomatic and spleensomatic indices using these equations: Hepatosomatic Index (HSI) = ([M\(_{liv}\) / (M\(_{40}\))] x 100); Spleensomatic Index (SSI) = ([Mspl / (M\(_{40}\))] x 100) according to the method by Pennycuick (1971c).

4.3.4 Molecular determination of Eda genotype

4.3.4.1 DNA extraction from skin swab samples

To obtain DNA samples for Eda genotyping, individual fish were chosen randomly from stock tanks and skin-swabbed without using any anaesthetic procedures following the procedure by Breacker et al. (2017). In brief, fish were blotted dry on a piece of tissue paper and swabbed with a sterile cotton swab stick (swab virus
transport plastic stick, VWR International Ltd, UK) for at least ten times from the operculum to the end of the body. The swab was then returned to the sterile container and immediately taken through the DNA extraction procedure. The swab sample was placed in a 1.5 mL microcentrifuge tube containing 400 µl DNA extraction buffer (200 mM TRIS pH 7.5, 25 mM ethylenediaminetetraacetic acid pH 8.0, 250 mM NaCl, and 0.5% w/v sodium dodecyl sulphate) warmed to 55°C, and incubated at room temperature for 15 min. The swab was then removed and 400 µL of chilled isopropanol was added to the DNA solution and mixed three times. The DNA solution was then chilled at -80°C for 10 min, after which it was centrifuged for 10 min at 13,000 RPM, and the pellet washed with 190 µL 70% EtOH. After a further centrifugation step (2 min at 13,000 RPM) the DNA pellet was air-dried and resuspended in 30 µL ultra-pure H₂O. DNA was quantified using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific, UK).

4.3.4.2 Optimisation of PCR protocols for Eda

The PCR protocol used to amplify Eda genotype the following the protocol modified given by Colosimo et al. (2005) using STN382 primers. According to Simmonds (2015), PCR with the original Colosimo et al. (2005). STN382 primer failed to give any bands on electrophoresis gels; this failure to amplify the target was attributed to genetic differentiation between North American and United Kingdom stickleback populations. Therefore, the sequence of the forward STN382 primer was modified by Simmonds (2015) in a few bases that match the stickleback genome using Ensembl genome browser after (Fliceck et al., 2012, Jones et al., 2012). To become CCCTTAGAGACTGCTAGCAG, the reverse primer sequence was CTTGTCCGGATCATACGC, which produced the characteristic bands on agarose gels as described by Colosimo et al. (2005): a 150 bp single band in low plated fish; a single 218 bp band in completely plated fish; and double bands in partially plated heterozygous fish (Figure 4.3).

PCR reactions were in a 10 µl volume containing 5 µl Red Taq (Sigma, U.K.), 0.5 µl of forward primer, 0.5 µl of reverse primer at 10 µM concentration, 3 µl H₂O and 1 µl DNA sample (depending on the amount of DNA in the extracted sample). The PCR was found after trials to be optimal using 1 cycle at 94°C for 5 minutes, 35 cycles of
95°C for 30 s, 56°C for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min before returning to 4°C. A 4% agarose gel (high resolution for DNA/RNA fragments 10–500 bp; Sigma UK) was used to differentiate the 68 bp difference between the bands. A volume of 50 ml 1X TAE (Tris base, acetic acid and Ethylenediaminetetraacetic acid, EDTA) was used with adding 2 g high definition agarose and heating to dissolve; 0.5 μl ethidium bromide was added before pouring the gel. PCR products were run on a gel tray at 60 V 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 distinction between the bands from the low- and complete-plated alleles.

![Gel image](image.png)

**Figure 4. 3** Gel image showing the bands of PCR amplification products from the *Eda* gene obtained with primer STN 382 from three-spined stickleback DNA. LL: low-plated genotype. CL: partially plated genotype. CC: completely plated genotype.

### 4.3.5 Experiment 2: Morphological analysis of plates number

To allow the identification of plates, fish preparations were stained with Alizarin (Peichel et al., 2001), following the protocol of Dingerkus and Uhler (1977). After both eyes were removed to detect any *Diplostomum* infection, fish were dissected from one side of the body under a dissection microscope, after which they were fixed in 10% NBF (Formalin Neutral Buffer) for a minimum of 14 days. After this time, they were washed in ddH₂O for 10 minutes before keeping them in H₂O overnight. Specimens were immersed in Alcian blue stain (20 mg Alcian blue 8GX, 70 ml 100%
Ethanol, 30 ml glacial acetic acid) for 2 h 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. Specimens were then rinsed twice for 5 min in 30% saturated di-sodium tetraborate (Na\(_2\)B\(_4\)O\(_7\).10H\(_2\)O) solution (from saturated stock). Then immersed in 1% trypsin (Sigma, U.K.) in 30% saturated sodium borate, and incubated at 30°C for 48 h. Samples were then washed in 2% KOH twice for 5 min before immersion in Alizarin red solution (0.002g Alizarin complexone (C\(_{19}\)H\(_{15}\)NO\(_8\)), 100 ml 2% KOH) overnight. Specimens were cleared for visual analysis by immersing in bleaching solution (500 μl 60% H\(_2\)O\(_2\), 150 ml 0.5% KOH, 50 ml glycerol) for 48 h. Then transferred through a 0.5% KOH: glycerol series of 1:1, 1:3 and finally 100% glycerol for 2 h each. Specimens were stored in fresh 100% glycerol.

Digital photographs of the lateral plates of some fish were taken and plates were counted by using a dissection microscope. The method of Reimchen (1994) was followed to confirm the presence or absence of each of the possible 36 lateral plates. In this study, fish from completely plated genotypes were very rare so only a few fish were available for photographing. Low-plated fish frequently possessed only the cleithrum and plates 5-9, partially plated fish had 11–32 plates (See Figure 4.9 in Results).

4.3.6 Eda gene and stickleback plate morphology

All fish used in this study have bred from originally wild parents that showed substantial variations in armours number of selective gene. I was able to bred fish that varied in Eda genotype whilst controlling the offspring age to allow two generations to use. Juvenile three-spined sticklebacks were used from lab-bred stock bred in June 2014 and July 2015 from wild caught parents originally captured at Carsington Reservoir (CAR), Derbyshire (53°3’30”N 1°37’50”W). Experiments were carried out in 2016 using 160 fish from Carsington Reservoir (lab bred fish), divided between two genotype and two fish ages 80 for each genotype, these 80 fish were subdivided to a group of 40 fish each belonging to 0+ juveniles of the year and 1+ year for adult fish have used, 30 fish were exposed and 10 as a sham exposed.

After cercariae were emerged, fish exposed to 50 cercariae. Each fish was kept in a plastic tank with 1 L of water, then cercariae were added by using glass pipette, fish
were left for 24 h under aquarium conditions. Fish were exposed in May 2016, then housed in 100 L glass aquaria (41 cm x 60 cm x 40 cm large tanks) on a recirculating system maintained at 17 ± 1°C under a 16 L:8 D photoperiod to match natural conditions and fed daily with bloodworms (*Chironomus* sp. larvae).

After 60 days of exposure, fish were euthanized using an overdose of Benzocaine anaesthetic (stock solution: 10 mg L⁻¹) according to UK Home Office Schedule 1 methods. Fish were blotted dry, measured using a dial calliper (standard length, SL₆₀, to 0.1 mm), weighed (M₆₀, to 0.001 g) and dissected. Eyes were removed directly, metacercariae were counted per eyes, fish were kept directly in 10% NBF (Stock: 4 g Sodium dihydrogen orthophosphate dehydrate (NaH₂PO₄·H₂O), 6.5 g di-Sodium hydrogen orthophosphate anhydrous (Na₂HPO₄), 100 ml 37% Formaldehyde, 900 ml dH₂O) ensuring the entire body was covered for next staining procedure.

### 4.3.7 Statistical analysis

All statistical analysis was carried out in Minitab17 statistical software. Data were tested for normality and homogeneity of variance using Anderson-Darling and Kolmogorov-Smirnov analyses. Non-parametric data were normality transformed using logarithm, square root; if normality was still not achieved non-parametric statistical tests were used.

A 2-way ANOVA model was used to test the effect of both sex and population on fish susceptibility to *D. spathaceum*. A Tukey test within ANOVA was used then to compare the number of developing metacercariae between population and sex group. A series of multivariable linear models (LM) regression test were used to test the effect of province; sex and infection status on fish body conditions. Kruskal-Wallis test was used to detect differences in infection intensity between *Eda* genotype. A series of multivariable linear models (LM) regression analyses were used to test the relationship between the number of plates and infection status, host growth and other body conditions. ANOVA models were then employed to test the relationships between genotype, sex and age of fish host with other variables measured. By using ANOVA mixed effect model the correlation between the mean abundance of infection in right and left eye were tested. For all boxplots in this chapter the dark
line represents the median, the box shows the Q1-Q3 interquartile range (IQR) and the whiskers represent variability outside the upper and lower quartiles, outliers are shown as asterisks in figures.

4.4 Results

4.4.1 Host susceptibility in relation to spatial variations
To investigate whether the susceptibility of three-spined sticklebacks to *D. spathaceum* infection depends on host sex or provenance, I carried out a controlled infection set of experiments on laboratory-bred fish stock derived from two natural populations (Carsington Reservoir and Llyn Frongoch). The number of successfully established metacercariae in the eye lenses of three-spined sticklebacks after being exposed to 100 cercariae per fish was ranked by level of infection and is shown in Figure 4.4.

4.4.1.1 Host provenance and susceptibility to *Diplostomum spathaceum* infection
To investigate whether the susceptibility of three-spined sticklebacks to *D. spathaceum* infection depends on host sex or provenance. I counted the number of metacercariae in the eyes of male and female fish from two populations (Carsington Reservoir and Llyn Frongoch). The ANOVA analysis revealed that host susceptibility was affected by both sex and population, but differed between sexes in a population-dependent way, sex*population interaction (F1,80 = 10.85, P = 0.001; Figure 4.5, Table 4.1). In the Llyn Frongoch population, the infection intensity was more than three times higher in males than in females (mean number of metacercariae in females: 16.05 ± S.D 18.83.; in males: 56.33 ± S.D. 18.56); (GLM, Tukey's post-hoc P < 0.0001, Figure 4.5; Table 4.2). Males also had higher infection intensities than females in the sample from Carsington Reservoir, but the sex difference was much less pronounced (mean = 45 ± S.D. in males, 40 ± S.D. in females) and did not reach significance (GLM, Tukey's host-hoc P = 0.291, Figure 4.5; Table 4.2).
Figure 4. The number of successfully established *Diplostomum spathaceum* metacercariae in the eye lenses of three-spined sticklebacks belonging to two populations (Llyn Frongoch: Black Bars) and (Carsington Reservoir: White Bars) after being exposed to 100 cercariae per fish ranked by level of infection.
Table 4. 1 ANOVA table with the number of *D. spathaceum* metacercariae that established in the eye lenses of experimentally exposed three-spined stickleback as the response variable.

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Figure 4. 5 The effect of host sex and provenance on the intensity of *Diplostomum spathaceum* infection in experimentally exposed three-spined sticklebacks from two populations (Carsington Reservoir and Llyn Frongoch).
Table 4. 2 Post-hoc comparison using Tukey’s test showing the means of \textit{D. spathaceum} infection in the lenses of experimentally infected three-spined sticklebacks. CAR: Carsington Reservoir, LF: Llyn Frongoch, F: Females and M: Males; significant values ($P < 0.05$) are shown in bold.

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<th>$P$ value</th>
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<tr>
<td>(LF M) - (LF F)</td>
<td>40.28</td>
<td>(23.88, 56.68)</td>
</tr>
</tbody>
</table>

4.4.1.2 \textit{D. spathaceum} infection and fish body condition

To examine whether the host body condition of three-spined sticklebacks differed between infected and non-infected fish and male and female fish in two populations (Carsington Reservoir and Llyn Frongoch) multivariable linear models (LM) were used. The LF fish had a significantly smaller hepatosomatic index (HSI; $F_{1, 115}= 5.67$, $P < 0.019$) compared to Carsington, and across both populations males had a significantly smaller HSI than females regardless their infection status ($F_{1, 115}= 4.47$, $P < 0.037$). There was no significant interaction between population and fish sex ($F_{1, 115}= 0.58$, $P = 0.450$). Infection development had no effect on HSI on fish regardless their both sex and population ($F_{1, 115}= 1.14$, $P = 0.708$).
There was no evidence that sex-specific differences had an effect on HSI between infected and control fish (Sex*Infection Status: $F_{1,115} = 1.18$, $P = 0.280$). However, fish population had a strong effect on HSI in the term of infection status (Population*Infection Status: $F_{1,115} = 4.62$, $P = 0.034$; Figure 4.6). In the non-infected fish, HSI was lower than in infected fish in both populations (Post-hoc, $P = 0.028$; Figure 4.6), the mean number of HSI was LF: Non-infected: $-0.526 \pm 1.148$ S.D.; infected: $0.026 \pm 0.966$ S.D.; CAR: Non-infected: $-0.194 \pm 0.653$ S.D.; infected: $0.386 \pm 0.867$ S. D.

Figure 4.6 Boxplot showing the effect of *Diplostomum spathaceum* infection on hepatosomatic index (HSI) of all females and males Carsington Reservoir and Llyn Frongoch three-spined sticklebacks.
Among all the fish used in the experiment, there was no significant difference in spleensomatic (SSI) index between fish in both populations and sex regardless their infection status (Population: $F_{1,115} = 0.04$, $P = 0.840$); (Sex: $F_{1,115} = 0.01$, $P = < 0.931$), there was no interaction between populations and fish sex ($F_{1,115} = 0.04$, $P = 0.836$). Infected fish in both populations had higher SSI than control fish ($F_{1,115} = 6.09$, $P = 0.015$). There was no significant interaction between infection status and population ($F_{1,115} = 2.61$, $P = 0.109$; Figure 4.7), however, significant interaction between sex and infection status has recorded ($F_{1,115} = 4.11$, $P = 0.045$; Figure 4.7). Infected fish within both sexes had higher SSI than control fish (Post-hoc, $P = 0.002$) with a SSI mean in infected fish (Females: $0.514 \pm 1.003$ S.D.; Males: $-0.195 \pm 0.776$); control fish (Females: $0.051 \pm 0.665$; Males: $-0.562 \pm 1.146$).

**Figure 4.7** Boxplot showing the effect of *Diplostomum spathaceum* infection on spleensomatic index (SSI) of Carsington Reservoir and Llyn Frongoch three-spined sticklebacks used in the study.
4.4.1.3 Infection and fish length

There was a significant differences in final length related to both host provenance and sex. LF fish were significantly smaller than CAR fish ($F_{1,118} = 14.11, P < 0.0001$; Figure 4.8), females had the higher length values than males ($F_{1,118} = 7.07, P < 0.009$). Fish developing infections were smaller at the end of the experiment than control fish ($F_{1,118} = 20.89, P < 0.0001$; Figure 4.8). There was no significant interaction between fish population; sex and infection status (Population*Fish sex: $F_{1,118} = 0.001, P = 0.995$); (Population*Infection Status: $F_{1,118} = 1.52, P = 0.221$) and (Fish sex*Infection Status: $F_{1,118} = 0.96, P = 0.330$). Suggesting that the effects of infection on growth were similar between populations, the reduction in final length caused by infection was similar in both sexes.

Figure 4. 8 Boxplot showing the effect of *Diplostomum spathaceum* infection on final fish length of all females and males Carsington Reservoir and Llyn Frongoch three-spined sticklebacks used in the study compared with control fish.
4.4.2 *Eda*, plates number and susceptibility to *D. spathaceum* infection

4.4.2.1 Genotypes present, plates morph and fish growth

There were abundant variations in the number of plates according to their *Eda* genotype. Partial plated fish showed considerable range in mean number of plates between individuals and it exhibited higher plates number than low plated. All low plated fish had the cleithrum and between 2-9 plates (the median= 6, Q₁= 5, Q₃= 7) except one fish was recorded with 13 plates the (Figure 4.9 C). Partial plated fish had a much more variable number of plates ranging from 9 plates in some fish to highest number 32 in one individual (the median= 19, Q₁ = 14, Q₃ = 26) (Figure 4.9 B). As a result of rarely having fish with completely plated genotype (Figure 4.9 A), there were not enough fish in the two age groups for a meaningful statistical analysis (a few fish were used for staining only). Therefore, only partial and low plated fish were used in these experiments.

4.4.2.2 *Eda* genotype, plates number and susceptibility to infection

Carsington fish provide an ideal specific opportunity to examine experimentally whether variation in *Eda* genotype and plates number affected parasite load. The *Eda* genotype can be determined easily by PCR to give three genotypes: completely CC, partial CL and low plated LL. In this study, fish with completely plated genotypes were very rare so only CL and LL were exposed to infection.
Figure 4. Variation in the number of lateral plates in three-spined stickleback. Alizarin-stained, cleared preparations, one half of body and both eyes removed. A: completely plated fish. B: partially plated fish. C: low plated fish.
In this part of the study fish were experimentally infected by exposure to a constant number of *D. spathaceum* cercariae (50 per fish). After 60 days of exposure, I measured the intensity of infection by counting the number of metacercariae that had successfully established in the eye lenses.

Parasite intensity was found to be significantly associated with *Eda* genotype (Kruskal-Wallis: $H = 15.32, P < 0.0001$; Figure 4.10). Partial plated fish CL were more susceptible to *D. spathaceum* infection than LL fish.

![Box plot showing the number of *D. spathaceum* metacercariae established in the lenses of experimentally exposed three-spined sticklebacks presented by *Eda* genotype.](image)

**Figure 4.10** The number of *D. spathaceum* metacercariae that established in the lenses of experimentally exposed three-spined sticklebacks presented by *Eda* genotype as a significant predictor of infection; LL: Low plated CL: Partial plated.
The plates number differed between genotypes and there was also much more variability in plates number within partial plated fish (Figure 4.11). The plates number was used to detect whether having more plates could influence fish susceptibility to *D. spathaceum* infection. Among exposed fish, there was also a significant correlation between plates number and parasite level of the infection (Spearman's correlation: rs = 0.421, n= 119, P < 0.0001; Figure 4.12). Fish with higher numbers of plates were significantly more infected than those with fewer plates.

**Figure 4.11** Probability density plot for the plates number of experimentally exposed three-spined sticklebacks to *D. spathaceum* infection, separated by their *Eda* genotype; LL: Low plated CL: Partial plated.
4.4.2.3 The effect of infection on fish length

In order to determine whether *D. spathaceum* infection might influence fish size and if there is a difference between individual fish length based on their *Eda* genotype the effect of infection status and genotype on the fish standard length was tested. There was no significant differences between infected and sham exposed fish in their length (F$_{1,158} = 0.93$, $P = 0.336$). Fish length was not significantly differed between low and plated fish (F$_{1,158} = 2.48$, $P = 0.432$; Figure 4.13) There was also no interaction between *Eda* genotype and *D. spathaceum* infection, suggesting that the effects of infection on fish length did not differ between *Eda* genotypes (F$_{1,158} = 1.98$, $P = 0.171$).
4.4.2.4 Effect of host age and sex on fish susceptibility to *D. spathaceum* infection

All fish (N=120) were divided into two ages groups, young-of-the-year (i.e., less than 1 year old) and adults (1 year or older), these fish were of sufficient size to develop a full set of plates. Among exposed fish, there was a significant difference in infection level between the adult and juvenile fish that used in experiment (F₁, 119= 5.34, P =0.023, Figure 4.14). Adults fish seemed to be more susceptible to *Diplostomum* infection, (Median= 32, Q₁ = 27, Q₃ =40) than the juveniles fish, (Median= 28, Q₁ = 17, Q₃= 35).
Figure 4.14 The number of *D. spathaceum* metacercariae that established in the lenses of experimentally exposed three-spined sticklebacks presented by fish age as a significant predictor of infection.

As mentioned previously, fish with higher numbers of plates were more likely to become infected than those with a few plates. When host age was added as a factor into the linear model, plates number was still a significant factor (F$_{1, 119}$ = 3.94, $P$ = 0.050, Figure 4.15) and there was no significant interaction between plates number and age (F$_{1, 119}$ = 0.25, $P$ = 0.620, Figure 4.15).

There was no significant difference between sexes in the intensity of infection (F$_{1, 119}$ = 2.10, $P$ = 0.15; Figure 4.16) Females: (Median = 32, Q$_1$ = 23, Q$_3$ = 38). Males: (Median = 28, Q$_1$ = 21, Q$_3$ = 34). There was also no interaction between fish sex and plates number (F$_{1, 119}$ = 0.01, $P$ = 0.906; Figure 4.17). This suggests that the positive correlation between plates number and parasite load that recorded among all infected fish was not linked to fish age and sex but it was related to *Eda* genotype.
Figure 4. 15 Plates number of three-spined sticklebacks grouped by fish ages: adults and juveniles of the year, showing regression of *D. spathaceum* intensity of infection for each individual fish and their plates number.

Figure 4. 16 The number of *D. spathaceum* metacercariae that established in the lenses of experimentally exposed three-spined sticklebacks presented by fish sex as a predictor of infection.
4.4.3 Bilateral asymmetry of *D. spathaceum* infections in the eyes of exposed three-spined stickleback

In order to determine if there were bilateral symmetry arises when *D. spathaceum* cercariae had invaded fish body and finally established in the eye lenses. Statistical comparison between right and left eyes was used as a measure of whether parasites invasion showed one side consistently favoured in one eye than the other with given host factor (i.e. genotype, age and sex). Among all experimentally infected fish, the mean number of metacercariae developed in the lenses of three-spined stickleback was compared by ANOVA mixed effect model. Significant side (left-right) difference in infection severity, independent of age, sex or genotype was reported between exposed fish (Figure 4.18), showing that fish developing more infection in their left
eyes than the right. Although significant difference in metacercariae severity was reported between *Eda* genotype, there was no significant difference in severity between both eyes side regarding fish *Eda* genotypes (Table 4.3; Figure, 4.19 A).

![Intensity of Infection](image)

**Figure 4.18** Individual value plot showing distribution of differences in the abundance of *D. spathaceum* metacercariae between left and right eyes for each infected fish.

Age-dependent difference in *D. spathaceum* distribution between eyes was not significant between young and old fish (Table 4.3; Figure, 4.19 B). Fish sex had shown no statistical difference in the number of metacercariae between left and right eye (Table 4.3; Figure, 4.19 C). A significant sex-dependent difference in severity of parasite distribution between both eyes was found between adults and juveniles. According to GLM, Tukey’s post-hoc test, there is clear evidence that infection severity increases with age in females (*P* = 0.001), but no evidence for an age-related increase in males (*P* = 0.901).
Table 4. 3 ANOVA mixed effects model for the *D. spathaceum* metacercariae infection severity of exposed Carsington Reservoir three-spined sticklebacks measured as a parasite number in left and right eyes as the response. The side of infection (left or right eye), *Eda* genotype, fish age and sex were each used as predictor variables; significant values (*p* < 0.05) are shown in bold.

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</tbody>
</table>
Figure 4. The number of *D. spathaceum* metacercariae that established in left and right lenses of experimentally exposed three-spined sticklebacks presented by: A- Genotype; LL: Low plated, CL: Partial plated; B- Age: Adults and Juveniles and C- fish sex: Females and Males as a predictors of infection.
4.5 Discussion

4.5.1 Infection spatial variations

Fish susceptibility to eye flukes may be driven by many factors such as fish species, host immunity, host genotype, parasite infectivity level and parasite genotype (Sweeting, 1974, Lively and Dybdahl, 2000, Kalbe and Kurtz, 2006, Karvonen et al., 2011). In the current study, the three-spined stickleback’s susceptibility to the trematode *D. spathaceum* was found to be affected by both sex and population origin, but differed between sexes in a population-dependent way. In the Llyn Frongoch population, the infection intensity was more than three times higher in males than in females. Males had also higher infection intensities than females in the population from Carsington Reservoir, but the difference due to sex was much less pronounced.

Local adaptation in host-trematodes parasite interactions has been recorded in several studies (Krist et al., 2000, Voutilainen et al., 2009). Nonetheless, parasites are expected to be better at infecting individuals drawn from their local host population than individuals from an allopatric host (Lively, 1999, Krist et al., 2000). In trematodes, the migration distance of the definitive host might play a crucial role in determining gene flow between parasite populations, as low migration rate and high parasite abundance increase local intermediate hosts’ adaptation (Lively, 1999). As a result, sympatric hosts species are more susceptible to local parasite infection from the same geographical area than the parasites from allopatric locations. Voutilainen et al. (2009) reported that *Diplostomum* sp. and *Tylodelphus clavata* infections were higher in their local Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*) than other great geographically distant hosts were.

All fish in the current experiment were three-spined sticklebacks bred from wild parents under aquarium conditions, cercariae used were uniform in quantity and quality, and emerged from the same snail within a short time not exceeding 3 h which is within the timeframe recommended by Karvonen et al. (2003) for high infectivity. Therefore, the possibility of using inactive (long-time emerged cercariae) or other genetically different sources of infection were excluded.
Fishwerebelongedtow allopatricpopulationbackgrounds. Thedistancebetweentwo fishpopulationswereestimated as170kmusingGoogleEarth(GoogleEarth, 2018),whichisconsideredalongspatialscale. Inaddition,thecercaireaeusedwere shedfromnaturallyinfectedsnailswhichwerecollectedfromadifferentlocation withintheSoarRiverareathatwasgeographicallyisolatedfrombothfish populations. The current study did not investigatessympatrichost-parasiteadaptation sincethesnailswereinfectedwithparasitesfromthedifferentpopulationsthatthefishbelongedto. Therefore,itisdifficulttoidentifys thereasonsbehindparasiticload variationsbetweenallopatrichostbackgrounds. Thisdifferencein*Diplostomum* infection susceptibilitymaybethedue tothegeneticdifferencesbetweenthetwopopulations. Thisdifferences inparasiticloadbetweenthepopulationsmightbe attributedtothegeneticdifferencesinhost-parasitecoevolutionaryprocesses, or otherwise reflectthevariationintheadaptationtoenvironmentalconditionsby individualsinallopatricpopulations. However, to some extent theresult presented here mightsuggestthat* Diplostomum*parasitesareabletoadapttonon-localhost populations, asrepresentedbytheirabundanceinexperimentallyexposedfish. However, furtherinvestigationwillbeneededtoexaminehowgeneticchangesoccur inthreespinedsticklebacksand*Diplostomum*coevolutionaryprocesseswithin manygenerations. A recent study by ElNagar andMacColl(2016)suggestedthat laboratory-rearedthreepinedsticklebacksmoreresistanttoexperimental *Diplostomum* sp. infection fromtheirownpopulation, andtheircross-hybridsare moreresistantтопarentalpopulationparasiticinfection.

Theeffectofsexonsusceptibilitytoinfectionweretested, withthefemalefishwere morelikelytodevelopresistance to*Diplostomum*infectionthanmalesand CAR’sfish,whichcouldbeduetoothergeneticpredispositionfactorsandlevelof immune geneexpressionwhichmightdifferbetweentheindividualsofeach population,particularlyfemales. Stresslevelsduringindividualexposureprobably variedbetweensexestheLFpopulation, whichcouldleadtoincreased/ decreasedabilitytoavoidthecercaireaeencounter. Parentalinfectionexperience backgroundmightaffectoffspringsusceptibilitytoparasiticinfection, andtherefore
how this may increase or decrease the new generation’s resistance to infection. This area certainly needs further investigation.

Lake and river sticklebacks have been shown to differ in their resistance to parasites. As suggested by Kalbe and Kurtz (2006), three-spined sticklebacks from lake habitats were less susceptible to *D. pseudopathaceum* infection and higher respiratory burst activity in head kidney and larger spleen size samples than fish from river in Germany. In this current study, as parental fish were collected from lake ecosystems they possibly have developed a genetic innate immune basis against *D. spathaceum* for many generation. It is possible that other physiological factors might cause sex-related variation in *D. spathaceum* infections in this study. For example, physiological trade-offs between reproduction, breeding activity and immunity have occurred, causing one sex to become more susceptible to pathogen infection (Sheldon and Verhulst, 1996). Since this experiment was run during stickleback breeding season, males’ 11 Ketotestosterone levels could have been relatively high, which correlated positively with ornamentation but negatively with immunocompetence (Kurtz et al., 2007). This may suggest that lake populations are generally less susceptible, but that the corresponding difference in males was affected by strong immunosuppression due to their hormonal status during the breeding season, leading to an increase in susceptibility to *D. spathaceum* infection in both populations.

Terminal HSI was higher in CAR-infected fish than LF and is generally higher in infected than non-infected fish in both populations. One possible explanation for having a large liver mass in infected fish is that they tend to consume more food, reflecting the cost of developing infection resistance. Similarly, developing resistance to *S. solidus* infection in wild three-spined sticklebacks has been suggested to be energetically costly (Tierney et al., 1996). These authors suggested that newly infected fish consumed more food due to a pathological response, leading to the development of greater HSI than in non-infected fish.

In the current study, females were generally longer than males in both populations, regardless of their infection status. This might be attributed to the heavily energy investment in reproduction to develop their eggs which may positively correlated with
their rapid growth as females host increased energy investment in size than males. Llyn Frongoch fish normally showed slower growth rates than other populations in the UK, as recorded by Allen and Wootton (1982), which might explain the significant differences in length between LF and CAR fish.

Several studies found a positive correlation between parasitic intensity and fish length (Poulin, 2000). *Diplostomum* sp. show an accumulation pattern in the host lenses with time that can be associated with host age and the larger body area that older hosts provide for penetration (Pennycuick, 1971a, Hoglund, 1995).

At the end of the study, it was found that infected fish showed a greater reduction in length than the controls. This result is likely to be related to the cataracts level, which are negatively correlated with fish growth. It is possible that impaired vision was caused by the parasites’ feeding process, leading to slow growth rates. *Salvelinus alpinus* experimentally infected with *D. spathaceum* trematodes showed a slower feeding reaction to zooplankton than non-infected fish from the same school (Voutilainen et al., 2008). Moreover, body size and the rate at which weight declined in cataract-bearing rainbow trout, *Oncorhynchus mykiss*, lead to slow growth rates in a fish farm in Finland, as reported by Kuukka-Anttila et al. (2010).

**4.5.2 Eda genotype, plate morph and infection**

In this study, I used an experimental model approach to examine whether fish susceptibility to *D. spathaceum* was associated with the *Eda* gene haplotype or the number of plates developed by three-spined sticklebacks. Previous studies have shown the *Eda* genotype to be associated with growth, behaviour and skin pigmentation in three-spined sticklebacks (Barrett et al., 2009b, Barrett et al., 2009a). Here, I demonstrated another link with the *Diplostomum* infection. A high number of plates was found to significantly increase the development of infection. This finding is contrary to a previous wild study by Simmonds (2015), which suggested that *D. spathaceum* infection is comparable between genotypes and plate morphs. The result of the current experimental study also contrasted with two recent studies by Morozińska-Gogol et al. (2011) in wild marine fish, and Simmonds (2015) in lab-bred freshwater fish. Both authors found that low-plated genotype fish were more susceptible to *Schistocephalus solidus* infection.
Morozińska-Gogol et al. (2011) thought that due to *leiurus* (low-pated form) marine stickleback feeding habits, they have shown similar infection rate to those in freshwater through feeding on infected crustaceans during migration for breeding. *Semiarmatus* (partial-plated form) preferred to stay in shallow waters, whereas *trachurus* (fully-plated form) spawned in shallow water then migrated to the open sea. Simmonds (2015) suggested different possibility for the variation in *S. solidus* infection between *Eda* genotypes of experimentally infected three-spined sticklebacks. Since the number of procercoids fed to each fish in Simmonds (2015) was the same, and they were fed the same diet, different levels of exposure have been excluded as the main reason behind genotype-specific infection differences. Simmonds (2015) attributed the difference between *Eda* genotypes to other factors related to lateral plate development.

By generating F2 offspring from eight families via crossing individuals between salty water (completely plated) and freshwater fish (low plated) in North Uist, Scotland, F2 low-plated fish were found to be more heavily infected with the ectoparasite *Gyrodactylus arcuatus* than highly plated fish (Robertson et al., 2017). The authors hypothesized that this may be due to the increased probability of parasites attaching to hosts without plates. This might lead to a difference in immune gene expression levels, particularly in low-plated fish who showed inability to drive their immunity against infection. The authors suggested that F2 low-plated fishes’ underlying immunity was due to reduced expression of protective genes, which might make them more susceptible to infection as the low-plated allele is not adaptive to the saltwater where the parasite *G. arcuatus* is common.

The results presented here differs from the findings presented in their findings, since all fish were exposed to 50 cercariae, and partially plated fish showed greater levels of infection. This suggests that having more plates may not offer greater protection against parasite invasion. Therefore, infection is more likely to be correlated with other protective mechanisms including non-immunological factors; for example, thickness and viscosity of the skin mucus, the thickness of muscle layers under the skin, and possibly other behavioural resistance traits that might present a strong barrier to cercariae penetration (Betterton, 1974). Another possible explanation for
partially plated fish being more heavily infected than low-plated is that plates might present an effective obstacle against cercariae penetration, and therefore parasites might prefer other host body sites for entry such as the head and gill regions. While the present study supports the hypothesis that possessing more plates may not protect against parasitic invasion, however, this would need further examination of completely plated fish to confirm this theory. Moreover, other innate immunity components, probably other intrinsic genetic basis variations between two genotypes and their immune genes expression have in several studies been shown to lead to noticeable fish susceptibility differentiation to parasitic infections (de Roij, 2011, Robertson et al., 2017).

The results presented here also indicate variations between individuals in the number of plates for a given genotype, in agreement with previous studies (Colosimo et al., 2005, Lucek et al., 2012). Armoured plate development has been driven by environmental constraints under divergent selection between lakes in Iceland (Lucek et al., 2012). Since the fish used in this experiment were lab bred and reared under standard temperature light and water quality controlled aquarium conditions, the environmental factors have been excluded as a factor in plate development within the same genotype.

Host age is an essential factor in any assessment of parasitic effect studies (Horton and Okamura, 2001). In the current study, adult (1+ year) fish showed greater levels of metacercariae infection with Diplostomum in their lenses than juveniles. By increasing breeding hormone levels associated with age and stress (from exposure to parasites), immunological defence against pathogens is dramatically reduced, which is in agreement with the study by Schalk and Forbes (1997). The authors suggested that immune response differs between the sex and age of mammal hosts due to hormonal profile and stress level variations between adults and juveniles infected with arthropod parasites. Trade-offs play a key role in host-parasite evolution, particularly when infections may differ between host age categories; young hosts were found to be more suitable for parasite development than older ones (Lahmar et al., 1999, Colinet et al., 2005). The contribution of host age to parasite accumulation might be linked to the parasitic transmission processes. In
trophically transmitted parasites, sharp increases in infection that could be positively associated with age occurred with the ingestion of infected larval stages in the host’s diet (Pennycuick, 1971a, Zelmer and Arai, 1998). When parasites were non-trophically transmitted through their larval stage, host infection aggregation could be increased with age. Pennycuick (1971a) studied *Diplostomum gasterostei* infections in three-spined sticklebacks from Priddy pool in Somerset, where mean parasite intensity increased with host age due to accumulation. However, in this experimental study, parasites were introduced to the fish only once, and therefore the number of metacercariae that developed in the eyes of the adult fish could not reflect parasitic aggregation. Nevertheless, this could be attributed to age-related changes such as either a decrease in the host’s ability to develop resistance mechanisms to new parasitic challenges, especially in fish that had not been previously exposed or showed age-dependent changes in exposure to parasites, such as avoidance behavioural factors (Wilson et al., 2002).

In many fish-parasite research studies, age and size represent an essential element in development/ resistance to infection. The establishment of age-dependent parasite species should not be affected by host size, and they should therefore be present in the majority of host ages and size classes (Zelmer and Arai, 1998). However, host length and age had significant effects on the structure of *Diplostomum* in fish lenses (Désilets et al., 2013). Ryce et al. (2005) found that rainbow trout at less than 9 weeks post-hatch and that were at least 40 mm in fork length to be more susceptible to whirling disease, becoming more disease resistant when older and larger.

In this current study, sticklebacks that varied in age and body size were exposed to the *Diplostomum* parasite. The age of fish generally had a significant influence on susceptibility to infection; however, length was not an influence. The explanation for this could be that bigger fish may provide a larger surface area for cercariae penetration than juveniles or some fish may also have a greater genetic predisposition to infection, making them more susceptible to the parasite.
4.5.3 Bilateral asymmetry of D. spathaceum infection

The degree of asymmetry in infection between the eyes of the fish used in this study varied significantly, with increased levels of infection being recorded more often in the left eye than the right, regardless of Eda genotype, age and sex. This is in agreement with a number of studies that have reported asymmetrical distributions of Diplostomum sp. in the eyes of fish (Rau et al., 1979, Ching, 1985, Graczyk, 1991). Rau et al. (1979) suggested three reasons for the asymmetrical distribution of Diplostomum in eyes of lake whitefish (Coregonum clupeaformis). First, any damage to the choroidal gland due to bacterial infection in natural population fish might lead to increased asymmetry in the metacercariae distribution. Second, disorganization in the ocular blood supply might similarly lead to asymmetric distribution, and finally the high levels of infection that fish might have exposed to.

The current results do not support the first supposition, since the fish in this experiment were lab bred and have not been previously exposed to any other source of infection, suggesting that other ocular infections cannot be the only reason behind asymmetry. However, host morphological and congenital differences (Lee et al., 2012, Johnson et al., 2014) might explain bilateral asymmetry in infection within hosts. For example, in their experimental study, Johnson et al. (2014) found that anatomical measurements from a subset of larval amphibian Pacific chorus frogs (Pseudacris regilla) and wood frogs (Lithobates sylvaticus) revealed that the position of the right kidney explained 83% of the variation in the trematode Echinostoma trivolvis; however, generally neither kidney nor host size affected parasitic infection bias.

It is possible that the asymmetric distributions is due to host histological structure influencing metacercariae distribution, particularly where exposure is extensive. In this present study, fish were exposed once to 50 active cercariae, so their penetration through the sticklebacks' tissue was dependent on the rapidity of host tissue reaction, and this might be in agreement with Graczyk (1991) results where four species of experimentally exposed fish showed an asymmetric D. pseudosphathaceum distribution, after fish were exposed to a large number of cercariae in a short time.
It is possible that the asymmetric distribution is a density-short time dependent bias. As Diplostomum cercariae usually reached eye lenses within 24 h of contact with the skin, this suggests that parasitic migration is energetically costly. Migration through host tissue is distance-length dependent, and is estimated to be about 5 mm/hr in the fish as an intermediate host (Ratanarat-Brockelman, 1974). The results presented in this study essentially correspond with those reported by Ratanarat-Brockelman (1974), which due to the small body area of three-spined sticklebacks that possibly provide cercariae with a short migration route and less energetic consuming penetration through short distance of fish tissue. As possibly energy have not totally consumed by migration through sticklebacks tissue, faster parasite establishment could be achieved successfully, this makes it likely that a number of cercariae will penetrate the eye lenses more quickly.

It is possible that the metacercariae distribution inside fish eyes is relatively species dependent. In some species challenged with natural D. spathaceum infection, biased symmetry has been recorded in several studies. For example, Muzzall and Peebles (1988), in Rainbow smelt in Lake Huron and Lake Michigan; Rintamäki-Kinnunen et al. (2004), in farmed salmonid fish northern Finland; and Karvonen and Seppälä (2008) found three out of ten fish species in Bothnian Bay showed highly asymmetric distributions when heavily infected. However, in some naturally infected fish species, a more symmetrical distribution is likely to be more closely associated with the lower levels of cercariae that the fish faced. Graczyk (1991) found four wild species that showed no particular differences in Diplostomum sp. metacercariae between lenses in fish from Poland compared to experimentally exposed fish with a highly biased asymmetry. Marcogliese et al. (2001) found no significant differences in Diplostomum spp. abundance in the eyes of walleye and white suckers from the St. Lawrence River, Canada. Similarly, Machado et al. (2005), in the case of D. (Austrodiplostomum) compactum in six fish species from the Parana River, Brazil.

It is possible that the factors discussed above might result in an asymmetric distribution of D. spathaceum inside the lenses of the three-spined sticklebacks in the current study. Therefore, more experimental studies are needed to investigate each factor separately with at different levels of parasitic exposure.
4.5.4 Conclusions and future directions

This study indicates that the *Diplostomum* parasite is able to infect non-local host populations. The results indicated that fish populations differ in their resistance to *Diplostomum* infection depending on individual age, sex and genetic background. This study identified that ocular parasitic infections differ in a sex-population dependent manner. The role of plate morphology in determining three-spined sticklebacks’ susceptibility to *Diplostomum spathaceum* infection was examined, the results of which supported Simmonds’ (2015) finding whereby Carsington fish showed a wider range of morph plates than expected for their genotype. However, the current study contradicts Simmonds findings due to the observation that a greater number of plates negatively influences fish susceptibility to *D. spathaceum* infection, particularly as fish aged.

Further investigation into the wild fish from both Carsington and Llyn Frongoch populations is required, especially with cross-hybrid generations from infected and non-infected parents using infection from their own and allopatric populations to test if parasites are able to adapt to local and non-local host populations.

For an appropriate experimental study, it would be necessary to expose the completely plated form to the *D. spathaceum* parasite to give a better indication of the host-parasite interaction between this parasite and the *Eda* genotype.
4.6 References


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Chapter 5

Effect of paternal infection status on offspring susceptibility to *Schistocephalus solidus* infection

Image courtesy of I. Barber ©
5.1 Abstract

The influence of parental genotype or phenotypes on offspring phenotype is complex. The consequences of paternal infection status for the performance of offspring including their ability to withstand infections and subsequent progression of disease are poorly understood. In this study, I used male three-spined sticklebacks *Gasterosteus aculeatus* that were either infected, or non-infected, with the parasite *Schistocephalus solidus*, as sires to generate families of offspring that were later exposed to a controlled dose of *S. solidus* procercooids. I found that the proportion of offspring sired by infected males that became infected following controlled parasite exposure was significantly higher than the proportion of offspring sired by non-infected fathers.

Male sticklebacks build nests and use an endogenous protein glue, called spiggin, as an adhesive in nest construction, and spiggin gene expression is routinely used as an endpoint for male reproductive development in sticklebacks. The relative expression of spiggin genes in the kidney of experimentally infected and control (non-infected) male offspring that had been sired by infected or non-infected males was measured using qPCR. The expression of *spiggin C1* and *spiggin C2* among male offspring was significantly affected by their infection status, with infected fish showing increased expression compared to non-infected fish. However, the level of *spiggin B* expression did not differ significantly between infected and non-infected fish. There was no apparent effect of sire infection status on spiggin gene expression. The study may serve as a first step towards developing a greater understanding the potential long-term consequences of relaxed sexual selection that may arise under environmental change.
5.2 Introduction

Male three-spined sticklebacks Gasterosteus aculeatus is well known to have a characteristic reproductive behaviour. Sticklebacks, infected with plerocercoids of the cestode *Schistocephalus solidus*, often show reduced sexual development by do not develop sexual organs and the majority of infected fish are unable to engage in reproductive behaviour such as build nests, defend territories, chosen as mates and spawn (Arme and Owen, 1967, Tierney et al., 1996). Reproduction reduced in males appears to result as a side effect of infection, possibly by the parasite nutrient theft, low levels of 11-ketotestosterone (11KT), infected males poor competitors, females do not choose infected males or low-quality (small, less colourful) males as mates (Bakker and Milinski, 1991, Bakker and Mundwiler, 1994, Macnab et al., 2011).

However, even though studies have mainly focused on the effect of *S. solidus* infections as a castrator to their host reproduction, some infected males in some of the UK populations might be more capable of developing sexually (Macnab et al., 2009), and in a number of Alaskan populations are known to produce gametes and are able to spawn (Heins and Baker, 2008).

Furthermore, the global changes that associated with anthropogenic activities are dramatically influencing host parasite interaction. However, *S. solidus*-infected male sticklebacks male shows clearly that in degraded ecosystems, low quality and parasitized male sticklebacks may be more likely to be successful in developing sexually, defending territories, building nests and getting females to spawn with them (Candolin et al., 2007; Heuschele & Candolin, 2010). Therefore, under normal environmental condition, it is unlikely that infected males have much reproductive success, but this might be changed under degraded conditions when female sticklebacks have to mate with parasitized males. Therefore, it is unclear what are the consequences of paternal infection status for the performance of offspring survival and fitness. The aim of this study was therefore to understand how sire infection status impacts offspring performance including their ability to withstand infections and subsequent progression of disease.
5.2.1 What are parental effects, and how can they be associated with parasite infections?

Parental effects, including genetic and non-genetic inheritance, have become a point of considerable interest in ecology and evolutionary biology (Badyaev and Uller, 2009, Bonduriansky et al., 2012). Wolf and Wade (2009) define parental effects as the influence of the parental genotype or phenotype on the offspring phenotype. Marshall and Uller (2007) suggested that parental effects might have adaptive significance, either through positive or negative impacts on offspring fitness. The authors suggested the adaptive parental effect should to be beneficial to parents, offspring, or both in terms of selection for a new generation.

Parasites are ubiquitous components of all natural ecosystems and some of the adult hosts might face the same parasite infection once or more in their life and in the same ecological condition. Inheritance effect from parental animals to their offspring is of such fundamental importance in biology especially when the susceptibility/resistance to animal parasites of offspring that generated from infected parents have been investigated (Culbertson, 1938, Webster and Woolhouse, 1999). Following experimental exposure, Webster and Woolhouse (1999) found that the resistance of snails (*Biomphalaria glabrata*) to *Schistosoma mansoni* parasites was heritable, with significant differences in susceptibility of offspring generated from resistant and susceptible parents after exposed each individually to five miracidia of *S. mansoni*, when susceptible parents offspring snails exhibited more susceptibility than resistant parent offspring.

The immune system is probably the most efficient defence that animal hosts have evolved in order to manage the continuous threat from parasites, so as to reduce the fitness costs of parasitism (Sheldon and Verhulst, 1996). Variation in immune responses may also have genetic components and be under genetic control, leading to heritable variation in host resistance to parasites (Christe et al., 2000). Heritable parasite resistance could be driven by host-parasite coevolution, for instance Hamilton and Zuk (1982) hypotheses suggested parasite-mediated sexual selection across host taxa. By choosing a “good” male gene, females mate on the basis of
criteria that the chosen male is able to successfully cope with parasitic infections within the population.

Recent evolutionary studies have added an additional perspective to the host-parasite interaction by investigating the parental infection effect on offspring infection susceptibility, and the three-spined stickleback has emerged as a key model for studying the genetic basis of parasite resistance (Wegner et al. 2003; Eizaguirre et al., 2012, Barber 2013) and non-Mendelian modes of inheritance (Kaufmann et al., 2014). Kaufmann et al. (2014) revealed the costs and benefits of the paternal infection status effects in an experimental host-parasite system. The authors found that reproductive success disruption was reported in three-spined sticklebacks that had been experimentally exposed to the nematode *Camallanus lacustris* through reduced success in sperm competition in fertilization trials. In addition, this study found that the offspring of exposed males showed high mortality rates after exposure to the parasite; however, they had a better body condition than fish spawned from an unexposed father. The authors suggested that offspring resistance and tolerance are shaped by processes involving both genetic and non-genetic inheritance (Kaufmann et al., 2014).

This current experiment was designed to examine the consequences of paternal *Schistocephalus solidus* infection status for the infection susceptibility and development of offspring, as well as studying the impacts of experimental infections on aspects of their biology. As the infected and non-infected sires used to generate the offspring were wild-caught, it was not possible to know whether infection status reflected a resistance / susceptible genotype, or simply exposure / non-exposure to the parasite. It was therefore not possible to determine whether any association between sire infection status and offspring performance arose as a result of genetic, epigenetic or non-genetic mechanisms. Nonetheless, the study may serve as a ‘first step’ towards developing a greater understanding the potential long-term consequences of relaxed sexual selection that may arise under environmental change.
5.2. 2 The effect of Schistocephalus solidus infection on the reproduction of male sticklebacks

Male three-spined sticklebacks Gasterosteus aculeatus is well known to have a characteristic reproductive behaviour, namely that of nest building. By using an endogenous glue produced in the kidney, called spiggin, male fish are able to glue together collected plant and algae filaments to build their own breeding nest (Wootton, 1976). Spiggin is a multimeric glycoprotein, encoded by a multigene family (Jones et al., 2001, Kawahara and Nishida, 2006, Kawahara and Nishida, 2007) that is produced in kidney and stored in the urinary bladder (Jakobsson et al., 1999).

Spiggin production is under the control of androgens 11-ketotestosterone (11KT) (Borg, 2007). In one UK population infected males harboured heavy infections and showed significantly 11KT titres reduction and kidney spiggin content compared to non-infected fish. However in a second population infection levels were more variable particularly when some males with smaller infections recorded 11KT and spiggin titres that equivalent to non-infected fish (Macnab et al., 2011).

Three-spined sticklebacks, infected with plerocercoids of the cestode Schistocephalus solidus, often show reduced sexual development and the majority of infected fish are unable to engage in reproductive behaviour and spawn (Arme and Owen, 1967, Tierney et al., 1996). Furthermore, even though studies have mainly focused on the effect of S. solidus infections as a castrator to their host reproduction, some infected males in a number of Alaskan populations are known to produce gametes and are able to spawn (Heins and Baker, 2008).

Infected males from Llyn Frongoch, Wales, UK, were reported as being unable to overcome the effects of S. solidus infection, even following a period of rearing them under favourable laboratory conditions (Rushbrook and Barber, 2006). The authors reported that infected males had significantly reduced kidney development courtship levels, nesting activity, and nuptial colouration than non-infected males. However, another study has reported population variation in the reproductive capacity under laboratory housing, with naturally infected male sticklebacks from two UK populations showing different levels of reproductive success (Macnab et al., 2009).
Regulation of spiggin gene expression may lead to adjustment of nest structure in response to environmental variables by altering the quality and quantity of spiggin synthesized by the kidney (Rushbrook et al., 2010). Seear et al. (2014) showed that the expression of spiggin genes could be significantly influenced by environmental conditions, with nesting fish reared under flowing water showing higher expression levels of three genes (Spiggin B, Spiggin C1, and Spiggin C2) than those reared in still water. This study was designed to investigate increase/decrease in spiggin gene expression prior to the start of nest construction, providing a useful model for studying the effect of infection status and paternal infection status on future nest-building and reproductive success.

5.2.3 Aims

Here, three-spined stickleback and S. solidus were used as a model host-parasite system to examine whether paternal infection status might predict offspring susceptibility to parasite infection. By generating families of offspring sired by males that were either S. solidus-infected or non-infected, and then exposing the new generation of fish to a controlled dose of parasite procercoids, I was able to test for association between sire infection status and offspring infection susceptibility, and also investigate whether there was any beneficial effect on the condition of infected offspring, which might indicate a sire influence on parasite tolerance.

I also investigated whether sire infection status was associated with infection phenotype among male offspring that acquired infections. The expression of three different spiggin genes was quantified using RT-qPCR, as a proxy for male reproductive development, which was also assessed using kidney-somatic and gonadosomatic indices. This might help to understand some of the evolutionary aspects of breeding behaviour and our results might offer a data that provide insights for developing a greater understanding the potential long-term consequences of relaxed sexual selection that may arise under environmental change.
5.3 Methods

5.3.1 Fish collection and husbandry
Thirty adult three-spined sticklebacks were caught in April 2016 from the River Soar, Leicestershire, UK (N 52°37'42.7", W 1°08'33.0"). Fish were held in stock tanks (40cm x 60cm x 40cm) in a recirculating system, under temperature and day length regimes that matched those in the natural environment to promote reproductive development. Fish were fed *ad libitum* daily with frozen bloodworms (*Chironomus* sp.).

5.3.2 Fish breeding programme
In June, 12 gravid females were selected from the tanks as female parents for the IVF breeding programme. Six males, which showed no external signs of infection, and six males with obvious *S. solidus*-induced swellings (Barber, 1997) were selected as sires. Twelve families of juveniles – each the product of a single female x male cross – were generated using *in vitro* fertilisation (IVF) techniques, as described in Chapter 3, following the method of (Barber and Arnott, 2000). In brief, male sticklebacks were euthanized using a Home Office approved Schedule 1 technique and dissected under a stereomicroscope. Paired testes were removed and placed in a watch glass over ice before being macerated using sterile forceps. Eggs were stripped from females into a watch glass before adding the macerated testes solution to the aquarium water and being left for 30 minutes. A stereomicroscope was used to check for fertilisation, which was confirmed by the appearance of the fertilisation membrane. Fertilised eggs were kept in a 1 L plastic aquarium with constant aeration and 2 ml/L methylene blue solution (stock solution: 2 mg/L) as an anti-fungal agent.

Hatched fry were kept in the same 1 L aquaria and fed daily with Liquify No. 1™, until they were capable of consuming 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. They were fed daily *ad libitum* with *Artemia* sp. nauplii and bloodworm as they got older. Since it was sometimes difficult to fertilize eggs using testes from infected sires, the number of offspring that generated by infected sire so obtained was less than the number of

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non-infected sire offspring. Therefore, the number of offspring obtained from infected sire was somewhat limited and depending on fertilisation success.

![Experimental design schematic](image)

**Figure 5.** Experimental design schematic illustrating the two paternal infection status batches of *Schistocephalus solidus* exposure from the same set of three-spined stickleback families.

### 5.3.3 Experimental parasite infection procedure

*Schistocephalus solidus* plerocercoids were recovered from naturally-infected three-spined sticklebacks collected from the River Soar, Leicester, UK. Infective plerocercoids (i.e., those weighing > 50 mg) (Tierney and Crompton, 1992) were cultured in pairs using techniques adapted from (Smyth, 1954). Plerocercoids were
placed into a loop of 6.3 mm diameter dialysis tubing (Visking, UK) suspended in a 70 ml Pyrex screw-top glass tube (Fisher, UK) that contained 30 ml RPMI medium, 30 ml horse serum (Sigma, UK) and 0.5 ml of penicillin-streptomycin-glutamine solution (Thermo Scientific, UK). Culture tubes were placed in a shaking water bath at 40°C for 6 d as previously described ( Arnott et al., 2000, Macnab, 2012). Eggs were collected from the dialysis tubing and the culture liquid was removed and the eggs washed with ddH2O to remove any remaining parasite tegument. Collected eggs were stored in ddH2O in a 9 cm diameter sealed Petri dish, which was then covered in aluminium foil and incubated in the dark at 20°C for 21 d to embryonate.

Eggs were then taken from the incubator and exposed to daylight for 24 h to stimulate hatching. Laboratory-reared copepods (Cyclops strenuus abyssorum) were placed in 100 ml conical flasks and exposed to hatching coracidia for approximately 24 h in natural daylight before being moved to a new flask and stored into fresh, filtered aquarium water under aquarium conditions. Three weeks after being exposed to the parasites, copepods were screened individually under a compound microscope at 300x magnification to determine the level of infection. The infection status and intensity of infection in each copepod was scored, as was the infective status of any procercoids, based on the presence or absence of a cercomer.

Copepods containing infective (i.e., cercomer bearing) procercoids were fed to lab-bred juvenile sticklebacks, which had been previously sex-determined non-invasively by PCR analysis of a sex-linked marker (see Chapter 3). Fish were measured (Standard Length, SL0, to 0.1 mm), blotted and weighed (M0, to 0.001 g). To maximise the likelihood, they ingested the infected copepod, food was withheld for 2 d prior to parasite exposure. Experimental fish were selected from the parental background stock tanks. Each fish was fed a total of three infective procercoids. Each known-sex fish was either exposed to a controlled dose of infective procercoids, by fed an infected copepod or was sham-exposed by feeding a non-infected copepod via a glass pipette in a small 1 L plastic aquarium (15.5 x 9.5 x 8.5 cm) filled with 500 ml of filtered system water. Parasite-exposed and sham-exposed fish were left undisturbed for 24 h before being transferred to separate 1.25 L plastic aquaria (15 cm x 14 cm x 11 cm), which were held on a recirculating system and fed
blood worms *ad libitum* to excess for 90 d. The water temperature was maintained at aquarium temperature between 10-13°C which was changed on each of three consecutive months and the lighting regime shifted from 8L:16D to 13L:11D to mimic the natural length of a day.

A total of 100 fish (50 males and 50 females) were used in the experiment; 15 males and 15 females from each paternal infection status were exposed to a controlled dose of procercoid parasites, and 10 males and 10 females were sham-exposed from each paternal infection status (Figure 5.1). Exposure to infective parasite stages was carried out under the authority of a UK Home Office licence (Project licence: 80/2327, Personal Licence: IAD9DF470).

For the spiggin gene expression experiment, a total of 60 male fish were used in this experiment. Twenty-four per paternal infection status were exposed to a control dose of *S. solidus* as described above, and 6 per paternal infection status were sham exposed. Aquaria were held on a recirculating system and fish were fed bloodworms *ad libitum* to excess for 70 d. Water temperature was maintained at 17°C ± 1.4 and the day length regime used was 14L:10D. Exposure to parasite infective stages was carried out under the authority of a UK Home Office licence (Project licence: 80/2327, Personal Licence: IAD9DF470).

**5.3.4 Molecular analysis of spiggin expression**

**5.3.4.1 Fish dissection and tissue sampling**

At the end of the study, each fish was euthanized by an overdose of Benzocaine anaesthetic (stock solution: 10g L⁻¹ in 70% EtOH) according to UK Home Office Schedule 1 methods. Fish were blotted dry, measured using a dial calliper (Standard Length, SL₉₀, to 0.1 mm) and weighed using an analytical balance (wet mass, Mₚ, to 0.001 g) before dissection. Plerocercoids recovered from infected fish were blotted and weighed (wet mass, to 0.001 g); in the case of multiply infected fish, the mass of each individual plerocercoid was recorded separately and total parasite mass (Mₚ) calculated. Parasite index was calculated as PI = Mₚ / (M₉₀-Mₚ), where (M₉₀-Mₚ) is the mass of the infected fish following subtraction of parasite mass (Pennycuick, 1971a). Kidney mass (Mkid) and gonad mass (Mgon) were weighed (all to 0.0001 g). Body condition factor (BCF) was calculated as BCF= [(M₉₀ - Mₚ) L⁻³] × 10⁵
(Pennycuick, 1971b); Gonadsomatic Index (GSI) was calculated as $\text{GSI} = \left( \frac{\text{M}_{\text{gon}}}{(\text{M}_{90}-\text{M}_{p})} \right) \times 100$ and Kidney-somatic Index (KSI) calculated as $\text{KSI} = \left( \frac{\text{M}_{\text{kid}}}{(\text{M}_{90}-\text{M}_{p})} \right) \times 100$. The kidney was then snap frozen in liquid nitrogen before being stored at -80°C prior to RNA extraction for quantification of spiggin gene expression.

5.3.4.2 RNA Isolation

RNA was extracted using a Sigma GenElute™ Mammalian Total RNA Miniprep kit following manufacturer instructions. Kidney 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 12,000 RPM for 2 min. Then, 500 µl of 70% ethanol was added to the sample and the resultant solution transferred to a binding column. 500 µl wash solution was pipetted into the column and centrifuged at maximum speed for 15 seconds, after which the binding column was transferred into a fresh 2 ml collection tube and wash solution 2 added, after which the mixture was centrifuged at maximum speed for 15 seconds. Then, second 500 µl volume of wash solution 2 was added into the column and centrifuged at maximum speed for 1 minute. 50 µl of elution solution was added into the column and centrifuged at maximum speed for 1 minute. 50 µl of DNasel (Sigma, UK) was used to remove genomic DNA from the RNA samples by incubating samples with 5 µl reaction buffer and 5 µl DNasel enzyme for 15 min at 70°C before adding 5 µl stop solution. RNA was quantified using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific, UK).

5.3.4.3 Reverse Transcription Quantitative PCR analysis

Reverse Transcription qPCR was used to detect the expression of spiggin genes. The three genes under investigation were spiggin B, spiggin C1 and spiggin C2 in the kidney tissue of experimentally-infected and sham-infected male sticklebacks. The housekeeping gene used for reference was ribosomal protein L8 (rpL8), as this gene has previously been used by Seear et al. (2014) to investigate spiggin expression in stickleback populations.

First strand cDNA was reverse transcribed from 0.5 µg of total RNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, UK), and then diluted to
2 µg concentration. The RT-qPCR mixture consisted of 10 µl SYBR Green JumpStart Taq ReadyMix (Sigma, UK), 1 µl of 5 µM forward and reverse primer (Table 3.1), 7 µl of water and 1 µl diluted cDNA to a total volume of 20 µl. The RT-qPCRs were performed in duplicate on a Chromo4 qPCR thermocycler (BioRad Laboratories, Hercules, CA) under the following cycling conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec, 60–66°C (depending on gene amplified) for 30 sec, and 72°C for 30 sec. A melting curve step (50–95°C) was then performed to ensure that only a single product had been amplified in each reaction. For each spiggin gene, cDNA from each of the infected and non-infected sticklebacks was run in duplicate qPCRs. “No template” and “no reverse transcriptase” controls were performed for each primer and cDNA combination, respectively. To normalize the gene expression data, ribosomal protein L8 was used as a reference gene. The CT value is the cycle at which amplification of the fluorescence level reaches or crosses the threshold.
Table 5.1 Primers used for qPCR on three-spined stickleback cDNA for spiggin genes, after Seear et al. (2014).

<table>
<thead>
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<th>Primers Name</th>
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<th>Annealing temp.</th>
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<td>66°C</td>
</tr>
<tr>
<td>Spiggin B R</td>
<td>TTTAGGAATACAGCGATAGCCCTTTT</td>
<td>66°C</td>
</tr>
<tr>
<td>Spiggin C 1 F</td>
<td>AAGAAATCAAGGACTGTGTGCAAT</td>
<td>65°C</td>
</tr>
<tr>
<td>Spiggin C 1 R</td>
<td>ACTGCTGGACCCTTTTCCCTATAT</td>
<td>65°C</td>
</tr>
<tr>
<td>Spiggin C 2 F</td>
<td>AACCAATCCAAGTCCGATGACA</td>
<td>60°C</td>
</tr>
<tr>
<td>Spiggin C 2 R</td>
<td>TCGGAAAGAACCCTTTCGTTTC</td>
<td>60°C</td>
</tr>
<tr>
<td>Ribo L8 F</td>
<td>CGACCCGTACCGCTTCAAGAA</td>
<td>60°C</td>
</tr>
<tr>
<td>Ribo L8 R</td>
<td>GGACATTGCCAATGTCAGCTGA</td>
<td>60°C</td>
</tr>
</tbody>
</table>
5.3.5 Statistical analysis

Proportional data (BCF, GSI, KSI, PI) were arcsine square-root transformed, and normality was tested using the Kolmogorov-Smirnov statistic. Non-normally distributed data was transformed using the Cox-Box transformation; if normality was still not achieved, non-parametric statistical tests were used. ANOVA used to examine the effect of infection status (infected/non-infected); paternal infection status (infected/non-infected sire) and fish sex on final length; mass and body condition factor (BCF).

Gene expression data 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 the Minitab 17 software. Multivariable analysis of variance (MANOVA) was used to examine the effects of infection status and paternal infection status and gene identity on spiggin expression. Then ANOVA was used to analyse each gene individually, again to determine the effects of infection status and paternal infection status on the expression of the gene of interest. For all boxplots in this chapter the dark line represents the median, the box shows the Q1-Q3 interquartile range (IQR) and the whiskers represent variability outside the upper and lower quartiles, outliers are shown as asterisks in figures.

5.4 Results

5.4.1 Effect of paternal infection status on offspring infection susceptibility

Among parasite-exposed fish, the probability of developing a *Schistosomephalus solidus* infection was significantly associated with the infection status of the sire (Chi-square: \( \chi^2 = 9.643, \text{ df} = 1, P = 0.002 \); Figure 5.2). At the end of experiment, the proportion of infected offspring sired by infected males was significantly higher than that sired by non-infected fathers. Parasites established in 20 of the 30 exposed fish (67%) sired by infected fathers, but in only 8 out of 30 exposed fish (26%) sired by non-infected fathers. Infection rates were similar across both sexes (Chi-square: \( \chi^2 = 0.268, \text{ df} = 1, P = 0.605 \)).
Figure 5.2 Frequency of *Schistoscephalus solidus*-infected three-spined sticklebacks of two paternal infection status separated by their sex.

A non-parametric Kruskal-Wallis test was employed to investigate whether paternal infection phenotype affected the number of parasites that established in offspring exposed to three proceroids. A significant effect of paternal infection status on the number of plerocercoids established was found (Kruskal-Wallis: $H = 10.66, df = 1, P = 0.001$; Figure 5.3); among exposed individuals, being sired by an infected male was associated with a higher number of worms establishing.
5.4.2 Fish body size and mass as determinants of infection susceptibility

5.4.2.1 Effect of paternal infection phenotype on offspring length

Prior to experimental parasite exposure or sham exposure, offspring sired by infected males were found to be longer than offspring sired by non-infected males (ANOVA: $F_{1,92} = 10.13, P = 0.002$; Figure 5.4 A). Offspring length prior to sham exposure / parasite exposure did not differ between sham exposed fish, parasite exposed fish that went on to develop infections or those that did not (ANOVA: $F_{1,92} = 1.30, P = 1.10$; Figure 5.4 B). No interaction between paternal infection status and
offspring infection status was found ($F_{1.92} = 0.49$, $P=0.615$). Fish length did not significantly vary between the sexes (ANOVA: $F_{1.92} = 0.82$, $P=0.369$).

**Figure 5.4** The pre-exposure standard length ($SL_0$) of three-spined sticklebacks. A: fish separated by their paternal infection status and B: fish represented by their *Schistocephalus solidus* infection status and paternal infection status. IS: Infected Sire, NIS: Non-Infected Sire.
At dissection, the length (SL$_{90}$) of infected offspring (43.7 ± 3.3 S.D.) did not differ significantly from that of exposed, non-infected offspring (43.5 ± 2.5 S.D.) or sham-exposed offspring (43.2 ± 3.6 S.D.) (ANOVA: $F_{1,92} = 0.66$, $P = 0.520$; Figure 5.5). Male fish did not significantly differ from the females in terms of their final length (ANOVA: $F_{1,92} = 0.001$, $P = 0.973$). However, there was a significant effect of sire infection status, with offspring sired by infected males being significantly longer (44.6 ± 2.2 S.D.) than those sired by non-infected fathers at the end of the study (42.6 ± 3.2 S.D.) (ANOVA: $F_{1,92} = 5.24$, $P = 0.025$; Figure 5.5). There was no significant interaction between paternal infection status and offspring infection status ($F_{1,92} = 2.77$, $P = 0.068$) and paternal infection status and sex ($F_{1,92} = 0.84$, $P = 0.361$).

**Figure 5.5** The effect of *Schistocephalus solidus* infection status and paternal infection status on the final length (SL$_{90}$) of exposed three-spined sticklebacks. IS: Infected Sire, NIS: Non-Infected Sire.
5.4.2.2 Effect of paternal infection status on offspring mass

At the start of experimental infection status, offspring sired by infected males were found to have higher body mass than offspring sired by non-infected males (ANOVA: $F_{1,92} = 8.97, P = 0.004$; Figure 5.6 A). However, fish that went on to develop infections did not significantly vary in terms of body mass than those that did not develop infections and sham-exposed fish (ANOVA: $F_{1,92} = 0.88, P = 0.419$; Figure 5.6 B). No interaction between paternal infection status and offspring infection status was found (ANOVA: $F_{1,92} = 1.16, P = 0.854$). Fish mass did not vary significantly between the sexes (ANOVA: $F_{1,92} = 2.62, P = 0.109$).

At dissection, fish mass ($M_{90} - M_P$) (i.e., excluding the mass of plerocercoids removed from infected fish) of offspring sired by infected males was significantly greater than for that of offspring sired by non-infected males (ANOVA: $F_{1,92} = 8.05, P = 0.006$; Figure 5.7), with no interaction between paternal infection status and offspring infection status (ANOVA: $F_{1,92} = 1.10, P = 0.337$). The mass of infected offspring did not differ significantly from that of non-infected offspring and sham-exposed (ANOVA: $F_{1,92} = 0.50, P = 0.610$; Figure 5.7), and male mass was not significantly different from female mass at the end of the study (ANOVA: $F_{1,92} = 4.94, P = 0.209$).
Figure 5.6 The pre-exposure mass ($M_0$) of three-spined sticklebacks. A: fish separated by their paternal infection status and B: fish represented by their *Schistosome solidus* infection status and paternal infection status. IS: Infected Sire, NIS: Non-Infected Sire.
The effect of *Schistocephalus solidus* infection status and paternal infection status on final fish mass ($M_{90-M_P}$) of exposed three-spined sticklebacks. IS: Infected Sire, NIS: Non-Infected Sire.

### 5.4.3 Host body condition among exposed fish

The effect of sire infection status, fish sex and parasite infection on offspring body condition was tested. There was no significant effect of sire infection status (ANOVA: $F_{1,59} = 0.13$, $P = 0.722$), fish sex (ANOVA: $F_{1,59} = 2.13$, $P = 0.150$) or *S. solidus* infection (ANOVA: $F_{1,59} = 0.02$, $P = 0.887$; Figure 5.8) on body condition. There were also no significant interactions between sex and paternal infection status ($F_{1,59} = 0.09$, $P = 0.759$; Figure 5.8); sex and infection status ($F_{1,59} = 1.54$, $P = 0.221$) and paternal infection status and offspring infection status ($F_{1,59} = 0.58$, $P = 0.449$; Figure 5.8).
Figure 5. 8 The effect of *Schistocephalus solidus* infection status, fish sex and paternal infection status on the fish body condition factor (BCF) of exposed three-spined sticklebacks. I: Infected fish, NI: Non-Infected fish.

5.4.4 Analysis of parasite growth

The mass of plerocercoids recovered from infected fish after 90 d was not found to be significantly affected by paternal infection status (Kruskal-Wallis: $H = 1.49$, df =1, $P = 0.222$; Figure 5.9 A). When the mass of plerocercoids was controlled for the mass of the fish by calculating the parasite index (PI), there was no significant difference in PI between infected offspring sired by infected and non-infected fathers ($t$-test: $t = 0.34$, df = 1, $P = 0.744$; ; Figure 5.9 B).
Figure 5.9 The effect of paternal infection status on A: total parasite mass and B: parasite index (PI).
5.4.5 Male sexual development

5.4.5.1 Effects of *S. solidus* infection and sire infection status on male GSI and KSI

To investigate if *S. solidus* infection or paternal infection status influenced male sexual development, a 2-way ANOVA was used. This analysis revealed a marginally non-significant effect of *S. solidus* infection on kidney somatic index (KSI), with non-infected fish showing a trend towards higher KSI values than infected males ($F_{1,29} = 3.52, P = 0.071$; Figure 5.10 A). There was no significant effect of sire infection status on KSI ($F_{1,29} = 0.64, P = 0.430$; Figure 5.10 A), and there was also no interaction between *S. solidus* infection and sire infection status in terms of resultant KSI ($F_{1,29} = 1.46, P = 0.238$).

*S. solidus* infection had no effect on GSI (2-way ANOVA: $F_{1,29} = 0.04, P = 0.841$; Figure 5.8 B), though a significant effect of sire infection status was found ($F_{1,29} = 5.08, P = 0.032$; Figure 5.10 B), with offspring sired by non-infected males having higher GSI values than those sired by infected males. No significant interaction was found between *S. solidus* infection and sire infection status ($F_{1,29} = 0.62, P = 0.439$).
Figure 5. 10 The effect of *Schistocephalus solidus* infection status and paternal infection status on the A: Kidney Somatic index (KSI) and B: Gonadosomatic index (GSI) of exposed male three-spined sticklebacks.
5.4.6 Spiggin gene expression experiment

5.4.6.1 Effect of paternal and *S. solidus* infection status on KSI of male offspring used in gene expression analysis

Differences in KSI were investigated using a 2-way ANOVA, which revealed a marginally non-significant effect of infection status ($F_{1,34} = 3.50$, $P = 0.071$; Figure 5.11), with infected fish tending to have a higher KSI than non-infected males. Sire infection status had no effect on KSI ($F_{1,34} = 0.22$, $P = 0.643$; Figure 5.11) and there was no interaction between the two variables ($F_{1,34} = 1.83$, $P = 0.185$).

![Box plot showing the effect of infection status on KSI](Figure 5.11)

*Figure 5.11* The effect of *Schistocephalus solidus* infection status and paternal infection status the Kidney Somatic index (KSI) of exposed male three-spined sticklebacks used in spiggin gene expression.
5.4.6.2 RT-qPCR analysis of spiggin gene expression of male offspring

Analysis by multivariate analysis of variance (MANOVA) of delta-Ct values was undertaken to investigate the role of paternal infection status, fish infection status and gene identity on spiggin gene expression in the kidney tissue of experimentally exposed male sticklebacks. This analysis revealed that spiggin gene expression among male offspring was significantly affected by fish their infection status. The expression of spiggin C1 and spiggin C2 among male offspring was significantly affected by their infection status, with infected fish showing increased expression compared to non-infected fish. However, the level of spiggin B expression did not differ significantly between infected and non-infected fish. However, there was no significant effect of paternal infection status and gene identity, and no interaction between these variables was found (Table 5.2).

Table 5.2 Multivariate analysis of variance MANOVA of three-spined stickleback spiggin gene expression level relative to L8, when testing the effect of Schistocephalus solidus infection status, paternal infection status and gene identity. Significant values ($p < 0.05$) are shown in bold.

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<th>df</th>
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<tr>
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</tr>
<tr>
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<td>0.07</td>
<td>0.929</td>
</tr>
</tbody>
</table>
The delta-Ct values were then analysed using a multivariate analysis of variance (MANOVA) to test the effect of infection status and paternal infection status on each gene separately. There was a marginally non-significant effect of infection on *spiggin B* expression ($F_{1,34} = 3.36$, $P = 0.077$), with infected fish showing a trend towards lower levels of gene expression than non-infected fish. There was no significant effect of paternal infection status on delta-Ct values for *spiggin B* ($F_{1,34} = 0.30$, $P = 0.591$; Figure 5.12) and no interaction between infection status and paternal infection status was found ($F_{1,34} = 0.029$, $P = 0.594$). This meant that the *S. solidus* infection status of the sire was not associated with the expression of *spiggin B* among male offspring.

Expression of *spiggin C1* among male offspring was associated with their infection status, with infected fish showing increased expression compared to non-infected fish ($F_{1,34} = 4.60$, $P = 0.040$). There was no association with paternal infection status ($F_{1,34} = 0.72$, $P = 0.404$), and no interaction between infection status and paternal infection status ($F_{1,34} = 0.72$, $P = 0.402$, Figure 5.12).

The expression of *spiggin C2* gene among male offspring was closely associated with their infection status ($F_{1,34} = 12.81$, $P = 0.001$), with *spiggin C2* being considerably upregulated among infected fish. Paternal infection status was not associated with the *spiggin C2* expression of male offspring ($F_{1,34} = 0.03$, $P = 0.855$) and there was no interaction between the two variables ($F_{1,34} = 0.04$, $P = 0.850$; Figure 5.12).
**A- spiggin B**

**B- spiggin C 1**

**C- spiggin C 2**

**Figure 5.** Association between *Schistocephalus solidus* infection status, paternal infection status and the expression of the genes A) *spiggin B*, B) *spiggin C1* and C) *spiggin C2* relative to that of the reference gene, ribosomal protein L8, by three-spined sticklebacks in the study. Sample sizes: Infected fish sired by infected male = 14; Non-Infected fish sired by non-Infected male = 9; Control fish sired by infected male = 6 and Control fish sired by non-Infected male = 6.
5.4.6.3 Parasite burden, paternal infection status and gene expression

Parasite mass relative to fish mass, as represented by PI, was investigated to see if it had a significant effect on spiggin gene expression among infected male fish. Paternal infection status was used as a factor in the ANCOVA analysis, and PI as a covariate. The analysis revealed that neither PI nor parental infection status were significantly associated with gene expression among infected male fish (Table 5.3, Figure 5.13).

Table 5.3 ANCOVA table of Delta-Ct values of *Schistocephalus solidus* infected three-spined sticklebacks with parasite index (PI) as a covariate.

<table>
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<tr>
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<td>2.09</td>
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<td>Paternal Infection Status*PI</td>
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Figure 5.13 Gene expression of Schistocephalus solidus infected three-spined sticklebacks for each gene of interest (A: Spiggin B, B: Spiggin C1, C: Spiggin C2) plotted against parasite index (PI). Solid and dashed lines show the regression between PI and delta-Ct values for each gene for male fish sired by infected (open symbols) and non-infected males (closed symbols).
5.5 Discussion

5.5.1 Main findings of the study

The main aim of this study was to examine whether the susceptibility of male offspring to *Schistocephalus solidus* infection, or the progression of the infection in terms of the subsequent growth rates of plerocerocids, was associated with paternal *S. solidus* infection status. This question is relevant because, although *S. solidus* infected male sticklebacks typically have low reproductive success (Arme and Owen, 1967, Tierney et al., 1996), processes of natural and sexual selection can be relaxed under environmental degradation, potentially benefiting the success of parasitized fish (Candolin et al. 2008). For example, under conditions of human-induced eutrophication, both low quality and *S. solidus*-infected male sticklebacks may be more likely to build nests, defend territories and be chosen as mates by females than under non-impacted conditions (Candolin et al., 2007; Heuschele & Candolin, 2010). The usual preferences of female sticklebacks for high quality, non-parasitized males (Milinski & Bakker 1990) may therefore be lessened under degraded conditions, with a greater number of offspring arising from mating with parasitized males. At present it is unclear what the long-term impacts of these altered mate choice decisions may be largely (Candolin et al., 2014), because the consequences of paternal infection status for the performance of offspring are poorly understood. The aim of this study was therefore to gain a better understanding of the likely implications for male offspring of having an infected father for their susceptibility to infection, and subsequent progression of disease.

In the study, paternal infection status was closely associated with offspring susceptibility to infection which could summarised by:

(1) Offspring sired by infected males were more susceptible to infection, with the offspring of infected males being more than twice as likely to become infected following challenge as the offspring of non-infected males.

(2) Among the infected offspring, those sired by infected males developed more parasites than those sired by non-infected males.
Offspring sired by infected males were longer and heavier than those sired by non-infected males at the start of the study, and this pattern continued to the end of the study.

Male offspring sired by non-infected males had higher GSI than those sired by infected males (but there was a relatively weak effect and there was no sire effect on KSI).

Among all offspring, *S. solidus* infection was associated (non-significantly) with lower KSI values and (significantly) with Spiggin C1 and C2.

### 5.5.2 Paternal infection status and offspring susceptibility to infection

There are a number of possible explanations for the association between sire infection status and the infection susceptibility of offspring discovered in this study. First, and most straightforwardly, the patterns might reflect a simple genetic basis to infection susceptibility, with the probability of offspring infection being linked to natural variation in immunocompetence among the sire population.

The cestode *S. solidus* has a large impact on its host's fitness and has a selection pressure on stickleback defence mechanisms particularly when fish had shown genetic diversification of immune function. For example, sticklebacks with low MHC diversity suffered more from *S. solidus* than individuals with very high allelic diversity (Kurtz et al., 2004). Although, there are indications that *S. solidus* could manipulate sticklebacks immune system, nothing is yet known about the specific interactions of this cestode with the stickleback immune system (Scharsack et al., 2007). The authors suggested that the respiratory burst activity which has the potential to destroy early small parasites is upregulated very late in the course of *S. solidus* infection (Scharsack et al., 2007).

There is a strong evidence that physiological trade-offs between reproduction, breeding activity and immunity have occurred, in order to reduce the fitness costs of parasitism (Sheldon and Verhulst, 1996). Since some males used in the current experiment for generating the offspring were infected, it is possibly that their 11 Ketotestosterone levels could have been relatively high, which correlated positively with ornamentation but negatively with immunocompetence (Kurtz et al., 2007). This
may suggest that those males are generally affected by strong immunosuppression
due to firstly their hormonal status during their breeding season and secondly by *S. solidus* infection, meaning that the overall low immune status of infected parent fish is genetically transferred. Therefore, fathers’ derived immunity is very much required for their offspring to survive and protect against parasite invasion which was probably quite weaker in infected sire than the immunity has derived from non-infected fathers.

Experimental infection of three-spined sticklebacks to the nematode *Camallanus lacustris* reduced reproductive success in sperm competition for fertilization (Kaufmann et al., 2014). Furthermore, offspring sired by exposed males had a lower rate of hatching and survival, even though they had a better body condition value than individuals from unexposed fathers after experimental infection. The authors suggest that paternal infection had positively increased resistance and tolerance to the parasite, as driven by both genetic and non-genetic inheritance.

The offspring fish used in this study were in experimental groups of a similar age and parasitic burden (each fish exposed to three procercoids only). Therefore, the possibility of having individual variation in the body size and mass between offspring was the more improbable factor in the analysis of susceptibility between paternal groups. Standard length and mass prior to parasite exposure of the offspring varied significantly in this experiment to record higher values between infected sire offspring than non-infected sire individuals. Interestingly, the analyses also supported the observation that increased both body length and mass were observed in infected sire offspring at the end of the study that can be associated with infection-induced paternal effects. As each animal has a specific immune genetic variation for resistance against parasites and tolerance (the ability to limit parasitic burden) (Råberg et al., 2007, Sorci, 2013), and the results of this current experiment do not differentiate the occurrence of parents genetic and non-genetic effects on their offspring. Therefore, further work is required to investigate if offspring resistance and tolerance to *S. solidus* infection are driven by processes involving both genetic and non-genetic inheritance from their parents.
5.5.3 The effect of S. solidus infection and paternal infection status on sexual development in male sticklebacks

As in the result of the present study, a trend of statistically significant differences in KSI between infected and non-infected fish was observed, with a slightly higher KSI in infected than non-infected males. This was in contrast with previous studies, which suggested kidney mass was reduced in infected male fish compared to non-infected males in some Alaskan and UK populations (Heins and Baker, 2008, Macnab et al., 2009).

On the other hand, other studies reported a population variation in the reproductive capacity through the development of large kidneys and involvement in courtship behaviour when naturally infected males from two different populations of G. aculeatus were reared under lab housing (Macnab et al., 2009). S. solidus infection levels did not correlate significantly with relative kidney mass (Rushbrook and Barber, 2006).

Among lab-bred fish in current study that experimentally exposed to a controlled dose of S. solidus proceroids and reared under controlled laboratory conditions, infected fish were able to develop kidneys with a slightly higher KSI than non-infected males. RT-qPCR was then used to quantify the expression of three major spiggin genes in the kidney tissue of infected and non-infected males which had been divided into two groups as per their sire infection status. Interestingly, I found that spiggin C1 and spiggin C2 expression was significantly higher in infected fish than non-infected fish; however, paternal infection status had no effect on the expression of any of the genes under investigation. These results indicates that sticklebacks may be able to adjust spiggin gene expression patterns, even when they developed infection.

As expected, expression of spiggin genes is significantly higher among nesting than non-nesting males (Seear et al., 2014). Therefore, this current study was designed to investigate spiggin genes expression prior to the start of nest construction, which might help us evaluate the effects of infection status and paternal infection status on the nest-building process in the future. It seems that the genetic basis for stickleback nesting behaviour is to exhibit a plasticity in response to S. solidus infection. This
were represented in stickleback’s spiggin gene expression adjustment patterns in response to the infection.

There are two possible explanations for high *spiggin C1* of *spiggin C2* expression in upregulation in infected fish. First, fish have been abundant in the River Soar for a long time, therefore males might be showing a unique evolutionary interaction with the parasite through increased reproductive capacity even when infected. Second is parasite infection level, it has been suggested that spiggin levels could be strongly correlated with parasite intensity and mass, and also spiggin concentration declined significantly when a large number of parasites have developed with larger mass (Rushbrook and Barber, 2006). Since the fish in this present experiment developed between 1-3 plerocercoids only, it is possible that higher spiggin concentrations are produced in only lightly infected males and further studies will be needed regarding the spiggin concentration level and the number of parasites that will be developed.

Relatively few studies have considered spiggin gene expression in sticklebacks, and none to my knowledge have studied this in relation to *S. solidus* infection. This is despite the role of reproductive disruption that this parasite plays in some natural stickleback populations.

In a recent study, Seear et al. (2014) demonstrated that *spiggin B*, *spiggin C1* and *spiggin C2* expression is higher in nesting fish than non-nesting. Furthermore, the expression of these three genes was significantly affected by water condition, where fish reared under flowing-water conditions showed significantly increased levels of spiggin gene expression compared to those reared in still water.

Seear et al. (2014) provided evidence of stronger *spiggin B* expression in flowing water than for the other two genes, even within non-nester males. In contrast to Seear et al. (2014), there was no differential expression of *spiggin B* between infected and non-infected males in this study. It is possible that the infected sires provided certain benefits to their offspring’s KSI and spiggin gene, when paternal infection status had no effect on increase spiggin gene expression. Further studies that investigate the level of spiggin gene expression across paternal infection status
that vary in their degree of infection and under variable environmental conditions are required.

5.5.4 Weaknesses of the study and suggestions for future study

I have shown that paternal infection status has the potential to affect offspring susceptibility to *S. solidus* infection.

On the other hand, there were some limitations of the experimental design represented by the lack of control for susceptible/resistance genotype in the parents as they were wild caught infected/ non-infected fish. An experimental study that considers the sires were experimentally infected is needed, to determine the resistance/susceptibility genotype of the sires to *S. solidus* infection. As I am unable to prove/rule out that the increased in infected sire offspring susceptibility to *S. solidus* results mainly from the transgenerational epigenetic effect, rather than from the inheritance of greater susceptibility in their offspring *per se*, further investigations are still mainly required.

Furthermore, I faced another difficulties during the breeding season when performing *in vitro* fertilization, it was quite difficult to ensure a sufficient number of gravid females and large egg clutch size. Only a few eggs from the same female were fertilised with the testes of the male. Therefore, it was difficult to have maternal half-sibship mothered by the same female and sired separately by infected and non-infected fathers. Therefore, an experimental study that considers maternal half-sibship will be needed to accurately assess paternal infection effects between siblings.

As infection status strongly affected the expression of two spiggin genes to upregulation in infected males, generally speaking the level of infection was low for each fish. Further experimental studies with a variation in the level of infection that associated with varied parasitic mass per fish will be required. In addition, the use of male fish from different UK populations to allow for a comparison of spiggin gene expression needs to be conducted.
5.6 References


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Chapter 6

Discussion
6.1 Main Finding

Parasites are ubiquitous, and have the potential to interfere with host biology. The outcome of a given parasitic infection can change depending on host phenotypic and genotypic factors. Therefore, the pre-existing variations between particular hosts species might influence the susceptibility or resistance to parasitic infection. Understanding how these host morphological and genetic factors affect host-parasite interactions are important in shaping host-parasite coevolution models. In this thesis, the three-spined stickleback and both Schistocephalus solidus and Diplostomum spathaceum were used as a host-parasite model to investigate a range of related questions. Firstly, identifying the effects of parasite infection on host biology, and secondly investigating how host factors can affect host-parasite interactions. I will summarise the host factors identified as important to host-parasite interactions and key results will be summarised, limitations highlighted, and suggestions as to future work will be given.

6.1.1 Spatial and seasonal variation in parasite infection

6.1.1.1 Parasites occurrence and seasonality in sticklebacks

The field survey of stickleback populations over one year examined the variation in macroparasite community composition and their seasonality in the River Soar, Leicestershire (Chapter 2). A survey of 271 fish revealed 12 different species belonging to various taxa; I found considerable differences among the populations in terms of both the overall diversity and the richness of the parasite community. Three species were present throughout the year: Argulus sp., Schistocephalus solidus and Diplostomum spathaceum. The number of D. spathaceum metacercariae was higher in June than in other months due to the abundance of a new generation of suitable intermediate hosts (snails) (Pennycuick, 1971b). Infected male fish had a poorer body condition than non-infected and infected females, furthermore, S. solidus plerocercoid masses varied between both host sexes with a heavier plerocercoids mass being found in the males. It is possible that both sexes are probably varied in their response to nutrient theft by S. solidus, even the mechanisms by how each sex response to nutrient theft and the factors responsible for variation in their body condition remains debatable.
The seasonal cycle of particular parasitic species might attribute to the short annual cycle and seasonal variation in the environment of their host. This suggests that stickleback populations in the River Soar may experience divergent parasite-mediated selection, which would exert varying degrees of pressure on the evolution of their resistance and immunity.

6.1.1.2 Are populations different in their infection resistance?

To examine spatial variation in host susceptibility/resistance to parasitic infection, lab-bred fish from two stickleback populations (Carsington and Llyn Frongoch) were experimentally exposed to the common eye fluke *Diplostomum spathaceum* (Chapter 4). Populations differed significantly in their susceptibility, which could be due to genetically predisposition factors and a level of immune gene expression in the individuals of each population. Stress levels during individual exposure probably varied between sexes, which could also lead to an increase/decrease in the fishes' ability to avoid cercariae encounters. Since the fish used in this study were bred from lakes through habitat-wild parents, parental background might play role in increased offspring immunity via genetic inheritance. As suggested by Kalbe and Kurtz (2006), three-spined sticklebacks from lake habitats were less susceptible to *D. pseudospathaceum* infection after a single exposure as their innate immunity defended them against infection to a greater extent than river fish in Germany. Males had also higher infection intensities than females, which could be related to immune competence differences among the host sexes, especially when this experiment was run during the stickleback breeding season (Kurtz et al., 2007). Host-parasite interactions are known to vary geographically within the same host and parasite species, and host resistance might be strongly affected by environmental factors such as temperature and resource differences. Hence, it seems unlikely that there are any environmental factors that might affect fish susceptibility to *D. spathaceum* infection as the experiment was conducted under controlled conditions.

In terms of local adaptation in host–parasite interactions, parasites have to adapt to host evolution, leading to oscillatory dynamics in both host and parasite allele frequencies (Krist et al., 2000). Sympatric host species are more susceptible to local parasite infection in the same geographical area than the parasite from allopatric
location (Krist et al., 2000, Voutilainen et al., 2009a). In this study, the differences in parasite burdens between stickleback populations possibly contributed to divergent selection in life history and immunity that lead to reproductive isolation and host speciation (MacColl, 2009).

The fish in this study differed greatly in size, and there are several lines of evidence to indicate that Llyn Frongoch fish normally have a slower growth rate than other populations in the UK, as recorded by Allen and Wootton (1982), due to an adaptation to lake foraging habitats. Infected fish from both population were shown length reduction than control individuals at the end of this study. This result is likely to be related to amount of cataracts and opaqueness which are both negatively correlated with fish growth, in agreement with other fish studies (Voutilainen et al., 2008, Kuukka-Anttila et al., 2010).

6.1.2 Host factors and parasite infection
6.1.2.1 Host genotype and phenotype
Individual variation in host phenotype should lead to differences in infection; in three-spined sticklebacks, parasitic infections have been associated with phenotype-dependent traits such as habitat use (benthic and limnetic morph) and diet (Stutz et al., 2014). Within populations, individuals with benthic or limnetic phenotypes were more likely to be infected with a benthic or limnetic parasite; however, across populations, the relationship between morphology and infection rate was absent in limnetic or reversed benthic parasites, which might suggest phenotype-dependent exposure due to foraging habits (Stutz et al., 2014).

In this study, three-spined sticklebacks were experimentally exposed to a control dose of *D. spathaceum* cercariae. Significant differences were reported in host susceptibility to infection, where the host phenotype for lateral plates and the ectodysplasin *Eda* genotype were found to be associated with susceptibility to infection. Fish with a high number of plate phenotypes were found to increase infection development (Chapter 4). This finding is contrary to a previous wild study by Simmonds (2015), which suggested that the *D. spathaceum* infection was evenly distributed between genotype and plate morphs. In the present study, these results were also contrary to the results of two recent studies by Morozińska-Gogol (2011),
in a wild marine fish, and Simmonds (2015), in lab-bred freshwater fish. Both authors found low-plated genotype fish were more susceptible to *Schistocephalus solidus* infection. Therefore, in the present study, high levels of infection in partially plated phenotypes might be related to non-immunological factors. For example, the thickness and viscosity of the skin mucus, the thickness of muscles layers under the skin the and possibly of other behavioural resistance traits that might present a strong barrier to cercariae penetration (Betterton, 1974). Another possible explanation is that having more plates might present an effective physical obstacle to cercariae penetration; therefore, parasites might prefer other points of entry such as the head and gill regions instead, and then subsequently establish in the eyes. Moreover, other innate immunity components, most likely other intrinsic genetic basis variations between genotypes and expression levels of fish immune genes lead to noticeable differences in fish susceptibility (de Roij et al., 2011, Robertson et al., 2017). Adult (1+) fish showed a higher intensity of metacercariae with *Diplostomum* in their lenses than juveniles; this result contributed to increasing breeding hormone levels associated with greater age and stress (from exposure to parasites), and an immunological defence that was dramatically reduced against pathogens, which is in agreement with a previous study by (Schalk and Forbes, 1997). Older fish might show a decreased ability to develop resistance mechanisms to new parasites, especially when they have not previously experienced such an infection.

6.1.2.2 Host sex and body size

Body size can play an important role in determining host susceptibility to parasitic infection, and may influence parasitic development and, further, potentially influence host interactions with the parasite (Poulin, 2011). Environmental conditions might have a direct effect on host body size and growth, which in turn might be expected to have a significant effect on transmission rates of parasites and pathogens (Marcogliese, 2008a). Body size variations may have important implications for parasite infection between host sexes, where larger body size may play an important role in determining the pattern of infection by presenting an easy target for parasitic invasion (Haas, 2003). There are a number of differences between males and
females in terms of behaviour, immunity, physiology and ecology that might give rise to one sex becoming more susceptible to parasitic infection than the other (Barger, 1993, Zuk and McKean, 1996). Growth and overall body size may be altered in some species as they reach maturity, leading to sexual size dimorphism. It has been found that parasites may interact with both host hormones and immunity, resulting in sexual size dimorphisms in some animals (Potti and Merino, 1996). Therefore, understanding the effect of host sex and size at the time of parasite exposure might have important implications in terms of interpreting the dynamics of infection and the host-parasite ecology.

Interestingly, evidence was found that the *S. solidus* plerocercoid exhibited more rapid growth in a faster-growing fish host (Barber, 2005). In Chapter 3, fish that varied in size but were same age did not show any differences in their susceptibility to *S. solidus* infection. This is in contrast with a previous experimental study, where the body sizes of three-spined sticklebacks were shown to be an important factor in significantly increasing infection rates and parasite growth; however, age is not (Simmonds, 2015). In the present study, all fish were eight months old at the time of exposure, meaning that it is possible that older fish were better able to withstand infection than younger.

Host sex was not found to be an important factor in determining fish susceptibility to *S. solidus* infection. Variations in parasitism between both sexes have been reported in a number of studies including wide ranges of animals hosts such as fish (Tierney et al., 1996) amphibians (Tinsley, 1989), birds (Poulin, 1996) and mammals (Folstad et al., 1989). In the present study, since the experiment was conducted during the fish breeding season, and despite the cost to reproduction associated with immunity reduction (Kurtz et al., 2007), it has been suggested that there are other important ecological factors that might create a fish sex-biased *S. solidus* infection in natural populations, such as habitat use, diet and behavioural traits, which is consistent with other gender difference studies (Pennycuick, 1971a, Reimchen and Nosil, 2001).

The results show that infected three-spined stickleback females and males had reduced gonadosomatic (GSI), and males kidneysomatic (KSI) indices than non-infected individuals, which is consistent with the results of other studies, suggesting
that *S. solidus*-infected males are unable to breed (Arme and Owen, 1967, Tierney et al., 1996). By contrast, recent studies have investigated the potential reproductive ability of infected wild males from two populations in the UK (Macnab et al., 2009, Macnab et al., 2011). Furthermore, Heins and Baker (2008) observed that infected females showed the ability to produce egg clutches in Alaskan populations. One possible explanation for this could be due to the time of the evolutionary interaction between *G. aculeatus* and the cestode *S. solidus* (Macnab et al., 2009), leading to reproductive differences between stickleback populations in the UK.

Another reason for reproductive variations between populations probably arises as a result of nutrient theft as a higher food demand will be the consequence of the parasite increasing in size, and can be associated with decreasing metrics of reproduction (Heins et al., 2010). However, further studies are clearly needed to fully understand the reasons behind population differences in terms of their associated reduction in reproduction. For example, the hypothesis that the parasite or host is locally adapted (host-parasite specificity), or how the combination of $G_h \times G_p$ interactions are influenced by the consequences of infection. If a host population is locally adapted to its own parasite population, it is unlikely to show reproductive reductions. A greater susceptibility may put the populations’ growing dynamic under threat of reproductive castration (Heins et al., 2010).

6.1.3 Does paternal infection status affect offspring susceptibility to *S. solidus* infection and spiggin gene expression?

Parental effects are defined as the influence of the parental genotype or phenotype on the offspring phenotype (Wolf and Wade, 2009a). Male three-spined sticklebacks *Gasterosteus aculeatus* is well known to have a characteristic reproductive behaviour. Reproduction reduced in *S. solidus*-infected males appears to result as a side effect of infection (Macnab et al., 2011). However, some infected males in some populations showed the ability to develop sexually (Heins and Baker, 2008, Macnab et al., 2009). Therefore, the reproductive success of males sticklebacks under normal environmental/ degraded conditions is of particular interest that might help to understand how sire infection status impacts offspring performance including
their ability to withstand infections and subsequent progression of disease especially when female sticklebacks have to mate with parasitized males.

Host innate immunity against pathogen are strongly affected by previous parasitic outcome experienced condition which possibly leading to enhance same host and his new offspring immunity (Little et al., 2003). In three-spined sticklebacks’ infection, paternal effects can have both a significant cost and benefit (Kaufmann et al., 2014). As the parental derived immunity is essential in the offspring early life against pathogen in fish (Swain and Nayak, 2009). In the present study, the number of S. solidus-infected offspring sired by infected males was significantly higher than individuals from non-infected males at the end of the experiment. It is suggested that this attributed to the variation in their paternal immunity genetic background particularly when the offspring used in this experiments were generated from wild caught parents. However, infected sire offspring had increased in both body length and mass at the end of the study, meaning that paternal infection can also have clear benefits on offspring condition and increased tolerance.

Reproductive success disruption has been reported by Kaufmann et al. (2014) in three-spined sticklebacks that have been experimentally exposed to the nematode *Camallanus lacustris* due to reduced success in sperm competition in fertilization trials. In addition, the authors found that the offspring generated from exposed males showed high mortality rates after exposure to the parasite; however, they had better body condition than fish generated from unexposed fathers.

The expression of the spiggin gene is routinely used as an endpoint of male reproductive development in sticklebacks. I found that *spiggin C1* and *spiggin C2* expression was significantly higher in infected fish than non-infected fish; however, paternal treatment had no effect on the expression of all genes under investigation. As there was trend of statistically significant differences in KSI between infected and non-infected fish with a slightly higher KSI in infected than non-infected males, this result indicates that the sticklebacks were able to adjust spiggin gene expression patterns, even when they developed the infection.
There are two possible explanations for high spiggin C1 of spiggin C2 expression in males that show a unique evolutionary interaction with the parasite by showing an increased reproductive capacity, even when they are infected. The second is lower parasite intensity, so fish can cope with lower levels of infection and produce spiggin. Lower spiggin concentration was found for a higher parasite number and mass (Rushbrook and Barber, 2006).

6.2 Ideas for further work

This thesis illustrates the potential for various host phenotypic and genotypic factors to have substantial effects on disease progression. It is well known that the factors influencing parasitic infections are complex and the combined effects of these factors might significantly influence disease outcome in infected hosts. However, as global warming affect wildlife, a number of questions still need to be investigated regarding the combination of the effects of variation in host factors and environmental conditions on disease phenotype.

The experimental infection study yielded the observation that host sex and body size had no effect on increasing fish susceptibility to *S. solidus* infection. However, naturally infected *S. solidus*-three-spined stickleback was shown sex biased in some studies. There would need to be further studies to validate our results under varied environmental condition and feeding preference choice are needed.

Since the reduction in reproduction in males varied between populations (Macnab et al., 2009), and infection had a strong influence on the GSI of both sexes, further research is required to investigate whether other infected wild populations are able to spawn. Future studies should focus on population variations, and how genetic and environmental factors interact to create a sex bias in this host-parasite system.

The results in Chapter 5 give an indication that paternal effects were expressed as increased offspring susceptibility to *S. solidus* infection. Therefore, an experimental study that considers the sires were experimentally infected is needed, to determine the resistance/susceptibility genotype of the sires to *S. solidus* infection. Further study with more focus on transgenerational epigenetic effect/inheritance of greater susceptibility in the infected sire offspring is therefore suggested. There were a
limited number of eggs from the same female that were fertilised with the testes of
the male. Therefore, it was difficult to have a maternal half-sibship mothered by the
same female. Furthermore, maternal half-sibship would be needed to accurately
assess infection paternal effect between maternal half-sibship siblings. The
expression of spiggin genes may differ between male fish regarding their *S. solidus*
infection status.

In present study, the level of infection was low, meaning that the effect of infection
intensity could not be tested. Further experimental studies into infection with a
variation in the level of infection and parasitic mass per fish will be required. In
addition, the use of males from different UK populations and a comparison of spiggin
gene expression need to be investigated.

This study indicates that the *Diplostomum* parasite is able to infect non-local host
populations. The results demonstrated that each population could be somewhat
varied in their resistance to *Diplostomum* infection depending on individual age, sex
and other such genetic background. In order to test for geographic variation, and the
host-parasite local adaptation theory associated with fish resistance to this parasite,
fish from various areas in the UK should be examined.

The role of plate morphology in determining three-spined sticklebacks’ susceptibility
to *Diplostomum spathaceum* infection was noted in Chapter 4. The results found that
Carsington fish show a wider range of morph plates than expected for their genotype.
However, having a higher number of plates probably influences fish susceptibility to
*D. spathaceum* infection, particularly when fish become older. In terms of an
experimental study it would necessary to use completely plated genotype, so as to
give a better indication of host-parasite interaction, would be needed to support our
results. Additionally, further studies would be needed to investigate the association
between the *Eda* genotype with the immune function of *D. spathaceum*-infected fish,
in particular when there is evidence from previous studies that reported low-plated
fish experienced higher burdens of a common ectoparasites, and there was a
significant overall effect of the *Eda* genotype on the immune gene expression levels
(Robertson et al., 2017).
6.3 Concluding comments

The major conclusion of this thesis is that host-parasite interactions are influenced by host factors and some of the ecological and evolutionary traits in which they interact. Host-parasite interactions have the potential to reshape host responses and disease phenotype. The pathogenic effects of *S. solidus* and *D. spathaceum* infection have helped to shed some light on the ecology and evolution of stickleback-parasite interactions.

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