

Evolutionary ecology of virulence in a fish parasite

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Dedicated to the residents of North Uist

Abstract

Virulence (parasite- induced host fitness reduction) is thought to correlate positively with pathogen reproduction rates, but its relationship with pathogen transmission is likely to be determined by a trade- off between the costs and benefits of harming hosts. This project aims to investigate factors which affect host-parasite interactions and particularly those which may play a role in virulence evolution. In doing so, it describes experiments carried out using a monogenean ectoparasitic flatworm (*Gyrodactylus arcuatus*) and its three-spined stickleback (*Gasterosteus aculeatus*) host. Populations of this fish species experience a range of both environmental and ecological conditions. Such environmental heterogeneity has been found to drive changes in fish phenotypic traits such as morphology, behaviour, life history and physiology which may consequently affect parasite fitness. I demonstrate that under these conditions, parasite strains from different host populations evolve variation in virulence levels. I also reveal that coevolution in this host parasite system is likely to lead to local adaptation of *G. arcuatus* at the host population level. I show that competition between parasite species sharing a single host leads to increased parasite reproduction rates, but it shortens the infection time which may be due to earlier stimulation of host immune responses. I show that virulence is neither influenced by the population density, immunity (epidermal mucus), social behaviour of fish hosts nor the natural parasite life expectancy. Lastly, I find that virulence in this system is negatively influenced by the density of stickleback predators and positively associated with loch water pH. Taken together, these results suggest that in this host parasite system, both ecological and environmental factors which drive phenotypic changes in fish hosts may evolutionarily feedback to affect parasite virulence.

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Chapter 1.

General introduction

Chapter 1. General introduction

In this chapter, I first briefly introduce host- parasite interactions, definitions of virulence and the most recent theories on virulence evolution. I review some empirical studies that have been carried out to address the question of what factors are assumed to drive changes in virulence. I introduce the three-spined stickleback-*Gyrodactylus* sp.- host-parasite system used in this study project and why the North Uist populations are important in investigating virulence evolution. Then, I present an outline of the chapters of this thesis describing the experiments performed to investigate the importance of ecological and evolutionary conditions that are predicted to affect virulence in this host- parasite interaction.

1.1 Host- parasite interactions and infectious diseases

The most obvious characteristic of a parasitic organism is that it harms its host. Parasite-induced harm (virulence) can involve decreased growth, reproductive success and longevity of hosts and, therefore, it is considered a driving factor in host-parasite coevolution (Zhan *et al.*, 2002). Parasites extract resources from hosts to gain basic requirements for their growth, and their virulence is considered as an unpreventable side effect of parasitism. As the health status (and mobility) of hosts may be important for the transmission of parasites with a direct life cycle, therefore, virulence may directly associate with fitness components of these parasites (Choo *et al.*, 2003; Hammerschmidt and Kurtz, 2005). Both innate and adaptive immune responses of hosts are responsible for fighting parasites at the molecular and macroscopic level (Schmid-Hempel, 2009). Parasite- induced damage to hosts may cause the induction of host immune responses and that may also generate selection on parasite virulence (Schmid-Hempel, 2009).

Consequently a strong reciprocal selection is exerted by both the host and the parasite on each other that leads to coevolution races in many host- parasite systems.

Understanding the interactions between parasites and their hosts and how changes are induced in parasite virulence remain an important objective in evolutionary biology. Insights from adaptation of parasite pathogenicity may potentially contribute to the application of biological controls, management of virulence and controlling of infectious diseases in human and wildlife (Bull, 1994; Woolhouse *et al.*, 2002; Ebert and Bull, 2003).

1.2 Virulence in theory and in empirical studies

Recent theories of virulence evolution began in the last four decades after the publication of two research papers that used mathematical models to describe host parasite interactions (Anderson and May, 1979; May and Anderson, 1979). These studies explain the relationship between ‘host density’ and ‘parasite transmission’ using two ordinary differential formulas (Best, 2010):

$$dX/dt = aX + \gamma Y - qXN - \mu X - \beta XY \quad (1.1)$$

$$dY/dt = \beta XY - (\mu + \alpha + \gamma)Y \quad (1.2)$$

They assume that host populations are of three types: uninfected (X), infected (Y) and recovered hosts are immune to infection, but immunity is quickly lost. Host population density is temporally increased by the rate at which the host reproduces (aX) and the rate at which infected hosts recover (γY), but it is decreased by natural host mortality (μX) and crowding (qXN). When a directly transmitted parasite causes infection, its transmission

coefficient (β) is determined by the total density of the two host types ($N = X + Y$). Some of the infected hosts die due to the parasite virulence at rate ' α ', but others recover to become uninfected at rate ' γ '. Anderson and May (1981) and Anderson and May (1982) have later used these mathematical formulas to describe parasites' basic reproductive rate (R_0):

$$R_0 = \beta S / (\mu + \alpha + \gamma) \quad (1.3)$$

Where, S is the density of uninfected susceptible hosts ($S = (a - \mu)/q$). For a parasite to persist, the value of its R_0 must be larger than '1'. This model, which predicts that virulence should evolve to an optimum dependent on circumstances, has become the alternative to the classical view on virulence which suggests that parasites evolve toward becoming ever more benign. The new model clearly rejected the traditional view on virulence evolution and instead, suggests that the outcomes of coevolution depend on the relationship between virulence and transmission of parasites (Anderson and May, 1982). The recent theory assumes that parasite reproductive fitness positively correlates with virulence and with transmission rates to a certain level (Levin and Pimentel, 1981; Anderson and May, 1982; May and Anderson, 1983). Nonetheless, beyond that level (when parasite transmission is reduced by host mortality), a parasite may evolve to optimize its reproductive fitness and this is likely to happen, when there is a trade-off between the parasite transmission and virulence (Figure 1.1).

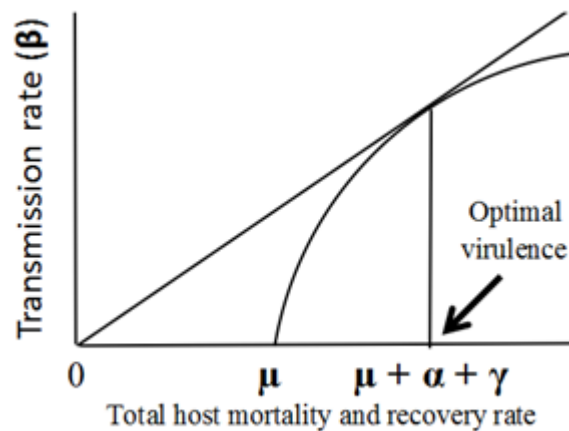


Figure 1.1. Trade-off curve showing the relationship between parasite transmission (β) success (Y-axis) and the duration of parasite infection (X-axis). Infection duration is thought to be determined by the total host mortality rate (induced by the parasite (α) or other background factors (μ)) and host recovery rate (γ). R_0 is thought to be maximized at optimal virulence level (where straight and curved lines meet), adapted from Alizon *et al.* (2009).

The existence of a trade-off between parasite virulence and transmission has been supported by several empirical studies (Messenger *et al.*, 1999; Davies *et al.*, 2001; Thrall and Burdon, 2003; de Roode *et al.*, 2008a), but some recent theory suggests that it is not the whole story. For example, Ebert and Bull (2008) argue that virulence evolution in some parasites cannot be explained by only a ‘two-dimensional trade-off’ model. Multiple abiotic and biotic factors can directly or indirectly cause selection on parasite fitness. For example, the behaviour, immune response and changes in the life history of hosts can affect parasite fitness (Ebert and Bull, 2003, 2008). Environmental conditions under which host parasite interactions take place may indirectly affect a parasite’s fitness by inducing changes in biological traits of its host (Patz and Olson, 2006; Wolinska and King, 2009; Mostoway and Engelstadter, 2011; Lopez Pascua *et al.*, 2012). Abiotic environments can also affect host survival and the transmissibility of directly transmitted parasites (Blanford

et al., 2003; Ewald, 2004; Walter and Ewald, 2004), although this possibility is not well explored (Lively *et al.*, 2014). Furthermore, while most of these theories explain host parasite interactions assuming infections with a single parasite genotype, empirical findings suggest that this may be simplistic (Sharp *et al.*, 1997; de Roij and MacColl, 2012). Concurrent infections with more than one parasite genotype make the prediction of virulence more difficult because coinfecting parasites affect each other's fitness through resource competition, stimulation of immune response and increased mortality rate of their common host (Nowak and May, 1994; Petneya and Andrews, 1998; Jackson *et al.*, 2006).

The modern theories on virulence evolution were mostly inspired by Fenner's classic example of European rabbits- myxoma virus coevolution (Fenner, 1983). To test the hypotheses of virulence evolution, a large body of empirical studies has been carried out on invertebrate hosts (Anderson and May, 1981; Ebert, 1994; Haine *et al.*, 2005; de Roode *et al.*, 2008a; Duffy and Hall, 2008). Invertebrates are known to lack a complex adaptive immunity (Beck and Habicht, 1996; Rinkevich, 1999) and therefore, the impact of hosts' response on parasites' fitness, may not be evolutionarily as strong as vertebrate hosts (Hedrick, 2004). Vertebrate hosts have been poorly used in studies of host- parasite coevolution. Specifically, there are no examples of environment affecting vertebrate host- parasite interactions (Wolinska and King, 2009). In all the studies presented by this project, I used the three-spined stickleback (*Gasterosteus aculeatus*) and its monogenean flatworm ectoparasite, *Gyrodactylus arcuatus* to: (1) investigate whether virulence differs among spatially isolated strains of the parasite and (2) explain what factors shape parasite virulence.

1.3 The host, three-spined stickleback

The three-spined stickleback (*Gasterosteus aculeatus*) is a small scale-less fish of the teleost group, with a broad geographical distribution. This species is divided into oceanic and freshwater types. Oceanic sticklebacks are protected by bony lateral plates along their body sides and dorsal and pelvic spines. They commonly migrate to freshwater to breed. It has been obvious that in freshwater, they evolve loss of these bony parts (Cresko *et al.*, 2004) which results in rapid and frequent phenotypic divergence. Sticklebacks have been increasingly used as vertebrate models to study aspects such as animal behaviour (Barber, 2003), immunology (Scharsack *et al.*, 2007), evolution (MacColl *et al.*, 2013) and host-parasite interactions (Raeymaekers *et al.*, 2011). Besides that, these fish are ubiquitous, simple to catch, easy to breed in the laboratory and with a relatively short life cycle, while a large body of literature is available on their ecological and evolutionary history (Whoriskey and FitzGerald, 1994; Katsiadaki *et al.*, 2007). More importantly, the entire genome from an individual stickleback has been sequenced (Jones *et al.*, 2012) making them an excellent model to understand different aspects of the genetics of host-parasite interactions. With such valuable background available, these interesting fish have also become a perfect vertebrate host to study virulence evolution in parasites (Barber and Nettleship, 2010).

Both field and experimental studies suggest that sticklebacks are potentially subject to natural selection caused by their local parasites. For example, several studies have shown that naïve fish which had never been exposed to any parasites show local adaptation to a range of parasite species that naturally exist in their parental environment (Scharsack *et al.*, 2007; MacColl and Chapman, 2010; de Roij *et al.*, 2011). Parasite-mediated selection at least in these examples may be responsible for a considerable change in the phenotypes

and population density of sticklebacks. This may result in a potential selective pressure on parasites and subsequently lead to selection for changes in virulence.

1.4 The parasite, *Gyrodactylus arcuatus*

Individuals of the genus *Gyrodactylus* are viviparous monogenean flat worms of the Phylum Platyhelminthes. They commonly live on the fins, gills and skin of fresh- and saltwater fish. These parasites feed on epithelial cells and mucus of fish skin (Kearn, 1979; Cone and Odense, 1984). They cause damage to the skin epidermis (Sterud *et al.*, 1998) which consequently may lead to host death by reducing the respiratory function of epithelium and rendering the host susceptible to secondary infections by other pathogens (Cone and Odense, 1984). Their unusual viviparity is thought to be an extremely effective form of reproduction (Fig. 1.2). Each individual parasite contains a mature daughter within the uterus, which already contains another growing embryo ordered one inside another (Cable and Harris, 2002). They have been reported to use both sexual and asexual modes of reproduction (Schelkle *et al.*, 2012). They are highly infectious because they can establish a complete population from one individual worm (Cable and Harris, 2002). In optimum conditions, within a day of her parent's birth, a new daughter could be born (Cable and Harris, 2002). Short generation time of *Gyrodactylus* sp. can quickly lead to large population size and together with their direct life cycle enhance the parasite's potential to infect new hosts. This may also quicken their population differentiation and speciation (Huyse and Volckaert, 2005).

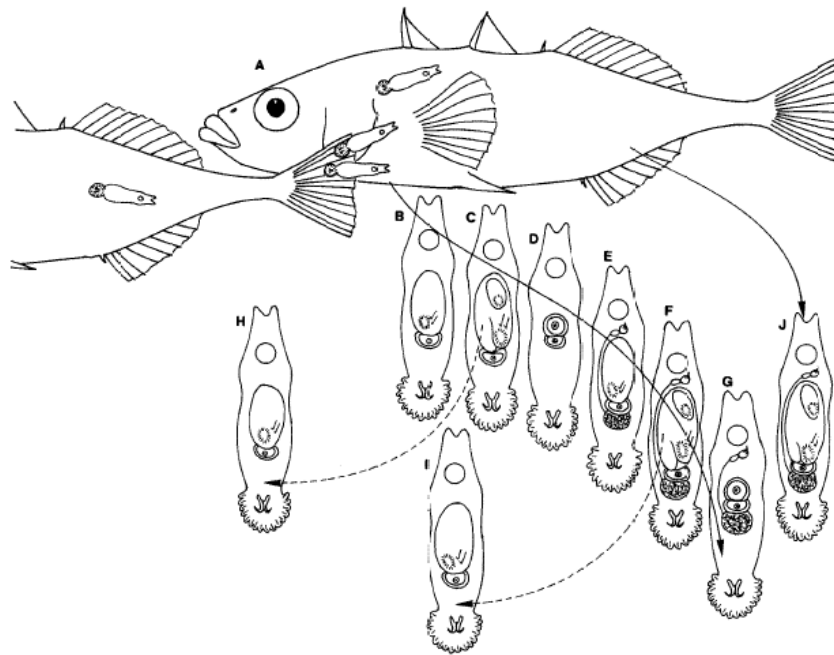


Figure 1.2. The life cycle and host-host contact transmission of *Gyrodactylus* sp. A: parasite transfers from infected to uninfected fish when the hosts physically contact each other. B – I: show sequential stages of an embryo development. The parasite gives first birth (H) at stage C and second birth (I) at stage F. Male reproductive system is not developed when the first-embryo is born, but the subsequent-born individual is developed from an oocyte/uterus. Male reproductive system is developed at the end of stage E and all subsequent daughters are developed by either parthenogenesis or sexual mating. Mating can only occur after first birth between stages E, F, G and another mature worm J. The figure was taken from Kearn (1994).

Compared to their hosts, parasites in general have higher evolutionary potential because of their shorter generation time, bigger population size and higher mutation rate (Kochin *et al.*, 2010). This increases the likelihood of rapid genetic recombination and evolution of parasites. As *Gyrodactylus* sp. are obligate ectoparasites, they can only use specific hosts to derive energy requirements (Bakke *et al.*, 1992; Bakke *et al.*, 2002; Raeymaekers *et al.*, 2011). Hence, it must have a specific life strategy to successfully deal with the structures of microhabitats on its hosts. Buchmann and Lindenstrøm (2002) describe several constraints which may determine the host specificity of monogenean parasites. First the parasite recognises the molecules released by its host. Second, there should be

compatibility between the molecules released by the host and the parasite. Third, the host surface (skin structure) should anatomically fit the attachment apparatus of the parasite. Fourth, after a successful attachment, the parasite reproduction depends on the type of stimuli perceived from the host. Fifth, the parasite must be able to utilise host- based nutrition for its reproduction. As a result, the parasite must be tightly linked to its hosts and has to follow all of these requirements to establish a successful infection. Hence, any response from the fish host can have a strong impact on the parasite's survival and consequently may exert a considerable selective pressure on it.

Although, no data are available to show whether or not *Gyrodactylus* sp. have immune evasion mechanisms, they efficiently endure a broad range of immune responses from their hosts. This has led researchers such as Buchmann and Lindenstrøm (2002) to believe that monogeneans should have some evasion strategies to cope with responses from their hosts.

Gyrodactylus sp. live on the external surface of their fish hosts, so they might face innate defences rather than specific responses at least during the initial stages of infections. Skin is thought to be the biggest immunologically functional organ in fish (Cainl *et al.*, 1996), but the role of its mucous contents in the resistance to gyrodactylid infections is not clear. In some fish species, findings suggest that skin mucus contains anti *Gyrodactylus* sp. agents (Lester and Adams, 1974; Buchmann and Uldal, 1997; Harris *et al.*, 1998), others suggest that the epidermal mucous cells are severely affected by the parasite (Cusack and Cone, 1986; Wells and Cone, 1990; Sterud *et al.*, 1998). Arguably, the epidermal mucus of fish skin may contain very different substances that can have opposite effects on *Gyrodactylus* sp.(Buchmann, 2000). Therefore, the association between these parasites and fish mucus may depend on fish species and environmental conditions (Gheorghiu *et al.*, 2012).

Gyrodactylus are generally identified to species level depending on both the morphological characteristics of their secretory system (bladder) and attachment apparatus (Geets *et al.*, 1999), and molecular analysis of ribosomal sequences (ITS1 and ITS2 rDNA and V4 region of 18S rRNA) (Huyse *et al.*, 2003; Huyse and Volckaert, 2005). Previous studies suggest that *G. arcuatus* is the native *Gyrodactylus* species on North Uist three- spined sticklebacks (de Roij *et al.*, 2011; de Roij and MacColl, 2012). The morphological characteristics of seven *Gyrodactylus* isolates used in this project were found to be very similar to *G. arcuatus*. Unpublished results of ITS sequences from two contemporary PhD students (S. Robertson, Nottingham University- UK and Anna Rahn, Theo Bakker- Germany) also agree that North Uist *Gyrodactylus* are very similar to *G. arcuatus* (personal communications). Although some literature suggests that *G. arcuatus* is a gill-specific parasite (see Bakke *et al.*, 2007), its distribution upon the stickleback body has been found to vary according to geographical or environmental conditions. In Belgium, *G. arcuatus* was generally found on gills (Raeymaekers *et al.*, 2008), but it was considered to be fin-specific on the same host species in Sweden (see Huyse *et al.*, 2003). On North Uist sticklebacks, this parasite species has been mostly recorded on the fins and body, but only rarely on the gills (in 2010, 23 worms/177 fish and in 2011, only 10 worms/434 fish, from Anna Rahn's unpublished data). Hence due to the morphological and genetic similarity of the North Uist *Gyrodactylus* studied in this thesis to other *G. arcuatus* strains, I refer to them as *G. arcuatus*. However, we also recognise that cryptic species complexes are common amongst parasites, including *Gyrodactylus* (Xavier *et al.*, 2015), and detailed future studies into the precise identity of North Uist *Gyrodactylus* could be fruitful.

In many parasites, virulence levels depend on the parasite population growth on individual infected host (Ebert, 1994; Messenger *et al.*, 1999; de Roode *et al.*, 2008a; Nidelet *et al.*,

2009). As the pathogenicity of *Gyrodactylus* sp. mainly depends on the on-host activity of individual parasites (Cone and Odense, 1984), the parasite population growth rate should provide an accurate measure for quantifying virulence. Under experimentally controlled conditions, increase in the population growth of this monogenean has been seen to result in an almost a linear increase in the mortality rate of infected hosts (Scott and Anderson, 1984; Madhavi and Anderson, 1985). Therefore, for all the studies conducted during the current project, I inferred virulence from parasite population size.

1.5 North Uist study system and sticklebacks

This research mostly used hosts and parasites sampled on North Uist, Outer Hebrides, Scotland. North Uist is a small island (approx. 303 Km²) containing a large number of fresh and saltwater lochs (Fig. 1.3). The lochs differ greatly in environmental conditions such as water pH, conductivity and water chemistry. Generally, freshwater lochs differ in water salinity, pH and water chemistry between the west and east sides of the island. Eastern lochs are often acidic with low water conductivity while western lochs are characterised with high water pH and alkaline metals. Giles (1983) suggested that these environmental variations are the most predictive driver in the phenotypic variation of these sticklebacks. The lochs also vary quantifiably in ecological conditions such as the density of a stickleback competitor, the nine-spined stickleback (*Pungitius pungitius*) and a stickleback predator, the brown trout (*Salmo trutta*) (see MacColl *et al.*, 2013). The prevalence and abundance of ecto- and endoparasites, including *G. arcuatus*, consistently vary among these fish populations (de Roij and MacColl, 2012). Environmental stressors, such as competitors, predators and parasites, constitute selective agents that may drive adaptive changes in populations' phenotypes (eg. body size, Fig. 1.4) (MacColl *et al.*,

2013) including resistance of sticklebacks to gyrodactylid infections (de Roij *et al.*, 2011). Some of the lochs are geographically isolated from other aquatic ecosystems which may make resident stickleback populations evolutionarily independent. Under such conditions, these fish populations may represent biological islands for directly transmitted parasites. This is particularly interesting because such evolutionary processes in the fish hosts may feedback to affect evolutionary processes in their parasites (Cable and Oosterhout, 2007a). Therefore, these fish may provide a valuable host model meeting the needs of the current project.



Figure 1.3. Map of the North Uist, Outer Hebrides, Scotland. The arrows point to lochs which were included by studies of this project. Unlike the three lochs on the left side of the island which are alkaline, lochs on the middle and right side are acidic, except Obse, which is linked to the sea and is alkaline. The image was adapted from Google Maps (<https://www.google.co.uk/maps>).



Figure 1.4. The host model species, three- spined stickleback (*Gasterosteus aculeatus*). The middle individual is a marine (anadromous) stickleback, while the other two come from two geographically isolated freshwater populations (Scadavay: bottom left and Hosta: top right). All fish are scaled to the average size of adult females in their population. Photographs taken by Andrew MacColl.

1.6 Thesis outline

The overall aim of this body of work is to investigate whether strains of *G. arcuatus* differ in virulence and if they do, what factors play a role in the evolution of the parasite's virulence. I have written the data chapters of this thesis in the style of a manuscript and therefore some of the important biological descriptions of the host and the parasite may have been repeated. The following provides an outline and objectives of each chapter:

- Chapter 2 compares virulence among strains of *G. arcuatus* and investigates the extent of parasite local adaptation using a reciprocal cross infection between four strains of the parasite and their lab raised hosts. This chapter provides insights about the host-parasite coevolution outcomes in heterogeneous environments and how that may affect virulence evolution.
- Chapter 3 focusses on the effects of abiotic and biotic environments on the survival time of detached parasites and investigates the adaptation of parasite strains to the water quality of their local habitat. The aim is to compare average survival time

among parasite strains and to understand the immunological functions of the epidermal mucus of host skin against the survival and performance of *G. arcuatus*.

- Chapter 4 quantifies and compares the schooling (swimming in polarized groups) and shoaling (aggregation) behaviours among populations of three-spined sticklebacks. It investigates the influence of infections with *G. arcuatus* and *Schistocephalus solidus* on these behaviours. The aim is to evaluate effects of environmental and parasite-induced behaviour alteration in stickleback hosts on the transmission of *G. arcuatus*.
- Chapter 5 investigates the interactions between sympatric and allopatric species of *Gyrodactylus* sp. (*G. arcuatus* and *G. gasterostei* respectively) when they concomitantly infect individual sticklebacks. This chapter compares virulence among sympatric, allopatric and mixed parasite species to understand how virulence associates with multiple infections.
- Chapter 6 assesses whether virulence consistently differs among the parasite strains on a susceptible allopatric host population. This chapter quantifies virulence for seven geographical strains of the parasite. The main aim is to investigate the association between virulence and different ecological and environmental factors which are commonly thought to directly or indirectly affect parasite fitness components especially transmission success.
- Chapter 7 integrates all the findings described in the previous chapters, discusses general trends and makes conclusions about what factors are likely to drive increase or decrease in the virulence of *G. arcuatus*.

Chapter 2.

Local adaptation of virulence in *Gyrodactylus arcuatus*

Chapter 2. Local adaptation of virulence in *Gyrodactylus arcuatus*

2.1 Abstract

Evolutionary divergence among parasite strains may lead to local adaptation which results in higher average fitness on sympatric rather than on allopatric host populations, but there are few examples of vertebrate host – parasite interactions. A monogenean ectoparasite (*Gyrodactylus arcuatus*) and its three-spined stickleback (*Gasterosteus aculeatus*) host were used to investigate local adaptation of virulence by four strains of the parasite. The strains were collected from wild sticklebacks (one saltwater lagoon connected with the sea and three isolated freshwater lochs) on the island of North Uist, Scotland. Three artificial infection experiments were conducted using lab-bred fish from the four North Uist lochs and from a population in Nottingham, UK (880 Km distant from North Uist) as a tester strain. I used allopatric host-parasite combinations to compare virulence among the parasite strains (virulence was inferred from growth rate of parasite populations) and reciprocal cross infections to examine the extent of local adaptation in host-parasite coevolution. The infection experiments were performed in dechlorinated Nottingham tap water (pH = 7.5) and under controlled conditions of temperature and photoperiod. Parasite strains varied significantly in their virulence on allopatric hosts and the fish population which was host to the least virulent parasite strain was significantly more susceptible to virulent parasites than those hosting such parasites. The three freshwater parasite strains had higher fitness on their sympatric hosts than on allopatric host populations, but the saltwater strain grew better on fish from the most susceptible host than on its native host. These results support the central theory on virulence evolution that virulence decreases when host resistance is low. These results also suggest that under limited gene flow

conditions (isolated environments), coevolution in the stickleback-*G. arcuatus* system leads to parasite local adaptation.

2.2 Introduction

The evolutionary outcome of host-parasite interactions has been intensively studied both theoretically (Anderson and May, 1982; Frank, 1996) and empirically (Ebert, 1994; Herre, 1995). Virulence is thought to positively correlate with parasite reproductive fitness and it is considered a driving factor in host-parasite coevolution (Zhan *et al.*, 2002). Studying the variation of virulence among strains of parasite species may reveal the cause of such variation and it may contribute to a better understanding of how to control parasitic infections (Bull, 1994). To date, most experimental studies of host-parasite coevolution have involved invertebrate hosts (Ebert, 1994; Herre, 1995; Lively, 1999; Kraaijeveld *et al.*, 2003), but vertebrate hosts have been little studied since the classic work of Fenner (1983). The recent studies in the published literature suggest different evolutionary outcomes of host parasite interactions (Table 2.1). Only a few number ($\simeq 6$) of these studies have focused on local adaptation in fish-parasite systems (Konijnendijk *et al.*, 2013), five used controlled experimental infections and one looked at naturally occurring infections. Experiments are more difficult in vertebrate host-parasite interactions, but findings may also differ between invertebrates and vertebrates because of the more sophisticated adaptive immune system of the latter (Hammerschmidt and Kurtz, 2005). Invertebrates lack any sophisticated adaptive immune system. The impact of hosts' defence is important in antagonistic coevolution (Hammerschmidt and Kurtz, 2005) because even small changes in hosts' resistance are thought to lead to large changes in virulence evolution (Best *et al.*, 2009).

The classical view on virulence suggests that all pathogens tend to maximize their fitness by evolving toward benignity (May and Anderson, 1983). Modern theories have increasingly challenged this idea, suggesting that parasite virulence is an evolutionary trait affected by a variety of factors. For example the trade-off hypothesis (Anderson and May, 1982) suggests that parasites have to make an optimal balance between their growth rate on a host and their transmission to new hosts. According to this theory, if parasite transmission depends on the mobility of its host, parasite local adaptation should favour a level of virulence that makes an ideal balance between the parasite transmission and longevity of its host.

Table 2.1. Summary of experiments on local adaptation in horizontally transmitted parasites of vertebrate hosts. These studies included either observations of natural populations (N) or experiments (E). Number of tested populations (pop) ranged between 1- 4 with different designs classified here to two common types: ‘a’ fully reciprocal and ‘b’ not fully reciprocal. The coevolutionary results were expressed as ‘1’ locally adapted, ‘-1’ locally maladapted and ‘0’ no significant difference found between sympatric and allopatric host parasite pairs). Most of these findings were cited and discussed in (a) Lajeunesse and Forbes (2002), (b) Hatcher *et al.* (2005) and (c) Konijnendijk *et al.* (2013).

parasite species	host species	study	pop	design	results	reference
<i>Ceratophyllus gallinae</i>	<i>Parus major</i>	E	2	b	0	(Dufva, 1996) ^{a, b}
<i>Clamator glandarius</i>	<i>Pica pica</i>	N	3	a	-1	(Soler and Moller, 1990) ^{a, b}
<i>Diplostomum phoxini</i>	<i>Phoxinus phoxinus</i>	E	2	a	1	(Ballabeni and Ward, 1993) ^{a, b, c}
<i>Diplostomum</i> sp.	<i>Salmo salar</i>	E	3	a	1	(Voutilainen <i>et al.</i> , 2009) ^c
<i>Gyrodactylus gasterostei</i>	<i>Gasterosteus aculeatus</i>	E	1	b	1	(Raeymaekers <i>et al.</i> , 2011)
<i>Gyrodactylus gasterostei</i>	<i>Gasterosteus aculeatus</i>	E	2	a	0	(Konijnendijk <i>et al.</i> , 2013)
<i>Gyrodactylus</i> sp.	<i>Poecilia reticulata</i>	E	4	a	0	(Perez-Jvostov <i>et al.</i> , 2015)
Haemogregarines	<i>Gallotia galloti</i>	E	3	b	-1	(Oppliger <i>et al.</i> , 1999) ^b
<i>Labratrema minimus</i>	<i>Pomatoschistus microps</i>	E	4	b	0	(Sasal <i>et al.</i> , 2000) ^c
<i>Molothrus ater</i>	<i>Dendroica petechial</i>	N	1	b	-1	(Briskie <i>et al.</i> , 1992) ^{a, b}
<i>Molothrus ater</i>	<i>Turdus migratorius</i>	N	1	b	-1	(Briskie <i>et al.</i> , 1992) ^{a, b}
<i>Tylodelphus clavata</i>	<i>Salmo salar</i>	N	3	a	1	(Voutilainen <i>et al.</i> , 2009) ^c

Parasites are conventionally expected to evolve faster than hosts due to their shorter generation time and bigger population size than their hosts, but subtler effects may also be important. Modern theories suggest that evolutionary outcomes in host-parasite interactions are determined by the process of natural selection, gene flow, genetic drift and temporal change in the environment (Kaltz and Shykoff, 1998; Kawecki and Ebert, 2004). These processes are not completely independent and do not have constant effects on local adaptation (Blanquart *et al.*, 2012). For example, it is thought that limited gene flow and inbreeding conditions contribute to maladaptation in parasites. Local adaptation can be evident when there is high within population genetic variation and bigger differences in the environmental and phenotypical characteristics between populations (see Hereford, 2009). Migration may generally increase in the rate of gene flow and hence population genetic variability (Blanquart *et al.*, 2012). Theories predict that only low migration contributes to parasite local adaptation (Lively, 1999; Blanquart *et al.*, 2012), but intermediate and high migration reduce local adaptation (Gandon *et al.*, 1998). Low migration may allow maintaining of certain levels of gene flow and natural selection favours adaption to local conditions (Slatkin, 1987) while high migration can hinder local adaptation because migrants will repetitively introduce new genes resulting in adaptation to various conditions (Gandon *et al.*, 1998). Thus, estimating the amount of gene flow can provide better understanding about the process of local adaptation (Kawecki and Ebert, 2004).

Aquatic ecosystems like lakes may be good places to examine host-parasite coevolution because their geographical isolation means that such systems are often evolutionarily independent (de Roij *et al.*, 2011). Lakes can vary substantially in ecological conditions (host density, competitors and predators) and environmental factors (water chemistry, pH and temperature). Even at a small scale, such differences have been found to lead to large

differences in parasite communities among populations of three-spined stickleback fish (de Roij and MacColl, 2012).

The interactions between three-spined stickleback and its parasite species have been used to understand several aspects of host-parasite coevolution. For example, in studying the impact of environmental variation on parasite infections of sympatric hosts (Karvonen *et al.*, 2013), the parasite role in evolutionary immunology (de Roij *et al.*, 2011; Franke *et al.*, 2014), divergence of hosts (MacColl, 2009), host local adaptation (Lenz *et al.*, 2013), and parasite local adaptation and virulence evolution (Hammerschmidt and Kurtz, 2005; Konijnendijk *et al.*, 2013). Raeymaekers *et al.* (2011) found that an isogenic strain of *G. gasterostei* could successfully infect its home stickleback hosts, but failed to infect an allopatric host population under the same environmental conditions. Reciprocal transplant experiments provide a useful tool for studying such coevolutionary patterns (Kawecki and Ebert, 2004). The design of such experiments has to include comparison of the average fitness of a parasite between sympatric and allopatric hosts within similar environmental conditions that mimic the parasite's natural environment.

The island of North Uist, Scottish Western Isles provides an ideal study system. It comprises many geographically separate brackish and freshwater lochs, most of which contain sticklebacks which experience large differences in parasitic infections (de Roij and MacColl, 2012). These lochs also exhibit large ecological and environmental variation (MacColl *et al.*, 2013) for example in environmental variables such as water chemistry, salinity and pH (Giles, 1983), as well as in the presence and density of fish predators and competitors (Soleng and Bakke, 1998; Stephenson *et al.*, 2015).

In this study, a monogenean ectoparasite (*Gyrodactylus arcuatus*) and its three-spined stickleback (*Gasterosteus aculeatus*) host were used to examine virulence variations among strains of the parasite and to assess the extent of local adaptation in fish-parasite coevolution. The study involved three isolated freshwater bodies (Maga, Scad and Reiv) and a saltwater lagoon (Obse) which is connected with the Sea allowing high influx of genes into the fish population and its parasites (Table 2.2). *Gyrodactylus* sp. are viviparous monogenean flatworms of Phylum Platyhelminths. Monogeneans are generally described as very host-specific parasites (Poulin, 1992; Whittington *et al.*, 2000; Mendlová and Šimková, 2014). The genus *Gyrodactylus* is commonly seen on the fins, gills and skin of many fish species. They can directly reproduce asexually and sexually on fish hosts (Harris, 1989; Schelkle *et al.*, 2012), can transmit directly between hosts and can survive on dead hosts for a short time (Olstad *et al.*, 2006). These parasites attach and feed on the cells and mucous of fish skin (Bakke *et al.*, 2007), consequently they cause epithelial damage which may lead to host death by reducing respiratory and barrier functions of epithelium and rendering the host susceptible to secondary infections by other pathogens (Cone and Odense, 1984). The degree of *Gyrodactylus* sp. virulence depends on the parasite growth rate on an infected host and host size (Cable and Oosterhout, 2007a). For example, there was a strong positive correlation between the growth rate of parasite infections and parasite induced host death using *G. turnbullis* on guppies (Scott and Anderson, 1984) and *G. salaris* on salmon (Bakke and MacKenzie, 1993). In this study, virulence was determined by estimating the growth rate of the parasite (number of parasite per host) and their infection success (percentage of hosts infected) on allopatric hosts, including a geographically distant host ‘tester’ stock. The measures were then compared between sympatric and allopatric hosts to assess the extent of parasite local adaptation.

Table 2.2. Location and conductivity of lochs on North Uist, Scottish Western Isles, those were included in this study.

Loch name	Loch code	Grid	Area (ha)	Conductivity (mS.cm ⁻¹)
nam Magarlan	Maga	57°35'16.9"N 7°26'38.7"W	6.5	261.8
Ob nan Stearnain	Obse	57°36'06.1"N 7°10'25.4"W	10	45100
na Reivil	Reiv	57°36'42.9"N 7°30'53.2"W	6.1	433.3
Scadavay	Scad	57°35'05.5"N 7°14'11.2"W	552	144.4

2.3 Materials and methods

Experimental design

This chapter involved three experiments: The first experiment was designed to quantify differences in virulence among four *G. arcuatus* strains on an allopatric (tester) fish population. The second experiment included a reciprocal cross infection between three strains of the parasite and their hosts and in the third experiment one strain of the parasite was tested on sympatric and two allopatric hosts to examine the extent of local adaptation among the parasite strains (Fig. 2.1). Throughout these experiments sticklebacks were raised in the laboratory from wild parents and their parasitic *Gyrodactylus* sp. from four isolated lochs on the island of North Uist which are referred to as: ‘Obse’, ‘Reiv’, ‘Scad’ and ‘Maga’ lochs (Table 2.2). In addition, sticklebacks raised in the laboratory from wild parents from a freshwater lake in Nottingham, Jubilee Lake (880 km away from North Uist) were used as an allopatric tester host for the four North Uist gyrodactylid strains. In experiment 1, the four parasite strains (Obse, Reiv, Scad and Maga) were used to separately infect four groups of Jubilee fish in order to ascertain the virulence of each North Uist parasite strain (measured as the growth rate of the *Gyrodactylus* sp. infection). This was a pilot experiment and therefore involved only 30 fish divided in to four groups

of 6, 8, 8 and 8. In experiment 2, a reciprocal cross infection was performed between Obse, Reiv and Scad parasites and their host populations. This experiment involved 102 sticklebacks using 34 individuals (including six uninfected controls) from each of the three fish populations. In experiment 3, the Maga parasite was used to infect its sympatric (Maga) host and two allopatric (Obse and Scad) host populations. This experiment involved 18 sticklebacks using 6 individuals from each of the fish populations.

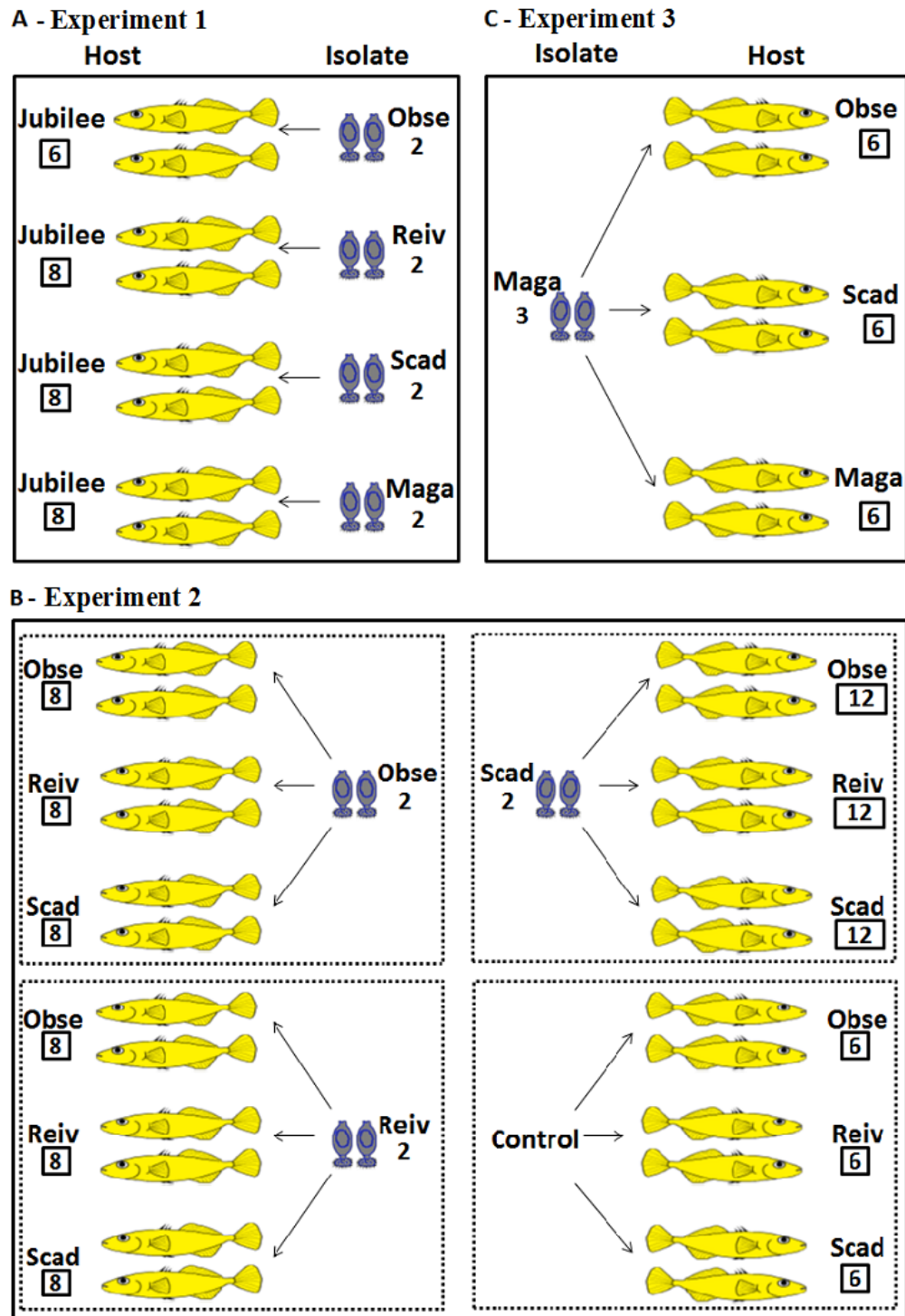


Figure 2.1. The schematic diagram depicting the experimental design. Each two adjacent sticklebacks represent a separate group of fish populations (Host) with different fish number (the bolded number in boxes) either were infected with parasite strain(s) or kept as uninfected control. Each individual fish was initially infected with 2 worms (bolded number) in the first (A) and second (B) experiments, but in the third experiment (C) 3 worms were used to infect an individual fish.

Study areas and fish sampling

Fish population samples were collected using minnow traps ('Gees', Dynamic Aqua, Vancouver) during April-May 2013 from four geographically isolated lochs on the island of North Uist in Scotland. For each of the fish populations, 36 minnow traps were set in the morning and left overnight. Traps were set in pairs around loch shores. Gaps of at least five meters were left between trap pairs. The four lochs were chosen because of their contrasting environmental conditions (MacColl *et al.*, 2013). Obse is connected with the sea at high tides and is saline, while the others are isolated freshwater lochs (Table 2.2).

Fish breeding and feeding

Approximately five fish families were raised for each of the Obse, Reiv, Scad, Maga and Jubilee fish populations. This was done by artificially cross breeding males and gravid females of three-spined sticklebacks on North Uist as described in de Roij *et al.* (2011). In brief, fertilised eggs were transported on ice to the aquaria of the School of Life Sciences at the University of Nottingham and incubated until day 10 in oxygen saturated dechlorinated tap water with 2 ppt salt and methylene blue. At day 10, each clutch was separately moved into one half of a 100L glass tank partitioned with fine mesh. Tanks were filled with dechlorinated Nottingham tap water (approx. pH 7.5) and provided with a biological filter (Fluval, Askoll, Italy) and an air source under controlled temperature and photoperiod conditions mimicking the fishes' natural habitat. After hatching, fry were fed on different regimes, starting with *Paramecium caudatum* until day 7 and then with a mixture of *P. caudatum* and freshly hatched brine shrimp nauplii until day 14. After 14 days, fry were fed on brine shrimp nauplii alone until day 30 and then changed to on a

mixture of Brine shrimp and chopped bloodworm defrosted from frozen (gamma blister bloodworm, Tropical Marine Centre, UK) for 60 days. After that, fish were fed on whole blood worm, defrosted from frozen, until the end of the experiment. Infected fish were housed individually in a 3L plastic tank containing 2L of dechlorinated tap water from the school aquarium. For each tank 50% of the water content was changed with clean water from the same source every 3 days. All the fish were housed in a room with controlled temperature ($13.5 \pm 1^\circ\text{C}$) and 16:8 of light/dark photoperiod mimicking the external conditions on North Uist isle. Infected fish were monitored twice daily and if a fish did not swim well or was not feeding properly, it was killed as mentioned above.

Parasite breeding and artificial infections

At the same time that fish were collected to make crosses, sticklebacks were also collected to establish lab populations of *G. arcuatus*. The parasite strains were identified to species level using morphological characteristics of the hard parts (opisthaptor) and excretory system (see chapter 1). These were passaged on naive allopatric lab fish (CHRU, CHRU-OBSM hybrid and Jubilee) for about six months, until parasites were required for infection experiments, according to the following protocol. Twenty wild fish were caught from each of Maga, Scad, Reiv and Obse (April- May 2013) and transferred in polystyrene boxes to the aquarium at the School of Life Sciences at the University of Nottingham. The boxes containing water from lochs where fish originated were supplied with a source of air using a battery air pump (JAD 200, Germany). At the aquarium, wild sticklebacks were moved to separate 100L glass tanks divided to two partitions containing dechlorinated water with air sources and biological filter in a temperature ($13.5 \pm 1^\circ\text{C}$) and photoperiod (16:8 light/dark) controlled room. After a week all the fish were killed using an overdose of MS222 (400mg.L^{-1}). Parasites attached to small portions of skin or fin tissues were

isolated and placed into a Petri dish containing water from the school aquarium. Parasite samples collected from each donor fish population were pooled in a petri dish and left for few minutes to let parasites detach from the infected tissues. Several individuals of an allopatric lab bred stickleback populations were separately infected with the parasite strains to continue the parasite life cycle. This was carried out by gently bringing the caudal fin of each receiver into proximity with the parasites in a dish until 2-3 worms were successfully attached. The growth of infections on passage fish was regularly examined at least once a week and whenever parasite load became high (>50 worms per fish), fish were humanely euthanized as described above and 2-3 worms were passed to restart the infection on a freshly selected lab bred stickleback. Newly infected fish were then kept in small groups in 100 l aquaria divided into two partitions. In this manner, parasites were serially passaged until they were needed in infection experiments.

Experimental fish were infected in a similar way to passage fish. For the first and second experiments there was a limited number of parasites available and hence two worms were used to start infections, but the in the third experiment individual fish received 3 worms. At the start and end of the experiments, standard length and wet weight were measured for the fish. Total worm number (including the initial worms) on each fish was counted approximately every 4 days in the first experiment until day 36, every 3 days in the second to day 28 and on days 5, 13 and 20 in the third experiment. The procedures of infection and monitoring were carried out under a weak concentration of MS222 (100mg.L^{-1}), sufficient to induce gentle anaesthesia in the experimental fish. The first experiment was set up about months earlier (Dec. 2013) than the two subsequent experiments (Feb. 2014). During the course of infections, parasite counting was carried out by scanning fins, spines and body surface including head parts (mouth, eyes, operculum, and on gills) and at the

end of the experiments fish were dissected and both oral cavity and gills were thoroughly examined for the parasite. All the fish were eventually killed at the end of the experiments and dissected for gender identification. I designed and conducted all the experiments under a UK Home Office Project License 40/2886, and in accordance with the authorization of the Ethical Review Committee of Nottingham University.

Statistical analysis

Two response variables were derived from the data from each experiment. ‘Infection success’ took a value of one if any *Gyrodactylus* sp. were present on a fish at the first count (for the first exp: on day 4, second exp: day 3 and third exp: day 5) following infection, and zero otherwise. Infection success was analysed as a binary variable using a Generalized Linear Model (GLM) with binomial error and logit link function. ‘Total parasite count’ was calculated as the total of all counts for each fish from day ‘0’ to the last day of the experiment (de Roij *et al.*, 2011). Total parasite count for each experiment was separately analysed using a univariate General Linear Model. Main effects for each of total parasite load and infection success included only parasite strain for the first experiment, and only fish population for the third experiment. For the second experiment, data were analysed in two ways; first, excluding data for sympatric infections, with parasite strain as the only explanatory variable to look at the effect of parasites’ origin on their average performance on allopatric hosts and second, including all data, with parasite strain, fish population and their interaction as explanatory variables to determine whether local adaptation was present (assessed from significance of the parasite strain x fish population interaction).

Effect size of local adaptation was estimated using an approach developed by Rosenberg *et al.* (2000) and used by other studies (Hoeksema and Forde, 2008; Konijnendijk *et al.*, 2013) to investigate parasite local adaptation. The effect size (E) was measured as natural log ratio of ' X_S/X_A ' where ' X_S ' is the mean fitness measurements of the parasite strains on their sympatric hosts and ' X_A ' is the mean fitness measurements of the strains on allopatric hosts. If the mean value of E value is positive, a parasite is thought to be adapted to its local hosts and if 'E' is negative a parasite is thought to be maladapted to its local hosts.

It was of interest to determine the effect of the parasite on the host, measurements of the infected fish at the beginning and end of experiments were used to calculate a daily growth rate for individual sticklebacks, and to see whether this was affected by infection (de Roij *et al.*, 2011). Fish daily growth rate was estimated using this equation: Specific growth rate (SGR) = $[(L_{End}) - (L_{Start})] / t$, where L_{Start} and L_{End} indicate the standard length measured at the start and at the end day of experiments respectively and "t" is the infection time (days). 'SGR' values were also compared between fish treatments using one way ANOVA for independent samples followed by a *post hoc* test of the difference between means using Bonferroni's multiple comparisons test. As the effect of *Gyrodactylus* sp. infections on fish growth is assumed to be negative which is one directional, a one tailed Pearson correlation was performed between the total parasite count and hosts' SGR values.

Non-normally distributed data sets were log transformed to achieve normality. Statistical tests were performed to examine the differences between groups using 0.05 as the level of significance using the SPSS package (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

2.4 Results

Performance of parasite strains on allopatric hosts

The time course of infections on allopatric tester hosts differed significantly between the parasite strains. The total worm load and infection success of the Scad parasite were consistently lower than the other strains on allopatric fish populations in the first and second experiments (Fig. 2.2).

In the first experiment, the four parasite strains were significantly different in the total parasite count ($F_{3, 26} = 3.14$, $P = 0.042$, Table 2.3A(i)), but no significant difference was found in paired comparison tests ($P > 0.05$, Fig. 2.2A). The four parasite strains differed significantly in infection success ($P = 0.041$, Table 2.3 B(i)) and paired comparison tests showed that the infectivity of Maga was significantly higher than that of the Scad parasite ($P < 0.01$, Fig. 2.2B). In the second experiment, measurements of parasite performance were also found to be significantly different among the three parasite strains on allopatric hosts. There was a large difference in the total parasite load (GLM, $F_{2, 53} = 10.93$, $P < 0.001$). *Post hoc* tests showed that both Obse and Reiv parasites had significantly larger population growth than the Scad strain ($P < 0.001$, $P < 0.01$ respectively, Fig. 2.2C). The infection success of both Obse and Reiv were significantly higher than the Scad parasite (GLM with binomial error and logit link, Wald $\chi^2 = 86.81$, $df = 2$, $P < 0.001$). *Post hoc* tests showed that Obse had higher infection success than Reiv and Scad parasite ($P < 0.05$ and $P < 0.001$ respectively) strains and the difference between Reiv and Scad was also significant ($P < 0.001$, Fig. 2.2D).

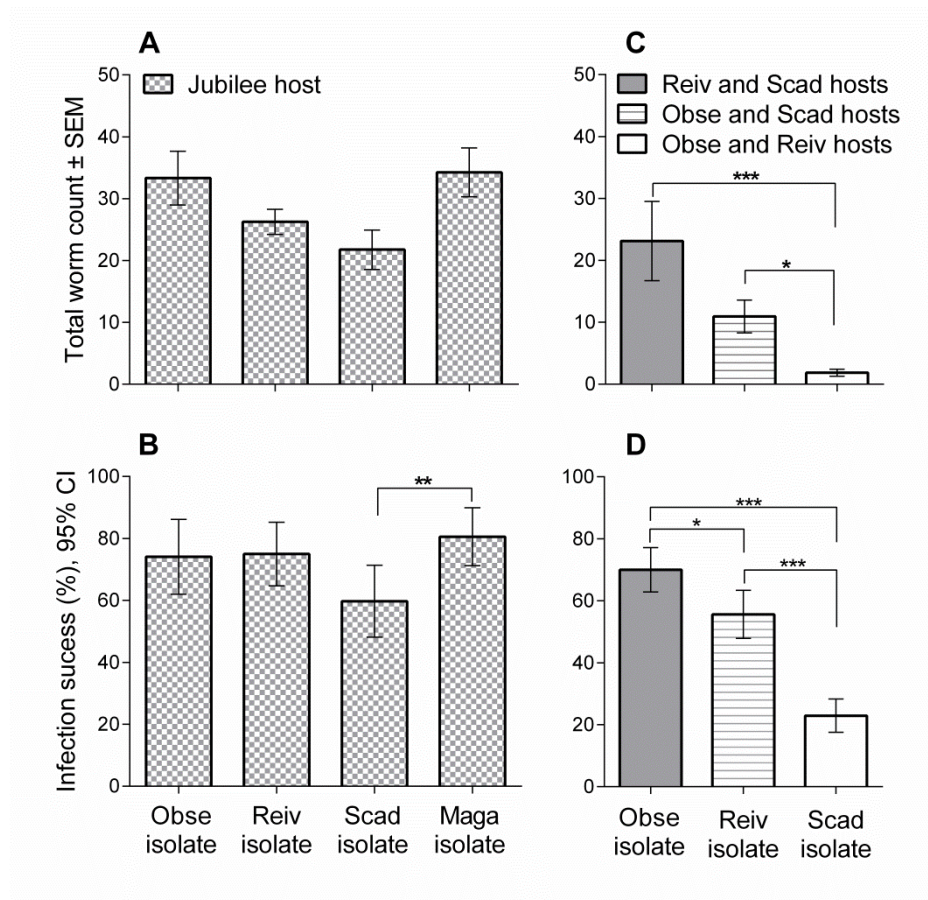


Figure 2.2. Virulence of parasite strains on allopatric hosts. Differences in the total worm load and average of infection success among Obse, Reiv, Scad and Maga parasites on one allopatric host (Jubilee fish: dotted) in the first experiment (A and B respectively) and among Obse, Reiv and Scad parasites on two allopatric host populations in the second experiment (C and D respectively). Note: In the second experiment, each of the three parasite strains was tested on its sympatric and two allopatric hosts, but only their average measures on allopatric hosts (Reiv and Scad: shaded; Obse and Scad: lined; Obse and Reiv: plain) are used in this figure. The values for total worm load are given with SEM and for infection success are given with 95% confidence intervals. Asterisks above the error bars represent results of *post hoc* tests indicating the presence of significant differences (* = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$).

Table 2.3. Statistical analysis of the outcome of three artificial infection experiments. GLM of the total worm count (A) and infection success (B) for (i) the four parasite strains (Obse, Reiv, Scad and Maga) on one allopatric host in experiment one, (ii) three parasite strains (Obse, Reiv and Scad) in a reciprocal cross infection between the parasites and their hosts in experiment two and (iii) one parasite strain (Maga) on its sympatric and two allopatric host populations in experiment three.

Source of variation	DF	(A) Total worm load		(B) Infection success	
		F	P value	χ^2	P value
(i) <i>Experiment one</i>					
Intercept	1	286.8	< 0.001	49.6	< 0.001
Parasite strain	3	3.1	0.042	8.2	0.041
(ii) <i>Experiment two</i>					
Intercept	1	178.1	< 0.001	12.7	< 0.001
Parasite strain	2	7.2	0.001	66.4	< 0.001
Fish population	2	4.8	0.011	40.9	< 0.001
Parasite strain * Fish population	4	3.1	0.021	45.5	< 0.001
(iii) <i>Experiment three</i>					
Intercept	1	446.8	< 0.001	8.7	0.003
Fish population	2	21.5	< 0.001	9.2	0.01

Is there local adaptation to the host population?

In experiment 2, the performance of parasite strains depended on the fish population that they infected (for parasite load, $P = 0.011$, for infection success, $P < 0.001$, Table 2.3A(ii), B(ii)). All parasites did best on their own host population, with the exception of Obse, which did best on Scad. Scad hosts supported the highest infection levels overall (Fig. 2.3). The total worm count of each of Reiv and Scad was significantly higher on sympatric than allopatric host populations (for Scad parasite on sympatric host, $P < 0.01$ vs Obse host and $P < 0.001$ vs Reiv host; for Reiv parasite on sympatric host, $P < 0.05$ vs Obse host and $P > 0.05$ vs Scad host, Fig. 2.3A). Interaction between parasite origin (strain) and fish origin (population) was significant ($P = 0.021$). For these two parasite strains, the average infection success was also significantly higher on their own hosts than allopatric

populations (Fig. 2.3B) and the effect of parasite strain and host origin interaction was extremely significant ($P < 0.001$, Table 2.3 B(ii)).

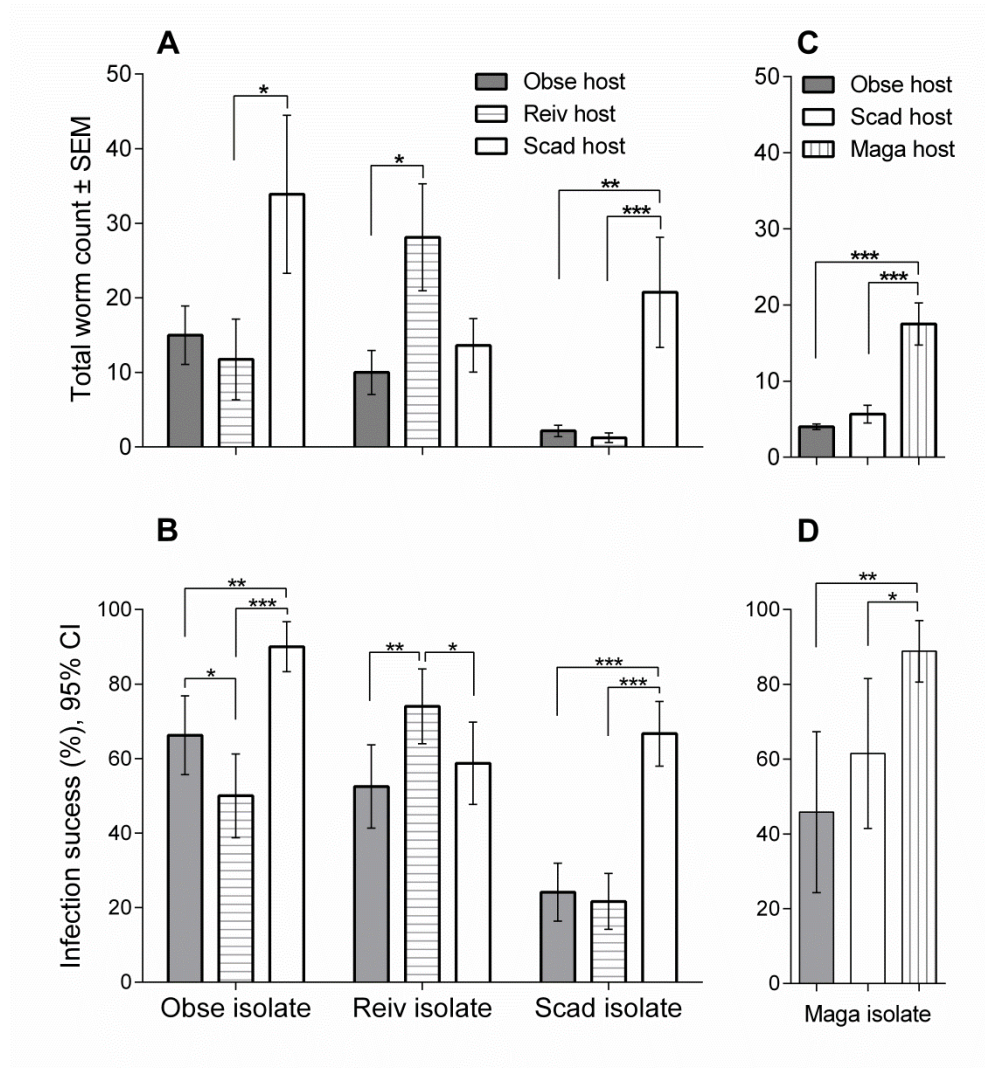


Figure 2.3. Differences in the total worm load and average of infection success measured for each parasite strain on its sympatric and two allopatric hosts. In the second experiment (A and B), each of Obse, Reiv and Scad parasites was tested on three fish populations (Obse: shaded; Reiv: horizontally lined and Scad: plain) and in the third experiment (C and D), Maga parasite was also tested on three fish populations (Obse: shaded; Scad: plain and Maga: vertically lined). Note: error bars and asterisks are same as fig. 2.2.

In experiment 3, Maga parasite strain performed better on its sympatric Maga fish than Obse and Scad host populations (Fig 2.3C, D). For this isolate, the total worm load and infection success significantly differed among Maga, Obse and Scad fish populations (for

total worm load, $P < 0.001$, Table 2.3A(iii) and for infection success, $P < 0.01$, Table 2.3B(iii)).

The three parasite strains (Reiv, Scad and Maga) consistently had positive values of the effect size 'E' measured for the total worm count and infection success, but the Obse parasite strain had negative 'E' values. The Obse parasite showed higher burdens on Scad fish than on other populations, but there was no statistically significant difference in total worm value between sympatric and allopatric treatments.

Specific growth rate (SGR) among fish populations

There was little overall effect of infection on growth. SGR values did not differ significantly between the control and infected treatments for Obse and Reiv fish populations (for Obse, $F_{3,30} = 2.23$, $P = 0.105$; for Reiv, $F_{3,30} = 0.90$, $P = 0.451$), but the difference was significant for the Scad fish population ($F_{3,30} = 4.15$, $P = 0.014$). *Post hoc* tests revealed that the Scad fish which were infected with sympatric parasites had significantly lower SGR value than the uninfected Scad treatment ($P = 0.026$), but the SGR of Scad fish which were infected with allopatric parasites was only slightly lower than the control group ($P > 0.05$). There was a strong negative correlation between SGR of Scad fish infected with Scad parasite and the total worm load (Pearson correlation, $r(18) = -0.60$, $P = 0.008$). Neither fish sex nor their size was significant in any of the statistical analysis the infection success and population growth of the parasites and their effect on the daily growth rate of stickleback populations.

2.5 Discussion

Parasite virulence differed consistently between parasite strains across the three experiments. Overall, results of the current chapter suggest that parasites are adapted to their local host fish population, although the most virulent parasites did better on the weakest host than on its sympatric host population. Although parasite local adaptation is a common prediction of theoretical models of host-parasite coevolution, there have been few reports of it in experimental studies of vertebrate host-parasites (but see Ballabeni and Ward, 1993; Voutilainen *et al.*, 2009). Sticklebacks may provide a model system in this regard, since the isolation of water bodies from one another may favour evolutionary divergence. Given the direct transmission of *G. arcuatus*, and its rapid reproductive strategy it is likely that gene flow between parasite populations will be higher than between host populations, and this may favour local adaptation of the parasite (Raeymaekers *et al.*, 2011).

Apparent lack of local adaptation in one of the parasite strains (Obse) has an obvious explanation. Two ecotypes of three-spined sticklebacks coexist in this saltwater lagoon which is flooded by the sea at spring tides. I used fish of (and parasites from) the ‘resident’ phenotype which inhabit this waterbody year-round. However, anadromous fish also enter this lagoon to breed in the spring. It seems very likely that the gene flow between fish or parasites that results may disrupt the potential for local adaptation (Lively, 1999). In this regard, my results agree with previous studies on the evolutionary outcomes of fish parasite combinations originated from non-isolated waterbodies. For example, Sasal *et al.* (2000) used four strains of a digenean flatworm (*Labratrema minimus*) and *Pomatoschistus microps* hosts and Konijnendijk *et al.* (2013) used two strains of *G. gasterostei* and three-spined stickleback hosts, Perez-Jvostov *et al.* (2015) used four

populations of *Gyrodactylus* sp. and guppy (*Poecilia reticulata*) hosts. In the three studies, the parasite strains in general did not show differences quantitatively between the sympatric and allopatric host infections. In such scenarios parasite local adaptation could be absent because gene flow in hosts is expected to be higher than in the parasite (Konijnendijk *et al.*, 2013). Compared to the other strains, Obse could successfully infect a range of host populations including its sympatric hosts. This might suggest another possible reason that high gene flow levels introduce new genes to the parasites population thereby contributing substantially to the parasite strain adaptation to different hosts and consequently lacks local adaptation (Gandon *et al.*, 1998). Experiments which do not find local adaptation are not failed experiments, but they are illustrations that populations might be blocked from reaching optimal adaptation (Hereford, 2009).

Generally *G. arcuatus* did not appear to have a large effect on the growth rate (SGR) of sticklebacks, although the short length of infections may have made such effects difficult to detect (de Roij *et al.*, 2011). The effect of *G. arcuatus* is likely to be greater in longer infection experiments, because the parasite reduces fish immunity to secondary infections by microbial pathogens (Cone and Odense, 1984). Nevertheless it is interesting that among all of the fish populations, Scad, which was least resistant to infection, was the one population that showed a significant negative correlation between fish growth and parasite growth load. Taking into account both susceptibility to the parasite and growth reduction due to the experimental infections might suggest that parasite induced fish mortality is likely to be higher in the Scad fish population, possibly increasing the selection pressure on Scad parasites toward low virulence. Alternatively, Scad parasite strain may have low virulence for other reasons, and this may have meant that resistance has not been selectively favoured in their Scad stickleback.

The idea of the influence of host ecology on the evolution of parasite life history traits has been well established (de Roode *et al.*, 2008a; Duffy *et al.*, 2012). Heterogeneity in the biotic and abiotic environments is arguably an important reason associating with genetically slower growth of Scad worms compared to the other three parasite strains on allopatric hosts. It is, however, not yet clear whether the adaptation of parasite life history traits is directly affected by environmental conditions which are experienced by hosts or is an adaptation to the changes in the life history traits of their hosts. Parasite virulence can be influenced by various aspects of hosts such as competition (Johnson *et al.*, 2009), predation (Ebert and Mangin, 1997; Choo *et al.*, 2003), life span (Nidelet *et al.*, 2009), growth conditions (Magalon *et al.*, 2010) and behaviour (Ezenwa, 2004; Ostlund-Nilsson *et al.*, 2005). Estimation of these factors and correlating them with parasite's virulence may provide an important insight on the evolution of the parasite life history traits and host exploitation.

Performance measurements (the total worm load and infection success) of the four parasite strains were generally higher in the first experiment, on Jubilee fish (i.e. an allopatric host population in the first experiment) than in subsequent experiments on the other host populations including their sympatric hosts. However it is difficult to compare results across separate experiments, they could arise from differences in host genotypes, but might also be the result of seasonal differences or small differences in experimental conditions. The parasites' performance seemed to gradually decrease with time during passaging on all host populations, from the date of sampling (May 2013) until the end of the third experiment (March 2014) (data are not shown). As the parasite strains were continuously passaged on several allopatric fish genotypes, their short generation time and artificial selection of inoculums on every passage may have reduced genetic variation within the lab

populations. Another possible reason for the difference in infection trajectories on the allopatric tester versus local allopatric North Uist populations (see Fig. 2.2A and B) is that the allopatric host population used in the first experiment (Jubilee fish) may be naturally susceptible to *G. arcuatus* infections. Fish populations can vary genetically in resistance to *Gyrodactylus* sp. (Madhavi and Anderson, 1985) which could be due to divergent selection caused by the parasite in their natural habitats (de Roij *et al.*, 2011). The natural prevalence of *Gyrodactylus* sp. on Jubilee fish was found to be very low (Mahmud's unpublished data) and that may also be a good reason why Jubilee fish are particularly susceptible to infection.

In conclusion, my study illustrates that strains of *G. arcuatus* vary importantly in their virulence levels on allopatric hosts and the parasite strain from the most susceptible host had the lowest virulence levels. These results support the trade-off hypothesis for virulence evolution which suggests that natural selection optimises parasite fitness by trading-off between the parasite reproduction rate and its host's ability to survive the infection (Alizon *et al.*, 2009). The overall fitness measurements of three freshwater parasite strains were higher on sympatric than allopatric hosts. As both the hosts and their parasites were raised in the lab, the difference among the parasite growth should be due to genetic makeup. These results are consistent with Raeymaekers *et al.* (2011) findings that coevolution in the stickleback-*Gyrodactylus* sp. system results in parasite adaptation to its local host fish. These results also suggest a role for environmental conditions in natural habitat in the evolutionary divergence of stickleback hosts (MacColl *et al.*, 2013) and this may indirectly feedback to affect parasite life history. These results may inspire more studies on virulence using this host parasite system.

Chapter 3.

Toward understanding the influence of environmental and host related factors on the survival and performance of *Gyrodactylus arcuatus*.

Chapter 3. Toward understanding the influence of environmental and host factors on the survival and performance of *Gyrodactylus arcuatus*.

3.1 Abstract

Ectoparasites must forge an existence at the interface of two hostile environments: the host that they exploit, and the environment in which it lives. Successful reproduction depends on ability to exploit the host, while successful transmission in directly transmitted parasites is predicted to correlate positively with parasite ability to survive in the external environment. Here, I examine some of the factors that affect the ability of both lab-bred and wild strains of *Gyrodactylus* ectoparasites of three-spined stickleback (*Gasterosteus aculeatus*) to exist at the interface between the host and its aquatic environment. I quantify local adaptation of the parasite to its external aquatic environment, in terms of its ability to survive away from the host in water from different lochs with contrasting pH. I also quantify the effect of host epidermal mucus, considered as an important defence mechanism against ectoparasites in fish, on parasite survival. In addition I examine variation between host populations in mucous cell morphology (size and density), and relate this to parasite density. Survival of *Gyrodactylus* sp. differed significantly among parasite strains, and this was reduced at higher temperature. Parasite strains from lochs with naturally higher pH survived for longer at 11.5°C, but this pattern reversed at 14.5°C. Epidermal mucus samples did not affect parasite longevity *in vitro* and fish experimentally infected with *Gyrodactylus* sp. responded to the infection by reducing their epidermal mucus cells. This was supported by field data in which the average density and size of host epidermal mucous cells was negatively associated with the natural abundance of gyrodactylids. These results may point to the existence of adaptive relationships both

between the parasites' life span and chemical properties of their natural environment and between fish mucous cells and the parasite natural abundance. Finally, I discuss the reduction of host epidermal mucous cells as a defence mechanism that could be used by sticklebacks to minimize infections with *Gyrodactylus* sp.

3.2 Introduction

Descriptions of the interaction between ectoparasites and their biotic and abiotic environments are essential to understand how ectoparasites sustain themselves in the wild. Obligate parasites permanently depend on their hosts for survival. Therefore, they have to cope with the environment in which their hosts live. The effect of environment on ectoparasites can be direct as they are exposed to the surrounding external physical and chemical variables and/or indirect through inducing changes in their biotic environment such as the host immune system. For aquatic ectoparasites, especially those which do not have a specific transmission stage (lack encystation and vectors), the environmental effects can be even more important (Bakke *et al.*, 2007). Long term exposure to geographically isolated environments with different conditions, populations may lead to changes in the biological traits of host-parasite interactions (Tinsley, 1999). Temporal and spatial differences in the natural environment may drive selection on hosts and parasites (Kaltz and Shykoff, 1998; Kawecki and Ebert, 2004). In many host parasite systems, it has been reported that environmentally induced changes in the life history traits of one partner can induce changes in the other (Vale *et al.*, 2008; Wolinska and King, 2009; Vale *et al.*, 2011). This phenomenon is commonly known as host- parasite coevolution (Anderson and May, 1982; Gandon and Michalakis, 2002; Best *et al.*, 2009).

Gyrodactylus sp. are microscopic monogenean ectoparasites of many species of marine and freshwater fish. They are viviparous, and a newly born individual contains more than one growing embryo located one inside another, a biological feature that allows the parasite to build an entire population from a single worm (Cable and Harris, 2002).

Gyrodactylus sp. are obligate parasites with direct lifecycles. They are characterized by high host specificity and thus often spend their entire life on one host species (Bakke *et al.*, 2002; Buchmann *et al.*, 2004). On infected fish, the parasite population continuously grows in size until the damage caused by infection results in host death or until the parasite population is controlled by host immune responses (Scott and Anderson, 1984). They live on hosts' skin, fins, and gills which often cause damage to skin tissues due to their feeding and movement (Kearn, 1979; Cone and Odense, 1984). The population size of *Gyrodactylus* sp. on individual hosts is highly variable, depending on host's body size (Cable and Oosterhout, 2007a) and defence strategies (Scott and Anderson, 1984; Bakke and MacKenzie, 1993; Buchmann and Uldal, 1997), and environmental conditions where the (Polo *et al.*, 2004; Steverding *et al.*, 2005; Gheorghiu *et al.*, 2007; Gheorghiu *et al.*, 2012). *Gyrodactylus* sp. lack a specific transmission phase. Like her mother, a newly born daughter is fully mature and attaches to its host regardless of its age. Transmission to new hosts is possible at any time (Bakke *et al.*, 2007). These parasites can transmit to new hosts using different routes such as contact with infected (live or dead) hosts and with detached worms in the water column or on the surface of substrates (Bakke *et al.*, 1992). Survival rate of detached *Gyrodactylus* sp. is an important factor affecting their opportunity for transmission, because detached parasites are thought to use the strategy of 'sit and wait' until a new susceptible fish host approaches (Bakke *et al.*, 2007). Understanding the relationship between parasite survival rate and water quality to which they are exposed

may therefore be important in making predictions about their transmission (Walter and Ewald, 2004).

Monogenean ectoparasites in general are thought to be affected by the surrounding habitat conditions, affecting their dispersal and population dynamics (Chubb, 1977). Several findings have reported the potential role of physico-chemical properties of water on the survival of *Gyrodactylus* sp. For example, Cable *et al.* (2002) suggested that detached *G. gasterostei* can survive until the depletion of energy resource and Soleng and Bakke (1997) suggested that survival time of *G. salaris* decreases with increasing salinity. Water temperature was negatively correlated with the survival time of detached worms in these two studies and with the survival of *Gyrodactylus* sp. on dead salmon (Olstad *et al.*, 2006). Other environmental factors such as water pH and aqueous aluminium (Soleng *et al.*, 1999), waterborne zinc (Gheorghiu *et al.*, 2012) and several metals (Polo *et al.*, 2004) also have been found to affect the growth and survival of *Gyrodactylus* sp. Additionally, some host related factors such as epithelial cells and epidermal mucous have been found to negatively affect the survival time of detached *Gyrodactylus* sp. (Harris *et al.*, 1998; Buchmann *et al.*, 2000). Despite their sensitivity to such environmental challenges under lab conditions, it is apparent that these parasites are able to survive, at least to some degree, in these hostile environments in the wild. Understanding how these monogeneans can cope with such variable environments may improve our knowledge about their infection dynamics and virulence.

The distribution of *Gyrodactylus* sp. on the bodies of fish is thought to vary depending on factors related to the parasite, the hosts and environmental conditions. For example, host immune responses such as epidermal mucous cell density (Buchmann and Uldal, 1997; Buchmann and Bresciani, 1998; Pie *et al.*, 2006), within population competition of

Gyrodactylus sp. (Anthony, 1969; Blažek *et al.*, 2008) and water temperature (Anthony, 1969). Buchmann and Bresciani (1998) suggest that mucous cells play a critical role in the site preference of *Gyrodactylus* sp. because these cells excrete substances that can potentially affect parasite behaviour. In some fish species, the distribution of mucous cell density has been reported to vary greatly among different body sites (Pickering, 1974) and it has been found to negatively affect *Gyrodactylus* sp. both *in vitro* (Harris *et al.*, 1998; Buchmann *et al.*, 2000) and *in vivo* (Buchmann and Uldal, 1997; Buchmann and Bresciani, 1998; Gheorghiu *et al.*, 2012).

Here, I examined the relationship between the survival time of *Gyrodactylus* sp. and environmental factors. I used strains of the parasite isolated from naturally infected three-spined stickleback (*Gasterosteus aculeatus*) populations from eight geographically separate water bodies (lochs) on North Uist island in north west Scotland. I reciprocally exposed these parasites to water collected from their own and different lochs to examine the extent of parasite local adaptation to the quality of their aquatic environment.

Reciprocal transplant experiments are highly effective for studying local adaptation in parasites (Kawecki and Ebert, 2004) because such experiments allow comparing the mean fitness of a parasite strain between local and different environments. I used wild and lab bred fish to study both the relationship between parasite survival time and epidermal mucus *in vitro* and the *in vivo* association between parasite population size and mucous cell morphology. Three-spined stickleback have frequently been used as a host model system in studies of survival, population growth, infection dynamics and transmission of *Gyrodactylus* sp. (see Lester and Adams, 1974; Soleng and Bakke, 1998; Brooker *et al.*, 2011; de Roij *et al.*, 2011; Raeymaekers *et al.*, 2011). This small fish (approx. 2-5 cm) is easy to breed, keep and experimentally infect with *Gyrodactylus* sp. under controlled lab

conditions. Populations of North Uist sticklebacks experience a range of both chemico-physical variables such as water conductivity, pH, and alkaline metals (Giles, 1983) and ecological conditions especially natural abundance of *G. arcuatus* (de Roij and MacColl, 2012). Commonly lab controlled experiments suggest that these fish populations are genetically variable in body size (MacColl *et al.*, 2013) and resistance to *G. gasterostei* infections (de Roij *et al.*, 2011). Host size is thought to be an important factor in the population growth and virulence in some *Gyrodactylus* sp. (e. g. *G. turnbulis* and *G. bullatrudis*) (Cable and Oosterhout, 2007a). These studies strongly suggest that aquatic ecosystems on North Uist island are evolutionarily independent and therefore offer an ideal environment for the current study goal. Here I hypothesized that strains of *Gyrodactylus* sp. will survive longer in their own than different lochwaters and their life expectancy will correlate negatively with epidermal mucus concentration *in vitro*. I predict a negative relationship between mucous cell density and size of fish epidermis and each of parasites' natural abundance and their population size of experimentally manipulated infections.

3.3 Materials and Methods

Source of fish and parasites

Strains of *Gyrodactylus* sp. were obtained from naturally infected three-spined stickleback caught from eight separate locations on North Uist, Scotland. Stickleback populations from these locations and two populations from Nottingham were involved in the study. Parasite strains and fish populations were denoted by the name of the water body (loch/stream) where they originated. These locations are characterised by different environmental variables (pH and conductivity) and natural abundance of *Gyrodactylus* sp. (Table 3.1).

Table 3.1. The grid reference, fish standard length, mean natural abundance of *Gyrodactylus* sp. in May 2014 (A) and over five years: 2007, 2008, 2011, 2013 and 2014 (B) and two environmental variables (water pH and conductivity) measured for 10 natural populations of stickleback. These included eight populations in experiment one (2-10), two populations in experiment two (5, 11) and 9 populations (1, 3-10) in experiment three.

No	Loch (population)	Code	Grid reference	N	Standard length ($\pm$ SE)	Natural abundance of <i>Gyrodactylus</i> sp. ($\pm$ SE)		Water pH	Water conducti vity (μ S)
						A	B		
1	Chadha Ruaidh	Chru	57°35'38.7"N 7°11'51.2"W	71	33.67 ($\pm$ 0.76)	0 ($\pm$ 0)	0.00($\pm$ 0.0)	6.7	182
2	Cnoc na Feille	Cnfe	57°36'23.1"N 7°10'38.3"W	18	34.95 ($\pm$ 0.94)	5.85 ($\pm$ 1.32)	-	6.9	447
3	Mhic Gille-bhríde	Gill	57°36'07.3"N 7°24'37.3"W	31	38.28 ($\pm$ 1.15)	6.48 ($\pm$ 0.96)	3.72($\pm$ 0.49)	6.8	158.5
4	Hosta	Host	57°37'38.1"N 7°29'24.8"W	37	40.21 ($\pm$ 1.16)	3.22 ($\pm$ 0.57)	2.48($\pm$ 0.34)	8.34	339.5
5	nam Magarlan	Maga	57°35'16.9"N 7°26'38.7"W	36	44.3 ($\pm$ 1.95)	4.72 ($\pm$ 0.82)	2.44($\pm$ 0.41)	7.75	261.8
6	Ob nan Stearnain	Obse	57°36'06.1"N 7°10'25.4"W	30	38.94 ($\pm$ 1.45)	13.29 ($\pm$ 2.30)	9.40 ($\pm$ 1.14)	9.15	45100
7	na Reivil	Reiv	57°36'42.9"N 7°30'53.2"W	36	47.06 ($\pm$ 1.44)	2.53 ($\pm$ 0.57)	2.35($\pm$ 0.50)	8.95	433.3
8	Scadavay	Scad	57°35'05.5"N 7°14'11.2"W	36	30.16 ($\pm$ 0.61)	1.42 ($\pm$ 0.23)	0.78($\pm$ 0.17)	6.14	144.4
9	Tormasad	Torm	57°33'46.8"N 7°18'54.4"W	37	29.24 ($\pm$ 0.58)	0.41 ($\pm$ 0.16)	0.17($\pm$ 0.05)	6.84	181.4
10	Jubilee	-	52°57'00.6"N 1°11'12.9"W	21	43.33 ($\pm$ 2.09)	0.05 ($\pm$ 0.05)	-	7.3	210
11	Tottle Brook	-	52°56'00.1"N 1°12'01.5"W	26	41.83 ($\pm$ 2.48)	10.58 ($\pm$ 2.31)	-	7.1	199

Factors affecting the survival of *Gyrodactylus* sp.

Experiment 1.

The survival time (hours) of detached *G. arcuatus* were estimated using a similar method to that employed by Harris *et al.* (1998). Survival time was estimated for parasite strains in water from their own loch (experiment 1a) and in reciprocal exposure to water from all lochs (experiment 1b). For experiment 1a, 12 individuals for each of seven parasite strains (Gill, Host, Maga, Obse, Reiv, Scad and Torm) were removed from freshly killed sticklebacks into wells of sterile 96 well Microplates (Nalge Nunc International, Denmark). Parasites were kept in refrigerator (4 °C) until the isolation of the parasite was completed ($\approx$ 2hrs). To compare the expected longevity of each parasite strain in its natural environment, each worm was individually placed in a well containing 100 μ l of water from the loch where the parasite had originated. The plate containing worms was kept at 11.5 °C ($\pm$ 1) mimicking the natural temperature of loch water. The survival of worms was recorded every three hours using a dissection microscope provided with fibre optic illumination. The distinction between live and dead worms was made based on movement and/or visible muscle contractions. Plate containing parasites was gently shaken under microscope (i.e. to stimulate the parasite) in order to confirm the distinction between dormant and dead individuals.

To quantify the extent to which worms were locally adapted to the water from their own loch, in experiment 1b, similar to the above procedure, eight parasite strains (Cnfe, Gill, Host, Maga, Obse, Reiv, Scad and Torm) were reciprocally exposed to water from their own and from seven other lochs. Each plate contained water from one of the eight lochs and was divided into eight columns of 12 wells. Twelve worms of one parasite strain were

individually placed in the wells of each column. Strains were randomly assigned to columns. The eight plates were incubated at a temperature of $14.5 (\pm 1) ^\circ\text{C}$. Survival of worms was tracked as in experiment 1a. In experiment 1a and 1b, the parasites obtained from infected wild fish and were not identified to species levels.

Experiment 2.

Does epidermal mucus of lab bred or wild sticklebacks contain anti- *Gyrodactylus* sp. agents to influence the parasite survival time *in vitro*? The survival rate of one strain (Maga) of the worm was simultaneously estimated in different concentrations of mucus (100%, 50%, and 25%), a low concentration of MS222 (100 mg L^{-1}) and dechlorinated tap water as control in a 96-micro well plate. The mucus samples were obtained from the epidermal surface of one naive (Maga, $n = 6$) and one wild caught (Tottle Brook, $n = 6$) population of three-spined stickleback. Fish were killed in an overdose of MS222 (400 mg L^{-1}), blotted with filter paper to remove excess water, and placed in a plastic bag. Approximately 5mL of dechlorinated tap water ($\text{pH} = 7.5$) was added to the bag and fish were rubbed against the inner surface of the bag until a mucus film became visible (Harris *et al.*, 1998). The initial concentration of the collected mucus was considered 1:1 and two fold dilutions with dechlorinated tap water were used to prepare 1:2 and 1:4 concentrations. The 96- well plate was divided to eight columns (of 12 wells) and each column was labelled for one combination. From each of the three concentrations of mucus samples, MS222 and control water, an amount of $100 \mu\text{L}$ was added to each of 12 wells of one column. After adding these contents, the plate was kept in refrigerator for half an hour. Then one worm was added to each well and the plate containing parasites was incubated at $19.5 ^\circ\text{C}$ until the end of the experiment. The parasite individuals had been raised on lab bred stickleback originally sampled from a naturally infected fish population (Maga).

Mucous cell morphology

Experiment 3

To examine mucous cell morphology, the entire caudal fin was selected to represent fins, and the lower jaw to represent the anterior part of the body for all the fish included in this study. These are both locations where *Gyrodactylus* sp. are commonly found in natural infections. The density and diameter of mucous cells were estimated in these locations (entire caudal fin and approximately 1.5 mm² of lower jaw skin) for all test fish included in this experiment. For simplicity, 'CF' was used to denote the caudal fin tissue and 'HS' to denote head skin tissue. Mucous cells parameters were estimated for three separate fish groups: a wild fish population (Chru) experimentally infected with wild strains of *G. arcuatus* (exp. 3a), a lab raised fish population (Jubilee) infected with four lab raised strains of *G. arcuatus* (exp. 3b), and eight geographically isolated wild stickleback populations with a range of abundance of *Gyrodactylus* sp. (exp. 3c).

For exp. 3a, 80 individuals from a naturally *Gyrodactylus* sp.-free fish population (Chru), were divided to eight groups of 10. One group was an uninfected control and each of the others was infected with one wild strain of *G. arcuatus* by placing two individual worms on the caudal fin of test fish. Parasites were obtained from naturally infected sticklebacks caught from seven isolated lochs (Gill, Host, Maga, Obse, Reiv, Scad or Torm). The total worm load was counted on individual infected fish every three days and 'attachment site' was recorded (the infection details are explained in chapter 6). To describe attachment site, the body surface of fish was divided into three zones of approximately equal area (Figure 3.1): (1) the 'anterior' included the area from the tip of nose back to a vertical line from the first spine (mouth, eyes, operculum and gills); (2) the 'middle' ran from the first dorsal

spine back to a vertical line from the third dorsal spine (body sides, pectoral fins, dorsal and ventral spines) and (3) the ‘posterior’ ran from third dorsal spine to the end of caudal fin (body sides, dorsal, anal and caudal fins).

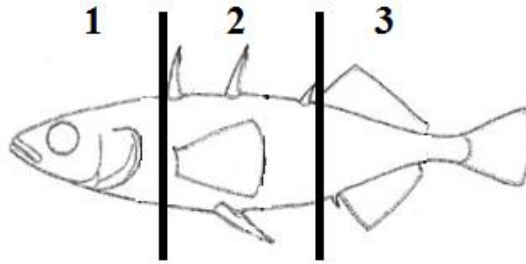


Figure 3.1. Diagrammatic illustration of the three zones ((1) anterior; (2) middle and (3) posterior on the fish's body used to quantify the distribution of *Gyrodactylus* sp. on artificially and naturally infected stickleback.

At the end of artificial infection experiments all the fish were killed by overdose of MS222 (400 mg L^{-1}), and death was confirmed by physical destruction of the brain. After taking CF and HS tissues from individual test fish, samples were fixed and preserved in 10% neutral buffered formalin. Tissue samples were kept in the fixative for a month, dehydrated in graded alcohol-xylene and embedded in paraffin wax using a Leica TP1020 Tissue Processor. Tissue samples were sectioned ($7\mu\text{m}$) using a SLEE Cut 4060 Microtome and stained using Alcian blue (pH 2.5)/Neutral red allowing goblet mucous cells (with mucus contents) to stain blue/purple against a pink tissue background. The slides were mounted in DPX (SIGMA-44581), covered with a coverslip (No. 1 borosilicate glass 22 x 50 mm) and left overnight to dry at room temperature. All the slides were photographed at 2.5 and 10 X magnifications using a light microscope (Leica DM4000B) supported with software packages and camera systems. The tissue size and cell diameter was measured using ImageJ 1.48v (Wayne Rasband National Institute of Health, USA <http://imagej.nih.gov/ij>).

The diameter and average number of goblet cells was counted per mm² of tissue for the whole caudal fin and the cell diameter for approximately 1 mm² for the tip of the lower jaw.

For exp. 3b, 24 individuals from a lab bred fish population (Jubilee) were divided into four groups and each group was infected with a lab raised strain of *G. arcuatus* (Maga, Obse, Reiv or Scad) (the infection details explained in chapter 2). The infection and tissue sampling procedure followed the same protocol (mentioned above) used for exp. 3a.

For exp. 3c, tissue samples were collected from eight geographically isolated wild fish populations (Chru, Gill, Host, Maga, Obse, Reiv, Scad and Torm). For each of the population, 10 individuals were haphazardly selected and tissue sampling was carried out according to the above mentioned protocol. The natural abundance of *Gyrodactylus* sp. was measured for the test wild fish and the average abundance of the parasite strains (measured over five previous years; 2007, 2008, 2011, 2013 and 2014) was obtained from Andrew MacColl (unpublished data from at least 20 fish per population per year). All the experiments were carried out under a UK Home Office Project License 40/2886, and in accordance with the authorization of the Ethical Review Committee of Nottingham University.

Statistical analysis

The survival of detached worms in Experiments 1 (a) and (b) was analysed using the log-rank test (Mantel-Cox test) and the Gehan-Breslow-Wilcoxon test with two tailed probability values. The log-rank (Mantel-Cox) test tends to emphasise on events that occur later in time courses, while the Gehan-Breslow-Wilcoxon test tends to compare the curves with slightly increased emphasis on events occurring earlier in time courses (Martinez and

Naranjo, 2010). Two tests were employed for all comparisons made among survival distributions in this study. A multiple linear regression with backward elimination was used to examine whether the response variable ‘median survival hours’ of the parasite strains in their loch water was related to three independent variables: ln water conductivity, water pH and fish size (standard length). Fish size represents the biotic environment that *Gyrodactylus* sp. occupy and is thought to associate with the parasite population growth (Cable and Oosterhout, 2007a). In experiment 1 (b) The extent of parasite local adaptation to the water from their own loch was examined using a Generalized Linear Model (GLM) with normal error and identity link function. The response variable was ‘estimated parasite survival time’ (in hours) while both parasite origin and loch water to which they were exposed were fixed factors in this analysis which had a fully factorial design. To understand better the effect of water chemistry differences on *G. arcuatus* survival, multiple regression with backward elimination method was used to examine the relationship between mean survival of different parasite strains in different loch waters, and the absolute difference (delta) in pH or conductivity between the water in which they were tested and their ‘home’ water.

Mucous cells in HS were patchy and overlapping which made counting them difficult and hence only nine individuals were randomly selected for exp. 3a and exp. 3b and 21 individuals for exp. 3c to compare the cell density between CF and HS tissues. The distribution of log transformed mucous cell density was compared between CF and HS, using a paired-samples t-test. The difference in the total worm load among the three body zones was compared using a one way ANOVA for repeated measures. The association between population size of the parasite and fish epidermal mucous cell properties was examined using a GLM with gamma distribution and log link function for exp. 3a (parasite

number+ 1) while for 3b and 3c GLMs with normal errors and identity link functions were used. The response variables included: mucous cell diameter in head skin (HS), mucous cell diameter in caudal fin (CF) and mucous cell density in caudal fin. For artificially infected (Chru and Jubilee) fish in experiment 3a and 3b, the full model included parasite strain as the only fixed factor and maximum worm load as a covariate. For the wild fish samples (exp. 3c), fish population was included as a fixed factor while total worm count was used as a covariate.

It was also of interest to test whether the measures of mucous cells in natural fish populations were associated with environmental or biotic variables. Here multiple linear regression with back elimination method was used to predict each of the dependent variables ‘CF cell density’, ‘CF cell diameter’ and ‘HS cell diameter’ based on three independent variables: water pH, fish body size (standard length) and average natural abundance of *G. arcuatus* in the population (measured in five previous years: 2007, 2008, 2011, 2013 and 2014). Each of the three dependent variables was square root transformed to achieve normality. The abundance of *Gyrodactylus* sp. was highly different among the populations and therefore, was also transformed to squared root. All the analyses were carried out in SPSS package, IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

3.4 Results

Experiment 1.

Survival differed greatly among the parasite strains and generally parasites survived longer at 11.5°C than 14.5°C (mean (hrs) = 47.11, SEM = 1.24 and mean = 33.47, SEM = 0.94

respectively, Fig. 3.2A, B). When incubated in their own loch water at 11.5 (± 1) °C (exp. 1a), the seven detached parasite strains (Gill, Host, Maga, Obse, Reiv, Scad and Torm) differed significantly in survival rates (Log-rank test, $\chi^2 = 20.4$, df = 6, $P = 0.002$ and Gehan-Breslow-Wilcoxon test, $\chi^2 = 23.4$, df = 6, $P < 0.001$). The difference remained significant after excluding 'Obse' the saltwater parasite strain (Log-rank test, $\chi^2 = 23.9$, df = 5, $P < 0.001$ and Gehan-Breslow-Wilcoxon test, $\chi^2 = 24.36$, df = 5, $P < 0.001$). When the experiment was repeated at 14.5 (± 1) °C (exp. 1b), the survival difference among the same parasite strains (plus the Cnfe strain) was also significant (Log-rank test, $\chi^2 = 87.1$, df = 7, $P < 0.001$ and Gehan-Breslow-Wilcoxon test, $\chi^2 = 73.5$, df = 7, $P < 0.001$). The difference remained significant when the data were reanalysed excluding Obse (Log-rank test, $\chi^2 = 80$, df = 6, $P < 0.001$ and Gehan-Breslow-Wilcoxon test, $\chi^2 = 67.6$, df = 6, $P < 0.001$). The survival time of five parasite strains (Gill, Host, Maga, Obse and Reiv) was significantly shorter at 14.5 °C than 11.5 °C, but the difference was not significant for Scad and Torm strains (Table 3.2). At 11.5°C (exp.1 a), median parasite survival in their own water was associated with the mean standard length of their host populations ($R^2 = 0.72$, $F_{1,5} = 12.9$, $P = 0.016$), but not environmental variables (water pH and conductivity) while at 14.5 °C (exp. 1b), median survival time was associated with water pH only ($R^2 = 0.53$, $F_{1,6} = 6.8$, $P = 0.040$; Fig. 3.3A, B and C).

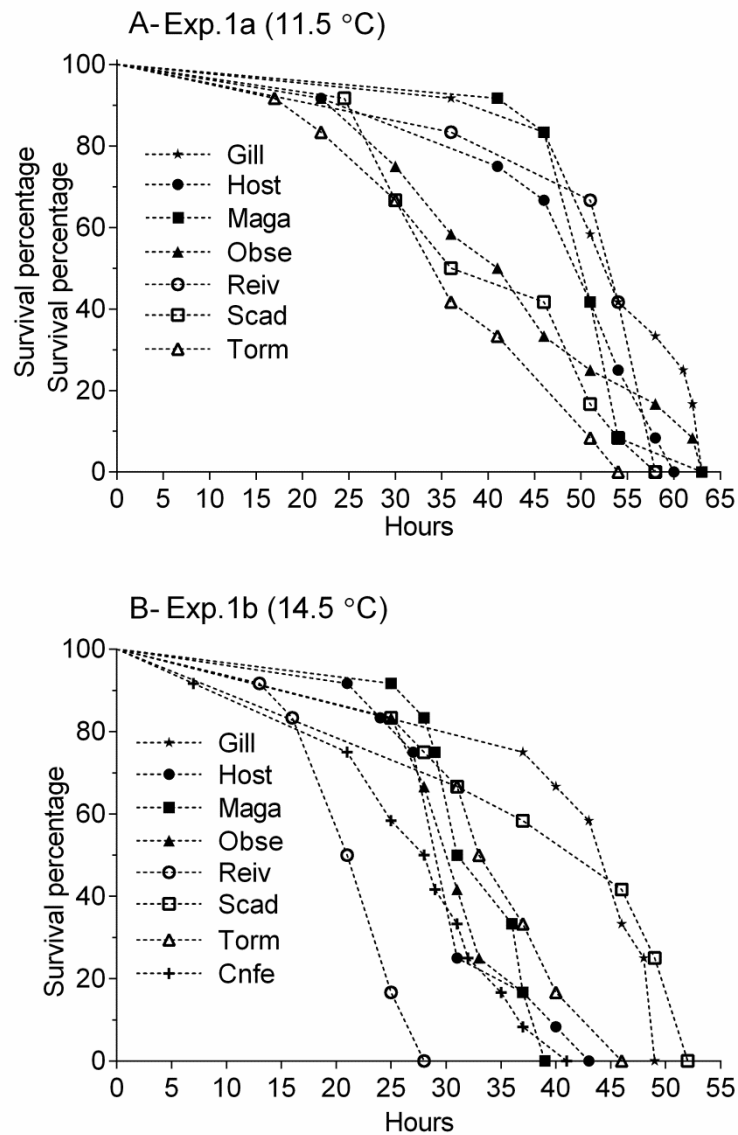


Figure 3.2. Survival of different strains of *Gyrodactylus arcuatus* in water from their own loch, when detached from the host. (A) Seven strains incubated at 11.5 (± 1) °C, from an experiment carried out in May 2014. (B) Eight strains incubated at 14.5 (± 1) °C, from an experiment carried out in October 2014. Dotted lines intercepted with: starlets = Gill, filled circles= Host, filled squares= Maga, filled triangles= Obse, open circles= Reiv, open squares= Scad, open triangles= Torm and plus symbols= Cnfe.

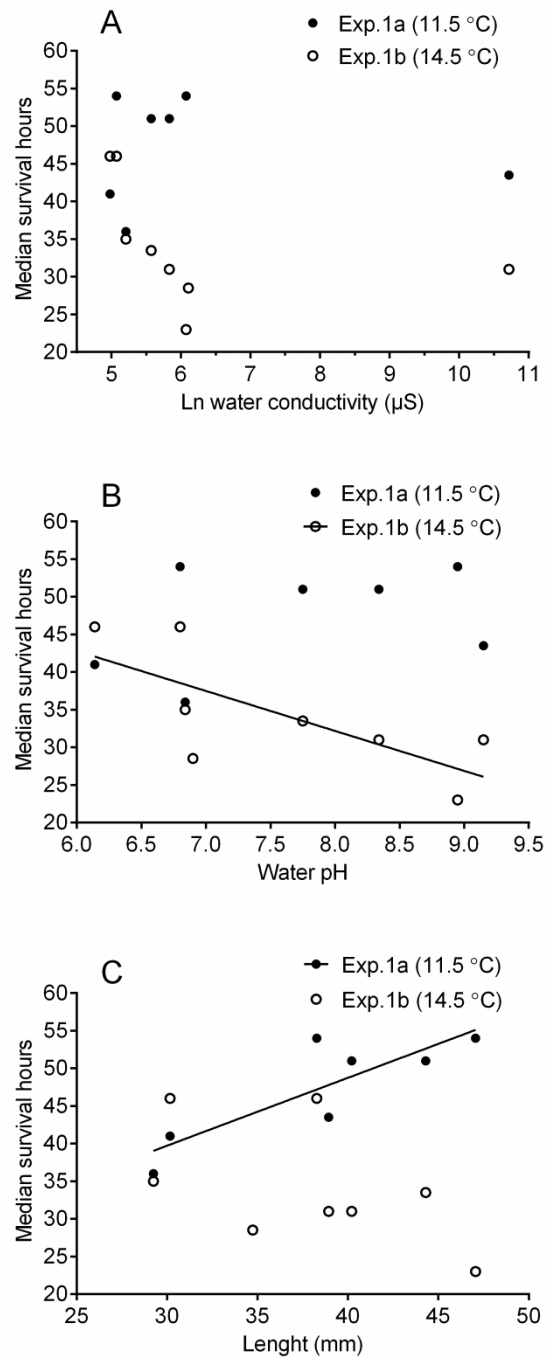


Figure 3.3. The relationship between median survival time (hours) of different strains of *Gyrodactylus arcuatus* in water from their own loch when detached from their host and (A) ln water conductivity, (B) water pH and (C) mean standard length of their fish populations in two separate experiments: exp. 1a at 11.5°C (filled circles) and exp. 1b at 14.5°C (open circles). The solid lines show the best fit regression line.

Table 3.2. Comparison of survival differences from two different experiments (1a and 1b), which happened to have been at two different temperatures (11.5 and 14.5 °C). Seven strains of *Gyrodactylus arcuatus* were included (Gill, Host, Obse, Maga, Reiv, Scad and Torm), the degree of freedom is '1' for each strain in both long rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests.

Parasite strain	Log-rank (Mantel-Cox) test		Gehan-Breslow-Wilcoxon test	
	χ^2	<i>P</i>	χ^2	<i>P</i>
Gill	14.95	<0.001	11.21	<0.001
Host	17.18	< 0.001	13.54	<0.001
Obse	7.8	0.005	5.08	0.024
Maga	25.51	< 0.001	21.67	< 0.001
Reiv	24.93	< 0.001	21.4	< 0.001
Scad	0.22	0.642	0.01	0.908
Torm	1.2	0.274	0.05	0.816

Note: Probability values which are < 0.05 are in bold type.

In exp. 1b, median survival time of the parasite depended on the combination (interaction) of the parasite population of origin, and the loch water to which they were exposed ($\chi^2 = 602.1$, $P < 0.001$, Table 3.3A). When the saltwater parasite (Obse) and its loch water were excluded from the analysis, the pattern remained the same ($\chi^2 = 88.2$, $P < 0.001$, Table 3.3B). In general the parasites survived for longer in their own than different loch waters (unpaired t- test for all the strains, $t = 6.10$, $df = 766$, $P < 0.001$, fig. 3.4A) and this remained true when only freshwater strains were considered ($t = 3.31$, $df = 586$, $P = 0.001$ fig. 3.4B). The parasite strains survived for less time in waters collected from Obse and Reiv than the other five lochs (Log-rank test, $\chi^2 = 401.9$, $df = 7$, $P < 0.001$ and Gehan-Breslow-Wilcoxon test, $\chi^2 = 442.9$, $df = 7$, $P < 0.001$). Multiple regression showed that median survival time was negatively associated with the absolute difference (delta) in ln water conductivity, but not pH (fig. 3.5A and B) when all the eight parasite strains were included ($R^2 = 0.42$, $F_{1, 62} = 45.8$, $P < 0.001$). When the saltwater parasite (Obse) and its lochwater were excluded from the analysis, the model equation was not significant ($P > 0.05$).

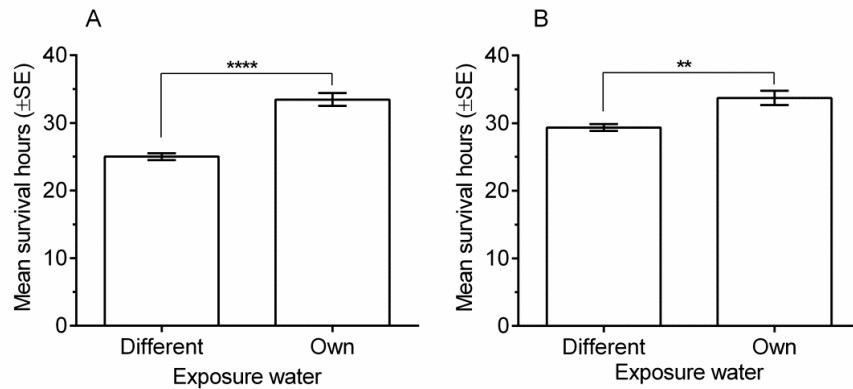


Figure 3.4. The difference in survival time (hours) of *Gyrodactylus arcuatus* detached from their host, when in water from their own or different lochs in exp. 1b. A- all eight parasite strains and, B-seven freshwater strains only. Note: asterisks indicate significant difference (**: $P < 0.01$, ****: $P < 0.0001$).

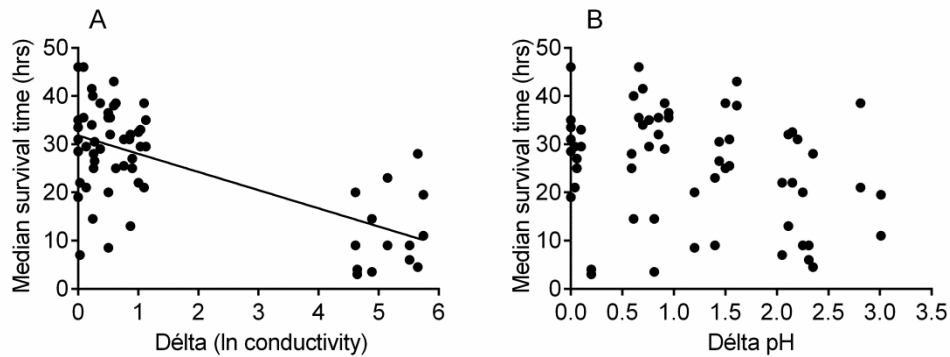


Figure 3.5. The relationship between median survival time of the eight parasite strains (exp. 1b) and mean differences (delta) in (A) water conductivity and (B) pH between water from their own loch, and that to which they were exposed. The solid line is the most fit regression line.

Table 3.3. Analysis of the association between the survival time of detached *Gyrodactylus arcuatus*, strain of parasite, water to which they were exposed and their interaction from a GLM for (A) all eight lochs in exp. 1b and (B) for the seven freshwater lochs only in the same experiment.

Effects	χ^2	df	Sig.
<i>(A) For all the strains</i>			
Parasite origin	295.5	7	< 0.001
Water origin	1071.9	7	< 0.001
Parasite origin * Water origin	602.1	49	< 0.001
<i>(B) For freshwater strains only</i>			
Parasite origin	88.2	6	< 0.001
Water origin	498.3	6	< 0.001
Parasite origin * Water origin	210.8	36	< 0.001

Experiment 2.

Generally, mucus did not affect the survival rates of the Maga strain *in vitro* (Fig. 3.6).

Compared to aquarium water (control), parasite survival was not reduced in any of the concentrations of the two mucus samples (Log-rank test, $\chi^2 = 4.51$, df = 6, $P = 0.609$ and Gehan-Breslow-Wilcoxon test, $\chi^2 = 2.857$, df = 6, $P = 0.654$). The parasite survival in 100 mg.L⁻¹ of MS222 was markedly reduced compared to the control and other mucus solutions and the difference was highly significant (Log-rank test, $\chi^2 = 41.97$, df = 7, $P < 0.001$ and Gehan-Breslow-Wilcoxon test, $\chi^2 = 39.05$, df = 7, $P < 0.001$).

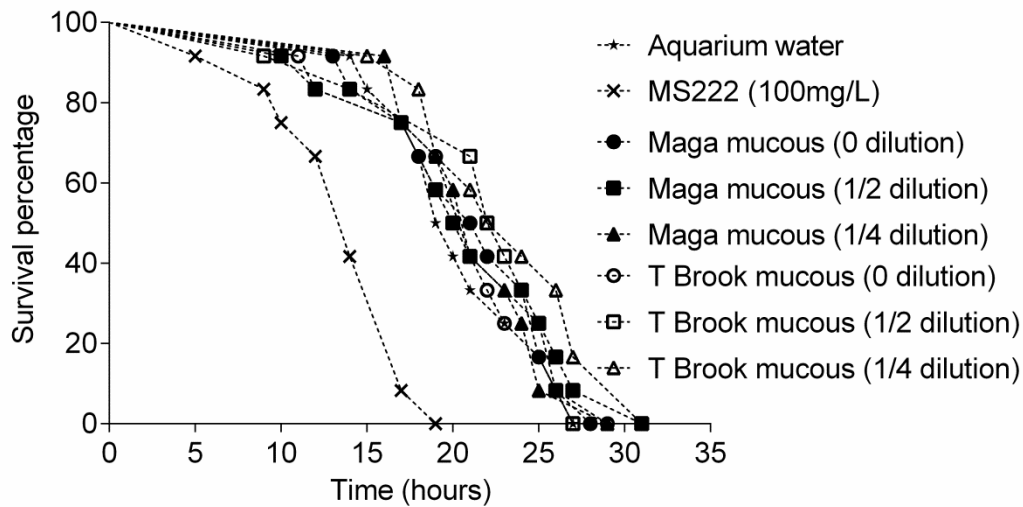


Figure 3.6. The effect on survival time of detached *Gyrodactylus arcuatus* of different concentrations of stickleback mucus [dotted lines intercepted with: starlets= aquarium water, crosses= MS222 (100mg/L), filled circles= Maga mucus (1:1), filled squares= Maga mucus (1:2), filled triangles= Maga mucus (1:4) open circles= Tottle Brook mucus (1:1), open squares= Tottle Brook mucus (1:2) and open triangles= Tottle Brook mucus (1:4). The experiment was carried out at lab temperature (19.5 ± 1) °C.

Experiment 3.

The distribution of mucous cell density differed markedly between the two zones on fish body (Fig. 3.7A and B). Mucous cell density was much higher in HS than CF tissue for both experimentally infected and wild fish populations (A paired-samples t-test for log transformed mucous cell density in exp. 3a, $t(8) = 7.84$, $P < 0.001$ fig. 3.8A, in exp. 3b, $t(8) = 5.90$, $P = 0.004$, fig. 3.8B, in exp. 3c, $t(20) = 20.59$, $P < 0.001$, fig. 3.8C). The distribution of parasites did not differ among the body zones on artificially infected wild fish from Chru (Fig. 3.9A, $F_{2, 156} = 0.21$, $P = 0.807$, exp. 3a) or naturally infected wild fish (Fig. 3.9B, $F_{2, 280} = 0.28$, $P = 0.664$, exp. 3c), but it was strongly skewed toward the anterior part on the artificially infected lab raised Jubilee fish population (Fig. 3.9C, $F_{2, 50} = 126.1$, $P < 0.001$, exp. 3b).

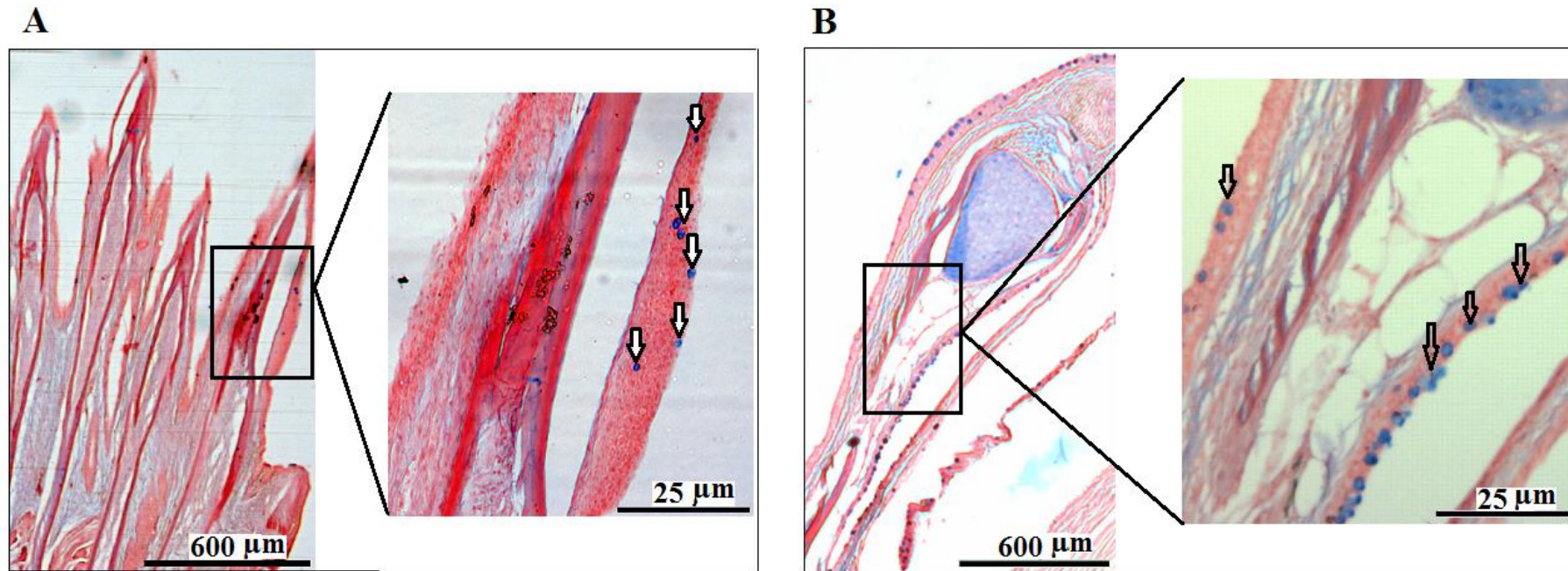


Figure 3.7. Light microscopy images showing the mucin content of goblet mucous cells in the fish caudal fin (A) and head skin tissue (B) of a Jubilee fish experimentally infected with *Scad gyrodactylids*. Mucus content is stained with Alcian blue stain and the images were taken at the magnifications of 2.5X (Scale bar = 600 μm) and 10X (Scale bar = 25 μm). Arrows point to goblet cells filled with mucin and stained pale blue.

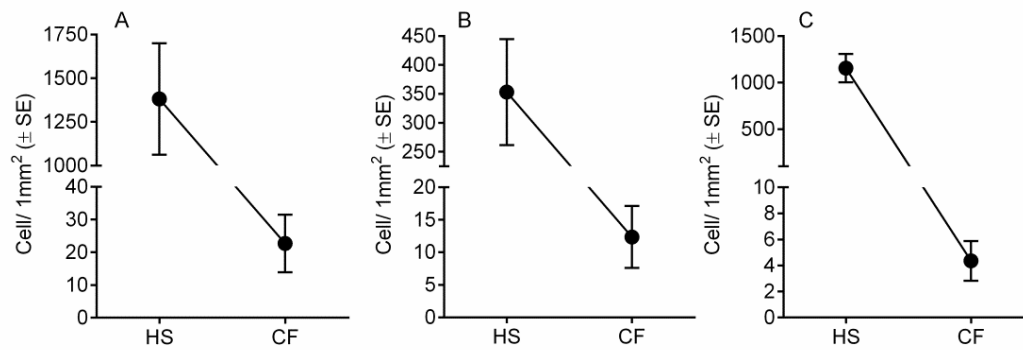


Figure 3.8. Difference in the mean mucous cell density (dots) between head skin (HS) and caudal fin (CF) tissues for experimentally infected Chru fish (A) in exp. 3a and Jubilee fish (B) in exp. 3b and wild fish populations (C) in exp. 3c (see texts for the details).

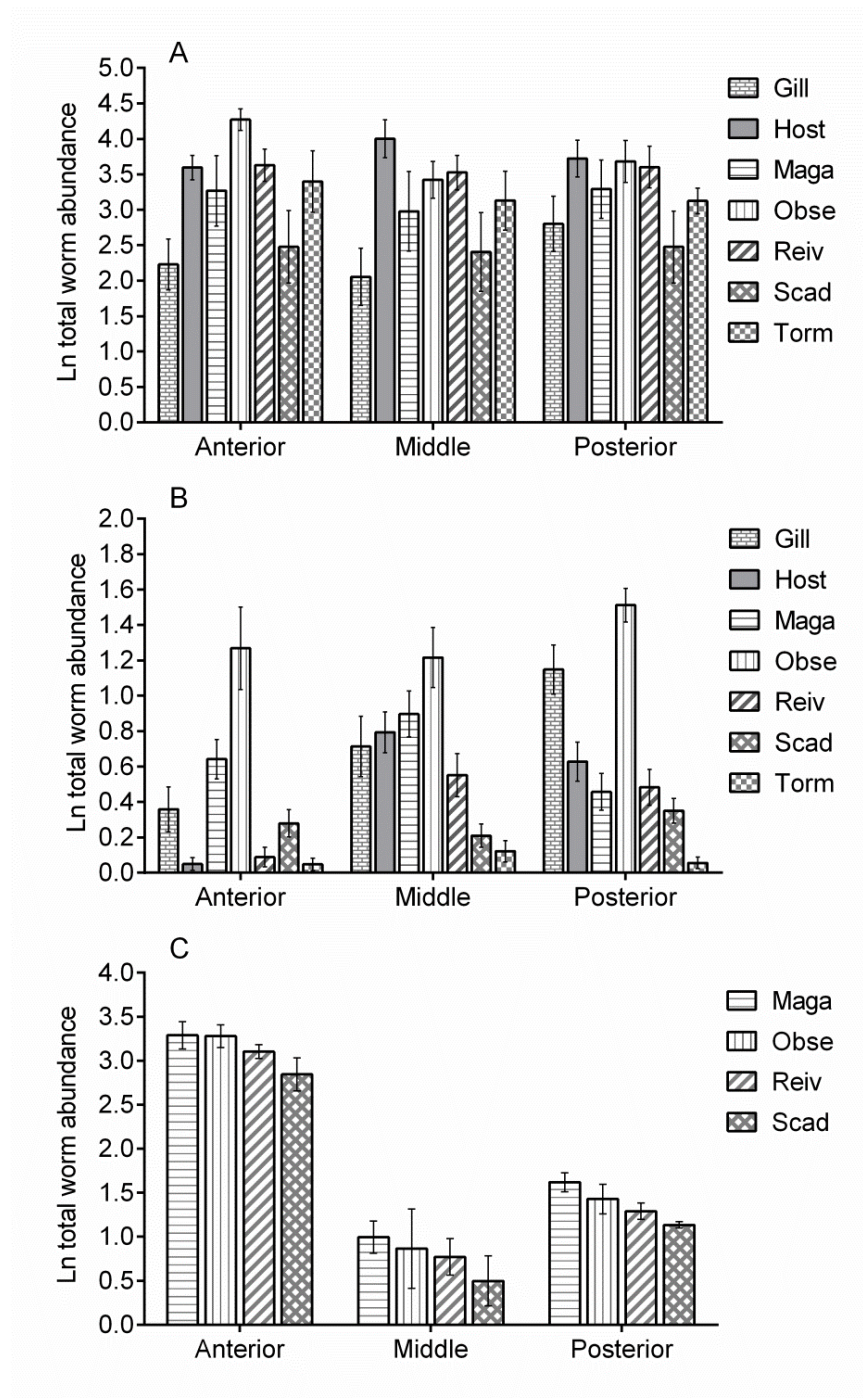


Figure 3.9. The distribution of log total worm load ($\pm$ SE) on fish body zones (anterior, middle and posterior) in artificially infected Chru fish (A), wild fish populations (B) and artificially infected Jubilee fish (C). Legends indicate the parasite strains (left to right: bricks= Gill, shaded= Host, horizontal lines= Maga, vertical lines= Obse, curved lines= Reiv, crossed lines= Scad and dotted= Torm). The distribution of worm load significantly differed among the three zones for the four strains (Maga, Obse, Reiv and Scad) on Jubilee fish, for wild fish populations.

In artificially infected Chru fish (exp. 3a), mucous cell density in CF varied according to the parasite strain with which they were infected, but was unrelated to the maximum parasite load experienced by individual fish (Table 3.3A (i)). Infected fish generally had lower CF mucous cell density than control fish (Fig. 3.10A). Pairwise comparison tests showed a significant difference between control fish and those infected by Host ($P = 0.034$) and Scad ($P = 0.044$) parasites. The diameter of CF and HS mucous cells was not affected by the strain of parasite or the maximum parasite load (Table 3.4A (ii) and A (iii), Fig. 3.10 B and C). There was a positive correlation between mucous cell diameter in CF and mucous cell diameter in HS (Pearson correlation, $r = 0.47$, $df = 68$, $P < 0.001$), but mucous cell density in CF did not relate to the cell diameter in either of the two tissues ($P > 0.05$).

Table 3.4. GLM outcomes for mucous cell diameter in head skin (HS) and caudal fin (CF) and mucous cell density in caudal fin. The effects included *G. arcuatus* strain and maximum worm load for artificially infected wild Chru fish (A) and artificially infected lab bred Jubilee fish (B), but for the wild fish populations (C), the main effects involved fish population and gyrodactylid abundance. *P* values which are lesser than 0.05 are given in bold.

Effects	χ^2	Df	<i>P</i> value
A- Exp. 3a (Chru fish)			
(i) CF cell density			
<i>G. arcuatus</i> strain	18.9	7	0.009
Maximum worm load	0.6	1	0.457
(ii) CF cell diameter			
<i>G. arcuatus</i> strain	6.2	7	0.514
Maximum worm load	0.1	1	0.783
(iii) HS cell diameter			
<i>G. arcuatus</i> strain	10.9	7	0.145
Maximum worm load	0.0	1	0.871
B- Exp. 3b (Jubilee fish)			
(i) CF. cell density			
<i>G. arcuatus</i> strain	2.9	3	0.408
Maximum worm load	0.01	1	0.952
(ii) CF. cell diameter			
<i>G. arcuatus</i> strain	1.3	3	0.728
Maximum worm load	0.03	1	0.855
(iii) HS cell diameter			
<i>G. arcuatus</i> strain	2.2	3	0.530
Maximum worm load	8.3	1	0.004
C - Exp. 3c (wild fish populations)			
(i) CF. cell density			
Fish population	72.1	7	<0.001
Total worm count	0.03	1	0.853
(ii) CF. cell diameter			
Fish population	33.1	7	<0.001
Total worm count	1.4	1	0.245
(iii) HS cell diameter			
Fish population	9.9	7	0.201
Total worm count	0.6	1	0.457

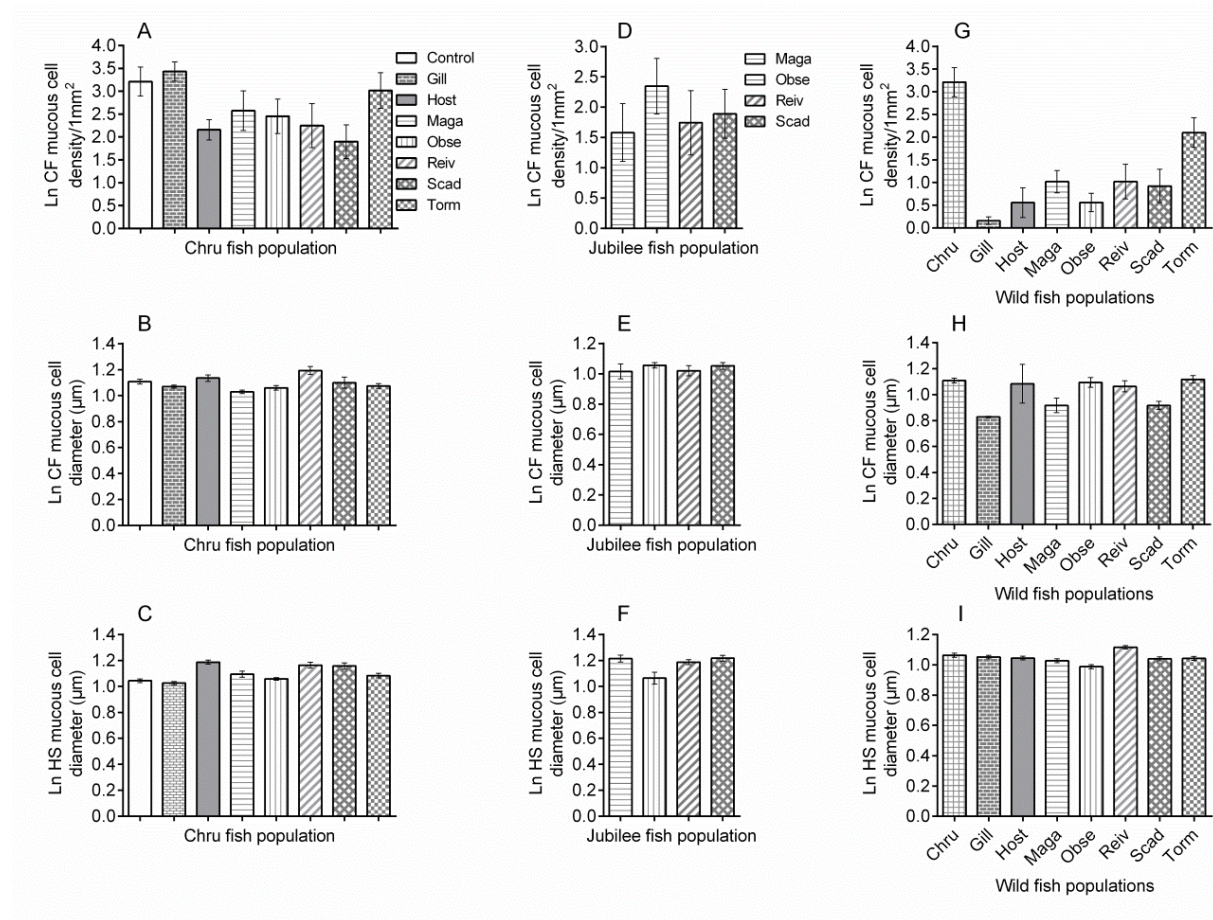


Figure 3.10. The ln mucous cell density ($\pm$ SE) in caudal fin and ln mucous cell diameter ($\pm$ SE) in caudal fin (CF) and head skin (HS) measured for artificially infected Chru fish (A, B and C respectively), artificially infected Jubilee fish (D, E and F respectively) and naturally infected wild fish populations (G, H and I with bar titles respectively). Bars represent different parasite strains. For Chru fish, strains are (left to right): Control, Gill, Host, Maga, Obse, Reiv, Scad and Torm; Jubilee fish, strains are (left to right): Maga, Obse, Reiv & Scad and for wild fish, populations are (left to right): Chru, Gill, Host, Maga, Obse, Reiv, Scad and Torm.

In the artificially infected Jubilee fish (exp. 3b), the density and diameter of mucous cells of CF were unrelated to the strain of parasite or the maximum parasite load on individual fish (Table 3.4B (i) and B (ii), Fig. 10D and E). The diameter of mucous cells in head skin (HS) was negatively associated with maximum worm load during infection, but unrelated to parasite strain (Table 3.4B (iii), fig. 3.10F, fig. 3.11). There was no correlation between mucous cell diameter in CF and mucous cell diameter in HS ($P > 0.05$), but mucous cell density in CF correlated positively with mucous cell diameter in CF ($r = 0.46$, $df = 24$, $P = 0.025$) and negatively with mucous cell diameter in HS ($r = -0.46$, $df = 21$, $P = 0.035$).

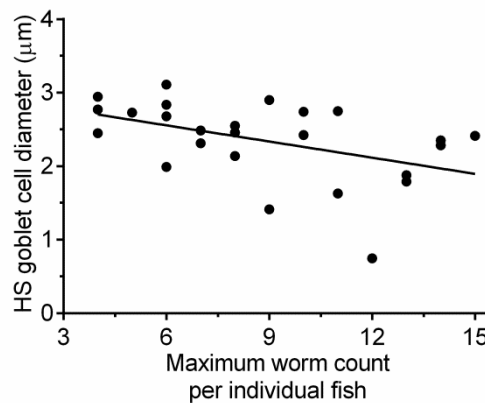


Figure 3.11. The relationship between the diameter of mucous cells in head skin (HS) and the maximum parasite count on individual fish in artificially infected Jubilee fish (exp. 3b). The solid line represents the best correlation fit.

In fish caught from wild populations (exp. 3c), the density and diameter of CF mucous cells varied significantly between populations, but were not related to the observed parasite load of the individual fish. HS cell diameter did not vary between fish populations or with parasite load (Table 3.4C (i), C (ii), and C (iii), Fig. 3.10G, H and I).

At the population level, the morphology of mucous cells in CF and HS were not associated with either fish length or water pH, but both CF mucous cell density and HS mucous cell diameter decreased in populations with higher natural abundance of *Gyrodactylus* sp. (for CF density, $R^2 = 0.67$, $F_{1,6} = 12.9$, $P = 0.012$; for HS diameter, $R^2 = 0.72$, $F_{2,5} = 6.3$, $P = 0.043$, fig. 3.12). None of the three predictors explained statistically significant variation in CF mucous cell diameter, ($P > 0.05$).

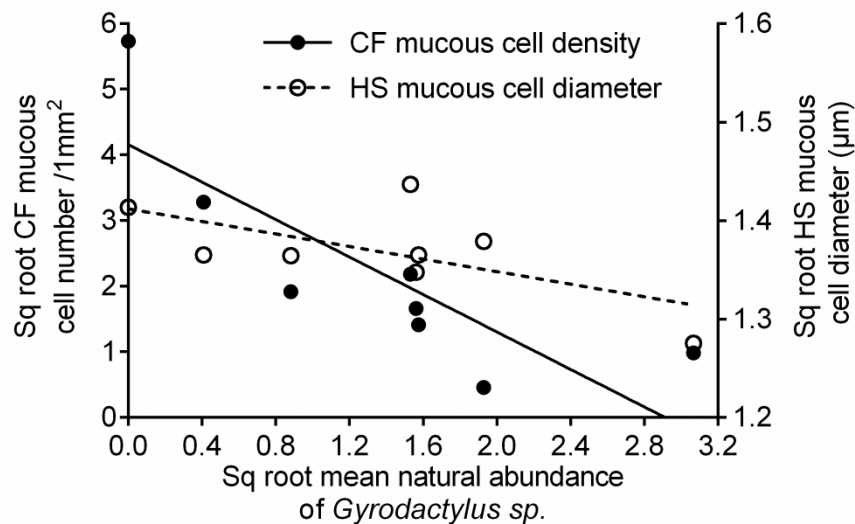


Figure 3.12. The relationship between the squared- root transformed natural abundance of *Gyrodactylus* sp. and mucous cell density in CF (filled circles) and mucous cell diameter in HS (open circles) of wild fish (exp3 c). The solid and dashed lines represent the best fit regression lines.

3.5 Discussion

The role that biotic and abiotic environments played in the survival of detached *G. arcuatus* was examined. Specifically, I tested the hypotheses that strains of the parasite isolated from different lochs would show higher survival time in water from their own loch, their survival would correlate negatively with mucus concentrations *in vitro* and

population size would correlate negatively with mucous cell density *in vivo* respectively. These results showed that the survival ability of detached *G. arcuatus* differs among the eight strains in their natural environments. In the reciprocal exposure experiment, detached parasites clearly showed higher survival time in their own water than in different loch waters. Parasite survival time was not reduced by mucus *in vitro*, but there was some evidence for existence of a negative association between parasite intensity on individual fish and density and diameter of epidermal mucous cells. There was a strong negative correlation between the average natural abundance of *Gyrodactylus* sp. and each of density and diameter of epidermal mucous cell which may suggest an ultimate physiological association between the host and parasites.

In general, the survival time of detached *Gyrodactylus* sp. is thought to associate with the physiological status of parasites and environmental conditions. For example, Cable *et al.* (2002) found a negative association between the survival of detached *G. salaris* and time up until 60 hours at 10°C, followed by a sharp decrease in survival, suggesting that the parasite could stay alive until the depletion of energy reserves. Similarly, Scott and Anderson (1984) found that mortality of *G.* was negatively associated with the parasite population age (infection time on individual host) while Cable *et al.* (2002) found no association between parasite survival and parasite age or the developmental stage of their embryos.

A strong positive correlation was found between the survival time of detached *G. arcuatus* and the body size (standard length) of their fish hosts at 11.5 °C. Such a pattern could be explained either by the energy content of individual worm (Cable *et al.*, 2002) or possibly the quality (chemistry) of the parasites' lochwater. Fish body size may simply determine resource richness for the parasite and large hosts may provide more energy than small

hosts. It has been reported that body size of some monogenean parasite species positively correlates with the body size of their fish hosts (Sasal *et al.*, 1999), while a cestode parasite (*Schistocephalus solidus*) grows larger in faster growing stickleback hosts (Barber, 2005). Therefore, the association between the survival time of detached parasites and their host size might explain the quantity of energy reserves of the parasite in relation to the space they have on their host fish.

Compared to exp. 1a, the survival time of detached parasites was shorter in the exp. 1b which could be explained by the circumstances differed between the two experiments. For example there was a slight increase (3°C) in the environmental temperature. The negative influence of temperature on the survival of detached *Gyrodactylus* sp. has also been reported by other studies (Soleng and Bakke, 1997; Cable *et al.*, 2002), but the impact was lesser on strains of low water conductivity and pH. This may suggest that this level of temperature changes could only increase the influence of chemico-physical stressors on the survival of detached *Gyrodactylus* sp. Seasonal changes in the physiological conditions of the parasite could also be considered (Geets *et al.*, 1999; Blažek *et al.*, 2008).

The overall survival time of the parasites was longer in their local loch water than waters from other lochs. Environmental stress could be very important for ectoparasites like *Gyrodactylus* sp., as it could affect a large number of their biological processes (Russell *et al.*, 1995). Difference in the effect of water quality on parasite survival and transmission may be the key factor contributing to the local adaptation (Lopez Pascua *et al.*, 2012) and extinction of parasites (Tinsley, 1999). Under lab conditions, bacteria and fungi can adapt to environmental stressors and this makes the pathogens more resistant to such stressors both *in vitro* and *in vivo* (O'Driscoll *et al.*, 1996; Conte *et al.*, 2002; Davidson and Harrison, 2002). In this study, there was a negative correlation between median survival

time of *G. arcuatus* and mean difference in water conductivity. Previous studies have reported that survival of *Gyrodactylus* sp. is sensitive to changes in water salinity (Soleng and Bakke, 1997; Soleng *et al.*, 1998) and pH (Soleng *et al.*, 1999). However, like any organisms, once a parasite is adapted to an environment (water body), we should expect that this is the environment in which its performance will be maximum, regardless to the chemico-physical properties of that environment (Combes and Morand, 1999). In fact, the difference between survival time in own and different lochwater remained significant even when the saltwater strain (Obse) was excluded from the analysis, suggesting that even subtler differences between freshwater environments can drive local adaptation of the parasites.

Parasite longevity was neither influenced by concentrations nor origins of epidermal mucus samples. Extractions from skin tissues and mucus of some fish species, regardless of their infection status, have been reported to contain anti *Gyrodactylus* sp. agents *in vitro* (Harris *et al.*, 1998; Buchmann and Bresciani, 1999; Buchmann *et al.*, 2000). In this chapter, skin mucus sampled from both uninfected lab raised and infected wild stickleback populations had no statistically important influence on the survival time of detached *G. arcuatus*. Buchmann (2000) describes fish epidermal mucus as a “two-edged sword” because, on the one hand, it constitutes an important immune mechanism against pathogens including *Gyrodactylus* sp. and on the other, it seems to contain factors activating these monogeneans. Indeed, there was a slight increase in the survival time of detached parasites in water containing mucus than in the water (control) alone. *Gyrodactylus* sp. normally feed on skin mucus and epithelial cells of their living hosts (Kearn, 1979; Harris, 1982). As mucus samples were collected by rubbing epidermal surface of freshly killed fish, possibly mucus gradients could provide a source of nutrients

for the detached parasites. It has been found that the survival time of *G. salaris* on dead hosts was significantly higher than detached individual, suggesting that these parasites can utilize tissues of dead fish at least for a certain period (Olstad *et al.*, 2006).

The density of mucous cells was consistently higher in head skin than in caudal fin in experimentally infected lab raised and wild fish and in wild caught fish. These results are in the line with those of Pickering (1974) in which the anterior body parts of brown trout (*Salmo trutta*) and char (*Salvelinus alpinus*) contained more mucous cells than fins and posterior body parts. The author suggested that such a pattern of mucous cell distribution may result from the functions of epidermal mucus in fish. Mucous cell distribution is thought to influence the distribution and site specificity of *Gyrodactylus* sp. For example, Buchmann and Bresciani (1998) reported that the initial distribution of *G. derjavini* on rainbow trout (*Oncorhynchus mykiss*) was skewed toward sites which contained high density of mucous cells and at later time of infection the distribution was reversed. The authors suggested that presence of liable substances in fish mucus possibly attract the parasite at first, but reactive substances are also present in mucus and may act against the parasite's site preference. In the current study, I found no difference in the intensity distribution of *G. arcuatus* among the three zones on experimentally and naturally infected wild fish, but the parasite intensity was clearly higher on the anterior part (zone 1) of experimentally infected lab bred fish (exp. 3b). Compared to other fish populations, Chru fish generally had high mucous cell density in their caudal fin (and possibly in other fins) and these could have caused a balanced distribution of the parasite individuals among the three zones. On their sympatric lab raised hosts, the distribution of some of these parasites (Maga, Obse, Reiv and Scad) also did not differ among the three zones (Mahmud's unpublished data), therefore, the distribution of these parasites is very likely to have an

adaptive association with factors (mucous cell density) in host skin (Buchmann and Bresciani, 1998).

Generally, there was a negative relationship between the parasite intensity and the measures of epidermal mucous cells. In artificially infected Chru fish, six out of seven infected treatments had lower mucous cell density in their caudal fin than uninfected control, but in artificially infected Jubilee lab bred fish, there was a strong negative association between mucous cell diameter in head skin and intensity of parasites. In wild sticklebacks, the density of mucous cells differed greatly among populations and that the measures (density and size) of mucous cells negatively associated with the average natural abundance of *Gyrodactylus* sp. These results are in agreement with previous findings. For example, Cusack and Cone (1986) reported a negative effect of *G. salmonis* intensity on the density of goblet mucous cells in the skin of brook trout fry. Wells and Cone (1990) observed reduction in the density of mucous cells in the fins of *Oncorhynchus mykiss* fry infected with *G. salmonis* and *G. colemanensis*. Sterud *et al.* (1998) found that infection with *G. salaris* induced mucous cell reduction in the skin of hatchery-reared salmon, but not brook trout under the same experimental conditions. The authors also reported that compared to salmon, brook trout suffered lesser intensity of the parasite, suggesting that susceptibility (in salmon) was related to the loss of mucous cells. Other findings suggest a positive correlation between the concentration of surface mucus and fish resistance to infections with *Gyrodactylus* sp.. For example, Lester and Adams (1974) found that infections with *G. alexanderi* in sticklebacks cause continuous mucus shedding, suggesting that is an important defence mechanism against the fluke. Buchmann and Uldal (1997) found a negative relationship between susceptibility of four salmonid fish to *G. derjavini* and density of mucous cells in their tail fin. Collectively, these studies may suggest two

different views regarding to the role is played by skin mucous cells in the host susceptibility to infections with *Gyrodactylus* sp.: (first), it is supportive to the parasite (increases host susceptibility) and (second), it is repulsive to the parasite (decreases host susceptibility). Interestingly, my results appear to support the first view because: (1) detached gyrodactylids survived better in mucus gradients (at least to some extent) than tap water alone, (2) wild fish reduced mucus cell density when experimentally infected, (3) in naive fish, gyrodactylids preferred head skin where there were more mucus cells and (4) wild populations with more *Gyrodactylus* sp. abundance had less and smaller mucous cells.

In summary, the three experiments carried out in this chapter show that the monogenean trematode *G. arcuatus* can successfully adapt to both abiotic environmental variables and biotic factors present in hosts' skin. Geographically isolated strains of this parasite vary markedly in their off- host survival time and survival was substantially higher in their own than other lochwaters, suggesting an ultimate physiological adaptation to the abiotic factors of their environments. The parasite interaction with host epidermal mucous was not significant *in vitro* and hosts clearly reduced the mucus concentration of their epidermal skin in response to the parasite infections. The relationship between mucous cell abundance and parasite intensity was consistently negative across experimental and field data. These results suggest that stickleback's epidermal mucus may actually associate with susceptibility to infections with *Gyrodactylus* sp. Therefore, the natural abundance of *G. arcuatus* may be an important cause shaping the density and size of mucous cells in the caudal fin and head skin of wild sticklebacks.

Chapter 4.

Behavioural and ecological relationship between three-spined stickleback and two metazoan parasites

Chapter 4. Behavioural and ecological relationship between three- spined stickleback and two metazoan parasites

4.1 Abstract

The social behaviour of hosts is thought to be an adaptive trait that plays a role in the epidemiology of parasites with different modes of transmission. Trophically transmitted parasites are predicted to manipulate hosts' behaviour (e.g. predator avoidance) to mediate their transmission from one host (prey) to the next host (predator) species, but directly transmitted parasites might be expected to induce grouping behaviour in order to increase contact incidence between infected and uninfected hosts. In this chapter, I used wild and lab raised three-spined sticklebacks (*Gasterosteus aculeatus*) to examine the relationship between parasitic infections and fish social behaviour. Schooling and shoaling behaviour were estimated for seven wild fish populations sampled from separate lochs with different ecological and environmental conditions. I examined the effect of natural *Schistocephalus solidus* (cestode with complex life cycle) infection on shoaling and schooling of individual sticklebacks and the relationship between the fish population behaviour and the average natural abundance of *S. solidus* and *Gyrodactylus* sp. (monogenea with direct life cycle). I also examined the effect of experimental infection with single and double *Gyrodactylus* sp. (*G. arcuatus* and/or *G. gasterostei*) on schooling and shoaling of lab raised sticklebacks and whether that is likely to be mediated through energetic costs. Infection of wild fish with *S. solidus* and lab raised fish with *Gyrodactylus* sp. led to significantly less time schooling than uninfected conspecifics, but there was no association between average natural abundance of any of the parasite species and the behaviour of wild fish populations. I found that gyrodactylid -induced reduction in schooling time was not due to

energetic costs of infection. These results suggest that infections with *S. solidus* and *Gyrodactylus* sp. reduce grouping tendency in sticklebacks and that may make the host fish susceptible to predation. This may have fitness advantages for parasites with complex life cycle, but it is likely to have negative consequences for directly transmitted parasites.

4.2 Introduction

Pathogen transmission is one of the most important factors to control epidemic occurrence in both humans and animals (Reno, 1998). Parasites that need to go through different host species to complete their life stages have been commonly reported to manipulate the behaviour of their intermediate hosts to facilitate their transmission (Barber and Huntingford, 1995; Thomas *et al.*, 2002; Lagrue *et al.*, 2007; Seppälä *et al.*, 2008).

Trophically transmitted parasites are commonly transmitted from intermediate hosts to definitive hosts by predation. Therefore, studies suggest that selection pressure on trophically transmitted parasites is high to increase the susceptibility to predation of their hosts (Kuris, 2005). For parasites with direct lifecycles, transmission might be dependent on characteristics of hosts and parasites that mediate the direct contacts between the two partners (Johnson *et al.*, 2011). For example group formation can determine the probability of physical contacts between infected and uninfected hosts and hence parasite transmission efficiency (Richards *et al.*, 2012).

In fish, group formation may include a cluster of individuals staying in close proximity (aggregating) which is called ‘shoaling’ or swimming in a polarized group in one direction, called ‘schooling’. Although fish are thought to gain several advantages from grouping, such as increased antipredator responses, foraging efficiency, swimming efficiency and mate finding for reproduction (Pitcher *et al.*, 1982; Wagner *et al.*, 2000;

Larsson, 2009; Marras *et al.*, 2015), grouping is thought to increase the risk of acquiring infection with directly transmitted pathogens (Ezenwa, 2004; Johnson *et al.*, 2011). In the presence of contradicting effects of social grouping, individuals' fitness is likely to be maximized by a trade-off between the costs and benefits of forming groups (review in Domenici *et al.*, 2007).

The role of within population ecological interactions of hosts in parasite transmission is not clear. Generally, theoretical models use two assumptions to predict the probability of contact rates between infected and uninfected hosts (see Read and Keeling, 2003 for a review). These assumptions include: (1) populations are homogeneously mixed and the probability of infection incidence is equal for all the individuals (May and Anderson, 1979); (2) populations have non-random mixing patterns in which local processes (eg. social and sexual behaviours) influence the ecological interactions between hosts and parasites (Boots and Sasaki, 1999; Krause and Ruxton, 2002; Rifkin *et al.*, 2012). The latter assumption is biologically more acceptable in that directly transmitted parasites are thought to exploit different host contacts during social and sexual interactions (Ryder *et al.*, 2007; Croft *et al.*, 2011; Johnson *et al.*, 2011). For example several studies have shown a positive correlation between shoaling preference in fish and the transmission success of ectoparasites (Ward *et al.*, 2005; Johnson *et al.*, 2011; Richards *et al.*, 2012).

The role of social structure in the spread of infectious pathogens has become more evident since the development of social network theory (Potterat *et al.*, 1999; Eames and Keeling, 2002; Meyers *et al.*, 2005). This theory assumes that the social structure of populations comprises a network assembled by interconnecting individuals through their social associations (Croft *et al.*, 2011). Parasites can potentially interfere with host social networks for reasons that may include: (1) infected individuals are recognized and rejected

by uninfected mates leading to frequent shoal splitting (Croft *et al.*, 2011); (2) the social preference of infected individuals is altered and the alteration may be manipulated by parasites to facilitate transmission (Barber and Huntingford, 1995; Ward *et al.*, 2005) or by hosts to reduce the impact of infection (Poulin and FitzGerald, 1989; Poulin, 1995). Therefore, parasite infections may affect both the social associations of infected individuals and the social network structure of the host population (Croft *et al.*, 2011).

Here I used both wild and lab raised three-spined sticklebacks sampled from North Uist, Scotland to: (1) examine whether experimental infections with *Gyrodactylus* sp. affect individual sticklebacks' grouping tendency and if yes, investigate whether that happens through pathological effects of infections or is due to a behavioural response by individuals; (2) examine the influence of natural *Schistocephalus solidus* infections on shoaling and schooling of seven isolated populations; (3) examine the relationship between the natural abundance of *S. solidus* and *Gyrodactylus* sp. and the grouping behaviour of wild fish populations.

Stickleback populations on North Uist experience different levels of predation (MacColl *et al.*, 2013; MacColl and Aucott, 2014) and a range of parasite taxa (de Roij and MacColl, 2012). Among the parasites, *S. solidus* (cestode) and *G. arcuatus* (monogenean) have different life cycles and have frequently been found to share the same individual hosts (de Roij and MacColl, 2012). *S. solidus* is trophically transmitted from copepods (the first intermediate host) to sticklebacks (the second intermediate host) and from sticklebacks to different species of fish eating birds (definitive host). The abundance of *S. solidus* is likely to depend on biotic and abiotic factors affecting parasite transmission (Iqbal, 2014). This parasite is thought to induce behavioural and morphological changes making infected fish

less social and more prone to predation (Arme and Owen, 1967; Barber and Huntingford, 1995).

Schooling and shoaling are likely to affect the probability of host- host body contacts (Read and Keeling, 2003; Gudelj and White, 2004; Tildesley *et al.*, 2010) and hence transmission of ectoparasites such as *Gyrodactylus* sp. *Gyrodactylus* sp. are a group of monogenean ectoparasites that commonly live on fin and skin of a wide range of fish species. They have a direct lifecycle and give birth to completely mature worms. Newly born individuals are already pregnant with a fully grown embryo and this embryo contains another growing inside it. They lack a resting stage and a single worm is enough to seed a new population, a biological attribute that makes the parasite extremely infectious (Cable and Harris, 2002). This group of parasites attaches to surface tissues and mostly transmits from infected to uninfected individuals using direct body contacts between fish hosts (Winger *et al.*, 2012). Therefore, stickleback group preference may play a critical role in parasite transmission success.

Previous studies have demonstrated the existence of genetic differences in grouping tendency between isolated stickleback populations and shown that group structure is influenced by individual characters such as sex, size and health status. For example marine sticklebacks showed higher schooling tendency than a benthic morph when raised under uniform lab conditions (Wark *et al.*, 2011) and ‘mud’ morph fish formed more cohesive shoals than those of a ‘lava’ morph (Doucette *et al.*, 2004; Ólafsdóttir *et al.*, 2014).

Generally males have been found to be less social than females regardless of their reproductive status (Webster and Laland, 2011). Non- reproductive females showed increased preference to shoal with gravid females than non- gravid females (Frommen *et al.*, 2012). Here I test the hypothesis that generally, isolated wild stickleback populations

should differ in shoaling and schooling tendency. I expect that infection with *G. arcuatus* should lead to increased grouping tendency of sticklebacks that will improve the parasite's probability of transmission.

4.3 Materials and methods

Two experiments were performed to: (1) examine the effect of *S. solidus* infection on the grouping behaviour (shoaling and schooling) of seven wild stickleback populations (Table 4.1) and whether there is a relationship between group formation tendency and the natural abundance of *Schistocephalus solidus* and/or *G. arcuatus*, (2) examine the effect of *G. arcuatus* infection on the group-joining tendency of a lab bred fish population (Reiv).

Experiment 1.

An assay originally developed by Wark *et al.* (2011) was adopted to estimate the tendency of fish schooling and shoaling behaviours. For each of seven wild fish populations (Table 4.1) approximately 34 individuals which did not show breeding signs were kept for at least two weeks to acclimatize them to lab conditions prior to the experiment day. It has been well established that both predators and parasites drive adaptive changes in fish social behaviour (Barber and Huntingford, 1996; Ward *et al.*, 2005; Botham *et al.*, 2006; Bell and Sih, 2007; Croft *et al.*, 2011). Here these stickleback populations were chosen because they vary greatly in ecological conditions such as the prevalence and abundance of different parasite species especially *G. arcuatus* (monogenean), *S. solidus* (cestode) and *Glugea anomala* (microsporidian) as well as the density of predators (brown trout) (de Roij and MacColl, 2012; MacColl *et al.*, 2013). The lochs also differ in environmental conditions such as conductivity and pH (Table 4.1). Attempts were made to select only

those individuals which did not show signs of breeding such as nuptial colouration of the anterior body in males and signs of being gravid/ abdominal enlargement in females in order to exclude effects of sexual behaviour on the current study.

Table 4.1. The grid references, fish standard length, mean natural abundance of *Gyrodactylus* sp. and *S. solidus* measured for the seven stickleback populations used in this study.

Loch (population)	Code	Grid reference	Mean fish St. length ($\pm$ SE)	Mean <i>Gyrodactylus</i> sp. abundance ($\pm$ SE)	Mean <i>Schistocephalus</i> abundance ($\pm$ SE)
Mhic Gille-bhrìde	Gill	57°36'07.3"N 7°24'37.3"W	38.3 (1.15)	3.72 (0.49)	1.24 (0.68)
Hosta	Host	57°37'38.1"N 7°29'24.8"W	40.2 (1.16)	2.48 (0.34)	1.81 (0.20)
nam Magarlan	Maga	57°35'16.9"N 7°26'38.7"W	44.3 (1.95)	2.44 (0.41)	0.1 (0.05)
Ob nan Stearnain	Obse	57°36'06.1"N 7°10'25.4"W	38.9 (1.45)	9.40 (1.14)	0 (0.0)
na Reivil	Reiv	57°36'42.9"N 7°30'53.2"W	47.1 (1.44)	2.35 (0.5)	0.3 (0.07)
Scadavay	Scad	57°35'05.5"N 7°14'11.2"W	30.2 (1.61)	0.78 (0.12)	1.5 (0.29)
Tormasad	Torm	57°33'46.8"N 7°18'54.4"W	38.3 (1.15)	0.17 (0.05)	0.07 (0.05)

The assay involved 34 individuals (stimulator = 8, control = 10 and test fish = 16) for each population tested. The stimulator fish (from the 34) and were selected to represent approximately the average size of test fish. Attempts were made to select the healthiest individuals and minimum infections with ectoparasites. The stimulator individuals were killed using an over dose of MS222 (400 mg/ L) and the death was confirmed by destruction of the brain using a sharp needle inserted into the top of head. The dead fish were kept in a separate tank of clean tap water for not less than 10 min to confirm the absence of signs of bleeding around the needle insertion site. The stimulator fish were organized in a polarized group using fine metal wires similar to the organization of model stimulator fish developed by Wark *et al.* (2011) (Figure 4.1). The movement of stimulator fish was controlled by a six- volt battery motor (spinning at 6 RPM). In total, 119 (approx. 16 per populations) fish were individually tested for both schooling and shoaling behaviours in a 50L dark-edged plastic tank (54 x 36 x 10 cm) containing dechlorinated tap water. For saltwater fish (Obse), the tank was filled with saltwater brought from the fish loch. Individual fish was introduced into the testing tank at one side and videoed it for 6 minutes. The first two minutes were allowed for fish familiarization with the microhabitat and the second two minutes to estimate fish tendency toward joining the stationary stimulator group (shoaling). In the third two minutes stimulators were regularly moved in a circle (diameter = 20 cm), fish tendency toward joining the moving stimulator group (schooling) was estimated. The response variables ‘shoaling’ and ‘schooling’ were recorded by measuring the absolute time (number of seconds) spent within a ‘joining zone’ (5 cm distances to the stimulator individuals). As a control for schooling behaviour, the assay was repeated for ten naive fish, with stones (of similar size to the stimulator fish) suspended from the wires instead of fish to confirm that the any behavioural response was

due to a fish to fish interaction and/or simply a response to moving objects. For all seven populations, control fish showed no response to moving stones which were used instead of stimulator fish for estimating schooling tendency and hence I did not consider these results further. All the fish were killed and dissected at the end of the experiment to confirm their gender and record whether they harboured plerocercoids of *S. solidus* in the body cavity. For infected fish, the number and wet weight of *S. solidus* plerocercoids were recorded.

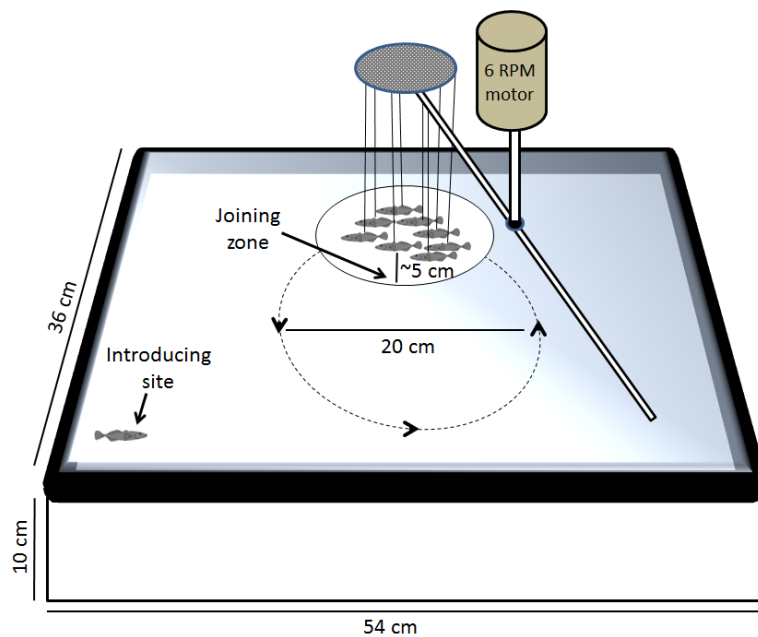


Figure 4.1. A schematic diagram depicting the design of schooling behaviour assay in the first experiment.

Experiment 2.

The shoaling and schooling tendencies of *Gyrodactylus*- infected stickleback were compared to uninfected conspecifics. In this experiment, fish were used from one population (Reiv) which had been raised under controlled conditions and had no previous exposures to parasites. In total, 47 individuals were used and divided among four

treatments (approximately 12 replicates per treatment). The treatments included: (1) uninfected control, (2) infected with a sympatric parasite species (*G. arcuatus*), (3) infected with an allopatric parasite species (*G. gasterostei*) obtained from naturally infected Tottle Brook fish and (4) coinfecting with both parasite species. For the three infected treatments, infection was initiated by placing a total of four worms on the caudal fin. These comprised either four individual worms from *G. arcuatus* (Treatment 2) or *G. gasterostei* (3) or two worms from each species (4). After initial exposure, infections were left for 21 days to develop (details of infection procedures are explained in chapter 5). The number of seconds spent schooling was recorded using the same protocol employed in experiment 1, but for shoaling, a different method was used (Fig. 4.2). In this method, stimulator fish comprised three uninfected live individuals of the same population with the same age and similar body size, raised under the same lab conditions as the tester fish. The stimulator fish were placed in a 3L transparent plastic tank (25 x 12 x 10 cm) and the tank was fixed in one corner of a larger testing tank (54 x 36 x 10 cm). An empty 3L tank (identical to that of the stimulator fish) was placed in the opposite corner of the large tank. Tester fish were individually introduced into the centre of the larger tank, and after two minutes acclimatization their behaviour was filmed for two minutes. Shoaling time was measured by recording the number of seconds spent in the joining zone (within 5cm distance of the stimulator tank).

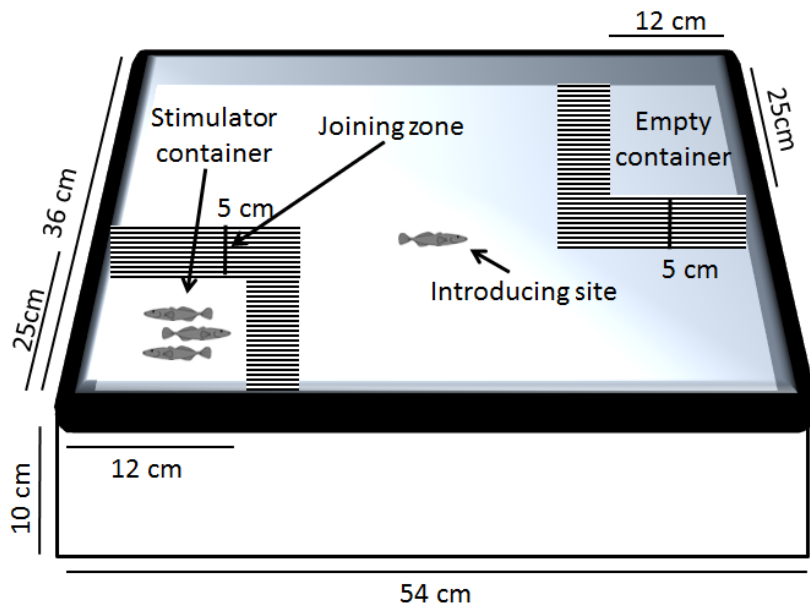


Figure 4.2. The diagram of shoaling behaviour assay in the second experiment.

After estimating the schooling and shoaling tendency, the fish were tested for swimming performance against water current using the output of an aquarium filter machine (Fluval 204, Askoll, Italy) to examine whether there was any change in the swimming performance of infected fish suggestive of energetic costs of infection (Fig. 4.3). A non-transparent plastic chamber (45 x 10 x 8 cm) was used to compare the dynamics of swimming efficiency (performance) among the fish treatments and to determine whether that was associated with the time spent schooling. The chamber received a continuous input of water (output = 680 L/hr) at one side and the water exited through a fine plastic mesh (1mm x 1mm mesh hole size) on the other side of the chamber. Each individual fish was introduced into the chamber at 12 cm far the water exit side and was videotaped for two minutes. The number of seconds spent in the active zone (distance of 35cm between the introducing site and water input point) was recorded. Performance test was not carried out in the first experiment because test fish were wild caught and it was not possible to

know about their infection history. In the current study, fish housing, using in the experiments and killing were done under a UK Home Office Project License 40/2886, and in accordance with the authorization of the Ethical Review Committee of Nottingham University.

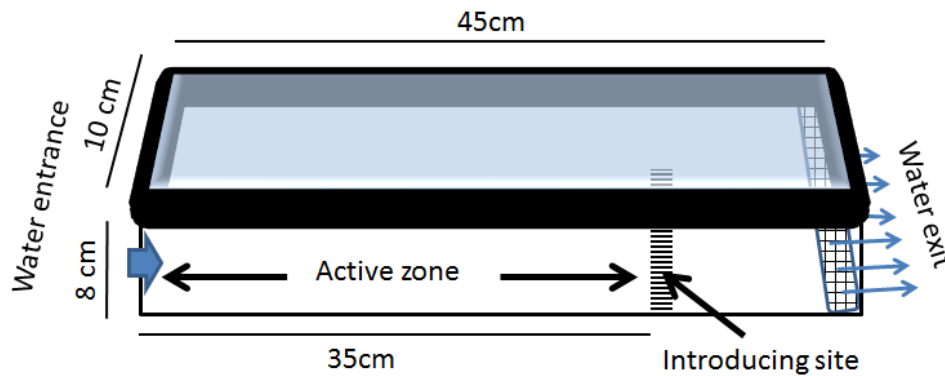


Figure 4.3. Fish performance assay in the second experiment.

Statistical analysis

A Generalized Linear Model (GLM) with negative binomial distribution and log link function was used to examine the natural abundance of *Schistocephalus* and *Gyrodactylus* sp. for at least 20 individuals for each of the fish populations. For the first experiment, each of the response variables; 'shoaling time +1' and 'schooling time +1' was analysed for all test fish (*S. solidus*- infected and uninfected individuals) using a GLM with a gamma probability distribution and log link function. Fish population and fish sex were included as fixed factors while fish standard length and wet weight of *S. solidus* were treated as covariates. Previous studies have revealed that natural prevalence and abundance of *S. solidus* and *Gyrodactylus* sp. vary across stickleback populations on North Uist (de Roij and MacColl, 2012), here these were examined whether associate with fish grouping

preference. A multiple linear regression with backward elimination was used to examine whether any of the response variables mean ln 'shoaling' or 'schooling' time of the fish populations were associated with any of three predictors (abundance of *S. solidus*, abundance of *Gyrodactylus* sp. or fish standard length). For the second experiment, each of the response variables; 'shoaling', 'schooling' and 'performance' time was analysed using a General Linear Model for univariate measure. The main effects included fish sex and infection status (treatment) as fixed factors and fish length and total worm load as covariates. All the statistical analyses were performed in SPSS package (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

4.4 Results

Experiment 1.

Post experimental dissection revealed that out of 119 individuals tested, 92 were females and 27 were males. From the 119 test fish, 22 individuals harboured plerocercoids of *S. solidus* and these included nine males and 13 females. The natural abundance of *Gyrodactylus* sp. and *S. solidus* differed significantly among the seven fish populations (for *Gyrodactylus* sp., $\chi^2 = 313$, df = 6, $P < 0.0001$ and for *S. solidus*, $\chi^2 = 62.9$, df = 6, $P < 0.0001$).

Generally, fish populations did not show large differences in time spent shoaling, but schooling tendency differed markedly among the populations (Fig. 4.4) and between *S. solidus* free and infected individuals (Fig. 4.5B). Schooling tendency differed significantly among fish populations ($\chi^2 = 25.8$, $P < 0.001$, Table 4.2A) and Bonferroni pairwise

comparison test showed that Reiv fish had significantly higher schooling tendency than Host fish population ($P < 0.05$). Neither fish standard length nor sex had effects on schooling and shoaling time ($P > 0.05$, Table 2.2, Fig. 4.5A), but the effect of *S. solidus* wet weight on schooling tendency was significant ($P = 0.015$). Indeed, *S. solidus* infected individuals had lower schooling and shoaling tendency than uninfected conspecifics. There was no correlation between the schooling and shoaling times for individuals (Spearman correlation, $r_s(119) = 0.17$, $P = 0.062$) and for populations ($r_s(7) = 0.29$, $P = 0.556$).

Table 4.2. GLM outcomes for schooling (A) and shoaling (B) response variables measured for seven wild fish populations. Fish populations and fish sex were the fixed factors while standard length and wet weight of *Schistocephalus solidus* were covariates.

Effects	χ^2	df	P value
<i>(A)- For schooling time</i>			
Population	25.8	6	<0.001
Fish sex	0.2	1	0.654
Fish standard length	0.3	1	0.603
<i>S. solidus</i> wet weight	5.9	1	0.015
<i>(B)- For shoaling time</i>			
Population	4.5	6	0.610
Fish sex	0.1	1	0.756
Fish standard length	0.1	1	0.753
<i>S. solidus</i> wet weight	2.8	1	0.093

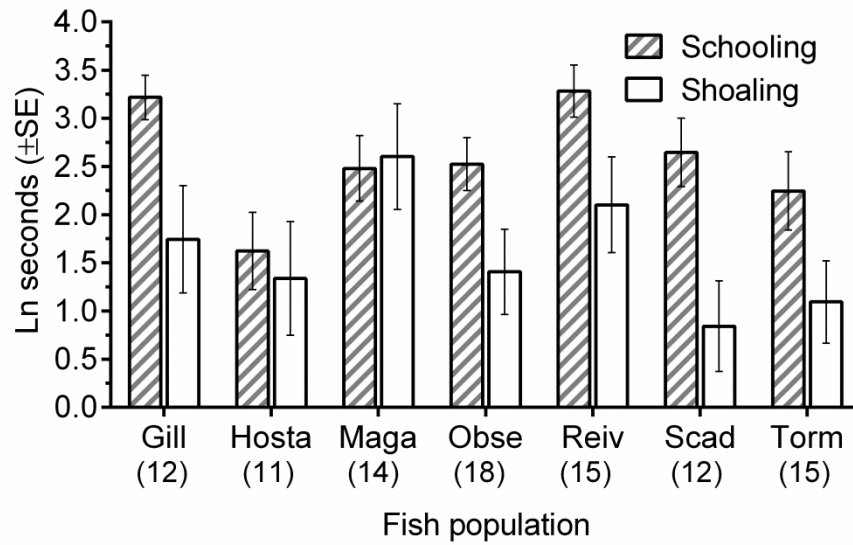


Figure 4.4. Contrast in the ln number of seconds spent schooling (lined) and shoaling (plain) for seven wild stickleback populations. Numbers in brackets represent the number of individuals (n) included in the analysis (i.e. *Schistocephalus solidus* free individuals only).

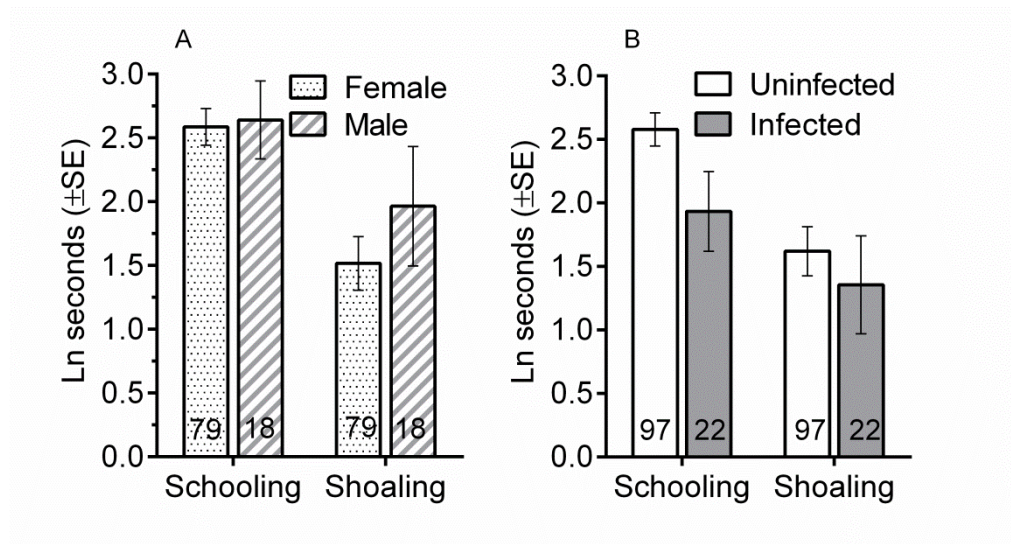


Figure 4.5. Difference in the ln number of seconds spent schooling and shoaling, between sexes for *Schistocephalus solidus*- free individuals only (A) and between *S. solidus*- free and infected individuals (B). Note: numbers in the bars represent the number of replicates used per treatment.

The mean shoaling time was positively associated with the average standard length of the fish populations, but not the natural abundance of either of the two parasite species (*G. arcuatus* and *S. solidus*). The regression model was statistically significant ($F_{1,5} = 6.72$, $P = 0.049$) and accounted for 57% of the variance of mean time spent shoaling ($R^2 = 0.573$, adjusted $R^2 = 0.488$, fig. 4.6). Mean abundances of *S. solidus* and *G. arcuatus* were not significant predictors ($P > 0.05$). The model was not significant for schooling tendency ($P > 0.05$).

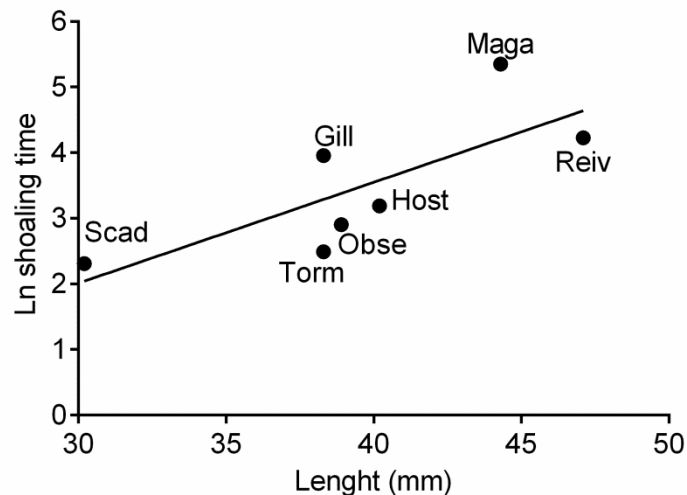


Figure 4.6. The relationship between mean 'ln shoaling' time and the average standard length of seven wild fish populations (dot labels). Note: only *Schistocephalus solidus*- free individuals were included (see texts for details).

Experiment 2.

Generally, there was some influence of *G. arcuatus* infections on schooling behaviour, but not on shoaling or swimming performance of lab bred fish (Fig. 4.7A- F). GLM revealed that schooling tendency significantly differed among the four fish treatments (Table 4.3).

Post hoc tests showed that uninfected control fish schooled significantly longer time than the group which was infected with allopatric *Gyrodactylus* sp. ($P < 0.05$). Generally, fish which had higher worm burdens had lower schooling tendencies than others, but the influence of total worm load on individual fish was not significant. Neither sex nor standard length had effects on the number of seconds spent schooling. However infection with sympatric worms (*G. arcuatus*) tended to slightly increase shoaling time in comparison to other treatments including control fish, but overall difference among the four treatments was not significant ($P > 0.05$). The four fish treatments were quite similar in swimming performance and their ability to swim against a water current was not influenced by any of infection status, fish sex, fish standard length or total worm load.

Table 4.3. Statistical analysis of the effect of *Gyrodactylus* sp. infection on (I) schooling, (II) shoaling and (III) performance stickleback in the third experiment.

Effects	F	Df	P value
<i>(I) For schooling time</i>			
Infection status	9.8	3	0.021
Fish sex	0.4	1	0.551
Fish standard length	0.01	1	0.920
Total worm load	0.1	1	0.702
<i>(II) For shoaling time</i>			
Infection status	7.1	3	0.068
Fish sex	1.2	1	0.273
Fish standard length	1.2	1	0.277
Total worm load	0.2	1	0.670
<i>(III) For swimming performance</i>			
Infection status	2.0	3	0.569
Fish sex	1.3	1	0.248
Fish standard length	1.5	1	0.223
Total worm load	1.1	1	0.294

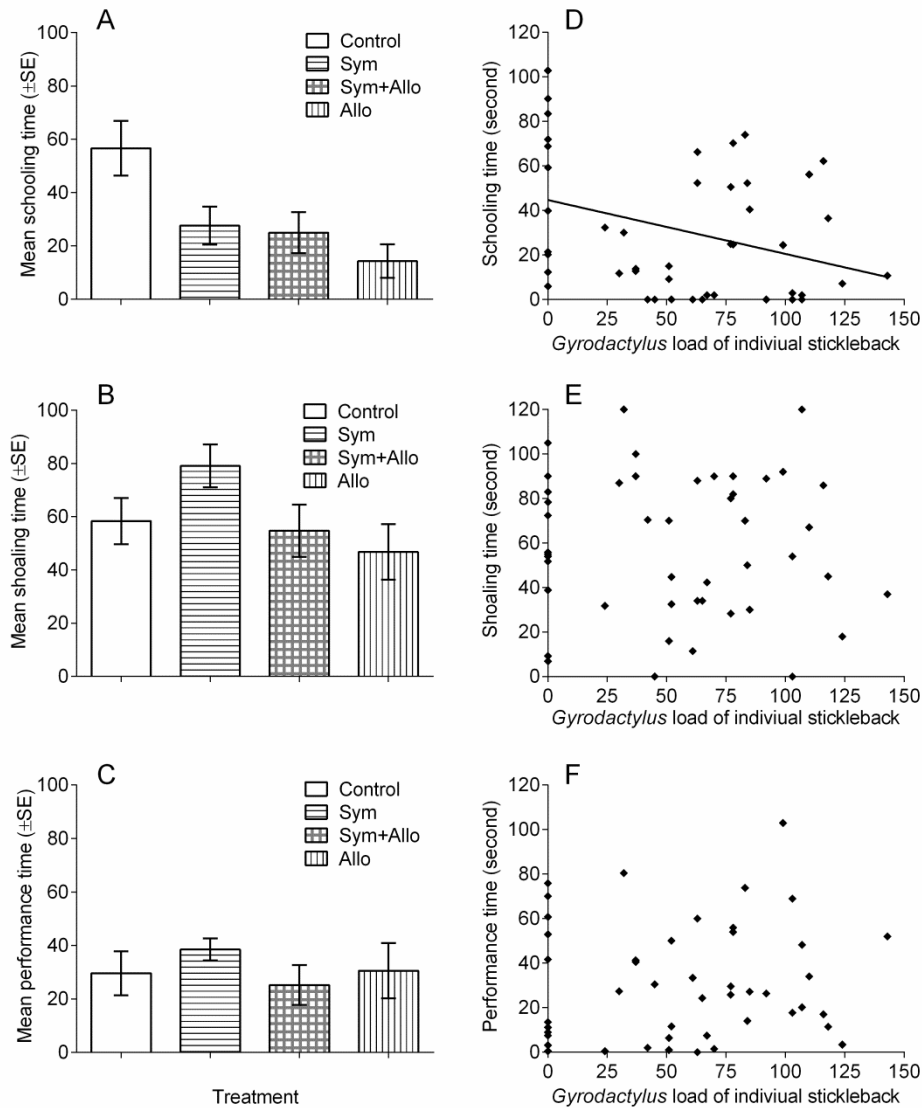


Figure 4.7. The right panel shows the contrast in fish schooling (A), shoaling (B) and swimming performance (C) among four treatments: control, sym (infected with *G. arcuatus* only), sym+allo (coinfected with both *G. arcuatus* and allopatric *G. gasterostei*) and allo (infected *G. gasterostei* only). The left panel indicates the relationship between each of the fish behaviour (schooling, shoaling and performance) and total worm load (see the texts for details).

4.5 Discussion

Wild fish populations did not vary in shoaling preference, but they clearly differed in schooling tendency and that time spent schooling was significantly affected by *S. solidus* infection. Presence of the parasite markedly reduced schooling, but not shoaling tendency.

Neither fish size nor their sex had an influence on the two behavioural measures.

Compared to uninfected controls, artificially infected lab bred sticklebacks with sympatric, allopatric and mixed *Gyrodactylus* sp. consistently reduced their schooling, but not shoaling tendency. The schooling time of lab raised fish was more influenced by infection rather than worm load and the group which was infected with allopatric parasites had the lowest schooling tendency. Both the control and infected lab fish did not differ in the time spent swimming against a water current (performance) and this may indicate that the schooling difference was more likely to be a behavioural rather than a pathogenic response of the infected fish.

Under heterogeneous environments, group formation behaviours in many aquatic vertebrates can be driven by environmental stressors such as predators (Huizinga *et al.*, 2009) and parasites (Poulin and FitzGerald, 1989; Barber *et al.*, 1998). Schooling is the most commonly used strategy by fish which do not have proper shelter against these environmental challenges (Pitcher, 1993; Wark *et al.*, 2011). On North Uist, geographically isolated stickleback populations experience a range of these environmental factors (de Roij and MacColl, 2012; MacColl *et al.*, 2013) and hence the divergence in schooling behaviour among the populations might be an adaptive behaviour that affords protection to stickleback (Larson, 1976; Doucette *et al.*, 2004; Wark *et al.*, 2011). Schooling performance may energetically be more demanding than shoaling because fish must precisely harmonize their location, motion angle and speed with their school mates (Pitcher, 1979). Therefore, fish may exhibit changes in schooling quicker than shoaling behaviour.

The negative influence of *S. solidus* infection on the schooling tendency of sticklebacks is in agreement with previous studies. For example, the cestode parasite has been found to

reduce grouping tendency in infected sticklebacks by increasing the demands for foods (Arber *et al.*, 1995; Barber and Huntingford, 1995). In addition to the energetic costs of parasitism, infected fish are likely to leave groups in order to avoid competition with uninfected conspecifics on foods (Barber and Huntingford, 1995; Barber and Huntingford, 1996). Such manipulation of behaviour in infected stickleback is thought to be advantageous to *S. solidus* as it makes the fish more vulnerable to predation by final hosts (fish-eating birds). *Gyrodactylus* sp. have a directly transmitted parasites and therefore increased predation on its stickleback hosts may have consequences for the parasite fitness (Choo *et al.*, 2003).

Shoaling tendency did not differ among the populations ($P > 0.05$), but unlike schooling, the average shoaling time of fish populations was positively correlated with their average body length. This observation is in line with previous findings on guppies (Paxton, 1996; Hockley *et al.*, 2014). Joining groups may be more advantageous to larger individuals because they can be more successful competitors compared to smaller shoal mates (Hoare *et al.*, 2000). It is also thought that larger fish are more visible to predators than smaller conspecifics, therefore, joining shoal may increase their protection (Rodgers *et al.*, 2011; Hockley *et al.*, 2014). In the lab, higher shoaling tendency in guppies is associated with contracting more *G. turnbullis* infections (Richards *et al.*, 2010, 2012). The observed association between fish body size and shoaling tendency may suggest that fish size is indirectly associated with the transmission of directly- transmitted parasites. Indeed, several studies have found a positive association between the abundance of *Gyrodactylus* sp. and fish body size (Barber, 2003; Cable and Oosterhout, 2007a; de Roij and MacColl, 2012).

In fish, it is thought that certain behaviour is selected based on its fitness advantages to the organism and it is likely to associate with other behaviours of the organism (Møller *et al.*, 1993; Bell, 2005). For example a negative correlation was found between aggression and boldness behaviours of a stickleback population, but such association was absent in another population of the same fish (Bell, 2005). The author suggested that association between different behaviours might be dependent on selective factors driven by certain ecological circumstances. In the current study, I found no relationship between shoaling and schooling in the two experiments.

The sex and body size of individual test fish did not affect schooling and shoaling and this was consistent through the two experiments. It has been found that female guppies show high shoaling tendency and therefore they contract more *G. turnbullis* than males (Richards *et al.*, 2010; Johnson *et al.*, 2011). Thus it was concluded that there is a trade-off between the possible advantages of high shoaling tendency and the impact of being infected by ectoparasites. In another study, (also using *Gyrodactylus*- guppy hosts), the opposite result was found in which males had a higher tendency toward grouping than did females (Richards *et al.*, 2012). The authors showed that higher shoaling tendency in males led to a ten-fold increase in the chance of the parasite infections compared with females. In sticklebacks, gravid females have been found to be generally preferred by other conspecifics because they are slower in movement and this may increase the survival chances for non-gravid shoal mates (Frommen *et al.*, 2012). In the current study, majority of the fish were females (77.3%), but only those individuals were tested which did not show breeding signs. Therefore, I did not expect any an influence of reproductive status on their behaviour.

Parasitized hosts usually display distinct behaviour which might be either mediated by the hosts to reduce the impact of pathogens or by pathogens to facilitate transmission (Klein, 2003). Some parasites which have complex lifecycles have been reported to modify their hosts' behaviour in order to serve their transmission to their next hosts (Poulin, 1993; Lafferty and Morris, 1996; Berdoy *et al.*, 2000). Results of the current chapter showed that *Gyrodactylus*-infected fish had markedly lower tendency to join schools. Hockley *et al.* (2014) also found that guppies had lower shoaling tendency when infected with *G. turnbulli*. The reason for such a pattern of reduced tendency toward schooling might be to avoid further infection, or such behavioural change could be an adaptive trait to limit the spread of infection (Møller *et al.*, 1993). Generally, there was a negative association between total parasite load and mean time spent schooling, but the effect of just being infected was more obvious than the load of parasites per infected individuals. For example, the group which was infected with allopatric worms (*G. gasterostei*) had the lowest worm load in comparison to the other two infected groups, but the schooling time of this group was the lowest.

Gyrodactylus sp. have a direct lifecycle and lack any specific vector, hence transmission is thought to require physical contacts between donor and receiver fish. Reduction in the schooling tendency of hosts might therefore decrease the spread of infections. However, as schooling is an important antipredator behaviour of fish (Magurran, 1990), this might also decrease the host and parasite survival rate. Parasites with complex lifecycles have frequently been reported to affect anti-predator responses of their intermediate hosts. For example, the eye fluke *Diplostomum* species is a trophically transmitted parasite which can potentially affect the visual ability of three-spined stickleback (Owen *et al.*, 1993). Presumably, such parasite- induced manipulation of stickleback behaviour may increase

the transmission probability of the parasite if bird predators are available. In sticklebacks which inhabit environments that are dominated by fish predators (eg. brown trout), such behavioural changes may negatively affect fitness components (survival and transmission) of parasites (Choo *et al.*, 2003).

There is a growing body of evidence that grouping behaviour in natural populations has potential to influence the transmission of parasites (Freeland, 1979; Richards *et al.*, 2010; Chapman *et al.*, 2012; Richards *et al.*, 2012). The influence of such behavioural tendencies on parasite transmission may lead to adaptive changes in other life history traits of parasites such as virulence (Boots *et al.*, 2004). The central hypothesis of virulence evolution suggests that there is a positive genetic association between transmission and virulence of parasites (Anderson and May, 1982). Therefore, understanding the relationship between transmission of pathogens and host behaviour can play an important role in the prevention and control of micro- and macro-pathogens (Macpherson, 2005). Stickleback populations exhibited large differences in their tendency toward schooling and schooling time was significantly reduced by *S. solidus* infections. These results revealed that gyrodactylids- infected sticklebacks have low schooling tendency, possibly to avoid further infections (Møller *et al.*, 1993). Under high fish predation environments, this behavioural change may have consequences for the transmission of these parasites and, therefore, may have consequences for parasite infection success and virulence (Ebert and Bull, 2003).

Chapter 5.

Association between the ecological interaction and virulence in two coinfecting species of a fish flatworm (*Gyrodactylus arcuatus* and *G. gasterostei*).

Chapter 5. Association between the ecological interaction and virulence in two coinfecting species of a fish flatworm (*Gyrodactylus arcuatus* and *G. gasterostei*)

5.1 Abstract

Theory predicts that local adaptation optimizes parasites' fitness on local hosts at the expense of decreased exploitation ability of foreign hosts. Multiple infections are generally thought to lead to competition between parasites and consequently higher host exploitation, but interactions between some helminths have been found to be antagonistic. In this chapter, species of a flatworm ectoparasite (*Gyrodactylus arcuatus* and *G. gasterostei* respectively) sympatric and allopatric with a population of three-spined stickleback (*Gasterosteus aculeatus*) were separately and jointly used to infect lab raised fish. Although all three parasite combinations successfully infected tester fish, sympatric and mixed treatments had higher worm loads than the allopatric line. Compared to their conspecifics in infections with single worm species, *G. gasterostei*, but not *G. arcuatus* had significantly lower burdens in the mixed treatment. Amongst all the fish including uninfected controls, the *G. arcuatus*- infected group had the lowest growth rate and this was associated with size of the worm burden. These results support previous studies which suggest that coevolutionary processes in this host-parasite system result in parasite local adaptation at host population levels. Ecological interaction between the two worm species in the mixed treatment could have resulted in the early stimulation of hosts' immune responses and hence expulsion of *G. gasterostei*. This might suggest that the impact of multiple infections in this system is dependent on the evolutionary association between hosts and parasites and local *G. arcuatus* evolves an immune evasion strategy.

5.2 Introduction

Understanding the evolutionary processes affecting virulence (host fitness reduction caused by parasites) is an important step in the control and future management of infectious diseases (Bull, 1994; Ewald, 2004). Theories of virulence evolution generally predict a positive genetic correlation between virulence of horizontally transmitted pathogens and their transmissibility (Fenner *et al.*, 1956; Anderson and May, 1982; Ewald, 1983). These theories suggest that pathogens optimize their average fitness by maintaining a balance between advantages and disadvantages of harming hosts. However these predictions are based on mathematical analysis of infections by single parasites; empirical findings suggest that natural populations are frequently infected with more than one parasite genotype (Read and Taylor, 2001; Woolhouse *et al.*, 2002). For example multiple infections have been commonly reported among wild hosts (Sharp *et al.*, 1997; Lord *et al.*, 1999; Bordes and Morand, 2011) especially aquatic vertebrates (Simkova *et al.*, 2008; de Roij and MacColl, 2012; Lhorente *et al.*, 2014).

When more than one parasite genotype simultaneously infects a host, simple single parasite models fail to make correct predictions about virulence because one parasite genotype can reduce the fitness of other genotypes by reducing the host's longevity (Sasaki and Iwasa, 1991). Therefore evolutionary biologists have recently added multiple infections to mathematical models to predict virulence evolution of coinfecting parasite genotypes. The models suggest that competitive and synergistic interactions between parasite genotypes affect the evolution of parasite transmission and evolution (Sasaki and Iwasa, 1991; Read and Taylor, 2001; Brown *et al.*, 2002) and hence multiple infections represent an additional selective level affecting virulence (Alizon, 2013).

The earlier view of coinfections suggests that more virulent clones tend to be more dominant and that they quickly eliminate less virulent ones (Levin and Pimentel, 1981), however, multiple infections do not always result in the emergence of more pathogenic forms. For example Schjorring and Koella (2003) suggest that if coinfecting parasites cause only sub-lethal effects (growth reduction) and especially if the effects feedback on parasites to influence their performance, multiple infections are likely to result in lower virulence. Coinfecting parasite genotypes can increase, decrease or have no effect on each other's virulence (Petneya and Andrews, 1998). It is suggested that host exploitation behaviour by coinfecting parasite individuals can influence the nature of selection on virulence (Brown *et al.*, 2002). For example if reproduction and host exploitation rate of an individual parasite is reliant on the joint behaviour of a coinfecting group, multiple infections can adaptively result in decreased virulence (Alizon and Lion, 2011).

The consequences of coinfections can vary massively, mostly depending on interacting parasite species. For example helminths are common parasites that have potential for concurrent infections with other parasite species. Several studies have reported that helminths can alter the risks of infections with other parasites. Partially, such interaction may happen because helminths can significantly affect host immune responses such as ability to change the activity of immunologically important cells (e.g. T_H1 and T_H2) (Maizels and Yazdanbakhsh, 2003). Studies have reported that such changes have potential effects on other helminths. For example, different species of a Monogenean (*Dactylogyrus extensus* and *D. vastator*) gill parasites were found to potentially exclude each other when coinfecting a single of carp host (Paperna, 1964). In mince, *Schistosoma mansoni* infections induced the host immune responses which had a strong antagonistic influence on the coinfecting *Hymenolepis diminuta* and *H. microstoma* (Andreassen *et al.*, 1990). Helminth

parasites can also impact other pathogen species such as the antagonistic effect of *Ascaris lumbricoides* on the risk and recovery dynamics of infections with *Giardia lamblia* in human (Blackwell *et al.*, 2013). Virulence of coinfecting parasites is likely to be dependent on the ecological interactions between parasites and evolutionary associations between the host and their parasites (Boots *et al.*, 2009; Alizon and Lion, 2011). When different parasite genotypes coinfect a host, inducing changes in host resistance or tolerance due to the existence of one parasite population may affect the dynamic of other genotypes (Read and Taylor, 2001). The adaptive outcome for a host may either go down a resistant or tolerant route (Maze-Guilmo *et al.*, 2014), but this is dependent on whether it is coinfecting or not (see Raberg *et al.*, 2009 for a review), since tolerance (which may work against one parasite) may open the host to exploitation by a second parasite (Buendía *et al.*, 2002; Furze *et al.*, 2006).

However, although findings suggest high prevalence of coinfections among wild fish (for eg. see Simkova *et al.*, 2008; de Roij and MacColl, 2012; Lhorente *et al.*, 2014), only limited empirical studies have been carried out in controlled lab conditions (but see Paperna, 1964; Jäger and Schjørring, 2006). In the present chapter, two monogenean ectoparasite species (*G. arcuatus* and *G. gasterostei* respectively) were separately and jointly used to infect lab raised three-spined sticklebacks (*G. aculeatus*). The genus *Gyrodactylus* has a direct life cycle and is characterized with some distinct biological features such as viviparity with polyembryony, using asexual, parthenogenetic and sexual modes of reproduction (Harris, 1989). A newly born *Gyrodactylus* sp. is always pregnant with a mature embryo and the unborn embryo is also pregnant with a younger embryo fixed one inside the other resembling ‘Russian dolls’(Cable and Harris, 2002). These features allow the parasite to successfully establish an entire population from a single

isolated worm. These parasites attach to surface tissues of fins, gills and skin and feed on the epidermis which may breakdown mucous cover increasing the possibility of secondary infections with other pathogens (Cone and Odense, 1984). A positive correlation has been found between the worm load on infected fish and parasite virulence (Scott and Anderson, 1984). The parasite population dynamic is thought to be affected by both innate and acquired immune responses of fish hosts (Scott and Anderson, 1984; Cable and Oosterhout, 2007b). The sensitivity of monogeneans to complement components (Harris *et al.*, 1998; Buchmann and Lindenstrøm, 2002) and other innate factors such as macrophages, lectins and acute phase reactants is evident, but the success of these parasites is likely to associate with their ability to evade host defence strategies (Buchmann and Lindenstrøm, 2002).

Gyrodactylus species are easily seen under the microscope and a large body of literature is available on their life history traits making them an excellent parasite model for various studies. Both *G. arcuatus* and *G. gasterostei* species can successfully colonize three-spined sticklebacks (Raeymaekers *et al.*, 2008). They are easily identified to species levels using morphological descriptions of secretory bladders and attachment apparatus as well as molecular analysis of ribosomal sequences of ITS regions (Huyse and Volckaert, 2005).

Gyrodactylus species are among the parasites presumed to be highly host specific (Whittington *et al.*, 2000) and coevolution in stickleback- *Gyrodactylus* sp. is likely to result in parasite local adaptation at the fish population levels (Raeymaekers *et al.*, 2011). Artificial infections using *Gyrodactylus* sp. and their fish hosts have been widely used to understand aspects of parasites such as population ecology (Scott and Anderson, 1984), epidemiology (Scott and Anderson, 1984; Winger *et al.*, 2012), host specificity (Bakke *et*

al., 2002), response to hosts defence mechanisms (Harris *et al.*, 1998; Buchmann and Bresciani, 1999) and virulence (Cone and Odense, 1984).

Sticklebacks inhabit marine, brackish and various freshwater habitats and experience different parasite burdens which may lead to adaptive divergence between populations (MacColl, 2009). This is particularly true for many freshwater populations of sticklebacks on North Uist which are hosts for at least 10 parasite species (de Roij and MacColl, 2012). They experience different levels of natural abundance and prevalence of *G. arcuatus* and they vary in their innate resistance to experimental infections by *Gyrodactylus* sp. (de Roij *et al.*, 2011). Therefore those fish parasites may represent an ideal study system to investigate the influence of ecological interactions between coinfecting parasite species on host fitness. Here, my hypothesis is that concomitant infection will lead to an increased burden of each of the parasite species compared to infection with either of the parasites alone. I predict that coinfection leads to delayed declining stage in the parasite growth curve and higher virulence.

5.3 Materials and methods

Study location and sampling

Fish and/or parasites were obtained from two locations in the U.K. Wild sticklebacks, used to establish a population of lab raised experimental fish (see below), and their *G. arcuatus* parasites were obtained from Loch na Reivil (pH = 8.95), North Uist, Scotland (57°36'42.9"N 7°30'53.2"W). In addition, *G. gasterostei* were obtained from wild stickleback caught in Tottle Brook (pH= 7.4), Nottingham (52°56'00.1"N 1°12'01.5"W). In both locations fish were caught using minnow traps ('Gees', Dynamic Aqua, Vancouver)

set in the morning and left overnight. Fish from Reivil for breeding were caught during April- May 2013. Approximately 40 fish from each location, for use as parasite donors, were caught during November 2014 and kept in aquarium dechlorinated tap water (approx. pH = 7.5) at temperature and photoperiod controlled conditions for two weeks to allow the parasites to increase in number prior the infection experiments. Parasite species were identified based on the presence and absence of secretory vesicles (bladders). The vesicle is noticeable in *G. arcuatus*, but it is completely absent in *G. gasterostei* (Harris, 1982; more details in chapter 1).

Fish breeding and housing

Artificial crossing was carried out by mixing eggs collected from gravid females with sperm of mature breeding males (de Roij *et al.*, 2011). Fertilised egg clutches were incubated in mesh-bottomed containers in oxygen rich dechlorinated tap water. Two days before hatching, tubes were moved into 100L glass tanks divided to two partitions containing dechlorinated tap water. Fish were kept in groups of approximately 10 under controlled conditions of temperature and photoperiod ($13\pm 1^{\circ}\text{C}$ and 16:8 light:dark). They were fed first with *Paramecium caudatum*, then brine shrimp, chopped bloodworm and finally whole blood worm by six months.

Artificial infection

Donor fish were killed using overdose of anaesthetic MS222 (400 mg.L^{-1}) followed by physical destruction of the brain and infected tissues such as fins and skin were placed into a clean petri dish containing dechlorinated tap water. Parasites were left for approximately 1 hour at 13°C to release from host tissues and then isolated in pairs in the petri dish using insect pins. Receiver fish were lightly anaesthetised (MS222, 100 mg.L^{-1}) and after

measuring their size (standard length and wet weight), the caudal fin of each individual was gently brought to the parasite containing petri dish to let worms attach. Each fish was infected with four worms (details in study design below). Infected fish were housed individually in 3L plastic tanks containing 2L of dechlorinated tap water. These were distributed randomly in a room with controlled photoperiod and temperature as mentioned above. All the fish were killed at the end of the experiment as above. To estimate the proportion of each parasite species in the mixed treatment at the end of the experiment, 10 individual fish with their attached parasites were separately scanned under a dissection microscope provided with fibre optic illumination. Parasites were counted *in situ* and worms were separated from each infected fish into a clean petri dish containing aquarium water. Finally, each individual worm was thoroughly examined for the presence or absence of secretory bladder. From 10 coinfecting fish with the two worm species, 1050 worms were isolated and mounted on clean glass slides and identified to species under 10X magnification.

Study design

The experiment involved 48 lab-raised fish descended from the Reivil population divided into four groups of 12 (Figure 5.1). The first group was infected with four worms from its sympatric parasite species (*G. arcuatus*), the second with four worms from the allopatric species (*G. gasterostei*) and the third with two sympatric and two allopatric parasites simultaneously. The fourth group was kept as uninfected control. Every fish in the first and second groups received four individual worms from sympatric and allopatric parasite species respectively, but in the third group, individual hosts received two worms from each parasite species. Parasites were counted on infected fish every three days from day 3 to the last day of the experiment (day 24). The experiment was performed under a UK Home

Office Project License 40/2886, and in accordance with the authorization of the Ethical Review Committee of Nottingham University.

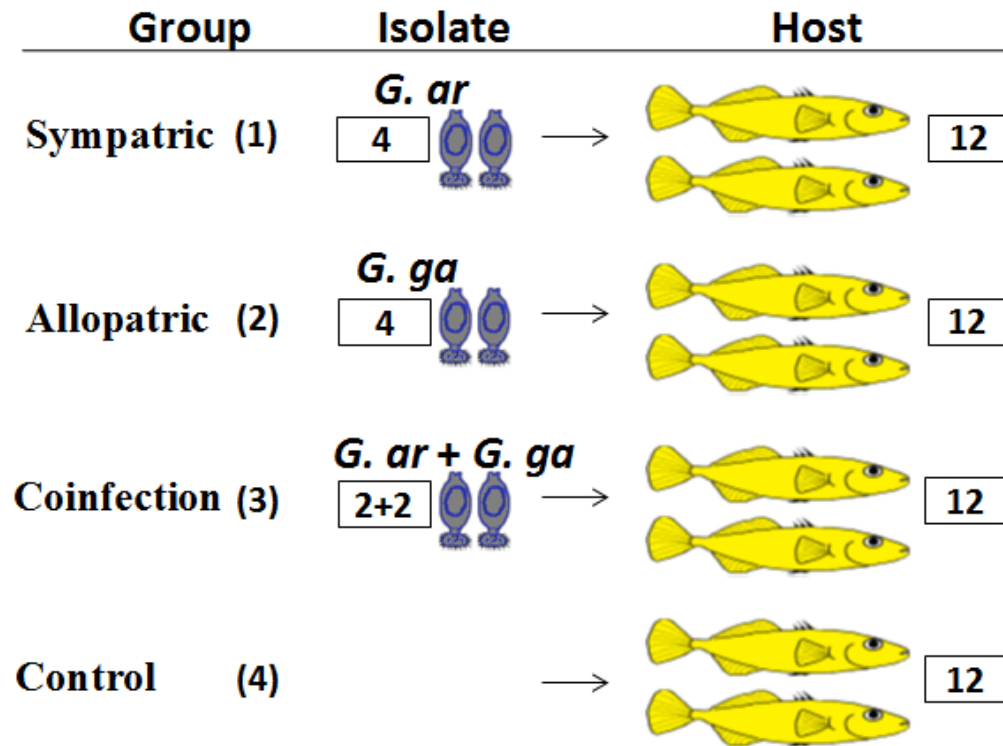


Figure 5.1. Schematic diagram showing the experimental design. Four groups of 12 fish, each host in group 1 received four individuals from their sympatric worm (*Gyrodactylus arcuatus*), in group 2 received four allopatric worms (*G. gasterostei*), in group 3 received two worms from each of the parasite species and the group 4 hosts were kept as control.

Statistical analysis

For the three infection treatments, the size of each parasite infra-population on individual fish was estimated by recording the total parasite counts on individual fish every three days from day 3 to day 24 of the infection period. ‘Total worm load’ was then measured by summing the overall worm counts throughout the infection time. The response variable

‘total worm load’ was compared among the three parasite combinations using a General Linear Model. Fixed factors included only fish sex and infection status (treatment) while fish length was treated as a covariate. The effect of fish length was not significant and hence it was dropped from the model. A paired t- test was used to examine the difference in the total worm burden between *G. arcuatus* and *G. gasterostei* in the mixed treatment. A chi- square test for association was used to examine whether infection status (single/mixed) associated with parasite virulence. The difference in the total worm load between single and mixed infections was examined using an unpaired t- test. To examine the influence of infection on hosts’ growth (as an index of virulence), specific growth rate (SGR) was estimated for tester fish. This was calculated using the formula ‘ $SGR = (L_{24} - L_0)/24$ ’, where ‘ L_{24} ’ was the standard body length measured on day 24 and ‘ L_0 ’ was the standard length measured on day 0 (de Roij *et al.*, 2011). The response variable ‘SGR’ was compared among the four fish treatments including the control group using a General Linear Model. Fish sex and infections status were the fixed factors and the total parasite load was treated as a covariate, but was not significant and hence was dropped from the model. A two- tailed Pearson correlation was used to examine the relationship between fish specific growth rate (SGR) and the total worm load. Square root or natural logarithm transformations were used to achieve normality in some response variables. Validity of GLMs was checked by visual inspection for residuals’ graphs and normality was confirmed using Shapiro- Wilk normality test ($P > 0.05$). All statistical tests were performed in SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

5.4 Results

Patterns of infection across parasite treatments

Generally, parasites in both sympatric and mixed treatments attained higher worm burdens than the allopatric parasite species (Figs. 5.2, 5.3). The population of worms in the mixed parasite treatment increased and peaked slightly faster with lesser fluctuation than both sympatric and allopatric strains, but started to decline earlier than the other two lines. Total worm load differed significantly among the parasite treatments ($F_{2, 32} = 4.0$, $P = 0.029$, Table 5.1A) and Bonferroni's multiple comparisons test showed that only the difference between mixed and allopatric treatments was significant ($P = 0.025$). There was a large difference in the proportion of sympatric and allopatric parasite species in the joint infection (Fig. 5.4). Sympatric parasites constituted the majority of the worms (72.6% with CI = 65.5, 78.3). The single infections suggest an expected ratio of 60.4:39.6 *G. arcuatus* to *G. gasterostei*. *G. gasterostei* in mixed infections had smaller population size than expected while *G. arcuatus* had larger population size than expectation ($\chi^2(1) = 41.1$, $p < 0.0001$). When separately compared to infections with single parasite species, the worm load of allopatric, but not sympatric *Gyrodactylus* sp. was significantly lower in the mixed treatment ((for sympatric alone, mean = 103.7, SE = 17.96 and sympatric in mixed treatment, $M = 76$, SE = 12.5; $t = 1.27$, df = 18, $P = 0.223$), but for allopatric alone ($M = 68$, SE = 6.38) and allopatric in mixed treatment ($M = 29$, SE = 3.7; $t = 5.29$, df = 18, $P < 0.0001$)).

Table 5.1. GLM outcomes for total worm count for the three parasite treatments (A) and fish specific growth rate (SGR) measured for the four fish groups (B).

Effects	DF	F	P value
<i>(A) Total parasite count</i>			
Parasite treatment	2	4.0	0.029
Fish sex	1	0.4	0.552
<i>(B) Fish growth rate (SGR)</i>			
Fish group	3	3.0	0.040
Fish sex	1	0.4	0.527

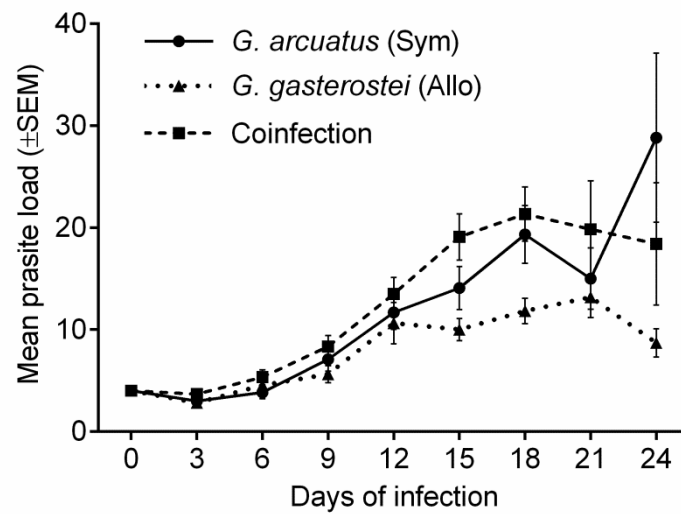


Figure 5.1. The mean daily abundance of the three parasite treatments. Legends: solid line intercepted with circles = sympatric strain, dotted line intercepted with triangles = allopatric strain and dashed line intercepted with squares = coinfection with the two parasites species.

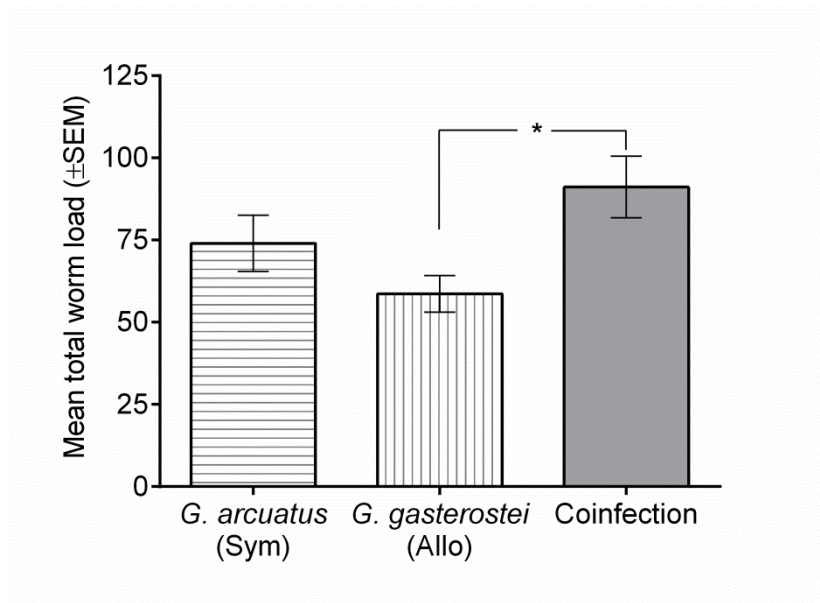


Figure 5.2. Difference in the mean of total worm load counted on infected test fish in the three infected groups (sympatric, allopatric and both parasite species) on day 21 of the experiment.

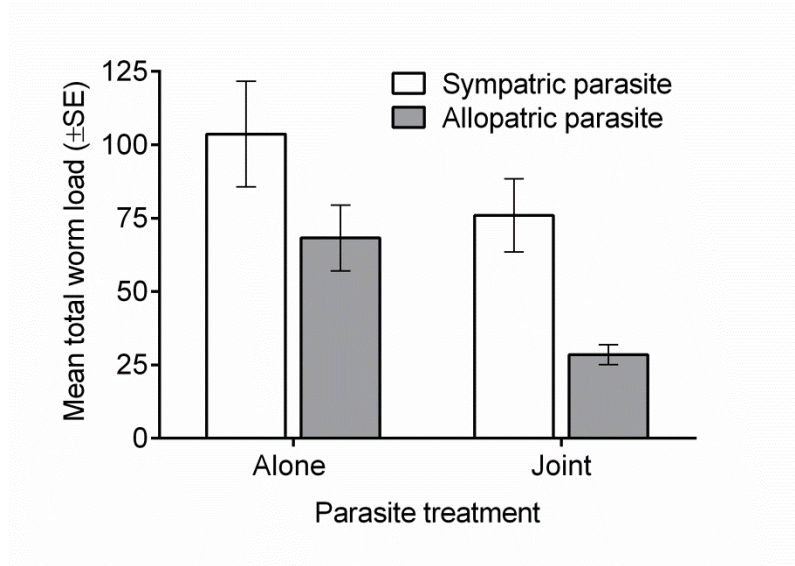


Figure 5.3. Difference in the mean total worm number counted for sympatric (plain) and allopatric (shaded) parasite strains in separate and joint infections on 10 individual fish for each treatment (details are given in the text) on day 24 of the infection time.

Growth differences among the fish groups

The infected fish treatments generally showed slower growth rates than uninfected controls (Fig. 5.5A). The control group had the highest specific growth rate (SGR) and the group which was infected with the sympatric strain had the lowest rate. The difference in the ‘SGR’ values among the four fish treatments was significant (Table 5.1B). However, Bonferroni's multiple comparisons test showed no statistically significant difference in pair comparisons, the SGR value of control fish was considerably higher than groups which were infected with sympatric ($P = 0.066$) and mixed parasite species ($P = 0.096$). GLM revealed that decrease in the SGR values of the infected fish groups was not associated with fish sex. However, the effect of total parasite was not significant in the model, generally, there was a significant negative relationship between worm load and fish SGR values (Pearson correlation, $r = -0.32$, $N = 47$, $P = 0.026$, fig. 5.5B).

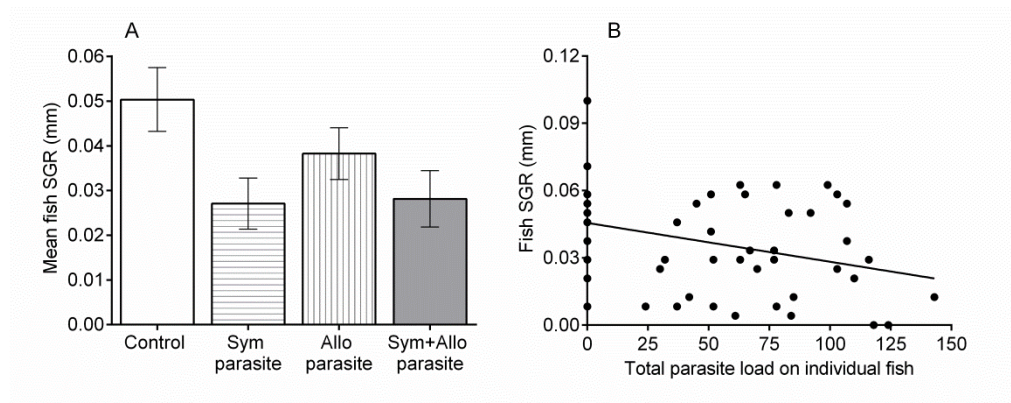


Figure 5.4. Difference in the SGR and its association with parasite infection. Left panel (A) shows the mean SGR ($\pm$ SE) among the four fish treatments (infected with sympatric, allopatric, both parasite species and uninfected control) and right panel (B) shows the association between the SGR value of individual fish and total worm load counted on that fish. Dots are meeting points between the two measures and solid line represents the most fit correlation line.

5.5 Discussion

These results plainly showed that *G. arcuatus* had higher population growth than *G. gasterostei* when they were separately and jointly used to infect lab bred three-spined stickleback. In coinfections, *G. arcuatus* suppressed the growth of *G. gasterostei*, suggesting that the former species is a better competitor in these conditions. *G. arcuatus* comprised a greater portion of worms in coinfections than did *G. gasterostei* and was expected from the performance of single infections, but the growth curve of coinfecting parasites began to decline simultaneously with allopatric parasites. Infected fish generally had lower SGR values than uninfected control individuals suggesting that the three parasite combinations were virulent. Single infection with *G. arcuatus* caused the highest reduction in the fish SGR while infection with *G. gasterostei* had the lowest impacts compared to the other two parasite treatments.

The host immune system plays an important role in parasite adaptation and virulence (Hammerschmidt and Kurtz, 2005; Babayan *et al.*, 2010). To fight off monogenean parasites, fish have various nonspecific immune components. These may include epithelial cells, epidermal mucous, phase reactants, lectins, complement and leukocytes (Buchmann and Bresciani, 1999; Buchmann *et al.*, 2000; Buchmann and Lindenstrøm, 2002).

Although these important parasiticide molecules have been detected in fish skin and are thought to have potential effects against monogeneans (see Buchmann, 1999; Buchmann *et al.*, 2000), *Gyrodactylus* sp. can survive a range of these attacks (Buchmann and Lindenstrøm, 2002). Infection success of *Gyrodactylus* sp. in the presence of these challenges strongly suggests that the parasite must have mechanism(s) to avoid or deal with hosts' immune responses (Buchmann and Lindenstrøm, 2002). Fish defence against monogenean parasites may be achieved by: (1) restricting the parasite growth and

achieving infection clearance by launching natural and/or specific immunity; (2) tolerating infection- induced diseases (Paperna, 1964; Buchmann, 1999; Buchmann and Lindenstrøm, 2002). Previous studies have found that sibships of *Schistocephalus solidus* which hid from immune responses in both copepod and stickleback hosts had the highest fitness rate (Hammerschmidt and Kurtz, 2005).

I have previously shown that *G. arcuatus* (sampled from Reivil fish) is locally adapted to its sympatric host population (chapter 2). There could be more than one possible reason for the success of sympatric parasite compared to allopatric parasites. There could be a direct interaction between the parasites that is not mediated by a host response. For example, *G. arcuatus* could have excluded *G. gasterostei* from feeding sites, or passed a disease to them. Other possibilities might either include the ability of sympatric parasites to avoid host defence strategies (Schmid-Hempel, 2009), or the evolution of host tolerance to local parasites (Miller *et al.*, 2005). If sympatric parasite success is associated with host tolerance to local parasites then I would not have expected to see a difference between the two allopatric parasite treatments (joint and alone), because the host should be less prepared to tolerate them in either case. From this view point, it seems possible that sympatric parasites have mediated the reduction in allopatric parasites through engagement of host immune response.

The phenomena of multiple infections is common, but as it is complex to understand, it has attracted only little attention (Cox, 2001). The influence of interacting parasites on each other's reproduction and virulence is controversial. The growth of interacting parasites may be increased for both species, increased for one and inhibited the for other, or inhibited for both species (Cox, 2001). In the current study, the burden of *G. arcuatus* in the mixed treatment did not significantly differ from its conspecifics in the single infection

treatment, but the burden of *G. gasterstei* in the mixed treatment was significantly reduced compared to its conspecific in the single infection group. Antagonism between species of fish parasites has been previously documented. For example one monogenean parasite species (*Dactylogyrus vastator*) of carp gills was found to potentially reduce the burden of another congeneric species (*D. extensus*) under lab infestation (Paperna, 1964). In the wild, carp which were infested with either of the *Dactylogyrus* species were mostly free of the other species. The author suggested that regulation of host's specific immune response was induced by one of the parasites to clear the host from the other species. In an artificial infection experiment using two populations of *S. solidus*, the overall growth of joint parasites was positively associated with the genetic relatedness between the coinfecting parasites (Jäger and Schjørring, 2006). In this context, results of the current study are in the line with the previous finding on *Dactylogyrus* sp.

The immune evasion hypothesis suggests that a smaller dose of pathogens initiating an infection is likely to be more successful than a large dose (see Schmid-Hempel, 2009). In the present experiment, all infections were initiated with four individual worms, although the inoculum in the mixed treatment involved two individuals from sympatric and two individuals from allopatric parasites. Our experimental design did not allow us to examine whether there was an association between inoculum size and parasite population growth. A negative association has been found between inoculum size and population growth in *G. turnbullis* (see Madhavi and Anderson, 1985) and *G. salaris* (see Ramirez *et al.*, 2015). Therefore population growth difference between each parasite species in mixed treatment compared to the population of its conspecific in infection with single species is not likely to be mediated by inoculum size. The precise reasons for the decreased growth of

allopatric parasites in the mixed treatment are not possible to be unravelled in this experiment, but require additional analysis.

The results of the current study are in agreement with previous work on mice and amphibians. Several studies have reported that in mice, infection with one helminth species usually leads to protection of the host against other helminths (reviewed by Cox, 2001). For example, mice which were concurrently infected with *Trichuris muris* and *Nematospiroides dubius*, could more actively reduce the survival of *T. muris* than hosts infected with only this parasite (Behnke *et al.*, 1984). Mice which were coinfecting with *Mesocostoides corti* and *Schistosoma mansoni* had lower fitness reduction than those infected with either of the parasites alone (Chernin *et al.*, 1988). In a separate study, *S. mansoni* stimulated mice immune responses to expel the cestode *Hymenolepis diminuta* (see Andreassen *et al.*, 1990). In natural amphibian populations, such antagonistic interactions also have been found among parasite species (Jackson *et al.*, 2006; Johnson and Hoverman, 2012). The outcome of competitive interactions among parasite species sharing a common host is expected to depend on the composition of mixed parasite species and genetic structure of hosts and parasites (Jackson *et al.*, 2006).

In the natural environment, isolated stickleback populations are often only infected by one species of *Gyrodactylus*. For example, freshwater populations on North Uist are only infested by *G. arcuatus*, while wild sticklebacks collected in some ponds and streams around Nottingham only harbour *G. gasterostei* (see Harris, 1998; de Roij *et al.*, 2011; de Roij and MacColl, 2012). *G. arcuatus* is a euryhaline, marine species, while *G. gasterostei* is a freshwater species, therefore, a possible reason for absence of coinfections in the wild could be a consequence of different species distributions. Alternatively, taking in to account that *Gyrodactylus* sp. are highly host specific (Huyse *et al.*, 2003) and the absence

of coinfections with the specific parasite; *G. arcuatus* and *G. gasterostei* on sympatric sticklebacks, such a pattern could be due to competition between the two parasite species on host individuals (Paperna, 1964; Jackson *et al.*, 2006). Given the reduction in growth of coinfecting *G. arcuatus* and *G. gasterostei* compared to their burden in a single infections, I expect that the competitive sympatric *G. arcuatus* initiated this reduction possibly through stimulation of host immune system (Scott and Anderson, 1984; Buchmann, 1999). Infection with sympatric parasites alone had a greater effect in reducing host growth (was more virulent) than infections with allopatric and mixed parasite treatments. These results support previous studies in this host parasite interaction that coevolutionary processes lead to parasite local adaptation at the level of fish populations (Raeymaekers *et al.*, 2011; chapter two of this thesis). Results of this chapter suggest that concurrent infections of three-spined sticklebacks with *G. arcuatus* and *G. gasterostei* have negative effects on the reproductive success of both parasite species and that may have consequences for their epidemiological dynamics and virulence.

Chapter 6.

Correlates of virulence variation in *Gyrodactylus arcuatus*

Chapter 6. Correlates of virulence variation in *Gyrodactylus arcuatus*

6.1 Abstract

Virulence, the ability of a parasite to damage its host, occupies a central place in host-parasite theory. Numerous theoretical studies have made predictions about the factors which should affect the evolution of virulence, but there are few tests of these predictions, and almost none in vertebrate host-parasite interactions. In this chapter, I study geographical variation in the virulence of a monogean trematode parasite, *Gyrodactylus arcuatus* of the three-spined stickleback (*Gasterosteus aculeatus*) and examine its association with variation in other characteristics of the parasite, host and their shared environment. Previous work in fish- *Gyrodactylus* interactions has shown that virulence depends on the growth rate of infections, and I use it here as a proxy of virulence. I used seven geographical strains of *G. arcuatus* to artificially infect one population (Chru) of three-spined stickleback, which itself experiences very little infection. Following experimental infection of hosts, the population growth of each strain was recorded. The strains were sampled from seven isolated lochs on the Scottish island of North Uist, which vary in environmental variables (water pH, conductivity and water chemistry) and ecological conditions (predation, competition, and parasite abundance). In addition, I quantified social behaviour of stickleback from the seven source lochs. To test the predictions of different virulence models, I examined the correlations between the virulence (population growth) of the seven strains, and different environmental, ecological and host social measures. Virulence was strongly positively correlated ($R^2 = 0.92$) with the pH of the water in the source populations of parasite strain, suggesting a substantial role for environment in the evolution of virulence. The only other variable that was correlated

(negatively) with virulence was host predator density. These results suggest that virulence is more likely to be driven by the external environment than by properties ‘internal’ to the host parasite relationship, such as prevalence, population density or transmission rate.

6.2 Introduction

During the last four decades, the topic of host- parasite interactions has been a central focus for a large body of theory, attempting to make predictions about conditions which cause evolutionary changes in parasite virulence (Anderson and May, 1982; Frank, 1996; Day and Proulx, 2004). Parasite virulence is assumed to have a positive association with parasite reproductive rates and it is thought to be an important factor driving host-parasite coevolution (Zhan *et al.*, 2002). Comparing virulence among strains of a parasite species with aspects of parasite and host biology and the biotic and abiotic environment may reveal what drives changes in parasite virulence and improve our knowledge on how to manage parasitic infections (Bull, 1994). To test theories of virulence evolution, many empirical studies have been carried out with invertebrate hosts (Ebert, 1994; Herre, 1995; Lively, 1999; Messenger *et al.*, 1999; de Roode *et al.*, 2008a; Little *et al.*, 2008; Nidelet *et al.*, 2009), but so far few studies have involved vertebrate hosts since the classical example of rabbit- myxomatosis host- parasite coevolution (Fenner, 1983). Using invertebrate host-parasite systems is easier than using vertebrate combinations, but due to the immunological complexity of the later (Buchmann, 2000; Lenz *et al.*, 2013), coevolutionary outcomes may also differ between the two host types.

Virulence is known as the level of harm that a pathogen causes to its host due to the pathogen’s growth and reproduction. The traditional view of virulence proposes that parasites evolve to being harmless (May and Anderson, 1983). However, more recent

theories suggest that pathogen fitness is controlled by a genetic trade-off between costs and benefits of harming hosts (Anderson and May, 1981, 1982; Ewald *et al.*, 1998; Woolhouse *et al.*, 2002; Ewald, 2004; Leggett *et al.*, 2013). These theories generally suggest that parasites evolve to maximize their basic reproductive rate (R_0). In directly transmitted parasites, the value of R_0 is increased by parasite transmission and host density and decreased by reduction in infection duration caused by host mortality and recovery rate (see chapter 1, equation 1.3 and fig. 1.1). Selection will act against parasites with high virulence that results in host death before transmitting to new susceptible hosts (Levin and Pimentel, 1981; Frank, 1996; Alizon *et al.*, 2009).

Studies of virulence evolution predict that any biological traits of hosts or parasites that mediate high parasite transmission can lead to evolution of high virulence and those which restrict transmission result in the emergence of low virulence. For example, the transmission of directly transmitted parasites can be increased by high density of hosts (Pulkkinen *et al.*, 2010; Donnelly *et al.*, 2013; Pollitt *et al.*, 2013), host social behaviour (Loehle, 1995; Boots *et al.*, 2004) and high durability of detached parasites in the environment (Walter and Ewald, 2004). Conversely, other factors such as host death due to parasites (Anderson and May, 1982) and host death due to other environmental stressors such as natural predators (Duffy and Hall, 2008) may reduce parasite transmission. The hosts' immune system is likely to determine host recovery and on-host parasite survival (Frank and Schmid-Hempel, 2008) that can shorten the duration of infection (i.e. reduces R_0) and may therefore, select against virulence (Alizon, 2008).

Environmental conditions can alter the dynamics of infections by either directly affecting parasite fitness or indirectly by inducing changes in hosts' life history traits. In aquatic ecosystems, water chemistry and temperature have been found to directly affect the

survival and population dynamics of parasites (Eisler, 1993; Soleng *et al.*, 1999; Gheorghiu *et al.*, 2007; Karvonen *et al.*, 2013). Environmentally induced changes in hosts' traits can include physiology and demography (Ebert and Mangin, 1997; Thompson, 1999; Barrett *et al.*, 2009; Laine, 2009). For example, it is suggested that water salinity, pH, dissolved oxygen and temperature shape resource conditions and the fish's immune system (Scott, 1988; Bowden, 2008). In fish, epidermal mucous cells play an important role in the defence against monogenean ectoparasites (Buchmann and Uldal, 1997; Buchmann and Bresciani, 1998; Gheorghiu *et al.*, 2012) and are affected by water chemistry (Gheorghiu *et al.*, 2012). Such conditions may have vital role in the fitness and life span of hosts which may in turn feedback to influence evolutionary processes in parasites (Cornet *et al.*, 2014). Therefore spatial differences in environmental conditions experienced by natural populations can exert different selection levels that may lead to adaptive divergence in host traits and parasite virulence (Thompson, 1999).

In the current chapter, I used a monogenean flat worm (*Gyrodactylus arcuatus*) and its host, the three-spined stickleback (*Gasterosteus aculeatus*) to compare virulence among seven strains of the parasite. Parasite strains were collected from naturally infected fish caught from geographically isolated lochs to represent a range of ecological conditions (Predators, competitors and parasitism) and environmental variables (water conductivity, pH and alkaline metals) on North Uist- Scotland. *Gyrodactylus* sp. are microscopic ectoparasites that commonly live on the skin and fins of both saltwater and freshwater fish and have a direct life cycle. They are viviparous and newly born individuals carry an already pregnant baby in uterus (Cable *et al.*, 2002). These parasites use sexual and asexual mode of reproduction (Schelkle *et al.*, 2012) and can generate an entire population

from a single worm, a feature which makes them highly infectious (Cable and Harris, 2002).

In many studies investigating virulence variation, the degree of virulence has been found to depend on the parasite load per individual host (Ebert, 1994; Messenger *et al.*, 1999; Nidelet *et al.*, 2009). This is very likely to be true in *Gyrodactylus*- fish systems because a strong positive correlation has been found between the parasite number and mortality rate of infected fish (Scott and Anderson, 1984; Madhavi and Anderson, 1985). The stickleback is a small fish species (approx. 2-5 cm) widely distributed throughout the northern hemisphere. Several findings have suggested that these fish can adaptively respond to changes in ecological and environmental challenges (Schluter, 1996; Scharsack *et al.*, 2007; Jones *et al.*, 2012; MacColl *et al.*, 2013) which may feedback to influence parasite epidemiology and virulence.

The populations of sticklebacks from North Uist experience very different environmental conditions such as water conductivity, pH and alkaline metals (Giles, 1983) and ecological status such as density of predators, competitors and parasite richness (de Roij and MacColl, 2012; MacColl *et al.*, 2013). Variation in these environmental stressors are expected to drive selection on behavioural (Wark *et al.*, 2011), phenotypic (MacColl *et al.*, 2013) and immunological (de Roij *et al.*, 2011) traits in these fish. Such biological features make this host – parasite interaction an outstanding study system to understand the role of biological and environmental factors in virulence variation. Here, I test different hypotheses for the underlying causes of virulence evolution by examining the correlations between virulence (measured in the lab as peak load in artificial infections) of seven *G. arcuatus* strains, and measures of environmental variation that are predicted to drive virulence. The environmental variables examined were: (a) host population density

(Donnelly *et al.*, 2013), (b) host social behaviour (Ezenwa, 2004), (c) the concentration of host skin mucus as innate immune response against the parasite (Gheorghiu *et al.*, 2012), (d) the density of host predator as a source of host mortality (Duffy and Hall, 2008), (e) the longevity expectancy of hosts (Nidelet *et al.*, 2009), (f) life expectancy of detached parasites (Walter and Ewald, 2004) and, (g) four abiotic variables (water pH, dissolved oxygen, zinc and temperature) which are thought to influence the survival expectancy of both hosts (Bowden, 2008) and parasites (Gheorghiu *et al.*, 2007; Lopez Pascua *et al.*, 2012; Karvonen *et al.*, 2013). I hypothesized that host population density, social behaviour and the life expectancy of detached parasites enhance parasite transmission and hence select for high virulence. Host immune efficiency is thought to enhance infection recovery, and the density of host predators (brown trout) is thought to be associated with increased host mortality rates. Therefore, I expected host immune efficiency and host predator density to negatively correlate with virulence.

6.3 Materials and methods

Source of hosts and parasites

I collected approximately 20 parasite donor fish from each of seven locations (Gill, Host, Maga, Obse, Scad, Reiv and Torm) and about 100 receiver fish from a population (Chru) which is found to be free of *Gyrodactylus* sp. (de Roij *et al.*, 2011; de Roij and MacColl, 2012). All the fish sampling was carried out using minnow traps (Gee traps, Dynamic Aqua, Vancouver) set overnight and lifted in the next morning during April-May 2014 on North Uist, Scotland. Lochs were chosen to represent a range of environmental (Table 6.1) and ecological conditions (Table 6.2) (MacColl *et al.*, 2013).

Table 6.1. Grid references and five environmental variables: loch surface area, water conductivity, pH, dissolved oxygen and the concentration of water zinc measured for the eight locations used in this study.

Loch (population)	Code	Grid reference	Loch area (ha)	Water conductivity (µS)	Water pH	Dissolved oxygen (mg/L)	Zinc availability (µg/L)
Chadha Ruaidh	Chru	57°35'38.7"N 7°11'51.2"W	2.1	182	6.7	9.7	0.596
Mhic Gille-bhrìde	Gill	57°36'07.3"N 7°24'37.3"W	14	158.5	6.8	9.08	0.369
Hosta	Host	57°37'38.1"N 7°29'24.8"W	26	339.5	8.3	9.10	0.307
nam Magarlan	Maga	57°35'16.9"N 7°26'38.7"W	6.5	261.8	7.8	9.45	0.515
Ob nan Stearnain	Obse	57°36'06.1"N 7°10'25.4"W	10	45100	9.2	8.65	0.023
na Reivil	Reiv	57°36'42.9"N 7°30'53.2"W	6.1	433.3	9.0	9.14	0.03
Scadavay	Scad	57°35'05.5"N 7°14'11.2"W	552	144.4	6.1	8.50	0.837
Tormasadh	Torm	57°33'46.8"N 7°18'54.4"W	21	181.4	6.8	9.05	0.777

Table 6.2. Seven ecological characteristics of stickleback populations and *G. arcuatus* strains in seven spatially isolated lochs on North Uist. These included stickleback body size, stickleback population density, stickleback's mean time spent shoaling, mean mucous cell density in stickleback's caudal fin (CF), stickleback longevity (percentage survived by two years), longevity of detached *G. arcuatus* (percentage survived by 51 h) and stickleback's predator density (brown trout catch rate).

Loch code	Mean stickleback standard length (mm $\pm$ SE)	Stickleback population density (CPUE)	Mean stickleback shoaling time (seconds $\pm$ SE)	Mean mucous cell density in stickleback's CF (cell/mm $\pm$ SE)	The percentage of stickleback survived by two years	Survival of detached gyrodactylids (%)	Predator density (brown trout catch rate)
Gill	38.28 (1.2)	0.81	25.15 (9.3)	0.21 (0.1)	30	58.33	0.30
Host	40.32 (1.2)	15.34	20.84 (11.9)	2.00 (1.6)	6.1	41.67	0.16
Maga	45.33 (2.0)	7.42	40.13 (10.3)	2.76 (1.0)	29.4	41.67	0.20
Obse	38.29 (1.2)	33.97	19.31 (8.0)	0.97 (0.4)	97.0	25.00	-
Reiv	47.37 (1.45)	34.73	28.35 (10.1)	4.76 (2.5)	31.3	66.67	0.00
Scad	30.16 (0.6)	27.78	12.47 (10.0)	3.67 (2.1)	6.1	16.67	1.29
Torm	29.24 (0.6)	30.07	10.81 (5.3)	10.76 (2.6)	0	8.33	0.25

Note: CPUE = catch per unit effort (fish per trap per night), CF = caudal fin, - = measurement was not available

To confirm the absence of ectoparasites on receiver fish (Chru), they were anaesthetised (MS222, 100 mg. L⁻¹) and their fins and body surface carefully searched under a dissection microscope. Fish with ectoparasites or signs of *Schistocephalus solidus* infections such as a marked abdomen enlargement (see Barber and Huntingford, 1995) were not included in the experiment. Among the 100 individuals, 80 most healthy fish were selected for the artificial infection experiment.

Artificial infection and experimental design

Gyrodactylid strains were sampled from infected wild fish and each strain was denoted by the loch name of its original host population. Previous research suggested that *G. arcuatus* is the native *Gyrodactylus* species on Uist sticklebacks (de Roij *et al.*, 2011; de Roij and MacColl, 2012). The morphological characteristics of parasite isolates used in the current study, were also found to be very similar to *G. arcuatus* (see chapter 1 for details).

Parasites of saltwater fish (Obse) were acclimatized to freshwater conditions by gradually diluting the salt concentration. This was done by hourly replacing approximately 10% of seawater with dechlorinated tap water over approximately 10 hours. Parasites together with small pieces of fin or a thin tissue layer where parasites attached to the skin surface were separately isolated into a clean disposable Petri dish containing approximately 50 ml of dechlorinated tap water. The Petri dishes were stored at 4°C for a maximum one hour until sample isolation was done for all the seven strains of the parasite. Two worms were gently moved toward a side in the dish using an insect pin and then the caudal fin of each receiver fish was brought into close contact until the two worms attached to it. If less than two parasites attached on the fin, the attempt was repeated and if there were more than two parasites found on the same fin, extra worms were removed.

In total, 80 tester fish were used in the experiment and divided to eight groups of 10 individuals. One of the groups was kept as uninfected controls and each of the remaining seven groups was infected with one of the seven strains. All the test fish were transferred to individual 3L transparent plastic tanks containing 2L of dechlorinated tap water (pH = 7). The tanks were randomly distributed in a room with a temperature of 12 (± 1) °C and photoperiod 16/8 (light:dark) hours, similar to the natural environment. For each tank, about 50% of the water content was regularly changed with clean dechlorinated tap water every 3 days. Fish were daily fed once on blood worm, defrosted from frozen (gamma blister bloodworm, Tropical Marine Centre, UK) until the end of the experiment (24 days). Three fish were euthanised during the course of the infection experiment and they were not included in any analysis. Both housing and experimentation were performed under a UK Home Office Project License 40/2886, and in accordance with the authorization of the Ethical Review Committee of Nottingham University.

Ecological variables

The density of three-spined stickleback populations was estimated by measuring the average number of fish caught per minnow trap over 24 h in May 2013. For each loch about 40 traps were set in pairs (2-5 m away from loch shore). The average abundance of *G. arcuatus* was estimated for the seven fish populations in May and October 2014 (Bush *et al.*, 1997) using approximately 20 individual fish per population. Fish standard length was estimated by measuring the distance between the end of the caudal peduncle and the tip of the snout. Mean brown trout catch rate (predator density) was estimated from the number of trout landed per angler per hour in competitions by the local angling club (MacColl and Aucott, 2014). The life expectancy of wild sticklebacks was estimated using otolith reading method by Abdul Rahman (MacColl's PhD student). Average life

expectancy of detached parasites and density of epidermal mucous goblet cells for each fish population are from chapter 3. Fish shoaling (aggregation) behaviour was estimated in chapter 4.

Environmental variables

Water chemistry and environmental variables such as area, water pH, conductivity, dissolved oxygen and concentration of chemical variables such as Zn^{2+} were recorded in April-May by MacColl *et al.* (2013). For all the experiments employed by this study, killing fish was carried out using an over dose of anaesthetic (400 mg.L^{-1} MS222, tricaine methane sulphonate) and death was confirmed by mechanical destruction of the brain.

Statistical analysis

In order to analyse differences in the natural abundance and prevalence of *Gyrodactylus* sp. between wild fish from the seven host populations, A Generalized Linear Model (GLM) was used. The prevalence was analysed using GLM with binomial error and logit link. The response variable was '1' for infected and '0' for uninfected individuals. The response variable 'total parasite abundance +1' was analysed using GLM with negative binomial errors and log link function. For both prevalence and abundance of parasites, the model included fish population as the only fixed factor and fish standard length as a covariate with a full factorial design.

Virulence in the artificial infections with the seven *G. arcuatus* strains was inferred from the maximum parasite number (peak) counted on individual fish throughout the infection course. In order to analyse differences in virulence between parasite strains a General Linear Model was used. The response variable, 'peak burden' was square root transformed

and the main effects included parasite strain as a fixed factor, fish standard length as covariate and their interaction. The effect of covariate was not significant and hence was dropped from the model. Pairwise contrasts were analysed with Bonferroni adjustment.

With the aim of comparing within population differences in virulence levels among the parasite strains, the variance to mean ratio of worm burden was measured as it has been shown by previous studies using *Gyrodactylus* sp.- fish interactions (Madhavi and Anderson, 1985; Buchmann and Uldal, 1997). A two- tailed Pearson correlation was used to test the relationship between mean parasite ‘peak’ and the average of each of the factors hypothesised to associate with virulence of directly transmitted parasites. All the statistical tests were performed in SPSS package (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

6.4 Results

Differences in the ecological conditions

In May, the natural abundance of *G. arcuatus* differed significantly among the seven fish populations ($\chi^2_6 = 19.5$, $P = 0.003$, fig 6.1A). Fish standard length was positively associated with *Gyrodactylus* sp. abundance ($\chi^2_1 = 10.8$, $P = 0.001$), but the interaction between the parasite abundance and fish length was marginally not significant ($\chi^2_6 = 11.7$, $P = 0.068$). In October, parasite abundance was much lower and did not differ among the strains ($\chi^2_6 = 3.0$, $P = 0.806$, fig 6.1A). The difference between log mean gyrodactylid abundance in October and May was significant for all the seven strains (Unpaired- samples t- test for Gill, $t = 3.6$, $df = 38$, $P = 0.001$, for Host, $t = 3.1$, $df = 50$, $P = 0.003$, for Maga, $t = 3.7$, $df = 45$, $P < 0.001$, for Obse, $t = 6.1$, $df = 43$, $P < 0.001$, for Reiv, $t = 3.2$, $df = 51$, $P =$

0.003, for Scad, $t = 5.4$, $df = 57$, $P < 0.001$ and for Torm, $t = 2.0$, $df = 61$, $P = 0.047$). There was a strong positive correlation in the abundance values between May and October (two-tailed Pearson correlation for log transformed mean abundance, $r = 0.95$, $n = 7$, $P < 0.001$).

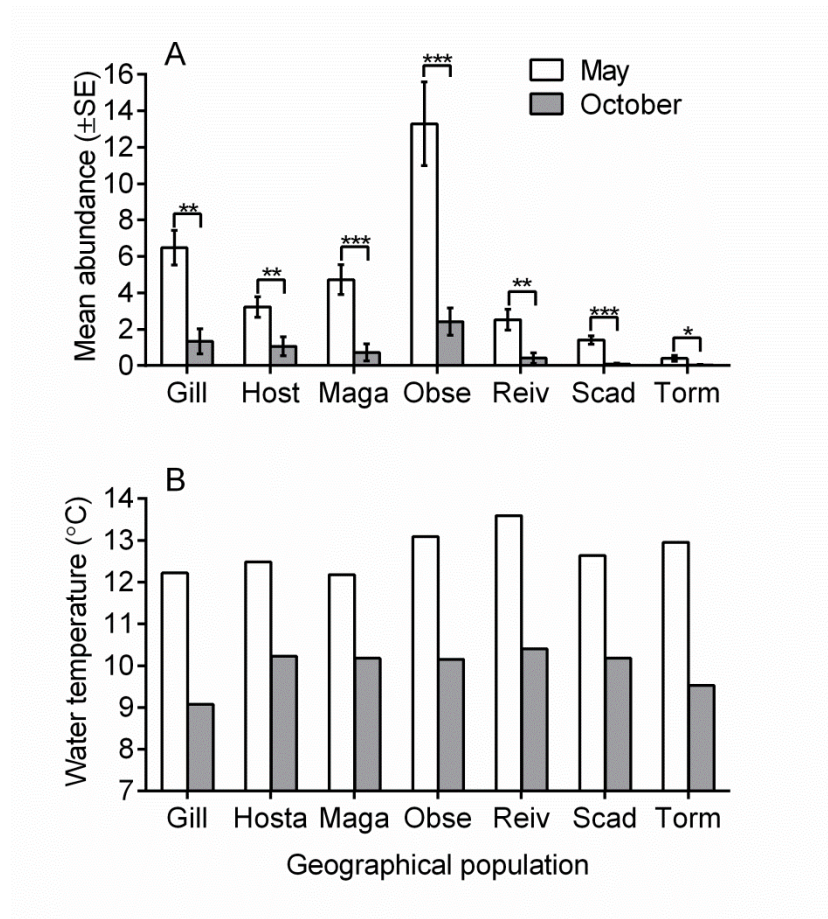


Figure 6.1. Natural abundance of *Gyrodactylus* sp. (A) and average water temperature (B) measured during May (plain) and October (shaded) for the seven parasite strains. Asterisks indicate the probability value for differences in the parasite abundance between the months. Note *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Virulence variation among the strains

All of the parasite strains successfully established infections on the allopatric tester fish (Chru) following the artificial infection. Generally, freshwater strains of the eastern lochs (Gill, Scad and Torm) had lower population growth than parasites originated from the west

side of the island (Host, Maga and Reiv) (Fig. 6.2A). Interestingly, strains with intermediate levels of population growth had lower mean to variance ratio than those which had low and high growth rates (Fig. 6.2B). GLM revealed a significant difference in peak burden among the parasite strains ($F_{6, 60} = 7.22$, $P < 0.001$, fig. 6.3). Bonferroni multiple comparisons' test showed that the Scad and Gill strains had significantly lower peak burdens than the Host, Obse and Reiv parasites ($P < 0.05$).

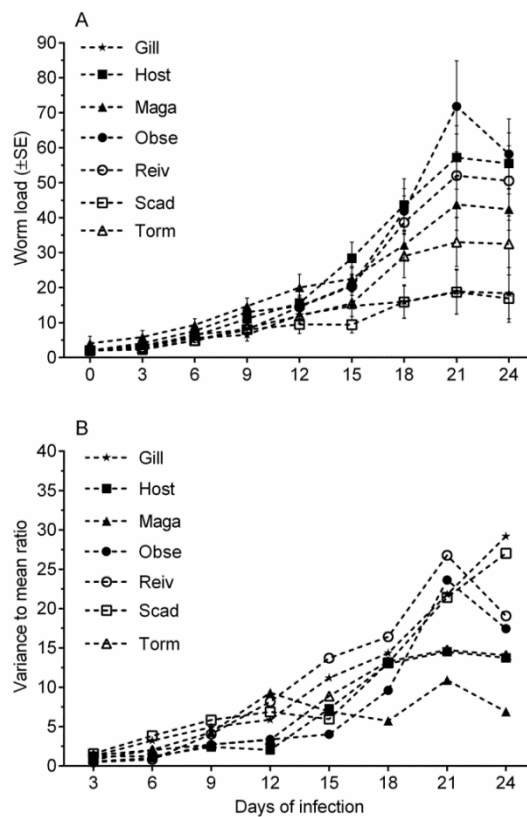


Figure 6.2. Mean daily worm burden (A) and variance to mean parasite load ratio (B) measured for the seven strains when used to artificially infect a *Gyrodactylus*- free stickleback population (Chru).

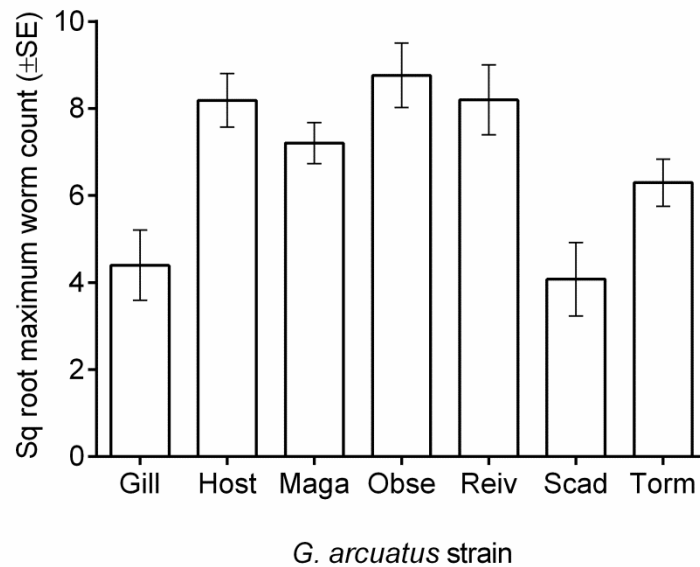


Figure 6.3. The peak burden of *Gyrodactylus* of the seven strains during their infection on the naturally uninfected Chru fish population.

Correlates of virulence

Apart from the density of brown trout (predator), there was no relationship between parasite virulence measured in the lab and any of the ecological characteristics of stickleback hosts or the life expectancy of detached parasites. A two-tailed Pearson correlation revealed that there was no significant association between the mean ‘squared root peak’ values and any of: mean stickleback populations’ density ($r = 0.19$, $n = 7$, $P = 0.682$, fig. 6.4A), shoaling tendency ($r = 0.29$, $n = 7$, $P = 0.532$, fig. 6.4B), fish epidermal mucous cell density in caudal fin ($r = -0.01$, $n = 7$, $P = 0.996$, fig. 6.4C), longevity ($r = 0.46$, $n = 7$, $P = 0.295$) and life expectancy of detached parasites ($r = 0.46$, $n = 7$, $P = 0.295$, fig. 6.4D). There was a strong negative correlation between the parasite peak and the density of stickleback predator ($r = -0.81$, $n = 6$, $P = 0.049$, fig. 6.4E). Among the abiotic environmental variables, water conductivity ($r = 0.66$, $n = 7$, $P = 0.105$), average

temperature ($r = 0.67$, $n = 7$, $P = 0.103$) and the concentration of dissolved oxygen ($r = 0.21$, $n = 7$, $P = 0.645$) were not significantly related to parasite virulence. The peak of parasite infection was strongly positively correlated with the pH of water in the loch from which the strain originated ($r = 0.96$, $n = 7$, $P < 0.001$, fig. 6.4F) and it was negatively correlated with the concentration of waterborne zinc ($r = -0.77$, $n = 7$, $P = 0.043$).

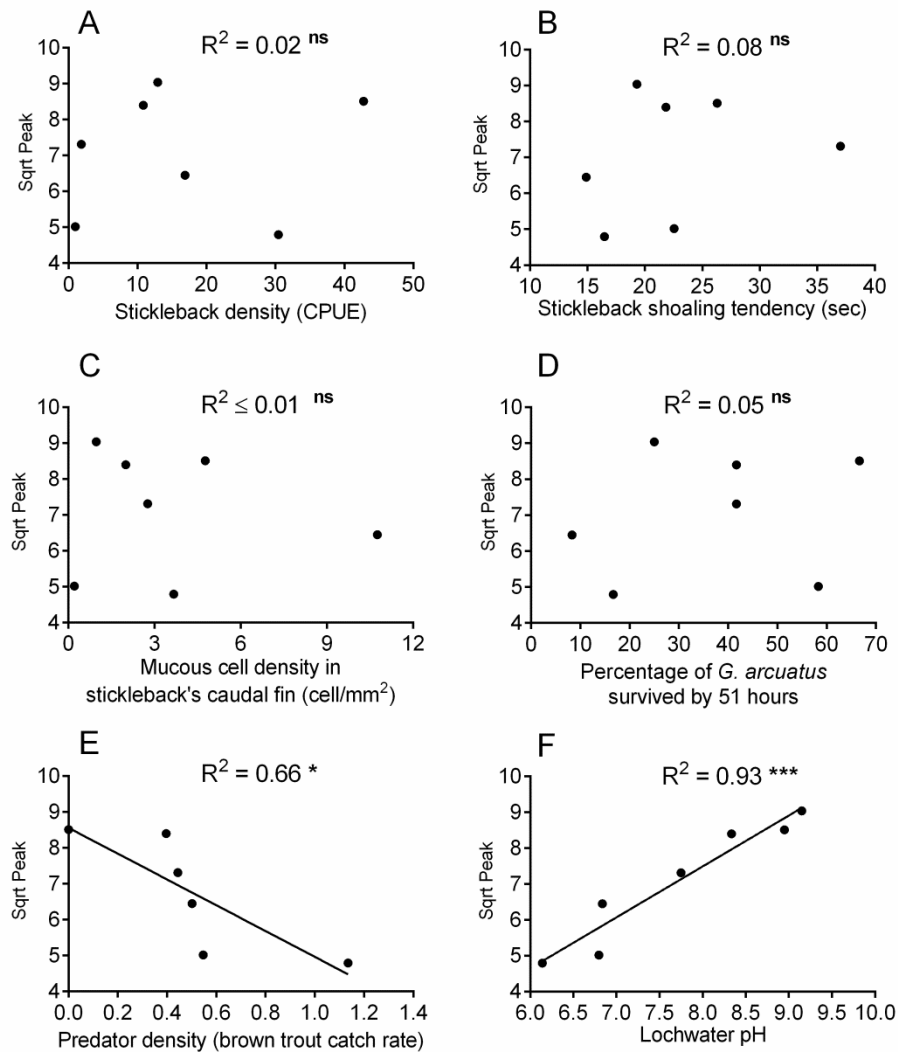


Figure 6.4. The relationship between squared root parasite 'peak' values measured in the lab and five ecological factors (right to left: A- host density, B- host shoaling behaviour, C- the density of mucous cells in hosts' caudal fin, D- life expectancy of detached parasites and E- host predator density) and water pH (F). R square was derived from Pearson correlation with two tailed probability, ns: not significant, *: $P < 0.05$, ***: $P < 0.001$.

6.5 Discussion

The seven *G. arcuatus* strains tested in this study differed in their virulence on a wild (*Gyrodactylus* sp. free) population of three- spined stickleback under uniform lab conditions. Despite the fact that host populations experience a range of ecological and environmental conditions, virulence differences among the parasite strains were not

associated with the biological characteristics of host populations or parasites (eg. host density, host social behaviour, epidermal mucous contents and life expectancy of detached *G. arcuatus*). Virulence was strongly linked to biotic and abiotic environmental stressors (host predator density and water pH). It seems likely that spatial isolation of the host and parasites in different environments has led to selection on parasite life-history traits. Such variation in the life history traits of *Gyrodactylus* sp. has been rarely studied (but see Cable and Oosterhout, 2007a; Robertsen *et al.*, 2007).

There was an almost linear relationship between the growth rate of parasites in the lab and pH of water in their natural environment. Given that *Gyrodactylus* is an ectoparasite, exposed to its environment, and that pH has many effects on organisms, it is quite possible that pH itself has driven divergent evolution of *G. arcuatus* among North Uist lochs. However, in these lochs, water pH is strongly associated with the availability of alkaline (eg. Ca^{2+} , Mg^{2+} and Na^{1+}) and transition (e.g. Zn, Cu) metals, and with overall water conductivity. Therefore, water pH may be a proxy for a wide range of water chemistry and resource conditions (MacColl *et al.*, 2013). The association between environmental pH and parasite virulence could be a direct result of selection on the parasite or an indirect result of changes in the life history traits of hosts. Lochs with low water pH probably have poorer resources for stickleback, and this may affect the evolution of the host-parasite relationship. For example, the availability of alkaline metals may affect the abundance of phytoplankton and hence zooplankton, which is an important food resource for sticklebacks (Campbell and Knoechel, 1990). In a study of bird- malaria interactions, the parasite was found to adapt to the nutritional conditions of its hosts and that was thought to genetically shape the parasite virulence (Cornet *et al.*, 2014). de Roode *et al.* (2008b) found a protozoan parasite of monarch butterfly selected for low virulence when the larvae

of its host fed on a plant containing a toxic substance. The authors suggested two possibilities for how environmental conditions could alter the virulence of the monarch parasite: (1) by reducing longevity of the butterfly host when this selected against virulent parasites or (2) by somehow directly affecting the parasite fitness and virulence.

Theoretical works on virulence evolution commonly suggest that ecological conditions play a vital role in the adaptation of parasite exploitation rates. In particular, a highly accepted view is that, parasites should evolve to quicker growing (higher virulence) when their hosts' life span is shortened by environmental factors (Baalen and Sabelis, 1995; Ebert and Weisser, 1997; Choo *et al.*, 2003). This view suggests that the influence of increased hosts' background mortality rate on a parasite's transmission is buffered by selection for higher virulence. In the current study, reversely, there was a strong negative correlation between the density of brown trout, which are potential predators of stickleback, and parasite virulence. The influence of predator body mass on predation efficiency is well known in many species (Tripet and Perrin, 1994; Vucic-Pestic *et al.*, 2010; González-Suárez *et al.*, 2011). The observed correlation is not in the line with the context of predation influence because *Gyrodactylus* with the lowest virulence was from stickleback populations (Gill, Scad and Torm) with high density of brown trout, but the body size of these predators are much smaller than those inhabit with other stickleback populations (Host and Maga) (MacColl and Aucott, 2014). Additionally, virulence was the highest in Reiv strain of the parasite, where vertebrate predators are absent. Arguably, these may suggest that predation has nothing to do with the parasite exploitation strategies, and that trout abundance and virulence are both associated with some third factor, like water chemistry resources, which gives rise to the correlation (MacColl *et al.*, 2013).

Based on the central theory of virulence evolution (Anderson and May, 1982), a parasite has to exploit its host to maximize its long term transmission rate. Therefore, to understand the factors that play a role in the evolution of parasite virulence, evolutionary biologists at least have stressed the role of factors internal to the host- parasite interaction, but the role of the external environment has been largely ignored. For example, many theoretical investigations into virulence evolution have explicitly focussed on hosts' background mortality, population density, and transmission as being important in the evolution of virulence (Bull, 1994; Lambrechts *et al.*, 2006; Alizon, 2008). Clearly, the results of the current study do not support these views, instead, they substantiate the role of geographical pattern of external environmental conditions (Hochberg *et al.*, 2000).

In theory, biological features of parasites and their hosts which are assumed to directly or indirectly influence parasites' epidemiology are predicted to drive changes in virulence. The transmission of *Gyrodactylus* sp. can happen by contact with infected live fish, infected dead fish, detached parasites on substrates and detached parasites drifting in the water column (Bakke *et al.*, 1992). High host density is thought to increase the opportunity of transmission of the parasite (Bakke and MacKenzie, 1993). Female guppies with high shoaling tendency have been found to have increased *G. turnbullis* transmission when compared to males, which were described with less shoaling tendency (Johnson *et al.*, 2011). Detached *Gyrodactylus* sp. in the water column and on substrates are thought to use 'sit and wait' strategy until a suitable host approaches (Bakke *et al.*, 2007) and hence off-host durability may be important for their transmission (Walter and Ewald, 2004). In this chapter, there was no association between the parasites' virulence and any of host population density, host shoaling tendency and the parasites' durability *in vitro*. Many ecological processes can interrelate in the natural environment and that may involve more

mechanisms than initially considered, affecting virulence transmission trade-off (Alizon *et al.*, 2009).

Virulence of the parasite strains did not appear to associate with the average density of epidermal mucous cells. Epidermal mucous cells are thought to constitute an important part of fish's resistance against *Gyrodactylus* infections (Buchmann and Bresciani, 1998; Buchmann, 1999). Indeed, there was a negative relationship between epidermal mucous cell density and the average abundance of *Gyrodactylus* sp. measured for eight wild stickleback populations (chapter 3). Two possibilities may explain the lack of association between virulence and the density of skin mucous cells of their hosts: (1) quantifying skin mucous cells may not represent a good measure for host resistance against the parasite infections or (2) when recovery rate is low, the denominator of R_0 equation (chapter 1, equation 1.3) is dominated by mortality rates and in such conditions, parasite transmission is likely to be limited by increased host mortality (see Bull and Llaure, 2014 for a review).

The abundance of *Gyrodactylus* sp. differed greatly among the fish populations in May, but not in October. Consistently, the abundance of all *Gyrodactylus* strains was markedly higher in May than October. The change in abundance of *Gyrodactylus* sp. within population between the two months may point to an important role of temperature on the parasite growth rate (Gelnar, 1990). Such fluctuation in parasite reproductive potential may also be associated with physiological changes in the hosts (Magnadóttir, 2006). It is thought that during breeding seasons energy allocation is shunted toward reproductive and secondary sexual characters especially in males, at the expense of their immune investment (Rohlenova *et al.*, 2011). These results are in line with those reported by Karvonen *et al.* (2013), suggesting that water temperature was positively associated with the abundance of

sticklebacks' parasites even out of breeding season. Although these results emphasize the proximate effect of temperature on the population growth of gyrodactylids, there was no association between parasite virulence in the lab and the average temperature of their loch water. Compared to mean temperature difference among the populations (less than 1 °C), the mean temperature difference between the two months was high (2.77°C). This may suggest that parasite strains are not affected by differences in temperature within the range I tested.

In conclusion, these results showed substantial differences in the virulence of *G. arcuatus* strains in the lab and that this was associated with the environmental conditions of their natural stickleback hosts. Studies have rarely investigated the role of environmental conditions on host- parasite interactions (but see Thompson, 1999; Hochberg *et al.*, 2000). The current study shows how environmental conditions can alter the evolutionary dynamics of virulence, an aspect of host- parasite interactions is seldom considered (Lively *et al.*, 2014). Similarly, the intensity of the interaction in the geographic mosaic of coevolution between *Gyrodactylus* sp. and stickleback seems to be driven by environmental conditions (Thompson, 1999).

Chapter 7.

General discussion and conclusions

Chapter 7. General discussion and conclusions

7.1 General discussion

In this thesis, I have focussed on virulence variation in strains of the monogenean ectoparasite, *Gyrodactylus arcuatus* and assessed how environmental conditions and the ecology of its stickleback host can shape parasite virulence. How and why virulence evolves, constitute arguably the most interesting research questions in the field of evolutionary biology (Frank and Schmid-Hempel, 2008; Lively *et al.*, 2014). I investigated virulence variation among geographical strains of the parasite and examined its correlates. In order to do this, some of the most important biological traits of hosts and parasites (Zhan *et al.*, 2002; de Roode *et al.*, 2008a; Little *et al.*, 2008) had to be quantified: (1) virulence levels and parasite local adaptation using transplant and reciprocal cross infections between strains of the parasites and their host populations under lab controlled conditions, (2) the survival expectancy of detached parasites and parasite adaptation to the biotic and abiotic properties of their local environment, (3) the morphology of host epidermal mucous cells and its effect on the parasite fitness both *in vivo* and *in vitro*, (4) the social behaviour of host populations and its role in parasite transmission, (5) the influence of ecological interactions between coinfecting *Gyrodactylus* species on the parasite virulence, and finally, (6) the most important ecological properties (the density of host population and host predator) and abiotic variables (water chemistry and pH) of the natural environment of host populations. Then, I examined the relatedness of these factors to virulence levels of seven strains measured on an allopatric host population. These requirements are addressed in below paragraphs, with particular focus on the results found in this project.

(1) Virulence levels and parasite local adaptation

To survive, obligate ectoparasites must cope with two hostile environments: abiotic variables of the habitat where their hosts live and immune responses of hosts which represent a biological challenge (Gandon and Zandt, 1998). These environments often vary spatially and temporally causing variable selection pressures directed on parasites or on their hosts which may in turn feedback to affect parasite evolution (Gandon *et al.*, 1996; Gandon and Zandt, 1998; Lopez Pascua *et al.*, 2012). Therefore, we might expect to find evolution of virulence as a result of arms races, local mosaics of coevolution (Thompson, 1999; Gallet *et al.*, 2009), and such patterns have been previously documented (Fenner and Ratcliffe, 1966; Ebert, 1994; Osnas and Lively, 2004). In chapter 2, I found that geographical strains of *G. arcuatus* vary greatly in virulence levels on allopatric host populations, but not on sympatric hosts. Additionally, the population size of three out of four of the parasite strains grew larger on their sympatric hosts than allopatric fish populations, suggesting that the parasite is generally locally adapted. Similarly, Raeymaekers *et al.* (2011) reported that population size of an isogenic line of *G. gasterostei* significantly differed on two lab raised stickleback populations. The authors reported that the parasite strain was successful on its sympatric fish population, but failed to establish on allopatric hosts, again suggesting that coevolution favours *G. arcuatus* local adaptation at stickleback population levels.

It has been suggested that host- parasite coevolution is influenced both by abiotic heterogeneity (Lopez Pascua *et al.*, 2012) and the resistance and genotypes of hosts (Little *et al.*, 2008; Andras and Ebert, 2013). In this chapter, parasite strain which came from the most susceptible host population showed the lowest level of virulence. It seems to be commonly assumed that this pattern of variation in virulence usually emerges purely as a

result of the dynamics of the host-parasite interaction. For example, Fenner's study on rabbits and myxomatosis, where virulence outcome depends partly on the mortality rate of the host, but also on the efficiency of transmission (flea or mosquito) (Fenner *et al.*, 1956). Transmission is also important in Herre's study on nematode virulence in fig wasps (Herre, 1993), and in barley stripe mosaic virus (Stewart *et al.*, 2005). This unstated assumption seems to run through other examples of coevolution such as the interaction between a snake (*Thamnophis sirtalis*) predator and its newt (*Taricha*) prey (Brodie *et al.*, 2002), but most work is unclear about why a similar interaction should have different outcomes (virulence) in different locations. The obvious answer is that some property of the interaction varies in the first place (i.e. the environment) (see Lively *et al.*, 2014 their question 3: about the effects of environmental factors on virulence).

(2) Adaptation of *G. arcuatus* to the abiotic and biotic properties of their local environment.

Environmental heterogeneity can influence a broad range of the physiology and behaviour of organisms (Bowden, 2008). Host- parasite associations represent long-term interactions between two biologically and genetically different organisms (Horwitz and Wilcox, 2005) and can potentially be affected by environmental conditions (Mostowj and Engelstadter, 2011). In chapter 3, I focused on the interaction between the parasite and its abiotic and biotic environments. The parasite strains differed markedly in survival time (hours) in their natural loch-water under a uniform lab temperature. Results of this chapter suggest that *G. arcuatus* struggles to exist in low pH and low conductivity environments. The survival time of detached worms from such environments was less affected compared to strains from lochs with higher pH and conductivity when exposed to a temperature level higher than their loch water. As these parasites and their stickleback hosts originated from the sea,

these results might suggest that the harsher environment (low pH and conductivity) makes them tougher in some sense which could be explained by countergradient selection (see Craig and Foote, 2001), but they still struggle to grow and make big virulent infections.

When reciprocally introduced into water from their own and different lochs, the parasite strains survived longer in their own water. In isolated populations on North Uist, it is expected to find local adaptation of the parasite because adaptation to a specific environment depends on temporal and spatial variation, suggesting that low gene flow rate enhances local adaptation (Vale *et al.*, 2008). In other microorganisms such as bacteria, it has been found that abiotic environmental stressors potentially select for adaptive genotypes (Garner *et al.*, 2006) and such a pattern of adaptation can have implications for parasite transmission and virulence (Walter and Ewald, 2004). The survival time of detached *G. arcuatus* could be important because in addition to their ability to transmit directly between hosts (Bakke *et al.*, 1992), most of gyrodactylids are also thought to make use of the ‘sit and wait’ transmission strategy (Bakke *et al.*, 2007).

(3) The relationship between host skin mucus and the parasite fitness both *in vivo* and *in vitro*

As *Gyrodactylus* sp. parasitize the outer surface of skin, parasite survival and infection success are thought to associate with the structure of the hosts’ epidermis especially the contents of skin mucus (Buchmann and Bresciani, 1998; Harris *et al.*, 1998). Chapter 3 assessed the effect of stickleback epidermal mucus on the survival time of detached *G. arcuatus* and the relationship between the intensity of parasite infections in natural populations and the morphology of the epidermal mucous cells of wild hosts.

Unexpectedly, parasite survival time was slightly higher in the presence of mucus *in vitro*,

and on lab raised fish, gyrodactylids showed higher preference for parts of the body surface where the density of mucous cells was high. These results suggest that in this system, the parasite somehow benefits from the mucus. Several studies have reported a similar influence of *Gyrodactylus* sp. on fish epidermal mucous cells (Cusack and Cone, 1986; Wells and Cone, 1990; Sterud *et al.*, 1998). In the current study, post-infection histological analysis showed a negative relationship between parasite intensity and the size and density of epidermal mucous cells. Strikingly, field data also showed a strong negative association between the average natural abundance of *Gyrodactylus* sp. measured over five previous years and fish skin mucous cell density. Collectively, these results suggest an involvement of epidermal mucous cells in the susceptibility of sticklebacks to *G. arcuatus* and that reducing these cells may be an adaptive response by the hosts to provide protection against the parasite.

(4) The social behaviour of host populations and its role in *Gyrodactylus* sp. transmission

Studies of virulence evolution suggest that changes in virulence depend on changes in parasite transmission success (Ewald, 2004; de Roode *et al.*, 2008a). Grouping behaviour of host populations can play an important role in the transmission of contact transmitted parasites (Freeland, 1979; Richards *et al.*, 2010; Chapman *et al.*, 2012; Richards *et al.*, 2012). Chapter 4 employed a recently developed technique by Wark *et al.* (2011) to quantify schooling and shoaling of wild and lab raised fish and to investigate how these behaviours are influenced by *Schistocephalus solidus* and *Gyrodactylus* sp. parasites. Stickleback infections with any of the parasites resulted in a clear decrease of schooling, but shoaling was not affected by any of the parasites. Previously studies have evidently reported that the cestode, *S. solidus* manipulates the behaviour of stickleback (second

intermediate) hosts (Ness and Foster, 1999). Although, it is yet not clear by what mechanisms the cestode causes such behavioural changes in stickleback (Barber and Scharsack, 2010), but altered group joining behaviour is very likely to increase its transmission success to fish-eating birds (final host) through predation (Barber and Huntingford, 1995; Barber and Scharsack, 2010).

In fish hosts, group formation increases their antipredator responses (Pitcher, 1979), but also mediates parasite transmission through increased contacts among individuals (Richards *et al.*, 2012). The group making tendency of juvenile sticklebacks was found to increase when the fish were subjected to a crustacean ectoparasite (*Argulus canadensis*) and that resulted in lower attack rate on individual host (Poulin and FitzGerald, 1989). In the wild, infections with a microsporidian (*Glugea anomala*) was also found to increase grouping tendency in sticklebacks (Ward *et al.*, 2005). In the current chapter, natural and artificial infection with *Gyrodactylus* sp. reduced schooling tendency in stickleback hosts. Such changes in the tendency of host social behaviour might be initiated to avoid additional infections and/or an adaptive attribute to the spread of infectious agents (Møller *et al.*, 1993). For directly transmitted parasites, such behavioural changes of hosts can have two consequences: (1) reduction in the probability of contact among hosts and reduced transmission rates, (2) increased mortality of hosts through enhanced predation on hosts (Seghers, 1974) and hence parasite survival. Such patterns of host-parasite interactions that reduce the group tendency of a host may reduce the transmission of its contact transmitted parasites and may therefore, select against virulence (Boots *et al.*, 2004).

(5) The influence of ecological interactions between coinfecting *Gyrodactylus* sp. on the parasite virulence

Virulence in concurrent infections with multiple parasite genotypes cannot be explained by the trade-off hypothesis because, coinfecting parasites differently affect the resistance and mortality of hosts (Sasaki and Iwasa, 1991; Gandon *et al.*, 2002). In chapter 5, I investigated the influence of ecological interactions between *G. arcuatus* (sympatric) and *G. gasterostei* (allopatric) parasites on their virulence. Some researchers suggest that under such circumstances different parasite genotypes compete on resources and that induces higher virulence (Levin and Pimentel, 1981). Results of this chapter have shown that *G. arcuatus* was more successful than *G. gasterostei* when separately and jointly used to infect their local stickleback population. This finding lends more supports to the result pattern of chapter 2, which suggests that coevolution in this host-parasite interaction, favours parasite local adaptation at the level of host populations (Raeymaekers *et al.*, 2011). Compared to population growth of parasites in single infections, the two *Gyrodactylus* sp. had smaller growth when they concurrently initiated the infection. Such antagonistic interactions among monogenean species have previously been reported (Paperna, 1964), but the exact mechanism is not clear. Induced changes in the life history traits of a host by one parasite are thought to induce changes in the virulence of all parasites sharing that host (Sasaki and Iwasa, 1991; Gandon *et al.*, 2002; Best *et al.*, 2009). In such interactions, a local parasite might induce a specific host resistance to the other parasite species (Paperna, 1964; Jackson *et al.*, 2006). *Gyrodactylus* sp. are commonly known as highly host specific parasites (Huyse *et al.*, 2003) and such associations often cause little stimulation of hosts' immune responses (Frank and Schmid-Hempel, 2008; Franke *et al.*, 2014), but coexistence with allopatric parasites might cause a different outcome (Simkova *et al.*, 2008). The results of this chapter suggest that concomitant infections with both *Gyrodactylus* species may lead to shortening infection duration and lower virulence.

(6) The abiotic and biotic associates of *G. arcuatus* virulence

Spatially distributed organisms often experience different stressors that result from local environments (Eisler, 1993) and that can impose variable selection on geographically isolated populations (Thompson, 1999). In chapter 6, I isolated seven strains of *G. arcuatus* from naturally infected, geographically disconnected stickleback populations and quantified their virulence on an allopatric host population. Interestingly, the results were in a complete agreement with those I found in chapter 2. Parasites from freshwater fish populations (Gill, Scad and Torm) located on the middle and east side of North Uist (experiencing low water pH and salinity) had lower virulence than those (Host, Maga, and Reiv) that were isolated from the west part of the island (experiencing alkaline water and higher salinity). It is commonly predicted that environments shape the ecology of many organisms including hosts and parasites (Herre, 1995; Zhan *et al.*, 2002). The influence of environmental conditions on ectoparasitic organisms may be highly important because it may have a direct impact on their fitness or indirectly by inducing changes in host life history traits that may induce changes in virulence evolution (Cornet *et al.*, 2014).

Experimental infections evidently showed that the parasite strains evolve virulence variations, but the question of “what drives such variations” may become clear by understanding the association between virulence and characteristics of local habitats. Chapter 6 tested the predictions of some models of virulence evolution by examining correlations between the virulence of geographical strains of the parasite, and some aspects of abiotic and biotic environments, and host social behaviour. Environmental stressors explained most of the variability in parasite virulence. In particular, water pH was strongly positively associated with parasite virulence levels, but virulence had a negative correlation with host predator density. Among the abiotic environmental variables, water

pH is an associate of a wide range of chemical properties which play a key role in shaping the life history traits of sticklebacks and the community structure of other fish species such as nine-spined stickleback (MacColl *et al.*, 2013), and are also likely to affect the parasite.

The exact mechanism of the association between *G. arcuatus* virulence and water pH is not clear, but at least three explanations are possible: (1) the concentration of zinc (and transition metals in general) in the water on North Uist is negatively associated with pH levels and presence of any concentration of this metal has been found to impact *G. turnbullis* (Gheorghiu *et al.*, 2007), (2) under alkaline environments, there are richer resources and sticklebacks grow larger and survive longer (MacColl *et al.*, 2013) which may result in higher abundance of, and therefore more virulent *Gyrodactylus* sp. (Cable and Oosterhout, 2007a), (3) fish diversity positively correlates with water pH (MacColl *et al.*, 2013) and some gyrodactylids (e.g. *G. salaris*) are thought to use different fish species as transmitters (Soleng and Bakke, 1998). These findings support the results of some recent theoretical analyses which suggest that under poor environmental conditions and high predation pressure, hosts develop increased susceptibility and this consequently selects for low virulent parasite (Choo *et al.*, 2003; Duffy *et al.*, 2012).

7.2 General conclusions

The results of this thesis suggest that geographical strains of *G. arcuatus* genetically vary in their life history traits and virulence and such variations are mainly driven by local environmental conditions experienced by the populations of their stickleback hosts. Similar to previous observations of the association between *Gyrodactylus* sp. abundance and the body size of natural guppy populations (Cable and Oosterhout, 2007a), in the wild, *G. arcuatus* abundance positively associated with stickleback length. As body length in

North Uist sticklebacks strongly associates with environmental pH and conductivity (MacColl *et al.*, 2013), these results point to the role that environmental conditions experienced by hosts and host resistance play in shaping the coevolution of virulence in *Gyrodactylus* sp. Generally, *Gyrodactylus* are known to be highly specific to their host genotype (Bakke *et al.*, 2002; Buchmann and Lindenstrøm, 2002) and their continuous infra-population growth can quickly result in host death (Scott and Anderson, 1984; Madhavi and Anderson, 1985). Therefore, the optimum fitness of *Gyrodactylus* strain is likely to reach an equilibrium between its reproduction rate and the costs result from damaging its host (Raeymaekers *et al.*, 2011).

To my knowledge, this project represents the first intensive study investigating coevolution outcomes in this host- parasite system. In this regard, it sheds the light on the importance of spatial and temporal differences, suggesting that, with low gene flow, coevolution processes lead to *Gyrodactylus* local adaptation at host population levels (Raeymaekers *et al.*, 2011). Geographic isolation is likely to limit gene flow rate in their host genotypes, but obviously the parasites' shorter generation time and larger population size compared their hosts can favour the parasite local adaptation (Gandon *et al.*, 1998; Gandon and Michalakis, 2002). The results of this project also suggest that the parasite strains have an adaptive relation with their abiotic environments. In some fish, skin mucus which has previously been found to be influenced by water conditions (Gheorghiu *et al.*, 2012), stickleback epidermal mucus appeared to attract gyrodactylids and, under high abundance of the parasite, selection is likely to work against hosts with high density of epidermal mucous cells.

In general, the results of this thesis highlight the sensitivity of *Gyrodactylus* sp. transmission to the social behaviour of sticklebacks and the presence of other parasite

species. For example, the schooling tendency of wild fish hosts was largely reduced by infections caused by *Schistocephalus solidus* and lab bred fish showed poor preference to schooling when separately or jointly infected with *G. arcuatus* and *G. gasterostei*. Barber and Huntingford (1996) suggest that such behavioural changes can increase the susceptibility of fish hosts to predation which might have important consequences for *Gyrodactylus* sp. transmission. Coinfections with the sympatric and allopatric *Gyrodactylus* sp. result in antagonistic interactions leading to a sharp reduction in the population growth of the latter. This pattern may explain why concurrent infections with the two parasite species are rare in the natural environment and could be important in the parasite transmission and infection recovery (Blackwell *et al.*, 2013).

Collectively, this body of works highlights the fact that environmental variables (especially water pH) can potentially alter the dynamic of this host- parasite interactions and may particularly determine virulence levels (Lively *et al.*, 2014). These findings support the geographic mosaic theory of coevolution, emphasizing on the role of geographic perspective in shaping the coevolutionary outcomes of continuous ecological interactions (Lively, 1999; Thompson, 1999). The present study also emphasizes on how hosts' phenotypes may contribute significantly to selection mosaics found in intraspecific variation in virulence, suggesting that the most virulent parasite strains exist where the hosts' environment is highly productive (Hochberg *et al.*, 2000).

7.3 Future directions

The current work has examined the ecological variations in virulence among geographically isolated strains of *G. arcuatus*. It made it clear that, under spatial and temporal variations, strains of this parasite species evolve virulence differences, but due to

time limitation, the mechanism stands for the present variation remained unknown. In future, it would be fascinating to understand the genetic differences among the parasite strains which might consequently increase our understanding of the mechanism of virulence in *Gyrodactylus* sp. Estimating the immune gene expressions of stickleback hosts under infections with sympatric, allopatric and mix species of the parasite could answer the questions of how geographically isolated strains of *G. arcuatus* grow better on their sympatric hosts than allopatric hosts. Quantifying host immune gene expression may also improve our understanding about the mechanism of ecological interactions between *G. arcuatus* and *G. gasterostei* when concomitantly infect sticklebacks.

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