

# The Ecological and Evolutionary Importance of Immune System Variation in the Three-Spined Stickleback

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# Abstract

Placing our understanding of the function of the immune system into a more natural setting remains a fundamental challenge in biology, particularly how natural variation shapes the immune response and what the evolutionary consequences of such variation are. In this thesis, I use the three-spined stickleback as a model system for wild immunological studies. First, I developed a set of markers to measure the expression levels of key immune system genes using quantitative real-time PCR, representing the innate and adaptive immune response, and then used them to address a number of questions. I demonstrated that there are underlying differences in innate and adaptive gene expression levels between populations, as well as in innate immune response potential, which may reflect the contrasting challenges faced in these populations. By sampling individuals from multiple wild populations, I was able to demonstrate how a range of factors contribute to shaping immune system function, including sex, reproductive status, and infection with the common parasites *Schistocephalus solidus* and *Gyrodactylus arcuatus*. Next, I exposed laboratory raised fish to natural conditions and examined their response. Again, a range of factors were identified which appear to shape immune expression levels, particularly reproductive investment and infection with *G. arcuatus*. I also used this approach to demonstrate that immune system variation can be linked to *Eda* genotype, the gene which controls lateral plate phenotypic divergence during adaptive radiations. Finally, I performed a controlled infection experiment in the laboratory to show that both the innate and adaptive systems respond to *Gyrodactylus* infection. This thesis provides the basis for further immunological studies in stickleback, and adds to our growing understanding of the relevance of natural variation in shaping the immune response.

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# Contributions

The work presented in this thesis is my own. A number of people, however, made contributions to individual chapters:

Chapter 2: Andrew MacColl and Jan Bradley helped to select the genes to target. Andrew MacColl helped to select populations to sample, and supplied the Icelandic fish.

Chapters 4 & 5: Andrew MacColl suggested using F2 hybrid fish for enclosure experiments.

Chapter 6: Muayad Mahmud helped to perform the infection experiment.

For chapters 2-6, Andrew MacColl and Jan Bradley gave advice on statistical analysis and contributed comments to the discussion.

All work involving animals was approved by the University of Nottingham ethics committee, under Home Office Project licence (PPL-40/3486). Controlled procedures were performed under Home Office personal licence (40/10860 and IFA287AAD).

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# 1. General Introduction

In this thesis, I develop a set of gene expression markers with which to measure the immune response of three-spined stickleback, and then use these to examine how various factors act to shape the function of the immune system in a natural setting. Here, I introduce the relevant background information, which places the work into a wider context. I begin by examining the possible role of parasites as drivers of adaptation, then look at the emergence of the study of wild immunology. Next, I examine the immune system of teleost fish, and introduce the three-spined stickleback, the North Uist study system, and the two common parasites on which my work will largely focus. Finally, I outline the main aims of the data chapters which follow.

## 1.1 Parasites as agents of selection

Adaptive evolution is the process by which populations become better adapted to the environment in which they live, and is driven by natural selection. Natural selection, however, is not the ultimate cause of adaptive evolution. It is in turn driven by any biotic or abiotic factor (an agent of selection) which results in differing fitness between individuals in a population. The role of a number of possible selective agents, such as competition (Pfennig *et al.* 2007; Schluter 1994) and predation (Nosil & Crespi 2006; Reznick 1982), has been well studied, but the potential role of parasites in this context has been largely overlooked (MacColl 2011).

Parasitism is one of the most widespread lifestyles in the animal kingdom (Price 1980; Windsor 1998), with at least one species of parasite for every species of host (Poulin & Morand 2000). Parasites have the potential to

influence the dynamics of a host population (Hudson *et al.* 1998), manipulate host behaviour (Thomas *et al.* 2005), and affect host life history (Agnew *et al.* 2000; Perrin *et al.* 1996). Where infection with a parasite has an impact on host fitness, there is the potential for parasites to drive the evolution of a host population. For this to occur, selection must be exerted on the genes underlying a host phenotype (Little 2002). The theoretical potential of parasites to drive various aspects of host evolution and diversification has long been suggested (Anderson & May 1978; Clarke 1979; Hamilton 1980), with evidence of parasite-mediated selection in the wild now growing (for example, see Little 2002; Summers *et al.* 2003; Vamosi 2005). Parasites have the potential to drive the evolution of a number of host traits, but those directly involved in defence against infection may be subject to particularly strong selective pressure. Genes of the immune system, for example, show evidence of adaptive evolution above that seen across the genome as a whole (Downing *et al.* 2009; McTaggart *et al.* 2012; Obbard *et al.* 2009; Tonteri *et al.* 2010). Changes in defence traits of the host can in turn affect the dynamics of the infecting parasite population (Anderson & Gordon 1982; Anderson & May 1978), and can have negative fitness consequences for infecting parasites. In this way, classic cyclical host-parasite evolutionary dynamics would be predicted to establish (Hamilton 1980; Nee 1989; Schmid-Hempel & Ebert 2003), with coevolving host and parasite populations becoming increasingly adapted to one another.

For parasite-mediated natural selection to occur, two criteria must be met. First, there must be fitness consequences for parasitized hosts, and second, there must be genetic variation in resistance or tolerance that results in the uneven distribution of parasites in a host population (Little 2002). In order to investigate whether a host population is evolving as a result of parasitism in a

given system, we need some suitable measure of these two criteria. Fitness consequences for infected hosts have been demonstrated for a range of parasite types, from intracellular viruses (Packer *et al.* 1999; Telfer *et al.* 2002) through to external macroparasites (Lehmann 1993; Richner *et al.* 1993). Uneven parasite distributions are common, with a small number of individuals commonly hosting a disproportionate burden relative to the rest of the population (De Roij 2010; Wilson *et al.* 2002). An uneven distribution may not, however, be the direct result of genetic variation in resistance (Wilson *et al.* 2002). Infection of laboratory raised hosts with the same initial parasite burden in a common garden setting can demonstrate whether genetic variation actually exists (Little 2002). This approach has been successfully employed in both invertebrate (Ebert *et al.* 1998; Tinsley *et al.* 2006) and vertebrate (De Roij *et al.* 2011; Jackson & Tinsley 2005) host systems, to show that a number of uneven parasite distributions have a heritable genetic basis. As one of the main host defences against infection, the immune system would be predicted to play a key role in determining the outcome of such host-parasite interactions, and thus the genes underlying immune system function may be the subject of strong selection pressure.

## **1.2 Wild Immunology**

Current knowledge of the vertebrate immune system is based largely on studies of humans and a few select model species. Such studies tend to be laboratory based, performed in highly controlled settings where variation of hosts and parasites, as well as the external environment, can be minimised. Laboratory study organisms are often lab-adapted and inbred over many generations, and infections performed with a single strain of parasite. By

minimising variation in this way, it is possible to identify the cellular and molecular mechanisms underlying the immune system (Babayan *et al.* 2011; Maizels & Nussey 2013; Pedersen & Babayan 2011). Whilst this approach has greatly expanded our understanding of how the immune system works, it is disconnected from what happens in a more natural setting.

Natural systems are characterised by genetic diversity of both hosts and parasites, and by variation in the biotic and abiotic environment. Factors such as resource availability, competition, seasonality, age and sex differences may all affect how the immune system functions (Lazzaro & Little 2009; Martin *et al.* 2011; Nussey *et al.* 2012), and the high degree of outbreeding often found in wild populations can create new genetic combinations that alter immune competence (Abolins *et al.* 2011). This wide ranging variation observed in wild populations is often overlooked in purely laboratory based studies, but may play an important role in the 'natural' function of the immune system. Placing our current knowledge of the functioning of immunological mechanisms into a real-world context is a fundamentally important challenge in advancing understanding of the immune system.

There has been a recent interest in taking this lab-based knowledge of the immune system and applying it to studies in a more natural setting (Bradley 2015; Pedersen & Babayan 2011). A range of wild immunological studies in vertebrates have been performed to date, typically focussing on rodent species where knowledge of immune function is well developed due to their close relation to laboratory model species, and where a range of immunological tools are readily available. The T cell response of wild deer mice *Peromyscus maniculatus* has been profiled using real-time PCR based assays (Okó *et al.* 2006), and variation in the development of regulatory T cells has been linked to chronic viral infections (Schountz *et al.* 2007).

Immune gene expression levels in wood mice *Apodemus sylvaticus* varied with parasite burdens (Friberg *et al.* 2011) whilst immune variation in field voles *Microtus agrestis* was associated with seasonal changes and tolerance to infection (Jackson *et al.* 2011; Jackson *et al.* 2014). Whilst applying immunological measures to wild rodent populations has provided new insights into the function of the immune system in the wild, there is still a desire to expand these approaches to other organisms.

The expansion of immunology into new systems has suffered from a lack of appropriate assays and potential study species (Fassbinder-Orth 2014), but recent advances in next generation sequencing and molecular tools have begun to address this issue. Next generation sequencing allows the whole transcriptome of an organism to be measured, but such approaches are still costly, and linking changes seen in a wide range of genes from multiple interconnected pathways to the function of the immune system can be difficult (Fassbinder-Orth 2014). Alternatively, primers can be designed to measure the expression levels of specific genes based on *a priori* knowledge of the function of the immune system, using quantitative real-time PCR (hereafter 'qPCR'). This approach uses PCR primers to amplify a short section of an mRNA molecule, produced as part of the expression of a specific gene. Special dyes are included in the reaction which fluoresce when bound to double-stranded PCR product, allowing the relative amount of a target molecule to be calculated, based on the amount of fluorescence. qPCR retains the advantage of being relatively cheap, allowing the high level of replication required for studies in wild systems, where levels of background variation are likely to be high. This approach has been widely used to measure the expression levels of candidate immune system genes across a wide range of species, especially of small rodents (for example, see Bertolotti

*et al.* 2013; Jackson *et al.* 2011; McWilliam *et al.* 2013). It is not easy to perform manipulative experiments in many of these rodent systems (Bradley 2015; Pedersen & Babayan 2011), thus they rely on correlations between expression levels and any factors of interest.

In order to combine the correlational-type studies which have so far been conducted in wild rodents with an experimental based approach, the development of study systems using new, non-model, organisms is required. Teleost fish may provide such systems. They have a long history of use in biological study, providing an extensive background knowledge on which further studies can be based. Many species can be readily caught in the wild, and live fish or fertilized eggs can be raised in laboratory conditions. Genomic resources allow immunological tools to be developed, and both lab-based and natural experiments are possible. Importantly, teleost fish possess the main components of the innate and adaptive immune system (Alvarez-Pellitero 2008; Magnadottir 2006; Whyte 2007), and may be one of the lowest branch of the vertebrates in which these are conserved (Uribe *et al.* 2011; Whyte 2007).

### **1.3 The teleost fish immune system**

A range of teleost fishes have been the focus of biological study. Some have been used as model organisms in classical behavioural, ecological and evolutionary work, such the three-spined stickleback *Gasterosteus aculeatus* (For review, see Bell & Foster 1994; Hendry *et al.* 2013), whilst others, such as zebrafish *Danio rerio*, are used to better understand developmental processes (For example, Lieschke & Currie 2007; Westerfield 2000). Other species have been studied due to their economic importance as a food source

and their use in the aquaculture industry, including the Atlantic salmon *Salmo salar* (For example, Austreng *et al.* 1987; Tacchi *et al.* 2011), rainbow trout *Oncorhynchus mykiss* (For example, Bailey *et al.* 1996; Ellis *et al.* 2002) and Atlantic cod *Gadus morhua* (For example, Mieszkowska *et al.* 2009; Perez-Casanova *et al.* 2008). This long history of biological study, combined with a basic understanding of the immune system, makes the teleost fishes an ideal group in which to further examine the immune system in a natural setting.

Traditionally, the innate and adaptive immune systems were considered as two separate arms, differing in their function and underlying mechanisms. Evidence from both fish and mammals, however, shows there is a high degree of cross-linkage, and that they form two components of the same general system (Benjamini *et al.* 2000; Eales 1997; Magnadottir 2006). For simplicity, I will consider these two arms of the immune system separately, but note where they interact with one another. I use the term 'parasite' to refer to any infection in general, from viruses through to external macroparasites.

Physical barriers act as the first line in a fish's defence against infection, and are particularly important as living in water leaves them constantly exposed to the risk of infection from all directions. The epidermal layer, scales and mucus all act as barriers to infection (Fast *et al.* 2002; Ingram 1980; Shephard 1994). As well as being a physical barrier to parasite attachment, mucus also contains a range of immunologically active molecules (Aranishi & Nakane 1997; Fast *et al.* 2002; Rombout *et al.* 1993). Variation in any of these external components may affect an individual's susceptibility to infection. If a parasite can pass these initial physical barriers, it will then be exposed to the immune system of the host.

In order for the immune system to be activated, it must first recognise the presence of a parasite. The innate system uses a number of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and C-type lectins, to bind to pathogen associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Benjamini *et al.* 2000; Eales 1997; Whyte 2007). PAMP is the collective term for any conserved parasite-associated molecule, such as bacterial cell walls or viral dsDNA, which are not normally found in the host (Elward & Gasque 2003). PRRs have been identified in a range of fish, including Atlantic cod (Star *et al.* 2011) and rainbow trout (Baerwald *et al.* 2008), and show high levels of conservation at the structural level (Aoki *et al.* 2013; Boltana *et al.* 2011; Poynter *et al.* 2015). The binding of a PAMP or danger signal by a PRR activates the immune response, with the type and magnitude of that response dependent upon the PAMP/DAMP and PRR involved.

One of the main responses associated with the innate system is inflammation at the site of infection. Activated PRRs, mainly from the TLR family, recruit macrophages to the site of infection which produce pro-inflammatory cytokines, such as tumour necrosis factor alpha (*TNF $\alpha$* ) and interleukin-1 $\beta$  (*IL-1 $\beta$* ). These cytokines act as signal molecules which recruit hepatocytes, which in turn 'mark' parasites for phagocytosis by macrophages and neutrophils when appropriate (Alvarez-Pellitero 2008; Magnadottir 2010). The level of inflammation is controlled by the expression levels of immune regulating genes, including interleukin-10 (*IL-10*) and transforming growth factor beta (*TGF $\beta$* ) (Benjamini *et al.* 2000). TLRs can also directly induce macrophages to phagocytose parasites, and some can induce apoptosis or programmed cell death (Salaun *et al.* 2007). The induction of inflammation can result in the formation of granulomas which encapsulate the parasite (Buchmann 2000),

isolating the parasite and reducing damage to the host. The inflammatory response is active in many fish against a wide range of pathogens (Braden *et al.* 2012; Buchmann 2012; Buchmann & Lindenstrom 2002). Induction of the innate response is extremely rapid, with changes in pro-inflammatory markers levels occurring within one day of infection in gilthead seabream *Sparus aurata* (Chaves-Pozo *et al.* 2005) and Atlantic salmon (Tadiso *et al.* 2011). As the first line of response to infection, the innate response plays an important role both directly fighting infection and in activating downstream adaptive immune mechanisms.

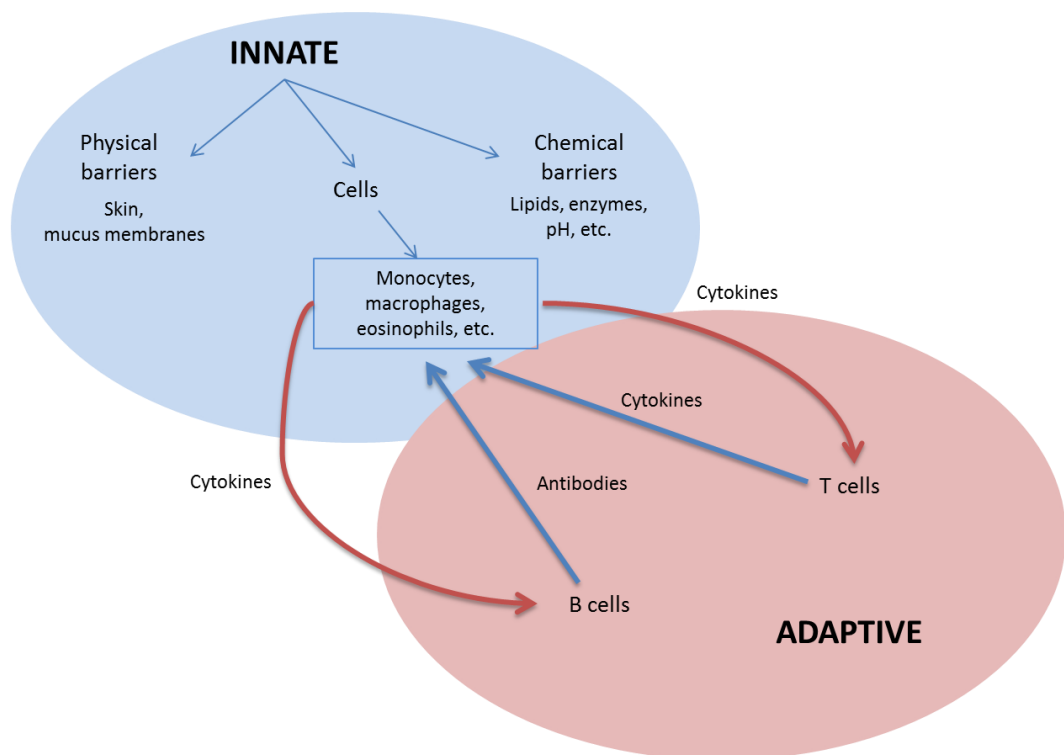


**Figure 1.1:** Outline of the main components of the innate (blue) and adaptive (red) immune systems, and the connections between them. Cytokines act as signals from the innate to the adaptive system (red arrows), whilst antibodies and cytokines produced by the adaptive system feed back to the innate (blue arrows).

While the innate response is a general response to infection, adapted to the challenges faced by a species over evolutionary time, the adaptive response develops over the life of an organism as a result of the specific challenges which it faces. The innate system responds rapidly as the first line of defence, whilst the adaptive response takes longer to be activated after infection. The innate and adaptive arms of the immune response interact through cytokines and cell surface signals (Figure 1.1, Benjamini *et al.* 2000; Uribe *et al.* 2011). The specific combination of signals produced by cells of the innate immune response determines the type of adaptive response which is triggered.

Three main cell types are involved in the adaptive response: T cells, B cells and antigen presenting cells. T cells and B cells differ in function, but both display specificity towards an antigen, therefore acting as the major recognition and reactive cells of the adaptive response (Benjamini *et al.* 2000; Uribe *et al.* 2011). Antigen presenting cells, such as macrophages and dendritic cells, present antigens to specific receptors on lymphocytes, with two classes of molecules on their surface involved in antigen presenting: the class I and class II major histocompatibility complex (MHC). Diversity of MHC class II molecules correlates with parasite resistance, in both fish (Kurtz *et al.* 2004; Uribe *et al.* 2011), and in wider vertebrates (For review, see Apanius *et al.* 1997; Bernatchez & Landry 2003). Whilst MHC diversity appears important, it represents only a small portion of the overall immune response (Acevedo-Whitehouse & Cunningham 2006). B cells become activated when an antigen binds to a B cell receptor on the cells surface, resulting in the activation of an antibody response. Activation also results in the production of more receptors onto the cell surface, making the B cell more responsive. Signals also activate the complement system, in which a series of serum enzymes result in the lysis of a target or enhanced phagocytosis. B cells can only bind a single

antigen type as they only display a single receptor type on their cell surface, making them specific in their response.

In contrast to B cells, T cells are activated by binding of antigen fragments already processed by other cell types. They can be broadly split into three types: inflammatory T cells which drive inflammation at the site of infection; cytotoxic T cells which directly mediate the death of infected cells; and helper T cells which orchestrate other parts of the immune response appropriate to the type of infection. Helper T cells can be further divided into sub-types (Th1, Th2, Th17 and Treg) which each act against a different broad type of challenge (Figure 1.2). These sub-types each have a characteristic set of signal molecules that drive their maturation and a further set of signals and transcription factors associated with their function once mature (Figure 1.2). These signal molecules and transcription factors can be measured, allowing us to see the type and magnitude of T cell response that is being mounted. Whilst much of our understanding of T cell function comes from humans and rodents, there is increasing evidence that the major components and mechanisms are present and functional in teleost fish (Laing & Hansen 2011; Somamoto *et al.* 2014; Toda *et al.* 2011; Wang *et al.* 2010; Wang & Secombes 2013).

By measuring the expression levels of key genes involved in these different immune processes, selected based on our *a priori* understanding of the immune response, we can get an overview of the function of the immune system at the time of sampling. This candidate gene based approach has been employed in the study of the immunology of a range of rodent species, including deer mice *P. maniculatus* (Okó *et al.* 2006; Schountz *et al.* 2007), wood mice *A. sylvaticus* (Friberg *et al.* 2011) and field voles *M. agrestis* (Jackson *et al.* 2011; Jackson *et al.* 2009). Similar approaches have also been

applied to immunological questions in fish, including rainbow trout *O. mykiss* (Baerwald *et al.* 2008), Atlantic salmon *S. salar* (Krasnov *et al.* 2012; Skugor *et al.* 2008), Atlantic cod *G. morhua* (Caipang *et al.* 2011; Chi *et al.* 2012) and gilthead sea bream *Sparus aurata* (Chaves-Pozo *et al.* 2005; Cuesta *et al.* 2007). It should, therefore, be possible to develop markers to measure the expression levels of key immune system genes in the three-spined stickleback, and use these to study the function of the immune system in this common model species in ecology and evolutionary research.

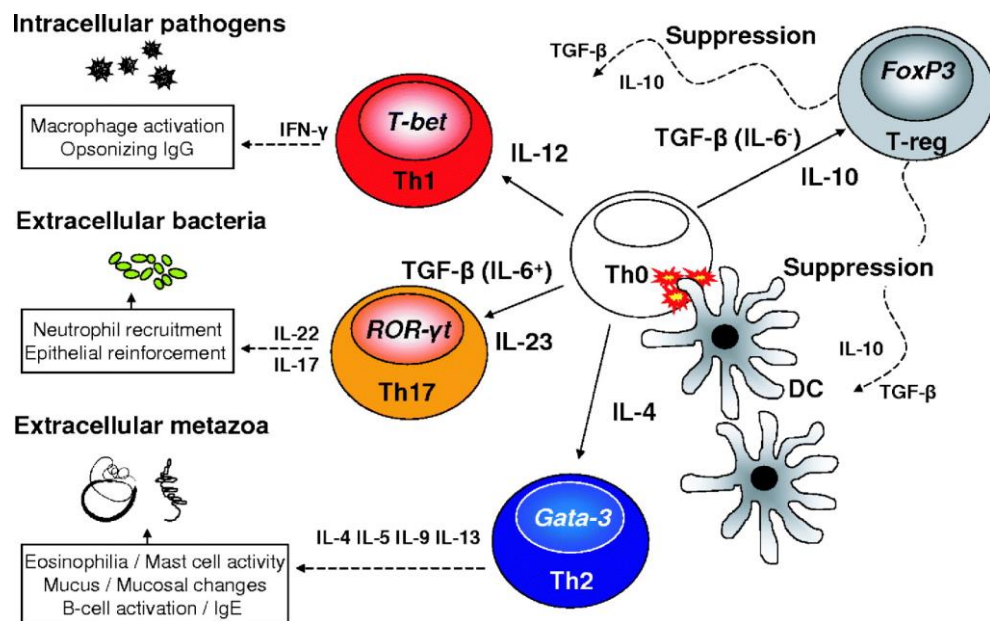


**Figure 1.2:** Maturation of naïve T cells (Th0) into one of four mature T cell types (Th1, Th2, Th17 or Treg) upon activation by dendritic cells (DCs). Each type acts towards a specific category of challenge, as shown. There are characteristic signal molecules associated with the development (solid arrows) and function (dashed arrows) of each type. The main transcription factors associated with the function of each cell are also shown within each depicted mature cell. Diagram from Bradley and Jackson (2008).

## 1.4 The host: three-spined stickleback

The three-spined stickleback *Gasterosteus aculeatus* (hereafter 'stickleback') is a small teleost fish which is widely distributed throughout the northern hemisphere. Sticklebacks have a long history of use in biological study, due to their wide-spread distribution, ease of capture, and the ability to keep and breed them in the laboratory. Past studies have focussed on a wide range of topics, from behaviour (Bakker 1986; Huntingford 1976; Tinbergen 1951) to ecology and evolution (Bolnick & Lau 2008; Schluter 1994) to development (Knecht *et al.* 2007; Shapiro *et al.* 2004). The publication of the stickleback genome has moved research on this species to the forefront of the molecular genetics of adaptation and development (Jones *et al.* 2012a; Jones *et al.* 2012b; Roesti *et al.* 2015).

The ancestral form of stickleback are marine, coming in to coastal areas or freshwater to breed. Following the end of the last ice age, approximately 10,000 years ago, these marine fish repeatedly colonised novel freshwater habitats formed by the retreating ice sheet, to create a large number of isolated, independent, populations; an adaptive radiation (Bell & Foster 1994; McKinnon & Rundle 2002). Adaptations to freshwater have therefore evolved in a relatively short period of time in response to local conditions (McKinnon & Rundle 2002), in a range of traits including morphology (Colosimo *et al.* 2004; Shapiro *et al.* 2004), life history (Heins & Baker 2003; Schluter 1995) and behaviour (Boughman 2001; De Winter *et al.* 2016). One of the most conspicuous changes to occur during adaptation to freshwater is the loss of lateral ectodermal bony 'armour' plates in the freshwater form, controlled by a mutation in the *Ectodysplasin (Eda)* gene (Bell & Foster 1994; Colosimo *et al.* 2005). Marine populations are commonly homozygous for the ancestral allele,

and have a full row of 32-36 plates, whilst many freshwater populations are homozygous for the derived allele and are low plated, with less than 10 anterior plates only (Bell & Foster 1994; Jones *et al.* 2012a; Jones *et al.* 2012b). The *Eda* locus controls around 80% of observed variation in plate number (Colosimo *et al.* 2004), and has become one of the most widely studied examples of a locus controlling a major adaptive trait (For review, see Barrett 2010).

The study of ecology and evolution in stickleback has typically focussed on the impact of competition and resource availability (Matthews *et al.* 2010; Svanbäck & Bolnick 2007), and predation (Marchinko 2009; Reimchen & Nosil 2004; Vamosi 2005) in driving divergence between populations during adaptive radiation. The diverse parasite fauna of stickleback has long been recognised, and is now well characterised (Barber 2007; Chappell 1969a, b; Zander 2007), with stable differences in parasite community composition observed between host populations (Barber & Scharsack 2010; De Roij & MacColl 2012; MacColl 2009). There is between population variation in parasite resistance (De Roij *et al.* 2011; Kalbe & Kurtz 2006; Scharsack *et al.* 2007a) which probably has a genetic basis (El Nagar 2014; Rauch *et al.* 2006), and infection with common parasites can have fitness consequences (Barber & Scharsack 2010; Barber *et al.* 2008; De Roij 2010; De Roij *et al.* 2011). The fitness consequences of infection, combined with an uneven distribution of parasites between hosts with a heritable genetic basis, means that stickleback have the potential to experience parasite mediated natural selection, particularly on mechanisms involved in defence against infection.

Some research has been performed on the underlying basis of parasite resistance in stickleback, with early focus on how immune-related cell populations change over the course of infections with the common cestode

parasite *Schistocephalus solidus* (Scharsack *et al.* 2004). Parasites have been shown to be a major selective pressure on MHC diversity and evolution (Eizaguirre *et al.* 2012a, b), with MHC haplotypes conferring resistance to the local parasite populations with which they have evolved (Kurtz *et al.* 2004; Wegner *et al.* 2006). Although MHC is important in determining infection levels, the overall genetic background of an individual also plays a role (Rauch *et al.* 2006), indicating that other mechanisms are also involved.

Studies of gene expression are becoming a more common way to quantify immune phenotypes. Whole transcriptome sequencing has shown changes in expression of a wide suite of genes, some with putative immune functions, during infection with a range of parasites (El Nagar 2014; Haase *et al.* 2014; Huang *et al.* 2016). A set of reference genes for use in qPCR based studies of stickleback have been developed (Hibbeler *et al.* 2008). Assays of immune gene expression, measured using qPCR, have shown that the immune system changes with temperature (Dittmar *et al.* 2014), MHC gene expression levels change with parasitic infection (Wegner *et al.* 2006) and that stickleback can modulate their immune system in response to local conditions (Stutz *et al.* 2015). Whilst these qPCR based studies have advanced our understanding of the function of the stickleback immune system, each study relies on a very limited set of markers which generally only measure a limited set of components of an individual's immune response. There remains a lack of a set of markers which provide an overview of immune system function, development of which would allow the stickleback to move to the forefront of wild immunological research.

## 1.5 The study system: North Uist

The work in this thesis concentrates predominantly on the stickleback and their parasites found on North Uist, a small island located in the Outer Hebrides, off the west coast of Scotland (Figure 1.3). The island is covered in a large network of freshwater and saltwater lochs (the Scottish term for a lake), most of which contain stickleback. As most of the lochs are not connected, most populations are geographically isolated and evolutionarily independent. There is a high degree of environmental variation between lochs, particularly in pH, with an east to west shift from acidic to alkaline. Conductivity, temperature, and loch size and depth also vary (Magalhaes and MacColl, unpublished data). There is variation in the phytoplankton and zooplankton found in each loch, which relates to diet and feeding morphology of stickleback (Chitheer, Rahman and MacColl, unpublished data). Morphological variation between populations has long been noted (Campbell 1985; Giles 1983; Giles & Huntingford 1984), with more recent work still discussing the relative importance of calcium availability and predation in driving this variation (MacColl & Aucott 2014; MacColl *et al.* 2013; Spence *et al.* 2012).

Parasites are ubiquitous in all populations, although there is a high degree of between population variation in parasite community composition (De Roij 2010; De Roij & MacColl 2012). Fish from different populations exhibit varying levels of resistance to infection with common parasite species (De Roij 2010; De Roij *et al.* 2011; El Nagar 2014; Mahmud 2016). Changes in gene expression associated with infection have been found and immune system genes show evidence of strong selection (El Nagar 2014). The wide range of environmental variation present, across a large number of replicate

populations, combined with our understanding of host-parasite interactions from previous studies, makes this an ideal system in which to further study stickleback host-parasite interactions, particularly in the context of the function of the immune system in a natural setting.

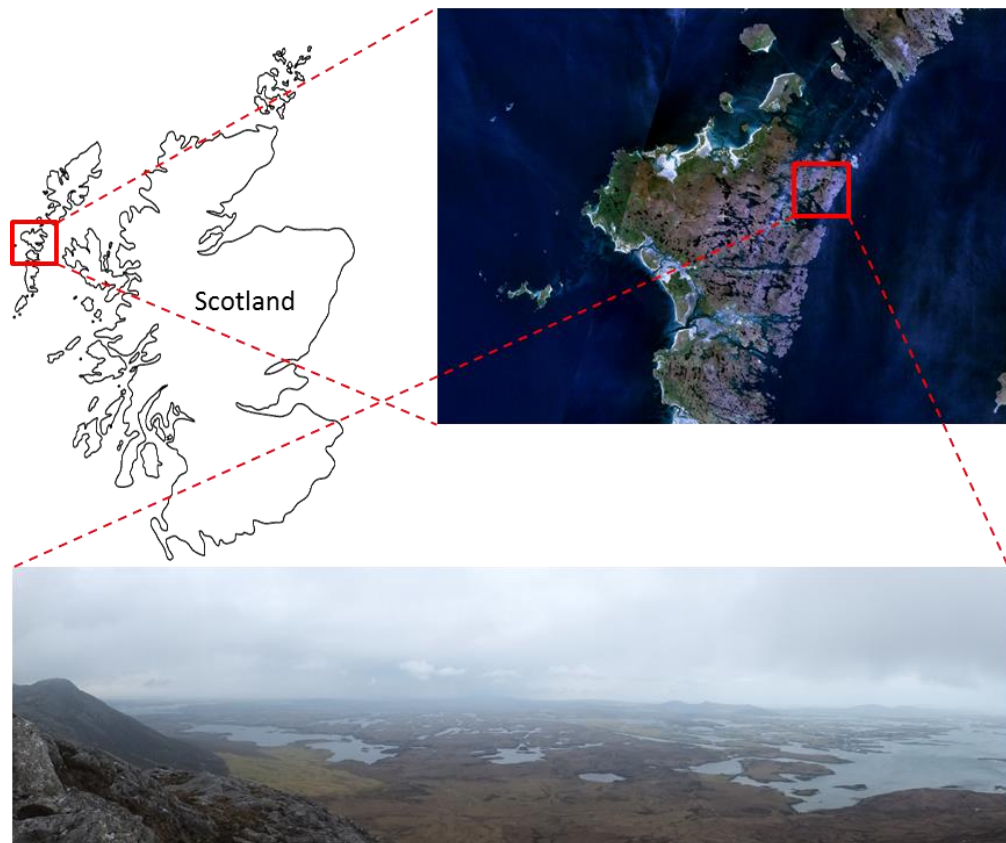


**Figure 1.3:** The study system – North Uist, Outer Hebrides, Scotland. The bottom photo shows the view looking west from the summit of Li a Tuath across the eastern side of the island. The sea is visible at the right hand edge, with connected saltwater lochs. The lochs towards the centre and left are freshwater. Scotland map obtained from [worldatlas.com](http://worldatlas.com), satellite image of North Uist from Google Maps (accessed February 2016).

## 1.6 The parasites: *Schistocephalus* and *Gyrodactylus*

Across the work in this thesis, two common stickleback macroparasites were found when surveying wild populations, *Schistocephalus solidus* and *Gyrodactylus arcuatus*, and two were used in an artificial infection experiment, *G. arcuatus* and *Gyrodactylus gasterostei*. Here, I give a brief description of the life cycle and life history of these species.

### 1.6.1 *Schistocephalus solidus*

*S. solidus* is a pseudophyllidean cestode which obligately infects stickleback as the second intermediate host (Barber & Scharsack 2010). The definitive host is any fish-eating bird ingesting an infected stickleback, where worms mature and reproduce, either sexually or asexually, in the gut. Eggs enter the water in bird faeces where they hatch into coracidia. This is a free living larval stage that is ingested by the first intermediate host, a cyclopoid copepod. In the copepod, coracidia migrate to the haemoceal where they develop into proceroids. Sticklebacks become infected by eating an infected copepod, where the proceroid migrates through the intestinal wall into the body cavity of the fish. Here they develop into plerocercoids, and remain and grow until the fish is eaten by a bird, completing the life cycle (Figure 1.4) (Barber & Scharsack 2010).

Infections in stickleback can be relatively long lived, and plerocercoids can attain a very large size in the body cavity, accounting for a large proportion of the total body weight of the host fish. Infection with *S. solidus* can have a serious negative impact on host fitness (Barber & Scharsack 2010; Barber *et al.* 2008), and there is between population variation in resistance to infection (Henrich *et al.* 2014). Changes in the immune response of infected stickleback

have been observed (Scharsack *et al.* 2004; Scharsack *et al.* 2007b), with growing evidence that the parasite can manipulate the immune response of the host to evade the immune system (For review, see Barber & Scharsack 2010).

#### **1.6.2 *Gyrodactylus arcuatus* and *Gyrodactylus gasterostei***

Parasites of the *Gyrodactylus* genus are monogenean ectoparasites of freshwater and saltwater fish. Both *G. arcuatus* and *G. gasterostei* are common, often dominant, parasites of stickleback (De Roij *et al.* 2011; Harris 1985; Raeymaekers *et al.* 2008). They vary in their geographical distribution, but have the same life cycle and a very similar mode of infection, usually attaching to the fins, gills and body. Both species are viviparous and lack a specific transmission stage. Parasites reproduce directly on the host with a very short generation time (2-4 days depending on water temperature). *Gyrodactylus* parasites give birth to a fully grown daughter, which itself already contains a developing embryo, often compared to 'Russian Dolls' (Bakke *et al.* 2007). Reproduction tends to be asexual (Harris 1998), and parasite population growth can be extremely rapid. Transmission occurs when hosts come into close physical contact.

*Gyrodactylus* parasites attach to the external surfaces where they feed on epithelial cells and mucus (Cone & Odense 1984). Infections can cause significant damage to the epidermis (Sterud *et al.* 1998) leading to host morbidity and mortality, and this damage also leaves the host open to secondary infections (Cone & Odense 1984). There are fitness consequences for the host, with infected stickleback having lower growth rates (De Roij 2010; De Roij *et al.* 2011), and a mortality rate of ~2.5% under laboratory conditions (Mahmud, Robertson & MacColl, unpublished data). Fish from different

populations vary in their resistance to infection, and parasite strains differ in their virulence (De Roij 2010; De Roij *et al.* 2011; Mahmud 2016). There is evidence from guppies that both the innate and adaptive systems are involved in resistance to infection with *Gyrodactylus* (Cable & Van Oosterhout 2007), although this study did not directly measure the immune response. Changes in expression levels of innate immune response genes with infection have been seen in rainbow trout (Lindenstrom *et al.* 2003; Lindenstrom *et al.* 2004) and Atlantic salmon (Lindenstrøm *et al.* 2006). This confirms that, even though *Gyrodactylus* are ectoparasites, they are still exposed to an immune response which is potentially adaptive for the host.

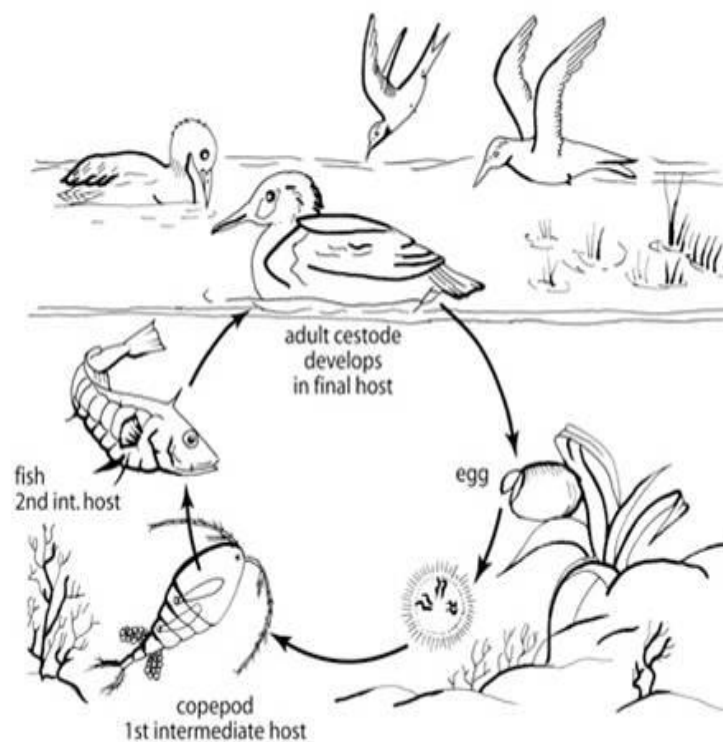


**Figure 1.4:** Lifecycle of the parasite *Schistocephalus solidus*, including the three-spined stickleback – its only obligate host. Figure from fishpathogens.net (accessed February 2016).

## **1.7 Thesis outline**

The overall aim of this project was to develop a set of qPCR markers to allow immunological studies of stickleback, and to employ these markers to examine how the vertebrate immune system functions in a natural setting. Each data chapter has been written in the style of an individual manuscript, so pertinent background information may be repeated. An outline of the contents of each chapter is given below.

### **Chapter 2 – Measuring the immune system: quantifying gene expression in the laboratory and the wild**

In this chapter, I describe the development of a set of qPCR assays to measure gene expression levels of key markers of the immune system, and an accompanying cell culture technique to measure an individual's immune response potential. I use these assays to then: 1) Compare expression profiles between wild and laboratory fish derived from the same populations, 2) Examine expression patterns across 6 wild populations, and 3) look for underlying differences in innate immune response potential.

### **Chapter 3 – Parallelism and divergence in immune responses between two lochs**

I use the newly developed assays to measure gene expression levels in two geographically distinct populations that suffer from high *S. solidus* infection rates, to examine whether: 1) infection with *S. solidus* corresponds to variation in immune gene expression levels, and 2) whether there are common factors shaping immune gene expression in these populations.

#### **Chapter 4 – Exposing naïve fish to natural challenges**

I used laboratory-raised fish to conduct a common-garden experiment, by placing them into semi-natural enclosures in a loch on North Uist. The fish were hybrids between two populations contrasting in *Gyrodactylus* resistance levels. They were left in place for eight weeks, and their immune response measured at the end. I use this experiment to examine what factors relate to immune gene expression levels in a natural setting, and look at whether immune function trades-off against growth and condition.

#### **Chapter 5 – Pleiotropic effects of the *Eda* gene are associated with immune variation**

I conducted a second common-garden experiment, again placing fish into semi-natural enclosures on North Uist. Fish were F2 hybrids between a low and a high plated population, with enclosures placed into both parental populations for four weeks. I again measured immune gene expression levels at the end of the experiment, and examined the relationship between: 1) *Eda* genotype, which controls armour plate phenotype, and the function of the immune system, 2) *Eda* genotype and parasite infection levels, and 3) *Eda* genotype and growth rates.

#### **Chapter 6 – Host-parasite adaptation and the immune response**

I conduct a factorial infection experiment in the laboratory, using lab-raised stickleback from two geographically distinct locations, North Uist and Nottingham. *Gyrodactylus* parasites were used from both host locations; *G. arcuatus* from North Uist and *G. gasterostei* from Nottingham. I measured gene expression levels to examine: 1) the type of immune response produced

during *Gyrodactylus* infection and 2) whether there is evidence of local associations in infection dynamics or the immune response.

## **Chapter 7 – General discussion**

I combine the findings from all of the data chapters, discuss general trends, and draw conclusions regarding factors which influence the function of the immune system in a natural setting. I finish by suggesting possible areas for future research.

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## Chapter 2: Measuring the immune system by quantifying gene expression in the laboratory and the wild

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### 2.1 Abstract

Current understanding of the immune system comes primarily from lab-based studies. There has been substantial interest in examining how it functions in the wild, but studies have been limited by a lack of appropriate assays and study species. The three-spined stickleback (*Gasterosteus aculeatus* L.) provides an ideal system in which to advance the study of wild immunology, but requires the development of suitable immune assays. In this chapter, I demonstrate that meaningful variation in the immune response of stickleback can be measured using real-time quantitative PCR, by quantify the expression of eight genes, representing the innate response and Th1, Th2 and regulatory type adaptive responses. Assays are validated by comparing the immune expression profiles of wild and laboratory raised stickleback, and by examining variation across populations on North Uist, Scotland. I also compare the immune response potential of laboratory raised individuals from two Icelandic populations by stimulating cells in culture. Immune profiles of wild fish differed from laboratory-raised fish from the same parental population, with immune

expression patterns in the wild converging relative to those in the laboratory. Innate measures differed between wild populations, whilst the adaptive response was associated with variation in age, relative size of fish, reproductive status and *S. solidus* infection levels. Laboratory-raised individuals from different populations showed markedly different innate immune response potential. The ability to combine studies in the laboratory and in the wild underline the potential of this toolkit to advance our understanding of the ecological and evolutionary relevance of immune system variation in a natural setting.

## **2.2 Introduction**

Current knowledge of the vertebrate immune system is based largely on studies of humans and a few select model species (Maizels & Nussey 2013). These studies are often lab-based, performed in highly controlled settings where variation is minimised, so that cellular and molecular immune mechanisms can be identified. Natural settings, however, where 'real' immune systems have to operate, are characterised by genetic diversity of both hosts and parasites, and by variation in the abiotic environment. Such diversity may play an important role in the natural function of the immune system (Lazzaro & Little 2009; Martin *et al.* 2011), but is often overlooked in purely lab-based studies. Placing our current understanding of the immunological mechanisms into a real-world context is a fundamentally important challenge in advancing our understanding of the immune system.

Studies examining immune variation in the wild have hitherto suffered from a lack of appropriate assays and model species (Fassbinder-Orth 2014). Focus to date has primarily been on a number of rodent species (For review, see

Babayan *et al.* 2011; and Pedersen & Babayan 2011) where knowledge of immune function is relatively well developed due to their close relationship to lab models, and a range of immune related assays are available. The three-spined stickleback represents a species with enormous potential for use in the study of the vertebrate immune system in a wild setting.

Sticklebacks are widely distributed across a range of habitats throughout the northern hemisphere, from the open ocean to freshwater lakes and streams, and show a high degree of both phenotypic and genetic variation across this range (Bell & Foster 1994; Colosimo *et al.* 2005; Jones *et al.* 2012a). Individuals can be sampled from a large number of replicate populations in the wild, crosses between populations are possible, and offspring can be raised in uniform laboratory conditions and controlled infection experiments performed. Sticklebacks have a well characterised parasite fauna (Barber & Scharsack 2010; De Roij & MacColl 2012; MacColl 2009), and a reference genome is also available (Jones *et al.* 2012b). This unique set of characteristics has made the stickleback an excellent model in the study of evolutionary and ecological parasitology (Barber 2013), and an ideal system in which to conduct a range of further immunological-related studies.

The sticklebacks found on the island of North Uist, Scotland, represent one well-studied system (De Roij & MacColl 2012; Giles 1983; MacColl & Aucott 2014). There is growing evidence for within and between population variation in parasite resistance (De Roij *et al.* 2011), which probably has a genetic basis (El Nagar 2014). Two common stickleback parasites found on North Uist are the ectoparasitic trematode *Gyrodactylus arcuatus* and the cestode *Schistocephalus solidus*. Individuals infected with *G. arcuatus* in controlled conditions have a 2.5% mortality rate (Mahmud, Robertson and MacColl, unpublished data), and grow slower than non-infected fish (De Roij *et al.*

2011). Infection with *S. solidus* can cause reduced egg production in females (Barber *et al.* 2008; Heins & Baker 2008), and reduced levels of 11-ketotestosterone, courtship behaviour and nest building in infected males (Barber & Scharsack 2010; Macnab *et al.* 2011; Rushbrook & Barber 2006). The impact of parasitism on host fitness has been well studied, but the link between infection and a host's immune defence is less clear.

Research has begun to examine the underlying basis of parasite resistance in stickleback, with early focus on how changes in immune-related cell types correspond to parasitic infection (Scharsack *et al.* 2004). The role of the major histocompatibility complex (MHC) in parasite resistance has been documented (Kurtz *et al.* 2004; Wegner *et al.* 2006), as has the relative importance of MHC diversity and genetic background in determining parasite load (Rauch *et al.* 2006). However, a wider overview of the immune system in stickleback is required to better understand its function in a natural setting.

Teleost fish possess components of both the innate and adaptive immune system (For review, see Magnadottir 2006; and Uribe *et al.* 2011), and may be the lowest branch of vertebrates in which these are conserved (Uribe *et al.* 2011; Whyte 2007). Inflammation at the site of infection is central to the innate response, and is non-specific to the type of challenge being faced. The adaptive response consists primarily of B cells, T cells and antigen presenting cells. T cells differentiate into sub-types (Th1, Th2, Th17 and Treg) which take longer to develop but mediate a response specific to the type of challenge being faced: Th1 cells act against intracellular pathogens; Th2 cells against extracellular metazoan parasites; Th17 cells against extracellular bacteria; and Treg cells act to suppress the other T cell sub-types. Each of these sub-types has a unique combination of signals and transcription factors associated with it (For a review in mammals, see Bradley & Jackson 2008) and these

signalling pathways appear active in teleost fish (Laing & Hansen 2011; Toda *et al.* 2011). Measuring these different components, which arise from linked immunogenetic pathways but differ in their response to infection, can give a general overview of the function of the immune system as well as indicate the nature of the challenge being faced.

Quantitative real-time PCR (hereafter 'qPCR') allows the measurement and quantification of a specific target sequence. Recent advances have made whole transcriptome sequencing more accessible, but qPCR retains the advantage of being relatively cheap, allowing a much higher level of biological replication, and primers can be designed based on *a priori* understanding of the function of the immune system (Fassbinder-Orth 2014). qPCR has been widely used in immunology to measure the expression levels of candidate immune system genes (Bertolotti *et al.* 2013; Jackson *et al.* 2011; McWilliam *et al.* 2013). Using qPCR in stickleback gives only a single time point, due to the destructive nature of sample collection. Such data can be combined with cell culture techniques, as described in Bradley and Jackson (2008), so that it is not only possible to measure the contemporary function of the immune system, but also the ability of an individual to respond to subsequent challenge. A set of qPCR reference genes are available for stickleback (Hibbeler *et al.* 2008), and some immune system assays have previously been used (Dittmar *et al.* 2014; Stutz *et al.* 2015b; Wegner *et al.* 2006). Whilst this previous body of work contains some assays, there remains a lack of a single set of markers which provide a cohesive summary of immune system function.

In this chapter, I aim to develop a set of assays which give a general overview of the function of the immune system at the time of sampling, and develop an accompanying cell culture technique to measure the potential of an individual's immune system to respond to challenge. Previous work in our

study populations has shown there to be a genetic basis to resisting parasitic infection (El Nagar 2014; Mahmud 2016), so I examine whether there are underlying differences in both immune expression profiles and innate response potentials in fish which have been raised in uniform controlled conditions. In order to show that the assays can capture relevant variation in the immune response, I compare immune expression profiles between laboratory raised and wild individuals from the same parental populations. Finally, I examine variation across six populations which contain contrasting parasite communities in order to identify factors which may contribute to shaping an individual's immune response. The toolkit presented will allow this common model species to move to the forefront of wild immunological research, and provide a base on which further measures can be added and refined over time.

## 2.3 Methods

### 2.3.1 Assay Development

I identified candidate genes representing different functional arms of the immune system based on the known roles of orthologous genes, and their presence in previous studies in other fish species. The innate immune system is characterised by the expression of *IL-1 $\beta$*  and *TNF $\alpha$* , which promote, and *IL-10* and *TGF $\beta$*  which regulate, inflammation. Th1-type responses are characterised by expression of *IL-12*, *T-bet*, *Stat4* and *IFN $\gamma$* , whilst Th2-type responses are characterised by *Gata-3*, *Stat6*, *CMIP*, and *IL-4*, -5, -9 and -13. Th17-type responses are characterised by *ROR- $\gamma$ t*, and *IL-6*, -17, -22 and -23, with the immunosuppressive Treg-type response characterised by *TGF $\beta$* ,

*FoxP3a* and *IL-10* expression (For further details, see Magnadottir 2006; Uribe *et al.* 2011; Whyte 2007).

In order to get sequences for candidate genes, the stickleback genome was accessed through ENSEMBL release 77 (Flicek *et al.* 2014). If a gene was present and named, the coding sequence was downloaded. A blastx search was performed using this sequence against the NCBI protein sequence database, to confirm that the correct gene had been selected. Where no named gene was found, the sequence of the target gene from another fish species was used to perform a tblastx search against the stickleback genome in ENSEMBL. The sequence of any unnamed stickleback gene showing high similarity was downloaded, and a blastx search performed against the NCBI protein sequence database to confirm the identity of the unnamed gene.

Three pairs of primers were designed for each gene using Primer3web v4.0.0 (Koressaar and Remm, 2007, Untergasser *et al.*, 2012), using default parameters with the following modifications: primer size 18-23 bp's, melting temperature 57°C-62°C, GC content 30%-70% and a product size of 90-150 bp's. Where possible, the product spanned an intron or one primer crossed an exon:intron boundary to avoid dsDNA amplification. A blastn search was performed against the NCBI nucleotide sequence library to ensure primer specificity to the gene of interest.

### **2.3.2 Primer Screening**

Initial screening for amplification was performed on pooled cDNA samples from multiple individuals, caught on North Uist in Spring 2013 (methods as described below), with each primer at a 2µM concentration. Primers were retained for further testing if logarithmic stage amplification occurred before 40 cycles. Efficiency tests were performed on five-fold serial dilutions of cDNA

from single individuals in triplicate replicates. Efficiency values were calculated, with a desired range between 75%-110% and an  $R^2$  value  $>0.98$ . The average efficiency of primers fulfilling these criteria was calculated across 3 separate efficiency runs. If multiple primers for each gene reached this stage the best pair was selected based on amplification plots, efficiency %, and  $R^2$  values. Primers which failed to meet the criteria were tested at a range of concentrations (2 $\mu$ M, 3 $\mu$ M, 4 $\mu$ M and 6 $\mu$ M), and retained if they met the specification at a new concentration.

### **2.3.3 Primer Validation**

The repeatability level of each primer pair was determined as the lowest cDNA concentration at which amplification was detected across triplicate replicates in the three efficiency runs. To confirm the specificity of amplification, PCR product from each primer pair was sequenced. A PCR reaction was performed on a pooled cDNA sample in a 20 $\mu$ l total volume, containing 10 $\mu$ l BioMix Red (Bioline), 6 $\mu$ l H<sub>2</sub>O, 1 $\mu$ l of each 10 $\mu$ M primer, and 2 $\mu$ l template cDNA. An initial step of 4 minutes at 95°C was followed by 35 cycles of 95°C for 15 seconds, 58°C for 15 seconds and 72°C for 30 seconds, with a final extension of 72°C for 4 minutes. Amplification was confirmed by visualization of 5 $\mu$ l of product on a 2% Agarose gel stained with Ethidium Bromide. Samples with positive amplification were Sanger sequenced in both forward and reverse orientation (Source Bioscience, Nottingham, UK). The sequences for each gene were aligned with the reference and primer sequences to ensure the correct amplicon was present.

### **2.3.4 Sample Collection**

For the examination of immune profiles in wild fish, I sampled individuals from six populations (Table 2.1) on the island of North Uist, Scotland, which contain

contrasting parasite communities, in October 2014. Fish were caught in Gee's Minnow Traps, set overnight for approximately 16 hours. All individuals caught in a population were pooled, and a subset was randomly selected for inclusion in this study. Live fish were transported directly to the laboratory in darkened boxes containing water from their population, with air from battery powered pumps, and processed in a random order within 3 hours of collection.

Fish were euthanized by overdose of MS-222 followed by destruction of the brain, in accordance with UK Home Office regulations, then measured and weighed. Their spleens, an immunologically important tissue in fish (Zapata *et al.* 2006), were removed and immediately placed in RNAlater (Life Technologies). Spleen samples were kept at 4°C for 24 hours, then at -20°C for no more than 6 months until RNA extraction. Sex was recorded along with reproductive status, defined as whether there was evidence of investment in attaining reproductive condition, determined by visual examination of the gonads. Males were deemed to be investing in reproduction if their testes and/or kidneys were enlarged, whilst females were deemed to be reproductive if any ovules were enlarged.

Macroparasites were identified and counted under a dissection microscope. Where individuals were infected with *S. solidus*, the total weight of all plerocercoids (the life stage infecting stickleback) was recorded, and the parasite index calculated as the total proportion of a fish's body weight accounted for by the parasite (Arme & Owen 1967). An individual's condition was calculated using Fulton's condition factor,  $K = 10^5 \times \text{Weight (g)} / \text{Length (mm)}^3$  (Cited in Nash *et al.* 2006), with a high K value indicating good condition. Calculations for individuals infected with *S. solidus* were adjusted by using individual weight minus the total weight of all plerocercoids. Age of fish

was determined by examination of the annuli in sagittal otoliths following the protocol of Jones and Hynes (1950).

To create families of laboratory raised fish, adults in breeding condition were caught in two lochs, Reiv and Obse (Table 2.1) in Spring 2013. Fish were crossed to create families following the protocol in Leinonen *et al.* (2011). Fertilized eggs were transported to an aquarium at the University of Nottingham, and after hatching, families were raised in 25L partitions of 100L tanks under uniform environmental conditions. Single fish from each of six families from Reiv and six families from Obse were euthanized when 11 months old, using the appropriate schedule 1 technique according to UK Home Office regulations. Their spleens were removed and immediately placed in RNAlater (Life Technologies) and kept at 4°C for 24 hours, then stored at -20°C for no longer than 6 months until RNA extraction was performed.

To compare innate immune responsiveness between populations, adults in breeding condition were caught from Holsvatn (64°04'N 21°52'W, hereafter 'Hols') and Vífilstaðavatn (54°31'N 22°08'W, 'Vifi') in Iceland in June 2014. Crosses were made between adults in breeding condition to create families following the approach of Leinonen *et al.* (2011). Fertilized eggs were transported to the University of Nottingham and raised as above. Single individuals from each of 17 families from Hols and 20 families from Vifi were euthanized in March 2015, using the appropriate schedule 1 technique according to UK Home Office regulations. Head kidneys, an important immune tissue in fish (Zapata *et al.* 2006), were removed and immediately stored in ice cold RPMI 1640 supplemented with 10% H<sub>2</sub>O for use in cell culture.

| Loch             | Short Name | Location              | n  | Gyrodactylus Prevalence | Gyrodactylus Intensity | Schistocephalus Prevalence | Schistocephalus Intensity |
|------------------|------------|-----------------------|----|-------------------------|------------------------|----------------------------|---------------------------|
| a'Bharpa         | BHAR       | 57°34'20"N; 7°18'11"W | 15 | 0 (0-21.8)              | 0 (0)                  | 60 (32.3-83.7)             | 0.191 (0.024)             |
| Hosta            | HOST       | 57°37'30"N; 7°29'8"W  | 16 | 68.8 (41.3-88.9)        | 2.55 (0.53)            | 43.8 (13.8-70.1)           | 0.054 (0.026)             |
| nam Magarlan     | MAGA       | 57°36'15"N; 7°28'51"W | 15 | 53.3 (26.6-78.7)        | 3.38 (0.68)            | 20 (4.3-48.1)              | 0.136 (0.070)             |
| unnamed          | MUAY       | 57°36'23"N; 7°10'38"W | 15 | 40 (16.3-67.7)          | 1.83 (0.40)            | 0 (0-21.8)                 | 0 (0)                     |
| Ob nan Stearnain | OBSE       | 57°36'9"N; 7°10'19"W  | 15 | 46.7 (21.3-73.4)        | 1.43 (0.20)            | 0 (0-21.8)                 | 0 (0)                     |
| Reivil           | REIV       | 57°36'38"N; 7°30'49"W | 16 | 56.3 (33.3-80.3)        | 2.44 (0.41)            | 0 (0-20.6)                 | 0 (0)                     |

**Table 2.1:** North Uist wild stickleback populations sampled in October 2014, including prevalence and intensity of the two main parasite species found. 'n' indicated the number of individuals sampled. Prevalence was calculated as the percentage of individuals infected, with 95% binomial confidence intervals in brackets. Intensity data are mean number of parasites on infected individuals, with standard errors.

| Gene Name    | ENSEMBL ID         | Primer Sequence (5' - 3')                                  | Amplicon Length | Working Conc | PCR Eff | Repeatability Level |
|--------------|--------------------|------------------------------------------------------------|-----------------|--------------|---------|---------------------|
| IL-1 $\beta$ | ENSGACG00000014611 | Fwd-ACGGCTCGAGTTGCTGAT<br>Rev-CTGCACAGCGTCACGATCTC         | 100             | 2            | 85      | 0.4                 |
| TNF $\alpha$ | ENSGACG00000013372 | Fwd-GCTTGGTTCTGCCAGGTTT<br>Rev-GCTGCTGATTTGCCCTCAACG       | 125             | 2            | 87.9    | 0.4                 |
| TGF $\beta$  | ENSGACG00000012798 | Fwd-TCCCGCTTCGTCAACAACA<br>Rev-ACGTCTGTCTGGCCACATTAC       | 140             | 2            | 96.5    | 0.08                |
| Stat4        | ENSGACG00000002684 | Fwd-CTCTCAGTTTCGAGGCTTGCTT<br>Rev-GGCAGTTGGCTCACATTGG      | 100             | 2            | 93.5    | 0.016               |
| T-bet        | ENSGACG00000003829 | Fwd-CACATCGTGGAGGTGAAGGA<br>Rev-CGGTGACGGCGATGAACT         | 99              | 4            | 79.8    | 0.08                |
| Stat6        | ENSGACG00000008477 | Fwd-CTCAGCCACAGTTCACACCGTTC<br>Rev-GTCGGATGTTCTGGACCTCGAGT | 104             | 3            | 100.9   | 0.08                |
| CMIP         | ENSGACG00000002527 | Fwd-GGCATGGAGGTCGTCAAGAA<br>Rev-TAGCAGGAGTAAATGGCGGC       | 119             | 3            | 100.6   | 0.08                |
| FoxP3a       | ENSGACG00000012777 | Fwd-GTTGACCCATGCAATTCCGA<br>Rev-CTGCTGTAGTTGTGTCCTG        | 94              | 2            | 98.9    | 0.4                 |

**Table 2.2:** Primer details for 8 immune system assays in three-spined sticklebacks, including working concentrations and average PCR efficiency values. 'ENSEMBL ID' data gives the gene on which primer designs were based. 'Amplicon Length' indicates the length of product in base pairs. 'Working Conc' gives the working concentration of each primer in  $\mu$ M, with 0.25 $\mu$ l each added to a 10 $\mu$ l total reaction volume. 'PCR Eff' gives the efficiency of amplification for each primer pair, in %, averaged over 3 runs with  $R^2 > 0.98$ . 'Repeatability level' indicates the lowest total cDNA concentration, in ng/ $\mu$ l, at which amplification was consistently detected across triplicate replicates in three efficiency runs.

### **2.3.5 Primary Culture of Stickleback Cells**

A full step-by-step cell culture protocol can be found in appendix 1, adapted from the protocol of Hibbeler *et al.* (2008). Briefly, head kidneys were disaggregated through a 40µm cell strainer into RPMI 1640 supplemented with 10% H<sub>2</sub>O. Cells were washed and four replicates per fish were cultured (20°C, 5% CO<sub>2</sub>) on 96 well plates at  $2 \times 10^6$  cells/ml in 100µl of culture media, containing: RPMI 1640 supplemented with 10% H<sub>2</sub>O; 10% heat-inactivated foetal calf serum; 2mM L-glutamine; 100µg/ml penicillin; and 100µg/ml streptomycin. Two replicates per fish served as a control whilst the others were stimulated with 12.5µg/ml zymosan (Sigma-Aldrich Z4250), a general agonist of the innate immune response. After 24 hours, cells were removed from culture. The supernatant was removed and the remaining cells were stored at -80°C until RNA extraction was performed.

### **2.3.6 Sample Preparation and Reaction Conditions**

All qPCR work was performed in accordance with the MIQE guidelines (Bustin *et al.* 2009; Taylor *et al.* 2010). RNA was extracted from whole spleens using the GeneJET RNA purification kit (Thermo Scientific) according to the manufacturers' protocol. Extractions from cultured cells were performed using the RNAqueous-Micro total RNA isolation kit (Ambion AM1931) following the manufacturers' protocol.

Purity of RNA was assessed on a NanoDrop 1000 spectrophotometer (Thermo Scientific), with a desired 260/280 absorbance ratio >1.80. RNA integrity was assessed by incubating 5µl of each sample at 65°C for 10 minutes, followed by visualisation on a 2% Agarose gel stained with Ethidium Bromide. All samples were DNase treated using Precision DNase (Primer Design), following the manufacturers' protocol. Reverse transcription reactions

were performed on approximately 1.5µg of total RNA using the nanoScript2 RT kit (Primer Design), with a combination of random nanomer and oligo-dT priming, following the manufacturers' protocol. Periodic no-enzyme controls indicated that genomic DNA contamination was negligible. All cDNA samples were diluted 1:10 with nuclease free water before further use.

All qPCR reactions were performed in 10µl total volumes, containing 5µl of PrecisionFAST mastermix with SYBR green (Primer Design), 0.25µl of each primer at working concentration (see Table 2.2), 2µl of template cDNA and 2.5µl of H<sub>2</sub>O in 96-well optical PCR plates with optical seals (StarLab). Reactions were performed in an ABI 7500 FAST real-time thermocycler (Applied Biosystems) at 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All runs included a post-PCR melt curve analysis.

### **2.3.7 Gene Expression Quantification**

Accurate normalization is essential for the production of reliable data from qPCR experiments, with the optimal reference genes being specific to a particular set of experimental conditions (Dheda *et al.* 2005). In order to select the most appropriate reference genes for this study, a geNorm analysis was performed on six candidate reference genes (*B2M*, *GAPDH*, *RPL13A*, *HPRT1*, *TBP* and *TOP1*) using a custom stickleback geNorm kit for SYBR green (Primer Design), following the manufacturers standard protocol. For the wild expression study, 12 spleen cDNA samples from wild fish were included, comprising two randomly selected individuals from each study population. Analysis of the stability of expression was performed in qbase+ (Biogazelle), with *B2M* and *RPL13A* identified as the most stable combination of reference genes. *TBP* and *HPRT1* were identified as the optimum combination for the

cell culture samples, after stability analysis on 7 random samples from Hols and 7 from Vifi.

To quantify immune gene expression in wild fish, a total of 92 samples were used. Numbers contributed by each population are shown in Table 2.1, plus 12 samples from laboratory raised control fish. A reference sample was made by pooling cDNA from all experimental samples, to control for between plate variation. Samples were randomly split between plates, with each plate also containing the reference sample and negative controls. Reactions were performed in duplicate for each sample.

37 cell culture cDNA samples were included for expression measurements of *IL-1 $\beta$* , *TNF $\alpha$*  and two reference genes. A reference sample was made by pooling cDNA from all samples. Reactions were performed in duplicate for each sample, and all plates included the reference sample and negative controls.

Relative expression values were calculated using the  $\Delta\Delta C_q$  method (Pfaffl 2001), adjusted for the amplification efficiencies of each primer pair and standardised against the geometric mean  $C_q$  of the two reference genes for each sample (Vandesompele *et al.* 2002). For cell culture data, the relative increase in expression in zymosan stimulated versus unstimulated control cells was calculated for both genes.

| Short Name | Full Name                                                          | Response          | Sequence | ENSEMBL Gene ID      | Amplification | Assay |
|------------|--------------------------------------------------------------------|-------------------|----------|----------------------|---------------|-------|
| IL-1B      | Interleukin 1 Beta                                                 | proinflammatory   | Full     | ENSGACG000000014611  | 1             | 1     |
| TNFa       | Tumour Necrosis Factor Alpha                                       | proinflammatory   | Full     | ENSGACG000000013372  | 1             | 1     |
| NFkB       | Nuclear Factor of Kappa Light Polypeptide gene enhancer in B cells | proinflammatory   | Full**   | ENSGACG000000016876  | 1             | 0     |
| IL-10      | Interleukin 10                                                     | Treg / regulatory | Partial  |                      | 0             | 0     |
| TGFB       | Transforming Growth Factor Beta                                    | Treg / regulatory | Full     | ENSGACG000000012798  | 1             | 1     |
| IFNy       | Interferon Gamma                                                   | Th1               | Partial  |                      | 1             | 0     |
| IL-12      | Interleukin 12                                                     | Th1               | Full***  | ENSGACG000000020455  | 0             | 0     |
| IL-18      | Interleukin 18                                                     | Th1               | No       |                      |               |       |
| Stat4      | Signal Transducer and Activator of Transcription 4                 | Th1               | Full     | ENSGACG000000002684  | 1             | 1     |
| T-bet      | T box transcription Factor                                         | Th1               | Full     | ENSGACG000000003829  | 1             | 1     |
| IL-4       | Interleukin 4                                                      | Th2               | No       |                      |               |       |
| IL-5       | Interleukin 5                                                      | Th2               | No       |                      |               |       |
| IL-9       | Interleukin 9                                                      | Th2               | No       |                      |               |       |
| IL-13      | Interleukin 13                                                     | Th2               | No       |                      |               |       |
| Stat6      | Signal Transducer and Activator of Transcription 6                 | Th2               | Full     | ENSGACG000000008477  | 1             | 1     |
| CMIP       | C-maf Inducing Protein                                             | Th2               | Full     | ENSGACG000000002527  | 1             | 1     |
| Gata3      | Trans-acting T-cell-specific Transcription Factor GATA-3           | Th2               | Partial  |                      | 0             | 0     |
| IL-17      | Interleukin 17                                                     | Th17              | No       |                      |               |       |
| IL-22      | Interleukin 22                                                     | Th17              | Partial  |                      | 0             | 0     |
| IL-6       | Interleukin 6                                                      | Th17              | Full     | ENSGACG000000006102  | 1             | 0     |
| ROR-γt     | RAR-related Orphan Receptor Gamma t                                | Th17              | Full     | ENSGACG0000000012239 | 1             | 0     |
| IL-10      | Interleukin 10                                                     | Treg / regulatory | Partial  |                      | 0             | 0     |
| TGFB       | Transforming Growth Factor Beta                                    | Treg / regulatory | Full     | ENSGACG0000000012798 | 1             | 1     |
| FoxP3a     | Forhead Box P3a                                                    | Treg / regulatory | Full     | ENSGACG0000000012777 | 1             | 1     |

**Table 2.3:** See over for legend.

**Table 2.3:** Summary of immune system genes targeted during primer design and development. 'Short Name' and 'Full Name' refer to names used for genes in the ENSEMBL genome database. 'Response' indicated the arm of the immune response for which each gene is a marker. 'Sequence' denotes whether the gene was found in the stickleback genome, with 'Full' genes being named in the genome, and 'Partial' being found using homology to the gene sequence from another fish species, with corresponding ENSEMBL Gene ID numbers. 'Amplification' showed whether any primers amplified for a given gene, whilst 'Assay' indicated whether a fully working assay could be produced. '\*\*' denotes a gene where 2 copies were present in the genome, and '\*\*\*' a gene where three copies were present.

|                | Reiv Lab v Reiv Wild |      |                  | Obse Lab v Obse Wild |      |                  | Reiv Lab v Obse Lab |      |                  |
|----------------|----------------------|------|------------------|----------------------|------|------------------|---------------------|------|------------------|
|                | F                    | df   | p                | F                    | df   | p                | F                   | df   | p                |
| <b>Overall</b> | 11.31                | 20,1 | <b>&lt;0.001</b> | 4.40                 | 19,1 | <b>0.011</b>     | 16.92               | 10,1 | <b>0.020</b>     |
| IL1B           | 6.51                 | 20,1 | <b>0.019</b>     | 0.72                 | 19,1 | 0.406            | 28.47               | 10,1 | <b>&lt;0.001</b> |
| TNFa           | 13.13                | 20,1 | <b>0.002</b>     | 2.30                 | 19,1 | 0.146            | 10.95               | 10,1 | <b>0.008</b>     |
| Stat4          | 17.38                | 20,1 | <b>&lt;0.001</b> | 15.67                | 19,1 | <b>&lt;0.001</b> | 0.04                | 10,1 | 0.855            |
| Tbet           | 73.45                | 20,1 | <b>&lt;0.001</b> | 19.70                | 19,1 | <b>&lt;0.001</b> | 0.01                | 10,1 | 0.919            |
| Stat6          | 3.81                 | 20,1 | 0.066            | 0.00                 | 19,1 | 0.991            | 10.22               | 10,1 | <b>0.009</b>     |
| CMIP           | 24.23                | 20,1 | <b>&lt;0.001</b> | 3.52                 | 19,1 | 0.076            | 21.71               | 10,1 | <b>&lt;0.001</b> |
| TGFB           | 4.09                 | 20,1 | 0.057            | 0.33                 | 19,1 | 0.572            | 14.49               | 10,1 | <b>0.003</b>     |
| FoxP3          | 14.61                | 20,1 | <b>0.001</b>     | 0.67                 | 19,1 | 0.422            | 12.73               | 10,1 | <b>0.005</b>     |

**Table 2.4:** Summary of pairwise comparisons of immune gene expression levels of 8 genes between wild caught and lab bred fish from two different populations (Reiv and Obse), and between lab bred fish only, using multivariate analysis of variance (MANOVA). Overall differences are shown, along with individual comparisons for each gene, calculated using the Pillai method. Significant p values (p<0.05) are indicated in bold.

### 2.3.8 Data Analysis

All relative expression values were  $\log_{10}$ -transformed prior to analysis, due the inherently skewed distribution of such data. All analysis was performed in R v.3.1.2 (R Core Team 2014).

*Lab vs Wild Comparisons* - In the first instance, I compared expression profiles of the eight immune genes between wild and lab-raised fish for the pairs of populations (Reiv and Obse) for which both were available. Multivariate analysis of variance (MANOVA) was used to test for differences between Reiv and Obse lab bred fish, Reiv lab and wild fish, and Obse lab and wild fish. Overall differences were assessed using the Pillai method, followed by examination of each gene separately. To simultaneously summarise the expression profiles of the groups in multivariate space, a principal components analysis (PCA) was performed on immune expression measures for all groups, using the 'prcomp' function on the variance-covariance matrix, and individual scores for the first and second principal components were plotted. To compare the separation of each group in multivariate space, the average Euclidean distance ( $\pm$ SE) was calculated between each group, based on the expression values of all immune genes.

*Expression Profiles in Wild Populations* - In order to examine which factors related to the expression of immune genes in the sampled wild populations, PCA was followed by the fitting of general linear models containing recorded factors which were expected to influence gene expression measurements. PCA is a widely used data reduction technique which converts a number of positively correlated variables into a smaller set of principal components which summarise the variation present in the original measures. In this way, multiple expression measurements of functionally related components of the immune system can be summarised by a single measure, composed of the individual

scores from a dominant first principal component (PC1), following the approach previously employed by Jackson *et al.* (2011). Measures were split into four functional groups based on their role: *IL-1 $\beta$*  and *TNFA* (innate); *Stat4* and *T-bet* (Th1-like); *Stat6* and *CMIP* (Th2-like), and *FoxP3a* and *TGF $\beta$*  (immunosuppressive).

PCA was performed using the 'prcomp' function, based on the variance-covariance matrix, with individual PC1 scores then used in subsequent analysis. *FoxP3a* and *TGF $\beta$*  expression was not positively correlated, so they were subsequently analysed separately. For other PCA's, the first component explained a high proportion of variation in the data (>60%) and both factors contributed positively to the loadings. To ensure that the time which fish were held before processing and the order in which they were processed did not affect the expression of immune system genes, the correlation between sampling order and each of the immune groupings was calculated. Values were calculated using the 'corr.test' function of the 'psych' package, with the Holm correction applied for multiple comparisons.

General linear models (glm) were fitted using PC1 score, or *FoxP3a* and *TGF $\beta$*  expression measures, as the response variable. Maximal models contained population (6 levels), sex (2 levels), reproductive status (2 levels) and *G. arcuatus* presence (2 levels) as factors, and adjusted length (calculated as individual length minus population average length), *S. solidus* parasite index, age, and condition score as continuous variables. Biologically relevant interaction terms were also fitted. Non-significant terms were sequentially dropped to generate a minimum adequate model. Significance of remaining components was determined by Wald F-tests.

*G. arcuatus* and *S. solidus* were the dominant parasite species found across populations, so the only parasites included in the full models. In one population, Obse, lice of the family Argulidae were found in high prevalence. In order to investigate their effect within the population, a model was fitted to Obse Innate, Th1, Th2, *FoxP3a*, and *TGFβ* measures separately, with the addition of Lice Count as a continuous variable replacing the *S. solidus* Parasite Index term and interactions.

*In Vitro Response Potential* - The difference in response potential of fish from Hols and Vifi was examined by comparing the change in expression of *IL-1β* and *TNFα* in stimulated versus control cultured cells. The distribution of expression values for both genes was not normal (Shapiro-Wilk test, *IL-1β*  $W=0.492$ ,  $p<0.001$ ; *TNFα*  $W=0.529$ ,  $p<0.001$ ), so the difference in responsiveness between populations for each gene was compared using Mann-Whitney U tests.

## 2.4 Results

### 2.4.1 Assay Development

Initially 22 potential candidate genes were identified, of which 16 were present in the stickleback genome. Primers for 12 genes showed consistent amplification across a number of cDNA samples (for full details of targeted genes, see Table 2.3). 8 genes met the required amplification criteria, with *IL-1β*, *TNFα*, *TGFβ*, *Stat4* and *FoxP3a* optimised at 2μM, and *Stat6*, *CMIP* and *T-bet* optimised at higher concentrations (Table 2.2). Primer efficiencies ranged from 79.8% to 100.9%, with repeatability levels of 0.4ng/μl to 0.016ng/μl of template cDNA. For each primer pair, PCR product was successfully sequenced and aligned back to the expected location, with blastx

searches against the NCBI protein sequence database further confirming that the intended targets were amplified.

#### 2.4.2 Sampling Wild Populations

*G. arcuatus* and *S. solidus* were found to be the dominant parasites across the wild populations sampled (Table 2.1). The prevalence of *G. arcuatus* differed significantly between populations ( $\chi^2_5=17.4$ ,  $p=0.004$ ), as did the prevalence of *S. solidus* ( $\chi^2_5=31.4$ ,  $p<0.001$ ). Lice of the Argulidae family were found only in Obse, with a prevalence of 86.7% (95% binomial confidence interval 59.5%-98.3%), and an average intensity on infected individuals of 2.92 (SE $\pm$ 0.84). No other parasite species were identified in large numbers. All populations contained both male and female fish, with individuals of both sexes varying in their reproductive condition.

#### 2.4.3 Lab vs Wild Comparisons

There was a significant difference in the immune profiles of lab raised fish from Reiv and Obse (MANOVA  $F_{1,10}=16.92$ ,  $p=0.020$ ), primarily driven by differences in innate, Th2-type and immunosuppressive measures. Wild fish from Reiv differed from Reiv laboratory fish (MANOVA  $F_{1,20}=11.31$ ,  $p<0.001$ ), with wild fish having higher expression of all genes. Wild fish from Obse also differed from Obse laboratory fish (MANOVA  $F_{1,19}=4.40$ ,  $p=0.011$ ), primarily driven by higher expression of Th1-type genes in wild fish. Full details of comparisons are shown in Table 2.4. PCA on all groups found a PC1 which explained 81.4% of variation in expression, with a high PC1 score indicating high expression of all immune genes. The PC2 axis explained 10.8% of variation, with a high score indicating high expression of *IL-1 $\beta$*  and *TNF $\alpha$* , and low expression of *Stat4* and *Tbet*. Laboratory-raised fish were widely separated in the PCA (Figure 2.1, average Euclidean distance  $1.797\pm0.076$ ),

whilst wild fish were not ( $0.534 \pm 0.018$ ). Obse lab and wild fish were closer in expression profile ( $1.701 \pm 0.054$ ) than Reiv lab and wild fish ( $2.601 \pm 0.039$ ).

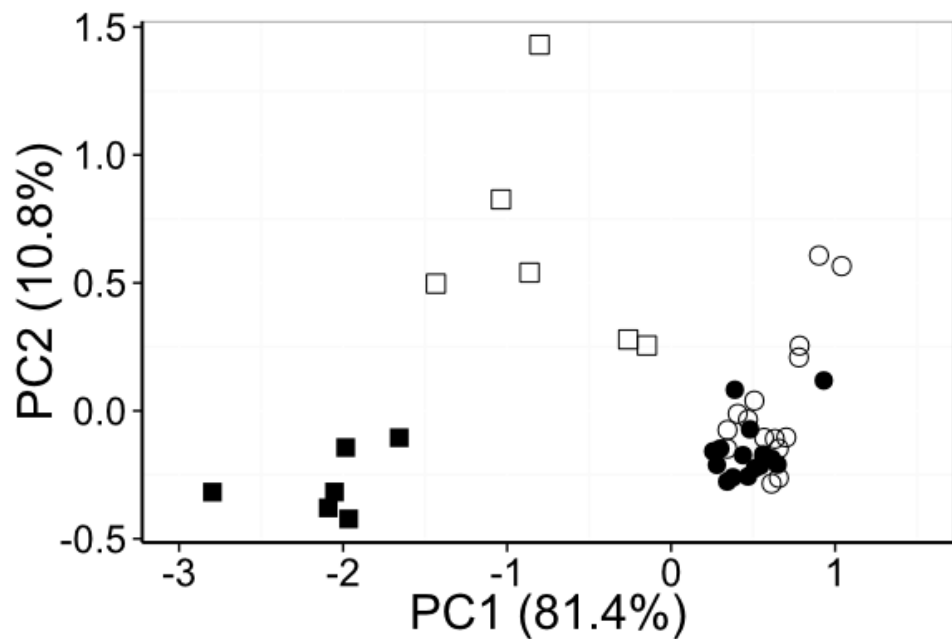


**Figure 2.1:** Laboratory raised and wild fish from two populations ( $\square$  – Obse Lab,  $\circ$  – Obse Wild,  $\blacksquare$  – Reiv Lab,  $\bullet$  – Reiv Wild) are split by expression levels of eight immune genes on the first two principal component axes (PC1 and PC2) from a Principal Components Analysis. Numbers in brackets indicate the percentage of variation in the original measures explained by each PC axis.

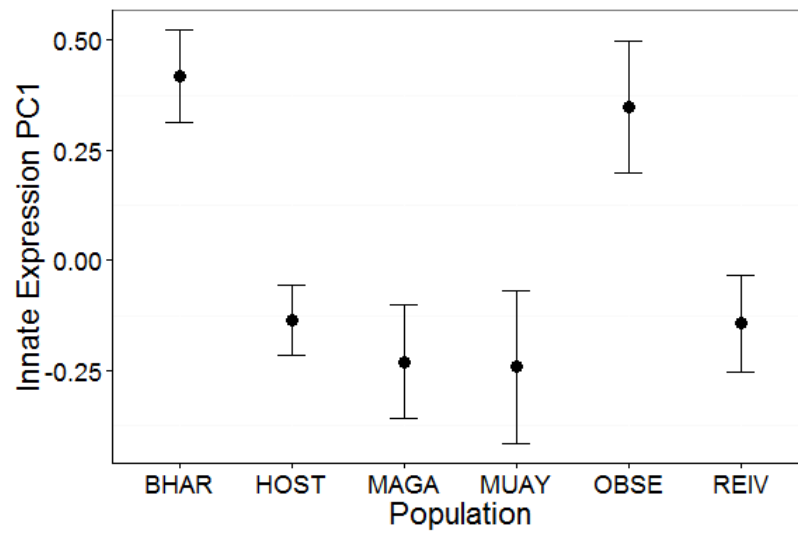


**Figure 2.2:** Innate immune expression scores (Mean PC1  $\pm$  S.E.) for each study population. A high PC1 score indicates high expression levels of IL-1 $\beta$  and TNF $\alpha$ .

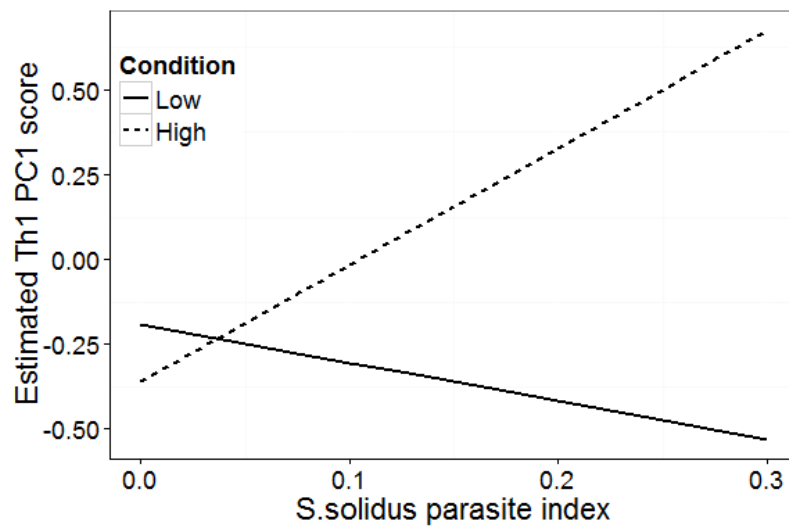


**Figure 2.3:** The effect of *S. solidus* parasite index on Th1 PC1 score is dependent upon condition. A high Th1 PC1 score indicates high expression levels of Stat4 and T-bet. Lines are estimates calculated from the fitted model, for the lowest ('Low', K=0.75) and highest ('High', K=1.40) Fulton's condition scores found in the sampled individuals.

#### 2.4.4 Expression Profiles in Wild Populations

No significant correlations were found between sampling order and innate expression ( $r^2=-0.28$ ,  $p=0.09$ ), Th1-type expression ( $r^2=0.11$ ,  $p=1$ ), Th2-type expression ( $r^2=0.12$ ,  $p=1$ ), *FoxP3a* expression ( $r^2=0.07$ ,  $p=1$ ), and *TGF $\beta$*  expression ( $r^2=-0.11$ ,  $p=1$ ), indicating that sampling order did not influence the expression levels of measured genes.

A summary of significant components in fitted models is shown in Table 2.5. Across all populations of wild fish, PC1 of innate measures explained 86.4% of variation in the data, with loadings of *IL-1 $\beta$* =0.987 and *TNF $\alpha$* =0.157. A high PC1 score indicates a high level of expression, with populations differing in their expression (Figure 2.2,  $F_{5,85}=4.97$ ,  $p=0.002$ ).

The Th1-type measures had a PC1 accounting for 62.4% of variation, with loadings of *Stat4*=0.802 and *Tbet*=0.597. Longer fish in each population had higher expression levels ( $F_{1,87}=7.19$ ,  $p=0.009$ ). An increasing *S. solidus* parasite index was associated with a decrease in expression ( $F_{1,87}=6.22$ ,  $p=0.015$ ), but this affect also varied with condition (Figure 2.3,  $F_{1,87}=5.18$ ,  $p=0.025$ ), such that in uninfected fish expression decreased with increasing condition, but in heavily infected fish expression increased with condition. PC1 of Th2-type measures explained 83% of variation, with loadings of *CMIP*=0.870 and *Stat6*=0.241. Individuals in their third year (2+) had lower Th2-like expression levels than those in their first (0+) or second (1+) ( $F_{2,84}=3.75$ ,  $p=0.028$ ). This affect also varied with condition ( $F_{2,84}=3.55$ ,  $p=0.033$ ), with individuals in good condition having lower expression when 0+ and 1+, but higher expression when 2+.

| System | Factor                                  | F     | df   | p            |
|--------|-----------------------------------------|-------|------|--------------|
| Innate | Population                              | 4.97  | 5,85 | <b>0.002</b> |
| Th1    | Adjusted Length                         | 7.19  | 1,87 | <b>0.009</b> |
|        | <i>S. solidus</i> Index                 | 6.22  | 1,87 | <b>0.015</b> |
|        | Condition (K)                           | 3.89  | 1,87 | 0.052        |
|        | Condition (K) : <i>S. solidus</i> index | 5.18  | 1,87 | <b>0.025</b> |
| Th2    | Age                                     | 3.75  | 2,84 | <b>0.028</b> |
|        | Condition (K)                           | 2.31  | 1,86 | 0.132        |
|        | Age : Condition (K)                     | 3.55  | 2,84 | <b>0.033</b> |
| FoxP3a | Adjusted Length                         | 1.17  | 1,84 | 0.283        |
|        | Repro. Score                            | 0.01  | 1,81 | 0.912        |
|        | Age                                     | 0.44  | 2,81 | 0.648        |
|        | <i>S. solidus</i> Index                 | 0.01  | 1,84 | 0.905        |
|        | Adjusted Length : Age                   | 5.42  | 2,81 | <b>0.006</b> |
|        | Repro. Score : <i>S. solidus</i> Index  | 6.24  | 1,81 | <b>0.015</b> |
| TGFβ   | <i>S. solidus</i> Index                 | 10.25 | 1,90 | <b>0.002</b> |

**Table 2.5:** Summaries of minimum adequate GLMs fitted to expression data of genes representing different functional arms of the immune system from wild fish caught on North Uist, Scotland. ‘Innate’, ‘Th1’ and ‘Th2’ response variables are first principal components of the following pairs of genes respectively: *IL1β* and *TNFα*; *Stat4* and *Tbet*; *Stat6* and *CMIP*.

Adjusted length had an effect on *FoxP3a* expression which was dependent upon age (Figure 2.4,  $F_{2,81}=5.42$ ,  $p=0.006$ ), with relatively large individuals having higher expression when 0+, but lower expression when 1+. There was an effect of *S. solidus* parasite index dependent upon reproductive condition ( $F_{1,81}=6.24$ ,  $p=0.015$ ), with no effect of *S. solidus* parasite index in reproductive individuals, but a decrease in expression with increasing parasite

index in non-reproductive individuals. *TGFβ* expression levels increased with increasing *S. solidus* parasite index ( $F_{1,89}=9.84$ ,  $p=0.002$ ).

When Obse data were analysed separately, no factors were found to be significant in models for Th1-like or Th2-type expression. Innate measures were summarised by a PC1 accounting for 78.7% of variation, with contributions of  $IL-1\beta=0.999$  and  $TNF\alpha=0.019$ . Males were found to have higher expression levels than females ( $F_{1,7}=6.414$ ,  $p=0.039$ ), and expression increased with condition ( $F_{1,7}=10.463$ ,  $p=0.014$ ). Presence of *G. arcuatus* was associated with an increase in expression ( $F_{1,7}=6.101$ ,  $p=0.042$ ), whilst increasing lice numbers were associated with a decrease ( $F_{1,7}=8.366$ ,  $p=0.023$ ). The relationship between size and expression varied with *G. arcuatus* presence ( $F_{1,7}=15.594$ ,  $p=0.006$ ), with uninfected fish showing a decrease in expression with increasing length, and infected fish showing an increase. The effect of size also varied with lice number ( $F_{1,7}=12.376$ ,  $p=0.010$ ), with an increase in length corresponding to a more negative relationship with expression when lice number increased. Individuals infected with *G. arcuatus* had lower *FoxP3a* expression levels ( $F_{2,13}=6.324$ ,  $p=0.012$ ). An increase in lice was associated with an increase in *TGFβ* expression levels ( $F_{1,11}=4.89$ ,  $p=0.049$ ), and this affect was also dependent upon age ( $F_{1,11}=5.75$ ,  $p=0.035$ ), with less of an increase seen in older individuals.

#### **2.4.5 In Vitro Response Potential**

Innate immune response potential, measured as the increase in gene expression in stimulated versus unstimulated cultured cells, was higher in individuals from Hols than those from Vifi (Figure 2.5) for both *IL-1β* ( $W=208$ ,  $p=0.009$ ) and *TNFα* ( $W=138$ ,  $p=0.018$ ). The average increase in relative expression of *IL-1β* was 4.81 (SEM±1.92) in Hols, but only 0.37 (±0.13) in Vifi.

The increase in relative expression of *TNF $\alpha$*  was 7.13 ( $\pm 3.34$ ) in Hols and 0.40 ( $\pm 0.33$ ) in Vifi.

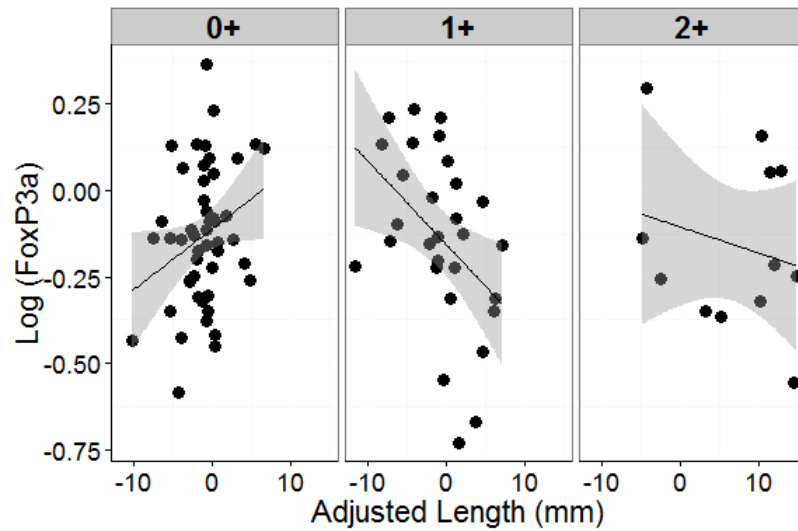


**Figure 2.4:** The relationship between relative size and *FoxP3a* expression level changes with age. Each box contains fish of a different age (0+,1+, or 2+), and shaded areas indicate 95% confidence levels of fitted lines. Patterns were significant at 0+ and 1+ but not at 2+.

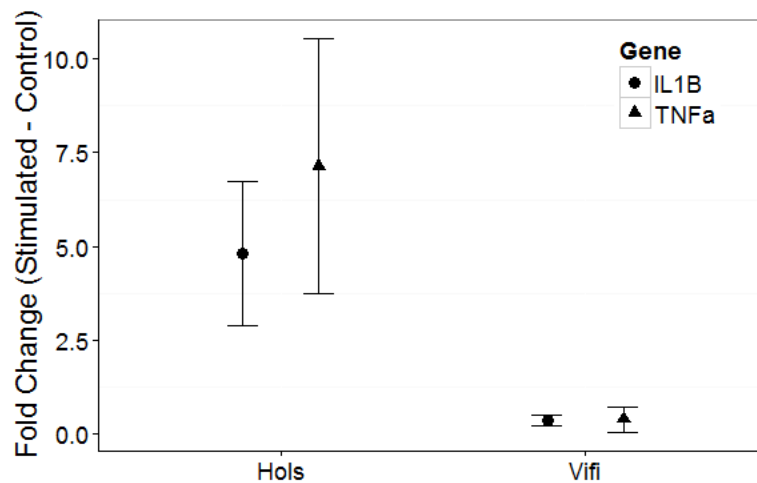


**Figure 2.5:** The relative fold change (stimulated minus control) in expression levels (Mean  $\pm$  S.E.) of two innate immune system genes in cultured head kidney cells. Fish from Hols show higher responsiveness in both genes than those from Vifi.

## 2.5 Discussion

Laboratory raised fish from alternate parental populations, kept in controlled conditions with no previous pathogen exposure, differ in both their immune response potential and immune expression profiles. Fish from different wild populations show contrasting immune expression profiles, and surveying multiple populations has identified a range of factors which may be involved in shaping the immune response. The suite of qPCR assays and accompanying cell culture technique developed have allowed us to study variation in the immune response in both the laboratory and the wild, and represents a significant advance in our ability to study immunology in a natural setting since hitherto the number of wild species with such a toolbox has remained small, and been largely confined to rodents. By selecting markers which represent different response types, and which respond to contrasting types of challenge, it is possible to get a general overview of an individual's immune phenotype. Our results were consistent with expectations in several respects, giving us confidence that they are meaningful and useful. It is hoped these tools will facilitate the use of stickleback to study a range of topics relating to the function of the immune system, with the addition of further assays allowing the panel to grow and refine over time.

Laboratory raised fish from Reiv and Obse showed significant differences in immune profiles. As these fish have been raised and maintained in uniform lab conditions with no exposure to any parasites, there appears to be some genetic basis for determining an individual's baseline immune profile. Comparisons between wild fish and laboratory individuals from the same parental population showed differences in immune profiles. Fish from Reiv showed higher expression of all genes in the wild, whilst wild fish from Obse

showed significantly higher Th1-type expression compared to their lab counterparts. Both PCA analysis and Euclidean distances show that the expression profiles of wild fish converge relative to their lab-raised counterparts (Figure 2.1). Conditions encountered in the wild appear to have a larger effect on shaping the immune profile than the underlying genetic differences controlling baseline immune expression, supporting previous work showing that immune expression profiles of stickleback converge during reciprocal transplant experiments (Stutz *et al.* 2015a). The increase in immune expression seen in the wild relative to lab-raised fish suggests that the immune system is being stimulated by a range of possible factors in the wild. For example, Obse fish have consistently high Th1-type expression, suggesting their immune system may be primed to respond to intracellular pathogens. Further studies are required to see if the possible genetic control of baseline expression is present across a larger number of populations, and to examine the link between this baseline expression and the common immune system antagonists present in the wild.

Wild individuals showed a high degree of variation in immune gene expression, both within and between populations, with a range of factors relating to the measured responses. The sampled populations differed in innate expression levels, but not in adaptive immune measures. The innate immune system responds rapidly to contemporary threats, whilst the adaptive response develops over a longer period to continued challenge; differences in innate immune response accounted for by the population term in the model may be due to additional within-population factors that were not measured in this study. Lochs may differ in environmental factors, such as temperature, pH and salinity (Giles 1983; MacColl *et al.* 2013), infection levels of microparasites, and stress levels, all of which may affect the innate immune

response (Castillo *et al.* 2009; Magnadottir 2010). Expression of the adaptive response appears to be better explained by factors which exist above population level variation.

A complex range of factors were found to shape an individual's immune profile within a single population. When fish from Obse were analysed alone, due to the presence of an ectoparasite not found in other populations, a number of factors including sex, condition, and the presence of *G. arcuatus* and lice, were found to be significantly correlated with innate immune expression levels. Due to the immediate and rapid nature of the innate immune system, it would be predicted that an individual's response will be shaped by the set of conditions currently being experienced at the time of sampling. Previous studies of Soay sheep (Nussey *et al.* 2014) and wood mice (Jackson *et al.* 2011) find similar complex sets of characters to be involved in shaping the immune response within single wild populations. Whilst studies in stickleback provide the ability to look for general patterns across multiple replicate populations, these findings reiterate the importance of not overlooking subtle within-population patterns which may exist.

Age was found to relate to the expression of the Th2-type response and of *FoxP3a*. 2+ year old fish showed lower expression of Th2 markers than 0+ or 1+ year olds. *FoxP3a* was associated with an age by relative length interaction (Figure 2.4), with larger individuals having higher expression in 0+ aged fish, whilst the reverse pattern was seen in 1+ fish. Changes in Th2-type and *FoxP3a* expression levels could be the result of a decline in immune potential with age, a pattern of growing interest in immunological research (Aw *et al.* 2007; Vleck *et al.* 2011). This pattern could also represent the transition from a resistant to tolerant immune strategy, as has been observed in wild populations of field voles (Jackson *et al.* 2014) and Soay sheep (Hayward *et*

*al.* 2014). Further studies are required to examine whether tolerant and resistant phenotypes exist in stickleback, and to further examine the changes in response which occur with age.

Infection with *S. solidus* was associated with changes in Th1-type, *TGFβ* and *FoxP3a* expression levels. Changes in the function of the immune system with *S. solidus* infection have previously been observed (eg. Scharsack *et al.* 2004; Scharsack *et al.* 2007), with growing evidence that there is parasite driven manipulation of the host immune system (For summary, see Barber & Scharsack 2010). To date there has been limited understanding of the role of Th1/Th2 immunity in this context, primarily due to a lack of appropriate assays. Work in mice on Schistosomes, a digenean trematode residing in the blood stream, has shown a shift towards an increased Th1-type response in order to avoid potentially harmful Th2-type or humoral responses (Herve *et al.* 2003). Fish in good condition had higher Th1-type expression when infected, which could be a result of parasites manipulation if driving the host to a Th1-type response decreases the potentially harmful Th2-type response. Possible patterns of host manipulation warrant further investigation; the assays presented provide an ideal toolkit to investigate the Th1/Th2 trade off in this context, and to shed new light on the role of *S. solidus* in manipulating the host immune system.

Fish from Hols showed higher innate immune responsiveness than those from Vifi. These fish were raised in common garden conditions, so differences in their ability to respond to challenge are likely to have a genetic basis. These results are consistent with previous work examining the respiratory burst response of different stickleback populations to varying parasite antigens (Franke *et al.* 2014). The high responsiveness of Hols fish could suggest they have evolved in an environment which requires a large, rapid innate response.

Further studies which examine innate immune responsiveness across multiple populations could identify general patterns which shape response potential. Antagonists are available for other arms of the immune system (see Jackson *et al.* 2011), which will allow a similar approach to be taken for other immune response types.

The assays developed represent both the innate and adaptive responses, and give a general overview of different functional arms of the immune system. A number of other genes were identified for which working primers could not be developed, which may be a result of sequence divergence between fish from North Uist and the stickleback reference genome (a fish from Bear Paw Lake, Alaska). Genes of the immune system have been shown to undergo higher levels of selection across a range of taxa (McTaggart *et al.* 2012; Nielsen *et al.* 2005), thus some immune genes may show high degrees of sequence divergence between two isolated lineages within a species. Combining new sequences from European stickleback with the reference genome should allow primers which function across a wide geographic range to be designed for more genes, thus allowing assays to be added and refined over time.

The primers developed were found to be reliable and repeatable, with amplification efficiencies all within the desired range of 75%-110%.  $R^2$  values  $>0.98$  indicate a low degree of variability at each concentration tested. Primers amplified across a range of cDNA concentrations, from 10ng/ $\mu$ l to between 0.4 and 0.016ng/ $\mu$ l. The actual concentration of target transcript in these samples is likely to be much lower, as the measured concentrations only indicate the total cDNA added to each reaction and not the concentration of template of interest. The primers used are sensitive to a wide range of template concentrations, allowing accurate quantification across varying expression levels. The primers have successfully amplified cDNA from fish from Scotland,

England, and Iceland, showing that they function across a wide geographic range.

To conclude, differences in immune expression profiles and response potential between laboratory raised fish show that there is a genetic basis controlling an individuals' baseline immune function and response potential, possibly reflecting the need for a more robust response in certain populations. Profiles in wild populations converge relative to their laboratory raised counterparts, with the conditions faced by an individual having a greater effect on shaping the immune response than genetic background. Variation in expression levels of different functional components in wild populations were related to a range of factors, including size, age and *S. solidus* infection levels. Measures of the innate response reflect the current set of challenges facing a population, whilst the adaptive response represents longer term trends above the population level. Observed patterns raise a number of potential questions for further study. The tools presented represent a significant advance in our ability to study the function of the immune system in a natural setting, and allow the study of multiple replicate wild populations to be combined with controlled lab and field based infection experiments.

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## Chapter 3: Parallelism and divergence in immune responses between two lochs

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### 3.1 Abstract

In the previous chapter, I developed a set of assays to measure the expression levels of genes which act as markers of the immune system in stickleback. In this chapter, I use these markers to examine the relationship between *S. solidus* infection and immune gene expression levels in more detail, by sampling individuals from two populations with high infection levels. Furthermore, by comparing the patterns of expression between populations, I examine whether common factors relate to the expression levels observed. Fish from each population differed significantly in their immune gene expression profiles. Within each population, multiple factors, including condition, reproductive status and *S. solidus* infection level, were found to correlate with expression levels of different arms of the immune system. Some factors were associated with the same expression pattern in both populations, whilst others had opposing effects or were only present in a single population. This chapter adds to our understanding of the immune response in a natural setting, and shows that studying the effects of a single factor in isolation may overlook potentially important and complex interactions which exist in the wild.

## 3.2 Introduction

Studies of the immune system to date have focussed primarily on humans and a few select model species (Maizels & Nussey 2013; Pedersen & Babayan 2011). Such studies are typically lab-based and performed in highly controlled settings where variation is minimised, so that cellular and molecular mechanisms can be identified. This approach, however, is disconnected from the natural world where ‘real’ immune systems have to operate (Pedersen & Babayan 2011). This natural setting is characterised by genetic diversity of both hosts and parasites, and variation in the abiotic environment. Such diversity may play an important role in shaping the function of the immune system in the wild (Lazzaro & Little 2009; Martin *et al.* 2011), and placing current knowledge of immunological mechanisms into this real-world context is a fundamentally important challenge in advancing our understanding of the immune system from an evolutionary perspective (Maizels & Nussey 2013).

The expansion of the study of the immune system in the wild is constrained by a lack of appropriate assays and study species (Fassbinder-Orth 2014). Studies applying modern immunological measurements to natural populations by simultaneously measuring multiple markers of the immune system are rare and primarily limited to rodents (Jackson *et al.* 2014; Oko *et al.* 2006; Schountz *et al.* 2007). Expression assays represent an obvious starting point for the study of the immune response in organisms that lack reagents (particularly for protein-level analysis), although results can be difficult to interpret and data from wild populations inherently noisy. One way to address the possible limitations of such an approach is to compare expression profiles between different populations in relation to candidate causal factors, for example parasitic infection levels, sex or size. If signals are consistent across

locations, we can have more confidence in any patterns identified. Due to the restricted set of previous studies, there is a limited understanding of the role of an individual's environment in shaping immune function in a natural setting; the three-spined stickleback *Gasterosteus aculeatus* is ideally suited to address this issue.

Sticklebacks are a commonly studied model species in evolution and ecology (eg, see Hendry *et al.* 2013). Populations of stickleback show stable differences in parasite community composition (De Roij & MacColl 2012; Scharsack *et al.* 2007a), and interactions with a range of parasites have been well documented (Barber 2013; Kalbe & Kurtz 2006; MacColl 2009). There is growing evidence for within and between population variation in parasite resistance (De Roij *et al.* 2011), which probably has a genetic basis (El Nagar 2014; Rauch *et al.* 2006). This long history of study, combined with the ability to sample replicate populations in the wild and perform controlled infection experiments in the lab, has made the stickleback an excellent model in the study of evolutionary and ecological parasitology (Barber 2013), and an ideal system in which to conduct immunological studies.

The sticklebacks found on the island of North Uist, Scotland, represent one well-studied system (De Roij & MacColl 2012; Giles 1983; MacColl *et al.* 2013). A number of parasite species infect stickleback on North Uist, including *Gyrodactylus arcuatus*, an ectoparasitic trematode, and *Schistocephalus solidus*, a pseudophyllidean cestode. Infections with *S. solidus* can have a negative impact on host fitness (Barber & Scharsack 2010; Barber *et al.* 2008), and parasites have been shown to modulate the immune response of the host (Barber & Scharsack 2010). Whilst the impact of infection on host fitness of a number of parasites has been well studied, the link between infection and a host's defence is less clear.

Past research has begun to examine the immune response of stickleback, particularly the role of immune-related cell populations (Scharsack *et al.* 2004) and the major histocompatibility complex (MHC) (Kurtz *et al.* 2004; Wegner *et al.* 2006) . The genetic background of an individual appears to play a role in determining parasite load (Eizaguirre *et al.* 2011; Rauch *et al.* 2006), suggesting adaptation to local parasite populations, which could be modulated through the immune response.

Recent advances allow the expression levels of immune-related genes to be measured directly. Next-generation sequencing of the transcriptome of stickleback has shown adaptation of expression levels of immune-related genes to local, co-evolved, parasite strains (Lenz *et al.* 2013), and changes in gene expression which are dependent upon the genotype of the infecting parasite (Haase *et al.* 2014) in controlled, laboratory-based, infection experiments. This whole-transcriptome sequencing approach has provided new insights into the response of individuals to controlled infections, but there is a relatively high cost associated with sequencing the large numbers of biological replicates required to study wild populations. An alternative approach to whole transcriptome sequencing is to use quantitative real-time PCR (qPCR) to measure the expression of pre-selected genes (Fassbinder-Orth 2014), chosen based on *a priori* knowledge of the function of the immune system. Such studies have proved fruitful in the study of rodents (Jackson *et al.* 2011; Jackson *et al.* 2009), and qPCR has successfully been applied to stickleback (Dittmar *et al.* 2014; Hibbeler *et al.* 2008; Stutz *et al.* 2015).

In order to give a cohesive overview of the function of the immune system, I developed a set of qPCR assays which measure the innate response, the Th1-type and Th2-type adaptive responses, and the regulatory response (Chapter 2; Robertson *et al.* 2015). In this chapter, I employ these assays to

measure the immune response of wild stickleback from two populations on the island of North Uist, Scotland. I selected populations that typically have similarly high rates of infection with *S. solidus* (MacColl, unpublished data), but are geographically isolated from one another. In this way, I could investigate whether changes in immune gene expression levels that were associated with *S. solidus* infection could be detected, above the inherent natural variation expected when sampling wild individuals. Furthermore, I examined which factors related to immune gene expression levels within each population, and looked at whether there were common factors across both populations which may be involved in shaping the immune response in the wild.

### **3.3 Methods**

#### **3.3.1 Sample Collection**

I sampled fish from Loch a'Bharpa ('Bhar', 57°34'20"N; 7°18'11"W) and Loch Hosta ('Host', 57°37'30"N; 7°29'8"W) on the island of North Uist, Scotland, in May 2015. Forty six fish were sampled from each population, split between two sampling days per population. Fish were caught in Gee's Minnow Traps, set overnight for approximately 16 hours. All individuals caught in a trapping session were pooled, and a subset was selected for inclusion in this study. Fish were transported directly to the laboratory in darkened conditions and processed in a haphazard order within four hours of collection, with all fish collected and processed before midday.

Fish were euthanized using the appropriate schedule 1 technique according to UK Home Office regulations, then measured and weighed. Whole spleens, an immunologically important tissue in fish (Zapata *et al.* 2006), were removed,

weighed, and placed into RNAlater (Life Technologies). Sex was recorded along with reproductive status, determined by visual examination of the gonads in females and the gonads and kidneys in males. The kidneys of males enlarge during reproduction as they secrete spiggin, a proteinaceous substance required for nest building (Jakobsson *et al.* 1999). Male (M) and female (F) reproductive status was split into two categories: individuals with no apparent investment in reproduction (small gonads and kidneys in M, small ovules only in F), versus individuals investing in reproduction or ready to breed (gonads and/or kidneys showing enlargement in M, some or all ovules enlarged in F). The liver was removed and weighed, as was any adipose tissue in the body cavity.

Parasites were identified and counted under a dissection microscope. Where individuals were infected with *S. solidus*, the total weight of all plerocercoids (the life stage infecting stickleback) was recorded, and the parasite index calculated as the total proportion of a fish's body weight accounted for by the parasite (Arme & Owen 1967). The relative weights of the spleen (spleen somatic index, SSI), liver (hepato-somatic index, HSI), and adipose tissue (adipose index, AI) were calculated. Individual condition scores were calculated using Fulton's condition factor,  $K=(10^5 \times \text{weight(g)})/\text{Length(mm)}^3$  (cited in Nash *et al.* 2006), adjusted for individuals infected with *S. solidus* by using individual weight minus the total weight of all plerocercoids.

### **3.3.2 Sample Preparation**

All qPCR work was performed in accordance with the MIQE guidelines (Bustin *et al.* 2009; Taylor *et al.* 2010). Spleens were stored in RNAlater (Life Technologies) at -20°C for no longer than 3 months. RNA was extracted using the GeneJET RNA purification kit (Thermo Scientific) according to the

manufacturers' standard protocol. Purity of RNA samples was assessed on a NanoDrop 1000 Spectrophotometer (Thermo Scientific), with a desired 260/280 absorbance ratio >1.80. Integrity of RNA was assessed by incubating 5µl of sample at 65°C for 10 minutes, followed by visualisation on a 2% Agarose gel stained with Ethidium Bromide.

All samples were DNase treated using Precision DNase (Primer Design), following the manufacturers' protocol. Reverse transcription reactions were performed on approximately 1.5µg of total RNA using the nanoScript2 RT kit (Primer Design), with a combination of random nanomer and oligo-dT priming, following the manufacturers' standard protocol. Periodic no-enzyme controls indicated that genomic DNA contamination was negligible. cDNA samples were diluted 1:10 with nuclease free water before further use.

### **3.3.3 Gene Expression Quantification**

All qPCR reactions were performed in 10µl total volumes, containing 5µl of PrecisionFAST mastermix with SYBR green (Primer Design), 0.25µl of each primer, 2µl of template cDNA and 2.5µl of H<sub>2</sub>O in 96-well optical PCR plates with optical seals (StarLab). Reactions were performed in an ABI 7500 FAST real-time thermocycler (Applied Biosystems) at 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All runs included a post-PCR melt curve analysis.

Accurate normalization of gene expression is essential for the production of reliable data in qPCR experiments, with the optimal reference genes being specific to a particular set of experimental conditions (Dheda *et al.* 2005). To select the most appropriate normalization strategy, a geNorm analysis was performed with six candidate reference genes (*B2M*, *GAPDH*, *RPL13A*, *HPRT1*, *TBP* and *TOP1*) on 12 cDNA samples, randomly selected from all

experimental samples, using a custom stickleback geNorm kit for SYBR green (Primer Design), following the manufacturers' standard protocol. Analysis of the stability of expression was performed in qbase+ (Biogazelle) with *B2M* and *RPL13A* identified as the most stable combination of reference genes for this study.

Expression levels of eight genes of interest were measured, along with two reference genes. Genes of interest were *IL-1 $\beta$* , *TNF $\alpha$* , *Stat4*, *Tbet*, *Stat6*, *CMIP*, *FoxP3*, and *TGF $\beta$*  (Chapter 2; Robertson *et al.* 2015). A reference sample was made by pooling cDNA from each experimental sample, to control for between plate variation. A total of 92 cDNA samples were split randomly between two plates, with reactions performed in duplicate for each sample, and each plate also contained the reference sample and negative controls.

Relative expression values were calculated using the  $\Delta\Delta C_q$  method (Pfaffl 2001), adjusted for the amplification efficiencies of each primer pair and standardised against the geometric mean  $C_q$  of the two reference genes for each sample (Vandesompele *et al.* 2002).

### **3.3.4 Data Analysis**

All expression values were  $\log_{10}(x+1)$  transformed prior to analysis, due to the inherently skewed distribution of relative expression data. Analysis was performed in R v.3.2.2 (R Core Team 2014). To ensure that the order in which I sampled individuals did not influence gene expression levels, the pairwise correlations were calculated between sample order and gene expression levels using 'corr.test' function of the 'psych' package, with Holm correction applied for multiple comparisons. I then examined whether any measured factors related to variation in individual condition, and whether any factors related to immune gene expression variation in each population.

*Variation in Individual Condition.* Individual condition was summarised by using principal components analysis (PCA), a widely used dimension reduction technique which converts a number of variables into principal components which summarise the variation present in the original measures. PCA was performed on K, HSI, SSI and AI, based on the correlation matrix. Both populations were combined for this analysis to ensure that each condition principal component (PC) was summarising the same variation in both populations, to allow direct comparisons of the relationships between condition measures and immune gene expression levels during subsequent modelling. PCs were retained for use in further analysis if they had a standard deviation  $\geq 1$  and explained  $\geq 10\%$  of variance in the data. In order to examine what factors related to the condition measures, general linear models (glm's) were fitted separately to each population, with condition PC1 or PC2 as the response variable. Sex, reproductive status, *G. arcuatus* presence and *S. solidus* presence were included as factors, and length as a continuous variable, along with the sex by reproductive status interaction term. Non-significant terms were sequentially dropped from the full model to give a minimum adequate model. Significance of remaining components was determined by Wald F-tests.

*Summarising Immune Expression.* Profiles of the eight immune response genes were compared between Host and Bhar using multivariate analysis of variance (MANOVA). Overall differences were assessed using the Pillai method, followed by examination of each immune type separately using analysis of variance (ANOVA).

The expression of groups of functionally related components of the immune system was summarised using PCA. By using this approach, the expression of the genes from each arm of the immune response can be summarised in a

single measure encompassing the majority of variation present in the original data. Genes were split into four functional groups based on their role: *IL-1 $\beta$*  and *TNFA* (Innate response); *Stat4* and *Tbet* (Th1-type adaptive response); *Stat6* and *CMIP* (Th2-type adaptive response); and *FoxP3* and *TGF $\beta$*  (immune regulatory response). PCA was performed separately on each population on each immune group using the 'prcomp' function, based on the variance-covariance matrix. In most cases, except Th2-type expression in Bhar, the dominant first principal component (PC1) explained a high proportion of variation in the data (>69%), with both genes having positive loadings. Individual PC1 scores were used for subsequent analysis, except for the Th2-type response where *Stat6* and *CMIP* relative expression values were fitted instead.

*Factors Relating to Immune Expression.* Glm's were fitted to both populations separately using each immune grouping PC1 score, or *Stat6* and *CMIP* relative expression, as the response variable, to examine which factors related to expression levels. Maximal models were fitted with sex, reproductive status, *G. arcuatus* presence and *S. solidus* presence as factors, and condition PC1 and PC2 scores and length as continuous variables. Biologically relevant interaction terms were also fitted. Non-significant terms were sequentially dropped from the full model to give a minimum adequate model, and the significance of remaining components was determined by Wald F-tests.

### **3.4 Results**

*S. solidus* was found to be the dominant parasite species in both populations, with a prevalence of 54.3% (95% binomial confidence interval 39.0%-69.0%) and an average intensity in infected individuals of 8.6 (SE $\pm$ 1.7) in Bhar and a

prevalence of 43.5% (95% binomial confidence interval 28.9%-58.9%) and intensity of 4.5 (SE±1.0) in Host. *G. arcuatus* was also found, with a prevalence of 6.5% (95% binomial confidence interval 1.4%-10.8%) and an intensity on infected individuals of 2 (SE±0.6) in Bhar and a prevalence of 19.6% (95% binomial confidence interval 9.4%-33.9%) and an average intensity of 2.3 (SE±0.3) in Host. No other parasite species were identified in the sampled fish at sufficient rates to include in the analysis.

In Bhar, sample order did not correlate with expression levels of *IL-1β* ( $r^2=0.13$ ,  $p=1$ ), *TNFα* ( $r^2=0.28$ ,  $p=1$ ), *Stat4* ( $r^2=0.16$ ,  $p=1$ ), *Tbet* ( $r^2=0.31$ ,  $p=0.970$ ), *Stat6* ( $r^2=0.06$ ,  $p=1$ ), *CMIP* ( $r^2=-0.13$ ,  $p=1$ ), *FoxP3* ( $r^2=-0.08$ ,  $p=1$ ) or *TGFβ* ( $r^2=0.22$ ,  $p=1$ ). In Host, there were no significant correlations between sample order and expression levels of *IL-1β* ( $r^2=0.02$ ,  $p=1$ ), *TNFα* ( $r^2=-0.22$ ,  $p=1$ ), *Stat4* ( $r^2=0.07$ ,  $p=1$ ), *Tbet* ( $r^2=-0.12$ ,  $p=1$ ), *Stat6* ( $r^2=0.07$ ,  $p=1$ ), *CMIP* ( $r^2=-0.04$ ,  $p=1$ ), *FoxP3* ( $r^2=0.14$ ,  $p=1$ ) or *TGFβ* ( $r^2=0.04$ ,  $p=1$ ).

|                      | Factor                     | Bhar  |      |        | Host  |      |        |
|----------------------|----------------------------|-------|------|--------|-------|------|--------|
|                      |                            | F     | df   | p      | F     | df   | p      |
| <b>Condition PC1</b> | Sex                        | 4.50  | 1,39 | 0.040  | 0.05  | 1,39 | 0.826  |
|                      | Reproduction               | 4.69  | 1,39 | 0.036  | 12.08 | 1,39 | <0.001 |
|                      | <i>S. solidus</i> presence | 37.57 | 1,39 | <0.001 | 10.59 | 1,39 | <0.001 |
|                      | Sex : Reproduction         | 4.89  | 1,39 | 0.033  | 10.53 | 1,39 | <0.001 |
| <b>Condition PC2</b> | Length                     | 6.86  | 1,44 | 0.012  | 13.51 | 1,40 | <0.001 |
|                      | Sex                        |       | n/a  |        | 6.49  | 1,40 | 0.005  |
|                      | Reproduction               |       | n/a  |        | 6.09  | 1,40 | 0.015  |
|                      | <i>S. solidus</i> presence |       | n/a  |        | 5.01  | 1,40 | 0.031  |

**Table 3.1:** Summaries of minimum adequate models fitted to condition data from wild fish from Bhar and Host. Terms that were not included in the final minimum model for a population, but which were retained in the minimum model for the other population, are marked 'n/a'.

### 3.4.1 Variation in Individual Condition

PCA was used to summarise the four different measures that reflect individual condition. Condition PC1 accounted for 41% of variance, with loadings of K (Fulton's condition factor) = 0.134, HSI (hepato-somatic index) = 0.641, SSI (spleen somatic index) = -0.489 and AI (adipose index) = -0.577, whilst PC2 accounted for 24.9% of variance, with loadings of K = 0.973, HSI = -0.119, SSI = 0.186 and AI = -0.062 (Figure 3.1a).

Factors relating to condition PC1 and PC2 scores are summarised in Table 3.1. In both Bhar and Host, individuals investing in attaining reproductive condition had higher PC1 scores (Bhar  $F_{2,39}=4.69$ ,  $p=0.036$ , Host  $F_{2,39}=12.08$ ,  $p<0.001$ ), which also varied with sex (Bhar  $F_{(2,39)}=4.89$ ,  $p=0.033$ , Host  $F_{2,39}=10.53$ ,  $p<0.001$ ) such that females had higher condition PC1 scores for a given reproductive condition score. Infection with *S. solidus* was associated with a decrease in condition PC1 scores (Figure 3.1b) in both Bhar ( $F_{1,39}=37.57$ ,  $p<0.001$ ) and Host ( $F_{1,39}=10.594$ ,  $p=0.002$ ). In both Bhar and Host, larger fish had lower condition PC2 scores (Bhar  $F_{1,44}=6.86$ ,  $p=0.012$ , Host  $F_{1,40}=13.51$ ,  $p<0.001$ ). In Host, males had lower condition PC2 scores than females ( $F_{1,40}=6.49$ ,  $p=0.015$ ), scores were higher in fish in reproductive condition ( $F_{2,40}=6.09$ ,  $p=0.005$ ) and infection with *S. solidus* was associated with an increase in condition PC2 score ( $F_{1,40}=5.01$ ,  $p=0.031$ ).

### 3.4.2 Summarising Immune Expression

Fish from Bhar and Host differed significantly in their overall immune profiles (Figure 3.2; MANOVA  $F_{1,90}=10.169$ ,  $p<0.001$ ). The populations differed in expression of *TNF $\alpha$*  ( $F_{1,90}=13.58$ ,  $p<0.001$ ), *Stat6* ( $F_{1,90}=10.29$ ,  $p=0.002$ ), *CMIP* ( $F_{1,90}=14.30$ ,  $p<0.001$ ), *FoxP3* ( $F_{1,90}=1.38$ ,  $p=0.039$ ) and *TGF $\beta$*

( $F_{1,90}=27.13$ ,  $p<0.001$ ), but not  $IL-1\beta$  ( $F_{1,90}=1.23$ ,  $p=0.270$ ),  $Stat4$  ( $F_{1,90}=0.55$ ,  $p=0.461$ ) and  $Tbet$  ( $F_{1,90}=0.69$ ,  $p=0.409$ ).

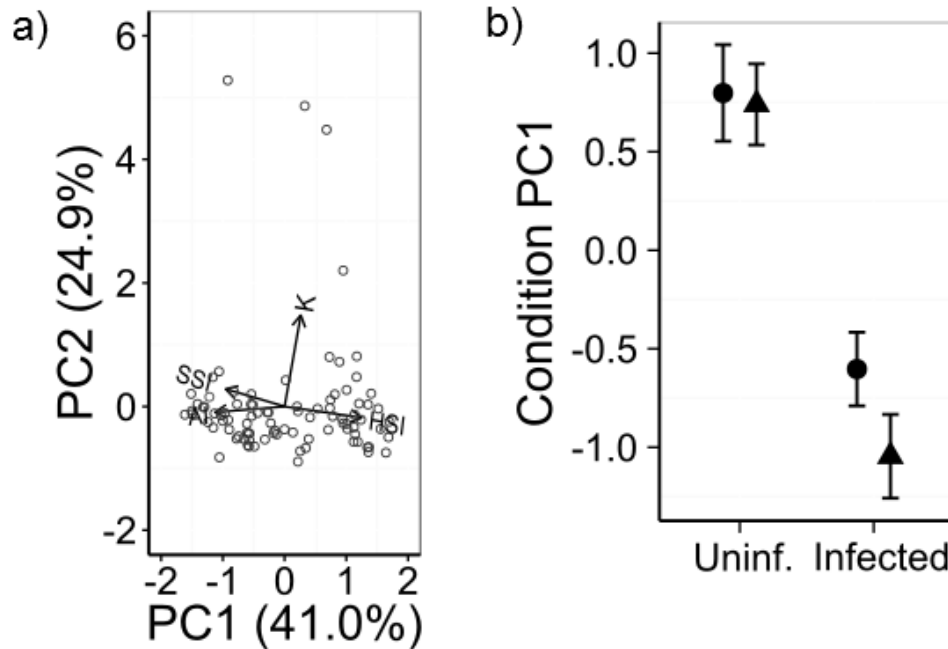


**Figure 3.1:** a) Individual scores of the first and second principal components of condition measures (with % variation) on fish from Bhar and Host. PC1 and PC2 summarise variation in Fulton's condition factor (K), adipose index (AI), spleen-somatic index (SSI) and hepato-somatic index (HSI). b) Fish infected with *S. solidus* ('Infected') had lower condition PC1 scores than uninfected fish ('Uninf.') in both Bhar (●) and Host (▲).

Grouped immune gene expression measures were summarised in each population using PCA, with high PC1 values in all groups indicating high expression levels of included genes. In Bhar, PC1 of innate immune measures explained 69.6% of variation in the data, with loadings of  $IL-1\beta=0.856$  and  $TNF\alpha=0.516$ . For the Th1-type measures, PC1 explained 79.7% of variation, with loadings of  $Stat4=0.267$  and  $Tbet=0.964$ . The Th2-type measures were found to give PC1 loadings in opposite directions, so were kept separate for

subsequent analysis. The regulatory measures gave a PC1 explaining 81.7% of variation, with loadings of *FoxP3*=0.965 and *TGFβ*=0.263.

|        | Factor                                     | BHAR  |      |        | HOST  |      |        |
|--------|--------------------------------------------|-------|------|--------|-------|------|--------|
|        |                                            | F     | df   | p      | F     | df   | p      |
| Innate | Length                                     | 14.11 | 1,40 | <0.001 | 8.20  | 1,38 | 0.007  |
|        | Sex                                        |       |      | n/a    | 4.84  | 1,38 | 0.034  |
|        | Condition PC1                              |       |      | n/a    | 12.94 | 1,38 | <0.001 |
|        | Condition PC2                              | 5.48  | 1,40 | 0.024  | 11.32 | 1,38 | 0.002  |
|        | <i>G. arcuatus</i> presence                |       |      | n/a    | 5.19  | 1,38 | 0.028  |
|        | <i>S. solidus</i> presence                 | 0.39  | 1,40 | 0.534  | 6.59  | 1,38 | 0.014  |
|        | Length : Sex                               |       |      | n/a    | 5.84  | 1,38 | 0.021  |
|        | Length : Condition PC2                     | 5.30  | 1,40 | 0.027  |       |      | n/a    |
|        | Condition PC2 : <i>S. solidus</i> presence | 6.85  | 1,40 | 0.012  |       |      | n/a    |
|        |                                            |       |      | n/a    | 3.89  | 1,39 | 0.056  |
| Th1    | Length                                     |       |      |        |       |      | n/a    |
|        | Sex                                        | 10.59 | 1,37 | 0.002  |       |      | n/a    |
|        | Reproduction                               | 0.14  | 1,37 | 0.714  | 0.46  | 1,39 | 0.540  |
|        | Condition PC1                              | 9.03  | 1,37 | 0.005  | 3.69  | 1,39 | 0.062  |
|        | Condition PC2                              | 0.15  | 1,37 | 0.697  |       |      | n/a    |
|        | <i>S. solidus</i> presence                 | 13.51 | 1,37 | <0.001 | 11.07 | 1,39 | 0.002  |
|        | Length : <i>S. solidus</i> presence        |       |      | n/a    | 5.70  | 1,39 | 0.022  |
|        | Sex : Reproduction                         | 5.90  | 1,37 | 0.020  |       |      | n/a    |
|        | Sex : Condition PC2                        | 7.71  | 1,37 | 0.009  |       |      | n/a    |
|        | Reproduction : Condition PC1               | 8.87  | 1,37 | 0.005  | 5.15  | 1,39 | 0.029  |

**Table 3.2:** See over for legend.

|                   |                                            |      |      |              |   |       |      |                  |   |
|-------------------|--------------------------------------------|------|------|--------------|---|-------|------|------------------|---|
| <b>Stat6</b>      | Length                                     | 0.52 | 1,36 | 0.476        | + | 7.61  | 1,40 | <b>0.009</b>     | + |
|                   | Sex                                        | 4.77 | 1,36 | <b>0.036</b> | - |       | n/a  |                  |   |
|                   | Reproduction                               |      | n/a  |              |   | 11.87 | 1,40 | <b>0.001</b>     | + |
|                   | Condition PC1                              | 3.42 | 1,36 | 0.072        | + | 2.60  | 1,40 | 0.115            | - |
|                   | Condition PC2                              | 0.07 | 1,36 | 0.793        | + |       | n/a  |                  |   |
|                   | <i>S. solidus</i> presence                 | 0.87 | 1,36 | 0.358        | + |       | n/a  |                  |   |
|                   | Length : Reproduction                      |      | n/a  |              |   | 14.28 | 1,40 | <b>&lt;0.001</b> | - |
|                   | Length : Condition PC1                     | 4.43 | 1,36 | <b>0.042</b> | - |       | n/a  |                  |   |
|                   | Sex : Condition PC1                        | 8.35 | 1,36 | <b>0.007</b> | - |       | n/a  |                  |   |
|                   | Sex : Condition PC2                        | 4.91 | 1,36 | <b>0.033</b> | - |       | n/a  |                  |   |
| <b>Regulatory</b> | Reproduction : Condition PC1               |      | n/a  |              |   | 5.95  | 1,40 | <b>0.019</b>     | + |
|                   | Condition PC1 : <i>S. solidus</i> presence | 6.31 | 1,36 | <b>0.017</b> | + |       | n/a  |                  |   |
|                   | Length                                     | 2.29 | 1,38 | 0.139        | + | 6.20  | 1,43 | <b>0.017</b>     | - |
|                   | Condition PC1                              | 2.53 | 1,38 | 0.120        | - |       | n/a  |                  |   |
|                   | Condition PC2                              | 3.79 | 1,38 | 0.060        | + | 6.06  | 1,43 | <b>0.018</b>     | - |
|                   | <i>S. solidus</i> presence                 | 6.33 | 1,38 | <b>0.016</b> | + |       | n/a  |                  |   |
|                   | Length : <i>S. solidus</i> presence        | 5.91 | 1,38 | <b>0.020</b> | - |       | n/a  |                  |   |
|                   | Condition PC1 : <i>S. solidus</i> presence | 6.33 | 1,38 | <b>0.016</b> | + |       | n/a  |                  |   |
|                   | Condition PC2 : <i>S. solidus</i> presence | 7.11 | 1,38 | <b>0.011</b> | - |       | n/a  |                  |   |
|                   |                                            |      |      |              |   |       |      |                  |   |

**Table 3.2 Continued:** See over for legend.

**Table 3.2 Continued:** Summaries of minimum adequate models fitted to expression data from wild fish from two lochs ('Bhar' and 'Host') on North Uist, Scotland. 'Innate', 'Th1', and 'Regulatory' response variables are first principal components of the following pairs of genes respectively: *IL-1 $\beta$*  and *TNFA*; *Stat4* and *Tbet*; *FoxP3* and *TGF $\beta$* . Significant p values ( $P < 0.005$ ) are marked in bold. The direction of the effect (+ or -) of each factor or interaction term is shown (M relative to F for 'Sex', reproductive relative to non-reproductive for 'Reproduction'). Terms that were not included in the final minimum model for a population, but which were retained in the minimum model for the other population, are marked 'n/a'.

In Host, PC1 of innate measures explained 78.5% of variation, with loadings of  $IL-1\beta=0.961$  and  $TNF\alpha=0.277$ . For the Th1-type measures, PC1 explained 84.8% of variation, with loadings of  $Stat4=0.439$  and  $Tbet=0.898$ . PC1 of the Th2-type measures explained 81.4% of variation, with loadings of  $Stat6=0.222$  and  $CMIP=0.975$ . The regulatory measures PC1 accounted for 82.2% of variation, with loadings of  $FoxP3=0.969$  and  $TGF\beta=0.245$ .

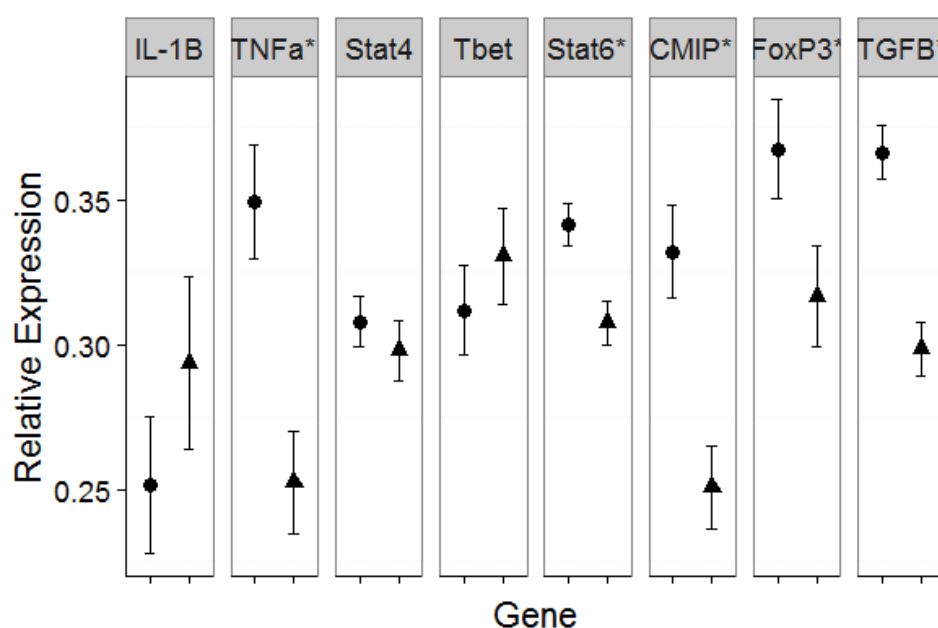


**Figure 3.2:** Relative gene expression levels (Mean  $\pm$  SE) of Bhar (●) and Host (▲). Genes with significant expression differences are indicated (\*).

### 3.4.3 Factors Relating to Immune Expression

A range of factors were found to relate to expression levels of different arms of the immune system, as summarised in Table 3.2. Some factors relating to innate and Th1-type expression levels had the same effects in both populations, whilst others relating to innate, Th1-type, Th2-type and immune regulatory measures either had opposing effects or were only related to expression levels within a single population.

*Innate Expression.* In Bhar, larger fish had lower innate expression levels (Figure 3.3,  $F_{1,40}=14.11$ ,  $p<0.001$ ). An increase in condition PC2 was associated with an increase in expression ( $F_{1,40}=5.48$ ,  $p=0.024$ ), with the effect being less pronounced as fish get bigger ( $F_{1,40}=5.30$ ,  $p=0.027$ ). Individuals infected with *S. solidus* had lower innate expression levels at a given condition PC2 score ( $F_{1,40}=6.85$ ,  $p=0.012$ ). Larger fish in Host also had lower innate expression levels (Figure 3.3,  $F_{1,38}=8.20$ ,  $p=0.007$ ), but this also varied by sex ( $F_{1,38}=5.84$ ,  $p=0.021$ ), with males showing a larger decrease in expression with increasing size. An increase in condition PC1 score was associated with an increase in innate expression ( $F_{1,38}=12.94$ ,  $p<0.001$ ), whilst an increase in condition PC2 was associated with a decrease ( $F_{1,38}=11.32$ ,  $p=0.002$ ). In contrast to Bhar, infection with both *G. arcuatus* ( $F_{1,38}=5.19$ ,  $p=0.028$ ) and *S. solidus* ( $F_{1,38}=6.59$ ,  $p=0.014$ ) were associated with an increase in innate expression levels in Host.

*Th1-type Expression.* In Host, fish infected with *S. solidus* had lower Th1-type expression levels (Figure 3.4,  $F_{1,39}=11.07$ ,  $p=0.002$ ), with the difference increasing as fish got larger ( $F_{1,39}=5.70$ ,  $p=0.022$ ). Individuals in reproductive condition had higher Th1-type expression levels for a given condition PC1 score ( $F_{1,39}=5.15$ ,  $p=0.029$ ). In Bhar, fish infected with *S. solidus* also had lower Th1-type expression than uninfected fish (Figure 3.4,  $F_{1,37}=13.51$ ,  $p<0.001$ ). Males had lower expression levels than females ( $F_{1,37}=10.59$ ,  $p=0.002$ ), and this effect varied with reproductive status ( $F_{1,37}=5.90$ ,  $p=0.020$ ), with the difference between sexes much greater in non-reproductive individuals. Sex also altered the relationship between condition PC2 score and Th1-type expression ( $F_{1,37}=7.71$ ,  $p=0.009$ ), with the difference between sexes decreasing as condition PC2 score increased. As in Host, the effect of condition PC1 score also varied with reproductive status in Bhar ( $F_{1,37}=8.87$ ,

$p=0.005$ ), with reproductive individuals having lower Th1-type expression at a given condition PC1 score.

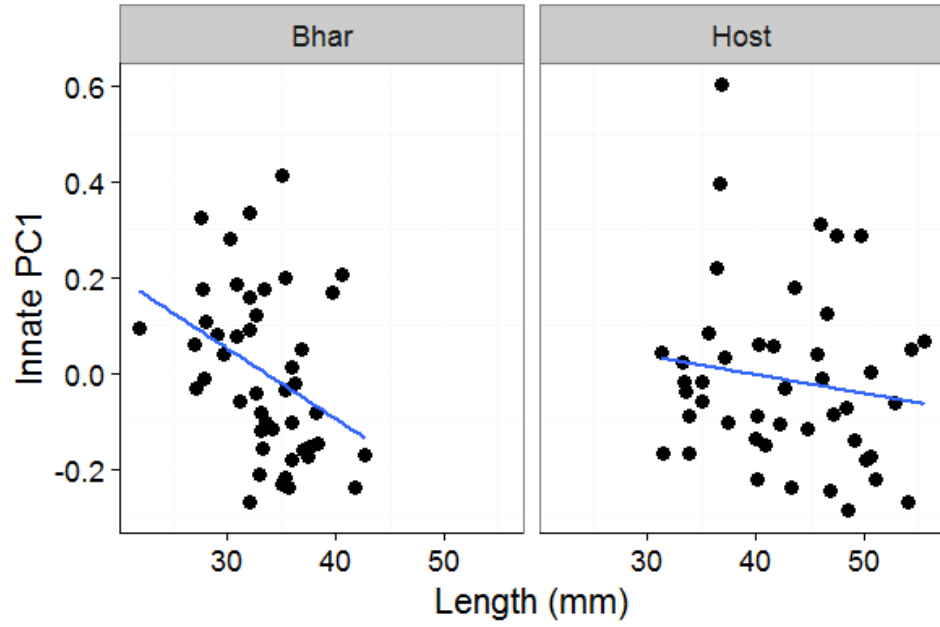


**Figure 3.3:** Larger fish had lower innate gene expression levels (Mean  $\pm$  SE) in Bhar and Host (including linear best fit lines). The ‘Innate’ response variable is the first principal component scores of *IL-1 $\beta$*  and *TNF $\alpha$*  gene expression levels, with PCA performed separately on fish from each population. A high PC1 score indicates high expression levels of both genes.

*Th2-type Expression.* No significant factors were found in the models for *CMIP* expression in Bhar or Host. In Bhar, males showed lower *Stat6* expression levels than females ( $F_{1,36}=4.77$ ,  $p=0.036$ ). Sex influenced the relationship between condition PC1 and *Stat6* expression ( $F_{1,36}=8.35$ ,  $p=0.007$ ) and condition PC2 and *Stat6* expression ( $F_{1,36}=4.91$ ,  $p=0.033$ ), with males having lower expression than females for a given condition PC score. The effect of condition PC1 score on *Stat6* expression also varied with length ( $F_{1,36}=4.43$ ,

$p=0.042$ ), with larger fish having lower *Stat6* expression levels at a given condition PC1 score. The effect of condition PC1 score on *Stat6* expression also varied with *S. solidus* infection ( $F_{1,36}=6.31$ ,  $p=0.017$ ), with infected fish having higher *Stat6* expression levels than uninfected fish at a given condition PC1 score. In Host, larger individuals had higher *Stat6* expression levels ( $F_{1,40}=7.61$ ,  $p=0.009$ ), as did individuals in reproductive condition ( $F_{1,40}=11.87$ ,  $p=0.001$ ). The effect of length also varied with reproduction ( $F_{1,40}=14.28$ ,  $p<0.001$ ), with the difference between reproductive and non-reproductive individuals decreasing with increasing length. Individuals in reproductive condition had higher *Stat6* expression levels than non-reproductive individuals at a given condition PC1 score ( $F_{1,40}=5.95$ ,  $p=0.019$ ).

*Regulatory Expression.* In Host, larger fish had lower regulatory gene expression levels ( $F_{1,43}=6.20$ ,  $p=0.017$ ), whilst an increase in condition PC2 score was associated with a decrease in expression levels ( $F_{1,43}=6.06$ ,  $p=0.018$ ). In Bhar, individuals infected with *S. solidus* had higher regulatory gene expression levels than uninfected individuals ( $F_{1,33}=6.33$ ,  $p=0.016$ ), but this difference decreased with increasing fish length ( $F_{1,33}=5.91$ ,  $p=0.020$ ). The difference in regulatory gene expression levels between *S. solidus* infected and uninfected individuals decreased with increasing condition PC1 score ( $F_{1,33}=6.33$ ,  $p=0.016$ ), and with increasing condition PC2 score ( $F_{1,33}=7.11$ ,  $p=0.011$ ).

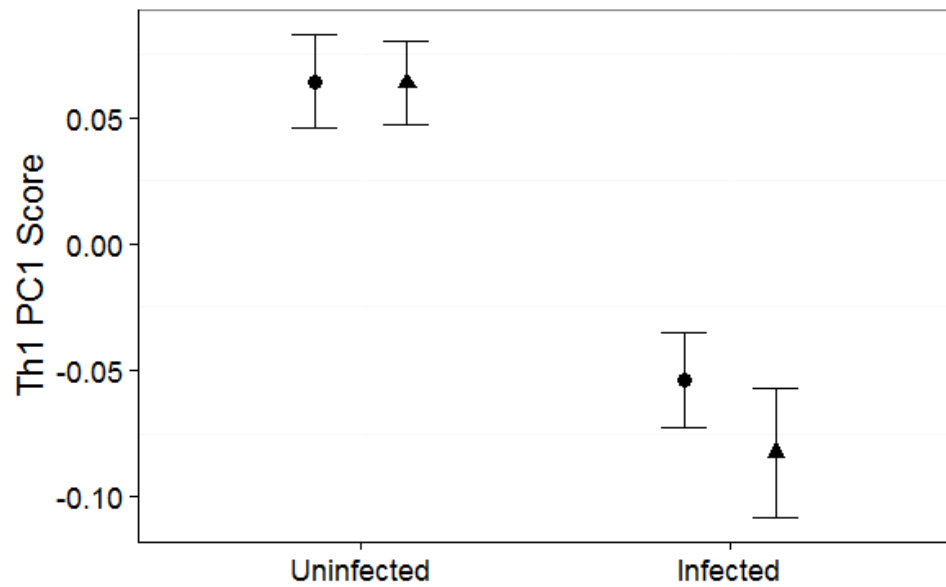


**Figure 3.4:** Fish infected with *S. solidus* had lower Th1-type gene expression levels (Mean  $\pm$  SE) in Bhar (●) and Host (▲). The 'Th1' response variable is the first principal component scores of *Stat4* and *Tbet* gene expression levels, with PCA performed separately on fish from each population. A high PC1 score indicates high expression levels of both genes.

### 3.5 Discussion

In this chapter, I demonstrate that there are differences in immune gene expression levels between two populations infected with high levels of *S. solidus*, and a range of factors appear to contribute to shaping this variation. No correlations were found between sampling order and gene expression levels, indicating that our sampling and holding procedure had no effect on gene expression levels, and therefore did not influence the results. Data collected from wild populations is inherently noisy, yet variation in immune gene expression levels that was associated with infection with two common parasite species, *S. solidus* and *G. arcuatus*, as well as a range of other factors, was still detectable. Overall gene expression profiles differed between fish from Host and Bhar, primarily driven by higher expression levels of Th2-type and immune regulatory genes in Bhar. Previous work has shown that underlying gene expression levels differ between populations (Chapter 2; Robertson *et al.* 2015), and an individual's ability to respond has some genetic basis (Chapter 2; El Nagar 2014; Rauch *et al.* 2006; Robertson *et al.* 2015), thus the measured variation in expression here could represent underlying genetic differences in immune function, or differences in the challenges being faced in each population.

A range of factors were found to contribute to shaping expression of each response type, relating to different arms of the immune system in different ways. Some explanatory factors were found to have common effects in both populations, including size, sex and condition, whilst others had opposing effects or only related to expression levels in a single population. Interactions between the factors also varied. Recent thinking in immunology suggests that controlled lab-based studies of the immune system lack complexity when

compared to a natural setting (Bradley 2015; Pedersen & Babayan 2011). The broad set of factors found here which relate to gene expression levels, and the interactions between them, confirm that immune function in the wild is complex, and shows that studies in immunology should consider multiple factors simultaneously.

Infection with *S. solidus* was associated with variation in expression of all immune response types, but some of these effects differed between the studied populations. This could potentially represent between-population differences in immune response to infection. The immune response of stickleback has been shown to change with *S. solidus* infection in experimental studies (Scharsack *et al.* 2004; Scharsack *et al.* 2007b), with growing evidence of parasite driven manipulation of the host immune system (For review, see Barber & Scharsack 2010). The role of Th1/Th2 differentiation during *S. solidus* infection is not well understood due to a previous lack of appropriate assays. Here we see a decrease in Th1-type expression with infection in both populations, as previously observed in wild populations on North Uist (Chapter 2; Robertson *et al.* 2015), and an increase in Th2-type and immune regulatory expression in Bhar only. Work in mice on Schistosomes, a digenean trematode residing in the blood stream, has shown a shift towards an increased Th1-type response in order to avoid the potentially harmful Th2 or humoral type responses (Herve *et al.* 2003), an approach that may be employed by *S. solidus* (Barber & Scharsack 2010). Our data, however, shows the opposite change in Th1-type response. Our results support the involvement of the adaptive response during infection, but a better understanding of the Th1/Th2 mechanism in teleost fish is required to shed further light on the role of *S. solidus* in manipulating the host's immune system.

Both sex and reproductive status correlated with an individual's immune expression profile. Previous studies in vertebrates have found that there are general differences in immune function between the sexes (Hawley & Altizer 2011; Restif & Amos 2010), and we expected males to have lower expression levels in general than females. Sex correlated with innate expression levels in Host, and with Th1-type, Th2-type and *Stat6* expression levels in Bhar; in all these cases, males had lower expression levels. Investing in reproduction can affect immune response ability (Downs *et al.* 2014). Individuals investing in reproduction had lower Th1-type expression levels in both Bhar and Host, and higher *Stat6* expression levels in Host. During the breeding season, the immune response can be affected by circulating hormone levels (Cuesta *et al.* 2007; Maule *et al.* 1996), and other factors, such as sex specific behavioural changes, may also play a role. Further studies at different times of year could indicate whether such sex differences are transient, and longitudinal studies could show whether different investment strategies affect breeding success, and ultimately fitness.

Condition measures were found to relate to expression levels of all response types in both populations, although the effect and interactions varied between populations. The condition PC1 axis represents opposing changes in liver size against spleen size and adipose fat tissue, and was associated with differences between sexes, in reproductive condition, and *S. solidus* infection levels. Changes in liver, spleen and adipose tissue weight were expected to correspond with *S. solidus* infection (Arme & Owen 1967). The condition PC2 axis represents general body condition, measured as Fulton's condition factor, and decreases with increasing length. In turn the condition PC1 and PC2 scores interact with a number of factors in the gene expression models for both Bhar and Host, again including sex, reproduction and *S. solidus* infection.

There appears to be a complex interplay between factors, where the direction of causality in the relationship between factors determining condition, condition itself, and immune gene expression levels is an area open to further research. Condition may affect an individual's ability to respond, or response may determine condition. Furthermore, factors such as reproductive investment and *S. solidus* infection may have direct effects on immune gene expression levels, whilst also acting indirectly through condition.

In conclusion, studying the immune expression profiles of individuals from two wild populations of stickleback has provided new insights into the function of the immune system in a natural setting. The two sampled populations differ in their immune gene expression profiles, and a complex interplay of multiple factors correlates to individual immune expression levels within each population. There appear to be important roles of sex, reproductive status and individual condition. Infection with a parasite with fitness consequences to the host, *Schistocephalus solidus*, relates to the innate and adaptive responses, and may represent parasite manipulation of the host. Whilst some factors have the same pattern in both populations, others only relate to gene expression levels in a single population. This chapter adds to our understanding of the immune response in a natural setting, and shows that studying the effect of single factors in isolation may overlook important and complex interactions in the wild.

### 3.6 References

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## Chapter 4 – Exposing naïve fish to natural challenges

### 4.1 Abstract

In the previous chapters, I examined variation in the immune response across wild individuals from multiple populations. One potential limitation of only examining wild individuals is that the immune system at the time of measurement may be shaped by unmeasurable past experiences of each individual. In order to control for this variation, fish raised in controlled laboratory conditions can be exposed to natural conditions, therefore removing such variation and allowing us to better examine the factors which shape the function of the immune system. In this chapter, we expose F2 hybrid fish, between two parental populations which vary in their parasite resistance, to semi-natural conditions in replicated enclosures in a loch on North Uist, Scotland. We measured their immune profiles at the end of the experiment using the markers developed in chapter 2, in order to examine which factors contribute to shaping immune function. Exposure to natural conditions resulted in a change in immune gene expression levels, with variation associated with sex, reproductive condition, growth rate, and infection with the common parasite *Gyrodactylus arcuatus*. Growth rate varied with *Eda* genotype, the gene underlying a major adaptive phenotypic polymorphism, whilst change in condition differed with reproductive investment. This chapter adds to our growing understanding of the function of the immune response in a natural setting.

## 4.2 Introduction

An individual's immune system faces a complex set of challenges in the wild, from genetic diversity of multiple infecting pathogens to a constantly changing abiotic environment (Pedersen & Babayan 2011). This variation, combined with host genetic diversity, plays a key role in shaping the function of the immune system in a natural setting (Lazzaro & Little 2009; Martin *et al.* 2011). Understanding of the immune system has come primarily from performing lab-based studies in highly controlled settings, so that underlying cellular and molecular mechanisms can be identified (Maizels & Nussey 2013; Pedersen & Babayan 2011), but this approach is disconnected from the real-world setting where immune systems actually have to operate. Placing our current understanding of the immune system into a more natural context is a fundamentally important challenge in advancing our understanding of the immune system from an evolutionary perspective (Maizels & Nussey 2013), and in particular we need to understand what factors shape an individual's response, and how the type of response may in turn affect fitness.

Expansion of the study of the immune system in a wild context has been constrained by a lack of appropriate assays and study species (Fassbinder-Orth 2014). Modern immunological measurements have been applied to natural populations, but have been primarily limited to rodents (Jackson *et al.* 2011; Oko *et al.* 2006; Turner *et al.* 2014), due to the availability of reagents developed in closely related laboratory-based species. Whilst such studies have shown seasonal changes in immune activity and identified possible factors which influence immune function, they are unable to control for previous, unknown, variation in individual experience prior to the point of sampling. Individuals may differ in their previous exposure to pathogens, or

past differences in foraging success may have caused variation in growth or energy availability which can influence immune function. To control for such variation we could expose individuals raised in a controlled laboratory environment to natural conditions, and examine how they respond to the challenges they face; the three-spined stickleback *Gasterosteus aculeatus* provides an ideal model species in which to perform such work.

Sticklebacks are a commonly studied model species in evolution, ecology and parasitology (Barber 2013; Hendry *et al.* 2013). They are widely distributed throughout the northern hemisphere, where the ancestral marine form has repeatedly colonised novel freshwater habitats since the end of the last ice age to create a large number of isolated populations (Bell & Foster 1994). One of the most conspicuous changes to occur during adaptation is the loss of bony armour plates in the freshwater form, controlled by a mutation in the *Ectodysplasin (Eda)* gene, which has become one of the most widely studied examples of a gene controlling a major adaptive phenotype (Barrett 2010; Colosimo *et al.* 2005). Fish can be sampled from a large number of replicate populations that have adapted to different biotic and abiotic conditions, crosses between these populations are possible, and offspring can be raised in uniform laboratory conditions for use in controlled experiments.

Sticklebacks have a well characterised parasite fauna, with stable differences in parasite community composition observed between host populations (Barber & Scharsack 2010; De Roij & MacColl 2012; MacColl 2009). There is within and between population variation in parasite resistance (De Roij *et al.* 2011; Kalbe & Kurtz 2006; Scharsack *et al.* 2007) which has some genetic basis (Chapter 2; El Nagar 2014; Rauch *et al.* 2006; Robertson *et al.* 2015). One of the dominant parasites infecting wild stickleback are ectoparasitic trematodes of the *Gyrodactylus* genus (De Roij & MacColl 2012; Harris 1985;

Raeymaekers *et al.* 2008). Individuals infected with *Gyrodactylus* grow slower than non-infected fish (De Roij *et al.* 2011) and have a mortality rate of ~2.5% (Mahmud, Robertson & MacColl, unpublished data) in controlled conditions, indicating that *Gyrodactylus* may represent a significant selection pressure in wild populations. Whilst the impact of parasitic infection on host fitness has been well studied in the lab, the link between infection and a host's defence in the wild is less clear.

Previous immunological research on stickleback has focussed on the role of immune-related cell populations during infection (Scharsack *et al.* 2004) and the link between infection and the major histocompatibility complex (MHC, Kurtz *et al.* 2004; Wegner *et al.* 2006). It has been found that stickleback populations differ in expression levels of immune related genes, and that wild fish exhibit higher expression levels than those raised in controlled conditions (Chapter 2; Robertson *et al.* 2015). Patterns of expression across multiple wild populations have been examined (Chapters 2 & 3; Robertson *et al.* 2015, 2016), and experimentally transplanted fish have been shown to modulate their immune response to the challenges being faced (Stutz *et al.* 2015). It is possible to expose lab-raised fish to natural conditions (Eizaguirre *et al.* 2012; MacColl & Chapman 2010; Scharsack *et al.* 2007), but previous work on the immune response has concentrated on a single response measures, or multiple measures of the same response type. A range of possible factors which may influence the function of the immune system in a natural setting have been identified (Chapters 2 & 3; Robertson *et al.* 2015, 2016), but these patterns are confounded by previous, unknown, variations in individual experience.

In this study, we address this issue by exposed laboratory raised fish to the natural conditions experiences by wild individuals, in order to investigate the

factors which may influence an individual's immune response. Fish were exposed in replicated semi-natural enclosures in Loch Ob nan Stearnain ('Obse'), which has a naturally high prevalence of infection with *Gyrodactylus arcuatus*. We created F2 hybrids between fish from Obse, which show relatively high levels of resistance to *G. arcuatus* infection, and fish from a nearby loch which show little natural resistance (De Roij *et al.* 2011; Mahmud 2016). By using this approach, we randomise each individual's genetic background whilst creating fish which vary in their ability to resist infection, irrespective of the rest of their genetic background and their adaptation to local conditions. After eight weeks, we measured the expression levels of eight genes which give an overview of the function of the immune system (Chapter 2; Robertson *et al.* 2015). By individually marking each fish, we tracked growth rates, changes in condition, and the level of reproductive investment over the course of the experiment. We identify factors which relate to growth rates and condition, and those which may play a role in shaping an individual's immune expression profile.

The function of the immune system may be influenced by a wide range of factors, and we can predict their effect on immune gene expression profiles, based on previous research across vertebrates, both in the lab and in the wild. Trade-offs could exist between immune system function and other energetically costly activities, for example growth and maintaining body condition, as individuals will only have a limited set of resources to allocate (Benjamini *et al.* 2000; Lazzaro & Little 2009; Magnadottir 2010). The function of different arms of the immune response may trade-off against one another, due to different effector mechanisms and antagonistic effects between response types (Benjamini *et al.* 2000), although wild stickleback show general positive correlations in expression levels (Chapter 3; Robertson *et al.*

2016). Males would be expected to have reduced immune function relative to females (Hawley & Altizer 2011; Klein 2004), but this effect could vary with reproductive investment. Circulating sex hormone levels can alter immune function (Cuesta *et al.* 2007), as can differential investment in attaining reproductive condition, which may be more costly to females due to the high energy expenditure required to produce eggs relative to sperm. Differences in expression profiles between the sexes and with reproductive condition have been seen across multiple populations of wild stickleback (Chapter 2 & 3; Robertson *et al.* 2015, 2016). By exposing individuals to natural conditions, we can control for unknown past variation in individual experience, allowing us to better understand how multiple factors interact to immune gene expression levels, growth and condition.

## **4.3 Methods**

### **4.3.1 Fish Crossing**

I created eight F1 families in May 2012 by crossing individuals from Obse (57°36'9"N; 7°10'19"W), a saltwater lagoon containing fully armour plated resident fish with high levels of resistance to *G. arcuatus* infection, with individuals from Loch à Chadha Ruaidh ('Chru', 57°35'38"; 7°11'51"W), a freshwater population with low armour plating with little natural resistance to *G. arcuatus*, both located on North Uist, Scotland. Each population contributed half of the males and half of the females, and crosses were performed following the procedure of De Roij *et al.* (2011). Fertilized eggs were transported to aquarium at the University of Nottingham where all eggs from each family were placed into individual half-tank partitions of 100L tanks. After hatching, families were split between multiple tank partitions to give 15

fish per partition, in order to ensure that all fish were maintained at the same density. The aquarium was temperature controlled, with a lighting regime mirroring the natural photoperiod of North Uist. All fish were reared in freshwater containing 2 parts per thousand (ppt) salt. In May 2013, I then created 4 F2 families by crossing unrelated F1 fish, following the same method.

#### **4.3.2 Experimental Exposure**

Seven fish from the first three families, and four from the fourth family, were randomly assigned to each of ten treatment groups, giving 25 fish per group and 250 fish in total. A second set of fish, comprising five fish from each family, were assigned to the control group. Experimental fish were to be exposed to semi-natural conditions in enclosures in Obse, where infection with *G. arcuatus* is common, whilst control fish remained in the laboratory. As Obse is a saltwater loch, all fish were gradually acclimatised to 20 ppt saltwater conditions three months prior to the start of the experiment. We tagged all fish with visible implant elastomer tags (VIE, Northwest Marine Technology) so that each individual could be identified within its group. Tagging was performed two months prior to the start of the experiment to allow sufficient recovery time and to ensure that immune response was not affected by the tagging procedure (Henrich *et al.* 2014). Length and weight were also recorded.

Experimental fish were transported to North Uist in May 2014. I constructed ten enclosures using 6mm square mesh, with each enclosure being cylindrical in shape, 90cm long and 68cm in diameter, giving a total volume of 330cm<sup>3</sup>. This mesh size was selected to contain fish within the enclosures whilst still allowing the free flow of water and suspended materials. All fish from each

treatment group were added to a single enclosure and were then left in place for eight weeks. The control fish remained in the aquarium at the University of Nottingham, kept in a light and temperature regime mirroring that on North Uist.

At the end of the experiment, I removed all fish from a single randomly selected enclosure at a time. Fish were transported to the laboratory in darkened boxes containing fresh loch water, with air supplied from a battery powered pump. All fish were processed in a random order within two hours of collection. Control fish were sampled 3 days after the end of the experimental exposure in North Uist.

Fish were euthanized by overdose of MS222 followed by destruction of the brain, in accordance with UK Home Office regulations, then measured and weighed. Spleens, an immunologically important tissue in fish (Zapata *et al.* 2006), were removed and immediately placed in RNAlater (Life Technologies). Each fish was identified using its unique VIE tag, and I recorded sex and reproductive status by visually examining the gonads under a low powered microscope. Male and Female fish were split into two reproductive status categories: no reproductive investment (small gonads and kidneys in M, small ovules in F), or some investment in reproduction (gonads and/or kidneys enlarged in M, some or all ovules enlarged in F). I identified and counted any macroparasites under a dissection microscope, and a sample of fin tissue was removed and preserved into 100% ethanol for *Eda* genotyping.

I calculated the growth rate of each individual as the daily increase in length over the course of the experiment. Each individual's condition, at the start and end of the experiment, was calculated using Fulton's condition factor,  $K=10^5 \times \text{Weight(g)}/\text{Length(mm)}^3$  (Cited in Nash *et al.* 2006), with a high K value

indicating good overall body condition. The change in condition over the course of the experiment was calculated as End K minus Start K.

All individuals were genotyped for *Eda* using the *Stn382* primers, which flank an indel polymorphism in intron 1 of the gene (Colosimo *et al.* 2005). DNA was extracted from fin clips using DirectPCR Lysis Reagent for Mouse Tails (Viagen Biotech), by incubating for 12 hours at 55°C followed by 45 minutes at 85°C. PCR reactions were performed in a 20µl total reaction volume, containing 10µl BioMix Red Mastermix (Bioline), 6µl H<sub>2</sub>O, 1µl forward and 1µl reverse primer at 10µM, and 2µl template DNA, with an initial step of 94°C for 4 minutes, followed by 35 cycles of 94°C for 15 seconds, 60°C for 15 seconds and 72°C for 15 seconds, and a final extension of 72°C for 4 minutes. PCR product was visualised on a 2% Agarose gel containing Ethidium Bromide, with the complete plated allele (C) giving a product 218bp in length, and the low plated allele (L) being 150bp in length.

#### **4.3.3 Sample Preparation**

All qPCR work was performed in accordance with the MIQE guidelines (Bustin *et al.* 2009; Taylor *et al.* 2010). From the samples available, I selected a subset of 82 individuals for inclusion in the gene expression analysis study, with 68 coming from experimental fish, and 14 from control fish. I extracted RNA from Spleens using the GeneJET RNA purification kit (Thermo Scientific) according to the manufacturers' standard protocol. Purity of RNA was assessed on a NanoDrop 1000 spectrophotometer (Thermo Scientific), with a desired 260/280 absorbance ratio >1.80. RNA integrity was assessed by incubating 5µl of each sample at 65°C for 10 minutes, followed by visualisation on a 2% Agarose gel stained with Ethidium Bromide. All samples were DNase treated using Precision DNase (Primer Design), following the

manufacturers' protocol. Reverse transcription reactions were performed on approximately 1.5µg of total RNA using the nanoScript2 RT kit (Primer Design), with a combination of random nanomer and oligo-dT priming, following the manufacturers' protocol. We included periodic no-enzyme controls, which indicated that genomic DNA contamination was negligible. All cDNA samples were diluted 1:10 with nuclease free water before further use.

#### **4.3.4 Gene Expression Quantification**

qPCR reactions were performed in 10µl total volumes, containing 5µl of PrecisionFAST mastermix with SYBR green (Primer Design), 0.25µl of each primer, 2µl of template cDNA and 2.5µl of H<sub>2</sub>O in 96-well optical PCR plates with optical seals (StarLab). Reactions were performed in an ABI 7500 FAST real-time thermocycler (Applied Biosystems) with an initial step of 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All runs included a post-PCR melt curve analysis.

Accurate normalization is essential for the production of reliable data from qPCR experiments, with the optimal reference genes being specific to a particular set of experimental conditions (Dheda *et al.* 2005). In order to select the most appropriate reference genes for this study, I performed a geNorm analysis on six candidate reference genes (*B2M*, *GAPDH*, *RPL13A*, *HPRT1*, *TBP* and *TOP1*) using a custom stickleback geNorm kit for SYBR green (Primer Design), following the manufacturers standard protocol. Analysis of the stability of expression was performed in qbase+ (Biogazelle) with *TBP* and *RPL13A* identified as the most stable combination of reference genes.

I measured the expression levels of eight genes of interest, along with the two reference genes. Genes of interest were: *IL-1β* and *TNFα* which represent the innate response; *Stat4* and *Tbet* which represent the Th1-type response;

*Stat6* and *CMIP* which represent the Th2-type response; and *FoxP3a* and *TGFβ* which have an immunosuppressive, regulatory, role (Chapter 2; Robertson *et al.* 2015). A reference sample was made by pooling cDNA from all experimental samples, to control for between plate variation. Samples were split randomly between two 96-well plates for each gene, with each plate also containing the reference sample and negative controls.

Relative expression values were calculated using the  $\Delta\Delta C_q$  method (Pfaffl 2001), adjusted for the amplification efficiencies of each primer pair and standardised against the geometric mean  $C_q$  of the two reference genes for each sample (Vandesompele *et al.* 2002).

#### **4.3.5 Data Analysis**

All relative expression data was  $\log_{10}(x+1)$  transformed prior to analysis, due to the inherently skewed distribution of relative expression data. I performed all analysis in R v.3.2.2 (R Core Team 2014).

*Growth and Condition.* An analysis of variance (ANOVA) was performed to compare the growth rates of control and exposed fish. A general linear model (glm) was fitted to the growth of exposed fish only, with growth rate as the response and sex, reproductive status, *G. arcuatus* presence and *Eda* genotype as factors. Start K and start length were fitted as continuous variables, along with biologically relevant interaction terms. Non-significant terms were sequentially dropped to give a minimum adequate model, with the significance of remaining components calculated using Wald F tests.

Change in condition over the course of the experiment was compared between exposed and control individuals using ANOVA. A glm was fitted to the exposed fish only with change in condition as the response, and sex, reproductive status, *G. arcuatus* presence and *Eda* genotype as factors and

start K and growth rate as continuous variables, along with biologically relevant interaction terms. Non-significant components were dropped to give a minimum model, with the significance of remaining components calculated using Wald F tests.

*Control v. Exposed.* First, I examined whether the expression levels of the eight immune genes differed between control and exposed fish, using a multivariate analysis of variance (MANOVA). Overall differences were calculated using the Pillai method, followed by separate examination of expression levels of each immune gene.

*Expression Patterns in Exposed Fish.* Principal components analysis (PCA), a dimension reduction technique which summarises the variation present in a set of measures, was used to summarise the expression levels of pairs of functionally related genes in exposed fish. Genes were split into four functional groups based on their role: *IL-1 $\beta$*  and *TNF $\alpha$*  (innate response); *Stat4* and *Tbet* (Th1-type adaptive response); *Stat6* and *CMIP* (Th2-type adaptive response); and *FoxP3a* and *TGF $\beta$*  (regulatory). PCA was performed on each immune group using the 'prcomp' function, based on the variance-covariance matrix. The dominant first principal component (PC1) of each pair of measured explained a high proportion of variation (>60%), with both genes having positive loadings. To ensure that the time fish were held before processing, and the order in which they were processed, did not affect the expression of immune system genes, I calculated the correlation between sampling order and each immune grouping PC1 score using the 'corr.test' function of the 'psych' package, with the Holm correction applied for multiple comparisons. Next I examined whether variation in the expression levels of each response type were correlated, by calculating the pairwise correlations between PC1 scores as above.

To examine which factors relate to the gene expression levels seen, I fitted residual maximum likelihood (REML) linear mixed models (LMMs) with each immune grouping PC1 score as the response. Fish family was included as a random term to control for underlying differences in expression levels. Enclosure was initially included as an additional random term, but was dropped from the model as it did not account for a significant amount of variation. Maximal models were fitted with sex, reproductive status, *G. arcuatus* presence and *Eda* genotype as factors, and growth rate and K change as continuous variables. Biologically relevant interaction terms were also fitted. Non-significant terms were sequentially dropped from the full model to give a minimum adequate model, and the significance of remaining components was determined by Wald F tests.

## 4.4 Results

Of the 250 fish exposed in Obse, 68 survived to end of the experiment, giving an overall survival rate of 27.2%. All control fish survived. Storm damage resulted in no fish being recovered from one enclosure (25 fish), whilst no fish that were less than 30mm at the start of the experiment (19 individuals) were recovered, suggesting they may have been able to escape from the enclosures. Removing these 'lost' fish gives a survival rate of 33%. Only *G. arcuatus* infections were common on exposed fish, with a prevalence of 17.7% and an average intensity on infected fish of 4.92. Growth rates of control fish were higher than those of exposed fish (control= $0.066 \pm 0.005$  mm/day, exposed= $0.046 \pm 0.003$  mm/day).

| Response      | Factor                                               | F     | df   | p                |
|---------------|------------------------------------------------------|-------|------|------------------|
| <b>Innate</b> | Sex                                                  | 50.72 | 1,55 | <b>&lt;0.001</b> |
|               | Reproductive Condition                               | 43.41 | 1,55 | <b>&lt;0.001</b> |
|               | Growth Rate                                          | 7.86  | 1,55 | <b>0.007</b>     |
|               | K Change                                             | 0.01  | 1,55 | 0.921            |
|               | Sex : Growth Rate                                    | 31.58 | 1,55 | <b>&lt;0.001</b> |
|               | Sex : K Change                                       | 5.58  | 1,55 | <b>0.022</b>     |
|               | Reproductive Condition : Growth Rate                 | 36.36 | 1,55 | <b>&lt;0.001</b> |
|               | Reproductive Condition : K Change                    | 5.92  | 1,55 | <b>0.018</b>     |
| <b>Th1</b>    | No significant factors                               |       |      |                  |
| <b>Th2</b>    | Reproductive Condition                               | 0.58  | 1,60 | 0.449            |
|               | <i>G. arcuatus</i> presence                          | 1.01  | 1,60 | 0.319            |
|               | K Change                                             | 0.03  | 1,60 | 0.863            |
|               | Reproductive Condition : <i>G. arcuatus</i> presence | 5.39  | 1,60 | <b>0.024</b>     |
|               | Reproductive Condition : K Change                    | 5.12  | 1,60 | <b>0.027</b>     |
| <b>Reg</b>    | Sex                                                  | 8.92  | 1,56 | <b>0.004</b>     |
|               | Reproductive Condition                               | 0.14  | 1,56 | 0.710            |
|               | <i>G. arcuatus</i> presence                          | 0.25  | 1,56 | 0.619            |
|               | Growth Rate                                          | 5.02  | 1,56 | <b>0.029</b>     |
|               | K Change                                             | 8.67  | 1,56 | <b>0.005</b>     |
|               | Sex : Growth Rate                                    | 4.55  | 1,56 | <b>0.037</b>     |
|               | Reproductive Condition : <i>G. arcuatus</i> presence | 8.81  | 1,56 | <b>0.004</b>     |
|               | Growth Rate : K Change                               | 9.09  | 1,56 | <b>0.004</b>     |

**Table 4.1:** Summary of the minimum adequate models fitted to gene expression data of fish exposed to natural conditions. ‘Innate’, ‘Th1’, and ‘Regulatory’ response variables are first principal components of the following pairs of genes respectively: *IL-1β* and *TNFα*; *Stat4* and *Tbet*; *FoxP3* and *TGFβ*. Significant p values ( $P < 0.05$ ) are marked in bold.

#### 4.4.1 Growth and Condition

Control individuals grew faster than those exposed to natural conditions ( $F_{1,78}=5.96$ ,  $p=0.017$ ), with control fish growing an average of 0.066 ( $SE \pm 0.005$ ) mm/day and exposed growing 0.046 ( $SE \pm 0.003$ ) mm/day. Males grew slower than females ( $F_{1,59}=15.37$ ,  $p < 0.001$ ). Growth rate varied with *Eda* genotype ( $F_{2,59}=4.80$ ,  $p=0.012$ ), with CL heterozygotes growing slower than

homozygous high or low plated fish. Individuals with a higher condition score at the start of the experiment grew slower ( $F_{1,59}=4.29$ ,  $p=0.043$ ). Fish that were smaller at the start of the experiment grew faster ( $F_{1,59}=15.82$ ,  $p<0.001$ ). This also varied with *Eda* genotype (Figure 4.1,  $F_{2,59}=4.75$ ,  $p=0.012$ ), with CL heterozygotes showing no change in growth rate with start length, but complete or low plated homozygotes showing a decrease.

Change in condition over the course of the experiment differed between control and exposed fish ( $F_{1,78}=6.30$ ,  $p=0.014$ ), with control fish K change = -0.033 ( $\pm 0.025$ ) and exposed fish K change = -0.183 ( $\pm 0.026$ ). Reproductive fish lost more condition than non-reproductive fish ( $F_{1,64}=13.63$ ,  $p<0.001$ ). Loss of condition increased with start K ( $F_{1,64}=231.54$ ,  $p<0.001$ ), such that individuals in good condition at the start lost more condition over the course of the experiment.

#### **4.4.2 Control vs. Exposed Expression**

The expression profiles of exposed fish differed to those of the control fish (Figure 4.2, MANOVA  $F_{8,78}=5.86$ ,  $p<0.001$ ). This difference was driven by variation in the expression of *TNF $\alpha$*  ( $F_{1,78}=5.64$ ,  $p=0.02$ ), *Stat6* ( $F_{1,78}=8.55$ ,  $p=0.005$ ), *CMIP* ( $F_{1,78}=8.50$ ,  $p=0.005$ ), *FoxP3* ( $F_{1,78}=15.41$ ,  $p<0.001$ ) and *TGF $\beta$*  ( $F_{1,78}=10.03$ ,  $p=0.002$ ), but not the expression of *IL-1 $\beta$*  ( $F_{1,78}=1.42$ ,  $p=0.237$ ), *Stat4* ( $F_{1,78}=0.05$ ,  $p=0.831$ ) or *Tbet* ( $F_{1,78}=2.23$ ,  $p=0.140$ ). All significant differences involved down regulation of expression in fish in enclosures.

#### **4.4.3 Expression Patterns in Exposed Fish**

All immune response groupings were found to have significant positive correlations (Figure 4.3), with the exception of the innate and Th1-type

responses ( $r^2=0.08$   $p=0.53$ ). No correlations were found between sampling order and innate ( $r^2=-0.05$ ,  $p=1$ ), Th1-type ( $r^2=0.07$ ,  $p=1$ ), Th2-type ( $r^2=0.07$ ,  $p=1$ ), or regulatory ( $r^2=0.06$ ,  $p=1$ ) expression levels, indicating that sampling order had no effect on gene expression levels.

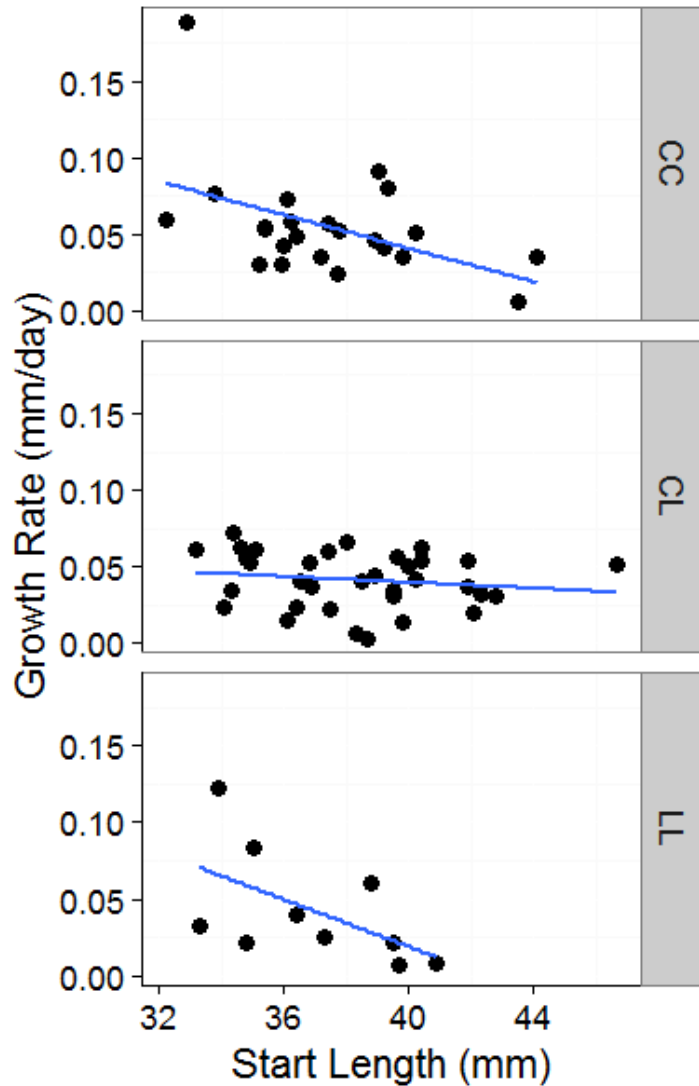


**Figure 4.1:** Growth rates (mm/day) of fish homozygous for *Eda* (genotype CC or LL) were lower in fish that were larger at the start of the experiment, whilst there was no change in the growth rates of *Eda* heterozygous fish (genotype CL).

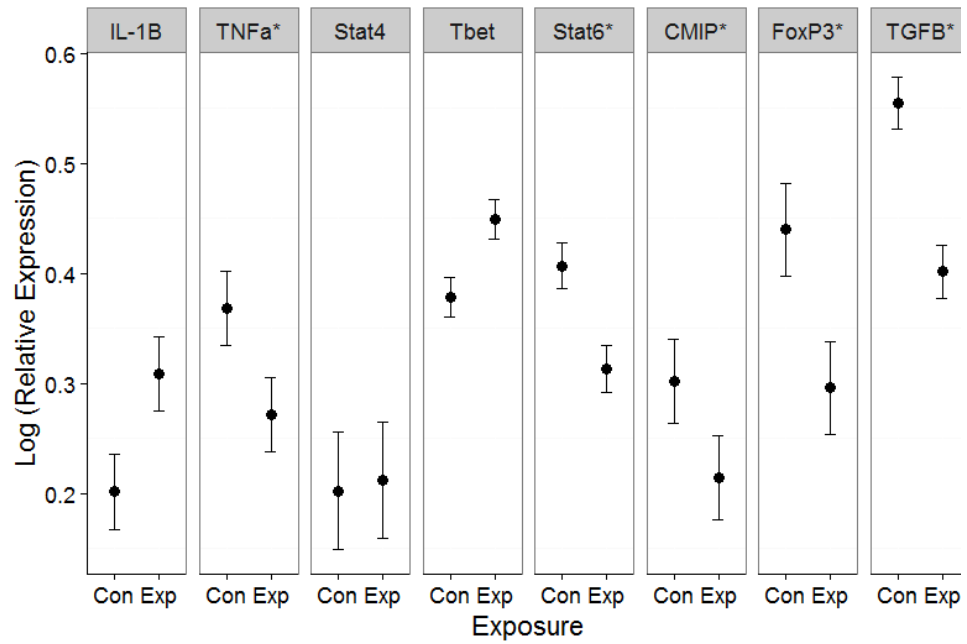


**Figure 4.2:** Gene expression levels (Mean Log(Relative Expression)  $\pm$  SE) of eight immune system genes, compared between control ('Con') fish, and those exposed ('Exp') to natural conditions.

A summary of the minimum adequate model fitted to each response type is shown in Table 4.1. The PC1 of innate measures explaining 90.0% of variation, with loadings of  $IL-1\beta = 0.964$  and  $TNF\alpha = 0.266$ . Females had higher innate expression levels than males ( $F_{1,55}=50.72$ ,  $p<0.001$ ), as did reproductive relative to non-reproductive fish ( $F_{1,55}=43.41$ ,  $p<0.001$ ). Individuals who grew faster had lower innate expression ( $F_{1,55}=7.86$ ,  $p=0.007$ ). This effect varied with both sex (Figure 4.4,  $F_{1,55}=31.58$ ,  $p<0.001$ ) and reproductive status ( $F_{1,55}=36.36$ ,  $p<0.001$ ), such that the decline in expression with growth was steeper for females relative to males, and for non-reproductive relative to reproductive individuals. The relationship between K change and innate expression also varied with sex ( $F_{1,55}=5.58$ ,  $p=0.022$ ) and reproductive status ( $F_{1,55}=5.92$ ,  $p=0.018$ ), with no change in expression with K

change in males and non-reproductive individuals, but females and reproductive individuals that gained condition had higher expression levels.

Th1-type measures had a PC1 explaining 60.7% of variation, with loadings of *Stat4* = 0.452 and *Tbet* = 0.892. No significant terms were found in the Th1-type model. The PC1 of Th2-type measures explained 64.8% of variation, with loadings of *Stat6* = 0.911 and *CMIP* = 0.41. The effect of *G. arcuatus* infection varied with reproductive condition (Figure 4.5,  $F_{1,60}=5.39$ ,  $p=0.024$ ), with non-reproductive individuals having the same expression levels with infection, but reproductive individuals showing a decrease in Th2-type expression levels with infection. The relationship between K change and Th2-type expression also varied with reproductive status ( $F_{1,60}=5.12$ ,  $p=0.027$ ). In non-reproductive individuals, those that gained condition had lower expression levels than those which lost condition, whilst the opposite pattern was true in reproductive fish.

PC1 of regulatory measures explained 69.4% of variation and had loadings of *FoxP3a* = 0.123 and *TGF $\beta$*  = 0.992. Females had higher expression levels than males ( $F_{1,56}=8.92$ ,  $p=0.004$ ). Individuals that grew faster had lower expression levels ( $F_{1,56}=5.02$ ,  $p=0.029$ ), and this varied between sexes (Figure 4.4,  $F_{1,56}=4.55$ ,  $p=0.037$ ) with a bigger decrease in expression with growth in females than in males. Individuals that gained condition had higher expression levels than those which lost condition ( $F_{1,56}=8.67$ ,  $p=0.005$ ), and the relationship between K change and regulatory expression also varied with growth ( $F_{1,56}=9.09$ ,  $p=0.004$ ), with the difference in expression levels increasing with growth. The effect of *G. arcuatus* infection varied with reproductive condition (Figure 4.5,  $F_{1,56}=8.81$ ,  $p=0.004$ ), with infected individuals having higher expression when not in breeding condition, but lower expression when in breeding condition.

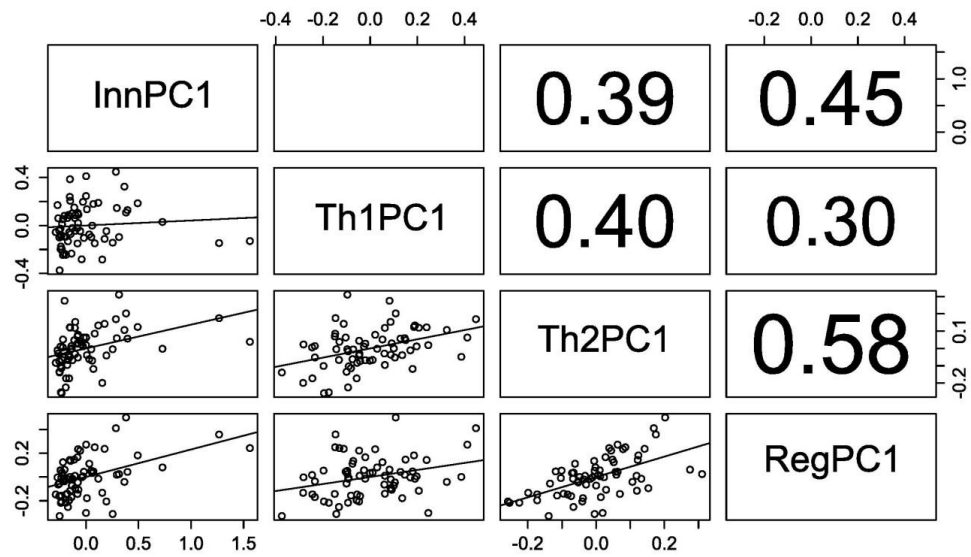


**Figure 4.3:** Pairwise correlations of expression levels of four functional arms of the immune system, with scatter plots and linear best fit lines below the diagonal, and  $r^2$  values of significant correlations (adjusted  $p < 0.001$ ) above the diagonal. Gene expression levels are the first principal component scores of the pair of genes representing each immune response type: 'InnPC1' - innate genes *IL-1 $\beta$*  and *TNF $\alpha$* ; 'Th1PC1' - Th1-type adaptive genes *Stat4* and *Tbet*; 'Th2PC1' - Th2-type adaptive genes *Stat6* and *CMIP*; 'RegPC1' – regulatory genes *FoxP3* and *TGF $\beta$* .

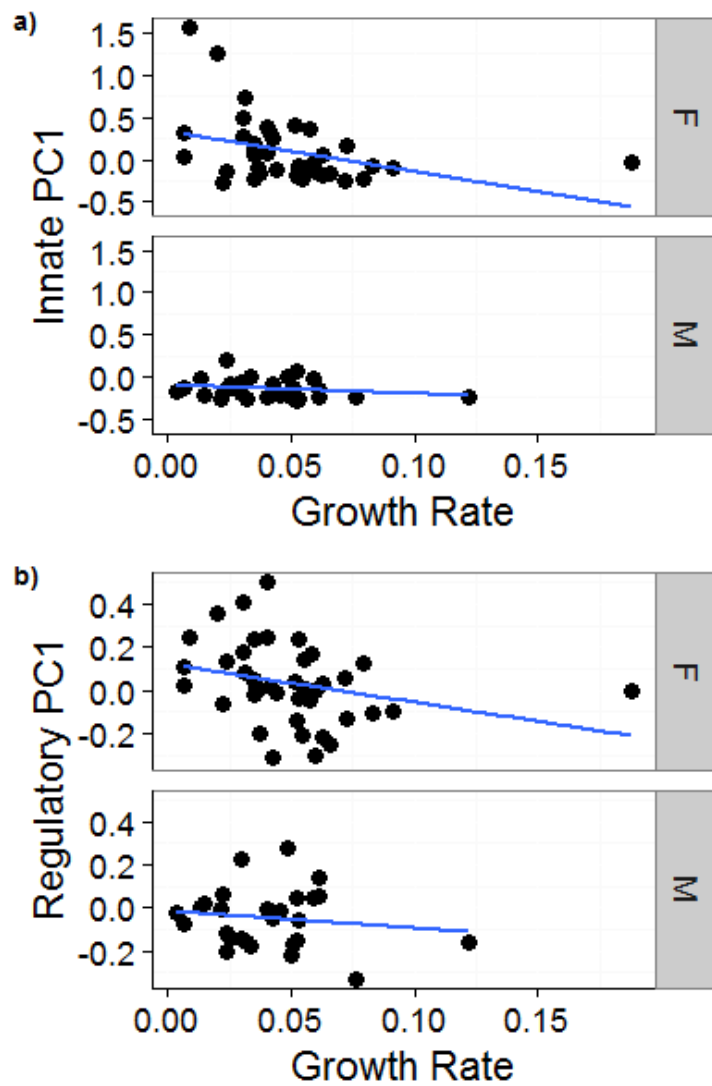


**Figure 4.4:** The relationship between the expression levels of the a) innate and b) regulatory immune responses and growth rate (mm/day) varies between the sexes. Males show a slight decrease in expression levels as growth rate increases, whilst there is a significant decrease in expression levels as growth rate increases in females. Innate and regulatory response PC1 axes represent the expression levels of the following pairs of genes respectively: *IL-1 $\beta$*  and *TNF $\alpha$* ; *FoxP3* and *TGF $\beta$* .

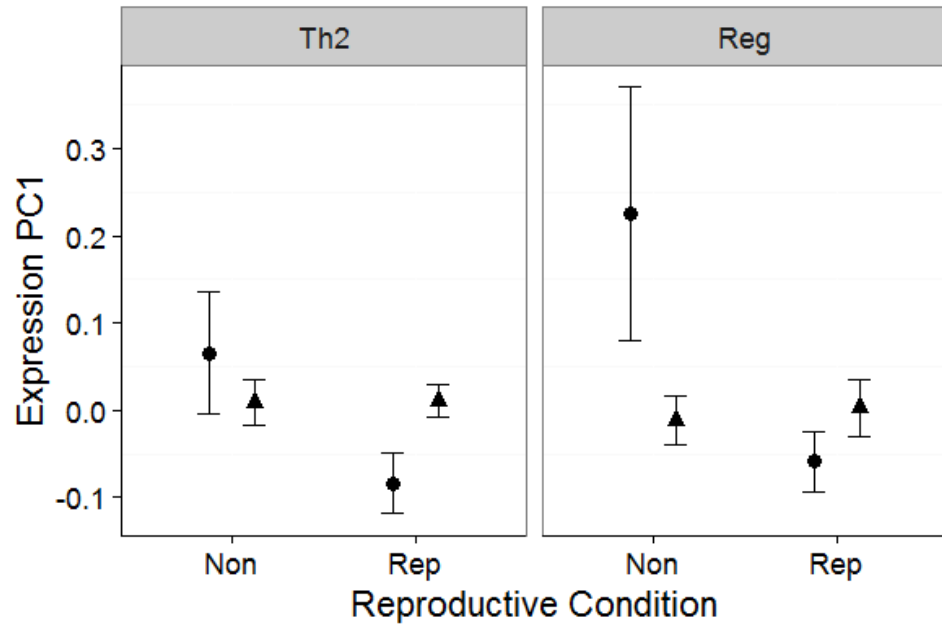


**Figure 4.5:** The Th2-type adaptive ('Th2') and regulatory ('Reg') immune gene expression levels of individuals in reproductive ('Rep') and non-reproductive ('Non') condition vary with *G. arcuatus* infection (● – Infected, ▲ - Uninfected). Th2 and Reg PC1 axes summarise the expression levels of the following pairs of genes respectively: *Stat6* and *CMIP*; *FoxP3* and *TGFβ*. High PC1 scores indicate higher expression levels of both genes.

## 4.5 Discussion

The work presented represents a novel immunological study in the wild, by measuring the response of multiple functional arms of the immune system in naïve fish exposed to natural conditions. By individually marking each fish we were able to track their growth and change in condition, and investigate which factors contribute to shaping the immune response in a natural context. During the experiment, overall survival at the end was 33%, which sits between recapture rates in other enclosure based experiments in stickleback of 23% (Stutz *et al.* 2015) and 90% (Rauch *et al.* 2006). Storm damage may have allowed some fish to escape from the enclosures, so this survival rate is a minimum estimate. Of the fish that were recaptures, infection with the dominant parasite species had established at a low level, and we were able to get high quality RNA samples to allow gene expression measurements to be made.

Exposing fish to natural conditions results in an overall change in the expression of immune system genes, driven by lower expression levels of the innate, Th2-type adaptive and immune suppressive (regulatory) responses in the exposed fish. Due to logistical constraints, control fish had to be kept in laboratory conditions in Nottingham rather than experience similar natural conditions similar to the exposed group. Ideally the control individuals would have undergone the same transport and exposure to natural conditions mirroring the exposed group as closely as possible, with the exception of exposure to *G. arcuatus*. A decrease in expression in exposed fish seems counter intuitive, as exposure to immune challenge in a natural setting would have been expected to result in up-regulation of the expression of immune system genes. This decrease could be a result of the release of stress

hormones, which can increase immune activity in fish in the short term (Demers & Bayne 1997; Fast *et al.* 2008) but tends to be suppressive in the long term (Castillo *et al.* 2009; Harris & Bird 2000; Watanuki *et al.* 2000), or the result of limited energetic resources being diverted from immune function to other costly metabolic processes. The difference we see between the exposed and control fish could thus be the result of the stress of movement and experimental exposure, rather than a direct response to the new conditions experiences. In the exposed fish, rather than seeing the expected trade-off between costly traits sharing a common resource pool (Stearns 1992), we instead find a positive correlation between immune gene expression levels. This fits the prediction of Van Noordwijk and de Jong (1986) that individuals within a population acquire different levels of resources and thus individuals with more resources can allocate more to all traits.

Both sex and reproduction can influence the function of the immune system of teleost fish (Cuesta *et al.* 2007; Magnadottir 2006, 2010). Here we found that males had lower innate and regulatory expression levels than females, although this pattern may be confounded by differences in reproductive status. No fish showed signs of investing in attaining reproductive condition at the start of the experiment, so reproductive status at the end represents investment made over the course of the experiment. At the end of the experiment, all males, but only 40% of females, were ready to breed. Getting ready to breed is energetically costly, so there may be a trade-off between attaining breeding condition and immune function, and this cost may vary between the sexes. Changes that occur during reproduction, particularly in circulating hormone levels, can affect immune system function (Cuesta *et al.* 2007; Downs *et al.* 2014; Maule *et al.* 1996), which could account for the sex differences observed. Repeating this experiment at different times of the year

could show whether there are general underlying differences between the sexes, or whether these changes are a result of investment in breeding.

In wild stickleback, infection with *G. arcuatus* is associated with an increase in innate and regulatory immune gene expression levels (Chapter 2 & 3; Robertson *et al.* 2015, 2016). In this instance, an effect of infection with *G. arcuatus* on Th2-type and regulatory expression was seen, which varied with reproductive condition. This pattern could again represent different strategies to maximise the limited energy resources available to each individual, with infected individuals in breeding condition having lower Th2-type expression levels and infected non-breeding individuals having higher regulatory expression levels. The direction of causality of such relationships is, however, not clear. The change in expression levels could be a response to infection, or changes in the immune system as a consequence of breeding could make individuals more vulnerable to infection. Controlled experiments in a laboratory setting are required to better understand the relationship between *G. arcuatus* infection and immune system function.

Growth is another energetically costly activity. We found that individuals which grew faster had lower innate and regulatory gene expression levels, which may represent a trade-off between growth and immune system function. Growth rate itself also varied, with control fish growing faster than exposed fish. This would be expected given the relatively benign conditions experienced by the control group. Males grew less than females, which again may relate to the cost of attaining breeding condition. Growth rate varied between *Eda* genotypes, with heterozygous fish growing slower than high or low plated homozygous fish. Fish with low plated *Eda* genotypes have previously been found to grow faster as juveniles, but this advantage disappears in adult fish (Barrett *et al.* 2009; Barrett *et al.* 2008). Here, we see

an apparent disadvantage to being an *Eda* heterozygote, even though the low plated *Eda* allele is not adaptive in the loch which fish were exposed in.

In conclusion, I exposed naïve laboratory raised fish to natural conditions in order to provide novel insights into the function of the immune system in the wild. The expression profiles of exposed and control fish differed, showing that the environment experienced can drive changes in the immune response. A range of factors were found to relate to immune gene expression levels in the exposed individuals, including reproductive investment, growth rate, and infection with the common parasite *G. arcuatus*. *Eda* genotype was associated with variation in growth rates, with an apparent disadvantage to heterozygotes. The results show that the patterns observed in previous work sampling wild individuals are not just the result of differences in experience prior to capture, but can be driven by the abiotic environment, and add to our growing understanding of the function of the immune system in a natural setting.

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## Chapter 5: Pleiotropic effects of the *Eda* locus are associated with immune variation

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### 5.1 Abstract

In previous chapters I measured immune gene expression and looked for associations with a range of factors which may shape expression levels. In this chapter, I used these assays to look for a link between a gene underlying a major adaptive change, *Ectodysplasin* (*Eda*), and the function of the immune system. Genes underlying adaptive changes are expected to be highly pleiotropic, but possible links with the immune system have never been investigated. The *Eda* gene directly controls bony lateral plate architecture and has been associated with a range of adaptations to freshwater. I used F2 hybrid fish to separate *Eda* genotype from genetic background, and exposed them to contrasting conditions in semi-natural enclosures. The low plated *Eda* allele was associated with lower expression of a marker of the Th1-type adaptive response, and higher expression of markers of the innate and regulatory responses. Furthermore, low plated fish grew less and suffered higher burdens of a common ectoparasite. This chapter demonstrates the potential for additional effects of adaptive genes, which may themselves in turn have adaptive consequences, and demonstrates the potential of these markers to address a wide range of biological questions.

## 5.2 Introduction

Evolutionary theory gives good reason to expect that genetic loci of large effect that are involved in adaptation and speciation will be highly pleiotropic, with effects on many additional aspects of an organisms phenotype even when they do not directly affect mate preference or reproductive isolation (Barton & De Cara 2009; Felsenstein 1981; Seehausen *et al.* 2014). Such effects can result from individual genes that are themselves pleiotropic, or because selection favours strong linkage disequilibrium and ultimately physical linkage of different genes that contribute to divergent adaptive phenotypes (Barton & De Cara 2009). This potentially results in a ‘supergene’ region, the close linkage and inheritance of gene with evolutionarily related function (Smith 1989). Understanding of such phenomena remains poor because of the difficulty in identifying the causal genetic variants driving adaptive phenotypic variation, which has long been a holy grail of evolutionary genetics. Big strides have been made in this enterprise in recent years, particularly with the advent of modern sequencing technology. A number of examples now exist across a range of taxa where the region or gene underlying phenotypic variation has been identified, including the *Agouti* locus for colour polymorphisms in deer mice (Linnen *et al.* 2009), the *RXFP2* gene for horn architecture in Soay sheep (Johnston *et al.* 2013; Johnston *et al.* 2011), the *Optix* gene controlling wing pattern in *Heliconious* butterflies (Reed *et al.* 2011), and two genes controlling petal colour morphs in annual phlox (Hopkins & Rausher 2011). The full range of adaptive consequences of these genes, however, is not yet understood.

One of the best documented examples of a gene underlying adaptive evolution is *Ectodysplasin* (*Eda*) in the three-spined stickleback, a small

teleost fish found throughout the northern hemisphere. The *Eda* gene controls a polymorphism in lateral ectodermal bony ('armour') plates (Colosimo *et al.* 2005). Two major alleles are present; the ancestral *Eda*<sup>C</sup> allele ('C') and the derived *Eda*<sup>L</sup> allele ('L'). Across the Holarctic, marine populations are commonly homozygous for the ancestral allele and have a full row of 32-36 plates, although the low plated allele is present at a low frequency (Colosimo *et al.* 2005). Many independently derived freshwater populations throughout the stickleback's range are homozygous for the derived allele and are low plated, with less than 10 anterior plates only (Bell & Foster 1994; Jones *et al.* 2012a; Jones *et al.* 2012b). The *Eda* locus accounts for approximately 80% of observed variation in plate number (Colosimo *et al.* 2004), with heterozygous individuals typically having an intermediate phenotype. Selection on *Eda* can be very strong (Barrett *et al.* 2008) and evolution can be extremely rapid (Bell *et al.* 2004). Numerous selective agents have been suggested, with predation probably at least partly driving change (Marchinko 2009; Nosil & Crespi 2006). Whilst there is strong selection on plate phenotype, the *Eda* gene itself experiences additional selection above this (Rennison *et al.* 2015), possibly as a result of pleiotropic effects of *Eda* or through linked genes (Barrett *et al.* 2009b; Mills *et al.* 2014).

The immune system represents a key defence against infection in vertebrates, but the potential for association between *Eda* genotype and immune phenotype has not been investigated. Stickleback experience major changes in the parasites they encounter when they colonise freshwater, and these have the potential to be a major selective agent (MacColl 2011; MacColl & Chapman 2010). Furthermore, several genes now in tight physical linkage with *Eda* have major roles in the function of the immune system in other vertebrates (Colosimo *et al.* 2005). These include *Baff* (*TNFSf13b*), a B cell

activating factor (Mackay & Browning 2002; Sun & Sun 2015), *Garp*, which is involved in *TGF $\beta$*  expression and regulation of T cell function (Chan *et al.* 2011; Probst-Kepper & Buer 2010; Tran *et al.* 2009), and *Dusp1*, which plays a role in the control of inflammation (Arthur & Ley 2013; Hammer *et al.* 2010). The presence of genes with major roles in the immune system in tight linkage with *Eda* provides a potential mechanism by which the function of the immune system could change in concert with *Eda* during adaptation.

I performed a common garden experiment in the field to examine the relationship between *Eda* genotype and immune system function, using F2 hybrids between two populations with contrasting *Eda* genotypes (and thus plate phenotypes). Fish were selected from two lake populations, a high plated saltwater resident population, Loch Ob nan Stearnain ('Obse') and a nearby low plated freshwater population, Loch à Chadha Ruaidh ('Chru'), on North Uist, Scotland. These populations show stable contrasts in parasite communities. Fish in Obse are typically infected with high levels of *Gyrodactylus arcuatus*, a monogenean ectoparasite that attaches to stickleback fins, skin and gills, whilst fish in Chru show high levels of infection with the digenean trematode *Diplostomum gasterostei*, which penetrates the skin and migrates to the eye. Both of these parasites have fitness consequences for infected hosts (Brassard *et al.* 1982; De Roij *et al.* 2011; Owen *et al.* 1993), and could therefore have significant impacts on host fitness in their respective habitats.

By using F2 crosses, the genetic background of each individual was randomised whilst creating fish with the three possible *Eda* genotypes, allowing me to focus on *Eda* genotype whilst controlling for possible confounding effects of genetic background or maternal effects (Lexer *et al.* 2003; Rauch *et al.* 2006). I exposed the fish to the two lakes where their

grandparents were caught by placing them into replicated semi-natural enclosures. After 4 weeks of exposure, I measured the expression levels of a set of eight genes which give an overview of the function of the immune system (Chapter 2; Robertson *et al.* 2015), representing the innate pro-inflammatory response (*IL-1 $\beta$*  and *TNF $\alpha$* ), Th1-type adaptive response (*Stat4* and *Tbet*), Th2-type adaptive response (*Stat6* and *CMIP*) and immune regulatory response (*TGF $\beta$*  and *FoxP3*). By employing this specific experimental design, I was able to examine the link between *Eda* genotype and immune gene expression levels under natural conditions, in two contrasting environments. This represents the first study to examine the link between a gene underlying adaptive phenotypic evolution and the key defences of an organism against potentially harmful pathogens and parasites, and adds to our growing understanding of the importance of unexpected additional effects of genes underlying adaptive evolution through pleiotropy or linkage disequilibrium.

## 5.3 Methods

### 5.3.1 Fish Crossing

I created eight F1 families in May 2011 by crossing individuals from Loch Ob nan Stearnain ('Obse', 57°36'9"N; 7°10'19"W), a saltwater lagoon containing fully armour plated resident fish, with individuals from Loch à Chadha Ruaidh ('Chru', 57°35'38"; 7°11'51"W), a freshwater population with low armour plating, both located on North Uist, Scotland. Each population contributed an even number of males and females, and crosses were performed following the procedure of De Roij *et al.* (2011). Fertilized eggs were transported to aquaria at the University of Nottingham, with families split between multiple tanks after

hatching to ensure all fish were maintained at the same density. Fish were kept in a temperature controlled room, with a lighting regime mirroring the natural photoperiod on North Uist. Four F2 families were then created in May 2012 by crossing F1 fish from unrelated families, following the same method.

### **5.3.2 Experimental Exposure**

Five fish from the first three families, and four from the fourth family, were randomly assigned to each of ten treatment groups, giving 19 fish per group and 190 fish in total. Five groups were exposed to semi-natural conditions in Chru, and five in Obse. Prior to exposure, I tagged all fish with visible implant elastomer tags (VIE, Northwest Marine Technology) so that individuals could be identified within a treatment group. Tagging was performed two months prior to the start of the experiment to allow sufficient time to recover, and to ensure that immune response was not affected by the tagging procedure (Henrich *et al.* 2014). Individual length and weight were recorded at the time of tagging. As Obse is a saltwater lagoon, fish assigned to an Obse enclosure were gradually acclimatised to saltwater conditions two months prior to the start of the experiment (from 2 parts per thousand [ppt] to 20 ppt), and all fish were kept in daylight conditions mirroring those on North Uist.

Fish were transported to North Uist in May 2013. I constructed ten enclosures using 6 mm square mesh, with each enclosure being cylindrical in shape, 90 cm long and 68 cm in diameter, giving a total volume of 330 cm<sup>3</sup>. This mesh size was selected to contain fish within the enclosures whilst still allowing the free flow of water and suspended materials. All fish from a treatment group were added to a single enclosure.

After 4 weeks, all fish were removed from a single, randomly selected, enclosure at a time. Fish were transported to the laboratory in darkened boxes

containing fresh loch water, with air supplied from a battery powered pump, and processed fish in a random order within two hours of collection. Fish were euthanized by overdose of MS222 (Sigma-Aldrich) followed by destruction of the brain, in accordance with UK Home Office regulations, then measured and weighed. The spleen, an immunologically important tissue in fish (Zapata *et al.* 2006), was removed and immediately placed in RNAlater (Life Technologies). Each fish was identified using its unique VIE tag, and I identified and counted macroparasites under a dissection microscope. Sex and reproductive status was determined by examination of the gonads. Fish were split into three reproductive status categories, reflecting the investment made over the course of the experiment: no apparent investment in reproduction (Small testes and kidneys in M, no enlarged ovules in F), investment in attaining reproductive condition (Enlarged testes but small kidneys in M, some enlarged ovules in F), or ready to breed (Enlarged testes and kidneys in M, ovules enlarged and ready to be laid in F). A sample of fin tissue was removed and stored in 100% ethanol for *Eda* genotyping.

### **5.3.3 Eda Genotyping**

Individuals were genotyped using *Stn382* primers, which flank an indel polymorphism in intron 1 of the *Eda* gene (Colosimo *et al.* 2005). DNA was extracted from fin tissue using DirectPCR Lysis Reagent for Mouse Tails (Viagen Biotech) by incubating for 12 hours at 55°C, followed by 45 minutes at 85°C. PCR reactions were performed in a 20µl total reaction volume containing 10µl BioMix Red Mastermix (Bioline), 6µl H<sub>2</sub>O, 1µl forward and 1µl reverse primer at 10µM, and 2µl template DNA, with an initial step of 94°C for 4 minutes, followed by 35 cycles of 94°C for 15 seconds, 60°C for 15 seconds and 72°C for 15 seconds, and a final extension of 72°C for 4 minutes. PCR products were visualised on a 2% Agarose gel containing Ethidium Bromide,

with the complete plated allele giving a product 218bp in length, and the low plated allele 150bp in length.

#### **5.3.4 Gene Expression Quantification**

I extracted RNA from Spleens using the GeneJET RNA purification kit (Thermo Scientific) according to the manufacturers' standard protocol. Purity of RNA was assessed on a NanoDrop 1000 spectrophotometer (Thermo Scientific), with a desired 260/280 absorbance ratio >1.80. RNA integrity was assessed by incubating 5µl of each sample at 65°C for 10 minutes, followed by visualisation on a 2% Agarose gel stained with Ethidium Bromide. All samples were DNase treated using Precision DNase (Primer Design), following the manufacturers' protocol. Reverse transcription reactions were performed on approximately 1.5µg of total RNA using the nanoScript2 RT kit (Primer Design), with a combination of random nanomer and oligo-dT priming, following the manufacturers' protocol. Periodic no-enzyme controls indicated that genomic DNA contamination was negligible. All cDNA samples were diluted 1:10 with nuclease free water before further use.

From the samples available, a subset of 45 was selected for inclusion in the gene expression analysis study, with 26 samples from fish exposed in Chru (*Eda* genotype n: CC=7, CL=14, LL=5) and 19 from Obse (*Eda* genotype n: CC=5, CL=10, LL=4). qPCR reactions were performed in 10µl total volumes, containing 5µl of PrecisionFAST mastermix with SYBR green (Primer Design), 0.25µl of each primer, 2µl of template cDNA and 2.5µl of H<sub>2</sub>O in 96-well optical PCR plates with optical seals (StarLab). Reactions were performed in an ABI 7500 FAST real-time thermocycler (Applied Biosystems) with an initial step of 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All runs included a post-PCR melt curve analysis.

Accurate normalization is essential for the production of reliable data from qPCR experiments, with the optimal reference genes being specific to a particular set of experimental conditions (Dheda *et al.* 2005). In order to select the most appropriate reference genes for this study, I performed a geNorm analysis on six candidate reference genes (*B2M*, *GAPDH*, *RPL13A*, *HPRT1*, *TBP* and *TOP1*) using a custom stickleback geNorm kit for SYBR green (Primer Design), following the manufacturers standard protocol. Analysis of the stability of expression was performed in qbase+ (Biogazelle) with *HPRT1* and *TBP* identified as the most stable combination of reference genes. Along with these two reference genes, I measured the expression levels of eight genes of interest (Chapter 2; Robertson *et al.* 2015). Relative expression values were calculated using the  $\Delta\Delta C_q$  method (Pfaffl 2001), adjusted for the amplification efficiencies of each primer pair and standardised against the geometric mean  $C_q$  of the two reference genes for each sample (Vandesompele *et al.* 2002).

### 5.3.5 Data Analysis

All gene expression data was  $\log_{10}(x+1)$  transformed prior to analysis, due to the inherently skewed distribution of relative expression data. I performed all analyses in R v.3.2.2 (R Core Team 2014).

*EDA genotype and immune gene expression.* To examine whether there was a relationship between *Eda* genotype and gene expression levels, I fitted a MANOVA with relative expression values as the response. Enclosure location, *Eda* genotype and their interaction were fitted as factors, with overall differences assessed using the Pillai method. For significant terms, the effect on each immune response type was then examined separately using ANOVA. As *Eda* genotype had three levels, we achieved the most parsimonious model

by combining levels which did not differ significantly in post-hoc Tukey HSD tests where appropriate.

*Survival and parasite infection dynamics.* The infection levels of the most common parasites in the study populations, *G. arcuatus* and *D. gasterostei*, were compared between the experimental fish in the enclosures and wild fish caught in the same location during the experiment (MacColl, unpublished data), along with survival rates of experimental fish in each location. Infection levels of *G. arcuatus*, on fish exposed in Obse, were compared between fish differing in armour plating. A generalised linear model (glm) was fitted with plate level as a factor (high or low plated) and infection intensity (parasite burden) as the response, with Poisson error structure and log link function. Significance of model components was calculated using  $\chi^2$  likelihood ratio tests.

*Growth variation.* As individual fish were marked, I could examine whether *Eda* genotype or other measured factors related to growth over the course of the experiment. A glm was fitted with growth rate as the response. Maximal models were fitted with enclosure location (2 levels), sex (2 levels), reproductive status (3 levels) and *Eda* genotype (3 levels) as factors. Non-significant terms were sequentially dropped to give a minimum adequate model, and the significance of remaining components was determined by Wald F-tests. To achieve the most parsimonious model, we combined levels within the reproductive status or *Eda* genotype factors (those with 3 levels) when it was appropriate to do so. If two levels of the factor did not differ from each other, the data was recoded so these levels were combined. The change in variance of the two models was tested using the F statistic, with the simpler model retained if the two models did not differ significantly. If a simpler model was retained, we recalculated the significance of all components in the model.

## 5.4 Results

### 5.4.1 *Eda* Genotype and Immune Gene Expression

We found large differences in immune gene expression levels between fish with different *Eda* genotypes (Figure 5.1). Overall expression profiles differed between *Eda* genotypes (MANOVA  $F_{2,39}=2.02$ ,  $p=0.024$ ), and between fish exposed in enclosures in different lochs (MANOVA  $F_{1,39}=20.10$ ,  $p<0.001$ ), but the effect of *Eda* did not change with enclosure location (MANOVA  $F_{2,39}=1.11$ ,  $p=0.367$ ).

The overall difference in expression between *Eda* genotypes was driven by underlying expression patterns of *IL-1 $\beta$*  (ANOVA  $F_{2,39}=4.98$ ,  $p=0.012$ ), *Tbet* (ANOVA  $F_{2,39}=4.00$ ,  $p=0.026$ ) and *FoxP3* (ANOVA  $F_{2,39}=4.60$ ,  $p=0.016$ ), as shown in Figure 5.1, but not *TNF $\alpha$*  (ANOVA  $F_{2,39}=1.69$ ,  $p=0.197$ ), *Stat4* (ANOVA  $F_{2,39}=0.91$ ,  $p=0.410$ ), *Stat6* (ANOVA  $F_{2,39}=0.49$ ,  $p=0.619$ ), *CMIP* (ANOVA  $F_{2,39}=2.15$ ,  $p=0.131$ ), or *TGF $\beta$*  (ANOVA  $F_{2,39}=1.89$ ,  $p=0.165$ ). *IL-1 $\beta$*  expression levels were highest in heterozygous fish (genotype CL) and lowest in high plated CC fish, with low plated LL fish having an intermediate expression level. Fish with one or more high plated allele (genotype CC or CL) had higher *Tbet* expression than low plated LL fish. There was an additive change in *FoxP3* expression levels, with LL fish having the highest expression, CC fish the lowest, and CL fish expressing *FoxP3* at an intermediate level.

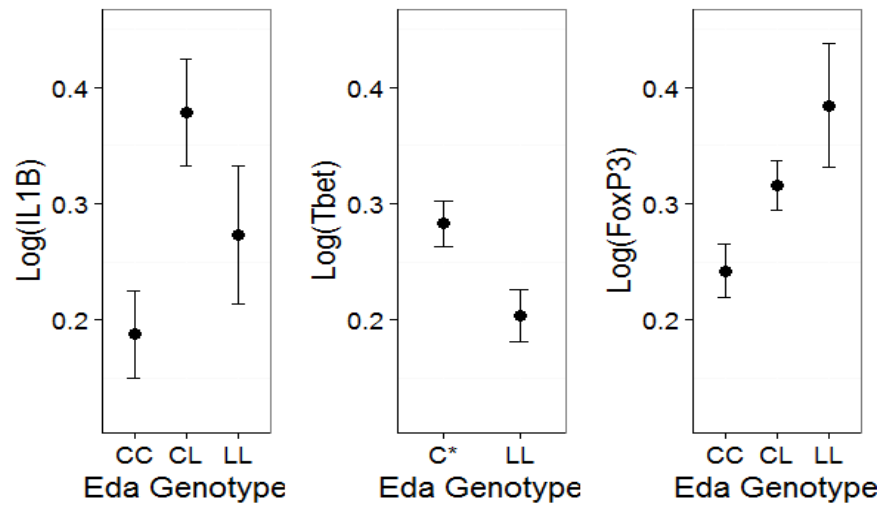


**Figure 5.1:** Relative expression levels (mean  $\pm$  SE) of three immune system genes vary with *Eda* genotype. *Eda* has two alternative alleles, denoted ‘C’ for the complete plated allele and ‘L’ for the derived low plated allele. For *Tbet* expression, individuals with the CC and CL genotypes have been combined to provide the most parsimonious model, and denoted ‘C\*’, as their expression levels did not differ significantly.

Fish exposed in Obse had higher *IL-1 $\beta$*  (ANOVA  $F_{1,39}=8.26$ ,  $p=0.007$ ), *TNF $\alpha$*  (ANOVA  $F_{1,39}=12.93$ ,  $p<0.001$ ), *Stat6* (ANOVA  $F_{1,39}=15.25$ ,  $p<0.001$ ) and *TGF $\beta$*  (ANOVA  $F_{1,39}=33.14$ ,  $p<0.001$ ) expression levels than those in Chru, whilst expression of *Tbet* was higher in Chru than Obse (ANOVA  $F_{1,39}=53.79$ ,  $p<0.001$ ), possibly reflecting the different challenges faced in these two contrasting environments. Expression levels of *Stat4* (ANOVA  $F_{1,39}=0.14$ ,  $p=0.712$ ), *CMIP* (ANOVA  $F_{1,39}=0.01$ ,  $p=0.975$ ) and *FoxP3* (ANOVA  $F_{1,39}=0.32$ ,  $p=0.574$ ) did not differ with enclosure location.

| Location             | n  | <i>G. arcuatus</i><br>Prevalence | <i>G. arcuatus</i><br>Intensity | Diplostomum<br>Prevalence | Diplostomum<br>Intensity |
|----------------------|----|----------------------------------|---------------------------------|---------------------------|--------------------------|
| Chru<br>Experimental | 52 | 0                                | 0                               | 5.8% (1.2-<br>16.0%)      | 1 ( $\pm 0$ )            |
| Chru<br>Wild         | 56 | 0                                | 0                               | 71.4% (57.8-<br>82.7%)    | 3.7 ( $\pm 0.4$ )        |
| Obse<br>Experimental | 34 | 67.6% (49.5-<br>82.6%)           | 5 ( $\pm 0.9$ )                 | 0                         | 0                        |
| Obse<br>Wild         | 55 | 92.7% (82.4-<br>98.0%)           | 10.1 ( $\pm 1.2$ )              | 0                         | 0                        |

**Table 5.1:** Infection data of two common stickleback parasites, *Gyrodactylus arcuatus* and *Diplostomum gasterostei*, in both wild fish ('Wild') and fish experimentally exposed to natural conditions ('Experimental') in Chru and Obse. Prevalence was calculated as the percentage of infected fish, with 95% binomial confidence intervals in brackets. Intensity is the mean number of parasites on infected individuals, with standard errors.

#### 5.4.2 Survival and Parasite Infection Dynamics

Over the course of the four week experiment, storm damage in both locations may have allowed some fish to escape, so survival data are minimum estimates. Of the 95 fish exposed in Chru, 52 (54.7%) survived to the end of the experiment. In Obse, 95 fish were also exposed, but storm damage resulted in no fish being recovered from one enclosure. From the remaining enclosures, 34 fish survived (44.7%).

Fish were infected with *G. arcuatus* in Obse and *D. gasterostei* in Chru, but infection levels were lower than those found in wild fish (Table 5.1). Low plated fish were infected with a higher number of *G. arcuatus* than high plated

fish (Figure 5.2,  $\chi^2=6.04$ ,  $p=0.014$ , mean high plated $\pm$ SE=2.96 $\pm$ 0.65, low plated=4.88 $\pm$ 2.14). As only 5.8% of fish were infected with *D. gasterostei* in Chru, it was not possible to test for any effects of *Eda* genotype.

### 5.4.3 *Eda* Genotype and Growth

Growth rates differed between locations (t test,  $t_{41}=2.12$ ,  $p=0.040$ ), with fish in Chru growing faster (0.084 $\pm$ 0.007 mm/day) than fish in Obse (0.063 $\pm$ 0.007 mm/day). Fish with two complete *Eda* alleles (genotype CC) grew faster than fish with a low plated allele (genotype CL or LL) irrespective of enclosure location (Figure 5.3, Wald  $F_{1,38}=6.95$ ,  $p=0.012$ ). There was also a difference in growth rates between populations that was dependent upon reproductive status (Wald  $F_{2,38}=3.93$ ,  $p=0.028$ ), with individuals ready to breed growing slower in Chru, and individuals investing in attaining reproductive status growing slower in Obse.

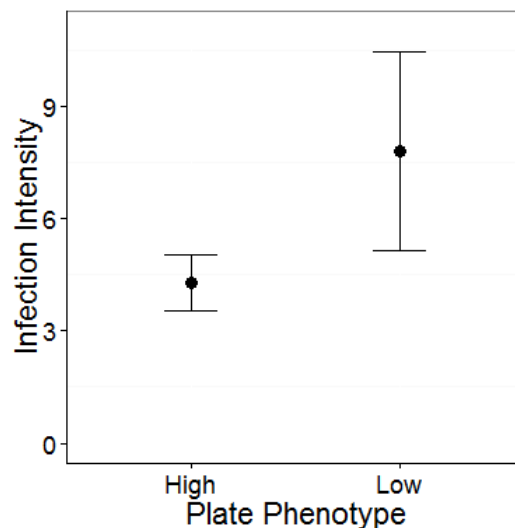


**Figure 5.2:** Individuals with the low plated phenotype were infected with a higher number of *G. arcuatus* parasites than individuals with the high plated phenotype in Obse. Infection intensity is the mean number of parasites found on infected individuals ( $\pm$ SE).

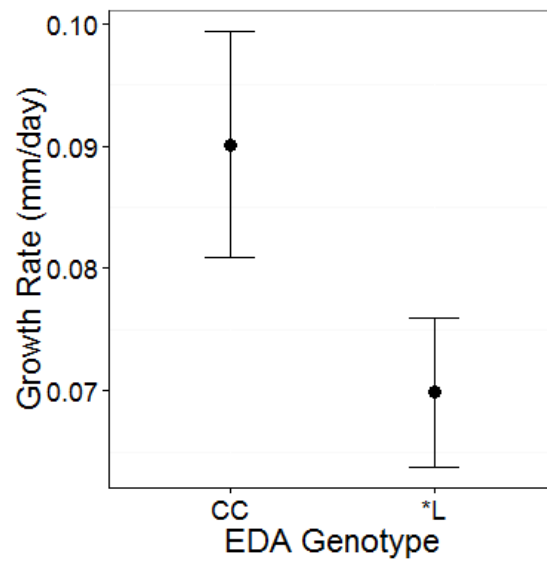


**Figure 5.3:** Growth rates (Mean  $\pm$  SE) of experimental fish, exposed to natural conditions, differ with *Eda* genotype. *Eda* has two alternative alleles denoted 'C' for the complete plated allele, 'L' for the low plated allele. CL and LL fish did not differ significantly in growth rate so their growth data was combined to produce the most parsimonious model, and marked '\*L'.

## 5.5 Discussion

In this study, I employed a common garden based approach, exposing laboratory raised fish to natural conditions, to show a link between *Eda* genotype and the function of the immune system. By using F2 hybrids, I was able to create fish that varied in *Eda* genotype whilst controlling for background genetic variation and maternal effects. Evolutionary genetic theory predicts that adaptive loci with large effects will have pleiotropic phenotypes (Barton & De Cara 2009; Seehausen *et al.* 2014). Previous work has shown *Eda* to relate to growth, behaviour, and sensory system development (Barrett *et al.* 2009a; Barrett *et al.* 2009b; Mills *et al.* 2014), and here I demonstrate an additional link with the immune system. *Eda* heterozygous fish had higher *IL-1 $\beta$*  expression levels, a marker of the pro-inflammatory innate response. Low plated, homozygous LL, fish had lower *Tbet* expression, a Th1-type adaptive response marker, and higher *FoxP3* expression, a gene with an immune regulatory role. As fish were laboratory raised with no previous exposure to stressful conditions, the patterns observed likely relate to *Eda* genotype, either directly or through the action of closely linked genes.

Whilst the link between *Eda* and immune gene expression levels is apparent, the possible underlying mechanisms are less clear. Differences could represent pleiotropic effects of *Eda* itself, possibly through the regulatory change which dictates plate number during development (O'Brown *et al.* 2015), or *Eda* genotype, through plate phenotype, could result in differing parasite burdens, which in turn result in expression differences. Alternatively, the pattern could arise from the tight linkage of *Eda* with other gene with roles in immune function pathways. The block of tight linkage surrounding *Eda*

contains a number of genes, including *Baff*, *Garp*, and *Dusp1* (Colosimo *et al.* 2005; Jones *et al.* 2012a; Jones *et al.* 2012b), which have important roles in the function of the immune system. *Garp* promotes *TGF $\beta$*  expression and regulates the T cell response (Chan *et al.* 2011; Probst-Keppler & Buer 2010; Tran *et al.* 2009), thus the decrease in *Tbet* expression, and increase in *FoxP3* expression levels in low plated fish could be associated with *Garp*, although there were no direct changes in *TGF $\beta$*  expression levels. *Dusp1* controls inflammation levels (Arthur & Ley 2013; Hammer *et al.* 2010), so the difference in expression of *IL-1 $\beta$* , a major driver of the inflammatory response, between fish with high or low *Eda* alleles could be driven by *Dusp1*. Changes in expression levels of genes in the same pathways as genes in linkage with *Eda* demonstrate the strong possibility of a link between immune function and this genomic region. The assays used here measured the overall immune response rather than the expression of the specific genes in this genomic region. Direct measurement of the expression of immune related genes in close linkage with *Eda*, along with examination of genomic sequence, could establish whether any of these have diverged between populations in concert with *Eda*.

In Obse, where infection with *G. arcuatus* is common, low plated fish were infected with a higher number of parasites than high plated fish. *G. arcuatus* attaches to the external surface of a fish, so it could be easier for parasites to attach to low plated fish. This difference in parasite burden could then drive the differences in immune expression levels seen with *Eda* genotype. Alternatively, low plated fish may be unable to mount a successful response to infection, or their underlying immune expression may make them more susceptible to infection, as the L allele is not adaptive in the saltwater environment where this parasite is common.

An individual's *Eda* genotype was also associated with growth, with CC genotype fish growing faster than fish with an L allele. Fish carrying the low allele have previously been found to grow faster as juveniles, but this advantage disappears in adult fish (Barrett *et al.* 2009a; Barrett *et al.* 2008). In the wild, fish from Chru are typically smaller than those in Obse. The lower growth of fish with the L allele from Chru could thus be the result of this difference in adult size, if adult size or growth rate are also determined by *Eda*. The slow growth of LL fish was particularly evident in Obse, where it could be due to the non-adaptive L allele, or a result of the higher *G. arcuatus* infection levels observed.

In conclusion, the stickleback-*Eda* system represents one of the best studied examples of an adaptive phenotypic change and its underlying genetic control, yet the full range of pleiotropic and linked effects of this adaptation have yet to be discovered. I found that *Eda* genotype was associated with immune gene expression levels, along with growth and parasite infection. Previous studies on *Eda* have shown pleiotropic effects on growth, behaviour and sensory system development (Barrett *et al.* 2009a; Barrett *et al.* 2009b; Mills *et al.* 2014). For the first time, we demonstrate a clear link between *Eda* genotype and the function of the immune system. Parasites have the potential to drive adaptive evolution (MacColl 2011), if they have a negative impact on fitness, and genetic variation in resistance with uneven parasite distributions (Little 2002). Parasites may represent an additional selective pressure on the *Eda* locus, either as a direct result of pleiotropy, or through selection on linked genes involved in the immune response. Further work is required to better elucidate the role and mechanisms underlying the observed variation in immune gene expression levels. I demonstrate a previously undescribed link between a major adaptive gene and the function of a vital organismal process,

showing the potential of loci of large effect to have a range of unexpected, and potentially important, pleiotropic effects.

## 5.6 References

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## Chapter 6: Host-parasite adaptation and the immune response during infection with *Gyrodactylus*

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### 6.1 Abstract

In the previous chapters, I examined the immune response in a range of wild populations and exposed laboratory raised fish to natural conditions. Increases in innate and adaptive gene expression levels were consistently associated with infection with the common parasite *Gyrodactylus arcuatus*, but the direction of causality was not clear. In order to directly attribute changes in expression levels to infection, I conducted a reciprocal cross infection experiment in the laboratory. I used laboratory raised fish from two geographically distinct parental populations, each with their own distinct *Gyrodactylus* species. By employing this design, it was also possible to examine whether there was local adaptation of infection dynamics and the immune response. Parasite burdens were monitored over the course of the experiment, and individuals were sampled at multiple time points for immune gene expression analysis. There were large differences in virulence between parasite species, irrespective of host. Some host local adaptation was present, with one parasite species having a lower burden on its sympatric host. The immune system responded to infection, with a decrease in

expression of innate and Th1-type adaptive response genes in fish infected with the less virulent parasite, representing a marker of a possible resistance mechanism. Our results add to the growing understanding of the extent of host-parasite local adaptation, and demonstrate the involvement of the immune response during infection.

## 6.2 Introduction

Parasitism is one of the most widespread lifestyles in the animal kingdom (Price 1980), with at least one parasite species for every species of host (Poulin & Morand 2000). Parasites have the potential to influence the dynamics of host populations (Hudson *et al.* 1998), manipulate host behaviour (Thomas *et al.* 2005) and affect host life history (Agnew *et al.* 2000; Perrin *et al.* 1996), and hosts in turn can impact parasite populations (Anderson & Gordon 1982; Anderson & May 1978). The majority of host parasite interactions, however, fail to result in successful infection (Antonovics *et al.* 2013), with parasites often less likely to establish infections on hosts from other populations (Ebert 1994; Lively 1989; Ward 1992). Such variation in infectivity on differing hosts may be the result of the local adaptation of host-parasite pairs through a shared evolutionary history (Dybdahl & Storfer 2003; Greischar & Koskella 2007; Kaltz & Shykoff 1998), but explicit tests of local adaptation are lacking in natural vertebrate populations.

The immune system is a key host defence against infection. Immune system genes show elevated levels of selection (Lazzaro & Clark 2012; McTaggart *et al.* 2012; Tiffin & Moeller 2006; Viljakainen *et al.* 2009), suggesting that parasites may have been a significant selective pressure. The expression levels of resistance genes can be determined by host-parasite genotype x

genotype interactions ( $G_H \times G_P$ ), or modulated by the external environment (Lazzaro & Little 2009). There is evidence from invertebrates that variation in the expression levels of immune response genes in a host can determine parasite resistance (Barribeau *et al.* 2014; Bonneaud *et al.* 2011), and that expression of resistance genes can vary with both host (Brunner *et al.* 2013; Sackton *et al.* 2010) and parasite (Barribeau & Schmid-Hempel 2013; Riddell *et al.* 2009) genotype. Modern molecular immunological techniques make it possible to measure the immune response of infected individuals, adding another level at which host-parasite local adaptations can be examined.

The three-spined stickleback and its *Gyrodactylus* parasites provide an ideal system in which to perform such work. Stickleback have repeatedly colonised novel freshwater habitats from their ancestral marine form since the end of the last ice age, creating a number of now isolated populations (Bell & Foster 1994). Adaptations to freshwater have evolved in a short period of time in response to local ecological conditions, including variation in parasites (McKinnon & Rundle 2002). Populations show consistent differences in parasite community composition (De Roij & MacColl 2012; Scharsack *et al.* 2007), and there is growing evidence for within and between population variation in parasite resistance (De Roij *et al.* 2011; Kalbe & Kurtz 2006). Parasites may be a significant selection pressure in these stickleback populations, and fish would be expected to evolve to resist their local parasite fauna.

Parasites of the *Gyrodactylus* genus are common monogenean ectoparasites of freshwater and saltwater fish. Infection can result in high rates of host mortality, with *Gyrodactylus salaris* responsible for the destruction of Atlantic salmon (*Salmo salar*) stocks in Norwegian rivers (Johnsen & Jensen 1991). *Gyrodactylus* infect fish by attaching to the external surfaces and feeding on

epithelial cells and mucous, where they can cause significant damage and leave fish susceptible to secondary infections (Cone & Odense 1984; Sterud *et al.* 1998). *Gyrodactylus gasterostei* and *Gyrodactylus arcuatus* are frequent, often dominant, parasites of stickleback populations (De Roij & MacColl 2012; Harris 1985; Raeymaekers *et al.* 2008). Infection with *Gyrodactylus* has fitness consequences to stickleback, with infected individuals having lower growth (De Roij *et al.* 2011) and a 2.5% mortality rate under laboratory conditions (Mahmud, Robertson and MacColl, unpublished data). *Gyrodactylus* thus have the potential to drive classic host-parasite evolutionary dynamics and adaptation of the hosts' immune response, although evidence for adaptation is mixed. There is evidence for local adaptation of both hosts (De Roij *et al.* 2011) and parasites (Raeymaekers *et al.* 2011), of local adaptation of both hosts and parasites (Mahmud 2016), or of no adaptation at all (Konijnendijk *et al.* 2013). These studies focus on parasite load as a measure of infection success, but have yet to examine the extent of host-parasite specificity in the immune response in this context.

Here I examine how infection dynamics on, and the immune responses of, naïve hosts from two widely separated populations vary when infected with two closely related parasite species, each of which is sympatric to one of the host populations. Whilst studies in the wild have shown changes in the immune response with *Gyrodactylus* infection (Chapters 2, 3 & 4; Robertson *et al.* 2015, 2016), such patterns are confounded by a wide range of additional external factors. By performing an experiment under controlled laboratory conditions, it is possible to look directly at infection dynamics and the response of the immune system to infection. I made F1 families of fish from a population in northern Scotland, and a population in the midlands of England, giving offspring with geographically distinct genetic backgrounds and no

previous parasite exposure. Parasites were collected from the same locations a year later, with fish in northern Scotland infected with *G. arcuatus* and fish from the midlands with *G. gasterostei*. These were used to perform a fully cross-factored reciprocal infection experiment, with the inclusion of uninfected control fish.

This experimental design was employed to allow me to address three main questions. First, do infection dynamics differ between the two parasite species used? Both species infect their host by superficially similar infection routes, but may differ in their infection success measured in terms of parasite load. Second, what immune response does *Gyrodactylus* infection produce? By measuring expression levels of genes of the innate and adaptive immune response (Chapter 2; Robertson *et al.* 2015), I can test whether the immune system plays a role during infection, and examine which pathways may be involved. Third, is there evidence of local adaptation of host and parasites? This could be reflected in either parasite load, fish immune response, or a combination of the two.

## **6.3 Methods**

### **6.3.1 Study Populations and Parasites**

Parental fish were first collected from two geographically separated populations, Loch Ob nan Stearnain ('Uist', 57°36'09"N; 7°10'19"W), a saltwater lagoon on the island of North Uist, Scotland, and Jubilee Lake ('Nott', 52°57'02"N; -1°11'13"W), a freshwater lake on the campus of the University of Nottingham, England. For each population, I bred F1 progeny in May 2014 for use in the infection experiment by making crosses between unrelated breeding adults to create full-sib families, following the procedure of

De Roij *et al.* (2011). Fertilised eggs were transported to aquarium at the University of Nottingham, with each family placed into a quarter-tank partition of a 100L tank. After hatching, I split families between multiple partitions to give 8 fish per partition, to ensure all fish were maintained at the same density. After six months, families were mixed in half-tank partitions, at 15 fish per partition, to give mixed groups of fish from the same source population. All fish were kept in a climatically controlled room, under a natural photoperiod and temperature regime.

*G. gasterostei* infect Nott fish, were they were the dominant parasite species found, whilst *G. arcuatus* infect Uist fish. Were again they were the dominant parasite species found. These fish populations were expected to have coevolved with these different but closely related parasite species. Two weeks prior to the start of the experimental infection, in May 2015, I collected wild fish to act as parasite donors. Fish were caught in Obse and the Tottle Brook (52°56'06"; -1°11'41"), a small stream running through the University of Nottingham campus. *G. gasterostei* infections were unusually low at the time of sampling in Jubilee Lake, so fish from nearby Tottle Brook were used instead. Parasites from Nottingham will be referred to as 'Nott' and from North Uist as 'Uist'. These donor fish were housed in groups of 20-25 for one week, to encourage growth of parasite populations.

### **6.3.2 Experimental Design**

Overall, 30 12-month-old stickleback were exposed to *G. gasterostei* and 30 to *G. arcuatus*, with 24 uninfected fish kept as controls, assigned to an experimental group at random in a fully cross-factored design (Table 6.1). All fish were housed individually in 3L tanks containing 2L of dechlorinated water, with 25% of the water changed every three days. By housing fish individually,

infections could be tracked on each individual. The number of parasites on each fish was counted at 7, 14, 21, 29, and 36 days post infection (dpi). At 14, 29 and 45 dpi, five fish from each treatment group and four from each control group were randomly selected to be sampled for immunological analysis. By employing this experimental design, all fish x *Gyrodactylus* combinations were present, allowing me to look at the extent of local adaptation of host-parasite infection dynamics and of the immune response. The addition of uninfected controls allowed me to examine how the immune system responded to infection.

| Fish Source  | Gyro. Source | Treatment  | n  | Time (days post infection) |    |    |    |    |    |
|--------------|--------------|------------|----|----------------------------|----|----|----|----|----|
|              |              |            |    | 7                          | 14 | 21 | 29 | 36 | 45 |
| Nott         | Nott         | Sympatric  | 15 | -                          | 5  | -  | 5  | -  | 5  |
| Nott         | Uist         | Allopatric | 15 | -                          | 5  | -  | 5  | -  | 5  |
| Nott         | -            | Control    | 12 | -                          | 4  | -  | 4  | -  | 4  |
| Uist         | Nott         | Allopatric | 15 | -                          | 5  | -  | 5  | -  | 5  |
| Uist         | Uist         | Sympatric  | 15 | -                          | 5  | -  | 5  | -  | 5  |
| Uist         | -            | Control    | 12 | -                          | 4  | -  | 4  | -  | 4  |
| <b>Total</b> |              |            | 84 | 28                         |    | 28 |    | 28 |    |

**Table 6.1:** Outline experimental plan showing sampling time points for immunological measures. Fish were from two sources (Nottingham, 'Nott'; North Uist, 'Uist'). Each fish was infected with *Gyrodactylus* sourced from Nottingham ('Nott') or North Uist ('Uist') to give all sympatric and allopatric infection combinations, along with uninfected control individuals. The number of parasites on each individual was counted at each time point given, with the number of individuals sampled for immunological analysis at a time point also indicated.

### 6.3.3 Infection Protocol and Sample Collection

Naturally infected fish from Obse and Tottle Brook were used as parasite donors. These were euthanized by overdose of MS-222 (400 mg L<sup>-1</sup>) followed by destruction of the brain, in accordance with UK Home Office regulations. Fish were placed into a petri dish containing a small amount of dechlorinated water, and any tissues with attached *Gyrodactylus* removed under low powered microscopy. Tissues were left for 10 minutes to allow *Gyrodactylus* worms to detach. I removed *Gyrodactylus* from a number of fish into the same petri dish, to ensure no fish contributed an excessive number of parasites to the overall infection procedure. Prior to infection, the length and weight of recipient fish were recorded. To infect a fish, it was lightly anaesthetised in MS222 (40 mg L<sup>-1</sup>), and its caudal fin was held near two unattached *Gyrodactylus* until the worms attached to the fin. All fish receiving Nott parasites were infected first. Fish from each population were infected alternately, to ensure exposure to worms from a single donor fish was as uniform as possible. All control fish were anaesthetised and handled in the same manner as infected fish.

After 7 days, I counted the numbers of parasites on each exposed fish. Fish were lightly anaesthetised as above, and the caudal, anal, dorsal and pectoral fins, as well as the dorsal spines, pelvic girdle, flanks and head were examined for parasites under a dissection microscope. Again I anaesthetised and handled control fish in the same manner as infected fish. This counting procedure was repeated at 14, 21, 29, 36 and 45 dpi on all remaining fish.

At 14, 29 and 45 dpi, a subset of fish were removed and sampled. Fish were euthanized in a random order, then measured and weighed. Their spleens, an immunologically important tissue in fish (Zapata *et al.* 2006), were removed and immediately placed in RNAlater (Life Technologies). Spleen samples

were kept at 4°C for 24 hours, then at -20°C until RNA extraction. I counted the number of parasites infecting each fish. Sex was recorded along with reproductive status, determined by visual examination of the gonads. Male and female reproductive status was split into three categories: no reproductive investment (1, small gonads and kidneys in M, small ovules only in F), some investment (2, gonads enlarging, but kidneys small in M, some enlarged ovules in F), or full investment (3, gonads and kidneys enlarged in M, ovules enlarged and ready to lay in F).

Of the 84 fish used in the experiment, three were euthanized prior to their pre-determined sample point due to deteriorating health, with one fish each coming from the Nott fish / Uist parasite group, one from the Nott fish / Nott parasite group, and one from the Uist fish control group. These fish were not sampled as the cause of their ill health could not be determined, giving a total of 81 spleen samples for use in the gene expression analysis.

#### **6.3.4 Gene Expression Quantification**

I extracted RNA from spleens using the GeneJET RNA purification kit (Thermo Scientific) according to the manufacturers' standard protocol. Purity of RNA samples was assessed on a NanoDrop 1000 Spectrophotometer (Thermo Scientific), with a desired 260/280 absorbance ratio > 1.80. Integrity of RNA was assessed by incubating 5µl of sample at 65°C for 10 minutes, followed by visualisation on a 2% Agarose gel stained with Ethidium Bromide.

All samples were DNase treated using Precision DNase (Primer Design), following the manufacturers' protocol. Reverse transcription reactions were performed on approximately 1.5µg of total RNA using the nanoScript2 RT kit (Primer Design), with a combination of random nanomer and oligo-dT priming, following the manufacturers' standard protocol. Periodic no-enzyme controls

indicated that genomic DNA contamination was negligible. All cDNA samples were diluted 1:10 with nuclease free water prior to further use.

qPCR reactions were performed in a 10µl total volume, containing 5µl of PrecisionFAST mastermix with SYBR green (Primer Design), 0.25µl of each primer, 2µl of template cDNA and 2.5µl of H<sub>2</sub>O in 96-well optical PCR plates with optical seals (StarLab). Reactions were performed in an ABI 7500 FAST real-time thermocycler (Applied Biosystems) at 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. All runs included a post-PCR melt curve analysis.

Accurate normalization of gene expression is essential for the production of reliable data in qPCR experiments, with the optimal reference genes being specific to a particular set of experimental conditions (Dheda *et al.* 2005). To select the most appropriate normalization strategy, I performed a geNorm analysis with six candidate reference genes (*B2M*, *GAPDH*, *RPL13A*, *HPRT1*, *TBP* and *TOP1*) on 12 cDNA samples, randomly selected from all samples, using a custom stickleback geNorm kit for SYBR green (Primer Design) following the manufacturers' standard protocol. Analysis of the stability of expression was performed in qbase+ (Biogazelle), which identified *RPL13A* and *HPRT1* as the most stable combination of reference genes for this study.

I measured the expression levels of eight genes of interest, along with the two reference genes. Genes of interest were *IL-1β*, *TNFα*, *Stat4*, *Stat6*, *CMIP*, *FoxP3a*, and *TGFβ* (For full details, see chapter 2 & Robertson *et al.* 2015). A reference sample was made by pooling cDNA from each experimental sample, to control for between plate variation. A total of 81 cDNA samples were split randomly between two plates, with reactions performed in duplicate

for each sample, and each plate also contained the reference sample and negative controls.

Relative expression values were calculated using the  $\Delta\Delta C_q$  method (Pfaffl 2001), adjusted for the amplification efficiencies of each primer pair and standardised against the geometric mean  $C_q$  of the two reference genes for each sample (Vandesompele *et al.* 2002).

### **6.3.5 Data Analysis**

All relative expression data were  $\log_{10}(x+1)$  transformed prior to analysis, due to the inherently skewed distribution of such data. All data analysis was performed in R v.3.2.3 (R Core Team 2014).

*Infection Dynamics.* The magnitude of infection was summarised in two ways. Peak abundance was defined as the highest number of parasites found during any count on an individual. Total abundance was calculated as the total number of parasites found over all counts. As individuals were removed from the experiment at different sample points, total abundance was standardised for the length of infection by dividing total abundance by infection length.

To examine whether infection dynamics differ between hosts or parasites, I fitted general linear models (glms) with peak abundance or relative total abundance as the response. Host type (Nott or Uist), parasite type (Nott or Uist) and the host by parasite interaction term were included as explanatory factors. Due to the skewed distribution of parasite count data, a quasipoisson error function and log link was included in the model of relative total abundance, with significance calculated using Wald F tests. For the peak abundance model, a Poisson error function and log link were used, with significance calculated using  $\chi^2$  likelihood ratio tests.

*Immune response to infection.* I examined variation in immune gene expression levels to see whether the immune system responded to infection, and whether there was evidence of local adaptation of the immune response. I performed a multivariate analysis of variance (MANOVA) with relative expression values as the response, and host type (Nott or Uist), parasite treatment (Nott, Uist or Control) and sample day (14, 29 and 45 dpi), along with their interaction terms as explanatory variables. As there were underlying differences in the immune expression profiles of Nott and Uist fish in uninfected controls, I fitted host type first in the model and used type 1 sum of squares to test the effects of treatment and sample day above these underlying host differences. Overall differences were calculated using the Pillai method and F statistic, followed by examination of expression levels of each immune gene separately. Where significant components had multiple levels, ANOVAs were fitted and Tukey's HSD tests were used to perform all possible pairwise comparisons of factor levels.

## **6.4 Results**

### **6.4.1 Infection dynamics**

Average parasite burdens over the course of the experiment are shown in Figure 6.1. Relative total abundance differed between parasite species ( $F_{1,44}=6.19$ ,  $p=0.017$ ), with an average of 0.43 (SE $\pm$ 0.07) Uist parasites and 1.46 (SE $\pm$ 0.28) Nott parasites per infection day. There was no difference in relative total abundance between host type ( $F_{1,44}=0.39$ ,  $p=0.538$ ), and no host by parasite interaction ( $F_{1,44}=0.47$ ,  $p=0.496$ ). Peak parasite abundance also differed between parasite species ( $LRT_{1,44}=82.09$ ,  $p<0.001$ ), with an average peak of 5.24 (SE $\pm$ 1.16) Uist parasites and 22.37 (SE $\pm$ 5.10) Nott parasites.

Peak parasite abundance also differed between hosts ( $LRT_{1,44}=14.17$ ,  $p<0.001$ ), with an average peak parasite abundance of 15.26 ( $SE\pm5.44$ ) on Nott fish, and 14.52 ( $SE\pm3.49$ ) on Uist fish. There was also a peak parasite abundance by host interaction (Figure 6.2,  $LRT_{1,44}=14.76$ ,  $p<0.001$ ), with no difference in Nott parasite peak abundance between hosts, but lower numbers of Uist parasites on Uist fish.

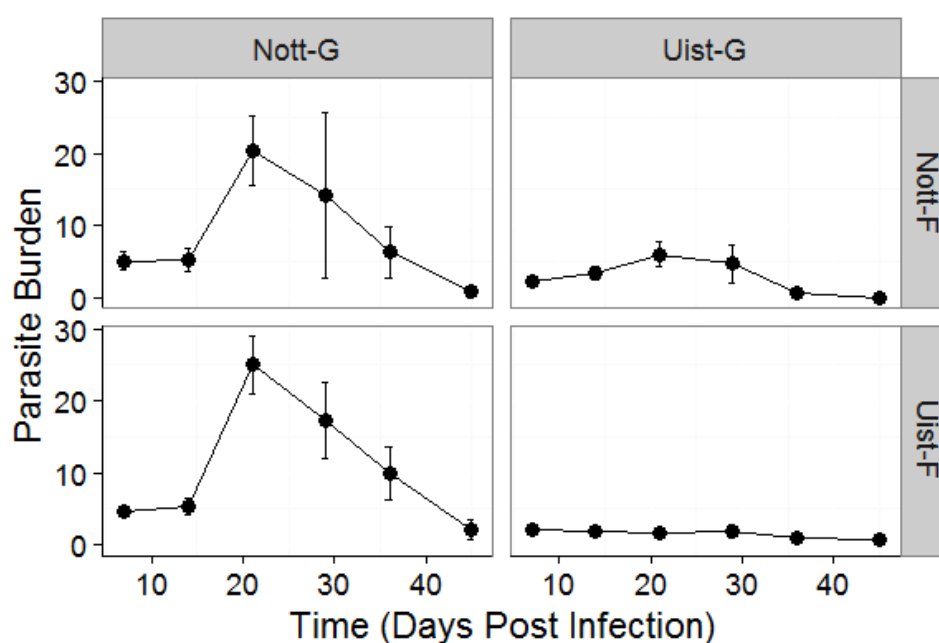


**Figure 6.1:** Mean parasite burden ( $\pm SE$ ) over the course of the infection experiment for each host by parasite combination. Infections with *G. gasterostei* from Nottingham ('Nott-G') are in the left column, and infections with *G. arcuatus* from North Uist ('Uist-G') are in the right column. Infections on fish from Nottingham ('Nott-F') are shown in the top row and infections on fish from North Uist ('Uist-F') in the bottom row.

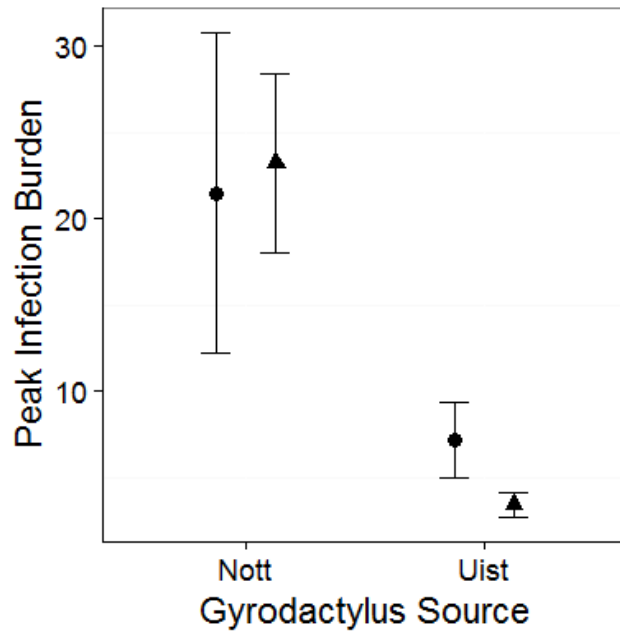


**Figure 6.2:** Peak parasite burden (Mean  $\pm$  SE) varies with parasite species ('Uist' *G. arcuatus* and 'Nott' *G. gasterosteii*) on hosts from Nottingham (●) and North Uist (▲). Peak infection burdens of Uist parasites were lower on Uist fish than those from Nottingham, but there was no difference in peak burden between fish source for Nottingham parasites.

#### 6.4.2 Immune response to infection

Overall immune profiles differed between fish (MANOVA  $F_{1,63}=8.69$ ,  $p<0.001$ ), driven by variation in expression of *IL-1 $\beta$*  ( $F_{1,63}=36.15$ ,  $p<0.001$ ), *TNF $\alpha$*  ( $F_{1,63}=4.66$ ,  $p=0.035$ ), *Stat4* ( $F_{1,63}=9.80$ ,  $p=0.003$ ), *Stat6* ( $F_{1,63}=21.00$ ,  $p<0.001$ ), *CMIP* ( $F_{1,63}=4.22$ ,  $p=0.044$ ), *FoxP3* ( $F_{1,63}=4.05$ ,  $p=0.049$ ) and *TGF $\beta$*  ( $F_{1,63}=27.64$ ,  $p<0.001$ ), but not *Tbet* ( $F_{1,63}=0.68$ ,  $p=0.411$ ).

The overall response also differed between treatment groups (MANOVA  $F_{2,63}=1.86$ ,  $p=0.032$ ). There was variation in *TNF $\alpha$*  expression (Figure 6.3,  $F_{2,63}=7.96$ ,  $p<0.001$ ), where levels in control fish were higher than in Uist parasite (Tukey  $p<0.001$ ) and Nott parasite (Tukey  $p=0.036$ ) infected fish, but did not differ between the two infection types (Tukey  $p=0.253$ ). Expression

levels of *Stat4* differed with treatment (Figure 6.3,  $F_{2,63}=4.54$ ,  $p=0.014$ ), with Uist parasite infected fish having lower expression than Nott infected (Tukey  $p=0.026$ ) or control fish (Tukey  $p=0.038$ ), but no difference between Nott infected and controls (Tukey  $p=0.999$ ). *Stat6* expression also differed with treatment (Figure 6.3,  $F_{2,63}=3.64$ ,  $p=0.032$ ), with Uist parasite infected fish having lower expression than control fish (Tukey  $p=0.034$ ), but no difference between Nott infected fish and Uist infected (Tukey  $p=0.131$ ) or control fish (Tukey  $p=0.756$ ). Expression levels of *TGF $\beta$*  varied with treatment (Figure 6.3,  $F_{2,63}=5.54$ ,  $p=0.006$ ), with Uist parasite infected fish having lower expression than Nott infected (Tukey  $p=0.039$ ) or control (Tukey  $p=0.008$ ) fish, but there was no difference between Nott infected and control fish (Tukey  $p=0.731$ ). Expression levels of *IL-1 $\beta$*  ( $F_{2,63}=1.30$ ,  $p=0.281$ ), *Tbet* ( $F_{2,63}=2.84$ ,  $p=0.066$ ), *CMIP* ( $F_{2,63}=1.18$ ,  $p=0.315$ ) and *FoxP3* ( $F_{2,63}=2.00$ ,  $p=0.144$ ) did not vary with between treatment groups.

Overall immune expression profiles varied with sample day (MANOVA  $F_{2,63}=2.11$ ,  $p=0.012$ ), although the sample day term was not significant when the expression of *IL-1 $\beta$*  ( $F_{2,63}=1.40$ ,  $p=0.253$ ), *TNF $\alpha$*  ( $F_{2,63}=0.86$ ,  $p=0.428$ ), *Stat4* ( $F_{2,63}=2.43$ ,  $p=0.097$ ), *Tbet* ( $F_{2,63}=2.05$ ,  $p=0.137$ ), *Stat6* ( $F_{2,63}=1.66$ ,  $p=0.198$ ), *CMIP* ( $F_{2,63}=0.03$ ,  $p=0.969$ ), *FoxP3* ( $F_{2,63}=2.59$ ,  $p=0.083$ ) and *TGF $\beta$*  ( $F_{2,63}=0.11$ ,  $p=0.897$ ) were analysed individually. No significant interaction terms were found in the MANOVA of overall expression levels, but the effect of treatment on *TNF $\alpha$*  expression levels varied between fish types (Figure 6.4,  $F_{2,63}=3.17$ ,  $p=0.048$ ), and there was also an effect of treatment on *Tbet* expression levels that varied between fish and with sample day (Figure 6.5,  $F_{4,63}=2.57$ ,  $p=0.046$ ).

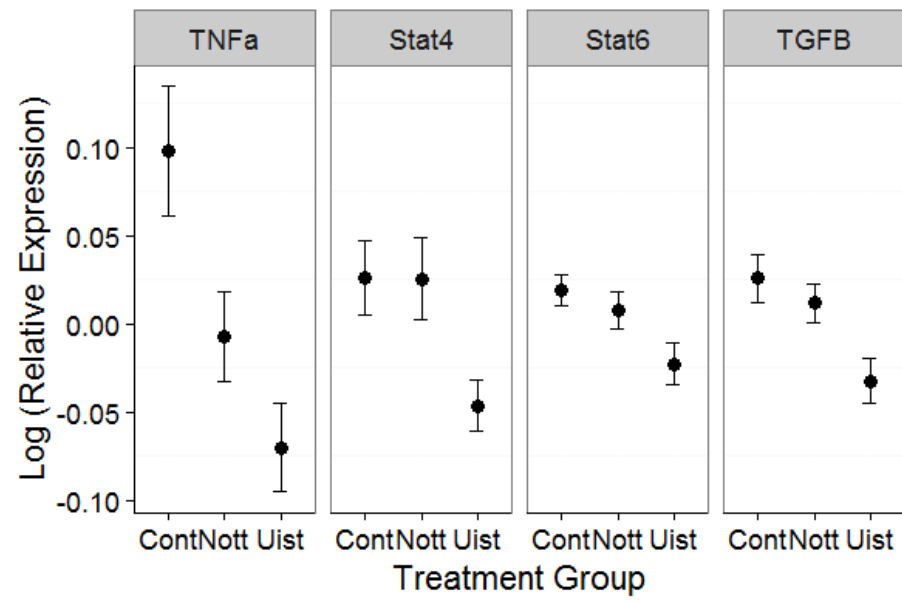


**Figure 6.3:** Relative gene expression levels (Mean  $\pm$  SE) of *TNFα*, *Stat4*, *Stat6* and *TGFβ* differ between treatment groups in a controlled infection experiment. Expression values have been standardized against the mean of each fish source population for display, to control for underlying expression differences. Individual fish were either uninfected controls ('Cont'), infected with *G. gasterostei* from Nottingham ('Nott') or infected with *G. arcuatus* from North Uist ('Uist').

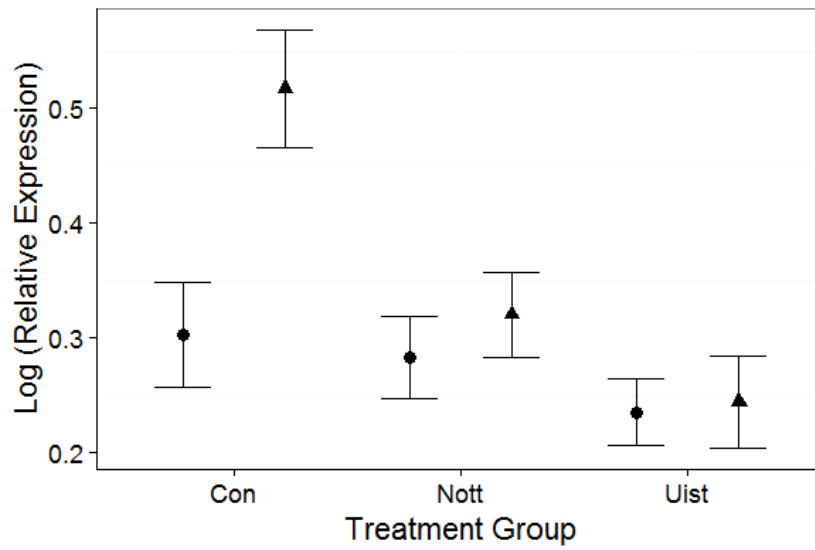


**Figure 6.4:** The effect of treatment on *TNFα* relative expression levels varies between source fish population in a controlled infection experiments. Individual fish from Nottingham (●) or North Uist (▲) were either uninfected controls ('Con'), infected with *G. gasterostei* from Nottingham ('Nott') or infected with *G. arcuatus* from North Uist ('Uist'). Control fish show underlying differences in gene expression levels, whilst Uist fish show a decrease in *TNFα* expression in response to infection and Nott fish do not.

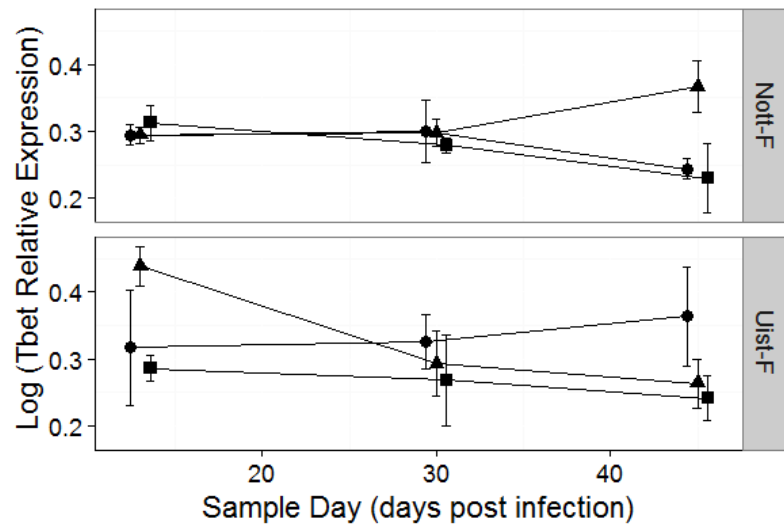


**Figure 6.5:** The effect of treatment groups on *Tbet* relative expression levels varied with fish source and across sample days. The response of fish from Nottingham ('Nott-F') is shown in the top graph, whilst fish from North Uist ('Uist-F') are in the bottom graph. Individual fish were left as untreated controls (●), infected with *G. gasterostei* from Nottingham (▲), or infected with *G. arcuatus* from North Uist (■). Fish were sampled at 14, 30 and 45 days post infection (dpi). In Nottingham fish, we see higher *Tbet* expression in Nottingham parasite infections at 45 dpi. Uist fish have higher expression when infected with Nottingham parasites at 14 dpi, and lower expression when infected with either parasite at 45 dpi.

## 6.5 Discussion

In this study, I performed a reciprocal cross infection experiment with two host-parasite pairs to examine the type of immune response *Gyrodactylus* infection elicits in stickleback, and to what extent we see local adaptation of infection dynamics and the immune response. Previous studies of stickleback and *Gyrodactylus* have found mixed evidence of the occurrence of local adaptation (De Roij *et al.* 2011; Konijnendijk *et al.* 2013; Mahmud 2016; Raeymaekers *et al.* 2011). Here, I found large differences in virulence between the two species, irrespective of whether they infect a sympatric or allopatric host type, even though both parasite species have very similar modes of infection. *G. gasterostei* from Nottingham had significantly higher peak and total abundance than *G. arcuatus* from North Uist. There was limited evidence of local adaptation, with *G. arcuatus* burdens lower on their sympatric than allopatric host, showing that North Uist fish have higher resistance to their sympatric parasite. This reflects the general pattern seen in guppies, where parasite virulence varies with strain and resistance varies between host populations, but without extensive host-parasite local adaptation (Pérez-Jvostov *et al.* 2012, 2015). As evidence for local adaptation is still mixed, the reciprocal cross-infection approach employed here could be expanded to include a larger number of host and parasite populations, giving a clearer understanding of the generality of host-parasite adaptation in this system.

Variation in immune gene expression levels were seen in response to infection. Expression levels differed between control fish derived from different populations, supporting previous work showing population level differences in underlying immune function (Chapter 2; Robertson *et al.* 2015). Above these

underlying differences, I found that infection with *G. gasterostei* from Nottingham caused a decrease in *TNF $\alpha$*  expression levels, whilst infection with *G. arcuatus* from North Uist caused a decrease in the expression of *TNF $\alpha$* , *Stat4*, *Stat6* and *TGF $\beta$* . The patterns of expression observed differ from those seen in other fish species during *Gyrodactylus* infection, suggesting that different resistance mechanisms may be acting in stickleback. Infection studies in guppies found evidence for both innate and acquired responses to infection (Cable & Van Oosterhout 2007). Whilst this study did not measure the immune response directly, here we find changes in markers of the innate, Th1-type adaptive, Th2-type adaptive and immune regulatory response pathways.

Expression levels of *IL-1 $\beta$*  and *TNF $\alpha$*  increase in the skin of *Gyrodactylus* infected rainbow trout (Lindenstrom *et al.* 2003; Lindenstrom *et al.* 2004) and Atlantic salmon (Lindenstrøm *et al.* 2006). Here, a decrease in *TNF $\alpha$*  expression occurred during infection with both parasites, and in *Stat4*, *Stat6* and *TGF $\beta$*  levels in fish infected with the less virulent parasite. Although a decrease in immune gene expression levels with infection is counterintuitive, they correspond to a high level of resistance in this instance. Decreasing expression levels may indicate that resources are being allocated to other arms of the immune response not measured here, which are actually fighting the infection. Here expression levels were measured in the spleen, which is an immunologically important tissue in fish, but not the only place where expression changes could occur (Zapata *et al.* 2006). A decrease in expression in a central immunological tissue could correspond to expression levels increasing in the periphery, at the site of infection. Sampling multiple tissue types during infection, along with a wider suite of genes, is required to better understand the changes observed here.

There was some evidence of local adaptation of the immune response to infection. In North Uist fish, I found a decrease in *TNF $\alpha$*  expression levels with infection, with the lowest levels seen when fish were infected with sympatric parasites. Expression levels of *Tbet* were higher in Nottingham fish infected with sympatric *Gyrodactylus* at 45dpi. In North Uist fish, *Tbet* expression was higher with Nottingham *Gyrodactylus* infection at 14 dpi, and lower with infection with either parasite at 45dpi. These fish by treatment variations in the expression levels of *TNF $\alpha$*  and *Tbet* show that there may be specific  $G_H \times G_P$  interactions which result in differing responses to infection.

In studies in wild three-spined stickleback using the same set of immune assays, infection with *Gyrodactylus* tends to correlate with increases in innate expression and decreases in regulatory gene expression levels (Chapter 2 & 3; Robertson *et al.* 2015, 2016). In the wild, individuals are likely to be faced by multiple simultaneous challenges, and trade-offs between costly immune function and other necessary activities may be required (Lazzaro & Little 2009; Pedersen & Babayan 2011; Schulenburg *et al.* 2009). Artificial infection experiments, where individuals are kept in benign conditions, struggle to replicate the variation associated with natural conditions (Pedersen & Babayan 2011), but do allow us to isolate the factor in which we are interested. Whilst the changes observed here can be directly attributed to infection, the difference in pattern seen when compared to data from wild individuals may represent the difference between healthy individuals able to cope with infection and individuals facing multiple challenges and a wide range of energetic demands. Furthermore, the direction of causality of infection is not clear in wild individuals as changes in immune function could be a response to infection, or may themselves have made an individual more susceptible to infection. Infection with *Gyrodactylus* can also increase the

chance of secondary infections (Cone & Odense 1984), possibly as a result of changes in immune system function. Controlled infection studies involving multiple parasite species are possible, and represent a next step to better understand how changes in response to one infection affect the ability of individuals to respond to subsequent challenge.

In conclusion, I found large differences in the virulence of two closely related parasite species, *G. gasterostei* and *G. arcuatus*. Infection with both parasites elicited changes in the innate immune response, whilst infection with *G. arcuatus* also elicited changes in the adaptive immune response. As *G. arcuatus* was the less virulent species, this may represent the marker of a possible resistance mechanism. There was evidence of differential expression of the innate and Th1-type adaptive response, dependent upon host, parasite and time, which may represent local adaptation of the immune response. Differences between patterns of expression observed in the wild and the laboratory demonstrate the importance of combining both approaches. The stickleback-*Gyrodactylus* system represents an ideal structure in which to advance our understanding of host-parasite local adaptation and the function of the immune response in a natural setting.

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## Chapter 7 – General discussion

In this thesis I developed a set of gene expression assays to measure the immune system of the three-spined stickleback, and used them to examine aspects of variation in the immune response in a natural setting. Here I review the main findings, highlight potential limitations, and suggest areas for further research.

I designed and validated eight gene expression assays, split between the innate, Th1-type adaptive, Th2-type adaptive and immune regulatory response types (Chapter 2). Using these assays I demonstrated that there are underlying differences in immune gene expression levels and in innate immune response potential between lab-raised fish derived from different parental populations. Expression levels in wild populations differ from lab-raised fish from the same populations, and there are expression differences between wild populations. I identify a range of possible factors which may contribute to this observed variation, including sex, reproductive investment and parasitic infection. Further examination of two wild populations which suffer from high levels of *S. solidus* infection but are otherwise superficially similar allowed me to identify factors which are common or divergent in relation to immune gene expression levels between populations (Chapter 3). Some factors, such as sex and reproductive effort, appear to have the same general relationship with immune function between populations, whilst others, including individual condition and parasitic infection, have population-dependent differences.

I performed two large experiments in which laboratory raised fish were exposed to semi-natural conditions in enclosures in lochs on North Uist. The first experiment further examined which factors may shape immune gene

expression levels whilst controlling for the unknown variation in past experience associated with sampling individuals in the wild (Chapter 4). This demonstrated the importance of a range of factors, including sex, reproductive investment, and infection with *G. arcuatus*, in shaping gene expression levels. This experiment confirmed that the patterns observed in wild populations are not just the result of differences in life history prior to capture, but can be driven by the biotic and abiotic environment and variation between individuals. Whilst fish in Chapter 4 were exposed to natural conditions in a single location dominated by a single parasite species, fish in Chapter 5 were exposed to two contrasting environments. This second experiment demonstrated a previously undocumented link between the *Eda* locus, which underlies the change in armour plate phenotype during adaptation to freshwater, and the function of the immune system (Chapter 5). *Eda* has pleiotropic effects on a range of factors, including growth and sensory system development. As parasite communities differ between salt and freshwater environments, changes in the immune system in concert with *Eda* could have possible fitness, and therefore adaptive, consequences. In Chapter 4, there was a growth disadvantage to *Eda* heterozygous individuals, whilst in Chapter 5 fish with one or two L alleles were found to grow slower. Whilst Chapter 5 identified a clear link between *Eda* genotype and immune gene expression levels, no such relationship was identified in Chapter 4.

In the final experiment, I returned to a more traditional laboratory setting to perform an infection experiment with *Gyrodactylus* (Chapter 6). This showed that expression levels of innate, Th1-type and regulatory genes decrease with infection, and could represent a possible marker of a resistance mechanism to *Gyrodactylus* infection. Furthermore, I demonstrated that two *Gyrodactylus* species differ in their virulence, and that there is some local adaptation of host

resistance to infecting parasites. A number of aspects of the work in this thesis warrant further discussion, particularly the general patterns seen across multiple chapters, and the potential future uses and limitations of the findings presented.

## **7.1 Measuring the immune response with qPCR**

Studies of the immune system in a natural setting require suitable markers of the immune response. Whilst protein level assays are available for lab-based model species, other approaches are required for immunological studies in non-model species. Measurement of gene expression levels provides one such approach, with the main requirement for such studies being the availability of a reference genome for the species of interest (For a full review of these approaches, see Fassbinder-Orth 2014). The use of qPCR to measure gene expression levels was chosen over a whole transcriptome RNA-seq approach in this instance for a number of reasons. RNA-seq remains a relatively expensive option, which limits the degree of biological replication possible. It has the advantage of providing a full transcriptome, but the large amount of data produced makes analysis and inference difficult. qPCR has the advantage of being relatively cheap, allowing the high number of biological replicates required to measure expression in a highly variable natural setting. Genes can be selected based on *a priori* knowledge of the function of the immune system, allowing a much more targeted approach to be employed. The main limitation of a qPCR approach is that only the expression levels of pre-selected genes will be known, meaning that any other relevant changes in gene expression will not be identified.

Both qPCR and RNA-seq measure gene expression by quantifying the number of mRNA transcripts present in a sample. Whilst mRNA levels do not directly determine the amount of final functional protein product, the two do tend to correlate (Fassbinder-Orth 2014; Munsky *et al.* 2012), thus measuring gene expression levels is a suitable proxy for measuring immune function when suitable protein assays are not available. As sampling of immune tissues from stickleback is destructive, it only gives a single time point for each individual. To add to our ability to measure the immune response, I developed an accompanying cell culture technique that allows the immune response potential of an individual to be measured. In this way, we can not only measure the response of an individual at the time of sampling, but the potential of the immune system to respond to subsequent challenge can also be examined. Together, these approaches provide an excellent toolkit with which to study the function of the immune system in a wild setting in stickleback.

Whilst a number of candidate genes were identified, it was not possible to develop working assays for them all. In this thesis, I was able to develop eight immune gene expression markers. Initially, 24 genes were identified as possible targets. Of those genes, 6 could not be found in the stickleback genome. This could be the result of these genes not being present in stickleback, or that the stickleback sequence has diverged so far from other fish species as to make it impossible to find them with the sequence similarity based approach employed here. A number of other genes were found, but primers did not amplify properly in North Uist samples. The stickleback reference genome on which primer design was based is the sequence of a single fish from Alaska. Gene sequences from fish from Alaska and North Uist could potentially be highly divergent, due to the relatively long time since

these populations split. This could be an issue for immune genes in particular, as they tend to show evidence of strong selection relative to other genes across a range of taxa (Downing *et al.* 2009; McTaggart *et al.* 2012; Nielsen *et al.* 2005; Obbard *et al.* 2009). Sequence divergence could thus account for the lack of amplification of a number of primer pairs. There is potential for more assays to be added over time as more sequence information becomes available for North Uist stickleback. The assays presented are most useful if they work on stickleback from a geographically wide range of locations. Here, only stickleback sampled from Scotland, England and Iceland were tested. Testing the primers on stickleback from across the northern hemisphere is an important next step in assessing the generality of the assays presented.

## **7.2 Factors shaping the immune response**

Previous work on North Uist stickleback has shown that parasite resistance has a genetic basis (De Roij *et al.* 2011; El Nagar 2014). Here I have shown that there are underlying differences in immune gene expression levels, by comparing the expression profiles of fish from two different populations which have been raised in the same controlled laboratory conditions. Furthermore, the potential of the innate immune system to respond to challenge also varies at both the individual and population level. As a common garden based approach was used, ensuring that fish were raised under uniform lab conditions, the differences observed are also likely to have a genetic basis. We cannot rule out entirely, however, the influence of other factors, particularly maternal effects, on shaping the immune response. Further work could examine whether profiles and responsiveness have been shaped by

parasitism levels experienced in the wild, and whether expression differences correspond to varying parasite resistance levels.

Broad expression differences seen between wild populations could represent the unique set of conditions experienced by each population. Lochs on North Uist vary in a number of ways (Giles 1983; MacColl *et al.* 2013), which may contribute to shaping immune function. Temperature is particularly important in ectotherms (Magnadóttir 2010), and this varies, especially between shallow and deep lochs. Temperature differences, however, are small (MacColl, pers. comms.), so further work is required to examine whether they are sufficient to shape expression levels. There is also between loch variation in plankton communities and fish diet (Chittheer, Rahman & MacColl, unpublished data). Nutrition affects immunity and resistance to parasites in rodents (Ing *et al.* 2000; Martin *et al.* 2008a; Martin *et al.* 2008b), and could also contribute to shaping the immune response between these populations. Laboratory based experiments which manipulate these factors in a controlled way would demonstrate whether they impact on immune gene expression levels in stickleback.

Further to these general between population differences, I found a number of factors which were consistently associated with individual variation in immune response, including sex, reproduction, condition and age. These factors often interacted with one another during statistical modelling, demonstrating the potentially complex relationships between multiple factors in shaping the immune response in the wild. Males had lower gene expression levels than females, both in wild fish and in laboratory raised fish exposed to natural conditions. Individuals investing in reproducing had lower Th1-type and higher Th2-type expression in the wild, whilst reproduction was associated with a decrease in expression levels in experimentally exposed fish. Breeding is

energetically costly, so individuals may have to trade this off against immune function if resources are limited. This effect could vary between the sexes if the cost of reproduction differs. Hormonal changes that occur during reproduction can affect immune system function (Cuesta *et al.* 2007; Downs *et al.* 2014; Maule *et al.* 1996), and these changes could again be sex specific. Further studies are required to better understand whether changes in expression with reproductive effort affect parasite resistance levels, and whether there are fitness consequences to such variation. Examining the effect of single factors in isolation may overlook potentially important interactions; performing studies which examine the interplay between multiple immune altering factors represent an important next step in advancing our understanding of the immune system in the wild.

Combining sampling of wild populations with the results of the common garden experiments has given confidence in the associations found. Whilst correlations between a factor and gene expression levels does not indicate any causation, the repetition of the same patterns across multiple data sets strongly suggests that these patterns are real and biologically relevant. Performing controlled experiments in a laboratory setting could confirm the link between the factors found. The link between declines in immune function with increasing age presents the opportunity for future work into senescence and immune function in a natural setting. Individual variation in immune gene expression levels associated with these factors could be significant in the evolution and divergence of these stickleback populations, but the link between immune function and fitness needs to be explored.

### 7.3 Parasites and the immune system

One of the main aims of this project was to examine the link between immune gene expression levels and parasite infection and resistance. I found variation in immune expression levels with parasitic infection in the wild, and expression changes in response to infection in a controlled setting, which may represent possible markers of parasite resistance. When examining wild populations, I found that infection with *S. solidus* was associated with a decrease in Th1-type adaptive expression levels. There is growing evidence of *S. solidus* manipulation of the host immune response (For review, see Barber & Scharsack 2010), although the role of Th1/Th2 differentiation in this context had not been examined to date due to a lack of appropriate assays. If parasite manipulation was occurring, we would have predicted a shift away from a Th2-type response towards a Th1-type response. Here I found a decrease in Th1-type expression levels and no relationship with Th2-type expression levels, suggesting that the Th1/Th2 adaptive system is partly changing during *S. solidus* infection. A better understanding of the role of the Th1 and Th2 adaptive responses in fish is required to better understand the changes seen, along with controlled infection experiments to demonstrate the specific responses occurring with *S. solidus* infection.

Infection with *Gyrodactylus* was associated with an increase in immune gene expression levels in the wild, but a decrease in gene expression levels was associated with lower parasite burdens in a controlled infection study in the laboratory. The difference in patterns observed between the lab and the wild highlight the shortcomings of either approach in isolation. Whilst fish in the lab were in benign conditions with easily available food and only exposed to parasites on a single occasion, wild individuals potentially face multiple

simultaneous challenges and chronic infections with limited resources. Furthermore, fish failing to control infections in the lab may be able to survive when in the wild they would not. The direction of causality of gene expression levels and parasite infection are not clear in the wild: infection may elicit an immune response; a change in immune expression levels may leave an individual open to infection; or other factors, such as food availability, may drive changes in both. Controlled infection experiments with multiple parasites could demonstrate how infection with one parasite affects the response to a second infecting parasite, how the response changes over the course of long-term chronic infections, and how resource availability affects an individual's ability to respond to infection.

## **7.4 Concluding remarks**

During this project, I developed a toolkit that gives an overview of an individual's immune response by measuring the expression levels of key immune system genes. These assays can be applied to a range of further research questions in their current form, and more genes can be added over time as our understanding of the teleost immune system continues to develop. I have shown that a range of factors, with potentially complex interactions, may contribute to shaping the immune response in a natural setting. I demonstrate that there is a genetic basis to underlying variation in the immune response, and in innate immune response potential. This could potentially reflect the challenges faced by these populations in the wild and the coevolution between hosts and parasites. A controlled infection experiment conducted in the laboratory demonstrated variation in parasite virulence, and identified changes in immune gene expression levels in response to infection.

Furthermore, I used the markers to demonstrate a previously unidentified link between the gene underlying a major adaptive phenotypic change and the function of the immune response. A range of issues remain to be addressed, particularly the impact of multiple infections on immune function, and the fitness consequences of immune variation.

## 7.5 References

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# Appendix 1

## A1.1 Protocol for the culture of stickleback cells

This protocol describes the process by which fresh whole tissues, dissected and stored at 4°C for no more than 4 hours, can be placed into primary culture. I adapted this protocol from the one described in Hibbeler *et al.* (2008), and published it as a supplementary methods file in Robertson *et al.* (2015). For all steps in the protocol, RPMI 1640 culture media (Sigma-Aldrich R0883) should be supplemented with 10% H<sub>2</sub>O to adjust the osmoregularity to match that of fish cells. Work should be conducted in a sterile flow hood following standard sterile working procedures. The protocol has been tested on both whole spleens and head kidneys, and works on both tissue types.

RPMI- is RPMI 1640 with 10% H<sub>2</sub>O, whilst RPMI+ is RPMI 1640 supplemented with 10% H<sub>2</sub>O, 10% Foetal Bovine Serum, 2µM L-glutamine, 100µg/ml Penicillin and 100µg/ml Streptomycin.

Upon dissection, spleens and/or head kidneys from each fish should be placed immediately into ice cold RPMI- in an individual 5ml Eppendorf tube until ready to be prepared for culture.

The procedure to create primary cell culture is as follows:

- Dissagregate cells by passing whole spleen or head kidneys through a 40µM cell strainer with a small quantity of RPMI-, then transfer into a new sterile 5ml Eppendorf tube and top up to ~4ml with RPMI-
- Centrifuge cell suspension for 2 minutes at 400rcf to pellet cells
- Pour off excess RPMI- and re-suspend pelleted cells. Top up with 3ml RPMI-

- Repeat the centrifugation and re-suspension step twice, using RPMI- for the first and RPMI+ for the second.
- After final centrifugation, carefully remove excess liquid with a pipette, and re-suspend pelleted cells in 400µl of RPMI+

From the 400µl cell suspension, count the number of cells by staining 20µl of cell suspension with 20µl Trypan Blue. Adjust the concentration of cells in each sample to give  $4 \times 10^6$  cells/ml. Cells should be cultured in a 100µl final volume, giving 40,000 cells/well.

Culture cells in 96-well flat bottomed cell culture plates. Add 75µl of cell suspension to 4 wells for each sample (2 control wells, 2 treatment wells). To the control wells, add 25µl of additional RPMI+. To the treatment wells, add 25µl of 50µg/ml Zymosan (Sigma-Aldrich Z4250, diluted in RPMI+).

Plates should be placed into an incubator at 20°C, with 5% CO<sub>2</sub> and saturated humidity.

For Zymosan stimulated cells, culture time should be approximately 24 hours to allow an immune response to develop. Zymosan is a general agonist of the immune system, here being used to promote an innate immune response. The use of other agonists is possible, and combined with for longer culture times, allows for the activation of other parts of the immune system. Cells have been successfully cultured for up to 92 hours, although passaging of cells into fresh culture media would allow this time to be extended.

When removed from incubator, cells can be pelleted out of suspension and stored at -80°C until ready to extract RNA.

## A1.2 References

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