Experimental investigations into the effects of Schistocephalus solidus on the reproductive biology of sticklebacks

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Abstract

Parasitism involves two distinct organisms, the parasite and the host. Parasite interactions add to the complexity of hosts' biology. Parasites frequently reduce reproductive success of hosts, often explained by simple energy theft. However, increasing evidence suggests parasitism causes altered host phenotypes in other ways. Changes have been categorised as; 1) direct manipulation of the host, 2) defensive adaptations by the host, or 3) side effects from infection.

The effect of *Schistocephalus solidus* infections on the three-spined stickleback's ability to undergo sexual development varies greatly. The mechanisms underlying this are still relatively unknown. In laboratory conditions, with unlimited food sources, effects of infection vary across populations. Suggesting adverse outcomes are not solely down to resource availability, and other factors are controlling fish development. Different populations of fish have been coexisting with the parasite for varying timescales; the level of adaptation to overcoming them is likely to differ. Variation between *S. solidus* could lead to different responses from their hosts. Fish may be able to overcome negative effects of infections. Alternatively, some parasite populations may have the ability manipulate their fish host so could have a greater impact on them.

The main aim is to investigate the mechanisms by which parasites alter host reproduction in the stickleback–*Schistocephalus* model; specifically focusing on differences observed between populations. The effect of the timing of infection and food supply on the sexual development of stickleback hosts is also investigated.

The clear outcome of this thesis is that variation between populations exists. The population background of the parasite plays an important role in determining the outcome of infections. Parasites from Carsington Water (CRS) have detrimental effects on male stickleback's nesting behaviour. This effect was seen in both wild-caught and lab bred males from all studied populations. Other parasite populations did not show this effect. The cross infections indicate that different mechanisms may be occurring. Fish and parasites were treated the same but differences in fish response are still observed. Analysis of the *CO1* gene revealed genetic variation between parasites, indicating greater mutation rates in the CRS genome, thus manipulation genes may develop.

The second outcome shows effects of parasitism on development and gene expression of key genes involved in reproductive development. Infections at key time points during the year showed younger fish are compromised to a greater extent. *Kiss2* expression was compared between infected fish with unlimited food and those on a low ration. Surprisingly *Kiss2* expression significantly increased in fish on limited diets. No change in expression was found in infected fish. Similar expression levels of *Kiss2* might be expected between ration fed and infected fish if the same mechanism between low nutrition and energy drains were in place.

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1 Introduction

Host-parasite interactions often lead to changes within the biology of the host organism. However the mechanisms underlying these alterations are often unknown or unclear. The purpose of this thesis is to further understand the mechanisms involved in the reduced reproductive capabilities of three-spined stickleback *Gasterosteus aculeatus* infected with the parasitic tapeworm, *Schistocephalus soldidus*.

1.1 Parasitism

Parasitism, as a way of life, is widespread and varies in form (Matthews, 1998). It is one of the most successful modes of life, as demonstrated by the large number of times it has evolved, creating huge diversity of parasite species and life cycles (Deaton, 2011). Parasites are ubiquitous, with estimates made that over half of all living species, including both plants and animals, are parasitic. Many organisms including plants, mammals, insects and fish can become infected and serve as hosts (Minchella, 1985, Matthews, 1998, Hurd, 2001, Moore, 2002).

Parasitism involves two distinct organisms: the parasite, generally defined as an organism that depends on another organism for its nutritional needs, and the host, the organism the parasite lives on or in. This dependence will inevitably have a negative effect on the host's fitness to some degree. Some parasites are relatively benign in their effects, whereas others result in host death (Clayton and Moore, 1997). Parasites can have simple life cycles, consisting of only one host organism on which the parasite completes its whole life, for example the human head louse *Pediculus humanus capitis* (Matthews, 1998). However, life cycles of parasites may require more than one host species, with the parasite developing its different life stages in different hosts (Price, 1980, Smyth, 1994, Clayton and Moore, 1997, O'Donnell, 1997, Matthews, 1998). When life cycles consist of more than one host, organisms in which parasites mature and reproduce are classed as **definitive** hosts, and those in which the parasites completes one of its developmental (larval) stages are **intermediate** hosts (Smyth, 1994).

Interactions with parasites add greatly to the complexity of the hosts' biology, and parasites are often observed to have significant and dramatic effects on morphology, physiology and behaviour (Santos and Santos, 2013). The ability of the host to carry out normal behavioural patterns, including reproductive behaviour, can be affected by parasite infection (Matthews, 1998, Barber *et al.*, 2000, Hurd, 2001, Moore, 2002). Being parasitized has been shown to alter host behaviour in many different ways, and the

greatest changes are often observed in intermediate hosts. There are two main reasons for this: (1) it is within the intermediate host that many parasites achieve the most extensive, and rapid rates of, growth which can impose enormous physiological burdens on intermediate hosts and affect behaviour. For example, the plerocercoid of the tapeworm, *solidus*, grows to an enormous size within its intermediate host, the three-spined stickleback, often contributing over half the mass of infected fish (Arme and Owen, 1967, McPhail and Peacock, 1983, Barber, 2007), and (2) many of the changes observed in the intermediate hosts often aid transmission of the parasite to its definitive host *Schistocephalus*. Parasites that alter behaviour might be selected to manipulate intermediate hosts to facilitate this (Santos and Santos, 2013). The two causes for changes within intermediate hosts may not be mutually exclusive.

Changes in host behaviour can influence activity. For example, three-spined sticklebacks infected with *S. solidus*, move more slowly (Arme and Owen, 1967) and have reduced flight response to predators (Godin and Sproul, 1988) compared to non-infected conspecifics. Changes can also alter microhabitat selection, with parasitized sticklebacks swimming closer to the water surface (Arme and Owen, 1967, Quinn et al., 2012), and rodents infected with *Toxoplasma gondii* choosing open habitat instead of staying under cover (Berdoy et al., 2000, Webster et al., 2006, Webster, 2007). Changes can be related to specific life history traits, such as reproductive behaviour, which is often reduced in parasitized organisms (Lehmann, 1993, Macnab et al., 2011, Lafferty and Shaw, 2013). Parasitism can also alter host morphology, such as the demelanisation of *S. solidus* infected three-spined sticklebacks (LoBue and Bell, 1993). How and why these alterations occur is often unclear, and a great deal of research into the underpinning mechanisms has been undertaken in recent years (Lafferty and Shaw, 2013).

1.2 Infection-associated host phenotype change: Parasite manipulation, host adaptation or simple side effect?

The changes within a host associated with parasite infections have been categorised as arising from one of three processes (Poulin (1994), Loot et al. (2002), Heins et al. (2004), Heins et al. (2010a), Schultz et al. (2006), Trubiroha et al. (2009) and Macnab et al. (2011)): (1) direct manipulation of the host by the parasite, (2) defensive adaptations by the host against the parasite, and (3) simple side effects that arise as a by-product of the infection. The molecular genetics and physiological mechanisms underlying the three

processes are not yet fully understood and remain of considerable scientific interest (Lafferty and Shaw, 2013). There are many examples by a parasite infection altering host phenotype, but the physiological mechanisms for the observed changes are often unclear, making it difficult to conclude whether such phenomena are adaptations of parasites, or hosts, or are simply not adaptive at all (Poulin, 1995, Hurd, 2001). Each parasite-host interaction must be assessed on an individual basis. Even when parasite species are closely related, infecting similar hosts, and the altered host phenotype appears to be the same, this may not be caused by the same mechanism. For example, studies comparing the tapeworms *Ligula intestinalis* and *S. solidus* have shown that they induce similar reproductive phenotypes in their intermediate fish hosts. Both infections result in a reduction in their host's ability to sexually mature and reproduce, but the underlying mechanism appears to differ. Whereas *L. intestinalis* manipulates its host by hormonal castration (Arme, 1997, Williams et al., 1998), research suggests that the changes caused by a *S. solidus* infection are more likely to be caused by the side effect of nutrient theft (Barber, 2007, Heins et al., 2010a).

1.2.1 Parasite manipulation

In cases of manipulation, the changes seen within the host are induced by the parasite for its own benefit. To be considered as a true adaptive manipulation, the altered host phenotype must be shown to increase the parasite fitness in some way, for example increasing parasite growth rate or reproductive success. For parasites infecting intermediate hosts, this typically means increasing the chance of successful transmission to the definitive host. When such life cycles involve trophic transmission the parasite is directly dependent on its host being consumed by the next host in its life cycle and manipulation by the parasite can aid this (Poulin, 1994, Poulin, 1995, Hurd, 2003, Poulin, 2010).

Over the past four decades adaptive parasite manipulation has generated considerable interest within the scientific community. The published research demonstrates many contrasting explanations and conclusions related to this hypothesis (Poulin, 2010). The support and evidence for the hypothesis ranges from clear, strong manipulation, through to weak, tenuous links, but many of the published examples of altered host phenotype are inconclusive regarding the underlying mechanisms (Hurd, 2003, Poulin, 2010).

Changes in host phenotype can clearly arise as a result of parasite manipulation. Some of the best evidence supporting true adaptive manipulation is the synthesis of molecules or biochemical agents by parasites that influence host phenotypes in a manner that benefits parasite fitness (Hurd, 2003). Warr et al. (2006) demonstrated that the metacestodes of the tapeworm *Hymenolepis diminta* secreted a manipulator molecule that decreased the ability of host beetles (*Tenebrio mollitor*) to synthesis Vitellogenin (Vg). The reduced Vg synthesis prevented beetles from developing eggs. The authors concluded that this reduced unsustainable energy demands on infected females, increasing host lifespan and therefore the likelihood of predation by definitive hosts. The parasite fitness indeed increased, indicating that adaptive manipulation is occurring (Poulin, 1995).

The mechanisms underpinning other parasite-host interactions are less obvious. Initially altered host phenotypes can appear to be caused by manipulation by the parasites, but further investigations may show they are due to side effects rather than manipulation. One example of an altered host phenotype which had previously been regarded as parasite manipulation but is now under question is the altered feeding behaviours in mosquito vectors when infected by malaria parasites. Field studies have shown that the feeding behaviour of infected mosquitos is altered in two ways: 1) mosquitoes infected with sporozoites (the stage transmitted to human hosts) take larger blood meals - 82% of infected mosquitoes engorged fully compared to 72% of uninfected mosquitoes, and 2) sporozoite-infected mosquitoes are more likely to bite several people per night than uninfected ones, suggesting their host seeking behaviour is also increased (Koella et al., 1998). Anderson et al. (1999) demonstrated a difference in the altered phenotype depending on the developmental stage the malaria parasite had reached. When the parasite is at the oocyst stage the mosquito biting behaviour is actually decreased compared to uninfected mosquitoes. However, once the malaria parasite has developed to the sporozoite stage the biting behaviour increases. It is often concluded that an altered host phenotype that differs depending on the developmental stage of the parasite must indicate parasite manipulation. This has led to the suggestion that different stages of malaria parasites manipulate their mosquito vectors in different ways (Koella et al., 1998, Anderson et al., 1999, Schwartz and Koella, 2001). But in a recent study, Cator et al. (2013) have questioned the manipulation role of the parasite in this system, suggesting that the observed changes in behaviour maybe a side effect of an immune response to the infection. They were able to obtain a similarly altered feeding habit by injecting mosquitoes with heat-killed *E. coli*, thus stimulating the immune response.

Altered foraging behaviour dependent on the developmental stage of the parasite is demonstrated in three-spined sticklebacks infected by *S. solidus*. It has been shown that the foraging activity and boldness of host sticklebacks is reduced in comparison to non-infected fish, when plerocercoids are below the threshold weight of 50 mg required to successfully infect definitive bird hosts. However, once plerocercoids exceed the threshold weight, foraging activity increases (Tierney et al., 1993). Furthermore Talarico et al. (2017) showed that infected sticklebacks returned to foraging faster than non-infected conspecifics following a stimulated bird strike, whereas in a novel environment no difference was seen in foraging, suggesting specific manipulation of the predator avoidance behaviour in the presence of predators.

Another classic parasite-host interaction, often been cited as an obvious case of parasite manipulation, is that of the *Toxoplasma gondii* in rodents. Infected rodents have been shown to prefer areas that are scented with cat urine, and this is considered to be manipulation by the parasite to aid transmission to the cat host (for example see (Berdoy et al., 2000, Webster et al., 2006, Webster, 2007). But in a recent review of the literature, Worth et al. (2013) question if this altered behaviour is indeed manipulation, finding inconsistences in the behavioural changes reported in the infected rodents. They also found no clear evidence that when infected rodents did spend more time in areas scented with cat odour it actually led to more of the infected rodents being caught by cats compared to non-infected individuals. Transmission to cats is not essential for T. gondii to complete its life cycle. Other related parasites – for which the cat is not a host – also produced the same cat odour-seeking behaviour in infected rodents. Therefore, they concluded that this is not an example of manipulation but rather a side effect of the infection. Although parasite-altered host phenotypes can be framed as fascinating and attractive stories suggesting active manipulation, clear evidence must be shown before these can be proven (Poulin, 1995, Worth et al., 2013).

1.2.2 Host adaptation

Another possibility is that the infection-associated changes seen in hosts are produced by the host itself to overcome or reduce the impact of being parasitized. The negative effects of infection could be reduced by either by ridding themselves of the parasite through resistance or by adopting a tolerance strategy. Therefore, the altered phenotypes should show clear benefits to the host by the preservation of, or increase in their fitness that would not exist if the changes did not occur (Poulin, 1995, Hurd, 2001). Unlike parasite manipulation of hosts, which has received much interest over the last four decades, the role of host adaptation as a hypothesis for explaining the altered host phenotypes has received less attention and has only more recently gained interest (Moore, 2013). As with the parasite manipulation hypothesis, evidence to support host adaptation is often unclear or inconclusive. Clear examples of adaptation by hosts against parasites include utilizing a defensive mechanism to prevent establishment of the infection, or mounting a successful immune response. Other host adaptations may lead to tolerance of infection, which involves the alteration of their life history traits to reduce the cost of parasitism (Agnew et al., 2000, Hurd, 2001). When hosts are unable to prevent infections from becoming established physically, or by mounting immune responses, they may be able to alter their life history traits to avoid the full cost of infections. Since the impact of infections are often not immediate but increase over time as the parasite grows and develops, the lag between infection and negative impacts may allow hosts to alter their phenotype in a beneficial manner (Agnew et al., 2000). Tolerance allows the host to maximise its fitness whilst being infected, for example, hosts may alter their reproductive strategy (Kutzer and Armitage, 2016, Franke et al., 2017). They may delay reproducing until after the infection has passed, or they may adaptively increase reproductive effort to reproduce early, thus compensating for the future loss of reproductive capability that might occur as the parasite burden increases and energy demands rise (Schultz et al., 2006). These altered life history patterns are less obvious as adaptation by the host.

1.2.3 Side effects

In the final type of explanation, neither the parasite nor the host control the observed changes, i.e. neither parasite manipulation, or host adaptation is involved, and there is no obvious benefit of the altered phenotype to either host or parasite. Many of the effects of parasitism stem from the fact that parasites divert energy resources from the host, either directly, e.g. by using host derived nutrition to fuel development, or indirectly by increasing host energy e.g. in mounting immune responses (Wedekind, 1992, Deerenberg et al., 1997). If an altered host phenotype in response to parasite infection occurs immediately after infection, before the parasite grows, it is often considered an adaptive manipulation. Whereas when changes observed increase as the parasite grows and

develops often indicates a side effect caused by nutritional theft (Hurd, 2001). Potential side effects have been observed in Alaskan populations of female three-spined sticklebacks. Early on in the breeding season *S. solidus* infected females were capable of producing clutches, whereas females were unable to develop clutches later in the season once plerocercoids had grown to a larger size (Heins et al., 1999).

How parasites affect host phenotypes can reflect both the level of energy drain from the parasite and the environmental conditions that the host is experiencing (Hurd, 2001). If infected hosts are brought into favourable laboratory conditions where the host is supplied with unlimited food and protected from competition, the negative consequences of infection might be expected to be overcome. This is the conclusion that Candolin and Voigt (2001) made about the nesting behaviour of *S. solidus*-infected male sticklebacks. In their study they observed that infected males in the wild rarely attempted to defend territories or build nests. However, after transfer to favourable laboratory conditions, infected males successfully built nests, suggesting that the non-nesting behaviour observed was due to a side effect of nutrient theft by the parasite and can be eliminated with unlimited food resources. However, both Rushbrook and Barber (2006) and Macnab et al. (2009) found non-nesting behaviour persisted in infected males despite being in favourable laboratory conditions.

1.3 Reproduction

Life history theory assumes that reproduction is costly to the individual and therefore competes with other activities that the individual needs to carry out (Roff, 1992, Stearns, 1992). Individuals cannot simultaneously maximise all life history traits as energy becomes a limiting resource, so they need to trade-off the demands of competing systems. Energy taken in is channelled into one of three sinks, as summarised in the dynamic energy budget (DEB) theory ((Leloutre et al., 2016) shown in Figure 1.1): 1) the basic maintenance of vital systems; 2) somatic growth; 3) sexual development and reproduction. The priority of any individual is to maintain its vital systems. Excess resources can then be channelled into growth or, depending on the age of the individual, reproduction. Reproduction is energetically costly and therefore only undertaken when an organism has enough energy available to do so. The basic assumption is that reproduction is limited by access to energy, meaning that nutritional status or condition affects fecundity (Hillgarth and Wingfield, 1997, Barber et al., 2000).



Figure 1.1 Schematic representation of the dynamic energy budget. Adapted from Leloutre et al. (2016)

1.3.1 Stickleback reproduction

Sticklebacks are ideal organisms to study the effects of parasite infections on reproductive cycles as they have very distinctive physiological and behavioural changes that indicate sexual maturation and development.

Sticklebacks typically have an annual reproductive cycle, which can be divided into two main phases (Wootton, 1976), although in some populations the distinction between the two is not clear (Mori, 1985). During the non-reproductive phase, males and females are difficult to distinguish in either morphology or behaviour (Craig-Bennett, 1931, Wootton, 1976, Mori, 1985). They live in mixed-sex schools in deeper water habitats. At the start of the breeding season, usually in late April (Craig-Bennett, 1931, Östlund-Nilsson, 2007), the schools move into shallower habitats and males and females develop distinct sexual dimorphism and can be clearly identified. The breeding season phase lasts for approximately 3-4 months. Females remain in schools, whilst the males begin to defend territories and develop their nuptial breeding colours; a distinctive red throat and belly and bright blue irises (Craig-Bennett, 1931). Once the male has successfully defended a territory from other males he will build a nest on the substrate. The nest is made from vegetation and sand, which the male glues together with Spiggin, a glycoprotein secretion from the kidney (Jakobsson et al., 1999). Once the nest is complete the male signals this to the females by 'creeping through' the nest (Wootton, 1976, Wootton, 1984).

Females may develop a contrasting concentration of dark pigment as they become gravid. They will also move out of the schools and into the males' territories. The male and female will then engage in courtship behaviour; the male zigzags and the female assumes the 'head-up' posture. Her contrasting pigmentation may also become more pronounced. Courtship continues until the female enters the nest and spawns her eggs. The male then follows her through the nest and fertilises the clutch. The male remains with the nest fanning it and caring for the young for a few days after they have hatched (Wootton, 1976).

At the end of the breeding season, usually around late July (Craig-Bennett, 1931, Östlund-Nilsson, 2007), surviving adult fish lose their breeding colours and along with the young fish migrate back to the deeper water habitats during autumn (Craig-Bennett, 1931, Wootton, 1976, Mori, 1985).

During development, sexual maturation is indicated by changes in the body somatic indices of fish. Somatic indices define organ weights relative to the total body weight of the individual, enabling comparisons to be made between fish differing in size. Brain size is linked to potential cognitive demands organisms face. For example, nine-spined stickleback (*Pungitius pungitius*) have larger brains when living in more complex habitats (Gonda et al., 2009). Performing complex parental care could require larger brains (Samuk et al., 2014). As male three-spined stickleback mature the brain somatic index increases as they partake in intricate nest building, courtship and care of offspring (Samuk et al., 2014). Sexual development in males is indicated by an increased kidney-somatic index as the kidney hypertrophies and produces Spiggin (Borg, 1982, Jakobsson et al., 1999). In females, maturation is indicated by an increased gonadosomatic index, as vitellogenesis occurs and eggs develop (Borg and Veen, 1982). In fish, the liver is an important energy store, as sexual development occurs and energy is diverted from maintenance and growth into reproduction energy stores may be drained and a reduction in the hepatosomatic index may occur (Hemre et al., 2002).

1.3.2 Physiological regulations of fish reproduction

Puberty, sexual development and maturity in vertebrates are under control of complex feedback loops which involve environmental cues such as photoperiod and temperature. These are the main environmental cues that determine the timing of the stickleback reproductive cycle (Sokolowska and Kulczykowska, 2006, Oakley et al., 2009) along with physiological factors such as nutritional status. These factors activate the Brain-Pituitary-Gonadal (BPG) axis, triggering hormone production, which stimulates gonad maturation and the production of sex steroids. Production of these signals all operate under feedback loops during the breeding season (Hellqvist et al., 2001, Hellqvist et al., 2004, Hellqvist et al., 2006, Shao et al., 2012). Teleost reproduction is known to be

regulated by the BPG axis in a similar way as in other vertebrates. Sex steroid secretion is controlled by gonadotropins (GtHs), which are pituitary hormones that stimulate gonad maturation (Shao et al., 2012). Two distinct hormones produced by the pituitary have been identified and recognized as gonadotrophic in both male and female fish: Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) (Ahsan and Hoar, 1963, Hellqvist et al., 2001, Hellqvist et al., 2004, Hellqvist et al., 2006, Kitano and Lema, 2013).

In males, FSH triggers spermatogenesis and in females it leads to ovarian development. In males LH activates the androgen-producing tissue in their testis, and in females it activates the estrogen-producing follicle cells within their ovaries. Ahsan and Hoar (1963) established the important role LH has in the stickleback reproductive cycle. They held adult fish, which would normally mature sexually in long-day conditions, under short photoperiods and treated them with LH and FSH. Their results showed that LH promoted gonad development in both males and females, yet FSH on its own produced no accelerated sexual development. Hellqvist et al. (2001) reported that castrated males had lower kidney-somatic indices, LH levels and *spiggin* expression compared to sham operated males, but FSH levels were unaffected. They also showed a positive correlation between 11-ketotestosterone (11-KT), Spiggin and LH. *LH* and *FSH* expression was lower in post breeding males compared to nesting males.

The BPG axis in fish is under control of both positive and negative feedback regulation (Hellqvist et al., 2001, Hellqvist et al., 2004, Hellqvist et al., 2006, Shao et al., 2012). The levels of LH and FSH exhibit annual cycles and the seasonal patterns vary between species, however FSH usually peaks earlier than LH. In three-spined sticklebacks, expression levels are under photoperiod control. Under short photoperiods, *LH* is expressed at low levels, with the highest expression levels being observed in fish kept under a 16L:8D photoperiod at 7°C. *FSH* is also minimally expressed under short photoperiods. A similar pattern was observed in 11-KT levels, which led Hellqvist et al. (2004) to suggest that gonadotropin hormones (GtHs) stimulate androgens. Mayer and Pall (2007) reported similar links between LH and FSH and androgen production in testes. In the three-spined stickleback significant seasonal changes occur. Hellqvist et al. (2006) measured levels over an annual cycle and their findings are shown in Figure 1.2.



Figure 1.2 Seasonal cycle of LH- β and FSH- β mRNA expression, and of Kidney-somatic index (KSI) in male sticklebacks and gonadosomatic index (GSI) in female sticklebacks. From Hellqvist et al. (2006)

In males, spermatogenesis and the development of secondary sexual characteristics are separated in time. Exogenous androgens probably inhibit GtH secretions (Hellqvist et al., 2006). Spiggin production is also under control of androgens (Borg, 2007) which in turn are controlled by gonadotropins; therefore Spiggin synthesis follows a similar seasonal pattern (Mayer and Pall, 2007).

In females, vitellogenesis starts in the winter and increases in the spring (Shao et al., 2012). As females repeatedly spawn through the breeding season vitellogenesis must continue to be active (Hellqvist et al., 2006). Katsiadaki et al. (2012) showed how plasma levels of Vitellogenin (VTG) cycles throughout the year in both females and males. Although the levels in males were considerably lower than in females, they follow the same pattern. The highest levels were seen in May and June, with significant drops between June and September. This cycle is also likely to be under control of GtHs.

GtHs are produced by the pituitary, which is stimulated by Gonadotrophin-releasing Hormones (GnRHs). GnRHs play a role in the regulation of seasonal reproduction. It has been shown that the increase in expression under long photoperiods is dependent on the presence of gonads; therefore, feedback effects on the BPG axis are critical to induce expression (Shao et al., 2015). GnRH was believed to initiate activation of the BPG axis, but recently the importance of Kisspeptin, a neuropeptide encoded by *Kiss1* and *Kiss2* genes, has been recognized in the early part of the pathway, as shown in Figure 1.3 (Felip et al., 2009, Oakley et al., 2009, Mechaly et al., 2011, Mechaly et al., 2012, Mechaly et al., 2013, Tena-Sempere et al., 2012, Zmora et al., 2012). Increases in Kisspeptin lead to increases in GnRH, LH and FSH level thus triggering reproductive development (Park et al., 2016a).

Kisspeptin expression is altered under different nutritional regimes and this is now thought to be how the physiological cue by which nutritional status triggers an organism's sexual development (Mechaly et al., 2011, Mechaly et al., 2013). Nutritional drain induced by parasitic infections could also change *kisspeptin* expression, and this may be the mechanism linking nutrient theft and reduced reproduction in parasitized animals.



Figure 1.3 Diagram of the neuroendocrine control of gonadal maturation in vertebrates via the brain-pituitary-gonadal (BPG) axis. From Mechaly et al. (2013)

1.4 Schistocephalus solidus life cycle

The life cycle of S. solidus is shown in Figure 1.4. During its life, S. solidus passes through three host stages (Smyth, 1946, Barber and Scharsack, 2010) and is a common parasite of three-spined sticklebacks in slow-flowing or still waters (Wootton, 1976, Barber, 2007). The definitive host for S. solidus is non-specific and can be any endothermal vertebrate, however this commonly tends to be a piscivorous bird (Tierney and Crompton, 1992). Within the definitive host, the parasite rapidly matures in response to the change in temperature, producing eggs within 2-3 days of entering the intestine. The eggs are then passed out in the host faeces (Barber and Scharsack, 2010) and after a period of development hatch to release free-swimming coracidia. Coracidia are then consumed by the first intermediate hosts, cyclopoid copepods, and develop into procercoids in the copepod body cavity becoming infective to the next host following the development of a cercomer (Smyth, 1969). When three-spined sticklebacks, the second intermediate hosts, feed on copepods harboring infective procercoids, infection can be established in the fish. The parasite passes through the intestinal wall into the body cavity of the fish where development takes place (Hammerschmidt and Kurtz, 2007). Within the fish the parasite stages are known as plerocercoids, and they can grow to enormous sizes compared to the fish host (Arme and Owen, 1967, McPhail and Peacock, 1983, Barber, 2007). Plerocercoids become infective to the definitive host once they have reached a mass of 50 mg (Tierney and Crompton, 1992). The life cycle is then completed when sticklebacks containing parasites of this size are consumed by birds (or other endothermal vertebrates).



Figure 1.4 The life cycle of *Schistocephalus solidus*. From Banner (2016)

1.5 Parasitic tapeworms and fish reproduction

Since parasites extract some or all of their resources from their hosts they negatively affect fitness (Hillgarth and Wingfield, 1997) and often reduce host reproductive success (Lehmann, 1993). Many examples of reduced reproduction in parasitized organisms can be explained by simple energy theft by the parasite. However, there has been increasing evidence over recent years that parasitism causes altered host reproductive success in other manipulative ways. These mechanisms can be adaptations that either the parasites have made to manipulate their hosts or that the host displays to survive the infection (Brown et al., 2001).

The tapeworms *Ligula intestinalis* and *S. solidus* have generated scientific interest due to the effects they have on their intermediate hosts' reproduction. More recently interest in the tapeworm *Schistocephalus pungitii* and its effect on the nine-spined stickleback *Pungitius pungitius* has also gained momentum (Merilä, 2013). All three species of tapeworm typically reduce their hosts' reproductive fitness, but despite being closely related, the parasites may utilize different mechanisms. Both *L. intestinalis* and *S. pungitii* appear to be capable of manipulating the reproductive development of their hosts whereas

changes caused by *S. solidus* may be due to nutrient theft, (Carter et al., 2005, Barber, 2007, Heins and Baker, 2010).

1.5.1 Ligula intestinalis

In a number of studies it has been shown that *L. intestinalis* suppresses gonad development and sterilizes fish hosts. *L. intestinalis* infects many species of cyprinid fish and similar negative effects have been observed across a number of species. Gonadal development is suppressed in roach *Rutilus rutilus* (Arme, 1968, Carter et al., 2005, Trubiroha et al., 2009, Trubiroha et al., 2010, Kroupova et al., 2012), bream *Abramis brama* (Hecker and Karbe, 2005), and spottail shiners *Notropis hudsonius* (Mahon, 1976). Loot et al. (2002) also found that in one population of roach infection caused prevention of reproduction and enhanced growth. Several of these studies have found that reproduction is prevented regardless of the size and number of parasites present. Even small infections that are unlikely to be of high energy demand impaired gametogenesis, yet fish do not resemble starved or food-deprived individuals. This suggests manipulation is occurring rather than a side effect of nutrient theft (Mahon, 1976, Kroupova et al., 2012).

Arme (1968) reported that the gonads of infected males and females were undistinguishable; female ovaries were very small with only first phase oocytes present, and male testes had no cell development beyond spermatogonia. Gamete development had been halted at precisely the point where pituitary activity should have had a controlling influence. Therefore, the author suggested that *L. intestinalis* was potentially manipulating its host by releasing a substance to depress pituitary function. Carter et al. (2005) also focused on the interaction with the BPG axis. They found that infected roach had 50% less LH within their pituitaries, with a corresponding reduction in *LH-* β mRNA expression. This evidence supports the hypothesis that a secretory product from the parasite interacts with the fish BPG axis. Furthermore, Hecker and Karbe (2005) showed evidence for interaction with the endocrine system in bream. In infected males 11-KT was significantly reduced and plasma levels of Vitellogenin (VTG) were also significantly lower in infected females. Moreover, gonad maturation was prevented by infections interacting with the BPG axis which Hecker and Karbe (2005) conclude is evidence for a secretory substance.

The influence of infection on levels of FSH and LH and gene expression of *VTG* and *androgen receptors* (*AR*) has also been demonstrated. Trubiroha et al. (2009) found that FSH and LH pituitary levels decreased in both males and females when infected. Trubiroha et al. (2010) also found that expression levels of *VTG* and *AR* decreased in infected females, but in infected males only *VTG* decreased. These studies also suggest manipulation of the host endocrine system by a secretory substance. Identification of such a substance would indicate clear parasite manipulation of hosts (Hurd, 2003) but as yet no such parasitic factors have been identified that act directly on the BPG axis so other mechanisms may be responsible (Hecker and Karbe, 2005, Trubiroha et al., 2009).

As similar responses have been observed many times this is also considered to be an indication that *L. intestinalis* are capable of manipulation. However, Loot et al. (2002) found that differences in effects from infection occurred when comparing three distinct populations of roach. Two populations had no significant growth enhancement in infected fish, whereas in the third population infected individuals grew to larger sizes than their non-infected counterparts. Growth enhancement, or gigantism, has been described as a result of either parasite manipulation, diverting energy away from reproduction for parasite growth with excess energy enhancing growth in the host, or host adaptation as a reproduction-delaying tactic to overcome the infection (Agnew et al., 2000, Heins et al., 2010b). These phenotypic differences may indicate possible differences in the probability of the completion of the parasites' life cycle or in the environmental conditions that the hosts are experiencing (Loot et al., 2002). Studies comparing laboratory and field conditions have also demonstrated that differences can occur. Trubiroha et al. (2011) found differences in expression levels of FSH and LH in infected fish from field and laboratory conditions. Wild infected fish had reduced expression of both hormones in both sexes, whereas under laboratory conditions FSH was reduced in infected females but no trend was shown in infected males. However, only three infected males were obtained in the laboratory set meaning that the results were not conclusive. Kroupova et al. (2012) compared feeding regimes in laboratory and field infected fish. Growth Hormone (GH) expression increased in infected male fish; however infected females from the field did not show this response. As the BPG axis is gated by metabolic and nutritional factors these differences between field and laboratory studies could indicate as role for nutrient theft as well as manipulation in producing the negative effects on reproduction.
1.5.2 Schistocephalus pungitii

Research on S. pungitii is limited, but so far the evidence points to it being capable of manipulating host reproduction. Heins et al. (2004) demonstrated that the nine-spined stickleback is even more severely affected by S. pungitii than three-spined sticklebacks infected with S. solidus. Female ability to produce a clutch significantly decreased, with 73% of infected females unable to initiate oocyte development and 96% unable to produce a clutch. Infected males had significantly reduced testis development, whereas body condition in infected individuals of both sexes was greater than non-infected. Heins et al. (2004) suggested this demonstrates manipulation, as body condition would be expected to decrease if reduced reproduction was a side effect of nutrient theft. Furthermore, Heins and Baker (2010) showed that both light and heavy infections of S. pungitii prevented reproductive development before females reached a size of sexually mature fish. Since reproduction was prevented even by small parasites unlikely to cause a high nutritional drain, Heins and Baker (2010) concluded that S. pungitii is a parasite manipulator. Yet, in a very recent study Heins (2017) investigated the effect of S. pungitii in a new study population and found contrasting results. Female capability to produce clutches was linked to parasite size. Therefore, for this population, it was concluded that S. pungitii causes reduced host reproduction by simple nutrient theft and not by acting as a true manipulator (Heins, 2017). These contrasting studies indicate that different mechanisms may be operating in different host-parasite populations.

1.5.3 Schistocephalus solidus

S. solidus has classically been viewed as an absolute castrator, a parasite that specifically targets reducing host reproduction by the destruction or prevention of gamete growth. *S. solidus* inhibits the three-spined sticklebacks production of gametes and ability to spawn (Arme and Owen, 1967, Pennycuick, 1971b, McPhail and Peacock, 1983, Tierney et al., 1996), which could be due to parasite manipulation via endocrine disruption (Poulin, 1995). However, recent research has shown the effects of infection on the capability of three-spined sticklebacks to reproduce varies greatly across its geographical range, with many studies demonstrating contrasting results. The effect on development and sexual maturation also differs between the two sexes, which may indicate a side effect of infection rather than manipulation or adaptation (Heins and Baker, 2008, Macnab et al., 2009).

Effect on male three-spined sticklebacks

In an early study, Arme and Owen (1967) reported that male nuptial coloration developed at the same time and to similar intensities in both infected and non-infected males. Folstad et al. (1994) obtained similar results with no significant difference in colour levels between infected and non-infected males. However, Rushbrook and Barber (2006) found that infected males were unlikely to display nuptial colours. Any coloration in infected males was less intense compared to non-infected individuals. Macnab et al. (2009) also found mixed results from two UK populations. Arme and Owen (1967) found that spermatogenesis was not delayed in infected males and they also successfully attempted to build nests. However, Heins et al. (1999) found spermatogenesis was delayed or even prevented; furthermore infected males were unsuccessful at building nests. Nest building was also not undertaken by infected males from Llyn Frongoch, even when provided with favourable laboratory conditions (Rushbrook and Barber, 2006). Mixed results were found by Macnab et al. (2009) when comparing two UK populations. Infected males from Kendoon Loch successfully build nests and courted females, whereas no infected male from Victoria Park Pond, Leicester showed this behaviour suggesting that different mechanisms exist.

Effect on female three-spined sticklebacks

Arme and Owen (1967) observed that infected females are more negatively affected by infection than males, with a delay in maturation of oocytes and vitellogenesis restricted to early stages so that no advanced yolky eggs developed. However, since no histological changes in the pituitary were observed, coupled with lower liver weight, this suggested a general metabolic drain. Similar results and conclusions were made by Heins and Brown-Peterson (2010). However, this negative effect is not observed in all populations. In several studies on Alaskan populations of three-spined stickleback it has been shown that a high proportion of infected females develop clutches, become gravid and attempt to spawn.

One possibility is that infection impacts female sexual development via a simple nutritional side-effect that can be modulated by ecological conditions experienced by the fish (Heins et al., 1999, Heins and Baker, 2008, Heins et al., 2010b). As female reproductive success is linked to food intake and body mass, enough energy to enable transfer of resources from somatic growth into ovary development must be available. The

body condition of fish may play a key role in their ability to reproduce whilst sustaining infection. Therefore, the environmental conditions experienced could account for differences observed between populations (Bagamian et al., 2004).

Stickleback body condition

Sexual development in three-spined stickleback is typically associated with reduced body condition which decreases progressively during the breeding season (Wootton, 1976). Reproductive investment by both sexes is strongly dependent on energy levels. Females invest large amounts of energy into egg production. Males use less energy to produce sperm, but they have high energy demands to produce secondary sexual characteristics, territory defence, nest building and parental care (Wootton, 1984, Rushbrook and Barber, 2006, Rushbrook et al., 2007). In their study, Tierney et al. (1996) found that stickleback body condition differed significantly in the autumn and spring in both infected and noninfected fish as well as between the sexes. Immature infected males had the lowest body condition whereas non-infected mature and immature males had a similar, good body condition. Mature, infected males had the best condition of all, along with larger testes. However, Bagamian et al. (2004) found that infected males had lower body conditions throughout all developmental stages. These differences once again could highlight the fact that different populations of sticklebacks are experiencing varying environmental conditions. As body condition influences gonadal development, and infected individuals are often in poorer condition, infection has a potential consequence for reproduction (Tierney et al., 1996).

Tierney et al. (1996) showed that females had similar body condition regardless of maturity or infection. This finding is supported by several studies that found a positive relationship between body condition and clutch production, meaning that females in better condition are more likely to have a clutch. A number of infected females had mature ovaries indicating infection does not completely remove breeding capabilities, which suggests that energy drain caused by the growth of *S. solidus* is the reason for reduced reproduction rather than manipulation (Kitano and Lema, 2013, Bagamian et al., 2004).

Energy allocation

Most studies have concluded that the effects of *S. solidus* on three-spined stickleback reproduction arise through side effects because the changes observed are not consistent

with manipulation. For example, it would benefit energetically demanding parasites to prevent their hosts from reproducing as early as possible; therefore, if manipulation by the parasite is occurring it would be expected that even small parasites with very low energy demands would cause castration as is seen with *L. intestinalis*. However, three-spined sticklebacks with small *S. solidus* are typically capable of maturing, and are only prevented from doing so by heavy infections, suggesting that simple nutrient theft is a more plausible explanation (Bagamian et al., 2004, Heins and Baker, 2008, Macnab et al., 2009, Macnab et al., 2011, Heins, 2012).

The amount of energy allocated to reproduction could also indicate if manipulation, host adaptation or side effect is occurring. If reproductive investment (RI, the absolute amount of energy devoted to reproduction) and reproductive effort (RE, the proportion of available energy devoted to reproduction) both decrease in infected fish this would support parasite manipulation. If there is a decrease in RI, but an increase in RE, this would indicate that reduced reproduction was a host tactic, whereas, a decrease in RI but no change in RE would indicate a side effect (Schultz et al., 2006, Heins and Baker, 2008). Schultz et al. (2006) found infected three-spined stickleback to have a reduced RI, but a similar RE compared to non-infected individuals, again indicating a side effect. Furthermore, the effect on egg size and number can also indicate which process is taking place. If infection causes a reduction in clutch mass, egg numbers and egg size this indicates a side effect of nutrient drain. A reduced clutch size with no relationship between egg numbers or size would indicate parasite manipulation. Finally, if increased egg numbers occur but of a decreased size, giving the same total clutch mass, this indicates fecundity compensation which may represent a host adaptation to lower energy availability (Heins and Baker, 2008).

Heins and Baker (2008) reported that fecundity reduction in females was due to an overall decrease in reproductive performance. Smaller egg sizes and numbers and overall clutch mass occurred with increasing parasite index (the parasite:host mass ratio). This was also shown to be the case by Heins et al. (2010b). These two studies suggest that a side effect of infection reduces the females' reproductive capabilities. However, Heins (2012) reported this not to be the case in all populations. In Scout Lake (Alaska, USA), infected females showed fecundity compensation, with a similar clutch mass to non-infected females, but composed of greater numbers of smaller sized eggs. Whereas infected

females in the other population in their study, Walby Lake (Alaska, USA) showed a reduction in clutch mass, egg number and size, indicating a possible side effect. Freshwater three-spined stickleback are known to become strongly adapted to local conditions, and perhaps only certain populations have evolved fecundity compensation, with the differences being down to the different environmental conditions experienced (Kitano and Lema, 2013, Heins, 2012). However, Walby Lake is known to be a high nutrient lake, so should provide females with enough energy to support both infection and reproduction. Therefore, reduced reproduction could indicate parasite manipulation in this population (Heins, 2012).

Population variations

The effect of S. solidus infections on the three-spined sticklebacks' ability to develop and reproduce varies greatly. These differences could indicate that different populations are experiencing different processes, and the role for parasite manipulation or host adaptation cannot be ruled out. However, all of the responses described could be from side effects and the differences observed between populations could be due to the environmental conditions the fish experience prior to, and during infection. Candolin and Voigt (2001) suggested that experiments carried out under favourable laboratory conditions would eliminate these differences. Under laboratory conditions infected fish would be expected to overcome the energy drain and mature sexually. However, in a number of recent studies it has been shown that fish from some populations are still not able to overcome the negative effect of infection despite being brought into favourable conditions for an extended time before the breeding season. For example, males from Llyn Frongoch and Victoria Park Pond, Leicester were unable to develop nuptial colours or construct nests (Rushbrook and Barber, 2006, Macnab et al., 2009). Furthermore, Heins (2012) stated that the nutrient-rich Walby Lake should have provided enough energy for females to develop despite infection, but they did not (however, no laboratory experiments were carried out on this population). These results could be evidence that another process is taking place.

Disruption to host endocrine system

Reduced reproductive capability could be a result of disruption to the host endocrine system, which could be evidence for a parasite adaptation to manipulate its host, since such complex mechanisms are unlikely to just be a side effect. Given the variation observed in three-spined sticklebacks, and the inconclusive evidence regarding the mechanisms involved, recent research has begun to focus on the expression levels of the genes and hormones involved in reproduction (Macnab et al., 2011). Rushbrook et al. (2007) reported that Spiggin content in kidneys was significantly reduced in infected males. The amount of Spiggin was shown to be more sensitive to infection than kidney size. This supports the hypothesis that the impacts of infection are mediated through the energy demands of the parasite. Spiggin production is induced by androgens, especially 11-KT, which attain their highest level during the early phases of reproduction. Energy drain may reduce circulating 11-KT levels (Rushbrook et al., 2007). This hypothesis is supported by Macnab et al. (2011), who observed that infection reduced plasma 11-KT levels although with differences between the two study populations. The variation in reproductive potential between the two populations was associated with differing circulating 11-KT levels. The level of 11-KT and Spiggin correlated with the nesting and courtship scores in both populations. Although the reduction of Spiggin and 11-KT may indicate energy drain, the variation between populations means different mechanisms of exploitation by the parasite cannot be ruled out (Macnab et al., 2011).

Shao et al. (2012) focused on gene expression, as S. solidus could be suppressing the actions of the BPG axis as seen with L. intestinalis-infected roach. Four key genes were studied; gonadotropin-releasing hormones 2 and 3 (GnRH2 and GnRH3), LH and FSH. GnRH3 was found to be more highly expressed than GnRH2 in all groups of fish (infected and non-infected males and females). There was no significant difference in expression of GnRH2 between infected and non-infected individuals, whereas infected fish had the highest expression level of GnRH3. Infected males expressed both FSH and LH more highly than non-infected males, whereas in females only FSH showed an increase. LH in females was expressed at similar levels in both infected and non-infected fish. These results are opposite to the findings in L. intestinalis-infected roach (see (Carter et al., 2005, Trubiroha et al., 2009)). Shao et al. (2012) conclude that inhibition of gonadal development was not due to lack of circulating gonadotropins, but because of another mechanism. Starvation is ruled out as the infected fish were relatively well nourished. However, in this study the fish were sampled in April at the very start, or possibly before the breeding season had begun. Shao et al. (2012) also stated that none of the males (infected and non-infected) displayed any nuptial colours. This study was therefore not looking at sexually mature males, so gonadal development may not have been completed in any individual. Different results may be obtained at different points during the session.

In summary, the research shows *S. solidus* typically have negative effects on the ability of sticklebacks, their second intermediate hosts, to sexually mature. However, the effects vary considerably between different host populations, and different mechanisms may be occurring. Such mechanisms underlying the effects of infections on host reproduction are still relatively unknown. Therefore, experimental infection studies, cross-population infections and common garden rearing experiments are required to identify the mechanisms behind the parasite effect on reproductive development of the three-spined stickleback (Macnab et al., 2009).

1.6 Aims and objectives of the thesis

The overall aim of this research was to investigate the physiological mechanisms by which parasite infections reduce host reproduction. The first half of the thesis aimed to investigate the importance of environmental conditions on the reproductive capacity of infected fish. To understand whether mechanisms leading to reduced reproduction are best explained as side effects of infection or as active manipulation by parasites. The second half of the thesis specifically focused on the differences in reproductive phenotypes of infected fish from different stickleback populations and aimed to investigate the role of genotype-genotype interactions.

The objective of Chapter 3 was to establish the annual cycle of sexual development in three-spined sticklebacks within the lab-bred fish in the aquarium facilities at the University of Leicester, to ensure patterns were similar to those reported in nature and determine the background baseline for future studies. With this information the objective of establishing whether the timing of infections is important in determining impact on host reproduction. Similar impacts might be observed at all points, or crucial developmental periods could be identified.

Chapter 4 compared the effects of food availability and parasite infections on sexual development, investigating whether genes that code for five key proteins important in the control of sexual development have similar expression profiles under limited food compared to infections with parasites. The prediction was that nutrient restriction and

nutrient theft would give similar outcomes, whereas other mechanisms might vary in response.

Chapter 5 studied the nesting behaviour of male sticklebacks from different UK populations and how the origin of both the parasite and the fish influenced the reproductive phenotypes of infected sticklebacks. To determine if genotype-genotype interactions are important or whether all combinations of host-parasite genotypes give similar results.

The final objective was addressed in Chapter 6 which examined the genetic diversity of parasites from the different UK populations, to investigate whether differences in the reproductive phenotypes associated with infection in different stickleback populations could be explained by the level of genetic difference between parasite populations.

2 Methods

In the course of undertaking the studies described in this thesis, a number of protocols are used repeatedly and hence are relevant to the majority of experimental chapters. The following sections therefore describe in detail these common protocols, which are later briefly referenced in the relevant experimental chapters. Additional materials and methods that are specific to particular chapters, and chapter-specific deviations from the basic protocols detailed here, are described within the relevant chapter. Methods include those for the maintenance of animals and for the experimental procedures.

2.1 Study organisms

2.1.1 Fish stocks and husbandry

Each Spring (March) from 2012 to 2015, a sample of wild three-spined sticklebacks *Gasterosteus aculeatus* were collected from Carsington Water (CRS), Derbyshire, UK (53°3'30" N 1°37'50" W). These samples included sticklebacks that were naturally infected with plerocercoids of the diphyllobothriidean cestode *Schistocephalus solidus*, as well as non-infected fish. In 2013, fish were also collected from Clatworthy Reservoir (CLT), Somerset, UK (51°03'57" N 3°22'42" W) and Inverleith Pond (INV), Edinburgh, UK (55°97'85'' N, 3°22'72'' W). In 2014, collections were made from Llyn Frongoch (FRN), Ceredigion, UK (52°21'46'' N, 3°52'26'' W) and River Welland (WEL), Leicestershire, UK (52°28'33'' N, 0°55'29'' W).

Prior to the sampling, the relevant permissions were gained from the owners of each water body, and the respective angling associations and landowners, as well as Natural England and the Environment Agency. Fish were collected using a combination of techniques, including hand netting, using 30 cm x 30 cm dip nets with a 1 mm mesh and trapping using unbaited, galvanised metal minnow traps with either a 3 mm or 5 mm mesh, and 20 mm aperture. After collection, fish were transferred in aerated, insulated containers (10 L) directly to the aquarium facilities at the University of Leicester. After a period of gradual thermal acclimation, fish were housed in 80 L (60 cm x 40 cm x 40 cm) glass aquaria and fed *ad libitum* on a mixture of frozen bloodworm (*Chironomus* sp. larvae) and ZM Medium Premium Granular pellets, prior to either being used in experimental procedures or as parental stock in breeding programmes.

Throughout the studies, day length and temperature regimes were adjusted to match the natural seasonal changes occurring in the field; day length was updated weekly to sunrise

and sunset times at the local latitude, and water temperature was adjusted each month to the mean external temperature.

2.1.2 Fish breeding

Stocks of lab bred fish were generated each year from the CRS population for experimental studies. In addition, in 2014, lab bred stocks of CLT and INV were generated, and in 2015 FRN and WEL were bred. In all cases, the IVF technique, as described by Barber and Arnott (2000), was used to generate lab-bred fish. In brief, male sticklebacks displaying classic nuptial colouration (red throats and blue eyes, (Craig-Bennett, 1931)) were selected and euthanized according to the UK Home Office approved Schedule 1 method (Benzocaine anaesthetic overdose (Stock solution: 10 g prepared in 1 L of 70% EtOH) followed by the destruction of the brain). Testes were dissected and placed in an ice cold watch glass and macerated in several drops of aquarium water. Ripe gravid females were selected and their eggs were stripped into cooled watch glasses and covered with the solution from the macerated testes. After 15 min, eggs were checked for successful fertilisation by viewing the development of the outer membrane via a dissection microscope. Following confirmation of fertilisation, the eggs were transferred to 1 L (15.5 cm x 9.5 cm x 8.5 cm) plastic aquaria. Methylene Blue (2 ml of 1 mg/ml stock solution) was added to the aquarium water to prevent the development of fungal infections, and the water was aerated using an airstone. At 5 days post fertilisation, a 75% water change was made, and then for the following days until the fry hatched 50% water changes were made to ensure all Methylene Blue was removed prior to hatching, as exposure to the chemical post-hatching can affect fry development (Perlberg et al., 2008, OECD, 2011). After hatching, the fry were fed on LiquidFry No1 (Interpet, Dorking, UK) for the first 5 days, before transferring to ad libitum 2 day old Artemia sp. nauplii for three months. Following this, fish were fed an ad libitum diet of bloodworm (Chironomus sp. larvae) until the start of experimental treatments.

2.1.3 Maintenance of lab bred copepods Cyclops strenuus abyssorum

Initially, cultures of copepods *Cyclops strenuus abyssorum* were supplied from Sciento (Manchester, UK). These cultures were maintained in the laboratory to increase numbers for experimental use. The initial culture was placed in a 1 L conical flask with 300 ml of aquarium water and kept in the aquarium room, being exposed to the same temperature and light regime as the fish. Once a week, approximately 100 ml of alfalfa/protozoa

infusion was introduced, as food for the copepods. Once a month the copepod cultures were sorted by size, to prevent cannibalism, by sieving into three groups; gravid females and adult males (separated using a 250 μ m sieve), copepodites (150 μ m sieve) and nauplii (45 μ m seive). The three groups were then separated into separate 1 L conical flasks as described above and maintained in this pattern.

Alfalfa/protozoa infusion stock was made by adding 1 g Alfalfa Powder (Naturally Green, Reading, UK) to 400 ml ddH₂0 in a 1 L conical flask and boiling on a hotplate for approximately 5 min. After boiling, the solution was filtered into a 400 ml Duran bottle and 0.2 g Na2HPO4 was added. The solution was then autoclaved. Once cooled approximately 20 ml of Yeast/Glucose solution (100 ml ddH₂0, 0.5 g Yeast, 0.02 g Glucose) and 50 ml of stock protozoa food *Colpidium striatum* (Sciento, Manchester, UK), was added and mixed in the 400 ml Duran bottle. Solution from the 400 ml bottle was then used to top up the stock food bottle. The solution was then left at room temperature for 24 h before being stored at 4°C until required for feeding.

2.1.4 Schistocephalus solidus culturing and experimental parasite exposure

2.1.4.1 Parasite cultures

Plerocercoids of *S. solidus* were dissected from the naturally infected wild sticklebacks from each population (Figure 2.1).



Figure 2.1 Schistocephalus solidus plerocercoid dissected from a three-spined stickleback

Each was blotted dry and weighed (to 0.0001 g). Any plerocercoids exceeding the infective weight of 50 mg (Tierney and Crompton, 1992) were transferred singly to an *in vitro* culture vessel to allow sexual maturation and eggs to be produced, using a method

adapted from Smyth (1946). The plerocercoids were placed in sterile 6.3 mm diameter dialysis tubing (Visking, UK), suspended in a glass screwtop tube filled with 30 ml of horse serum (Sigma-Aldrich, UK) and 30 ml of RPMI 1640 cell culture medium (Sigma-Aldrich, UK) and 500 μ l of penicillin-streptomycin-glutamine solution was added (Thermo Scientific, UK). The cultures were incubated at 40.2°C in a shaking incubator for 7 d. Eggs produced by the adult cestodes were then collected into a 9 cm diameter Petri dish and rinsed with dH₂0. Depending on the number of eggs produced by each worm, these were then transferred to between one and four 9 cm Petri dishes that were filled with dH20. These were then wrapped in aluminium foil and incubated in the dark at 20°C for at least 30 d.

2.1.4.2 Experimental exposure of copepods

Prior to exposure, laboratory reared copepods (*Cyclops strenuus abyssorum*) were starved for 7 d before being separated into the different developmental stages by passing the culture through various sized mesh sieves (see above). For parasite exposure, copepodites were selected and placed into 250 ml conical flasks with approximately 20 ml of water (four flasks for each parasite population were used). The day before copepods were due to be exposed, parasite eggs were removed and exposed to daylight for 2 h. The parasite eggs were then incubated overnight in the dark and exposed again to daylight the next morning, to facilitate hatching (Scharsack et al., 2007). Petri dishes were then screened for the presence of actively swimming parasite larvae (coracidia) (Figure 2.2).



Figure 2.2 Free swimming Schistocephalus solidus coracidia at 40 x magnification

Copepodites were exposed to coracidia by adding the contents of plates where active coracidia had been identified to each of the conical flasks ('batch exposure'). Exposed copepodites were then kept at 20°C for 21 d before screening for infections. Copepods that had acquired infections were identified by the presence of the procercoid stage in the

body cavity (Figure 2.3). Procercoids considered to be infective to sticklebacks, i.e. if they exhibited a cercomer (Smyth, 1969), were isolated into one of the 5 ml wells in a 24-well cell culture plate. Infected copepods collected following batch exposure harboured a mixture of single and multiple infections.



Figure 2.3 *Cyclops strenuus* abyssorum copepod at 10 x magnification, infected with a *Schistocephalus solidus* tapeworm (procercoid), which is visible in the back of the copepod. The procercoid exhibits the cercomer indicating it is infective to the next host, three-spined sticklebacks.

2.1.4.3 Experimental exposure of fish

Prior to experimental exposure, three-spined sticklebacks were fed *ad libitum* with freshly-hatched *Artemia* sp. nauplii for one week before being starved for 2 d prior to the being exposed. Each fish was exposed to two infective copepods, each of which harboured either one or two infective procercoids. The exposure level therefore varied between 2 and 4 procercoids. The fish and infected copepods were placed in a 1 L (15.5 cm x 9.5 cm x 8.5 cm) plastic aquarium for 16 h, overnight, to allow time for the fish to consume the copepods, after which the fish were returned to the aquaria where they would remain in until the end of the experimental period. Depending on the experiment, the exposed fish were either housed in groups, or individually.

2.2 Molecular techniques

2.2.1 Collection of DNA samples from sticklebacks via swabbing

Prior to experimentation, fish from the laboratory bred stickleback populations were swabbed to collect DNA samples (Le Vin et al., 2011, Sebire et al., 2015, Breacker et al., 2017), and sex was identified by PCR. Fish were randomly selected from the stock tanks and a swab from their external mucus layer was taken. A sterile rayon-tipped swab (Fisher Scientific, UK) was gently rubbed along the flank between the anal fin and the caudal fin, from head to tail approximately ten times. The fish were then isolated in 1 L tanks until identification had been made.

2.2.2 DNA extraction from swabs

DNA was extracted from the swabs taken from fish using an in-house method that had been adapted from the protocol described by Sambrook and Russell (2001). Swabs were placed into 400 μ l of pre-warmed (50°C) DNA extraction buffer (200 mM Tris pH 7.5, 25 mM EDTA pH 8, 250 mM NaCl, 0.5% sodium dodecyl sulphate (SDS)). The solution was then incubated at room temperature for 15 min before the swab was removed. After incubation the solution was then centrifuged at 13000 rpm for 2 min, following centrifugation 300 μ l of supernatant was transferred to a fresh microcentrifuge tube and 300 μ l of 4°C Isopropanol was added, mixed and then incubated at -80°C for 10 min. The solution was then centrifuged at 13000 rpm for 10 min. The resulting DNA pellet was dried by gently pouring off the supernatant and then a 70% ethanol wash was carried out by adding 200 μ l of 70% EtOH to the pellet and this was centrifuged at 13000 rpm for 2 min. The pellet was dried by removing the solution by pipette and then incubating the open tube at 50°C for 10 min. The DNA was then rehydrated by adding 30 μ l of ddH20 and incubated at 65°C for 5-10 min before use in the PCR reactions.

2.2.3 Collection of DNA samples from S. solidus plerocercoids

Plerocercoids of *S. solidus* from each population were dissected from the body cavity of infected sticklebacks, weighed as above and were placed individually into 1.5 ml centrifuge tubes containing 1 ml of 100% EtOH and stored at room temperature until DNA extractions were performed.

2.2.4 DNA extraction from S. solidus plerocercoids

Prior to DNA extraction the plerocercoids that had been stored in 100% EtOH were transferred to a 1.5 ml centrifuge tube containing 1 ml of ddH20 to prevent ethanol carryover to the extraction method. Following the washing step a 0.5 cm² section was cut from the plerocercoids and placed into 400 μ l of pre-warmed (50°C) DNA extraction buffer as detailed above for swabs (the remaining section of the plerocercoid was returned to the tube containing ethanol). For parasite tissue, 15 μ l of 20 mg/ml proteinase K was added. The sample was then vortexed for 10-15 s before being placed in a dry heat block at 55°C with periodic vortexing for 30 min or until the tissue had dissolved. The extraction then followed the same protocol of centrifugation and Isopropanal precipitation as detailed above for swabs. The resulting parasite DNA was rehydrated with 100 μ l of ddH20.

2.3 Fish sex identification by PCR

To identify fish sex Polymerase Chain Reactions (PCRs), were carried out to amplify sexlinked markers from DNA samples obtained from swabbing. This PCR uses primers designed against the sex linked marker, isocitrate dehyrogenase (IDH). This marker is closely linked to sex in sticklebacks, with females producing a single band of approximately 300 bp, whilst males produce two products of 270 bp and 300 bp due to a small deletion. The primers used are: STKSEX forward primer 5'GGGACGAGCAAGATTTATTGG 3'; STKSEX reverse primer 5'TATAGTTAGCCAGGAGATGG 3' modified from those described by Peichel et al. (2004). 10 µl reactions were set up, (5 µl Red Taq master mix (Sigma-Aldrich, UK), 0.5 μl each of 10 μM forward and reverse primers, 3 μl of DNA template and 1 μl ddH2O). The reaction conditions of the PCR were as follows: 94°C for 5 min, followed by 40 cycles of: 95°C for 30 s, 56°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 10 min. Products were visualised on a 5% (w/v) high strength agarose gel (Melford, UK).

2.4 Post mortem data

At the end of each study, fish were euthanized using an approved UK Home Office Schedule 1 method (anaesthetic overdose followed by severing of the spinal cord). Fish were blotted dry, weighed (total weight, to 0.0001 g) and measured using dial callipers (standard length, SL, to 0.1 mm). Any *S. solidus* plerocercoids were recovered and

weighed (to 0.0001 g). Fish weight was calculated as: total weight – total plerocercoid weight. The brain, liver, kidney and gonads were removed and weighed (to 0.0001 g) to calculate body condition indices before being placed into 1 ml of RNA*later*[®] solution (Sigma-Aldrich, UK), kept at 4°C overnight and stored at -20°C for subsequent gene expression analysis.

The following indexes were then calculated:

Parasite index $(I_P) = ([Total Plerocercoid Weight / Total weight] x 100).$

Brain somatic Index (I_B) = ([Brain weight / Fish weight] x 100)

Gonadosomatic Index $(I_G) = ([Gonad Weight / Fish weight] x 100)$

Kidney-somatic Index, $(I_K) = ([Kidney Weight / Fish weight] x 100)$

Hepatosomatic Index, (I_L) = ([Liver Weight/ Fish weight] x 100)

2.5 Gene expression analysis

2.5.1 Molecular cloning and sequencing of stickleback genes

For the study of gene expression patterns and potential changes caused by parasitism, five genes with key functions in reproductive development were selected for investigation; *luteinizing hormone (LH), follicle stimulating hormone (FSH), vitellogenin (VitAa), kisspeptin (Kiss2)* and *spiggin (SpgB)*. Of the genes of interest selected, *LH, FSH* and *SpgB* had published sequences available to which primers for qPCR analysis were designed; LH accession number AJ534969.1, FSH accession number NM_001267631.1 (Hellqvist et al., 2004), Spiggin accession number AB910012.1 (Seear et al., 2015). However, the sequences for the stickleback *vitellogenin* and *kisspeptin* have not been fully identified. Therefore, in order to use these genes within the planned qPCR study these sequences needed to be obtained.

Cloning was carried out by using the GeneRacer[™] with SuperScript[™] III RT and TOPO TA Cloning[™] Kit (ThermoFisher Scientific, USA) and gene-specific primers, following the manufacturer's instructions. Partial cDNA sequences for stickleback *vitellogenin* are available on GenBank (accession number EU399547.1 and EU481823.1) and specific primers were designed for these. For *kisspeptin*, no sequence was available at the start of this study (although since this work was carried out, a partial cDNA sequence has been

published on GenBank, accession number KT202354). *Kisspeptin* sequences from closely related species were used to design degenerate and specific primers. Sanger sequencing of clones was carried out by GATC Biotech (Konstanz, Germany) and nucleotide sequences were subsequently processed with Geneious Pro v9.1.8 (http://www.geneious.com, (Kearse et al., 2012)). Sufficient sequence for both *vitellogenin* and *kisspeptin* were obtained to design effective primers for qPCR.

vitellogenin

The two fragments of sequence available on GenBank are identified only as *'vitellogenin'*; however it is known that teleost fish, including sticklebacks, have multiple *vitellogenin* genes (Matsubara et al., 2003, Rawat et al., 2013) each with different functions in vitellogenesis. To ensure that only one gene was included in the subsequent gene expression studies there was a need to clone and further sequence the vitellogenin gene. Sticklebacks have three genes *VitAa*, *VitAb* and *VitC* (Finn and Kristoffersen, 2007) the GenBank sequences were found to match *VitAa* and *VitAb* (EU481823.1 and EU399547.1 respectively). After cloning and sequencing from these it was found that *VitAb* is highly variable, with possible alternative splicing occurring whereas *VitAa* was chosen as the gene for subsequent gene expression studies as differences found in expression would be unlikely to be caused by splice variants. A 1339 bp sequence for *VitAa* was obtained, and primers for qPCR were designed from it (Table 2.1):

kisspeptin

Sticklebacks only have one Kisspeptin protein – Kiss2 (Felip et al., 2009) – so degenerate primers were designed to the *Kiss2* sequence from other closely related species. The resulting sequence from the clones matched with other *Kiss2* sequences. A 203 bp sequence was obtained and primers designed from this (Table 2.1):

2.5.2 RNA extraction

Total RNA was extracted from the RNA*later*[®] preserved tissues using the GenElute[™] Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, UK) following the manufacturer's instructions. These samples were treated with TURBO[™] DNase (ThermoFisher Scientific, USA) to remove any contaminating genomic DNA. The concentration and purity was determined using a NanoDrop 2000 spectrophotometer (LabTech

International, UK) and 3 μ L of total RNA was electrophoresed on a non-denaturing 1.5% (w/v) agarose gel to check for degradation.

2.5.3 First strand cDNA synthesis

First strand cDNA was reverse transcribed from 0.5 μ g of total RNA using a RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA) following the manufacturer's instructions using both oligo dT and random primers in a 20 μ l reaction followed by a 1 in 4 dilution with ddH₂0.

2.5.4 Reverse-transcription quantitative PCR (RT-qPCR)

Reverse-transcription quantitative PCR (RT-qPCR) analysis was performed to examine the expression profiles of the five selected key genes in the sexual development pathways of females and males (primer details shown in Table 2.1). The RT-qPCR mixture consisted of 10 μ L SensiFASTTM SYBR[®] No-ROX Kit (Bioline, UK), 250 nM of forward and reverse primers (Table 2.1), 1 μ L diluted cDNA and sterile water in a total volume of 20 μ L. The RT-qPCRs were performed in triplicate on a CFX Connect qPCR thermocycler (BioRad Laboratories, CA) with the following cycling conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A melting curve step (50–95°C) was then performed, to verify that only single products had been amplified. No-template and no-reverse transcriptase controls were also performed for each primer pair and cDNA, respectively

Potential reference genes, successfully used by others in previous three-spined stickleback studies, were; *ribosomal protein L8 (rpL8)* (Geoghegan et al., 2008), *ribosomal protein L13A*, (*rpL13A*) and *ubiquitin (Ubiq)* (Hibbeler et al., 2008), (primer details shown in Table 2.1). To select the most stable reference gene from these, geNorm software (Vandesompele et al., 2002) was used. The *rpL8* gene was considered to be the most stable reference gene, and was used to normalize the data. The fold change was then calculated using the 2^{-ddCT} method (Livak and Schmittgen, 2001). The dCT value for each sample was determined by calculating the difference between the cycle time (CT) value of the gene of interest (GOI) and the CT value of the reference (REF) gene. This was determined for each unknown sample (sample) as well as for the control sample (calibrator). dCT (sample) = CT (sample GOI) – CT (sample REF), dCT (calibrator) = CT (calibrator GOI) – CT (calibrator REF). The ddCT value for each sample was determined by subtracting the

dCT value of the calibrator from the dCT value of the sample. ddCT = dCT (sample) – dCT (calibrator). The fold change of the normalized level of GOI expression was calculated by using the formula: 2^{-ddCT}

Primer name	5'-3' sequence	Purpose	Annealing
STKSEX Forward	GGGACGAGCAAGATTTATTGG	Sexing PCR	56°C
STKSEX Reverse	TATAGTTAGCCAGGAGATGG	Sexing PCR	56°C
Ribo L8 F	CGACCCGTACCGCTTCAAGAA	qPCR	60°C
Ribo L8 R	GGACATTGCCAATGTTCAGCTGA	qPCR	60°C
Ribo L13A F	CACCTTGGTCAACTTGAACAGTG	qPCR	60°C
Ribo L13A R	TCCCTCCGCCCTACGAC	qPCR	60°C
Ubiq F	AGACGGGCATAGCACTTGC	qPCR	60°C
Ubiq R	CAGGACAAGGAAGGCATCC	qPCR	60°C
Kiss2 F	CTGAGAGAGAACGAGGAACAGAGG	qPCR	60°C
Kiss2 R	ACGTCCAGTTATCGCAAGAAGAGA	qPCR	60°C
FSH F	CATCCACACCACCATCTGCTC	qPCR	60°C
FSH R	GTGTGTCACCTCGTAGGACCAGT	qPCR	60°C
LH F	GTACGACTGCACCTTCGAGAGC	qPCR	60°C
LH R	GGACTAGTAGTTGAAAGGGATGTCG	qPCR	60°C
VitAa F	CCAGGAATCCAGATGCTACTCCGT	qPCR	60°C
VitAa R	CCGACAGTGGAAGCCAATAGTGAC	qPCR	60°C
SpgB F	TGAAAACCAAGAACTGTCTGCAAG	qPCR	60°C
SpgB R	TTTAGGAATACAGCGATAGCCCTTTT	qPCR	60°C

Table 2.1 Primers used for various techniques throughout the studies. Primers were manufactured by Sigma-Aldrich (Dorset, UK).

2.6 Statistical analysis

All statistical analysis was carried out using SPSS (version 24). Data were tested for normality using the using the Shapiro-Wilk test and if necessary log transformed to allow for parametric tests to be performed.

2.7 Ethical statement

All work was carried out under the authority of Home Office project licences 80/2327 and 70/8148 held by Dr Iain Barber and personal licences 40/9987 and ID9FA25C3 held by Ceinwen A. Tilley, in accordance with local and national regulations.

3 How does the timing of *Schistocephalus* infections alter the disease phenotype in sticklebacks?

3.1 Introduction

The timing of parasite and disease challenges experienced by host organisms can have a profound effect on the outcome of infections (Altizer et al., 2006, MacIntosh et al., 2010, Lisovski et al., 2017). In many host-parasite systems infection levels are highest in juveniles, and the impact of infection is greatest in young organisms. Nematode parasites *Oesophagostomum aculeatum* are more prevalent in juvenile Japanese macaques *Macaca fuscata yakui* and produce greater egg numbers in the spring and summer, particularly in juveniles (MacIntosh et al., 2010). Similarly, a higher intensity of the blood parasite *Haemoproteus columbae* was found in juvenile pigeons *Columba livia*. Within juveniles the parasites are significantly more virulent, often fatally, whereas adults with similar infection levels survive (Sol et al., 2003).

Seasonal changes in the physiological, hormonal, immunological or other condition of host organisms also has the potential to impact the consequences of infections (MacIntosh et al., 2010). Post one-year old blue tits *Cyanistes caeruleus* have a high prevalence of *Plasmodium spp*. and also experience a spring relapse which may be due to changes in day length and host gonadal hormones and immune level (Cosgrove et al., 2008).

Mismatches in the effects of changing environments on the biology and life cycles of hosts and parasites can alter the timing of disease challenges and parasitic infections, with unpredictable outcomes for the biology and ecology of infections (Harvell et al., 2002, Thomas and Blanford, 2003). In particular, seasonal changes in reproductive physiology – which have significant consequences for fish energetics – may have important consequences for the outcome of metabolically costly infections.

In this chapter, I first quantified changes in the morphology and gene expression levels of a laboratory-housed population of three-spined sticklebacks held under natural seasonal environmental fluctuations. I then used this data to identify key developmental phases in fish that may be significantly affected by parasite infection and carried out experimental infections to measure this.

3.1.1 Seasonality of reproductive development in sticklebacks

Sticklebacks occupy a wide range of latitudes and habitat types across the northern hemisphere and populations vary in the speed of their development, typically undergoing sexual maturation in their first or second year. In UK freshwater populations, sticklebacks usually have an annual reproductive cycle which can be divided into two main phases: the non-reproductive phase (autumn and winter) and the breeding season (spring and summer). Sticklebacks are therefore typically classed as being long-day breeders (Craig-Bennett, 1931, Wootton, 1976, Sokolowska and Kulczykowska, 2006) although in some populations the distinction between these phases is less clear (Mori, 1985). This annual cycle of development is under the control of complex feedback loops which regulate the Brain-Pituitary-Gonad (BPG) axis. A combination of both environmental and physiological factors determine how and when sticklebacks develop and mature sexually, ensuring that breeding and hatching of offspring occurs at the most favourable time of year for fry growth and survival to be optimal (Baggerman, 1985, Borg, 2007).

As with most temperate ectotherms, sticklebacks are not reproductively active throughout the year. Due to occupying mid to high latitudes sticklebacks have a relatively short, clearly defined breeding season meaning that gonad development must be triggered by some factor (Baggerman, 1985). The main environmental cues are photoperiod and water temperature (Craig-Bennett, 1931, Baggerman, 1958, Baggerman, 1985, Sokolowska and Kulczykowska, 2006, Sokołowska and Kulczykowska, 2009) with a combination of long days and high temperatures promoting sexual maturation, though the significance of each factor appears to differ between the sexes (Sokołowska and Kulczykowska, 2009). In order to complete the normal process of gametogenesis and attain full sexual maturity it is crucial that sticklebacks experience the low temperatures and short days of winter (Sokolowska and Kulczykowska, 2006).

Secretion of gonadotrophic hormones is triggered by the increasing day length in the spring. The annual expression change in *kisspeptin*, a key regulator of reproductive development through the BPG axis in fish and other animals, is influenced by photoperiod (Oakley et al., 2009, Espigares et al., 2017); as is the expression of *luteinizing hormone* (*LH*) and *follicle stimulating hormone* (*FSH*) in this pathway (Borg et al., 2004, Espigares et al., 2017). Both sexes are similarly influenced by alterations in photoperiod.

Water temperature is also important for fish development. Although increases in expression of *LH* and *FSH* are delayed in cold temperatures it is not prevented (Baggerman, 1958), meaning that maturation takes place but at slower rates. Higher water temperatures in winter lead to earlier maturation particularly in male sticklebacks whereas

females did not show such a significant increase in development rate (Craig-Bennett, 1931, Baggerman, 1958, Sokołowska and Kulczykowska, 2009).

Another crucial factor to complete maturation is resource availability, which is thought to be a nutritional cue influencing the BPG axis by acting on the expression of *kisspeptin*. Limited food is linked to *kisspeptin* expression remaining low, thus preventing sexual development. Fish kept in low nutrition conditions, particularly females, often fail to mature which could be due to the inactivation of the BPG axis (Felip et al., 2009, Mechaly et al., 2011, Mechaly et al., 2013, Wang et al., 2014, Trombley et al., 2014, Escobar et al., 2016).

3.1.2 Schistocephalus infections and interactions with host sexual development

The effects of Schistocephalus solidus infections on sticklebacks between host populations are not consistent, particularly in terms of the consequences for host reproductive development (Arme and Owen, 1967, Folstad et al., 1994, Rushbrook and Barber, 2006, Macnab et al., 2009). These differences potentially arise as a consequence of varying environmental conditions experienced by different host stickleback populations that affect, for example, the nutritional resources available to infected fish and hence (potentially) the developing parasite (Candolin and Voigt, 2001, Heins et al., 1999, Heins et al., 2010b, Heins, 2017). However, inter-population variation in the time of year that fish become infected may also be crucial in terms of whether the fish can develop and overcome the negative effects of the infection. McPhail and Peacock (1983) hypothesised that S. solidus has evolved to delay causing harm to their stickleback hosts until after the fish had reproduced, as they found numbers of infections were low during the breeding season but increased significantly in post-reproductive adults. Important biological consequences for population dynamics of both host and parasite can be dependent on the timing of infections and could influence the survival and reproductive success of both the stickleback and S. solidus. Despite the importance for the interaction between sticklebacks and *Schistocephalus*, the timing of when infections occur between host populations is not well documented (Heins et al., 2016).

3.1.3 Variation in occurrence of *Schistocephalus* infections across populations

Three-spined sticklebacks tend to become infected with *S. solidus* in the first year of their life (Pennycuick, 1971a, Heins et al., 2011, Heins et al., 2016) when they primarily prey

on small-sized copepods, the first intermediate host for S. solidus. Consequently, by consuming large numbers of these organisms they have a high exposure rate to the parasite (Pennycuick, 1971a, Pennycuick, 1971c, Christen and Milinski, 2005, Morozińska-Gogol, 2011). However the time of year when the sticklebacks become infected appears to differ depending upon the population being studied. Large numbers of infected copepods were observed during the spring by Christen and Milinski (2005) which suggested that sticklebacks would be gaining infections at this point, whereas Pennycuick (1971c) and McPhail and Peacock (1983) found the majority of infections occurred in late summer and early autumn. Heins et al. (2016) also found large number of infections occurring during these time periods, with one third of the young of the year being infected by mid-summer. By October this had increased to half. Yet they also found that significant numbers of fish continued to gain new infections throughout the winter months. The timing of new infections is likely to vary between stickleback populations due to variations in climate (Heins et al., 2016) as these patterns would alter the period in the year that is optimal for S. solidus hatchings and infecting copepods to occur. The free living coracidia of S. solidus do not hatch in water temperatures below 8°C and high water temperatures (above 19°C) reduce their life span (Sakanari and Moser, 1985, Christen and Milinski, 2005). Therefore, copepods would only be exposed to and become infected with S. solidus when conditions are suitable for coracidia. Consequently, the number of fish to gain infections would also be highest shortly after this period when the developing procercoids in copepods become infective.

3.1.4 Environmental effects on *Schistocephlaus* infections in sticklebacks and consequences for host phenotype

Environmental conditions also affect how the plerocercoid develops within sticklebacks, which could influence host phenotype. The water temperature significantly affects how quickly parasites grow within their stickleback host – warm water temperatures allows for faster parasite growth (Macnab and Barber, 2012). *S. solidus* plerocercoids could reach the size required to be infective at a faster rate when the host fish are living in warm waters. This could suggest that the ideal time for *S. solidus* to infect their stickleback host would be in the summer when water temperatures are highest. Faster parasite growth could have a greater impact on the development of fish hosts compared to slower growing individuals.

The amount of resources available to the host can also have a significant impact on the developing parasite. In copepods, *S. solidus* procercoids grew to larger sizes when their host copepod was fed on a high ration (Benesh, 2010). When infected sticklebacks were given two different food rations, plerocercoids in fish on higher rations grew to larger sizes (Simmonds, 2015). The summer is likely to be the part of the year when resources available to stickleback fish would be highest, meaning that this could be the ideal time for infections to occur. This could help limit the negative effects on host development as enough resources maybe available to sustain both host and parasite growth.

Host size could also affect how quickly parasites can grow. At the point of infection larger hosts cause less restriction on parasite growth. Parasites can grow at a faster initial rate in larger hosts than in smaller individuals (Meakins and Walkey, 1973, Parker et al., 2003), and parasites grow faster in fish hosts that have a higher growth rate (Barber, 2005). This could suggest that the better time for *S. solidus* to infect their host would be when fish are older; not in the summer after the young of the year had hatched, but during the winter and subsequent spring when the sticklebacks would be of a greater size (Christen and Milinski, 2005). Older fish may have already developed sufficiently so, that the negative effect of infection in terms of sexual maturation may not occur due to energy reserves the fish have gained (Schultz et al., 1991, Heins et al., 1999).

3.1.5 Aims

The first aim of this chapter was to quantify seasonal patterns in the reproductive development of a lab population of three-spined sticklebacks, using both physiological and gene expression analysis across a 12 month cycle. This baseline data was used to identify different phases in stickleback reproduction, and subsequently directed the timing of experimental infections, to test the hypothesis that timing of exposure has a significant effect on the outcome of experimental parasite challenges. We addressed the following questions:

- (1) What are the patterns in stickleback development across a 12 month cycle?
- (2) Does the time point during the year when fish become infected alter their resulting reproductive phenotype?
- (3) Do different aged/sized plerocercoids produce different host reproductive phenotypes at different points during the year?

3.2 Methods

3.2.1 Fish stocks and husbandry

All fish used in this study were generated by IVF in the lab using wild caught Carsington parents as described in Chapter 2. For the baseline studies fish were aged 6 months old at the start; for the timed exposure fish were 4 months old. The fish were fed *ad libitum* on 2 day-old *Artemia sp.* nauplii for three months after hatching. After three months they were transferred to an *ad libitum* diet of bloodworm (*Chironomus sp.* larvae) until the end of the study. The aquarium conditions were altered throughout the experiment to reflect the seasonal changes occurring in the field.

3.2.2 Experimental procedure

3.2.2.1 Baseline studies

Fish were sampled at the beginning of each month across a calendar year (January to December). Six female and six male fish were selected each month and processed for physiological and gene expression profiles to generate baselines for development.

At each time point where no visible secondary sexual characteristics were displayed (January-March and October-December) fish sex was identified using a sex-linked molecular marker (technique described in Chapter 2); at other times sexes were identified visually. Due to limited numbers of fish, no sample was taken in August, and in November only females were sampled.

3.2.2.2 Timed exposure to Schistocephalus solidus

The experimental procedure for the timed exposure is summarised in Figure 3.1. After the seasonal patterns of reproductive development in fish had been identified, five key time points were selected across the year to investigate effects of infection. At most time points, 36 females and 36 males were selected and exposed to *S. solidus*-infected copepods as described in Chapter 2. Parasites were allowed to grow for either 9 weeks or 18 weeks before the fish were euthanized, with the aim to compare effects of small non-infective plerocercoids (under 50 mg) and large infective plerocercoids. Due to limited numbers of infected copepods available at the September dissection point, only 14 female and 14 male fish were exposed, and all resulting plerocercoids were allowed to grow for 18 weeks. A total of 316 fish were used for this study.





Figure 3.1 Schematic diagram of the timed exposure experiment over the annual cycle. Grey boxes indicate dissection points selected from the baseline data as being critical for stickleback development.

3.2.3 Post mortem analysis

At each time point in both studies fish were euthanized according to the UK Home Office approved Schedule 1 method of an overdose of Benzocaine anaesthetic followed by severing of the spinal cord. Measurements and tissue samples of brain, liver and kidney were collected as described in Chapter 2.

3.2.4 Molecular studies

RNA extraction, cDNA synthesis and qPCR were carried out on the three tissue sets according to protocols described in Chapter 2. Gene expression profiles of the five key genes, *kisspeptin* (*kiss2*), *luteinizing hormone* (*LH*), *follicle stimulating hormone*, (*FSH*), *vitellogenin* (*VitAa*) and *spiggin* (*SpgB*) were measured and the fold change normalised to the reference gene *ribosomal protein L8* (*rpL8*) was calculated using the 2^{-ddCT} method (Livak and Schmittgen, 2001).

3.2.5 Statistical Analysis

Factorial ANOVAs were used to analyse the baseline and timed exposure morphological data, total parasite mass, parasite index and gene expression data for each timed exposure. One way ANOVAs were used to analyse the baseline gene expression profiles for each gene and fish sex separately.

3.3 Results

3.3.1 Baseline Data

3.3.1.1 Annual pattern of fish growth

During the year the fish grew steadily both in length and mass as shown in Figure 3.2. There were significant main effects of fish sex and sampling month on both fish standard length and mass and also a significant interaction between the two (SL: sex: $F_{1,10}$ = 29.622, p < 0.001; month: F_{9.90} = 119.8, p < 0.001; interaction: F_{9.90} = 3.472, p = 0.001; Mass: sex: $F_{1,10} = 16.981$, p = 0.002; month: $F_{9,90} = 53.494$, p < 0.001; interaction: $F_{9,90} = 53.494$ 2.001, p = 0.049, Figure 3.2). Pairwise comparisons showed that across the year there were significant differences between male and female lengths (p < 0.001) and weights (p= 0.001). Parameter estimates showed that in January and February there was no significant difference in length (p = 0.895) or weight (p = 0.867) between the two sexes. In March, however there was a significant difference with females being both longer (p = 0.010) and heavier (p = 0.004) than males. This coincided with the first signs of male nuptial colours developing. Subsequent male growth rates appeared to be staggered, with periods of rapid growth followed by limited growth; in both May and October there were no significant differences between males and females but during the other months and at the final time point in December, males were significantly shorter (p = 0.006) and lighter than females (p = 0.026).





3.3.1.2 Annual changes in stickleback body indices

During the annual cycle there were significant changes within the body indices of sticklebacks as they developed and matured sexually.

There were significant main effects of both fish sex and month on the relative brain size (brain index, I_B), kidney size (kidney index, I_K) and gonad size (gonad index, I_G) of fish. Although there was a significant effect of month on the relative liver size (liver index, I_L) there was no significant main effect of fish sex. A significant interaction between the two factors was found in changes in I_L , I_K , and I_G , but not for I_B . This indicates that changes in I_L , I_K , and I_G each month differed between the two sexes, whereas the changes in I_B each month where similar for both (Table 3.1, Figure 3.3).

Variable	df	IB		\mathbf{I}_{L}		I _K		IG	
		F	р	F	р	F	р	F	р
Fish sex	1	43.822	< 0.001	4.152	0.069	184.434	< 0.001	71.883	< 0.001
Month	9	9.108	< 0.001	6.966	< 0.001	16.293	< 0.001	14.451	< 0.001
Sex*month	9	1.054	0.404	3.112	0.003	21.733	< 0.001	15.892	< 0.001
Residuals	90								

Table 3.1 ANOVA table for development in three-spined sticklebacks over the course of the study using I_B , I_L , I_K , and I_G as the response variables. Fish sex and sampling month were used as predictor variables. Significant values (p < 0.05) are shown in bold.

There were significant differences between male and female brain, kidney and gonad sizes (p < 0.001 for all), but not for liver sizes (p = 0.069) during the year. Males were found to have significantly larger brains than females throughout the year (p < 0.001). From March onwards the I_B decreased in both sexes, indicating that brain size does not keep increasing in proportion to fish growth. Female kidneys remained small and constant throughout the year, whereas male I_K significantly increased between March and June before steadily decreasing to a similar level compared to the females in October. Male kidneys remained significantly larger than females for all months from March. Whereas I_G had the opposite response to the I_K. Male gonads remained consistently small throughout the year, whereas female I_G increased significantly from March to June, before steadily decreasing to a stable level in September. For the last three months of the year there was no significant difference in gonad size between male and female fish. Liver sizes were largest in March for males and in April for females. Between June and September (the breeding season) I_L decreased. This was significant in the males (p < 0.001) but was only a non-significant trend in females (p = 0.087).



Figure 3.3 The changes in fish body indices over an annual cycle. A; Brain somatic index (I_B) B; Kidney-somatic index (I_K) C; Hepatosomatic index (I_L) D; Gonadosomatic index (I_G) Data points represent mean values, error bars represent SEM The dashed lines represent when no monthly sample was taken (August and November). * indicates a significant difference at *p* <0.05

3.3.1.3 Gene expression profiles across an annual cycle of development

There were significant changes in gene expression levels across the 12 month study as the fish matured and develop. Expression levels of *kiss2, FSH, LH* and *VitAa* varied

significantly in both sexes annually. *SpgB* expression was only analysed in males as Spiggin is only produced by male sticklebacks. This was also found to vary significantly across the 12 month cycle (Table 3.2, Figure 3.4).

Table 3.2 ANOVA table for changes in expression levels of *kiss2*, *FSH*, *LH*, *VitAa* and *SpgB* in three-spined sticklebacks over the course of the study. Significant values (p < 0.05) are shown in bold.

Variable		Male	S		Female	S
	df	F	р	df	F	р
kiss2	9	5.774	< 0.001	10	3.178	0.003
FSH	9	4.092	0.005	10	2.980	0.005
LH	9	3.572	0.002	10	8.091	< 0.001
VitAa	9	2.620	0.015	10	7.155	< 0.001
SpgB	9	4.633	< 0.001	-	-	-
Residuals	50			55		

Kisspeptin 2 (Kiss2)

In males *kiss2* expression increased significantly in March compared to January before decreasing to basal expression levels in April and remaining at this level for the rest of the year. Females had a similar pattern, however their expression peaked earlier with the highest expression level in February.

Follicle Stimulating Hormone (FSH)

The expression level of *FSH* in males increased significantly in February and March compared to January, returning to the basal expression level from April onwards, until December when expression started to increase significantly again. In females a similar pattern was seen, however in the spring the peak of expression existed for a shorter period as only February had a significantly higher level, with expression level returning to that of January by March. *FSH* expression then significantly increased in females in September before returning to the base level in December.

Luteinizing Hormone (LH)

LH expression increased significantly in April compared to January in both sexes. Expression peaked in May, then returned to the base level in June in males but remained highly expressed until September in females.

Vitellogenin Aa (VitAa)

In males *VitAa* significantly increased in expression from April when it reached its peak but remained high compared to January until September. In females, expression rose earlier, significantly increasing in March, with peak expression levels occurring in May, although the levels fell from May onwards the level remained significantly higher than January for the rest of the year.

Spiggin B (SpgB)

SpgB expression increased significantly from February onwards. This peaked in May, before decreasing across the rest of the year, although it remained significantly higher than the January base level for all months.



Figure 3.4 Changes in gene expression profiles across an annual cycle in three-spined stickleback. A; *kisspeptin (Kiss2)* B; *follicle stimulation hormone (FSH)* C; *luteinizing hormone (LH)* D; *vitellogenin (VitAa)* E; *spiggin (SpgB)*. Data points represent mean values and error bars SEM. Black lines = females and grey lines = males. The dashed lines represent when no monthly sample was taken (August and November).

3.3.2 Effects of parasite infection across an annual cycle and variation of changes in host depending upon parasite age

3.3.2.1 Parasite infections

Of the 316 fish exposed to infective *Schistocephalus* parasites, 108 harboured plerocercoids at the time of dissection. At least 6 infected and 6 non-infected fish were included in each sample point giving enough biological replicates to carry out qPCR analysis of gene expression. Factorial ANOVA showed that the month sampled, plerocercoid age and fish sex all significantly affected total plerocercoid mass.

There was a significant interaction between month and parasite age, indicating that parasites grew at different rates across the year. There was no significant interaction between fish sex and sample month or fish sex and parasite age, or between sample month, parasite age and fish sex (Table 3.3, Figure 3.6).

Pairwise comparisons revealed that 18 week-old parasites were significantly larger than 9 week-old parasites at each time point (p < 0.001). Plerocercoids were similar in size in February and April. In June, July and September they were significantly larger than those collected in February and April; however there was no significant difference in size between these three months. Female fish harboured significantly larger plerocercoids than males (p = 0.015) for all time points except September when they were similar in size.

Variable	df	F	р
Sampling month	4	38.583	< 0.001
Parasite age	1	76.705	< 0.001
Fish sex	1	4.453	0.038
Month*age	3	9.004	< 0.001
Sex*month	4	1.371	0.250
Age*sex	1	1.699	0.196
Month*age*sex	3	0.331	0.803
Residuals	90		

Table 3.3 ANOVA table showing the influence of predictor variables (fish sex, sampling month and parasite age) and their interactions with parasite mass. Significant values (p < 0.05) are shown in bold.
Similar results were found when expressing parasite mass as a proportion of host mass (i.e. parasite index I_P). However, the interaction between sample month and parasite age was marginally non-significant, indicating that the size of parasite in relation to the host fish did not depend on the time of year (Table 3.4, Figure 3.6).

Table 3.4 ANOVA table showing the influence of predictor variables (fish sex, sampling month and parasite age) and their interactions on parasite index. Significant values (p < 0.05) are shown in bold.

Variable	df	F	р
Sampling month	4	29.030	< 0.001
Parasite age	1	28.137	< 0.001
Fish sex	1	4.098	0.046
Month*age	3	2.676	0.053
Sex*month	3	1.875	0.140
Age*sex	1	1.284	0.261
Month*age*sex	3	1.624	0.190
Residuals	90		



Figure 3.5 Boxplots showing: total parasite mass (M_P), A; Females, B; Males and parasite index (I_P), C; Females, D; Males from the timed parasite exposure study. The median is indicated by the line across the box, the box extends from the 25th to 75th percentiles and the whiskers represent the maximum and 55 minimum value. Letters indicates a significant difference at *p* <0.05. The dashed line on the parasite mass boxplot represents the critical weight *S. solidus* plerocercoids need to reach to become infective to the the final host

3.3.2.2 Effects of parasite infection on body indices

Females

In female fish there was a significant main effect of sampling month on all four body indices, however, no significant effect of infection level (non-infected, infected with 9 week or 18 week-old parasites) was found for I_B , I_L or I_K . Infection level did have a significant effect on I_G . A significant interaction between month and infection was found for both I_B and I_G , but not for I_L or I_K (Table 3.5, Figure 3.6).

Table 3.5 ANOVA table for development in female sticklebacks over the course of the study using I_B , I_L , I_K , and I_G as the response variables. Sampling month and infection level were used as predictor variables. Significant values (p < 0.05) are shown in bold.

Variable	df	\mathbf{I}_{B}		Ι	$\mathbf{I}_{\mathbf{L}}$		Iĸ		IG	
		F	р	F	р	F	р	F	р	
Sampling month	4	14.433	< 0.001	6.137	< 0.001	3.712	0.007	14.497	< 0.001	
Infection level	2	0.265	0.768	1.719	0.181	2.047	0.135	13.149	< 0.001	
Month*infection	7	2.332	0.031	0.653	0.711	1.453	0.193	4.131	0.01	
Residuals	95									

Pairwise comparisons showed a significant difference between sampling months. Females dissected in February had significantly larger brains in proportion to their body size compared to the other time points (p < 0.001). The reduced I_B reflected the increased body weight of females as their gonads significantly increased in size across the study. Females also had significantly smaller livers in proportion to their body size in February compared to the rest of the year (p = 0.008). Kidney size did not significantly change across the time points. There was no significant effect from parasites of either age on the I_B, I_L or I_K at any time point. Females had significantly smaller (p = 0.030) compared to the other time points regardless of their infection status. In June and July females harbouring parasites of both ages had significantly smaller gonads than the non-infected fish (p = 0.041).



Figure 3.6 Bar graphs showing the body somatic indices of female three-spined sticklebacks in the timed parasite exposure study. A; Brain somatic index (I_B) B; Hepatosomatic index (I_L) C; Kidney-somatic index (I_K) D; Gonadosomatic index (I_G) Bars show the mean + SEM, black bars = non-infected fish, grey bars = infected fish. The month of dissection and parasite age (9 week or 18 week post exposure) is shown on the horizontal axis. Letters and * indicates a significant difference at p < 0.05

Males

In male fish there was a significant main effect of both sampling month and infection level on relative brain, liver and kidney size. There was also a significant interaction between the two factors, indicating that different effects of infection were seen at different time points. However, relative gonad size was only significantly affected by month and not infection level, with no interaction between the two factors (Table 3.6, Figure 3.7).

Table 3.6 ANOVA table for development in male sticklebacks over the course of the study using I_B , I_L , I_K , and I_G as the response variables. Sampling month and infection level were used as predictor variables. Significant values (p < 0.05) are shown in bold.

Variable	df	I _B		\mathbf{I}_{L}		Ι	K	IG	
		F	р	F	р	F	р	F	р
Sampling month	4	3.128	0.018	9.291	< 0.001	15.781	< 0.001	7.737	< 0.001
Infection level	2	24.504	< 0.001	8.265	< 0.001	37.082	< 0.001	0.821	0.443
Month*infection	7	4.382	< 0.001	4.937	< 0.001	6.163	< 0.001	1.581	0.151
Residuals	94								

There was a significant difference between sampling month for non-infected fish as well as a significant difference in the effect of parasites of different ages on the I_B, I_L and I_K but not on I_G. In February, 9 week-old parasites had no significant effect on I_B (p = 0.151), however males harbouring 18 week-old plerocercoids had a significantly smaller I_B (p =0.019). In April, males harbouring 9 week-old parasites had a larger I_B, whereas there was no effect from the 18 week-old parasites. I_B was significantly smaller in fish harbouring parasites of both ages in June (p < 0.001). Whereas in July fish harbouring 9 week-old parasites had similar I_B compared to non-infected fish, those with 18 week-old parasites had significantly smaller I_B (p < 0.001). In September males had significant smaller sized brains compared to the other time points regardless of infection status (p = 0.032).

In June, July and September males harbouring parasites of both ages had significantly larger livers (p = 0.029). Non-infected males also had significant larger I_L in September than at other time points (p = 0.040). In February and September males had significant smaller sized kidneys compared to the other time points, with no effect from infection. In April, males harbouring 9 week-old parasites had significantly smaller I_K compared to the non-infected fish (p = 0.009), those harbouring 18 week-old parasites also had smaller I_K; however this was not significant. In June and July, fish harbouring parasites of both ages had significantly smaller I_K (p < 0.001).



Figure 3.7 Bar graphs showing the body somatic indices of male three-spined sticklebacks in the timed parasite exposure study. A; Brain somatic index (I_B) B; Hepatosomatic index (I_L) C; Kidney-somatic index (I_K) D; Gonadosomatic index (I_G) Bars show the mean + SEM, black bars = non-infected fish, grey bars = infected fish. The month of dissection and parasite age (9 week or 18 week post exposure) is shown on the horizontal axis. Letters and * indicates a significant difference at p < 0.05

A summary of all the body indices changes in three-spined sticklebacks in relation to month and age of parasite are shown in Table 3.7

Month	Parasite age	I_B		$\mathbf{I}_{\mathbf{L}}$		Ι	I _K		I _G	
		9	0	9	0	9	5	9	0	
Feb	9 Wk	-	-	-	-	-	-	-	-	
	18 Wk	-	\downarrow	-	-	-	-	-	-	
April	9 Wk	-	Ť	-	-	-	\downarrow	-	-	
	18 Wk	-	-	-	-	-	-	-	-	
June	9 Wk	-	\downarrow	-	Ŷ	-	\downarrow	\downarrow	-	
	18 Wk	-	\downarrow	-	Ŷ	-	\downarrow	\downarrow	-	
July	9 Wk	-	-	-	ſ	-	\downarrow	\downarrow	-	
	18 Wk	-	\downarrow	-	ſ	-	\downarrow	\downarrow	-	
Sept	18 Wk	-	-	-	Ť	-	-	-	-	

Table 3.7 Summary table of changes in the body indices of three-spined sticklebacks in relation to time of year (month) and infection status (9 week-old or 18 week-old *S. solidus* parasites)

3.3.2.3 Effects of parasite infections on gene expression

Expression levels of the genes *kiss2*, *FSH*, *LH*, *VitAa* and *SpgB* (in males only) altered across the year, however the effect of parasite infection was not consistent and varied for each gene, fish sex and parasite age.

Kisspeptin 2 (kiss2)

Female and male fish had similar annual patterns of *kiss2* expression, however there was no significant effect of infection with parasites of either age (Females; sampling month; $F_{5,98} = 4.699$, p = 0.001; infection level: $F_{2,98} = 1.269$, p = 0.286; interaction: $F_{7,98} = 2.899$, p = 0.009, Males; sampling month; $F_{5,99} = 3.926$, p = 0.003; infection level: $F_{2,99} = 0.933$, p = 0.397; interaction: $F_{7,99} = 2.490$, p = 0.021, Figure 3.8). In both sexes the highest expression level occurred in April, which is later than the original baseline study.



Figure 3.8 Relative expression profile of *kisspeptin 2* normalised to the reference gene, *ribosomal protein L8* and relative to the January sample (1) in the timed parasite exposure study. Bars show the mean + SEM, black bars = non-infected fish, grey bars = infected fish A; female fish, B; male fish. The month of dissection and parasite age (9 week or 18 week post exposure) is shown on the horizontal axis. Letters indicates a significant difference at p < 0.05

Follicle Stimulating Hormone (FSH)

The annual pattern of *FSH* expression differed between the two sexes. There was also a significant effect of infection which varied between ages of parasites and fish sex (Females; sampling month; $F_{5,98} = 2.815$, p = 0.020; infection level: $F_{2,98} = 8.051$, p = 0.001; interaction: $F_{7,98} = 7.451$, p < 0.001, Males; sampling month; $F_{5,99} = 11.768$, p < 0.001; infection level: $F_{2,99} = 3.700$, p = 0.028; interaction: $F_{7,99} = 21.614$, p < 0.001, Figure 3.9). In non-infected females expression was significantly higher in April and September, whereas in males it was highest in February, July and September. In February, *FSH* in females was higher in infected fish, although not significantly, whereas males harbouring 9 week-old parasites had significantly lower expression, and the 18 week-old parasites caused a significant increase. In April, females harbouring infections with parasites of both ages had significantly lower expression, whereas in males, 9 week-old

parasites led to a significant increase, however the 18 week-old ones had no significant effect. In June and July, 9 week-old parasites had no significant effect on expression in females, whereas 18 week-old parasites caused a significant increase in expression. However, in June both ages of parasite caused a significant increase in *FSH* in males, and in July, 9 week-old parasites caused a significant increase Neek-old ones led to a significant decrease. In September there was no effect of infection in females, however a significantly lower of expression level occurred in males.



Figure 3.9 Relative expression profile of *follicle stimulating hormone* normalised to the reference gene, *ribosomal protein L8* and relative to the January sample (1) in the timed parasite exposure study. Bars show the mean + SEM, black bars = non-infected fish, grey bars = infected fish A; female fish, B; male fish. The month of dissection and parasite age (9 week or 18 week post exposure) is shown on the horizontal axis. Letters indicates a significant difference at p < 0.05

Luteinizing Hormone (LH)

LH expression also differed between the two sexes. A significant effect of infection was also found, this varied between fish sex and parasite age (Females; sampling month; $F_{5,98}$ = 47.283, *p* < 0.001; infection level: $F_{2,98}$ = 6.761, *p* = 0.002; interaction: $F_{7,98}$ = 44.272,

p < 0.001, Males; sampling month; F_{5,99} = 11.246, p < 0.001; infection level: F_{2,99} = 21.689, p < 0.001; interaction: F_{7,99} = 12.393, p < 0.001, Figure 3.10). In non-infected females *LH* was significantly higher in April, June and July, whereas in males it was significantly higher in all time points compared to January. In February, expression in both sexes was significantly higher in infected fish regardless of parasite age. In April infection by both ages of parasite led to significantly lower expression in both sexes. In June, 9 week-old parasites caused a significant increase in females, whereas a significant decrease was caused by 18 week-old parasites. Males harbouring both ages of parasites had significantly lower expression. In July, females harbouring 9 week-old had significantly lower expression whereas it was significantly higher in those with 18 week-old ones, however, this was the opposite way round in males. In September there was no effect of infection in females, however infected male fish had significantly lower *LH* expression.



Figure 3.10 Relative expression profile of *luteinizing hormone* normalised to the reference gene, *ribosomal protein L8* and relative to the January sample (1) in the timed parasite exposure study. Bars show the mean + SEM, black bars = non-infected fish, grey bars = infected fish A; female fish, B; male fish. The month of dissection and parasite age (9 week or 18 week post exposure) is shown on the horizontal axis. Letters indicates a significant difference at p < 0.05

Vitellogenin Aa (VitAa)

In non-infected fish *VitAa* expression was similar in both sexes, although the level of expression in male fish was less than in females. There was a significant effect of infection which also varied between fish sex and parasite age (Females; sampling month; $F_{5.98} = 4.869, p = 0.001$; infection level: $F_{2.98} = 0.597, p = 0.553$; interaction: $F_{7.98} = 0.970$, p = 0.457, Males; sampling month; $F_{5.99} = 6.658, p < 0.001$; infection level: $F_{2.99} = 12.393$, p < 0.001; interaction: $F_{7.99} = 2.417, p = 0.025$, Figure 3.11). In all non-infected fish expression was significantly higher at all time points compared to January, peaking in June. In February, females harbouring both ages of parasites had significantly lower expression. In April and June, expression was also significantly lower in females with 9 week-old parasites, whereas there was no significant effect in those with 18 week-old ones. In February, April and July, males harbouring parasites of both ages had significantly higher expression levels. In June, males with 9 week-old parasites also had significantly higher expression, whereas there was no significant effect from the 18 week-old parasite. This was also the case in September as although expression was higher, this was not significant.



Figure 3.11 Relative expression profile of *vitellogenin Aa* normalised to the reference gene, *ribosomal protein L8* and relative to the January sample (1) in the timed parasite exposure study. Bars show the mean + SEM, black bars = non-infected fish, grey bars = infected fish A; female fish, B; male fish. The month of dissection and parasite age (9 week or 18 week post exposure) is shown on the horizontal axis. Letters indicates a significant difference at p < 0.05

Spiggin B (SpgB)

SpgB expression was found to vary significantly over the year. There was a significant main effect of sampling month and of infection, as well as a significant interaction between the two. *SpgB* expression also varied between ages of parasites (sampling month; $F_{5,99} = 5.896$, p < 0.001; infection level: $F_{2,99} = 13.726$, p < 0.001; interaction: $F_{7,99} = 3.278$, p = 0.004, Figure 3.12). In non-infected fish expression was significantly higher at all time points compared to January, peaking in June. The effect of parasite infections differed between the ages of parasite. In February, 9 week-old parasites had no significant effect, whereas fish harbouring 18 week-old ones had significantly lower expression. In April, fish harbouring parasites of both ages had significantly reduced expression, this was also the case in July. In June, however, 9 week-old parasites caused a significantly lower expression, although reduction of expression caused by 18 week-old parasites was not significant. In September infected fish also had significantly lower *SpgB* expression.



Figure 3.12 Relative expression profile of *spiggin B* normalised to the reference gene, *ribosomal protein L8* and relative to the January sample (1) in the timed parasite exposure study. Bars show the mean + SEM, black bars = non-infected fish, grey bars = infected fish. The month of dissection and parasite age (9 week or 18 week post exposure) is shown on the horizontal axis. Letters indicates a significant difference at p < 0.05

A summary of all the changes in gene expression in three-spined sticklebacks in relation to month and age of parasite are shown in Table 3.8

Month	Parasite age	Ki	ss2	FS	SH	L	H	Vii	tAa	SpgB
		Ŷ	8	9	8	9	2	9	5	8
Feb	9 Wk	-	-	-	↑	ſ	↑	\downarrow	1	-
	18 Wk	-	-	-	ſ	ſ	ſ	\downarrow	1	\downarrow
April	9 Wk	-	-	↓	ſ	↓	\downarrow	\downarrow	↑	\downarrow
	18 Wk	-	-	\downarrow	-	\downarrow	\downarrow	-	↑	\downarrow
June	9 Wk	-	-	-	↑	1	1	\downarrow	↑	\downarrow
	18 Wk	-	-	1	↑	\downarrow	1	-	-	-
July	9 Wk	-	-	-	ſ	\downarrow	ſ	-	Ť	\downarrow
	18 Wk	-	-	\uparrow	-	↑	\downarrow	-	1	\downarrow
Sept	18 Wk	-	-	-	\downarrow	-	\downarrow	-	-	-

Table 3.8 Summary table of changes in gene expression of three-spined sticklebacks in relation to time of year (month) and infection status (9 week-old or 18 week-old *S. solidus* parasites)

3.4 Discussion

3.4.1 General patterns of stickleback growth and development over the year

This study has examined how sticklebacks allocate their resources to growth and development over the annual cycle with marked differences seen between the breeding season and the periods of non-sexual somatic growth. Throughout the year some of the energy available is directed to somatic growth as each month their SL and weight increased. Over the first three months of the study (January-March, in the run up to the breeding season) resources were also channelled into energy storage, as the relative liver size (I_L) increased; in fish the liver is the main organ for glycogen storage (Hemre et al., 2002). Relative liver size (and presumably energy stores) significantly reduced over the summer in males, with a similar non-significant trend being observed in females, and started to increase again from October after the end of the breeding has finished. From April onwards, the fish began diverting energy from storage to sexual development, as there was a decrease in I_L in both males and females. This coincided with rapid hypertrophy of the kidney (shown by increasing I_K values) and significant increases in SpgB expression in males, which indicates Spiggin production (Wootton, 1976, Jakobsson et al., 1999). In females there was significant gonad growth (shown by I_G values rising) and increases in VitAa expression, which indicates eggs development (Wootton, 1976). This also coincided with the males beginning to develop nuptial colouration as PCR was no longer required to identify the sexes from April onwards. From late summer (July to September) male kidneys reduce in size and SpgB expression decreases. As nest building ceases Spiggin is no longer being produced. In females, gonads reduce in size and VitAa expression reduces due to vitellogenesis ceasing (Wootton, 1976, Wootton, 1984). These results are similar to patterns observed in two wild populations studied in their natural environments in Poland, in which the breeding season lasts from the beginning of April until mid-August (Sokolowska and Kulczykowska, 2006). Sokolowska and Kulczykowska (2006) investigated the variation of gonad size in both female and male fish, liver size only in the females and kidney size in males only. They found that in females, gonads increased in size from March, peaking in May before decreasing to similar sizes as at the start of the year by September. Liver size remained fairly constant across the year, with a small peak in March and a slight reduction in May and June. In males they found gonad size did not fluctuate during the year, but kidney size changed significantly, increasing in size from February until May,

before decreasing again by August. Similar results for female gonads and male kidney sizes were seen in wild fish from Sweden that had been transferred to aquaria with simulated natural photoperiod and temperature (Hellqvist et al., 2006). Therefore, it can be concluded that lab-bred fish kept in the controlled environmental conditions in the laboratory aquarium is a good representation of conditions fish would experience in their natural wild habitats.

Throughout the year it was found that male fish had significantly larger brains than females, this is similar to results of previous studies in both three-spined sticklebacks (Kotrschal et al., 2012, Herczeg et al., 2015, Toli et al., 2017) and in the related nine-spined stickleback (Herczeg et al., 2014). The difference in brain size between the two sexes is thought to be due to the different cognitive requirements for the complicated courtship behaviour males' display, and the parental care of offspring that is carried out by male fish. Samuk et al. (2014) observed that brain sizes were reversed in the "white" form of three-spined sticklebacks, in which the males have limited nesting and parental care behaviours with females having larger brains.

Gene expression profiles of fish also varied across the year giving an indication of how sexual development is influenced by environmental conditions. In both female and male fish, *Kiss2* expression levels peaked early in the year. In females the highest expression level was found to occur in February, whereas in males this was in March. This pattern of expression for *Kiss2* is in line with those observed in European sea bass *Dicentrarchus* labrax (Migaud et al., 2012, Escobar et al., 2016) and Nile tilapia Oreochromis niloticus (Park et al., 2016a) and is consistent with the theory that it is a key player at an early stage of the pathway triggering sexual maturation (Felip et al., 2009, Oakley et al., 2009, Mechaly et al., 2013). FSH peaked in both sexes of fish in February and in females only in October. LH expression increased after FSH, in females the levels increased from April before reducing in September, following a similar pattern to that of female I_G. In males the peak in expression occurred for a shorter time period, increasing in April but decreasing in June. These expression patterns are similar to those described in stickleback by Hellqvist et al. (2006). FSH and LH have long been recognised as gonadotrophic for both sexes of stickleback; in males, FSH triggers spermatogenesis and in females it leads to ovarian development. LH in males activates the androgen producing tissue in their testes, and in females, it activates the oestrogen producing follicle cells within their ovaries (Ahsan and Hoar, 1963). VitAa expression also increased significantly from late spring to early autumn, spanning the breeding season in both females and males. However in females the changes in expression were several magnitudes higher than in males. The Vitellogen protein is a major egg yolk precursor and the VitAa type has a lipovitellin heavy chain that is degraded heavily during oocyte hydration associated with the final stages of egg maturation (Hiramatsu et al., 2002, Sokolowska and Kulczykowska, 2006). As female sticklebacks are repeat spawners, maturation of eggs would need to occur continuously over the breeding season, so vitellogenesis and expression of VitAa would be expected to remain high during this period (Hellqvist et al., 2006). Similar patterns of Vitellogenin levels matching changes in annual water temperature has been observed in male fish (Hotta et al., 2003, Ma et al., 2005) including wild caught male stickleback from populations in south-west UK (Katsiadaki et al., 2012). However, the cause of this and the function Vitellogenin has for males is unclear. Endogenous oestrogens are a candidate and Vitellogenin may have a role in gonad maturation within male fish as well as its clear function in females (Hotta et al., 2003, Ma et al., 2005). In males SpgB expression also significantly increased during the breeding season, peaking in May before reducing from July, corresponding to the period when male sticklebacks undertake nest building and produce the Spiggin glue (Borg et al., 1985, Jakobsson et al., 1999).

3.4.2 How does the timing of infections across the annual cycle affect hostparasite interaction?

From the baseline studies of both morphological and gene expression profiles, key time points in the stickleback development were identified and used as end dissection points for the annual timed exposure to parasites. The time points chosen were: February, prior to the breeding season with hormone levels increasing and gonads beginning to develop; April, at the beginning of the breeding season when female gonads start rapidly increasing and male kidneys start to hypertrophy; June, the peak of the breeding season; July, towards the end of the breeding season; and September, post-breeding. In the experimental infection study, non-infected control fish demonstrated similar patterns in terms of their annual development at each time point compared to those observed in the baseline study carried out 12 months earlier, although the timings were not identical (e.g. *Kiss2* expression peaked one month later). Gaining infections prior to these key time points had varying effects on how the fish continued to develop. Significant sex differences in the effects of infection were shown, both in terms of the effect on

morphological development and in gene expression. Effects of infection were also often found to be related to the size and infective status of the parasite at the sampling point.

3.4.2.1 Parasite infections

At each time point and for each age of parasite the fish harboured it was found that significantly larger plerocercoids were retrieved from female fish. Similar results were found by Pennycuick (1971a), suggesting that parasites find conditions more favourable for growth in female rather than male fish. However, despite the plerocercoids being smaller in males, the effects of infection on both morphological and gene expression changes were generally greater in males compared to female fish. The aim of studying the two ages of parasites was an attempt to compare non-infective plerocercoids of weights less than 50 mg to infective plerocercoids (those above 50 mg) (Tierney and Crompton, 1992, Barber and Svensson, 2003), as an indication of either manipulation by castration or side effect altering the stickleback development. However some of the 9 week-old plerocercoids retrieved in June and July had already attained sizes larger than 50 mg; despite this, differences could still be seen between the two different ages.

Across the year the parasites reached different weights, which can be linked to growth rates influenced by warmer waters, which were varied across the year to reflect natural conditions. In June and July the water temperature was set at $18 \pm 1^{\circ}$ C, whereas in February this was $10 \pm 1^{\circ}$ C. Such differences in growth related to water temperatures have been seen previously with significantly different growth rates between 15 and 20°C (Macnab and Barber, 2012). Therefore the time of year when fish become infected can be significant for the developing parasites. Infecting hosts during warmer periods allows faster growth, and the ability to reach the size of infectivity for the final fish- eating bird host sooner. Faster growing parasites, however, may impair their fish host to greater levels than slower growing ones (Antia et al., 1994) which could ultimately have negative consequences for both host and parasite if virulence is so high it causes host death before transmission (Poulin, 1994, Poulin, 2007). With predictions of raising water temperatures related to climate change being most significant in the northern hemisphere, where sticklebacks are found (van Vliet et al., 2013), seasonal restrictions on the suitable time of year for S. solidus to infect fish hosts could be reduced as faster parasite growth would occur across the whole year. Consequently if negative effects of infection on hosts are limited by low temperatures, these might also increase over different seasons in warmer water.

3.4.2.2 Effect on body indices and gene expression

Effects of infection on condition, sexual development and gene expression varied across the year. Early infections had limited effects on the condition and sexual development of fish, whereas fish exposed later on in the year had significant differences in body indices. However, gene expression levels were affected significantly across the year. Differences were also seen in the effects of infection between male and female fish.

Females

There was no significant effect from either age of parasite on female brain, hepato-, or kidney-somatic indices. However, females harbouring both sizes of plerocercoids at the middle of the breeding season (June) had significantly smaller gonads whereas in the post-breeding season infected females had significantly larger gonads. The opposite effect on *vitellogenin* expression was seen, with significant reduction in expression levels occurring in the earlier infections, whereas the later infections had no effect. Variation in the effects of infection on the development of female gonads has previously been seen. Many studies of wild caught fish show, as found here during summer months, that infected females had smaller gonads and were less likely to become gravid (McPhail and Peacock, 1983, Heins et al., 1999) although in Alaskan populations many heavily parasitized females attain sexual maturity (Heins et al., 1999, Heins and Brown-Peterson, 2010). However, in experimentally infected females Barber and Svensson (2003) found gonads to be larger, as found in the autumn in this study. Larger gonads in infected females during the autumn were also observed in wild caught fish by Arme and Owen (1967). Infection may delay vitellogenesis causing gonads to fail to develop until later in the year. Changes in timing of development of gonads could be influenced by the feedback loops of gene expression that are also altered due to parasite infections, such as the changes in both LH and FSH expression seen in infected females across the year (Hellqvist et al., 2001, Hellqvist et al., 2006, Shao et al., 2015), and the reduced expression levels of VitAa observed here. However, expression levels varied dependent on the age of parasites suggesting the timing of infection could influence this.

Males

Unlike in females, infections of males significantly affected all the body indices, except the gonadosomatic index. Furthermore the ages of parasites also had differing effects. Brain size was generally significantly reduced in infected males. This could be an indication of the potential decrease in reproductive development as larger brains in male sticklebacks can be an indication of the level of investment in parental care males take part in (Samuk et al., 2014). Another indicator of male reproductive condition is increases in kidney size (Wootton, 1976, Jakobsson et al., 1999, Hellqvist et al., 2001), however during the breeding season parasite infections of both ages led to significantly smaller kidneys. Surprisingly the 9 week-old parasites had a greater effect than the 18 week-old ones. Males harbouring the 9 week-old plerocercoids had smaller kidneys than both the non-infected males and those harbouring the 18 week-old ones, although during the nonbreeding period there was no significant effect. Similar changes within Spiggin expression across the year were also seen, with reduced expression in males harbouring parasites during the breeding season. The results of smaller kidneys and lower expression of Spiggin is consistent with previous studies (Rushbrook and Barber, 2006, Rushbrook et al., 2007, Macnab et al., 2009, Macnab et al., 2011) although kidney hypertrophy and Spiggin production is not always compromised within infected males (Tierney et al., 1996, Candolin and Voigt, 2001, Macnab et al., 2011). The opposite effect of infection was seen on liver sizes in infected males. During the breeding season I_L increased regardless of the size of parasite, suggesting that energy is being diverted away from sexual maturation and resources that are surplus to the parasite growth are being stored. As well as larger livers, an unexpected result in males is those that were infected have significantly increased expression of *vitellogenin*, occurring at all time points except in September. Males harbouring parasites of both ages showed this response including sizes that are non-infective to the final host. Males would not normally produce Vitellogenin and significant health effects occur when males undergo intense Vitellogenin synthesis (Macnab et al., 2016). In their study Macnab et al. (2016) found that oestrogenic pollutants caused an increase in Vitellogenin production in stickleback, and in infected males plerocercoids reached a larger size. Their hypothesis for these effects is that as males had no use for the vitellogenin the parasite utilised it as a resource for its own growth. The increased levels of *VitAa* expression could indicate a possible manipulation from the parasite to generate an additional energy source from the males, particularly as this effect is seen in fish with the smaller parasites. This could account for the greater parasite growth found in the female fish. Vitellogenin produced by the females may have already been available for the parasites to use without the need to manipulate this; therefore the additional energy can be used for parasite growth without utilising resources by host manipulation.

3.5 Conclusions

From this study it was found that fish hosts are affected in different ways when gaining a parasite infection at different points in their development cycle. It was also shown that different sizes of parasites also have differing levels of impact and some of these changes could indicate a form of manipulation by the parasite as the observed changes in host morphology and gene expression occurred at early stages when the parasites were of low weights, below the level of infectivity for the final host, when the energetic demand that would be placed on the host harbouring them would also be low. The most significant effects on the host fish occurred during the summer months, coinciding with the breeding season in sticklebacks. In this situation energy has been diverted from the host sexual maturation to parasite growth.

3.6 Limitations and further work

There are a number of limitations to this study, which could be investigated further to more fully answer the question. One major limitation is that the 9 week-old parasites in the warmer summer months grew to sizes larger than the desired 50 mg, therefore although differences were observed between the two ages of parasite at these time points the changes seen within some of the host-parasite pairings could all be due to nutrient theft. None of the fish were allowed to attempt courtship or breeding, therefore, the changes seen in the morphology and gene expression on the infected fish can only be assumed as indicators that reproduction would not occur. Fitness of hosts in terms of successfully rearing offspring cannot be determined from this study.

4 The effect of nutritional status and infection status on stickleback development and gene expression profile of Brain-Pituitary-Gonad axis

4.1 Introduction

The outcomes of life history and developmental trade-offs often rely on the amount of resources and energy available (Zera and Harshman, 2001). The resources available to an organism can either be directed into the maintenance of key life functions and vital systems, or – depending on the age of the individual – somatic growth or sexual development and reproduction (Wootton, 1994, Reyes and Baker, 2016). Reproduction is energetically costly to the individual, and life history theory predicts that sexual development competes for resources with other activities (Roff, 1992, Stearns, 1992, Zera and Harshman, 2001). It is therefore assumed that energy is a limiting factor for reproduction and that the nutritional status or condition of an individual will affect the extent to which it engages in reproduction (Hillgarth and Wingfield, 1997).

The hormonal control of sexual development in vertebrates includes complex feedback loops, with environmental cues and physiological factors influencing the Brain-Pituitary-Gonad (BPG) axis, triggering a hormonal cascade that stimulates sexual development and maturation (Hellqvist et al., 2001, Hellqvist et al., 2004, Castellano et al., 2005, Hellqvist et al., 2006, Shao et al., 2012, Ahmed et al., 2012, Wang et al., 2014). In recent years, the protein Kisspeptin has been recognised as an important component of the early part of the BPG axis pathway (Felip et al., 2009, Oakley et al., 2009, Mechaly et al., 2011, Mechaly et al., 2012, Tena-Sempere et al., 2012, Zmora et al., 2012, Mechaly et al., 2013, Shahi et al., 2017). *Kisspeptin* expression has also been shown to be influenced by the nutritional status of the individual (Mechaly et al., 2011, Mechaly et al., 2013), providing a potential mechanistic link between nutritional condition and sexual maturation. Under limited nutrition, the expression of *kisspeptin* is reduced and sexual development fails to take place (Wang et al., 2014).

Reproductive success is often reduced within parasitized organisms (Lehmann, 1993, Smyth, 1994, Macnab et al., 2011, Goater et al., 2013, Lafferty and Shaw, 2013). Many examples can be explained as a result of simple side effects from indirect nutrient theft by the parasite. Resources used by the parasite to grow are no longer available for host development (Minchella, 1985). However, evidence also suggests that parasites can prevent their hosts from sexually developing by some form of manipulation, such as direct castration through the destruction of gametes and/or interaction with host endocrine systems (Arme, 1968, Mahon, 1976, Heins and Baker, 2010, Kroupova et al., 2012) (but

see (Heins, 2017)). The interaction and mechanism between host and parasite can have significant ecological and evolutionary implications as changes can have an impact on the dynamics of food webs, competition between species and biodiversity of ecosystem (Weinersmith and Faulkes, 2014). Identifying the mechanism operating in the host-parasite system is of great interest. Understanding this can further understand how evolutionary pressures on both host and parasite are altering and adapting phenotypic traits across evolutionary time (Weinersmith and Faulkes, 2014). Nutrient theft versus manipulation make different predictions about what would happen to the reproductive success of infected and non-infected fish in a situation where food became more or less available (as a result of either changing food levels or changing density of fish). If food levels increase infected fish would be expected to have greater reproductive success under simple nutrient theft as more resources would be available, whereas parasite manipulation should prevent development even when food is unlimited (Candolin and Voigt, 2001, Hurd, 2001, Heins and Baker, 2010).

Nutritional condition is known to be an important predictor of the likelihood of subsequent sexual maturation in fish. Fish fed restricted rations typically fail to develop reproductively (Trombley et al., 2014, Escobar et al., 2016). Such effects could be due to changes in the upregulation of the *kisspeptin* gene. Parasites that alter the nutritional condition of their host might therefore impact reproductive development through purely resource effects. One such host-parasite model, which has been extensively studied in this context is the stickleback-Schistocephalus system. Infected fish typically do not develop sexually (Arme and Owen, 1967, Pennycuick, 1971b, McPhail and Peacock, 1983, Tierney et al., 1996). It is thought that S. solidus prevents host reproduction due to the side effect of nutrient theft from its fish host, rather than through (parasite) adaptive castration (Heins and Baker, 2008, Macnab et al., 2009, Macnab et al., 2011). However, since there is evidence that S. solidus is a manipulator of various aspects of stickleback behaviour (Milinski, 1985, Macnab and Barber, 2012, Talarico et al., 2017), the lack of reproductive development in the fish may not be just down to nutrient theft, but could be due to some other manipulative factor. The evidence for the mechanism within the Schistocephalus-three-spined stickleback system is mainly based on that of field observations, which indicate that the reduced reproductive development is caused by nutrient theft from the parasite (Heins and Baker, 2008), however, limited laboratorybased studies have been carried out to determine this.

There are two main explanations for the observation that infected fish do not typically reproduce sexually. Firstly, the simple energetic drain of the parasite may generate a reduced somatic condition which then prevents sexual development. Secondly, the parasite may directly interfere with sexual development, for example, by switching off the hormonal control of reproduction. These two scenarios make different predictions; in scenario 1, a) the effects of the parasites should be reversible if nutritional stress can be alleviated, and b) the physiological fingerprint of infection should match that of simple nutritional stress. In scenario 2, it might be expected a) that the alleviation of nutritional stress would not reverse the effects of infection, and b) the physiological fingerprint of infection would differ from that of nutritional stress. Although parasites negatively affect their host, it would often not be beneficial to the parasite to cause premature death of their host. This is particularly true for their intermediate host, as growth and successful transmission to the final host are essential for the parasite to complete its life cycle and reproduce. If the host were to continue normal life-history strategies that are energetically demanding, such as reproduction, and support the growing parasite the energy burden could be too great and ultimately lead to its and the parasite's death. Therefore, it would be advantageous to the parasite to manipulate its host to prevent it from attempting to reproduce allowing enough energy for the parasite to grow and to keep its host alive to maximise the likelihood of transmission to the next host (Poulin, 1994).

One approach to study the physiological mechanism of reduced reproduction associated with infection is to compare patterns of gene expression in experimentally infected and nutritionally-starved hosts. If the lack of development in parasitized individuals simply reflects nutrient theft, it would be expected that patterns of physiological development and expression profiles for key genes involved in sexual development would be similar in well-fed, but infected individuals when compared to nutritionally-limited, but non-infected individuals. In this chapter, I describe an experimental study in which the infection status and nutritional condition of laboratory-held fish is manipulated to test this hypothesis.

4.1.1 Aims

The main aim of this chapter was to compare the effect of food restriction on the physical development and expression profiles of key genes involved in sexual maturation in stickleback fish with those induced by *S. solidus* infection. If the mechanisms preventing

reproduction in food restricted fish match those of infected fish, then nutrient theft by the parasite is supported. Differences in the effects of the treatments would indicate that parasites limit host reproduction through different mechanisms. The study was carried out by using laboratory bred fish that were either given a rationed diet or were exposed to the *S. solidus* parasite. Endpoint fish physiological data were collected and gene expression profiles through quantitative reverse transcription PCR (RT-qPCR) were used to compare the effects of the two treatments.

4.2 Methods

4.2.1 Fish supply and husbandry

All fish used in this study were lab bred stocks from wild caught Carsington parents generated by IVF as described in Chapter 2. Both adults which had been through a breeding season (16 months old) and juveniles (4 months old) were used in the food rationing section of the experiment, whereas only juveniles were used in the parasite exposure part. The use of adult fish allowed investigation into the effect of food rationing on gene expression in fish that had previously completed a normal annual cycle without the fish needing to grow to a size where they could mature. Juvenile fish were used to make the comparison between food rationing and parasite infection.

4.2.2 Experimental procedure

Male and female fish were separated prior to the start of the experiments, as the annual development study in Chapter 3 demonstrated that there are differences in their growth patterns and sizes of internal organs over the year that could potentially mask effects of the experimental conditions. Juvenile fish were sexed using the technique described in Chapter 2, and adult fish were separated by the visual characteristics of the male red throats and blue eyes. At the start of the study all fish were netted, blotted dry, weighed to 0.0001 g and standard length (SL) was recorded to 0.1 mm, before being placed in their individual tank for future identification and feeding regime. The experimental procedure is summarized in Figure 4.1. The study lasted for 91 d – this time period was chosen to allow the parasites to reach approximately 50 mg of weight (Barber and Svensson, 2003), the size at which plerocercoids become infective to the next host (Tierney and Crompton, 1992). The end point of the experiment was chosen to correspond to the period maximum expression of *kisspeptin* expression. The annual development study (Chapter 3) had

shown this to be at the beginning of February for females, and the beginning of March for males; mid-February was therefore chosen as the end point.



Figure 4.1 Schematic diagram summarizing the nutritional versus infection study protocol. A; Adult three-spined sticklebacks fed on either excess or 4% body mass ration. B; Juvenile three-spined sticklebacks fed on excess rations that were either non-infected or exposed to *Schistocephalus solidus*, or non-infected fish fed on a 4% body mass ration.

4.2.2.1 Food rationing procedure

Non-exposed adult and juvenile fish were fed once per day on frozen bloodworms (*Chironomus* sp. larvae); either to excess or given a 4% of body mass, which is sufficient to fuel somatic growth but not sexual development (Allen and Wootton, 1982b, Inness and Metcalfe, 2008). Fish were weighed at 14 d intervals during the trial to allow recalculation of the 4% ration. A total of 24 adult and 40 juvenile fish were used with equal number of females and males split between the two conditions. Fish that had already reached the critical length for development of 40 mm (Wootton, 1984) would no longer need to direct energy into somatic growth, instead surplus resources would enable sexual development. Whereas the juvenile fish had not reached this critical size, therefore resources would need to be directed into somatic growth before sexual development. A

restricted diet would impact on somatic growth and have indirect effects on reproductive development. Therefore the effect of limited food on development in adult fish could be determined without the factor of fish being too small being a factor.

4.2.2.2 Experimentally infected fish

Juvenile fish were exposed to *S. solidus* infected copepods as described in Chapter 2. Thirty fish in total (15 females and 15 males) were exposed to parasites. All exposed fish were fed on an excess diet, as combining both infection and food ration may have caused potential for high mortality levels. Adult fish were not exposed to parasites as they are unlikely to gain infections at this stage. Adults were studied as a control for size of fish capable of reproducing.

4.2.3 Post mortem analysis

At the end of the 91 d experimental period all fish were euthanized according to the UK Home Office approved Schedule 1 method of an overdose of Benzocaine anaesthetic followed by severing of the spinal cord. Measurements and tissue samples of brain, liver and kidney were collected as described in Chapter 2. Body condition factor (BCF) was also calculated ([Total mass-plerocercoid mass/standard length³] x 100000). Mortality rates were low, with 2/40 juvenile fish (one female and one male) in the non-exposed low ration treatment group dying during the 91 d period. The data from these fish was excluded from the analysis. No other mortalities occurred in the other groups of fish.

4.2.4 Molecular studies

RNA extraction, cDNA synthesis and qPCR were carried out on the three tissue sets according to protocols described in Chapter 2. Gene expression profiles of the five key genes, *luteinizing hormone (LH)*, *follicle stimulating hormone (FSH)*, *vitellogenin (VitAa)*, *kisspeptin (Kiss2)* and *spiggin (SpgB)*. were measured and the fold change normalised to the reference gene, *rpL8* and relative to excess non-infected fish was calculated using the 2^{-ddCT} method (Livak and Schmittgen, 2001).

4.2.5 Statistical analysis

Two-way ANOVA were conducted to explore the effect of food rationing and parasite infections on body indices and body condition factor. These showed there was an interaction in the effect between fish sex and food rationing in the body indices (except for brain somatic index) and the body condition factor, therefore the data for female and male fish were reanalysed separately using the Student's t-test for adults (excess vs ration) and one-way ANOVA for juveniles (excess fed non-infected, ration-fed non-infected and excess fed infected). For juvenile fish, parasite mass and parasite index were analysed using the unpaired Student's t-test. The changes in growth over time, both total mass and standard length were analysed using a repeated measures ANOVA. Fold changes in gene expression were analysed separately for each gene and for each sex, using a Student's t-test for adults and one-way ANOVA for juveniles.

4.3 Results

4.3.1 The effect of limited food and parasite infection on fish growth rates

4.3.1.1 Mass

During the study all fish increased in mass (which included plerocercoid weight in infected juveniles) over the 91 d period (Table 4.1), however there were significant differences in the rate of growth between well-fed and rationed fish.

Table 4.1 Total mean mass (g) of fish and plerocercoid from the food ration study showing initial weight at the start of the study period and the final weight achieved at the point of dissection on 91 d

Age	Sex	Treatment	Initial W	eight (g)	Final W	eight (g)
			Mean	SD	Mean	SD
Adult	Female	Excess	1.290	0.358	1.549	0.300
		Ration	1.220	0.128	1.311	0.125
	Male	Excess	1.415	0.208	1.739	0.210
		Ration	1.420	0.157	1.499	0.165
Juvenile	Female	Excess Non	0.211	0.060	0.763	0.078
		Ration Non	0.195	0.067	0.300	0.059
		Excess Inf	0.233	0.078	0.989	0.214
	Male	Excess Non	0.220	0.057	0.669	0.067
		Ration Non	0.188	0.056	0.276	0.056
		Excess Inf	0.235	0.060	0.914	0.133

After the initial two weeks of being fed 4% of body mass, a difference in mass could be detected. The analysis by two-way repeated measures ANOVA demonstrated that there was an effect of both the period of time and treatment group (diet only in adults, diet or infection status in juveniles).

There was no significant main effect of ration on mass in adults, however there was a significant main effect of time, with a significant interaction between the two (Females; Ration $F_{1,5} = 0.865$, p = 0.395, Time $F_{7,35} = 38.896$, p < 0.001, Ration*Time $F_{7,35} = 6.854$, p < 0.001, Males; Ration F_{1,5} = 2.572, p = 0.170, Time F_{7,35} = 107.31, p < 0.001, Ration*Time $F_{7,35} = 31.074$, p < 0.001), whereas in juveniles there was also a significant main effect of treatment (Females; Treatment $F_{2,12} = 97.347$, p < 0.001, Time $F_{7,42} =$ 79.174, p < 0.001, Treatment*Time F_{14,84} = 22.132, p < 0.001, Males; Treatment F_{2,10} = 36.333, p < 0.001, Time F_{7,35} = 116.789, p < 0.001, Treatment*Time F_{14,70} = 17.311, p < 0.0010.001). Pairwise comparisons showed that all fish gained weight between each time point. Parameter estimates revealed fish fed a 4% ration grew at a slower rate. For adult females this was significant after 56 d; however in adult males a significant difference occurred earlier at 42 d. In juveniles, over the course of the 91 d fish fed on an excess diet (both non-infected and infected) increased in weight significantly compared to the fish fed on a 4% ration. The pairwise comparisons showed significant differences at each fortnightly weighing. There was a trend for the infected fish fed on an excess diet to increase in weight more than the non-infected excess fed fish. Parameter estimates showed that this was significant in females from 70 d and from 84 d in males.



Figure 4.2 Line graphs showing growth rates of fish in the nutrition vs infection status study A; adult females, B; adult males, C; juvenile females, D; juvenile males. The markers indicate the mean value and the error bars represent the SEM. * indicates a significant difference at p < 0.05.

4.3.1.2 Fish length

Fish in all groups also increased in standard length over the 91 d study period, Table 4.2

Table 4.2 Mean standard length (mm) of fish from the food rationing study showing initial length at the start of the study period and final length achieved at the point of dissection on 91 d

		Initial Stand (m	dard Length m)	Final Standard Length (mm)		
		Mean	SD	Mean	SD	
Adult Females	Excess	46.00	1.801	50.21	2.264	
	Ration	46.12	2.424	48.86	0.559	
Adult Males	Excess	47.21	1.941	51.96	2.134	
	Ration	46.59	2.216	49.20	2.346	

Juvenile	Excess Non	27.61	2.409	42.62	1.910
Females	Ration Non	27.88	2.694	31.54	2.272
	Excess Inf	27.59	1.688	41.60	2.754
Juvenile	Excess Non	27.32	2.242	41.12	2.470
Males	Ration Non	26.72	2.960	30.51	2.399
	Excess Inf	27.25	0.734	39.85	2.411

In adult fish repeated measures ANOVA showed there was a non-significant main effect of treatment group (Females; $F_{1,5} = 0.382$, p = 0.553, Males $F_{1,5} = 2.728$, p = 0.119). Pairwise comparisons showed all fish increased in length over time. Parameter estimates revealed no significant effect of ration on the end SL in females, whereas in adult males there was has a significant effect; males fed on the 4% ration grew to a shorter length compared to the excess fed ones. In the juveniles ANOVA showed there was a significant main effect of treatment group (Females; $F_{1,31} = 24.989$, p < 0.001, Males $F_{1,32} = 28.508$, p < 0.001). Pairwise comparisons showed all fish increased in length over time. However, parameter estimates showed differences between excess and ration fed fish; both the noninfected and infected fish fed on the excess diet increased their length at the same rate, whereas the non-infected fish fed on the 4% ration were significantly shorter in length (Figure 4.3).



Figure 4.3 Line graphs showing change in standard length (SL) in fish in the nutrition vs infection status study A; adult females, B; adult males, C; juvenile females, D; juvenile males. The initial SL was measured on 0 d and re-measured at the end of the study on 91 d. The markers indicate the mean value and the error bars represent the SEM. * indicates a significant difference at p < 0.05.

4.3.2 The effect of limited food or parasite infection on body condition in fish

The effects of food ration and parasite infections on the body condition factor (BCF) of males and females were also investigated. Among adult fish, those fed the 4% ration had lower BCF than those fed to excess (Females; $t_{10} = 3.192$, p = 0.013, Males; $t_{10} = 2.139$, p = 0.049. In juvenile fish the effect of both food ration and parasite infection were significant (Females; $F_{2,31} = 36.747$, p < 0.001, Males; $F_{2,32} = 39.160$, p < 0.001). Fish fed on the 4% ration had lower BCF, whereas infected fish actually had significantly higher BCFs than the non-infected fish (Figure 4.4).



Figure 4.4 Boxplots showing body condition factor (BCF) of fish in the nutrition vs infection status study. A; adult fish (rations only) and B; juvenile fish (both rationed and infected). Dark grey boxes represent excess fed non-infected fish, pale grey boxes represent 4% ration fed non-infected fish and mid grey boxes represent excess fed infected fish. The median is indicated by the line across the box, the box extends from the 25th to 75th percentiles and the whiskers represent the maximum and minimum values. * indicates a significant difference at *p* <0.05

4.3.3 Parasite infections

At dissection a total of 13 fish (7 females and 6 males) from the 30 exposed were found to been infected with *S. solidus* plerocercoids. Each infected fish harboured only one plerocercoid. Although these numbers are low this gave enough biological replicates to carry out qPCR analysis with the infected fish.

As was found in Chapter 3, host sex significantly affected parasite mass with female fish harbouring larger plerocercoids ($t_{11} = 2.452$, p = 0.032, Figure 4.5). The parasite index (I_P) was also significantly different between female and male fish ($t_{11} = 2.757$, p = 0.019, Figure 4.5).



Figure 4.5 Boxplots showing A; plerocercoid mass (M_P) and B; parasite index (I_P) of experimentally-infected juvenile male and female fish in the nutrition vs infection status study. The median is indicated by the horizontal line across the box, the box extends from the 25th to 75th percentiles and the whiskers represent the maximum and minimum values. * indicates a significant difference at p <0.05

4.3.4 Effects of ration and parasite infection on fish body indices

4.3.4.1 Adult fish

Among adult fish, the effects of food ration on body indices depended on the index studied and also varied between females and males.

4.3.4.1.1 Brain somatic index (I_B)

There was no significant difference in brain somatic index (I_B) in female fish compared to male fish (F_{1,23} = 0.515 p = 0.484), and only a non-significant trend for fish on low ration diet to have a larger brain size than those fed to excess (F_{1,23} = 3.801 p = 0.063). There was no significant interaction between fish sex and ration (F_{1,23} = 1.881 p = 0.184, Figure 4.6 A).

4.3.4.1.2 Kidney-somatic index (I_K)

Kidney-somatic index (I_K) was strongly affected by fish sex with male fish having larger kidneys than females ($F_{1,23} = 48.893 \ p < 0.001$). As male fish, but not females, develop larger kidneys during sexual maturation a decision was made to analyse data on the effect of ration on the two sexes separately. Whereas ration had no significant effect on the I_K of females ($t_{10} = 1.984$, p = 0.083), males fed the lower ration diet developed smaller kidneys than those fed *ad libitum* ($t_{10} = 4.162$, p < 0.001, Figure 4.6 B).

4.3.4.1.3 Hepatosomatic index (I_L)

Hepatosomatic index (I_L) was influenced by a significant interaction between fish sex and ration (F_{1,23} = 5.678 p = 0.026), with the effect of ration differing between male and female fish. T-tests were therefore used to analyse the effect of food rations on liver size in females and males separately. Females on the low ration had significantly smaller livers than females fed the *ad libitum* diet (t_{10} = 3.303, p = 0.011) whereas ration had no effect on male liver size (t_{10} = 1.687, p = 0.112, Figure 4.6 C).

4.3.4.1.4 Gonadosomatic index (I_G)

Gonadosomatic index was influenced by a significant interaction between fish sex and ration ($F_{1,23} = 4.559 \ p = 0.044$), fish sex was found to have a significant effect on I_G ($F_{1,23} = 239 \ p < 0.001$), females had larger gonads than males. As fish develop male gonad size remains similar throughout the year, whereas females with developing ovaries change dramatically having much larger gonads than males (see Chapter 3). This factor again could mask the effects of food rationing if compared together. T-tests used to analyse females and males separately showed that there was no effect of being fed a rationed amount on gonad size in either females $t_{10} = 1.661$, p = 0.135 or males $t_{10} = 1.853$, p = 0.918, Figure 4.6 D.



Figure 4.6 The effect of food rationing on body indices in adult three-spined sticklebacks. Bars show the mean + SEM. A; Brain somatic index (I_B), B; Kidney-somatic index (I_K), C; Hepatosomatic index (I_L) and D; Gonadosomatic index (I_G) For I_K, I_L and I_G fish sex was found to be a significant factor in the size of the body index, therefore the data has been analysed separately. * indicates a significant difference at p < 0.05

4.3.4.2 Juvenile fish

As with adult fish, the effects of food restriction on the body indices of juvenile fish depended on the index studied and varied between females and males. In juveniles, the effect of parasite infection was also investigated and compared with the effect of food restriction. Two-way ANOVA found interactions between fish sex, ration and infection. As female and males body indices naturally vary as they develop (see Chapter 3) it was more appropriate to analyse their data separately using one-way ANOVA.

4.3.4.2.1 Brain somatic index (I_B)

In both female and male fish there was a statistically significant difference in brain somatic index (I_B) between the three groups of fish: excess-fed non-infected, restricted
ration non-infected and excess-fed infected (Females $F_{2,31} = 27.182$, p < 0.001, Males $F_{2,32} = 30.870$, p < 0.001). Post-hoc comparisons with the Tukey HSD test revealed that restricted-ration fish of both sexes had a larger relative brain mass compared to both non-infected and infected fish fed to excess. However, there was no significant effect of parasite infection on I_B (Figure 4.7 A).

4.3.4.2.2 Kidney-somatic index (I_K)

There was a significant effect of treatment on kidney-somatic index (I_K) in both sexes (Females $F_{2,31} = 12.141$, p < 0.001, Males $F_{2,32} = 6.833$, p = 0.003). Post-hoc comparisons revealed that female fish fed the restricted ration had a smaller I_K than both infected and non-infected excess-fed females. In males, however, there was a significant effect of both food availability and parasitism, with both excess-fed, *S. solidus*-infected fish and non-infected fish on the restricted ration having smaller I_K than the non-infected males fed on the excess ration (Figure 4.7 B).

4.3.4.2.3 Hepatosomatic index (IL)

There was a significant treatment effect on hepatosomatic index (I_L) among both female and male fish, (Females $F_{2,31} = 6.324$, p = 0.005, Males $F_{2,32} = 13.09$, p < 0.001). Posthoc comparisons for both sexes showed that there was a significant effect of food availability but not infection status, with fish on the restricted ration having smaller livers compared to both the non-infected and infected fish fed the excess ration (Figure 4.7 C).

4.3.4.2.4 Gonadosomatic index (I_G)

The gonadosomatic index (I_G) of males and females was not affected by treatment. Posthoc comparisons showed that females had significantly larger I_G than males (Females $F_{2,31} = 2.311$, p = 0.116, Males $F_{2,32} = 1.798$, p = 0.182; Figure 4.7 D).



Figure 4.7 The effect of food ration and parasite infection on body indices in juvenile three-spined sticklebacks. Bars show the mean + SEM. A; Brain somatic index (I_B), B; Kidney somatic index (I_K), C; hepatosomatic index (I_L) and D; Gonadosomatic index (I_G) * indicates a significant difference at p < 0.05

4.3.5 The effect of food restriction or parasite infection on gene expression

Gene expression profiles were similar in adult and juvenile fish and between females and males; however, there were significant differences in expression levels when comparing fish fed on a restricted ration diet with those infected by *S. solidus* (Figure 4.8).

4.3.5.1 Luteinizing hormone (LH)

Restricted feeding had no significant effect on the level of *luteinizing hormone (LH)* expression in adult fish (Females; $t_{10} = 0.335$, p = 0.746, Males; $t_{10} = 0.911$, p = 0.377). Among juvenile females, there was no significant treatment effect on *LH* expression (F₂, $s_1 = 1.357$, p = 0.273); however, among juvenile males there was a significant treatment effect (F_{2,32} = 7.764, p = 0.003), and post-hoc comparisons showed that restricted ration

fish had significantly reduced *LH* expression compared to both excess fed non-infected and infected fish.

4.3.5.2 Follicle stimulating hormone (FSH)

In adult fish, there was no significant difference between restricted ration and excess-fed fish on expression levels of *FSH* (Females; $t_{10} = -0.093$, p = 0.928, Males; $t_{10} = 0.287$, p = 0.778). However, a significant treatment effect was found in *FSH* expression among juvenile females (F_{2, 31} = 3.991, p = 0.029), with post-hoc comparisons showing that excess-fed infected fish had higher *FSH* expression levels than both excess-fed and restricted ration non-infected females. There was no effect of food rationing on *FSH* as both the rationed and excess fed females had similar levels. Juvenile males exhibited a similar pattern with the infected males having increased expression levels, though the trend was non-significant (F_{2, 32} = 2.697, p = 0.090).

4.3.5.3 Kisspeptin 2 (Kiss2)

Food restriction had a significant effect on the level of *Kiss2* expression in both adults and juveniles, and in both males and females. Among adult males and females, those being fed the restricted ration exhibited significantly increased *Kiss2* expression levels (Females; $t_{10} = -3.789$, p = 0.005, Males; $t_{10} = -2.589$, p = 0.021). Among juvenile fish, there was a significant treatment effect in both sexes (Females; $F_{2, 31} = 14.547$, p < 0.001, Males; $F_{2, 32} = 12.279$, p < 0.001), with post-hoc comparisons indicating that the food restriction, but not infection, significantly increased *Kiss2* expression.

4.3.5.4 Vitellogenin Aa (VitAa)

The expression of *VitAa* was significantly affected by dietary restriction in adult female fish, and by parasite infection in male fish. Adult female fish on the restricted ration diet showed reduced *VitAa* expression compared to excess-fed fish, whereas this was not the case for males (Females; $t_{10} = 6.517$, p < 0.001, Males; $t_{10} = 1.129$, p = 0.285). There was a significant treatment effect on *VitAa* expression among juvenile fish of both sexes (Females; $F_{2,31} = 6.907$, p = 0.004, Males; $F_{2,32} = 4.321$, p = 0.023). Post-hoc comparisons showed that among females, both dietary restriction and parasite infection caused a significant reduction in *VitAa* expression, whereas in male fish infection was associated with a significant upregulation of *VitAa*.

4.3.5.5 Spiggin B (SpgB)

The expression of *SpgB* was only investigated in male fish. In adult males, restricted ration caused a significant reduction in *SpgB* expression ($t_{10} = 2.808$, p = 0.013). Among juvenile males, there was a significant treatment effect on *SpgB* expression (F_{2,32} = 47.394 p < 0.001), with post-hoc comparisons showing both restricted ration and parasite infection having a similar negative effect on expression levels.



Figure 4.8 The effect of food restriction and parasite infection on gene expression profiles in three-spined sticklebacks, A; Adult females, B; Adult males, C; Juvenile females, D; Juvenile males. Bars show the mean fold change in gene expression with error bars representing + SEM. Expression level is compared for each gene; *luteinizing hormone (LH), follicle stimulating hormone (FSH), kisspeptin (Kiss2), vitellogenin (VitAa)* and in male fish only *spiggin (SpgB)*, across the three treatment groups but are not compared against expression of other genes. Relative expression levels of genes were normalised to the reference gene, *rpL8* and relative to excess non-infected fish (1). Letters represent a significant difference at p<0.05.

4.4 Discussion

In this study, it was found that both food restriction and infection with the parasite *S. solidus* had different effects on both physiology and gene expression patterns of stickleback fish. While food restriction affected the physical characteristics (reduced growth and body indices), parasite infections generally affected gene expression patterns to a greater extent. These findings appear to support the hypothesis that reduced development and sexual maturation observed in fish infected by *S. solidus* occur as a result of mechanisms other than simple nutrient theft. While the masses of parasite infections in the experimental study were relatively small, and so may have placed only limited energetic demands on their hosts, a significant difference in the host gene expression profiles of non-infected restricted ration fish and infected but well-fed fish potentially supports active manipulation of host phenotype over a simple nutritional side effect.

4.4.1 Parasite development

Seven females and six males developed plerocercoids post-exposure, with male fish harbouring significantly smaller plerocercoids than females after 91 d. In females, parasites had reached the size that is considered infective to the final bird host (i.e. 50mg, (Tierney and Crompton, 1992, Barber et al., 2004); the mean mass of plerocercoids recovered from females was 72 mg whereas the mass of plerocercoids recovered from males was almost half this at 38 mg. Parasites therefore appeared to be more effective at gaining resources to support somatic growth from female rather than male hosts. These results are consistent with those found by Pennycuick (1971a) and from Chapter 3. As discussed in Chapter 3, increased growth rates within in female fish could be due to parasites utilising the Vitellogenin proteins.

4.4.2 Differing effects of reduced food and parasite infection on fish growth and development

There were significant negative effects on stickleback growth when maintained on restricted ration, but not from being infected with *S. solidus*. The final body mass and standard lengths were lower in fish fed the restricted diet than the fish fed to excess. Although both sexes were impacted in a similar way, juvenile fish were affected more than adults. The body weight and SL of all fish increased over the 91 d study, although restricted ration fish grew more slowly, particularly the juveniles. While the level of ration

chosen allowed for somatic growth, sexual maturation was unlikely, and this is comparable with previous studies that showed development was limited under restricted feeding regimes. Wootton (1973) found that varying food intake had an effect on female maturation; only 38% of females matured and produced eggs at the lowest ration, whereas the two higher rations in the study allowed 63% of females to mature. In their study, Stanley and Wootton (1986) found that rations of 6% and 18% had no effect on male nest building, whereas at 2% there was a significant reduction in the number of males attempting to build nests.

Before sticklebacks can sexually mature they need to reach a critical size. This varies between populations, but is on average 40 mm SL (Wootton, 1984). In the present study, the adult fish had already exceeded this size at the start of the study and had been through a breeding season. Therefore, the effects of limited food were likely to affect body condition rather than growth rates. Juvenile fish had not reached this size at the start of the study and so there was an effect on growth. Among the juvenile fish, differences were detected between restricted ration fish and parasite infected fish. Both non-infected and infected fish fed with the excess diet achieved an SL of approximately 40 mm, whereas the fish fed on the restricted ration only reached lengths of approximately 30 mm. This difference in length could indicate that fish on the restricted fish would be at a size where sexual development could occur.

4.4.3 Variation in body indices indicate maturation capability of fish

Restricted diet and parasite infections had significant effects on the body indices of fish. In male fish, both food restriction and parasite infection resulted in smaller Kidneysomatic indices compared to the non-infected excess fed males. In male sticklebacks the development of the kidney is crucial for to mature and successfully breed as it is from the kidney that male sticklebacks produce Spiggin glue to build nests (Jakobsson et al., 1999). Despite the small size of parasites in this study, they had an equivalent effect on kidney mass as the restricted ration diet, indicating the parasite had prevented the development of the kidney at an early stage of infection. Changes in the host at early stages of infection are often regarded as evidence for manipulation and not nutrient theft as the energy drain would not be significant (Hurd, 2001). No effect of diet or infection was observed in the gonadosomatic index of either sex. Gonads were present in all fish suggesting that they would be capable of developing if limited resources and potential manipulation were avoided.

4.4.4 Variation in response to restricted diet or infection may demonstrate manipulation and not just nutrient theft

4.4.4.1 Body condition factor is reduced in restricted feeding regimes but increases when infected suggests differing mechanisms

Significant differences were seen in the body condition factor of fish when comparing restricted-ration and excess fed infected fish. Fish under the restricted feeding regime, had significantly reduced condition factors since fish divert energy away from somatic growth and into maintenance of vital functions. This is comparable to other studies such as that on Atlantic salmon (Salmo salar) (Trombley et al., 2014). However, infected fish fed the excess diet actually had significantly higher condition factors; these fish apparently had enough energy to support the developing parasite as well as maintaining their own growth. In juvenile fish a lower hepatosomatic (I_L) index was also observed when fed low rations, indicating that energy is not available for storage. Conversely, infected fish had a similar IL to non-infected fish fed the excess ration, showing that enough energy was available to allow both fish and parasite growth. If significant nutrient theft from the parasite is occurring and the fish host is attempting to develop and mature normally a reduction in I_L might be expected. As this is not the case it could indicate that the parasite has caused the fish host to divert energy from, for example, sexual maturation for its own benefit (Arme, 1968, Carter et al., 2005, Heins et al., 2004). This is similar to the results observed in nine-spined sticklebacks infected with S. pungitii. Infected fish, whilst being prevented from maturing sexually, actually had higher condition factors than non-infected fish that had matured (Heins et al., 2004). As the nutritional drain of developing parasites would be expected to cause a reduction in condition this led to the suggestion that S. pungitii was manipulating its fish host in some way (Heins et al., 2004). The increased body condition shown here also suggests that parasites in this study are capable of manipulation.

4.4.4.2 Gene expression profiles differ between diet and parasite infection giving indication of different underlying mechanisms preventing reproduction

The effects of food restriction and parasite infection were investigated in five key genes involved in the BPG axis that are crucial for development and sexual maturation; *kisspeptin (Kiss2), luteinizing hormone (LH), follicle stimulating hormone (FSH), vitellogenin (VitAa)* and, in males, *spiggin (SpgB)*. This study showed that both restricted diet and infection with *S. solidus* had significant effects on the expression levels of these genes. However depending on the gene and the sex of fish the effects of *S. solidus* differed from responses to restricted food.

In adult fish food restriction did not affect the expression of LH or FSH but caused a significant downregulation of VitAa in females and SpgB in males. Since VitAa and SpgB are important for the final energetically demanding stages of reproduction (egg and Spiggin production) they could be more affected by low nutrient availability. More surprising was the significant upregulation of Kiss2 among both males and females held under restricted rations. Kisspeptin has recently been recognised as one of the key 'gatekeeper genes' involved in the switch of development in organisms that triggers puberty (Oakley et al., 2009, Pasquier et al., 2011, Park et al., 2016a, Shahi et al., 2017). It has also been identified as a potential link in signalling nutritional status and sexual maturation (Mechaly et al., 2011, Mechaly et al., 2013, Wang et al., 2014). In mammals, restricted diets consistently lead to the downregulation of kisspeptin and LH (Ahmed et al., 2012, Castellano et al., 2005, Wang et al., 2014) and treating animals that had been kept on a limited ration, which caused reduced kisspeptin and LH expression, with Kisspeptin protein rescued the expression of LH (Castellano et al., 2005, Chang et al., 2012, Park et al., 2016a). In fish there is greater diversity in changes in gene expression in response to restricted diet; fish are extremely diverse, inhabiting many different environments and adopting different life history and breeding strategies, so different patterns in response to reduced food might be expected (Mechaly et al., 2011). Restricted diets caused upregulation of kisspeptin and subsequently LH and FSH in Senegalese sole (Solea senegalensis) (Mechaly et al., 2011, Mechaly et al., 2012), European sea bass (Dicentrarchus labrax) (Escobar et al., 2016) and pejerrey fish (Odontesthes bonariensis) (Tovar Bohórquez et al., 2017). Conversely, there was no effect on expression on any of the genes when Atlantic salmon were held under food restriction regimes (Trombley et al., 2014), and in Nile tilapia (*Oreochromis niloticus*) food rationing led to down regulation of the genes similar to that seen in mammals (Park et al., 2016b).

It might be expected that nutrient theft from a parasite would lead to a similar response in gene expression level of genes as that triggered by limited food availability. However, restricted food and S. solidus infection led to variable expression levels at different points in the BPG axis. This implies that limited resources and parasite infection do not prevent maturation and sexual development by the same mechanism. In juvenile fish, limited food had similar effects on gene expression as in adults, with significant upregulation of Kiss2 (an early player in triggering sexual development), but no change in either LH or FSH, which act downstream of Kiss2. However, food restriction also caused significant downregulation of *VitAa* and *SpgB* which are expressed at the later stages of development. Conversely, S. solidus infections caused no change in the expression of Kiss2 compared to the non-infected fish. Infection also caused a trend for LH and FSH to be upregulated in males when infected. In females infection only caused a significant upregulation of FSH. These are similar to the results found by Shao et al. (2012), who found that infection increased expression of LH and FSH in males, but only FSH in females. The differences observed in Kiss2, LH and FSH expression indicates that limited food and infection are influencing the BPG axis, and subsequent development, in different ways. Prevention of maturation may not not simply be caused by nutrient theft, with some form of manipulation occurring.

Similar to food rationing, infection led to significantly lowered expression of *VitAa* for females and *SpgB* for males indicating that both restricted diet and parasite infection would prevent sexual maturation taking place; females would not develop eggs with the limited Vitellogenin and males would not build nests without producing Spiggin. However, in males infection caused a significant increase in *VitAa* expression. Males would not normally produce vitellogenin and significant health effects occur when males undergo intense Vitellogenin synthesis (Macnab et al., 2016). In their study Macnab et al. (2016) found that oestrogenic pollutants caused an increase in vitellogenin production in stickleback, and plerocercoids reached a larger size in infected males. Their hypothesis for these effects is that as males had no use for the Vitellogenin the parasite utilised it as a resource for its own growth. The increased levels of *VitAa* expression could indicate a possible manipulation from the parasite to generate an additional energy source from the

males. This could account for the larger parasites found in the female fish, as Vitellogenin may have already been available for the parasites to use in female fish without expending energy to generate it.

4.5 Limitations and further work

There are a number of limitations to this study which if investigated further may answer the question more thoroughly. One limitation is that infected fish were only kept on the excess diet; however different results may be found if infected fish were also maintained on a rationed diet. A second limitation is possibly due to the relatively small sizes of plerocercoids. Larger parasites may have more similar effects to food rationing than the smaller ones that developed in this study. Therefore future work could investigate the combination of ration and infection alongside older parasites. This study also only looked at one time point in the year. Since development and gene expression patterns vary across the year different effects of both rationing and infection might be seen. 5 Variation in reproductive phenotype of *Schistocephalus* infected male sticklebacks reflects parasite but not host provenance

5.1 Introduction

Schistocephalus solidus infections typically impair the reproductive development and behaviour of three-spined sticklebacks, and this parasite has been classically viewed as an absolute castrator, inhibiting gamete production and spawning in three-spined sticklebacks (Arme and Owen, 1967, Pennycuick, 1971b, McPhail and Peacock, 1983, Tierney et al., 1996). Pennycuick (1971b) and Tierney et al. (1996) found infected males fail to fully mature, possibly due to spermatogenesis being delayed or prevented. Although Arme and Owen (1967) found evidence for oogenesis being prevented or delayed in females as a result of infection, there was no significant effect on male gonad development. Similar differences between the sexes was seen by McPhail and Peacock (1983). However, recent research has shown considerable inter-population variation in the reproductive capability of infected fish (Macnab et al., 2009).

Variability in development in response to infection, might arise from environmental conditions. Different populations will experience varying resource availability, such as the low levels available in the oligotrophic Llyn Frongoch (Hanlon, 1982). Many contrasting results in different populations, and between the fish sexes, suggest that side effects of nutrient theft, rather than manipulation are preventing development (Heins and Baker, 2008, Macnab et al., 2009). However the role of parasite manipulation, altered host phenotype caused by the parasite that has fitness benefits to the parasite; or host adaptation, changes in host phenotype produced by the host itself to overcome or reduce the impact of being parasitized, cannot be ruled out (Macnab et al., 2009, Macnab et al., 2011).

Alternatively host or parasite genotype from different populations could account for the observed variation. Populations may differ in their period of evolutionary interaction and different reciprocal selection pressures on the host and the parasite may lead to regional differentiation (Poisot, 2015). A host's ability to resist parasites and to defend itself against the negative effects of infection is influenced by the genotype of both the host itself and the infecting parasite, with many interactions being highly specific to the spoecies involved leading to coevolution (Parker et al., 2017). Coevolution is likely to be oscillatory, as one strain of parasite may develop manipulative capabilities before the host evolves to counteract this. Interactions will be key to changes in coevolution and independent to neighbouring populations. (Carius et al., 2001). Therefore effects of

parasitism could be determined by genotype-genotype interactions and variation in host phenotype response to infection can reflect local adaptations (Barrett et al., 2008).

Local adaptation is likely to be more evident when parasites are detrimental to their hosts, due to stronger parasite-mediated selection on host populations leading to greater between-population divergence. High virulence may also lead to local adaptations within parasites even in the face of relatively low migration rates, and increase the genetic variation between different populations of parasites within the same species (Greischar and Koskella, 2007)

The greatest effects of parasitism across host species are often seen in reduced female reproduction. This could be explained by the theory that sexual development and reproduction is more costly to females due to the significant energy resource required to become gravid and produce mature eggs. However, in sticklebacks, successful reproduction also incurs significant energetic costs to males (Smith and Wootton, 1999). Reproductive success requires males to undergo development of secondary sexual characteristics (red throats and blue eyes), the defence of territories, along with the construction of nests (Wootton, 1976), which requires the synthesis of copious amounts of Spiggin 'glue' from the hypertrophised kidney (Jakobsson et al., 1999), and performing intense courtship displays. Moreover, the males also participate in parental care of eggs and hatched fry (Wootton, 1976). These are energetically demanding and costly to the males (Wootton, 1984, Frischknecht, 1993, Smith and Wootton, 1999) and essential for reproductive success. Males that fail to develop sexually, secure a territory and build a nest or engage in sufficient parental care are unlikely to be successful in terms of producing offspring. Furthermore, not only do male stickleback nests serve as functional structures for deposition and protection of eggs, they are also the significant focal point for courtship (Wootton, 1976) and are used by female fish to assess the quality of males (Barber et al., 2001, Östlund-Nilsson and Holmlund, 2003, Rushbrook et al., 2008).

Inter-population variation in the sexual development of naturally-infected *S. solidus* parasitized males in wild populations has been documented. Arme and Owen (1967) and Tierney et al. (1996) observed that infected males developed red nuptial colouration to similar intensities as the non-infected fish (at Farnley, Leeds, UK and Inverleith Pond, Edinburgh, UK respectfully). However, Pennycuick (1971b) and Folstad et al. (1994) found that infected males lacked colouration compared to the non-infected fish (at Priddy

Pool, Yorkshire, UK and Lake Takvatn, Norway, respectively). Furthermore, the extent to which infected males engage in nesting behaviour also varies between populations. Infected males in Fuller Lake on Vancouver Island (British Columbia, Canada) were found to regularly defend nests (McPhail and Peacock, 1983), whereas, despite exhibiting nuptial colours, males in a disused clay pit (Leeds, UK) typically failed to nest (Arme and Owen, 1967).

A study by Candolin and Voigt (2001) compared the reproductive phenotypes of S. solidus infected male sticklebacks in the field and after transfer to the laboratory. When observing fish in the field at Tvärminne (Finland), infected males tended to have less nuptial colouration and were unlikely to be engaging in nesting activity. However when fish were brought into favourable laboratory conditions, where they were provided with excess food and a suitable territory in an individual aquarium, both infected and noninfected males displayed similar nuptial colouration and successfully built nests. These results suggest that resource availability might be important in determining the reproductive phenotype of infected males, and therefore, that the parasite primarily exerts its effects through its nutritional demands. Studies on other populations have shown that infected males fail to build nests even after being kept under favourable laboratory conditions. Rushbrook and Barber (2006) showed that infected males from Llyn Frongoch (Wales, UK), failed to nest under such conditions, and divergent results for infected males from populations in Victoria Park (Leicester, UK) and Kendoon Loch (Scotland, UK) – held under identical experimental conditions in the same study – supported the hypothesis that reproductive impairment of infected males was not solely due to nutritional limitation (Macnab et al., 2009). Variation in the sexual maturation of males across populations still persists, despite being given favourable conditions within laboratory aquaria.

The population differences in reproductive phenotype of infected males after transfer to favourable laboratory conditions could suggest a mechanism other than side effects of nutrient theft from the growing *S. solidus* plerocercoids. Such a mechanism could involve either parasite manipulation or host adaptation. Nevertheless, limited resources cannot be ruled out as the energetically expensive nest building behaviour depends on the level of food intake and body condition during the period before the breeding season (Wootton, 1984, Tierney et al., 1996, Bagamian et al., 2004). All the studies with males that had

been brought in to the favourable conditions were for short term periods prior to their nesting trials. In the studies carried out by Candolin and Voigt (2001) and Rushbrook and Barber (2006) males were collected during the breeding season, and although fish in the Macnab et al. (2009) study were kept in the favourable laboratory conditions for longer periods, up to one month before trials started, this is still a short period of time. Triggers for switching from somatic growth to sexual maturation that are dependent on food resources could already be limited. For example, results from Chapter 3 showed that expression levels of the kisspeptin, which plays a key role in the timing of sexual maturation (Oakley et al., 2009, Zmora et al., 2012), and follicle stimulating hormone which triggers spermatogenesis in males and in females leads to ovarian development (Ahsan and Hoar, 1963) peak early on in the season (highest expression was seen in February and March – 2-3 months prior to the breeding season). In Chapter 4 reduced food availability was also shown to significantly alter kisspeptin expression at the time point when expression should be high and the triggering of the cascade of hormones to promote maturation occurs. Therefore, more long term experiments to try to eliminate these resource differences are required. By using laboratory bred F1 generations from different populations in experimental infection studies, cross-population infections and common garden rearing experiments, the mechanisms behind the negative effects of S. solidus infections on the reproductive development of three-spined sticklebacks could be identified, as all populations will experience the same level of nutrition from hatching to adulthood (Macnab et al., 2009).

5.1.1 Aims

The aim of this chapter was to examine how the reproductive phenotype of *S. solidus* infected male sticklebacks was affected by the provenance of the fish and the parasites involved. The study focussed on quantifying aspects of nesting behaviour and physiology, in order to determine whether genetic differences of either host or parasite influence the mechanism that prevents development.

In the first part of the study, the reproductive phenotype of naturally-infected, wild caught adult male sticklebacks from a range of populations was investigated. In the second part of the study, the reproductive phenotypes of F1 offspring – raised under favourable laboratory conditions and experimentally infected with *S. solidus* parasites – were studied. Parasites used in the experimental investigations were either taken from the

stickleback's own population, or from one of the other populations (cross-infections). Comparing the results of these studies generated insights into the most likely explanation for the observed differences. The aim was to determine if reduced development is due to nutrient theft from parasites, or from parasite manipulation and whether differences observed from wild fish in previous studies reflect the environmental conditions the various populations live in.

5.2 Methods

5.2.1 Fish stocks and husbandry

The aquarium conditions throughout the experiment were altered to reflect the seasonal changes occurring in the field.

Wild populations

Infected and non-infected sticklebacks were collected in March 2013 and 2014 from populations across the UK with endemic *S. solidus* populations. They were brought back to favourable laboratory conditions at the University of Leicester aquarium facilities. The fish were housed in large 80 L tanks (60 x 40 x 40 cm) and were fed *ad libitum* on a mixture of bloodworm and ZM Medium Premium Granular pellets, prior to nesting trial beginning in May of each year.

2013 populations

The three sites chosen were:

- 1. Carsington Water (CRS) (personal observation by V. Macnab (2009) indicated infected males did not build nests)
- 2. Clatworthy Reservoir (CLT) (personal observations by C.A. Tilley (2012) indicated infected males build nests)
- 3. Inverleith Pond (INV) (in their study Tierney et al. (1996) observed infected males successfully built nests)

2014 populations

Again three sites were used:

1. Carsington Water (CRS) (repeat sample to compare repeatability between years)

- 2. Llyn Frongoch (FRN) (Rushbrook and Barber (2006) observed infected males failed to build nest)
- 3. River Welland (WEL) (No observations on this population have been made).

Lab-bred populations

In June of the respective year, 15 families from each of the six populations were generated by IVF using wild parental fish as described in Chapter 2. All families from the same population were combined and housed in replicate 30 L (40 x 25 x 30 cm) tanks at a density of one fish per litre. The fish were fed *ad libitum* on 2 day old *Artemia sp.* nauplii for three months after hatching, after which they were transferred to an *ad libitum* diet of bloodworm (*Chironomus sp.* larvae) until the experimental parasite exposures were carried out in the following January.

5.2.2 Experimental procedure

The experimental procedure for the nesting trials and the experimental cross-infections are summarised in the schematic diagram in Figure 5.1 and described fully below.

5.2.2.1 Nesting trials

Starting in May each year, males were used in nesting trials. The same protocol was used for both naturally-infected wild fish and the lab-bred, experimentally-exposed males. Wild caught males (in 2013 and 2014) were identified by developing nuptial colours, and lab-bred experimentally exposed males (in 2014 and 2015) were selected randomly from the stock tanks. Fish were selected with a similar colouration from each population for each batch of males; both infected and non-infected males were selected. Nuptial colouration was scored at the start of the trial using the protocol described by Rushbrook and Barber (2006) (Table 5.1). Fish were also blotted dry and weighed (to 0.001 g) and their standard length (SL) measured using callipers (mm) before being placed into individual 14 L tanks (36 x 20 x 20 cm), one fish per tank. Tanks were separated from each other by an opaque divider in order to isolate the males. The tanks contained a sponge airlift biofilter; and were lined with a 2 cm layer of gravel with a plastic plant for shelter, along with nesting material consisting of a 10 x 10 x 1cm Petri dish filled with washed sand and 200, 7 cm long black polyester threads.

Five times a week, each male was presented with a gravid female for 10 min and the level of nest development and courtship behaviour was scored according to a protocol described by Rushbrook and Barber (2006) (Table 5.1). The trial continued either until the male had completed his nest (which was indicated when the male passed through during the female presentation) and was therefore counted as a 'Nester' or up to 30 d when the male was removed, and was counted as a 'Non-nester' as he had failed to complete a nest by this point.

Description						
Score	Nuptial colouration	Courtship behaviour	Nest activity			
0	No visible colouration	No response	No visible structure			
1	Blue upper eye	Occasional jar biting	Threads manipulated: separate from pile			
2	Pale blue eye	Regular bouts of jar biting, each with numerous bites	Visible nest structure: threads in pit			
3	Bright blue eye	Zig-zag dance	Completed nest with visible entrance			
4	Blue eye and red line along bottom of throat	Zig-zag and lead				
5	Blue eye and pale red throat	Zig-zag, lead and creep				
6	Blue eye and bright red throat					

Table 5.1 Score values and associated descriptions for nuptial colouration, courtship. FromRushbrook and Barber (2006)

5.2.2.2 Experimental Schistocephalus solidus infections

Prior to the experimental parasite exposures in January 2014 and 2015, male fish were identified using non-invasive DNA sampling and PCR protocols, described in Chapter 2. *S. solidus* parasites recovered from naturally-infected fish from the different populations were cultured; eggs were collected and hatched to yield infective coracidia, which were used to generate infective copepods. Male sticklebacks were then exposed to the parasite by being fed infected copepods, as described in Chapter 2. Thirty males from each stickleback populations were exposed to copepods infected with parasites from each parasite population. Unfortunately, in 2014, due to limited number of lab-bred Carsington fish, Carsington males were only exposed to Carsington parasites. No infective copepods that had been exposed to Inverleith coracidia developed infections, so no male fish were

exposed to Inverleith parasites. In 2015, only a limited number of copepods exposed to Welland coracidia developed infections, therefore only males from the Welland population were exposed to these. The final host/parasite infection combinations achieved in 2014 were CRS/CRS, CLT/CLT, CLT/CRS, INV/CLT and INV/CRS, and in 2015 were CRS/CRS, CRS/FRN, FRN/CRS, FRN/FRN, WEL/CRS, WEL/FRN, and WEL/WEL.

5.2.3 Post mortem analysis

At the end of each individual trial (after nest completion or 30 d had elapsed, whichever was sooner) fish were euthanized according to the UK Home Office approved Schedule 1 method. The fish were blotted dry and weighed, and their SL measured. The fish were then dissected, and any *S. solidus* plerocercoids were collected, blotted dry and weighed. The gonads were dissected and weighed. The kidney, liver and brain were dissected, weighed and stored in RNA*later*[®] and indices were calculated as described in Chapter 2.

5.2.4 Molecular studies

RNA extraction, cDNA synthesis and qPCR were carried out on the kidneys from males that had completed nests according to protocols described in Chapter 2. Expression levels of the *spiggin* (*SpgB*) gene were measured and the fold change, normalised to the reference gene, *rpL8* and relative to non-infected CRS males was calculated using the 2^{-} ddCT method (Livak and Schmittgen, 2001).



Figure 5.1 Schematic diagram of the nesting trials and the experimental cross-infections combination. Naturally infected wild-caught fish were initially tested in nesting trials. Plerocercoids from the wild fish were dissected and cultured for cross-infections. Lab-bred fish from the different populations were exposed to different parasite populations as indicated by the arrows and then tested in nesting trials. Carsington Water (CRS), Clatworthy Reservoir (CLT), Inverleith Park (INV), Llyn Frongoch (FRN) and River Welland (WEL).

5.2.5 Statistical Analysis

The percentage of males that completed nests and their infection status was calculated. Chi-squared tests for independence (with Yates Continuity Correction) were used to analyse association between nesting behaviour and infection status. Unpaired Student's t-tests were carried out to analyse the effect of parasite index (I_P) on nesting ability. Males were classified into three groups (non-infected nester; infected nester; infected non-nester), one-way, between-groups ANOVAs were used to analyse the impact of infection status and nesting status on the following physiological indices; brain somatic index (I_B), gonadosomatic index (I_G), kidney-somatic index (I_K) and hepatosomatic index (I_L). However, an unpaired Student's t-test was conducted for the wild populations CLT and INV in 2013 as all fish completed nests, therefore only two groups were present (non-infected or infected). Factorial ANOVAs were used to analyse the expression levels of *SpgB* of nesting males in relation to population or origin and infection status. The Tukey HSD method was then used for post-hoc testing to determine significance between groups.

5.3 Results

5.3.1 Nesting Trials

5.3.1.1 Wild populations (2013)

Nesting and infection status

The percentage of males that nested and their infection status is shown in Figure 5.2 There was a significant association between infection status and nest building among Carsington (CRS) males ($\chi^2_{1,n=20} = 4.904$, p = 0.027), with infected males often failing to nest. However, among Clatworthy (CLT) and Inverleith (INV) males, there was no association (CLT = $\chi^2_{1,n=21} = 0.397$, p = 0.529, INV = $\chi^2_{1,n=22} = 0.000$, p = 1.000), with almost all infected and non-infected fish building nests.



Figure 5.2 Nesting performance by wild caught males from Carsington Water (CRS), Clatworthy Reservoir (CLT) and Inverleith Park (INV) in the 2013 trials. * p < 0.05

Nesting behaviour in relation to parasite index (I_P) is shown in Figure 5.3. Statistical analysis could only be made for the data from the CRS fish, as all infected males from both CLT and INV completed nests. There was no significant difference in the mean I_P of nesting and non-nesting CRS males, though non-nesters tended to have a higher mean I_P, $t_7 = 1.44$, p = 0.193. This indicates that the ability to nest was not related to the size of plerocercoids males harboured. However, the low sample size of infected males reduces confidence in this conclusion.



Figure 5.3 Parasite index (I_P) and nesting status of wild caught males from Carsington Water (CRS), Clatworthy Reservoir (CLT) and Inverleith Park (INV) in the 2013 trials. Bar heights are the mean values, error bars show ± 1 SEM.

Body indices

The body indices for non-infected nesters, infected nesters and infected non-nesters are shown in Figure 5.4. No significant difference between the groups from any population for I_B , I_G or I_L was found and there was no significant difference in I_K between the groups from CLT and INV. However, among CRS males, I_K was affected by group. Infected non-nesters had lower I_K values than both non-infected and infected nesters (ANOVA results are shown in Table 5.2 and t-tests in Table 5.3, Figure 5.4).

Table 5.2 ANOVA results from comparing body indices in non-infected nesters, infected nesters and infected non-nesters from wild CRS males in 2013. Significant values (p < 0.05) are shown in bold.

Body Index	df	F	Р
I_{B}	2	1.904	0.186
I_{G}	2	0.191	0.829
I_L	2	0.116	0.891
I_K	2	7.987	0.005

Table 5.3 Student's t-test results from comparing body indices in non-infected nesters and infected nesters from wild CLT and INV males in 2013. Significant values (p < 0.05) are shown in bold.

Pop ⁿ	Body Index	df	t	Р
CLT	IB	19	-0.961	0.348
-	I _G	19	-0.599	0.556
	I_L	19	-0.169	0.867
	I_K	19	0.521	0.608
INV	I _B	19	-0.348	0.732
	I_G	19	0.480	0.637
	I_L	19	-0.211	0.835
	I_K	19	1.418	0.172



Figure 5.4 Body indices for wild caught males from Carsington Water (CRS), Clatworthy Reservoir (CLT) and Inverleith Park (INV) in the 2013 trials. Bar heights are the mean values, black bars = non-infected nesters, light grey bars = infected nesters and dark grey = infected non-nesters; error bars show ± 1 SEM and letters indicate significance at p < 0.05. A) Brain somatic index I_B B) Gonadosomatic index I_G C) Hepatosomatic index I_L D) Kidney-somatic index I_K

5.3.1.2 Wild populations (2014)

Nesting and infection status

The percentage of infected and non-infected males that nested is shown in Figure 5.5. There was significant association between infection and nest building in all three populations, with a higher proportion of infected males failing to build nests in each (CRS; $\chi^2_{1,n=20} = 10.208$, p = 0.001, FRN; $\chi^2_{1,n=21} = 13.902$, p < 0.001, WEL; $\chi^2_{1,n=21} = 11.172$, p = 0.001).



Figure 5.5 Nesting performance of wild caught males from Carsington Water (CRS), Llyn Frongoch (FRN) and River Welland (WEL) in the 2014 trials. * p < 0.05

The nesting behaviour in relation to mean parasite index (I_P) is shown in Figure 5.6 While non-nesting males from both CRS and FRN tended to have higher mean I_P, the differences were not significant (Nesters vs non-nesters; CRS; $t_{10} = 1.961$, p = 0.078, FRN; $t_9 = 1.282$, p = 0.232, WEL; $t_{10} = -1.135$, p = 0.283), indicating that the ability to nest was not related to parasite index.



Figure 5.6 Parasite index (I_P) and nesting status of wild caught males from Carsington Water (CRS), Llyn Frongoch (FRN) and River Welland (WEL) in the 2014 trials. Bar heights are the mean values, error bars show ± 1 SEM.

Body indices

The body indices for the three groups of males are shown in Figure 5.7. ANOVA results are shown in Table 5.4.

 I_G was found not to be significantly different among FRN and WEL males, but varied significantly between groups among CRS males. Infected non-nesters had a lower I_G than infected and non-infected nesting males.

Among CRS and WEL males there was no significant difference in I_L between the three groups, although there was a non-significant trend in both populations for infected nonnesters to have larger mean I_L . However, there was a statistically significant difference among the three groups from FRN. Non-infected nesting males have lower I_L than both groups of infected males.

There was no statistically significant difference in I_K for the three groups in the FRN population although there was a trend for the infected non-nesters to have a smaller I_K than the other two groups. However, in the CRS and WEL populations a significant difference was found, infected non-nesters had a lower I_K than both groups of nesting males.

Pop ⁿ	Body Index	df	F	Р
CRS	I_B	2	0.202	0.819
	I_G	2	4.791	0.022
	I_L	2	1.908	0.179
	I_K	2	36.000	< 0.001
FRN	I_B	2	0.389	0.683
	I_G	2	2.748	0.089
	I_L	2	4.873	0.020
	I_K	2	1.166	0.333
WEL	I_{B}	2	1.455	0.260
	I_G	2	0.077	0.926
	I_L	2	1.000	0.387
	I_K	2	14.781	< 0.001

Table 5.4 ANOVA results from comparing body indices in non-infected nesters, infected nesters and infected non-nesters from wild CRS, FRN and WEL males in 2014. Significant values (p < 0.05) are shown in bold.



Figure 5.7 Body indices for wild caught males from Carsington Water (CRS), Llyn Frongoch (FRN) and River Welland (WEL), in the 2014 trials. Bar heights are the mean values, black bars = non-infected nesters, light grey bars = infected nesters and dark grey bars = infected non-nesters; error bars show ± 1 SEM. Letters indicate significance at p < 0.05. A) Brain somatic index I_B B) Gonadosomatic index I_G C) Hepatosomatic index I_L D) Kidney-somatic index I_K

5.3.1.3 Experimental cross-infections of lab-bred fish (2014)

Nesting and infection status

The percentage of infected and non-infected males that nested is shown in Figure 5.8. There was a significant association between infection status and nesting among males from all populations when infected with CRS parasites. (CRS/CRS; $\chi^2_{1,n=30} = 15.880$, *p* < 0.001, CLT/CRS; $\chi^2_{1,n=30} = 7.827$, *p* = 0.005, INV/CRS; $\chi^2_{1,n=29} = 10.950$, *p* = 0.001). Whereas all non-infected males from each population nested, very few of those infected with CRS parasites did. In contrast, there was no significant association between infection status and nest building among males from populations that had been infected with CLT parasites (CLT/CLT; $\chi^2_{1,n=26} = 0.340$, *p* = 0.560, INV/CLT; $\chi^2_{1,n=29} = 2.290$, *p* = 0.130).



Figure 5.8 Nesting performance of lab-bred experimentally exposed males from Carsington Water (CRS), Clatworthy Reservoir (CLT) and Inverleith Park (INV) in the 2014 trials. * p < 0.05

Nesting behaviour of infected males in relation to their parasite index (I_P) is shown in Figure 5.9. There was no significant difference in the mean I_P of infected males that build nests and those that did not in four of the five experimental groups, despite a trend for non-nesters to have higher parasite indices (nesters vs. non-nesters; CRS/CRS; $t_{19} = 0.928$, p = 0.365; CLT/CRS; $t_{21} = 0.683$, p = 0.502, INV/CRS; $t_{21} = 2.041$, p = 0.064 INV/CLT; $t_{17} = 1.954$, p = 0.067). This indicates that in these host-parasite provenance combinations, the ability to nest is unrelated to parasite index. However, among CLT males infected with CLT parasites there was a significant difference in the mean I_P of fish that build nests and those that did not ($t_{19} = 2.808$, p = 0.011), suggesting that CLT males

infected with parasites from their own background failed to nest due to the large parasite burden.



Figure 5.9 Parasite index (I_P) and nesting status of lab-bred experimentally exposed males from Carsington Water (CRS), Clatworthy Reservoir (CLT) and Inverleith Park (INV) in the 2014 trials. Bar heights are the mean values, error bars show ± 1 SEM. * p < 0.05

Body indices

The mean body indices for the three groups of males from each of the five host-parasite combinations are shown in Figure 5.10. ANOVA results are shown in Table 5.5.

There was no significant variation in I_B between groups among the CRS/CRS males; however, I_B varied significantly between groups in all other host-parasite combinations. Among CLT/CLT and CLT/CRS males, infected non-nesters had a lower I_B than noninfected nesters. In INV/CLT infected non-nesting males had significantly lower I_B than both non-infected and infected nesters. In INV/CRS males there was a significant difference in I_B between the non-infected nesters and both groups of infected males but there was no significant difference between the infected males.

In males CLT/CLT, INV/CLT and INV/CRS I_L did not differ significantly between the non-infected and infected nesting males, however infected non-nesting males had significantly larger I_L values than both groups of nesters.

There was a statistically significant difference in I_K for the three groups in all exposure conditions. In CRS/CRS, CLT/CLT, CLT/CRS and INV/CRS males I_K did not differ significantly between the two groups of nesting males, however infected non-nesting males had significantly smaller I_K than either group of nesters. In INV/CLT males there was also a significant difference in I_K between the non-infected and infected nesters, non-infected males had the largest kidneys, whereas infected non-nesters had the lowest I_K .

Population background		Body Index	df	F	Р
Host fish	Parasite				
CRS	CRS	I_B	2	2.245	0.125
		I_G	2	1.018	0.375
		I_L	2	0.110	0.896
		I_K	2	14.341	< 0.001
CLT	CLT	I_B	2	4.312	0.026
		I_{G}	2	0.616	0.549
		I_L	2	6.700	0.005
		I_K	2	12.021	< 0.001
CLT	CRS	I_B	2	6.075	0.006
		I_G	2	0.279	0.759
		I_L	2	1.841	0.178
		I_K	2	7.313	0.003
INV	CLT	I_B	2	5.156	0.007
		I_{G}	2	0.968	0.393

 I_{L}

 I_{K}

 I_B

 I_{G}

 $I_{L} \\$

 $I_{K} \\$

INV

CRS

2

2

2

2

2

2

27.176

17.616

16.231

0.506

12.136

26.063

< 0.001

< 0.001

< 0.001

0.609

< 0.001

< 0.001

Table 5.5 ANOVA results from comparing body indices in non-infected nesters, infected nesters and infected non-nesters from cross-infections in experimentally exposed males in 2014. Significant values (p < 0.05) are shown in bold.



Figure 5.10 Body indices for lab-bred experimentally exposed males from Carsington Water (CRS), Clatworthy Reservoir (CLT) and Inverleith Park (INV), in the 2014 trials. Bar heights are the mean values, black bars = non-infected nesters, light grey bars = infected nesters and dark grey bars = infected non-nesters; error bars show ± 1 SEM letters indicate significance at p < 0.05. A) Brain somatic index I_B B) Gonadosomatic index I_G C) Hepatosomatic index I_L D) Kidney-somatic index I_K

5.3.1.4 Experimental cross-infections of lab-bred fish (2015)

Nesting and infection status

The percentage of males that nested and their infection status is shown in Figure 5.11 There was a significant association between infection and nest building when males were infected with parasites from the CRS background (CRS/CRS; $\chi^{2}_{1,n=27} = 19.673$, p < 0.001, FRN/CRS; $\chi^{2}_{1,n=28} = 18.101$, p < 0.001, WEL/CRS; $\chi^{2}_{1,n=27} = 19.368$, p < 0.001). Therefore, a greater number of males from all populations than would be expected were unable to nest when infected by *S. solidus* from Carsington Water. In the males infected with FRN and WEL parasites there was no significant association between infection and nest building (CRS/FRN; $\chi^{2}_{1,n=30} = 3.092$, p = 0.079, FRN/FRN; $\chi^{2}_{1,n=29} = 1.940$, p = 0.164, WEL/FRN; $\chi^{2}_{1,n=29} = 3.545$, p = 0.060, WEL/WEL; $\chi^{2}_{1,n=27} = 2.913$, p = 0.088).



Figure 5.11 Nesting performance of lab-bred experimentally exposed males from Carsington Water (CRS), Llyn Frongoch (FRN) and River Welland (WEL) in the 2015 trials * p < 0.05

The nesting behaviour in relation to parasite index I_P is shown in Figure 5.12. No significant difference between the mean I_P for infected males that build nests and those that didn't was found, (nesters and non-nesters; CRS/CRS; $t_{11} = 0.831$, p = 0.424, FRN/CRS; $t_5 = -0.579$, p = 0.580, WEL/CRS; $t_9 = -0.662$, p = 0.525, CRS/FRN; $t_7 = -1.626$, p = 0.130, FRN/FRN; $t_7 = -0.023$, p = 0.982, WEL/FRN; $t_{13} = -0.507$, p = 0.621, WEL/WEL; $t_{11} = -1.013$, p = 0.333). These indicate that the ability to nest is not related to parasite index.



Figure 5.12 Parasite index (IP) and nesting status of lab-bred experimentally exposed males from Carsington Water (CRS), Llyn Frongoch (FRN) and River Welland (WEL) in the 2015 trials. Bar heights are the mean values, error bars show ± 1 SEM.

Body indices

The body indices for the three groups of males are shown in Figure 5.13. ANOVA results are shown in Table 5.6.

There was a statistically significant difference in I_B between the three groups in all experimental conditions. I_B did not differ significantly between the groups of nesting males, however, the infected non-nesting males had significantly lower I_B than the two groups of nesters.

In CRS/FRN and WEL/WEL males there was a statistically significant difference in I_{G} . Infected non-nesting males had significantly larger I_{G} than either of the two groups of nesters, but there was no difference between the nesting males.

There was a statistically significant difference in I_L . In CRS/CRS and WEL/FRN males, I_L did not differ significantly between the infected nesting males and the other two groups; however there was a significant difference between the non-infected nesters and infected non-nesting males. In CRS/FRN, FRN/CRS and FRN/FRN males, infected non-nesting males had significantly larger I_L values than either of the two nesters, however I_L did not differ significantly between the nesting males. In WEL/CRS males I_L did not differ significantly between the nesting males. In WEL/CRS males I_L did not differ significantly between the two groups of infected males, however the non-infected nesting males had significant smaller I_L than the infected individuals. In WEL/WEL males there was a significant difference between all three groups of males, the non-infected nesting male have the smallest I_L and the infected non-nesting males had the largest.

In all cross-infection combinations there was a statistically significant difference in I_K between the three groups. In CRS/CRS and FRN/CRS males I_K did not differ significantly between the infected nesting males and the other two groups. The non-infected nesters had the largest kidneys and this was significantly different to the infected non-nesting males. In FRN/FRN, WEL/CRS and WEL/FRN males the infected non-nesters had significantly smaller I_K than the two groups of nesters, there was no difference between the nesters. In WEL/WEL males I_K did not differ significantly between the two groups of infected males, however there was a significant difference between non-infected nesting males and both groups of infected fish, with the non-infected nesters having a larger I_K . In the population CRS/FRN there was a significant difference between all three groups, the non-infected nesting males have the largest I_K and the infected non-nesting males had the smallest.

Table 5.6 ANOVA results from comparing body indices in non-infected nesters, infected nesters and infected non-nesters from cross-infections in experimentally exposed males in 2015. Significant values (p < 0.05) are shown in bold.

Population background		Body Index	df	F	Р
Host fish	Parasite				
CRS	CRS	I_B	2	20.295	< 0.001
		I_G	2	0.706	0.504
		I_L	2	26.641	< 0.001
		I_K	2	36.925	< 0.001
CRS	FRN	I_B	2	4.087	0.028
		I_G	2	7.103	0.003
		I_L	2	12.935	< 0.001
		I_K	2	18.523	< 0.001
FRN	CRS	I_B	2	27.696	< 0.001
		I_G	2	0.398	0.676
		I_L	2	35.344	< 0.001
		I_K	2	4.854	0.017
FRN	FRN	I_B	2	8.194	0.002
		I_G	2	1.040	0.368
		I_L	2	14.923	< 0.001
		I_K	2	7.090	0.003
WEL	CRS	I_B	2	24.112	< 0.001

		I_{G}	2	0.278	0.760
		I_L	2	15.840	< 0.001
		I_K	2	22.347	< 0.001
WEL	FRN	I_{B}	2	7.036	0.004
		I_{G}	2	0.264	0.770
		I_L	2	6.610	0.005
		I_K	2	13.762	< 0.001
WEL	WEL	I_{B}	2	3.781	0.037
		I_{G}	2	6.402	0.006
		I_{L}	2	22.692	< 0.001
		I_K	2	14.864	< 0.001



Figure 5.13 Body indices for lab-bred experimentally exposed males from Carsington Water (CRS), Llyn Frongoch (FRN) and River Welland (WEL), in the 2014 trials. Bar heights are the mean values, black bars = non-infected nesters, light grey bars = infected nesters and dark grey bars = infected non-nesters; error bars show ± 1 SEM. Letters indicate significance at p < 0.05. A) Brain somatic index I_B B) Gonadosomatic index I_G C) Hepatosomatic index I_L D) Kidney-somatic index I_K
5.3.2 Spiggin expression in nesting males and effects of parasite infection

Spiggin (*SpgB*) gene expression profiles were analysed in both non-infected and infected males that had completed nests.

5.3.2.1 Wild populations (2013 and 2014)

Expression levels from the two CRS samples from both 2013 and 2014 were compared and no differences were found so data for CRS were combined and all populations from both years were analysed together. There was a significant main effect of population and of infection status on *SpgB* expression, however there was no interaction between the two (population; $F_{4,70} = 2.684$, p = 0.038; infection; $F_{1,70} = 4.021$, p = 0.049; interaction; $F_{4,70}$ = 0.962, p = 0.434, Figure 5.14). Non-infected FRN males had lower *SpgB* expression than males from the other four populations – CRS, CLT, INV and WEL – which had similar levels of expression to one another. Infected males from CRS, CLT and INV had significantly lower expression levels compared to their non-infected counterparts. Infected WEL males also tended to have lower gene expression, however this was not significant. Infected FRN males had similar low *SpgB* expression levels compared to noninfected FRN males.



Figure 5.14 Relative expression levels of *SpgB*, normalised to the reference gene, *rpL8* and relative to non-infected CRS males (1) in nesting males from the wild population trials; Carsington Water (CRS), Clatworthy Reservoir (CLT), Inverleith Park (INV), Llyn Frong Frongoch (FRN) and River Welland (WEL). Bar heights are the mean values, black bars = non-infected nesters, light grey bars = infected nesters; error bars show ± 1 SEM and * indicate significance at p < 0.05

5.3.2.2 Lab-bred experimental cross-infection 2014

SpgB expression did not vary between fish populations in the 2014 cross-infection experiment, however there were significant differences between the males harbouring

parasites from different populations. There was no significant interaction between fish population and parasite population meaning that the effect of infections for each parasite population was uniform across fish populations, (fish population; $F_{2,60} = 0.058$, p = 0.944; parasite population; $F_{2,60} = 3.434$, p = 0.040; interaction; $F_{3,60} = 0.040$, p = 0.989, Figure 5.15). Pairwise comparisons showed that expression did not vary between non-infected males. However males from all populations harbouring CRS parasites had significantly lower expression levels of *SpgB* than the non-infected fish. Although males harbouring CLT parasites tended to have lower expression compared to the non-infected fish, this was found not to be significant.



Figure 5.15 Relative expression levels of *SpgB*, normalised to the reference gene, *rpL8* and relative to non-infected CRS males (1) in nesting males from the 2014 lab-bred cross-infection trials; Carsington Water (CRS), Clatworthy Reservoir (CLT) and Inverleith Park (INV). Bar heights are the mean values, black bars = non-infected nesters, light grey bars = CRS parasite infected nesters, dark grey bars = CLT parasite infected nesters; error bars show ±1 SEM and letters indicate significance at p < 0.05

5.3.2.3 Lab-bred experimental cross-infection 2015

Similar results were observed in the 2015, with no significant variation of *SpgB* expression between the fish populations. Furthermore parasite background again gave significantly different expression levels, although there was no interaction between fish population and parasite population (fish population; $F_{2,69} = 0.071$, p = 0.932; parasite population; $F_{3,69} = 4.719$, p = 0.005; interaction; $F_{4,69} = 0.472$, p = 0.756, Figure 5.16). As with the 2014 trials, pairwise comparisons showed that all non-infected males had similar levels of expression. This was an altered pattern compared to the wild fish in which FRN males had lower expression than other populations. Again males from all populations

harbouring CRS parasites had significantly lower expression levels of *SpgB* than the noninfected fish. No significant difference in expression was observed in males infected with FRN parasites, and although the fish harbouring WEL parasites had lower expression this was not significant.



Figure 5.16 Relative expression levels of *SpgB*, normalised to the reference gene, *rpL8* and relative to non-infected CRS males (1) in nesting males from the 2014 lab-bred cross-infection trials; Carsington Water (CRS), Llyn Frongoch (FRN) and River Welland (WEL). Bar heights are the mean values. Black bars = non-infected nesters, light grey bars = CRS parasite infected nesters, dark grey bars = FRN parasite infected nesters, horizontal striped bars = WEL parasite infected nesters; error bars show ±1 SEM and letters indicate significance at p < 0.05

5.4 Discussion

In this study the nesting behaviour of both wild-caught naturally-infected, and lab-bred experimentally-infected male sticklebacks were assessed to identify the role of environmental conditions and host/parasite provenance on the reproductive phenotype of *S. solidus* infected sticklebacks. Wild caught and lab-bred non-infected males from all fish populations successfully completed nests. However, across all studies, both wild-caught and lab-bred males infected with plerocercoids from the Carsington Water (CRS) population failed to nest. This was not due to CRS plerocercoids growing more quickly or achieving a larger size in host fish, since males infected with larger plerocercoids from other parasite populations frequently built nests in the studies. In the experimental infection study, all fish were housed in similar conditions may play a less important role than the parasite population differences in determining the outcome of infections. The smaller parasite sizes achieved by CRS parasites could also suggest that nutrient theft is

unlikely to be the main mechanism for the lack of reproductive development. The results of these studies suggests that parasite, rather than host provenance, appears to be the main factors determining reproductive phenotype of the male stickleback hosts.

5.4.1 Nesting behaviour of wild males in favourable laboratory conditions

Naturally infected males from the five stickleback populations varied in nesting behaviour. Infected males from CRS, FRN and WEL typically did not build nests, whereas infected males from CLT and INV often completed nests. These results were consistent across the two years of the study and also with previous studies - infected males from FRN where found not to nest by Rushbrook and Barber (2006), whereas Tierney et al. (1996) observed that INV males successfully build nests even when infected - suggesting long-term consistency in reproductive phenotypes of infected fish in these populations. As nest building is energetically costly, the ability to nest could simply be due to the resources available to the fish and the level of infection could influence this. Larger parasites would place a greater energy demand on their host, so failure to nest might be expected to reflect the size of plerocercoid. However, although fish that failed to nest tended to have larger parasites, there was no significant difference found in the parasite index (I_P) of nesting males compared to those that failed to nest. Infection status and nesting behaviour had limited effects on the body indices of the fish and similar patterns between the three groups of males were seen across populations. Although in the 2014 study CRS infected non-nesters had smaller gonads and FRN infected fish had larger livers irrespective of nesting status.

Infected males that failed to build nests, across populations, had significantly smaller kidneys than nesting males, whereas infected males that nested had similarly-sized kidneys to non-infected fish. From the wild studies it has been shown that the reproductive behaviour and development of infected males differs between host populations, and that ability to nest when infected is unlikely to be a lab artefact as suggest by Candolin and Voigt (2001) since all host populations experienced similar favourable conditions prior to the nesting trials. Similar results were found among wild-caught males from two different UK populations that were kept in favourable conditions prior to the breeding season (Macnab et al., 2009). However, studies of wild-caught fish are unable to answer fully the cause for this variation, as ecological conditions within the natural habitats can have a significant impact on patterns of early growth and development in advance of

sampling. Key periods of sexual development at gene expression level occur at early stages, as has been shown in Chapter 3, yet none of the published studies on wild-caught fish have provided fish with favourable conditions at these time points in the development cycle. Genetic differences between populations of either host or parasite could allow for host adaptation or parasite manipulation to occur, and may contribute to the variation in reproductive phenotypes observed (Macnab et al., 2009). The experimental cross-infections in lab-bred fish that followed on from wild population studies therefore have the potential to provide further insights into this.

5.4.2 Nesting behaviour of lab-bred males with experimental cross-infections

As with the wild-caught males, all non-infected lab-bred males nested successfully, therefore the laboratory rearing conditions did not impact negatively on the natural behaviour of male fish. The nesting behaviour of infected males depended on the provenance of the parasites they harboured. Whereas infected males from all host populations that harboured CRS parasites typically failed to build nests, those infected with either CLT, FRN or WEL parasites often completed nests. Since wild-caught, naturally infected males from FRN and WEL populations rarely nested, this may indicate that environmental conditions in natural habitats play an important role in the host's reproductive phenotype. Llyn Frongoch (FRN) is known to be highly oligotrophic (Hanlon, 1982) and fish growth rates from this reservoir are slower compared to other more nutritionally rich sites (Allen and Wootton, 1982a), perhaps suggesting that resource availability at early stages of parasite infection leads to a detrimental effect on host reproductive development later in the year. Favourable laboratory conditions during early life may allow fish from these populations to overcome detrimental effects of infection, as sufficient resources for sustaining both parasite growth and sexual development are available (Candolin and Voigt, 2001).

Therefore, nutrient theft by the parasite at an early stage might prevent infected males nesting. Fish harbouring larger plerocercoids might also be expected to be more nutritionally stressed and less likely to build nests. However, in almost all host-parasite combinations there was no significant difference in the I_P of infected nesters compared to non-nesters, with the exception being non-nesting infected CLT males with CLT parasites which had significantly larger I_P than those that nested. In the case of infections involving CRS parasites, despite ending the experiment with smaller infections than those involving other parasite populations, CRS-infected males typically failed to nest. These results could indicate that parasites from CRS have developed a strategy of manipulation to prevent their host fish from maturing and building nests that is independent of nutrient theft.

Similar effects of infection on body indices were seen, regardless of the host or parasite provenance. In contrast to the situation in wild-caught males, infected non-nesters were found to have smaller brain sizes than either group of nesters. The reasons for this is unclear, but could be an indication that due to underdevelopment the brain size is restricted and would lead to lack of future parental care (Samuk et al., 2014). Liver size was also affected by nesting status in lab-reared fish but not wild fish. Infected non-nesters had significantly larger livers than either of the nesting males, suggesting energy has been diverted away from sexual development and hypertrophy of kidneys, and excess resources that had not been utilized by the growing plerocercoids had been stored in the liver. As in wild-caught fish, lab-bred infected non-nesters had significantly smaller kidneys than the nesting males. This indicates that these males might be unable to construct nests, as their Spiggin production would be limited, since kidney size is known to be positively correlated with levels of Spiggin (Katsiadaki et al., 2002).

5.4.3 Spiggin expression in nesting males

An essential component of stickleback nest construction is the Spiggin glue produced in the male kidneys (Wootton, 1976, Jakobsson et al., 1999) the proteins of which are encoded by multigene families (Jones et al., 2001, Seear et al., 2015). As suggested by Rushbrook et al. (2007), although infected males may undertake nest building, the quality may be poor compared to non-infected males and could be avoided by females. For example, infection may reduce the male's capability to produce Spiggin, making the nest structure weak. The expression levels of genes encoding *spiggin* can be influenced by environmental conditions as shown by Seear et al. (2014) who found that flowing water caused an upregulation of the mucin-like *SpgB*, whereas *SpgC* was unaffected. This suggests that nesting glues with different functional properties may be produced by varying expression of the different genes, potentially allowing males to respond to changes in environmental conditions by altering the structural properties of the protein (Seear et al., 2015). Parasite infections also place additional energy demands on their host fish which may prioritise which form of Spiggin is produced. Alternatively manipulation could alter gene expression levels, thus changing the type- and amount of Spiggin produced and potentially the quality of the nest built.

In the wild populations, *SpgB* expression in non-infected males varied among populations, with FRN males having significantly lower expression levels than the other four populations. Among CRS, CLT and INV males, infected nesters had significantly lower expression than their non-infected counterparts, whereas infected FRN and WEL nesters had similar levels to non-infected nesting males. Infected WEL fish had significantly higher expression levels than infected fish from other populations, possibly suggesting that infected males from CRS, CLT and INV construct less structurally sound nests compared to the non-infected fish. Both infected and non-infected FRN males may also have built poorer quality nests with lower levels of Spiggin. WEL fish were caught from a flowing river; therefore *SpgB* may be preferentially expressed over the other types of *spiggin* even in infected males, as flowing habitats cause upregulation of *SpgB* but not *SpgA* or *SpgC* (Seear et al., 2014).

However, among lab-bred fish, all non-infected males had similar expression levels – including those from FRN – providing further evidence of the importance of early nutrition in determining reproductive potential for male fish, and the subsequent impact of infection. As shown in Chapter 4, it was demonstrated that reduced food levels before the breeding season led to lower levels of *SpgB* expression.

The parasite population also had a significant effect on *SpgB* expression. All males infected with CRS parasites, regardless of host population, had significantly lower expression levels, whereas infections by parasites from the other populations had no significant effect on *SpgB* expression levels in any population of fish. Therefore, even if males infected with CRS parasites may occasionally be capable of constructing nests, these nests may not be of as high a quality as those of non-infected fish or of those infected with parasites from other populations. However, in the nesting trials females were not released into the aquaria, so no information on the likelihood that nests would be chosen by the female fish is available.

5.5 Conclusions

The results are consistent with the hypothesis that *S. solidus* provenance, and possibly genotype, influences the reproductive development and behaviour of stickleback hosts. A

number of mechanisms may be responsible for the lack of sexual development and reproductive phenotype of the male fish. Nutrient theft may lead to infected males failing to construct nests in the CLT and FRN populations, whereas parasites from the CRS population may have developed manipulative mechanisms to prevent their host fish from developing.

5.6 Limitations and further work

There are a number of limitations to this study, which could be investigated further to answer the questions more fully. One limitation is that the full cross experimental infections were unable to be made, due to either limited fish numbers or unsuccessful culturing of infected copepods. A further limitation is that males were only scored as nesters based on the male behaviour of passing through their finished nest, and no element of female choice was investigated. So although nests had been completed there was no indication of the quality of the construction, or whether the males would be able to successfully raise young. 6 Patterns of genetic variation within and between *Schistocephalus* parasites in relation to their infection phenotypes

6.1 Introduction

Parasitism first originated several million years ago and has evolved independently many times since, such that extant parasitic taxa are taxonomically highly diverse (Poulin, 2007), with an enormous range of species and life cycles (Matthews, 1998, Deaton, 2011). The remarkable diversity in life history strategies, transmission modes and ecological structures drive the genetic dynamics within and between populations. Therefore genetic diversity both between and within parasite species is likely to be high (Barrett et al., 2008).

Many host-parasite interactions are characterised by being highly specific to the species involved, which can lead to coevolution and genetic variation within natural populations maintained by the cycling of allele frequencies (Parker et al., 2017). Arms races between hosts and parasites can generate extreme polymorphism in natural populations, and genetic systems control both the specific and non-specific host responses to parasite diversity resulting in a continual selection by a wide variety of parasite strains (Anderson and May, 1982).

Obligate parasites specialised to a single host species are more likely to experience frequent local extinctions and recolonization events than generalists, particularly in small fragmented host populations. Loss of genetic diversity within parasite populations may occur among such population processes and generate among population genetic differences through genetic drift (Barrett et al., 2008).

Local adaptations within parasites is predicted to be due to strong selective pressures within their host populations (Grant, 1994). High levels of gene flow are likely to homogenise populations, whereas low to intermediate gene flow can promote local adaptation as selection pressures can act on genetic variation of individuals. When populations of parasites are significantly more harmful to their hosts than neighbouring populations, it is expected that local adaptations are likely to be more pronounced. As the parasites place stronger selection pressure on their hosts to change and cyclic responses in turn occur within the parasites, population genetic divergences across sites will occur (Greischar and Koskella, 2007, Poisot, 2015).

Local interactions will most likely result in different reciprocal selection pressures on the host and the parasite leading to regional differentiation (Poisot, 2015). Reciprocal natural

selection on host resistance and parasite infectivity leads to coevolution. If interactions within populations are characterised by the genotype-specific resistance within hosts and the infectivity levels in parasites then the greatest arms races are likely to occur, as studies using the *Daphnia magna-Pasteuria ramosa* associations showed (Ebert et al. (1998). Low levels of among population variations existed relative to the strong genetic variation for resistance present within populations suggesting that local interactions are dominant. Selective outcomes and the ability to manipulate hosts are dependent on parasite gene frequency. Coevolution is likely to be oscillatory, interactions within the local species will be key to changes in coevolution and they will not be dependent upon neighbouring populations. However gene flow can introduce new virulence genes into local populations (Carius et al., 2001).

Parasites can evolve local adaptations when they have higher gene flow than their hosts as beneficial genes can be introduced (Greischar and Koskella, 2007). Intermediate fish hosts in both the *Ligula* and *Schistocephalus* systems will be constrained to the lake and river system they are located in, therefore the gene flow within fish will be low. However the final host in the system, birds, are capable of flying great distances potentially allowing the parasites to mix over larger areas and populations. Thus mixing of parasite genes is likely to be greater than mixing of stickleback genes, allowing parasites to adapt to differences within local hosts (Sprehn et al., 2015).

S. solidus shows high levels of host specificity at the fish host stage and is only capable of successfully infecting three-spined sticklebacks *Gasterosteus aculeatus*. They fail to establish lasting infections in nine-spined sticklebacks *Pungitius pungitius* following experimental challenge (Henrich et al., 2013). *S. solidus* is also genetically distinct, yet morphologically almost identical, to two other species; *S. cotti* which infects bullheads *Cottus gobio* (Chubb et al., 2006) and *S. pungitiii* which infects nine-spined sticklebacks (Nishimura et al., 2011). Differences in infection prevalence among host populations could result from differences in parasite specificity and genotype. Weber et al. (2017) found that when the fish host genotype was kept constant European and Canadian genotypes of *S. solidus* differed significantly in their infection success, indicating that genetic variation within the parasites influences the outcomes within the hosts.

S. solidus parasites have significant effects on their intermediate stickleback host, particularly on reducing the reproductive behaviour of the fish (Arme and Owen, 1967,

McPhail and Peacock, 1983). However the effects are variable across populations. In some cases infected fish participate in normal reproductive behaviour (McPhail and Peacock, 1983, Heins et al., 1999), whereas in other populations reproduction is limited and sexual maturation is prevented (Rushbrook and Barber, 2006). Macnab et al. (2009) suggested that a difference in the co-evolutionary association time may lead to the differing reproductive potential of *S. solidus* infected male three-spined sticklebacks observed in two UK populations. Populations exposed to parasites for longer periods may be better adapted to cope with the infections. Kendoon Loch (KL) was built in 1935, whereas Victoria Park Pond (VP) was constructed in the 1990s. In their study, males from KL successfully completed nest building, whereas VP males failed to do this. However the possibility that the host's ability to overcome negative effects of infection is due to genetic differences between parasite populations means that variation in infected host phenotypes related to parasite genotype cannot be ruled out (Macnab et al., 2009).

The reasons for the observed differences between populations could be due to a number of mechanisms; nutrient theft, parasite manipulation or host adaptation. Different mechanisms may also be occurring across populations as the results from studies in Chapter 5 suggest. In some populations (Llyn Frongoch and River Welland) infected fish in natural conditions fail to nest, which could be due to limited resources and nutrient theft, as lab bred infected fish from these populations, with unlimited access to food successfully completed nest building. Parasites from these populations had similar effects on their host fish regardless of the host background they were developing in. However Carsington (CRS) parasites prevented males of all host backgrounds from nesting even with unlimited resources, suggesting that CRS parasites may be capable of preventing three-spined sticklebacks from developing by manipulation for their own gain.

It is also possible that a speciation event had taken place and that the CRS parasites are a unique *Schistocephalus* species, and be genetically different to other populations. Spectiation has taken place within the *Schistocephalus* with the separation of *S. solidus* and *S. pungitii* occurring relatively recently (20 mybp) (Nishimura et al., 2011). Alternatively they could be genetically similar to other populations, but local selection pressures may favour particular alleles that give the parasite an advantage and have evolved to be able to carry out manipulations.

The process of host-parasite coevolution often means that a host speciation event is followed by a parasite speciation event, particularly if parasite dispersal is low. Any barriers between the sub-populations will prevent gene flow occurring, therefore both host and parasite will co-speciate. If a manipulative allele appears in the population and gives individuals an advantage this allele is likely to spread due to selective pressures. (Poulin, 2007, Poisot, 2015).

Genetic structure (the pattern of the genetic diversity across multiple populations) is determined by various evolutionary forces interacting together. At the molecular level, different populations of a single species can differ in regions of their genomes that are either under strong selection or experience selective neutrality (different alleles of a gene confer equal fitness). Novel alleles are created when mutations occur in the parasite's DNA; if the novel allele then produces some benefit to the organism, positive selection may increase the frequency of the mutant allele across the population. Genetically distinct assemblages could develop under these conditions; however, the extent to which this occurs will depend on the level of gene flow between adjacent populations. Where populations are isolated and limited gene flow takes place, genetic differentiation can rapidly occur (Poulin, 2007, Poisot, 2015).

Evolutionary arms races and trade-offs between hosts and parasites occur as each competes to gain resources or eliminate infections. The interacting life histories collectively determine disease epidemiology and the evolutionary trajectories of parasite populations which can generate genetic diversity. This can not only occur between closely related species of parasite, but also within in a species that has many isolated populations, leading to genetic variation in parasite strains of the same species. Differences can be reflected in the differing effects on hosts between strains of parasites (Barrett et al., 2008, Hawlena and Ben-Ami, 2015).

The winning strain will not only depend on the level of virulence, but also the ability to successfully transmit to the next host in their life cycle (Anderson and May, 1982). Advances in genotyping methods have revealed that hosts can be infected with more than one genotype of the same parasite species and these differing genotypes can have varying effects on the host (Read and Taylor, 2001).

Genetic variation among hosts for resistance to parasites, and among parasites for virulence within hosts, are an important assumption underlying co-evolutionary theory

for host-parasite evolution (Ebert et al., 1998). However, variation in host-parasite genomes does not begin and end at the susceptibility/infectivity loci; other genes may also influence the outcome of the interactions, by altering life-history patterns and leading to a variety of outcomes. Differences within the effects of the host-parasite interactions indicates intraspecific and genetic variation present in both parasites and host populations (Minchella, 1985).

Genetic diversity between strains within parasite species can be seen at the finer scale when comparing specificity. When different populations of the same parasite species exploit different populations of the same host species divergence can occur leading to greater host specificity and genetic diversity across parasite strains (Poulin, 2007).

In summary, genetic variation between parasites could influence changes in host phenotype, therefore identifying such variation within the genetic makeup of parasites would be useful to gain understanding of genes that may code for manipulative strategies by the parasite.

6.1.1 Aims

The aim of this chapter was to investigate the genetic diversity of *S. solidus* parasites from the different UK populations studied in Chapter 5. It aimed to establish whether all parasites were from the same species as there is a possibility that CRS parasites are from a different species of *Schistocephalus* given their significantly different effect on host fish (Chapter 5). Significant variation between allele numbers and richness of microsatellite markers would indicate speciation. Once this has been established the level of genetic differentiation can be assessed to determine how much variation exists between populations and whether this can be linked to host phenotype. CRS parasites may vary genetically and could have evolved a manipulation strategy that other populations have not. Ongoing population genetics processes such as gene flow would be determined to assess the level of isolation and past demographic events. This would be achieved by analysing mitochondrial DNA (mtDNA).

6.2 Methods

6.2.1 Schistocephalus solidus specimen collection

S. solidus plerocercoids were dissected from naturally infected wild caught fish from four UK populations; Clatworthy Reservoir (CLT), Carsington Water (CRS), Llyn Frongoch (FRN), and River Welland (WEL). Eight parasites from each population were singly cultured and experimental infections of copepods were made as described in Chapter 2. Male sticklebacks were then exposed to infected copepods. Four fish for each parasite and each population were exposed (32 fish altogether). The exposed fish were then used for the nesting trials in Chapter 5. At the end of the trials plerocercoids were dissected from the body cavity of male stickleback. Plerocercoids were blotted dry and weighed before being preserved in 1 ml of 100% ethanol prior to DNA extraction. Twenty four individual plerocercoids from the four fish population backgrounds were collected represented by three individual from each of the eight parent plerocercoids.

6.2.2 Experimental procedure

6.2.2.1 Genomic DNA extraction

Genomic DNA from the 96 plerocercoids was extracted using an in-house method, modified from an isopropanol precipitation protocol adapted from Sambrook and Russell (2001), as described in Chapter 2. The concentration and purity of DNA were quantified using a NanoDrop 2000 spectrophotometer (LabTech International).

6.2.2.2 Mitochondrial DNA (mtDNA) sequence data collection and analysis

Polymerase chain reactions (PCRs) were performed to amplify 530 base pairs (bp) of the mitochondrial *cytochrome oxidase 1* gene (*CO1*) from 96 individuals. Using primers Ss CO1 F1 (CCCCGATTAAAAGCACTGAG) and Ss CO1 R1

(AGCAGCCAGTAAAAGCCAAA) based upon partial CDS for *S. solidus* CO1 (GenBank: KP864727.1). PCR amplifications were carried out in a 25 μ l reaction containing 12.5 μ l Red Taq® ReadyMixTM (Sigma-Aldrich, UK), 1.25 μ l 10 mM Forward Primer, 1.25 μ l 10 mM Reverse Primer, 7 μ l ddH₂0, and 3 μ l DNA template. The reaction conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. To check for amplification, 5 μ l of the resulting PCR products were visualised on a 2% (w/v) agarose gel. Having confirmed that PCR products had been amplified for all 96 samples, the

remaining 20 µl of the PCR were purified using isopropanol precipitation prior to Sanger sequencing by GATC Biotech (Konstanz, Germany).

Nucleotide sequences were processed in Geneious Pro v9.1.8, (Kearse et al., 2012). Low quality sequence was trimmed leaving a high quality sequence of 510 bp for each individual. BLASTN (Altschul et al., 1990) was then used to search the NCBI nonredundant (nr) database for confirmation that the obtained sequences matched the *S. solidus CO1*. DNAsp v5.10.1 (Librado and Rozas, 2009) and Arlequin v 3.5 (Excoffier et al., 2005) were used to estimate the total number of haplotypes (linked single-nucleotide polymorphism (SNP) alleles that tend to occur together), haplotype diversity (the probability that two randomly sampled alleles are different), pairwise differences among haplotypes, nucleotide diversity among haplotypes, polymorphic sites and total number of mutations for each population. A phylogenetic gene tree was constructed from the 96 sequences to show how the populations clustered, using Geneious Pro v9.1.8 (Kearse et al., 2012). The following model options in the Geneious Tree Builder were selected; 1) global alignment with free end gaps, 2) cost matrix 65% similarity (5.0/04.0), 3) Jukes-Cantor genetic distance model, 4) Neighbor-Joining tree building method with no outgroup.

6.2.2.3 Microsatellite data collection and analysis

The 96 individual plerocercoids (24 from each population) were genotyped at 18 nuclear microsatellite loci; Scso1, Scso4, Scso7, Scso9, Scso18, Scso19, Scso22, Scso24, Scso29, Scso33, Scso34, Scso35, Scso36, Scso37, SsCAB6, SsCA25, SsCTA22, SsCTB24 (Binz et al., 2000, Samonte-Padilla and Kalbe, 2012, Sprehn et al., 2015). Forward primers were labelled with HEX or FAM fluorescent dyes, primer sequences, dye combinations and annealing temperatures are shown in Table 6.1. PCR amplifications were carried out in a 10 μ l reaction containing 1 μ l 10x PCR reaction buffer, 0.5 μ l 10 mM dNTP's, 0.3 μ l 50 mM MgCl₂, 0.15 μ l 10 mM forward primer, 0.15 μ l 10 mM reverse primer, 0.05 μ l Taq DNA polymerase, 5.85 μ l ddH₂0 and 2 μ l DNA template. The reaction conditions were as follows: 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing temperature for primers (Table 6.1) for 1 min 30 s, and 72°C for 1 min, with a final extension of 60°C for 10 min. Following amplification the samples were prepared for fragment analysis in a 10 μ l reaction mix consisting of 9 μ l Hi-DiTM formamide/GeneScanTM 500 LIZ® size standard mastermix (35 μ l 500 LIZ was mixed into 1 ml of formamide) and 1 μ l of

combined PCR products for multiplex reactions. Fragment analysis was performed in a 3730 DNA Analyzer (Applied Biosystems).

Allele genotyping was scored using Geneious Pro v9.1.8, (Kearse et al., 2012). Observed (H_0) and expected (H_E) heterozygosities, and effective number of alleles (the number of equally frequent alleles that would take to achieve the same expected heterozygosity) were calculated and tests for Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) were estimated using GENEPOP on the web version 4.2. Bonferroni corrections were applied to account for multiple comparisons in HWE and linkage disequilibrium tests. Allele richness (mean number of alleles per locus) was calculated using FSTAT.

6.2.3 Statistical analysis

One way ANOVAs were used to analyse the numbers of alleles and allele richness across between the four populations of parasites. A one sample t-test was used to analyse F_{IS} values compared to zero.

Table 6.1 Microsat	ellite loci I	PCR details
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Locus	GenBank	Primer sequence	Fluorescent	Annealing	Allele length	Repeat
	Accession no.		Label	T°C	(bp)	Structure
Scso1	JQ619705	F: CAGGGGACATCACCGTCGTAC	FAM	50°C	107-140	(CA) ₁₇
		R: TCGGATGAGTTGTGAATTGAC				
Scso4	JQ619707	F: CACCGCTGTCTCTCCAACGAC	FAM	53°C	88-109	(CAC) ₁₃
		R: GCATTGTTGGGCGGAGATAGC				
Scso7	JQ619706	F: TGATCTCCAACGCCAGGAGTC	HEX	50°C	104-121	(CAG) _{10.3}
		R: CACGTGGTGGTACTCCGACTG				
Scso9	JQ619714	F: AGCTGCTCATCAGCAAGATCG	FAM	53°C	122-137	(CAG) ₁₇
		R: GAATCCCAGTGTACAGCCGAG				
Scso18	JQ619711	F: ATAGTGTTCTCACCAGTGTGC	FAM	53°C	142-157	(ACC) _{8.7}
		R: TCAGCAGGGAGGTTCATTGTC				
Scso19	JQ619718	F: TTGCCATCCCACCAGTCAGAC	HEX	53°C	151-183	$(ACA)_{20}$
		R: TCAGCAGGGAGGTTCATTGTC				
Scso22	JQ619712	F: TGAGCCGAGTCTCTGGCTCAG	HEX	50°C	107-146	$(CA)_{24.5}$
		R: GAGCCGAAAATAGAGCTAGGC				
Scso24	JQ619713	F: CTCTCTTATTCATTCCACGTG	HEX	53°C	143-163	(AGG) _{9.7}
		R: GTTCAGAATCAAGGCCTTCAG				
Scso29	JQ619710	F: GTCGCAGAAGAATGTAAGGTC	HEX	53°C	92-101	$(CAC)_{8.3}$
		R: TTTCGGGTTCTACCCAGAGGC				
Scso33	JQ619709	F: TCGCAGTGGACCAGTGTTCAG	HEX	50°C	107-140	(GCA) ₉
		R: ACTGTTGTCCCGAGAATCCTG				
Scso34	JQ619715	F: ATCTGTCAGGCTTCCTCCAGC	FAM	50°C	119-135	$(GAG)_{12.7}$
		R: GCGTTCTCTCGTGCCTACCC				
Scso35	JQ619716	F: ATCCTTATATTGTAACGACTC	FAM	53°C	98-114	$(TCC)_{8.3}$
		R: TGTGCAACAATGGGTAGGGAC				
Scso36	JQ619717	F: CACCTGAAACACTGGAAGACC	FAM	55°C	75-88	(TTG) _{8.3}
~		R: GCTTATCATCATCTTGGCTTG				
Scso37	JQ619708	F: AACCTGGCGACAACAACAGTG	HEX	53°C	127-137	(TGA) _{26.3}
		R: GCITATCATCATCTTGGCTTG			0.4.4.0	
SsCAB6	AF247829	F:GTTGGTGATGGTCGAGAAAG	FAM	57°C	96-130	$(CA)_{11}$

		R:GAATGTGTGATTTTCAGGGAAC				
SsCA25	AF247832	F:CGCAATAAGGTTGGATCGTC	FAM	57°C	132–194	$(CA)_{19}$
		R:TAAATCACGCGCAAGTCATC				
SsCTA22	AF247830	F:TGATCCCAACCCTACTGCTG	FAM	57°C	146–160	$(CT)_{14}$
		R:GCACAAGTCACCGTCCCTG				
SsCTB24	AF247831	F:ACGCAGTCCGAGTTATACCG	FAM	57°C	162–196	$(CT)_{21}$
		R:CGGTGGTCTGATTGTGAATG				

6.3 Results

6.3.1 Microsatellite genetic diversity and differentiation

Inter-individual genetic diversity using microsatellite-based measures was found to be similar across all four parasite populations. Across parasite populations, the total observed heterozygosity was consistent (Table 6.2), ranging from 0.526 (CRS) to 0.659 (WEL). The mean number of alleles over 18 loci, effective allele number and allele richness were similar across populations; ANOVA analysis showed were no significant differences between populations (Allele numbers: $F_{3,68} = 0.186$, p = 0.906; Effective alleles: $F_{3,68} = 0.426$, p = 0.735; Allele richness: $F_{3,68} = 0.119$, p = 0.949; Table 6.2, Figure 6.1). Little variation in these values indicate that parasites from all populations are from the same species. Two loci, Scso36 and Scso37, contained null alleles (null alleles occur when mutations in the binding site of the targeted DNA sequence prevent the efficient annealing of at least one primer resulting in failure of amplification (Rico et al., 2017)). CRS parasites had the greatest level of null alleles present within these loci having no heterozygotes (Table 6.3). This could indicate that greater mutation rates are occurring within CRS parasites. An analysis of linkage disequilibrium (LD) showed non-significant differences, indicating no significant linkage between loci. Therefore genotypes at one locus are independent of genotypes at the other locus.

Table 6.2 Microsatellite diversity across four populations over 18 loci. Population backgrounds
are shown; Carsington Water (CRS), Clatworthy Reservoir (CLT), Llyn Frongoch (FRN) and
River Welland (WEL)

ed Expect ygosity hetero (H _E)	ted Avera zygosity alleles loci	ge no. of Effecti over 18 no. ove	ive allele Allele er 18 loci richness
0.708	6.611	4.432	5.355
0.749	6.056	4.369	5.248
0.699	6.056	3.976	5.102
0.675	5.944	3.835	4.995
-	ed Expect ygosity hetero (H _E) 0.708 0.749 0.699 0.675	edExpectedAveragesygosityheterozygosityalleles (\mathbf{H}_E) loci0.7086.6110.7496.0560.6996.0560.6755.944	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$



Figure 6.1 Boxplots showing allele information from four populations of *Schistocephalus solidus* parasites. A) Average number of alleles, B) Effective allele numbers, C) Allele richness. The median is indicated by the line across the box, the box extends from the 25th to 75th percentiles and the whiskers represent the maximum and minimum value. Population backgrounds are shown; Carsington Water (CRS), Clatworthy Reservoir (CLT), Llyn Frongoch (FRN) and River Welland (WEL)

The majority of loci were found to be in Hardy-Weinberg Equilibrium (HWE), with only a few instances of departure from HWE detected (Table 6.3), although F_{IS} values were significantly different to zero ($t_{17} = 3.668$, p = 0.002). Most of the values were found to be positive, indicating that individuals within populations were more related than would be expected under normal random mating, perhaps reflecting the fact that the parasites in the study were generated by selfing. However, allele richness was not significantly different between populations, indicating that all parent worms contributed equally to the offspring (Table 6.3, Figure 6.1).

Table 6.3 Estimates of Hardy-Weinberg Equilibrium H_E : expected heterozygosity; H_O : observed heterozygosity; F_{IS} : inbreeding coefficient. Bold H_O values indicate significant departures from Hardy-Weinberg equilibrium with Bonferroni correction (p < 0.00012). Departures from HWE and positive F_{IS} values significantly different to zero indicate individuals are more related than would be expected under normal random mating. Population backgrounds are shown; Carsington Water (CRS), Clatworthy Reservoir (CLT), Llyn Frongoch (FRN) and River Welland (WEL)

Locus/ Pop ⁿ .	Estimates of HWE	CRS	CLT	FRN	WEL
<u> </u>	H_{E}	0.858	0.747	0.760	0.795
Scso1	Ho	0.870	0.625	0.958	1.000
	F _{IS}	0.010	0.183	-0.054	-0.088
Saga	$H_{\rm E}$	0.588	0.676	0.278	0.414
SCS04	Ho	0.458	0.417	0.250	0.458
	F _{IS}	0.189	0.218	-0.040	-0.086
Seco7	$H_{\rm E}$	0.611	0.712	0.753	0.713
50507	Ho	0.583	0.391	0.304	0.417
	F _{IS}	0.067	0.223	0.147	0.375
Sacol	H_{E}	0.696	0.766	0.737	0.744
50509	Ho	0.917	0.750	0.833	1.000
	FIS	-0.176	0.062	-0.098	-0.123
Seco18	H_{E}	0.780	0.821	0.802	0.635
505010	Ho	0.625	0.667	0.870	0.458
	Fis	0.219	0.116	-0.063	0.092
Seco10	$H_{\rm E}$	0.809	0.695	0.491	0.553
565017	Ho	0.250	0.458	0.304	0.435
	F _{IS}	0.506	0.209	0.180	0.143
Seco22	$H_{\rm E}$	0.879	0.850	0.768	0.691
505022	Ho	0.522	0.565	0.478	0.625
	F _{IS}	0.425	0.143	0.200	0.117
Seco24	$H_{\rm E}$	0.798	0.711	0.719	0.572
505024	Ho	0.591	0.667	0.304	1.000
	Fis	0.281	0.083	0.278	-0.328
Seco20	$H_{\rm E}$	0.678	0.779	0.805	0.804
505029	Ho	0.217	0.542	0.792 0.500	0.500
	Fis	0.691	0.156	0.037	0.175
Seco33	$H_{\rm E}$	0.853	0.788	0.773	0.885
565055	Ho	0.708	0.739	0.542	0.708
	F _{IS}	0.191	0.085	0.237	0.088
Scso34	$H_{\rm E}$	0.794	0.735	0.778	0.612

	Ho	0.875	0.958	0.875	1.000
	F _{IS}	-0.078	0.139	-0.087	-0.280
Seco25	H_{E}	0.674	0.727	0.680	0.695
SCS035	Ho	0.708	0.750	0.542	0.875
	F _{IS}	0.040	0.019	0.111	-0.087
Saga26	$H_{\rm E}$	0.236	0.649	0.760	0.624
SCS030	Ho	0.000	0.000	0.458	0.174
	F _{IS}	1.000	0.674	0.414	0.444
Saca 27	$H_{\rm E}$	0.601	0.728	0.589	0.622
505057	Ho	0.000	0.542	0.304	0.083
	F _{IS}	1.000	0.276	0.500	0.629
SeCAR6	$H_{\rm E}$	0.861	0.882	0.885	0.873
SSCADU	Ho	0.667	0.667	0.833	1.000
	F_{IS}	0.246	0.169	0.079	-0.022
SaCA25	H_{E}	0.866	0.856	0.836	0.864
55CA25	Ho	0.792	0.875	0.792	0.625
	F _{IS}	0.107	-0.001	0.074	0.125
SeCTA22	$H_{\rm E}$	0.686	0.687	0.584	0.573
55C1A22	Ho	0.565	0.375	0.625	0.917
	F_{IS}	0.198	0.272	-0.049	-0.328
SeCTR24	$H_{\rm E}$	0.478	0.676	0.582	0.490
55CID24	Ho	0.125	0.130	0.348	0.583
	F _{IS}	0.748	0.517	0.244	-0.128

Pairwise F_{ST} values between populations are all low (Table 6.4). F_{ST} values can range between 0 and 1, 0 indicates no population structure and 1 indicates all genetic variation is explained by population. Low values indicate high gene flow between populations.

Table 6.4 Pairwise F_{ST} values between populations. Low values indicate high gene flow. Population backgrounds are shown; Carsington Water (CRS), Clatworthy Reservoir (CLT), Llyn Frongoch (FRN) and River Welland (WEL)

Pop ⁿ 1	Pop ⁿ 2	F _{ST}
CRS	CLT	0.053
CRS	FRN	0.045
CLT	FRN	0.046
CRS	WEL	0.060
CLT	WEL	0.047
FRN	WEL	0.037

6.3.2 mtDNA haplotype diversity and differentiation

The analysis of the 510 bp region of the mitochondrial *CO1* gene showed there is genetic variation between the four populations. Overall, 22 unique haplotypes were recovered

from 96 individuals, with a haplotype diversity of 0.881. The nucleotide diversity among haplotypes was Pi = 0.015. A total of 31 polymorphic sites and 37 mutations were found. Haplotype diversity and the number of unique haplotypes varied between populations (Table 6.5). The largest haplotype diversity was detected in the WEL population, with nine unique haplotypes recovered from 24 individuals. The lowest genetic diversity was found in FRN with five unique haplotypes. The lowest level of pairwise haplotype diversity occurred in parasites emanating from the CLT population, whereas those from FRN had the highest level. High haplotype diversity – above 0.6 – indicates little divergence between populations, this combined with low nucleotide diversity suggests a rapid demographic expansion across the UK has taken place.

Table 6.5 Haplotype diversity among populations Population backgrounds are shown; CarsingtonWater (CRS), Clatworthy Reservoir (CLT), Llyn Frongoch (FRN) and River Welland (WEL)

Pop ⁿ	Haplotype diversity	Unique No. of haplotypes	Nucleotide diversity	Pairwise diversity
CRS	0.772	8	0.010	5.884
CLT	0.717	6	0.012	2.670
FRN	0.544	5	0.007	14.318
WEL	0.862	9	0.015	7.290

Gene flow between the populations was found to be high, with a low G_{ST} value of 0.177. G_{ST} values theoretically range between 0 (no variation between populations, indicating high gene flow) to 1 (complete variation, indicating no gene flow). Despite this, the sequences of the *CO1* gene from each population clustered together with the majority of sequences from each population occupying close locations on the phylogenetic gene tree (Figure 6.3).



Figure 6.2 Phylogenetic gene tree of *S. solidus* mitochondrial DNA *CO1* sequences. Twenty-four individuals from each population were sequenced. Population backgrounds are shown; Carsington Water (CRS), Clatworthy Reservoir (CLT), Llyn Frongoch (FRN) and River Welland (WEL)

6.4 Discussion

The variable infection phenotype of *S. solidus*-infected male sticklebacks, and its dependence on parasite provenance under controlled laboratory conditions (Chapter 5), suggests that genetic variation among parasites may be responsible. To investigate whether patterns of genetic differentiation between parasite populations co-vary with the infection phenotypes they induce, the genetic diversity of *S. solidus* parasites from four UK populations was assessed using both mitochondrial DNA sequences and polymorphic microsatellite loci.

The analysis of 18 microsatellite loci found that all four populations were genetically similar at these locations, providing confirmation that parasites from four populations are from the same species and that all individuals were *S. solidus*. There is no evidence that a speciation event has taken place which could have led to the differing effects on host phenotype observed between the parasite populations. Speciation in the genus *Schistocephalus* has taken place in the past, with *S. solidus* and *S. pungitii* being significantly differentiated at the genetic level, even when taken from host fish (three-spined and nine-spined stickleback respectively) co-occurring in the same lake. Conversely, *S. solidus* from geographically distant locations have been found to be genetically similar (Nishimura et al., 2011).

Two of the loci studied were found to have null alleles present, Scso36 and Scso37. The highest levels of null alleles at these sites were found in CRS parasites, with no heterozygotes being detected. The high number of null alleles could indicate that mutation rates are occurring at a faster rate in CRS than other populations (Chapuis and Estoup, 2006, Hoeksema and Forde, 2008). Therefore due to rapid mutation levels, potentially beneficial alleles could be more likely to appear in this population than others and through local selection pressures may spread in this population. Thus CRS parasites may be evolving manipulative strategies to prevent their hosts from engaging in reproductive behaviour and utilising the energy for their own growth.

Genetic variation between populations was found in the mitochondrial *CO1* genes, with each population containing unique haplotypes. This variation was found to be composed of 31 polymorphic sites and 37 mutations. Isolated populations and local selection pressures are likely to increase this variation with the potential for beneficial alleles to develop.

Gene flow between these four UK populations has been demonstrated to be high, as both the G_{ST} values from mtDNA *CO1* sequence analysis and F_{ST} values from the microsatellite analysis were low. This is consistent with results from the study by Nishimura et al. (2011), that found evidence for significant gene flow between parasites recovered from geographically distinct regions of Oregon and Alaska. This is in contrast to a recent study of seventeen lakes in central Alaska in which the evidence for gene flow was limited (Sprehn et al., 2015). Under conditions of high gene flow, new alleles may quickly spread between populations and if local selection forces are particularly strong, even under such high gene flow, differentiation between populations may occur and may lead to genetic variability within the same parasite species across their geographical range. This may act to produce different host phenotype responses (Poulin, 2007, Hoeksema and Forde, 2008, Poisot, 2015).

Although the results show that high gene flow between the UK populations exists, the possibility that within the CRS population mutation rates could be higher than the others means different strains of parasites have the potential to evolve. Carsington Water was constructed and filled in 1992, therefore in terms of evolutionary time the populations of three-spined sticklebacks and the S. solidus parasites are relatively young, thus isolation and strain development may still be occurring. Different strains of the same species of parasite are known to have varying effects on their hosts. Parasitic nematode populations have been found to be genetically heterogeneous and respond to selective pressures. Although this is indirect evidence for parasite genetic variation causing varying host phenotypic responses, there are few examples that prove this genetic basis (Grant, 1994). Similarly the parasite Trypanosoma brucei have genetically distinct strains which correspond to variation in their effects on their hosts. Variation in transmission success and the resulting virulence have been observed (Morrison et al., 2010, Tait et al., 2011). To understand this variation better, it is important to identify the genes involved (Grant, 1994). Genetic maps of *T. brucei* have opened up the possibility of identifying loci that determine transmission and virulence phenotypes (Tait et al., 2011). Such identification of parasite genes is possible and has been successfully done in *Toxoplasma gondii*. Forward genetic approaches have been employed to identify loci controlling virulence (Su et al., 2002). Identification of such loci associated with parasite virulence within the S. solidus genome would be of significant interest as this could indicate where genes coding for manipulation occur.

6.5 Conclusions

Differences in infection phenotype dependent on parasite provenance have been observed (Chapter 5). As the study in Chapter 5 took place under controlled laboratory conditions this potentially indicates genetic variation between parasite populations. The study in this chapter showed that parasites from all host populations were of the same species (*Schistocephalus solidus*), with evidence of genetic variation between populations. The high frequency of null alleles in parasites from the Carsington population might indicate that greater levels of mutation are accumulating in this population, perhaps facilitating novel host manipulation strategies.

6.6 Limitations and further work

One of the limitations to this study is that the parasites used in the experimental nesting trials had been generated by selfing of single worms. This is likely to limit the genetic diversity seen within the experimental plerocercoids. Further studies should use a larger sample size and generate parasites by cross-fertilisation between two or more worms. Parasites from natural infections should also be used as these are likely to be offspring from cross-fertilisation and would represent wider genetic variation. Parasite numbers for each population were limited, due to the experimental constraints of the nesting experiments. Greater numbers of individuals could identify more variation between populations. Identification of loci associated with parasite virulence would be of significant interest as this could indicate where genes coding for manipulation occur in the parasite genome. Variation in such a locus could indicate how such a mutation is spreading across populations.

7 General discussion and conclusion

7.1 Main Findings

Parasites are ubiquitous, extremely diverse and negatively affect their hosts as they divert resources from them. Many parasites also cause significant changes to their host's development, morphology and/or behaviour, and often this disrupts the host's reproductive biology. Infection-driven changes in host biology occur through a number of diverse mechanisms, and it may be expected that different species of parasites use various mechanisms to alter host phenotype. However, population-level variation in host responses to the same species of parasite is also often observed, possibly indicating that even within the same species of parasites a variety of mechanisms can be used to alter host biology. The variable reproductive potential of S. solidus infected three-spined sticklebacks is one such example (Macnab et al., 2009, Heins, 2012) where the mechanism(s) responsible for altered host phenotype have yet to be conclusively identified. Variation in the infection phenotype of infected sticklebacks may be due to environmental differences, but may also arise because either parasites or hosts have evolved different manipulation or adaptation strategies in isolated locations. Understanding the mechanisms that generate infection-associated changes in host phenotype is not only interesting in itself, but is also important to determine how other external factors, such as environmental or anthropogenic stressors, may interact with the effect of parasite infections and influence changes in the host-parasite system. It has already been shown that both warmer water temperatures and exposure to endocrine disrupting chemicals such as 17ß estradiol (E2) lead to increased parasite growth (Macnab and Barber, 2012, Macnab et al., 2016) which combined with different mechnisms could alter host phenotype significantly.

In this thesis, the three-spined stickleback-*Schistocephalus* host-parasite system was used to investigate a range of questions about the mechanisms underlying variability in infection phenotype between populations. Initially, the work sought to identify the effects of two environmental factors – the timing of infection over an annual cycle, and the effect of food limitation – on infection outcomes. Secondly, the role of putative genetic factors were investigated, by investigating the outcome of infections on host reproduction following cross-infection studies in which both host and parasite genotypes were varied. The key results, interpretation and potential further research directions will be summarised in this general discussion.

7.1.1 Environmental factors effecting stickleback development

The consequences of acquiring infections at different time points during the annual cycle, and the effects of food limitation for the reproductive development of sticklebacks were investigated in Chapters 3 and 4.

7.1.1.1 The importance of fish age at infection, and potential effects on host sexual development

The timing of parasite infections can have important biological consequences for population dynamics of hosts and parasites, and so could influence the survival and reproductive success of both the stickleback and S. solidus. Chapter 3 showed that the time point at which fish were exposed to infections and subsequent parasite development took place plays an important role in determining host fish development. The greatest impacts of infection occurred when fish were exposed in late winter/early spring, allowing the parasites to develop during the normal breeding season. Both fish condition and gene expression were significantly altered by infection. Changes in the various condition indices of the fish used as indicators of sexual development; gonadosomatic index (females) and kidney-somatic index (males) showed that sexual maturation was limited or prevented as a result of infections gained in early spring, whereas later infections had less of an impact on maturation. Expression levels of genes that code for developmental hormones in the brain-pituitary-gonad axis also varied depending on the time of infection. Similarly to body indices, gene expression was most effected by infections gained in the spring, and less impact was seen in infections gained later on in the year. This suggests that fish go through a key development period in the spring prior to the breeding season and that infections at this point have significantly detrimental effects, whereas once the fish have matured to a later stage infections have less negative impacts. Changes in host development were seen even in host fish harbouring plerocercoids that were below the 50 mg threshold weight for infectivity to the final host (Tierney and Crompton, 1992). This may support the manipulation hypothesis, as parasites that castrate their host at an early stage are often considered to be displaying manipulative capacity (Hurd, 2001, Kroupova et al., 2012). Parasites attained greater mass in female fish compared to male fish regardless of the time of year infections took place. However male fish were compromised in their development to a greater extent despite harbouring smaller plerocercoids, particularly from infections gained in the spring that developed over the breeding season.

Fish were not given the opportunities to attempt courtship or breed in these studies, therefore the changes seen in the morphology and gene expression in the infected fish can only be assumed to be indicators that reproduction would not occur. The fitness of hosts in terms of successfully rearing offspring cannot be determined from these results which is a limitation to the study. Fish that become infected later on in the year may have developed physically, however they may not engage in courtship as energy demands could still be too great. Therefore, further investigations into the full extent of infections at different points in the annual developmental cycle of three-spined sticklebacks are required. Longer-term infection periods and common-garden breeding behavioural experiments could determine if parasitic castration is truly occurring. For example, if infection is only delaying the onset of sexual development, studies over longer periods may show that sexual maturation occurs at later points in the year. However, S. solidus parasites continue growing to large sizes within sticklebacks, and so become a greater energetic burden on the fish. It therefore does seem that fish development is likely to be permanently impaired by early infections. Further studies investigating longer-term infections will more fully identify how stickleback reproduction is influenced over time.

In nature, the timing of infections is likely to be influenced by local environmental and climatic conditions. Further studies are required to determine the infection time period in natural populations as currently data on this is limited (Heins et al., 2016). With this information, a greater understanding of the factors controlling the timing of infections could be achieved. The greatest influence is likely to be temperature, as this factor places significant restrictions on when the early infective stages of *S. solidus* are present in the environment (Christen and Milinski, 2005). Thus, potential future climatic changes could play an important role as to when *S. solidus* infects its fish host. Detrimental effects of infection may therefore be increased or decreased depending upon when the infection occurs. Rises in water temperature could also influence the timing of the fish breeding season (Hovel et al., 2017), which could exaggerate any impact of altered timings of parasite infections. Changes in the temperature of water bodies are evident. Warming trends across the Northern Hemisphere have been observed (Magnuson et al., 2000),

which have led to faster growth rates in sticklebacks (Hovel et al., 2017), so mismatches between parasite infection and breeding seasons for fish are a real possibility.

7.1.1.2 The importance of food resources on development and comparisons between energy drain from parasites

Preventing hosts developing and maturing sexually is often explained as a consequence of simple nutrient theft by parasites. If the energy required to support both the growing parasite and the sexual development of hosts is too high, parasites and hosts will compete for available resources with the likely consequence that host reproduction is negatively impacted. Nutrient theft by parasites may impact host biological pathways in the same way as limited nutrient intake, since restricted diets in non-infected individuals are also known to prevent sexual maturation.

As expected, the results of studies described in Chapter 4 showed that a low (maintenance) ration of 4% body mass per day limited fish growth in non-infected fish, both in terms of length and mass increase. However such negative effects were not observed in infected fish fed *ad libitum*. Among *ad libitum* fed fish, infected individuals were slightly shorter in length than the non-infected conspecifics, but this was not significant. Infected fish also increased in mass by a greater amount than their non-infected conspecifics. Interestingly however, parasite infection and food restriction had differing effects on both the physiology and gene expression patterns of stickleback fish.

One key finding was the difference in gene expression levels between infected and food restricted fish, in particular for the neuropeptide *kisspeptin* (*Kiss2*). Kisspeptin has an important role in the early stages of the BPG axis and pathway and in triggering development (Mechaly et al., 2013). *Kiss2* expression level has also been linked to the nutritional status of animals; those that experience low levels of nutrition fail to express *kisspeptin* and also do not mature sexually (Mechaly et al., 2011, Mechaly et al., 2013). If similar mechanisms were occurring in both fish held under restricted rations and infected fish, expression levels might also be expected to be similar. However, the results of Chapter 4 showed that expression levels between the two classes of fish were very different. Surprisingly, fish held on low ration diets had significantly higher expression levels of *Kiss2* whereas infection caused no change. These results indicate that the underlying mechanism for delayed or prevented development may be different for infected fish compared to those on a restricted diet. This may suggest that nutrient theft

is unlikely to be the process that prevents sexual maturation in *S. solidus*-infected threespined sticklebacks. One caveat to this conclusion is that infection and food rationing may influence the stickleback via different signals. Lower energy input due to low food rations and reduced energy reserves due to nutrient theft may act through different underlying mechanisms (Heins and Brown-Peterson, 2010). Fish that are kept on a low ration would be experiencing 'empty stomach' signals, which may lead to maintenance strategies for resource distribution being employed as predicted by the dynamic energy budget (DEB) theory (Leloutre et al., 2016). On the other hand, infected *ad libitum* fed fish would experience 'full stomach' signals, and so would anticipate enough energy to be available for development.

Further comparison studies of the two conditions would be extremely useful to identify the mechanism by which energy levels are detected by fish. Future studies could use alternative methods to food restriction, such as increased activity levels or pharmacological methods to allow fish to experience similar energy drains or lack of glucose uptake. Comparisons in the levels of sexual maturation could be made between fish infected with *S. solidus* and non-infected fish experiencing a similar energetic drain, for example by forced increases in activity. Alternatively, administration of a drug such as sinigrin which reduces glucose levels in zebrafish (Abbas et al., 2017) could potentially mimic the diversion of energy from the host to the parasite. However, although both these methods would allow similar stomach content signals, they could both potentially influence other mechanisms that prevent maturation which would need careful consideration.

7.1.1 Population variations and parasite provenance influence host development

The differences in reproductive phenotype of *S. solidus* infected fish from different populations might be influenced by genetic differences either in the hosts or parasites involved in the infection. The effects of host and parasite provenance (used as a proxy for genetic background) on host reproductive phenotype were investigated in Chapter 5, focusing specifically on the male stickleback's ability to perform courtship and nesting behaviours whilst infected. The main outcome of this study appears to be that the parasite population involved in the infection, rather than the fish population, determines whether male sticklebacks can sexually mature. Following laboratory cross-infection studies, parasites from Carsington Water (CRS) consistently prevented host fish from developing

and building nests, regardless of the provenance of the host fish. In contrast, when infected with parasites from the other four UK provenances were studied, males from all population backgrounds successfully completed nests. As common laboratory conditions were used for all experiments, and all fish were fed *ad libitum* and to excess, variable environmental factors can be ruled out as an explanation for the variation in infection outcomes. The results suggest that there is likely to be genetic differentiation between the populations of parasites, perhaps arising from divergent strategies of parasite exploitation that have evolved in different host-parasite populations.

Despite this clear result it is still not completely evident that infected fish build good nests. In these studies, males were only scored as 'nesters' based on male behavioural indicators (i.e. passing through their finished nest) and no element of female choice was investigated. Although nests had been completed there was no indication of the quality of the construction, or whether the infected males would be able to successfully raise young. Further studies into the quality of nests built by infected males are now required to assess the total effect of infection on host fitness in terms of successful reproduction. Female choice experiments may reveal that nests built by infected males are rejected, and as a result infected males may be unable to attract females to spawn within their nests. Future paternal care for offspring may also be inhibited by infection; the energetic demands of maintaining a nest with developing eggs and protecting hatched fry when combined with supporting an increasing parasite load may be too great for breeding males. In addition, this study also only investigated effects on male fish. It would also be of interest to determine if different parasite populations have similarly variable effects on female fish. However, the study does demonstrate that cross-infection studies can be used to gain insights into these questions, and further similar studies are now needed to investigate how female fish develop and successfully produce clutches and how these outcomes are influenced by the combination of host and parasite genotypes. The idea that different effects on females can occur due to population variations is suggested by intensive field sampling by Heins (2012) who demonstrated that infected females from a Scout Lake population showed characteristics of fecundity compensation by producing a greater number of eggs, whereas Walby Lake females had reduced clutch sizes despite occupying a habitat high in nutrient levels.

7.1.2 Genetic variation within the same parasite species

The results of Chapter 5 indicated that differences in the infection phenotype induced by parasites from different population backgrounds are likely to be due to genetic differences between the parasites. Chapter 6 therefore focused on investigating the levels of genetic variation between four of the UK populations studied in the thesis. While analysis of mitochondrial DNA sequences of the CO1 gene suggested that there was genetic variation between the populations, the analysis of microsatellite diversity across 18 loci showed little variation, confirming that all populations were from the same species, S. solidus. No speciation events have occurred that could account for the different host responses seen. The result also demonstrated that a high level of gene flow occurs between the populations studied. This finding supports previous results of significant gene flow between geographically distant populations such as Alaska and Oregon (Nishimura et al., 2011), undoubtedly facilitated by the inclusion of highly mobile avian definitive hosts in the life cycle. Nonetheless, parasites from one population, Carsington Water (CRS) had higher levels of null alleles at two of the loci studied, which could indicate that mutation rates are occurring at a faster rate in CRS than other populations. This could mean that beneficial alleles are more likely to appear in this population than others, and through local selection pressures may spread in this population. CRS parasites, which significantly reduced male hosts nesting behaviour regardless of host background, may be evolving manipulative strategies to prevent their hosts from engaging in reproductive behaviour and utilising the energy for their own growth. Further studies into the genetic variation within parasites are needed to identify the differences in populations. This could be done by fully sequencing the genomes of the different parasite populations and comparing them to highlight further genetic differences. Identification of specific loci associated with manipulative behaviour has the potential to determine how parasites affect hosts.

7.1.3 Suggestions for future work

This thesis investigated variability in host-parasite interactions and illustrates the potential for different *S. solidus* populations to evolve differential capacity to alter host infection phenotypes, including manipulative capabilities. The existence of different mechanisms appears to be dependent on the population. Clatworthy Reservoir (CLT), Inverleith Pond (INV), Llyn Frongoch (FRN) and the River Welland (WEL) *S. solidus*
appear to prevent their hosts from developing and sexually maturing indirectly as a result of nutrient theft, whereas parasites from Carsington Water (CRS) appear to affect development more directly, potentially through manipulation strategies. It is now clear that the effect of *S. solidus* varies considerably across its geographical range and different mechanisms of host exploitation may be evolving within local populations. This makes the stickleback-*Schistocephalus* system an ideal model for studying co-evolution between host and parasite. Understanding the mechanisms that lead to population differences in host reproductive phenotype when infected is important, as this determines factors that influence parasite virulence. This is crucial for future studies involving combined effects of infection and other factors, such as environmental cues of temperature, or anthropogenic stressors and ecotoxicology.

7.2 Main Conclusions

The key finding of the work presented in this thesis is that parasite provenance and not host genotype significantly affects host development. The control of environmental conditions within the laboratory studies suggests this is most likely due to genetic variation between parasites from different provenances. The significance of this is that it provides further evidence that the parasite *S. solidus* varies greatly across its geographical range. In some populations it may be evolving manipulative strategies that prevent its host fish, the three-spined stickleback, from developing and maturing sexually. All three mechanisms of nutrient theft, parasite manipulation and host adaptation could potentially exist in this host-parasite system as the fish and parasite co-evolve together and continued research is required to identify this.

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