The Evolutionary Consequences of Genetic Adaptation to Parasitism

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Abstract

The processes driving and maintaining variable immune responses are poorly understood compared with other aspects of an organism's ecology. This is particularly true from an evolutionary perspective, as the evolutionary relationships between immune responses and other traits and processes in nature remain inadequately explored. I investigated these associations in this thesis using the three-spined stickleback system as an evolutionary and immunological model. I combined sampling of wild individuals with genomic analyses to demonstrate phenotypic and genomic associations between immune responses and life history evolution across multiple populations. I also observe how experimental changes in daylength, a seasonal cue, modulate immune responses and increase parasite susceptibility in a controlled laboratory experiment. These findings occur independently of natural variation in parasite resistance. Stickleback are also a model for studies of speciation. I used sampling of wild hybrids to assess the significance of immune variation in postzygotic isolating mechanisms between diverging ecotypes; although my findings suggest a minor role. Finally, I demonstrate that genomic responses to parasitism and abiotic environmental variation are repeatable across independent, intercontinental adaptive radiation events in sticklebacks; conferring a repeatability of the evolutionary relationships of immune variation documented in this thesis. The findings within this thesis therefore provide novel insights into poorly explored or open areas of research regarding how variable immune responses evolve in nature.

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Contributions

The work presented in this thesis is my own, however certain people made contributions within various chapters:

Chapter 2: Life history data for populations was kindly provided by Abdul Rahman. All members of the MacColl group assisted with sampling. RAD-seq data was kindly provided by Isabel Magalhaes.

Chapter 3: Muayad Mahmud assisted during artificial experiments.

Chapter 4: Fish staining and morphometric measurements were taken by Reuben Fakoya (described in section 4.3.2). Laura Dean provided advice regarding DFA analysis.

Chapter 5: Sampling of adaptive radiations was conducted by Isabel Magalhaes and Daniele D'Agostino (section 5.3.1). RAD-sequencing was conducted by Isabel Magalhaes with assistance from Paul Hohenlohe (section 5.3.2).

General contributions

Andrew MacColl assisted with sampling/experimental design throughout and provided advice regarding statistics and analysis. Jan Bradley provided advice regarding immunological aspects of the project. Shaun Robertson assisted with molecular protocols and qPCR.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Ecoimmunology: The interaction between environment and immune variation

Given the ubiquity of parasites in nature (Poulin & Morand, 2000; Windsor, 1998), it is surprising that there remain gaps in our knowledge regarding the ecological and evolutionary relationships of immune responses in the wild. The evidence for the strength and significance of parasitism in evolutionary processes, such as the evolution of sex (Jokela et al., 2009; Morran et al., 2011) and mate choice (Folstad & Karter, 1992; Hamilton & Zuk, 1982; Penn & Potts, 1999), warrants the heightened study of parasitism as a significant source of natural selection for hosts. Whilst there has been a great research effort into understanding the functions and mechanisms of immune responses to parasitic offence, the wider evolutionary implications of such adaptations remain less understood. Furthermore, most of this mechanistic work has largely taken place in a laboratory setting. On the one hand, this has allowed for the controlling of confounding environmental variables, permitting the study of specific immune responses in isolation. However, recently there has been a call to return the field of immunology to the wild in order to understand how the immune system is influenced by ecological parameters (Jackson, 2015; Martin et al., 2011; Pedersen & Babayan, 2011). These parameters include but are not limited to; abiotic conditions; intra and interspecific competition for

both hosts and parasites; predation regimes; co-infection; and life history (Boughton et al., 2011). To fully understand a host's response to parasitism therefore, it is important to contextualise the host-parasite interaction within the natural environment of infection. In doing this, the field of eco-immunology aims to answer the question of why there is variation amongst immune responses, both at the individual and population level (Martin et al., 2011; Pedersen & Babayan, 2011).

The existence of immune variation in the wild is significant, because it indicates that immune responses must interact with the environment in such a way as to generate and maintain diversity. In comparison to the laboratory, natural environments are complex and dynamic, changing hugely over various degrees of time whilst constantly cycling through seasonal extremes. However, environments are also predictable and structured according to general ecological principles. In this sense therefore, our understanding of how immune responses evolve in the wild must take account of how trade-offs and costs manifest with respect to environmental influence, whilst also looking to understand how predictions can be made given environmental structuring.

The immune system, like any trait, comes with costs and trade-offs that must be balanced and optimised against the local environment and parasite community (Lochmiller & Deerenberg, 2000; Sheldon & Verhulst, 1996). Immune responses are one of the ways hosts can evolve to cope with parasitism, along with physical barriers (Fast et al., 2002), behavioural adaptations (Hart, 1990; Karvonen et al., 2004), and microbiota associations

(Honda & Littman, 2016; Koch & Schmid-Hempel, 2012), and thus parasites are a significant source of selection for immune responses (Little, 2002). However, in the absence of parasitism, the relative costs of immune responses are increased with respect to reduced benefits in fighting infection, which should lead to the evolution of weaker responses. This scenario is a cornerstone of evolutionary ecology theory based upon conservation of energy, and is well documented in the loss of other traits such as flightlessness in island birds (McNab, 1994). Further, multiple populations subjected to equal parasitism pressure can only evolve responses in line with their local environment. For example, populations may vary in aspects such as available nutritional resources, predation regimes and competition that limit hosts in their ability to evolve more substantial responses (Moret & Schmid-Hempel, 2000; Navarro et al., 2004). This is not to say that such evolved responses are suboptimal, but rather that the local environment is a key determinant of optimality.

1.2 The costs and trade-offs of immune responses

In this section, I will provide empirical evidence for the costs and trade-offs associated with adaptations to parasitism. These typically fall into three categories; 1) Pleiotropic interactions in which a limited resource base enforces nutrient allocation between traits that boost fitness, with fitness defined in the quantitative genetic sense; as a trait of an individual akin to its reproductive success and contribution of genes to the next generation, which is influenced by both natural and sexual selection (Orr, 2009); 2) Pleiotropic interactions

within the immune system itself, in which antagonisms between response types can skew the immune system to favour resistance to one type of parasite and increase susceptibility to another type during multiple infections; 3) Immunopathology (autoimmunity) in which the use of the immune system in response to infection directly damages the host. I will also note that some host responses to parasitism may appear similar to trade-offs but are in fact deliberately induced by parasites and should not be confused with trade-offs of immune adaptations. Whilst the evidence presented here suggests trade-offs and costs are common, the mechanisms by which they evolve and function are poorly understood (Long & Nanthakumar, 2004).

Evidence for pleiotropic trade-offs in immune genes has been demonstrated empirically in many taxa, such as single-celled organisms (Lenski & Levin, 1985), arthropods (Ahtiainen et al., 2005; Luong & Polak, 2007; Rivero et al., 2010), birds (Loiseau et al., 2008) and mammals (Kamath et al., 2014). As stated, pleiotropic trade-offs can typically take two forms, the first of which involves a reduction in fitness, through diversion of resources away from other fitness boosting traits such as growth or reproduction, or through increases in mortality. For example, Luong and Polak (2007) demonstrated a pleiotropic trade-off in the *Drosophila-Macrocheles* system, through artificially selecting lines for resistance; in this case a behavioural avoidance trait. In this study, resistant *Drosophila* exhibited a reduction in fecundity through egg reduction and resorption. Furthermore, a negative correlation between the ratio of melanin, a sexually selected trait in the wings of the damselfly, *Calopteryx*

splendens, and the level of immune response in the haemolymph suggests that allocation of resources shared by immune responses and other traits can be genotypically fixed for individuals (Siva-Jothy, 2000).

These ideas are based on the foundations that resistance is expensive and resources are limited. This is supported by Moret and Schmid-Hempel (2000), who found the only difference between workers of the bumblebee, *Bombus terrestris*, that survived an immune challenge and those that died prematurely was access to sugar water. Restriction of certain nutrients can also have specific effects. It has been demonstrated that Vitamin A deficiency can result in a reduced T-helper Cell Type 2 (Th2) response, zinc deficiency can result in a reduced Th1 response, and leptin has a number of regulatory effects on the immune system (Long & Nanthakumar, 2004). However, how expensive immunity is remains unclear, with some authors such as Rigby et al. (2002) suggesting that immunity is largely inexpensive, whilst others claim the costs are widely apparent (Lochmiller & Deerenberg, 2000). A summary of some energetic costs of immune responses can be seen in Table 1.1. Energy costs may come in the form of protein synthesis as the immune response unfolds, but also through processes such as glucogenesis to produce glucose for the metabolism of proteins, carbohydrates and lipids (Schmid-Hempel, 2011).

Table 1.1: Estimates of the energetic costs of immune defences. Table edited from (Schmid-Hempel, 2011) see reference for original studies.

Organism	Immune Response or Challenge	Associated Response (if applicable)	Estimated Cost
Drosophila	Infection by *Mycobacterium*	Humoral response	Loss of metabolic store in form of glycogen and fat
Cabbage Butterfly (*Pieris brassicae)*	Nylon filament implanted in diapausing pupae	Humoral and cellular immunity, specifically melanization and/or encapsulation of foreign particles	Standard metabolic rate increased by 8% on day 3 after challenge
Collared Dove (*Streptpelia decaocto)*	Humoral response upon Sheep red blood cell (SRBC)-challenge	Humoral response	8.5% increase in basic metabolic rate (BMR) and loss of body mass
Great Tit (*Parus major)*	Overwintering birds in the wild: Humoral response upon SRBC-challenge	Humoral response	8.6% increase in BMR (day 7). Loss of body mass
Blue Tit (*Parus caeruleus)*	Diphtheria-tetanus vaccine	Antibody response (Humoral response)	8-13% increase in BMR (day 7), generally low costs
House Sparrow (*Passer domesticus*)	Cell mediated	T-cell mitogenesis (Cell-mediated immunity)	29-32% increase over resting metabolism
Laboratory Rat	IL-infusion	Acute phase response (innate)	18% increase over resting metabolism
Laboratory Rat	Inflammation	*NA*	28% increase over resting metabolism
Laboratory Rat	Isolated mitochondria stimulated with TNF-α or IL-1	Inflammation	30% increase over resting metabolism

Laboratory Mouse	Keyhole limpet haemocyanin injection	Antibody response (Humoral response)	27-30% increase over resting metabolism
Gerbils (*Gerbillus*)	Infection by fleas	Cell-mediated immunity	Increased average daily metabolic rate of 3% (*G. andersoni*) to 16% (*G. dasyrus*)
Sheep	Endotoxin	Inflammation	10-49% increase over resting metabolism
Human	Shivering and illness	*NA*	Resting metabolism 300% of normal
Human	Sepsis	Inflammation (alters innate and adaptive immune responses)	30% increase over resting metabolism
Human	Sepsis and injury	Inflammation (alters innate and adaptive immune responses)	57% increase over resting metabolism
Human	Typhoid vaccination	Acute phase response (innate)	16% increase over resting metabolism
Human	Sickle cell disease	Increased whole body protein breakdown/synthesis	15% increase over resting metabolism
Human	Infusion of IL-6 to healthy volunteers	Inflammation	25% increase over resting metabolism

In the second type of trade-off, pleiotropic trade-offs may also manifest through increased resistance and susceptibility during multiple infections. This is a particularly important argument for studying immunology in the wild, and has been demonstrated in the plains zebra, *Equus quagga*. In this system, rare alleles at the MHC class II locus ELA-*DRA* were associated with increased gastrointestinal parasite loads whilst common alleles predicted increased ectoparasite loads (Kamath et al., 2014). Similarly, Loiseau et al (2008) found that the MHC class I allele *pado123* was maintained in populations of the house sparrow, *Passer domesticus*, despite it conferring a 2.5-fold increase in infection risk with *Plasmodium*. This was hypothesised to be due to a pleiotropic trade-off involving a 6.5-fold reduction in risk of harbouring another protozoan, *Haemoproteus.* In these instances, a contextualised equilibrium must be reached based on the selection pressures elicited by the harboured parasite community. Similarly, the general immune response in vertebrates to intracellular parasites is categorised by a Th1 response, whilst extracellular parasites elicit a Th2 response (Figure 1.1). Upon recognition of intracellular foreign antigens, Th1 cells release Interferon Gamma (IFNγ), which inhibits the Th2 response. Similarly, Th2 cells when activated release Interleukin-4 (IL-4) which inhibits the Th1 response (Long & Nanthakumar, 2004; Wakelin, 1996). Because of this inherent mechanism, the immune system can become skewed to respond to a certain type or species of parasite, allowing parasites that require an alternative response to proliferate.

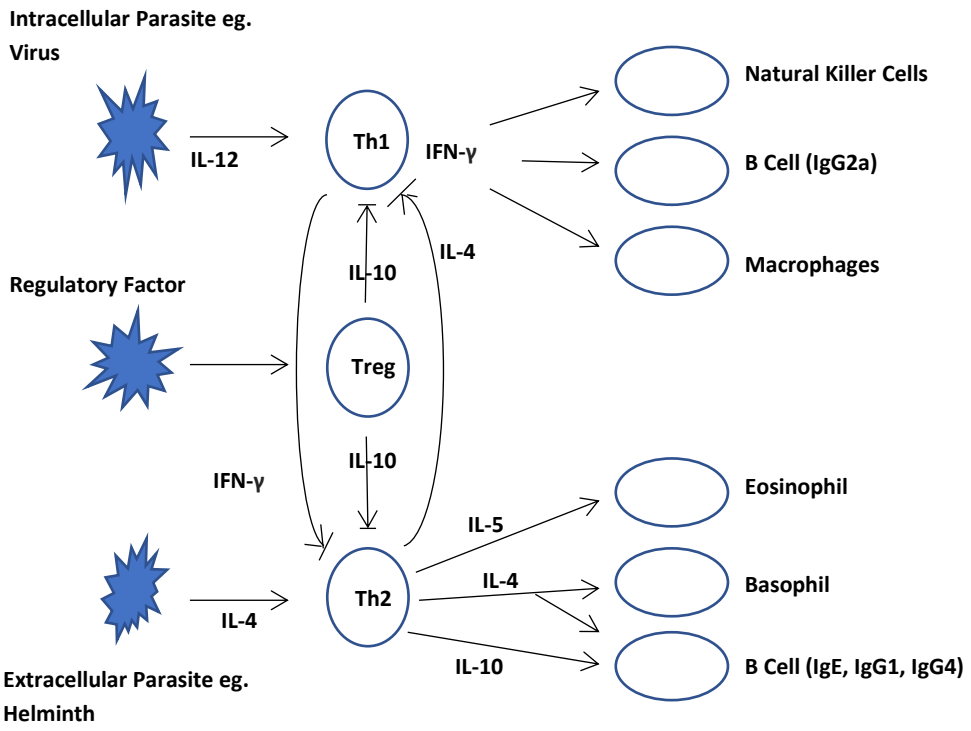


Figure 1.1: Differentiation of the T-helper response types illustrates the mechanisms behind the antagonism between the Th1 and Th2 responses. Arrows signify activation whilst blocked arrows signify inhibition. Adapted from (Long & Nanthakumar, 2004).

Immunopathology, or autoimmunity, represents the third way in which host adaptations may be deleterious. In these instances, the immune response is either over-, or inappropriately, expressed leading to pathological instances of self-damage to surrounding tissues or cells. Immunopathology has been extensively studied in humans, however its study in other vertebrates and particularly invertebrates has been somewhat lacking (Rolff & Siva-Jothy, 2003; Schmid-Hempel, 2011). The complexity of the immune system and autoimmunity is beyond the scope of this review; however, it feels pertinent to discuss some mechanisms of immunopathology. As opposed to parasite-

specific skewing of the immune system as seen in the Th1-Th2 antagonism, cases of immunopathology are non-parasite specific and more general. For example, cytokines, a large, variable group of signalling molecules used by the immune system in cell-cell signalling (Wakelin, 1996), can set into motion immune responses that unless strictly modulated can damage the host. A demonstration of this occurs during malaria infection, where over-activity of Tumor Necrosis Factor Alpha (TNF-α) expression degrades blood cells required by *Plasmodium* spp., but also results in a loss of vigour or anaemia in mice (Graham et al., 2005). Furthermore, the encapsulation immune response to *Schistosoma* infection, induced by IL-13 leads to the formation of granulomas and fibrosis in infected tissues resulting in the clinical symptoms of Schistosomiasis (Graham et al., 2005). It has also been suggested that cytokines inducing inflammation such as TNF-α, IL-1 and IL-6 (Kopp & Medzhitov, 2009) are associated with cancers following parasitic infection (Lin & Karin, 2007). In humans for example, the risk of developing gastric carcinomas increases following infection by *Helicobacter pylori* (Coussens & Werb, 2002), as does the risk of developing bladder and colon carcinomas following *Schistosoma* infection (Hussain et al., 2003), as a result of innate inflammatory responses by the immune system (Lin & Karin, 2007). Inflammation can also inadvertently modify lipid and lipoprotein metabolisms which, if left unchecked, can promote development of Atherosclerosis (Khovidhunkit et al., 2004).

It is important to distinguish the kinds of trade-offs mentioned thus far from those that are induced solely by the parasite. In these cases, a detrimental autoimmune effect of parasitism may not be a result of adapted immune responses at all, but another aspect of infection evolved to boost the fitness of the parasite. In the relationship between *Plasmodium* and its host, the mosquito *Culex pipiens*, infection is associated with a decrease in host fecundity and increase in host longevity. In comparison to pleiotropic life history trade-offs with resistance, this is likely to be an induced effect by the parasite, allowing for an increased development time and increased chance of transmission through *C. pipiens* biting (Vézilier et al., 2012). This is but one of many examples of host manipulation observed in nature (Moore, 2002) however it is important to separate these from pleiotropy and autoimmune trade-offs because they are not genetically determined by the host itself and therefore will not behave in the same way from an evolutionary perspective in the host-parasite interaction. As opposed to the way in which a trade-off may mediate the evolution of a host response, host-manipulation traits are another symptom of disease and should increase selection on host responses.

1.3 Evolutionary relationships between immune responses and other host traits

One of the best means by which we can understand immune variation in the wild is to examine how that variation correlates with the variation of other traits (Zuk & Stoehr, 2002). Selective agents in the wild are often

multidimensional, affecting and manipulating phenotypes along a cumulative axis of variation (MacColl, 2011). For some traits, we have a good understanding of how this process works. The life history of an organism defines several traits that tend to correlate in the wild; traits including developmental period, body size, fecundity and longevity. Typically, larger organisms tend to take longer to develop, produce fewer offspring with greater per offspring investment and senescese slower leading to longer lifespans (Promislow & Harvey, 1990; Ricklefs & Wikelski, 2002).

Through what has been discussed regarding costs and trade-offs we can begin to formulate hypotheses regarding life history interactions. For example, senescence is a component of life history variation involved with longevity. Senescence is, at least partly, genetically determined (Reznick et al., 2004) and varies among populations and closely-related species (DeFaveri et al., 2014; Nussey et al., 2008). Theories to explain the evolution of senescence are varied. Medawar's mutation accumulation theory (Medawar, 1952) suggests senescence manifests through the build up of deleterious mutations in genes involved in post-reproductive traits. This is because selection against such deleterious alleles should decline with the age a trait is expressed. Similarly, Williams' antagonistic pleiotropy theory (Williams, 1957) argues that weaker selection post-reproduction can favour the evolution of pleiotropic traits that are advantageous in early life but deleterious in post-reproductive stages.

Whilst there is evidence to support these ideas, their validity in natural systems has come under fire, as an overemphasis on senescence as a function

of exclusively the chronological age of an organism fits in poorly with observations from the wild (Williams et al., 2006). Kirkwood's disposable soma theory (Kirkwood, 1990) provides an alternative to age-centric theories, suggesting senescence is the product of resource partitioning between fitness-boosting traits and somatic maintenance. In the wild, chronological age without the consideration of environmental ageing factors, such as winters survived or previous parasite infection has proved insufficient to explain senescence (Hayward et al., 2009, 2011; Reznick et al., 2004; Sherratt et al., 2010). This idea of mortality factors interacting has also been proposed by Williams and Day (2003), who suggested through modelling that increasing interaction between environmental factors associated with mortality should select for minimal senescence in early life and rapid senescence in later life.

This emphasis on senescence interacting with the environment has drawn interactions with immune responses and parasitism into the fold. Taking what we know about costs and trade-offs, we can make predictions regarding how we might expect immune responses to coevolve with senescence according to various theories centred around age and interactions with the environment. For example, immune responses associated with autoimmunity represent a trait that may contribute towards senescence through antagonistic pleiotropy. Here, selection for stronger inflammatory responses, for example, in response to local parasitism may lead to an increased likelihood of autoimmune, inflammatory-associated conditions in later life and thus deteriorate condition and increase senescence (Franceschi et al., 2000, 2017).

Empirically, the costs of resistance are demonstrable given that animals such as field voles, *Microtus agrestis*, shift towards less autopathological tolerance responses in older age classes (Jackson et al., 2014). There are fitness advantages associated with tolerance phenotypes rather than resistance seen in other species (reviewed in (Kutzer & Armitage, 2016)), which are in keeping with current hypotheses. Additionally, selection for immune responses that are more energetically taxing should divert resources away from somatic maintenance, incurring more rapid senescence. For example, in a short-lived organism, earlier maturation and reproduction at the expense of resources available for immunity may be the optimal strategy (Lee, 2006; Williams et al., 2006). Pauwels et al. (2014) showed that in the presence of fish kairomones, *Daphnia* prey experienced an increased susceptibility to rotifer parasites as a result of increased investment in early reproduction.

The rate of declining condition, which is a product of the cumulative senescence of various organs and tissues, constitutes a single aspect of an individual's life historty. However, we can introduce other life history traits to see how immune variation correlates with wider life history. From the hypotheses above, stronger immune responses should be associated with a more rapid onset of senescence, assuming they incur increased autoimmunity and resource demands. Therefore, in scenarios in which shorter life histories are favoured by selection, we may see two scenarios. Firstly, we may expect to see stronger, developmentally cheap but use-costly responses (Lee, 2006). This is because the costs of expensive or autopathological responses can be offset

by a shorter lifespan. Alternatively, shorter life histories may favour weaker immune responses to funnel limited resources into development and reproduction (Pauwels et al., 2014). The adaptive potential of each scenario is likely determined by the selection pressure of parasitism. In terms of causality in these relationships, the reality is that we should observe effects in both directions. For example, where parasite pressure is extreme enough to select for costly immune responses, we may expect to see increased senescence or other life history changes as a result. Similarly, where there is strong selection pressure for shorter life histories, we would expect to see immune responses change in accordance. The goal here therefore is to incorporate parasitism into a multi-dimensional understanding of selection in natural environments.

In Chapter 2, I investigate these hypotheses and provide empirical evidence by utilising a genetics-based methodology, combining gene expression analyses with genomics to ascertain how immune responses vary across multiple populations of a single species with contrasting life history strategies. In doing so, I provide some of the strongest empirical support in the literature for the evolutionary relationships between immune responses and host life history traits.

1.4 Evolutionary relationships between immune responses and wider evolutionary processes

Interactions between immune variation and host traits impose evolutionary constraints that help explain how immune variation persists in the absence of

variation in parasitism. However, immune variation should also interact with parasite selection pressures themselves, through sculpting the host adaptive landscape and in turn modifying selection for parasites (Mackinnon & Marsh, 2010; Thompson, 2002). Because parasites exist either inside or on their hosts, the host environment represents a substantial source of selection for parasites, and principle among these are how host defences are deployed to cope with infection. Demonstrating this, Cornet et al. (2014) showed that the host immune environment could be modulated through experimental nutrient supplementation, which in turn modulated the virulence of malaria parasites taken from birds within each treatment group.

Parasites are significant agents of selection on immune responses (Little, 2002), but the inverse is also true. These interactions are exemplified by Red-Queen models of host-parasite co-evolution (Decaestecker et al., 2007; Penn & Potts, 1999), whereby hosts may cycle through a diverse pool of immune genes maintained through negative frequency dependence of parasite resistance. These biotic, stochastic interactions influence numerous measures of parasite fitness, such as virulence (reviewed by (Cressler et al., 2015)); defined generally as the reduction in host fitness attributable to infection and a product of parasite density and per parasite virulence (Little et al., 2010).

A prominent example of the way host immune environments influence parasite evolution regards the differential selection imposed on parasites from hosts with resistant or tolerant phenotypes. Tolerance defines the relationship between host fitness and parasite density, whereby more tolerant hosts exhibit

higher fitness at specific parasite densities (point tolerance) or reduced gradients of declining fitness with increasing parasite density (range tolerance) (Little et al., 2010). In contrast, resistance defines the host's ability to limit parasite burden (Råberg et al., 2007), and therefore differs from tolerance in directly interacting with the parasite.

Models of parasite virulence suggest that in tolerant hosts, parasites are selected to reduce their virulence. Such predictions are based on the trade-off between parasite virulence and transmission, whereby if virulence is too high and host mortality is too high, transmission will decline in accordance. This trade-off is generally accepted (Cressler et al., 2015; Frank, 1996), although some studies suggest it may not be ubiquitous (Alizon et al., 2009). This reduction in virulence is accompanied by an increase in prevalence of the parasite within the population. Host-parasite dynamics are further predicted to reach stable states (Best et al., 2014) at which point parasites are no longer selected to further exploit their hosts and hosts are no longer selected to resist the parasite. This can result in a state of apparent commensalism (Miller et al., 2006).

In contrast to this, populations that evolve resistance mechanisms are predicted by models to cycle with their parasites, with virulence selected to increase with resistance (Kubinak & Potts, 2013). Assuming resistance is costly, models by Miller et al. (2007) envisage that resistance should be selected for when parasite prevalence and thus risk of infection is high. As infection risk declines as resistance spreads through the population, so does the selection

pressure associated with resistance. Eventually resistance becomes too costly to maintain and so resistance should decline in the population, allowing the parasite to infect more individuals and increase in prevalence. This cycling is predicted to go on indefinitely.

Autoimmunity associated with resistance is predicted to further drive parasite evolution (Day et al., 2007). Depending on the mechanisms of resistance, immunopathology may boost parasite fitness or be detrimental. Effects are also dependent on whether immunopathology is a function of parasite virulence or occurs at levels irrespective of virulence (Day et al., 2007). For example, immune cell damage may hinder disease clearance benefitting the parasite (Carrero et al., 2004), or inflammation may increase transmission (Best et al., 2012; Dao et al., 2004). On the other hand, if the parasite's target cells are damaged by the immune response, this may represent an added detrimental effect for the parasite of the host's response (Moret & Schmid-Hempel, 2000). Models by Best et al (2012) predict that if immunopathology is beneficial to parasites, selection for parasitic exploitative traits should decline, particularly if immunopathology is associated with increased host mortality. Put simply, the more hosts damage themselves, the less parasites can afford to also damage them. This would manifest within a population as a reduced number of disease deaths but an increase in individual case severity. However, if immunopathology is detrimental then the reverse is predicted, as parasites are selected to compensate for their reduced fitness with increased host exploitation. Best et al. (2012) also provide a number of examples for which

immunopathology has been correlated negatively with parasite fitness, such as: *Staphylococus aureus*, *Histoplasma capsulatum* and *Bacillus anthracis*. Also enumerated are parasites for which parasite fitness correlated positively with immunopathology including: *Streptococcus pyogenes*, *Streptococcus pneumonia*, *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Toxoplasma gondii*. Macroparasite examples are harder to come by in the literature, although it is probable that these parasites are similarly affected.

Even environmental interactions between the host and other organisms can impact parasite evolution. For example, Morozov and Adamson (Morozov Yu. & Adamson, 2011) modelled the effects of predation on the relationship between parasite virulence and host mortality. Their models predict that the introduction of predation increases the evolutionary stable level of virulence within the parasite. Additionally, the level of predation is predicted to modify the evolutionary stable level of host resistance (Toor & Best, 2015). Further, predation on hosts may promote evolutionary branching of parasite strains and promote polymorphic virulence (Morozov & Best, 2012).

The evolutionary relationships between hosts and their parasites have been modelled extensively, however empirical support from the literature highlights how the complexity of natural systems may hinder the usefulness of models limited by simplified assumptions. For example, in a study of Monarch butterflies, *Danaus plexipus*, from three populations, and their protozoan parasite, *Ophryocystis elektroscirrha*, researchers failed to observe the expected relationship between increased tolerance and increased prevalence

(Sternberg et al., 2013). However, the most virulent population did harbour the most resistant parasite strain.

The above examples demonstrate that the host immune response is a key determinant of host-parasite evolutionary relationships. Thus, any environmental event that alters the host immune response should have evolutionary implications for both hosts and their parasites. Whilst divergent selection regimes between environments can generate immune variation, the immune responses of individuals also vary across the year, displaying annual rhythms. This temporal variation in host immune responses and its effect on host-parasite relationships represents a fruitful, underexplored theme of research in this area (Martinez-Bakker & Helm, 2015). Recent research has demonstrated that vertebrate immune responses exhibit some annual rhythm whereby certain responses, such as inflammation, are upregulated or downregulated at certain times of year (Brown et al., 2016; Dopico et al., 2015; Morey et al., 2016). Daylength has been experimentally shown to be a causative environmental cue in eliciting changes to immune responses (Stevenson & Prendergast, 2015), and indeed the neuroendocrine pathways that communicate changes in daylength to the immune system are conserved across vertebrate taxa (O'Brien et al., 2012). Daylength therefore should modulate immune responses and lead to temporal changes in susceptibility and resistance. It is well-documented that parasite infection dynamics display seasonal incidences (Altizer et al., 2006; Nelson, 2004), and thus this mechanism seems probable. However, to date, a causative relationship

between daylength modulation of immune responses and changes in parasite resistance has yet to be demonstrated experimentally. Crucially, such an observation would demonstrate that the selection imposed on parasites from their host's immune responses should display seasonal patterns, and therefore parasite traits such as virulence should evolve temporally in concert with seasonal host changes. This relationship is examined in detail through a common-garden artificial infection experiment with factorial design in Chapter 3.

Additionally, there are many outstanding questions in this field regarding population-level differences in these interactions. For example, if populations vary in their natural resistance to a parasite, how does seasonal modulation interact with this population-level variation? This is important, given that seasonal immune modulation affects certain responses differently (Dopico et al., 2015; Stevenson & Prendergast, 2015). In Chapter 3, I include two populations that have shown differential levels of natural resistance in the laboratory to study this. Additionally, what are the sources of selection for host responses to be modulated by daylength? Current theories include suggestions such as seasonal resource variation or usage and seasonal parasitism. The latter is considered in Chapter 3, as my naturally susceptible population does not tend to encounter the parasite in the wild. This therefore permits me to postulate on how seasonal cues and host susceptibility have evolved in the presence and absence of the parasite in the wild.

Immune variation is therefore important for parasite evolution, but divergent immune responses may also contribute towards the speciation process of hosts. In ecological speciation, traits that improve an organism's fitness within its local environment are selected for and should become fixed. In an environment with multiple niche spaces, there are also numerous peaks within the adaptive landscape, and therefore divergent selection processes should operate to separate populations (Nosil, 2012). Assuming only one adaptive peak can be utilised (specialisation rather than generalisation), selection acts to hinder random mating between groups and favour assortative mating within ecological specialists. Following ecological specialisation therefore, selection favours the development of barriers to gene flow, which are prezygotic or postzygotic (Rundle & Nosil, 2005; Schluter, 2009). Prezygotic barriers represent anything that inhibits mating, and more specifically fertilisation, such as behavioural (Maan & Seehausen, 2011; Martin & Mendelson, 2016; Merrill et al., 2011) or mechanical traits (Ueshima & Asami, 2003). Postzygotic barriers constitute post-fertilisation, negative implications for hybrids, such as genomic incompatibilities (Orr & Turelli, 2001; Seehausen et al., 2014) or hybrid maladaptation (Peccoud et al., 2014).

Immune responses represent traits that are ecologically adaptive and thus capable of adaptively diversifying between populations or ecotypes (Lenz et al., 2013; Scharsack et al., 2007a; Sparkman & Palacios, 2009). Further, adaptations to parasitism have been suggested to represent potential 'magic traits' (MacColl, 2009b); traits that are both ecologically adaptive and promote

assortative mating (Gavrilets, 2004; Servedio et al., 2011). Other proposed magic traits suggested in nature include body size in three-spined stickleback, *Gasterosteus aculeatus* (Conte & Schluter, 2013; Head et al., 2013), and opsin divergence in cichlids (Maan & Seehausen, 2010; Seehausen et al., 2008). For immune genes, one such magic trait theory posits that genes of the major histocompatibility complex (MHC), a constituent of the vertebrate adaptive immune response responsible for parasite resistance through the recognition and presentation of foreign antigens (Kurtz et al., 2004), may also be involved in mate choice (Eizaguirre et al., 2011; Milinski, 2006). In a recent study, Andreou et al. (2017) demonstrated the potential of this mechanism in an experiment with diverged stickleback ecotypes, and found female stickleback recognised and preferred within group males when presented by olfactory signals from males presenting MHC signals (males that had produced nests (Milinski et al., 2010)). However, the prominence and strength of MHC-based mate choice in vertebrate taxa is unresolved (Kamiya et al., 2014), with methodological inconsistencies cited as a concern within the literature, whilst an inability to distinguish unknown olfactory signals and MHC-associated olfactory signals may be similarly confounding.

From a postzygotic reproductive isolation perspective, immune responses in animals have received little attention. Interestingly, it is well established that the breakdown of immune regulation in plant hybrids can result in necrosis because of rampant autoimmunity (Bomblies et al., 2007; Bomblies & Weigel, 2007; Chae et al., 2014; Świadek et al., 2017), however

whether such processes occur in animal hybrids in the wild is unstudied. Such breakdowns in regulation occur due to Bateson-Dobzhansky-Muller incompatibilities, whereby hybridisation brings together incompatible diverged alleles (Dobzhansky, 1936; Muller, 1942). Within diverging populations, function is maintained through balancing selection, however balancing selection is unable to act on novel hybrid genotypes, with the result being improper gene function. For immune genes, dysregulation may represent reduced fitness in the form of poorer immunocompetence or increased autoimmunity, although the former seems unlikely based on recent evidence (Baird et al., 2012; Krasnovyd et al., 2017; Wiley et al., 2009). Both scenarios represent potential postzygotic isolating barriers and therefore the role of immune genes in the speciation process may be more prominent than currently thought. Immune genes have been highlighted as being improperly expressed in laboratory-produced hybrid embryos (Hill-Burns & Clark, 2010; Mavarez et al., 2009), however whether such transgressive patterns occur in nature is unknown. Chapter 4 expands upon these thoughts using sampling data from a naturally occurring hybrid zone.

1.5 Using the environment to predict evolutionary relationships

One of the aims of this thesis is to integrate our understanding of immune response variation into the general and well established principles of evolutionary ecology. I have discussed how the local environment interacts with costs and trade-offs of immune responses to constrain resistance and

maintain variation, however the local abiotic environment also directly manipulates parasite communities (Figure 1.2). Whilst host immune responses are a dominant source of variation for parasites, how local parasite communities are assembled can be directly or indirectly affected by environmental characteristics irrespective of host immune responses.

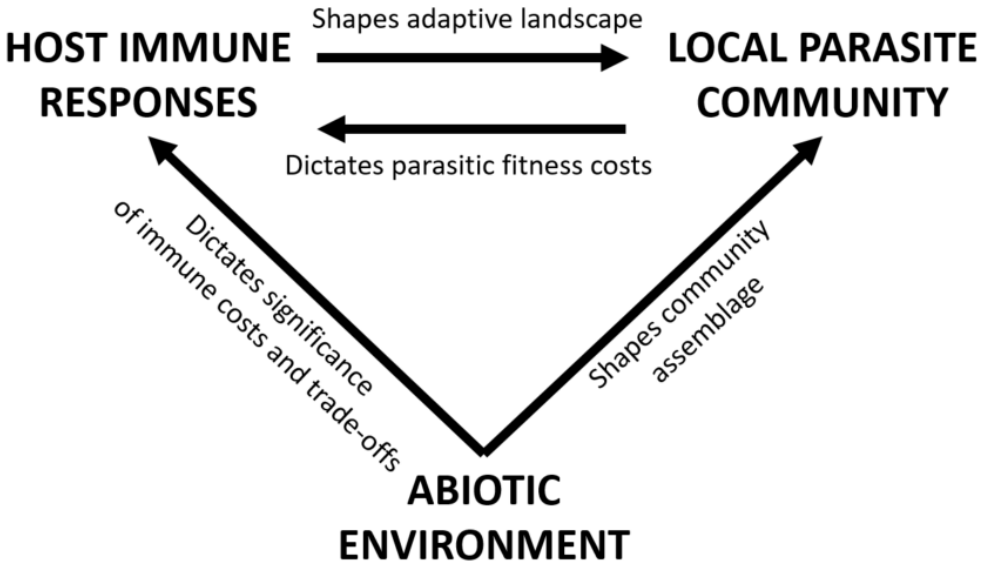


Figure 1.2: Interactions between host immune responses, local parasite community and the abiotic environment are predicted to display some degree of repeatability.

There are numerous examples of such interactions in the literature. Malaria parasites and others transmitted by vectors are affected by environmental patterns of temperature and precipitation that modulate vector populations (Zamora-Vilchis et al., 2012). In addition, there is evidence to suggest that the diversity of human pathogens follows latitudinal patterns associated with climatic variables such as annual precipitation variation

(Guernier et al., 2004). Whilst humans are by no means a model system for general host-parasite inferences, these effects do appear consistent in spite of confounding anthropological factors such as urbanization and modernization. A prediction of environmental influence on parasite assemblage would be that effects should be more pronounced in parasites with an external life stage. Indeed, this appears to be the case for human pathogens such as helminths and vector-transmitted pathogens as opposed to bacteria (Guernier et al., 2004). We might expect therefore that immune responses that are adapted to deal with certain parasites should be predictable according to spatial patterns of climate.

It should be unsurprising that parasite communities are affected by latitudinal spatial clines, given that many animal and plant taxa generally show similar patterns of increased diversity towards the equator (Pianka, 1966). Latitudinal clines represent correlated changes in many environmental variables and thus community effects may be inevitable, however even at small spatial scales the effect of local environmental variables in shaping parasite communities still appears strong. For example, anthropogenic land use around roosts of big brown bats, *Eptesicus fuscus*, is more important than spatial proximity in explaining correlated helminth community structuring (Warburton et al., 2016). Consistent with predictions from human pathogens, Warburton et al. (2016) highlight the effects of urbanization on helminth (predominantly trematodes) intermediate host abundances, such as freshwater snails and caddisfly. There are further examples of parasites with complex multi-host life

cycles varying with the environment. For example, following the egg stage, free swimming miracidium of the trematodes, *Diplostomum sp.*, infect aquatic snail intermediate hosts, before further infecting fish intermediate hosts and reaching sexual maturation in final avian hosts. Abiotic influences at any of these stages will affect the prevalence of infection for all hosts. Calcium availability is an important environmental constituent for snail shell development, and therefore limits the range of gastropods to some extent (Briers, 2003b). We might thus expect calcium availability to influence *Diplostomum* prevalence for all hosts in lakes with contrasting calcium ion concentrations. This has been demonstrated in some studies (Curtis & Rau, 1980), but not others (Rahn et al., 2016a).

However, direct interactions between the environment and parasites are also documented in the literature. For example, climatic variation, along with anthropogenic factors, is important for determining haemosporidian parasite communties of avifauna in the Canary islands (Padilla et al., 2017). Here, factors such as temperature limit the range of *Plasmodium* species due to the effect of low temperatures and developmental retardation of the parasite. Further, in aquatic systems, the pH and salinity of water influences the prevalence (Bakke et al., 2002) and virulence (Mahmud et al., 2017) of ectoparasites such as the monogenean flatworm genus *Gyrodactylus*, which infects many taxa of fish.

To further emphasise the interaction between host-parasite relationships and the local environment, we can examine how parasitism

changes across a hosts range. For example, at the edge of their range, hosts can find themselves limited by their environment. For *Geckobia* mites of the Asian house gecko, *Hemidactylus frenatus*, a reduction in host density at host range edges results in lower prevalence (Coates et al., 2017) , highlighting another mechanism by which the changing environment across a host's range may affect parasites. In the same host, prevalence of the tapeworm, *Raillietiella frenata,* declines similarly at range edges, however here the authors ascribe reduced prevalence to the absence of an intermediate cockroach host at range edges (Coates et al., 2017). The inverse can also occur, such as increased trematode prevalence at range edges of the freshwater snail, *Lymnaea stagnalis* (Briers, 2003a). Here, the author suggests host immune responses may be traded-off against an increased metabolic effort to source lower levels of environmental calcium for development, resulting in increased prevalence at range limits.

It is thus apparent the environment can be a key determinant of parasite community, either directly or indirectly. Because of the close interactions between the environment, hosts, and their parasites, it offers up the tantalising prospect that host responses should evolve in repeatable ways given similar environmental conditions and parasitism pressures. Such repeatability should stem from the homogenization of selection regimes, but at the same time should be dependent on genomic constraints such as shared genetic variation for selection to act on (Conte et al., 2012), as well as shared genomic architecture that affects the evolvability of certain genomic regions

(Renaut et al., 2014). Yet, there are surprisingly few examples of convergent evolution of immune responses in the literature, an observation that likely reflects the relative infancy of ecoimmunology. Srithayakumar et al. (2012) report convergent evolution of the MHC gene *MHC-DRB* in response to shared parasite communities in sympatrically occurring North American mustelid species, the striped skunk, *Mephitis mephitis*, and raccoon, *Procyon lotor*. Elsewhere Jacquin et al. (2016) demonstrate parallel behavioural responses to *Gyrodactylus* sp. in Trinidadian guppies, although this is only phenotypic. Whilst these studies highlight that convergent evolution of adaptations to parasitism can occur, it remains unclear how prevalent such repeatability is in nature and how predictable it is based on the environment.

In addition to the lack of research into repeated evolution of immune responses to parasites, there has been little research that has sought to contextualise this repeatability alongside environmental variation. Given the role of the environment in organising parasite selection regimes, this omission offers a fruitful avenue for research. In light of this, I investigate the repeatability, or parallelism, of genomic evolution in response to parasitism alongside environmental variation in chapter 5. To do this, I examine genomic parallelism across intercontinental adaptive radiations. Adaptive radiations represent isolated, independent diversification events whereby a myriad of diverse, adaptive forms originate from a single founding population. This therefore allows me to assess the role of environmental variation in eliciting adaptive variation. Examining these patterns across adaptive radiations

permits the examination of whether shared environmental variation independently associates with parasite community assemblage, and whether genomic responses to shared selection regimes results in mutual adaptation. Ultimately, such an understanding aids in the coming together of the theories discussed thus far regarding the interactions between host immune evolution, parasites, and the local environment.

1.6 The study system

The work conducted in this thesis involved sampling wild individuals (Chapters 2, 4 and 5) or using laboratory-bred individuals from wild populations (Chapter 3) of three spined stickleback, *Gasterosteus aculeatus*, hereafter referred to as 'stickleback'. The populations studied are found on the outer Hebridean island, North Uist, Scotland (Chapters 2, 3, 4 and 5) (Figure 1.3) with the addition of populations from Alaska, British Columbia and Iceland (Chapter 5).

Stickleback are a model system for studying adaptive evolution (Gibson, 2005; Hendry et al., 2013). Stickleback colonised freshwater habitats across the Northern Hemisphere at the end of the last glaciation ~10,000 years ago (Colosimo, 2005; Jones et al., 2012b). Since then, they have rapidly adapted from marine ancestors into a myriad of adaptive freshwater forms (Figure 1.4).

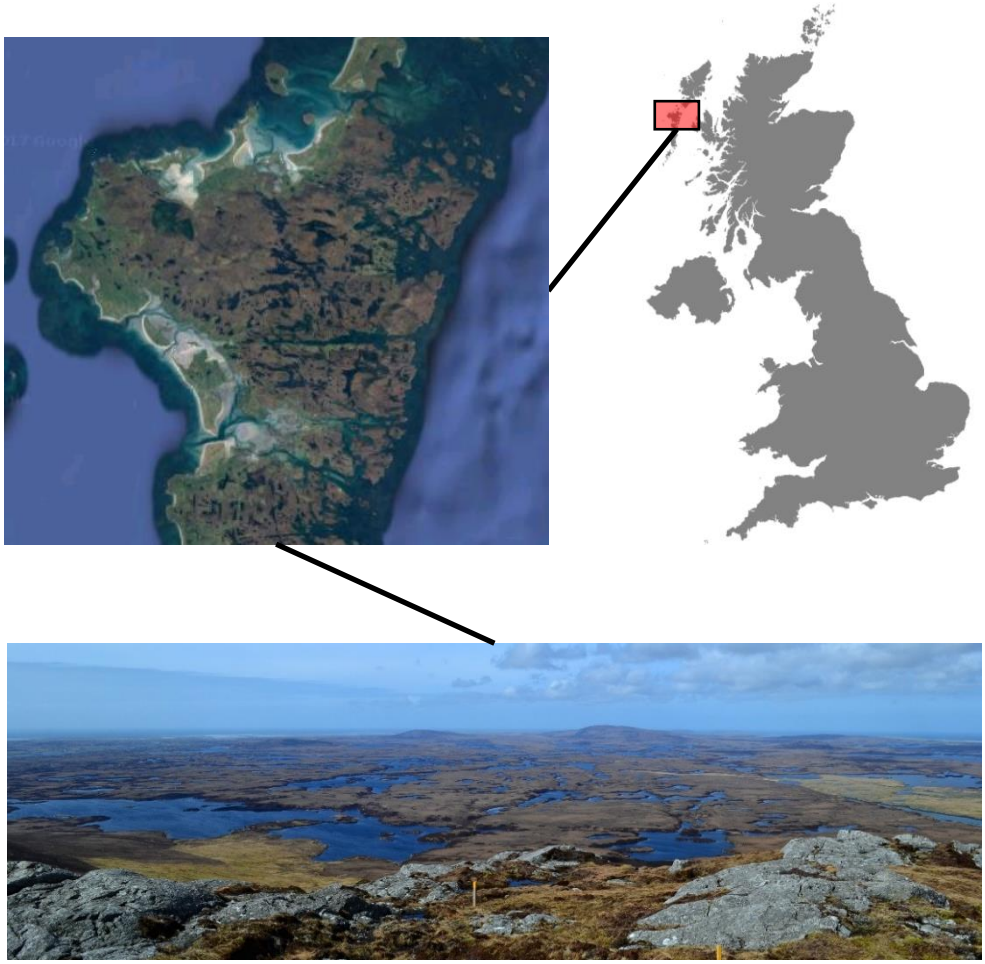


Figure 1.3: The isle of North Uist lies off the North-West coast of Scotland. Its low-level network of freshwater and saltwater lochs harbours an adaptive radiation of *G. aculeatus*. The bottom photo shows the distribution of lochs over the Eastern side of the island.

Through studying the associations between adaptive diversity and environmental variation, stickleback systems allow researchers to examine the role of natural environmental variation in shaping adaptive evolution. A large amount of research has been undertaken regarding the role of environmental factors such as calcium availability, salinity and predation in the repeated loss of morphological bony armour and plating reduction in freshwater fish (Barrett et al., 2008; Colosimo, 2005; MacColl et al., 2013). However, stickleback are

also a promising ecoimmunology system (Barber, 2013). Like all teleost fish, they possess a full vertebrate immune system, allowing inferences to be drawn across vertebrate taxa (Foey & Picchietti, 2014; Wilson, 2017). They also encounter a diverse array of parasites (MacColl, 2009a; Poulin et al., 2011) and have evolved divergent immune responses in response to differential environmental and parasite pressures (Huang et al., 2016; Jones et al., 2012a; Lenz et al., 2013; Milligan-Myhre et al., 2016; Robertson et al., 2016a; Scharsack et al., 2007a).

Like most stickleback systems, the adaptive radiation of stickleback on North Uist has long been studied from a morphological perspective (Campbell, 1984; Giles, 2010). More recently, the ecology of the island radiation has been studied extensively, and we now know that the lochs inhabited by populations vary in aspects such as conductivity, temperature, depth and predation (Magalhaes et al., 2016). There also exists variation in resource availability through differential phytoplankton and zooplankton communities (T. Chitheer, A. Rahman and A.D.C MacColl, unpublished data). There is a strong pH gradient across the island, with a shift from acidic to alkali as one moves from East to West. This longitudinal pH gradient also predicts a life history gradient, as Eastern populations are smaller and tend to exhibit short-lived strategies whilst Western populations are longer-lived (Rahman, 2017). Additionally, populations tend to exist in allopatry, as North Uist is made up of a network of fresh and salt water lakes (‘lochs’), resulting in strong genetic differentiation and population structuring between lochs (Magalhaes et al., 2016; Rahn et al.,

2016b). This plethora of environmental variation has yielded rich adaptive diversity in many traits, including immune responses. Parasite communities across lochs vary considerably (De Roij & MacColl, 2012; Rahn et al., 2016a; Young & MacColl, 2017) and diverged immune responses and resistance with regards to various macroparasites are also reported (De Roij et al., 2011; El Nagar & MacColl, 2016; Robertson et al., 2016a, 2016b). The previous work conducted in this system thus provides the foundation for the studies outlined here, providing well-documented information on a range of environmental variables, parasite communities and levels of natural resistance between populations.

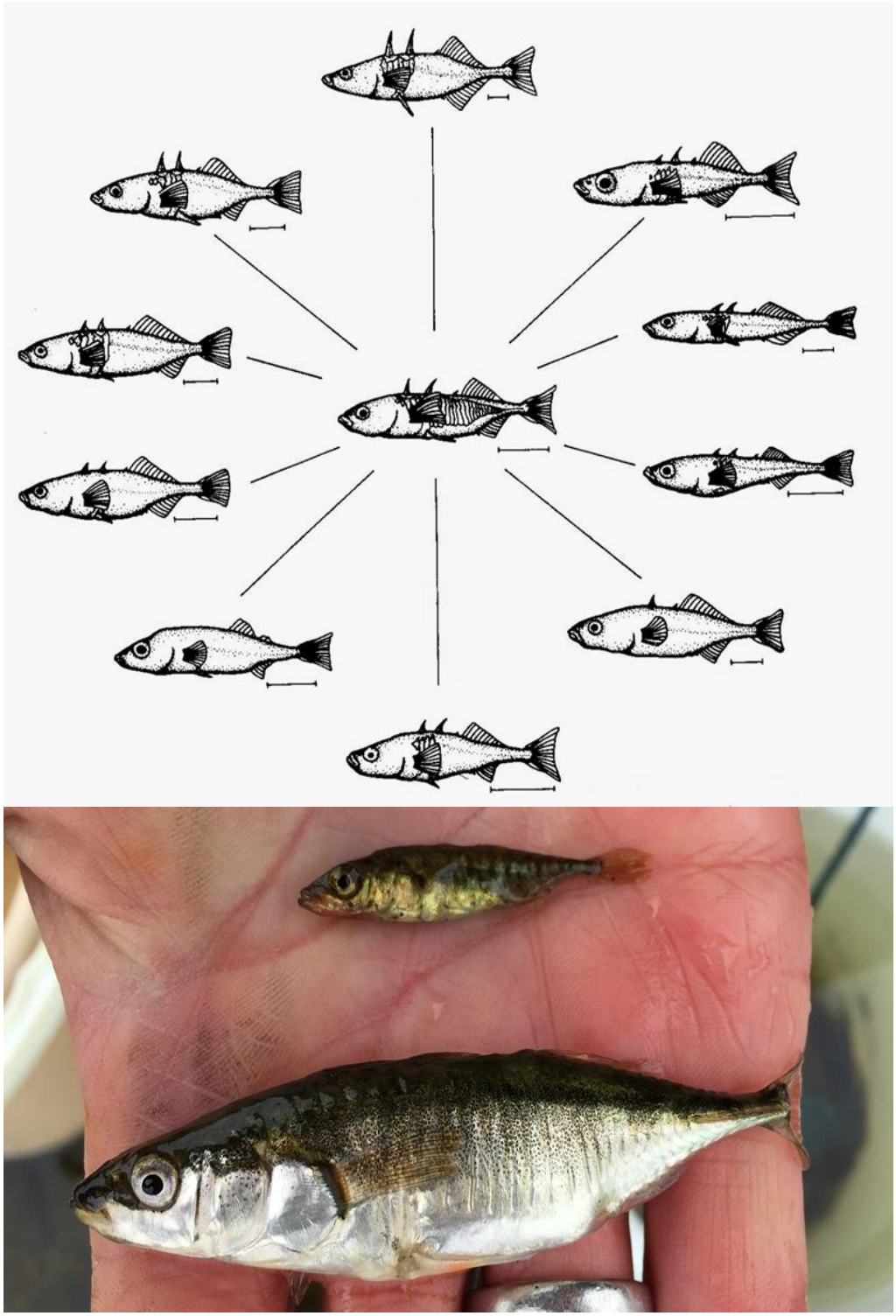


Figure 1.4: Rapid evolution of freshwater diversity from marine ancestors. Diagram at the top displays morphological diversity depicted in (Bell & Foster, 1994). Photograph at the bottom shows a diverged saltwater resident alongside the larger anadromous marine ecotype (photograph by Laura Dean).

1.7 Thesis outline

The aim of this project was to investigate how wild immune variation interacted with other evolutionary processes, with a goal of increasing our understanding of how immune variation evolves and is maintained in the wild. Each chapter in this thesis is presented as an individual manuscript which posits on a novel question or research theme regarding the evolutionary consequences of adaptation to parasitism. These chapters are outlined as follows:

Chapter 2 – The relationships between life history and immune responses.

In this chapter, I use qPCR to quantify immune responses between wild-sampled individuals experiencing different life history events, namely reproductive investment, within natural populations. I also examine how immune responses vary across populations of contrasting life history strategies. I also incoporate bioinformatics techniques using RAD-seq data, to assess genomic associations between life history strategies and immune responses in order to validate my findings and contexualise my qPCR results in an evolutionary framework. In short, this chapter asks: 1) How do immune responses vary plastically during life history events? 2) How do immune responses vary across similar stage individuals from populations of contrasting life history strategies? 3) Is there evidence that these relationships have a heritable genetic/genomic basis?

Chapter 3 – Daylength modulation of immune responses and its effect on parasite susceptibility

Here, I use laboratory-raised fish in a common-garden experiment to assess how experimental manipulation of daylength affects condition, immune responses, and parasite susceptibility. This study incorporates a factorial artificial infection design and asks: 1) How are immune responses affected by summer and winter photoperiod treatments? 2) How is fish condition and reproductive investment affected by summer and winter photoperiod? 3) Do these changes affect parasite susceptibility? 4) What is the interaction between photoperiod-treatment group and population-level differences in natural levels of resistance?

Chapter 4 – The potential of immune gene divergence for postzyogtic isolation between speciating ecotypes.

In this chapter, I return to sampling wild fish, this time from a single loch on North Uist where three distinct ecotypes coexist and hybridize. I use morphometric analyses of body shape and armour variation to quantify hybridisation in this system, and examine its affect on immune gene expression through qPCR. Here I ask: 1) Does hybridisation lead to transgressive immune gene expression, suggestive of genomic incompatibilities? 2) Do hybrids suffer reduced fitness through measures of condition or parasitism?

Chapter 5 – Intercontinential genomic parallelism in response to parasitism and water chemistry.

Here, I use RAD-seq data with Bayesian linear models to correlate allele frequency changes across populations with parasite prevalence for two common macroparasites and measures of water chemistry. This analysis is repeated across four independent adaptive radiations of stickleback. I then examine whether associated genes appear parallel in 2 or more radiations, and whether this parallelism occurs at a higher degree than expected by chance. This study asks: 1) Is genomic parallelism detectable across independent adaptive radiations? 2) What factors are important for determining the strength of genomic parallelism? 3) How do measures of parasite prevalence correlate with water chemistry across independent adaptive landscapes? 4) Is structuring of environmental variables consistent across radiations and is this important for promoting genomic parallelism?

Chapter 6 – General Discussion

Finally, I bring together my findings across chapters. I discuss the validity of methods used and how results from each chapter compliment one another and how this crosstalk relates to the literature. I also discuss the implications of my results for understanding evolutionary consequences of immune variation across other taxa, and discuss potential avenues of future research in the field.

CHAPTER 2: THE RELATIONSHIP BETWEEN LIFE HISTORY AND IMMUNE RESPONSES

2.1 Abstract

Understanding evolutionary correlates of wild immune variation can aid understanding of how costs and trade-offs shape immune evolution in the wild. Varying life history strategies may increase or alleviate immune costs, helping shape immune variation in a consistent, testable way. The relationship between life history strategies and immune variation has been investigated, but largely analysing phenotypes at the species-level, which can be confounding. I investigated the relationship between life history and the immune system within an island radiation of three-spined stickleback, which play host to a diverse range of parasite communities and display discrete populations with varied life history strategies. I sampled multiple populations of short-lived, long-lived and ancestral anadromous fish to examine consistent differences in wild immune responses between strategies. I used a genetics-based approach to aid in evolutionary inference, examining gene expression as a measure of current immune profile and using RAD-seq data to study the distribution of immune variants within our assays and across the genome. Short-lived populations exhibited significantly increased expression of all immune genes, which was accompanied by a strong association with population-level variation in local alleles. Genome-wide analyses further

revealed population-level divergence of inflammatory and complement genes that separated populations by their life history strategy. Across North Uist, immune genes show evidence of having diverged alongside life history strategies. I also observe various environmentally-driven variations in gene expression both within and between populations that highlight the cautiousness required when assaying immune responses in the wild. These results provide strong, gene-based support across multiple populations of a vertebrate model for current hypotheses linking life history and immune variation.

2.2 Introduction

Since its emergence, the field of ecoimmunology has revealed an abundance of variation in the ways that organisms respond to parasitic infection at both the genotypic and phenotypic level (Martin et al., 2011; Pedersen & Babayan, 2011). It is likely that variation exists due to costs and trade-offs associated with the immune system, such as energy expenditure (Schmid-Hempel, 2011), autoimmunity (Graham, 2002; Graham et al., 2005; Kopp & Medzhitov, 2009) and antagonisms between different branches of the immune response (Kamath et al., 2014; Loiseau et al., 2008), (e.g. different T-helper cell subsets such as Th1 and Th2 (Long & Nanthakumar, 2004)). Whilst our knowledge of the existence of immune variation is longstanding, our understanding of its implications for host evolution remains relatively poor. This gap in the

evolutionary synthesis is significant given the near ubiquity of parasitism in the wild (Poulin & Morand, 2000; Windsor, 1998).

One of the principle aims of ecoimmunology is to identify the ultimate causes of immune variation (Lee, 2006), which can be understood through identifying important correlates of the variation. Life history represents a highly variable, but correlated suite of host attributes, that has been suggested to associate with the immune system (Zuk & Stoehr, 2002). Typically, life history is described as a continuum, whereby 'short-lived' species exhibit rapid growth, and have many low-cost offspring and high mortality, whilst 'long-lived' species have characteristically long developmental periods, low numbers of high cost offspring and low mortality (Promislow & Harvey, 1990; Ricklefs & Wikelski, 2002).

There exist contrasting theories regarding the relationship between immune responses and life history. In terms of immune phenotypes, Lee (2006) argues that short-lived species should adopt developmentally cheap, but usage-costly immune defences that are associated with autoimmunity and are more energetically demanding, such as innate inflammatory and Th1-mediated responses. This is because the costs are offset by generally increased extrinsic mortality. Longer-lived species conversely should invest more in specific, less damaging and energetically cheaper adaptive responses (Lee, 2006). Alternatively, shorter life history strategies may favour an absence of immune investment, resulting in weaker responses as resources are partitioned into life history traits, such as rapid development and reproduction (Norris & Evans,

2000). Resource partitioning away from immune responses in favour of life history traits has been demonstrated in *Daphnia magna* in the presence of predator signals (Pauwels et al., 2014).

Studies of closely-related species appear to support the theories of Lee (2006), with empirical evidence existing from a diverse range of taxa; including birds (Lee et al., 2008; Pap et al., 2015; Tella et al., 2002; Tieleman et al., 2005), mammals (Previtali et al., 2012), and even corals (Pinzon et al., 2014). However, there are examples in which life history-immune relationships are weak or contrary at the species level (Horrocks et al., 2015; Martin et al., 2007; Matson et al., 2006). It therefore remains unclear as to how these relationships evolve in nature. Such disagreement may stem from a failure to account for evolutionary history. For example, Bech et al (2016) note that ancestral phenotypes explain indistinguishable basal metabolic rates (BMR) between sympatrically occurring short and long-lived Australian passerines, despite the expectation of a negative association.

Confounding evolutionary history complicates inferences drawn from between-species comparisons, and therefore within species variation may be more informative. However, individual or population-level analyses are less frequent in the literature, (although see (Rantala & Roff, 2005; Sparkman & Palacios, 2009)). In addition to this, few studies have examined life history-immune associations from a genetic perspective. The consensus has generally been to use biomarkers and immune assays to quantify an immune phenotype, but such measures can be strongly susceptible to plasticity in the wild (Galli et

al., 2011; Luoma et al., 2016). This risks leading to alternative explanations for life history associations (Palacios et al., 2013), and variable conclusions being drawn depending on the marker sampled (Christensen et al., 2016). From an evolutionary perspective, this empirical gap is particularly concerning, and linking heritable, genetically-determined immune variation to life history within species remains a challenge to overcome to understand wild immune variation.

There therefore exist two barriers to empirically examining the evolutionary relationships between life history and immune variation. First, evolutionary history needs to be accounted for and I do this by examining populations within a species, the three-spined stickleback, *Gasterosteus aculeatus*, with contrasting life history strategies. Second, when dealing with wild individuals the issue of unknown plasticity must be considered. I do this is in two ways, using genetics-based approaches. (1) I focus on immune gene expression assays in adult males, including measures of reproductive condition, which are likely to have comparable phenotypes. As male stickleback overwhelmingly have a single breeding season, and perform all parental care, incurring costs associated with nest-building, territoriality, ornamentation and fry rearing (Whoriskey & Fitzgerald, 1994), fish displaying reproductive traits are likely to be stressed and verging on senescent. Stressed individuals within a population should reveal resource-based plastic effects on the immune response. I assess how immune gene expression varies with measures of reproductive investment, to estimate the likely extent of plasticity. (2) Gene

expression is also plastic, so to draw evolutionary conclusions I compare my measures of population-level variation in immune expression to a genetic approach based on SNPs in immune-related genes that avoids issues of plasticity. To understand the genetic basis for any expression variation, I assess the allelic diversity of SNPs associated with assay genes. I also incorporate a GWAS approach to look for immune-linked genes across the genome that may have diverged between short-lived and long-lived populations. Finally, I expand the analysis to a larger group of populations and assess the comparative relationship between life history strategy and genetic variation around a larger number of immune genes. Such an approach introduces a novel level of genetic accountability for immune covariance with life history that will help reveal the potential for evolutionary constraints or associations to occur.

Three-spined stickleback represent an excellent study system for questions of evolutionary biology due to their rapid and repeated adaptive radiations from marine to diverse freshwater forms. Still extant marine populations are commonly assumed to be a good representation of the ancestor of freshwater populations which diverged following colonisation from the sea ~10,000 years ago (Colosimo, 2005). Diverged freshwater stickleback exhibit strong life history variation in keeping with known short-lived and longer-lived strategies (DeFaveri & Merilä, 2013; Gambling & Reimchen, 2012; Magalhaes et al., 2016). Stickleback are also hosts to a plethora of parasites (De Roij & MacColl, 2012; MacColl, 2009a; Poulin et al., 2011; Young & MacColl, 2017), which have helped drive immune variation between wild freshwater

populations (De Roij et al., 2011; Robertson et al., 2016a, 2016b; Scharsack et al., 2007a). This system therefore allows us to ask questions about covariation between life history and immune phenotypes across multiple populations within a single radiation, promoting our understanding of how life history and immune responses diverge alongside one another in response to local environmental variation. To do this, I assessed fish from two short-lived populations, two long-lived populations, and a proxy 'ancestral' anadromous population.

2.3 Materials and methods

2.3.1 Study site and sample collection

Three-spined stickleback were collected from the island of North Uist, Scotland, in April and May 2015. Fish were caught in unbaited Gee's Minnow Traps, set overnight in 5 lochs that harbour populations with varying life histories (Rahman, 2017). Fish in TORM and BHAR are mostly annual, but fish in REIV and HOSTA can live as long as 3 years and often survive for two breeding seasons (T. Chitheer, A. Rahman and A.D.C MacColl, unpublished data). Anadromous OBSM fish are largely 2 years old when they breed. I selected males from contrasting life stages from HOSTA (N = 35) and REIV (N = 36), and only mature males from lochs BHAR (N = 21), OBSM (N = 23) and TORM (N = 22), yielding a total of 137 fish. Life stage was assessed based on sexual maturity according to testes and kidney condition and sexual ornamentation

according to characteristic nuptial colouration (carotenoid based red-throat ornamentation). Fish were transported back to the laboratory in darkened boxes and sampled in a random order within 6 h of collection. Sampling order was found to have no effect on results (Appendix 2.1).

Fish were euthanized by MS222 overdose followed by destruction of the brain (in accordance with schedule 1 techniques described by UK Home Office regulations). Fish were then weighed and measured, before having their spleen removed and placed in RNAlater (Life Technologies). Samples of spleen, which is an important immune tissue in fish (Zapata et al., 2006), were kept at 4 °C for 24 h and then kept at -20 °C with RNA being extracted within 3 months. Reproductive condition was scored according to a qualitative scale based on size of testes and kidney (the kidney of breeding male stickleback becomes enlarged); 1 (small testes and kidney); 2 (enlarged, melanised testes, small kidney); 3 (enlarged, melanised testes and enlarged kidneys). Testes and adipose tissue were also weighed. Somatic weight was calculated by subtracting testes and adipose tissue weight from total weight. This value was then used to calculated Gonadosomatic Index (GSI) and Adiposomatic Index (ASI) according to: Testes or Adipose Weight/Somatic Weight x 100. Otoliths were extracted to estimate fish age and were stored in 100% ethanol before being mounted on slides and photographed under a light microscope. Age was estimated from otoliths according to (Jones & Hynes, 1950).

Macroparasite abundance for each fish was recorded for the ectoparasitic monogenean trematode *Gyrodactylus arcuatus* and the cestode

Schistocephalus solidus, which both have well-studied effects on host fitness (Barber et al., 2008; Barber & Scharsack, 2010; De Roij et al., 2011; MacNab et al., 2011; Rushbrook & Barber, 2006). All plerocercoids found in fish with *S. solidus* infections were also weighed, and a Schistosomatic Index (SSI) was calculated as above.

2.3.2 RNA isolation and qPCR

All qPCR work was conducted in accordance with the MIQE guidelines (Bustin et al., 2009). Sampling order was randomised and RNA was extracted from stored, whole spleens using the Genejet RNA purification kit (Thermo Scientific) according to the manufacturer's protocol. RNA was DNase treated using Precision DNase (Primerdesign) following the manufacturer's protocol.

RNA purity was assessed on a NanoDrop1000 spectrophotometer (Thermo Scientific) blanked using DNase (Primerdesign) treated nuclease-free water incubated under equivalent conditions. RNA integrity was assessed following DNase treatment by visualisation of 4 µl of sample on a 2% agarose gel stained with ethidium bromide. Reverse transcription was performed using nanoScript2 RT kit (Primerdesign) according to the manufacturer's protocol using approximately 1.5 µg of template. This protocol uses a combination of random nonamer and oligo-dT priming. Genomic DNA contamination was assessed via light PCR using intron-spanning primers. All cDNA samples were diluted 1:10 with nuclease free water before use.

A total reaction volume of 10 µl was used to perform qPCR reactions consisting of 5 µl of PrecisionFAST low ROX mastermix with SYBR green (Primerdesign), 2.5 µl of nuclease-free water, 0.25 µl of each primer at working concentration and 2 µl of cDNA template. Reactions were performed in 96-well optical PCR plates with optical seals (StarLab) in an ABI 7500 Fast real-time thermocycler (Applied Biosystems). Samples were incubated at 95 °C for 20 s, followed by 45 cycles of 95 °C for 3 s and 60 °C for 30 s. A melt-curve analysis was also included to confirm product.

2.3.3 Gene expression quantification

A custom stickleback geNorm analysis with SYBR was conducted to select appropriate housekeeping genes for this experiment. The analysis was conducted according to the manufacturer's protocol using 15 randomly selected samples made up of 2/3 fish from each population or sexual maturity group within populations HOSTA and REIV. Of the 6 candidate reference genes supplied (*b2m*, *gapdh*, *rpl13a*, *hprt1*, *tbp* and *top1*) *b2m* and *hprt1* were the most stably expressed combination.

In total, 137 spleen samples were used from 5 populations. A reference sample, comprised of a pool sample of all individuals, was made up and used as a control reference across all plates. Samples were randomly assigned wells with 46 duplicated samples to a 96-well plate, along with a duplicated reference and negative controls. In total, 5 genes were amplified (Table 2.1)

using primers published in (Robertson et al., 2016a). These genes were selected to characterise different arms of the immune response and were identified based on previous studies in other fish and known roles of orthologous genes. The pro-inflammatory gene *tnf*α is a key component of innate immunity in teleosts and other vertebrates (Secombes & Wang, 2012; Uribe et al., 2011), activating macrophages, eliciting inflammation and increased respiratory burst activity. The Th1 transcription factor *stat4* promotes the differentiation of Th1 cells and has been identified in teleost genomes. Expression of Th1-associated genes in infection studies suggest that fish may possess a full and conserved Th1 pathway (Secombes & Wang, 2012). Th1 adaptive immunity is associated with intracellular parasites, whilst Th2 adaptive immunity is associated with extracellular infection. Th2 cell differentiation is associated with up-regulation of *cmip* and *stat6* in mammals, which is also upregulated alongside other markers of Th2 responses in zebrafish head kidney and spleen cells in response to immunostimulation (Mitra et al., 2010). Finally, *foxp3a* expression characterises the regulation of immune responses by Treg cells. This gene has been identified in many teleost genomes and has demonstrable roles in immune regulation in fish and mammals (Secombes & Wang, 2012). The genes included in this study therefore serve to capture immune variation in innate (*tnf*α), Th1 adaptive (*stat4*), Th2 adaptive (*cmip, stat6*) and Treg regulation (*foxp3a*).

Relative expression values were calculated according to the ΔΔCq method (Pfaffl, 2001) and adjusted for the amplification efficiencies of each primer pair. Expression values were standardized against the geometric mean Cq of two reference genes, selected via a GeNorm kit (Primerdesign) according to the manufacturer's protocol.

Table 2.1: Primer details for qPCR assays

Gene	ENSEMBL ID	Immune Role	Primer Sequence (5'-3')	Amplicon length
tnfα	ENSGACG00000013372	Pro-Inflammatory cytokine	Fwd-GCTTGGTTCTGGCCAGGTTT Rev-GCTGCTGATTTGCCTCAACG	125
stat4	ENSGACG00000002684	Transcription factor for differentiation of Th1 cells	Fwd-CTCTCAGTTTCGAGGCTTGCTT Rev-GGCAGTTGGCTCACATTGG	100
cmip	ENSGACG00000002527	Signalling protein in Th2 pathway	Fwd-GGCATGGAGGTCGTCAAGAA Rev-TAGCAGGAGTAAATGGCGGC	119
stat6	ENSGACG00000008477	Involved in mediating Th2 cytokines IL-4 and IL-3 signalling	Fwd-CTCAGCCACAGTTCCAACCGTTC Rev-GTCGGATGTTCTGGACCTCGAGT	104
foxp3a	ENSGACG00000012777	Promotes development and function of Treg cells	Fwd-GTTGACCCATGCAATTCCGA Rev-CTGCTGTAGTTGTGGTCCTG	94

2.4 Data analysis

2.4.1 Relative expression of immune genes

All data were analysed in R (version 3.3.1)(R Core Team, 2016) and data for the 137 individuals were subsetted into two analyses. The first subset comprised all males from lochs HOSTA and REIV, totalling 71 individuals. The second subset included mature males from all lochs, totalling 102 individuals.

For each subset, the following analysis was conducted. Log-transformed relative expression values were assessed for covariance in a principal component analysis. PC1 and PC2 scores were then modelled as dependent variables in generalized linear models (GLM). For the HOSTA-REIV subset, these were modelled with the independent variables: sampling loch (LOCH), qualitative reproductive score (REPRO), breeding colouration (RED), age (AGE), *Gyrodactylus* load (GYRO) and infection status (GYRO_X), schistosomatic index (SSI) and infection status (SCHISTO_X), gonadosomatic index (GSI), adiposomatic index (ASI), and length standardised by population mean (LENGTH). ASI was also modelled using a Gamma family and log link by the other independent variables to clarify the assumption that reproductive investment is negatively associated with condition.

Expression data from the subset of all lochs were modelled using the same independent variables apart from RED. Variables were modelled additively and removed sequentially using a top-down approach. The model with the best fit, assessed using the Akaike Information Criterion (AIC), was then modified to include biologically plausible interactions between independent variables that may improve fit. If model fit was significantly improved interactions were added, if not the simplest model was retained. Variable significance was assessed using ANOVA and post-hoc Tukey tests for categorical variables where appropriate.

2.4.2 RAD-Seq data handling

RAD sequence alignments were acquired with permission for all 5 populations from those published in (Magalhaes et al., 2016), with all lochs being sampled in Spring 2013. In total, 91 fish were included from the 5 lochs sampled in this analysis, split between HOSTA (19), REIV (19), TORM (19), BHAR (18) and OBSM (16). The STACKS pipeline (Catchen et al., 2013) was used to analyse mapping files and population genetics statistics were calculated using the POPULATIONS program in STACKS. The following filters were applied: SNPs that were not present in all populations were removed; SNPs present in <80% of individuals within a population were removed; SNPs with a minor allele frequency below 0.05 were removed; the first SNP of each RAD locus was retained to avoid linked loci; and data from the sex chromosome (XIX) were also removed. Following filtering, the final output consisted of 11,558 SNPs. Population genetics statistics were output to GENEPOP format and converted to PLINK and ARLEQUIN formats using PGDSpider2 (Lischer & Excoffier, 2011).

2.4.3 Population structure analysis

PLINK files were used with FASTSTRUCTURE (Raj et al., 2014) to assess genetic structure between the 5 populations under a Bayesian framework for posterior inference from large SNP genotype data. The algorithm was run for 1-5 populations (K) using FASTSTRUCTURE's default conversion criterion of 10e-6 and the simple prior. FASTSTRUCTURE is packaged with a default algorithm for estimating maximum likelihood for K, which was used in this instance.

2.4.4 Outlier analysis

Outlier analysis was performed on the unlinked 11,558 SNPs spread across the genome using ARLEQUIN (Excoffier & Lischer, 2010) and BAYENV2 (Günther & Coop, 2013). For ARLEQUIN, population structure was input using results from FASTSTRUCTURE and loci were detected using 20,000 simulations with 100 demes per group, and a minimum and maximum expected heterozygosity of 0.1 and 1.0 respectively. To identify loci under selection I examined F_{ST} per SNP. Varying from 0 to 1, F_{ST} represents the fixation index, a measure of correlations between alleles within populations relative to the entire population. Low values of F_{ST} denote minimal structure (high within and low between population-level variance) and high values denote increased divergence (low within and high between population-level variance) (Holsinger & Weir, 2009). Loci that exhibit higher or lower F_{ST} in comparison to a null distribution are deemed to be under either directional or balancing selection respectively. P-values were corrected for multiple testing using the R package *qvalue* (Storey et al., 2015). To find loci under selection ARLEQUIN uses the fdist2 method, which has been demonstrated to exhibit a high rate of false positives (Whitlock & Lotterhos, 2015). To account for this, I also used the X^TX statistic packaged with BAYENV2 to cross-examine the data. The X^TX statistic is analogous to F_{ST} and is a Bayesian measure of population differentiation. Population structure was accounted for using a covariance matrix calculated according to the author's instructions (Günther & Coop, 2013). BAYENV2 was run independently 5 times over 100,000 iterations and final X^TX values were averaged across runs. Loci that were deemed to be under selection had P-values < 0.05 (after FDR corrections)

according to ARLEQUIN and were in the 95th percentile of X^TX scores according to BAYENV2. SNPs were mapped back to genes by comparing location information within the alignment files to gene locations extracted from Ensembl's BioMart (Smedley et al., 2015) (BROAD S1, release 87) using a custom bash script. Gene Ontology was also inferred from BioMart.

2.4.5 Genetic variance of assay genes

To investigate genetic variability of my 5 qPCR assay genes, I repeated the POPULATIONS analysis above with a subset of RAD-loci found within the 5 genes or within 50 kB flanking regions. Filtering conditions were also relaxed to increase the number of SNPs available: SNPs present in at least 2 of the 5 populations were retained; SNPs present in <50% of individuals were removed; minor allele frequency was maintained at 0.05; all SNPs within whitelisted loci were permitted. In total this analysis used 51 SNPs across 18 RAD-loci. PCAs were performed using *adegenet* (Jombart, 2008) in R and visualised using *ggplot2* (Wickham, 2016). PC1 and PC2 scores were extracted and averaged across populations; these were included in Pearson's correlation analyses against PC1 and PC2 population averages of relative gene expression. This analysis was performed using the 'corr.test' function in the R package *psych* with 'holm' multiple comparison corrections. The relative contributions of SNPs towards these correlative relationships were assessed by means of PC loadings from individual SNPs in the genetic variance PCA.

2.4.6 Comparative relationship of immune gene genetic variance and life history

To expand the analysis to comparative levels, I analysed genetic variation in and around genes annotated with the GO term 'immune system process' (GO:2376) and assessed the relationship with life history strategy across 15 diverged population. GO:2376 is a parent term for all GO terms associated with immune responses, and covers 389 genes with various immune functions in the stickleback genome (BROADs1, Ensembl release 90). RAD-seq data for the additional 11 freshwater populations was provided with consent from (Magalhaes et al., 2016) (Appendix 2.2). SNPs were filtered as being within the 389 immune genes or within 10kb flanks. POPULATIONS was run with the following filters: SNPs present in at least 8 of the 15 populations were retained; SNPs present in <50% of individuals were removed; minor allele frequency was maintained at 0.05; all SNPs within whitelisted loci were permitted. In total, this analysis used 597 SNPs across 360 RAD-loci, which captured variation around 166 of the 389 immune genes annotated in the genome. PCAs were performed using *adegenet* (Jombart, 2008), with PC1 scores extracted for further analysis.

Life history data for the populations was acquired with permission from (Rahman, 2017). This included: % in wild over 1 year old; absolute growth rate in 1 year; age at maturity; and size at maturity. This data was compiled into a single PCA axis with PC1 scores for each loch modelled alongside immune gene genetic variation PC1 scores using linear mixed models. A random factor of

LOCH was included to account for multiple genetic variation PC1 scores per population.

2.5 Results

2.5.1 Relative expression of immune genes

HOSTA and REIV (Males of contrasting life stage)

For a summary of GLM results, see Table 2.2. In my HOSTA-REIV comparison, PC1 and PC2 represented 59.1% and 18.2% respectively (77.3% cumulatively) of the total variation in relative expression across the 5 genes. PC1 represented covariance of all genes' expression in the same direction, whilst variation in PC2 was driven by increased expression of the inflammatory gene *tnfα* at the expense of relative expression of the other 4 genes (Table 2.3). These PCs were therefore summarised to signify relative expression of all genes (PC1) and increased relative expression of *tnfα* (PC2). Expression of all genes was best predicted by a GLM composed of the variables REPRO, SSI and a one-way interaction between SCHISTO_X and LENGTH. Within the model, sexually immature fish (REPRO = 1) had significantly higher expression of all genes relative to partially mature (REPRO = 2) or fully mature (REPRO = 3) individuals (Figure 2.1a). Additionally, overall immune gene expression declined significantly with increasing *S. solidus* infection intensity (SSI (Figure 2.1b). Larger fish had significantly greater overall expression, however infection with *S. solidus* inverted this relationship (Figure 2.1d).

Table 2.2: Model effects for minimum adequate GLMs fitted for the REIV & HOSTA analysis and analysis of all lochs. 'All Expression' PC represents PC1 for both analyses and represents the correlated relative expression of all immune genes. 'Relative *tnfα* Expression' represents PC2 for both analyses and represents increased relative expression of the pro-inflammatory gene *tnfα*

Analysis	PC	Factor	*F*	d.f.	*P*[1]	Effect
HOSTA & REIV	Overall Expression	Reproductive Index	9.599	2,64	**< 0.001**	*NA*
		Schistosomatic Index	4.095	1,64	0.047	-
		Modified Length : Schisto Infection	11.205	1,64	**0.001**	*NA*
HOSTA & REIV	Relative *tnfα* Expression	Loch	11.632	1,68	**0.001**	*NA*
		Breeding Colouration	4.957	1,68	**0.029**	*NA*
All Lochs	Overall Expression	Loch	9.108	4,92	**< 0.001**	*NA*
		Age	2.256	1,92	0.137	+
All Lochs	Relative *tnfα* Expression	Loch	5.208	4,96	**< 0.001**	*NA*
		Schisto Infection	6.162	1,96	**0.015**	*NA*

[1]Values in bold denote significance at < 0.05 threshold

Table 2.3: PC loadings for log-transformed qPCR relative expression ratios for the five immune genes included in this study. Loadings are grouped according to the two analyses conducted.

	HOSTA & REIV		All Lochs	
Gene	PC1	PC2	PC1	PC2
tnfα	0.568	0.816	0.474	0.856
stat4	0.488	-0.295	0.499	-0.451
stat6	0.197	-0.064	0.168	-0.067
cmip	0.501	-0.438	0.504	-0.205
foxp3a	0.387	-0.226	0.495	-0.133

The model selected for PC2 included the additive variables LOCH and RED. Fish sampled from loch REIV expressed the inflammatory gene *tnfα* more in comparison to their other immune genes than did fish sampled from HOSTA (Figure 2.1d). Interestingly, fish that displayed breeding colouration were also found to express *tnfα* more in relation to their other immune genes than fish that were not displaying breeding colouration.

Condition, approximated through ASI, varied with various factors. Crucially, sexually mature fish (REPRO = 3), were in significantly poorer condition than sexually immature (REPRO = 1) (Tukey, $p < 0.001$) and partially mature individuals (REPRO = 2) (Tukey, $p < 0.001$) (GLM, $F_{2,65} = 9.957$, $p < 0.001$). Additionally, fish from REIV were in poorer condition than HOSTA fish (GLM, $F_{1,65} = 5.715$, $p = 0.020$), and condition declined significantly with age (GLM, $F_{1,65} = 7.949$, $p = 0.006$).

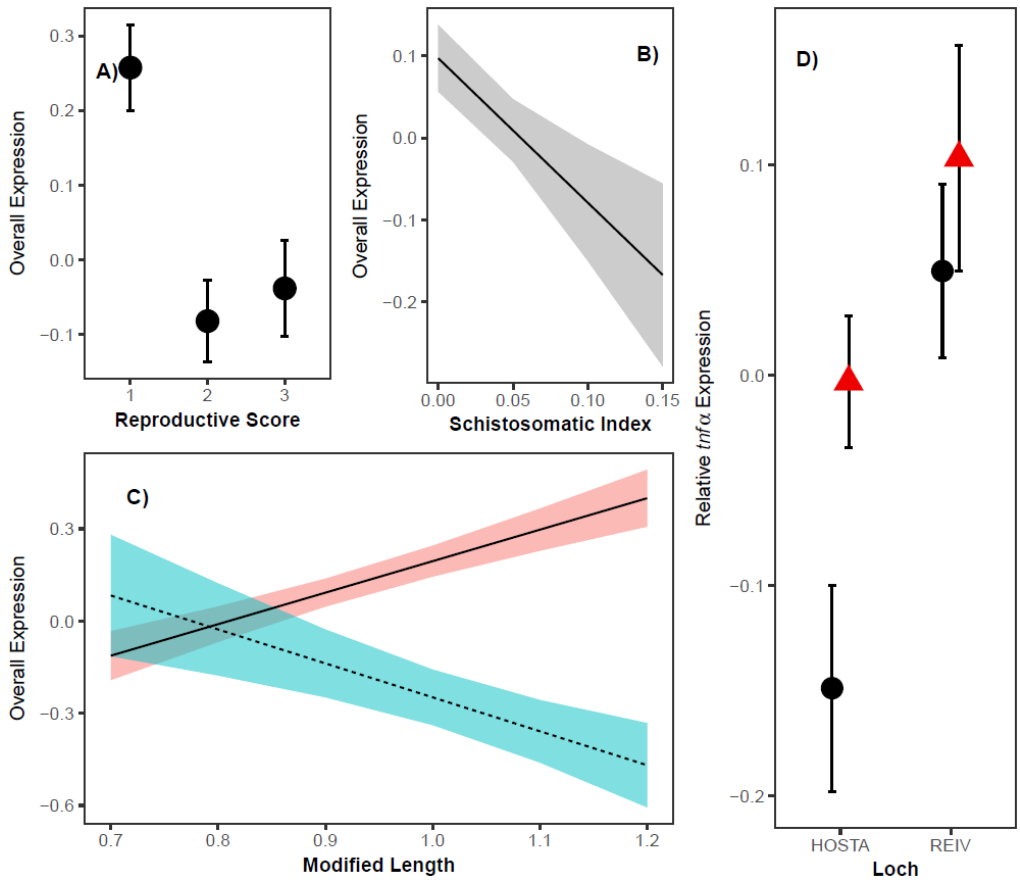


Figure 2.1: Significant factors in minimum adequate GLMs fitted to overall expression component scores (**A**-**C**) and relative *tnfα* expression component scores (***D***) for fish sampled from REIV and HOSTA. Effect plots (**A**-**C**) visualise the mean effect of an individual factor with standard error whilst other factors within the model are maintained at a constant. (**A**) The effect of reproductive condition on overall immune expression. (**B**) The effect of *S. solidus* infection intensity on overall immune expression. (**C**) The effect of the interaction between length (Modified Length) and *S. solidus* infection on expression of all immune genes. Line colour and type represent infection group; red solid line shows uninfected fish; blue dashed line shows infected fish. (***D***) The effect of sampling location and breeding colouration (● = No colouration, ▲ = Red throat) on relative *tnfα* expression. Points here show group mean PC2 scores with standard error.

All lochs (Short-lived vs long-lived)

For a summary of GLMs used, see Table 2.2. When comparing all mature fish across the short-lived lochs TORM and BHAR, the long-lived lochs REIV and HOSTA, and the ancestral marine fish OBSM, I found as above that PC1 (55.8%) represented the correlated expression of all immune genes and PC2 (21.5%)

represented increased *tnfα* expression at the expense of the other immune genes. PC1 was best explained by additive effects of LOCH and AGE. LOCH was a significant predictor of relative expression of all immune genes, driven by the fact that fish from the short-lived loch TORM had significantly higher relative expression compared with the other 4 lochs (Tukey, all comparisons $p < 0.05$) (Figure 2.2a). Age did not significantly predict immune gene expression, although its inclusion within the model improved fit significantly.

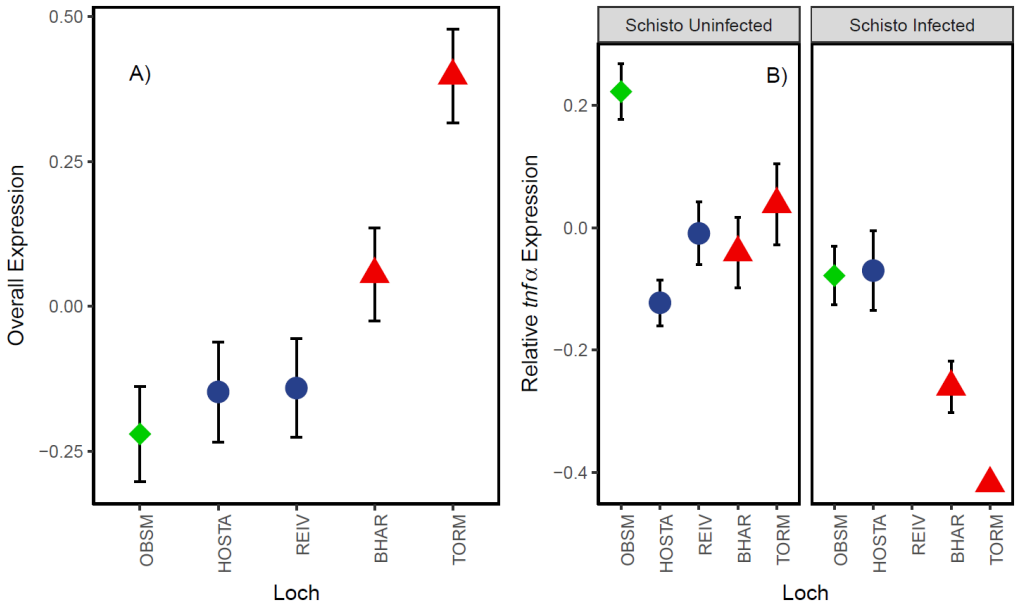


Figure 2.2: Significant factors in minimum adequate GLMs fitted to overall expression component scores (**A**) and relative tnfα expression component scores (**B**) for mature males sampled from all lochs. Point shapes and colours denote life history strategy ● = Long-lived, ▲ = Short-lived, ◆ = Anadromous. (**A**) Model effect plot for sampling location on expression of all immune genes. (**B**) The effect of sampling location and infection status with *S. solidus* on the increased relative expression of pro-inflammatory *tnfα*. Points denote group means for PC2 with standard error.

Increased relative *tnfα* expression was best predicted by an additive model including LOCH and SCHISTO_X. Anadromous fish had significantly higher relative expression of *tnfα* with respect to their other immune genes when compared to all freshwater populations (Tukey, all comparisons $p < 0.05$) (Figure 2.2b). Furthermore, infection with *S. solidus* resulted in a reduction in relative expression of *tnfα* across the four populations in which it was found (Figure 2.2b), although numbers of infected fish were substantially lower in lochs OBSM (N =2/23) and TORM (N = 1/22), compared with HOSTA (6/18) and BHAR (9/21).

To clarify that *Gyrodactylus* did not influence gene expression, expression variables PC1 and PC2 were modelled in *Gyrodactylus*-infected fish by *Gyrodactylus* burden. Neither expression variable was significantly associated with *Gyrodactylus* burden in infected fish (PC1, $F_{1,50} = 0.501$, $p = 0.482$; PC2, $F_{1,50} = 2.677$, $p = 0.108$).

2.5.2 RAD-seq analysis

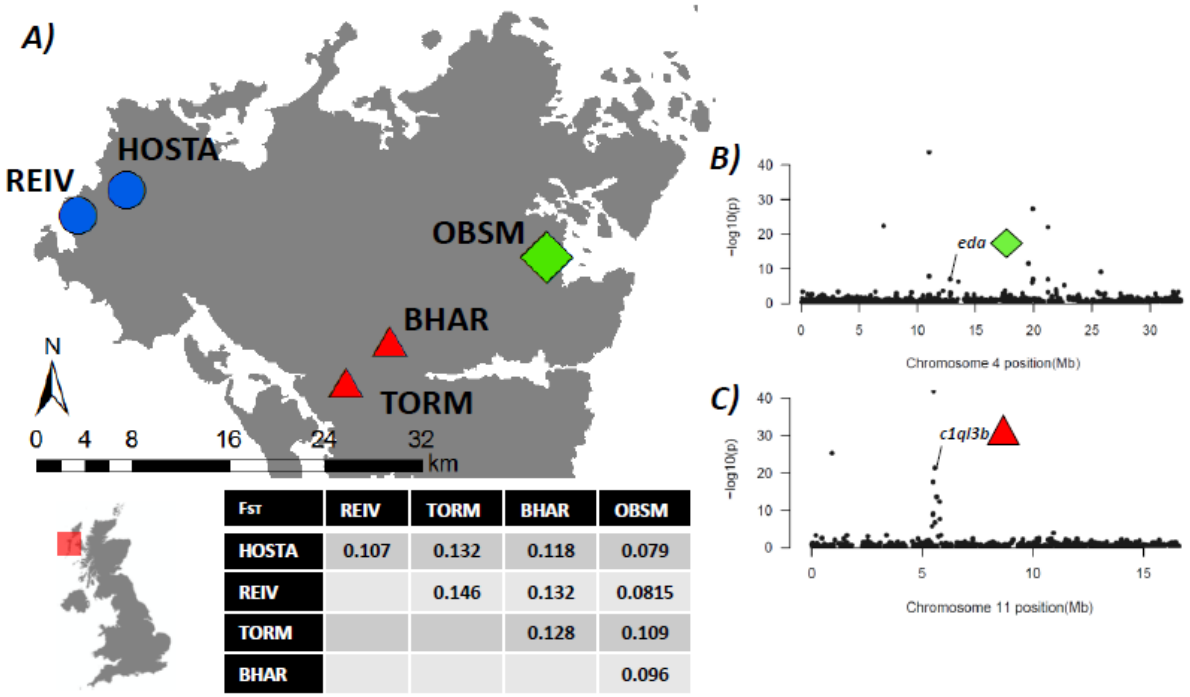
Population structure

FASTSTRUCTURE indicated a *k* value of 5, confirming that each of the 5 lochs sampled harbours a genetically distinct population of stickleback. As *k* increased from 1 to 5, it revealed an initial long-lived, short-lived divide at *k* = 2, and at *k* = 4 there were 4 distinct freshwater populations with remnants of all 4 freshwater populations found in each individual from the ancestral

anadromous population. Pairwise F_{ST} values calculated from POPULATIONS in STACKS supports this structuring (Figure 2.3a).

Outlier analysis

ARLEQUIN highlighted 82 SNPs of the 11,558 total to be under significant selection. All 82 of these SNPs had higher than expected F_{ST} values and can therefore be assumed to be under directional selection as opposed to balancing selection. Of these 82 SNPs, 79 were also found to be in the 95th percentile of X^TX values yielding an error rate of 3.66% for my analysis. When mapped back to genes, the roles of these SNPs varied considerably, with genes associated with bone development, fin morphogenesis, calcium and other metal ion management, protein binding and membrane processes. Two genes were associated with immune processes, *eda* and *c1ql3b*, which are involved in tumor necrosis factor binding and the complement arm of the innate response respectively. Variants associated with *eda* on chromosome IV (Figure 2.3b) were fixed in the anadromous OBSM fish and fixed for the alternative in all freshwater populations. Variants of *c1ql3b* on chromosome XI (Figure 2.3c) were strongly associated with either the short-lived populations TORM and BHAR or longer-lived REIV and HOSTA, with the REIV/HOSTA preferred allele fixed within the ancestral OBSM. These results are summarised in Figure 2.3.



Fst	REIV	TORM	BHAR	OBSM
HOSTA	0.107	0.132	0.118	0.079
REIV		0.146	0.132	0.0815
TORM			0.128	0.109
BHAR				0.096

Figure 2.3 Genetic differentiation between lochs and life history strategies. (***A***) visualises sampling locations and life history strategies for the 5 lochs sampled with accompanying genome-wide pairwise F_{ST} scores. Shape and colour of points denotes life history strategy; green (◆) represents the anadromous population, blue (●) represent long-lived populations, and red (▲) represent short-lived populations. (***B***) Genetic differentiation scores for Chromosome IV SNPs, plotted as –log10 *P*-values of F_{ST} scores given by ARLEQUIN. The *eda* gene is highlighted as having potential associations with *tnfα* and is fixed in anadromous and freshwater comparisons. (***C***) Genetic differentiation scores for Chromosome XI SNPs, plotted as –log10 *P*-values of F_{ST} scores given by ARLEQUIN. The *c1ql3b* gene is highlighted as having potential roles within the complement system and has variants strongly associated with either short-lived or long-lived populations, with the long-lived variant fixed in the anadromous fish.

Genetic variance of assay genes

A PCA for genotypic variance in the 18 loci associated with the 5 immune assay genes between individuals reveals population separation within genotypic space. PC1, which represents 41% of the total variation, completely separates long-lived and ancestral fish from short-lived populations at the 90% confidence interval (Figure 2.4). This axis was driven predominantly by genetic variability in the regulatory gene *foxp3a* and the Th1-activator *stat4*. Short-lived populations were predominantly heterozygous (TORM = 0.559, BHAR = 0.385) for a T/C variant found in *foxp3a* (chrXII:16894963) whereas long-lived and ancestral populations had high levels of homozygosity for the T variant (REIV = 0.833 ,HOSTA = 0.947, OBSM = 1.0). Short lived populations also had high levels of homozygosity for 2 SNPs found in and around stat4: the C variant of a C/G SNP (chrXVI:6372531) (TORM = 0.947, BHAR = 0.917) (REIV = 0.526, HOSTA = 0.474, OBSM = 0.633) and the A variant of an A/G SNP (chrXVI:6418158) (TORM = 0.868, BHAR = 0.972) (REIV = 0.553, HOSTA = 0.184, OBSM = 0.281).

There was slight population separation on PC2 which represented 28.4% of the variation (Figure 2.4). Separation on this axis was driven by genetic variability in *foxp3a*, *stat4* and the Th2-activator *cmip*. Lochs BHAR, HOSTA and REIV had high levels of homozygosity for two SNPs found in *foxp3a*: the G variant of a G/A SNP (chrXII:16925720) (BHAR = 0.972, HOSTA = 0.974, REIV = 0.974) and the C variant of a C/T SNP (chrXII:16925570) (BHAR = 0.972, HOSTA = 0.947, REIV = 0.974). In *stat4*, the short-lived populations showed high levels

of homozygosity for the A variant of an A/G SNP (chrXVI:6418158) (TORM = 0.868, BHAR = 0.972), whilst in *cmip*, TORM fish were fixed for the A variant of an A/G SNP (chrXIX:1516826).

Taken together, these results suggest that short-lived populations have diverged most significantly from long-lived and ancestral fish predominantly in the genes *foxp3a* and *stat4*, whilst the additional divergence of *cmip* serves to partially separate TORM from other lochs in genotypic space.

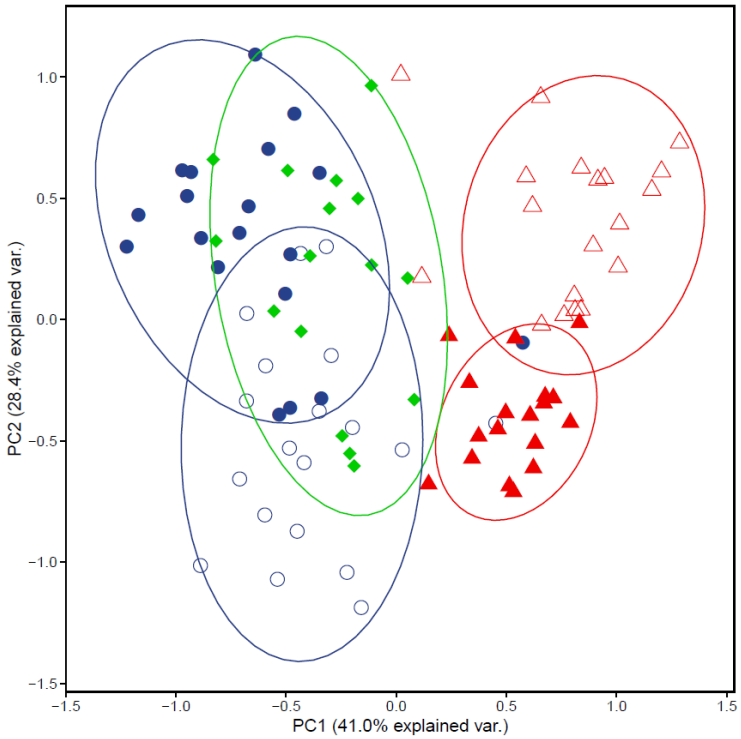


Figure 2.4 PCA for genetic variability of 51 SNPs located within 18 local (± 50kb) RAD-loci in and around our 5 assayed immune genes. PC1 represents 41% of the total variability within the data and PC2 represents 28.4% of the variability, giving a combined explanatory power of 69.4% for these two axes. Shape and colour of points denotes life history strategy; green (◆) represents the anadromous population, blue (●) represent long-lived populations, and red (▲) represent short-lived populations. Colour fill of points (filled = BHAR and HOSTA, unfilled = TORM and REIV) denotes populations. Ellipses represent 90% confidence intervals, which fully separate short-lived populations from the other 3.

When PC scores for SNP variability were averaged across populations and compared to average PC scores for expression, I observed a correlation for SNP PC1 averages and expression PC1 averages (Pearson's, $r = 0.943$, $p = 0.06$) (Figure 2.5), which was just outside the 5% confidence interval after correcting for multiple comparisons. The strength of this relationship, given the small number of data points, suggests a potentially strong association between genetic variance in *foxp3a* and *stat4* between short-lived and other populations and relative expression of the 5 immune genes assayed. No other significant correlations were detected for other comparisons between SNP PC1, SNP PC2 and expression PC1 and expression PC2 (Pearson's, $p = 1.00$).

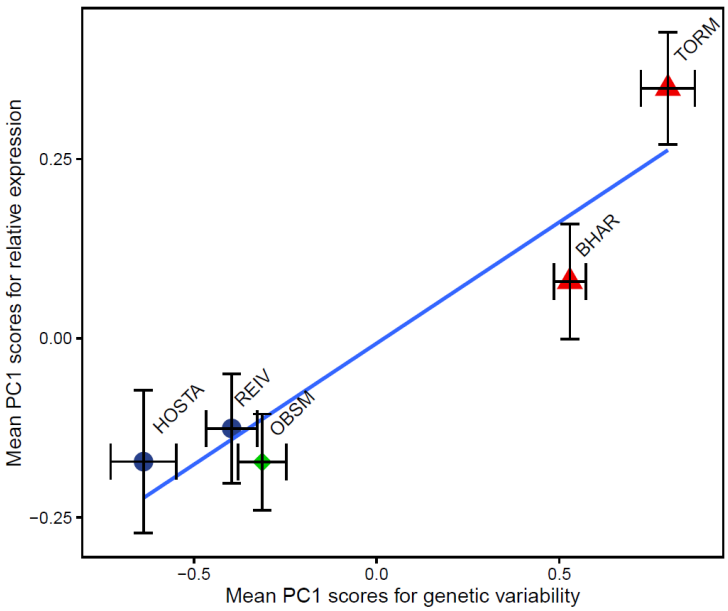


Figure 2.5: Association between population means for PC1 of relative expression (Expression Component scores) and PC1 of Genetic Variability of local SNPs with standard error represented along the x and y axes. Population means were used as relative expression of immune genes was ascertained from fish sampled in this study's sampling effort (2015), whilst genetic variability was ascertained through RAD-sequencing of fish from the same populations in a previous sampling effort (2013). The data shows a strong association ($r = 0.943$), which for the number of points present (N = 5) is likely substantial, despite an FDR-corrected *P* value of 0.06.

Comparative relationship of immune gene genetic variance and life history

PC1 for life history variation across the 15 populations represented 61.9% of the total variation and constituted the correlated variation of all 4 variables. There was a significant linear relationship between genetic variation around immune genes (GO:2376) (PC1 scores) and life history PC1 scores (Mixed model, $\chi^2_{1,13} = 4.261$, $p = 0.039$) (Figure 2.6). This relationship highlights that populations with similar life history strategies are also more genetically similar in and around their immune genes, suggesting a heritable evolutionary relationship between life history and immune responses is apparent on North Uist.

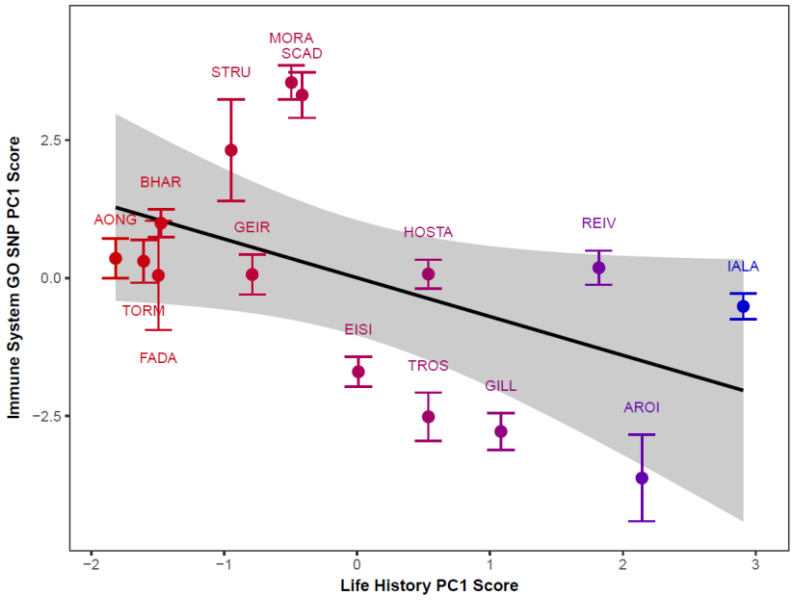


Figure 2.6: Linear regression between immune gene evolution and life history evolution across North Uist. Points denote population means ± standard deviation for immune gene genetic variation PC1 scores. Genetic variation PC1 scores are based on variation at 597 SNPs across 166 immune genes. Point colour gradient represents life history strategy denoted by Life history PC1 scores (red = short, blue = long). Life history PC1 scores are per population and represent common variation across: % wild individuals > 1 year old; mean growth rate in 1 year; mean age at maturation; mean size at maturation.

2.6 Discussion

I have documented substantial differences in immune gene expression between populations with contrasting life history characteristics, and these are associated with strong variation in allele frequencies near the same genes. Additionally, comparative analysis across freshwater populations on North Uist reveals that genetic variation around immune genes is associated with life history strategy. Consistent with hypotheses, overall expression was higher in the short-lived populations, while the ancestral, anadromous population exhibited higher relative expression of *tnfα*, a gene that is associated with inflammatory immune responses. This agrees with the fact that anadromous fish are semelparous, with a short breeding season, and may thus be showing signs of senescence (Franceschi et al., 2000, 2017).

Across life stages, reproductive investment in testes and kidneys reduced overall immune variation, whilst fish in breeding colouration had a higher relative expression of the inflammatory gene *tnfα* compared to other genes assayed. Additionally, fully sexually mature fish were in significantly poorer condition according to adiposomatic index. These patterns were consistent across the two populations involved in this sampling (REIV and HOSTA). This reveals how immunity might be affected by increased stress, and thus how the immune response is modulated in a plastic sense. Breeding male stickleback exhibit extreme resource demands as a result of sexual ornamentation, nest-building, territoriality and fry rearing and demonstrate senescence and high mortality as the breeding season progresses (Dufresne et

al., 1990), in the wild and experimentally (Kim et al., 2016; Pike et al., 2010). Consistent with my finding that anadromous fish had low overall expression and a higher relative *tnfα* expression, I found that potentially reproductive individuals, denoted by testes/kidney condition and colouration, displayed similar patterns. An explanation therefore for the patterns observed may be that regulation of immune gene expression and inflammatory processes triggered through *tnfα* signalling are traded off against reproductive investment, with the autoimmune costs of inflammation offset by a boost to reproduction at the expense of high mortality. This might explain the patterns observed in my qPCR data through an under expression of genes associated with processes capable of moderating inflammation, such as Treg and Th2 type.

Hegemann et al. (2012) found that innate immunity is maintained through periods of resource stress in wild skylarks, *Arlauda arvensis*, suggesting that alternative immune arms are downregulated. However, if such trade-offs increase investment in other fitness-boosting traits, then such a response may be adaptive. In support of this, there is evidence to suggest that seasonal variation, which characterises winter as a period of resource limitation and stress, results in increases in the expression of pro-inflammatory genes in humans (Dopico et al., 2015) and sticklebacks (Brown et al., 2016). Similarly, growing evidence in mammals indicates that somatic maintenance of oxidative stress, a self-damaging consequence of the rapid production of reactive oxygen species during acute phase inflammation (Ashley et al., 2012), is traded off against reproductive investment. As expected, this process occurs in the most

invested sex, such as female Soay sheep, *Ovis aries* (Nussey et al., 2009) and male Northern elephant seals, *Mirounga angustirostris* (Sharick et al., 2015).

This reinforces the notion that deleterious immune responses may manifest in demographics for which a fitness trade-off must be made. In such an event, evolved immune responses that rely more heavily on innate immunity may contribute towards shorter life history strategies as the increased innate autoimmunity acts as an additional source of health deterioration in stressful environments. This idea is in keeping with notions of environmental ageing and senescence in the wild (Hayward et al., 2009). Such mechanisms may be responsible for shaping mortality regimes across age distributions in the wild. This functional immune-linked ageing mechanism has been observed in the free-ranging bat species, *Saccopteryx bilineata*, whereby the numbers of individuals with higher white blood cell counts and immunoglobulin G (IgG) concentrations decline in older age classes (Schneeberger et al., 2014).

I cannot rule out however that variation in relative *tnfα* expression is not associated directly with breeding colouration as opposed to indirectly through reproductive resource allocation. Carotenoid-allocation into red sexual ornamentation is important in stickleback (Wedekind et al., 1998) and can elicit trade-offs with the immune response. For example, fighting fish, *Betta splendens*, supplemented with carotenoids trade off immunological benefits for red sexual ornamentation (Clotfelter et al., 2007). Similarly, guppies have revealed associations with carotenoids, sexual ornamentation and immune

responses (Grether et al., 2004). Away from fish, supplementation of carotenoids in zebra finch, *Taeniopygia guttate*, also results in redder sexual ornamentation and improved cellular and humoral immunity (Blount et al., 2003). Trade-offs between the immune response and sexual ornamentation have also been revealed in invertebrates, such as the calopterygid damselfly, *Calopteryx splendens xanthostoma* (Siva-Jothy, 2000). In addition, an association between low-carotenoid diet, high reproductive investment and increased senescence has been experimentally demonstrated in stickleback (Pike et al., 2010), strengthening the suggestion that increased relative *tnfα* expression may be associated with senescing individuals.

Environmentally driven variation in expression was also observed linking expression of all qPCR genes and infection with the cestode *S. solidus*. *S. solidus* excretory products (Scharsack et al., 2013) are typically suppressive, and the ability of *S. solidus* to modulate host immune responses is well-documented (Barber & Scharsack, 2010; Scharsack et al., 2004, 2007b). Patterns of immune modulation by *S. solidus* were suppressive here generally, with overall expression reduced with infection intensity in REIV and HOSTA, whilst across lochs relative *tnfα* expression was suppressed in infected individuals.

The patterns observed at the individual-level were largely consistent across the two populations. Plasticity appears to manifest as reduced overall expression and an increase in inflammatory profile under potential reproductive investment, suggesting these responses may be energetically

cheaper, whilst local parasites can further modulate responses. Stressed individuals should plastically adopt cheaper responses, whilst populations that evolve shorter life history strategies should evolve costlier responses (Lee, 2006). This pattern was observed across all 5 populations when the analysis was scaled up to include all mature males. The loch from which fish were sampled was the strongest predictor of both overall expression and relative *tnfα* expression, with short-lived populations adopting costlier patterns of overall expression as defined in the previous analysis. Variation at the population-level may be environmental, genetic, or both. The analysis of individuals at various life stages suggests a strong role for the environment in mediating expression. I did however find a strong relationship between genotypic variance of SNPs found in and around assayed genes and their overall relative expression across populations that suggests a strong genetic component in explaining population-level differences. Prominent SNPs driving this relationship were found in and around the Treg gene *foxp3a* and the Th1 gene *stat4*, with variants being associated with short-lived life history strategies displayed by fish from TORM and BHAR.

Given the predictions of life history and immunity, this relationship between *foxp3a* and *stat4* genetic diversity and overall expression of all qPCR genes is particularly interesting. Divergence of *foxp3a*, which encodes the Treg activator Forkhead Box P3, has the potential to modify the way in which the host's immune system is regulated, because Treg cells are capable of suppressing all arms of the fish immune system (Long & Nanthakumar, 2004;

Quintana et al., 2010) Treg cells are also important for balancing the immune response between a resistant and a tolerant phenotype, with increased Treg cell activation being associated with increased tolerance (Dejaco et al., 2006). Tolerance represents strategies employed by hosts to minimise fitness costs caused by infection, as opposed to potentially costlier resistance that actively reduces parasite burdens, and shows variation in natural populations (Råberg et al., 2007). It has been shown that *foxp3a* is essential for immune tolerance in zebrafish, *Danio rerio*, as individuals lacking a functional ortholog suffer increased mortality through autoimmunity (Sugimoto et al., 2017). If parasites are weakly virulent or hosts are more susceptible to immune costs, tolerance represents an adaptive alternative to costly resistance (Downs et al., 2014; Hayward et al., 2014). We expect tolerance therefore to be associated with individuals of older demographics and longer-lived populations. This has been demonstrated recently in wild mammals (Jackson et al., 2014), however evidence to the contrary also exists (Mayer et al., 2015) and the extent to which tolerance in older age classes is controlled by Treg cells is unclear (Nussey et al., 2012). In my populations, it is known that fish from TORM are more resistant to *Gyrodactylus* than are fish from HOSTA (De Roij et al., 2011), but tolerance has yet to be investigated in these populations. Understanding the divergence of Treg processes and their roles in mediating resistance and tolerance strategies between diverging life histories represents a fruitful avenue for future research.

The relationship between general immune expression and divergence of *stat4* between short-lived and other populations is not obvious. Life history strategy and *stat4* divergence may be associated with an additional unmeasured environmental variable, such as water chemistry, as the metal ion managing genes *adat3* and *fkbp7* lie within 40 kb upstream of *stat4*. Disentangling life history and immune associations from environmental variation in wild populations is a challenge, however laboratory selection studies and sampling of other adaptive radiations, with contrasting environmental patterns, will aid in providing stronger evidence.

At the genomic level, my outlier analysis revealed a SNP in the *c1ql3b* gene that showed strong divergence between short-lived and all other populations. The *c1ql3b* gene encodes a q-subcomponent of complement component 1, an active constituent of the fish innate inflammatory response (Ghai et al., 2007; Wang et al., 2017). Divergence of this response may be in keeping with shorter life histories ameliorating the costs associated with innate and inflammatory processes, and indeed in garter snakes, complement responses are stronger in ecotypes that exhibit shorter life histories (Palacios et al., 2011), although these effects were not observed in all age classes.

Whilst a strong association is observed between SNP variants within and around my qPCR genes and their correlated expression, as well as SNP variants that have diverged in lochs with shorter life histories, the functional significance of these SNPs is unclear. Resequencing of these genes within these populations would help assess the functional divergence of proteins,

facilitating the identification of the mechanisms linking genotypic variance and expression. However, at this stage my results represent a promising indication of some genes that may underpin life history correlates with immunity. A failure to link genotypic variance of our immune assays with increased relative expression of the inflammatory gene *tnfα* suggests two possibilities; either that variable expression patterns between populations are encoded elsewhere in the genome, or that this variation is plastic and environmental variation between my populations is responsible for modifying expression. Addressing the first of these scenarios; my outlier analysis did reveal fixed SNP variants separating anadromous and freshwater fish within the proximity of inflammatory genes.

The *eda* gene provides a well-documented example of parallel evolution, where freshwater colonisation events by stickleback across the northern hemisphere have been accompanied by fixation of alleles within this gene (Albert et al., 2008; Colosimo, 2005; Mäkinen et al., 2008). The *eda* gene is best known for its role in modifying skeletal morphology, and indeed anadromous OBSM fish display a fully plated 'marine' phenotype, whilst the 4 freshwater populations are low-plated. The protein encoded by *eda* harbours a tnf-domain that suggests it may also play a role in regulating the immune response. Whilst *eda* generally appears to be more important for development than immunity in humans (Sadier et al., 2014), there are other genes in close-proximity that are likely to influence innate and inflammatory immune responses, including *tnfsf13b* (Baff), *garp*, and *muc5b* (El Nagar & MacColl,

2016; Jones et al., 2012a). Indeed, *eda* haplotypes in stickleback are associated with immune responses and parasite resistance when separated out from genetic background in F2 crosses (Robertson et al., 2017a).

Alternatively, anadromous fish may be the most energetically stressed population. Anadromous stickleback develop in marine environments before migrating inland to reproduce, which leads to predicted modifications of their life history (Snyder & Dingle, 1990). Once there, they must compete with diverged, resident stickleback for resources, or may reduce feeding to concentrate on reproduction. This introduces a migratory element and increased competition that may intensify the potential effects of resource limitation on the skewing of the immune system towards pro-inflammation. Extreme mortality of male anadromous fish is well-documented (Dufresne et al., 1990), and it would be interesting to compare breeding season survival in freshwater populations. Few studies have examined the effects of migratory strategies on immune investment, although Eikenaar and Hegemann (2016) found innate immunity to be reduced in migratory blackbirds (*Turdus merula*), whilst Carbó-Ramirez and Zuria (2015) found migratory species of sparrows to have stronger immune responses than non-migrants. Contrasting patterns may occur as migration may either select for weaker immune responses to conserve energy, or stronger immunity to counteract a wider array of parasites.

To address issues associated with low population replication and low number of genes assayed, I expanded my approach to include 15 freshwater populations and genetic variation around 166 immune genes. Here, I found

that the principal axis of genetic variation was associated with life history strategy, indicative that populations that have evolved similar life history strategies have also evolved similarly in and around immune genes. This relationship cannot be explained by population structure as freshwater populations on North Uist exhibit strong population structure (Magalhaes et al., 2016; Rahn et al., 2016b) and are presumed to have been colonised independently. This analysis compliments patterns of expression and genetic variation in my sampled populations by confirming the expected evolutionary association between life history and immune responses.

In conclusion, I have been able to demonstrate consistent differences between the differential expression of immune genes between 'short' and 'long' life history strategies with a probable, heritable genetic determinant. Furthermore, by examining multiple populations, this study presents evidence with a degree of repeatability that is rarely available in the field of ecoimmunology. Disentangling plasticity and genetic determination is a challenge for ecoimmunology, however I have attempted to address it here by examining within and between population variation. Within populations, differential expression patterns are consistent with resource-mediation hypotheses of immune variation, as stressed individuals downregulate overall expression and inflammatory-moderating arms of the immune response. These patterns were observed at the population-level as well between anadromous and fresh water residents, although I cannot rule out a genetic basis for this. The similarities suggest a strong role for the environment in skewing the

stickleback immune system to increased *tnfα* expression. Strong agreement between local allele frequencies and overall expression of the assayed genes, as well as divergence of complement genes, seem to indicate that short-lived populations may have diverged towards costlier responses, a concept in-keeping with current hypotheses. In particular, Treg and Th1-mediated responses may represent promising avenues of targeted research to further understand the interactions between life history and immune variation in the wild. Finally, I reveal a general association across North Uist between life history trait evolution and genetic variation around immune genes across the genome, which confirms an evolutionary association between the two.

CHAPTER 3: DAYLENGTH MODULATION OF IMMUNE RESPONSES AND ITS EFFECT ON PARASITE SUSCEPTIBILITY

3.1 Abstract

The annual cycle of the seasons drives dramatic changes in living organisms. Many of these are evolved, inherent responses mediated through organisms' internal physiological clocks, using a variety of cues, especially daylength. Other, less predictable, changes may result from the direct effect of seasonally related fluctuations in environmental variables such as temperature and precipitation, or from wider social, epidemiological and ecological effects in the surrounding community of other organisms. Seasonal patterns in disease incidence and parasitic infection are a common occurrence across many organisms including humans, as is seasonal variation in immune responses. While the effect of daylength on immune responses has been demonstrated, we do not know whether seasonal changes in infectious diseases result from these inherent organismal responses to daylength or are a consequence of changes in the occurrence of disease in the surrounding community. Here, for the first time, I examine the effect of daylength on susceptibility to parasitic disease by exposing three-spined stickleback, a model aquatic vertebrate, to contrasting daylengths followed by artificial infection with the trematode ectoparasite *Gyrodactylus gasterostei* in a factorial experiment. Lab-reared fish

were housed under summer (16L) or winter (8L) photoperiods for 5 months before being infected by *G. gasterostei*. Infections were monitored over 20 days before expression analysis of 8 immune genes, representing different arms of the vertebrate immune response, using qPCR. Fish housed under summer daylengths suffered worse infections and reduced expression of mucosal immune genes important for managing infection. These effects were generally more pronounced in stickleback from a population that is naturally susceptible to the parasite, than in one that is resistant. These results are the first to demonstrate under controlled conditions that daylength, probably mediated through its effects on host immune responses, directly influence parasite infection dynamics, offering empirical insight into the evolutionary relationship between seasonal host-parasite associations.

3.2 Introduction

The ability of organisms to regulate processes in response to predictable, seasonal changes in their environment is an adaptive response to maximise fitness. Temporal variation is exhibited in a wide range of phenotypes, for example; reproduction can be timed so offspring are born when resources are plentiful (Rubenstein & Wikelski, 2003); metabolism and food intake varies temporally to correspond with availability (Ebling, 2015); fur growth and colour coincides with seasonal changes in temperature and conditions (Hoffmann, 1973; Martinet et al., 1992). Seasonal changes to organisms may result as a direct response to seasonal changes in their environment, such as changes to

temperature and precipitation or seasonal patterns of social behaviour and ecological interactions in their environment.

However, if seasonal selection pressures are truly predictable, the biological clock and annual rhythm represents one of the most reliable cues for time of year experienced by all organisms. Annual rhythm is maintained through internal responses to daily incremental changes in daylength. Thus, photoperiod as an environmental cue for temporal variation is a recognised phenomenon, and has been documented in humans and most vertebrates, with an emphasis on mammals and birds, and in particular Siberian hamsters, *Phodopus sungorus* (Stevenson & Prendergast, 2015). It has recently become clear that photoperiod changes interact with thyroid hormones in a conserved way across vertebrate taxa (O'Brien et al., 2012). In this mechanism, changes in photoperiod lead to increased production of deiodinase 2 (dio2) enzyme, which catalyses the conversion of the thyroid hormone thyroxin (T4) into bioactive triiodothyronine (T3). T3 is an important signalling molecule for initiating seasonal changes, such as the secretion of gonadotropic hormones from the pituitary gland, for example luteinizing hormone (LH), which in turns signals the start of the breeding season (O'Brien et al., 2012). Additionally, T3-signalling by lymphoid cells is directly altered by day length, conferring seasonal immunomodulation (Stevenson & Prendergast, 2015).

Indeed, immune responses demonstrably display an annual rhythm, with different immune responses exhibiting predictable, seasonal patterns. For example, human populations tend to upregulate inflammatory innate

immunity in winter months, a period which is characterized by increased incidences of inflammatory-linked autoimmune conditions such as arthritis (Dopico et al., 2015). Brown et al. (2016) demonstrated that wild three-spined stickleback, *Gasterosteus aculeatus*, populations also upregulate innate immunity and suppressors of adaptive T-cell responses in late winter compared with late summer. There are similar patterns in dolphins (Morey et al., 2016), and even coral species have been shown to modify innate immune activity in response to seasonal temperature changes (Van de Water et al., 2016).

There are numerous hypotheses as to why host immune responses vary seasonally. Firstly, the maintenance and deployment of immune responses are energetically taxing (Schmid-Hempel, 2011). In this regard, costly immune responses may be downregulated during energetically demanding times, such as the breeding season or overwinter. Immune responses may also vary seasonally in response to seasonal parasite dynamics. In this way, hosts should increase investment in immune responses when parasite infections are predictably severe. For example, malaria infections in humans increase at certain times of the year, likely because of interactions between seasonal temperature and rainfall and vector population size (Hoshen & Morse, 2004). Such parasitic rhythm creates a strong selection pressure to which host temporal immunity can respond.

There has been a plethora of work documenting how different arms of the immune system respond to photoperiod in controlled studies (reviewed in (Nelson & Demas, 1996; Stevenson & Prendergast, 2015)). Likewise, there have

been many incidences of host-parasite seasonality being recorded in the wild (reviewed in (Altizer et al., 2006)). However, to my knowledge, no studies have investigated how photoperiod modulation of the immune response affects the ability of a host to respond to its parasites in a controlled experiment. Such knowledge is imperative to understand the processes driving temporal plasticity of immune responses. Additionally, host populations vary in their resistance to parasites (Martin et al., 2011), and few studies have examined intraspecific temporal variation and the interactions between immune variation and photoperiod-immune modulation. These gaps in our knowledge have implications for understanding seasonal host-parasite interactions and how temporal selection regimes are imposed on parasites whose hosts display contrasting immune phenotypes.

I sought to test these questions in a controlled, laboratory experiment. My aim was to demonstrate a direct link between photoperiod cues and infection dynamics, as well as to examine how this relationship interacted with intraspecific immune variation. I studied the three-spined stickleback, *Gasterosteus aculeatus*, and its naturally-occurring monogenean flatworm parasite, *Gyrodactylus gasterostei*. Like all teleost fish, stickleback have a full vertebrate immune system and have repeatedly adapted to a wide variety of freshwater habitats displaying environmental variation in parameters such as water temperature, food availability, predator density and parasite prevalence, virulence and diversity (Colosimo, 2005; Jones et al., 2012b). They also exhibit substantial variation in immune responses (Robertson et al., 2016a) and display

seasonal immunomodulation (Brown et al., 2016). *Gyrodactylus* sp. have also been shown to display seasonal infection dynamics and modulation of reproduction rate (Koskivaara et al., 1991; Özer et al., 2004; Winger et al., 2008). I used lab-bred fish originating from lochs on the Outer Hebridean island of North Uist, Scotland, that have been shown to vary greatly in their *Gyrodactylus* prevalence and burdens (De Roij et al., 2011; Mahmud et al., 2017; Young & MacColl, 2017), both in the wild and in artificial infections (De Roij et al., 2011). I housed fish in alternative photoperiods for an extended period before conducting a controlled infection experiment in a factorial design. In this way, I was able to temporally disrupt host immune responses and test the subsequent effects on their resistance to a naturally-occurring parasite. I also measured how infection dynamics were affected differentially between fish of contrasting natural resistance.

3.3 Materials and methods

3.3.1 Fish culturing and photoperiod treatment

Gravid females and reproductive males were collected from two lochs with contrasting *Gyrodactylus arcuatus* prevalences and resistance, Chadha Ruaidh (CHRU - susceptible) and nan Strùban (STRU - resistant) on North Uist, Scotland (Table 3.1) in May 2014. F1 progeny were produced by *in vitro* crossing according to (De Roij et al., 2011) before being transported to the University of Nottingham. Fish were housed in family groups of equal density for 21 months,

under 6-month photoperiod regimes of 16L:8D summers (February-July) and 8L:16D winters (August-January) to simulate natural conditions. At the beginning of month 22 (March 2016), 40 fish from each population were randomly assigned to a control, winter photoperiod room (8L:16D) or summer photoperiod room (16L:8D). Each room was air-conditioned at 14°C, although temperatures fluctuated (16L, Mean Max = 16.1°C, Mean Min = 13.8°C; 8L, Mean Max = 15.4 °C, Mean Min = 13.4°C). Daylight hours in the 16L photoperiod room were steadily increased at 2 hours per week from 8 hours to 16 hours to mimic natural conditions. Fish were housed individually in 10 L compartments of 20 L tanks for a total of 153 days.

Table 3.1: Source populations for lab bred fish. Parasite information sampled May 2013, see (Magalhaes et al., 2016)

Loch	Location	*G. arcuatus* Burden[1]	*G. arcuatus* Prevalence	Immune Phenotype
Chadha Ruaidh (CHRU)	57°36"N; 7°12"W	0.00±0.000	0.000	Susceptible
nan Strùban (STRU)	57°34"N; 7°21"W	2.857±1.078	0.686	Resistant

[1]: Denotes mean±SE for N = 32 (CHRU) and 35 (STRU) fish

During the infection treatment, fish were housed for a further 20 days in a single room with an intermediate photoperiod (12L:12D) in individual 3 L tanks, with a 25% water change done every 3 days. Temperature over this period varied between a mean max of 17.4°C and a mean min of 14.0°C Photoperiod treatments are summarised in Figure 3.1.

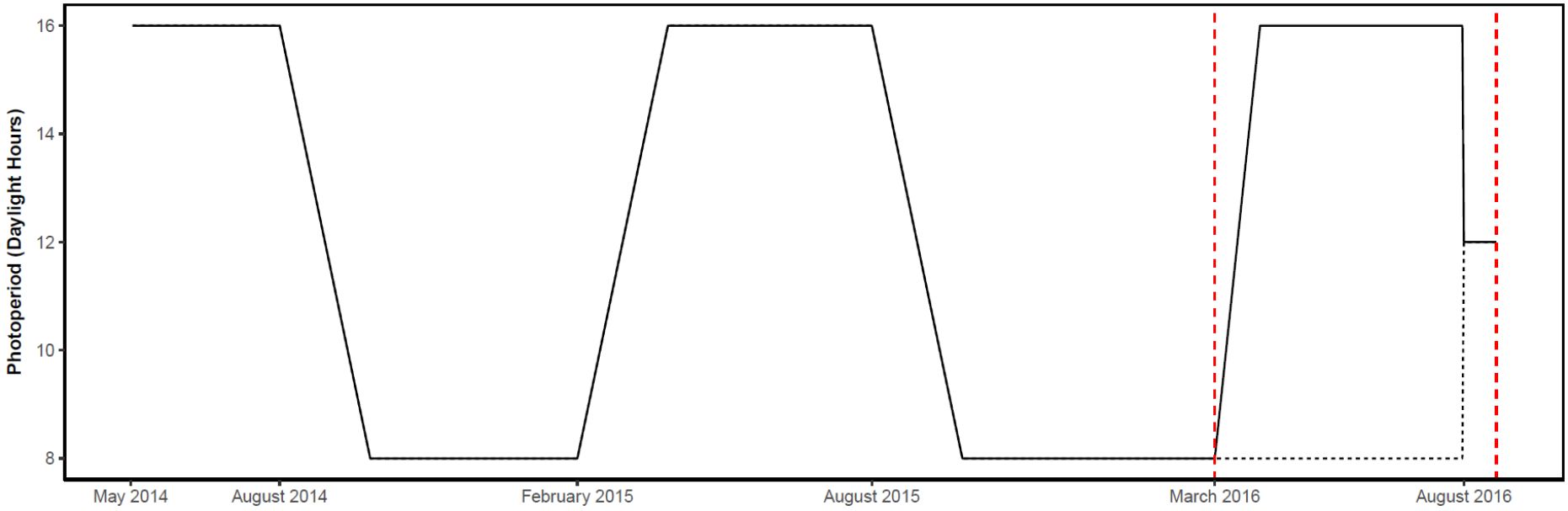


Figure 3.1: Photoperiod conditions experienced by fish since birth and experimental conditions. Eggs fertilised *in vitro* hatched under natural summer 16L photoperiod conditions in May 2014. As fish developed, natural conditions were emulated by gradually altering photoperiod between summer 16L and winter 8L conditions. Red dashed lines indicate experimental time frame from March 2016 – August 2016. After this period, 16L conditions are presented by a solid line, whilst 8L fish are shown as a dashed line. For infection, all fish were housed under 12L in a single room.

3.3.2 *Gyrodactylus* infections and sampling

Prior to photoperiod treatment, 20 fish from each photoperiod room were randomly assigned to undergo a further infection treatment, creating a full factorial experimental design. However, over the course of the 173 days, 8 fish died from the 16L room due to unknown causes, resulting in final sample sizes of: 8h Photo & Infected N = 20; 8h Photo & Uninfected N = 20; 16h Photo & Infected N = 15; 16h Photo & Uninfected N = 17). Elevated mortality rates of aquarium housed fish during periods of summer daylength are quite normal in our experience of stickleback husbandry (A. Lowe and A.D.C. MacColl, unpublished data).

On the morning prior to infection, donor stickleback were collected from Tottle Brook, a small stream on the University of Nottingham campus (52°55'58.4"-1°12'05.8"). Stickleback from this location are infected with *Gyrodactlyus gasterostei*. Donor fish were euthanized by an overdose of MS222 (400 mg L^{-1}) in accordance with Home Office regulations. To minimise the likelihood of parasites coming from the same individual host, all caudal and pectoral fins were clipped under a low powered microscope from donor fish and collected in a single petri dish with a small amount of dechlorinated water. Fins were left for 10 minutes to allow worms to detach.

Prior to infection, fish were anaesthetised by a low-dose of MS222 (280 mg L^{-1}) and had their weight and standard length recorded. Fish were infected by placing the caudal fin into close proximity with donated worms in a petri dish, under a low-powered microscope, until they attached; each fish was

infected with 3 worms. Fish that were not infected were exposed to the same dosage of MS222 for 30 seconds and were weighed, measured and handled to simulate the infection protocol. Following treatment, fish were transferred to individual 3L tanks in a single room.

After 2 days, infected fish were fully checked under anaesthetic for the presence of the parasite on all surfaces where it is known to occur (skin, caudal fin, anal fin, pectoral fins, spines and gills). Fish from the infection group that were found to be uninfected were re-infected using the same protocol as above. Again, uninfected fish were anaesthetised and handled to simulate infection treatment protocol. On days 7 and 14 post initial infection (pi), infected fish were anaesthetised and weighed (to track condition) before having their parasite burdens recorded. Uninfected fish were also anaesthetised and weighed.

On day 20 pi, all fish were euthanized with an overdose of MS222 (400 mg L^{-1}) before being weighed and, if from the infected group, having final parasite burdens recorded. Fish that had been infected were also classified as 'infected' if they still had *Gyrodactylus* on them or 'cleared' if they no longer had worms. From each fish, I took the spleen and left operculum (skin) and put both immunologically relevant tissues (Foey & Picchietti, 2014; Zapata et al., 2006) immediately into RNAlater (Life Technologies). Tissue samples were stored at 4 °C for 24 h and then kept at -20 °C, with RNA being extracted within 3 months. From each fish I also recorded the sex and weight of gonads, liver and adipose tissues, which were subtracted from the total weight to yield

somatic weight. I then calculated Gonadosomatic Index ('GSI'), Hepatosomatic Index ('HSI') and Adiposomatic Index ('ASI') according to Tissue Weight/Somatic Weight x 100. I also noted the reproductive condition ('REPRO') of males using a qualitative scale based on size of testes and kidney (the kidney of breeding male stickleback becomes enlarged); 1 (small testes and kidney); 2 (enlarged, melanised testes, small kidney); 3 (enlarged, melanised testes and enlarged kidneys). Reproductive condition of fish was determined through GSI for females, and REPRO for males. These were selected because there were no females in the experiment that were fully gravid, therefore GSI was the best predictor of reproductive investment. For males, the condition of the testes and kidneys is a better indicator of reproductive investment compared to GSI.

3.3.3 RNA isolation and qPCR

All qPCR work was conducted in accordance with the MIQE guidelines (Bustin et al., 2009). Sampling order was randomized and RNA was extracted from stored, whole spleens and opercula using the Genejet RNA purification kit (Thermo Scientific) according to the manufacturer's protocol. RNA was DNase treated using Precision DNase (Primerdesign) following the manufacturer's protocol.

RNA purity was assessed on a NanoDrop1000 spectrophotometer (Thermo Scientific). RNA integrity was assessed following DNase (Primerdesign)

treatment by visualisation of 4 µl of sample on a 2% agarose gel stained with ethidium bromide. Reverse transcription was performed using nanoScript2 RT kit (Primerdesign) according to the manufacturer's protocol using approximately 1.5 µg of template. This protocol uses a combination of random nonamer and oligo-dT priming. Genomic DNA contamination was assessed via light PCR using intron-spanning primers. All cDNA samples were diluted 1:10 with nuclease free water before use.

A total reaction volume of 10 µl was used to perform qPCR reactions consisting of 5 µl of PrecisionFAST low ROX mastermix with SYBR green (Primerdesign), 2.5 µl of nuclease-free water, 0.25 µl of each primer at working concentration and 2 µl of cDNA template. Reactions were performed in 96-well optical PCR plates with optical seals (StarLab) in an ABI 7500 Fast real-time thermocycler (Applied Biosystems). Samples were incubated at 95 °C for 20 s, followed by 45 cycles of 95 °C for 3 s and 60 °C for 30 s. A melt-curve analysis was also included to confirm product.

3.3.4 Gene expression quantification

Genes for which assays had been previously developed (Robertson et al., 2016a) were selected to characterise different arms of the immune response and were identified based on previous studies in other fish and known roles of orthologous genes. The pro-inflammatory gene *tnfα* is a key component of innate immunity in teleosts and other vertebrates (Secombes & Wang, 2012;

Uribe et al., 2011), activating macrophages, eliciting inflammation and increased respiratory burst activity.

The Th1 transcription factor STAT4 (*stat4*) promotes the differentiation of Th1 cells and has been identified in teleost genomes. STAT4 activates the expression of *tbet*, which in mammals represents the master transcription factor for Th1 cell differentiation, activating other genes required to promote differentiation and suppressing the development of other T-cell lineages such as Th2 and Th17 (Wang & Secombes, 2013). Fish *tbet* has been cloned in a number of teleosts and its existence and function are supported by conserved synteny across teleosts and humans and expression studies demonstrating its importance in fish T cell-mediated immunity (Wang & Secombes, 2013). Expression of Th1-associated genes in infection studies suggest that fish may possess a full and conserved Th1 pathway (Secombes & Wang, 2012). Th1 adaptive immunity is associated with intracellular parasites, whilst Th2 adaptive immunity is associated with extracellular infection. Th2 cell differentiation is associated with up-regulation of *cmip* and *stat6* in mammals, which is also upregulated alongside other markers of Th2 responses in zebrafish head kidney and spleen cells in response to immunostimulation (Mitra et al., 2010).

Th17 cells, a subset of pro-inflammatory T cells, are regulated by the transcription factor ROR-γt (*rorc*) in mammals, which increases in mRNA levels during the differentiation of naïve CD4+ lymphocytes into Th17 cells (Yang et al., 2008). *rorc* is present in fish genomes, albeit in several isoforms (Wang &

Secombes, 2013) along with the other major components of Th17 cell development, however expression studies in trout both support and contradict the notion that *rorc* in fish behaves like its mammalian counterpart (Monte et al., 2012)

Mucins represent constituent molecules of mucosal immunity and are secreted within mucus, forming a protective gel-like layer to protect epithelial surfaces (Linden et al., 2008). The mucin gene *muc2* is typically expressed at intestinal and respiratory surfaces and has been characterised in carp (van der Marel et al., 2012), showing high similarity to its mammalian and avian equivalent. Of specific interest here, mucin genes exhibit strong divergence between marine and freshwater stickleback, suggesting significant ecological associations with adaptation (Jones et al., 2012a).

Treg activity, which regulates the immune response in general, is characterised by *foxp3a* expression. This gene has been identified in many teleost genomes and has demonstrable roles in immune regulation in fish and mammals (Secombes & Wang, 2012). The genes included in this study therefore serve to capture variation across a range of immune responses; specifically, in the expression of an innate inflammatory cytokine (*tnfα*); markers for T-cell differentiation for Th1 adaptive (*stat4, tbet*), Th2 adaptive (*cmip, stat6*), Th17 adaptive (*rorc*) responses; mucin production as a measure of mucosal immunity (*muc2*); and Treg cytokine activity (*foxp3a*).

A custom stickleback with SYBR geNorm analysis was conducted to select appropriate housekeeping genes for this experiment for each tissue

type. The analysis was conducted per the manufacturer's protocol using 15 randomly selected samples, consisting of a random male and female from each population within each treatment group. Of the 6 candidate reference genes supplied (*b2m*, *gapdh*, *rpl13a*, *hprt1*, *tbp* and *top1*) *b2m* and *rpl13a* were the most stably expressed combination for spleens and *hprt* and *tbp* were the most stably expressed combination for opercula.

In total, 69 spleen and 69 operculum samples were analysed in duplicate. A reference sample, comprised of a pool of all 138 samples, was made up and used as a control reference across all plates. Three plates were run per gene. In total, 8 genes were amplified (Table 3.2) using 6 primers published in (Robertson et al., 2016a) and 2 primers (*rorc*, *muc2*) designed and tested by Primerdesign Ltd. Relative expression values were calculated per the ΔΔCq method (Pfaffl, 2001) and adjusted for the amplification efficiencies of each primer pair. Expression values were standardized against the tissue-specific geometric mean Cq of two reference genes.

Table 3.2: Primer details for qPCR genes

Gene	ENSEMBL ID	Immune Role	Primer Sequence (5'-3')	Amplicon length
tnfα	ENSGACG00000013372	Pro-Inflammatory cytokine	Fwd-GCTTGGTTCTGGCCAGGTTT Rev-GCTGCTGATTTGCCTCAACG	125
stat4	ENSGACG00000002684	Transcription factor for development of Th1 cells	Fwd-CTCTCAGTTTCGAGGCTTGCTT Rev-GGCAGTTGGCTCACATTGG	100
tbet	ENSGACG00000003829	Transcription factor modulates expression of Th1-cell cytokines eg. IFNγ	Fwd-CACATCGTGGAGGTGAAGGA Rev-CGGTGACGGCGATGAACT	99
cmip	ENSGACG00000002527	Signalling protein in Th2 pathway	Fwd-GGCATGGAGGTCGTCAAGAA Rev-TAGCAGGAGTAAATGGCGGC	119
stat6	ENSGACG00000008477	Involved in mediating Th2 cytokines IL-4 and IL-3 signalling	Fwd-CTCAGCCACAGTTCCAACCGTTC Rev-GTCGGATGTTCTGGACCTCGAGT	104
foxp3a	ENSGACG00000012777	Promotes development and function of Treg cells	Fwd-GTTGACCCATGCAATTCCGA Rev-CTGCTGTAGTTGTGGTCCTG	94
rorc	ENSGACG00000012239	Promotes differentiation of pro-inflammatory Th17 cells	Fwd-TTGACTTTGCCCACGGTATG Rev-TGCTGAACTCTGCCTCTGT	121
muc2	ENSGACG00000014109	Principal organic constituent of mucus	Fwd-AGAATGGCGAGTCCTGGAA Rev-AGATGGGTTGTTGTGGTGTG	105

3.4 Data analysis

3.4.1 Effect of photoperiod/infection on relative expression of immune genes

All data were analysed in R version 3.3.2 (R Core Team, 2016). Relative expression values were $\log_{10}$-transformed to account for the inherent skewness of relative expression data. A principal component analysis (PCA) of

transformed relative expression values revealed a split of mucosal immunity (*muc2)* from systemic immunity (other genes). Therefore, *muc2* expression was handled separately and a PCA of the seven systemic genes was made up for each tissue type, with the first 2 PCs taken for each. This left me with 6 expression variables: Immune PC1, Immune PC2 and *muc2* expression, all for both spleens and opercula.

I then fitted Gaussian family GLMs to each expression variable to understand causes of variation in the way that genes were expressed in all fish. The maximum models included the treatment variables photoperiod group ("PHOTO") and infection group ("INFECTED") and the control variables of population ("POP") and sex ("SEX"). Variables were modelled additively and removed sequentially using a top-down approach. Biologically plausible interactions between independent variables were assessed at each level to see if model fit could be improved. If model fit was significantly improved interactions were added, and top-down model selection was continued until the simplest, best fitting model was found. Model fit was assessed through AIC (Akaike Information Criterion) and residual diagnostic plots. The significance of remaining factors was inferred through ANOVA F-tests.

3.4.2 Effect of photoperiod/infection on fish condition

I used the first principal component ("CONDITION") of HSI and ASI as an approximation of fish condition as the two were correlated (Spearman's R =

0.458, $p < 0.001$)). CONDITION was modelled using GLMs as above using a Gaussian distribution and identity link and variables were selected from an initial model consisting of PHOTO, POP, INFECTED and SEX. Significance of remaining variables was inferred through ANOVA F-tests.

Female GSI was $\log_{10}$-transformed to meet the assumptions of a Gaussian fitted GLM, with the most complex model comprised of PHOTO, POP, and INFECTED. Males were divided into those that were fully reproductive (REPRO = 3) and those that were not (REPRO < 3). A binomial-fitted GLM was deemed inappropriate due to the extreme biasing effect of PHOTO, therefore a contingency table and chi-squared test was used to assess the reproductive status of males from each room.

3.4.3 Effect of photoperiod/infection on infection dynamics

To assess the effect of treatment group on peak *Gyrodactylus* burdens of infected fish, I used Generalized Linear Models (GLMs) fitted with a negative binomial distribution and logarithm link function. These were shown to fit the data better than models with Poisson distribution. I also used GLMs with binomial errors ('cleared' = 1, 'infected' = 0) to model the probability that individual fish cleared their infection. Models were fitted with the independent variables PHOTO, POP and SEX. Models were fitted by the above methods and the significance of remaining factors was inferred through Wald's Tests using the R package *survey*.

The relationships between the six immune gene expression variables and *Gyrodactylus* infection were modelled individually using GLMs with negative binomial distributions. Day 20 burdens were modelled as the dependent variable in each case. Significance of relationships was determined through Wald's Tests as above.

3.5 Results

3.5.1 Effect of photoperiod/infection on relative expression of immune genes

I ran PCAs for the seven systemic immune genes (Figure 3.2) and found that for spleens (summarised in Table 3.3), PC1 represented 48.8% of the variation, and constituted the correlated expression of all 7 genes. However, this effect was primarily driven by the pro-inflammatory genes *tnfα* (innate), *tbet* (Th1), and *rorc* (Th17), suggesting this is likely an inflammatory axis. PC2 for spleens represented antagonistic expression of the Th17 gene *rorc* and the pro-inflammatory gene *tnfα* and explained a further 24.8% of the variation in spleen gene expression. For opercula tissues, PC1 accounted for 42.6% of the variation and represented the correlated expression of all 7 genes, and in particular variation in *rorc* expression. Operculum PC2 represented decreased relative expression of *rorc* compared with the other 6 genes and characterised 29.0% of the variation in operculum gene expression.

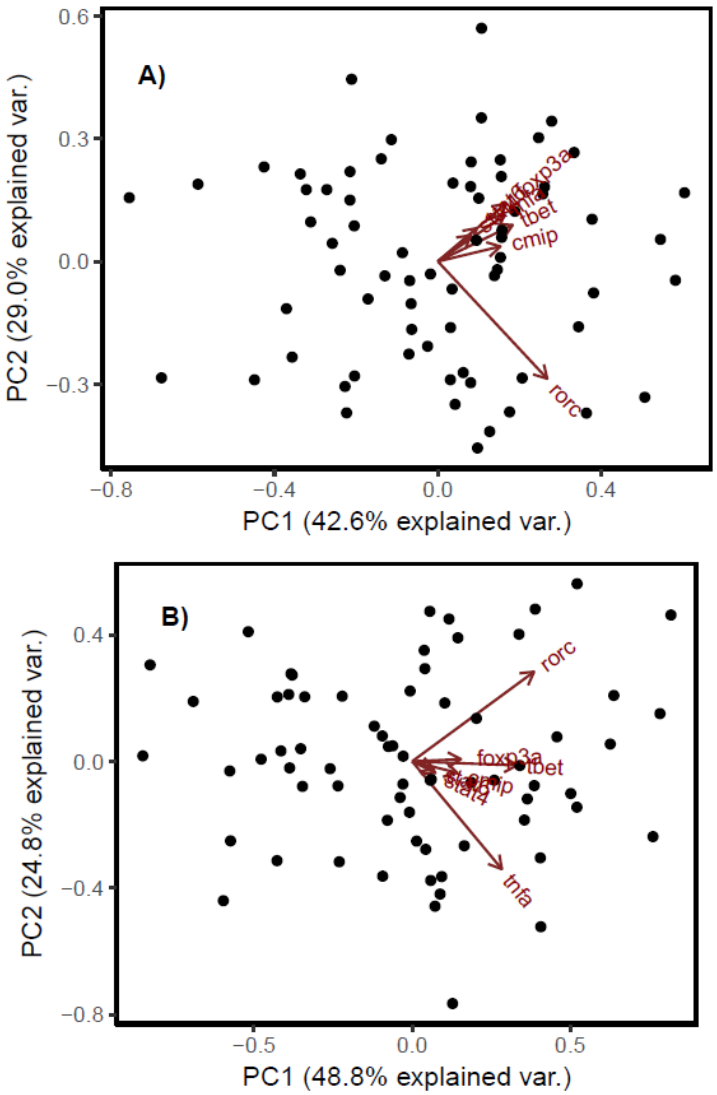


Figure 3.2: PCAs for the seven systemic immune genes for opercula tissues **A)** and spleen tissues **B)**. Points denote individual fish. Arrow direction denotes direction of variation along PCs 1 and 2, and length denotes loading of each immune gene to PCs 1 and 2.

Table 3.3: PC loadings for systemic immune genes across tissue types

Gene	Operculum PC1	Operculum PC2	Spleen PC1	Spleen PC2
tnfα	0.366	0.307	**0.452**	**-0.762**
stat4	0.181	0.175	0.117	-0.082
tbet	**0.411**	0.243	**0.529**	-0.028
stat6	0.214	0.222	0.109	-0.054
cmip	0.344	0.100	0.226	-0.081
foxp3a	0.374	0.384	0.246	0.016
rorc	**0.601**	**-0.781**	**0.615**	**0.634**

*Bold values denote those > 0.4

These expression variables, along with *muc2* expression in each tissue, were modelled to understand their response to the experimental treatments of photoperiod and infection, with the additional potential sources of variation in population and sex. Results are summarised in Table 3.4. Operculum *muc2* expression was strongly reduced in fish that had been housed in summer 16L conditions (Figure 3.3a). In addition, this difference was significantly exaggerated in susceptible CHRU fish compared to resistant STRU fish, and in males compared with females (Figure 3.3c). Interestingly, the model also included an interaction between photoperiod and infection treatments, which indicated that infected 16L downregulated operculum *muc2* whilst infected 8L fish increased expression (Figure 3.3b).

Photoperiod treatment had no effect on operculum PC1, the correlated expression of all systemic genes. Infection however significantly reduced expression of systemic genes in the skin, an effect which was significantly more exaggerated in more naturally susceptible CHRU fish (Figure 3.3d).

Similarly, photoperiod did not affect operculum PC2 and was dropped from the model. Although infection treatment was the dominant source of variation in this instance, differences between uninfected and infected fish were nonsignificant. Both sexes and population also displayed similar operculum expression of *rorc*.

Table 3.4: Model effects for response variables. Models were reduced from a maximum model including photoperiod treatment, infection group, population and sex (if applicable).

Analysis	Response Variable	Factor[1]	Family	ΔAIC[2]	*F*	d.f.	*P*[3]	Effect[4]
Gene Expression	Spleen Inflammation Expression	Photoperiod * Sex	Gaussian	6.72	8.619	1,64	**0.005**	*NA*
	(Systemic Immune PC1)	Population		12.43	15.731	1,66	**< 0.001**	STRU > CHRU
		Photoperiod		NA	2.067	1,67	0.155	
		Sex		NA	10.746	1,65	**0.002**	M > F

		Infection Group		1.74	2.384	1,65	0.128	
	Spleen *rorc*Up, *tnfα* Down	Population	Gaussian	20.19	25.419	1,67	**< 0.001**	CHRU > STRU
	(Systemic Immune PC2)	---						
		Photoperiod		-1.5	0.121	1,67	0.729	
		Infection Group		-2	0.003	1,65	0.955	
		Sex		-2	0.000	1,64	0.989	
	Spleen *muc2*	Infection Group * Sex	Gaussian	4.85	6.783	1,65	**0.011**	*NA*
		Sex		NA	2.008	1,66	0.161	
		Infection Group		NA	0.174	1,67	0.678	

		Population		-1.94	0.203	1,67	0.654	
		Photoperiod		-1.99	0.022	1,67	0.883	

	Operculum Overall Immune Expression	Population * Infection Group	Gaussian	3.35	5.238	1,65	**0.025**	*NA*
	(Systemic Immune PC1)	Population		NA	0.726	1,67	0.397	
		Infection Group		NA	12.479	1,66	**< 0.001**	UnInf > Inf

		Photoperiod		1.06	2.208	1,67	0.142	
		Sex		-1.14	2.339	1,64	0.131	
	Operculum *rorc* Down	Infection Group	Gaussian	-1.15	0.717	1,67	0.400	
	(Systemic Immune PC2)	---						
		Sex		-1.67	0.320	1,66	0.573	
		Photoperiod		-1.75	0.146	1,67	0.704	
		Population		-1.99	0.018	1,66	0.894	
	Operculum *muc2*	Photoperiod * Infection Group	Gaussian	15.75	23.035	1,63	**< 0.001**	*NA*
		Photoperiod * Sex		10.71	14.583	1,62	**< 0.001**	*NA*
		Photoperiod * Population		8.69	10.217	1,61	**0.002**	*NA*
		Photoperiod		NA	50.120	1,67	**< 0.001**	8L > 16L
		Population		NA	20.195	1,66	**< 0.001**	CHRU > STRU
		Infection Group		NA	0.001	1,65	0.975	
		Sex		NA	12.638	1,64	**< 0.001**	M > F
Condition	Condition	Photoperiod * Population	Gaussian	20.82	24.996	1,67	**<0.001**	*NA*

	(PC1 of HSI and ASI)	Sex		13.67	9.715	1,68	**0.003**	F > M
		Photoperiod		NA	4.514	1,70	**0.037**	8 > 16
		Population		NA	23.569	1,69	**<0.001**	CHRU > STRU

		Infection Group		0.6	1.870	1,68	0.176	*NA*
	Female GSI	Photoperiod * Population	Gaussian	4.3	6.167	1,42	**0.017**	*NA*
	(log10-transformed)	Photoperiod		NA	20.968	1,44	**< 0.001**	8L > 16L
		Population		NA	0.133	1,43	0.718	

		Infection Group		-2	0.001	1,42	0.978	
Gyrodactylus	Day 20 pi burden	Photoperiod	Negative	4.01	6.710	1,32	**0.014**	16L > 8L
		Population	Binomial	5.34	8.566	1,32	**0.006**	CHRU > STRU

		Sex		-2	0.002	1,31	0.962	
	Clearing Infection	Photoperiod	Binomial	3.64	3.898	1,33	0.057	8L > 16L

		Sex		0.84	2.505	1,32	0.123	
		Population		-1.89	0.115	1,31	0.737	

[1] Variables are ordered such that those removed first from the model are lowest down. Variables included in final models are those above "---".

[2] Values show changes in AIC based on that variable being removed from the model. Typically, increases in AIC of >2 result in poorer fit. "NA" represents variables included in an interaction for which changes in AIC could not be assessed outside of the interaction.

[3] Significance at $p < 0.05$ is denoted in bold.

[4] Effects are included where factors are significant and outside of interactions.

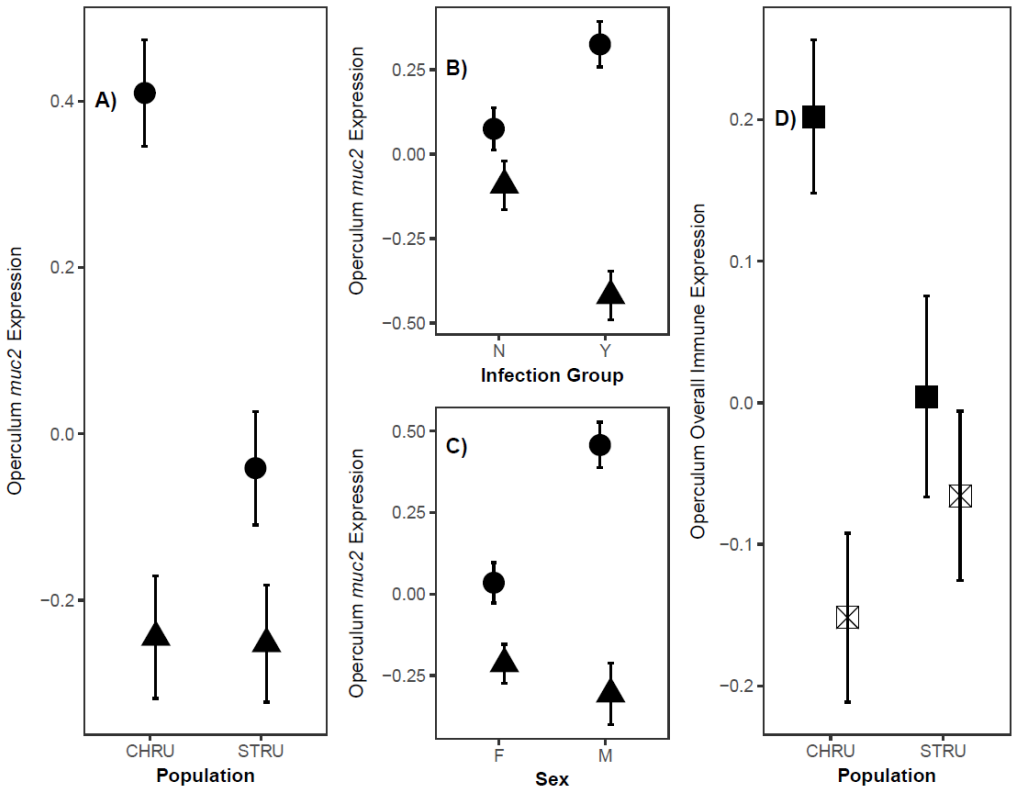


Figure 3.3: Experimental outcomes for significant model interactions with photoperiod treatment (16L = ▲ and 8L = ●) for relative expression of *muc2* in opercula tissues. Values plotted are group means for log10-transformed relative expression ratios with standard errors. **A)** illustrates interaction with population whereby *muc2* expression was lower in 16L compared with 8L fish, a decline which was greatest in more susceptible CHRU fish. However, 8L CHRU fish had the greatest levels of expression. **B)** shows interaction with infection group where 8L fish increased *muc2* expression in response to infection, whilst 16L fish sharply decreased expression. **C)** shows that males had a sharper decrease in *muc2* expression in response to 16L treatments, but 8L males had generally higher levels of expression. Also shown are model effects for operculum overall systemic immune gene expression **D)**. Points represent group means for PC1 scores with standard error. Point fill denotes infection status (■ = uninfected, ☒= infected).

Spleen *muc2* expression was unaffected by photoperiod treatment but was influenced by infection treatment in a sex-specific way; infected males displayed increased *muc2* expression, whilst infected females downregulated *muc2*. Aside from differing in their response to infection, males and females

displayed similar levels of spleen *muc2* expression, and population similarly had little effect on expression.

The response of spleen systemic inflammatory gene expression was significantly affected by photoperiod treatment in a sex-specific manner; males housed under summer 16L photoperiods showed increased expression relative to 8L males, whilst the opposite was observed for females (Figure 3.4a). Infection however had a negligible effect on spleen inflammatory expression and was dropped from the final model with trivial effect on fit. Population was an additional important source of variation for spleen inflammatory expression, which was found to be significantly greater in fish reared from the more resistant STRU population.

Antagonistic expression of spleen *tnfα* and *rorc* was unaffected by both photoperiod and infection treatments. Rather, this unbalance between pro-inflammatory *tnfα* and the Th17 promoter *rorc* appeared driven by population effects, with more naturally resistant STRU fish having increased relative *tnfα* expression and lower *rorc* expression whilst susceptible CHRU fish displayed lower *tnfα* expression and increased relative *rorc* expression (Figure 3.4b).

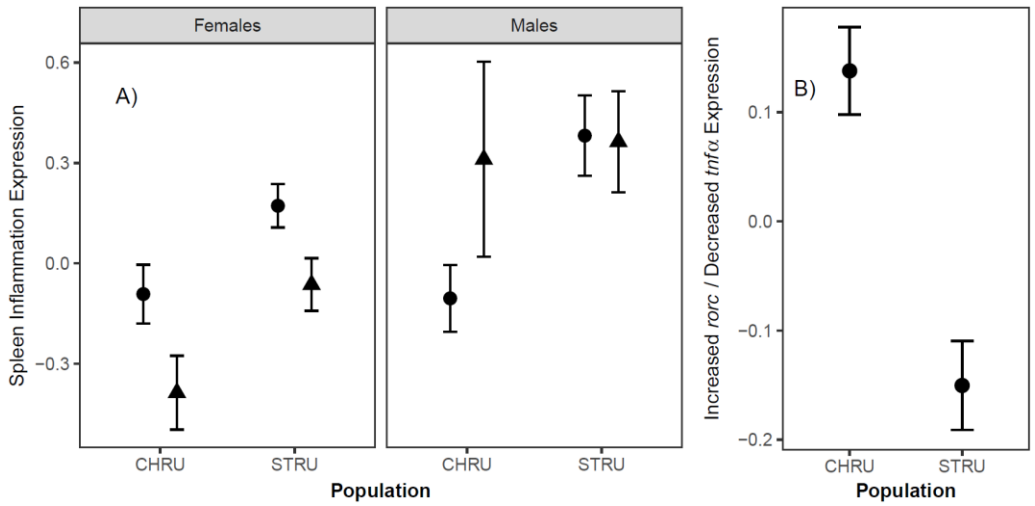


Figure 3.4: Experimental outcomes for significant model factors for the spleen immune variables, inflammation expression (S PC1) **(A)** and antagonistic *rorc* and *tnfα* expression **(B)**. Values plotted are group means for multivariate PC scores with standard errors. **A)** shows how spleen inflammation expression increased in males housed under 16L (▲) conditions compared to 8L (●), but decreased in response to 16L conditions in females. Spleen inflammation expression was also significantly greater in resistant STRU fish. **B)** illustrates that susceptible CHRU fish had increased relative *rorc* expression compared with *tnfα* whilst the opposite was true for resistant STRU fish.

3.5.2 Effect of photoperiod treatment on fish condition

Results of all GLMs are summarised in Table 3.4. A PCA analysis of HSI and ASI revealed correlation of both on PC1, which accounted for 65.4% of the variation between these proxies of fish condition. Fish housed under 16L were in significantly poorer condition than those housed under 8L in general, although this effect was substantially greater in susceptible CHRU fish and slightly inverted in STRU fish (Figure 3.5d). Surprisingly, infection had negligible effect on condition. Sex was also an important indicator of fish condition, with females displaying superior condition to males (Figure 3.5d).

There was a dramatic effect of photoperiod treatment on the reproductive condition of fish from each room. Of the 26 males included, all those found to be in full reproductive condition were housed in the 8L room (N = 15). The remaining non-reproductive males were found to have been housed largely in the 16L room (16L N = 9, 8L N = 2) (Figure 3.5b). This distribution of male reproductivity indicated that photoperiod regime had a large, significant effect on male reproductive timing (Chi-squared, $\chi^2 = 15.329$, $df = 1$, $p < 0.001$). In agreement with this, female GSI was also found to be significantly greater in females from the 8L photoperiod room (Figure 3.5a), however the strength of this effect was not the same for each population. The difference between 8L and 16L reproductive investment was found to be significantly more extreme for resistant (STRU) fish compared with susceptible (CHRU) fish. There was however no significant effect of population outside of this interaction. Similarly, infection treatment had no influence on female reproductive investment.

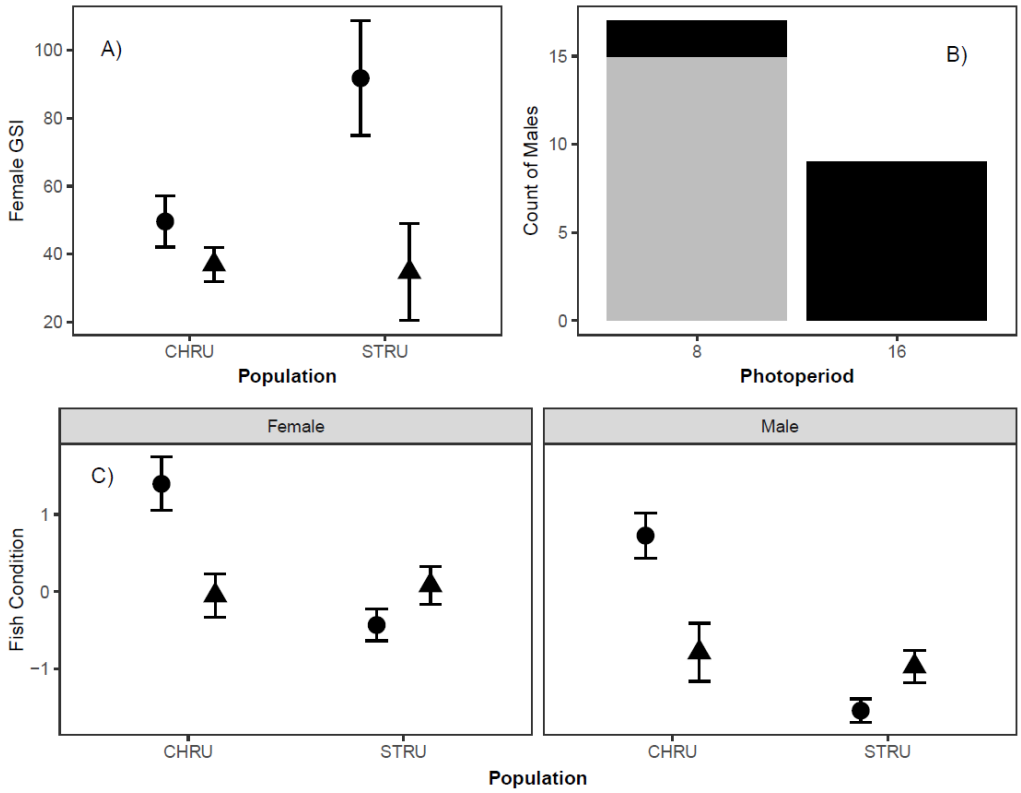


Figure 3.5: Experimental outcomes for measures of fish reproductive state for females **(A)** and males **(B)** and fish condition **(C).** Values plotted for **A)** and **C)** are group means with standard errors. Stacked bars in **B)** represent counts of individuals. **A)** demonstrates that 8L (●) females were significantly more reproductively invested, as measured by GSI, than 16L (▲) females. This effect was particularly strong for resistant STRU fish. **B)** The majority of 8L males were in full reproductive condition (grey-shaded) whilst 16L were not reproductive (black-shaded). **C)** shows interactions between photoperiod, population and sex. Condition declined in response to 16L (▲) treatment compared with 8L (●) in susceptible CHRU fish but increased slightly in resistant STRU fish. Males were consistently in poorer condition than females.

3.5.3 Effect of photoperiod treatment on infection dynamics

Results of models are summarised in Table 3.4 and infection dynamics are summarised in Table 3.5. Fish that were housed in the summer (16L) photoperiod room had significantly higher parasite burdens 20 days post infection compared to those housed in winter (8L) conditions (Figure 3.6a). This

effect was consistent across both the susceptible (CHRU) and resistant (STRU) populations (Figure 3.6b), however as expected CHRU fish had significantly greater burdens than STRU fish (Figure 3.6c). The likelihood of fish clearing their infection during the 20 day period was also dependent on their photoperiod during housing, with fish from the 8L room being more likely to clear their infection, although this was deemed nonsignificant (p = 0.057) in a conservative Wald's Test (Figure 3.6d). This effect was represented by 9 of the 20 infected fish from the 8L room clearing their infection, compared with 0 16L fish clearing their infection.

Table 3.5: *G. gasterostei* infection dynamics for infected fish from each treatment group

Population	N	Photoperiod Treatment	Day 7 Burden[1]	Day 14 Burden[1]	Day 20 Burden[1]
CHRU	10	8L	3.20±0.71	4.40±1.24	7.70±2.31
	8	16L	4.00±1.04	14.88±3.82	41.50±10.95
STRU	10	8L	0.90±0.38	1.40±0.75	3.60±2.85
	7	16L	4.29±1.25	3.57±1.09	5.57±1.38

[1] Values show mean±SE to 2dp

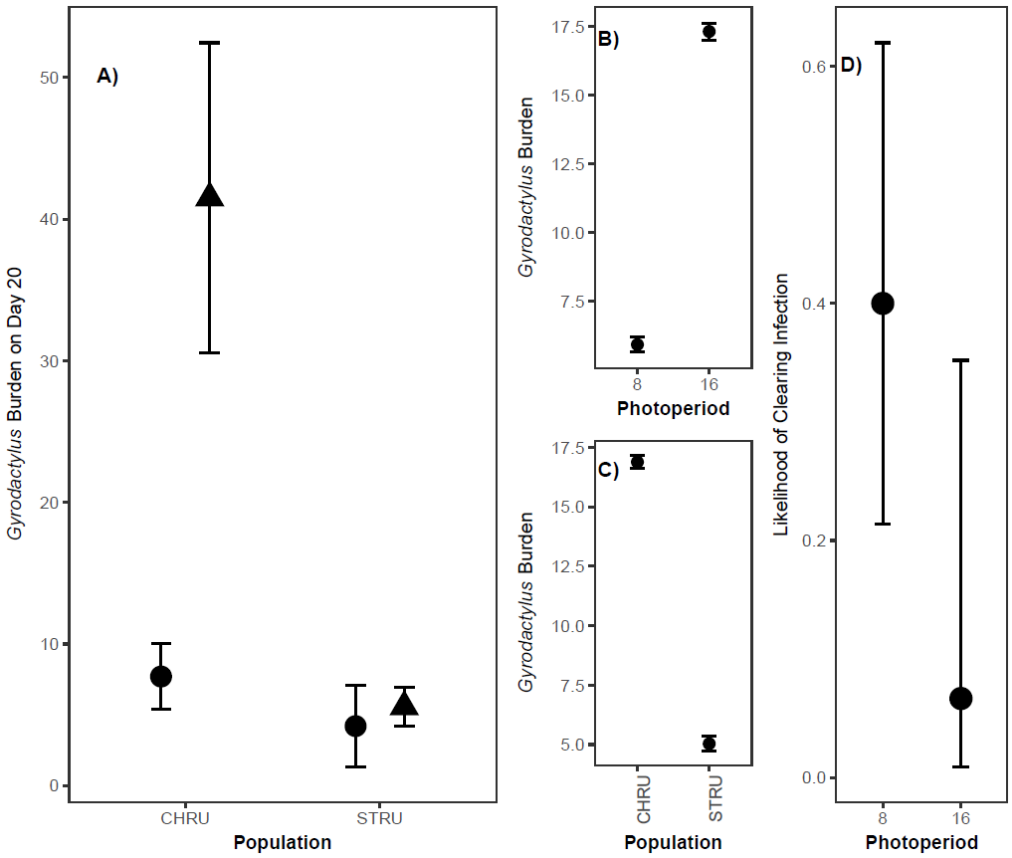


Figure 3.6: Experimental outcomes for measures of parasite susceptibility. **A)** demonstrates that Day 20 *Gyrodactylus* burdens were greater in 16L (▲) fish compared with 8L (●) fish, and were greatest in fish bred from the more susceptible CHRU population. Values plotted are group means with standard errors. These model effects are visualised for photoperiod treatment **(B)** and population **(C)**, whereby group means are plotted with standard error whilst averaging variation across the absent model factor. **D)** represents probability of clearing infection by day 20 with binomial error distributions for 8L and 16L fish. 8L fish were more likely to clear infection than 16L fish.

There were linear relationships between day 20 *Gyrodactylus* burdens and several immune variables (Figure 3.7). Fish with lower *muc2* expression in skin tissues suffered significantly greater infections (Wald's, $F_{1,31} = 4.603$, $p = 0.040$), in agreement with *muc2* expression being lower and infections being greater in 16L treated fish. There was also a weakly significant negative relationship between correlated expression of systemic immune genes in the

skin (*rorc* in particular) (operculum PC1) and *Gyrodactylus* burdens (Wald's, $F_{1,31} = 4.262$, $p = 0.047$). Attempting to model *Gyrodactylus* burdens by spleen inflammatory gene expression (spleen PC1) resulted in a failure of the model to converge, which was the result of an extreme outlier (*Gyrodactylus* burden = 106). Removal of this fish from the analysis permitted model fitting and revealed a highly significant relationship in which fish with reduced spleen inflammatory gene expression suffered increased infections (Wald's, $F_{1,30} = 8.327$, $p = 0.007$). The removed outlier in this instance displayed the highest splenic expression of inflammatory genes along with the highest parasite burden, contradicting the relationship observed in the remaining 30 fish. I cannot rule out the biological significance of this outlier, and thus the negative association may be weaker and less important than described above. If true however, this relationship may explain population-level differences in infection, given that resistant STRU fish displayed more inflammatory expression profiles. Relationships between *Gyrodactylus* burdens and other immune variables were nonsignificant (Wald's, $F_{1,31} \leq 3.198$, $p \geq 0.084$).

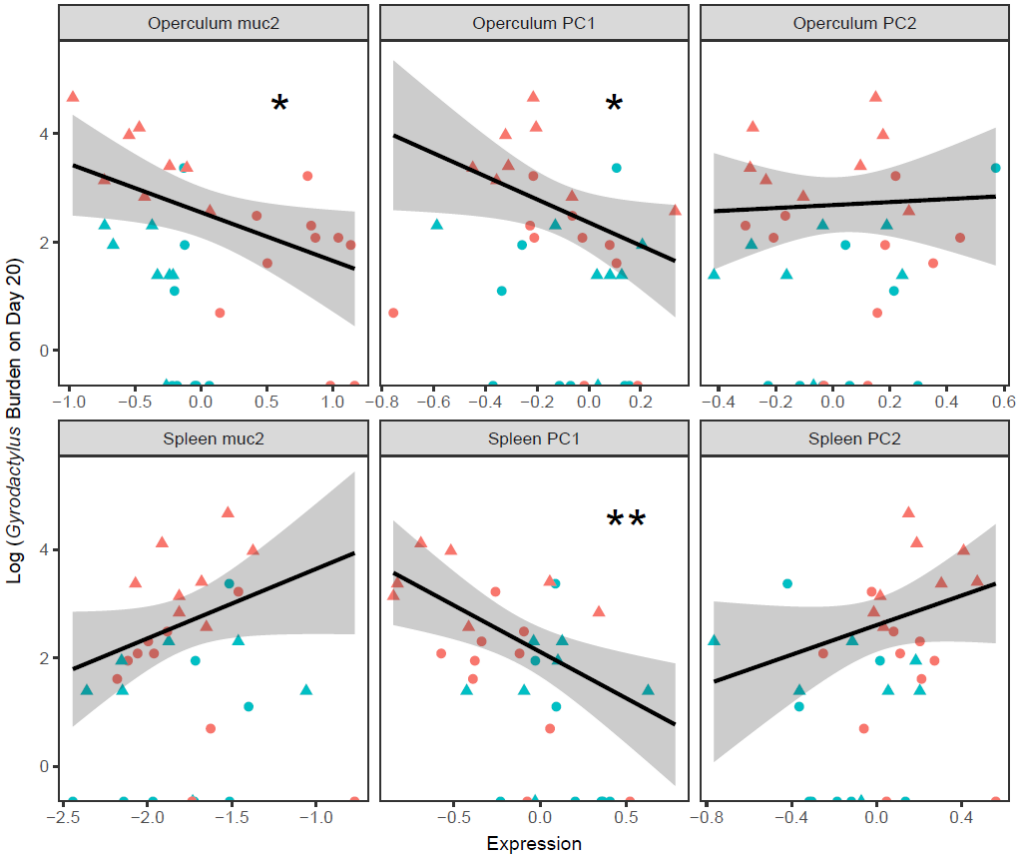


Figure 3.7: Logarithmic regressions between day 20 *Gyrodactylus* burdens and each of the six immune gene expression variables. Point shape denotes photoperiod treatment (16L = ▲ and 8L = ●) and point colour denotes source population (red = CHRU, blue = STRU). Fitted negative binomial models are represented by lines with shaded 95% confidence intervals. Asterisks denote significance level of relationship (* < 0.05; ** < 0.01). Absence of asterisks denotes non-significant relationships.

3.6 Discussion

This is, to my knowledge, the first-time photoperiod-modulation of the immune response has been investigated in conjunction with the infection dynamics of a naturally occurring parasite in a controlled setting. My results demonstrate that long-term exposure to alternative photoperiod regimes has

a dramatic effect on the ability of fish to cope with parasitic infection, and that this effect varies depending on differences in evolved resistance between populations. Measures of fish reproductive condition suggest that summer photoperiod (16L) treated fish were in a post-reproductive state, whilst 8L fish appeared to be in an early reproductive state. This makes sense given that sexual maturation in stickleback is known to be controlled by photoperiod (O'Brien et al., 2012). Summer photoperiod 16L fish had experienced 5 months of summer-like conditions, which would have induced maturation, while reproduction is likely to have been suppressed by short day length in 8L fish until the beginning of the infection stage of the experiment, when they were switched to 12L conditions. Fish housed long-term under a summer photoperiod regime experienced increased *Gyrodactylus* burdens and were less likely to clear their infections over the course of the experiment. This was true for fish from both the 'resistant' and 'susceptible' populations, however as expected, fish bred from a more susceptible population harboured greater parasite loads than fish bred from the more resistant population in both photoperiod treatments.

There are many examples of host-parasite systems experiencing seasonal patterns of increased infection. In humans, we observe seasonality in devastating diseases such as influenza, polio, cholera and measles (Altizer et al., 2006; Grassly & Fraser, 2006). At a macroparasitic level, schistosomiasis infections show annual temporal patterns (Samie et al., 2010) and wild ungulates (Cizauskas et al., 2015) and rabbits (González et al., 2016) display

seasonality of gastrointestinal parasites and ticks respectively. *G. aculeatus* are no different and experience annual seasonal infections by several macroparasites, in particular towards the end of their breeding season (Pennycuick, 1971). Likewise, *Gyrodactylus* sp. have been shown to display seasonality across several fish hosts (Koskivaara et al., 1991; Winger et al., 2008) including *G. aculeatus* around the breeding season (Özer et al., 2004). Proposed explanations for seasonality of parasite infections include behavioural and ecological causes as well as temporal variation in host immunity. For example, Reimchen and Nosil (2001) demonstrated that temporal changes in feeding habit dictate sex-biased parasitism in *G. aculeatus*, whilst suspension of territorial mating behaviours can facilitate infection in several taxa (Nelson, 2004).

However, my results demonstrate a strong link between experimentally modulated temporal immune responses and infection dynamics of *Gyrodactylus*. Expression of *muc2* in the skin and expression of inflammatory genes in the spleen were both important determinants of final parasite loads. Functionally this highlights the importance of both mucus as a delivery system and the role of the spleen in producing inflammatory agents. Of particular interest is the interaction between infection group and photoperiod treatment, demonstrating that 8L fish, which harboured lower parasite burdens, increased *muc2* expression in response to infection whilst 16L fish decreased skin *muc2* expression. Chronic exposure to longer photoperiods significantly decreased expression of *muc2* in the skin, and affected expression of inflammation genes

in the spleen in a sex-specific manner. Crucially therefore, differences between 16L and 8L fish appear driven by photoperiod-induced decreases in expression of *muc2* in the skin, which appeared significant in infected fish and led to increased *Gyrodactylus* burdens in 16L fish compared with 8L fish.

The *muc2* gene encodes the Mucin-2 protein, which is an important constituent molecule of mucosal secretions, and in mammals is tasked with separating pathogens and commensals from the epithelia (Gomez et al., 2013). Mucus secretions contain a cocktail of humoral and cellular innate components and humoral adaptive immunity components, and in fish, mucosal immunity of the skin is the first line of defence (Gomez et al., 2013). This is particularly important as an aquatic environment facilitates infection. Indeed, increased mucus secretion has been suggested to help stickleback 'shed' *Gyrodactylus* infections, through detaching the hooklets used by the parasite to ground itself to the host (Lester, 1972). Rubio-Godoy et al. (Rubio-Godoy et al., 2012) also demonstrated the importance of mucosal immunity for managing Gyrodactylid burdens, showing that *G. cichlidarum* aggregated away from fins with high mucus cell density on tilapia hosts. Likewise, experimental inhibition of mucus cell discharge in Atlantic salmon, *Salmo salar*, increases burdens of *G. derjavani* (Olafsdottir & Buchmann, 2004). Increased mucus secretion is not purely beneficial however, as some constituents of mucus have been suggested to attract Gyrodactylids in the initial stages of infection (Buchmann & Bresciani, 1998; Mahmud, 2016). The evidence in general indicates that increased mucus secretion may attract Gyrodactylids in early stages, but ultimately mucus and

its immune constituents are an integral response used by hosts to clear infection (Jones, 2001). My results here are consistent with a classic trade-off, whereby mucosal immunity to *Gyrodactylus* may be offset against reproduction in our 16L fish.

Temporal patterns in mucosal immunity have been recorded across taxa. For example, ground squirrels, *Spermophilus tridecemlineatus*, show increased mucosal levels of IFN-g, TNF-a, IL-10 and IL-4 during periods of hibernation (Kurtz & Carey, 2007). In fish, seasonal variation of mucosal immunity constituents has been observed in olive flounder, *Paralichthys olivaceus*, in which mucosal antibody activity were found to increase in warmer months, whilst protease and haemagglutinin decreased (Jung et al., 2012). Lysozyme activity of mucus also varies seasonally in farmed salmonids, showing marked declines in late summer and winter (Papezikova et al., 2016), in keeping with our observations here for post-reproductive 16L fish. These studies are both observations taken from farmed fish, and although photoperiod is mentioned as a seasonal cue, it receives little attention. Here, I demonstrate experimentally that mucins, an important constituent of host mucosal immunity (Linden et al., 2008) may be under direct control of photoperiod.

Correlated expression of the seven systemic immune genes, and in particular *tnfα*, *rorc*, and *tbet*, in the spleen was likewise a good predictor of parasite burdens, with parasite burdens declining with increased expression. The significance of these three genes specifically suggests this variable to be an inflammatory axis; *tnfα* encodes the pro-inflammatory cytokine tumor necrosis

factor alpha (TNFα); *rorc* encodes RAR-related orphan receptor gamma (RORγ), which promotes the differentiation of pro-inflammatory Th17 cells (Ivanov et al., 2006; Yang et al., 2008); and *tbet* encodes T-BET, an important signalling molecule for co-ordinating pro-inflammatory Th1 cells (Lazarevic et al., 2013). The correlation between *rorc* and *tbet* is surprising, given the active role of T-BET in suppressing *rorc* expression in human Th-precursor cells (Lazarevic et al., 2013).

Evidence indicates inflammatory responses are important for reducing Gyrodactylid infections in fish. Extensive research into the *G. derjavini* and rainbow trout, *Oncorhynchus mykiss*, system has shown an important role for the local expression of inflammatory genes such as *tnfα* and *il-β* during primary Gyrodactylid infection (Lindenstrøm et al., 2003, 2004). Likewise, recent analyses of wild populations of stickleback from North Uist have shown the same genes to increase their expression with *G. arcuatus* burden in some populations, whilst other immune responses were found to show little interaction (Robertson et al., 2016a). However, evidence from this system has also shown that in laboratory infections, *tnfα* is downregulated in response to Gyrodactylid infection (Robertson et al., 2017b). In the same study, *tbet* expression was shown to vary significantly across the infection period, and was particularly high until around 30 days pi. In sampling wild fish on North Uist, I have further found that eastern populations, which display increased correlated expression of similar immune genes, harbour lower *G. arcuatus* burdens and prevalences (chapter 2). This suggests that in the wild expression

of these genes may be under selection to control parasites. These results indicate that inflammatory responses, mediated through a variety of immune arms are important host responses to Gyrodactylids, particularly in the early stages of infection. Seasonality of inflammation has been detected genome-wide in humans (Dopico et al., 2015) and in stickleback (Brown et al. 2016), however I failed to detect a consistent effect of photoperiod on inflammatory gene expression for all fish, instead finding immunomodulation to be sex-linked.

The mechanisms underpinning how these immune responses were affected by photoperiod are unclear, and beyond the scope of this study, however I can speculate. There are two scenarios regarding immunomodulation in response to photoperiod. The first scenario is that modulation is deliberate and direct; with responses being boosted to match predictable parasite rhythms or reduced during resource expensive periods, such as breeding. In support of the latter, Li et al (2014) starved blue catfish, *Ictalurus furcatus*, for seven days and noted significant modulation of several immune processes, as well as significant downregulation of the mucin genes '*Mucin-2-like*' and '*Mucin-5AC-like*' in the skin. Downregulation of these genes in starved fish is in keeping with our observation that *muc2* was expressed less in 16L fish, which were in significantly poorer condition and had likely already been through reproductive investment. Whilst it is difficult to compare a short-term starvation to a long-term photoperiod treatment, both studies suggest that mucosal immunity may be modulated through nutritional stress.

Alternatively, immunomodulation may be consequential and indirect, with the immune system reacting in concert with changes elsewhere in the host, such as sex-specific hormonal shifts. Inflammatory expression in the spleen was affected by our photoperiod treatment, however the direction of the effect was dependent on sex; females downregulated when housed in 16L conditions, whilst males upregulated. This may reflect differential photoperiod-induced modulation of sex-linked neuroendocrine pathways important for timing breeding and reproductive investment. The link between photoperiod changes and the timing of reproduction is a common phenomenon across many taxa including mammals (Goldman, 2001; Lincoln et al., 2003), birds (Yoshimura et al., 2003) and fish (Baggerman, 1985). Accompanying temporal dynamics of important reproductive hormones can also secondarily modulate immune responses (Foo et al., 2016), as the two processes are often co-expressed (Milla et al., 2011). These neuroendocrine-immune associations appear highly conserved in vertebrates, including humans where male and female reproductive hormones have been shown to produce gender-specific immune responses (Bouman et al., 2005) and directly impair adaptive immune responses when received as part of contraceptives (Hall et al., 2017). Reproductive hormones are such important modulators of the immune response that some helminths have evolved the ability to produce or alter host hormone concentrations (Romano et al., 2015).

In stickleback, 11-ketotestosterone is the dominant circulating androgen; increasing in blood plasma levels during reproduction and

decreasing during the parental phase (Mayer et al., 2004). It also acts as an immunosuppressive (Kurtz et al., 2007), which may explain the patterns observed for 8L and 16L males given their altered reproductive timings. The decline in expression observed for 16L females may be resource-associated, as female gonadal investment is extreme in the breeding season. 8L females had significantly increased GSI compared with 16L fish, which had most probably fully undergone sexual maturation upon instigation of increased photoperiod treatment, and were now in a post-reproductive state. The fact that temporal variation in inflammatory expression did not lead to sex-specific incidents of increased parasitism may reflect its status as a secondary, indirect effect associated with reproductive condition rather than photoperiod specifically. When comparing parasitism between female black howler monkeys, *Alouatta pigra*, in varying stages of reproduction, parasitism was observed to be seasonal but not specifically linked to reproductive state (Martínez-Mota et al., 2017), suggesting that reproductive condition alone is a poor predictor of parasitism when decoupled from seasonality.

The reason 8L fish were shown to be in greater reproductive condition may stem from the changes in photoperiod between days, rather than chronic exposure. My infection experiment was run under 12L, therefore 8L fish experienced a 4-hour increase in photoperiod during infection treatment relative to photoperiod treatment, whilst 16L fish had their photoperiod reduced. In Japanese quails, *Coturnix japonica*, and Siberian hamsters, a single long day is enough to increase secretion of luteinizing hormone and promote

sexual maturation (Finley et al., 1995; Nakao et al., 2008). In sticklebacks, secretion of LHβ rises above baseline after 5-10 days (O'Brien et al., 2012), which is well within the scope of my 20-day infection period.

The intraspecific variation in susceptibility of populations is an additional area of interest rarely included in studies of photoperiod and the immune system. Its inclusion here permits me to posit on several outstanding questions regarding host-parasite interactions (Martinez-Bakker & Helm, 2015). If host temporal immunomodulation is an adaptive response to temporal parasite pressure, then we might expect populations with variable parasite pressures to exhibit differences in their degree of temporal immunomodulation. However, I observed no significant interactions between population and photoperiod for any of the immune variables, despite fish being bred from populations with contrasting parasite communities and diverged resistance to Gyrodactylids. Whilst this evidence is tenuous, it agrees with previous discussion points regarding immunomodulation as a result of resource constraints or interaction with the neuroendocrine system. Additionally, *muc2* expression in the skin was the only immune variable that displayed a consistent directional photoperiod effect across all fish. This effect was irrespective of population, despite my susceptible population being uninfected by Gyrodactylids in the wild. The persistence of temporal host immunity even in the absence of the parasite in natural conditions suggests that temporal host immunity is not a response to temporal *Gyrodactylus* dynamics.

I cannot however rule out that temporal observations here are adaptive in response to another unknown, commonly occurring parasite or pathogen that displays seasonality on North Uist. In such an instance, Gyrodactylids may benefit from temporal host immunity adapted to cope with temporal dynamics of a parasite or pathogen with greater fitness implications. Parasite communities on North Uist are complex (De Roij & MacColl, 2012; Rahn et al., 2016a; Young & MacColl, 2017). Such trade-offs do exist in complex natural systems, for example Loiseau et al (2008) found that the *Plasmodium*-susceptible MHC class I allele *pado123* gene is maintained in populations of the house sparrow, *Passer domesticus*, due to a pleiotropic trade off with a 6.5-fold reduction in risk of harbouring another protozoan, *Haemoproteus*.

Even if host temporal variation is not parasite-driven, its existence generates selection pressure for parasites to capitalise (Martinez-Bakker & Helm, 2015), through timing their own virulence and reproduction strategies. Host temporal variation thus may contribute towards temporal local adaptation between *Gyrodactylus* populations. Local adaptation has been demonstrated for *G. gasterostei* on stickleback hosts in Belgium (Konijnendijk et al., 2013) and on North Uist, local adaptation of virulence of *G. arcuatus* for stickleback populations has been described (Mahmud et al., 2017), although evidence for local adaptation of host responses is less forthcoming (Robertson et al., 2017b). This is true except for environments where two morphs, resident and anadromous, exist. Diverged temporal host immunity between morphs may contribute towards a weaker selection pressure for temporal parasitism,

and thus weaken selection for local adaptation. Kitano et al. (2010) demonstrated that marine and freshwater stickleback have diverged thyroid hormone signalling pathways because of divergence around the thyroid-stimulating hormone-β2 (*tshβ2*) locus. This pathway is essential for reproductive timing (O'Brien et al., 2012) and diverged morphs thus present parasites with a complex, multi-peaked, temporal selection environment, which may hinder local adaptation for infection dynamics. Furthermore, if temporal immunity is linked with energy expenditure, migratory anadromous morphs may display more extreme temporal immune variation. This has been demonstrated to be an intrinsic trait of migratory stonechats, *Saxicola torquata* (Versteegh et al., 2014). Investigating inter-population and inter-morph variation in temporal immunity, and its subsequent effects on parasite timing and fitness will aid in the understanding of immune seasonality and host-parasite interactions. Promising research may also focus on how host temporal immune responses vary across latitudinal clines, and whether temporal host-parasite interactions are affected by the strength of seasonal environmental variation. It has already been shown that latitude affects seasonal incidences of conditions such as Type 1 diabetes in children (Moltchanova et al., 2009), but whether such patterns exist for pathogen-induced conditions remains to be seen.

In conclusion, I have demonstrated experimentally that photoperiod represents a significant environmental cue for mediating host seasonal immune responses. My results confirm previous observations that different

immune responses respond to seasonal cues in different ways. In addition to this, by experimentally infecting photoperiod-treated fish, I have demonstrated how photoperiod cues affect immune responses that are directly involved in managing parasitic infection in the wild. In this respect, I find non-specific mucosal immunity to be affected across all fish, and demonstrate that diverged immune responses associated with more resistant phenotypes are maintained at the population level despite seasonal cues. Seasonal host-parasite interactions are poorly understood (Martinez-Bakker & Helm, 2015), however I have provided some evidence that host seasonality can occur irrespective of parasite seasonality. I have provided examples demonstrating that neuroendocrine processes regarding how photoperiod modulates host responses are well conserved across vertebrates. My findings therefore have implications for understanding seasonal parasite infections across taxa, including humans. The evidence provided here suggests that infection risk due to modulation of immune responses by seasonal cues is an important consideration for understanding disease epidemiology. More directly, this research will be of interest to the aquaculture industry, in which photoperiod is a commonly used method for modifying growth rates. I have demonstrated how such strategies may have knock-on effects for economically important parasites, such as *Gyrodactylids*, but also other ectoparasites, for which similar immune responses are mounted.

CHAPTER 4: THE POTENTIAL OF IMMUNE GENE DIVERGENCE FOR POSTZYGOTIC ISOLATION BETWEEN SPECIATING ECOTYPES

4.1 Abstract

The prevalence of ecological speciation in nature is more substantial than first thought. Bateson-Dobzhansky-Muller genetic incompatibilities (DMIs) are an important component of postzygotic isolating mechanisms during speciation, but are thought to be difficult to develop during speciation with gene flow. The rapid divergence in response to environment of immune genes, coupled with the negative fitness consequences that come from dysregulation-associated autoimmunity may make them an important source of DMIs in the incipient stages of ecological speciation. This has been demonstrated in wild plant hybrids that suffer autoimmune hybrid necrosis, but has yet to be investigated in wild animal hybrids. Quantifying such effects in the wild is an important step towards understanding the relevance and prevalence of laboratory results. I used morphometrics and armour variation to quantify hybridisation in a stickleback hybrid zone between three diverged ecotypes. I examined through qPCR the expression of 8 immunologically important genes and sought to test the hypothesis that hybrids suffering from DMIs should display transgressive patterns of expression. I did find substantial gene expression variation between ecotypes, but contrary to my hypothesis, gene expression patterns were in

keeping with introgression and additive effects. In addition, I found no evidence that hybridisation reduced fish condition or increased parasitism. These results suggest the vertebrate immune system may be resistant to DMIs during ecological speciation, however I discuss the pitfalls of quantifying such effects in the wild and compare my results to those acquired in the laboratory.

4.2 Introduction

Selection against hybrids is an evolutionary process capable of maintaining incipient species during speciation, by restricting gene flow between diverging populations or ecotypes that come into secondary contact (Burke & Arnold, 2001; Naisbit et al., 2001; Schluter & Rambaut, 1996). One such mechanism of selection against hybrids involves the coming together of incompatible alleles from diverging parentals. Within populations, balancing selection maintains molecular function as genes diverge, either through selection or drift, however hybridisation brings together novel genotypic combinations that have not been tested by selection (Dobzhansky, 1936; Muller, 1942). This is a powerful and often permanent form of postzygotic isolation which can maintain incipient species in the face of gene flow and environmental stochasticity (Seehausen et al., 2014). However, Bateson-Dobzhansky-Muller genetic incompatibilities (DMIs), can take time to accumulate sufficiently to hamper hybrid fitness, particularly in drifting loci (Seehausen et al., 2014), and therefore are generally assumed to be most prevalent in allopatric models of speciation. This means

that for many genes, DMIs are unlikely to contribute towards the initial stages of speciation, although see (Sá-Pinto et al., 2013).

Immune genes represent a potential pool of genes whereby DMI may play a role in the initial stages of speciation. The reasoning behind this assertion is two-fold. Firstly, the ubiquity of parasitism in nature (Hedrick, 2004), along with other sources of selection such as resource availability (Schmid-Hempel, 2011), and evolutionary constraints imposed upon immune responses (Loiseau et al., 2008), results in the rapid evolution (Eizaguirre et al., 2012) of variation in immune responses between populations and even ecotypes (Lenz et al., 2013; Scharsack et al., 2007a; Sparkman & Palacios, 2009). Secondly, immune responses are complex networks that evolve optimal responses to current environments. Sub-optimal immune responses because of improper regulation result in immediate fitness costs for hosts, due to increased parasitism or increased autoimmunity. The latter of these scenarios is a well-documented phenomenon in inter and intra-specific plant hybridisation (Bomblies et al., 2007; Chae et al., 2014), whereby rampant autoimmunity leads to hybrid necrosis (Bomblies, 2010; Bomblies & Weigel, 2007; Rieseberg & Blackman, 2010; Świadek et al., 2017).

However, these principles have yet to be investigated in wild, naturally occurring animal systems. The vertebrate immune system requires inflammatory, innate and acquired immune responses to operate within a carefully regulated equilibrium (Littman & Rudensky, 2010). As such, autoimmune conditions resulting from regulation deficiencies in vertebrates

can also incur high fitness costs for hosts (Graham et al., 2005). For example in the laboratory, mice deficient in the anti-inflammatory cytokine interleukin-10 (IL-10) suffer toxic shock and increased mortality following *Trypanosoma cruzi* infection due to the unregulated production of pro-inflammatory Tumor Necrosis Factor Alpha (TNF-α) (Hölscher et al., 2000). Previous studies have shown that immune genes do suffer from dysregulation when animal inter and intraspecific hybrids are formed in the laboratory (Hill-Burns & Clark, 2010; Mavarez et al., 2009), but whether these are detectable in naturally occurring hybrids remains an open question. Observing fitness costs associated with immune DMIs in wild hybrids would reinforce the notion that these genes are important for maintaining incipient species. Alternatively, if fitness costs are limited to a subset of laboratory-reared or very young hybrids, then those which survive to adulthood can facilitate gene flow. This distinction is therefore important, as increasing levels of gene flow limit genome divergence to regions of low recombination or those under strong selection (Feder et al., 2012; Pedersen et al., 2017), limiting speciation as a process.

I investigated these ideas in a naturally-occurring hybrid zone of the three-spined stickleback, *Gasterosteus aculeatus*, in the outer Hebrides, Scotland. Stickleback are a model system for evolutionary questions, including those associated with speciation. This is due to the parallel evolution of freshwater adaptive radiations from bony-armoured marine ancestors at the end of the last glaciation (Colosimo, 2005; Jones et al., 2012b). Stickleback hybrid zones between ecotypes are well-documented around the world, such

as lake-stream hybrids (Berner et al., 2009; Hanson et al., 2016; Hendry et al., 2002), benthic-limnetic hybrids (Behm et al., 2009; Ólafsdóttir et al., 2007; Taylor et al., 2012), and anadromous-freshwater hybrids (Hay & McPhail, 1999; Jones et al., 2006; Pedersen et al., 2017), as are diverged immune responses between ecotypes (Huang et al., 2016; Robertson et al., 2016a).

In my study, I examined hybrid immune responses across a three-ecotype hybrid zone, consisting of anadromous marine fish, brackish/freshwater residents, and freshwater 'anomalous' fish, which are characterized as being fully armour plated, partially plated, and lacking in any armour respectively. Differences in resistance between these ecotypes to parasitic infection have already been documented (El Nagar & MacColl, 2016), and these appear to be largely under additive genetic control. I sought to test the hypothesis that hybrids suffer an intrinsic cost of hybridization associated with autoimmunity and improper regulation of the immune system. To achieve this, I measured immune responses through qPCR assays, condition of individuals and macroparasite loads. I used morphometrics associated with body shape and armour variation to categorise fish into ecotypes and to determine hybrids. Morphometrics are a powerful tool to determine stickleback ecotypes (Hanson et al., 2016; Jones et al., 2006; Ólafsdóttir et al., 2007; Pedersen et al., 2017) and have been used previously to determine hybridization in stickleback and other systems (Nolte & Sheets, 2005).

4.3 Materials and methods

4.3.1 Study sites and sample collection

Fish were collected during April 2016, using minnow traps, from a hybrid zone in loch Ciste (CIST) (Figure 4.1) (Table 4.1) on the outer Hebridean island North Uist, Scotland. CIST is the last loch before the sea in the largest freshwater catchment on North Uist (Figure 4.1). Just below the level of mean highwater spring tides, it is fresh, or low salinity brackish for much of the time, but is flooded by the sea at spring tides. The loch is populated at all times by resident ecotype fish, but anadromous fish also return annually to the loch to reproduce in spring. Such sharing of saltwater lochs by resident and anadromous ecotypes is not uncommon on North Uist, however typically hybridisation between these ecotypes is rare on the island. Upstream and inland, CIST is connected to a second, freshwater loch, loch Sgealtair (SGEA). SGEA is populated by freshwater 'Anomalous' fish that lack all bony armour and pelvic structures, representing the typical freshwater ecotype for populations found in the island's eastern acidic lochs (Magalhaes et al., 2016). The presence of anomalous fish in loch CIST makes it unique on the island (and probably in Scotland) as the only known location where three ecotypes coexist (Campbell, 1979). Furthermore, the addition of anomalous fish appears to facilitate hybridization between all 3 ecotypes, either directly or through hybrid back crossing (L.L. Dean & A.D.C. MacColl, unpublished data).

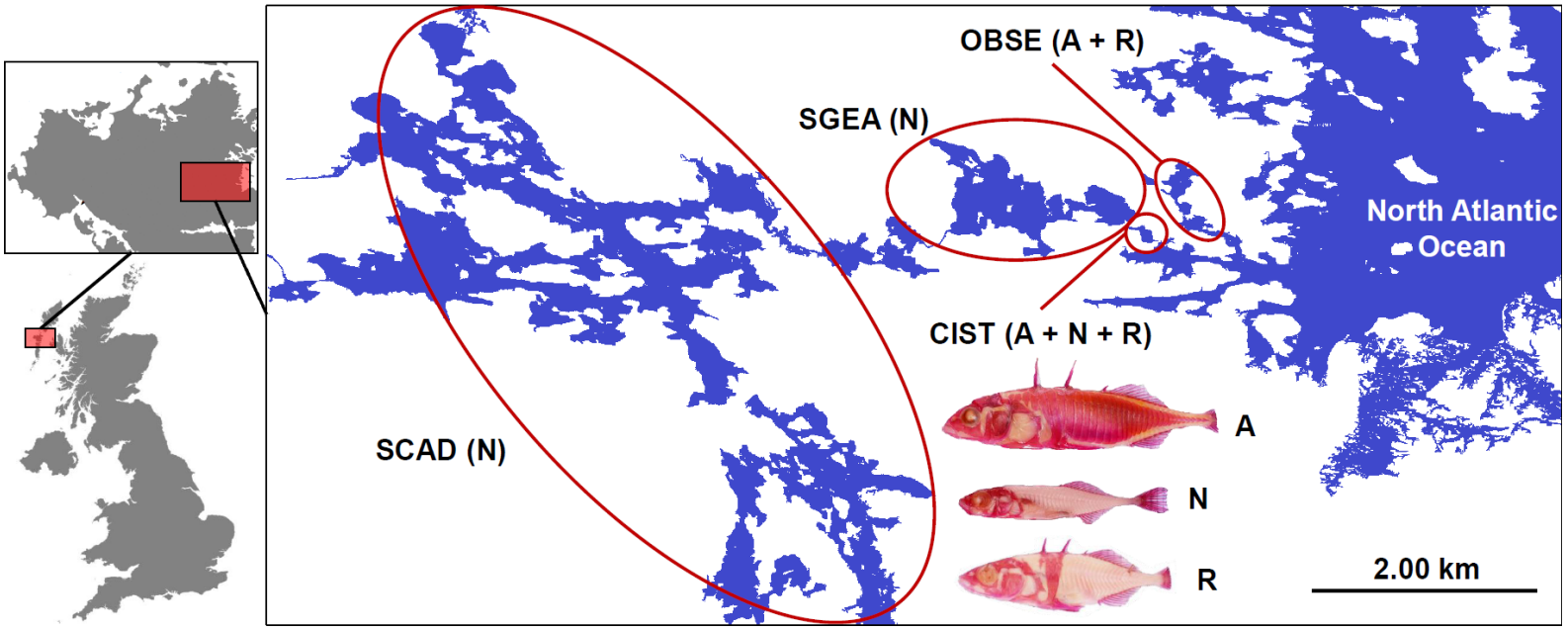


Figure 4.1: Map of lochs sampled on the Eastern side of North Uist and the ecotypes present in each. Loch OBSE is saltwater due to its connection to the North Atlantic and contains partially-plated resident (R) ecotypes and fully-plated anadromous (A) breeders. CIST is connected to the North Atlantic during Spring high tides and contains residents, anadromous breeders and non-plated anomalous (N) fish, which enter from the freshwater loch SGEA. Freshwater loch SCAD is further inland and contains only anomalous fish.

Table 4.1: Sampling locations for all lochs

Loch	Short Name	Ecotypes[1]	Latitude	Longitude
Ciste	CIST	A + N + R	57.598541	-7.177400
Sgealtair	SGEA	N	57.600468	-7.182283
Ob nan Stearnain	OBSE	A + R	57.601568	-7.172755
Sgadabhagh	SCAD	N	57.585060	-7.235588

[1] A = Anadromous, N = Anomalous, R = Resident

Sampling was conducted across all areas of the loch ranging from the point closest to the sea to furthest inland. Northing and Easting GPS co-ordinates were recorded at the closest land point to where traps were set. A total of 116 fish were collected and had their ecotype identified in the field according to whether they were fully plated ('Anadromous' N =28), low plated ('Resident' N = 32), non-plated and lacking a pelvis ('Anomalous' N = 37) or otherwise ('Hybrid' N = 19). It is likely that reliance on these simple morphological differences underestimates the true number of hybrid individuals. A further 16 Anomalous fish were sampled from around the area of SGEA that feeds into CIST. These fish were added to those sampled from CIST, yielding a total sample of 132 fish.

Fish were euthanized by MS222 overdose followed by destruction of the brain in accordance with UK Home Office regulations. Each had their weight and standard length recorded before having their spleen and one operculum

removed from their left side and placed into RNAlater (Life Technologies). Spleen and skin sample, which represent important immune organs for fish (Foey & Picchietti, 2014; Zapata et al., 2006), were kept at 4 °C for 24 h and then kept at -20 °C with RNA being extracted within 3 months. Fish were photographed on their right side for morphometric analysis. Fish were recorded as being in either full reproductive condition (males – enlarged, melanised testes and enlarged kidneys; females – fully gravid) or otherwise before having gonads, liver and adipose tissue removed and weighed. Somatic weight was calculated by removing these weights and the weights of any *Schistocephalus solidus* parasites from overall weights, and was used to calculate the following condition indices. Adiposomatic Index (ASI) was calculated as Total Adipose Weight/Somatic Weight x 100. Hepatosomatic Index (HSI) was calculated similarly using liver weight. Finally, fish were placed in 70% ethanol for preservation until staining (see below).

During processing, fish were comprehensively searched for macroparasites, and had their infection status and number of each parasite recorded.

Further groups of fish were collected for morphometric training sets during April 2015 from lochs Ob nan Stearnain (OBSE) for anadromous (N = 29) and resident (N = 30) ecotypes and from loch Sgadabhagh (SCAD) (N = 30) for anomalous fish (Figure 4.1). These lochs were selected due to their geographical proximity to loch CIST and because there is little (OBSE) or no

hybridisation (SCAD) between the ecotypes that are present (only anomalous fish are found in SCAD).

4.3.2 Morphometrics and hybrid classification

A total of 22 landmarks were used to quantify variation in fish shape using morphoJ (Klingenberg, 2011) for all 132 CIST fish and 89 training set fish from lochs OBSE and SCAD. Residual deviance in the x and y plane was calculated and output for the 22 landmarks within morphoJ along with centroid size yielding 45 shape variables.

Skeletal structures were stained according to the following. Whole fish were fixed in 10% buffered formalin for 2 weeks, rinsed and soaked in dH_2O for 1 hour, transferred to KOH for 2 hours, bleached by 1% H_2O_2, stained with 0.02% alizarin red in 1% KOH, rinsed with dH_2O and placed in 40% isopropanol for final storage. Fish were photographed using a macro lens on their left side. The following measurements were obtained: first spine length, second spine length, longest plate length, pelvis height, pelvis width and pelvic spine length. Allometric relationships were calculated and residual deviation for each variable from its standard allometry was recorded for each individual to account for variation in fish size.

I also genotyped all fish for the *eda* locus, which tends to be fixed for alternate alleles in anadromous and freshwater ecotypes, using light PCR and

Stn382 primers, which border an indel polymorphism in intron 1 of *eda* (Colosimo, 2005).

4.3.3 RNA extraction and qPCR

qPCR work was completed in accordance with the MIQE guidelines (Bustin et al., 2009). Of the 132 fish sampled, 92 were selected due to 96-well plate design and to achieve approximately equal representation across the three ecotypes and hybrids as identified in the field (Anadromous N = 25, Anomalous N = 24, Resident N = 25, Hybrid N = 19). These fish were selected at random within each ecotype subset. Sampling order was randomized and RNA was extracted separately from stored whole spleen and operculum cuttings using the Genejet RNA purification kit (Thermo Scientific) according to the manufacturer's protocol. RNA was eluted into 100 µl of nuclease-free water and had its purity assessed using 1 µl on a NanoDrop1000 spectrophotometer (Thermo Scientific). RNA was then DNase treated using Precision DNase (Primer Design) following the manufacturer's protocol. RNA integrity was assessed by visualisation of 4 µl of sample on a 2% agarose gel stained with ethidium bromide. Approximately 1.5 µg of template was reverse transcribed using nanoscript2 RT kit (Primerdesign) according to the manufacturer's protocol, which uses a combination of random nonamer and oligo-dT priming. Contamination by gDNA was ruled out through light PCR using intron-spanning primers. cDNA samples were diluted 1:10 with nuclease free water and aliquoted prior to use/storage at -20 °C.

To 2 µl of diluted template, 5 µl of PrecisionFAST low ROX mastermix with SYBR green (Primer Design), 2.5 µl of nuclease-free water, and 0.25 µl of each primer at working concentration was added to make up a total reaction volume of 10 µl. Reactions were performed in duplicate in 96-well optical PCR plates with optical seals (StarLab). Plate design consisted of 46 samples, a negative, and a pooled reference sample, which was consistent across the 4 plates (92 individuals duplicated across 2 tissues). Samples were incubated at 95 °C for 20 s, followed by 45 cycles of 95 °C for 3 s and 60 °C for 30 s. A melt-curve analysis was also included to confirm product. In total, 8 genes were amplified representing different immune responses: inflammatory (*tnfα*), Th1 (*stat4*, *tbet*), Th2 (*stat6*, *cmip*), Th17 (*rorc*), Treg (*foxp3a*) and mucus (*muc2*) (Table 4.2).

Table 4.2: Primer details for qPCR genes

Gene	ENSEMBL ID	Immune Role	Primer Sequence (5'-3')	Amplicon length
tnfα	ENSGACG00000013372	Pro-Inflammatory cytokine	Fwd-GCTTGGTTCTGGCCAGGTTT Rev-GCTGCTGATTTGCCTCAACG	125
stat4	ENSGACG00000002684	Transcription factor for differentiation of Th1 cells	Fwd-CTCTCAGTTTCGAGGCTTGCTT Rev-GGCAGTTGGCTCACATTGG	100
tbet	ENSGACG00000003829	Transcription factor modulates expression of Th1-cell cytokines eg. IFNγ	Fwd-CACATCGTGGAGGTGAAGGA Rev-CGGTGACGGCGATGAACT	99
cmip	ENSGACG00000002527	Signalling protein in Th2 pathway	Fwd-GGCATGGAGGTCGTCAAGAA Rev-TAGCAGGAGTAAATGGCGGC	119
stat6	ENSGACG00000008477	Involved in mediating Th2 cytokines IL-4 and IL-3 signalling	Fwd-CTCAGCCACAGTTCCAACCGTTC Rev-GTCGGATGTTCTGGACCTCGAGT	104
foxp3a	ENSGACG00000012777	Promotes development and function of Treg cells	Fwd-GTTGACCCATGCAATTCCGA Rev-CTGCTGTAGTTGTGGTCCTG	94
rorc	ENSGACG00000012239	Promotes differentiation of pro-inflammatory Th17 cells	Fwd-TTGACTTTGCCCACGGTATG Rev-TGCTGAACTCTGCCTCTGT	121
muc2	ENSGACG00000014109	Principal organic constituent of mucus	Fwd-AGAATGGCGAGTCCTGGAA Rev-AGATGGGTTGTTGTGGTGTG	105

4.3.4 Relative gene expression quantification

Genes for which assays had been previously developed (Robertson et al., 2016a) were selected to characterise different arms of the immune response and were identified based on previous studies in other fish and known roles of orthologous genes. The pro-inflammatory gene *tnf*α is a key component of

innate immunity in teleosts and other vertebrates (Secombes & Wang, 2012; Uribe et al., 2011), activating macrophages, eliciting inflammation and increased respiratory burst activity.

The Th1 transcription factor STAT4 (*stat4*) promotes the differentiation of Th1 cells and has been identified in teleost genomes. STAT4 activates the expression of *tbet*, which in mammals represents the master transcription factor for Th1 cell differentiation, activating other genes required to promote differentiation and suppressing the development of other T-cell lineages such as Th2 and Th17 (Wang & Secombes, 2013). Fish *tbet* has been cloned in a number of teleosts and its existence and function are supported by conserved synteny across teleosts and humans and expression studies demonstrating its importance in fish T cell-mediated immunity (Wang & Secombes, 2013). Expression of Th1-associated genes in infection studies suggest that fish may possess a full and conserved Th1 pathway (Secombes & Wang, 2012). Th1 adaptive immunity is associated with intracellular parasites, whilst Th2 adaptive immunity is associated with extracellular infection. Th2 cell differentiation is associated with up-regulation of *cmip* and *stat6* in mammals, which is also upregulated alongside other markers of Th2 responses in zebrafish head kidney and spleen cells in response to immunostimulation (Mitra et al., 2010).

Th17 cells, a subset of pro-inflammatory T cells, are regulated by the transcription factor ROR-γt (*rorc*) in mammals, which increases in mRNA levels during the differentiation of naïve CD4+ lymphocytes into Th17 cells (Yang et

al., 2008). *rorc* is present in fish genomes, albeit in several isoforms (Wang & Secombes, 2013) along with the other major components of Th17 cell development, however expression studies in trout both support and contradict the notion that *rorc* in fish behaves like its mammalian counterpart (Monte et al., 2012)

Mucins represent constituent molecules of mucosal immunity and are secreted within mucus, forming a protective gel-like layer to protect epithelial surfaces (Linden et al., 2008). The mucin gene *muc2* is typically expressed at intestinal and respiratory surfaces, and has been characterised in carp (van der Marel et al., 2012), showing high similarity to its mammalian and avian equivalent. Of specific interest here, mucin genes exhibit strong divergence between marine and freshwater stickleback, suggesting significant ecological associations with adaptation (Jones et al., 2012a).

Treg activity, which regulates the immune response in general, is characterised by *foxp3a* expression. This gene has been identified in many teleost genomes and has demonstrable roles in immune regulation in fish and mammals (Secombes & Wang, 2012). The genes included in this study therefore serve to capture variation across a range of immune responses; specifically, in the expression of an innate inflammatory cytokine (*tnf*α); markers for T-cell differentiation for Th1 adaptive (*stat4, tbet*), Th2 adaptive (*cmip, stat6*), Th17 adaptive (*rorc*) responses; mucin production as a measure of mucosal immunity (*muc2*); and Treg cytokine activity (*foxp3a*).

Custom stickleback geNorm (Primerdesign) analyses with SYBR were conducted separately for each tissue type across 12 randomly selected individuals assuring equal representation across the 3 ecotypes. Analyses were conducted according to the manufacturer's protocol to ascertain the most stably expressed pair of housekeeping genes for each tissue type from 6 candidate genes: *b2m*, *gapdh*, *rpl13a*, *hprt1*, *tbp* and *top1*. For spleens, *b2m* and *rpl13a* were selected. For opercula, *b2m* and *tbp* were selected.

Relative expression values were calculated according to the ΔΔCq method (Pfaffl, 2001) and respective of the amplification efficiencies of each primer pair as described in (Robertson et al., 2016a). Ratios for *rorc* and *muc2* were calculated assuming 100% amplification efficiency. Expression values were standardized against the geometric mean Cq of the two most stably expressed housekeeping genes for the relevant tissue type.

4.4 Data analysis

All data were analysed in R (version 3.3.2)(R Core Team, 2016).

4.4.1 Morphometrics and hybrid classification

Shape and armour variables were combined in a discriminant function analysis (DFA) to explain morphometric differences between the three ecotypes. The DFA model was trained using the 89 training fish from lochs OBSE and SCAD for

which ecotype was known, to maximise variation between the defined groups. The trained model was then used to predict x and y co-ordinates and ecotypes of each CIST fish based on morphometrics, armour variation and size. This information was used to calculate a Hybrid Index by finding the distance to the closest training set ecotype mean according to a Pythagorean root of the sum of the squared x and y deviations. This was done for all fish including the training set, and a 95% confidence window was ascertained for training set fish of known ecotype according to a one-tailed hypothesis. CIST fish which exceeded this confidence in terms of their Hybrid Index were scored as a hybrid or otherwise in a binary classifier. Predicted ecotype of CIST fish was also ascertained from the DFA model. CIST fish had their Hybrid Index modelled and were further classified primarily by ecotype, and secondarily by whether they were 'pure' or 'hybrid'.

4.4.2 Generalized Linear Models

Relative expression ratios of all genes were $\log_{10}$-transformed prior to analysis. Immune genes were separated into mucosal (*muc2*) and systemic (other genes) categories to simplify interpretation (inclusion of all genes did not change analysis as *muc2* expression dominated PC1). Relative expression ratios of systemic immune genes were combined in covariance matrix-based principal component analyses (PCA) for each tissue type with the top 4 PC scores (accounting for >90% of total variation) for each fish retained. I therefore

modelled 10 immune variables in total: *muc2* expression and PCs 1-4 for systemic immune genes across spleen and opercula tissues.

Generalized linear models (GLMs) were used to explain each immune variable and were fitted using the independent variables: *Thersitina* load (the most common parasite) (THERS), Closeness to the sea (DISTANCE) (calculated as PC1 scores between Northing and Easting measurements), Sex (SEX), Reproductive Condition (REPRO), Hybrid Index (INDEX), Hybrid classification (HYBRID) and predicted ecotype (ECOTYPE). Models were simplified using a top-down approach and transformed and refitted if necessary to meet family conditions. Best fitting models were retained and fit was determined by residual plots and AIC.

Condition of fish was approximated through ASI and HSI, which were similarly modelled using the same independent variables. Parasitism variables were modelled and were comprised of THERS and Coinfection level (COINFECTION) (determined as number of macroparasite species present). GLMs were fitted as above with the removal of THERS as an independent variable.

4.5 Results

4.5.1 Parasitism of ecotypes

The most common parasite by far was the copepod *Thersitina gasterosteii*, located around the gills. A total of 10 macroparasite species/groupings were

recorded in generally low prevalences (Table 4.3) across the three CIST ecotypes.

Table 4.3: Summary of parasite measures for each ecotype for CIST fish

	Anadromous (N = 29)		Anomalous (N = 68)		Resident (N = 35)	
Parasite	Burden*	Prevalence	Burden*	Prevalence	Burden*	Prevalence
Schistocephalus solidus	0±0	0.000	0.059±0.036	0.044	0±0	0.000
Gyrodactlyus arcuatus	0.207±0.152	0.069	0.029±0.020	0.029	0.286±0.145	0.143
Thersitina gasterostei	1.655±0.638	0.276	11.426±1.313	0.941	8.114±1.089	0.971
Proteocephalus	0.069±0.048	0.069	0.0147±0.0147	0.015	0±0	0.000
Diphyllobothrium latum	0.172±0.172	0.034	0.338±0.154	0.176	0.029±0.029	0.029
Apatemon sp.	0.138±0.065	0.138	0.044±0.033	0.029	0±0	0.000
Glugea sp.	0±0	0.000	0.044±0.025	0.044	0±0	0.000
Unknown copepod	0.034±0.034	0.034	0±0	0.000	0±0	0.000
Unknown nematode	0.276±0.098	0.241	0±0	0.000	0±0	0.000
Unknown cestode	0.069±0.048	0.069	0.132±0.066	0.088	0.171±0.119	0.086

4.5.2 Morphometrics and hybrid classification

The DFA model scaled variation between the training ecotypes across the 51 morphometric measurements to 2 axes which accounted for 76.2% and 23.8% of the total variation (Figure 4.2a). Scaling of standardized morphometric variables is summarized in (Table 4.4). Linear Discriminant 1 (LD1) separated out anomalous fish from resident and anadromous ecotypes based predominantly on size, with additional influence of eye position, head shape, length of longest plate and caudal fin position. Fish that were smaller generally had shallower heads, shorter longest plates, and caudal fins closer to the peduncle. LD2 separated resident ecotypes from anadromous and anomalous ecotypes and was influenced by how caudal peduncle length, ventral spine position, head shape and dorsal fin position scaled with size. For their size, residents had shorter caudal peduncles, spines closer to their heads, deeper head shapes and raised dorsal fins suggesting deeper body shape.

Table 4.4: DFA scalings for all standardized morphometric and armour variation data included in the DFA analysis

Landmark	Plane	Variable	LD1[1]	LD2[1]
1	x	Upper Lip	-0.1046	0.6396
1	y	Upper Lip	0.4096	-0.6278
2	x	Top of Head	0.0215	0.3164
2	y	Top of Head	**-1.1924**	-0.9071
3	x	Dorsal Fin Start	-0.5017	0.4031
3	y	Dorsal Fin Start	-0.1058	**1.2142**
4	x	Dorsal Fin End	-0.0112	0.3507
4	y	Dorsal Fin End	-0.2608	-0.0657
5	x	Caudal Fin Dorsal Start	-0.7501	0.2947
5	y	Caudal Fin Dorsal Start	-0.5426	-0.0422
6	x	Caudal Peduncle	0.7678	**-1.5381**

6	y	Caudal Peduncle	0.3864	-0.2253
7	x	Caudal Fin Ventral Start	**1.1080**	0.0018
7	y	Caudal Fin Ventral Start	0.0937	-0.0961
8	x	Anal Fin Posterior	-0.3501	-0.2645
8	y	Anal Fin Posterior	0.0442	0.7615
9	x	Anal Fin Anterior	-0.4943	1.1214
9	y	Anal Fin Anterior	-0.0347	0.2669
10	x	Ventral Spine	1.0892	**-1.2481**
10	y	Ventral Spine	0.4029	-0.9247
11	x	Pelvis Anterior	-0.5135	0.2924
11	y	Pelvis Anterior	0.6312	-1.1013
12	x	Bottom of Head	0.3387	0.6581
12	y	Bottom of Head	0.6308	**1.6069**
13	x	Lower Jaw	-0.3327	-0.2337
13	y	Lower Jaw	-0.8392	-0.3311
14	x	Lower Lip	0.5200	-1.1341
14	y	Lower Lip	0.4167	0.4894
15	x	Corner of Mouth	-0.1949	-0.2135
15	y	Corner of Mouth	-0.1373	0.3254
16	x	Nostril	-0.4741	0.4544
16	y	Nostril	-0.3123	0.3261
17	x	Top Point of Eye	-0.6124	0.2739
17	y	Top Point of Eye	**1.2410**	-1.0253
18	x	Anterior Point of Eye	0.4710	-0.5560
18	y	Anterior Point of Eye	-0.2237	0.1952
19	x	Bottom Point of Eye	-0.4977	-0.2481
19	y	Bottom Point of Eye	0.2848	0.5013
20	x	Posterior Point of Eye	0.5017	0.4570
20	y	Posterior Point of Eye	-0.3128	-0.2083
21	x	Pectoral Fin Upper	0.1970	-0.5826
21	y	Pectoral Fin Upper	0.2972	-0.5315
22	x	Pectoral Fin Lower	0.4927	-0.4836
22	y	Pectoral Fin Lower	-0.4359	0.1639
		Spine 1 Length Residual	1.0547	-0.3852
		Spine 2 Length Residual	-0.8306	-0.1718
		Longest Plate Length Residual	**-1.1231**	0.3356
		Pelvis Height Residual	-0.7070	0.1431
		Pelvis Length Residual	0.1626	-0.2658
		Pelvic Spine Length Residual	-0.4284	0.5768
		Centroid Size	**-3.9427**	**-1.8915**

[1] Values in bold denote top 5 contributors to LD axes

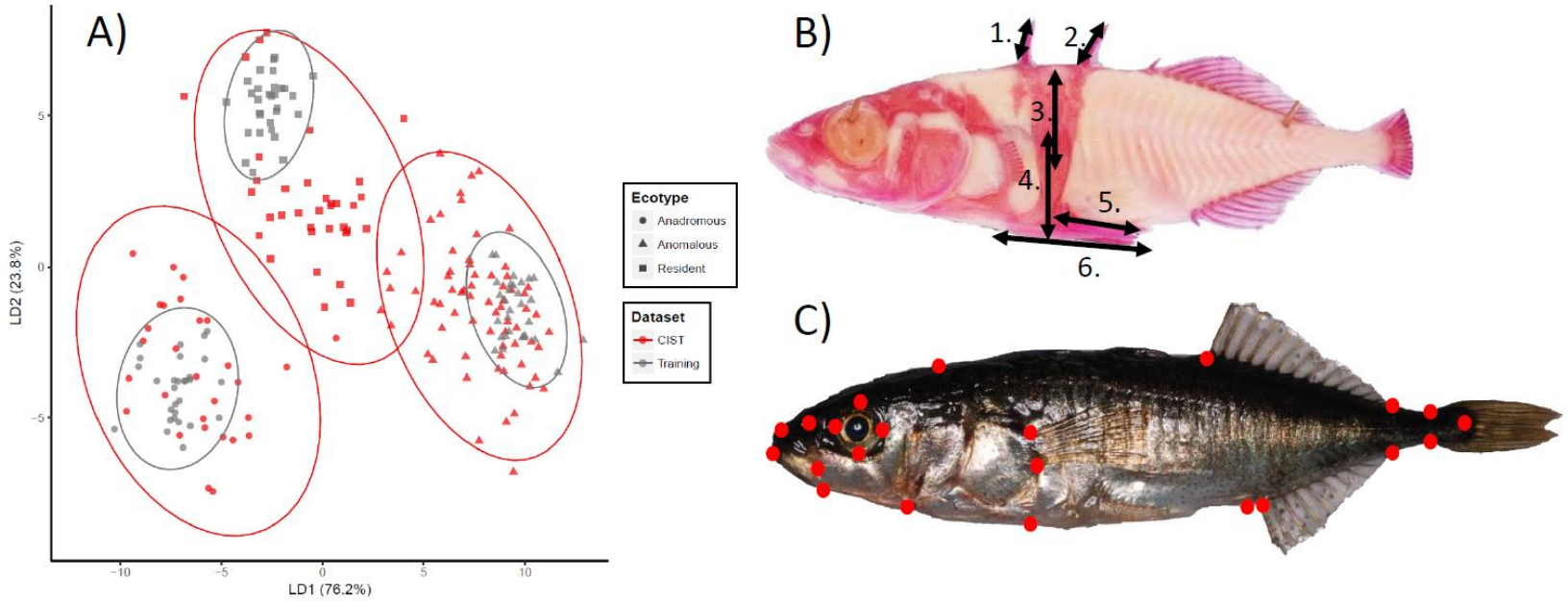


Figure 4.2: Results from DFA analysis **(A)** alongside armour variation measurements **(B)** and points used to determine morphometric variation **(C)**. DFA variation is comprised of two linear discriminant axes, with training fish (OBSE, OBSM and SCAD) coloured grey and CIST fish coloured red. LD1 and LD2 scores were predicted for CIST fish based on a DFA model trained using armour and morphometrics variation in training fish. Ellipses represent 95% confidence levels for each ecotype within each dataset (CIST and Training). Point shape represents ecotype: ● = Anadromous, ▲ = Anomalous, ■ = Resident. **(B)** Armour variation of stained fish is comprised of 6 measurements: 1 = first spine length, 2 = second spine length, 3 = longest plate length, 4 = pelvic height , 5 = pelvic spine length, 6 = pelvic length. **(C)** Morphometrics were determined through x and y plane variation of 22 landmarks, along with centroid size.

Hybrid statistics are summarised in (Table 4.5). Of the total 132 CIST fish sampled, 29 were predicted to be Anadromous, 68 Anomalous and 35 Residents. However, 90 of these were suggested to be hybrids according to the training set confidence intervals. According to the ratios of fish classified as either 'Hybrid' or 'Pure', anomalous ecotypes were significantly more likely to be pure compared with resident ecotypes (Wald's Test, $F_{2,129} = 5.182$, $p = 0.007$/Tukey A-N $p = 0.343$, A-R $p = 0.291$, N-R $p = 0.006$), suggesting hybridization is most common in resident ecotypes. Sampling of ecotypes and hybrid statistics are visualised for SGEA and CIST in Figure 4.3.

Table 4.5: Hybrid statistics calculated from DFA analysis.

Dataset	Predicted Ecotype	N	Mean Hybrid Index ± SE	Hybrid Proportion[1]
All CIST (N = 132)	Anadromous	29	2.980 ± 0.271	0.724
	Anomalous	68	2.937 ± 0.210	0.559
	Resident	35	4.499 ± 0.261	0.886
qPCR subset (N = 92)	Anadromous	25	2.849 ± 0.294	0.680
	Anomalous	39	3.268 ± 0.286	0.641
	Resident	28	4.493 ± 0.264	0.929

[1] Hybrid proportion determined as the ratio of fish classified as 'Hybrid' (Hybrid Index > 2.39) to 'Pure' (Hybrid Index ≤ 2.39)

Genotyping at the *eda* locus, which controls bony armour plating and is fixed for anadromous and freshwater alleles, did reveal 5 anadromous-freshwater hybrids, confirming that hybridization does occur between anadromous ecotypes and either resident ecotypes, anomalous ecotypes, or resident-anomalous hybrids.

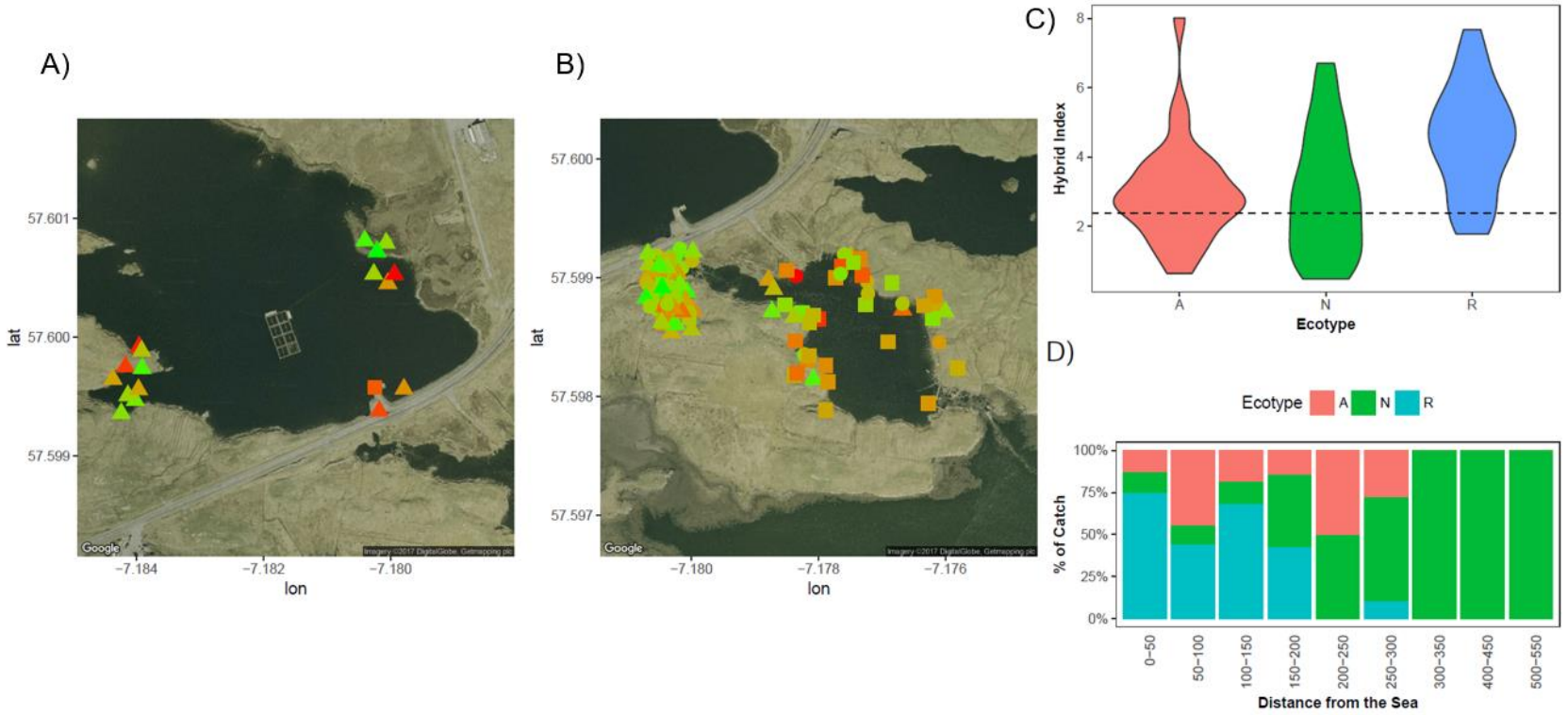


Figure 4.3: Sampling locations and hybrid data for lochs SGEA **(A)** and CIST **(B).** Point shape represents DFA-assigned ecotype: ● = Anadromous, ▲ = Anomalous, ■ = Resident. Point colour represents the Hybrid Index for each individual, ranging from 0 (green) to 8 (red). **(C)** Violin plots visualise variation in Hybrid Index scores by ecotype, with a dashed line signifying the cut off for binary hybrid classification according to the training dataset. **(D)** Percentage of ecotypes in catch at various distances from the sea, shown at 50m intervals.

4.5.3 Generalized Linear Models

GLM results are summarised in Table 4.6. Hybridization was found to affect various immune response variables, producing patterns that were generally in keeping with introgression (additive effects of alleles producing an intermediate phenotype) as opposed to transgression (extreme phenotypes outside the parental ranges produced through incompatibility of parental alleles). Spleen PC3 (19.3%) was a multivariate composite consisting of correlated expression between inflammatory *tnfα*, Treg-promoter *foxp3a*, and Th1 transcription factor *stat4*. Anomalous fish with low hybrid scores displayed increased expression of these 3 genes compared with similarly pure anadromous fish. However, these differences dampened and disappeared as Hybrid Index increased (Figure 4.4a). This dampening effect was significant, despite there being no detectable differences between the three ecotypes explicitly. Spleen PC4 (8.4%) described antagonistic expression of inflammatory *tnfα* and regulatory *foxp3a*. Here, fish that were classified as hybrids tended to show increased expression of inflammatory *tnfα* compared with their pure counterparts (Figure 4.4b). Increases however were within the scope of variation observed between ecotypes. In this regard, expression of *tnfα* relative to *foxp3a* was significantly greater in anadromous fish compared with freshwater ecotypes. A similar pattern was observed for operculum PC1 (48.7%), which, in agreement with spleen PC3, represented correlated expression of *foxp3a* and *stat4*. Pure anadromous fish had significantly lower expression of these 2 genes in their skin tissues, however anadromous hybrids

exhibited RNA-levels similar to those of freshwater ecotypes (both pure and hybrid) (Figure 4.4c).

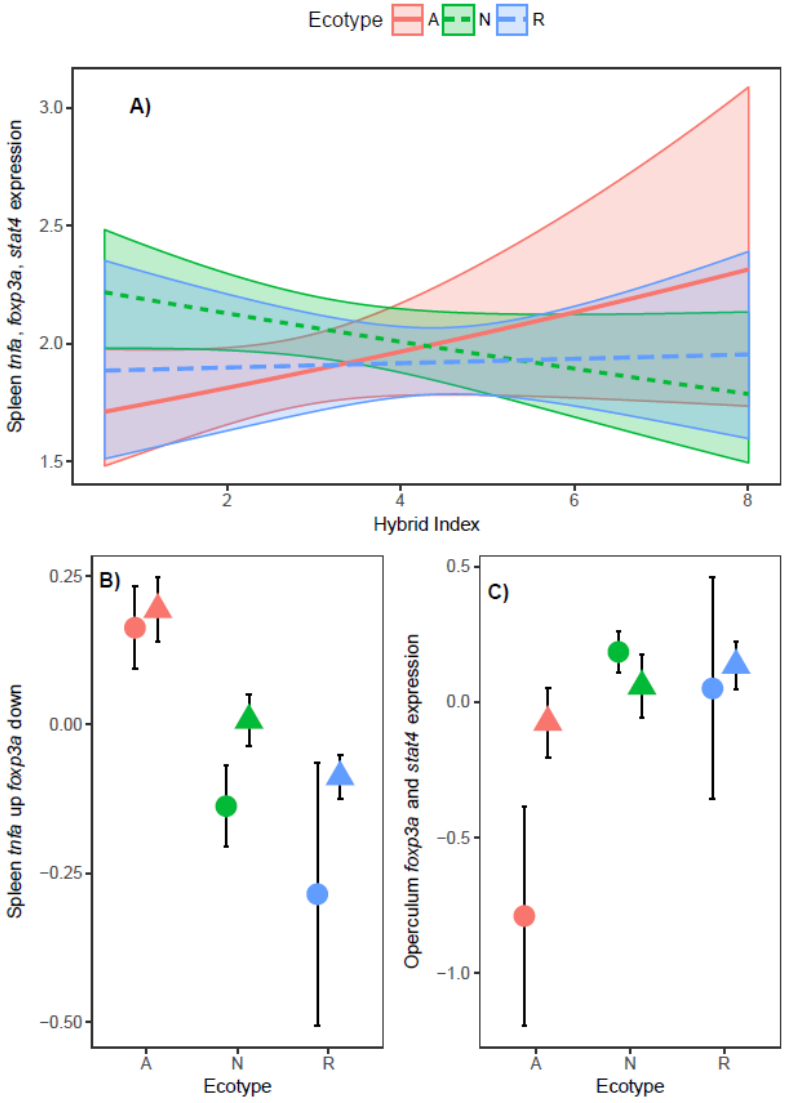


Figure 4.4: Variation in immune gene expression variables significantly affected by hybridisation. **(A)** Covarying spleen expression of *tnfα*, *foxp3a*, and *stat4* showed some variation between ecotypes at low hybrid index scores but differences disappeared at intermediate to high hybrid index scores. Data plotted are model simulated linear relationships with 95% confidence regions. **(B)** Fish classed as hybrids (▲) had increased splenic expression of *tnfα* relative to *foxp3a*, compared to pure (●) fish although differences were within the scope of variation between ecotypes. Points show actual group means with standard error. **(C)** Anadromous hybrids (▲) displayed more similar covarying expression of *foxp3a* and *stat4* in opercula tissues to freshwater ecotypes than to pure anadromous fish (●). Points show actual group means with standard error.

Table 4.6: Summary of GLMs for immune variables, condition variables and parasitism variables

Variable	Gene(s)[1]	PC Var[2]	Independent Variable	Family	df	F	*p*[3]	Effect[4]
Spleen PC1	*rorc* up *tbet* down	34.30%	Ecotype (DFA Assigned)	Gaussian	2,89	8.0886	**0.0006**	A > N = R
Spleen PC2	*rorc* down	30.30%	Ecotype (DFA Assigned)	Gaussian	2,89	2.1266	0.1253	
Spleen PC3	*tnfa* down	19.30%	Hybrid Index	Gaussian	1,90	0.0880	0.7670	
	foxp3a down		Ecotype (DFA Assigned)		2,88	2.0971	0.1290	
	stat4 down		Hybrid Index * Ecotype		2,86	3.7149	**0.0284**	*NA*
Spleen PC4	*tnfa* up	8.40%	Ecotype (DFA Assigned)	Gaussian	2,89	13.1574	**<0.0001**	A > N = R
	fox down		Hybrid?		1,88	4.4564	**0.0376**	Hybrid > Pure
Spleen *muc2*	*NA*	*NA*	Ecotype (DFA Assigned)	Gamma (link=log)	1,89	13.6220	**<0.0001**	A = N < R
Operculum PC1	*foxp3* down	48.70%	Ecotype (DFA Assigned)	Gaussian	2,89	4.8735	**0.0099**	A > N = R
	stat4 down		Hybrid?		1,88	1.5889	0.2109	
			Ecotype (DFA) * Hybrid?		2,86	3.6933	**0.0289**	*NA*

Operculum PC2	*tbet* down	23.50%	Ecotype (DFA Assigned)	Gaussian	2,89	5.8741	**0.0040**	A = N > R
Operculum PC3	*rorc* up	10.20%	Reproductive State	Gaussian	1,90	0.4987	0.4819	
Operculum PC4	*tnfa* up	7.90%	Sex	Gaussian	1,90	4.8137	**0.0310**	F > M
			Ecotype (DFA Assigned)		2,89	1.9852	0.1625	
			Thersitina N		1,87	1.5677	0.2145	
			Ecotype * Thersitina N		2,85	4.2858	**0.0169**	A ≠ N ≠ R
Operculum *muc2*	*NA*	*NA*	Ecotype (DFA Assigned)	Gaussian	2,89	5.4359	**0.0060**	A = N > R
Adiposomatic Index (log_{10})	*NA*	*NA*	Thersitina N	Gaussian	1,130	19.3160	**<0.0001**	-
			Sex		1,129	36.2120	**<0.0001**	M > F
			Ecotype (DFA Assigned)		2,127	9.0300	**<0.0001**	A > N = R
Hepatosomatic Index	*NA*	*NA*	Distance From Sea	Gaussian	1,130	1.3534	0.2469	
			Sex	(link=log)	1,129	145.7742	**<0.0001**	F > M
			Ecotype (DFA Assigned)		2,127	27.2506	**<0.0001**	A > R > N
Thersitina N	*NA*	*NA*	Reproductive State	Negative	1,126	4.1237	0.9949	
			Ecotype (DFA Assigned)	Binomial	2,126	12.0655	**<0.0001**	A < N = R
			Reproductive State * Ecotype		5,126	8.0590	**<0.0001**	*NA*

Coinfection N	*NA*	*NA*	Ecotype (DFA Assigned)	Poisson	2,129	1.5540	0.2153

[1]: Genes are noted for multivariate systemic immune gene expression PCs

[2]: Variation of systemic immune gene expression represented by each PC

[3]: Values in bold denote those significant at $p < 0.05$

[4]: Effects are recorded for significant effects and where interpretation can be simplified to a single expression

Where hybridization did not have an effect, ecotype was the major source of variation in gene expression. Expression of the mucin gene *muc2* in spleen tissues was unaffected by hybrid measures, but did vary between ecotypes and was significantly greater in resident ecotypes compared with anadromous and anomalous fish. Spleen PC1 (34.3%) represented antagonistic expression of *rorc* and *tbet*. Anadromous fish had significantly increased expression of *rorc* relative to *tbet*, whilst freshwater ecotypes had increased relative expression of *tbet* relative to *rorc*. Spleen PC2 (30.3%) was dominated by variable *rorc* expression, but was poorly explained by any of my measured variables.

Expression of *muc2* in skin tissues was significantly greater in anadromous and anomalous ecotypes compared with residents. Operculum PC2 (23.5%) represented *tbet* expression, which was found to be significantly increased in resident fish compared with anadromous and anomalous ecotypes. Expression of *rorc* in opercula was represented by PC3 (10.2%), however was poorly explained by all my measured variables. Operculum PC4 (7.9%) represented *tnfα* expression in skin tissues and varied between ecotypes depending on macroparasitic *Thersitina* burden. Anadromous and anomalous ecotypes with greater parasite burdens generally exhibited lower expression of inflammatory *tnfα* compared to fish with lower parasite burdens. This pattern was inverted however in resident ecotypes. I also found that *tnfα* expression on this PC was greater in females compared with males.

Condition indices were found to vary considerably but were generally not associated with hybrid measures. Male fish had significantly more stored fat tissue (represented by ASI) compared with females (Figure 4.5c) and anadromous ecotypes also had significantly greater ASI than anomalous and resident fish (Figure 4.5b). ASI also decreased significantly as *Thersitina* load increased (Figure 4.5a) suggesting poorer condition fish harboured greater parasite burdens. Conversely, females had significantly greater HSI scores (Figure 4.5f), signifying that whilst ASI and HSI are both proxies for condition, they likely represent different fitness advantages. However, as with ASI, anadromous fish had significantly greater HSI scores than the freshwater ecotypes (Figure 4.5e). Interestingly, HSI generally declined in fish sampled further inland, although this effect was non-significant (Figure 4.5d).

Hybridisation also had little effect on levels of parasitism recorded in my wild fish. However, *Thersitina* burdens were found to be significantly lower in anadromous fish compared with residents and anomalous (Figure 4.5g). In addition, fish that were fully reproductive tended to display greater *Thersitina burdens*, particularly in freshwater ecotypes (Figure 4.5g). Coinfection levels were generally poorly predicted by all measured variables.

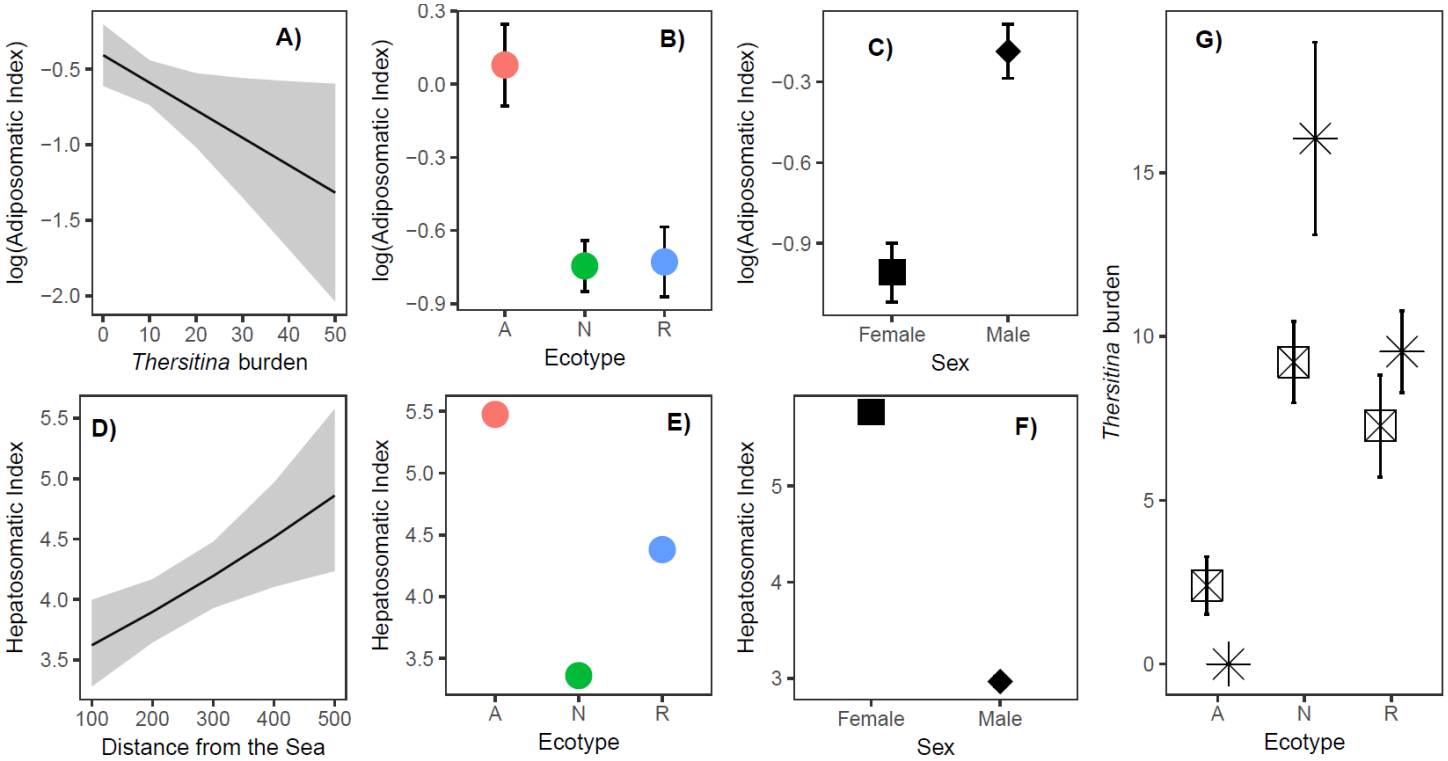


Figure 4.5: Variation in measures of fish condition and parasite burdens of *Thersitina gasterostei* were caused by various factors, but not hybridisation. Model effect plots for ASI show simulated $\log_{10}$-transformed values with SE of predicted values for the effects of *Thersitina* burden **(A)**, ecotype **(B)** and sex **(C)**. Model effect plots for HSI show simulated values with SE of predicted values for the effects of distance from the sea **(D)**, ecotype **(E)** and sex **(F)**. **(G)** Actual group means with SE for the effects of reproductive condition (sexually immature = ☒, fully reproductive = ✳) and ecotype on *T. gasterostei* burdens.

4.6 Discussion

Morphometrics indicate that significant hybridization has occurred between the three ecotypes existing in sympatry, which has affected the expression of immunologically relevant genes that are expressed differentially between 'pure' ecotypes. Interestingly, all expression variables in both spleen and skin tissues found to be affected by hybrid measures included variable expression of the Treg-promoter *foxp3a*. In spleen tissues, I observed that increased Hybrid Index scores reduced differences between ecotypes in terms of expression of inflammatory *tnfα*, Th1 transcription factor *stat4* and *foxp3a*. Further, fish classified as hybrids showed increased *tnfα* relative to *foxp3a* expression in spleen tissues compared with fish classified as pure. Crucially however, these changes were within the scope of variation between ecotypes, and are therefore indicative of introgression as opposed to transgression. In agreement with this, pure anadromous stickleback had significantly lower levels of *foxp3a* and *stat4* expression compared to freshwater ecotypes, however expression levels of anadromous hybrids were indistinguishable from freshwater fish. Taken together, these results suggest that hybrid dysregulation does not manifest in wild hybrid zones. Rather, my results suggest that parental alleles contribute additively to hybrid gene expression in the wild.

In general, the changes I found in fish classified as hybrids are in accordance with the small amount of previous work that has been conducted in wild animal hybrid zones, which suggests that rather than display transgressive phenotypes, as seen in plants, wild vertebrate hybrids tend to

display introgression and additive phenotypes. Additive immune responses have been observed in the laboratory for stickleback hybrids from separate lake populations with contrasting parasite communities (El Nagar & MacColl, 2016). In the wild, we see intermediate responses for hybrids of the pied flycatcher species, *Ficedula albicollis* and *F. hypoleuca* in response to malaria parasites (Wiley et al., 2009). Similar patterns have been observed even across intergeneric hybrids. Šimková et al. (2015) report intermediate leukocyte counts and complement activity in cyprinid hybrids between *Cyprinus carpo* and *Carassius gibelio*. Observations of intermediate responses across intergeneric hybrids hints at a robustness of the vertebrate immune response to resist hybrid dysregulation, at least phenotypically.

I would expect immune gene dysregulation to manifest as gene expression that falls outside of the variation observed between parentals. In this regard, I did observe that expression of *tnfα* relative to *foxp3a* in spleen tissues did increase in hybrids relative to pure fish when factoring in ecotype, hinting that herein may lie dysregulation. However, effect sizes here are small, and although there is an overall pattern for increased *tnfα* expression, patterns are non-significant when comparing hybrids against pure fish classified into the same ecotype grouping. Whilst evidence for immune gene dysregulation is lacking in wild systems, the phenomenon has been observed in large scale transcriptomic analyses of laboratory-reared organisms. For example, Hill-Burns & Clark (2010) observed dysregulation of functional groups associated with innate immune response pathways in *Drosophila* hybrids. Interestingly in

that study, whilst hybrid dysregulation was fairly substantial, there was no evidence for fitness costs in terms of immunocompetence, as bacterial loads of *Serratia marcescens* in artificial infections were unaffected by hybridization (Hill-Burns & Clark, 2010). Whilst these findings must be contextualised within a laboratory setting, they are supported by my observations here that neither hybrid index, nor hybrid classification appeared important for macroparasite measures. In a vertebrate system, Mavarez et al. (2009) produced hybrids in the laboratory between anadromous and resident ecotypes of brook charr, *Salvelinus fontinalis* Mitchill, and found large scale downregulation of a number of immune-related genes and genes associated with other processes when hybrids were compared to parentals. These results suggest that anadromous-resident brook charr hybrids should experience significant outbreeding depression because of transgressive expression. It therefore remains unclear as to why patterns observed in the laboratory do not match those observed in the wild.

At this stage, there are several caveats that must be considered when sampling hybrids from a natural hybrid zone. Firstly, I sampled adult fish, and it is possible that individuals with severe immune dysregulation have already died by this stage. Mavarez et al. (2009) sampled their laboratory hybrids at the first feeding stage shortly after hatching. In a natural setting, sampling adults allows ample time for selection to remove poorly performing hybrid individuals at younger stages. Indeed, Renaut & Bernatchez (2011) separated F2 backcrosses between lake whitefish species pairs, *Coregonus* spp., based on those

displaying abnormal signs of development at the 60-day stage. These embryos had significantly higher levels of transcriptome misexpression compared with developmentally normal backcross embryos and parentals, and experienced 100% mortality shortly after hatching. These data present the possibility of selective disappearance of hybrids exhibiting significant dysregulation, which may make such laboratory results impossible to detect in the wild.

There is also evidence to suggest that natural surroundings may dampen differences in expression observed in the laboratory. Empirically, population-level gene expression differences between laboratory-reared stickleback have been shown to be substantially greater than population-level differences between fish sampled from the same populations in the wild (Robertson et al., 2016a). This interaction between environment and gene expression may therefore make my results predictable if differences between parental ecotypes and hybrids are minimised by environmental influence. Such an effect would also make hybrid immune dysregulation difficult to detect in the wild, but critically would also remove or minimise selection against hybrids, as opposed to a selective disappearance mechanism. The significance of genotype x environment interactions and the incorporation of plasticity into our understanding of evolutionary processes is a prominent current focus of research. For immune genes, transplant experiments in stickleback have revealed a dominant effect of environment in shaping population-level variation in expression (Stutz et al., 2015). However, a study of hybrids between the killifish species *Lucania goodei* and *L. parva* found that, in general, genetic

incompatibilities were unaffected by experimentally manipulated rearing environment, suggesting DMIs are not environmentally dependent (Fuller, 2008).

Furthermore, my analysis is potentially confounded by different degrees of hybridization between ecotypes, namely significantly greater hybridization in residents. This finding is in keeping with what we would expect, given that CIST represents the meeting point of all three ecotypes, and both anomalous and anadromous ecotypes also exist in exclusive regions where diverged genotypes can be maintained and reintroduced into CIST. It therefore becomes unclear as to whether differences between ecotypes represent differences between ecotypes themselves or differences between purer and more hybridized ecotypes. This distinction becomes particularly interesting when considering that I detected numerous instances in which resident fish exhibited differential gene expression compared with anomalous and anadromous ecotypes. This was apparent in *muc2* expression in both tissue types as well as *tbet* expression in opercula (O PC2). There remains the possibility that these differences are ecotype-specific or a result of increased hybridisation producing transgressive expression. Differences between ecotypes are to be expected however, and I also observed numerous instances in which anadromous fish differed from both freshwater ecotypes. In terms of gene expression, anadromous fish had increased *rorc* expression relative to *tbet* in spleen tissues (S PC1), greater splenic inflammatory *tnfα* expression

relative to regulatory *foxp3a* (S PC4) and lower expression of *foxp3a* and Th1-associated *stat4* in skin tissues (O PC1).

An assumption often made of wild hybrid zones is that hybrids should harbour increased parasite loads due to combining each parental's locally adapted immune responses into a suboptimal middle ground. In an adaptive landscape, we would predict that increased hybridization between ecotypes may hinder their ascension towards optimal peaks, and therefore reduced condition and increased parasitism may manifest between hybrids and other fish. My data provides little support for this prediction however, as differences in condition and parasite burdens were most prominent between ecotypes rather than hybrids and pure fish. Specifically, I found that anadromous fish harboured the lowest densities of copepod parasites, *Thersitina gasterostei*, and were in the best condition according to both measures of fish condition, ASI and HSI.

My evidence is generally in agreement with empirical data from natural systems, with hybrids typically harbouring intermediate parasite loads (Vallender et al., 2012) or even lower parasite loads than parentals, as found in *Melanopsis* snails (Guttel & Ben-Ami, 2014) and house mice, *Mus musculus* (Baird et al., 2012). In the latter example, the authors speculate that immune genes may be 'immune' to Bateson-Dobzhansky-Muller type incompatibilities because of transitive relationships between new alleles formed in allopatry and a diverse array of current genotypes. Whilst such an explanation may fit for MHC genotypes, in which the maintenance of a diverse allele pool contributes

to immunocompetence (Spurgin & Richardson, 2010) and is driven by local parasite community (Eizaguirre et al., 2012), the applicability of such a hypothesis to other immune genes is unclear. Red-Queen host-parasite evolutionary dynamics invoke that rare genotypes are advantageous, and therefore a diverse pool of genotypes is maintained within a population. However, these models are oversimplified and assume immune gene evolution to be driven primarily or exclusively by parasites. Ecoimmunology has demonstrated the complex and diverse selection pressures which maintain and drive variation in natural immune responses (Martin et al., 2011; Pedersen & Babayan, 2011), and therefore the notion that immune genes should be immune to DMIs when other genes are not seems counterintuitive. It is also unclear how DMI-immunity operates under alternative models of immune gene evolution, such as arms race models that are dependent on novel genotypes and *de novo* mutations as opposed to maintenance of current polymorphisms (Brunner & Eizaguirre, 2016).

Differences in immune gene expression between anadromous and freshwater ecotypes may explain why these fish harboured lower parasite burdens. Anadromous fish displayed increased expression of the inflammatory cytokine gene *tnfα*, a gene that has been shown to be important for resistance against ectoparasites in fish (Lindenstrøm et al., 2004). Other studies have also shown that expression of inflammatory genes varies between anadromous and freshwater stickleback (Milligan-myhre et al. 2016; Robertson et al. 2017; J.R. Whiting & A.D.C. MacColl (unpublished)). Further, reduced *tbet* expression

relative to *rorc* expression may suggest a shift towards Th2 responses in the Th1-Th2 antagonism (Long & Nanthakumar, 2004), with Th2 helper cells acting as effectors of host immunity against extracellular parasites, such as those recorded here.

It is interesting however that some differences between ecotypes persisted despite hybridization, particularly given that other responses homogenized. This finding may highlight that distinct parts of the immune system are affected differentially by hybridization, a possibility alluded to by Hill-Burns & Clark (2010). There is the prospect that these genes may be where dysregulation occurs, if it occurs at all, and therefore immune dysregulation is cryptically contributing towards reduced hybridization. The ability of some genes to diverge to the point of incompatibility whilst others do not in the face of gene flow may occur through numerous prospective mechanisms. Firstly, if selection is strong enough and variable across the genome then we might expect genes associated with particularly strong selection to diverge more rapidly and become dysregulation hotspots. Here, one such source of variation may be migration in anadromous fish. Migration endows selection upon immune genes through various means. Firstly, migration is energetically taxing, and increased resource expenditure must be balanced alongside immune responses (Eikenaar & Hegemann, 2016). Secondly, migration results in hosts moving between different environments and parasite communities, meaning hosts must be able to respond to a potentially more diverse array of parasites (Carbó-Ramírez & Zuria, 2015). By this mechanism, selection removes hybrid

genotypes under migratory selection, whereas other immune genes may be free to hybridise and recombine.

Recombination is the undoing of divergence, and thus an alternative genomic mechanism could permit differential dysregulation through association with major adaptation genes, such as *eda* for example. *Eda* is an essential gene for adaptation from marine to freshwater habitats in sticklebacks (Colosimo, 2005; Jones et al., 2012b), and as such alleles are strongly differentiated between anadromous and freshwater ecotypes. It has also been shown that *eda* 'super gene' haplotypes influence expression of immune genes (Robertson et al., 2017a), demonstrating that at some level there is an association between immune gene expression and *eda*. The significance of *eda* for adaptation makes it, and genes associated with it, more resistant to recombination (Nosil et al., 2009; Samuk et al., 2017). These genes would become 'hotspots' for sources of dysregulation, as resistance to recombination can maintain diverging haplotypes even in the face of gene flow (Marques et al., 2016; Samuk et al., 2017). Such a mechanism would facilitate certain immune genes to diverge more rapidly, permitting DMI to develop in recombinant-resistant hotspots. This hypothesis is nevertheless speculative, and dependent upon an absence of transgressive expression patterns as an indicator for presence of DMIs in the wild, however such 'supergenes' may offer fruitful avenues for future research into the significance of immune gene DMIs.

In conclusion, I have attempted to detect dysregulation of immune genes in a naturally-occurring hybrid zone between three distinct ecotypes. My results provide evidence for additive expression profiles, suggesting that the vertebrate immune system may be robust and resistant to DMI, at least in the incipient stages of speciation. However, such phenomena are difficult to detect in the wild, due to the potential for selective disappearance and environmental influence on gene expression, the latter of which draws into question the prevalence of immune gene dysregulation found in laboratory studies. These two confounding effects have opposing implications for the significance of immune genes within postzygotic mechanisms of speciation, and therefore future work separating them out should help elucidate the matter. Crucially, despite hybridization affecting gene expression, I found no evidence that hybridization reduced host fitness in terms of condition or parasitism. Rather, these measures varied between the ecotypes themselves, with anadromous fish performing better than their freshwater counterparts.

CHAPTER 5: INTERCONTINENTAL GENOMIC PARALLELISM IN RESPONSE TO PARASITISM AND WATER CHEMISTRY

5.1 Abstract

If the 'tape of life' was rewound and played back, would we hear the same music? Whether or not evolution is repeatable has been studied extensively at the phenotypic level, but less so at the level of individual genes. Independent adaptive radiations provide an excellent opportunity to assess adaptive genomic parallelism in response to common ecological conditions. I used RAD-seq data gathered from four independent adaptive radiations in Alaska, British Columbia, Iceland, and North Uist to assess levels of genomic parallelism in response to water chemistry (Ca, Na, pH and Zn) and parasitism (*Gyrodactylus* and *Schistocephalus* prevalence). I used Bayesian linear models to correlate allele frequency changes within each radiation to environmental variation to identify genes with signals of adaptation. I then used probability calculations and bootstrapped simulations to examine whether repeated association of adaptive genes with an environmental variable occurred more often than expected in 2 or more radiations. I found significantly higher levels of genomic parallelism for all 6 environmental variables. Although no individual genes were parallel across all 4 radiations, signals of parallelism were strongest between geographic radiation pairings, suggesting shared genetic ancestry and environmental similarity have significant implications for the likelihood of

genomic parallelism. My results demonstrate that despite the inherent randomness associated with evolution by natural selection, there is indeed repeatability in the genetic basis of adaptation to common ecological conditions. In particular, observations here of parallelism in response to parasite prevalences represent some of the first data to show repeated genetic adaptation to parasitism.

5.2 Introduction

Whether evolution is repeatable, given the same starting conditions, remains a prominent question in evolutionary biology. This question is exemplified by Stephen Jay Gould's 'tape of life' analogy (Gould, 1990), which questions whether if life was rewound, erased and played back, we would observe the same forms and functions each time. Patterns of morphological convergence across taxa appear to support the notion that we would. In these examples, selection to optimise similar niches results in the independent evolution of similar adaptive traits. Convergence has been described for African lake cichlid trophic traits (Albertson et al., 2003; Muschick et al., 2012), *Anolis* lizard ecomorphs on Caribbean islands, (Mahler et al., 2013), and marsupials on the Australian continent (Forget & Vander Wall, 2001; Wroe & Milne, 2007). These cases demonstrate how natural selection can mould form in response to ecological pressure, however whether the genomic underpinnings of such moulding are equivalent remain uncertain.

Convergence upon a common phenotype from an equivalent evolutionary starting point, such as a shared common ancestor, constitutes parallel evolution (Conte et al., 2012) and represents the most probable scenario under which we would expect common evolution of the same genes. Evidence from individual traits would suggest that parallel evolution does occur. Investigations of three-spined stickleback, *Gasterosteus aculeatus*, have shown that for traits such as salt tolerance and bony armour plating, marine to freshwater radiations have repeatedly and independently modified allele frequencies at genes such as *eda* (armour plating) (Colosimo, 2005; Hohenlohe et al., 2010; Jones et al., 2012b) and *atp1a1a* (salt tolerance) (Defaveri et al., 2013; Jones et al., 2012a; Shimada et al., 2011; Terekhanova et al., 2014). However, whilst these patterns are repeated across the Northern hemisphere, they are not universal, as demonstrated in Japan, where reduced armour plating has been achieved through phenotypic convergence when freshwater *eda* alleles are absent or at very low frequencies in marine forebears (Leinonen et al., 2012). Similarly, where standing genetic variation varies around *Mc1r*, some mice populations have evolved lighter coat pigmentation through alternative genetic mechanisms (Hoekstra et al., 2006), despite the repeated modification of *Mc1r* with regards to pigmentation across diverse taxa (Arendt & Reznick, 2008). These studies suggest therefore that whilst evolution may be repeatable, there remains idiosyncrasy with regards to the genetic and genomic mechanisms.

Whether such patterns can be detected at the wider genomic scale remains a relatively open question however, although see (Ravinet et al., 2016; Soria-Carrasco et al., 2014). Such evidence can only be shown in the lab through experimental evolution studies (Graves et al., 2017) or through spatially independent adaptive radiations within species. The latter present optimal study systems to examine parallel evolution in response to common, natural ecological pressure. Adaptive radiations produce a myriad of ecologically adaptive forms from a single starting point, however whether the same genes underpin such adaptive variation in independent radiations remains untested. I examined whether genomic parallelism could be detected across four independent freshwater adaptations of *G. aculeatus* (stickleback hereafter) in response to radiation-specific environmental variation in measures of water chemistry and parasitism.

Whilst numerous studies have examined parallel evolution between population pairs, such as freshwater adaptations from marine ancestors, limnetic-benthic adaptations and lake-stream adaptations (Berner et al., 2008; Deagle et al., 2012; Jones et al., 2012a, 2012b), few have sought to understand the replicated genomic basis for the variation between freshwater forms themselves. Current understandings of this system therefore are limited to binary comparisons, which limits our ability to make inferences regarding the role of environmental variation, which is continuous rather than dichotomous. For example, my analysis here allows us to test whether the genes involved in the evolution of freshwater tolerance in marine-freshwater comparisons are

the same as those involved in fine scale variation in salinity between freshwater habitats. Analysing genomic parallelism between adaptive radiations allows us therefore to compare evolution across independent environmental continuums, elaborating on our current understanding whilst also providing a fresh perspective on a model evolutionary system.

Additionally, few studies have sought to investigate whether parallel evolution occurs with regards to common ecological pressure from parasites, although see (Jacquin et al., 2016). This is surprising, given the ubiquity of parasites (Poulin & Morand, 2000; Windsor, 1998), and their role in significant evolutionary processes such as the evolution of sex (Jokela et al., 2009) and mate choice (Folstad & Karter, 1992; Hamilton & Zuk, 1982). There is evidence to suggest parasite communities are partly structured by the local environment (Marcogliese, 2002; Padilla et al., 2017; Warburton et al., 2016; Young & MacColl, 2017; Zamora-Vilchis et al., 2012), and therefore we would predict that measures of local parasitism should be structured similarly and repeatedly with abiotic variation experienced within adaptive radiations. As such, we should expect to observe parallel genomic evolution to some extent between adaptive radiations for evolution in response to parasitism.

My aims in this chapter therefore were to examine genomic signals of parallelism in response to common ecological conditions across four independent adaptive radiations. In addition, I sought to find some of the first empirical evidence for parallel evolution in response to common parasitism pressures. My hypothesis here is that parallelism should be detectable at the

genomic level through allele frequency changes across the genome in accordance with common environmental gradients. However, parallelism should be dependent on factors such as shared ancestry, and thus shared standing genetic variation, and also common structuring of environmental variation.

5.3 Materials and methods

5.3.1 Sampling

Eighteen freshwater lakes from North Uist Scotland were sampled between April and June 2013, 18 lakes from Iceland between May and June 2014, 18 lakes from British Columbia (B.C.) between April and May 2015 and 19 lakes from Alaska in June 2015. Between 10 and 30 unbaited minnow traps (Gee traps, Dynamic Aqua, Vancouver, Canada) were set in water approximately 0.3–3m deep, along a 100–400m stretch of shoreline. Lake names, geographic coordinates and numbers of samples used in the study are shown in (Appendix 5.1).

Each lake had its pH measured using a calibrated pH meter (Multi 340i, WTW, Weilheim, Germany). The concentrations of metallic cation concentrations sodium (“Na”), calcium (“Ca”) and zinc (“Zn”) were obtained by collecting in the field a filtered water sample acidified with 2% nitric acid from each lake. These samples were then analysed at the Division of Agriculture & Environmental Science at the University of Nottingham for metallic cation

concentrations by inductively coupled plasma mass spectrometry (ICP-MS). Two parasites (*Gyrodactylus arcuatus* and *Schistocephalus solidus*) were chosen to be examined that are relatively common and widespread, with the potential to cause selection (MacColl, 2009a). The prevalence of *Gyrodactylus arcuatus* and *Schistocephalus solidus* per fish were counted during dissections and parasite numbers were averaged by lake. Concentrations of cations, pH and parasite prevalence per lake are shown in Appendix 5.1).

In total, 1,304 individuals were sampled across 73 populations spanning 4 independent radiations. This worked out at N = 18 or 19 populations for each radiation, with N = 10-21 fish per population (mean = 17.8).

5.3.2 DNA extractions, RAD library preparation and sequencing

Genomic DNA was purified from 10 to 21 individuals from each of the populations, chosen to represent a widely distributed subset of the most environmentally and phenotypically variable lakes. Extracted genomic DNA was normalized to a concentration of 25 ng /µL in 96-well plates.

In 2014, RAD sequencing was conducted on samples from North Uist and from Iceland. Sequencing libraries were prepared and processed into RAD libraries according to Etter et al. (2011), using the restriction enzyme SbfI-HF (NEB). Each sample was individually ligated to adaptors with 6bp in-line barcodes and multiplexed in libraries of 192. RAD libraries were sequenced in

four lanes on an Illumina HiSeq sequencer at the University of Oregon, producing 100-bp single-end reads.

In 2015, RAD-sequencing was conducted on samples from B.C. and from Alaska. Sequencing libraries were prepared following the modified single-digest RAD protocol of Ali et al. (2016), using the restriction enzyme SbfI, which digests DNA at an 8bp recognition sequence and is expected to cut roughly 22,000 locations across the stickleback genome. Each sample was individually labeled using one of 96 unique 8bp barcodes, and each pool of 96 samples received a different 8bp inline index using the NEBNext Ultra DNA Library Prep Kit for Illumina (Ali et al., 2016).

These RAD libraries were sequenced in two lanes of Illumina NextSeq high-output sequencing at the University of Oregon, using paired-end 75bp reads. After filtering for the presence of a correct index, barcode, and SbfI cutsite, these two lanes of sequencing produced a total of 256 million and 267 million read pairs, respectively.

5.3.3 Population genetics statistics

Raw sequence reads were demultiplexed using Stacks – 1.35 (Catchen et al., 2013, 2011). The retained reads were aligned to the three-spined stickleback reference genome (version BROAD S1, Ensembl release 82) using GSnap (Wu & Nacu, 2010; Wu & Watanabe, 2005). Reference mapping with GSnap took sequence quality information into account, allowed for up to five mismatches

and up to 2 indels between each read and the reference sequence and ignored reads which mapped against more than a single position in the genome. The STACKS pipeline was used to analyse mapping files and population genetics statistics were calculated using the POPULATIONS program in Stacks. POPULATIONS was run independently for each radiation with the following filters were applied: SNPs that were present in less than 8 populations were removed; SNPs present in < 50% of individuals within a population were removed; SNPs with a minor allele frequency below 0.05 were removed; all SNPs within a locus were retained; and data from the sex chromosome (XIX) were also removed. These filters were chosen to maximise SNP count whilst still providing enough information for allele frequencies and environmental variables to be correlated. After filtering, I retained 26,990, 26,937, 29,111, 26,169 SNPs within a GENEPOP formatted output for North Uist, Iceland, B.C. and Alaska respectively. GENEPOP files were converted to BAYENV2 format using PGDSpider2 (Lischer & Excoffier, 2011).

To assess genetic similarity between radiations, alignment files for every population were pooled into a single POPULATIONS analysis with the following filters applied: SNPs present in < 50% of individuals within a population were removed; SNPs with a minor allele frequency below 0.05 were removed; only a single SNP within a locus was retained; and data from the sex chromosome (XIX) were also removed. Following filtering, 20,278 SNPs were retained. Populations were grouped by radiation in a mapping file which was used to calculate bootstrapped F_{ST} scores across radiations. F_{ST} scores were also

calculated for each individual population pairing to understand the strength of within radiation divergence relative to between radiation divergence.

5.3.4 Correlating allele frequencies with environmental variation

Environmental variables were selected that were likely to cause natural selection on the fish, and that could be precisely measured. Abiotic water chemistry variables have been linked previously to the evolution of body shape, size and armour in stickleback (Giles, 1983; Magalhaes et al., 2016; Spence et al., 2013). I used the prevalence of the parasites *Gyrodactylus* sp. (ectoparasitic trematodes) and *Schistocephalus solidus* (endoparasitic cestode) that are likely to have a significant impact on the reproduction and life cycle of stickleback (Barber & Scharsack, 2010; De Roij et al., 2011; MacColl, 2009a; MacNab et al., 2009, 2011). Within radiation variation for these 6 environmental variables was standardised prior to analysis relative to the mean and standard deviation across populations in a single radiation.

Environmental associations with allele frequencies using Bayesian linear models in BAYENV2 (Günther & Coop, 2013). For each radiation, a random subset of 5000 unlinked SNPs was used to calculate a matrix of genetic covariance between populations according to the author's instructions (Günther & Coop, 2013). This covariance matrix was used to account for unknown population structuring whilst allele frequencies for each SNP were correlated against standardized environmental variation. BAYENV2 was run independently 8 times and final results were averaged across runs.

Environmentally associated loci were selected as having a $\log_{10}$(BayesFactor) > 1.5 and a Spearman's rank coefficient within the 95th percentile. These cut-offs were selected due to their use elsewhere in the literature (Guo et al., 2016). The combination of BayesFactor and non-parametric measure of correlation helps to avoid selecting SNPs with high BayesFactors due to potential outlier populations (Günther & Coop, 2013). SNPs associated with environmental variables were mapped to genes using location information acquired through BioMART (Ensembl) and a custom bash script that output a list of associated genes for each environmental variable. Genes were associated with environmental variables if at least one associated SNP was found within its exons or introns.

I plotted Bayenv2 results across the genome in stacked Manhattan plots constructed in ggplot2 (Wickham, 2016). To account for both measures of $\log_{10}$(BayesFactor) and Spearman's P, I multiplied the two together (absolute values for Spearman's P) to generate an arbitrary environmental association score, which was plotted against genome position. I also highlighted the position of genes that were found to be environmentally associated, and thus parallel, in 2 or more radiations. In doing so, I hoped to identify potential linkage regions where nearby parallel genes might represent parallel regions across all 4 radiations. Independent stacked Manhattan plots were made for each environmental variable.

5.3.5 Parallelism statistics

For each radiation, I located all the obtained SNPs in the genome and made a list of genes detectable by my methodology. These lists consisted of 4651, 4621, 5008 and 4719 genes for North Uist, Iceland, B.C. and Alaska respectively. These lists were then combined and subsetted to highlight genes that were detectable in all 4 radiations (1 subset - NIBA), to 3 radiations (4 subsets – NIB, NIA, NBA, IBA) or only 2 radiations (6 subsets – NI, NB, NA, IB, IA, BA).

Also, for each radiation and environmental variable, I located all environmentally-associated SNPs highlighted in my Bayenv2 analysis and made a list of genes that contained at least one environmentally-associated SNP. I will refer to these genes as "environmentally-associated genes". Environmentally-associated genes were grouped according to the subsets defined above to highlight environmentally-associated genes that were detectable in all 4 radiations (1 subset), 3 radiations (4 subsets) or 2 radiations (6 subsets). From these subsets, I recorded the numbers of environmentally-associated genes shared between 2, or more radiations.

I then conducted the following analysis for each environmental variable separately. I used the total number of genes detectable in all 4 radiations and the number of environmentally-associated genes for each radiation that were detectable in all 4 radiations to calculate the expected-by-chance number of genes shared between 2, 3 and 4 radiations. This was achieved by multiplying the probability of each scenario by the total number of detectable genes and pooling the 'Expected' outcomes. The probability equations for genes

detectable in all 4 radiations (NIBA) are as follows, where "P(x)" represents the probability of a gene being environmentally associated in radiation 'x', "Env(x)" represents the number of environmentally-associated genes for radiation 'x', "Total(NIBA)" represents the total number of genes detectable in all 4 radiations:

$$P(N).P(I).P(B).P(A) = \frac{Env(N)}{Total(NIBA)} \, x \, \frac{Env(I)}{Total(NIBA)} \, x \, \frac{Env(B)}{Total(NIBA)} \, x \frac{Env(A)}{Total(NIBA)}$$

$$P(N).P(I).P(B).P(A') = \frac{Env(N)}{Total(NIBA)} \, x \, \frac{Env(I)}{Total(NIBA)} \, x \, \frac{Env(B)}{Total(NIBA)} \, x \, (1 - \frac{Env(A)}{Total(NIBA)})$$

$$P(N).P(I).P(B').P(A) = \frac{Env(N)}{Total(NIBA)} \, x \, \frac{Env(I)}{Total(NIBA)} \, x \, (1 - \frac{Env(B)}{Total(NIBA)}) \, x \, \frac{Env(A)}{Total(NIBA)}$$

$$P(N).P(I').P(B).P(A) = \frac{Env(N)}{Total(NIBA)} \, x \, (1 - \frac{Env(I)}{Total(NIBA)}) \, x \, \frac{Env(B)}{Total(NIBA)} \, x \, \frac{Env(A)}{Total(NIBA)}$$

$$P(N').P(I).P(B).P(A) = (1 - \frac{Env(N)}{Total(NIBA)}) \, x \, \frac{Env(I)}{Total(NIBA)} \, x \, \frac{Env(B)}{Total(NIBA)} \, x \, \frac{Env(A)}{Total(NIBA)}$$

$$P(N).P(I).P(B').P(A') = \frac{Env(N)}{Total(NIBA)} \, x \, \frac{Env(I)}{Total(NIBA)} \, x \, (1 - \frac{Env(B)}{Total(NIBA)}) \, x \, (1 - \frac{Env(A)}{Total(NIBA)})$$

$$P(N).P(I').P(B).P(A') = \frac{Env(N)}{Total(NIBA)} \, x \, (1 - \frac{Env(I)}{Total(NIBA)}) \, x \, \frac{Env(B)}{Total(NIBA)} \, x \, (1 - \frac{Env(A)}{Total(NIBA)})$$

$$P(N).P(I').P(B').P(A) = \frac{Env(N)}{Total(NIBA)} \, x \, (1 - \frac{Env(I)}{Total(NIBA)}) \, x \, (1 - \frac{Env(B)}{Total(NIBA)}) \, x \, \frac{Env(A)}{Total(NIBA)}$$

$$P(N').P(I).P(B).P(A') = (1 - \frac{Env(N)}{Total(NIBA)}) \, x \, \frac{Env(I)}{Total(NIBA)} \, x \, \frac{Env(B)}{Total(NIBA)} \, x \, (1 - \frac{Env(A)}{Total(NIBA)})$$

$$P(N').P(I).P(B').P(A) = (1 - \frac{Env(N)}{Total(NIBA)}) \, x \, \frac{Env(I)}{Total(NIBA)} \, x \, (1 - \frac{Env(B)}{Total(NIBA)}) \, x \, \frac{Env(A)}{Total(NIBA)}$$

$$P(N').P(I').P(B).P(A) = (1 - \frac{Env(N)}{Total(NIBA)}) \, x \, (1 - \frac{Env(I)}{Total(NIBA)}) \, x \, \frac{Env(B)}{Total(NIBA)} \, x \, \frac{Env(A)}{Total(NIBA)}$$

I then repeated this analysis using the lists of genes detectable in 3 radiations (4 combinations), to calculate the expected-by-chance number of genes shared between 2 and 3 radiations. I repeated this analysis for genes detectable in only 2 radiations (6 combinations) and calculated the expected-by-chance of genes shared between 2 radiations. Finally, I summed all the expected-by-chance quantities to obtain a Poisson-distribution mean for environmentally-associated genes in at least 2 radiations.

This analysis is dependent on the assumption that each gene has an equal probability weighting, however this may not be true as the Bayenv2 analysis is run per SNP rather than per gene. Therefore, genes with more SNPs will be more likely to be have a SNP associated with an environmental variable by chance than genes with fewer SNPs, producing artefactual 'statistical parallelism' as opposed to true biological parallelism. To account for this, I created new lists of genes detectable in 4, 3 (4 lists) and 2 (6 lists) radiations, like the ones described above, but with the addition that the number of occurrences in the lists of each gene for each radiation was determined by the number of SNPs within that gene within each radiation.

I then performed the following analysis separately for lists of genes detectable in all 4 radiations, 3 radiations, or 2 radiations as described above. I performed bootstrapped simulations by first randomising genes in the list, then removing duplicates, and finally taking the number of genes from the randomised list that matched the number of environmentally-associated genes

(Env[x] see equations above) for each environmental variable and each radiation.

Simulated gene lists were then shortened to include only those genes which appeared in 2 or more radiations. Results from each subdivided analysis were pooled to find the simulated number of parallel environmentally-associated genes across all radiations. The analysis was simulated 100,000 times for each environmental variable, allowing me to calculate the peak of the simulated distribution as the mean number of parallel genes given the conditions. I also calculated the mean number of SNPs per parallel gene and used this information to construct a simulated distribution of the ratio of "Number of Parallel Genes" : "Mean SNP N per gene". This distribution was used to determine whether the ratio of parallel genes to average SNP number in my observed parallel gene lists was significantly greater than expected, indicative of biological parallelism, or not, indicative of statistical parallelism.

Simulations were repeated with the omission of SNP weightings to clarify expected values calculated through probability calculations. Expected values for each environmental variable calculated according to probability calculations and those generated through 100k simulations were thoroughly consistent (varying by 0.016 genes at most), verifying my expected values calculated through probability calculations. Because simulations also yielded null distributions, these were used to assess the statistical significance of observed numbers of parallel genes. P-values were calculated according to the

proportion of simulated parallel gene counts greater than or equal to observed count. P-values were then FDR-corrected across the 6 environmental variables.

As well as detecting an overall signal of genomic parallelism in 2 or more radiations, I was interested in whether certain groupings of radiations exhibited greater parallelism than others. I modified the probability calculation equations to analyse which particular combinations of radiations had the highest incidences of genomic parallelism, and thus which combinations had the highest relative contribution to the overall signal of parallelism. This involved calculating the expected number of shared genes for each combination of 2 or more radiations. For example, to understand the relative contribution towards overall parallelism of parallelism between North Uist and Iceland, I calculated the expected number of parallel genes between North Uist and Iceland for each of the above subsets in which North Uist and Iceland parallelism could occur (i.e. NIBA, NIB, NIA, NI). As before, expected values were summed to generate a Poisson-distribution expectation which was tested against observed values using traditional poisson methods. For North Uist and Iceland, probability calculations were as follows:

$$P(N).P(I).P(B').P(A') = \frac{Env(N)}{Total(NIBA)}\ x\ \frac{Env(I)}{Total(NIBA)}\ x\ (1 - \frac{Env(B)}{Total(NIBA)})\ x\ (1 - \frac{Env(A)}{Total(NIBA)})$$

$$P(N).P(I).P(B') = \frac{Env(N)}{Total(NIB)}\ x\ \frac{Env(I)}{Total(NIB)}\ x\ (1 - \frac{Env(B)}{Total(NIB)})$$

$$P(N).P(I).P(A') = \frac{Env(N)}{Total(NIA)}\ x\ \frac{Env(I)}{Total(NIA)}\ x\ (1 - \frac{Env(A)}{Total(NIA)})$$

$$P(N).P(I) = \frac{Env(N)}{Total(NI)}\ x\ \frac{Env(I)}{Total(NI)}$$

P-values, calculated as the probability of observing equal or greater numbers of parallel genes relative to expected values, were subtracted from 1 to yield parallelism scores for each combination of radiations for each environmental variable. Parallelism scores were then analysed using Kruskall-Wallis tests to determine heterogenous effects of parallelism.

5.3.6 Environmental structuring and variable correlations

To understand environmental similarities between adaptive radiations I conducted two principal component analyses (PCA) based on correlation matrices. Firstly, I included all 73 populations to understand absolute variation between all sampling sites and radiations. Secondly, I grouped populations into the 4 radiation groups and ran 4 separate PCAs to understand how the 6 environmental variables were structured within each radiation. To test for similarity in environmental structuring between radiations, the environmental variable correlation matrices were extracted from each radiation and analysed using Kzranowski matrix covariance tests. These analyses assessed whether the loadings of each environmental variable in each radiation were consistent, and thus indicative of repeated environmental structuring.

5.3.7 Estimates of linkage

To examine whether proximal parallel genes were in physical linkage, I first selected genomic regions where environmentally associated genes, shared

across 2 or 3 radiations, were in close proximity to other environmentally associated genes shared in alternative combinations of 2 or 3 radiations, resulting in the region appearing shared in all 4 radiations. Close-proximity of genes was determined by looking at Manhattan plots and identifying regions where lines of parallelism appeared to overlap in whole-genome Manhattan plots, with 50k flanks added upstream and downstream of the first and last occurring parallel genes within the defined region respectively.

Physical linkage between SNPs was calculated within individual radiations due to cut-site variation between radiations. For each radiation linkage was calculated using PLINK (Chang et al., 2015) for all SNPs found within the region, including those which were not found to be environmentally associated, across all populations within the radiation. PLINK returns an R^2 correlation for allele frequency changes between two SNPs. Linkage between SNPs was visualised using ggplot2 (Wickham, 2016) and plotted according to the location of SNP A (first SNP on 5' strand) and SNP B on the x and y axes respectively.

5.4 Results

5.4.1 Population genetics statistics

Predictably, genetic differentiation between radiations revealed North American and European groupings for radiations: radiation-level F_{ST} scores N-I = 0.029, N-B = 0.193, N-A = 0.199, I-B = 0.188, I-A = 0.193, B-A = 0.053 (Figure

5.1). Based on F_{ST} scores calculated between individual populations, relatively low F_{ST} scores calculated between radiations likely reflect high degrees of genetic differentiation within radiations as opposed to low differentiation between radiations.

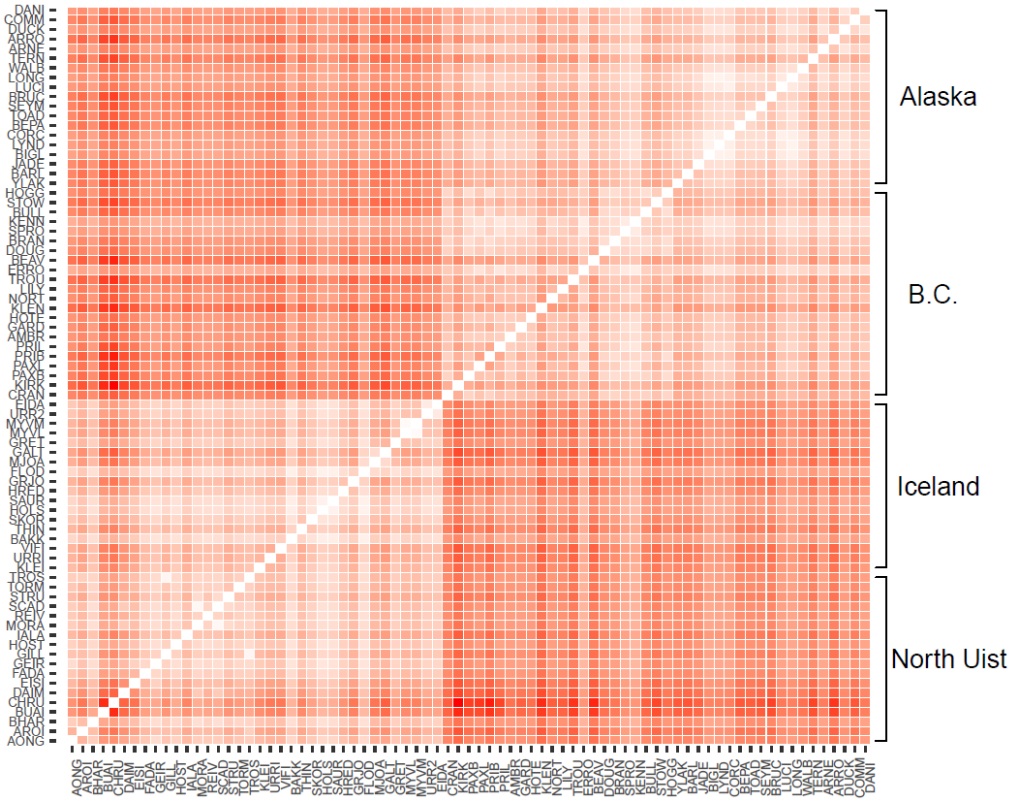


Figure 5.1: Heatmap of pairwise-F_{ST} scores for each population. F_{ST} vary between 0 (white) and 0.562 (KIRK (B) – BUAI (N), red). Lower general F_{ST} scores between radiations likely reflect accompanying high levels of intra-radiation divergence as well as inter-radiation divergence.

5.3.2 Correlating allele frequencies with environmental variation

I identified large numbers of SNPs as being associated with variation in environmental measures (Table 5.1). Total numbers of associated SNPs varied by radiation: 2741 SNPs for Alaskan populations, 2521 SNPs for B.C., 1507 SNPs for Iceland and 1762 SNPs for North Uist (values pooled together across

environmental variables. These environmentally associated SNPs mapped to 865 'environmentally-associated genes' in Alaska, 899 in B.C., 374 in Iceland and 581 in Scotland (values pooled together across environmental variables).

In terms of absolute values, I found no environmentally associated genes parallel in all four radiations, but several genes were parallel in groups of 3 radiations (Figure 5.2). The highest numbers of parallel genes in two or more radiations were associated with the prevalence of *Schistochephalus solidus* (37) and Na (28) (Figure 5.3) and the lowest with Zn (16). Alaska and B.C. shared the highest numbers of environmentally associated genes (71), followed by Alaska and North Uist (36) and Iceland and North Uist (31). B.C. and Iceland (24) and B.C. and North Uist (23) shared the lowest numbers of environmentally associated genes.

Table 5.1: Numbers of environmentally-associated SNPs (A) and genes (B) for each environmental variable within each radiation

	Environmental Variable						
Radiation	Ca	Gyro	Na	pH	Schisto	Zn	Total radiation^{-1}
a) SNPs							
N Uist	215	263	449	293	435	107	1762
Iceland	175	253	183	263	354	279	1507
B.C.	479	424	506	402	470	240	2521
Alaska	583	345	478	499	476	360	2741
Total SNPs Env^{-1}	1452	1285	1616	1457	1735	986	
b) Genes							
N Uist	84	106	144	58	154	35	581
Iceland	60	95	20	48	67	84	374
B.C.	212	174	163	125	163	62	899
Alaska	227	132	131	128	132	115	865
Total Genes Env^{-1}	583	507	458	359	516	296	

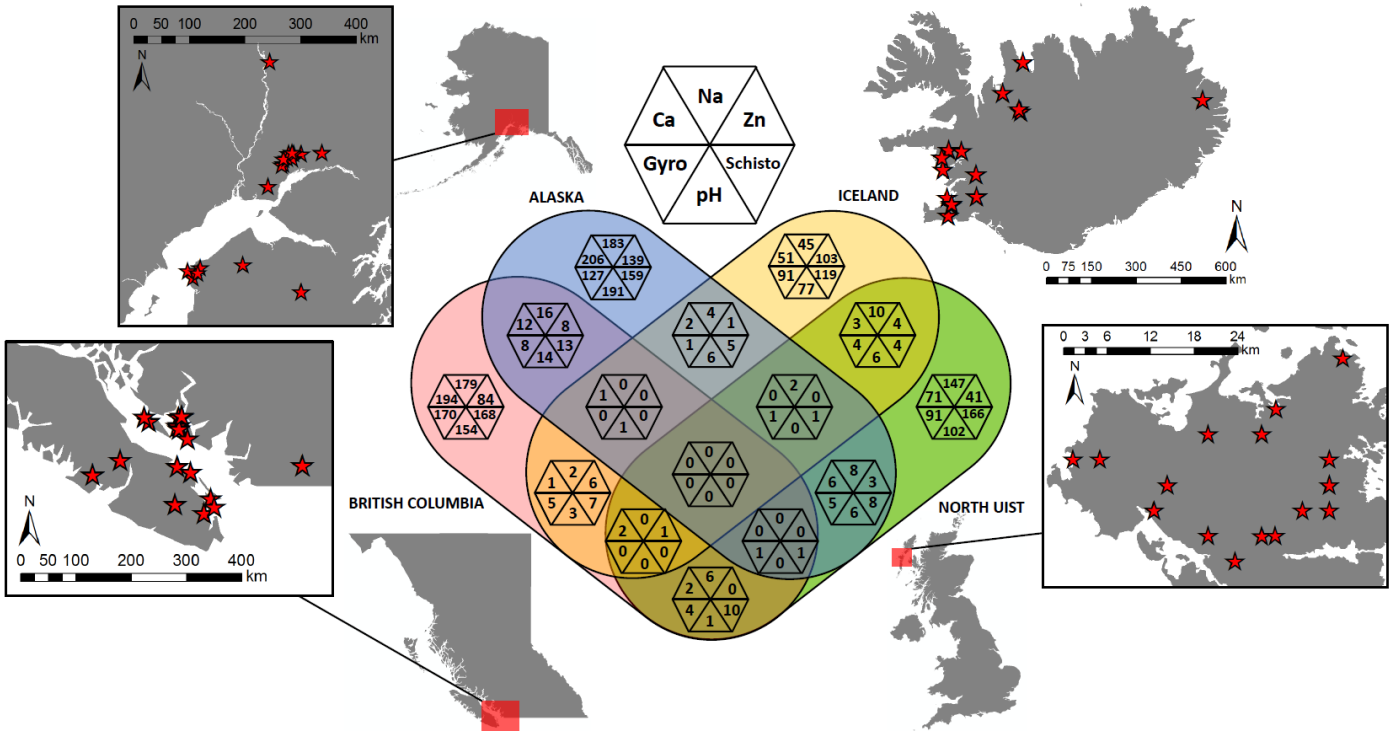


Figure 5.2: Numbers of genes with SNPs associated with each of the 6 environmental variables that are shared and unique among the four adaptive radiations: there are no genes that are shared by all four adaptive radiations. A total of 11 genes with SNPs correlated with an environmental variable are shared between three adaptive radiations, 174 are shared between two and 3058 have SNPs correlated to an environmental variable in only one radiation. Stars represent lakes sampled.

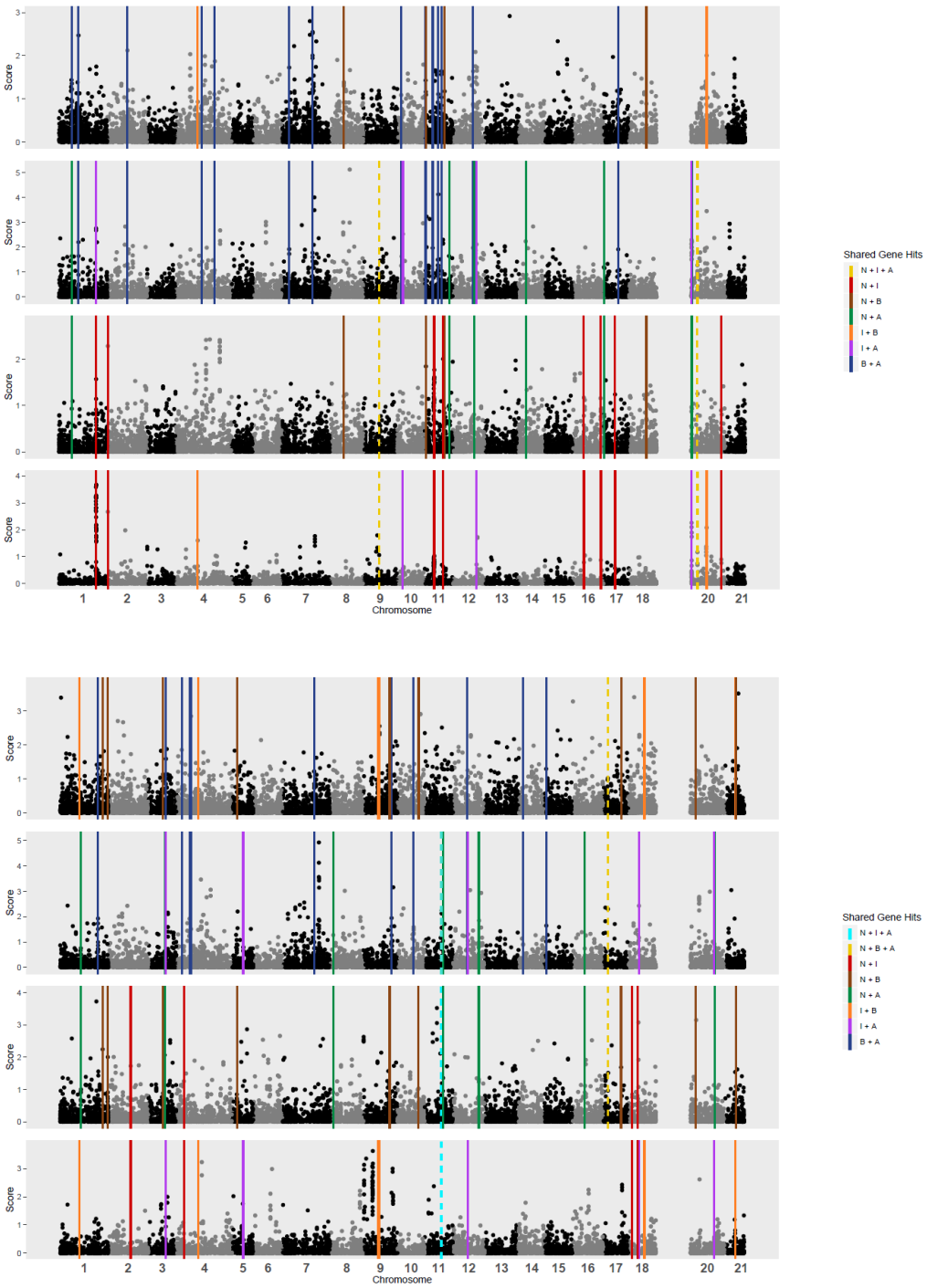


Figure 5.3: Manhattan plots summarizing correlations of allele frequency with Na (top) and prevalence of *Schistocephalus solidus* (bottom) for each of the 4 adaptive radiations (from top to bottom: B.C, Alaska, North Uist, and Iceland). Points illustrate the score (log10 (BayesFactor) * Absolute (Spearmans P)) per SNP. Shared genes are highlighted by vertical coloured lines. Each line represents the middle bp of the shared gene (see Appendix 5.2 for Manhattan plots of 4 other environmental variables). Manhattans are ordered BC (top), Alaska, North Uist, Iceland (bottom).

5.4.3 Parallelism statistics

Levels of parallelism were found to be significantly greater than expected-by-chance for all environmental variables, but to varying degrees (Figure 5.4). Generally, parallelism appeared strongest for measures of water chemistry. Genomic parallelism was strongest along salinity (Na) gradients (FDR < 0.001), followed by Zn (FDR = 0.002), pH (FDR = 0.006), *Schistocephalus* prevalence (FDR = 0.026), *Gyrodactylus* prevalence (FDR = 0.030) and Ca (FDR = 0.046).

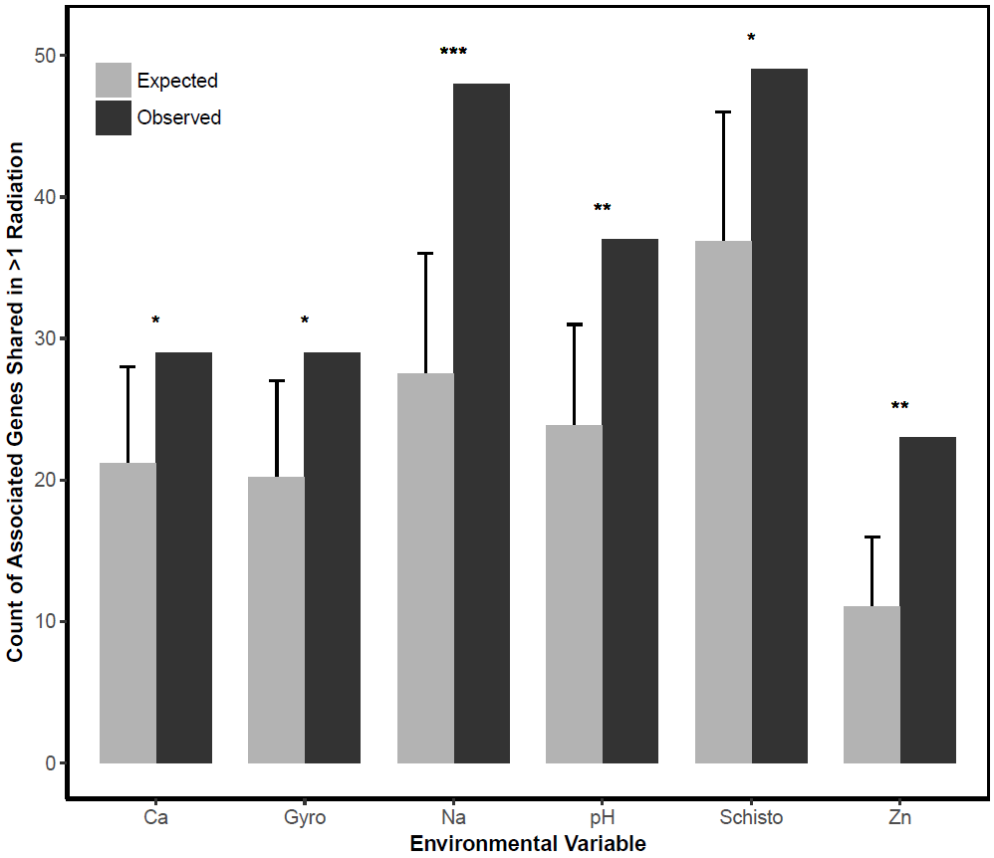


Figure 5.4: Expected and observed counts of parallel environmentally-associated genes in at least 2 radiations. Expected bars represent mean counts across 100,000 simulated outcomes with 95% confidence intervals per a one-tailed hypothesis. Asterisks denote significance of FDR-corrected one-tailed tests between the observed counts and the 100,000 simulated counts at the <0.05 (*), <0.01 (**) and <0.001 (***) levels.

In ascertaining whether the above results reflected biological genomic parallelism or a statistical artefact of the methodology, I found that for all variables I could discount statistical parallelism (Table 5.2). As expected, weighting the probability of genes being found to be parallel by their count of intragenic SNPs, and thus the number of Bayenv2 analyses conducted on each gene, greatly increased the expected number of parallel genes. Statistically speaking, simulated groups of parallel genes should tend to include those genes that have greater SNP weightings, as these will be randomly sampled more often than those with lower SNP weightings. When I compared the ratio of observed parallel genes, given their SNP weightings, to the null distribution of simulated ratios, I found that for all variables observed parallel genes had lower SNP weightings and were thus significantly more shared than expected. This over representation of 'rarer' genes in our observed sample of parallel genes supports the notion of biological parallelism over statistical parallelism. As above, the strongest effect of biological parallelism was observed for Na variation (FDR < 0.001), followed by *Schistocephalus* prevalence (FDR = 0.002), Zn (FDR = 0.009), Ca (FDR = 0.009), pH (FDR = 0.022) and *Gyrodactylus* prevalence (FDR = 0.038).

Relative contributions towards the overall signal of parallelism were found to fall unevenly across combinations of radiations. I assessed the strength of parallelism signal across all combinations of 2 or 3 radiations (no genes were observed to be parallel across all 4 radiations). Parallelism scores varied significantly across the possible combinations of radiations (K-W, χ^2 =

17.689, df = 9, p = 0.039) (Figure 5.5). North American (B.A) and European (N.I) pairings demonstrated the strongest effects of parallelism, although parallelism was also relatively high between North Uist and Alaska.

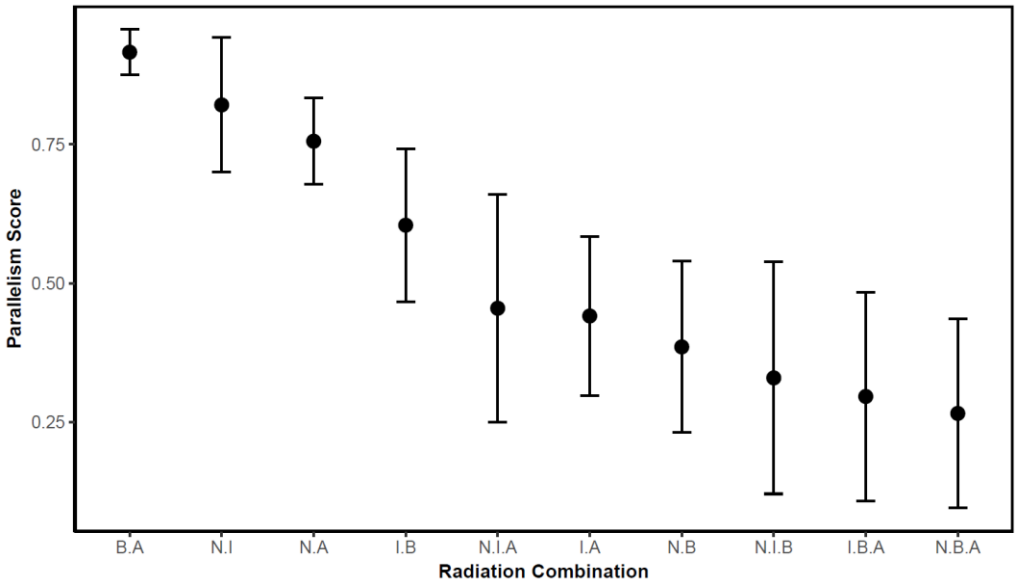


Figure 5.5: Relative contributions to overall parallelism signals for each grouping of 2 and 3 radiations. Parallelism scores were calculated as 1-P(greater or equal number of parallel genes being found in that grouping). Scores were calculated for each environmental variable with means ± SE plotted. In general, parallelism was strongest for geographic, continental groupings of Alaska and B.C (B.A) and North Uist and Iceland (N.I).

Table 5.2: Results from SNP weighted simulations to clarify whether parallelism is statistical or biological

		Environmental Variable					
Run	Measure	Ca	Gyro	Na	pH	Schisto	Zn
Simulation 1	Mean Parallel N	21.193	20.155	27.520	23.839	36.877	11.052
Simulation 2	Mean Parallel N with SNP Weighting	29.380	27.841	37.354	32.795	49.590	15.564
	Mean SNP Weighting	16.793	16.455	17.197	16.676	17.254	15.426
	Parallel N : SNP Weighting	1.750	1.692	2.172	1.967	2.874	1.009
Observed	Parallel N	29.000	29.000	48.000	37.000	49.000	23.000
	Mean SNP Weighting	10.902	12.683	12.663	13.520	11.210	13.128
	Parallel N : SNP Weighting	2.660	2.286	3.790	2.737	4.371	1.752
	Simulated : Observed *p*-value	0.006	0.038	0.000	0.018	0.001	0.005
	Simulated : Observed FDR-value	0.009	0.038	< 0.001	0.022	0.002	0.009

5.4.4 Environmental structuring and variable correlations

I observed striking similarity between radiations in the way my 6 environmental variables covaried within radiations that was not associated with geographic distance (Figure 5.6). Structural similarity was greatest between Alaska, B.C. and North Uist (Kzranowski matrix correlations: A.B = 0.994, A.N = 0.938, B.N = 0.943). Icelandic environmental structuring was most similar to North Uist's (Kzranowski matrix correlations: I.A = 0.622, I.B = 0.621, I.N = 0.785) but was less similar to the other 3 radiations than they were to each other.

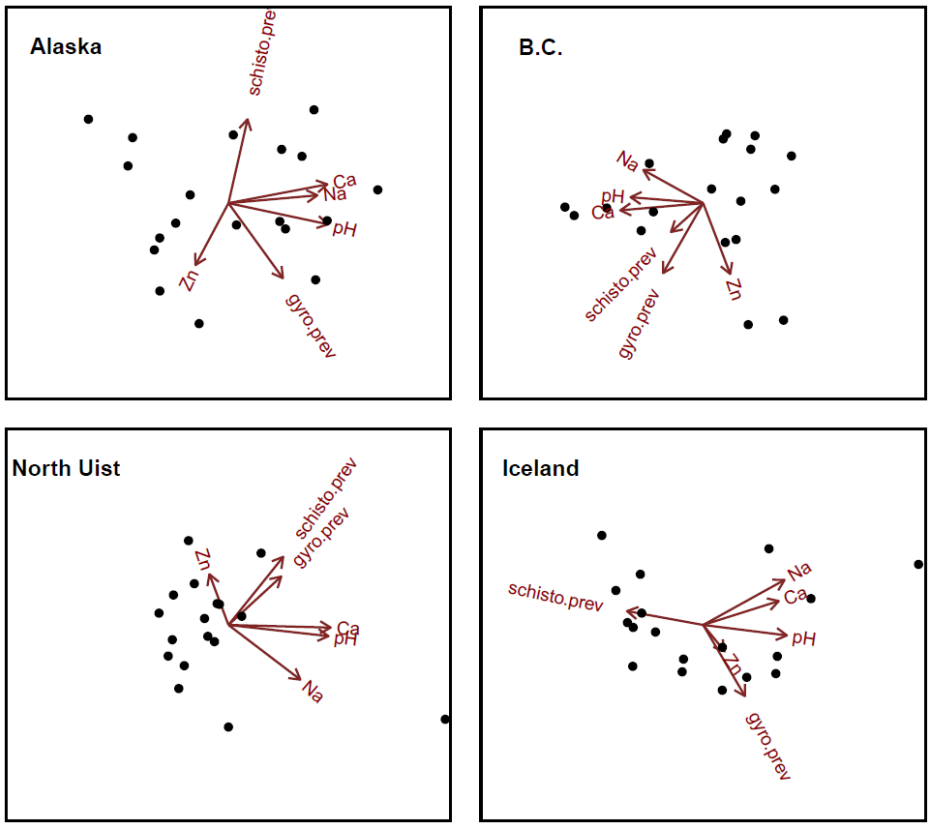


Figure 5.6: PCA loadings for environmental variation within each radiation. Arrows depict major correlative direction of variation and strength of variation (arrow length). Points are individual lakes within radiations. PCA loading matrices showed remarkable consistency across radiations, particularly between North Uist, Alaska and B.C. suggesting highly similar structuring of water chemistry and parasitism measures across radiations.

Whilst the variation between environmental variables measured here was structured similarly within each radiation, absolute values between radiations varied substantially (Figure 5.7). pH of lakes was found to be the most dominant source of variation between all 73 environments (PC1 35.26%) (Table 5.3). There was a general trend for Icelandic lakes to be more alkali than those found in other radiations. On PC2 (26.1%), there was significant separation of radiations. European lakes tended to have greater Zn ion concentrations, greater prevalence of *Schistocephalus* and lower Ca ion concentrations, whilst the opposite was true for North American lakes. Along these two principal components, I observed good separation between Icelandic lakes, North Uist lakes, and North American lakes. Alaskan and B.C. lakes were highly similar (Figure 5.7), indicating that as well showing highly comparable environmental structuring, in absolute terms the environments of these adaptive radiations are indistinguishable.

Table 5.3: PCA loading scores for environmental variables

	PC1	PC2	PC3	PC4	PC5	PC6
a) All Lakes						
Ca	0.440	-0.445	-0.212	-0.514	0.217	0.503
Gyro	0.391	-0.351	0.380	0.582	0.480	-0.103
Na	0.235	0.348	-0.731	0.115	0.444	-0.279
pH	0.590	-0.187	-0.129	0.035	-0.650	-0.420
Schisto	0.306	0.425	0.499	-0.543	0.251	-0.345
Zn	0.396	0.585	0.099	0.296	-0.202	0.603
b)Nuist						
Ca	0.574	0.023	-0.103	0.194	-0.328	0.717
Gyro	0.296	-0.430	0.534	-0.659	0.066	0.062
Na	0.404	0.485	-0.210	-0.233	0.709	0.019
pH	0.561	0.099	-0.182	-0.066	-0.445	-0.663
Schisto	0.307	-0.605	0.009	0.566	0.432	-0.180
Zn	-0.107	-0.451	-0.792	-0.386	0.006	0.094

c) Iceland						
Ca	-0.457	-0.257	0.177	-0.626	0.212	-0.507
Gyro	-0.253	0.765	0.278	-0.314	0.154	0.390
Na	-0.492	-0.484	-0.169	-0.053	0.017	0.701
pH	-0.506	0.112	0.225	0.281	-0.754	-0.184
Schisto	0.461	-0.149	0.143	-0.609	-0.570	0.223
Zn	-0.130	0.284	-0.890	-0.239	-0.195	-0.123
d) B.C.						
Ca	-0.600	0.066	-0.353	0.305	-0.039	-0.645
Gyro	-0.290	0.641	-0.177	0.228	-0.330	0.560
Na	-0.433	-0.304	0.451	0.477	0.429	0.325
pH	-0.525	-0.054	-0.180	-0.748	0.293	0.210
Schisto	-0.234	0.263	0.774	-0.262	-0.359	-0.282
Zn	0.195	0.649	0.093	0.011	0.701	-0.203
e) Alaska						
Ca	0.553	-0.143	0.120	-0.448	0.237	-0.635
Gyro	0.305	0.569	0.161	-0.402	-0.584	0.233
Na	0.497	-0.060	-0.492	0.560	-0.396	-0.190
pH	0.557	0.160	0.055	0.164	0.571	0.555
Schisto	0.106	-0.637	-0.380	-0.472	-0.172	0.432
Zn	-0.184	0.470	-0.755	-0.273	0.300	-0.104

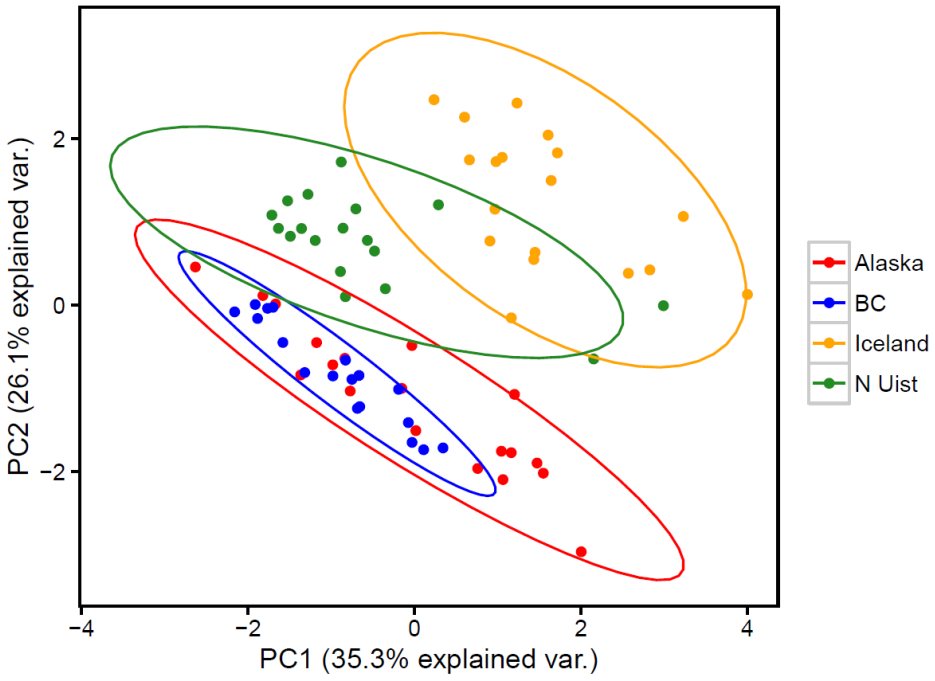


Figure 5.7: PCA of environmental variables revealed that PC1 of environmental variance separates the lakes along a gradient of pH and Ca. The second axis of environmental variance distinguishes between Zn and largely separates the European from the North American lakes. European radiations are more distinct than North American radiations, which fully overlap environmentally.

5.4.5 Estimates of linkage

Whilst I did not find any evidence of a single gene being associated with an environmental variable across all 4 radiations, I did observe 2 physically linked gene groups that were. The first region was identified on group I between the genes *igfbp2a* and an unnamed gene (*ENSGACG00000014345*) (Figure 5.8) and was associated with variation in Ca, Na and pH. This region contained 11 genes (Table 5.4), 5 of which were identified as being environmentally associated in at least 2 radiations (*igfbp5a*, *stk11ip*, *ENSGACG00000014323*, *atp1a1a.2* and *ENSGACG00000014345*). However, relatively strong linkage ($R^2 > 0.2$) between intragenic SNPs within these 5 genes was identified in North Uist, Iceland, and B.C. (Figure 5.8). I was unable to identify linkage in this region in Alaskan populations due to an absence of SNPs (Figure 5.8). To find such strong linkage between genes relatively far apart (~0.2 Mbp) across independent adaptive radiations suggests that the adaptive significance of genes in this region must be substantial between freshwater habitats.

I also identified a pair of linked genes that cumulatively were environmentally associated across all radiations. On group IX, *acy3.2* (Iceland and B.C.) and *tacr3* (North Uist, B.C., Alaska) were found to be environmentally associated with variation in *Gyrodactylus* prevalence (Figure 5.9). Unsurprisingly given their proximity, linkage between SNPs in these genes was relatively strong ($R^2 > 0.2$) in all radiations, with the exception of Alaska. As opposed to the linkage region on group I, this could not be attributed to an absence of identifiable SNPs.

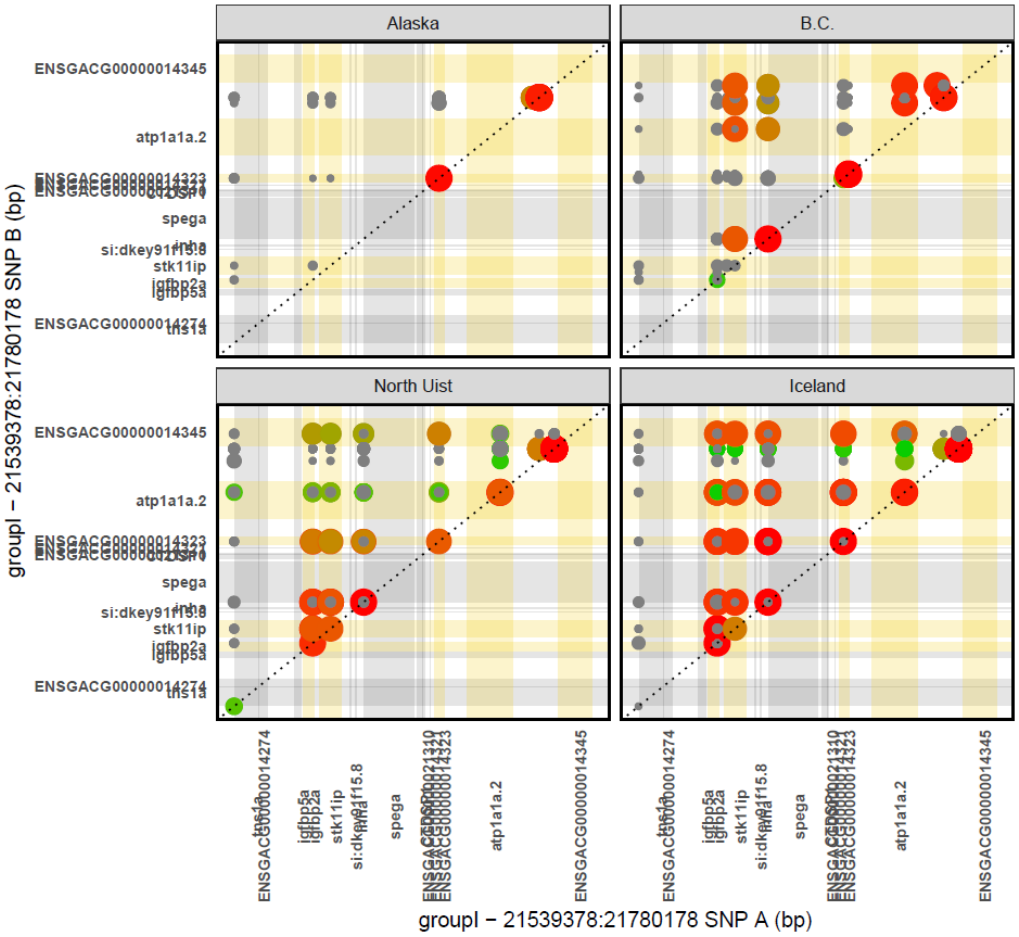


Figure 5.8: Group I linkage region. Points represent linkage between 2 SNPs (size = R^2 range 0:1; colour = grey < 0.2, green:red > 0.2-1.0). Shaded regions show gene regions (introns and exons). Yellow shaded regions show genes associated with Ca, Na and pH in at least 2 radiations. Dotted line represents equal chromosomal position of both SNPs, therefore chromosomal distance between SNPs increases with perpendicular distance from y = x.

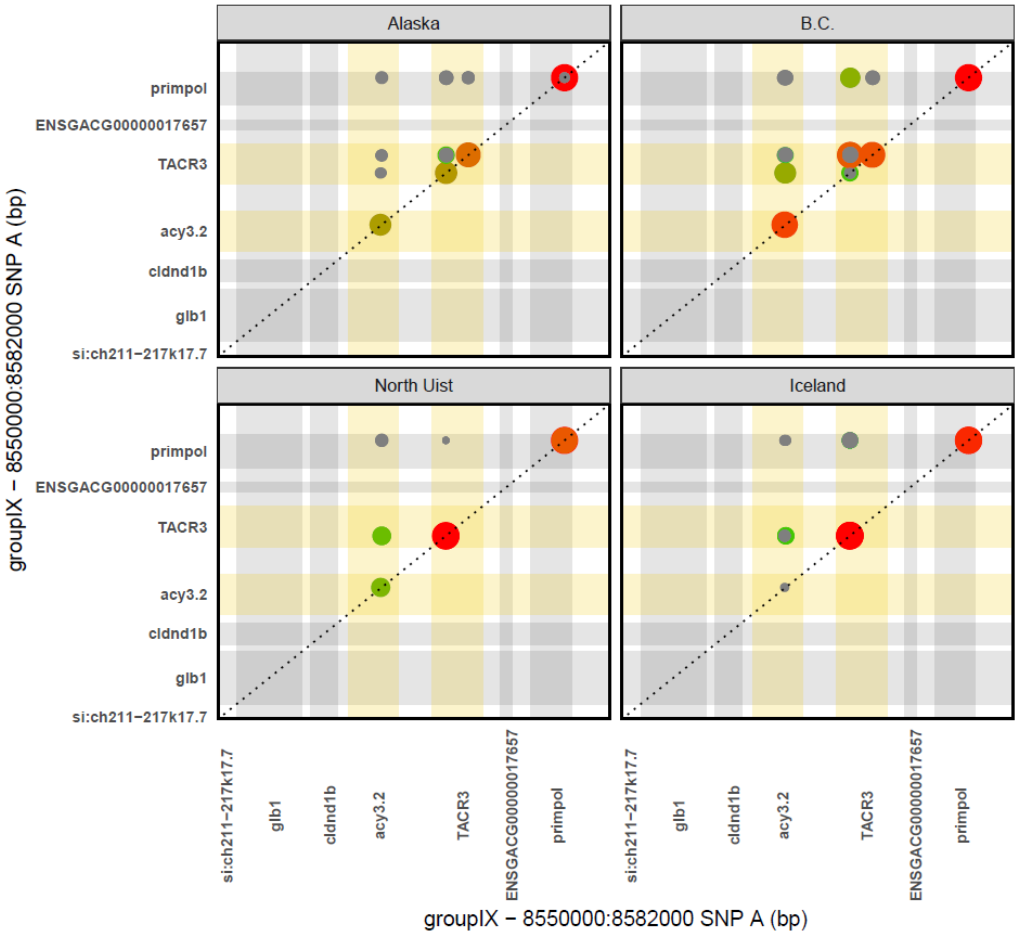


Figure 5.9: Group IX linkage region. Points represent linkage between 2 SNPs (size = R^2 range 0:1; colour = grey $R^2 < 0.2$, green:red $R^2 > 0.2$-1.0). Shaded regions show gene regions (introns and exons). Yellow shaded regions show genes associated with *Gyrodactylus* prevalence in at least 2 radiations. Dotted line represents equal chromosomal position of both SNPs, therefore chromosomal distance between SNPs increases with perpendicular distance from y = x.

Table 5.4: Information for genes found in linkage regions parallel across all 4 radiations

Gene stable ID	Gene name	Linkage Region	Environmental Association[1]	Gene start (bp)	Gene end (bp)	GO term accession	GO term name
ENSGACG00000014280	igfbp2a	groupI	Ca (N.I.B), Na (I), pH (I)	21589378	21596657	GO:0001558	regulation of cell growth
						GO:0005576	extracellular region
						GO:0005520	insulin-like growth factor binding
ENSGACG00000014289	stk11ip	groupI	Ca (N.I.B), Na (I), pH (N.I)	21600545	21614802	*NA*	*NA*
ENSGACG00000014294	si:dkey-91f15.8	groupI	*NA*	21620622	21621984	GO:0030054	cell junction
						GO:0005922	connexin complex
						GO:0005921	gap junction
						GO:0007154	cell communication
						GO:0005886	plasma membrane
						GO:0016021	integral component of membrane
						GO:0016020	membrane
ENSGACG00000014296	inha	groupI	*NA*	21624062	21625496	GO:0005576	extracellular region
						GO:0008083	growth factor activity
ENSGACG00000014299	spega	groupI	*NA*	21630258	21663404	GO:0006468	protein phosphorylation
						GO:0004674	protein serine/threonine kinase activity
						GO:0004672	protein kinase activity
						GO:0005524	ATP binding
ENSGACG00000014313	CTDSP1	groupI	*NA*	21665938	21670669	GO:0016791	phosphatase activity
						GO:0016311	dephosphorylation
ENSGACG00000021310	*NA*	groupI	*NA*	21669702	21669788	*NA*	*NA*
ENSGACG00000014321	*NA*	groupI	*NA*	21674393	21675279	*NA*	*NA*

ENSGACG00000014323	*NA*	groupI	Ca (I.A), Na (I.A), pH (I)	21677574	21684080	*NA*	*NA*
ENSGACG00000014324	atp1a1a.2	groupI	Ca (N.I), Na (N.I), pH (N.I)	21699651	21730178	GO:0006811	ion transport
						GO:0006810	transport
						GO:0005524	ATP binding
						GO:0000166	nucleotide binding
						GO:0006813	potassium ion transport
						GO:0016787	hydrolase activity
						GO:0046872	metal ion binding
						GO:0016021	integral component of membrane
						GO:0016020	membrane
						GO:0006814	sodium ion transport
						GO:0005391	sodium:potassium-exchanging ATPase activity
						GO:0010248	establishment or maintenance of transmembrane electrochemical gradient
ENSGACG00000014345	*NA*	groupI	Ca (I), Na (I), pH (I)	21760544	21783366	GO:0003677	DNA binding
ENSGACG00000017646	acy3.2	groupIX	Gyro (I.B)	8560149	8564640	GO:0046872	metal ion binding
						GO:0008152	metabolic process
						GO:0016788	hydrolase activity, acting on ester bonds
						GO:0016811	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides
ENSGACG00000017653	TACR3	groupIX	Gyro (N.B.A)	8567630	8572176	GO:0007186	G-protein coupled receptor signaling pathway

	GO:0007165	signal transduction
	GO:0004930	G-protein coupled receptor activity
	GO:0004871	signal transducer activity
	GO:0016021	integral component of membrane
	GO:0016020	membrane
	GO:0005886	plasma membrane
	GO:0004995	tachykinin receptor activity
	GO:0007217	tachykinin receptor signaling pathway

[1] Information in brackets relates to radiations in which association was detected

5.5 Discussion

The aims of this chapter were to assess whether adaptations in response to environmental variation are repeatable at the genomic level, and to identify factors that are important to promote parallelism. To achieve this, I used Bayesian linear models to correlate allele frequencies of loci across the genome with variation in six environmental measurements across four independent adaptive radiations. Through this, I identified genes apparently evolving in response to environmental variation and could test whether the same genes were identified in multiple radiations more often than expected by chance. My results provide evidence for significantly more incidences of parallel genes than predicted by chance, for all four measures of water chemistry and both measures of parasitism variation. These results were confirmed using two bootstrapped simulation methods, reinforcing that genomic parallelism observed here is a genuine biological phenomenon. Whilst I failed to identify any individual genes parallel across all four radiations, I noted that genes were associated with environmental variables in 2 or more radiations significantly more often than expected. This effect was predominantly driven by parallelism between my two North American radiations in Alaska and B.C., and my two European radiations in North Uist and Iceland. Nevertheless, parallelism scores were also high for other pairings, such as North Uist and Alaska, and Iceland and B.C. to a lesser extent. These findings join a slowly growing body of literature that has shown parallel evolution to occur at the genomic level in spite of Gould's predictions (Graves et al., 2017; Renaut et al., 2014; Soria-

Carrasco et al., 2014). This is the first example of genomic parallelism between adaptive radiations. Thus, my results here provide illuminating insights into the ability of environmental variation to work in concert with genetic material to produce adaptive phenotypes in repeatable, predictable ways.

These findings are in keeping with what I would expect given predictions of parallel evolution. For parallel evolution to occur at a genetic level, there are two requirements which must be met. Firstly, shared allelic variation in the genes underlying traits should exist between radiations for selection to act on, or else it must be achieved through independent *de novo* mutations (Conte et al., 2012; Hoekstra et al., 2006; Leinonen et al., 2012). The latter of these scenarios is less likely, thus parallel evolution should be expected to occur more frequently between radiations that are more genetically similar, and thus share similar 'starting points' for parallel evolution. In a review of the literature, Conte et al. (2012) demonstrated that the probability of parallelism at the genetic level was directly linked to time since lineages split, highlighting the probable influence of genetic similarity. Secondly, where radiations experience similar environmental structuring and variation, stronger parallelism is predicted, as selection itself homogenizes across radiations. Genomic repeatability in North American sunflower species-pairs, *Helianthus*, has been shown to be strongest between pairs sharing latitudinal distributions, and thus probable shared divergent selection (Renaut et al., 2014). Where both these conditions are met, genomic parallelism should be strongest. My results align with these predictions, as parallelism was strongest between more

genetically similar radiation pairings, and strongest overall between Alaska and B.C., which were more environmentally similar than European radiations. It is also interesting to note that following geographic pairings, North Uist and Alaska displayed the third highest parallelism effect. Whilst genetically more dissimilar than geographic pairings, these radiations displayed highly similar structuring of variation between environmental variables, and therefore parallelism here may reflect similar selection regimes in agreement with the second assumption of parallelism. North Uist and B.C. also displayed highly similar environmental structuring, but relatively low genomic parallelism however, an observation that may reflect greater absolute differences between the environments of these adaptive radiations compared with North Uist and Alaskan lakes.

It is unsurprising that geographic pairings were more genetically similar, given what we know about founding populations. Whilst the ages of these adaptive radiations are relatively young, having likely been founded at the end of the last glaciation; European radiations ~10 kya (Colosimo, 2005; Mäkinen et al., 2006), North American radiations ~15 kya (Liu et al., 2016), divergence of founding lineages stretches back far further. In general, marine stickleback are considered panmictic (Colosimo, 2005), although some structuring is apparent in diverging adaptive gene regions (Defaveri et al., 2013). Traditionally, two lineages of marine stickleback have been identified across the Northern hemisphere, the Japanese/Pacific lineage and the Atlantic/European lineage, the latter of which can be further divided into three

distinct lineages: Trans-Atlantic, European, and Black Sea (Mäkinen & Merilä, 2008). Previous phylogeographic studies have shown that North American stickleback populations harbour both major lineages (Lescak et al., 2015), whilst European populations show only signs of Atlantic/European ancestry (Orti et al., 1994), albeit varying according to the three sub-lineages (Mäkinen & Merilä, 2008). These major lineages have been estimated at separating ~90-250 kya (Orti et al., 1994) and demonstrate unique demographic histories (Liu et al., 2016), indicating there may be more ancient separation between our North American and European radiations. As well as finding lower levels of genetic differentiation between geographic pairings, I also found approximately 10-fold greater numbers of genes uniquely detectable in geographic pairings, indicative of shared cut-site variation. This suggests that both potential for parallelism to occur and the ability to detect it using RAD-seq are affected by shared ancestry. The majority of parallel genes identified were detectable across all 4 radiations however, and therefore shared cut-site variation is a poor explanation for increased parallelism in geographic pairings.

In terms of common covariance of environmental variation within radiations, it is perhaps unsurprising that Icelandic populations were more dissimilar to our other 3 radiations given Iceland's distinctively volcanic landscape. Volcanism would be expected to create unique habitat differences. For example, lava creates three-dimensional structures that modify habitats for fish and invertebrates (Malmquist et al., 2000), and freshwater systems are fed by groundwater flowing through porous lava rocks (Sigursteinsdóttir &

Kristjánsson, 2005). Ecological pressures of volcanic freshwater systems are strong enough to have produced parallel adaptive lava-mud forms in stickleback (Kristjansson et al., 2002) and arctic charr, *Salvelinus alpinus* (Sigursteinsdóttir & Kristjánsson, 2005).

The data presented provides evidence for a genomic signal of parallelism; therefore, it is pertinent to address what a genomic signal of parallelism may represent. A genomic signal of parallelism may characterise several scenarios. Firstly, adaptations in response to environmental variation may be underwritten by complex, multi-locus genetic architecture, and therefore loci are detected across the genome evolving in response. However, current genome-scan techniques, including Bayenv2, tend to be biased against traits with polygenic architecture (Haasl & Payseur, 2016; Hoban et al., 2016; Laporte et al., 2016), therefore this scenario seems unlikely. Alternatively, variation in important environmental variables may cause the simultaneous adaptation of many traits, and thus I detect evolution of many single loci of large effect evolving in response to a single environmental variable. Finally, variation in a single environmental variable may constitute a shared environmental axis with other correlated environmental measures. Here, we would detect multiple traits across the genome evolving in response to various correlated environmental measures. Given the strikingly similar environmental structuring observed between three of our radiations, this outcome seems probable. Under this scenario, I would expect to identify the same parallel genes as being associated with multiple covarying environmental measures.

This was observed for parallel genes associated with water chemistry. Of the 29 parallel genes identified for Ca variation, 9 were also associated with Na variation, 4 were associated with pH variation and 1 (*atp1a1a.2*) was associated with variation in Ca, Na and pH. Such common occurrence of parallel genes across different environmental variables suggests a common cumulative axis of water chemistry.

I also observed other incidences of common parallel genes across environmental variables, such as 2 shared between *Gyrodactylus* prevalence and Na variation, 2 between *Gyrodactylus* and *Schistocephalus* prevalence, 2 between *Schistocephalus* prevalence and Na variation, and 1 gene (*vma21*) shared across all 3. The commonality of parallel genes across these three measures suggests that parasitism measures may share a common environmental axis with salinity. Salinity has been shown to influence commonality of stickleback parasite communities (Poulin et al., 2011), and influences survival of *Gyrodactylus* sp. (Bakke et al., 2002) and *Schistocephalus solidus* (Simmonds & Barber, 2016), hence these results are unsurprising, albeit interesting. It is also possible that common parallel genes simply confer adaptive advantages to multiple environmental measures. The location of *vma21* in the genome is significant, as it spans *eda* (introns included) on the opposite strand. *Eda* is instrumental in reduced bony armour development in the parallel evolution of marine sticklebacks to freshwater environments (Colosimo, 2005; Jones et al., 2012b). Further, evidence is emerging to suggest *eda* may have an immune role (Robertson et al., 2017a), with inflammatory

genes downstream in close linkage including *baff* (*tnfsf13b*), *garp* and *dusp1* (Jones et al., 2012a; Robertson et al., 2017a). Here, I provide evidence that a gene in this region is evolving in parallel in response to fine-scale salinity and parasitism variation between freshwater environments, and thus the adaptive significance of this region may be even more substantial than already thought.

Whilst I can make some inferences on the role of genes identified in our analysis, the identification of specific parallel genes was not the main aim of this study. Limitations based on restriction enzyme cut-site variation make exhaustive searches of adaptive loci less informative than analyses based on full genomic sequencing methods (Lowry et al., 2017). However, whilst some adaptive loci may be overlooked, those that are identified are still of biological significance (McKinney et al., 2017). Aside from genomic signals of parallelism, I identified two linkage regions on groups I and IX that were associated with environmental variation across all 4 radiations. The adaptive significance of these genes is likely substantial given their repeated parallel evolution. Linkage on group I was strong across surprisingly large genomic regions. The most probable candidate for adaptive significance in this region is *atp1a1a*, given that genes in this region were associated with variation in water chemistry (namely Ca, Na and pH). *atp1a1a* encodes Na^+/K^+ ATPase's catalytic α1, which constitutes part of a membrane-bound enzyme responsible for moving K^+ in and Na^+ out of cells (Kaplan, 2002). As such, it is important for osmoregulation in freshwater fish (Evans et al., 2005), experiences selection regarding salinity (Hohenlohe et al., 2010; Terekhanova et al., 2014) and has rapidly evolved in

response to freshwater colonisation by marine stickleback (Jones et al., 2006; Shimada et al., 2011). a*tp1a1a* also demonstrates strong linkage disequilibrium with *eda* in cline analyses between anadromous marine and freshwater stickleback (Vines et al., 2016). Its detection here reinforces the adaptive significance of *atp1a1a*, not just in the evolution of freshwater forms from marine ancestors, but between freshwater habitats themselves. The adaptive significance of genes in the group IX linkage region are less clear-cut. This region contained the genes *acy3.2* and *tacr3.*

Whilst there has been some work undertaken regarding parallelism and convergence of parasitism (reviewed by (Poulin & Randhawa, 2013)), little research has been done regarding whether immune responses show parallelism with regards to common parasitic pressure. In studies of benthic-limnetic adaptation, stickleback studies have highlighted genes with an immune role showing some repeated evolution (Jones et al., 2012a). However, the dearth of research into parallel adaptation in response to parasitism may reflect the inherent complexities of host-parasite systems. For example, Jacquin et al. (2016) note that behavioural adaptations to *Gyrodactlyus* parasites of Trinidadian guppies, *Poecilia reticulata*, show phenotypic parallelism (present F0, absent in F2) with regards to shoaling behaviour. A lack of genetic parallelism, which they detect for predation adaptations, is attributed to either weaker selection or inconsistencies in parasitism within and between populations. Parasitic selection regimes are likely less stable than abiotic pressures, due to biotic interactions and constantly evolving nature of

host-parasite systems. Such stochasticity therefore may select for plasticity, to allow adaptations to parasitism to respond rapidly with changes in virulence or infection intensity. That said, I do detect signals of genomic parallelism in response to prevalence gradients for *Gyrodactylus* and *Schistocephalus*, albeit weaker effects generally than for water chemistry. Further, prevalence gradients structure consistently with abiotic environmental variation here, and thus whilst stochasticity presumably exists within and between populations, at the level of a whole adaptive radiation there is an apparent level of consistency regarding parasitism selection regimes, at least in terms of prevalence.

Given the novelty of the work undertaken here, despite the issues associated with identifying adaptive loci with RAD-seq, it seems relevant to highlight some of the parallel immune genes associated with parasitism in this study. There were two genes identified as having an immune function through gene ontology associated with *Gyrodactylus* prevalence in two radiations. *ENSGACG00000014598* is found on group I and was associated with *Gyrodactylus* in North Uist and Iceland. Assessment of gene homology suggests this gene likely encodes an Immunoglobulin V-set domain, and is involved with regulation of immune responses (GO:0043031) and negative regulation of macrophage activation (GO:0050776). *ENSGACG00000018312* is found on group IV and was associated with *Gyrodactylus* prevalence in North Uist and Alaska. This gene is immediately downstream of *eda*, is involved in immune responses (GO:0006955) and contains the transcripts for the inflammatory genes *dusp1*, *garp*, and *baff*. These genes have been implemented in

freshwater adaptation in stickleback before (Jones et al., 2012a), and further, their close proximity to *eda* has implicated their involvement in haplotype effects that lead to immune response changes and increased burdens of *Gyrodactlyus arcuatus* in low-plated stickleback (Robertson et al., 2017a).

In conclusion, I have demonstrated that genomic signals of parallelism are detectable across independent, intercontinental adaptive radiations of three-spined stickleback. I have highlighted how these genomic signals are likely dependent on shared ancestry, which influences standing genetic variation and the genetic architecture that can favour repeated adaptation of similar genomic regions. I have also shown that environmental structuring is important, due to the probable effects of homogeneity of selection within and between adaptive radiations. I provide some of the first evidence for detected genomic parallelism in response to parasitism gradients, although little can be inferred regarding the specific genes involved in adaptation. Detection of parallelism in the wild is complicated by repeated correlated environmental measurements, which concatenate selection in response to various environmental measures into cumulative environmental axes. However, this research provides some of the most compelling evidence to date for repeated adaptation at the genetic level in response to environmental gradients in the wild.

CHAPTER 6: GENERAL DISCUSSION

6.1 Summary of results

The aims of this thesis were to assess the evolutionary consequences of immune variation in the wild. Such an understanding is imperative if we are to gain further insights into the causes and consequences of natural variation in immune responses, which in turn should allow us to contextualise and predict where and how variation should arise. Evolutionary relationships can arise at the level of the individual, whereby traits impose evolutionary constraints on one another and evolve in concert, or at the more general level of the ecosystem. Here, variable host responses interact with the local environment to shape selection regimes imposed upon other individuals and other organisms. Finally, understanding the repeatability of immune variation at the genetic level informs on the repeatability of the evolutionary relationships in question. Put simply, if immune responses evolve in the same ways to repeated environmental conditions, we can assume that evolutionary relationships are partly consistent across independent systems.

In Chapter 2, I analysed how variable immune responses evolve in relation to host life history strategies. I found evidence to suggest populations with shorter life history strategies have also evolved stronger, potentially costlier immune responses. Genomic data from separate individuals of the same populations highlighted outlier loci between life history strategies in a

gene associated with the complement response, whilst a closer inspection of genetic variability in and around my five qPCR genes pointed towards importance of variation in the regulatory gene *foxp3a* and the Th1-adaptive type gene *stat4*. In addition to this, a comparative analysis of genetic variation in and around genes associated with 'immune system processes' GO terms revealed a linear relationship with the life history strategies across 15 freshwater populations. Taken together, these results highlight that shorter life history strategies are associated with stronger, costlier immune responses, potentially as costs associated with autoimmunity and fewer resources for somatic maintenance are offset by shorter lifespan and increased mortality. There does appear to be an underlying genetic basis for these observations from the wild, suggesting that such costlier responses are at least partly heritable. These results highlight evolutionary constraints between shorter and longer life history strategies associated with strength of response and associated parasitism.

With regards to wider evolutionary relationships, I investigated seasonal host-parasite associations and the effect of immune variation (Chapter 3). Here, I sought to demonstrate how photoperiod modulation affected immune responses of lab-bred fish and how these treatments influenced the infection dynamics of *G. gasterostei*. Fish were bred from either a resistant or a susceptible population, allowing me to assess how natural resistance variation interacted with photoperiod effects and seasonal parasitism. I measured expression of eight immune genes across two tissue

types (skin and spleen), and found expression of the mucosal gene *muc2* in the skin to decline significantly in fish housed under summer (16L, 8D) photoperiod conditions. This translated to increased susceptibility to *G. gasterostei* infection. However, this effect appeared consistent across resistant and susceptible-bred fish, suggesting that although susceptibility to *Gyrodactylus* increased, photoperiod-modulation of immune responses occurs irrespective of seasonal *Gyrodactylus* parasitism. Resistant fish displayed increased splenic expression of inflammatory genes (*tnfα* – innate, *tbet* – Th1, *rorc* – Th17), which is a probable source of natural variation in resistance given its close association with peak burdens. Results here demonstrate that seasonality of immune responses and parasitism can occur irrespective of parasitism in the wild, highlighting the complexity of natural systems and the adaptive consequences of seasonality in the stickleback-*Gyrodactylus* host-parasite system. Seasonal increases in susceptibility of hosts has implications for seasonal virulence evolution of parasites.

I also explored how the divergence of immune responses may facilitate speciation through the generation of Bateson-Dobzhansky-Muller type incompatibilities (DMI) between ecologically divergent ecotypes (Chapter 4). The potential for autoimmunity along with the rapidity with which immune responses can diverge as a result of ecological differences makes the genes underlying such divergence a prime candidate for DMIs (Bomblies et al., 2007; Bomblies & Weigel, 2007; Chae et al., 2014) that have yet to be studied in wild animal hybrids. Here, I showed that three stickleback ecotypes exist

sympatrically at the meeting place of the North Atlantic and the largest freshwater catchment on the east side of North Uist. Through morphometrics and armour variation I identified hybridization in this system and assessed variable parasitism and fish condition between ecotypes and how these were related to the expression of eight targeted immune genes in skin and spleen tissues. Whilst substantial expression variation between ecotypes was observed, contrary to predictions hybridization produced expression profiles that were introgressive rather than transgressive. Hybridization also had a negligible effect on fish condition and measures of parasitism, suggesting immune DMIs may not be at work in this hybrid zone, or are at least difficult to detect in wild hybrids.

Finally, I examined four independent adaptive radiations (Alaska, British Columbia, Iceland and North Uist) to test for the repeatability of genetic responses to six environmental measures, including two measures of parasitism: *Gyrodactylus* prevalence and *Schistocephalus* prevalence (Chapter 5). This intercontinental analysis included 73 populations and was one of the largest assessments of genomic signals of parallel evolution in response to common ecological conditions in the literature to date. I found significant signals of genomic parallelism for all six environmental variables, which were generally strongest for measures of water chemistry (Na^+, Zn^{2+} and pH gradients). Correlation matrices of environmental variation, including *Gyrodactylus* and *Schistocephalus* prevalence, showed remarkable consistency across 3 of the 4 radiations, highlighting repeatable environmental structuring

of parasitism measures with water chemistry across independent adaptive radiations. Along with shared genetic ancestry between European and North Atlantic radiation pairings, environmental structuring was likely a causative factor behind relatively high parallelism between intercontinental pairings (Alaska and North Uist). This study revealed that adaptive genomic responses to parasitism and water chemistry are indeed repeatable, but are probably dependent on factors such as shared ancestry and environmental structuring.

The results above provide empirical support to the notion that relationships between immune variation and other evolutionary processes shape the evolution of immune responses into repeatable, predictable patterns. Given the complexity of natural systems, in which environments can be hugely variable with regards to parasite communities, predation regimes, available resources and competition, demonstrating even a small degree of repeatability in evolutionary patterns is surprising and promising. For the remainder of this section I intend to discuss some interesting observations from across my chapters and highlight potentially fruitful avenues for future research.

6.2 Measuring immune responses in the wild

A large body of data in this thesis was collected using qPCR with wild (Chapters 2 and 4) and laboratory-reared fish (Chapter 3). This method was chosen for several reasons that warrant discussion. Quantitative-PCR (qPCR) is a method

that involves extracting mRNA transcripts from biological samples and using reverse-transcription to convert these into cDNA, which is then amplified and quantified through fluorescence of fluorophore probes (in this thesis, non-specific fluorochrome SYBRgreen). The goal is to quantify a proxy for post-translational gene products of biological interest. Whilst using mRNA as a proxy for gene products does introduce the potential for unknown variation in post-translational processes (Munsky et al., 2012), there are advantages to its use over direct measures of immune products. Chief amongst these is ease of assay development for non-model organisms. Immune model species such as mice, *Mus musculus domesticus*, come ready as the 'work horses of immunology' with an immunological toolkit for the quantification of immune responses in wild individuals (Viney et al., 2015), and indeed recent research has used these toolkits to highlight the comparative differences between laboratory and wild mice (Abolins et al., 2011, 2017). However, individual measures can offer contrasting conclusions (Christensen et al., 2016), and mice toolkits are often unusable in nonmodels, even in other rodents within the subfamily Murinae (Jackson, 2015). For nonmodels therefore, measures of RNA are a promising alternative (Downs et al., 2014; Jackson, 2015; Pedersen & Babayan, 2011; Robertson et al., 2016a).

Of course, qPCR is not the only means by which to quantify RNA, however it does hold several advantages over alternatives such as microarrays, multiplex assays and RNA-seq. Whilst analysis of genes must take a more targeted approach, qPCR assays can be developed with relatively little

sequencing information, as amplicons are relatively short (~100-150 bp). Compared with multiplex and microarrays, which require larger, highly specific sequence data (Fassbinder-Orth, 2014), qPCRs represent a more approachable method of assay development for non-models. Whilst genomic resources for *G. aculeatus* are relatively good, the majority of resources come from North American populations. Given the specificity of sequence data required for the development of microarrays and multiplex methods, divergence of immune gene sequences between North American and North Uist would have hindered their usage here (Robertson et al., 2016a). RNA-seq can be undertaken using a reference or *de novo* transcriptome (Wang et al., 2009), however it remains expensive in comparison to qPCR and requires a complex analysis with considerable computing power and storage (Fassbinder-Orth, 2014). These characteristics make RNA-seq less accessible and hinder biological replication, which when sampling individuals from the wild is an important means of controlling for environmental noise.

qPCR has been used consistently for ecoimmunology studies in the past (reviewed in Fassbinder-Orth, 2014; but see also Jackson et al., 2014), and in particular stickleback studies (Dittmar et al., 2014; Robertson et al., 2016a; Stutz et al., 2015), resulting in an extensive literature for methods and analyses to complement its usage. Whilst the trade-off of using qPCR is a lower number of genes analysed, the genes I have analysed here either come from immunological first principles (*tnfα*, *stat4*, *stat6*, *cmip*, *tbet*, *foxp3a*, *rorc*), which were developed and published by Robertson (2016) and Robertson et al.

(Robertson et al., 2016a) respectively, or have been demonstrated to be important for stickleback immune responses to parasites included here such as *Gyrodactylus* (*muc2*) (A. El Nagar, A.D.C. MacColl unpublished data). Thus, given that the questions addressed in this thesis are novel in the literature (Chapters 3-4), or are novel in their level of scope (Chapter 2), qPCR of targeted candidate genes represents an accessible means of confidently quantifying immune variation in a non-model.

6.3 Sources of variation for immune gene expression

To explain variation in immune gene expression, I measured several factors of individual stickleback that may have an effect. Put simply, these factors fall into three groupings: parasites, host condition and population effects. The significance of these grouped factors will be discussed across results from chapters 2-4.

Immune responses are adaptive responses to parasitism in the wild, and thus measures of infection should associate with immune gene expression. In chapter 2, infection with the endoparasitic cestode *Schistocephalus solidus* resulted in lower levels of relative expression for all five immune genes measures. Additionally, across all five lochs I found that infection with *S. solidus* reduced relative expression of inflammatory *tnfα* expression and increased relative expression of the other four genes. However, these results may reflect immunomodulation by the parasite as opposed to an adaptive response of the

host, in keeping with previous studies from the literature (Scharsack et al., 2004, 2007b, 2013). My results here also complement the findings of Robertson et al. (Robertson et al., 2016a), in which *S. solidus* was implicated in immunosuppression of Th1 type responses in fish of good condition. Modelling of Schistosomatic Index (weight of *S. solidus* worm(s) relative to somatic weight of fish) by relative gene expression variables in infected individuals failed to reveal any clear linear relationship between infection and gene expression. Unfortunately, *S. solidus* was largely absent from stickleback sampled from loch Ciste in chapter 4, thus comparisons here are unavailable.

Gyrodactylus arcuatus (chapter 2) and *Gyrodactylus gasterostei* (chapter 3) were both included as potential sources of variation for gene expression. In chapter 2, *G. arcuatus* burdens were less important than other factors such as source population in explaining expression variation. However, measures of parasitism, in particular prevalence, varied substantially between the five lochs sampled, a result mirrored by previous studies on North Uist (De Roij & MacColl, 2012; Rahn et al., 2016a; Young & MacColl, 2017). Given that source loch was often a dominant factor in expression variation, population-level differences in gene expression may correspond to variable prevalence or variable virulence (Mahmud et al., 2017) of *G. arcuatus*. There were relationships between expression and *Gyrodactylus* elsewhere however. In chapter 3, *Gyrodactylus*-resistant bred fish displayed more inflammatory expression profiles, and suffered lower burdens of *G.gasterostei* in laboratory infections. Here, there was a linear relationship between inflammatory gene

expression and *G. gasterostei* burdens. Whilst *G. arcuatus* was largely absent from loch Ciste sampled in chapter 4, there was another ectoparasite prevalent, the copepod *Thersitina gasterostei*, which infects the gills of stickleback. Here, results were in agreement with chapter 3, as fish with lower inflammatory gene expression harboured increased *T. gasterostei* burdens.

Discrepancies in the data for *Gyrodactylus* and *Thersitina* resistance may reflect pitfalls involved with comparing gene expression from fish in the wild and fish under controlled laboratory conditions. The importance of inflammatory signalling for *Gyrodactylus* resistance is well established (Buchmann, 1999) and has been demonstrated in other teleosts, such as rainbow trout, *Oncorhynchus mykiss* (Lindenstrøm et al., 2003, 2004) and atlantic salmon, *Salmo salar*, (Lindenstrøm et al., 2006). Similar changes in inflammatory gene expression are deployed by the carp, *Cyprinus carpio L*, in response to the ectoparasitic protozoan *Ichthyophthirius multifiliis* (Gonzalez et al., 2007), and by hosts in the early stages of sea lice (copepod) infection (reviewed by (Fast, 2014)), suggesting these responses may be typical of teleost hosts coping with ectoparasites. In the wild, such patterns become difficult to detect, given that immune response to *Gyrodactylus* sp. vary over the course of infection (Lindenstrøm et al., 2003, 2004; Robertson et al., 2017b), and a single time point attained through wild sampling provides no evidence regarding stage of infection. Thus, wild sampling results may be misleading as fish with low burdens will represent infections at both the early and late stage. Additionally, wild fish are undoubtedly subject to multiple infections, which is

confirmed by the data from chapters 2 and 4, as well as studies of the parasite communities on North Uist (De Roij & MacColl, 2012; Rahn et al., 2016a; Young & MacColl, 2017). This further confounds my ability to demonstrate relationships between immune gene expression and parasite burdens of individual parasites in wild individuals, as immune gene expression interacts with other parasites at various stages of infection at the same time. Robertson et al. (Robertson et al., 2016a) does describe an increase in innate immune gene expression (*tnfα* and *il1β*) in *G. arcuatus* individuals sampled from the wild in North Uist, but only when analysing a single loch (Obissary) in isolation. Across lochs, this study mirrors my findings of chapter 2, as *G. arcuatus* was generally a poor explanatory factor for immune gene expression.

Host condition was approximated as a qualitative score of reproductive investment (chapters 2-4), adiposomatic index (chapters 2-4) and hepatosomatic index (chapters 3-4). In chapter 2, there was an effect of reproductive condition of males, whereby immature males (score = 1) displayed increased relative expression of all five assay genes compared with partially (score = 2) or fully reproductive (score = 3) males. Males displaying red nuptial colouration also displayed increased relative *tnfα* expression in comparisons between fish of contrasting life stages, further suggesting that immune responses vary with measures of reproductive condition. Reproductive condition was affected significantly by photoperiod treatment in chapter 3, and was likely an important explanatory factor for immune gene expression differences seen between post-reproductive and early reproductive

stickleback from summer and winter photoperiods respectively. With regards to gene expression, I observed post-reproductive fish to display reduced expression of an important mucosal immune gene *muc2*, and sex-dependent changes to correlated inflammatory gene expression. Whilst both chapters agree reproductive condition influences gene expression, a crucial difference is the interaction with age. Experimental design in chapter 3 removed the possibility of age-related effects, however these may confound results from chapter 2 due to the likelihood of sexually immature fish being younger. In chapter 4, I observed no effect of reproductive condition on immune gene expression, although there was an effect in which fully reproductive individuals displayed higher burdens of *T. gasterostei* in freshwater ecotypes.

With regards to host condition, in chapter 2, I observed little influence of adiposomatic index on immune gene expression when including this variable as an explanatory factor in models, although adiposomatic index was lowest in males that scored highest in qualitative reproduction measures. In chapters 3 and 4, adiposomatic index and hepatosomatic index were instead modelled as dependent variables, to understand how they varied alongside gene expression changes; as opposed to defining a directional relationship between condition and expression. In this instance, I observed that changes in host condition because of photoperiod treatment (chapter 3) were closely associated with changes in *muc2* expression in the skin, which was important for *G. gasterostei* resistance. Additionally, I found that females and susceptible bred fish were in better condition than males and resistant bred fish. These observations

complimented differences in inflammatory gene expression between sexes and populations, which showed males and resistant bred fish to be more inflammatory. This opens the door to the possibility that reduced condition may be associated with increased inflammation. Such a hypothesis is supported by literature findings that suggest stressed individuals, such as those undertaking migration, utilise inflammatory responses (Hegemann et al., 2012) and is consistent with the notion that adipose tissue and adipocytokines modulate inflammation (Tilg & Moschen, 2006). However, such a finding would contradict predictions by Lee (Lee, 2006) that stressed individuals within species should downregulate inflammation. In chapter 4, anadromous ecotypes were in better condition than both freshwater ecotypes according to both indices of condition. Unlike my results from chapter 3, here there was little evidence for a close association between condition and *muc2* expression in the skin. Similarly, anadromous fish displayed the most inflammatory expression profiles (high splenic *rorc* and high *tnfα* expression variables) despite being in the best condition of the three ecotypes. This contradiction between results may reflect difficulties with comparing laboratory studies and wild sampling as described previously. Alternatively, expression differences between anadromous and freshwater ecotypes may reflect divergent selection regimes between anadromous and freshwater fish. There is evidence to suggest anadromous stickleback do display diverged immune responses compared with freshwater fish (Milligan-Myhre et al., 2016; Robertson et al., 2016a). As freshwater ecotypes were of similar condition, I may not expect to see condition-dependent expression differences here, however it is worth

noting that expression differences in the laboratory can be more heterogenous than those observed in the wild, even from fish sampled from the same populations (Robertson et al., 2016a).

The final source of variation for immune gene expression should be genetically-determined differences between populations. Within this thesis I include three scenarios in which population-level effects may be important: 1) Different populations in different environments (chapter 2); 2) Different populations in a shared, controlled environment (chapter 3); 3) Different populations (ecotypes) in a shared, natural environment (chapter 4). Population structuring is strong between lochs on North Uist (Magalhaes et al., 2016; Rahn et al., 2016b), thus in relative allopatry divergence should be facilitated. Indeed, in chapters 2 and 3, source population was consistently a dominant source of variation for immune expression variables. Similarly, ecotype was a dominant source of variation in chapter 4, suggesting that even in the face of gene flow there remains divergence between immune responses. In chapter 2, differences between lochs are likely also linked to differences in available resources (T. Chitheer, A. Rahman and A.D.C MacColl, unpublished data) and parasite communities (De Roij & MacColl, 2012; Rahn et al., 2016a; Young & MacColl, 2017). However, there is also good evidence that population effects are genetically determined, given the close relationship between expression variation and genetic variation around qPCR genes, as well as the linear relationship between genetic variation around 'immune system process' GO genes and life history strategy.

The common garden design employed in chapter 3 acts to minimise the effect of environmental influence on gene expression, and here I found strong differences between populations for immune gene expression, particularly for inflammatory genes. The real-world implications of these differences should be interpreted with caution, given the role of the environment in altering and potentially homogenising immune responses (Lenz, 2015; Robertson et al., 2016a; Stutz et al., 2015).

In chapter 4, I showed that fish of contrasting ecotypes display diverged immune responses despite being sampled from the same loch. This result may stem from different ecotypes occupying different niches within loch Ciste, and thus experiencing differential habitat-specific environmental variation. For example, Stutz et al. (2014) report repeated patterns of habitat-specific infection rates of benthic and limnetic stickleback ecotypes within lakes. There is however evidence to support the notion that armour plating morphology and immune response divergence are genetically linked. Robertson et al. (2017a) demonstrated this by separating out *eda* haplotypes from genetic background in F2 crosses and finding an association with immune responses and parasite resistance. Major adaptation genes such as *eda* are highly resistant to recombination (Marques et al., 2016; Samuk et al., 2017), thus immune responses associated with this haplotype persist in the face of gene flow and hybridization. Given our ecotypes varied in their plate morphs, this association between *eda* and immune responses is a probable source of some of the

variation for diverged immune responses between ecotypes in the Ciste hybrid zone.

My results from chapter 5 emphasise that many genes exhibit allele frequency changes across prevalence gradients for *S. solidus* and *G. arcuatus* in freshwater lochs across North Uist. This compliments the notion that populations should display diverged adaptive responses to parasitism. In addition, there is evidence that these adaptive responses are repeated across independent radiations, and thus probably biologically significant. That said, given the strong covariance between variation in parasite prevalence and water chemistry, it is difficult to ascertain specific genes that are evolving directly in response to parasitism or indirectly through selection along water chemistry gradients.

6.4 Unknown variables and potential effects

An important part of sampling populations in the wild is an appreciation of the unmeasured. The complexity of natural systems is a double-edged sword, producing the only 'real-world' data available whilst also creating noise and uncertainty. Natural environments are more stressful and variable, thus the manifestation and measurement of costs and trade-offs within the immune system can often only occur under natural conditions (Boughton et al., 2011; Martin et al., 2011; Pedersen & Babayan, 2011). The previously discussed sources of variation were measured to collect as much data as possible to

explain natural variation in immune phenotypes. However, further sources of variation were outside the scope of this thesis and warrant further discussion with regards to their effects, and avenues of future research that they present.

Whilst macroparasites were recorded, microscopic parasites were not, and have not been in previous studies of parasites on North Uist (De Roij & MacColl, 2012; Magalhaes et al., 2016; Rahn et al., 2016a; Robertson et al., 2016a; Young & MacColl, 2017). By microparasites, I refer specifically to intracellular pathogens associated with Th1-adaptive responses (Long & Nanthakumar, 2004), such as bacteria, viruses and protozoa. There has been little work undertaken regarding stickleback microparasites, relative to the macroparasite literature, most likely due to the difficulties involved in sampling microparasites. Moving forward, microparasites can be screened using classical culturing methods or through molecular techniques such as PCR-identification or qPCR-quantification (Boughton et al., 2011). This gap in our knowledge is important given both the sickness responses induced by microparasites and the interactions between microparasites, the immune response, and macroparasites. A prominent example involves the interaction between *Schistosoma* infection and increased susceptibility to HIV in humans (Chenine et al., 2008; Mbabazi et al., 2011). Associations may also be negative however, such as the negative relationship between bovine tuberculosis (TB) prevalence and gastrointestinal nematode prevalence in free-ranging African water buffalo, *Syncerus caffer* (Jolles et al., 2008). Interactions between intracellular microparasites and extracellular macroparasites can occur within the

vertebrate immune system due to antagonistic polarization between Th1 and Th2 T-cell differentiation (Long & Nanthakumar, 2004), which shows conservation across other vertebrate taxa such as birds (Degen et al., 2005). Without a comprehensive understanding of microparasite communities and their possible interactions, it may be difficult to draw accurate conclusions between immune responses and macroparasites from sampling data.

Microparasites may also be important for the data presented here for several reasons. Consequent microparasite infection following skin disruption by *Gyrodactylus* sp. (Cone & Odense, 1984) should be considered an additional source of mortality, and thus selection for host fish. The strength of selection on host immune responses to *Gyrodactylus* sp. should be linked to associated microparasite virulence, and thus a knowledge of microparasite communities would aid in understanding population-level differences in *Gyrodactylus* resistance. Microparasitic seasonality may also be important for explaining seasonal immune responses observed in chapter 3. Whilst these could not be attributed to *G. arcuatus* seasonality in the wild, seasonality of unknown microparasites may select for seasonal immune responses that subsequently increase susceptibility to *Gyrodactylus*. Seasonality of Vibrionaceae bacteria has been observed in marine stickleback (Schade et al., 2016), therefore microparasite seasonality is possible in this system.

In this thesis, Th1-adaptive responses were quantified through expression of *stat4* and *tbet*. The former is an important transcription factor for the development of Th1 cells (Kaplan, 2005), whilst the latter is a

transcription factor involved in regulating Th1-associated cytokines such as IFNγ (Lazarevic et al., 2013). In chapter 3, baseline splenic *tbet* expression was greater in stickleback bred from a population in which *G. arcuatus* is present compared with where it is absent; consistent with the idea that Th1 responses may be linked to *Gyrodactylus* infection in the wild. Splenic *tbet* expression also varied between ecotypes in chapter 4, highlighting that freshwater ecotypes were polarized towards *tbet* expression in the *tbet-rorc* antagonism (Lazarevic et al., 2013), whilst the opposite was true of anadromous fish. Microparasite infection rates between marine and freshwater fish may explain these differences, and thus their sampling in the future is of great interest. There was generally minor variation in *stat4* expression across all studies, although allelic variation of 2 SNPs associated with this gene was linked with overall gene expression variation between lochs of contrasting life history (chapter 2).

I also present my findings with little understanding of variable microbiomes (the collective community of commensal bacteria on host surfaces) between populations and individuals, and their effects on immune responses. The interaction between the microbiome and immune responses in the wild is currently a blossoming field of research. Organisms acquire their microbiota either directly from their parents or through contact with their environment, through ingestion for example (Boughton et al., 2011). Microbiomes are important for both the development and regulation of vertebrate immune responses (Hooper et al., 2012; Round & Mazmanian, 2009), and their presence or absence can influence costs of infection. For

example, the experimental removal of skin surface microbiota from redback salamanders, *Plethodon cinereus*, results in a 3-fold increase in morbidity associated with Chytridiomycosis (Becker & Harris, 2010). Moreover, commensals may compete with pathogenic microbes for resources, with competition impeding colonization by the pathogen. For example, Kamada et al. (2012) observed that germ-free mice suffered a 10-fold increase in infection intensity of the bacterial pathogen *Citrobacter rodentium* compared with pathogen-free reared mice, and were unable to clear the infection. This ability of the microbial commensals to outcompete the pathogen was confirmed experimentally through their growth on carbohydrates (Kamada et al., 2012).

In nature, microbiomes vary between populations and between life stages (Evans et al., 2017). Population-level variation reflects a combination of environmental factors associated with colonization and prevalence of commensals (summary for fish in (Ghanbari et al., 2015)) and interactions with the host immune genotype. For sticklebacks, microbiota of the gut may be accumulated through contact with microbes in the water, or through ingestion. Smith et al. (2015) suggest the latter may be a more significant contributor, as among-lake variation in stickleback gut microbiome closely associates with among-lake variation in invertebrate prey microbiome. However, within-lake variation exists as well, highlighting that habitat use and niche separation are important determinants of gut microbiome (Bolnick et al., 2014b; Smith et al., 2015). Smith et al. (2015) also note that among-lake microbiome variation correlates with among-lake genetic distance, which may reflect differences in

host immune genotypes, eg. MHC diversity and mucosal immunity (Bolnick et al., 2014a) or genetically based differences in host ecology (Bolnick et al., 2014c). Crucially, gut microbiome communities do influence the expression of immune genes in stickleback (Small et al., 2017).

In chapter 2, I sampled wild fish from five separate lochs. Here, microbiome variation should be substantial, due to both population genetic variation and lochs varying abiotically (Magalhaes et al., 2016) and in terms of prey communities (T. Chitheer, A. Rahman and A.D.C MacColl, unpublished data). Similarly, I expect microbiomes to vary between ecotypes in chapter 4, through potential niche separation in loch Ciste and genetic divergence, although it would be interesting to note how the effect of the latter is influenced by hybridization in this hybrid zone. Additionally, recent studies have highlighted that marine stickleback mount a stronger inflammatory response against resident gut microbiota (Milligan-Myhre et al., 2016), which should influence persistence of microbes and community assemblage of anadromous stickleback when compared to freshwater ecotypes. Abiotic variation in variables such as salinity is highly important for microbe community assemblage (Lozupone & Knight, 2007), and in light of this, microbiomes may vary along the predicted salinity gradient in loch Ciste moving inland. Additionally, if microbiome communities vary across salinity gradients, then microbiomes represent an additional source of selection to be interpreted within my parallelism analysis (chapter 5). I may therefore find genes associated with salinity, or other variables, to be evolving in relation to

microbiome-related traits such as nutrition or immune responses. Whilst my laboratory-reared fish from chapter 3 should have minimal microbiome variation (although some is expected due to the difficulties in rearing germ-free stickleback (Milligan-Myhre et al., 2016)), how well these results translate to the wild should be influenced by microbe community variation. These effects are likely a causative factor in transplanted stickleback displaying environment-specific immune responses (Stutz et al., 2015). However, it is interesting to note that Robertson et al. (2016a) report the immune responses of wild-sampled fish to be more similar at the population-level than laboratory comparisons, given that we would expect microbiomes to vary substantially more in wild than laboratory comparisons.

To conclude, micro-organisms, both pathogenic and commensal, are expected to significantly influence immune responses. Our understanding of the interactions between both groups of microbes and wild immune variation (commensals in particular) is lagging substantially behind other known sources discussed previously. The continual development of sequencing technologies will aid in their study and incorporation into ecological immunology (Ghanbari et al., 2015). The work presented highlights areas in which future studies may wish to assess the influence of micro-organisms on stickleback immune variation.

6.5 The relevance of findings across taxa

The North Uist stickleback system represents an excellent model system for asking questions of an evolutionary nature. This is due to several factors discussed in depth in chapter 1 (section 1.6). Briefly, the rapid evolution of numerous and diverse adaptive populations in response to measurable environmental variation has provided a natural evolution experiment, whereby the end results (current freshwater populations) and starting point (ancestral marine) are known. However, how applicable are results from this system to other taxa?

From an immunological perspective, stickleback as teleost fish possess a full vertebrate immune system with adaptive and innate responses (reviewed extensively, for example in (Foey & Picchietti, 2014; Uribe et al., 2011; Wilson, 2017; Zapata et al., 2006; Zhu et al., 2012)). Generally, this makes stickleback a good model for studying the evolution of vertebrate immune responses. Whilst the vertebrate immune system is generally well conserved (summary in (Wilson, 2017)), for example in features such as toll-like receptor (TLR) homology, complement activation, antimicrobial peptides (AMPs), chemokines, and some cytokines (Zhu et al., 2012), there are differences that warrant discussion. Chiefly, whilst teleosts do possess features of an adaptive immune response, the emphasis falls on innate immunity (Foey & Picchietti, 2014). This is demonstrated by a richer abundance of innate immune genes and networks in teleosts than in mammals (Zhu et al., 2012). This occurs in part due to lower temperatures in aquatic environments and the poikilothermic nature

of fish reducing the reaction time of adaptive processes (Uribe et al., 2011; Whyte, 2007), but also due to the nature of infection in aquatic environments. Innate responses are characterized as being non-specific and fast-acting, relative to adaptive responses. This is beneficial in aquatic environments that result in the constant exposure of teleost surfaces to various parasites such as bacteria, viruses, fungi and macroparasites. There is an emphasis therefore on barrier and surface defences, which can be compartmentalized into physical barriers (comprised of mucus and microbiomes), innate cellular responses and humoral responses (Uribe et al., 2011).

This emphasis on innate immune responses may explain why consistently in measuring the immune responses of stickleback, I failed to find significant variation in expression of several genes associated with adaptive immunity, such as *cmip*, *stat4*, and *stat6* (chapters 2-4). Conversely, expression of *tnfα* (associated with innate inflammatory signalling) (chapter 2-3) and *muc2* (encodes mucin 2, associated with mucosal immunity) (chapters 3-4) were consistently highly variable. In comparing the findings presented here to terrestrial vertebrate systems therefore, caution must be taken regarding the dependence on innate immune responses, which may downplay the potential for variable adaptive immune responses.

Ecologically, I expect there to be differences regarding the structuring of aquatic systems compared with terrestrial systems. The lochs of North Uist provide discrete study sites in which fish sampled show high-levels of population structuring (Chapters 2 and 5) (Magalhaes et al., 2016; Rahn et al.,

2016b). The combination of physical barriers to gene flow between populations with contrasting environments, with potential interactions between habitat area and population size, has promoted rapid adaptation. For example, where population sizes are restricted by area, novel mutations and alleles at low standing frequencies have a greater chance of proliferating through populations, promoting adaptation. These features have resulted in the rapid evolution of many traits, including those associated with immune responses (Eizaguirre et al., 2012; Marques et al., 2016; Terekhanova et al., 2014). The discrete nature of lake systems also aids in quantifying environmental variation, such as abiotic variation (Magalhaes et al., 2016; Mahmud et al., 2017) or differences in parasite communities (De Roij & MacColl, 2012; Rahn et al., 2016a; Young & MacColl, 2017). The level of diversity observed between stickleback populations is perhaps unique, which makes studies into genomic parallelism such as chapter 5 irreplicable in other vertebrate systems. Taken together, these features make stickleback an exemplary system for studying questions about population-level variation in evolved immune responses.

In contrast, terrestrial systems should be far less discrete. For example, prominent mammalian study systems for ecoimmunology include wild field voles, *Microtus agrestis*, in Kielder Forest (Northumberland, UK) (reviewed by (Turner et al., 2014)) and Soay sheep, *Ovis aries* (St. Kilda, Scotland, UK) (Clutton-Brock & Pemberton, 2004). In the absence of discrete population structuring (Turner et al., 2012), these systems have sought to investigate the evolution of wild immune variation longitudinally from a single population

perspective. This has advantages in providing insights into cyclic patterns and the effects of annual environmental variation and how selection changes over time (Clutton-Brock & Sheldon, 2010). However, these study systems are limited by an inability to replicate observations at the population-level.

A significant characteristic of stickleback systems is the inability to quantify the immune response of a single individual over time. This is due to the destructive nature of current sampling methods, and small size of sticklebacks limiting extraction of blood samples. Populations or groups can be sampled over time, through random sampling of subsets of individuals at time points, as in Robertson et al. (2016b), however this requires much larger starting population sizes, which may be unworkable in laboratory studies such as chapter 3. The ability to sample individuals over time is particularly informative for understanding factors associated with within-individual variation in immune responses, such as condition (Nussey et al., 2014) and age (Jackson et al., 2014; Schneeberger et al., 2014). Such sampling has been undertaken in mammalian systems. For example, Nussey et al. (Nussey et al., 2012) used longitudinal sampling of individual Soay sheep to reveal age-related declines in T-cell subsets and inflammatory markers in wild individuals.

The stickleback system therefore is particularly informative for population-level inference, however is limited in terms of individual level variation. Research in this field should be viewed as complimentary to research of terrestrial mammal systems, in which longitudinal sampling of individuals

and individual populations is informative in other regards, but limited by an absence of population-level variation.

6.6 Concluding Remarks

In conclusion, I have examined several relationships between immune responses and other evolutionary processes. In doing so, my hope has been to demonstrate that immune responses can fall predictably in line with facets of evolutionary ecology that are well studied. Empirical support presented here reinforces hypotheses regarding how variation in immune responses evolves and is maintained in wild populations, and how costs and trade-offs mediate such variation. Several aspects of this thesis represent novel work within the field. In analysing the relationships between life history and immune responses, I have provided some of the most evolutionarily-relevant data in the literature. The was achieved by combining measures of phenotype with genomics to demonstrate that immune variation between life history strategies is repeated across populations and likely heritable. My analysis of immune DMIs in wild hybrid zones is the first in the literature to examine wild animal hybrids and highlights that the influence of immune DMIs may be less substantial than laboratory studies predict. My common garden experiment is also the first in the literature to examine the causative relationships between experimental photoperiod-modulation of immune responses and host-parasite dynamics during infection. Here, I provide compelling evidence that seasonality of immune responses, mediated through daylength cues, directly influences

seasonal infection dynamics. This study has significant implications for the fields of parasite evolutionary biology, disease epidemiology and aquaculture. Finally, I demonstrate that adaptive evolution along environmental gradients, including parasitism, is underwritten by common genomic changes more often than expected by chance. This study validates the notion that evolution is repeatable, although repeatability is associated with factors such as shared genetic variation and environmental structuring. Common changes in the genes underlying adaptive evolution reinforce the notion that the findings within this thesis should be repeated outside of the North Uist stickleback system, and that my observations regarding the evolutionary relationships between immune responses and other processes are potentially prevalent across wild, natural environments.

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APPENDIX

1.1 BBSRC DTP Statement

The University of Nottingham BBSRC Doctoral Training Partnership (DTP) is undertaken in partnership with Rothamsted Research. Its aim is to develop an innovative, integrated, student-led programme of individualised research training in the Biosciences through an effective cohort-based programme. Students are not recruited to an individual project but, through a series of lab rotations, identify a PhD programme that they wish to pursue in one of three BBSRC priority research areas (Food Security; Industrial Biotechnology and Bioenergy; World Class Biosciences)

Research Training Structure

The research training structure is framed by individual development needs analysis and has the following core components:

- Generic and research skills development
- High-level modular training
- Laboratory rotations
- Experiential learning through public engagement and outreach activity

Generic and research skills development comprises the following: induction, introductory training programme, annual 'Spring School' conference-style training event, participation in Sysmic (online systems and mathematical biology course), specialist training days at partner organisations, twilight (after-lab) training which is arranged on an *ad hoc* basis to meet identified needs, and seminar series.

In Year One there is an initial period of intensive training which takes place over the course of the first six months. This comprises a two-week introductory training programme, 24 weeks of laboratory rotations, followed by a week-long 'Spring School' (conference-style training event for all DTP cohorts) and ending in a science outreach event. Each lab rotation is assessed focussing on a different form of scientific communication (Report, Poster, Presentation) and students receive feedback from supervisors. The PhD project begins in Month 7. Students are also encouraged to choose from a portfolio of relevant Master Courses which they can attend alongside their lab rotation projects.

In Years Two to Four training is mainly concentrated in the week long annual Spring School, although additional training and seminars are delivered where required throughout the year. A three month Professional Internship for PhD Students (PIPS) placement is usually taken in Year Three and, as BBSRC specifies, this is not in an area related to the students' PhD research programme.

The integrated programme of training is mapped to BBSRC priority areas, BBSRC Skills Statement: Scientific and Core Skills for UK Bioscience and the Researcher Development Framework (RDF) to ensure breadth and depth of provision, and to encompass vulnerable skills and strategic competencies. It builds on the best practice principles outlined in the Statement of Expectations for Doctoral Training, RCUK (2013) and the 'Good Practice Elements in Doctoral Training', LERU (2014).

It offers a wide range of skills development opportunities to prepare students for a successful bioscience-related career and supports the exchange of ideas and experiences within and across cohorts. It also generates a strong community of postgraduates and supervisors to stimulate the formation of cross-disciplinary links through cohort development, which prepares students for a career in an increasingly multidisciplinary environment.

1.2 Professional Internships for PhD Students Reflection Form

Name of Organization

The Royal Institution

Details of Placement

Please describe your main activities during the placement (150-200 words)

My role involved assisting on all media-related aspects of the Christmas Lectures 2015 “How to survive in space”, as well as other media jobs within the RI. Such tasks involved devising pitches for media pieces to promote the Lectures, which would involve devising ways of disseminating the science within the lectures to capture people’s attention. I also wrote press releases for events and giveaways and I led on the media promotion of the online accompaniment to the Christmas Lectures; the daily “Advent” promotions. I was also involved with day-to-day management of the RI’s media management including media monitoring and social media. In addition to organizing articles to be written by the Christmas Lecturer I also did some ghost-writing of articles published under the names of other RI staff.

Placement Achievements

Please detail all outcomes from the placement, including any publications, presentations given and reports written etc. (150-200 words)

During our media campaign, we achieved promotional and educational material that I was directly involved with in:

Laboratory News: “Over the moon for extreme lecture”

BBC News: “Medic Kevin Fong explains how space harms your body”

TES Global Education: “It’s the final countdown… lift off to space odyssey”

The Metro: “The Great British Take-Off”

Observer: “Humans to conquer Mars and beyond? Here’s how…”

Buzzfeed: “13 ways your body can go wrong in space”

BBC Focus: “The Royal Institution Christmas Lectures”

Independent: “The man who wants to put kids in space”.

My ghost-writing was published in Huffington Post and The Memo.

I also authored a report on the Royal Institution’s unconference entitled “Is science a land of equal opportunities?”. The event focused on young people discussing their views on equal opportunities in STEM careers across gender, race,

religion and economic background. The report was published online and given to the science minister Jo Johnson as part of the RI's 2015 annual review.

My blog of my experiences is published on the RI's blog entitled: "From the lab to the front page".

Skill development

Has this Placement helped you developed any new skills or enhanced your previous skill set? (100-150 words)

My placement helped me to understand how science is communicated to the public. This included having to learn how to find interesting stories within the science itself, as well as finding exciting and interesting ways to present those stories, such as using animations, videos or other media. I also acquired more general skills, such as learning how to write press releases and how to find and engage with journalists. My placement helped me to see the value and enjoyment in the challenge of taking real, cutting-edge science and translating it into a medium that can be informative and of interest to anyone.

Future Work

Has this Placement influenced your future career aspirations? If so, in what way? (150-200 words)

This experience taught me about how to work within the media and communicate science efficiently. I will use this information throughout my career to communicate and disseminate future research into general media pieces for public consumption.

2.1 Sampling order effects

The effect of individual fish sampling order on gene expression was tested using a Pearson's correlation in the R package '*psych*' using 'holm' corrections for multiple testing. Sampling order of individual fish had no effect on relative

expression of immune genes when studying all fish from REIV and HOSTA for PC1 (Pearson's, r = -0.13, P = 0.38) and PC2 (Pearson's, r = -0.01, P = 0.93) and all breeding fish for PC1 (Pearson's, r = -0.12, P = 0.63) and PC2 (Pearson's, r = -0.08, P = 0.63).

2.2 Life history and sampling information for comparative analysis

Appendix 2.2: Sampling locations and life history information for lochs included in comparative analysis. Data acquired with consent from (Magalhaes et al., 2016; Rahman, 2017)

Loch	Latitude	Longitude	Older than 1 year (%)	Size at maturity (mm)	Absolute growth rate (mm year^{-1})	Age at maturity	Life History PC1
AONG	57°39"N	7°16"W	2.94	29.22	27.94	1.04	-1.82
AROI	57°35"N	7°25"W	8.82	48.41	41.99	1.40	2.14
BHAR	57°34"N	7°17"W	11.76	30.66	28.51	1.00	-1.48
EISI	57°38"N	7°21"W	14.71	36.08	31.87	1.30	0.01
FADA	57°36"N	7°12"W	5.88	30.22	27.75	1.13	-1.50
GEIR	57°38"N	7°17"W	17.14	32.02	29.50	1.16	-0.79
GILL	57°36"N	7°24"W	16.67	42.27	34.79	1.40	1.08
HOST	57°37"N	7°29"W	3.03	47.68	36.56	1.00	0.54
IALA	57°37"N	7°12"W	66.67	37.99	30.27	2.04	2.91
MORA	57°34"N	7°16"W	11.76	34.22	31.21	1.20	-0.49
REIV	57°37"N	7°31"W	27.27	46.84	34.92	1.43	1.82
SCAD	57°35"N	7°14"W	14.71	34.21	31.58	1.18	-0.41
STRU	57°34"N	7°21"W	8.82	33.91	31.70	1.00	-0.95
TORM	57°33"N	7°19"W	0.00	32.14	29.19	1.00	-1.61
TROS	57°35"N	7°25"W	3.03	43.46	38.62	1.06	0.54

Values are population means

5.1 Sampling data for all adaptive radiations

Appendix 5.1: Sampling and environmental data for each population across four adaptive radiations

Population name	Code	Geographic region	Area	Date	Latitude	Longitude	N	Ca(mg/L)	Na(mg/L)	Zn(µg/L)	pH	Gyro[1]	Schisto[1]
Aonghais	AONG	North Uist	W	16.05.13	57°38'39.89"N	7°16'27.49"W	18	4.54	19.17	103.40	6.97	0.20	0.17
Mhic a'Roin	AROI	North Uist	E	09.05.13	57°35'27.90"N	7°25'10.86"W	19	2.90	24.32	56.18	6.48	0.66	0.03
a'Bharpa	BHAR	North Uist	S	14.05.13	57°34'23.33"N	7°17'32.19"W	18	1.47	18.63	82.63	6.03	0.43	0.51
na Buaile	BUAI	North Uist	NE	13.05.13	57°38'48.94"N	7°11'52.85"W	17	2.55	36.14	58.44	6.73	0.03	0.00
Chadha Ruaidh	CHRU	North Uist	SE	29.04.13	57°35'38.63"N	7°11'51.84"W	17	2.20	18.77	62.70	6.58	0.00	0.17
an Daimh	DAIM	North Uist	S	30.04.13	57°35'35.40"N	7°12'33.27"W	20	2.31	24.56	56.30	6.50	0.00	0.00
Eisiadar	EISI	North Uist	N	17.05.13	57°37'54.97"N	7°21'14.53"W	17	2.81	22.54	79.14	6.82	0.54	0.14
Fada	FADA	North Uist	E	17.05.13	57°37'3.69"N	7°12'33.92"W	15	1.81	17.29	90.41	6.71	0.17	0.00
nan Geireann	GEIR	North Uist	N	16.05.13	57°38'24.56"N	7°17'24.93"W	17	1.76	17.17	47.29	6.70	0.74	0.06
Mhic Gille-bhride	GILL	North Uist	W	09.05.13	57°36'7.11"N	7°24'35.29"W	17	2.71	21.85	46.04	6.80	0.88	0.16
Hosta	HOST	North Uist	NW	15.05.13	57°37'37.65"N	7°29'27.28"W	19	30.56	27.47	66.52	8.34	0.89	0.77
Iala	IALA	North Uist	E	16.05.13	57°37'11.28"N	7°12'20.41"W	18	2.73	21.40	65.90	6.36	0.14	0.00
na Moracha	MORA	North Uist	S	07.05.13	57°34'24.75"N	7°16'33.73"W	21	2.51	24.03	58.85	6.35	0.27	0.00
na Reival	REIV	North Uist	W	04.05.13	57°36'40.78"N	7°30'52.84"W	19	28.60	40.12	58.52	8.95	0.51	0.06
Scadavay	SCAD	North Uist	S	06.05.13	57°35'4.09"N	7°14'9.56"W	17	1.42	19.75	78.89	6.14	0.57	0.09

nan Strùban	STRU	North Uist	SW	17.05.13	57°33'24.72"N	7°20'29.98"W	16	3.72	25.05	93.42	7.06	0.69	0.42
Tormasad	TORM	North Uist	SW	01.05.13	57°33'48.37"N	7°18'55.03"W	19	4.01	25.43	86.88	6.84	0.29	0.03
Trosavat	TROS	North Uist	SW	19.05.13	57°35'3.85"N	7°24'48.40"W	20	2.85	26.12	93.32	6.71	0.69	0.03
Bakkatjorn	BAKK	Iceland	SW	02.06.2014	64° 9'19.29"N	22° 1'6.99"W	19	34.33	58.67	204.13	9.17	0.89	0.00
Eidarvatn	EIDA	Iceland	E	17.06.2014	65°23'17.26"N	14°21'35.94"W	19	5.13	5.04	229.69	7.73	0.71	0.29
Flodid	FLOD	Iceland	N	09.06.2014	65°29'27.95"N	20°21'33.59"W	20	5.58	6.07	194.57	8.05	0.77	0.69
Galtabol	GALT	Iceland	N	09.06.2014	65°15'40.89"N	19°44'24.30"W	19	6.02	5.01	203.80	7.70	0.69	0.89
Grettislaug	GRET	Iceland	N	13.06.2014	64°46'36.39"N	22° 0'6.89"W	19	2.49	49.94	227.63	8.59	0.14	0.00
Grjotarvatn	GRJO	Iceland	W	08.07.2014	64°46'36.39"N	22° 0'6.89"W	18	3.93	4.34	213.52	7.45	0.29	0.66
Holsvatn	HOLS	Iceland	W	26.05.2014	64°30'48.00"N	22° 8'43.28"W	18	3.57	10.52	191.94	7.53	0.66	0.57
Hredavatn	HRED	Iceland	W	26.05.2014	64°45'56.66"N	21°34'21.72"W	18	3.90	6.93	206.90	7.50	0.97	0.23
Kleifarvatn	KLEI	Iceland	SW	22.05.2014	63°55'27.55"N	21°59'56.42"W	16	9.88	10.76	186.01	7.44	0.46	0.65
Mjoavatn	MJOA	Iceland	N	09.06.2014	65°15'41.45"N	19°48'4.60"W	20	5.08	4.61	213.41	7.38	1.00	0.86
Myvatn-lava	MYVL	Iceland	NE	14.06.2014	65°37'41.49"N	16°55'31.57"W	19	22.46	42.50	211.87	8.47	0.91	0.37
Myvatn-mud	MYVM	Iceland	NE	14.06.2014	65°39'7.67"N	16°58'8.95"W	20	8.35	14.69	210.34	9.60	1.00	0.29
Sauravatn	SAUR	Iceland	W	26.05.2014	64°39'47.23"N	22° 7'32.61"W	16	4.91	13.40	181.47	7.08	0.14	0.66
Skorradalsvatn	SKOR	Iceland	W	30.06.2014	64°28'57.48"N	21°19'2.22"W	19	4.02	6.80	209.83	7.11	0.65	0.53

Thingvallavatn-lava	THIN	Iceland	SW	23.05.2014	64°14'38.59"N	21° 5'31.42"W	20	3.74	7.12	163.35	8.72	0.91	0.00
Urridavatn	URR2	Iceland	SE	17.06.2014	65°17'59.45"N	14°27'21.82"W	19	8.40	7.08	232.63	7.84	1.00	0.17
Urridakotsvatn	URRI	Iceland	E	19.05.2014	64° 4'5.41"N	21°54'34.08"W	19	5.69	18.73	223.85	8.05	1.00	0.03
Vífilsstaðavatn	VIFI	Iceland	SW	18.05.2014	64° 4'45.72"N	21°52'38.86"W	20	9.79	19.50	201.48	9.24	0.98	0.21
Ambrose	AMBR	B.C.	Sechelt P.	20.05.2015	49°44'4.70"N	124° 1'32.52"W	17	2.23	1.91	10.19	6.88	0.45	0.00
Beaver	BEAV	B.C.	Vancouver I.	12.05.2015	48°48'41.96"N	124° 4'51.38"W	18	3.87	1.21	31.20	6.82	1.00	0.04
Brannen	BRAN	B.C.	Vancouver I.	13.05.2015	49°12'51.16"N	124° 2'59.50"W	18	7.43	3.16	8.89	7.82	0.96	0.00
Bullocks	BULL	B.C.	Saltspring I.	10.05.2015	49°41'31.85"N	124°30'27.00"W	15	10.46	8.55	7.80	7.58	0.74	0.06
Cranby	CRAN	B.C.	Texada I.	29.04.2015	49°41'31.85"N	124°30'27.00"W	20	15.63	3.86	13.05	7.23	1.00	0.00
Dougan	DOUG	B.C.	Vancouver I.	12.05.2015	48°42'51.73"N	123°36'38.44"W	16	20.01	6.98	15.88	7.52	0.92	0.00
Errock	ERRO	B.C.	Fraser Valley	22.05.2015	49°13'28.73"N	122° 0'41.42"W	18	3.03	2.00	45.61	7.00	0.83	0.00
Garden Bay	GARD	B.C.	Sechelt P.	26.04.2015	49°39'3.62"N	124° 0'59.67"W	10	4.70	4.77	12.68	6.91	0.00	0.00
Hoggan	HOGG	B.C.	Gabriola I.	14.05.2015	49° 9'12.09"N	123°49'41.94"W	18	6.77	6.54	10.60	6.09	0.88	0.00
Hotel	HOTE	B.C.	Sechelt P.	25.04.2015	49°38'19.46"N	124° 3'9.01"W	18	2.86	4.99	9.13	7.36	0.00	0.00
Kennedy	KENN	B.C.	Vancouver I.	15.05.2015	49° 7'35.72"N	125°25'35.84"W	18	5.89	1.08	11.87	7.00	1.00	0.00
Kirk	KIRK	B.C.	Texada I.	01.05.2015	49°44'24.21"N	124°34'59.82"W	19	19.73	2.96	8.88	7.60	0.78	0.00
Klein	KLEN	B.C.	Sechelt P.	26.04.2015	49°44'20.34"N	123°58'0.93"W	18	5.10	1.49	7.85	6.65	0.00	0.00
Lily	LILY	B.C.	Sechelt P.	25.04.2015	49°36'44.02"N	124° 1'16.73"W	19	5.67	6.50	11.85	6.97	0.03	0.00

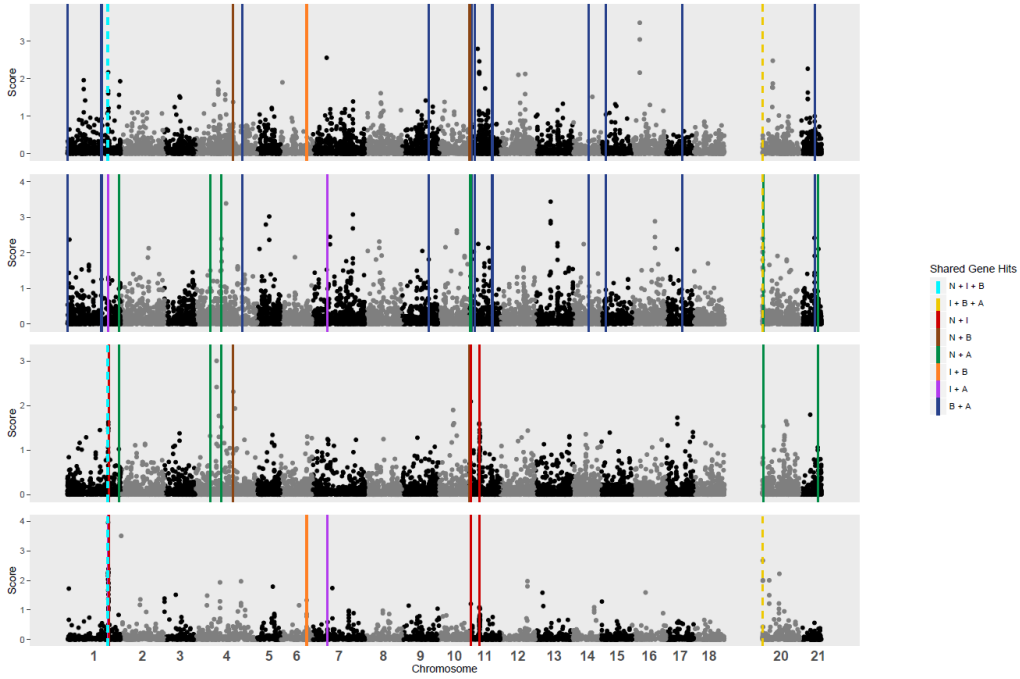
North	NORT	B.C.	Sechelt P.	26.04.2015	49°44'52.60"N	123°58'31.91"W	14	4.36	2.19	3.42	7.11	0.00	0.00
Sproat	SPRO	B.C.	Vancouver I.	15.05.2015	48°46'55.12"N	123°26'41.46"W	18	8.65	1.15	15.46	7.04	0.88	0.00
Stowell	STOW	B.C.	Saltspring I.	10.05.2015	48°46'55.12"N	123°26'41.46"W	17	8.99	5.94	8.91	7.61	0.48	0.00
Trout	TROUT	B.C.	Sechelt P.	25.04.2015	49°30'25.02"N	123°52'27.28"W	18	6.26	3.68	8.69	7.12	0.64	0.00
Arness	ARNE	Alaska	Kenai	15.06.2015	60°38'45.73"N	151°18'7.36"W	17	8.11	4.19	48.26	7.41	0.93	0.00
Arrow	ARRO	Alaska	Kenai	24.06.2015	60°45'1.29"N	150°29'21.07"W	15	0.74	1.80	31.58	6.92	0.90	0.00
Barley	BARL	Alaska	Mat-Su	08.06.2015	61°21'40.09"N	150° 5'1.20"W	18	42.21	2.75	8.34	8.37	0.97	0.10
Bear Paw	BEPA	Alaska	Mat-Su	03.06.2015	61°36'50.38"N	149°45'11.91"W	17	0.21	0.59	9.24	5.70	0.17	0.17
Big	BIGL	Alaska	Mat-Su	08.06.2015	61°31'58.86"N	149°50'3.79"W	15	21.96	3.55	5.08	8.07	0.90	0.07
Bruce	BRUC	Alaska		12.06.2015	61°36'31.97"N	149°33'4.77"W	16	1.11	0.66	6.78	6.61	0.86	0.00
Community	COMM	Alaska	Kenai	15.06.2015	60°42'8.72"N	151°23'1.32"W	17	0.33	1.84	26.34	6.80	0.77	0.07
Corcoran	CORC	Alaska	Mat-Su	05.06.2015	61°34'23.07"N	149°41'30.40"W	16	29.20	4.64	5.15	8.18	0.70	0.23
Daniel	DANI	Alaska	Kenai	15.06.2015	60°43'28.79"N	151°10'49.99"W	18	13.52	4.53	14.59	7.51	0.53	0.33
Duck	DUCK	Alaska	Kenai	15.06.2015	60°41'7.33"N	151°13'25.41"W	16	12.68	4.84	13.62	9.68	1.00	0.00
Jade	JADE	Alaska	Mat-Su	08.06.2015	61°31'29.25"N	149°52'1.50"W	18	0.83	0.65	15.43	6.57	0.42	0.17
Long	LONG	Alaska	Mat-Su	11.06.2015	61°34'33.76"N	149°46'29.47"W	18	12.84	2.43	4.97	7.80	0.83	0.03
Luci	LUCI	Alaska	Mat-Su	03.06.2015	61°34'11.93"N	149°28'53.26"W	18	29.94	11.98	7.56	8.17	0.89	0.17

Lynda	LYND	Alaska	Mat-Su	08.06.2015	61°34'13.44"N	149°50'24.75"W	17	11.64	2.01	7.69	7.42	0.47	0.00
Seymour	SEYM	Alaska	Mat-Su	03.06.2015	61°36'49.54"N	149°40'9.92"W	17	25.91	3.53	11.67	8.28	0.86	0.07
Tern	TERN	Alaska	Kenai	24.06.2015	60°32'2.70"N	149°32'49.59"W	18	32.13	3.04	7.94	7.60	0.77	0.30
Toad	TOAD	Alaska	Mat-Su	03.06.2015	61°37'12.12"N	149°41'56.11"W	19	0.60	0.69	6.64	5.99	0.57	0.27
Walby	WALB	Alaska	Mat-Su	22.06.2015	61°37'13.18"N	149°12'42.59"W	18	25.47	7.31	10.21	8.53	0.46	0.37
Y	YLAK	Alaska	Mat-Su	22.06.2015	62°18'21.42"N	150° 3'52.34"W	19	2.82	1.30	16.37	6.98	0.83	0.13

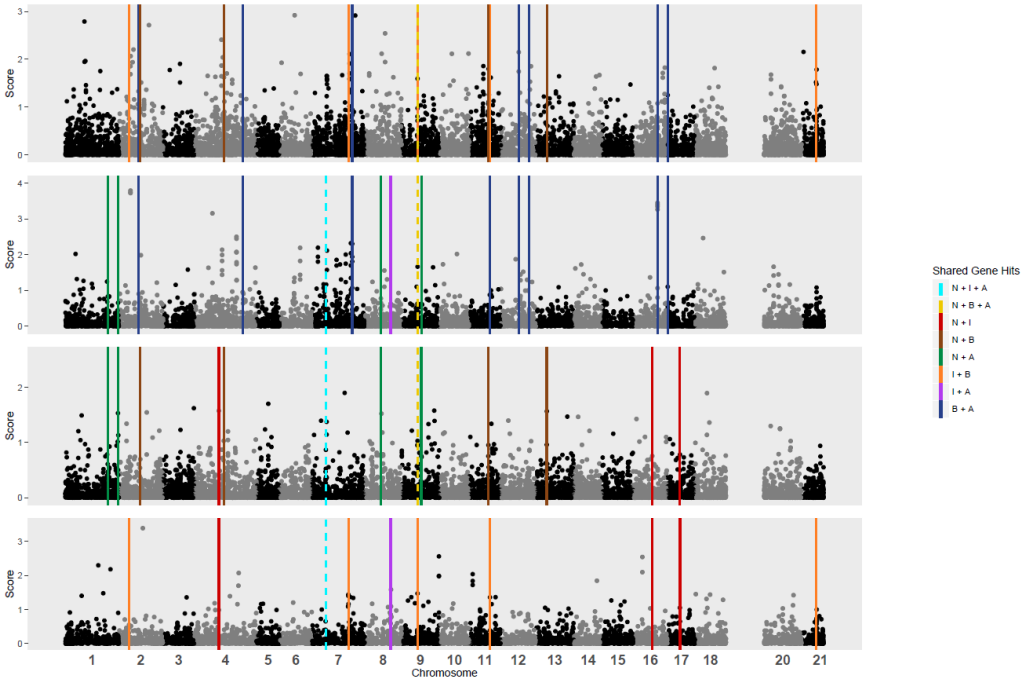
[1] Prevalence of parasite recorded as proportion of infected individuals

5.2 Manhattan plots for remaining 4 environmental variables

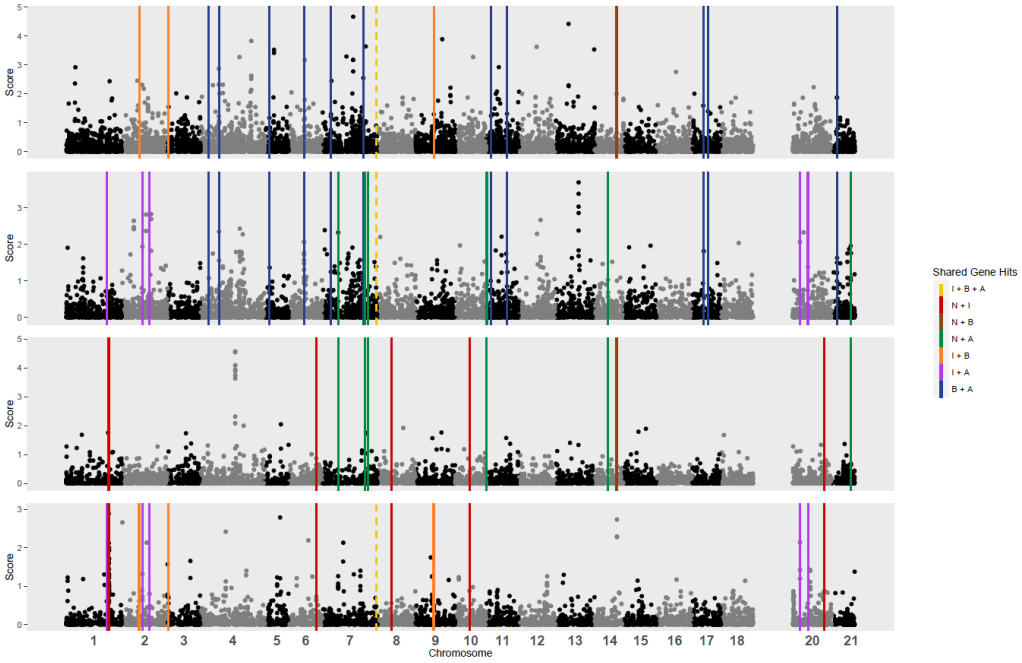
A)



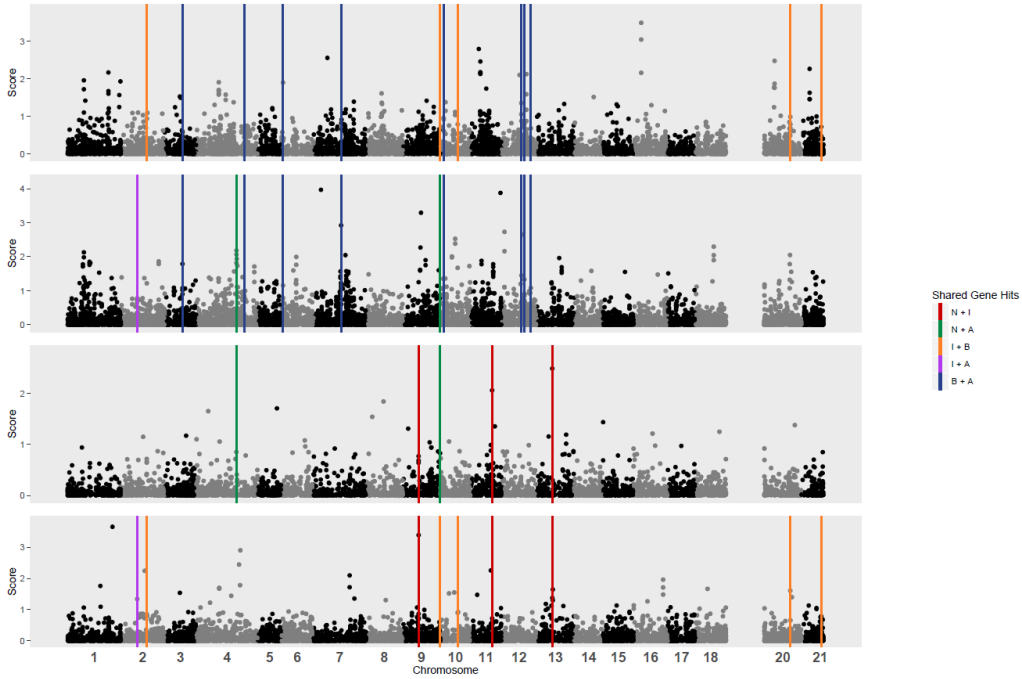
B)



C)



D)



Appendix 5.2: Manhattan plots summarizing correlations of allele frequency with Ca (**A**)**,** Gyro (**B**), pH (**C**)**,** and Zn (**D**) for each of the 4 adaptive radiations (from top to bottom: B.C, Alaska, North Uist, and Iceland). Points illustrate the score (log10 (BayesFactor) * Absolute (Spearmans P)) per SNP. Shared genes are highlighted by vertical coloured lines. Each line represents the middle bp of the shared gene (see Appendix 5.2 for Manhattan plots of 4 other environmental variables). Manhattans are ordered BC (top), Alaska, North Uist, Iceland (bottom).