Eco-immunology:

Thermal Variation and Parasitology

of the Three-Spined Stickleback

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A thesis submitted to Cardiff University for the degree of

Doctor of Philosophy (PhD) in the School of Biosciences

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Collaborators

The Leverhulme Trust funded this PhD and some of the key experiments presented in this thesis (Chapter 3 and 4) were outlined in this original grant held by my supervisors. Chapters 5 and 6 arose as extensions to the original grant with the experiments presented in Chapters 7 and 8 designed outside the original grant. Chapter 2 arose from the information gap identified during preparation of the other chapters, producing a single methods article that will hopefully be useful for furthering stickleback and associated parasite studies.

Initial *Argulus foliaceus* specimens and the *Saprolegnia parasitica* cultures were provided by Chris Williams and Amy Reading, and Pieter van West, respectively.

Chapter 2: AS wrote this chapter, with additional input and suggestions from Jo Cable. Experts commented on individuals sections: Chris Williams, the argulid section; Rachel Paterson the *Diplostomum* section; Pieter van West the *Saprolegnia* section; and Iain Barber the *Schistocephalus* section. Other authors may provide additional information before submission of this manuscript for publication.

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"It is well known that a vital ingredient of success is not knowing that what you're attempting can't be done."

Terry Pratchett

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Definitions of Terms

Biological terms

Term	Definition
Argulus foliaceus	Generalist ectoparasitic crustacean, commonly referred to as the fish louse.
AUC	Area Under Curve. A measure of parasitic infections with a time component. Calculated by plotting the parasite intensity over time and calculating the area under the curve produced.
Camallanus lacustris	Parasitic gut nematode, paratenic in sticklebacks. Definitive hosts include perch and pike, intermediate hosts include copepod species.
Definitive Host	Host in or on which a parasite reaches sexual maturity.
Diplostomum spp.	Digenean trematode occurs as a metacercaria in the lens or vitreous humour of the fish eye, the 2nd intermediate host. Piscivorous birds are the definitive hosts and snails the 1st intermediate hosts.
Ectoparasite	Parasite which lives on the exterior of a host e.g. on the skin or fins.
Endoparasite	Parasite which lives inside the body e.g. in the gut or body cavity.
Gasterosteus aculeatus	Three-spined stickleback. Ray fined fish in the Infraclass Teleostei of the Order Gasterostiformes.
Glugea anomala	Microsporidian parasite of the three-spined stickleback.
Gyrodactylus gasterostei	Monogenean ectoparasite of the three-spined stickleback.
Intermediate Host	A host required for larval development or growth of a parasite before it is infective to another intermediate host or the definitive host.
Parasite Intensity	Number of individual parasites of a particular species infecting a host.
Peak of Infection	The highest intensity of parasites found in/on a host over a period of time.
Prevalence	Number of individuals of a host species infected with a species of parasite. Expressed as a percentage.
Saprolegnia parasitica	Generalist oomycete freshwater pathogen that is both saprotrophic and necrotrophic. Causes white/grey cotton-like mould on fish.
Schistocephalus solidus	Cestode, plerocercoid stage in the 2nd intermediate host the three-spined stickleback. The definitive hosts are piscivorous birds and the 1st intermediate hosts copepods.
Sham Infection	Exposing an individual to an infection procedure without the inclusion of the infective stage of a parasite.
Time until Peak Infection	Period of time between date of an infection and Peak of Infection.

Immunological Terms

Term	Definition
cd8a	Cluster of Differentiation 8a, marker of cytotoxic T-cells.
IgM	Immunoglobulin M, tetrameric antibody in teleosts. First class of antibody usually secreted during an immune response, receptor of naïve B-cells. When referenced in immune data primer is specifically for the heavy chain.
IgZ	Immunoglobulin Z, referred to as IgT in some literature. Specialised to gut and mucosal immunity. Present in the serum as a monomer and in gut as a polymer. Anti-bacterial antibody of teleosts thought to be similar to human IgA.
foxp3b	Transcription factor localised to the nucleus, marker of regulatory T-cells.
il1r-like	Teleost cytokine receptor gene analogous to human IL-1R, marker of pro-inflammatory response.
il-4	Cytokine that is a marker of the Th2 immune phenotype, typically an anti-helminth response. Stimulates growth and differentiation of B-cells.
il-17	Cytokine that is a marker of the Th17 immune phenotype, marker of the pro-inflammatory response. In humans also linked to autoimmune diseases. Stimulates other pro-inflammatory cytokines such as IL-1.
il-12ba	Cytokine important in differentiation of Th1 T-cells.
orai1	Calcium release-activation calcium channel protein 1. Calcium ion channel necessary for T-cell proliferation.
β-def	B-defensin. Antimicrobial peptide active against bacteria, fungi and enveloped viruses.
tbk1	TANK-binding kinase 1. NFκB activator.
lyso	Lysozyme. Enzyme that digests bacterial cell walls, non-specific antibacterial.
tirap	Toll-interleukin 1 receptor domain containing adaptor protein. Protein involved in Toll-like receptor signalling and NFκB and JNK activation.
gpx4a	Glutathione peroxidase, housekeeping gene. Protects cells against lipid peroxidation.
NFκB	Transcription factor associated with the pro-inflammatory response
Innate Immunity	Non-specific and rapid immunity involving phagocytic, antimicrobial and inflammatory activity.
Adaptive Immunity	Host defences mediated by T and B-cells. Response is specific, diverse, has memory and is normally able to discriminate between self and non-self. Takes longer to become active than the innate response.
B-cells	B-lymphocytes. Humoral immune component of adaptive immunity. Primarily involved in secretion of immunoglobulins (antibodies) and immune memory.
T-cells	T-lymphocytes. Mediators of immune response separated into different T_H subtypes, see below.
T_H1	Helper type-1 CD4+ cells. Aids immune response against intracellular pathogens.
T_H2	Helper type-2 CD4+ cells. Enhance B-cell production and aids elimination of helminths.
T_H9	Helper type-9 CD4+ cells. Enhance antibody production, mostly antihelminthic.
T_H17	Helper type-17 pro-inflammatory CD4+ cells. Effective against bacteria and fungi.

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Thesis Abstract

Global warming and temperature variation are likely to have profound impacts on fish as ectotherms that are heavily reliant on environmental temperature for growth, development, metabolism and immunity. This study addresses the impact of climate change on the development of infection and immunity in three-spined sticklebacks (*Gasterosteus aculeatus*) and their common parasites. In addition to thermal consequences on host parasite interactions, the study also addressed the effects of co-infection on parasite intensities and pathology on host swimming ability.

Experiments were designed to mimic global warming, temperature variability and stochasticity (Chapters 3-5). Temperature during exposure to *Saprolegnia parasitica* was the major determinant of high infection prevalence and intensity with historical temperature exposure having little impact (Chapters 3-5). A further contributor to infection risk was higher host body condition (Chapter 3 and 5), attributed to a trade-off between host immunity and condition, higher condition individuals investing less in immunity supported by a decline in *β-def* expression in high condition fish (Chapter 5). Peak infection intensities in *Gyrodactylus gasterostei* were dependent on temperature variability and the host's immune response. In variable conditions, an established *G. gasterostei* was better able to adapt to a changing environment than the host's response causing higher peak infection intensities (Chapter 3 and 4).

Temperature, and not photoperiod, was the major cause of circannual rhythm in host immunity (Chapter 6). Co-infection between *G. gasterostei* and *A. foliaceus*, revealed higher gyrodactylid infection peaks compared to hosts infected with *G. gasterostei* alone suggesting that immunomodulation by *A. foliaceus.* Lastly, pathology, rather than drag, reduced burst and long-term swimming performance of sticklebacks infected with *A. foliaceus*.

Many of the factors highlighted have implications for aquaculture. High aquaculture feeding regimens, resulting in higher body condition, co-infection and temperature, all could severely increase morbidity and mortality of fish in a parasite species dependant manner.

Thesis Layout

This thesis consists of a general introduction on the parasites, immunology and general biology of three-spined sticklebacks (*Gasterosteus aculeatus*) and the consequences of climate change on ectotherms (Chapter 1). There then follows a methods chapter on the culturing procedures used for three-spined sticklebacks and their parasites (Chapter 2). There are then six experimental chapters, the first four are on the effects of temperature, climate change and photoperiod on the parasites, *Saprolegnia parasitica* and *Gyrodactylus gasterostei*, and immunology of sticklebacks (Chapters 3-6). Chapter 7 focuses on the consequences of co-infection for two ectoparasites of sticklebacks and Chapter 8 on the impact of fish louse (*Argulus foliaceus*) infection for stickleback swimming preferences and performance (Chapter 8). There is then a general discussion (Chapter 9). The two appendices include information that has been used across multiple chapters, but for the most part each chapter is self-contained and could form the basis of a publication.

Chapter 1 - General Introduction

Climate change

The Intergovermental Panel on Climate Change (IPCC) states that between 1880 and 2012 global surface temperature has risen by 0.65 to 1.06°C at an increasing rate, largely the result of the increasing concentration of greenhouse gases making up the atmospheric composition (Hansen *et al.*, 20 A summary of gene expression 10; Jones *et al.*, 2012; IPCC, 2013a; Rohde *et al.*, 2013). The average global temperature in 2100 is expected to have risen by 1.5-2.0°C on the 1850-1990 global average (Taylor *et al.*, 2012; IPCC, 2013b). In addition to warming, there has been a change in temperature extremes with a decrease in the number of cold extremes and a corresponding increase in the number of warm extremes, a global trend likely to continue (Alexander *et al.*, 2006; Kenyon & Hegerl, 2008; Seneviratne *et al.*, 2012; Donat *et al.*, 2013; IPCC, 2013b).

Long term studies on the phenological consequences of climate change might be considered rare if were not for the diligent recordings collected by enthusiasts stretching back several hundred years. These records typically focus on seasonal changes such as the first signs of spring, the appearances of migratory birds or agriculturally linked events such as harvest times. For example, springtime blossoming and autumn harvests have been steadily getting earlier in the year (Menzel & Dose, 2005; Menzel, 2005); a tend likely to continue based on the Representative Concentration Pathways of the Intergovernmental Panel on Climate Change (IPCC) (Hur *et al.*, 2014). Shorter term studies focusing on the consequences of carbon dioxide and warmer temperatures, found that the length of the vegetative growing season is increasing (Keeling *et al.*, 1996; Myneni *et al.*, 1997; Menzel & Fabian, 1999; Menzel *et al.*, 2003). From an animal perspective climate change has advanced the laying dates of multiple species of bird (Crick *et al.*, 1997; Brown *et al.*, 1999). The migration patterns of birds have also changed, with some migrating to their summer nesting grounds earlier to take advantage of improved conditions (Inouye *et al.*, 2000; Hüppop, 2003). Earlier migration is also a phenomenon linked with conditions such as precipitation and temperature in their over-wintering grounds (Cotton, 2003; Gordo *et al.*, 2005) and climatic variability caused by the North Atlantic Oscillation (Hüppop, 2003).

Climate change has also affected the habitat range of species, there being a general trend of plant and animal species shifting either towards the poles or to higher altitudes (Parmesan & Yohe, 2003; Kelly & Goulden, 2008; Chen *et al.*, 2011). These range

shifts pose a particular problem for human health and disease, as for instance, the habitat range of *Plasmodium falciparum* may also shift in latitude or elevation putting a greater percentage of the global population at risk (Martens *et al.*, 1995; Caminade *et al.*, 2014); a problem further compounded by the continued presence of mosquitoes able to transmit malaria in Europe, predominantly *Anopheles maculipennis* (see Kuhn *et al.*, 2002). The risk from malaria is further increased as climatic variability aids the rate of transmission at lower average temperatures by reducing the length of the gonotrophic cycle; the time between blood meal and oviposition (Paaijmans *et al.*, 2010).

Climatic variability has also been linked to the spread of other infectious diseases, such as *Batrachochytrium dendrobatidis* (chytrid fungus) and *Saprolegnia ferax* (oomycete). The spread of the chytrid and subsequent frog population crashes has been linked with abnormally dry conditions, thought to be caused by increasing temperatures in the tropics (Pounds *et al.*, 1999; Alan Pounds *et al.*, 2006). Recent evidence has, however, suggested that a further cause of the chytrid spreading dry periods is climatic variability brought about by El Niño events (Anchukaitis & Evans, 2010; Rohr & Raffel, 2010; Raffel *et al.*, 2015). El Niño events have also been linked to the spread of *S. ferax.* Reduced water pools caused by El Niño cause frog embryos to become exposed to a higher level of ultraviolet-B radiation and increasing their vulnerability to infection with *S. ferax* (see Kiesecker *et al.*, 2001).

A further consequence of climate change and variability, particularly for aquatic species, is the increased frequency at which harmful algal blooms (HABs) are occurring. Such blooms have been tightly linked with eutrophication and often cause mass fish mortalities though toxin production and anoxia (Trainer *et al.*, 2003; Burkholder & Glibert, 2006; Heisler *et al.*, 2008; Moore *et al.*, 2009). Changes in precipitation as a result of climatic variability, particularly in extreme events such as El Niño, have the potential to cause HABs; as reduced freshwater discharge increases eutrophication (Kedong *et al.*, 1999; Howarth *et al.*, 2000). Such events have important consequences for human health, as shellfish which ingest the neurotoxin producing dinoflafellate *Alexandrium catnella* cause paralytic shellfish poisoning in humans upon consumption, which has been known to be fatal (Trainer *et al.*, 2003).

Climate change is likely to alter many other aspects of aquatic systems including temperature, dissolved oxygen, pollutant toxicity and hydrology. Fish in particular are subject to climate change with many aspects of fish physiology affected by temperature

including activity, growth, reproduction and metabolism (see Schurmann & Steffensen, 1997; Handeland *et al.*, 2008; Donelson *et al.*, 2014; Md Mizanur *et al.*, 2014). The availability of dissolved oxygen for all aquatic species is also likely to change with increasing temperature, as there is a direct negative relationship between temperature and the quantity of dissolved oxygen water can hold (Kalff, 2002). The stratification of larger freshwater systems may also increase in terms of the magnitude of difference between each strata and the duration of stratification which is seasonally dependant; such a change further complicated dissolved oxygen availability as each layer of strata determines dissolved oxygen concentration (Boehrer & Schultze, 2008). The effect of increased temperature in the upper strata, and the expansion of deoxygenated water in the lower strata, is to narrow the range of available oxygenated habitat (Magnuson *et al.*, 1990). Oxygen deprivation at warmer temperatures is further exacerbated by enhanced metabolic processes at higher temperatures; meaning more oxygen is required but less is available potentially causing hypoxia and death. The toxicity of many pollutants, e.g. heavy metals and organophosphates, increases at higher temperature (Howe *et al.*, 1994; Eddy, 2005; Sokolova & Lannig, 2008). This increase in toxicity is attributed to changes in the equilibrium of toxic compounds (Eddy, 2005), the increased production of free radicals making the compound more toxic (Nemcsók *et al.*, 1987), and the increased uptake of these compounds by organisms which process them faster as a result of their increased metabolism (Jezierska & Witeska, 2006). Local hydrology and the ebb and flow of water is likely to change in timing and magnitude; as changes in precipitation, snow melt and climatic events, such as El Niño and El Niña, drastically affect habitats (Ficke *et al.*, 2007). The migration patterns of species such as Chinook salmon (*Oncorhynchus tshawytscha*) may be disrupted by low flow that blocks waterways (Mcdowall, 1992). The warming of groundwater and a corresponding reduction in cold water refugia is also likely to reduce available habitat for species such as trout (Meisner *et al.*, 1988; Ebersole *et al.*, 2001).

Changes in climate are therefore likely to have profound consequences, particularly for ectotherms such as teleosts, as change to growth rate, metabolic and immune processes are drastically altered (Schurmann & Steffensen, 1997; Handeland *et al.*, 2008; Watts *et al.*, 2008; Uribe *et al.*, 2011). The same can be said of parasites infecting ectotherms that are heavily reliant on temperature for growth, transmission or reproduction (e.g. Chappell, 1969; Powell *et al.*, 1972; Lester & Adams, 1974; Harris, 1982; Riberio, 1983; Jansen & Bakke, 1991; Bly *et al.*, 1992; Bruno & Wood, 1999; Marcos-López *et*

al., 2010; Macnab & Barber, 2012). The impacts of climate change on fish production could therefore be a major hurdle to food sustainability, particularly among the world's least developed countries where aquaculture provides 14% more dietary protein compared to developed countries (Allison *et al.*, 2009). It is therefore necessary to utilise a small, easily maintained model of temperate teleosts that can be easily manipulated in the laboratory, this thesis therefore turns to the three-spined stickleback (*Gasterosteus aculeatus*) to achieve this aim.

Study Species

The three-spined stickleback (*Gasterosteus aculeatus*) is a hardy temperate fish found throughout costal marine, brackish and freshwater environments located north of 30°N latitude. Unlike larger fish species of economic importance: cod, salmon and catfish, sticklebacks are easy maintained in the laboratory as a result of their general hardiness, relatively small size and low maintenance cost. Additionally, within the habitat range, sticklebacks can be easily caught from the wild either by netting or trapping. Sticklebacks have in the past provided a model for the study of behaviour (Giles, 1983), adaptive radiation (Schluter, 1996; MacColl, 2009) and parasitology (Arme & Owen, 1967). The publication of the stickleback genome (Kingsley, 2003; Hubbard *et al.,* 2007; Jones *et al.*, 2012) coupled with advanced post-genomic techniques now also make this small temperate fish an ideal model for parasite immunology (Gibson, 2005).

The oomycete parasite, *Saprolegnia parasitica*, is endemic to freshwater environments and is responsible for significant aquacultural losses (Jeney & Jeney, 1995; van West, 2006). *Saprolegnia* is a heterotrophic fungal-like pathogen that causes characteristic fluffy white or grey patches primarily on the epidermis with isolates that vary in pathogenicity (Hatai & Hoshiai, 1992; Fregeneda Grandes *et al.,*2001; Hussein & Hatai, 2002). Control of this oomycete has been hampered by the recent banning of treatments, such as malachite green and formalin, because of their carcinogenic properties (Srivastava *et al.,* 2004; van West, 2006; Sudova *et al.*, 2007; Defra, 2015). A 'perfect storm' has therefore been created, of high density fish stocks and reduced efficacy of control methods, which could potentially cripple aquaculture and become a major threat to food security.

Gyrodactylus species are virtually ubiquitous ectoparasites of fish with over 400 described species and are the best described genus of monogeneans (Bakke *et al.,* 2007;

Harris *et al.,* 2008). *Gyrodactylus* species are a major economic problem that can ravage salmon populations and are commonly found on ornamental fish (Bakke *et al.,* 2007; Linaker *et al.,* 2012). The viviparous reproduction strategy of these parasites makes them an ideal system for studying population growth over time on a single host where typically a fish will either succumb to the infection but the immune system will rid the host of the infection, if no further parasites are introduced, or a continuous low intensity infection occurs (Scott & Anderson, 1984). The ability to count the number of infecting gyrodactylids on a single host over time, without euthanasia, makes them an ideal system to study population dynamics that are dependent on external factors such as: temperature (Chappell, 1969; Lester & Adams, 1974; Harris, 1982; Jansen & Bakke, 1991), toxins (Khan & Kiceniuk, 1988; Soleng *et al.,* 1999; Poléo *et al.,* 2004; Gheorghiu *et al.,* 2007; Smallbone *et al.,* 2016), fish population size (Bagge *et al.,* 2004) and season (Chappell, 1969; Mo, 1997; Winger *et al.,* 2007; You *et al.,* 2008).

Argulids, fish lice, are generalist ectoparasitic crustaceans of sticklebacks and can be found infecting fish on every continent with the possible exception of Antarctica (Poly, 2008). Although not a major problem at low densities, deterring only anglers because of aesthetics, at high densities of infection they can cause fish appetite loss and mortality (Taylor *et al.,* 2005; Pekmezci *et al.,*2011). Advances in genomic techniques have recently uncovered the immunomodulatory nature of these parasites (Forlenza *et al.,* 2008; Saurabh *et al.,* 2010; Kar *et al.,* 2013). Given their additional ubiquitous nature, immunomodulative abilities and ease of breeding these parasites (see Chapter 2) make argulids an attractive model for co-infection studies.

Teleost Immunology

Teleosts are the largest group of vertebrates (ca. 30,000 species) with many morphological and behavioural characteristics having diverged from other vertebrates some 333-285 million years ago (Near *et al.,* 2012). Despite this there remains a remarkable degree of conservation between the immunology of teleosts and higher vertebrates. For an overview of vertebrate immunology see Murphy *et al.* (2012) and Owen *et al.* (2013).

Teleost immune genes orthologous to the mammalian system highlight the level of conservation across the vertebrates: immunological pathways, genes and recognition mechanisms including the Major Histocompatibility Complex (Hashimoto *et al.,* 1990; Hordvik *et al.,* 1993), cytokines (Secombes *et al.,* 1996), and Toll-like receptors (Stafford *et al.,* 2003). Many of the migratory and non-migratory cells present in the

mammalian immune system are also present in teleosts: T- and B-lymphocytes, macrophages, monocytes granulocytes, mast cells, dendritic cells, thrombocytes and eosinophils (Ellis, 1977; Press & Evensen, 1999; Balla *et al.*, 2010; Takizawa *et al.*, 2011). This is a remarkable level of conservation given the fact that physiologically teleosts lack some of the higher vertebrate primary and secondary lymphoid organs, including bone marrow and lymph nodes. In teleosts, the head kidney is analogous to the bone marrow and shares a number of the same properties, including the presence of B-cells (Kaattari & Irwin, 1985; Meseguer *et al.*, 1995). The gills are also a site of importance in immunity being exposed to the environment, and are found to include immune factors such as: Toll-like receptor 9 (Takano *et al.*, 2007), MHC class I and II (Koppang *et al.*, 1998a; Koppang *et al.*, 1998b), Mx protein (Jensen *et al.*, 2002), iNOS (Campos-Perez *et al.*, 2000) and antibody (Davidson *et al.*, 1997). Other immune relevant organs such as the thymus and spleen remain major immunological sites, as they are in mammals.

Teleost lymphocytes, as with mammals, are sub-divided into multiple types: immunoglobulin positive B-cells and immunoglobulin negative T-cells (Warr, 1995; Miller, 1998). T-cells maintain the same complexity as in mammals; having CD8+ cytotoxic cells (Nakanishi *et al.*, 2011; Takizawa *et al.*, 2011) and CD4 positive T-helper cells, which themselves are divided based on their cytokine production: T_H1, T_H2, T_H17 and Treg subtypes (Laing *et al.*, 2006; Laing & Hansen, 2011; Toda *et al.*, 2011). The genes that stimulate these differences including the major transcription factors for the T-helper cells such as *Tbx21* (Tbet in mice) and *Gata3*, are present within the genome of most teleosts. There is, however, at least one exception to the level of conservation between higher vertebrates and teleosts in cod (*Gadus morhua*); this fish has lost the *MHCII, CD4* and invariant chain genes required for CD4+ T-helper cell responses (Star *et al.*, 2011). Much research has also been conducted upon the divergence in resistance of sticklebacks of separate ecotypes with parasite communities differing between freshwater habitats (Kalbe *et al.*, 2002; Kalbe & Kurtz, 2006; Scharsack *et al.*, 2007; Eizaguirre *et al.*, 2011); finding that parasite driven diversity in MHC may be the basis for variation in parasite communities (Eizaguirre *et al.*, 2011; Eizaguirre *et al.*, 2012).

Unlike endothermic mammals, the body temperature of ecotothermic teleosts is reliant on the ambient temperature of their habitat. This has impacts for cellular, enzymatic and immunological processes, which rely on optimal temperatures (Schurmann &

Steffensen, 1997; Watts *et al.,* 2008). Temperatures within the physiological range of teleosts are capable of altering immune function with the adaptive response becoming non-functional at the lower end of the physiological spectrum of the species (Bly & Clem, 1992). The teleost adaptive response also only develops relatively late in life. In zebrafish the T-cell receptor alpha region and immunoglobulin light chain only reach an adult level of expression 4-6 weeks post fertilisation, some 3-5 weeks after hatching (Lam *et al.,* 2004). For a relatively long period during juvenile growth teleosts have to cope without a fully functioning adaptive response, suggesting that it is not vital to survival, at least during the early stages of life. In the case of the stickleback the lack of an active adaptive response in fry has resulted in the evolution of the antimicrobial nest building material, spiggin (Little *et al.,* 2008). Newly hatched stickleback fry cluster around their nest for about 1-2 weeks and it is thought that the antimicrobial properties of spiggin protect the fry from infection (Little *et al.,* 2008). Unlike tetrapods, teleosts do not have conserved linkage between the *MHC I* and *MHC II* genes (Bingulac-Popovic *et al.,* 1997; Klein & Sato, 1998), potentially allowing independent evolution of the two systems (Kelley *et al.,* 2005; Star & Jentof, 2012). The lack of linkage in teleost *MHC I* and *MHC II* appearing to have occurred as a result of disintegration by means of translocation, rather than the development of linkage in tetrapods (Kuroda *et al.,* 2002; Kelley *et al.,* 2005).

Aspects of the innate response are flexible across teleosts, for example the C3 component of complement is present in multiple copies in the teleost genome. Sticklebacks are no exception to this, and currently have eight annotated copies of *C3* in the genome (Forn-Cuni *et al.,* 2014). The presence of multiple copies does not appear to be the result of the tetraploid nature of some fish, evidence for this can be found in the medaka (*Oryzias latipes*). The genome of this diploid fish contains multiple copies of the *C3* gene which are closely linked indicating tandem duplication, rather than multiple copies having arisen as a result of the tetraploid genome (Kuroda *et al.,* 2000). Further evidence in favour of specific adaptation towards multiple *C3* copies can be found when looking at divergent teleost species where *C3* gene copies form their own individual clusters rather than the single conserved cluster that would have arisen as a result of a genome duplication event (Boshra *et al.,* 2006; Nonaka & Kimura, 2006). Evolution of multiple *C3* copies has provided teleosts with a greater range of binding efficiencies, due to the loss of His^{1126} and Glu^{1128} residues, essential in human *C3* binding specificity (Boshra *et al.,* 2006). This allows teleosts to recognise a wider range

of foreign molecules and function over a broader temperature range (Zarkadis *et al.,* 2001; Claire *et al.,* 2002). Further to the expansion of the *C3* genes other innate factors, such as Toll-like receptors (TLRs), have been driven towards a greater diversity. A broad spectrum of TLRs has been conserved between the higher vertebrate and teleost systems including: TLR-1, 2, 3, 5, 7, 8 and 9 (Takano *et al.,* 2011). Novel TLRs are found in teleosts and amphibians, including TLR14, however their function is considered to substitute for mammalian TLRs not present in the genome e.g. TLRs 6 and 10 (Takano *et al.,* 2011). TLRs 19, 20 and 23 are also novel, but their function has yet to be fully elucidated (Takano *et al.,* 2011).

In contrast to the greater diversity of innate responses of teleosts, the adaptive response appears to be more limited compared to the higher vertebrates. While higher vertebrates have a range of immunoglobulins: IgA, IgD, IgE, IgG and IgM, some of which can be further sub-divided, with four sub-classes of IgG alone. Teleosts, in contrast, have only IgD, IgM (tetrameric in teleosts) and IgZ (sometimes called IgT), but the specific functions of these immunoglobulins have yet to be fully discerned. The basic monomer unit of IgD in higher vertebrates is comprised of equimolar amounts of heavy and light chain with a disulphide bridge between the two heavy chains (Wilson & Warr, 1992; Watts *et al.,* 2008). IgD in teleosts is structurally quite variable, particularly in length of the heavy chain. IgD in humans for example contains three lengths of the Igδ domain while teleosts vary with five domains in Atlantic cod (*Gadus morhua*) and 13 in fugu (*Takifugu rubripes*) (see Edholm *et al.,* 2011). Teleost IgM is a tetrameric molecule present mostly in the plasma (Solema & Stenvik, 2006) but also in mucus (Fletcher & Grant, 1969; Ourth, 1980) and is thought to be functionally similar to mammalian IgM. IgT cells are specialised to the gut and respond to both bacterial and helminth infections (Zhang *et al.,* 2010). The loss of some components of the adaptive response might then not have the same drastic affect that would be felt in mammals as a result of a more flexible innate immune response.

Thesis Aims

The objective of this study was to investigate the consequences of temperature, climate change and infection on the model three-spined stickleback (*Gasterosteus aculeatus*). Through the use of mesocosms and molecular techniques at Aberystwyth University, and aquatic facilities and parasitic cultures at Cardiff University, a cross-disciplinary collaboration was utilised to investigate the impact of abiotic and biotic factors on stickleback behaviour and immunity. Of particular interest was the impact of climate

change and temperature variability on the economically important parasites *Saprolegnia parasitica* and *Gyrodactylus* spp. for which *Gyrodactylus gasterostei* was used as a model. As a result of these investigations, a circannual rhythm in immune genes expression was discovered but the root cause was unknown; as a result a further experiment tested whether temperature or photoperiod was the cause of this variation. In addition to the consequences of temperature on infection and immunity, studies on the immunomodulating parasite *Argulus foliaceus* were performed to understand how this parasite affects population trajectories when co-infected with *G. gasterostei* and what implications its drag and pathology has on stickleback swimming ability.

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Chapter 2 - Hook, line and infection: culture methods for common parasites of the three-spined stickleback and associated immunological responses

Abstract

The three-spined stickleback (*Gasterosteus aculeatus*) is model organism with extensive data on its evolutionary adaptations, behaviour, and parasitology coupled with a published genome. Sticklebacks provide an ideal system in which to study common fish diseases or orthologs of fish diseases that can be used as models of infection. However, information on the culture of specific stickleback infections have often been split between primary and grey literature resources, some of which are not easily accessible. Here, knowledge on basic biology, capture, maintenance of sticklebacks and their commonly studied parasites is drawn together highlighting recent advances in understanding of the associated immune responses.

Introduction

Molecular and immunological approaches are becoming increasingly common in mitigating the impacts of disease. The difficulty and importance of being able to maintain parasite populations in the laboratory is often underestimated and partly hampered by the lack of published information on the practicalities of establishing and maintaining host-parasite systems. This review aims to provide a comprehensive and standardised approach to support new research on parasites of the three-spined stickleback (*Gasterosteus aculeatus*), while also increasing awareness of the impact of parasites on behavioural, immunological and ecological studies if not controlled.

The three-spined stickleback is found throughout coastal marine, brackish and freshwater environments located north of 30°N latitude. It has been utilised as a model of adaptive radiation as a result of its many morphological variations including: size, shape and protective armour (see Schluter 1993; Reimchen, 1994; Walker, 1997). Exploitation of different resources is thought to be a primary cause of stickleback adaptive radiation (Schluter, 1993). Of particular interest are the Canadian limnetic and benthic forms of sticklebacks, which are thought to be ‘good’ biological species although not yet defined as such, even though they are reproductively isolated with only low levels of hybridisation (Mcphail, 1992; Gow *et al.*, 2006). Both the limnetics (inhabiting the pelagic zone) and benthics (from littoral zone) are believed to have evolved from independent marine ancestors (Mcphail, 1993). Supporting predictions of adaptive radiation, limnetics and benthics both have growth advantages in their native

habitats, which are lost in the alternative habitat while hybrids are intermediate, and the efficiency of this exploitation matches the observed morphological differences (Schluter, 1993, 1995).

The parasitic fauna of sticklebacks is remarkably diverse covering nine phyla to date (Barber, 2007), largely as a result of their wide geographical distribution, diverse habitat exploitation, varied diet and central position in food webs. Virtually all niches of the stickleback have been exploited by at least one parasite species, this includes the skin and fins, gills, muscle, eye lens and humour, body cavity, swim bladder, liver, intestine, kidney and urinary bladder. Over 200 parasite species have been described infecting the three-spined stickleback, although many of these are cross-species infections from other teleosts (for complete list see Barber, 2007).

In addition to their diverse parasite fauna, for several other reasons sticklebacks are a favourable model for host-parasite studies. First, the host is easily maintained in the laboratory as a result of their general hardiness, small size and low maintenance cost. Second, within the habitat range, sticklebacks can be collected easily from the wild, and can be bred under parasite-free conditions. Third, unlike many vertebrates, there is comprehensive knowledge of stickleback parasitology (Arme & Owen, 1967; Barber & Scharsack, 2010; Macnab & Barber, 2012), evolutionary history (Schluter, 1996; MacColl, 2009), physiology (Taylor & Mcphail, 1986; Pottinger *et al.,* 2002) and behaviour (Tinbergen & van Iersel, 1947; Giles, 1983; Milinski, 1985; Barber *et al.*, 2004). Fourth, publication of the stickleback genome (Kingsley, 2003; Hubbard *et al.*, 2007; Jones *et al.*, 2012) coupled with advanced post-genomic techniques make this fish an ideal model for molecular study, including host immunology. All of this allows us to focus, not on a single aspect of the system, but to take a holistic systems approach to studying host-parasite interactions.

Teleosts are the largest group of vertebrates (ca. 30,000 species) with many morphological and behavioural characteristics having diverged from other vertebrates some 333-285 million years ago (Near *et al.,* 2012). Teleost diversity is attributed in part to the whole-genome duplication event some 320-404 million years ago (Amores *et al.,* 1998; Hoegg *et al.,* 2004), but despite this event there remains a remarkable degree of conservation between the immunology of teleosts and higher vertebrates. For an overview of vertebrate immunology see Murphy *et al.* (2012) and Owen *et al.* (2013), and of teleost immunology (see Miller, 1998; Morvan *et al.,* 1998; Press & Evensen,

1999; Claire *et al.,* 2002; Watts *et al.,* 2008; Takano *et al.,* 2011; Forn-Cuni *et al.,* 2014). It is becoming increasingly important to understand fully fish parasites and immunology in order to produce a sustainable, high yield, food supply.

Aquaculture is currently the fastest growing animal-food producing sector responsible for 66.6 million tonnes of fish food in 2012 having increased from 51.7 million tonnes in 2006 (FAO, 2014). In order to maintain the current level of consumption global aquaculture production will have to reach 93 million tonnes by 2030 in order to make up for the shortfall from fisheries, which have reached their maximum potential output (The World Bank, 2013). As with common agriculture, production can be increased via two main approaches; increased surface area turned over to aquaculture to increase bulk supply, or improved yield. With land and aquatic environment use reaching its sustainable maximum, focus is now firmly set on yield improvement. It is possible to improve yield by a number of methods: selective breeding, genetic modification, disease elimination and feed conversion efficiency. One of the major inhibitory factors to improved yield is the presence of infectious disease (Meyer, 1991). The stickleback provides a useful study species being susceptible to diseases problematic in aquaculture, such as *Pseudomonas* spp., *Saprolegnia parasitica* and *Gyrodactylus* spp. Although such parasites have been studied extensively, until now there has been no single resource that summarizes all available culture methods.

Given the overwhelming tendency for sticklebacks taken from the field to be co-infected there is relatively little knowledge about how parasitic species might be interacting. It has been known for some time that one stickleback gyrodactylid species can alter the distribution of a separate co-infecting gyrodactylid species (Harris, 1982). The intestinal parasites *Proteocephalus filicolis* and *Neoechinorhynchus rutili* are also known to alter their distribution when co-infecting (Chappell, 1969). Experimental infection with *Schistocephalus solidus* will also alter the prevalence of co-infecting species if infected hosts are transferred into an environment allowing natural infection (Benesh & Kalbe, 2016), although immunomodulation may be involved the mechanism behind this change in prevalence is currently unknown (Scharsack *et al.,* 2007).

For this review, we have focused on the parasites that are most frequently used in research projects and routinely observed in our own studies: species of *Gyrodactylus* spp., *Diplostomum* spp., *Schistocephalus solidus*, *Camallanus lacustris*, *Saprolegnia*, *Glugea anomala*, and *Argulus* spp. For each taxon, culture and use the parasites in

experimental infections and the associated stimulated host immune response is outlined. *G. anomala*, although not widely used experimentally is a common infection of sticklebacks, and is therefore included in this review although the means of culture are not yet fully elucidated. Prior to parasite culture the basic husbandry of the host is outlined.

Stickleback Husbandry

Here methods for the collection, maintenance and breeding of three-spined sticklebacks are described. In some instances multiple methods are provided, the suitability of which is dependent on the focus of a particular study.

Ethics

All protocols carried out are subject to the relevant regulatory authority. Care, maintenance and infection of vertebrates in UK laboratories are governed by local animal ethics committees and the Home Office under The Animals Scientific Procedures Act 1986. EU member states are subject to Directive 2010/63/EU on the protection of animals used for scientific purposes. The Animals Scientific Procedures Act outlines humane methods for animal euthanasia referred to as 'Schedule 1 Procedures'. This nomenclature is used throughout the manual, but international colleagues should be aware that their regulatory authority may have different guidelines in place. At Cardiff University all work was approved by Cardiff University Ethics Committee and performed under Home Office Licence PPL 302357.

Collection

While some experiments require naïve hosts, for others, previous experience of background infections or specific ecotypes might be critical. Information on fish from all sources including provenance, parasite history and exposure to anti-parasitic treatments, is essential for most studies. When acquiring sticklebacks it is advised that multiple screens for ectoparasites and dissection for macroparasites is conducted; the latter may not be necessary, particularly for breeding, as many parasites require the presence of intermediate hosts to persist. Regardless, the presence of any parasites should be reported for any behavioural study, and it should never be assumed that an animal is uninfected unless bred in specific pathogen free conditions.

Sticklebacks can be acquired from other researchers actively breeding these fish, possibly holding multiple inbred and/or outbred lines. Alternatively they can be purchased from a commercial fish supply company. Given the diversity of stickleback parasites, when purchasing sticklebacks, be aware that rarely can a supplier guarantee

'parasite-free' fish and that they may have been treated chemically. Fish suppliers may be willing to supply infected sticklebacks for research or teaching, particularly overt infections, such as *Glugea anomala* or *Schistocephalus solidus*.

A third option is to collect wild fish. Most institutions in Europe and North America probably neighbour a water body containing sticklebacks, particularly around coastal regions, obtaining permission from the landowner and appropriate regulatory authority before fishing. Wild sticklebacks will almost certainly harbour an infection (see 'Treating Common Infections' for parasite control options). Minnow traps are an easy capture method and are easily purchased. Tie string to each minnow trap and secure the other end to a well-marked, but concealed object. Put the trap into the water and leave for <24 h; otherwise fish may become stressed and even die in the traps. Bait is not required as sticklebacks are inquisitive, and catching one fish incites others to follow. Alternatively, invert the end of a 2-3 L soft drinks bottle and reattach it using cable ties so that the spout faces into the bottle to make a minnow 'like' trap; add pebbles and drill holes into the bottle to aid with sinking. A hand net search is also effective, targeting areas of vegetation along the bank or under bridges.

Maintenance

Sticklebacks should be kept at densities not exceeding 1 fish/litre to reduce fish stress. Only dechlorinated water should be used, 0.1-0.3 ppm (parts per million) chlorine is lethal to the majority of fish (Wedemeyer, 1996), although brief exposure to chlorinated water (1-2 h) can be beneficial in removing some parasites. For the hobbyist, dechlorinated water is typically obtained either though an activated charcoal filter, use of a dechlorinating solution (follow manufacturer's instructions) or vigorous aeration of tap water for 24 h before use. Dechlorinated water should not be fed through copper pipes as high concentrations of copper ion can kill the fish. Although sticklebacks are kept in fresh water, routine addition of 0.5 % salt water (aquarium or marine grade) inhibits some infections. Aeration to each tank can be provided by means of an air stone or filter. The physiological temperature range of sticklebacks is 0-34.6°C (Jordan & Garside, 1972; Wootton, 1984a); fish in the lab are typically maintained between 10-20°C, 15-17°C being optimal. Lower temperatures result in slower growth rates and above 20°C populations are subject to higher stochastic mortality. Sticklebacks are typically kept on a summer light cycle 16 h light: 8 h dark, altered to induce breeding (see below).

For filtration, external or internal pump biological filters provide good water quality. Under-gravel filtration systems or other biological filters are only suitable for fish kept at lower densities as faeces can build up rapidly. If using a biological filter, the tanks should be set up two weeks in advance to allow colonisation by denitrifying bacteria. 'Seeding fish' can be used to speed up nitrifying bacteria generation, but only if the parasite status of these fish is known, remove the seeding fish a couple of days prior to introduction of the new fish. Starter bacteria cultures can also be used following the manufacturer's instructions or from other established filters, but only if the source tank is confirmed parasite free. Ammonia, nitrite and pH tests should be conducted regularly ensuring levels do not exceed 0 ppm ammonia, 0.5 ppm nitrite and 40-50 ppm nitrate. Alternatively, Recirculation Aquaculture Systems (RAS) can be used to house sticklebacks; however, because the tanks are not isolated infections may spread rapidly, even with the best in built treatment facility.

Adult sticklebacks can be fed on live, frozen or freeze dried: bloodworm (larvae of the non-biting midge in the family Chironomidae), *Tubifex* spp. or *Daphnia* spp. The preferred laboratory food is frozen bloodworm, as this is the most nutrient dense. Wild fish prefer live food due to dietary conservatism (Thomas *et al.*, 2010) and they may not feed immediately after capture. Commercial flake food can be used to supplement the diet particularly if used during fish rearing. Optimal diets for stickleback fry are outlined in Table 2.1. Precautions should be taken with live food with may contain parasites, although laboratory culture will remove many of these risks. For experimental protocols, sticklebacks can be isolated in pots at 1 fish/L, with water changed at least every 48 h to prevent increased ammonia and nitrite levels. Avoid chemical cleaning products, which can impact parasite infections.

Breeding

Breeding sticklebacks has a major advantage in that it produces naïve uninfected fish mitigating the risks associated with infection, but is time consuming. Females carrying eggs can be identified by their swollen abdomens, sharply angled in the region of the cloaca, sometimes with a single egg protruding from the cloaca. Breeding condition in males is apparent by change in colouring: blue of the eye sclera and the bright red/orange of the jaw and belly.

The major stimulus for stickleback breeding is a change in photoperiod. Sticklebacks must be exposed to a winter lighting cycle (8 h light: 16 h dark) for 2-3 months. The length of daylight must then be increased to a summer light cycle (16 h light: 8 h dark).

Wootton (1984b) describes additional light cycles to induce reproduction. When bringing laboratory sticklebacks into breeding condition, males and females should be housed together for reciprocal stimulation. Males that become aggressive should be separated and placed in a tank with a mesh divider that allows visual and chemical stimulation with 1-3 females or euthanised for *in vitro* fertilisation. In order that the fish have sufficient nutrients for pigment and egg development they should be fed at least 2-3 times a day, on bloodworm. Extra care should be taken to clean these tanks as a result of extra food waste and faeces.

Male and female sticklebacks can be kept separated in a tank divided with a mesh net allowing reciprocal stimulation. Environmental enrichment such as gravel and pipes, rocks or plant pots for refugia should be provided. In the male half of the tank submerge a Petri dish containing aquarium grade sand and 50-100 x 2-5 cm threads of cotton with which the male stickleback will build its nest. It is also possible to supply the male with pondweed and other natural nest building material but this may introduce unwanted pathogens and plant growth into the tank. Once tanks are set up, once or twice a day introduce the most egg bound female into the male half of the tank for 30 min. If breeding does not occur within this period it is unlikely to do so. Stickleback courtship goes through a series of stages (see Wootton, 1984b; Ostlund-Nilsson *et al.,* 2006), when the female has laid eggs she will swim out of the nest and the male will immediately enter and fertilise the eggs. At this stage remove the female from the tank and either leave the male to raise the clutch of eggs or remove the eggs into a hatchery (described below) by lifting the Petri dish and nest out of the tank transferring the nest into the hatchery. In order to use the male for breeding again supply it with more nest building material and it will produce another nest within 1-3 days.

If *in vivo* breeding, it is possible to breed the male more than once (up to 2-4 times typically) producing half siblings. With a gloved hand dipped in stress coat (API™), gently squeeze the abdomen of a gravid female, moving fingers posteriorly from the pectoral girdle to the cloaca allowing the eggs to fall into a 25 mm sterile Petri dish with Hanks' solution (see Hanks' solution with phenol red; Appendix 1). The eggs should be released easily; excessive force can damage the female. The eggs should form a clump, if the egg mass dissociates, then they should be discarded. To collect sperm euthanize a coloured male using a Schedule 1 procedure (see Ethics) and place into a sterile Petri dish filled with Hanks' solution. Make an incision from the pelvic girdle cutting posteriorly, or at the anus cutting anteriorly, and a second incision just behind the

operculum, pull the flap off tissue back exposing the gut. Make an incision in the vas deferens to remove the testes (Fig. 2.1) and place in fresh Hanks' solution. Shred the testes into multiple pieces using forceps, releasing the sperm in a small dish of Hanks' solution and transfer to an eppendorf containing Hanks' solution, store in a fridge (4°C) for up to 2-3 days. Large testes can be cut into 2-3 sections using a sterile blade for multiple fertilisations. To fertilise the eggs, stir the shredded testes around the egg mass, remove the testes after a few minutes replacing the lid of the Petri dish. Check for separation of the inner and outer membranes of the eggs after 30 min at 15°C using a low power microscope (x10-60) to confirm fertilisation; cell division should begin within 45-60 min and then the egg mass transferred into a hatchery (described below).

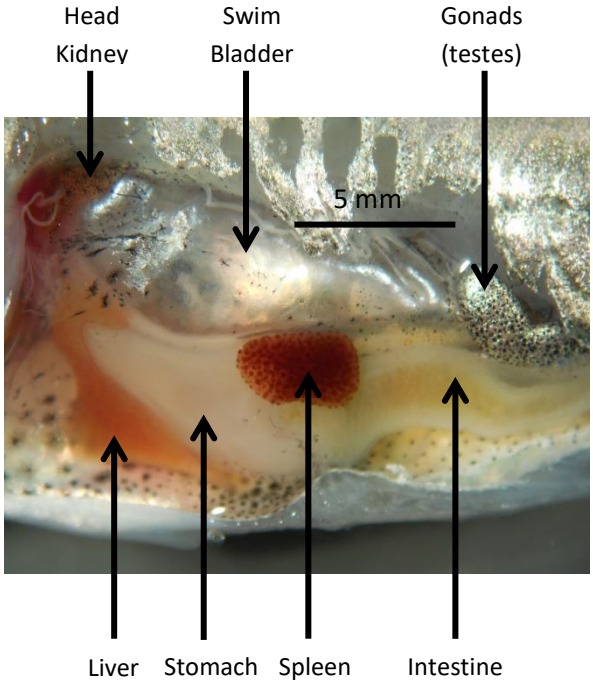

Figure 2.1: Body cavity of partially dissected three-spined stickleback showing major internal organs.

Hatchery

For the hatchery, use a small tank (20-30 x 40-50 cm x 10-20 cm deep) containing 10-20 L of Hatchery Water (Appendix 1), which inhibits bacterial, fungal and oomycete growth, particularly *Saprolegnia declina*. Methylene blue fades over time and will need to be replenished until the water is again a pale blue. Hatcheries should be cleaned and re-made every 2-3 weeks to reduce infection risk. Place the newly fertilised eggs from *in vivo* or *in vitro* breeding into plastic cups in the hatchery, with the cups suspended from the edge of the tank and the edges out of the water. Replace the bottom of each cup with a fine mesh (0.5 mm) so that the eggs are suspended with sufficient water circulation. An aquarium silicone sealant can be used to attach the mesh to the cup, or the mesh sandwiched between two cups. Position an air stone under the cups to provide oxygen and water circulation; avoid fine streams of bubbles which will float the egg

mass and dry them out. Eggs take 7-8 days to hatch at 15°C. After 7 days, remove cups and suspend from the edge of a standard 100 L tank; low salt concentration and methylene blue can be used to inhibit infection of the fry (see Table 2.3). Once hatched, the fry are normally small enough to fall through the mesh or they can be tilted out. Fry sink to the tank bottom and remain there for 1-3 days before establishing neutral buoyancy and will shoal in tank corners or around environmental enrichment. Cover filters in a mesh or sponge and run at the lowest setting, to prevent the newly hatched fry being drawn into the filter, or turn off entirely; turn on the filters again 1-2 weeks post-hatching. Feed the newly emerged fry as indicated below (Table 2.1).

Time/Size (standard length) Post-hatching	**Food**
1-7 days	Infusoria (microbiota bloom), place handful of hay in 100L water, leave for ca. two weeks, check for protozoa before feeding to fish
	Flake food (100 μm dia.)
2 days - < 2cm SL	Brine-shrimp (*Artemia*) (live, frozen or dried), ensure any egg shells removed, which can kill fish
	Flake food (200-300 μm)
> 2 cm onwards	Bloodworm (live or frozen), slice for small fish
	Daphnia spp. (live or frozen)
	Tubifex spp. (live or frozen)
	Flake food (crushed standard aquarium flake)

Table 2.1: Laboratory diet for three-spined stickleback fry.

Common Stickleback Parasite Cultures

Gyrodactylus spp.

Introduction

Gyrodactylus species are ubiquitous monogenean parasites of teleosts with over 400 described species (Harris *et al.,* 2008). Identification of species was typically conducted by sequencing the rDNA internal transcribed spacer (ITS) region supplemented by morphological characteristics of the marginal hooks and hamuli (Shinn *et al.,* 2010), although now it is recommend at least mtDNA gene sequencing to reveal potentially cryptic species (Xavier *et al.,* 2015). The viviparous nature of their reproductive life cycle means that they are capable of uncontrolled infrapopulation growth that at high densities becomes pathogenic (e.g. Scott & Anderson, 1984; Bakke *et al.,* 1990) although this is limited in most species by host immune responses (e.g. Bakke *et al.,* 1992; Harris *et al.,* 1998; Lindenstrøm *et al.,* 2004; Lindenstrøm *et al.,* 2006; Kania *et al.,* 2010). *Gyrodactylus salaris* (Malmberg, 1957) is of particular economic importance as it infects salmonids and has been the focus of intensive eradication schemes particularly in Norway since the 1980s (Linaker *et al.,* 2012). Studies on salmon are

often costly and their fry are particularly sensitive to stressors (Barton *et al.,* 1986). Therefore, many studies have used model fish, including the guppy and stickleback (reviews by Cable, 2011 and Barber, 2013, respectively) to assess potential environmental, pathological or immunological effects of this genus of parasite on tropical and temperate fish species (Bakke *et al.,* 2007).

Source, culture and infection

Stickleback *Gyrodactylus* spp. can be obtained from research institutions or the wild. The most common species found infecting sticklebacks are: *G. gasterostei* (Glaser, 1974), *G. arcuatus* (Bychowsky, 1933), *G. alexanderi* (Mizelle & Kritsky, 1967) and *G. branchicus* (Malmberg, 1964), other species such as *G. salaris* or *G. punitii* (Malmberg, 1964) may infect the three-spined stickleback but are not specific to it; for a full list see Harris *et al.* (2008). To infect a fish anesthetize a donor and recipient fish in 0.02% MS222, the donor fish may be euthanized by Schedule 1 procedure which can improve transmission. Fin clipping, the removal of a small fragment of the fin inhabited by a single *Gyrodactylus* and overlapping with a recipient fin allowing the parasite to cross onto the recipient, can also be performed. Under a dissection microscope with fibre optic illumination, overlap the caudal fins of both donor and infected fish and allow *Gyrodactylus* to cross onto the recipient fish. Variable numbers of *Gyrodactylus* can be used to infect the fish depending on experimental outcomes, although typically two worms are added to the caudal fin to initiate an infection. Under experimental conditions fish can be screened regularly by anesthetizing them as above and counting the number of worms under a dissection microscope, although it is suggest not more than once every 48 h as handling can cause undue stress.

To produce an isogenic culture of any *Gyrodactylus* species, infect a fish with a single gyrodactylid worm. Several fish should be infected as the *Gyrodactylus* worms may be at the natural end of their short life-span. Leave the infected fish for a week at 15-20°C to allow the parasite to reproduce on the stickleback. An infected fish should be transferred to a single tank with at least three other fish to allow natural transmission. Fish should be kept at densities of one fish per litre for adults or one juvenile (<20 mm standard length) per 250 ml. To avoid parasite extinction, 2-3 tanks of the culture should be maintained, adding new naïve fish in the event of mortality (Schelkle *et al.,* 2009). Water should be changed regularly, every 48 h if unfiltered, as nitrates and nitrites can have a detrimental effect on *Gyrodactylus* survival (Smallbone *et al.,* 2016). Additionally every 2 weeks the fish should be screened for the number of parasites on

each fish. Anaesthetise the fish in 0.02 % MS222 and count the number of *Gyrodactylus* on each fish under a dissection microscope with fibre optic illumination. If additional tank replicates are needed, remove 1-2 fish with a total of 40 parasites from the screened tank and place in a fresh tank with sufficient naïve fish to make the numbers up to four. If there are greater than 40 parasites per fish, treat the fish to prevent mortality (see Schelkle *et al.,* 2009).

Immunology

Gyrodactylus salaris infections of salmon (*Salmo salar*) can be broadly divided into susceptible Norwegian salmon and resistant Baltic salmon (Bakke *et al.,* 1990; Dalgaard *et al.,* 2003; Lindenstrøm *et al.,* 2006; Kania *et al.,* 2010) although there are some intermediate populations (see Bakke *et al.,* 2004) and as with all gyrodactylids considerable variation within strains (Hansen *et al.,* 2003; van Oosterhout *et al.,* 2006). Susceptible salmon show a marked increase in the mucus secretgogue *il-1β* in the epidermis, compared to uninfected salmon 24 h post-infection, in contrast resistant salmon show no increase in *il-1β* (Lindenstrøm *et al.,* 2006; Kania *et al.,* 2010). Resistant salmon also have increased *il-10, mhc II* and *serum amyloid A* transcript 3-6 weeks post-infection (Kania *et al.,* 2010). Likewise rainbow trout (*Oncorhynchus mykiss*), exposed to primary *G. derjavini* infections and then a secondary infection 35 days after parasite clearance, demonstrated susceptibility in the primary infections linked with increased *il-1β* transcript in the skin while resistant secondarily infected fish showed no increase in *il-1β* (Lindenstrøm *et al.,* 2003). Gyrodactylids feeding on the mucus and epithelium will therefore be at a disadvantage on any host able to suppress the increase in *il-1β* production. Serum and mucus taken from salmon and trout was also found to kill gyrodactylids with the mechanism being mediated by via the alternative complement system (Buchmann, 1998; Harris *et al.,* 1998). Immuno-cytochemical assays demonstrated binding of C3 to the cephalic gland opening, parasite body and hamulus sheath of the parasite but found no immunoglobulin binding (Buchmann, 1998). *Gyrodactylus* immunity is therefore mediated by a 'scorched earth strategy' where parasites are starved of nutrients and by the more active role of complement (Kania *et al.,* 2010).

***Diplostomum* spp.**

Introduction

Diplostomum (von Nordmann, 1832) species are one of the most common parasite infections in sticklebacks (e.g. Karvonen *et al*., 2013; 2015), especially for stickleback

populations inhabiting lentic environments (Kalbe *et al*., 2002). Historically, three species have been frequently noted; *D. spathaceum* (Rudolphi, 1819), *D. pseudospathaceum* (Niewiadomska, 1984) and *D. gasterostei* (Williams, 1966). The reliance on morphological features (e.g. Niewiadomska, 1986; Williams 1966) to distinguish between morphologically cryptic larval stages has, however, come under much scrutiny (Georgieva *et al.,* 2013; Locke *et al.*, 2010). Molecular approaches have revealed an ever expanding assemblage of *Diplostomum* species complexes spanning the geographic range of sticklebacks (e.g. Loche *et al.*, 2010; Georgieva *et al.,* 2013; Blasco-Costa et al. 2014).

Diplostomum utilises a complex, three stage lifecycle comprising freshwater snails (Family Lymnaeid) and fish as first and second intermediate hosts, respectively, and a range of piscivorous birds as definitive hosts (e.g. common gulls *Larus canus*; Karvonen *et al.,* 2006a). Sticklebacks obtain *Diplostomum* infections by encountering free swimming cercariae shed from infected snails (commonly of the Genera *Lymnaea* or *Radix*). Whilst *Diplostomum* are commonly considered as eye flukes in the fish host, forming metacercariae in the lens, vitreous humour, and/or retina; specific *Diplostomum* lineages may also be present in the brain tissues of fish (see Blasco-Costa *et al.,* 2014; Faltynkova *et al*., 2014).

Source, culture and infection

If an infection of *Diplostomum* has been identified within a stickleback population, it is highly likely that *Lymnaea* or *Radix* snails from the same habitat will be infected. *Diplostomum* prevalence, however, may vary considerably between seasons, localities and snail species (e.g. Karvonen *et al.*, 2006b,c; Faltynkova *et al.*, 2014). To optimise *Diplostomum* collection, select individual snails of larger size classes (e.g. *Lymnaea stagnalis* shell length > 40 mm) during late summer/early autumn to coincide with high prevalence and fully developed cercarial infections (Karvonen *et al.*, 2006c). Infected snail populations may be maintained in the laboratory in aquaria containing continuously aerated water (dechlorinated tap or filtered from source locality), fed *ad libitum* on washed lettuce in controlled climate facilities (reflecting source environment or 18 h light: 6 h dark cycle, ca. 15°C). Light stress is commonly used to stimulate cercarial release, by placing snails individually into beakers of water (ca. 20 ml) under a light source. Cercariae will be shed within 2-4 h provided that fully developed *Diplostomum* cercarial infections are present in snail hosts. Cercariae release rates are

highly temperature dependent, varying between 400 - 2400 cercariae per hour from snails housed in 10-20°C water (Lyholt & Buchmann, 1996).

Identification of cercariae released from snails is necessary since aquatic snails may harbour single or multiple infections of other trematode species. Whilst *Diplostomum* cercariae can be distinguished from other trematode cercariae based on their morphology and resting posture (see Niewiadomska, 1986) at x100 under a compound microscope, molecular techniques are essential to identify specific *Diplostomum* species and/or lineages. Such identification methods are increasingly important for experimental infections in second intermediate host fish since multiple *Diplostomum* lineages, which vary in their capacity to infect sticklebacks or other sympatric fish species, may be present in natural snail populations (Blasco-Costa *et al*., 2014; Faltynkova *et al*., 2014).

To infect sticklebacks, individually housed fish (~ 1 L water) are typically exposed to freshly emerged cercariae at exposure doses ranging from 20 - 100 cercariae per fish (see Haase *et al.,* in press; Scharsack & Kalbe, 2014). High doses of 5,000 - 10,000 cercariae per fish have been used for infection of other fish species (see Sweeting, 1974; Rintamäki-Kinnunen *et al.,* 2004). Whilst *Diplostomum* rapidly reach the ocular tissues (within 24 h post-infection; Chappell *et al.,* 1994), metacercariae establishment success is best assessed after approximately 1 week, since small, early infections may be overlooked (Rauch *et al.,* 2006); however Kalbe & Kurtz (2006) have demonstrated that 2 day and 8 week old metacercariae may be identified when sticklebacks are exposed to repeated cercarial infections.

Immunology

Given the immune privileged status of the eye (i.e. no localised immune response), assumed to be the same in teleosts as in mammals (Niederkorn, 2006; Sitjà-Bobadilla, 2008), in which *Diplostomum* species reside as metacercariae, the immune response is limited to the migratory period between epidermal penetration of the metacerariae and their arrival in the eye. Given this short window of vulnerability it is generally acknowledged that the adaptive response plays no role in resistance against the parasite (Rauch *et al.,* 2006). Instead, reactive oxygen species and oxidative burst are thought to be the key components of the innate immune response against these pathogens; head kidney lymphocyte respiratory burst activity is upregulated in fish 1.5 days post infection but not 5 days post infection (Kalbe & Kurtz, 2006; Scharsack & Kalbe, 2014). There is however a level of concomitant immunity as sticklebacks that receive a primary infection of *D. pseudospathaceum* acquire lower levels of metacercariae in a

secondary infection compared to controls that did not receive the primary infection (Scharsack & Kalbe, 2014). In addition, sonicated metacercariae injected into sticklebacks induce antibody responses capable of providing immunity to subsequent infection (Bortz *et al.*, 1984).

Schistocephalus solidus

Introduction

Plerocercoid larvae of *Schistocephalus solidus* (Müller, 1776) commonly infect three-spined sticklebacks in ponds, lakes and slow flowing rivers (Wootton, 1976; Barber 2007). *S. solidus* is one of the most studied stickleback parasites, and was the first parasite for which a complex, multi-host life cycle was demonstrated experimentally (Abildgaard, 1790). Experimental culture techniques, which permit physiological and developmental studies of the maturing plerocercoid, have been in existence for many decades (Hopkins & Smyth, 1951; Clarke, 1954; Smyth, 1954, 1959, 1962; Arme & Owen, 1967) and are now well-established (Jakobsen *et al.*, 2012). The stickleback-*Schistocephalus* host-parasite model has been widely used for studying the impacts of infection on host energetics (Barber *et al.*, 2008), growth and reproductive development (Heins & Baker, 2008) as well as on host behaviour (Milinski, 1985, 1990; Barber & Scharsack, 2010; Hafer & Milinski, 2016). Recently, experimental infection studies have been used to investigate evolutionary aspects of host-parasite interactions (MacColl, 2009; Barber, 2013) and host immune responses (Scharsack *et al.*, 2004; Scharsack *et al.*, 2007; Barber & Scharsack, 2010), as well as the impacts of changing environments on patterns of infection (Macnab & Barber, 2012; Macnab *et al.*, 2016).

Source, culture and infection

Naturally infected sticklebacks, which are readily identifiable by their swollen profile (Barber, 1997) can be collected from the wild and used as a source of infective parasites for experimental culture. Whilst sticklebacks can harbour multiple *S. solidus* plerocercoids, infected fish often support a single, large plerocercoid. The total mass of plerocercoids can approach that of the host fish (Arme & Owen, 1967). Plerocercoids can be successfully cultured *in vivo* from sizes of 20 mg (Tierney & Crompton, 1992; Dörücü *et al.*, 2007) but plerocercoids are only reliably infective to avian hosts at a body size of ≥ 50 mg (Tierney & Crompton, 1992).

Infective *S. solidus* plerocercoids are readily recovered from the body cavity of euthanised, naturally-infected sticklebacks following ventral incision. Complete, whole plerocercoids should be transferred using sterilised laboratory forceps to a pre-

autoclaved culture vessel containing a loop of narrow-diameter semi-permeable membrane suspended in *S. solidus* culture media (Appendix 1). As they are hermaphroditic, worms can be cultured successfully either individually (i.e. 'selfed') or in pairs, which permits the production of outcrossed offspring (Milinski, 2006). The compression of the worm by the cellulose tubing simulates the situation experienced by the worms in the intestine of the bird definitive host, and provides suitable conditions for fertilisation (Smyth, 1990). The worms, suspended in this 'model gut' inside the culture vessel are then incubated at 40°C in darkness, ideally in a water bath with lateral shaking at a frequency of 80 cycles per minute, which helps to dissipate metabolic products. To reduce bacterial and fungal infections, antibiotics and anti-fungal chemicals can be added to the culture medium (Jakobsen *et al*., 2012). Plerocercoids are progenetic (i.e. exhibit advanced sexual development in the larval stage) and the morphological transition to the adult worm is rapid, with fertilised eggs being produced from day two onwards. Egg production continues for several days, after which the adult worm dies (Dörücü *et al.*, 2007).

The eggs, along with the senescent or dead adult worm(s), can be then be washed from the cellulose tubing into a 12 cm dia. Petri dish by flushing with dH_20. To clean the egg solution, excess distilled water is added to the dish and a gentle swirling movement used to concentrate the eggs; this is best achieved whilst viewing under low power using a dissecting microscope with cold light illumination. Because the eggs are negatively buoyant, they are readily aggregated in the centre of the Petri dish. A pipette can then be used to remove detritus, including tegument of the adult worm, from the egg solution. Repeated iterations of this process, interspersed with dispersing the egg mass, generate a sufficiently clean egg solution for subsequent incubation. Eggs can then be split between multiple sterile Petri dishes, filled to a depth of 5 mm with dH_2O, sealed with Parafilm and wrapped in aluminium foil to restrict premature exposure to light.

Eggs are incubated for 21 d at 20°C in the dark before being exposed to natural daylight to induce hatching (Scharsack *et al.*, 2007). Pre-exposure to a short (ca. 2 h) period of light, the evening before desired hatching, may improve subsequent hatch rates (Dubinina, 1966). Hatched eggs release coracidia, which are spherical, ciliated, free-swimming first stage larvae. Coracidia move actively for ca. 12-24 h after hatching at normal laboratory temperatures, but apparently senescent (i.e. motionless) coracidia can establish infections in copepods hosts (unpublished data). Coracidia are collected using a Pasteur pipette and transferred to a drop of dH_2O on a watch glass, Petri dish or

microscope slide, or in a well of a 96-well microtitre plate. An individual cyclopoid copepod (typically *Cyclops strenuus abyssorum* or *Macrocyclops albinus*) is then added to the water drop containing the hatched coracidium (coracidia) to allow ingestion. It is important to cover the water droplet during this time to prevent evaporation. The water droplet is visually inspected under a dissection microscope to check that the coracidium has been ingested, after which the exposed copepod can be transferred to a larger volume of water and fed under normal culture conditions for 7 d, fed either newly-hatched *Artemia* spp. nauplii or a few drops of *Spirulena* feed (Appendix 1). Copepods are then screened at 7 d post-exposure for infection status. The procercoid stage that develops within the copepod is infective to sticklebacks when it develops a hooked cercomer – a caudal appendage used by the parasite during invasion of the fish host.

Sticklebacks become infected after ingesting infective procercoids (Dubinina 1966). In order to infect sticklebacks experimentally in the laboratory, it is therefore necessary to feed experimentally-infected copepods to sticklebacks. A range of techniques can be used to facilitate this, including feeding by gavage, or by allowing free feeding of isolated sticklebacks. One technique that can be used successfully is to hold individual sticklebacks in 15 cm diameter crystallising dish filled to 3 cm with aquarium water, illuminated from above using a cold light source and surrounded by black paper to improve contrast. A copepod harbouring an infective (i.e. cercomer-bearing) procercoid is moved up and down within the neck of a long-form Pasteur pipette, immediately in front of a stickleback that had food withheld for 24 h, before releasing it into the water. Alternatively, fish can be introduced to a small (1 L) plastic aquarium along with a small number of newly-hatched *Artemia* spp. nauplii and an infected copepod and left to forage for a 6 h period. Exposure can be confirmed either by direct observation of the ingestion event, or by sieving the water to confirm the removal of the copepod. Such techniques can be used to generate singly- or multiply infected sticklebacks for use in experimental studies.

A number of useful tools have been developed to increase the utility of the stickleback-*Schistocephalus* host-parasite model. The growth of the plerocercoid stage *in vivo* can be estimated non-invasively using image analysis based on the infection-induced swelling (Barber, 1997), facilitating longitudinal studies of infection and parasite growth. Individual coracidia can be stained using persistent fluorescent dyes (Kurtz *et al*., 2002), allowing differentiation of individual parasites in mixed infections. Finally, there now exist a wide range of microsatellite markers (Binz, 2000) and other genomic

resources, which facilitate taxonomic studies (Nishimura *et al.,* 2011) as well as ecological and biogeographical studies of genetic diversity of the parasite (Sprehn *et al.,* 2016), as well as a reference transcriptome (Hébert *et al.,* 2016).

Immunology

A rapid host immune response is thought to be crucial for *S. solidus* resistance, halting establishment within the body cavity. Infection prevalence drops from 60% in the first week to 54-52% one month post-infection, but with no further decline thereafter (Scharsack *et al.,* 2007). In addition, no dead *S. solidus* were detected in the body cavity after 17 days post-infection suggesting that this is the limit at which the immune response is effective against the parasite (Scharsack *et al.,* 2007). *S. solidus* resistance is associated with early proliferation of head kidney monocytes and lymphocyte proliferation 7 days post-infection (Barber & Scharsack, 2010).

Camallanus lacustris

Introduction

Camallanus lacustris (Zoega, 1776) is a nematode parasite of predatory fish, such as perch or pike, which can use the stickleback as a paratenic host (Kalbe *et al.,* 2002; Krobbach *et al.,* 2007). As adults, camallanids attach to the anterior section of the gut causing an inflammatory reaction (Meguid & Eure, 1996). *C. lacustris* exhibits a seasonal reproductive life cycle with L1 larvae only produced during the summer months (Skorping, 1980; Nie & Kennedy, 1991). The larvae are free moving within the parental uterus and are easily identified by their coiling and uncoiling motion. These L1 larvae are subsequently released; presumably shed (although the process has never fully described) from the vulva of the adult worm and released into the environment within fish faeces. Free-living L1 larvae in water are viable for 12 days at 22°C and 80 days at 7°C (Campana-Rouget, 1961) and are ingested by copepods. A range of Cyclopidae copepods act as intermediate hosts in which the larvae develop into L2 larvae after 3 days at 25° or 5 days at 20°C. The second moult into L3 occurs after 6 days at 25°C or 10-12 days at 20°C (Campana-Rouget, 1961; Stromberg & Crites, 1974; Stromberg & Crites, 1975). Only at L3 stage, coiled in the haemocoel of the copepod after migration from the digestive tract (De, 1999), is the worm infective to the definitive host on ingestion of the intermediate host (Moravec, 1969). The camallanid is relatively large within the haemoceol and although unstudied it would be remarkable if the parasite did not shorten the life-span of the copepod, particularly if infected with two or more worms. Similar to *Schistocephalus solidus* infections, copepods infected with *C.*

lacustris reduce predation risk prior to infectivity and increasing predation risk after it has reached infectivity (Wedekind & Milinski, 1996; Hafer & Milinski, 2016). Direct transmission from the copepod to the definitive host by ingestion is possible (Chubb, 1982), although more likely the copepods are eaten by planktivorous fish (such as sticklebacks) which act as paratenic hosts and in which the worms do not become reproductive. Predatory fish will prey upon the stickleback and ingestion of the camallanid will allow final development into the reproductive adult. Within the definitive hosts, L3 larvae of *C. lacustris* attach to the mucosa in the anterior section of the intestine and mature within 69 day, producing *in utero* L1 larvae (Chubb, 1982).

Source, culture and infection

Gravid *C. lacustris* adults can be collected most commonly from the anterior intestine of perch (*Perca fluviatilis*) during summer in the UK; although Salmonidae, Gadidae, Esocidae and Siluridae may also act as hosts (Moravec, 1971)

The characteristic red adult *Camallanus* worms (Fig. 2.2A) will survive for 1-2 weeks *in vitro* at 4°C in 50% PBS. L1 larvae (Fig. 2.2B) can be removed from the adult worm, held in a watch glass with 50% PBS, by puncturing the uterus with watchmakers forceps and allowing uterine contractions to force out the larvae. The L1 larvae are visible using a dissection microscope (x10-60), conspicuous due to their high motility, likely an adaption to increase predation. L1 larvae survive for a minimum 2-3 days *in vitro* at 4°C in tank water. The L1s can be transferred using a *Caenorhabditis elegans* worm pick or P2 pipette to a non-treated culture dish or watch glasses with glass cover lids containing copepods from the Family Cyclopidae. Previous experiments have used many copepod species as hosts for camallanids, including *Mesocyclops, Thermocyclops* (see Bashirullah & Ahmed, 1976), *Macrocyclops* (see Krobbach *et al.,* 2007), *Acanthocyclops* (see Chubb, 1982) and *Cyclops* spp. The larger of the *Macrocyclops* spp. has been used as a host for up to six larvae of *Camallanus lacustris* (see Krobbach *et al.,* 2007). Smaller copepod species may be less able to survive such intense infection.

Macrocyclops spp. should be fed on *Artemia* spp. (see Krobbach *et al.,* 2007) although species such as *Cyclops strenuus* survive well on a daily mixture of *Spirulena* and yeast (approximately 1 ml to an 10 L litre tank of copepods; see Appendix 1). For copepods kept in culture dishes, remove half their water and replace with a dilute feed mixture (100 µl in 100 ml) every 2-3 days.

Figure 2.2: (A) Adult and (B) L1 *Camallanus lacustris*.

C. lacustris develop into the L3 in about two weeks at 15-18°C on a 16: 8 h light/dark cycle. The L3 stage can be checked by sacrificing a copepod or using a recently deceased host, squashed on a glass slide with a cover slip and a drop of water under a compound microscope (x100). Striations on the buccal capsule, which itself is apparent first in the L2, are characteristic of the L3 (Fig. 2.3). To infect sticklebacks with *C. lacustris*, starve the fish for 24 h then release infected copepods into a crystallising dish containing the intended host, the optimal number of camallanids to feed each stickleback is six, which will give an infection rate of 40-50% (Krobbach *et al.,* 2007). Prior to infection, sticklebacks should be acclimated to feeding on copepods.

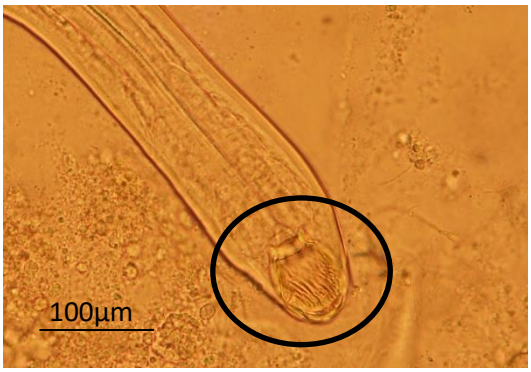

Figure 2.3: L3 *Camallanus lacustris* with buccal capsule (circled). The striations denoting the L3 are apparent running laterally along the buccal capsule.

Immunology

Stickleback immunity against *Camallanus* spp. infection is largely unknown with the majority of papers predating the genomic revolution. Granulocyte/lymphocytes are elevated during infection, but not respiratory burst and leucocyte responses (Krobbach *et al.,* 2007). Given the similarity between teleost and mammalian immune systems, it is likely that a nematode infection in teleosts will produce the same response to infection as a mammal. As such we might predict elevated, alternatively, activated macrophages and T-lymphocyte activity, particularly of the T_H2 and T_H9 subtypes.

Saprolegnia parasitica

Introduction

Oomycetes present a major threat to food security in aquaculture, but also terrestrial food sources, the most prominent being *Phytophthora infestans*, which caused the 19th Century Irish potato famine and continues to causes economic losses of €100 million annually (Haverkort *et al.,* 2008). In freshwaters, oomycetes from the genera *Saprolegnia*, *Achlya* and *Aphanomyces*, Order Saprolegniales, Sub-class Saprolegniomycetidae, are responsible for significant losses of fish (Jeney & Jeney, 1995; van West, 2006). Species identification typically depends on sequencing of the rDNA Internal Transcribed Spacer (ITS) region (Sandoval-Sierra *et al.,* 2014). As fungal-like heterotrophs they have branching tip-growing mycelia, typically thicker than fungi at 10 μm dia., and unlike fungi they have a cellulose, rather than chitin, cell wall. The chitinase gene is present but is thought only to have a role in hyphal tip growth (Baldauf *et al.,* 2000; Guerriero *et al.,* 2010; Beakes *et al.,* 2012).

The *Saprolegnia* lifecycle, as with other oomycetes, has an asexual stage including the development of sporangia and zoospores, and a sexual stage resulting in the production of oospores (see van West, 2006). The asexual stage is the primary method of infecting new hosts as free-swimming zoospores are released into the environment (Hatai & Hoshiai, 1994; Willoughby, 1994; Bruno & Wood, 1999). The sexual production of oospores is thought to enhance survival under acute stress conditions, such as temperature extremes or desiccation, until conditions become more favourable. Some *Saprolegnia* species (including most strains of *S. parasitica* Coker), however, seem to lack a sexual cycle and do not produce oospores, at least under laboratory growth conditions.

Two of the major oomycetes of fish infect adults, *S. parasitica*, or eggs, *S. diclina* (Humphrey) (see Van Den Berg *et al.,* 2013). *Saprolegnia* species were controlled using the organic dye malachite green or formalin up until 2002 and 2015, respectively, when they were banned in aquaculture for their carcinogenic and toxicological properties (Srivastava *et al.,* 2004; van West, 2006; Sudova *et al.,* 2007; Defra, 2015). Current control methods for salmonid eggs include formaldehyde, salt and ozone (Fornerisa *et al.,* 2003; Khodabandeh & Abtahi, 2006; van West, 2006) of which formaldehyde can also be used to treat or reduce mortality in adult fish (Ali, 2005; Gieseker *et al.,* 2006). Low concentration sodium chloride can be used to inhibit *Saprolegnia* germination but is not an efficient treatment as it will not fully arrest growth.

During infection, *S. parasitica* secretes a SpHtp1 protein, which is able to translocate independently into fish cells via an interaction with the cell surface tyrosine-O-sulphated molecule of the fish (van West *et al.,* 2010; Wawra *et al.,* 2012). The precise function of SpHtp1 is unknown, but it likely plays a role in the infection process. This finding and the immunomodulation capabilities (see Belmonte *et al.,* 2014) of *S. parasitica* suggest that the interaction is more complex than previously considered. Indeed one could argue that *S. parasitica* is a primary pathogen rather than a secondary pathogen as has often been assumed (e.g. Hoole *et al.,* 2001).

Source, culture and infection

Cultivated strains of *S. parasitica* are held at various institutions but the parasite can also be isolated from wild fish. The mycelia can be maintained on potato dextrose agar (PDA) (Appendix 1) in 140 mm Petri dishes indefinitely at 15-25°C (light cycle and humidity unimportant). Cultures should be re-plated every month, to protect against bacterial and fungal contamination, by transferring a 5 mm dia. plug of healthy (white/grey in colour with no yellowing or other fungal growth) mycelium from one Petri dish to another. To isolate a wild strain, scrape mycelium off an infected fish and inoculate onto a potato dextrose agar plate containing chloramphenicol at 50mg/ml. Re-plate the *Saprolegnia* mycelium repeatedly, taking 5 mm dia. plugs from the leading edge until a pure culture is obtained devoid of bacteria and fungi. The *Saprolegnia* mycelium is cotton like and white/grey in colour, all other growth should be avoided when taking the plug for culture.

To infect sticklebacks from a stock culture, take 3 plugs of mycelium (5 mm dia.) and place them in a 140 mm Petri dish with 70 ml of pea broth (Appendix 1) for 72 h at 25°C. Following incubation, remove the agar plugs from the broth using sterile forceps and the pea broth using a sterile syringe or pipette. The cultures are then washed three times with 70 ml of a 50/50 mixture of distilled and tank water in the Petri dish. During each wash, after the addition of the water mix, the mycelium should be agitated before the water mix is removed. Finally, add 30 ml of the 50/50 distilled and tank water mixture to the Petri dish and incubate for a further 24-48 h @15°C (Powell *et al.,* 1972; Riberio, 1983). Alternatively, dispense the clean mycelium from one Petri dish into 500 ml of 50/50 distilled and tank water, incubating for 24-48 h @15°C. The cultures should be checked for spore production under a microscope (x100); acquire the spores for infection by straining the *Saprolegnia* though a 40 μm cell strainer using a cell scraper to remove encysted spores from the Petri dish. Calculate the spore density using a

haemocytometer. If necessary, concentrate the sample by centrifuging at 3000 g for 5 min at room temperature, remove the excess supernatant and re-suspend the spores in distilled water. Fish can be infected by the ami-momi technique, in which fish are typically shaken in a net for 2 min (Hatai & Hoshiai, 1994), this duration of shaking is excessive for sticklebacks instead we recommend 30s shaking. After the ami-momi technique has been applied expose the fish, ideally individually, to $3x10^5$ spores per litre.

Immunology

Upon infection with *S. parasitica*, fish undergo a rapid acute phase response including upregulation of genes transcripts encoding all three complement pathways: C1r, C2, mannose-binding lectin, C3 and C6 (Roberge *et al.,* 2007). Other immune related genes including *ATP-binding cassette transporter* (required for MHC class I antigen presentation), and the cytokine receptors *CXCR4* (chemokine of importance in humoral immunity) and *cd63* (cell development and growth of multiple immune cells) are also upregulated (Roberge *et al.,* 2007). Induction of proinflamatory cytokines *il-1β, il-6, tnf-α* and *cox2* likely occur as a response of tissue damage by the oomycete (Kales *et al.,* 2007; Bruijn *et al.,* 2012; Belmonte *et al.,* 2014). In addition to upregulation of inflammatory genes, the parasite is capable of immunomodulation by means of prostaglandin E_2, causing suppression cellular immunity including a reduction in gene transcripts *cd8a, ifn-γ, il-4* and *il-17* (Belmonte *et al.,* 2014). It is clear then that there is a complex exchange between host and parasite that results in suppression of potentially protective cellular responses.

Glugea anomala

Introduction

Glugea anomala (Moniez, 1887) is a microsporidian that causes white tumour-like growths, ca. 1-4 mm dia., known as the xenoparasitic complex. This complex is formed of many polypoid host cells, in which the microsporidian replicates and grows, by stimulation of hypertrophic growth of the host tissue (Lom & Dyková, 2005). For *G. anomala* infecting sticklebacks, the xenoparasitic complex was re-named the 'xenoma' (Weissenberg, 1968). *G. anomala* acquire nutrients by producing a hyposome with rhizoids that extends into the host cell cytoplasm (Lom & Dyková, 2005).

Source, culture and infection

There are multiple published methods for infection of fish with *G. anomala* and other microsporidians, including *Tetramicra brevifilum* (see Figueras *et al.,* 1992). It is

assumed that *G. anomala* is transmitted orally during cohabitation of infected and uninfected fish (Lom & Dyková, 2005). Other methods of experimental infection include intraperitoneal, intramuscular or intravascular injection, and anal or oral gavage (Shaw & Kent, 1999). Crustaceans, including *Artemia salina* (brine shrimp) and *Corophium spinocorne* (amphipods), may act as intermediate hosts for *Glugea stephani* (see Olson, 1976). Preliminary testing of several infection methods in our laboratories including: oral transmission of extracted spores in the water column, oral gavage, intramuscular injection, co-habitation of infected and uninfected fish and exposure of putative intermediate hosts (*A. salina*, *Cyclops strenuous* and *Daphnia magna*) to *Glugea* spores for 48 h, to date, have not resulted in parasite transmission 90 days post treatment, despite xenomas reportedly developing 3-4 weeks post infection (Lom & Dyková, 2005).

Immunology

To date, there is only preliminary data on the immune response to *Glugea*. There is little or no detectable host response to the microsporidian until the xenoma is fully developed. Macrophage aggregates occur around the outside of the xenoma wall with eosinophils and neutrophils being recruited to reduce the mass of spores within the xenoma (Dezfuli *et al.,* 2004; Lom & Dyková, 2005).

Argulus foliaceus

Introduction

Argulus foliaceus (Linnaeus, 1758) is an ectoparasitic crustacean of the sub-class Branchiura. It is a generalist parasite with a widespread distribution across much of Europe being recorded on most freshwater fishes including: carp (*Cyprinus carpio*), bream (*Abramis brama*), brown trout (*Salmo trutta*), perch (*Pera fluviatilis*), pike (*Esox lucius*), sticklebacks (*Gasterosteus* spp.) and tench (*Tinca tinca*) (see Bower-Shore, 1940). *A. foliaceus* attaches to its host by means of circular sucking disks, contraction of the disk muscles results in adhesion (Møller *et al.,* 2008). Alternate relaxation and contraction of these two disks allows the louse to move around the host's surface. Further support is provided by a series of spines on the underside and edges of the carapace. *A. foliaceus* have two compound eyes used for detection of the host in light conditions in which the parasite uses ambush behaviour to find a host (Mikheev *et al.,* 2000). This behaviour switches in dark conditions to a 'cruising search strategy' accompanied by a mean increase in swim speed allowing the parasite to cover an area 3-4 times greater (Mikheev *et al.,* 2000). Argulids feed by means of a stylet and proboscis,

which is inserted into the skin of the host, a cytolytic toxin is injected, and blood is drawn up the stylet (Hoffman, 1977; Walker *et al.*, 2011).

Species	**Temperature (°C)**	**Hatching time (days)**	**Lighting (Light:Dark)**	**Reference**
A. americanus	22	16	Unknown	Hoffman (1977)
	18	18	Unknown	Hoffman (1977)
A. catostomi	22	35	Unknown	Hoffman (1977)
A. coregoni	21	30	Unknown	Hakalahti *et al.* (2004)
A. foliaceus	Natural 18-23	25-51	Natural summer	Pasternak *et al.* (2000)
	21	14-30	16:8	Our own cultures
	26	8	Unknown	Fryer (1982)
	Unknown	30-35	Unknown	Bower-Shore (1940)
	Unknown†	45	0:24	Bai (1981)
	Unknown†	38	11:13 (1750 lux)	Bai (1981)
	Unknown†	29	11:13 (100,000 lux)	Bai (1981)
	Unknown†	24	24:0 (69,000 lux)	Bai (1981)
A. japonicus	30	12	Unknown	Hoffman (1977)
	15	60	Unknown	Hoffman (1977)
	15	61	Unknown	Shafir & As (1986)
	35	10	Unknown	Shafir & As (1986)
A. lepidostei	22	10	Unknown	Hoffman 1977)
A. siamensis	0	Nil	Unknown	Sahoo *et al.* (2013)
	4	Nil	Unknown	Sahoo *et al.* (2013)
	15	Nil	Unknown	Sahoo *et al.* (2013)
	28	15	Unknown	Sahoo *et al.* (2013)
	32	10	Unknown	Sahoo *et al.* (2013)

Table 2.2: *Argulus* species and their time until hatching at various temperatures and photoperiods. †Although not specifically mentioned it is assumed temperature is the same in all cases.

Adult females lay strings of eggs, typically in 2-4 rows totalling between 20 and 300 eggs per string. Egg-laying is seasonal in the wild and generally occurs between July and August but can be conducted year round in the laboratory (Pasternak *et al.*, 2000; Harrison *et al.*, 2006). The eggs are commonly laid on hard substrates such as rocks, sticks and other hard substrates. The eggs are 0.3-0.6 mm in length and coated in cement, which anchors them firmly to the substrate. Egg hatching times are variable but strongly influenced by temperature. The first life stage is the nauplii which, depending on *Argulus* spp., will develop to the metanauplii or first copepodid stage within the egg

before hatching. *Argulus* spp. will then go through the 2nd-7th copepodid stages, subadult and adult stage (Hoffman, 1977). Below 9°C, the adults and sub-adults enter a hibernative state and development will cease (Hoffman, 1977). Development time is strongly reliant on temperature with eggs in the wild being laid before winter, over wintering and hatching in spring. Time to hatching is strongly reliant on both the species and temperature (Table 2.2). *A. foliaceus* egg development arrests below 10-12°C and oviposition of the adults will not occur below 10-12°C (Hoffman, 1977; Pasternak *et al.*, 2000; Taylor *et al.*, 2009).

Source, culture and infection

All life stages of *A. foliaceus* can be maintained in the laboratory. Although the methodology outline below refers specifically to this species, it can probably be applied to most *Argulus* species (e.g. *A. coregoni*, see Hakalahti *et al.* 2004).

A. foliaceus is a generalist and adults may be sampled from virtually any freshwater fish species, although carp are often a good source. Individual lice should be sexed: males have a larger and darker region defining the testes (Fig. 2.4A) while the females' egg sacks lack this darker region (Fig. 2.4B) and eggs may be visible within the egg sacks. Adult *A. foliaceus* suffer from poor survival in the lab. Although adult *A. foliaceus* are generally too large for sticklebacks to eat, their swimming style makes them vulnerable and fish will attack detached individuals. Therefore, abundant refugia (plant pots, fake or real weed, netting and/or plastic pipes) should be provided in the tank.

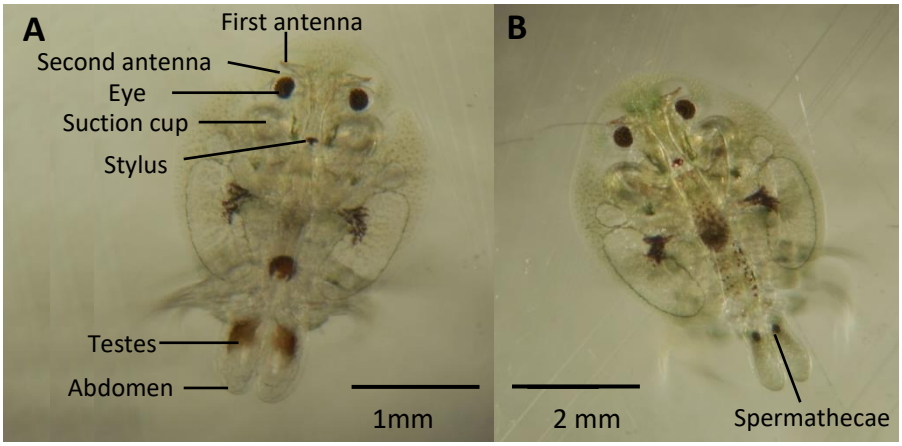

Figure 2.4: *Argulus foliaceus*. (A) Male 3-5 mm in length, with visible brown testes on the abdomen. (B) Female 5-7 mm in length, with small brown spots that are the spermathecae.

Infections with all *A. foliaceus* life stages can be performed by anaesthetising a stickleback in 0.02% MS222. The fish should then be transferred to 100 ml of dechlorinated water to allow the argulid to find the host as rapidly as possible. Adult

sticklebacks can be infected with 1-2 individuals of adult *A. foliaceus* (infecting with more than two is possible but not recommended as this may reduce stickleback survival). Alternatively, place in the dark and add refugia to reduce predation and allow natural infection, works well with metanauplii and copepodid.

For *A. foliaceus* breeding, infected fish should be kept at 15-25°C (optimally 20°C), transfer a single adult male and female *Argulus* to one host. The argulids will breed on the host with the female detaching in order to lay their eggs. *A. foliaceus* will lay eggs on hard substrate in shaded areas (Pasternak *et al.,* 2000; Taylor *et al.,* 2009) so the underside of rocks, stones and wood within a tank, or even the walls or bottom of the tank itself, provide a suitable habitat. Tanks should be regularly checked for eggs to prevent unwanted microbial infections when nauplii hatch. Ideally infected fish will be kept in 1 L pot and fish simply transferred to a new tank leaving the parasite eggs that can be stored or left for hatching. An advantage of using refugia, is that if eggs are laid here they can simply be moved to a pot of fresh water leaving the fish *in situ*. For fish in glass tanks, eggs laid on the tank glass can be removed with a razor blade or cell scrapper, and transferred to a 1 L pot or petri dish containing dechlorinated water. Alternatively, females can be removed from the fish when they develop large ovaries and placed into Petri dish (90 mm dia.) containing dechlorinated water for 24 h allowing them to lay their eggs.

Argulus spp. eggs can be stored at 4-5^{o}C, which arrests embryo development, causing the nauplii to go into 'over winter' state. Photoperiod may also alter hatching. *A. siamensis* in dark conditions take up to 15 days to hatch (Bai, 1981). It is unknown how long eggs can be maintained in an arrested state, but successful hatching of eggs up to 4 months old has been achieved in our laboratory. To induce hatching, transfer the eggs to a 1 L container of freshwater with aeration, timing is temperature and species dependent (Table 2.2). Once hatched the metanauplii will survive off the host for 2-3 days, but survival rate decreases *in vitro*. The metanauplii and copepodids should be kept on sticklebacks or carp, a maximum of 5 for a stickleback and 20 on a 20 g carp. Infected fish should be maintained at 15-20°C; warmer temperatures will increase *A. foliaceus* growth rate but also stochastic fish mortality. When copepodids reach the sub-adult stage remove all but two argulids from the fish, by gently encouraging them off the fish with a pipette tip or blunt forceps, and infect other fish with the detached argulids.

Immunology

The immunology of *A. foliaceus* infection has been little studied; there are however some closely related species for which the host immune phenotype has been documented. *A. siamensis*, infecting rohu (*Lebero rohita*), for example, stimulate elevated lysozyme, β2 macroglobulin, natural killer cell enhancing factor and superoxide dismutase (Kar *et al.*, 2013). Additionally *A. siamensis* appears to modulate the immune system down-regulating α2 macroglobulin, *tnf-α, TLR22* and serum complement (Saurabh *et al.*, 2010; Kar *et al.*, 2013). *A. japonicus*, which infects common carp (*Cyprinus carpio*), induces a slightly different response including up-regulation of *tnf-α* and the chemokines *CXCa* and *CXCR1* (Forlenza *et al.*, 2008).

Treating common infections

Not all infections can be eliminated and treatment choice is dependent on infection history and the planned fate of the fish. A list of common treatments for common aquarium infections is provided in Table 2.3.

The most common endemic infections to occur in laboratory tanks of sticklebacks are microparasites, commonly *Aeromonas* spp., *Flavobaterium* spp., *Pseudomonas* spp., *Ichthyophthirius multifiliis* and *Saprolegnia parasitica*. These infections typically establish when fish are stressed, for example by experimental procedures, following capture and/or transportation. The pathogens are ubiquitous, present in most water bodies and therefore are difficult to eliminate from aquatic systems. Additionally *Gyrodactylus* spp. and *Trichodina* spp. are easily introduced into tanks. Most *Trichodina* spp. and other ecto-commensals including *Epistylis* spp. and *Apiosoma* spp. are asymptomatic at low numbers but may become pathogenic at high intensities (see Collymore *et al.*, 2013). Other infections such as *Schistocephalus solidus*, *Diplostomum* spp. and *Camallanus lacustris* may infect wild sticklebacks but cannot be transmitted without the presence of their intermediate hosts. Although *Glugea anomala* may be transmitted directly, the details of transmission are unknown. Transfer of water between tanks should be avoided in all cases. Nets are a common source of water transfer and should be sterilised in Virkon or sodium metabisulfite (as per manufacturer's instructions), rinsed and dried before reuse. If fish are infected, isolate them and treat for infecting species as indicated (Table 2.3); early detection and rapid treatment is key for the majority of these infections.

Aeromonas spp. and *P. fluorescens* cause red ulcers, small white/grey marks fins and head, fin rot and death; if severe damage occurs the fish should be euthanized using a

Schedule 1 Procedure. Infections with these pathogens should be treated using an antibiotic; consult with a veterinarian. Because it is often difficult to distinguish these two infections without biochemical or molecular techniques, a broad-spectrum antibiotic can be used following the manufacturer's instructions.

I. multifiliis is a highly contagious protozoan parasite that causes small white spots on the fins and skin of the fish. The simplest method of treatment is increasing water salinity and adding methylene blue (see Table 2.3). A low concentration formalin or malachite green treatment may also be used following the low and prolonged immersion dose (see Table 2.3) or using an off-the-shelf solution following manufacturer's instructions. Given the complexity of the life-cycle, and the fact that resistance is common, multiple treatment doses are likely to be required.

Saprolegnia spp. and the closely related *Achyla* spp. are oomycetes that cause cotton like white/grey fungal-like mycelia. In the case of these infections, prevention (0.5% saline water) is better than cure; once a fish is infected it may survive no more than a few days, occasionally even hours, or be irreparably damaged, in these cases euthanize using a Schedule 1 procedure. If *Saprolegnia* infection does occur use high dose malachite green in formalin solution treatment (see Table 2.3), a low concentration formalin treatment may also be effective. After malachite green treatment, keep the fish in salt solution (0.5-1 %, 5-10 ppt) with the possible addition of methylene blue (see Table 2.3).

Gyrodactylus spp. are monogenean ectoparasites that can only be seen under a low powered microscope. Gyrodactylid treatments are problematic because 100% efficacy is required and transmission can easily occur between adjacent tanks by water or net transfer. The only tested treatment that works consistently for stickleback gyrodactylids is a high concentration formalin bath (Table 2.3). Other less damaging treatments, such as praziquantil and levamisole, are not as efficacious; follow manufacturers or veterinary advice when using these treatments. After treatment, screening for the parasite should be performed three times, no more than once per day, to ensure the parasite has been removed effectively from the entire host population (see Schelkle *et al.*, 2009).

Trichodina spp. are ciliated protists only visible under a low powered (x10-60 mag.) microscope. They appear as 'flying-saucer' shaped disks attached to the epidermis, fins and gills, but often moving freely over the surface of the fish. Changing tank water

regularly and keeping the water crystal clear effectively eliminates most *Trichodina* spp. If the clean water treatment fails, which is rare, low dose malachite green treatment is usually successful after 2-3 doses (Table 2.3). Other infections, *G. anomala*, *Diplostomum* spp. and the macroparasitic internal parasites are either difficult to treat, cannot be treated or may not need to be treated. *Diplostomum* spp. found in the lens and vitreous humour may be treated with praziquantel, although removal is not guaranteed. Likewise, once *S. solidus* worms have migrated through the intestine and into the body cavity they cannot be treated. *G. anomala* cannot be cured, although some success has been achieved in reducing spore survival using benzimidazole treatments (Schmahl & Benini, 1998).

Treatment	**Concentration**	**Time**	**Dosage**	**References**
Antibiotics	-	-	Consult with a vet	
Formalin† (Low)	0.015-0.025 ml/L	72-96 h	Once	Chinabut *et al.* (1988) Francisn-Floyd (1996)
Formalin† (High)	0.15-0.25 ml/L	30 min	Twice in 24 h Allow fish 30 min rest in freshwater between treatments	Chinabut *et al.* (1988) Francisn-Floyd (1996)
Malachite Green ‡ (Water-Low)	0.1-0.25 mg/L	72 h	1-3 times	Alderman (1985) Srivastava *et al.* (2004) Sudova *et al.* (2007)
Malachite Green ‡ (Water-High)	1-2 mg/L	30-60 min	1-3 times Allow 24 h between treatments	Alderman, (1985) Srivastava *et al.* (2004) Sudova *et al.* (2007)
Malachite green‡ (Formalin†)	0.015 ml/L of stock solution §	24 h	3 times every 24 h	Alderman (1985) Srivastava *et al.* (2004) Sudova *et al.* (2007)
Methylene blue§	2 mg/L	>24 h	Re-administer every 2-3 days	Tieman & Goodwin (2001)
Salt	0.5-3% (5-30 ppt)	>24 h	Increase concentration at 0.5% daily	Matthews (2005) van West (2006)
Stress coat (API™)	Follow manufacturer's instructions	>24 h	Follow manufacturer's instructions	Harnish *et al.*(2011)

Table 2.3: Common treatments for stickleback infections, indicating treatment dose, duration, repeated treatments and frequency. †Formalin is a 40% formaldehyde solution, wear appropriate personal protective equipment (PPE) and use in a well ventilated area. Paraformaldehyde is a white precipitate highly toxic to fish, discard solutions with white precipitate. Formalin depletes oxygen from water, so it is important to supply additional aeration for fish. Monitor fish for gasping, fin whitening, unusual swimming; if these symptoms occur discontinue treatment. Formalin treatments can be purchased from pet shops, follow instructions in these cases. ‡Malachite green is a suspected carcinogen and respiratory poison, wear appropriate PPE and conduct in a well ventilated area. A zinc free grade must be used. For a formalin stock add 3.3g malachite green per 1 L of formalin (†).§Methylene blue can reduce bacterial concentrations of tanks. Monitor biological filter systems and test for nitrate, nitrite, ammonia and pH.

Summary

Despite the wealth of literature on stickleback parasites, it is apparent that there are knowledge gaps for the basic biology of even some of the more common parasites. There are also many other parasites for which laboratory culture methods do not yet exist (e.g. myxozoans and many of the intestinal stickleback parasites such as *Proteocephalus filicolis*). Yet other studies have turned their attention to molecular techniques seeking out new methods of studying stickleback biology (e.g. Hibbeler *et al.*, 2008; Brown *et al.*, 2016). This chapter unites much of our knowledge about the culture techniques of this system providing a basis for future research. With new and improved culture and molecular methods stickleback biology is entering a new era of study that will increase our depth and diversity of knowledge and provide insights into the importance of parasites in ecologically and economically important systems.

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Chapter 3 - The impact of global warming and resource availability on three-spined sticklebacks (*Gasterosteus aculeatus*) and *Saprolegnia parasitica* and *Gyrodactylus gasterostei* infections

Abstract

Climate change is often cited as the largest threat to sustainable food production. As ectotherms, teleosts are heavily reliant on ambient temperature which alters metabolic and cellular functions, and resource availability. In this study, the three-spined stickleback (*Gasterosteus aculeatus*) was utilised to examine the effects of circannual temperature variation, predicted 2°C increase in climate, and resource availability, on parasitic infections and immunity. Sticklebacks, kept in mesocosms at ambient temperature and 2°C above ambient on both high or low feeding regimes, were experimentally exposed to one of two parasites: *Saprolegnia parasitica* or *Gyrodactylus gasterostei*. Unexpectedly, *S. parasitica* infections were not reliant on cold temperatures, with little circannual variation in prevalence or intensity of infection. Instead, prevalence and intensity were positively correlated with fish body condition; the former potentially occurring as a result of a trade-off in host body condition vs. immunity, the latter reflecting the parasite benefitting from exploitation of higher levels of host resources. No effect of increased temperature or feeding regime could be found on *S. parasitica* infections. *G. gasterostei* infection intensity was reliant on historical parasite and temperature exposure explained by the Foothold Hypothesis; whereby parasites established on the host are able to adapt to a variable environment faster than the host allowing higher infection intensities to be reached. In addition, fish infected in warmer months were better able to control infection, despite *G. gasterostei* population growth being enhanced at these temperatures. Fish on a low food treatment infected with *G. gasterostei* were less able to clear infections, likely occurring due to a lack of resources to invest in immunity. This study has wide ranging implication for aquaculture where high levels of feeding, increasing fish mass, could be exacerbating *S. parasitica* infections. Also highlighted is the interaction between variable temperature conditions and their effect on *G. gasterostei* infection intensity.

Introduction

Climate change is unequivocal: rising sea levels, warming atmosphere and oceans, dwindling sea ice and elevated greenhouse gases are all anthropogenic challenges to be faced by our and future generations. The Intergovernmental Panel on Climate Change

(IPCC) predicts that global atmospheric and surface temperature is likely to increase by 1-3.7°C by 2100 (IPCC, 2013a). Localised bodies of water, such as rivers and lakes, have a thermal behaviour heavily dependent on temporal, spatial, meteorological, hydrological and anthropogenic forces. The increase in mean temperature of local bodies of groundwater is expected to track a similar pattern to atmospheric temperature (Kothandaraman & Evans, 1972; Webb & Nobilis, 2007). A study of rivers in Devon, 1977-1990, identified significant increases in water temperature and predicted a rise in water temperature of between 1.3-1.6°C by 2050 (Webb & Walling, 1992; Webb & Walling, 1993). Likewise, ocean temperatures in the upper 75 m are warming at a mean rate of 0.11°C per decade (IPCC, 2013b).

Temperature increases of 1-3.7°C predicted globally by climatologists, are likely to have a significant impact on disease emergence and host immunity. In mammal cells small elevations in body temperature, causing hyperthermia, can affect the immune response causing changes in membrane fluidity, clustering of receptors and enhanced antigen-specific conjunction (Mace *et al.,* 2011). For ectotherms heavily reliant on environmental temperature, with enforced hypo- or hyperthermia caused by external influences, responses to infection could be drastically transformed by altered metabolic and immune processes (Schurmann & Steffensen, 1997; Watts *et al.,* 2008; Uribe *et al.,* 2011). For example, increases in body temperature of teleosts can directly affect their growth rate (Handeland *et al.,* 2008), the growth rate of their parasites (Macnab & Barber, 2012) or their immune system. The adaptive immune response functions better at warmer temperatures (Miller & Clem, 1984; Bly & Clem, 1992; Lillehaug *et al.,* 1993; Alcorn *et al.,* 2002), but becomes less active at temperatures below 4-10°C resulting in loss of T-cell and immunoglobulin function dependant on species (Avtalion, 1969; Avtalion *et al.,* 1970; Bly & Clem, 1994; Morvan *et al.,* 1998). The innate immune response conversely functions better at lower temperatures (Brown *et al.,* 2016), increasing respiratory burst activity (Miles *et al.,* 2001; Nikoskelainena *et al.,* 2004).

Parasites and their interactions with the host's immune system are severely restricted by thermal tolerance. Viral infections, such as viral haemorrhagic septicaemia, infectious haematopoietic necrosis virus and spring viraemia of carp, only establish when water temperatures drop below 14-17°C (Marcos-López *et al.,* 2010). Likewise, *Saprolegnia parasitica* infections typically occur during colder conditions (Bly *et al.,* 1992; Bruno & Wood, 1999), 15°C being optimal for zoospore production (Powell *et al.,* 1972; Riberio,

1983). In contrast to *S. parasitica*, gyrodactylid species, fair better at warmer temperatures were their reproductive rate is accelerated (e.g. Chappell, 1969; Lester & Adams, 1974; Harris, 1982; Jansen & Bakke, 1991). In addition, a long term study on temperature warming over 25 years found protozoan diseases including *Ichthyophthirius multifiliis* have increased in prevalence while *Ichthyobodo necator* has decreased in prevalence (Karvonen *et al.*, 2010). This means that parasite ecology and interactions with temperature, regardless of taxonomy, can drive disease emergence in either a positive or negative direction with implications for how the host might resist a changing diversity of infections.

Resource availability is another environmental factor that can influence fish health and immunity (Ortuno *et al.*, 2001; Magnadóttir, 2006). For example, the availability of dietary carotenoids, which cannot be synthesised by the majority of animals (Goodwin, 1986), has been associated with enhanced immune stimulation in zebra finches (*Taeniopygia guttata*) and guppies (*Poecilia reticulata*) (see Blount *et al.*, 2003; Mcgraw & Ardia, 2003; Grether *et al.*, 2004; Kolluru *et al.*, 2006). A simple increase in food availability may therefore lead to improved parasite resistance; greater nutrient abundance being directly linked to the condition of an animal (Wootton, 1973). Conversely, animals in weaker body condition are often predisposed to infection as a result of immune suppression (Nelson & Demas, 1996; Møller *et al.*, 1998; Bakker & Mundwiler, 1999; Beldomenico & Begon, 2010).

The oomycete *S. parasitica* and the monogenean *Gyrodactylus gasterostei* were assessed in the current study for variation in prevalence and intensity of infection. *S. parasitica* is prevalent during winter, being affected by variable temperature and seasonal conditions (Bly & Clem, 1991; Bly *et al.*, 1992). Variable temperatures also have an indirect effect on *S. parasitica* infections, inducing host stress which increases susceptibility to infection by means of immune suppression (Carballo *et al.*, 1995; Tort, 2011). *S. parasitica* is of particular importance because of its high virulence and morbidity costs in aquaculture, partly as a result of the ban on effective but carcinogenic treatments (reviewed by West, 2006). Likewise, many *Gyrodactylus* species are capable of uncontrollable population growth causing serve pathology (e.g. Scott & Anderson, 1984; Bakke *et al.*, 1990), making control and eradiation both difficult and costly as reinfection is common (Linaker *et al.*, 2012). There are over 400 described species of viviparous gyrodactylids, with many more described species, probably infecting most teleosts (Harris *et al.*, 2008). As gyrodactylids can be monitored non-invasively,

parasite infra-population growth can be tracked over time making it an ideal parasite for long term seasonal studies. Field studies of temperate *Gyrodactylus* spp. typically find higher prevalence and intensity of infection during the spring months, attributed to warmer temperatures and immunosuppression in breeding fish enhancing population growth; the parasite population then undergoes a crash during the summer usually attributed to host mortality (Chappell, 1969; Mo, 1997; Winger *et al.,* 2007; You *et al.,* 2008).

In this mesoscosm experiment, three-spined sticklebacks were maintained under two temperature and food treatments to mimic the effects of climate change and resource availability to understand how such variation might affect parasite prevalence and intensity. Fish were exposed to two parasites, *S. parasitica* or *G. gasterostei,* as two species with varied life-histories, virulent and rapid or relatively benign and long-lived respectively, that may respond very differently to changes in environmental and host condition. Cyclical annual variation in parasite prevalence and intensity was expected to match field studies (see Chappell, 1969; Bly *et al.,* 1992; Mo, 1997; Bruno & Wood, 1999; Winger *et al.,* 2007; You *et al.,* 2008). Reduced food availability was expected to reduce fish body condition and reduce infection prevalence and intensity, as fewer nutrients are available for investment in immunity. The increase in climatic temperature was expected to improve host adaptive immune response and reduce infection.

Materials and Methods

Three-spined sticklebacks (*Gasterosteus aculeatus*) were collected from Llyn Frongoch (SN 72248 75206) on the 9-14th October 2014 and transported to Aberystwyth University and fed on bloodworm in 12, 300 L, stand-alone flow-through outdoor mesocosms exposed to natural temperature and photoperiod variation, at a density of 0.35 fish per 1 L in 1% salt water with recirculating filter units. Half of these mesocosms were heated to 2°C above the ambient temperature. Average temperature over the course of the experiment was 0.4°C above the 1981-2010 long term average; although November and December of 2014 were unseasonably warm (Fig. 3.1) (Met Office, 2016). Fish were treated with praziquantil to remove *Gyrodactylus* spp. (fluke-solve; 4 g of 50% praziquantil per 1000 L for 24 h), allowed to recover in freshwater for a further 24 h before another 24 h treatment bath. A sub-sample of the fish was then screened for the absence of *Gyrodactylus* spp. (see Schelkle *et al.,* 2009). Mesocosms maintained four different regimens of three replicates each: ambient temperature and low food, ambient temperature and high food, 2°C above ambient and low food, and

2°C above ambient and high food. Temperature in the +2°C treatment was maintained by means of an automatic electronic thermometer system and pond heaters. Fish in the high food treatments were fed on frozen chironomid larvae (mini bloodworm, Tropic Marine) once per day until satiety up to a maximum of 8 g of food per tank. Low food treatment fish were fed half the ration of those in the high food tanks. Once per week all fish were fed on frozen cladocerans (*Daphnia* spp., Tropic Marine) in place of bloodworm. Both diets were sufficient to maintain good health in the fish.

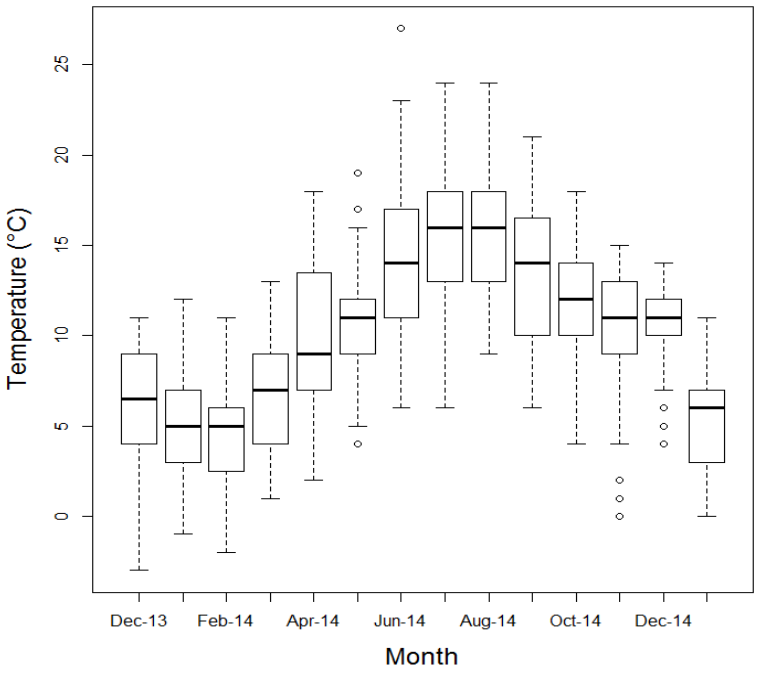

Figure 3.1: Ambient temperature variation in Cardiff over the course of stickleback exposure (Dec 2013-Jan 2014) to both *Saprolegnia parasitica* and *Gyrodactylus gasterostei*.

Fish were sampled from each mesocosm, monthly (n=378) and quarterly (additional n=116), transported to Cardiff University and experimentally infected with *Saprolegnia parasitica* or *Gyrodactylus gasterostei*, respectively. Here, fish were weighed and measured for standard length then kept individually in a litre of brackish, 0.5% salinity, dechlorinated water outside at ambient temperature for the remainder to the experiment; brackish water was kept outside for 24 h to allow temperature to adjust to ambient conditions. The salt concentration of the water was reduced by 0.5% per day over two days, and hosts were infected after 1 day in fresh water. Fish were infected 3 days after removal from the mesocosms. At Cardiff, all fish were maintained on bloodworm until satiety, all effects of Aberystwyth's food and temperature treatment would therefore be

residual. Parasitological dissection and analysis was only performed on fish infected with *G. gasterostei* and was not possible for *S. parasitica* infected fish.

Saprolegnia infection

Fish sampled monthly were exposed to *S. parasitica* (isolate CBS223.65 from Northern Pike (*Esox lucius*) in 1965). Spores were obtained using culture methods, outlined in Chapter 2; 1 plate of *S. parasitica* spores was produced for every 1-2 fish from a single culture with all spore plates being produced at a single time. Fish were subjected to an adjusted, 30 s exposure, ami-momi technique (Hatai & Hoshiai, 1994; Chapter 2) and exposed to *Saprolegnia* spores at a concentration of $3x10^5$ spores per litre for 24 h. Sham-infected fish were only exposed to the adjusted ami-momi technique. A total of 165 fish were exposed to a sham infection of which no fish developed infection. Fish that were exposed to ami-momi and spores but did not develop an infection were classed as asymptomatic and those that developed an infection as symptomatic. At 72 h post-infection, fish were killed by concussion and pithing, re-weighed and measured for standard length (mean length = 35.3 mm, range = 20.05 to 53.91 mm; mean mass = 0.640 g, range = 0.099 to 2.093 g), photographed using a Nikon S3600 camera to measure intensity of infection and the body cavity was opened to remove and weigh any *Schistocephalus solidus* (n=21 infected fish). Using the freehand selection tool in ImageJ (Abramoff *et al.*, 2004), the overall area of the fish and any infected areas were measured from the imported photographs. Intensity of infection was estimated using the following formula: $\text{Intensity of infection} = \frac{\text{Total infected area}}{\text{Total body area}}$.

Gyrodactylus infection

An isogenic line of *G. gasterostei* obtained from Roath Brook (ST 18897 78541), 14/10/2015 was used for infection. The species was identified based on the absence of an excretory bladder, morphometrics (Harris, 1982) and rDNA Internal transcribed spacer sequencing (GenBank AJ001841.1; Cable *et al.*, 1999) following the methods of Harris *et al.* (1999) and Shinn *et al.* (2010). Fish removed from the mesocosm quarterly were individually exposed to 0.02% MS222. Then, using a dissecting microscope and fibre optic lighting, the caudal fins of an infected donor and recipient fish were overlaid until 2 individuals of *G. gasterostei* transferred to the caudal fin of the recipient. The infected fish were then screened 24 h later by anesthetising them in 0.02% MS222 and the fins and body checked for infection, any uninfected fish were re-infected. Fish were screened every 4-5 days (Monday, Friday, Wednesday) for 58 days. Infected fish were

then euthanized by concussion and pithing, and dissected to check for co-infections of the body cavity, gut, gills, swim bladder and eyes. The only co-infecting parasite recovered was *S. solidus* (n=8 infected fish).

Animal ethics

All animal work was approved by the Aberystwyth and Cardiff University Ethics committees. Maintenance and infection was performed under Home Office Licence PPL 302357. Any fish exhibiting stress responses including sucking at the water/air boundary or unusual swimming, as a result of parasite burdens (>200 *G. gasterostei* per fish) were euthanized using a Schedule 1 method.

Statistical analyses

All data were analysed in R v3.2.2 (R Core Team, 2015) with the additional use of 'car' (Fox & Weisberg, 2011), 'MASS' (Venables & Ripley, 2002), 'survival' (Therneau & Grambsch, 2000), 'mgcv' (Wood, 2004, 2011) and 'gamlss' (Rigby & Stasinopoulos, 2005) packages. All models were check for normally distributed residuals and over-dispersion.

Analysis of *S. parasitica* intensity as the dependent variable was carried out using Zero Inflated Beta Distributions (BEZI) in a generalized additive model for location, scale and shape (GAMLSS) (Rigby & Stasinopoulos, 2005). Independent variables for mu (μ - intensity of infection) and nu (ν - likelihood of an infection being zero) parameters included: temperature treatment, food treatment, ambient temperature, month, length (length after experiment was used: regression of length after and length before R^2=0.965), body condition, sex, naturally acquired *S. solidus* infections (not removed following anti-helminthic praziquantil treatment) and an interaction between temperature and food treatments. The third parameter, Tau, was set to be constant. No other infections were found to be present in dissected fish. Body condition was calculated as a quadratic regression of fish length against mass. A *post-hoc* analysis, with Bonferroni correction, of month within the nu parameter was performed to look for variation between months.

Variation between *G. gasterostei* infected fish that were either, euthanized, cleared their infection or were still infected after 58 days, were analysed by season using a chi-squared test. A survival analysis (Therneau & Grambsch, 2000) was conducted comparing between months for fish that either survived until 58 days post-infection or were euthanized. Total burden of *G. gasterostei* (the number of worms on a fish at any

given screening period) was analysed, using a Generalized Additive Mixed Model (GAMM) and negative binomial errors (Wood, 2004, 2011) with fish identification (I.D.) included as a random term, to assess how *G. gasterostei* burden between months varies over time. Fish on which an infection did not establish 3 days post-infection were removed from the analysis (n=6). Fish that were euthanized early (n=21) were left in the model with all cases having their peak infection on the day of death. The removal of these fish from the experiment means measurements of peak burden and time of peak burden are probably conservative. Fish that cleared their infections were included, but the data after the day of clearance were removed to avoid zero inflation and dragging the plots towards zero. This means that the data are only representative of infected fish. Model independent variables included: food treatment, temperature treatment, month, maximum temperature change (the largest temperature change over a 12 h period in the interval since the last screen), length of fish, body condition (residuals from a quadratic regression of length against mass), sex, the presence/absence of *S. solidus* infection and an interaction between food and temperature treatments. Splines were fitted for number of days post infection by the month of infection. The use of the spline allows the model to plot non-linear prediction curves of the dependant variable 'total *G. gasterostei*' against time 'days post-infection' and with splines separated by 'month'. The plots were then assessed visually to determine the differences between the plots.

G. gasterostei population-level parameters were assessed using linear models. Population-level parameters included: Area Under Curve (AUC), the total numbers of *G. gasterostei* on the host over a given time calculated using the Trapezoid rule; the peak of infection, the highest number of *G. gasterostei* on a host; day of peak infection, the number of days post infection with the highest number of *G. gasterostei*. In each case independent variables were: food treatment, temperature treatment, month, length, body condition (as above) sex and presence/absence of *S. solidus* infection and an interaction between food and temperature treatment. *Post-hoc* analysis with Bonferroni correction for the month variable was used to look for differences between factor levels. For the AUC parameter an additional variable of 'infection duration' was used to account for fish that were euthanized early (n=21), but was not required in the final model because the majority of fish were still infected 58 days post-infection.

Body condition of all fish combined was assessed using a linear model with independent variables: temperature treatment, food treatment, infection treatment, month, length, sex and presence/absence *S. solidus* infection.

Results

Saprolegnia parasitica

Of the 213 three-spined sticklebacks exposed to *Saprolegnia parasitica*, 59 fish displayed symptomatic infection (Fig. 3.2). The intensity of infection (proportion of fish's body covered in mycelium) among symptomatic fish was mostly consistent over time, except when only a small number of fish became infected and intensity of infection was typically lower (Fig. 3.3).

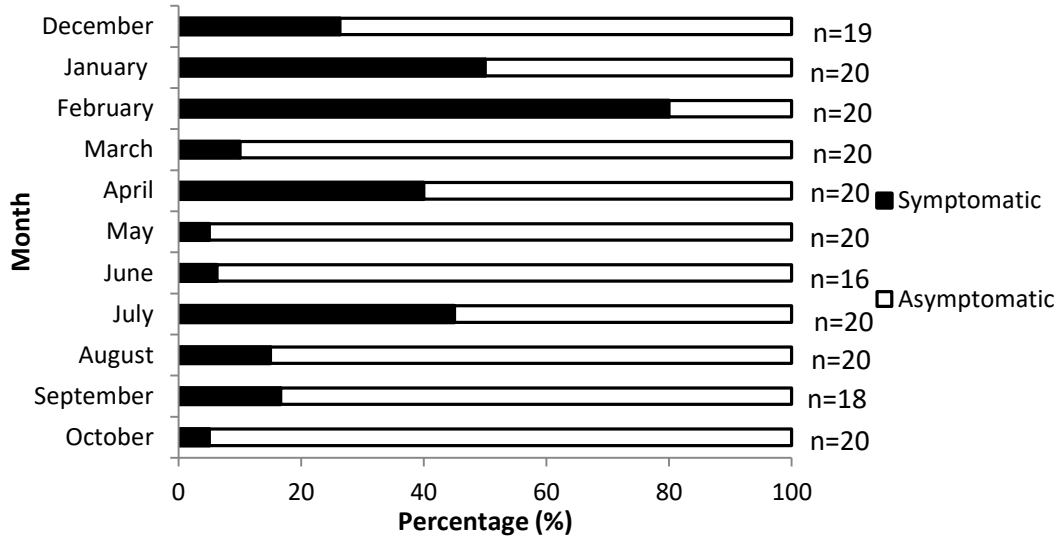

Figure 3.2: The percentage of three-spined sticklebacks (*Gasterosteus aculeatus*) 'symptomatic' (black bars) or 'asymptomatic' (white bars) for *Saprolegnia parasitica* 58 days post-infection.

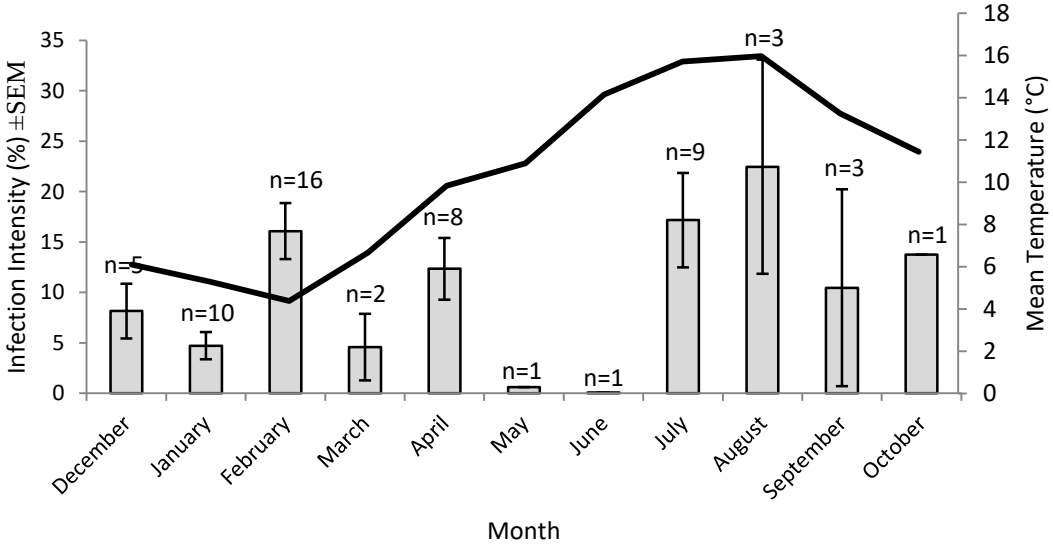

Figure 3.3: Intensity of *Saprolegnia parasitica* infection (the percentage of the fish's body covered with hyphae) among symptomatic three-spined sticklebacks (*Gasterosteus aculeatus*). Error bars are standard errors of the mean. Black line is the mean monthly temperature.

Month partially affected the likelihood, but not intensity of infection. Only February had a higher likelihood of infection when compared to March, May or August (*post-hoc* analysis Fig. 3.4). The higher likelihood of infection in February is not the result of low temperature causing immunosuppression because temperatures were also below 7°C for the previous two months (Fig. 3.1) and a similar affect was not observed. Host condition had the largest impact on infection with increasing body condition increasing the likelihood (ν: t=-2.723, d.f.=195, p=0.007; Fig. 3.5) and intensity of infection (μ: t=5.019, d.f. =195, p=<0.001; Fig. 3.5). Fish length also had a small negative impact on infection intensity (t-value: d.f.=195, p=0.049; Fig. 3.6). Independent variables: sex, *Schistocephalus solidus* infection, temperature or food treatments and their interaction, were non-significant (p>0.05) and were removed to produce the minimal model.

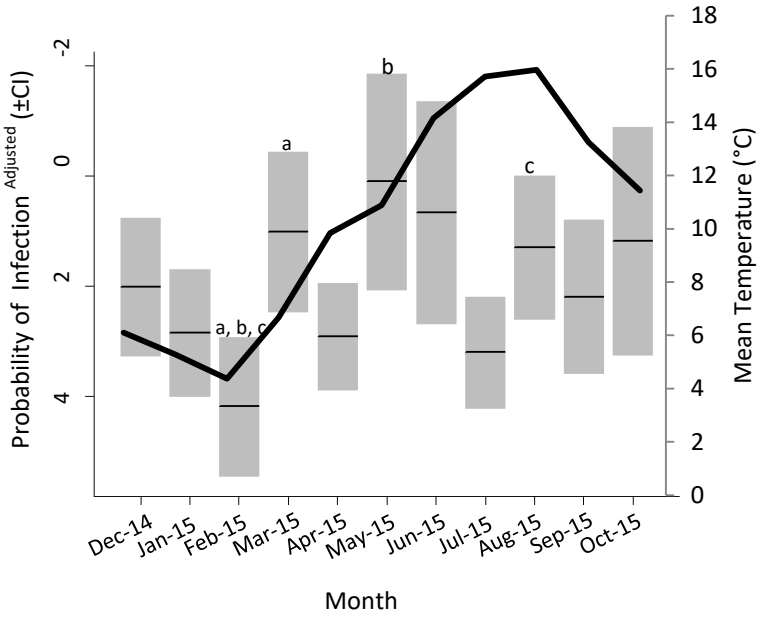

Figure 3.4: The likelihood of infection for those sticklebacks (*Gasterosteus aculeatus*) exposed to *Saprolegnia parasitica* each month. Those cases where letters are the same indicate significance in host-hoc analysis with a Bonferroni correction (p<0.0045). Black horizontal lines are the adjusted mean of the likelihood of an infection being zero; shaded areas are 95% confidence intervals. An axis with an adjusted prevalence of infection is also supplied. Note the un-inverted y-axis. The black line is the mean monthly temperature.

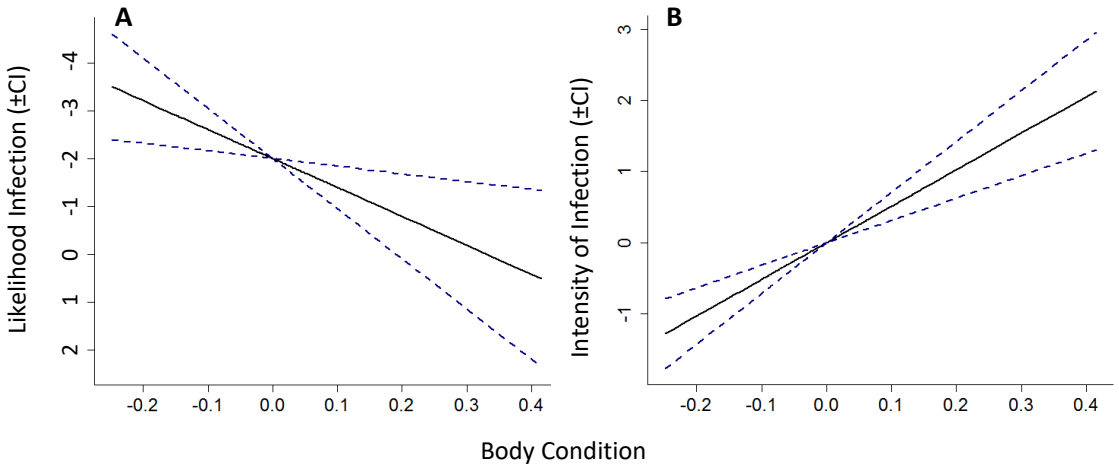

Figure 3.5: The effect of three-spined stickleback (*Gasterosteus aculeatus*) body condition on the likelihood of a fish having zero infection with *Saprolegnia parasitica* (A) and the intensity of that infection (B) measured as the percentage of the fish epithelium covered in mycelium. Black lines are regressions lines of likelihood or intensity of infection; dashed lines are 95% confidence intervals. An axis with an adjusted prevalence of infection is also supplied for the Nu parameter. Note the un-inverted y-axis (A).

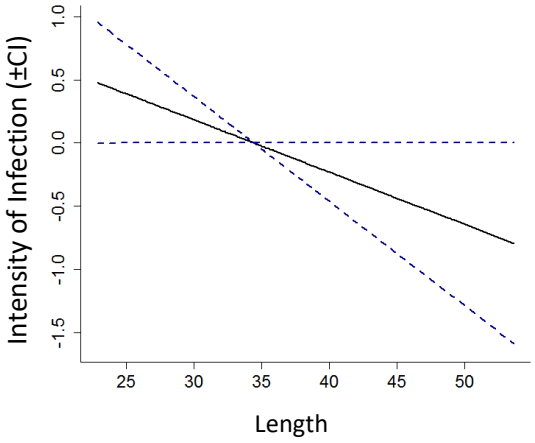

Figure 3.6: The effect of three-spined stickleback (*Gasterosteus aculeatus*) length on the intensity of *Saprolegnia parasitica* infection measured as the percentage of the fish epithelium covered in mycelium. Black line is a regression line of infection intensity; dashed lines are 95% confidence intervals.

Gyrodactylus gasterostei

In all 116 fish infected with *Gyrodactylus gasterostei* on a quarterly basis, the largest differences in the response to infection was between the summer months of May and August where >30% of fish cleared their infection with <45% remaining infected, compared to <12% clear of infection and >80% still infected in the winter months (χ^2=48.939, df=2, p<0.001) (Fig. 3.7). There was no significant difference in number of fish removed and euthanized, or that survived until the end of the experiment between months (survival analysis: χ^2=5.2, df=3, p=0.158; Fig. 3.7).

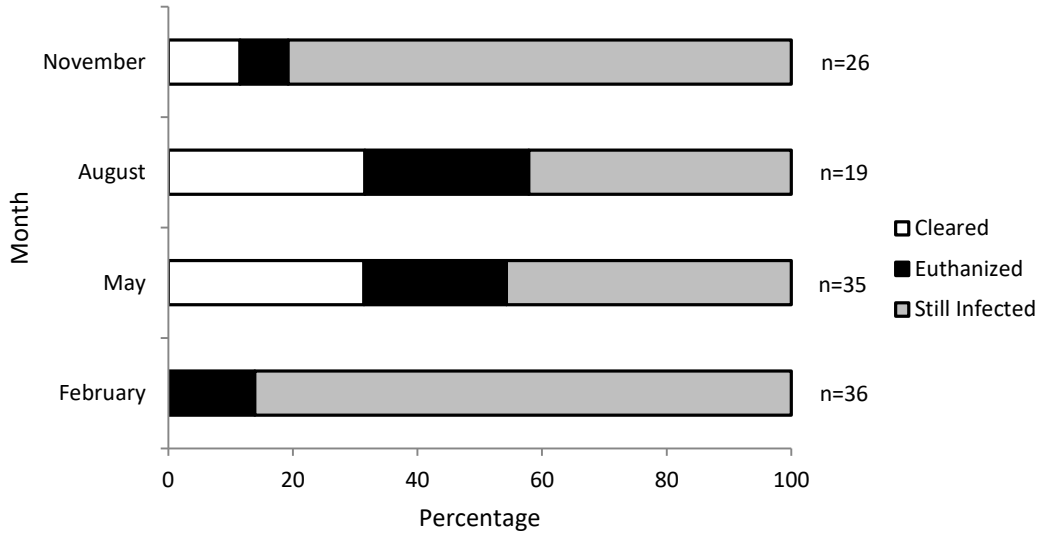

Figure 3.7: The percentage of three-spined sticklebacks (*Gasterosteus aculeatus*) infected with *Gyrodactylus gasterostei* that cleared their infection, were euthanized (as a result of high parasite >200 burden or stochastic mortality) or were still infected 58 day post-infection, at four time points in the year.

Rates of initial population growth, overall infection burden, the peak of infection and the day of peak infection (Fig. 3.8) varied over time. Fish infected in the warmer months of May (mean over 58 days of infection 14.1°C) and August (14.5°C) had higher initial rates of parasite population growth compared to winter months, November (8.5°C) and February (7.7°C) (Fig. 3.8). The warmer months (May and August) had lower peaks of *G. gasterostei* infection between 20-30 days post-infection while November and February infected fish peaked at a higher burden 30-40 or 40-50 days post-infection, respectively. All months had smooth terms with a high level of significance (February $F=164.1$, $p<0.001$; May $F=33.8$, $p<0.001$; August $F=36.1$, $p<0.001$; November $F=118.5$, $p<0.001$). Of the independent variables, only fish length correlated negatively with *G. gasterostei* intensity ($t=-3.006$, $p=0.003$). All other independent variables had no impact on the model: food treatment, temperature treatment, maximum temperature change (over 12h prior to screening), fish condition, sex, *S. solidus* infection and an interaction between food and temperature treatment.

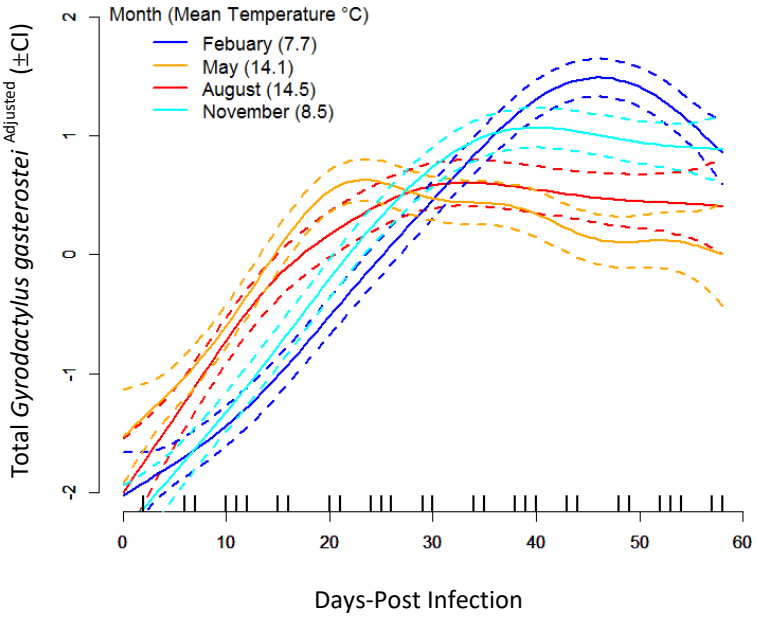

Figure 3.8: A GAMM of total *Gyrodactylus gasterostei* burden on the three-spined stickleback over time (days post infection) separated by the month of infection. Solid lines are the adjusted mean and the dotted lines confidence intervals .

Higher overall burdens, higher and later peaks of *G. gasterostei* infection from the GAMM are supported by additional analysis of parasite population dynamics: Area Under the Curve (AUC), peak of infection and day of peak infection (Fig. 3.9A-C). AUC was higher in November and February relative to May and August (Fig. 3.9A). In the February sample, the higher peak of infection was offset by slower initial population growth (Fig. 3.8). The peak of infection was highest in the November fish (Fig. 3.9B); the higher peak of infection for February fish in the GAMM (Fig. 3.8) is a result of an interaction between the time of peak and size of peak. Day of peak infection was also higher in the February and November samples compared with the May and August, or May samples, respectively (Fig. 3.9C). AUC (t=-2.275, df=108, p=0.025) and peak of infection (t=-2.445, df=108, p=0.016) were also negatively correlated with fish length. Additionally, fish on the lower food treatment had an earlier peak of infection (t=-2.347, d.f.=106, p=0.021). Month had an impact on body condition of sticklebacks with fish later in the year having a higher body condition (Fig. 3.10A) suggesting resources were dedicated to growth rather than fat storage. Condition was also higher in the high food treatment (Fig. 3.10B). Other independent variables (month, length, body condition, sex and presence/absence of *S. solidus* infection and an interaction between food and temperature treatment) were non-significant (p>0.05) and removed to create the minimum adequate model.

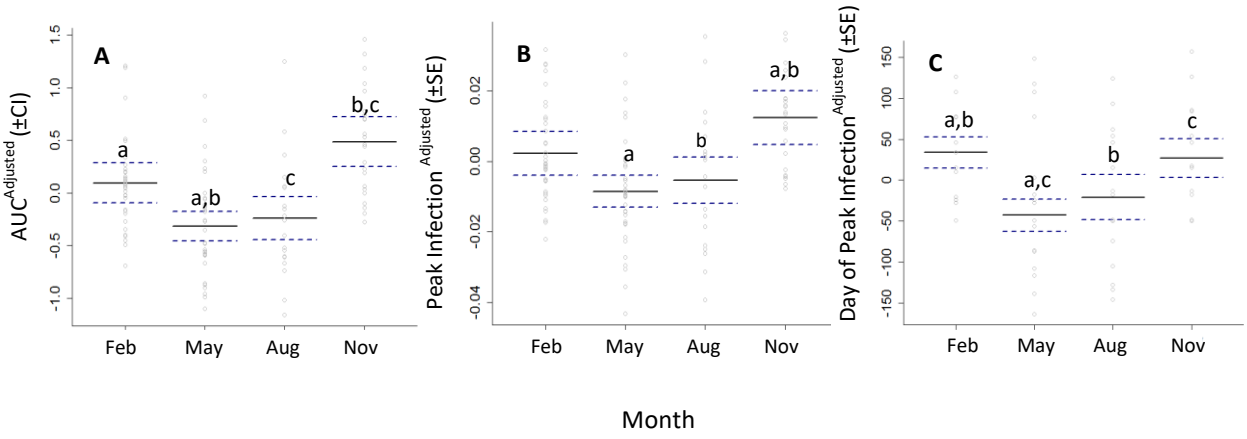

Figure 3.9: The effects and partial residuals of month for three-spined sticklebacks (*Gasterosteus aculeatus*) infected with *Gyrodactylus gasterostei* from linear models looking at (A) average number of *G. gasterostei* per unit time (Area Under Curve – AUC) (B) number of *G. gasterostei* at peak infection and (C) the day of peak infection. Identical lettering indicates significance with Bonferroni correction (p<0.0125). Black lines are the adjusted mean of the parameters; dashed lines are 95% confidence intervals.

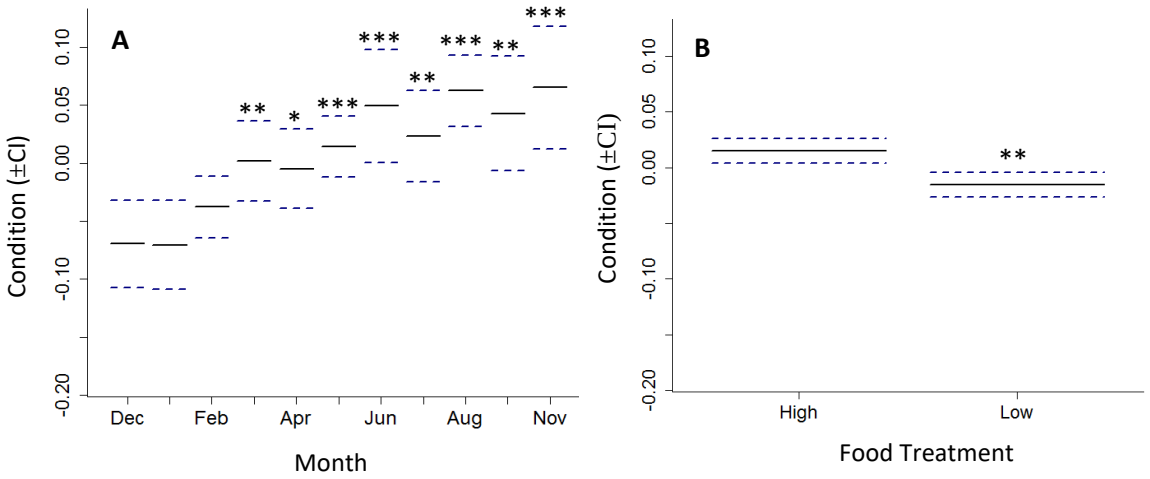

Figure 3.10: The effect of month on the body condition of three-spined sticklebacks (*Gasterosteus aculeatus*) (A) and the effect of food treatment on fish condition (B). Solid lines are means and dotted are 95% confidence intervals. p<0.001 = ***, p<0.01 = **, p<0.05 = *.

Discussion

Saprolegnia parasitica infections of three-spined sticklebacks (*Gasterosteus aculeatus*) did not vary as considerably with month and temperature as was predicted based on laboratory studies by Bly *et al.* (1992). The current study found remarkably little circannual variation of *S. parasitica* infections with no effect of a historical 2°C increase in ambient mean temperature over the course of the experiment. Instead host body condition was the most important predictor of both prevalence and intensity of *S. parasitica* infection. In contrast, *Gyrodactylus gasterostei* infections varied considerably in intensity between months in a manner that cannot simply be explained by temperature variation; but is instead explained on prior exposure to *G. gasterostei* interacting with variable temperatures and host immunity described as the 'Foothold Hypothesis'.

Saprolegnia parasitica

Likelihood and intensity of infection increased with high host body condition, partially as a result of feeding regime. Typically, low body condition individuals tend to be predisposed to infection particularly as a result of immune suppression (Nelson & Demas, 1996; Møller *et al.*, 1998; Bakker & Mundwiler, 1999; Beldomenico & Begon, 2010). However, the current study is not the only study to find increased prevalence of infection in higher condition individuals. Among common toads (*Bufo bufo*) over wintered and infected with *Batrachochytrium dendrobatidis*, those toads that lost relatively little mass and can be classified as higher condition, experienced a higher likelihood of infection (Garner *et al.*, 2011). Such a relationship might be attributed to the effect of higher mass in breeding individuals leading to a higher perceived condition and supressed immunity (see Sheldon & Verhulst, 1996; Lochmiller & Deerenberg, 2000; Norris & Evans, 2000). In the current experiment that is unlikely, as very few fish were in breeding condition, there was no effect of seasonality and no outliers on a weight to length regression were observed that would be associated with higher mass linked to egg production. Instead what might be occurring here is an investment trade-off between body condition and immunity; something normally associated with tolerance (Jackson *et al.*, 2014).

Increasing intensity of infection with increasing body condition, as found with *S. parasitica* infections, typically occurs when measures of intensity of infection are based on size or weight of the parasites rather than number of parasites infecting the host (Bean & Winfield, 1991; Barber, 2005; Blanchet *et al.*, 2009). In these previous studies

measures of superior host fitness including: higher growth rates (Barber, 2005; Blanchet *et al.,* 2009) or larger body sizes (Bean & Winfield, 1991), can lead to larger parasites. In addition, production of more or better quality transmission stages can be affected by the presence of higher quantities of food or reduced stress leading to improved condition (Ebert *et al.,* 2000; Bedhomme *et al.,* 2004; Seppälä *et al.,* 2008). Such relationships are likely caused by the availability of more abundant resources in fitter hosts, which the parasites can exploit for their own means. The relationship between larger fish length and lower *S. parasitica* infection is, in this case, unrelated to fish age because all fish were less than one year old. It likely that this relationship has arisen as a result of basic allometry and the fact it will take the *S. parasitica* mycelium longer to cover a fish of larger size (see Cable *et al.,* 2007). This relationship will occur even if the parasite growth rate is somewhat exponential, particularly given the short infection duration (72 h).

The lack of variation in *S. parasitica* infection across months was unusual considering that the majority of wild infections tend to occur during winter (Bly *et al.,* 1992) when fluctuating temperatures and cold conditions stress fish making them more susceptible to infection (see Carballo *et al.,* 1995; Tanck *et al.,* 2000; Donaldson *et al.,* 2008; Tort, 2011). In the current study, the higher likelihood of infection in February cannot be attributed to colder conditions as both previous months had similar temperatures but lower infection likelihoods. Such a result could have been the result of batch production of *S. parasitica,* although this is unlikely given that individual plates of spores were produced per 1-2 fish infection. Instead, month-by-month variation in fish could be causing this result.

Gyrodactylus gasterostei

This study proposes a new 'foothold hypothesis' to explain *G. gasterostei* population trajectories whereby *G. gasterostei* establishes itself on the host at low intensities, which then enables the parasite population to expand rapidly under variable environmental conditions. Such temperature variability causes changes in the immune competence of the host (see Miller & Clem, 1984; Bly & Clem, 1992; Lillehaug *et al.,* 1993; Alcorn *et al.,* 2002; Magnadóttir, 2006) as well as altering growth and survival of the parasite (Lester & Adams, 1974; Sereno-Uribe *et al.,* 2012); a rapidly adaptable parasite might therefore be expected to take advantage of both situations. There are two major reasons in this study to suspect the effects of a foothold hypothesis.

First, the current study indicates that fish exposed to temperature variation after infection develop higher intensity infections. Sticklebacks infected at warmer (November ca. 10°C, mean of the first 4 days of infection) and colder (February ca. 6°C) temperatures, which then experienced variable temperature, had higher peak infection intensities than fish held at the more stable temperatures over 10°C in May and August. These variable temperatures cause changes in immune gene expression which, as temperature passes the threshold for permissive immunity, are likely to result in higher infection intensities as either innate or adaptive immunity is supressed (see Chappell, 1969; Lester & Adams, 1974; Harris, 1982; Jansen & Bakke, 1991; Magnadóttir, 2006). Higher infection intensities in November compared to May/August are atypical as *G. gasterostei* population growth should be suppressed at these temperatures (see Chappell, 1969; Lester & Adams, 1974; Harris, 1982; Jansen & Bakke, 1991); these higher infection intensities can be explained by the temperature dropping rapidly at 30 days post-infection, corresponding with a plateau in *G. gasterostei* intensity. The continued high intensity of infection in November fish can therefore be explained by reduced mortality of *Gyrodactylus* spp. (Lester & Adams, 1974; Sereno-Uribe *et al.*, 2012), possibly as a result of reduced metabolic stress. In addition, reduced adaptive immune expression at colder temperatures might have favoured the parasite in the November infected fish (see Miller & Clem, 1984; Bly & Clem, 1992; Lillehaug *et al.*, 1993; Alcorn *et al.*, 2002). Higher peaks of infection in February compared to November are explained by an increase in *Gyrodactylus* spp. population growth rate at higher temperatures (e.g. Chappell, 1969; Lester & Adams, 1974; Harris, 1982; Jansen & Bakke, 1991). *G. gasterostei* establishing a foothold on the February sticklebacks in cold conditions (mean 5.5°C in first week of infection) therefore allows the parasite population to grow more rapidly. In summary, on either the February or November fish the immune response is overrun by *G. gasterostei* population growth when temperature variation occurs, no matter the direction of that temperature change.

Secondly, the ratio of fish that cleared their *G. gasterostei* infection was higher in the warmer months (May and August) corresponding with lower infection intensities compared to November and February fish. In field studies, the highest prevalence and intensities of *Gyrodactylus* spp. infection typically occur in spring-summer followed by a crash in the parasite population often attributed to host mortality (Winger *et al.*, 2007; You *et al.*, 2008). The contrast between the current and previous field studies suggests

that an enhanced immune response at warm and relatively stable temperatures is the cause of lower infection metrics in this study. These stable conditions therefore provide an environment in which the parasite cannot gain an upper hand and the host is at an advantage.

Higher peak infections of *G. gasterostei* in the November fish compared to field studies (see Chappell, 1969; Mo, 1997; Winger *et al.*, 2007; You *et al.*, 2008) may also be the result of longer lived wild infections and the development of resistance by the wild host. The foothold hypothesis might therefore be reliant on 'recent' infections, although the timescale for which it has an effect cannot be estimated from this study. The basis of the foothold hypothesis may be the result of rapid plastic genetic or epigenetic responses that surpasses or alters more rapidly than any plastic response of the host's genotype. The current study also highlights lower rates of initial *G. gasterostei* population growth on sticklebacks infected at colder temperatures (7.7 and 8.5°C) over the first month of infection, are consistent with previous studies which find a lower reproductive rate at colder temperatures (e.g. Chappell, 1969; Lester & Adams, 1974; Harris, 1982; Jansen & Bakke, 1991). In addition, the higher infection intensities in the November fish cannot be attributed to poor body condition in colder temperatures as body condition was actually at its highest level in these samples.

Analysis of *S. parasitica* and *G. gasterostei* infections has revealed a seasonal dynamic not based solely on temperature and host mortality effects but on interactions between parasites, host immunity and temperature allowing the parasite to establish an infection and then surpass host responses when conditions become more variable. This study revealed an unexpected effect of host body condition on the outcome of *S. parasitica* infection, both prevalence and intensity of infection increasing with better body condition. The former relationship might be explained by a trade-off between investment in immunity or body condition. The latter as a result of body condition fuelling the fire of infection, causing an increase in infection intensity that is likely based on the availability of resources to the parasite. The effect of body condition on infection highlights a potential problem within aquaculture which is geared towards high mass or high condition fish which may be predisposed to infection with *S. parasitica*, although no effect of resource availability was found in this study. The current results indicate that it is not simply seasonal variation in temperature that affects the likelihood and intensity of infection with *S. parasitica*. Indeed the lack of seasonal variation with this infection was unexpected and may be the result of month on month

variation in fish, batch dependent effects (which should have been minimal given that 1 spore plate was used to infect only 1-2 fish) or lower stocking density than would normally be experienced in aquaculture.

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Chapter 4 – Always winter, never Christmas: Winter length and its influence on sticklebacks, parasites and immunity.

Abstract

Variability in temperature is still an underestimated aspect of climate change. Moreover, changes in variability are not only affecting hosts but also their parasites. This study focuses on the effects of longer or shorter periods of temperature variability, in this case winter length, on the three-spined sticklebacks (*Gasterosteus aculeatus*) and generalist oomycete parasite *Saprolegnia parasitica* and the specialist monogenean *Gyrodactylus gasterostei*. With divergent life histories the responses of these parasites to temperature variability could be quite different. Sticklebacks were exposed to variable winter lengths and then to one of two parasites, *S. parasitica* or *G. gasterostei*, at regular intervals in a warming environment. Thus, the effects of winter length and temperature at time of infection were investigated in terms of host immunity and parasite development. The temperature at time of infection, rather than any residual effects of historical temperature, had the greatest impact on infection. *S. parasitica* infections increased in prevalence and intensity at higher temperatures. Additionally, at colder temperature or in fish that experienced winter, pro-inflammatory responses (*tbk1, il-17, il-1rlike*) were upregulated possibly explaining the lack of overt infection at colder temperatures. Infections with *G. gasterostei* reached higher overall intensities if the fish were exposed to a change in temperature after infection. For this reason a 'Foothold Hypothesis' is suggested whereby establishment of an infection allows it population to expand faster in variable conditions, while the host's immune response is unable to counter the increasing parasite intensity. This hypothesis would also explain the seasonally driven increase in wild gyrodactylid infections during spring.

Introduction

The IPCC predicts that global atmospheric temperature could increase by 1-3.7°C by 2100 (IPCC, 2013). Climate change is also expected to increase variability, with an increasing number of events that exceed threshold values for temperature and precipitation (Easterling *et al.,* 2000b). Such variability will likely be geographically dependent. The US, for example, has experienced a contraction in the number days exceeding threshold cold and warm temperatures (Degaetano, 1996; Easterling *et al.,* 2000a; Robeson, 2004) whilst parts of Australasia have experienced an increase in the number of days exceeding the upper threshold (Plummer *et al.,* 1999; Alexander & Arblaster, 2009). This climate variability has already begun reshaping the distribution

and dynamics of infectious disease with profound consequences on disease control; be it adverse or advantageous (Rohr *et al.,* 2011).

Many studies on climate change and infection have predominantly focused on average increases in temperature, particularly with regard to malaria, which is likely to increase in distribution (Martens *et al.,* 1995; Caminade *et al.,* 2014) and transmission (Lindsay & Birley, 1996; Tanser *et al.,* 2003) as the planet warms. Such research has been criticised for its focus on mean temperature increases and temporally confounded variables. A growing body of research considers temperature variability, for example; an increase in variability results in higher malaria transmission at lower temperatures but may hinder transmission at higher temperatures (Patz *et al.,* 2005; Paaijmans *et al.,* 2010). Additionally, El Niño events, which drive variability, have aided the spread of the chytrid fungus, *Batrachochytrium dendrobatidis*, by increasing outbreak frequency and compromising amphibian immunity as changes in temperature generate suboptimal immunity (Rohr & Raffel, 2010). Brunner & Eizaguirre (in press) suggests that if temperature variability fluctuates rapidly this could lead to selection for a plastic phenotype while a lower level of fluctuation could lead to population divergence and promote local adaptation of the host-parasite dynamic. Relatively little however is known about how contraction or amplification of variability (e.g. shorter/longer winters), might affect infectious disease, with one exception, West Nile Virus. This disease appears to have spread as a result of milder winters and drought conditions which improve mosquito breeding and the abundance of larvae food (Epstein, 2001). In vertebrates, including teleosts, many factors contribute to or hinder their ability to resist disease including: genetics (Kurtz *et al.,* 2006), epigenetics (Reiner, 2005), ecotype (Scharsack *et al.,* 2007) and environmental factors including temperature (Watts *et al.,* 2008).

Localised water bodies tend to track, but lag behind predicted atmospheric temperature increases, with an expected 2°C increase in water temperature by 2100 (Kothandaraman & Evans, 1972). As ectotherms, teleost body temperature is dependent on environmental conditions. Thus, global warming and associated seasonal changes could lead to a rise in the average body temperature of fish affecting metabolism and immunity (Schurmann & Steffensen, 1997; Watts *et al.,* 2008). These changes to basic metabolic functions also have implications for aquaculture in terms of fish and parasite growth rate and fecundity (Handeland *et al.,* 2008; Soleng *et al.,* 1999; Macnab & Barber, 2012; Denholm *et al.,* 2013).

It is generally considered that the innate immune system functions better at lower temperatures whereas the adaptive immune response is more effective at higher temperatures (Miller & Clem, 1984; Bly & Clem, 1992; Lillehaug *et al.,* 1993; Alcorn *et al.,* 2002). Temperature is a major contributing factor to the function of the adaptive response, which at low temperatures results in loss of specific T-cell function (Bly & Clem, 1994) and suppression of primary antibody responses (Avtalion, 1969; Avtalion *et al.,* 1970; Morvan *et al.,* 1998). In Atlantic cod (*Gadus morhua*), for example, immune factors of the innate response, haemolytic and antiprotease activity, are more active at 1-7°C than at 14°C (Magnadóttir *et al.,* 1999; Magnadóttir, 2006). In sockeye salmon (*Oncorhynchus nerka*), factors of the innate response, phagocytic and complement activity, were higher in fish reared at 8°C compared to 12°C (Alcorn *et al.,* 2002). In contrast, IgM activity, a factor of the acquired immune response, is reduced at these lower temperatures (Magnadóttir *et al.,* 1999).

Long term exposure of fish to high environmental temperature enhances adaptive immune function (Ahne, 1986; Alcorn *et al.,* 2002). In carp, antibodies to 'spring viremia carp virus' were detected after 2-8 weeks at 20°C (Ahne, 1986). In contrast, infection persisted for 12 weeks in fish kept at 10°C, although antibodies were present in the blood from 8 weeks post-infection (Ahne, 1986). Similarly, *O. nerka* reared at 12°C compared to 8°C had more lymphocytes and their antibodies were more responsive to r-p57 protein (Alcorn *et al.,* 2002). This sensitivity of the adaptive immune response to environmental temperature has major implications for disease eradication (Ahne, 1986).

The phenomenon of adaptive response non-permissiveness and a more active innate response at colder temperatures is ultimately important in aquaculture and preservation of wild stocks. Periods of low temperature or rapid changes in temperature can result in an immunocompromised fish, increasing the likelihood of infection. One of the major causes of cultured fish death in winter is infection by oomycetes of the genus *Saprolegnia* (see Bly & Clem, 1991; Bly *et al.,* 1992). The recent European Union ban on malachite green, a toxic but effective control method for *Saprolegnia*, has caused increased fish losses (van West, 2006). *Saprolegnia* now possess a colossal threat to the aquaculture industry and food security with no means of control. It is becoming increasingly important that we understand how this parasite interacts with its host, at the level of the immune system, under different temperature stresses.

Another common group of fish pathogens greatly influenced by temperature are the viviparous gyrodactylids (Harris, 1982; Jansen & Bakke, 1991). These monogenean ectoparasites with greater than 400 described species (Harris *et al.,* 2008) are capable of uncontrollable population growth on susceptible hosts, causing severe pathology (Scott & Anderson, 1984; Bakke *et al.,* 1990). The *Gyrodactylus* genera includes species that have been the focus of costly eradication schemes, primarily in Norway (Linaker *et al.,* 2012), but population growth of *Gyrodactylus* spp. is usually limited by the host's immune system (Bakke *et al.,* 1992; Harris *et al.,* 1998; Lindenstrøm *et al.,* 2004; Lindenstrøm *et al.,* 2006; Kania *et al.,* 2010). Given the temperature dependent nature of teleost immunity, however, mounting an effective response against *Gyrodactylus* spp. might not be possible if thermal variation immunocompromises the host.

This study utilises the three-spine stickleback and its parasites *Saprolegnia parasitica* and *Gyrodactylus gasterostei* as models for assessing how variability in winter length affects parasite infections and host immune response. The hypothesis being that changes in winter length will adversely affect fish resistance to infection. A rise in temperature, to simulate spring warming temperatures, was also used to assess how the host immune response recovers from cold conditions and whether this might also affects parasite infections; the expectation being that fish emerging from longer winters will take longer to recover.

Materials and methods

Host Origin

Wild three-spined sticklebacks (*Gasterosteus aculeatus*) were captured from Roath Brook, Cardiff (ST 18897 78541) on 16/02/14 (mean length = 39.9 mm, range = 25.86 to 51.66 mm; mean mass = 0.859 g, range = 0.201 to 1.625 g) and 14/10/14 (mean length = 38.3 mm, range = 30.71 to 44.62 mm; mean mass = 0.708 g, range = 0.276 to 1.583 g) for the *Saprolegnia parasitica* and *Gyrodactylus gasterostei* experiments, respectively. Fish were transported to the aquarium facility at Cardiff University, and maintained in 10 L tanks at a density of <1 fish/litre, on an 18 h light: 6 h dark cycle at 15(±0.5)°C and fed daily on frozen bloodworm. The sticklebacks were treated for common parasites using formaldehyde and praziquantel. First, fish were exposed to 0.004% formaldehyde solution for 1 h, with a mid-way 30 min rest period in freshwater. After a 24 h recovery period from formaldehyde treatment, fish were then treated with fluke-solve (Vetark) praziquantel according to the manufacturer's instructions (4 g of

50% praziquantel in 1000 L) for 48 h. Treatment with formaldehyde can cause epidermal damage; to prevent secondary infection, fish were then left to recover for 1 week in 1% salt solution and methylene blue (0.002 g/L) after praziquantel treatment.

Treated fish were 'screened' for ectoparasites by anaesthetising them in 0.02% MS222 and removing any remaining parasites with watchmaker's forceps following the protocol outlined by Schelkle *et al.* (2009). The fish were screened clear of ectoparasitic infections three times before defined as clear of infection. The fish continued to be maintained in 10 L tanks and were given 1 month of recovery/acclimatisation at 15(±0.5)°C and monitored for bacterial, fungal or oomycete infections.

Experimental design

The basic experimental design was repeated using two parasites, *S. parasitica* and *G. gasterostei*. Fish were exposed to different lengths of winter with successive groups of fish then exposed to infection at regular time intervals, to compare the effects of variable winter length and immune system recovery from cold temperatures. The design also encompassed temperature effects whereby sampling during the cold/warming/warm periods allowed an assessment of the direct effects of a given temperature.

For both the *S. parasitica* and *G. gasterostei* experiments, fish were allocated to one of four treatments. The first group was maintained at 15°C for 3 months, the second group at 15°C for 2 months and then 7°C for 1 month, the third group at 15°C for 1 month and then 7°C for 2 months, and the fourth group at 7°C for 3 months (Fig. 4.1). The temperature of all winter (7^{o}C) fish was then raised over a period of 1 week to 15°C at a rate of 1°C/day with a 2°C increase on the fourth day (Fig. 4.1) for the *S. parasitica* experiment; fish already at 15°C remained at that temperature. For the *G. gasterostei* experiment, the temperature was raised at a rate of 0.5°C/day, with 3 days of 1°C increase every fourth day, over two weeks; again fish at 15°C remained at that temperature. This slower warming period for *G. gasterostei* infections, taking 1 week longer than the *S. parasitica* experiment, ensured that all checks for total number of parasites were synchronous and on the same week day; thus allowing for infections half way through the warming period. Two thermostatically controlled rooms at 7 and 15°C (±0.5°C) were used to control temperature, to simulate spring warming a water bath and tank heaters were used to steadily increase the temperature in the 7°C room.

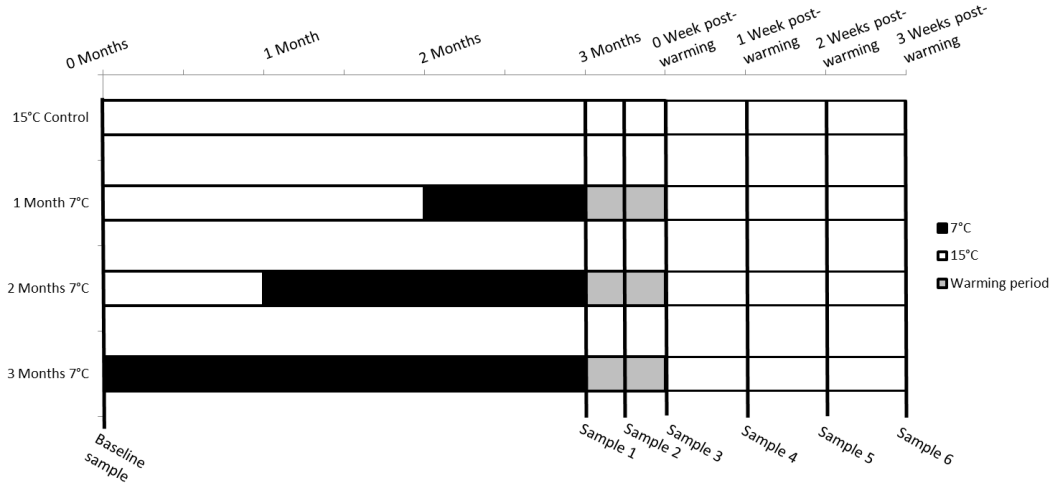

Figure 4.1: Summary of the Experimental Design. Horizontal bars are the 4 winter length treatment groups with temperatures represented by shading. The vertical lines represent sampling points 'Sample 1-6', 3 days prior to these sampling points the sticklebacks (*Gasterosteus aculeatus*) were infected/sham-infected with *Saprolegnia parasitica* or *Gyrodactylus gasterostei*. At these sample points, fish infected with *S. parasitica* were euthanized, morphometrics taken and tissue stored in RNAlater; fish infected with *G. gasterostei* had their first total burden recorded. A baseline sample was only taken for *S. parasitica* fish for comparative genetic analysis.

Slight changes to the overall experiment design, including different warming periods, were necessary to account for differences in the two parasite life cycles and the fact that fish immunology was only performed on the *S. parasitica* experiment. A baseline immunological sample of host tissue was taken for the *S. parasitica* experiment before winter treatments and stored in RNAlater at -80°C. Full dissection and additional parasite screening was only possible for the *G. gasterostei* infected fish, because immunological analysis of the *S. parasitica* infected fish was performed on RNA extracted from whole, non-degraded, fish. Infections of *S. parasitica* and *G. gasterostei* fish occurred at the same time points, relative to the end of the simulated winters, in order that infections would be established synchronously for sampling (Fig. 4.1), meaning that all samples of fish tissue or the first screen for total *G. gasterostei* burden began at the same temperature. Due to the different duration of warming periods and rate of warming, average temperatures on the day of infection were 7, 7.5, 12.5 and 15°C for *S. parasitica* infections, and 7, 9.5, 13 and 15 °C for *G. gasterostei* infections.

Fish were infected with either *S. parasitica* or *G. gasterostei*, described below, and sampled at the following sampling time points: (1) at 7°C after 3 months of winter length treatments; (2) halfway through the infections warming period, (3) at the end of the warming periods and (4-onwards) every week for 2 weeks post warming for the *G.*

gasterostei fish and 6 weeks post-warming for the *S. parasitica* fish (Fig. 4.1). During infection, fish were incubated at their current temperature (7°C or 15°C) for 72 h with the exception of samples in the rising temperature period, these fish continued to be exposed to that rising temperature while infected. In total, 260 fish were used in this study: n=6 or 4 per sampling point for the *S. parasitica* and *G. gasterostei* experiments, respectively.

Saprolegnia infection

S. parasitica strain CBS223.65, isolated from pike (*Esox lucius*) in 1965, was provided by the University of Aberdeen. The strain was maintained on potato dextrose agar (39 g/L) and re-plated monthly to maintain growth. Spores were obtained using the pea broth culture methods outlined in Chapter 2. Spores were collected and density was calculated using a Neubauer haemocytometer. Fish were infected using the ami-momi technique, net shaking for 30 s (Hatai & Hoshiai, 1994), and then individually exposed to $3x10^5$ spores per litre in 1 L pots for 24 h. Fish exposed to a sham infection underwent the ami-momi technique with the same degree of handling and were then put in 1 L pots of dechlorinated water. After 72 h, fish were euthanized by concussion and pithing. Fish were measured for standard length, weight and photographed with a Nikon S3600 camera in order to estimate the extent of *Saprolegnia* infection. Photographs were imported into ImageJ (Abramoff *et al.*, 2004) and the freehand selection tool used to outline the fish and any areas of infection. Infection intensity was calculated: $\text{Intensity} = \frac{\text{Total infected area}}{\text{Total body area}}$. For all sampling the body cavity of the fish was opened, sex noted, and the fish stored in RNAlater at 4°C for 24 h and then at -80°C prior to subsequent RNA processing.

Gyrodactylus infections

An isogenic line of *Gyrodactylus* sp. was obtained from Roath Park Stream on the 14/10/2015, the species was identified as *G. gasterostei* (see Chapter 3). Infections with *G. gasterostei* were conducted by anesthetising the fish individually in 0.02% MS222 and, under a dissection microscope using fibre optic illumination, two gyrodactylids were manipulated onto the caudal fin by overlapping the fins of a donor and recipient; standard length and weight were also taken. Fish were screened after 24 h to confirm establishment of infection and re-infected if not (n=5); these re-infected fish were screened again after 24 h. Fish were then screened twice a week until infections naturally cleared or fish had been euthanized (up to 91 days post-infection) as a result of

high burdens (>200 *G. gasterostei*) (see Schelkle *et al.*, 2009). All fish after the experimental procedure were dissected to check sex as well as screen for other parasites in the gills, eyes, gut and swim bladder.

Animal ethics

All animal work was approved by a Cardiff University Ethics Committee and performed under Home Office Licence PPL 302357. Any fish exhibiting stress responses, such as sucking at the water/air boundary or unusual swimming, as a result of parasite burdens (>200 *G. gasterostei* per fish) were euthanized using a Schedule 1 method.

Immunological profiling

RNA extractions were carried out using Bioline ISOLATE II RNA mini kits according to the manufacturer's instructions with the following amendments. Sticklebacks stored in RNAlater were defrosted at 4°C for 24 h and removed from the RNAlater. Whole sticklebacks cut up into 100 mg sections and placed into 3 ml RLY buffer with 1% β-mercaptoethanol, were homogenised in a bead beater at 50 Hz for 5 min. The volume of homogenate equivalent to 15 mg of tissue was calculated and made up to 1 ml with additional RLY buffer (with 1 % β-mercaptoethanol) and vortexed for 10 s. In order to improve filtration through the spin columns, lysate was passed through a 20 gauge needle three times. Clearing columns were loaded with 250 µl of the lysate and centrifuged at 11,000 g for 1 min and repeated a further two times until 750 µl of lysate had been passed through the clearing column. Washing of the clearing columns was performed as instructed. To elute RNA, 60 µl of RNase-free water was added directly to the filter and spun at 11,000 g for 1 min. RNA quality and quantity was check on a nanodrop using 260/280 and 260/230 ratios. RNA samples were then stored at -80°C. RNA samples were converted to cDNA using RNA to cDNA high capacity kits (Applied Biosystems) following instructions. Samples were stored in the RT-PCR plates at -20°C.

For qPCR, assay master mixes for each immune marker were prepared in Eppendorf SafeLock tubes with: 140 µl of Bioline SensiFAST SYBR LoROX mix, 72.8 µl DNAse/RNAse free water and 11.2 µl of 10 µM primer pair stock (see Appendix 2 for primer sequences). A total of 14 primer pairs were selected based on meaningful variation in gene expression from Brown *et al.* (2016) and to produce minimal redundancy across immunological phenotypes. Master mixes, samples plates and

control samples placed in a QuantStudio 12K machine and samples distributed into the 384 well MicroAMP optical plates in a randomized cohort fashion with three replicates of each fish's gene. Plates were briefly spun, checked for air bubbles and sealed with an ABI optical adhesive film and placed in the QuantStudio 12 K machine. A cycling protocol of 95°C for 2 min; 40 cycles of 95°C for 5 s, 62°C for 15 s; melt curve from 60°C to 95°C in 0.1°C increments was run. The delta-delta Ct method (ΔΔCt) (see Livak & Schmittgen, 2001) was used to assess relative transcript abundance.

Statistical analyses

All data were analysed in R v3.2.2 (R Core Team, 2015) with the additional use of 'car' (Fox & Weisberg, 2011), 'GAMLSS' (Rigby & Stasinopoulos, 2005), 'lme4' (Bates *et al.*, 2015) and 'MASS' (Venables & Ripley, 2002) packages. All model selection and model averaging was conducted using Akaike Information Criterion (AIC). All model residuals were assessed for normality and over-dispersion.

A global analysis of factors that affect *S. parasitica* intensity was conducted using GAMLSS (Rigby & Stasinopoulos, 2005) using the Zero Inflated Beta Family (BEZI) to correct for zero inflated data. Only fish exposed to *S. parasitica* were used in the model, no sham infected fish developed infections. The GAMLSS BEZI required two parameters: μ (mu), which models distribution (intensity) and ν (nu), which models the probability at zero (likelihood of having zero intensity - prevalence). Model nu and mu independent variables included: winter length degree days (calculated from point at which the fish entered the 1 month acclimatisation until sampled - used as a measure of relative age), temperature at infection, host sex, standard length at sampling, body condition after infection (as above) and an interaction between winter length and temperature at infection. Addition of the random effect 'tank' had no effect on the model outcomes.

Expression of immunological markers was determined by the ΔΔCt (see Livak & Schmittgen, 2001) and analysed using linear mixed effect models (lme4) (Bates *et al.*, 2015) and refined to linear models if the random factor, tank, had no effect on the model. Baseline samples were included in the 15°C and no winter treatments in order to adjust for fish age. Box-cox power transformations were used to normalise the dependent variables. Model independent variables were: winter length degree days (as above), infection (sham, asymptomatic, symptomatic), temperature at infection, host sex, standard length at sampling, and body condition after infection (calculated as a

quadratic regression of length against mass) and an interaction between winter length and temperature at infection. Degree days, host length and body condition were standardised by subtracting the mean and dividing by the standard deviation. A principle components analysis of all 14 genes produced 2 principle components with proportions of variance of 0.345 (*il-4, il-17, tirap*) and 0.268 (*il-1rlike, IgM)*; linear model analysis of these principle components with the above independent variables did not produce any significant results (p>0.05).

For analysis of *G. gasterostei* intensity, fish that were infected but on which an infection did not establish after two days were removed from the analysis (n=3) with the data limited to 62 days post-infection because of high errors thereafter. A chi-squared test was used to look for a difference between euthanized fish or those that cleared their infection A global model for *G. gasterostei* burden (the total number of *G. gasterostei* on a fish at any given screening event) was conducted using a GAMM with negative binomial errors (Wood, 2004, 2011); fish I.D. was used as the random variable. Model independent variables included: winter length, body condition (as above), length after experiment and sex with splines fitted for days post infection by the temperature of infection. GAMMs for WL were also fitted but with no visible effect and did not match data from *G. gasterostei* population dynamic analysis. All GAMM residuals were assessed for normality using the gam.check function.

GAMMs were supported with linear models, using Box-Cox power transformations which addressed the individual dynamics of *G. gasterostei* infection including: peak number of *G. gasterostei*, day of maximum burden of *G. gasterostei* and total *G. gasterostei* burden (Area Under the Curve - AUC) calculated using the Trapezoidal Rule to determine infection intensity with respect to time. Model independent variables included: winter length, temperature at infection, overall duration of infection, length after experiment, sex, body condition (as above), number of *Diplostomum* spp. and an interaction between winter length and temperature at infection. The inclusion of overall duration of infection was used to control for fish that were removed from the experiment (because they cleared the infection or were euthanized) when using AUC. Correlation of AUC with duration of infection means that statistics using AUC value actually analysed the average number of worms per unit time over the course of infection. Model residuals were checked for normality and assessed for over dispersion.

Our data suggested that fish exposed to *G. gasterostei* at low temperatures suffered higher burdens of infection than at 15°C. A GAM with negative binomial errors was

then fitted, looking for the difference within fish infected only at 15°C and between fish that were exposed to winter or not. Residuals for this model were checked as above. A GAMM, fish identification as the random term was tried but a sufficient number of PQL iterations could not be reached. Results

Saprolegnia intensity

Prevalence of *Saprolegnia parasitica* in three-spined sticklebacks was 47.4% within the control 15°C (no winter) treatment and 20.4% in all winter exposed fish. With increasing temperature, the likelihood of no infection (nu) decreased (Table 4.1; Fig. 4.2A) while the intensity of infection (mu) increased (Table 4.1; Fig. 4.2B). Other independent variables, winter length, degree days, sex, standard length and an interaction between winter length and temperature at infection had no impact an on the model and were removed to produce the minimum adequate model.

Dependent factor	Mu - Predictors	Std. Error	t- value	*p* - value	Nu - predictors	Std. Error	t-value	*p*- value
Intensity	**Intercept**	1.138	-4.529	<0.001	**Intercept**	2.109	2.706	0.010
	Infection Temperature	0.077	3.332	0.002	**Infection Temperature**	0.123	-2.695	0.010
					Body Condition	0.870	-1.806	0.077

Table 4.1: Summary of the averaged model for intensity of *Saprolegnia parasitica* infections in sticklebacks (*Gasterosteus aculeatus*) that experienced different lengths of winter. Mu parameter measures intensity of infection, Nu parameter measures likelihood of the fish having zero infection.

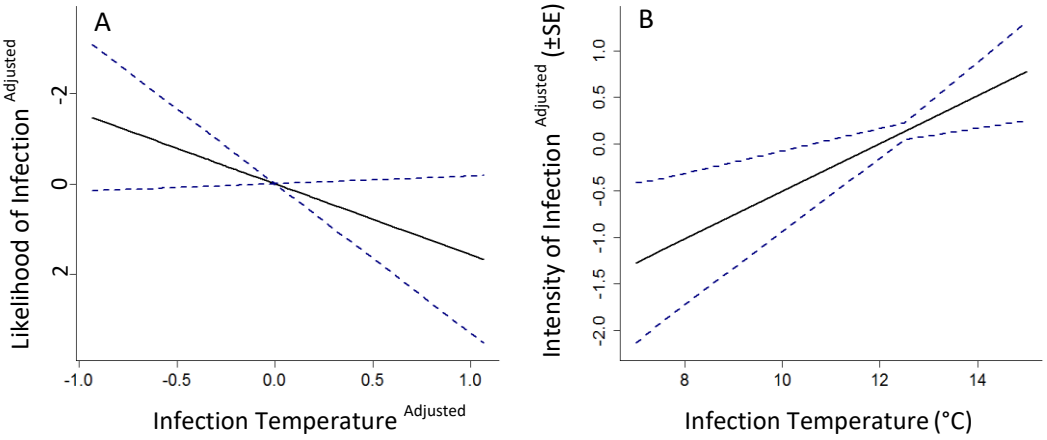

Figure 4.2: The effect of temperature on *Saprolegnia parasitica* infection generated from a non-random GAMLSS. The probability of infection decreases (A) and the intensity of infection increases (B) with increasing temperature. Solid lines are adjusted regression, dotted lines 95% confidence intervals. NB: (A) the inverted axis.

Immunological responses to Saprolegnia parasitica *infection*

Analysis of 14 immune genes from the fish infected with *S. parasitica* revealed significant variation in expression levels in response to infection, temperature at infection, degree days from acclimatisation until euthanasia, and winter length, in addition to host body condition, length and sex (Table 4.2). Independent variables not included in Table 4.2 were removed to produce the minimum adequate models and the interaction between winter length and temperature at infection had no impact on any model.

Symptomatic infections significantly reduced *foxp3b* and lysozyme expression with a trend towards reduction in *cd8a*, there was also a reduction in *foxp3b* and *cd8a* expression in asymptomatic fish (Fig. 4.3). Expression of *tbk1* was enhanced in symptomatic fish with a non-significant trend of increased expression in the asymptomatics (Fig. 4.3).

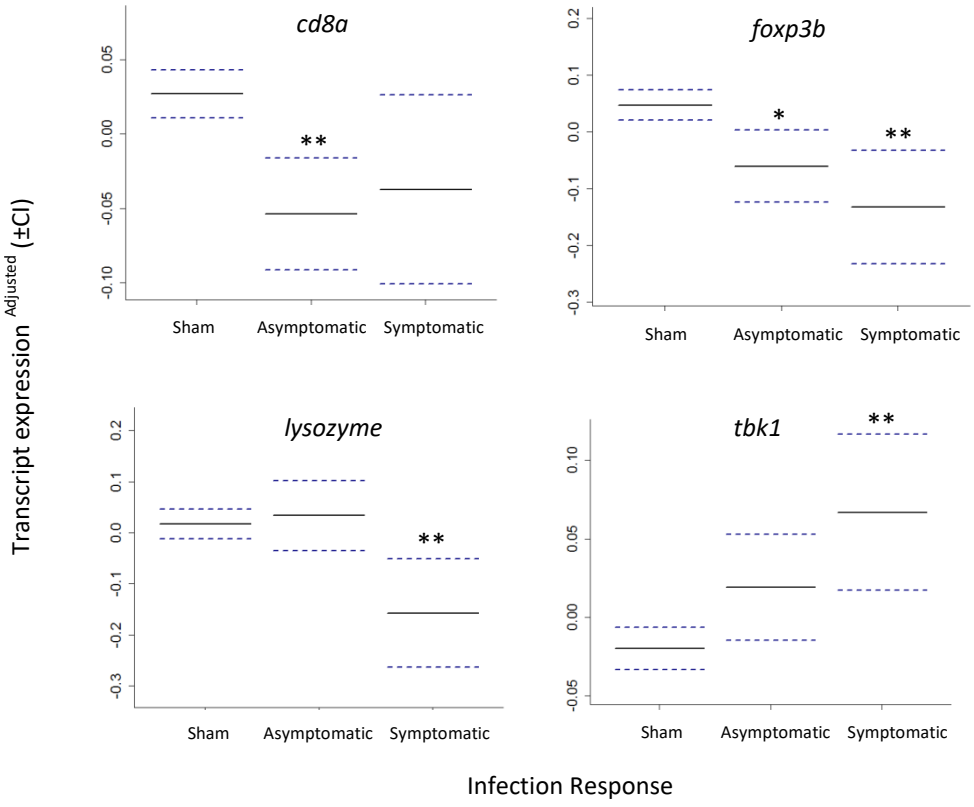

Figure 4.3: The infection treatment that each stickleback (*Gasterosteus aculeatus*) received, with those that were sham infected (n=60) received *Saprolegnia parasitica* spores (n=39) being separated into fish that did not develop *S. parasitica* (asymptomatics, n=26) and those that developed overt infection (symptomatics, n=12) plotted against the gene expression (black lines) and the 95% confidence intervals (dotted lines) (see Table 4.2). $p<0.001$ = ***, $p<0.01$ = **, $p<0.05$ *.

Dependant variable	Model	Independent Variable	Reference Factor	Compared Factor	t-value	*p*-value	
cd8a	lm	Infection	Sham	Asymptomatic	-3.176	0.002	**
				Symptomatic	-1.780	0.078	·
		Sex	Male	Female	-3.279	0.001	**
IgM	lm	Body Condition	-	-	-2.737	0.007	**
		Infection Temperature	-	-	2.621	0.010	*
IgZ	lm	Sex	Male	Female	-2.668	0.009	**
foxp3b	lm	Degree Days	-	-	4.601	<0.001	***
		Infection	Sham	Asymptomatic	-2.512	0.014	*
				Symptomatic	-3.106	0.002	**
		Sex	Male	Female	-2.862	0.005	**
il-1rlike	lm	Infection Temperature	-	-	2.051	0.043	*
		Length	-	-	2.236	0.028	*
		Winter Length	No Winter	1 Month	1.624	0.107	
				2 Months	2.371	0.020	*
				3 Months	3.475	<0.001	***
il-4	lm	Degree Days	-	-	-1.891	0.061	·
		Sex	Male	Female	-3.322	0.001	**
il-12ba	lm	Infection temperature	-	-	2.569	0.012	*
		Length	-	-	1.915	0.059	·
		Sex	Male	Female	5.852	<0.001	***
il-17	lm	Body Condition	-	-	2.394	0.018	*
		Degree Days	-	-	2.825	0.006	**
		Winter Length	No Winter	1 Month	1.273	0.206	
				2 Months	2.421	0.017	*
				3 Months	3.675	3.675	**
orai1	lm	Infection Temperature	-	-	-3.326	0.001	**
		Sex	Male	Female	2.462	0.015	*
β-def	lm	Body Condition	-	-	-2.736	0.007	**
		Infection Temperature	-	-	3.145	0.002	**
		Length	-	-	-2.532	0.013	*
		Sex	Male	Female	-4.213	<0.001	***
lysozyme		Degree Days	-	-	4.828	<0.001	***
		Infection	Sham	Asymptomatic	0.359	0.720	
				Symptomatic	-2.826	0.006	**
tbk1		Infection Temperature	-	-	-4.182	<0.001	***
		Infection	Sham	Asymptomatic	1.739	0.085	·
				Symptomatic	3.040	0.003	**
		Sex	Male	Female	-2.663	0.009	**
tirap	lmm	Sex	Male	Female	-2.205	0.030	*
gpx4a	lm	Infection Temperature	-	-	-2.712	0.008	**
		Winter Length	No Winter	1 Month	1.354	0.179	
				2 Months	2.818	0.006	**
				3 Months	1.988	0.0497	*

Table 4.2: A summary of models for each gene in sticklebacks (*Gasterosteus aculeatus*) analysed by RT-qPCR as part of the winter length experiment and the independent variables that predict gene expression. For factorial variables the reference and comparative factors are provided. $p<0.001$ = ***, $p<0.01$ = **, $p<0.05$ = *, $p<0.1$ = ·.

Interestingly, temperature at infection had a larger impact on immunity, although the effects were only minor except in the case of *tbk1* and *gpx4a* (Table 4.2), than any residual effect of winter (Fig. 4.5). Higher temperatures at infection corresponded with higher cytokine (*il-12ba*), cytokine receptor (*il-1rlike*) and *IgM* expression but a reduction in *orai1*. In the innate system, the effect of temperature was mixed with abundance of *β-def* expression enhanced at warmer temperatures and *tbk1* supressed. Higher temperature also reduced expression of the housekeeping gene *gpx4a*. Longer winters caused increases in gene expression of *il-17*, *il-1rlike* and *gpx4a* (Fig. 4.4).

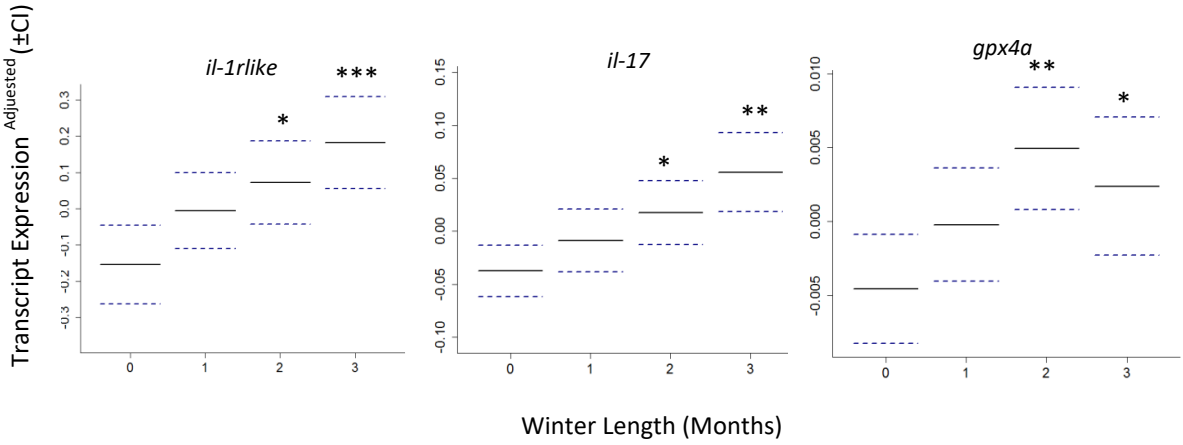

Figure 4.4: Gene expression of sticklebacks (*Gasterosteus aculeatus*) was plotted against winter length (0 months n=25; 1 months n=28; 2 months n=26; 3 months n=20) with 95% confidence intervals (dotted lines) (see Table-4.2). $p<0.001$ = ***, $p<0.01$ = **, $p<0.05$ *.

Others factors that affected gene expression included host sex, which caused higher expression in males of *cd8a, IgZ, foxp3b, il-4, β-def, tbk1* and *tirap*. Females had higher expression of *orai1* and *il-12ba*. Degree days caused an increase in *foxp3b*, *il-17* and *lysozyme* expression and a decrease in *il-4* expression. Body condition caused an increase in *il-17* and decrease in *IgM* and *β-def* expression. Length caused an increase in *il-1rlike* and a decrease in *β-def* expression.

Gyrodactylus infections

In total, 80 sticklebacks were infected with *Gyrodactylus gasterostei* in order to assess the effects of winter length and infection temperature on infection trajectories. A larger number of fish that were infected at <15°C were removed from the experiment and euthanized, because of high *G. gasterostei* burdens, compared to fish infected at 15°C (χ^2=16.545, df=2, p<0.001; Fig. 4.5A). A larger, but not significant, percentage of fish

were also removed and euthanized from the winter treatments (Fig. 4.5B), although this is likely caused by the fact that only winter fish were infected at any temperature other than 15°C. Survival of winter treated fish infected at 15°C (95%, n=20) was not appreciably different to survival of fish that did not receive a winter (87.5%, n=24); by this measure alone it is temperature at infection that appears to have the greatest impact on infection intensity rather than the host's historical temperature experience.

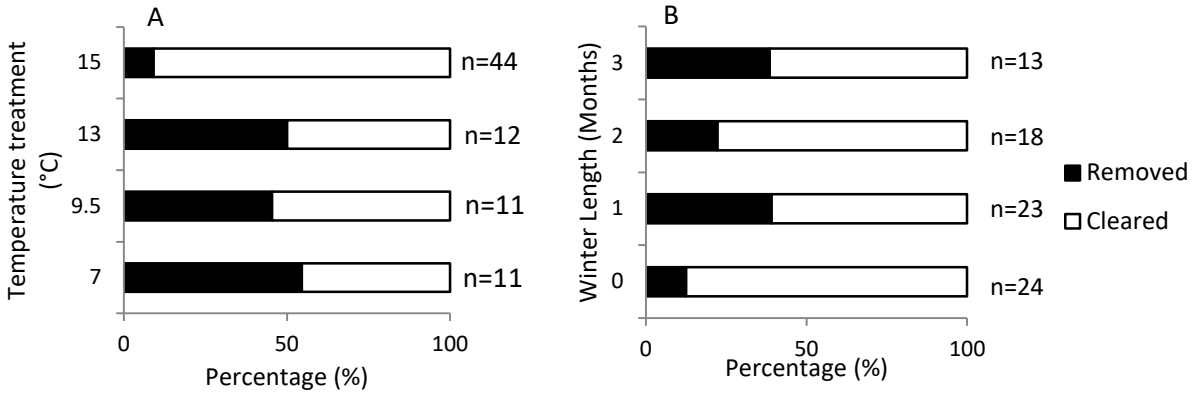

Figure 4.5: The percentage of sticklebacks (*Gasterosteus aculeatus*) euthanized (removed from the study due to high, >200, worms) or that cleared *Gyrodactylus gasterostei* infections at different (A) temperature treatments or (B) winter length treatments until all fish were either dead or cleared of infection.

In all treatments combined, fish infected at colder temperatures acquired higher infection intensities with later infection peaks than fish infected at 15°C (Fig. 4.6). Additionally *G. gasterostei* population growth was initially slower at 7°C compared to 15°C. Fluctuations in total *G. gasterostei* intensity were in general caused by fish that were removed and euthanized because of their high burdens (Fig. 4.7A-D). Given that *G. gasterostei* population growth from removed fish was likely to continue increasing, (Fig. 4.8) our results are conservative estimates of population growth as those hosts removed (Fig. 4.7C-D) would likely have continued supporting an upward parasite trajectory making the peak infection intensity and time of peak infection larger in 7, 9.5 and 13°C fish. Variation between splines was significant in all cases (7°C $F=41.27$, $p<0.001$; 9.5°C $F=15.04$, $p<0.001$; 13°C $F=14.79$, $p<0.001$; 15°C $F=11.15$, $p<0.001$), independent variables (length, body condition, sex and winter length) were non-significant ($p>0.05$).

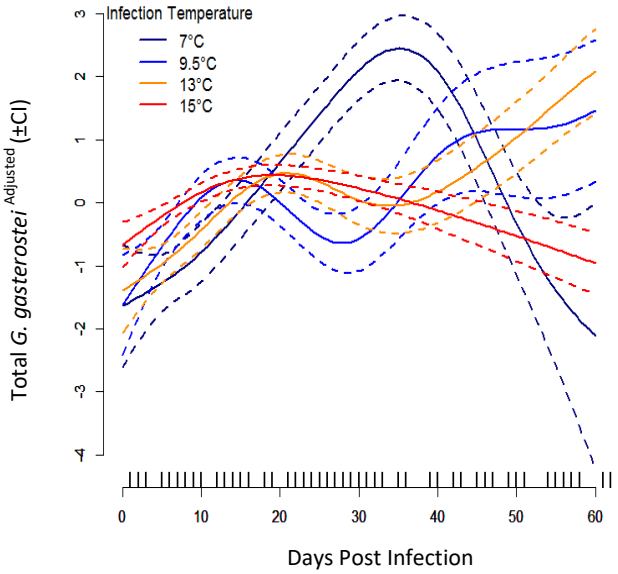

Figure 4.6: A GAMM of *Gyrodactylus gasterostei* burden on three-spined sticklebacks (*Gasterosteus aculeatus*) over time when infected at 4 different temperatures (7, 9.5, 13 and 15°C). Solid lines are adjusted means and dotted lines 95% confidence intervals.

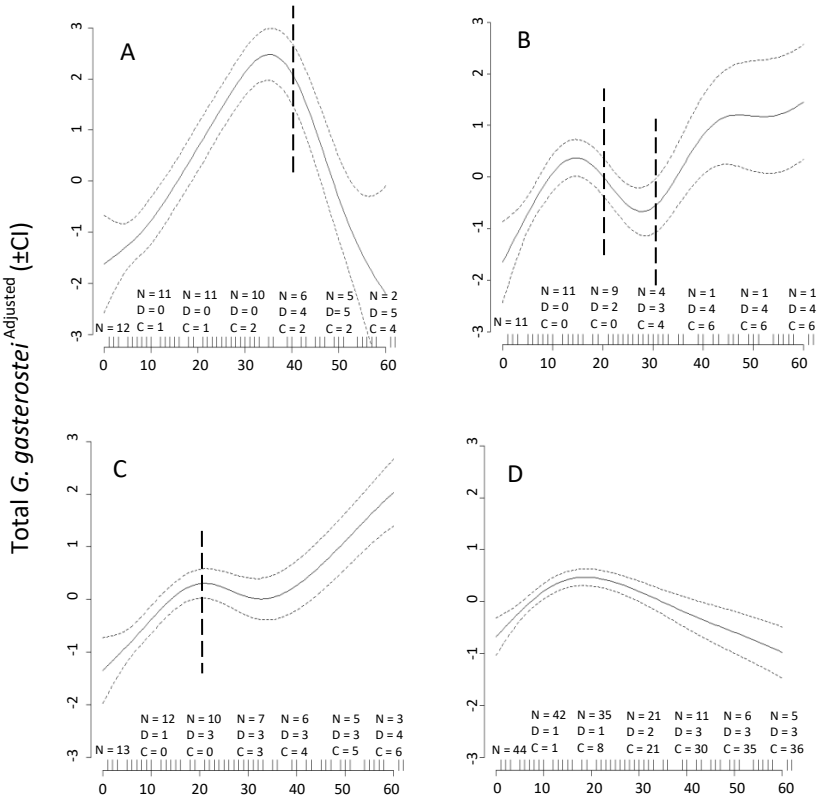

Figure 4.7: *Gyrodactylus gasterostei* burden on three-spined sticklebacks (*Gasterosteus aculeatus*) over time when infected at four different temperatures (A-D: 7, 9.5, 13°C and 15°C respectively). N = number of the fish in the experiment at that time point, D = the number of deaths (euthanized or stochastic) up until that time point, C = the number of fish screened clear of infection up until that time point. Vertical stripped lines indicate that in the previous 10 days, fish were removed from the experiment because of high *G. gasterostei* burdens resulting in the loss of >100 worms from the experiment. Bracketed numbers are the number of fish removed at a given time point each carrying >100 *G. gasterostei*. No fish in the 15°C treatment were euthanized as a result of high *G. gasterostei* burdens. Solid lines are adjusted means and dotted lines 95% confidence intervals derived from a GAMM.

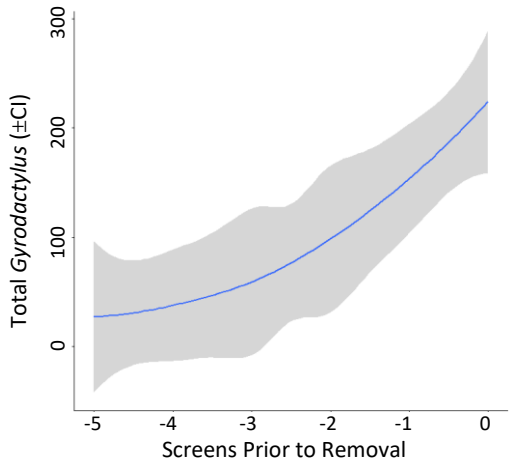

Figure 4.8: The total number of *Gyrodactylus gasterostei* on sticklebacks (*Gasterosteus aculeatus*) in the 5 days before it was removed from the experiment and euthanized (n=21, z=10.9, p<0.001). Blue line is the mean, shaded grey errors are 95% confidence intervals.

Results from the global GAMM models were supported by analysis of *G. gasterostei* infection dynamics which found a lower average number of *G. gasterostei* per unit time (t=-2.800, p=0.007; Fig. 4.9A), a lower peak of infection (t=-2.193, p=0.032; Fig. 4.9B) and earlier infection peak (t=-3.224, p=0.002; Fig. 4.9C) for fish infected at 15°C when compared to other temperatures. As expected, fish with a longer duration of infection had a higher AUC (t=5.520, df=69, p<0.001). Independent variables: winter length, temperature at infection, overall duration of infection, length after experiment, sex, body condition, number of *Diplostomum* spp. and an interaction between winter length and temperature at infection, were non-significant (p>0.05).

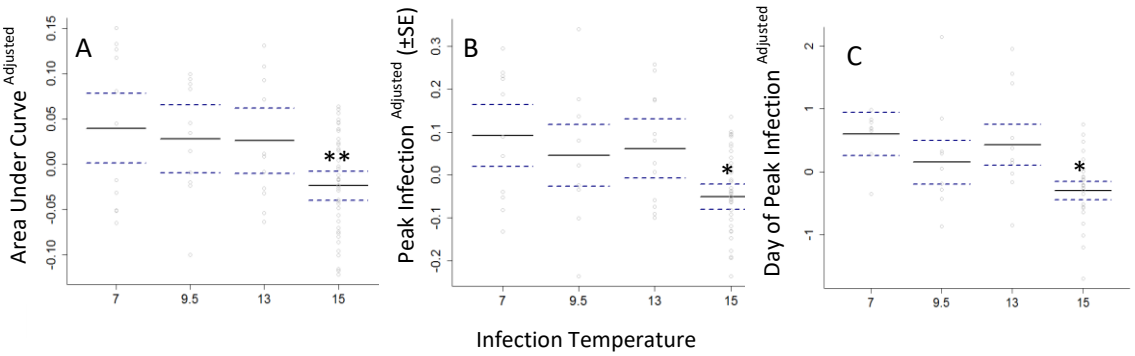

Figure 4.9: The partial residuals for temperature derived from linear models for a stickleback (*Gasterosteus aculeatus*) of (A) average number of *Gyrodactylus gasterostei* per unit time, (B) number of *G. gasterostei* at peak infection and (C) the day of peak infection. p<0.001 = ***, p<0.01 = **, p<0.05 *. Black lines are adjusted means and dotted line 95% confidence intervals.

For fish infected at 15°C there was no difference in those that were and were not exposed to winter; large error margins after 35 days post-infection in the winter group are an artefact of only one remaining fish with an infection (Fig. 4.10). Within the No Winter treatment (n=24), one fish was euthanized due to high parasite burden and 2 succumbed to stochastic mortality with infections not exceeding 10 worms. Within the Winter treatment (n=21), only one fish was euthanized for having a high infection at 32 days post-infection, all other fish cleared their infections.

Discussion

Rather than examining the response of animals exposed to constant elevated temperatures as has often been done previously (see Avtalion *et al.*, 1970; Bly & Clem, 1992; Morvan *et al.*, 1998; Uribe *et al.*, 2011), this study assessed the effects of temperature variability on teleosts. For these endothermic hosts it is the current, rather than historical, temperature that drives infection and immunity. In the case of *Saprolegnia parasitica*, a lower prevalence and infection intensity occurred at colder temperatures, somewhat in contrast to previous studies (Bly & Clem, 1991; Bly *et al.*, 1992). A number of factors might have caused such a phenomenon, not least of which is that winter Saprolegniasis tends to occur when there are rapid drops in temperature, typically >5°C over 24 h (Bly & Clem, 1991; Bly *et al.*, 1992), where this study utilised constant cold temperatures and a relatively slow increase in temperature. Rapid temperature drops are well known to cause cold shock stress to fish (Donaldson *et al.*, 2008) inducing cortisol production (Tanck *et al.*, 2000) associated with Saprolegniasis (Carballo *et al.*, 1995). Stress also suppresses immunity, potentially causing increase susceptibility to *S. parasitica* (see Tort, 2011). Although the ami-momi technique used during the infection process is a stressor it does not replicate natural conditions, in wild or farmed populations, where rapid temperature change, density, predators and other factors may have a multiplicative effect on the fish's susceptibility to infection. Exposure to consistent cold temperature in the current study demonstrates that cold conditions alone are not sufficient to induce infection and that higher rate of infection and intensity at warmer temperatures may simply be a reflection of favourable growth conditions for *S. parasitica* in a laboratory setting (Powell *et al.*, 1972).

A supplementary theory, to a purely temperature driven response by *S. parasitica*, was revealed in our immunological data. Increased transcript abundance of *tbk1* an NF-κB inducer (Hayden *et al.*, 2006) at colder temperatures or *il-17* (Jin & Dong, 2013) and *il-1rlike* (Garlanda *et al.*, 2013) during winter, consistent with Brown *et al.* (2016), could

have provided host resistance to *S. parasitica*. Indeed the pro-inflammatory response, to which *tbk1, il-17* and *il-1rlike* all belong, was previously found to be upregulated in trout cell lines and fish exposed to *Saprolegnia* and may be protective (see Bruijn *et al.*, 2012; Belmonte *et al.*, 2014). In particular, *tbk1* expression was considerably higher in our symptomatic fish suggesting it may play a key role in resistance. While *il-17* and other antimicrobial responses were not apparent in symptomatic fish this would be consistent with the delay in response by the adaptive immune system over the 72 h of infection. It is also the case that immunomodulation by *S. parasitica* (see Belmonte *et al.*, 2014) could be responsible for a lack of adaptive response, *cd8a* and *foxp3b* both being supressed in fish that were exposed to *S. parasitica*.

Infections with *Gyrodactylus gasterostei*, as with *S. parasitica*, were problematic for fish exposed at colder temperatures. The initial growth phase of *G. gasterostei* at 15°C was rapid over the first 10-15 days compared to fish infected at 7°C, but not 9.5 or 13°C. This trend was apparent despite fish infected at 7°C being exposed to continually warming water over 2 weeks up to 15°C. This lower initial population growth phase at 7°C was consistent with previous studies, which found reduced reproduction in *Gyrodactylus* spp. at low temperatures (Chappell, 1969; Lester & Adams, 1974; Harris, 1982; Jansen & Bakke, 1991). After the initial growth phase, fish infected at 15°C were better able to control infections which peaked earlier and at lower numbers. It is also the case that fish infected at 15°C suffered a far lower mortality as a result of high parasite burden. These results may also be quite conservative; as fish euthanized early, as a result of high infection burdens within the <15°C temperature group, would likely have continued to experience high population growth making peak infection intensity higher and later.

Fish infected at 15°C showed evidence of an effective immune response with reductions in their *G. gasterostei* infection by about 20 days post-infection. Despite being in a warming environment that reached 15°C, 14, 10 or 3 days post-infection, fish infected at below 15°C were not able to supress their infections after 20 days at 15°C, in contrast to fish infected at 15°C. This is particularly noticeable considering that fish which experienced the colder temperatures, but were infected at 15°C, suffered no differently to fish kept at 15°C for 3 months. This may explain why temperate field studies typically find the highest prevalence and intensities of gyrodactylids from May to July, 1-2 months after temperatures in the respective country has increased above 10-15°C, a phenomenon typically attributed to the effect of temperature on the reproductive rate of

Gyrodactylus spp. alone (Chappell, 1969; Mo, 1997; Winger *et al.*, 2007; You *et al.*, 2008). Our results offer an alternative explanation for these seasonal patterns; that immune system suppression at low temperatures allows the parasite population to establish and that in warming temperatures the gyrodactylid population can adapt faster than the host responses providing the parasite with an advantage, this has previously been described as the 'foothold hypothesis' (see Chapter 2).

In summary, this study finds that lower *S. parasitica* prevalence and intensities of infection at colder temperature are the result of heightened pro-inflammatory responses and that predicted contractions of winter length will not affect infections. There was also evidence for a new theory of gyrodactylid infections, the Foothold Hypothesis, focused around historical infection history that allows gyrodactylids to acquire an advantage over the host in changeable temperature conditions. This theory has implications for fisheries and aquaculture industry that previously had attributed seasonal changes in gyrodactylid numbers to temperature and fish mortality.

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Chapter 5 - The immunological responses of three-spined sticklebacks (*Gasterosteus aculeatus*) exposed to constant and shock temperature treatments and *Saprolegnia parasitica* infection

Abstract

Independently, the effects of temperature on teleost immunology and *Saprolegnia parasitica* have been well explored, but never in combination. Here, this knowledge gap is addressed using the three-spined stickleback (*Gasterosteus aculeatus*) as a model host. Sticklebacks were exposed to constant temperatures (7, 15, 23°C), or cold or warm shocks (+/-8°C) before being exposed to *S. parasitica* spores and sampled for immunological analysis. Likelihood of infection was increased in sticklebacks at a constant 23°C, whilst the impact of temperature shock on infection was dependent on the direction of change rather than absolute change; +8°C thermal shock decreased infection likelihood, -8°C did not change infection likelihood compared to controls. Unexpectedly, temperature shock had little effect on immune response 72 h after the temperature change. Likelihood of *S. parasitica* infection was reduced at colder temperatures ($7<15<23^{o}$C) and may be explained by enhanced pro-inflammatory expression (*tbk1* & *il-17*) at 7°C. Intriguingly, high host body condition had a major impact on increasing the likelihood and intensity of infection; the former is attributed to a trade-off between condition and immunity, the anti-microbial peptide *β-defensin* being supressed in high condition fish. Greater abundance of resources in the higher condition hosts might explain increase infection intensity on these fish promoting improved parasite growth rates. These results reveal that shock temperature treatments are dependent on the direction of temperature change and that host body condition is a major contributory factor in *S. parasitica* infection. Such information is key for timing pre-emptive control treatments in aquaculture.

Introduction

Climate change and the oomycete, fungal-like heterotroph, *Saprolegnia parasitica* are current and major threats to aquaculture and food security. The banning of effective treatments has increased the risk of this pathogen to food security (van West, 2006; Defra, 2015). With limited options available for treating *S. parasitica* it is imperative that we understand its biology, and that of the host, better in order to prevent infection. A number of factors are typically associated with susceptibility of teleosts to *Saprolegnia* spp. infection: temperature at infection, rapid changes in temperature,

water toxicity, handling and stocking density (Pickering & Willoughby, 1982; Bly *et al.,* 1992; Carballo *et al.,* 1995; Iguchi *et al.,* 2003). The common theme behind all of these factors is that they are potential stressors that enhance cortisol production (Barton *et al.,* 1980; Mazur & Iwama, 1993; Carballo *et al.,* 1995; Tanck *et al.,* 2000; Iguchi *et al.,* 2003) supressing the immune system and predisposing fish to infection (Pickering & Duston, 1983; Pickering & Pottinger, 1985; Maule *et al.,* 1987; Espelid *et al.,* 1996; Tort, 2011). Immune suppression resulting from stress may arise as a result of resource redirection: fight or flight responses (e.g. higher breathing/heart rate), and potentially catabolic activity directed at immune cells to redistribute protein (Sapolsky *et al.,* 2000). Alternatively, immune suppression could be the result of immune regulation to prevent auto-immunity, in the presence of increased novel self-antigens produced as part of the stress response (Råberg *et al.,* 1998). Rapid drops in temperature may have an additive effect on infection caused by immune suppression, as a result of cortisol production, and the suppressive effects of temperature on T-cell function (Bly & Clem, 1994) and immunoglobulin responses (Avtalion, 1969; Avtalion *et al.,* 1970; Morvan *et al.,* 1998).

The majority of studies investigating the effects of temperature variation on infections have focused on non-model teleosts and lack immunological analysis (see Bly *et al.,* 1992; Szalai *et al.,* 1994), while those that have focused on immunology have not considered variable temperatures (see Kales *et al.,* 2007; Roberge *et al.,* 2007; Bruijn *et al.,* 2012; Belmonte *et al.,* 2014). Typically lower body condition hosts are predisposed to infection because of immune suppression, due to lack of resources for investment in either condition or immunity (Nelson & Demas, 1996; Møller *et al.,* 1998; Bakker & Mundwiler, 1999; Beldomenico & Begon, 2010). There are also multiple trade-offs between life history traits and immunity, which may influence infection outcomes (see Schmid-Hempel, 2008). Such trade-offs typically result in paradigms such as tolerance, where investment in body condition is enhanced at the expense of immunity (Jackson *et al.,* 2014).

This current immunological study uses the three-spined stickleback to explain the effects of constant or rapid changes in temperature on infection and teleost immunity. With both an immune and infection component this study will also allow an in-depth look at the relationship between body condition, infection and immunity. Sticklebacks were exposed to three temperatures within their physiological range (7, 15 and 23°C), predicted to elicit varying immunological responses.

Materials and Methods

Host origins and maintenance

Wild three-spined sticklebacks (*Gasterosteus aculeatus*) were captured from Roath Brook, Cardiff (ST 18897 78541) on the 14/10/14 and the 10/09/15. Fish were transported to the aquarium facility at Cardiff University and maintained in 30 L tanks at a density of <1 fish/litre on a 18 h light: 6 h dark cycle at 15±0.5°C and fed daily on frozen bloodworm. Sticklebacks were treated for parasites by submerging them in 0.004% formaldehyde solution for 1 h, with a halfway 30 min rest period in fresh water. Fish were left in freshwater to recover for 24 h and then treated with fluke-solve (Vetark) praziquantel according to the manufacturer's instructions (4 g of 50% praziquantel in 1000 L) for 48 h. After praziquantel treatment, fish were left to recover for 1 week in 1% aquarium salt solution and 0.002 g/L methylene blue to prevent secondary bacterial or fungal infection. Treated fish were briefly anaesthetised in 0.02% MS222 and checked visually under a dissection microscope with fibre optic illumination for infecting ectoparasites. Any infecting parasites were removed with watchmaker's forceps following the protocol outlined by Schelkle *et al.* (2009); fish were checked in this manner three times before being defined as clear of infection if nothing was found on three consecutive checks. Fish were then maintained in 30 L freshwater tanks (<1 fish/L) at 15°C for 1 month in order to acclimatise and recover.

Saprolegnia *spores*

Saprolegnia parasitica cultures of isolate CBS223.65 from Northern Pike (*Esox lucius*, 1965) were maintained on potato-dextrose agar. Spores were obtained using the pea broth culture method (see Chapter 2 for full details): three mycelia plugs, 5 mm dia., were taken from the stock *S. parasitica* PDA cultures and placed in 140 mm Petri dishes, 3 plugs per plate, with 70 ml of pea broth for 72 h at 25°C. Cultures were then washed vigorously with distilled water removing excess pea broth and the Petri dishes topped up with a 50/50 mixture of distilled and tank water, dishes were left for a further 48 h at 15°C. Spores were collected through a 40 µm cell strainer and spore concentration determined using a haemocytometer.

Experimental design

Sticklebacks were separated into three different temperature groups, standard length and weight recorded, and they were acclimatised to 7, 15, 23°C for 3 weeks in three thermostatically controlled rooms (±0.5°C). Then, fish were further separated into

'shock' or 'constant temperature' groups of equal numbers of fish; constant or shock treatments are referred to as 'ΔT treatment'. In the shock temperature group, fish had their ambient water temperature changed rapidly over a period of 6 h either going from 7°C to 15°C, from 23°C to 15°C, or from 15°C to either 23°C or 7°C. Constant temperature groups continued to be maintained at 7, 15 or 23°C (Fig. 5.1). After the 6 h shock temperature treatment, fish in all treatment groups were exposed to one of four 'infection treatments': placed in freshwater, exposed to a 30 s ami-momi technique (Hatai & Hoshiai, 1994), exposed to *S. parasitica* spores at a concentration of $3x10^5$ spores per litre for 24 h or exposed to both 30 s of ami-momi and *S. parasitica* spores at a concentration of $3x10^5$/L (Fig. 5.1). The temperature at which fish were exposed to *S. parasitica* is hereafter called 'infection temperature'. Multiple blocks of this experiment were conducted to increase replicate number per treatment; as such multiple spore batches were introduced into the experiment.

At 72 h post-treatment, fish were euthanized by concussion and pithing, standard length and mass taken (mean length = 33.2 mm, range = 22.91 to 49.24 mm; mean mass = 0.535 g, range = 0.154 to 1.742 g) and photographed using a Nikon S3600 camera. The freehand selection tool in ImageJ (Abramoff *et al.*, 2004) was used to measure the overall area of the fish and the surface area of the fish that was covered in mycelium. Intensity of infection was estimated using the formula: $\text{Intensity of infection} = \frac{\text{Total infected area}}{\text{Total body area}}$. The body cavity of the fish was then opened, sex determined, and the fish stored in RNAlater at -80°C.

Immunological profiling

RNA extractions were performed on homogenised fish gills using Bioline ISOLATE II mini kits according to the manufacturer's protocol (outlined in Chapter 4 and Brown *et al.*, 2016). A total of 14 genes were selected for qPCR (see Appendix 2) to reduce redundancy and a produce a high level of meaningful variation based on Brown *at al* (2016). Quality of RNA was checked using a NanoDrop1000 at 260/280 and 260/230 ratios; RNA was then stored at -80°C. RNA to cDNA conversion was performed using Applied Biosystems High Capacity cDNA reverse Transcription kits according to manufacturer instructions. Bioline's SensiFAST SYBR LoROX was used to produce qPCR master mixes with primers (Appendix 2) to manufacturer's instructions in a QuantStudio 12K machine using 384 well MicroAMP optical plates in a randomized cohort fashion with three replicates of each fish's genes. The cycling protocol was 95°C for 2 min; 40 cycles of 95°C for 5 s, 62°C for 15 s; melt curve from 60°C to 95°C in

0.1°C increments. ΔΔCt values (see Livak & Schmittgen, 2001) were calculated to measure relative transcript abundance.

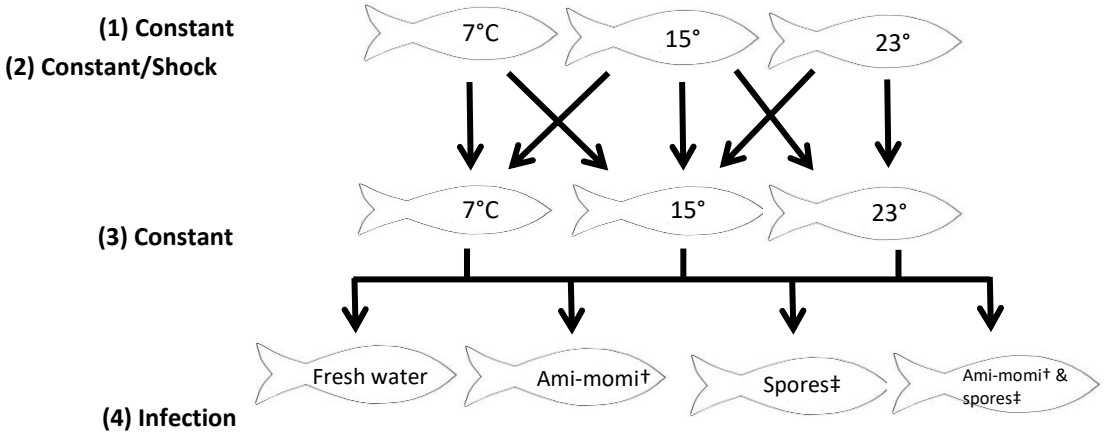

Figure 5.1: Flow diagram of the experimental outline. (1) Three-spined sticklebacks (*Gasterosteus aculeatus*) were kept at a constant temperature for 3 weeks. (2) Fish were exposed to constant or rapid temperature change (ΔT treatment) (3) and then maintained at the resulting temperature for the remaining 72 h of infection 'infection temperature' (4) and exposed to one of four infection treatments. †Ami-momi technique is an adjusted 30 s exposure based on Hatai and Hoshiai (1994). ‡Spores are *Saprolegnia parasitica* zoospores.

All data were analysed using R v3.2.2 (R Core Team, 2015) with the additional use of 'car' (Fox & Weisberg, 2011), GAMLSS (Rigby & Stasinopoulos, 2005), lme4 (Bates *et al.*, 2015) and 'MASS' (Venables & Ripley, 2002) packages. Model selection and averaging was conducted using Akaike Information Criterion (AIC) and residuals were assessed for normality and over-dispersion. *S. parasitica* intensity was analysed using a GAMLSS (Rigby & Stasinopoulos, 2005) and the Zero Inflated Beta Family (BEZI) to correct for zero inflated data. As such model parameters used were: mu (μ) which assesses intensity of infected individuals and nu (ν) which assesses the probability of an individual acquiring an infection. Model parameter independent variables included: infection temperature, ΔT treatment, infection treatment, *S. parasitica* infection batch, fish sex, standard length and body condition (calculated as a quadratic regression of mass against length) and an interaction between infection temperature and ΔT treatment. Batch of *S. parasitica* spores had an impact on infection with the last batch of four having a lower prevalence ($t=3.211$, $df=128$ $p<0.001$) and lower intensity of infection ($t=-3.616$, $df=128$ $p<0.001$); the other three batches did not vary significantly ($p>0.05$).

Immune gene expression was quantified via the ΔΔCt method (see Livak & Schmittgen, 2001) and was analysed using linear mixed effect models (lme4) (Bates *et al.*, 2015)

and refined to linear models if the random factors, RT-qPCR optical plate and *S. parasitica* spore batch, had no effect on the model. Box-Cox power transformations were used to normalise data. Model independent variables included: infection treatment, intensity of infection, fish sex, length and body condition (as above). Fish length and body condition were rescaled by subtracting the mean and dividing by the standard deviation. Principle components analysis of all 14 genes resulted in principle components were the proportion of variance was made up predominantly of single gene variation; further analysis was therefore redundant.

Ethics

All animal work was approved by a Cardiff University Ethics Committee and performed under Home Office Licence PPL 302357. Any fish exhibiting stress responses, such as sucking at the water/air boundary or unusual swimming were euthanized using a Schedule 1 method.

Results

From a total of 275 sticklebacks, 7.1% became infected when exposed to *Saprolegnia parasitica* spores and 29.5% became infected if pre-exposed to the ami-momi technique (see Hatai & Hoshiai, 1994) before exposure to spores. No unexposed control fish, with or without net shaking, became infected. Within both the temperature at infection and ΔT treatments, there was also a small positive relationship between temperature at infection and likelihood of infection ($t=-1.965$, $df=128$, $p=0.051$; Fig. 5.2). There was also a higher likelihood of infection if the temperature remained constant ($t=-3.265$, $p=0.001$) or if there was an 8°C decrease ($t=-3.368$, $p<0.001$) compared to an 8°C increase in temperature (Fig. 5.2B). The interaction between temperature at infection and ΔT was marginally significant ($t=1.972$, $p=0.049$) although not biologically meaningful. An increase in the relative body condition of fish increased the likelihood ($t=-3.160$, $p=0.002$; Fig. 5.3A) and intensity of infection ($t=5.421$, $p<0.001$; Fig. 5.3B).

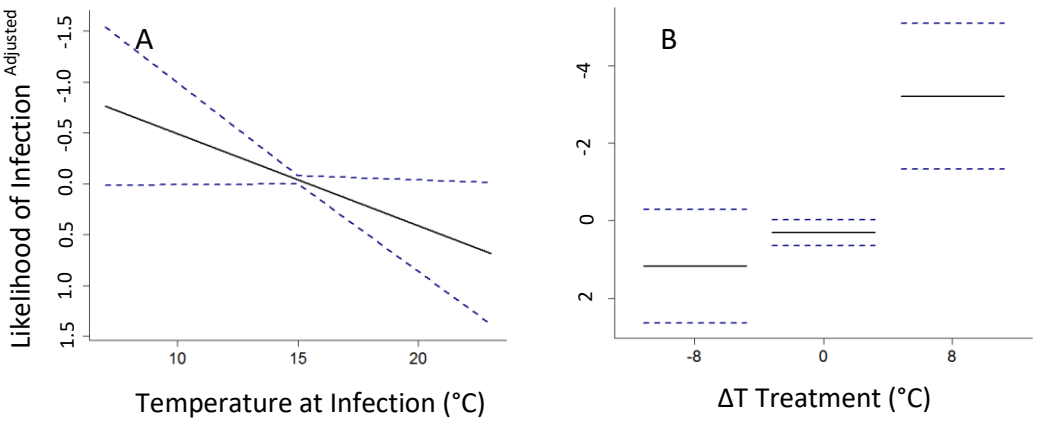

Figure 5.2: The Likelihood of Infection for sticklebacks that experienced a temperature change prior to infection 'ΔT' (A) and the temperature at which they were infected 'T' (B) when exposed to *Saprolegnia parasitica* spores. Solid black lines are the adjusted means or regression from the GAMLSS and the dotted lines 95% confidence intervals, note the inverted Y-axis.

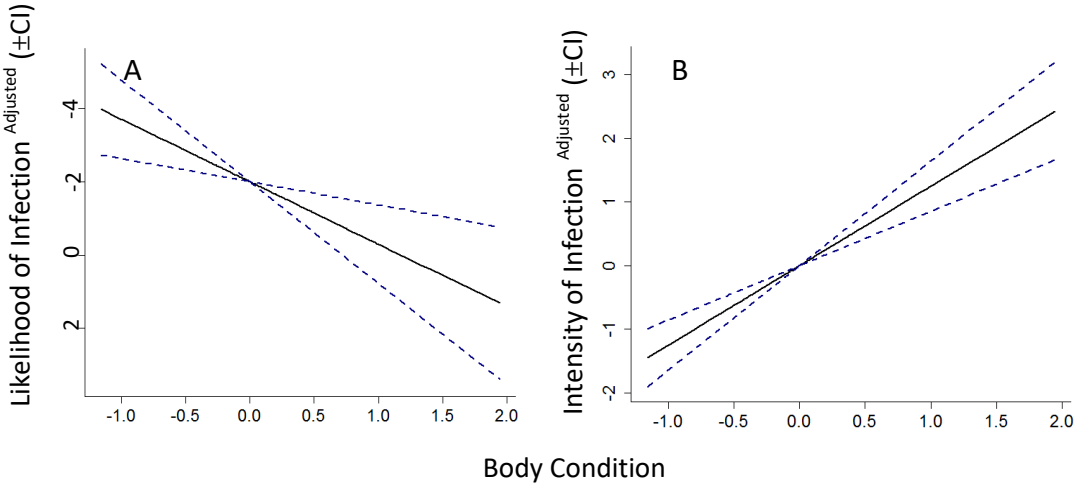

Figure 5.3: The relationship of stickleback (*Gasterosteus aculeatus*) body condition on the likelihood of a fish developing an infection (A) and the intensity of infection (B) within fish that were exposed to the spores of *Saprolegnia parasitica*. Solid black lines are the adjusted regression from the GAMLSS and the dotted lines 95% confidence intervals. Note the inverted Y-axis (A).

Analysis of 14 immune gene transcripts extracted from fish gills (Table 5.1) revealed significant effects of: relative fish body condition, infection treatment, *S. parasitica* intensity, standard fish length, fish sex, temperature at infection and ΔT treatment (Table 5.1). By far the most common predictor of transcript abundance was infection temperature with higher temperature causing upregulation of predominantly adaptive immune genes *cd8a, IgM, foxp3b* and *il-4* along with innate genes *lysozyme* and *tirap* while causing downregulation of *il-17* (adaptive), *orai1* (adaptive) and *tbk1* (innate) transcript (Fig. 5.4). Surprisingly the ΔT treatment had a relatively minor impact on

immune gene expression that was not dependent on absolute change but on the direction of change; *IgM* and *foxp3b* expression increased if temperature dropped but was unaffected by a temperature increase (Fig. 5.5). High fish body condition positively affected *cd8a*, *IgZ* and *tbk1* expression while decreasing *β-def* expression (Table 5.1). Increased intensity of *S. parasitica* infection caused a decrease in *cd8a*, *foxp3b*, *lysozyme* and *tirap* expression and an increase in *β-def* expression (Table 5.1).

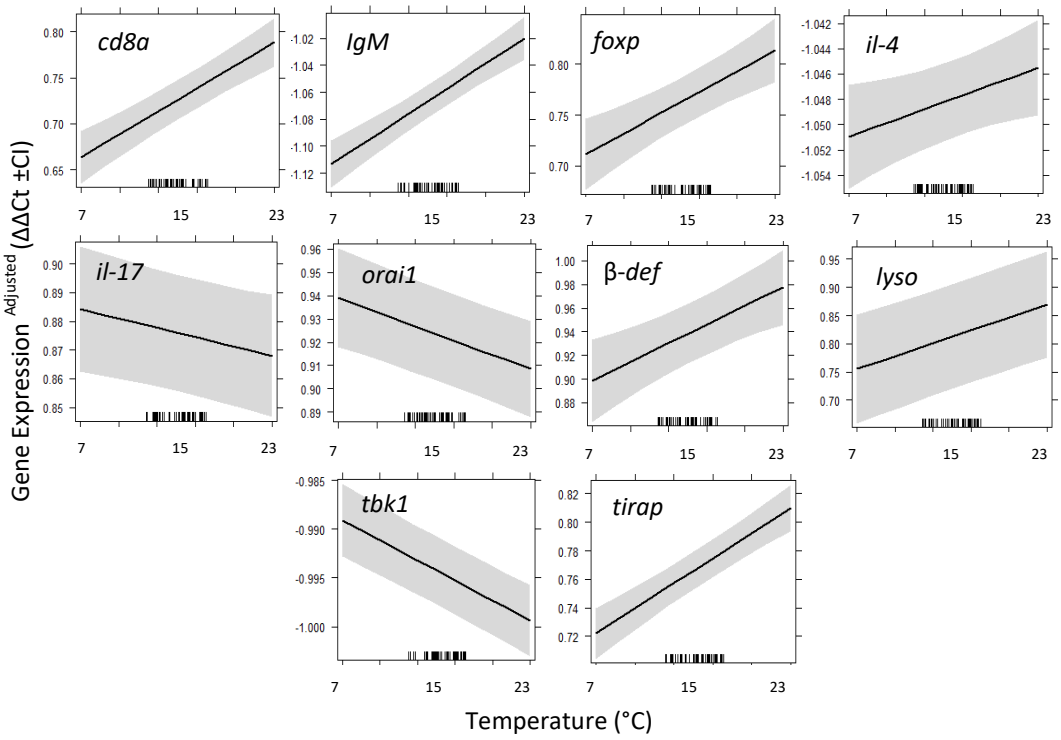

Figure 5.4: The effect of temperature at which sticklebacks (*Gasterosteus aculeatus*) were infected (7, 15, 23°C) with *Saprolegnia parasitica* on gene transcript abundance extracted from gills. Black lines are the relative mean transcript abundance (ΔΔCt) and the shaded areas the 95% confidence intervals from the linear or linear mixed models (see Table 5.1).

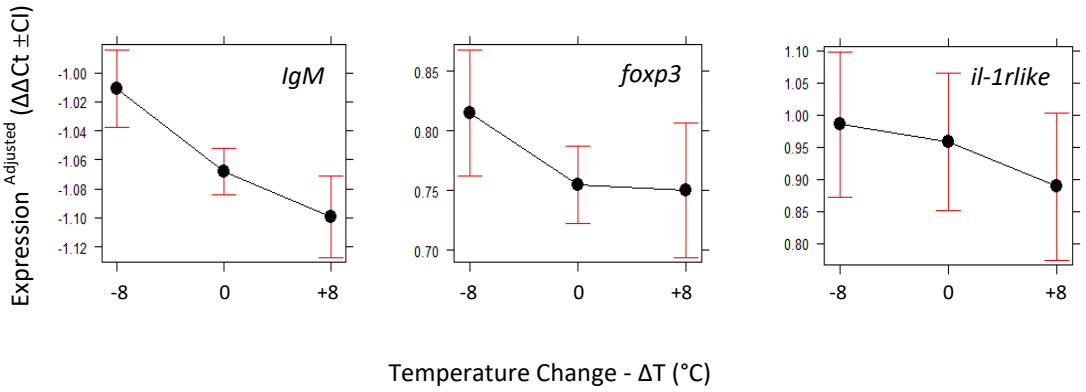

Figure 5.5: The effect of rapid change in temperature (+/-8°C or constant) over 6 hours (ΔT treatment) on stickleback (*Gasterosteus aculeatus*) gene transcript abundance extracted from gills. Black circles are the relative mean transcript abundance (ΔΔCt) and red error bars are the 95% confidence intervals from the linear or linear mixed models (see Table 5.1).

Dependant Variable	Model	Independent Variable	Reference Factor	Compared Factor	t-value	*p*-value	
cd8a	Lm	Intensity			-2.371	0.019	*
		Length			-2.120	0.036	*
		Sex	Male	Female	-4.935	<0.001	***
		InfTemp			6.080	<0.001	***
IgM	Lm	Sex	Male	Female	-4.256	<0.001	***
		InfTemp			8.042	<0.001	***
		ΔT Treat	0	+8	-1.893	0.061	
				-8	3.649	<0.001	***
IgZ	Lm	Body Condition	Male	Female	2.824	0.006	**
foxp3b	Lm	Inf. Treatment	1	2	0.573	0.568	
				3	-0.978	0.330	
				4	-1.938	0.055	·
		Intensity			-3.373	0.001	**
		Sex	Male	Female	-2.663	0.009	**
		InfTemp			4.269	<0.001	***
		ΔT Treat	0	+8	0.167	0.868	
				-8	1.984	0.050	*
il-1rlike	Lm	Sex	Male	Female	-3.151	0.002	**
		InfTemp			1.913	0.058	
		ΔT Treat	0	+8	-2.182	0.031	*
				-8	0.934	0.934	
il-4	Lm	Sex	Male	Female	-3.563	<0.001	***
		InfTemp			2.171	0.32	*
il-12ba	Lm	Sex	Male	Female	8.382	<0.001	***
il-17	lmm†	Sex	Male	Female	-3.662	<0.001	***
		InfTemp			-2.193	0.030	*
β-def	Lm	Body Condition			-4.616	<0.001	***
		Intensity			2.147	0.034	*
		Length			-7.895	<0.001	***
		Sex	Male	Female	-8.450	<0.001	***
		InfTemp			3.393	<0.001	***
lysozyme		Intensity			-3.107	0.002	**
		Length			2.706	0.008	***
		InfTemp			5.237	<0.001	***
tbk1	lmm‡	Body Condition			2.254	0.026	*
		Inf. Treatment	1	2	0.807	0.421	
				3	0.556	0.580	
				4	2.620	0.010	*
		Sex	Male	Female	-6.074	<0.001	***
		InfTemp			-8.377	<0.001	***
tirap	Lm	Intensity			-2.061	0.041	*
		Inf. Treatment	1	2	1.508	0.134	
				3	-1.154	0.250	
				4	0.076	0.939	
		Sex	Male	Female	-4.143	<0.001	***
		InfTemp			8.030	<0.001	***
gpx4a	Lm	Sex	Male	Female	-2.821	0.006	**
orai1	lmm§	InfTemp			-4.473	<0.001	***

Table 5.1: A summary of model for each gene analysed in sticklebacks using RT-qPCR and the ΔΔCt method in an experiment looking at the effects of rapid/no thermal change on *Saprolegnia parasitica* infections and immune gene expression. For factorial variables the reference and comparative factors are provided. $p<0.001$ = ***, $p<0.01$ = **, $p<0.05$ = *, $p<0.1$ = ·. Random factors: †Plate (χ=24.8, p<0.001); ‡ Plate (χ=21.28, p=<0.001) Batch (χ=4.84, p=0.03); § Plate (χ=24.3, p=<0.001).

Discussion

This study revealed a higher likelihood of infection with *Saprolegnia parasitica* in warmer temperatures in contrast to previous studies, which found higher prevalence of *S. parasitica* infection in colder temperatures (Bly & Clem, 1991; Bly *et al.,* 1992). Thermal shock decreased the likelihood of infection when the increase was positive (+8°C), suggesting the direction of temperature change is important when determining infection likelihood and could be an important factor when assessing the effects of climate change. High body condition was the main predictor of increased likelihood and intensity of infection. Host immunity at warmer temperatures was typically enhanced for adaptive genes, however, *il-17* and *tbk1* both had improved expression at lower temperatures.

Infection with *S. parasitica* induces pro-inflammatory markers associated with resistance (see Bly & Clem, 1991; Bruijn *et al.,* 2012). Given that both *il-17* and *tbk1* are markers of the pro-inflammatory response expressed at colder temperatures this may explain the reduced likelihood of infection, as an increased pro-inflammatory response could be providing some measure of resistance. Indeed, certain pro-inflammatory genes, including other innate responses (Lillehaug *et al.,* 1993; Alcorn *et al.,* 2002; Magnadóttir, 2006; Dios *et al.,* 2010), may be upregulated in colder conditions providing resistance when there are a greater number of parasite infection stages present in the colder conditions, including *S. parasitica* (see Powell *et al.,* 1972; Riberio, 1983; Thoen *et al.,* 2016).

S. parasitica infections have, in previous studies predominantly focused on catfish, tended to occur when there are rapid drops in temperature and temperature shock has been cited as a cause of infection (Bly & Clem, 1991; Bly *et al.,* 1992; Szalai *et al.,* 1994; Bruno & Wood, 1999). The current study does not demonstrate higher infection prevalence at colder temperatures but it does suggest that constant or decreasing temperatures are more likely to induce infection than warming conditions. As such temperature induced stress alone may not be sufficient for infection and the direction of that temperature change is also important; warm shock having a very different response to the classical cold shock (Tanck *et al.,* 2000; Donaldson *et al.,* 2008). Rapid temperatures changes had surprisingly little effect on immunity with *IgM* and *foxp3b* having enhanced expression if temperature dropped while *il-1rlike* had marginally reduced expression if temperature rose. This could be the result of sampling interval with fish euthanized 72 h after rapid temperature change the immune system may have

stabilised; the majority of heat stress markers returning to normal within 12-48 h after heat shock in grass carp (*Ctenopharyngodon idellus*) (see Cui *et al.*, 2014). In addition low levels of *cd8a*, *foxp3b* and *lysozyme* transcript in infected fish are the result of immune suppression by *S. parasitica* (see Belmonte *et al.*, 2014).

The higher likelihood of infection in fish with a high body condition is unusual as typically fish with a low body condition are susceptible to infection (Nelson & Demas, 1996; Møller *et al.*, 1998; Bakker & Mundwiler, 1999; Beldomenico & Begon, 2010). It is likely that this relationship exists because of a trade-off between condition and immunity as with tolerance (Jackson *et al.*, 2014), indeed *β-defensin* (an antimicrobial peptide) was found to be supressed in high condition hosts and could be responsible for increased susceptibility in high condition hosts. It is unlikely that this relationship exists because of a fertility/immunity trade-off (see Sheldon & Verhulst, 1996; Lochmiller & Deerenberg, 2000; Norris & Evans, 2000); firstly no fish developed into noticeable breeding condition during the current study and ovaries were not enlarged when dissected; secondly any breeding female would have an intrinsically higher mass for a given length an no such outliers were present, and thirdly the induction of a 16 h L: 8 h D light cycle is not always sufficient to induce breeding (Wootton, 1976). Instead, higher infection intensities as measured by greater parasite mass or transmission stage quality/quantity commonly occur in fish with higher growth rates or that have been on high resource diets; more host resources are therefore available to the parasite for growth (Bean & Winfield, 1991; Ebert *et al.*, 2000; Bedhomme *et al.*, 2004; Barber, 2005; Seppälä *et al.*, 2008; Blanchet *et al.*, 2009). Higher intensities of *S. parasitica* infection with increasing body mass in the current experiment are then the likely result of greater resource abundance available for the parasite to exploit and grow at a faster rate.

Overall, the current study indicates that temperature at time of infection is not sufficient to induce 'wild like' *S. parasitica* infections and that the effect of rapid temperature change is dependent on the direction of change and not on the induction of stress alone. In addition, there was very little effect of historical shock temperature treatments on fish immunology with current infection temperature having the dominate impact on gene expression; this result may be due to sampling 72 h after treatment, which is sufficient time to allow the immune system to stabilise. Higher likelihood of infection in high condition individuals may well be the result of a trade-off between immunity and condition with severe suppression of the antimicrobial *β-defensin* in high condition

hosts being a contributory factor. Lastly, higher intensities of infection in high condition individuals may be explained by greater host resource availability, which the parasite is able to exploit to enhance its own growth rate. Such information is likely to be key to combating infection in aquaculture with production geared towards high mass individuals and when taking into account the effects of temperature change in fish that are not house in thermo-stable conditions.

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Chapter 6 - I hear the ticking of the clock: the cause of circannual immune rhythms in an ectothermic vertebrate

Abstract

Circannual rhythms impact vertebrate immunity, but there is limited knowledge on how this is controlled. In this study, the three-spined stickleback (*Gasterosteus aculeatus*) was exposed to a normal photoperiod cycle or 'accelerated' photoperiod at two temperatures (7 and 15°C). Stickleback gene expression appeared unaffected by photoperiod, suggesting no effect on the circannual rhythms of immune gene expression seen in wild fishes. Instead, temperature was the major cause of changes in immune gene expression with upregulation of winter-biased gene in colder condition and summer-biased gene in warm condition; suggesting temperature is a major cause of circannual variation in ectothermic vertebrates.

Introduction

Circadian and circannual rhythms can affect immune gene expression in vertebrates (Martin *et al.*, 2008a; Curtis *et al.*, 2014; Zhang *et al.*, 2014; Dopico *et al.*, 2015; Paynter *et al.*, 2015; Brown *et al.*, 2016). While immune circadian rhythms are controlled largely as a result of differential expression of clock genes (Curtis *et al.*, 2014; Zhang *et al.*, 2014), comparatively little is known about what causes circannual rhythms, particularly in ectothermic vertebrates. This is perhaps surprising given the relative importance of circadian and circannual rhythms in mammalian medicine (Macmurray *et al.*, 1983; Zhang *et al.*, 2014). In vertebrates, availability of nutrition, melatonin production (linked to photoperiod variation) and temperature are all cited as possible explanations for immune circannual rhythms (Demas & Nelson, 1996; Schurmann & Steffensen, 1997; Martin *et al.*, 2008a; Watts *et al.*, 2008; Dopico *et al.*, 2015; Paynter *et al.*, 2015). In the three-spined stickleback (*Gasterosteus aculeatus*), cyclical immune rhythms have not convincingly been related to seasonal variations of any clock gene other than *timeless* (Brown et al., 2016). Understanding the causes of circannual rhythm could shed light on infections that demonstrate annual cycles in aquaculture systems (Bly *et al.*, 1992; You *et al.*, 2008).

The current study utilises the three-spined stickleback, exposing them to an annual photoperiod, either at a normal rate or at twice the normal rate, at either summer or winter temperatures in an attempt to disentangle the effect of these two confounding

variables on circannual immunity. If photoperiod were the main driver, a 'normal' seasonal immune pattern is predicted in fish exposed to the natural photoperiod and an immune pattern occurring at twice the rate in the accelerated photoperiod fish. Conversely, if temperature were the main driver it is expected that immune gene expression would be stable in either temperature but biased in favour of particular genes depending on whether they are summer or winter biased.

Materials and Methods

Sticklebacks were collected form Roath Brook, Cardiff (ST 18897 78541) on the 6/01/15. Fish were transported to Cardiff University aquarium and kept in 75 L tanks at a density of <1 fish/L in ambient temperature and lighting conditions prior to the experiment; ranging from 8 h to 9 h 45 min of daylight per day. Fish were treated with 0.004% formaldehyde solution for two 30 min periods, separated by a 30 min rest period in freshwater. Fish were then kept in 0.5% salt solution and screened for ectoparasites at least three times by briefly anesthetizing them in 0.02% MS222 and visually checking for ectoparasites under a dissecting microscope. Any ectoparasites found were removed using watchmaker's forceps following the procedure of Schelkle *et al.* (2009). All animal work was approved by the Aberystwyth and Cardiff University Ethics committees and conducted under Home Office Licence PPL 302357.

A baseline sample of four sticklebacks was taken one month after treatment (11/02/15). Fish were then separated into four treatment groups; normal 'natural' photoperiod (7 or 15°C) and accelerated photoperiod (7 or 15°C); fish were kept in 8, 30 L tanks containing water at 0.5% salinity, each with 25 fish with two tank replicates per treatment. Thermostatically controlled rooms were used to maintain temperature at 7 and 15°C (±0.5°C). A stickleback was then sampled from each of these treatment groups once per week for 30 weeks (n=30 per treatment). Lighting was provided by fluorescent full spectrum bulbs (6500K) and controlled by an electric timer (±2.5 min). Light levels were >10,000 Lux during daylight periods and <10 Lux at night turned on/off at sunrise/set; the light change was immediate with no dawn or dusk. In the natural photoperiod treatment, lights were consistent with Cardiff's normal daily light cycle (Fig. 6.1). The accelerated photoperiod treatment followed the natural photoperiod but at twice the normal rate allowing a yearly cycle to be completed within 6 months; this was calculated by skipping alternate days, thus a year's photoperiod cycle would be completed within 6 months (Fig. 6.1). Each week after 11/02/15 on the same day between 1200-1300, four fish were selected randomly from each treatment and

euthanized. All fish were euthanized by concussion and pithing, standard length and weight measured, body cavity opened, and sex and gravidity or colouration noted. Samples were then submerged in RNAlater, kept at 4°C for 24 h and stored at -80°C. Sticklebacks were fed daily on bloodworm until satiety between 1200-1230 based on the electric photoperiod timers; those fish taken for euthanasia were not fed.

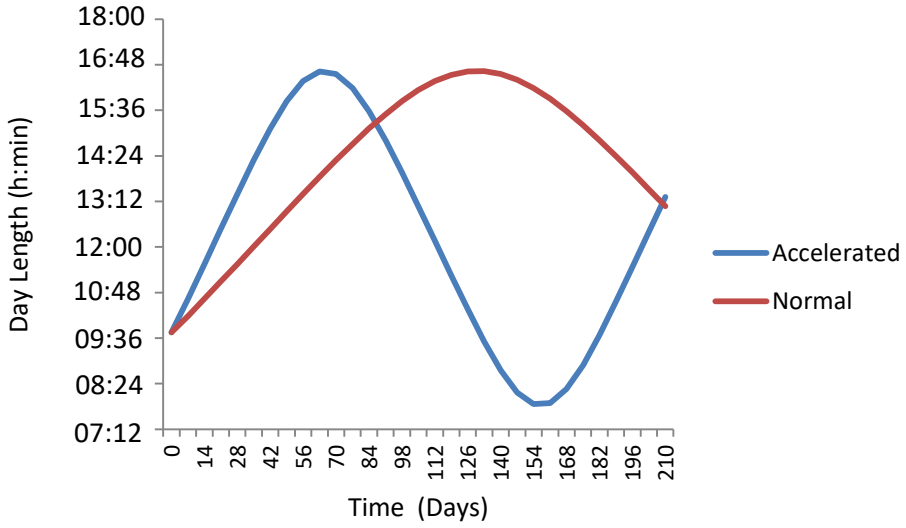

Figure 6.1: Three-spined sticklebacks (*Gasterosteus aculeatus*) were exposed to two photoperiod treatments: normal and accelerated, in which day length varied over the course of the experiment.

RNA extractions were conducted on fish gills using Bioline ISOLATE II RNA mini kits as per manufacturer's instructions. A nanodrop was used to check quality and quantity of RNA using 260/280 and 260/230 ratios; RNA was stored at -80°C. RNA to cDNA conversion was conducted using the Applied Biosystems High Capacity cDNA Reverse Transcription Kits according to instructions. qPCR assays were prepared using Bioline's SensiFAST SYBR LoROX master mixes according to instructions in a QuantStudio 12K machine using 384 well MicroAMP optical plates in a randomized cohort fashion; primers used are provided in Appendix 2 (see also Brown *et al.*, 2016). A selection of 14 genes were analysed including one housekeeping genes, four innate genes and nine adaptive genes comprising most T-cell phenotypes (see Bradley & Jackson, 2008). The cycling protocol of 95°C for 2 min; 40 cycles of 95°C for 5 sec, 62°C for 15 sec; melt curve from 60°C to 95°C in 0.1°C increments was used. Relative Quantification values were calculated against and control sample.

All data were analysed using R v3.2.2 (R Core Team, 2015) with additional use of the 'lmtest' (Zeileis & Hothorn, 2002) and 'mgcv' (Wood, 2004, 2011) packages. A

photoperiod effect was first looked for using cosine models to determine if the data had a temporal pattern that might be caused by photoperiod. These models were also used to assess the effect of temperature on immune gene expression with summer-biased genes expected to be upregulated at 15°C compared to 7°C and *vice versa* for winter-biased genes. Visual analysis was then also conducted using General Additive Models to determine whether any temporal effect followed seasonal patterns as observed in Brown *et al.* (2016); the predicted trends for gene expression of summer (Fig. 6.2A) and winter biased genes (Fig 6.2B) over the course of the experiment. Splines were divided into the accelerated and normal photoperiod groups with the two splines also expected to diverge in order to follow the seasonal pattern if photoperiod was causing a change in gene expression (Fig. 6.2); all genes were inspected in this manner.

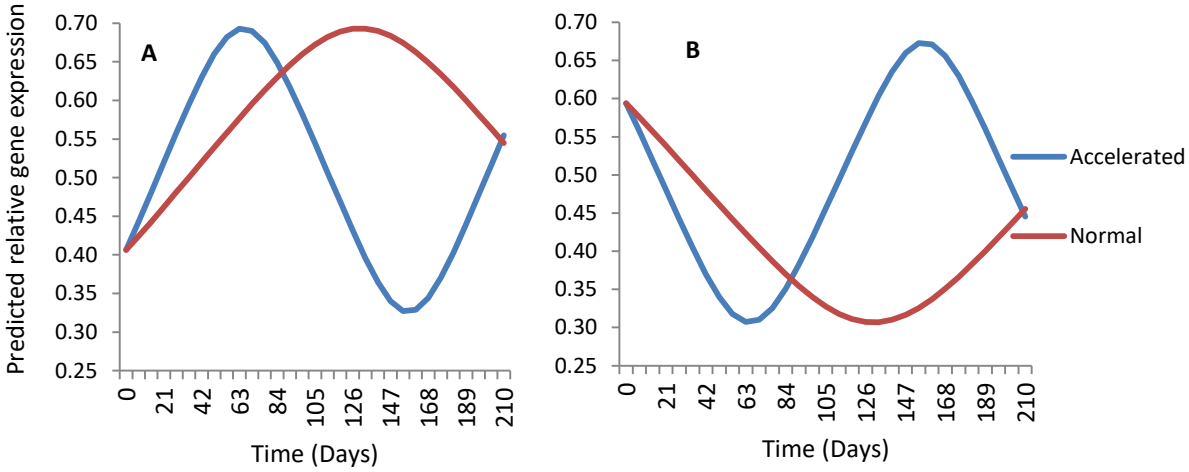

Figure 6.2: The predicted changes in genes expression for summer-biased (A) and winter-biased (B) stickleback (*Gasterosteus aculeatus*) genes over the course of the experiment separated by photoperiod treatment group. Normal photoperiod tracks the natural photoperiod in Cardiff, the accelerated photoperiod occurs at twice the rate of the natural photoperiod.

'Minimal models' of factors affecting individual expression of genes were produced using linear models with model selection conducted using Akaike Information Criterion (AIC); Box-Cox transformations were used to obtain a normal distribution. Independent variables included temperature treatment, photoperiod treatment, fish sex, body condition (residuals from a quadratic regression of fish mass and standard length), length and breeding condition. Sine and cosine models were produced to assess curvature of the data in order to look for seasonal effects that would be sinusoidal in nature. To achieve this, the independent variables 'cos((2*pi*time)/365))' and 'sin((2*pi*time)/365))' were both added to the minimal model; 'time' being the number of days since the baseline sample (11/02/14). A second sinusoidal model was then

produced using a second time variable 'time2', which was calculated as the time from the baseline (11/02/15) until sampling on the given date for the 'normal photoperiod' fish and twice that time for the 'accelerated fish' in days; thus, a fish sampled on the 2nd week in the normal photoperiod would then have a value of 7 while an accelerated fish would have a value of 14. The time2 function was added to the minimal model as independent variables 'cos((2*pi*time2)/365))' and 'sin((2*pi*time2)/365))'. Both time and time2 sinusoidal models were compared using AIC to test whether gene expression tracked photoperiod in real time (time) or with a time lag (time2). The sinusoidal models were compared to the minimal model using a likelihood ratio test to assess whether the sinusoidal model captured the variability in the data better. A significant likelihood ratio test meaning that the relationship is not merely linear; instead a curve in the model is required to assess the data, suggesting an annual cycle. General Additive Models (GAMs) (Wood, 2004, 2011) were also used to assess the data visually with splines set for time by the photoperiod treatment. Independent variables included: temperature treatment, fish sex, body condition (calculated as residuals from a quadratic regression of fish mass on standard length), length and breeding condition. Visual interpretations were based on predicted outcomes from Brown *et al.* (2016).

Results

Of the 14 gene transcripts analysed, six had sinusoidal models that described the data better than the minimal model: *foxp3b* (time: χ^2=4.239, p=0.120; time2: χ^2=6.008, p=0.049), *il-1rlike* (time: χ^2=7.613, p=0.022; time2: χ^2=7.674, p=0.022), *β-def* (time: χ^2=2.287, p=0.319; time2: χ^2=6.572, p=0.0374), *tbk1* (time: χ^2=12.246, p=0.002; time2: χ^2=6.214, p=0.045), *tirap* (time: χ^2=9.616, p=0.008; time2: χ^2=8.109, p=0.017), and *orai1* (time: χ^2=13.897, p<0.001; time2: χ^2=18.304, p<0.001). Of these six genes, *β-def*, *tbk1* and *tirap* had time models that tracked real time better, while the time lag model fitted the *foxp3b* data better; this indicates that these six genes have a temporal trend. Visual and statistical analysis of genes that had an indicated temporal pattern based on sinusoidal models revealed high levels of overlap between the accelerated and normal photoperiods (p>0.05 on all smooth terms), indicating that photoperiod had no effect on gene expression (Fig. 6.2), if photoperiod did have an effect the splines would have diverged to follow the genes seasonal pattern. In addition, the expression of these genes does not follow the seasonal patterns as described by Brown *et al.* (2016) (winter-biased genes: *il-1rlike, tbk1, orai1*) (summer-biased genes: *cd8a, foxp3, tirap*). The only gene that follows the wild seasonal expression pattern (Brown *et al.*, 2016) is *il-1rlike* in the accelerated photoperiod group, however, given that expression in the normal

photoperiod group does not display any temporal effect, this result is unlikely the result of photoperiod. All other genes were also visually inspected but no temporal tends observed. At 15°C the expression of *cd8a* (t=2.718, *p*=0.008), *IgM* (t=3.676, *p*<0.001), *foxp3b* (t=2.675, *p*=0.009), *il-1rlike* (t=3.773, *p*<0.001), *β-def* (t=2.515, *p*=0.013), *tirap* (t=2.718, *p*=0.008) and *gpx4a* (t=4.564, *p*<0.001) increased compared to 7°C. In contrast, *tbk1* (t=-3.819, *p*<0.001) and *orai1* (t=-2.300, *p*=0.023) expression were supressed at 15°C. These temperature results demonstrate a much greater effect of temperature on genes expression than photoperiod. Given the higher expression of summer biased genes *cd8a*, *foxp3* and *triap* in warmer condition and winter biased genes *tbk1* and *orai1* in colder conditions temperature is probably a component in causing circannual gene expression.

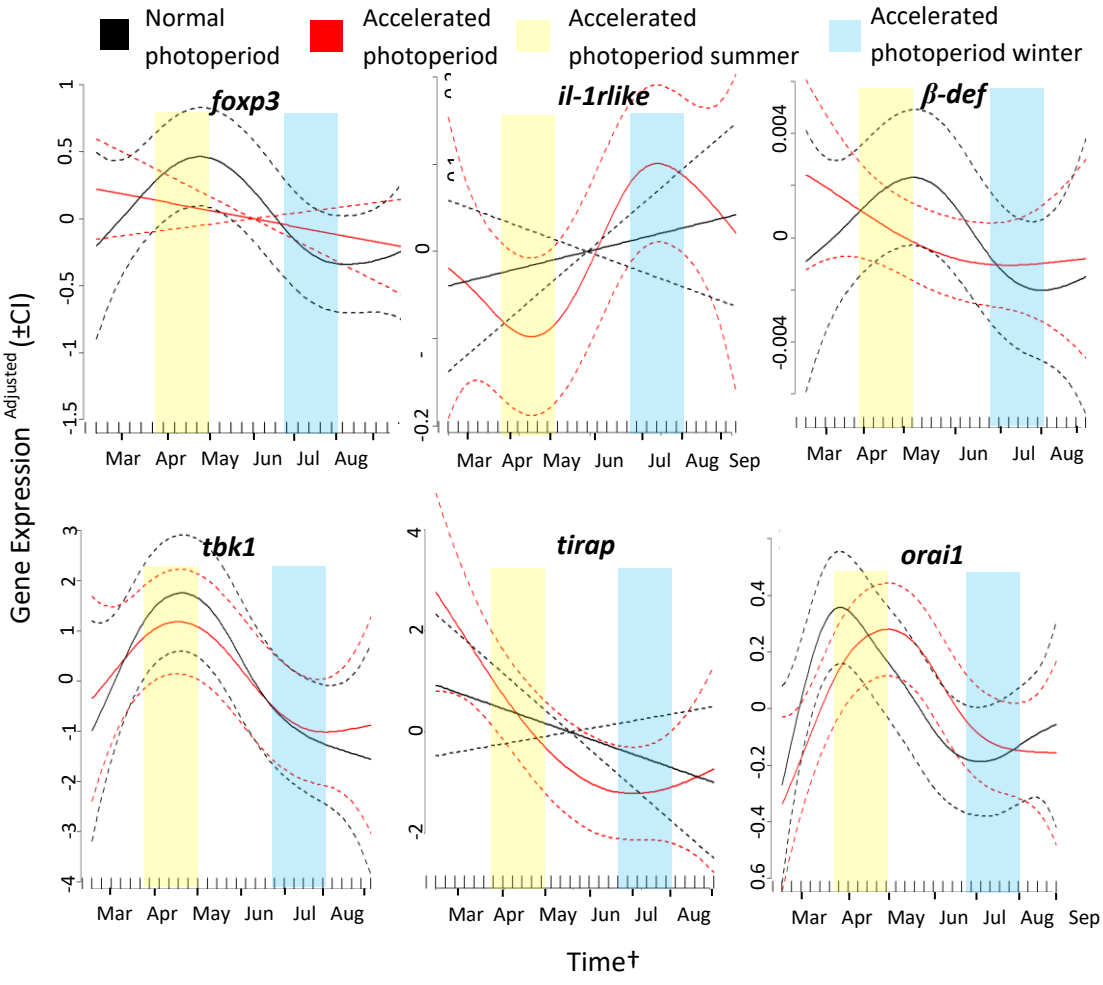

Figure 6.3: Stickleback (*Gasterosteus aculeatus*) gene expression over time separated by normal photoperiod (black) and accelerated photoperiod (red). Solid lines are adjusted means from a GAM model with dotted lines representing 95% confidence intervals. Yellow and blue bars represent the respective summer and winter months for the accelerated fish based on actual photoperiod experienced. †Time is calculated as the number of days from the first fish being sampled (18/02/15) until the date at which the fish is euthanized.

Discussion

The current study highlights temperature, rather than photoperiod, as a better predictor of circannual gene expression in sticklebacks (*Gasterosteus aculeatus*). Expression of winter-biased gene *tbk1*, *orai1* were upregulated in colder temperatures (7°C) and summer-biased genes *cd8a*, *foxp3*, *tirap* were upregulated in warmer temperatures (15°C), consistent with wild expression patterns (Brown *et al.*, 2016). Unusually, *il-1r*like (winter biased) was upregulated at warmer temperatures, likely the result of better adaptive immunity in ectotherms at this temperature (Miller & Clem, 1984; Bly & Clem, 1992; Lillehaug *et al.*, 1993; Alcorn *et al.*, 2002), suggesting another variable that could be causing circannual variation in this gene. While *foxp3*, *il-1rlike*, *β-def*, *tbk1*, *tirap* and *orai* all had sinusoidal models that suggested some level of temporal variation it was not consistent with the expectations of a gene affected by photoperiod. Firstly, the trends in gene expression were not consistent with seasonal circannual variation in wild fish (see Brown *et al.*, 2016). Secondly, given two photoperiods, one progressing at twice the rate of the other, gene expressions would be expected to diverge (see Fig. 6.2) but this was not the case.

Temperature has previously been linked to circannual and daily variation in vertebrate gene expression (Demas & Nelson, 1996; Podrabsky & Somero, 2004).The effect of temperature on food availability, as a result of altered growth rates, has also cited as a reason circannual variation (Martin *et al.*, 2008b). In ectothermic vertebrates the effect of temperature is pronounced, given their reliance on environmental temperature for growth, metabolism and immunity (Schurmann & Steffensen, 1997; Gillooly *et al.*, 2001; Handeland *et al.*, 2008; Watts *et al.*, 2008; Uribe *et al.*, 2011). This did appear to be the case as the temperature treatment strongly resembled predictions based on wild circannual immune rhythms and other experiments (see Brown *et al.*, 2016; Chapters 4 and 5). The absences of a photoperiod effect on gene expression could be the result of an internal clock that is difficult to alter. It has also been demonstrated that removal of environmental factors, including photoperiod, are not always sufficient to break the internal clock (Gwinner, 1996, 2003). This however is unlikely to be the case, given that *tbk1* and *orai1* had gene expression profiles (higher in April/May) which were the reverse of what would be expected if the clock had continued as under natural conditions (increasing going into winter) while other genes demonstrated no circannual rhythm thereby ruling out endogenous oscillators. The current study also did not simulate dawn and dusk, while the genetic basis of circannual rhythms is unknown, expression of *Per* and *Cry* genes at dawn and dusk, respectively, could contribute to the

circannual clock (Hazlerigg & Loudon 2008; Paul *et al.*, 2008; Visser *et al.*, 2010). Given the speculative nature of the relationship between these genes and the causes of circannual rhythms, it is difficult to say how a lack of dawn and dusk might affect circannual rhythms, if at all.

Knowledge of circadian rhythms in mammalian systems has underlined the importance of diurnal variation in cardiovascular and metabolic diseases, and the timely administration of drugs for improved efficacy (Curtis & Fitzgerald, 2006; Levi & Schibler, 2007). Similarly, circannual variation in immunity may well be having a large impact on immune related disorders such as allergic rhinitis (Reinberg *et al.*,1988) or rheumatoid arthritis (Cutolo, 2011). In aquaculture, infectious disease is often circannual in nature with species such as *Gyrodactylus* spp. (see Chappell, 1969; Mo, 1997; Winger *et al.*, 2007; You *et al.*, 2008) and *Saprolegnia parasitica* (see Bly *et al.*, 1992) being subject to seasonal fluctuations. It is therefore important that we begin to understand the mechanisms behind seasonal patterns of infection, including circannual immune variation, in order to combat emergent diseases better. This study suggests that temperature is a major driver of circannual variation in ectothermic vertebrates, while alterations in photoperiod do not match wild rhythms, indicating it is not a predictor of circannual variation.

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Chapter 7 - Love thy neighbour: co-infection of *Argulus foliaceus* and *Gyrodactylus gasterostei* on the three-spined stickleback (*Gasterosteus aculeatus*)

Abstract

While many experimental studies focus on the consequences of infection with a single parasite, in wild populations co-infection is the norm. Co-infection has consequences for both the host and the parasites that may be competing for resources or immunosuppressing the host and therefore enhancing the survival of one or both parasite species. This study utilised an as yet unexplored co-infection between two ectoparasites, the generalist crustacean *Argulus foliaceus* and the specialist monogenean *Gyrodactylus gasterostei*, on the three-spined stickleback (*Gasterosteus aculeatus*). Recording of both parasites on localised regions on the stickleback allowed assessment of trends potentially associated with immunomodulation, localised immune response or competition. Co-infection of *A. foliaceus* with *G. gasterostei* caused higher infection intensities of the latter, probably due to immunomodulation. Surprisingly, there was no evidence of localised inter-specific competition. As with previous studies, the *G. gasterostei* population was unevenly distributed on the host, showing preferences for the head and pectoral and caudal fins. In contrast, *A. foliaceus* avoided the fins, occurring most frequently on the body and head. This study highlights the consequences of co-infection for wild and aquacultural diseases where co-infection of generalist immunomodulating ectoparasites may alter infection dynamics of other parasites potentially leading to greater host morbidity and mortality.

Introduction

In the wild it is rare to find a host infected by a single parasite species; instead co-infection is the norm (Esch *et al.,* 1990). As with other ecological niches if two parasites co-occur on a single host, perhaps even isolated to a single tissue or organ, inter or intra-specific competition can occur. To avoid such competition, in rare cases macroparasites may avoid already infected hosts (Schmid-Hempel, 2011). Alternatively, parasites may prevent establishment of competitors, such as *Railietina cesticillus* inhibiting *Hymenolepis diminuta* infection (Gordon & Whitfield, 1985). When co-infection does occur there are often substantial effects on both host and parasite (see

Bentwich *et al.,* 1999; Correa-Oliveira *et al.,* 2002; Fleming *et al.,* 2006; Ezenwa *et al.,* 2010; Telfer *et al.,* 2010).

Parasites have the remarkable ability to manipulate host behaviour and immunity. A classic example of behavioural manipulation is found in three-spined sticklebacks (*Gasterosteus aculeatus*) infected with *Schistocephalus solidus*. This cestode alters the host's foraging, feeding, swimming and anti-predator responses; ultimately making the stickleback easier prey for the definitive host, piscivorous birds (Giles, 1983; Tierney *et al.,* 1993; Barber & Huntingford, 1995). Such manipulative abilities may also benefit co-infecting parasites, such as *Diplostomum spathaceum* or *D. gasterostei*, sharing the same intermediate and definitive host as *S. solidus*; thereby 'hitch-hiking' and saving the costs of manipulation while benefiting from the increased likelihood of transmission (Thomas *et al.,* 1998, 2005). Indeed, some manipulation may be co-beneficial, *D. spathaceum* also alters the foraging behaviour of the stickleback potentially enhancing transmission of co-infecting *S. solidus* (see Crowden & Broom, 1980). Parasites may also manipulate the host's immune response, often to benefit their own establishment, growth, survival and fecundity. *Heligomosomoides polygyrus bakeri*, for example, can supress T_H2 immunity to facilitate its own survival (Wahid *et al.,* 1994; Maizels *et al.,* 2012). Immunomodulation also has consequences for co-infecting parasites, including *Plasmodium chabaudi,* which benefits from increased parasitemia as a result of *H. p. bakeri* T_H2 suppression (Su *et al.,* 2005). Conversely, other parasites may be suppressed as a result of interspecific immunomodulation and benefit from the termination of co-infection (Lello *et al.,* 2004).

The most common studies of teleost co-infections have typically focused on ectoparasites including *Gyrodactylus* spp. and *Argulus* spp. co-infecting with bacterial or viral disease; where co-infection increases host mortality or enhances the pathogens' prevalence, partially the result of ectoparasites acting as vectors (see Ahne, 1985; Cusack & Cone, 1985; Busch *et al.,* 2003; Xu *et al.,* 2007; Shoemaker *et al.,* 2008). Fewer studies have assessed the relationship between eukaryotic parasites, but competitive exclusion from the host or host niches is known to occur amongst co-infecting species of gyrodactylid (Paperna, 1964; Harris, 1982). Also, immunisation of fish by exposure to *Gyrodactylus derjavini* resulted in reduced infection with the protozoan parasite *Ichthyophthirius multifiliis* (see Buchmann *et al.,* 1999). Experimental infection of sticklebacks with *Schistocephalus solidus*, which were then placed in the field, altered the co-infecting community by enhancing or diminishing

infection prevalence of different species (Benesh & Kalbe, 2016). Field studies of sticklebacks have also revealed competition between the two endoparasites *Proteocephalus filicolis* and *Neoechinorhynchus rutili* (see Chappell, 1969). To the best of my knowledge this study is the first to have co-infected fish with two different genera of ectoparasites that may be interacting via direct competition for resources, or indirectly via the host's immune system.

Argulus foliaceus is a generalist ectoparasitic crustacean of teleosts. Infection with this parasite has a broad range of pathological effects on fish including changes in feeding, lethargy, anaemia and epidermal necrosis (Taylor *et al.,* 2006; Tokşen, 2006; Noaman *et al.,* 2010). Many of these effects are linked to the feeding mechanism of argulids; puncture of the epidermis by means of a stylus, injection of cytolytic toxins and blood feeding (Hoffman, 1977; Walker *et al.,* 2011). Some *Argulus* species have been described as immunomodulators (Ruane *et al.,* 1999; Saurabh *et al.,* 2010; Kar *et al.,* 2013), however, no study has yet described the effect of this immune modulation on co-infection. To better understand the effects of *A. foliaceus* co-infection the current study assessed its impact on another ectoparasite, *Gyrodactylus gasterostei*. *Gyrodactylus* spp. infra-population growth is rapid due their progenetic and viviparous life-cycle and this growth can be tracked over time non-invasively (Scott & Anderson, 1984; Bakke *et al.,* 1990; Bakke *et al.,* 1991; Bakke *et al.,* 2007). Counting *G. gasterostei* on specific host body regions therefore provides the possibility of studying localised inter-specific competition on the host. Both *A. foliaceus* and *G. gasterostei* are in direct competition for host resources, particularly space, and will both be affected by any immune responses directed at the epidermis.

By studying *G. gasterostei* population growth on the stickleback it is possible to assess whether increases or decreases in the total population are the result of *A. foliaceus* driven immune suppression or competition. In this instance a higher *G. gasterostei* population would suggest immune suppression aids population growth while a localised decrease in population could be the result of inter-specific competition.

Materials and Methods

Three-spined sticklebacks (*Gasterosteus aculeatus*) were captured from Roath Brook Cardiff (ST 18897 78541), on the 07/10/2014 and transported to the aquarium facility at Cardiff University; mean standard length = 33 mm (range = 23.1 to 48.3 mm), mean mass = 0.570 g (range = 0.111 to 1.863 g). Fish were maintained in 30 L tanks at 15°C

at a density of <1 fish/litre on an 18 h light: 6 h dark cycle and fed daily on frozen bloodworm. Sticklebacks were treated for ectoparasites by submersion in 0.004% formaldehyde solution for 1 h with a halfway rest period of 30 min in freshwater. Fish were then maintained in 1% aquarium salt water and 0.002 g/L methylene blue for 48 h to prevent secondary infection. Treated fish were 'screened' visually three times for remaining ectoparasites by anaesthetising them in 0.02% MS222 and checking for infections under a dissection microscope with fibre optic illumination; any remaining parasites were removed with watchmaker's forceps following methods of Schelkle *et al.* (2009). Fish were then kept in dechlorinated water for 4 weeks to recover from treatment. All animal work was approved by the Cardiff University Ethics committees and conducted under Home Office Licence PPL 302357.

An isogenic line of *Gyrodactylus gasterostei* was used for infections. Founding parasites were identified by morphometrics following Shinn *et al.* (2010) and Harris (1982), and sequencing of rDNA internal transcribed spacers I and II (GenBank AJ001841.1; Cable *et al.*, 1999). The culture of *Argulus foliaceus* was originally obtained from a common carp (*Cyprinus carpio*) at still water fishery in North Lincolnshire, July 2014. For methods on generation of isogenic *G. gasterostei* and *A. foliaceus* lines see Chapter 2. During the experimental procedure sticklebacks were isolated in dechlorinated water 1 L pots, light cycle and temperature remained unchanged. Infections were conducted on individually anesthetised fish, using 0.02% MS222, by manipulating two individuals of *G. gasterostei* onto the caudal fin of a recipient fish by overlapping with the caudal fin of a donor fish, under a dissection microscope with fiber optic illumination. Fish were checked for infection 24 h later and any fish not infected with two worms were re-infected and checked again for infection 24 h later; any fish without two infecting gyrodactylids at this point were removed from the experiment. Subsets of fish were subsequently infected with the laboratory line of *A. foliaceus*, after the second *G. gasterostei* population count Day 3 post-infection, allowing the fish to be infected under anesthetic. Fish were exposed to either one (n=19) or three (n=8) individuals of *A. foliaceus* or sham infected (n=34). A control group of uninfected fish (n=10) was also kept as a mortality control, giving four treatments in total. Fish were screened for infecting *G. gasterostei* twice a week up to 25 days post infection. Location on the host (see Fig. 7.1) and number of *A. foliaceus* and *G. gasterostei* were recorded every 4 days. Twice daily checks were carried out for *A. foliaceus* detachment; any detached *A. foliaceus* were restricted in 100 ml of water with

the fish allowing it to reattach without the use of anesthetic and with minimal stress. A reciprocal treatment in which fish were first infected with *A. foliaceus* and then *G. gasterostei* was considered but not conducted because the procedure involving experimental infection of fish with *G. gasterostei* causes the *A. foliaceus* to detach from the host.

All data were analysed in R v3.2.2 (R Core Team, 2015) with the additional use of 'car' (Fox & Weisberg, 2011), 'mgcv' (Wood, 2004, 2011), 'survival' (Therneau & Grambsch, 2000), 'GAMLSS' (Rigby & Stasinopoulos, 2005) and 'MASS' (Venables & Ripley, 2002) packages. Any fish that cleared their infection, after the day of clearance, was removed from all models in order to avoid zero inflation. In order to look for variation in death, or clearance, between treatments a survival likelihood analysis (Therneau & Grambsch, 2000) was conducted using the number of days post infection or the number of days till clearance of infection as endpoints; co-infection treatment, fish sex, body condition (the residuals from a quadratic regression of fish length on mass) and length were used as independent variables. The infection totals of *G. gasterostei* population over time on individual fish were analyzed using a General Additive Mixed Model (GAMM) with negative binomial errors (Wood, 2004, 2011), with fish identification included as the random term using splines fitted as Days-Post Infection by the co-infection treatment. Model independent variables included host body condition, length and sex. *G. gasterostei* population-level parameters were assessed using negative binomial generalized linear models with co-infection treatment, fish body condition, length and sex as independent variables. Population-level parameters included Area Under the Curve (AUC - the total number of *G. gasterostei* over time calculated using the Trapezoid rule), the Peak of Infection (highest number of *G. gasterostei* to occur on the host) and Time until Peak of Infection (the number of days post-infection; up to a maximum of 25 days when the experiment ended). The relationship between presence/absence of *A. foliaceus* and the number of *G. gasterostei* on given body regions (Fig. 7.1) was modeled using a GAMLSS type I Negative Binomial (Rigby & Stasinopoulos, 2005) with *G. gasterostei* count on a single body region as the dependent variable, a mixed model was used with fish identification as the random variable. An interaction between the numbered body regions (Fig. 7.1) and the presence/absence of *A. foliaceus* was used to determine where the argulids were on the fish's body. Additional independent variables included the presence/absence of *A. foliaceus*, body region (thereby determining where the *G. gasterostei* where on the

fish's body; Fig. 7.1), fish body condition, sex and length. A chi-squared model was also used to determine the preference for *A. foliaceus* on different body regions using presence/absence and body region (Fig. 7.1) from all time points as observations with an expected likelihood ratio of 1 for each body region.

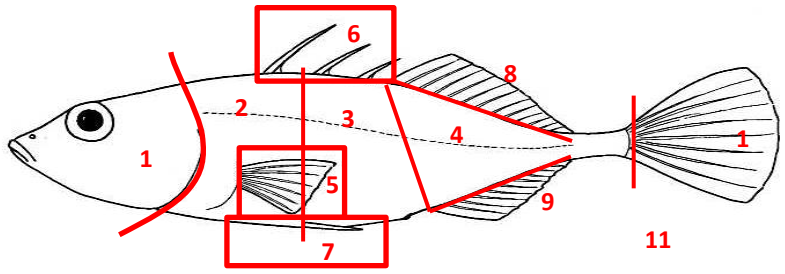

Figure 7.1: The individual body areas of a three-spined stickleback (*Gasterosteus aculeatus*) on which individuals of *Argulus foliaceus* were located: 'head' (1) from the gill operculum anteriorly, the 'anterior body' (2) and 'posterior body' (3) from operculum to tail divided at the pint of the pelvic spine, the 'tail' (4) from the anterior section of the 'dorsal fin' (8) and 'anal fin' (9) to the posterior end of the caudal peduncle, 'pelvic fin' (5), 'spines' (6), 'under belly' (7), 'caudal fin' (10) and free swimming off the host (11). Image outline courtesy of Mackean and Mackean (2004).

Results

In sticklebacks (*Gasterosteus aculeatus*) infected with *Gyrodactylus gasterostei* alone at 15°C, prevalence decreased from the first week of infection with the peak of infection (mean intensity of 26, range 2-175, worms) typically occurring between 20-40 days post-infection (Raeymaekers *et al.*2011; Chapters 3 and 4). Sticklebacks infected with 3 individuals of *Argulus foliaceus* reached a higher peak *G. gasterostei* infection (t=3.531, $p<0.001$; Fig. 7.2A), and higher total infection over time (t=5.414, $p=0.003$; Fig. 7.2B) and time to peak infection was delayed (t=2.874, $p=0.006$; Fig. 7.2C) compared to both other infection treatments.

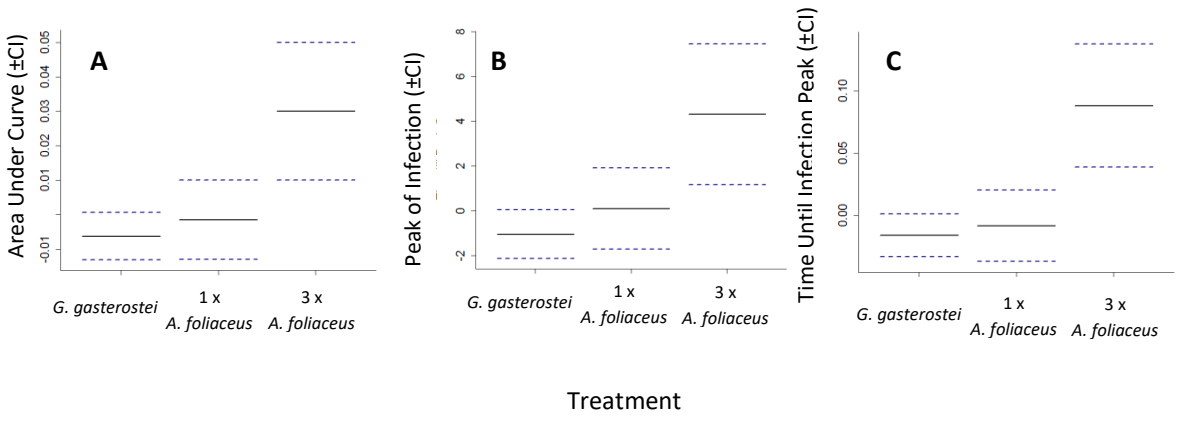

Figure 7.2: *Gyrodactylus gasterostei* population dynamics by co-infection treatment on sticklebacks (*Gasterosteus aculeatus*). Solid lines are means, dotted 95% confidence intervals from generalized linear model.

While co-infection with a single *A. foliaceus* did not significantly increase *G. gasterostei* in individual models assessing intensity, there was a clear trend towards higher and later infection peaks compared to *G. gasterostei* only infected fish in generalized additive mixed model (Fig. 7.3). As such, exclusively *G. gasterostei* infected fish were the only animals that reduced their infection intensity from Day 15 onwards; suggesting immune suppression in both co-infected treatments, one or three argulids (Fig. 7.3).

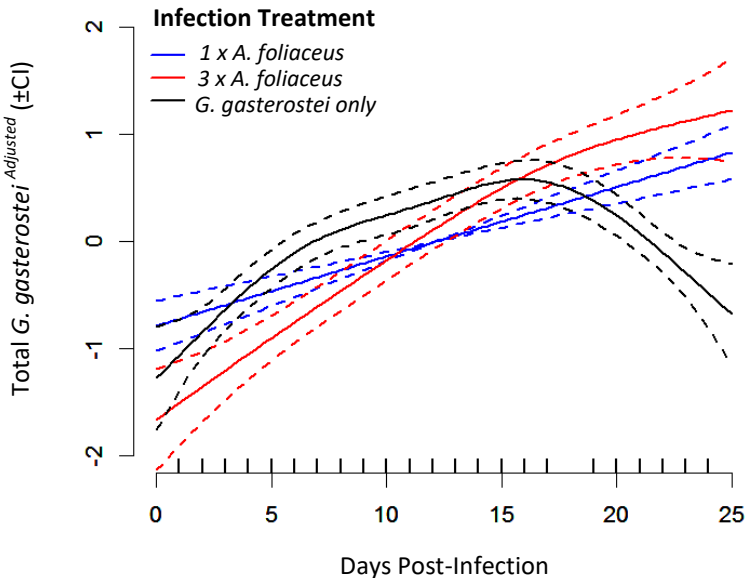

Figure 7.3: The *Gyrodactylus gasterostei* population trajectories on sticklebacks (*Gasterosteus aculeatus*) split by co-infection treatment as calculated in a GAMM. Solid lines are the adjusted mean and dotted the 95% confidence intervals.

A preference for the head, pectoral and caudal fins of the host was demonstrated by *G. gasterostei* (Fig. 7.4A), while *A. foliaceus* showed a preference for the body over fins (χ^2=210.86, df=10, p<0.001) (Fig. 7.4B). The presence/absence of *A. foliaceus* on particular regions of the host only affected *G. gasterostei* population totals on the head; here infections were higher in the presence of an argulid (t=2.675, p=0.008). Among a total of 61 fish there was no significant difference in stickleback survival or *G. gasterostei* clearance (p>0.05) between treatment groups using a survival likelihood analysis. Unusually there was no indication that host length had any effect on *G. gasterostei* infection intensity (p>0.05).

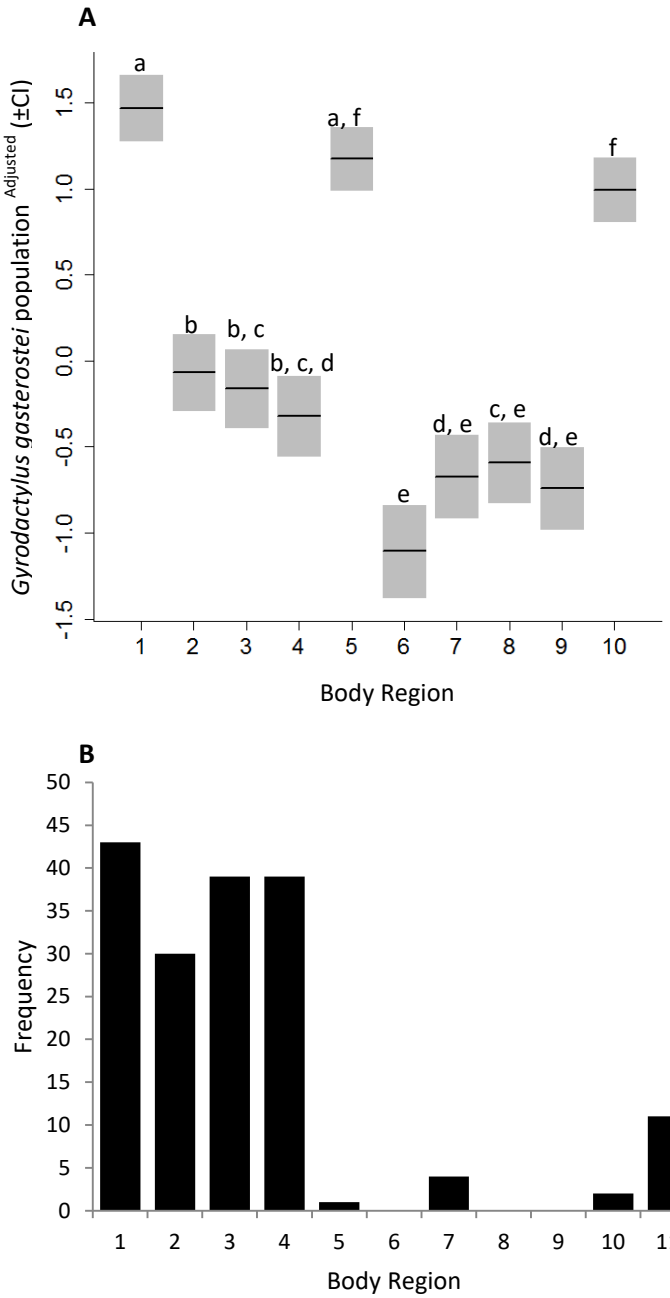

Figure 7.4: Distribution of parasites on the three-spined stickleback body.

(A) The number of *Gyrodactylus gasterostei* on individual three-spined stickleback (*Gasterosteus aculeatus*) body regions; derived from a negative binomial GAMLSS and adjusted for presence absence of *Argulus foliaceus*. Solid black lines are means, grey shaded areas are 95% confidence intervals. Where two letters are the same there is no significance between regions; Bonferroni corrected.

(B) The frequency at which *Argulus foliaceus* was on different body regions of three-spined stickleback (*Gasterosteus aculeatus*) over the course of the experiment being recorded at each *Gyrodactylus gasterostei* population check.

(C) Regions of the three-spined stickleback body: (1) head, (2) anterior body, (3) posterior body, (4) tail, (5) pectoral fins, (6) spined, (7) under belly, (8) dorsal fin, (9) anal fin, (10) caudal fin, and (11) detached from the host.

Discussion

Among wild infections there is often great diversity in the response to co-infection with species-specific interactions resulting in increases and decreases in parasite infection metrics (Lello *et al.*, 2004). This study focused on *Argulus foliaceus* and *Gyrodactylus gasterostei;* finding that co-infection increased the intensity of *G. gasterostei* likely the result of immunomodulation by *A. foliaceus*.

The majority of studies on teleost co-infection have focused on bacterial, fungal or viral pathogens infecting with one other genus of parasites, which have tended to show increased prevalence of the pathogen and higher fish mortality. For example, co-

infections of *Ichthyophthirius multifiliis* and *Edwardsiella ictaluri* on catfish (*Ictalurus punctatus*), and *Streptococcus iniae* and *G. niloticus* on Nile tilapia (*Oreochromis niloticus*) both resulted in higher host mortality (Shoemaker *et al.,* 2008; Xu *et al.,* 2012a). *I. multifiliis* enhances invasion of *Aeromonas hydrophila* in catfish (Xu *et al.,* 2012b), and *Argulus coregoni* or *G. derjavini* enhance *Flavobacterium* spp. prevalence and again increases host mortality in rainbow trout (*Oncorhynchus* mykiss) (see Busch *et al.,* 2003; Bandilla *et al.,* 2006). Such studies typically focus on larger ectoparasites acting as vectors or creating routes of infection by epidermal abrasion for bacterial diseases (Ahne, 1985) and little attention has been paid to the effect of such ectoparasites upon each other with the exception of gyrodactylid or dactylogyrid infections (Paperna, 1964; Harris, 1982; Buchmann *et al.,* 1999). *Gyrodactylus* spp. studies have also found that co-infection alters the habitat distribution of species across a host in comparison to fish infected with only a single species (Harris, 1982) or inhibits co-infection (Paperna, 1964), but until now no study has looked at how another genera of ectoparasites might affect *Gyrodactylus* spp. population distributions. This then is the first case where two co-infecting fish ectoparasites of different genera have been found to impact on fish disease progression.

When sticklebacks (*Gasterosteus aculeatus*) were co-infected with three individuals of *A. foliaceus* and two worms of *Gyrodactylus gasterostei*, peak *G. gasterostei* infection was on average 305% higher and infection intensity over time (AUC) 227% higher, compared to controls. Additionally resistance to gyrodactylid infection in sticklebacks, typically occurs after 20 days at 15°C (see Raeymaekers *et al.,* 2011; Chapters 3 and 4), but was delayed such that within the 25 days of this experiment, it was not observed in both co-infected treatment groups. A likely explanation for this relationship is that protective immune-modulation by *Argulus* is also supressing the gyrodactylids' immune response. Argulid immune-modulation is known to include the suppression of serum complement (Saurabh *et al.,* 2010) and other innate factors (Saurabh *et al.,* 2010; Kar *et al.,* 2013), some of which are known to be associated with *Gyrodactylus* spp. resistance (Buchmann, 1998; Harris *et al.,* 1998). This immune suppression explains the higher levels of infection, later infection peaks and lack of resistance to *G. gasterostei* up until Day 25 post-infection. Alternatively, the feeding mechanism of *A. foliaceus,* which results in necrosis of the host epithelium, might provide a more suitable nutrient resource for *G. gasterostei;* hence, the increased numbers of *G. gasterostei* around the head where argulids more commonly resided.

There was little evidence to support inter-specific competition in the current study as the presence of *A. foliaceus* on specified body regions did not coincide with reduced *G. gasterostei* burdens. In fact, on head of the fish, which was most commonly populated by *A. foliaceus*, there were higher burdens of *G. gasterostei*. The preference of adult *A. foliaceus* for the body of the fish over the fins is likely driven by easier adhesion to the host and a better blood supply. Lastly, the current study supports the observations of Harris (1982) that *G. gasterostei* favours certain body regions, namely the head, pectoral and caudal fins.

In summary, this study found that immune suppression as a result of *A. foliaceus* infection increases the intensity of *G. gasterostei* infection in co-infected sticklebacks. This finding has implications for disease control. *Gyrodactylus* spp. are virtually ubiquitous (see Harris *et al.,* 2008) and a major problem for aquaculture and the aquarium trade (see Mo *et al.*, 2006; Linaker *et al.*, 2012). The impact of a generalist parasite, like *A. foliaceus,* on co-infecting gyrodactylids could be drastically increasing infection rate; a relationship that was until now unknown. Similar to *A. foliaceus* serum complement suppression (Saurabh *et al.*, 2010), another problematic ectoparasite the salmon louse (*Lepeophtheirus salmonis*) is an immune-modulator capable of supressing expression of some complement genes in the skin (Tadiso *et al.*, 2011); suggesting that like *A. foliaceus* other ectoparasites may be responsible for high gyrodactylid infection intensities on teleosts.

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Chapter 8 - *Argulus foliaceus* infection and the consequences for three-spined stickleback (*Gasterosteus aculeatus*) swimming performance

Abstract

Many studies have utilised flumes as water tunnels to study the energetics, mechanics and kinematics of fish in flowing water, however, there have been few such studies that have assessed the impact of infection on fish swimming ability. The consequences of infection with large parasites could be twofold; affecting the mechanics of swimming (including drag) and the pathological costs of infection. This study utilised the model three-spined stickleback (*Gasterosteus aculeatus*) and the relatively large fish louse, *Argulus foliaceus*, to assess both the mechanical and pathological effects of infection. Both sustained (prolonged swimming) and burst (C-start) swimming performance were measured on each fish over five trials with infection occurring shortly before the third trial. Pathology had a significant effect on both prolonged distance and C-start swimming while there was no observable effect of form drag on sticklebacks. Additionally, sticklebacks displayed a preference for flow refuging, swimming in low velocity regions of the flume closer to the flume bed, and this preference increased with flow rate and with infection. Sticklebacks with larger pectoral fins performed more station holding swimming, which typically used labriform (pectoral sculling) over subcarangiform (caudal fin and body beating) locomotion. This study demonstrates that a large ectoparasite has pathological consequences on stickleback swimming performance as well as demonstrating the preference for low velocity areas when infected.

Introduction

The physics of drag on standard objects such as cylinders and aerofoils are well understood (Douglas *et al.,* 2011), and the use of live animals in flumes or water tunnels for the study of fish metabolomics, energetics, and swimming mechanics is common (see Brett, 1964, 1967; Jain *et al.,* 1998; Plaut, 2000; Reidy *et al.,* 2000; Blake, 2004). Only a limited body of work has utilised these flumes to study the effects of parasitism under flow action (Wagner *et al.,* 2003; Hockley *et al.,* 2014) and the change in profile shape of the fish due to parasitism have not yet been considered. If the parasite is large relative to the body size of the fish, the streamlined hydrofoil shape of a fish is likely to be compromised and this may alter the fish's swimming performance. Furthermore the

swimming stability of the fish may also be affected by parasite mass causing instability and the fish's ability to remain perpendicular to the bed, as is the case with external tagging (Lewis & Muntz, 1984; Steinhausen *et al.*, 2006). An estimate of the likely increase in hydrodynamic drag due to the parasite attachment can be calculated using the classical drag force formula: $F = \frac{1}{2} C_D \rho U_0^2 A$ where F is the drag force, C_D the drag coefficient which is a function of the Reynolds number and body profile, ρ the fluid density, U the velocity and A is the projected area of the body (Douglas *et al.*, 2011). Although the relative change in the drag coefficient is unknown, a rough estimate of the increase in drag can be calculated based solely on the projected area.

Perhaps surprisingly, only one study has utilised a large species of parasite, *Lepeophtheirus salmonis* (sea lice) an ectoparasitic crustacean, which could have a measurable impact on swimming performance at a hydrodynamic level as well as at a pathological one (Wagner *et al.*,2003). The impact of sea lice pathology (see Johnson & Albright, 1992; Jónsdóttir *et al.*, 1992) is however likely to be a major cause of swim performance reduction and may be masking any hydrodynamic effect, particularly over the ca. 4.5 weeks that these fish were infected with the juvenile stage and three days with the adult in the Wagner *et al.'s* (2003) study. Instead the fish louse, *Argulus foliaceus*, a generalist parasite known to infect a large number of commercially important fish, might be utilized to study drag effects as a result of its low level of pathology when present at low infection intensities (Taylor *et al.*, 2006; 2009; Steckler & Yanong, 2012; Menezes *et al.*, 1990). Such an effect is, however, likely only to be transient as the feeding mechanism of *A. foliaceus*, blood sucking by means of a stylet which injects cytolytic toxins (Hoffman, 1977; Walker *et al.*, 2011) does result in pathology (Lester & Roubal, 1995; Walker *et al.*, 2004). *A. foliaceus* might therefore be used to study the effects of parasitic drag on prolonged distance swimming by placing fish in a flume shortly after infection, with the pathological effects on swim performace becoming apparent some days later. Fish infected with parasites may also exhibit 'flashing' or 'twisting' behaviour whereby the fish rubs up against hard substrates or violently summersaults in an attempt to dislodge and remove the parasite and stop it feeding (Walker *et al.*, 2004). These pathological effects and energetically costly behaviours may also be affecting the swimming performance of fish.

In addition to the prolonged swimming performance of fish, infection with *A. foliaceus* may also have an impact on anti-predator escape responses. Depending on the species of

fish these responses are characterised by the shape the fish makes in the first few milliseconds of the escape, commonly a 'C' or an 'S' shape (Jayne & Lauder, 1993; Domenici & Blake, 1997). The velocity of this response is proportional to the likelihood of escape and is therefore a good measure of host fitness (Walker *et al.*, 2005; Blake *et al.*, 2006).

This study uses the small three-spined stickleback (*Gasterosteus aculeatus*) infected with *A. foliaceus* to maximise the effects of hydrodynamic drag, to study the effects of infection at both a mechanical and pathological level. Projected area is used as a measure of increased form drag; while *A. foliaceus* not increasing drag, because they are hidden in the wake of the fish's head, may also be having an effect on the swimming stability of the fish as a result of their mass. Sticklebacks were infected with *A. foliaceus* shortly before a third swim performance test, the first two trials were conducted as controls, this minimised the effect of pathology at the third trial fish were then trialled twice more to assess longer term pathological effects of infection. Alongside this, the stickleback C-start (Taylor & Mcphail, 1986) performance was measured as a further measure of fitness. If drag were to have an effect on swim performance there would be a drop off in either prolonged distance or C-start performance in the third test with pathology causing a further drop by the fourth or fifth trial.

Materials and Methods

Fish and parasite origin

Three-spined sticklebacks (*Gasterosteus aculeatus*) were initially collected from Roath Brook (ST 18897 78541) on the 02/07/15 and transported to the aquarium facility at Cardiff University. Fish (mean length = 31.5 mm, range = 26.1 to 37.3 mm; mean mass = 0.471 g, range = 0.249 to 0.655 g) were maintained in 30 L tanks at 15°C at a density <1 fish/L on a 18 h light: 6 h dark cycle and fed daily on frozen bloodworm. Prior to performance tests, fish were treated for ectoparasites by submersion in 0.004% formaldehyde solution for 1 h with a 30 min rest period in freshwater after 30 min (see Chapter 2). Fish were then maintained in 1% salt solution with 0.002 g/L of methylene blue for 48 h to inhibit secondary infection. Treated fish were checked visually for infecting ectoparasites at least three times under a dissection microscope with fibre optic illumination by anaesthetising them in 0.02% MS222. Any remaining ectoparasites were removed with watchmaker's forceps following the methods of Schelkle *et al.* (2009). Any fish found to have ectoparasites was checked a further three times to ensure

it was clear of infection. Sticklebacks were then maintained for 2 weeks prior to swim performance tests to allow recovery in dechlorinated freshwater. All animal work was approved by the Cardiff University Ethics committees and conducted under Home Office Licence PPL 302357. The *Argulus foliaceus* were a culture produced from specimens originally obtained from a carp (*Cyprinus carpio*) still water fishery in North Lincolnshire, July 2014.

Experimental design

A total of five prolonged swimming performance tests, each separated by 3 days, were performed on each fish with the first two acting as controls and allowing the fish to acclimatise to tests within the flume. The third performance test was conducted a maximum of 30 min after infection with *A. foliaceus*; this was to ensure minimal pathology and allow measurement of any potential effects of hydrodynamic drag on the fish as the result of infection. Infection was conducted by exposing fish to two individuals of *A. foliaceus* in 100 ml of water (n=8) or a sham infection (n=5), the *A. foliaceus* had been starved for 48 h prior to infection to facilitate natural attachment without the use of anaesthetics. The remaining two trials (3 and 6 days post-infection) were used to measure the effects of pathology on swimming performance; totalling 65 prolonged distance performance trials. C-start 'burst swimming' responses of each fish were additionally recorded 24 h after each prolonged distance flume run (as below). After all trials had been conducted the fish was euthanized in 0.002% MS222 and standard length, pectoral fin length, caudal fin width and length, mass, sex and gravidity recorded.

The Flume

Prolonged swim performance tests were conducted in a unidirectional recirculating open channel Armfield C4 multi-purpose flume (4 m length, 7.6 mm width and 150 mm depth) set with a negative bed gradient of 1/1000. A weir gate at the downstream end of the flume was used to control the longitudinal water surface profile and a 20 mm length of honeycomb flow straightener were used to contain fish within a 1 m length of the flume. Haloex chloride treatment was used at 0.02 ml/L to remove chlorides and additional air bubbled into the flume reservoir using a mains operated stone aerator. A 20 mm^2 grid was placed along the back sidewall of the flume to facilitate behavioural observations.

The flume was divided into four areas based on preliminary observation of the 13 sticklebacks over the course of the trials. Sticklebacks demonstrated a preference for Area-3. The velocities were measured using a Nixon propeller meter using a sampling time of 1 min at 20 mm intervals along the centreline of the flume (Fig. 8.1). Variation in water velocity was analysed using a linear model with velocity (cm/s) as the independent variable and interactions between flowrate (L/s) and area as the independent variables were conducted: the downstream boundary immediately upstream of the flow straightener (Area-2: t=7.689, p<0.001) and as would be expected, the area close to the water surface (Area-1: t=7.166, p<0.001) had significantly higher velocities than the area close to the flume bed (Area-4) or the upstream boundary (Area-3) which did not statistically differ from one another, the velocity at the downstream boundary was higher than that of the top of the flume (t=2.586, p=0.010) (Fig. 8.2).

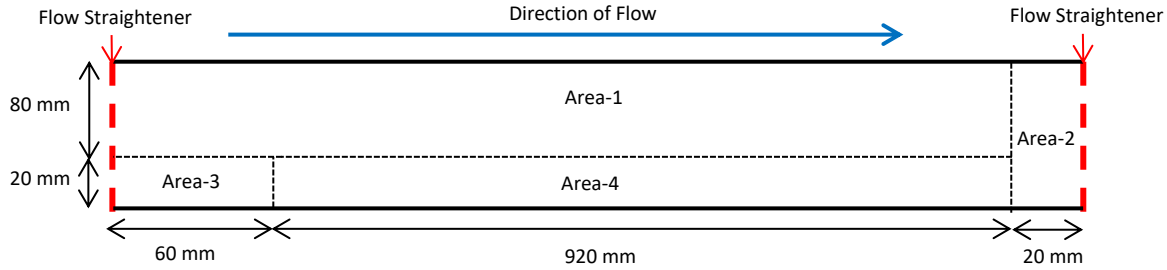

Figure 8.2: Flume elevation diagram showing the flume used for the prolonged swim performance tests and the characterised flow areas. Areas-1 represents the area with a reasonably uniform velocity. Area-2 is the higher velocity downstream boundary, against which fish exhausted. Area-3 is the upstream boundary in which fish were observed to spend a preferential amount of time. And Area-4 is the low velocity bed boundary layer. Flume width is 7.6 mm. Not to scale.

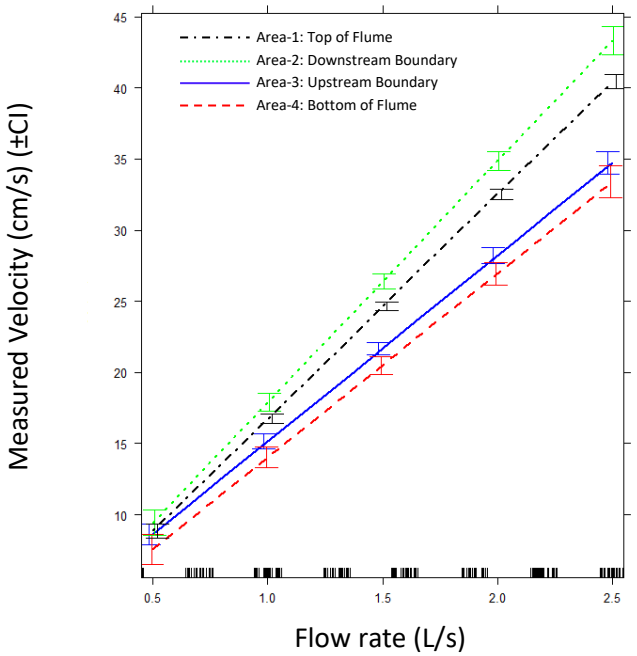

Figure 8.1: The measured volume-averaged velocities of different flume areas. Lines represent means and error bars 95% confidence intervals.

Prolonged distance swim performance test

Each stickleback was placed in the flume running at 0 L/s for 5 min of acclimatisation. The flow rate was then increased every 5 min to 0.4, 0.7, 1.0, 1.3, 1.6, 1.9, 2.2 to a maximum of 2.5 L/s at which fish were maintained for 20 min or until fish exhausted. Fish were considered exhausted when pushed up against the downstream flow straightener. Fish were recorded using a Swann DVR8-3425 960H resolution CCTV system. The videos were analysed in Jwatcher 0.9 (Blumstein *et al.,* 2007) for time spent in the four separate regions of the flume over the 55 min trial (Fig. 8.1) and assessed for five different behaviours: being pushed backwards (movement downstream but while facing upstream), swimming downstream, station holding (head maintained in the same 20 mm^2 space of the flume - see Gerstner & Webb (1998), swimming upstream and twisting or flashing behaviours in an attempt to remove *A. foliaceus*. In addition, photographs of each fish were taken head on using a Nikon S3600 with a ruler in the frame of reference. Photographs were imported into ImageJ (Abramoff *et al.,* 2004) to calculate the projected area using the freehand selection tool.

C-start performance test

The C-start response of each fish was conducted in a 300x400 mm glass experimental arena filled with dechlorinated water to a depth of 30 mm, allowing fish to move only along a horizontal plane. A Nikon D3200 camera was used to film each trial at a frame rate of 50 fps. Upon introduction to the tank fish were acclimatised for 5 min. A net was then thrust into the water of the tank near the head of the fish in order to initiate the response; a 2 min recovery period was allowed and three trials of C-start conducted (Harper & Blake, 1990; Brainerd & Patek, 1998; Bergstrom, 2002). A frame-by-frame analysis was performed in Tracker v4.87 (Brown, 2015) with the velocity of the C-start calculated from the 20 ms proceeding initiation of the response; an average of the three C-start velocities was then taken.

Statistical analysis

All data were analysed using R v3.2.2 (R Core Team, 2015) with the additional use of 'car' (Fox & Weisberg, 2011) and 'MASS' (Venables & Ripley, 2002), 'lme4' (Bates *et al.,* 2015) and 'lmerTest' (Kuznetsova *et al.,* 2016) and 'effects' (Fox, 2003) packages. All model selection and model averaging was conducted using Akaike Information Criterion. Least-squared means was used to compare within any 2-way factorial interactions. All models utilised the following independent variables unless otherwise stated: trial, temperature, fish body condition (residuals from a quadratic regression of

mass and length), sex, fish length, caudal fin size (principle component of fin width and length) and pectoral fin size (fin length).

Swimming performance, both prolonged distance and C-start, were analysed using linear mixed models with additional independent variables including: infection treatment, and an interaction between trial and infection group as the independent variables; fish identification was used as a random factor when applicable. 1) The effect of infection on prolonged stickleback swimming ability was analysed using time spent in the flume as a proportion of the total possible time in the flume (55 min – not including acclimatisation) as the dependant variable with a logit transformation. Any change drop in performance on the third trail would therefore be the result of instability in fish swimming caused by the presence of *A. foliaceus* on the fish. A linear mixed model using Trials 2 and 3 (as pathology may have a confounding impact after Trial 3) was used to look for an effect of projected area on long term stickleback swimming ability using the logit transformed proportion of, as calculated above, and projected area as an added independent variable. 2) The velocity of the fish's escape response from three repeats per trial were averaged and used to analyse variation in initial C-start velocity; a square root transformation was used on the dependant variable.

Stickleback preferences for the four areas of the flume were analysed by dividing the total time spent in each area in a given trial by the areas proportional size, therefore adjusting for the inherent biased in larger areas given a random distribution, and turned into a proportion of the total time spent in the flume. A linear mixed model was used with fish I.D. as the random variable and a logit transformation used on the dependant variable. Additional independent variables included the flume area, flow speed (L/s^{-1}), infection status, and 2-way interactions with area and all other independent variables and a 3-way interaction of infection: flow rate: area.

Stickleback behaviours were analysed using a linear mixed model with fish identification as the random variable. The dependent variable was the proportion of time each fish spent performing the 5 behaviours in each trial; a logit transformation was used. Additional independent variables included the fish behaviour, flow rate (L/s), infection status, temperature, and interactions between the given behaviour and all other independent variables. Argulid removal behaviour, flashing or twisting in order to dislodge the parasite (see Walker *et al.,* 2004), had a lack of statistical significance as a

result of zero inflation, only a few individuals actually exhibited this behaviour and for very little time, therefore this was removed from the analysis.

Results

Three-spined sticklebacks (*Gasterosteus aculeatus*) infected with *Argulus foliaceus* for 6 days demonstrated a significant reduction in prolonged distance swim performance by trials 4 (t=-3.134, p=0.003) and 5 (t=-3.892, p<0.001), but not Trial 3 (p>0.005) in comparison to the uninfected controls (Fig. 8.3). In addition, no effect of projected area on fish prolonged swimming performance was observed between Trials 2 and 3 (p>0.05). Within the C-start trials the escape velocity of these same sticklebacks by the 4th and 5th trials had also reduced significantly (p>0.05) in comparison to the 1st and 2nd control trials (Fig. 8.4). The reduction in swim performance by Trials 4 and 5 suggests that pathology has a negative impact on fish swimming ability. The lack of reduction in the third trial when compared with the first two trials and the uninfected fish suggests that there was no impact of hydrodynamic drag or instability caused by the parasite on fish swimming performance. This is despite the fact that when on the head of the fish the projected area was increased by as much as 27% (mean 26.4%).

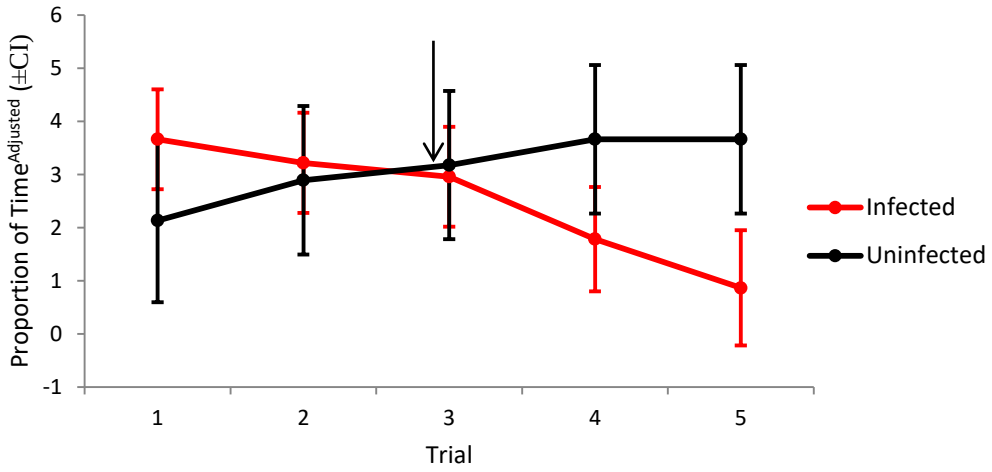

Figure 8.3: The proportional length of time the infected and uninfected three-spined sticklebacks (*Gasterosteus aculeatus*) were able to maintain prolonged distance swimming. Sticklebacks were infected with *Argulus foliaceus* or sham infected a maximum of 30 min before the third flume trial (arrow). Data are split by infection group rather than infection status; therefore infected fish are only infected from Trial 3 onwards. Points represent the mean and error bars are 95% confidence intervals extracted from a linear mixed model of logit transformed data.

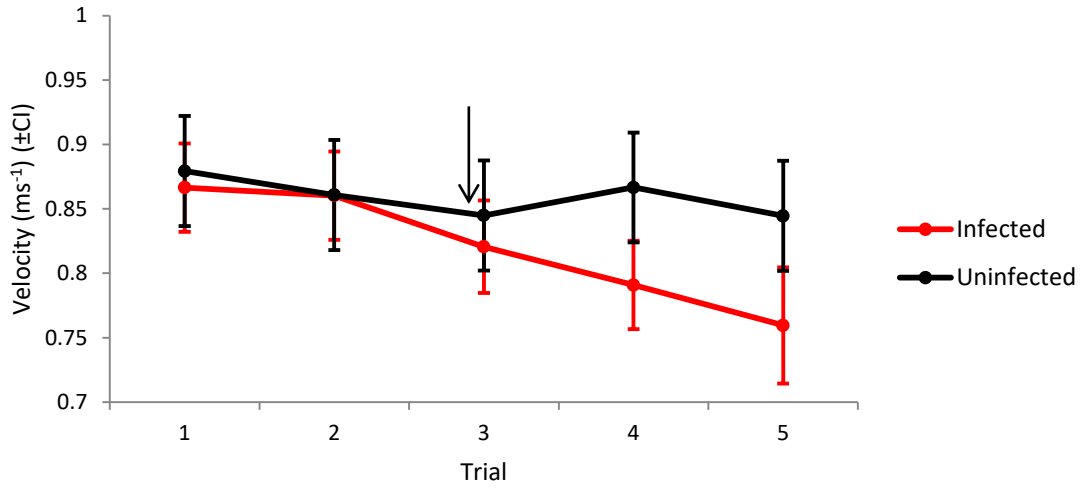

Figure 8.4: The velocity of three-spined sticklebacks (*Gasterosteus aculeatus*) in the first 20 ms of a C-start escape response when infected with *Argulus foliaceus* and uninfected. Sticklebacks were infected with *A. foliaceus* or sham infected before the third flume trail (arrow). Data are split by infection group rather than infection status; therefore fish are only infected from Trial 3 onwards in the infected group. Points represent the mean and error bars are 95% confidence intervals extracted from a linear mixed model with a square root transformation.

Sticklebacks demonstrated a preference for swimming in Area-3 (upstream boundary, low velocity area) whether infected or uninfected (t=2.042, p=0.041; Fig 8.5). Sticklebacks also had an increasing preference for Area-3 in higher flow rate conditions for both infected and uninfected fish (t=11.660, p<0.001; Fig. 8.5), and this increase in preference was stronger in the infected rather than uninfected fish (t=2.911, p=0.004; Fig. 8.5). Infected fish spent more time holding station in the flume than uninfected fish (t.ratio=-5.361, p<0.001; Fig. 8.6). All sticklebacks demonstrated a preference for station holding (t=3.254, p=0.001) over upstream (t=-2.328, p=0.20) or downstream (t=-2.407, p=0.016) swimming in high flow rate conditions. Additionally, sticklebacks with larger pectoral fins performed more stationary swimming (t=2.378, p=0.018) and less downstream swimming (t=-2.392, p=0.017); this is to be expected as the majority of swimming to hold position in the flume was labriform locomotion (pectoral sculling) which larger pectoral fins would make energetically favourable.

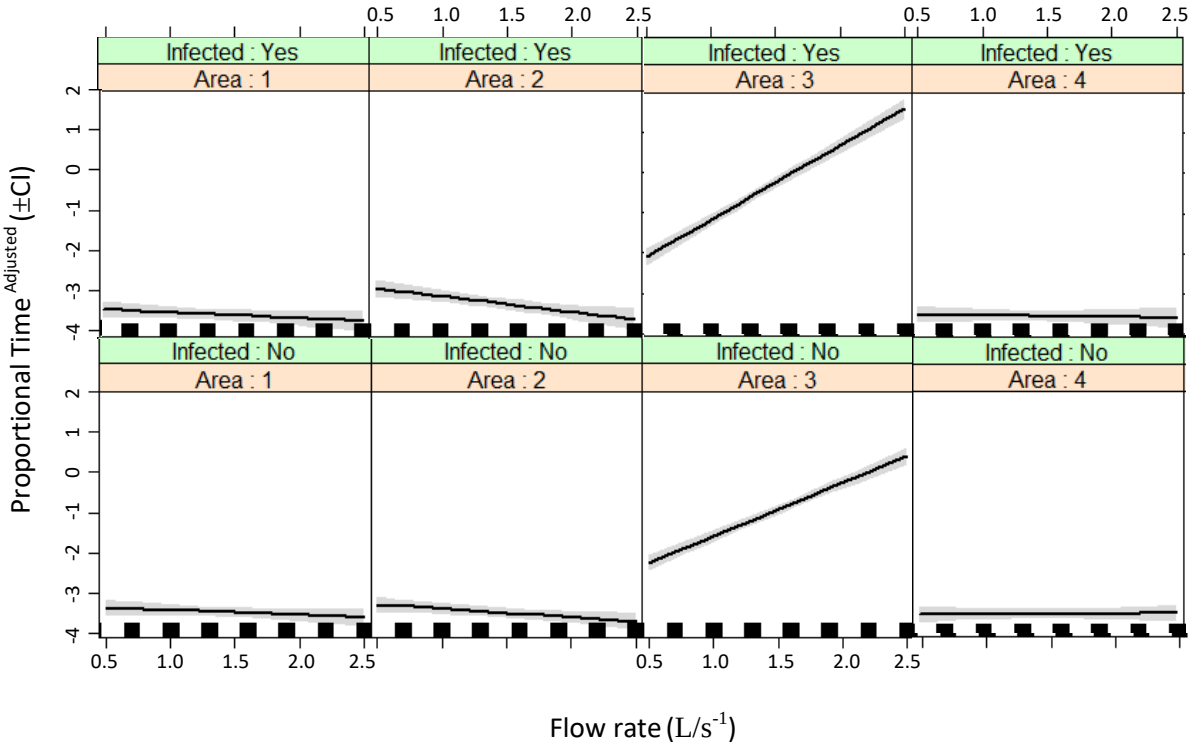

Figure 8.5: The relative length of time three-spined sticklebacks (*Gasterosteus aculeatus*), uninfected or infected with *Argulus foliaceus*, spent in each area of the flume with increasing flow rate and separated by infection status. Black lines are regression lines and shaded areas 95% confidence intervals extracted from a linear mixed model.

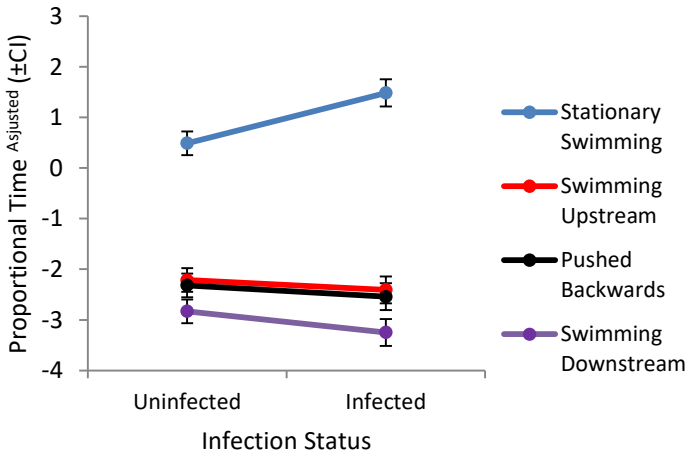

Figure 8.6: The proportional length of time three-spined sticklebacks (*Gasterosteus aculeatus*), infected with *Argulus foliaceus* or not spent performing certain swimming behaviours. Spots are means and error bars 95% confidence intervals extracted from a linear mixed model with a logit transformation.

Discussion

This study utilised sticklebacks infected with *Argulus foliaceus* and both prolonged distance and C-start swimming, finding that *A. foliaceus* pathology had a negative impact on both forms of swimming. Despite the parasites increasing the projected area of the fish by as much as 27% (mean 26.4%), no effect of hydrodynamic drag or instability was observed in either test. In comparison, external fish tags reduced

swimming performance (Lewis & Muntz, 1984; Steinhausen *et al.*, 2006), *A. foliaceus* has a more streamlined profile holding itself close to the fish body and, depending on the type of fish tag, may be more neutrally bouyant. The continued presence of an *A. foliaceus* is likely to compound the pathological effect on swim performance. Such an effect will occur over time with continued reduction in swim performance from the point of infection; as this study demonstrates by the greater magnitude of performance reduction at 6 days post-infection compared to 0 or 3 days post-infection. This performance reduction is likely derived by the feeding and attachment mechanisms of the argulid which is reliant on blood feeding by means of a stylet and cytolytic toxins with attachment by large maxillae suckers and numerous spines on the ventral surface (Bower-Shore, 1940; Hoffman, 1977; Walker *et al.,* 2011). The effect of these two mechanisms is to cause necrosis and apoptosis (Pottinger *et al.,* 1984; Ruane *et al.*,1999; van Der Salm *et al.,* 2000), which are likely to be a major cause of fish swimming performance reduction. A reduction in swim performance as a result of pathology is consistent with a similar study performed using sea lice, although drag was not examined in this study (Wagner *et al.,* 2003).

The velocity and turbulence of water in a flume is subject to variation along the length, width and height of the flume. Along the bottom and sides of an open channel flume, the velocity is reduced due to boundary friction and the velocity gradient is higher in these zones with greater turbulent shear stress. Multiple studies have demonstrated that fish use this boundary layer as a shelter from higher velocities and allowing them to attain higher swim performance (Barbin & Krueger, 1994; Gerstner, 1998; Hoover *et al.*, 2011). The current study also observed a bias in fish behaviour towards swimming in a lower velocity area of the flume upstream of the flow straightener, in a process known as flow refuging (Gerstner, 1998). The preference of sticklebacks for this low velocity area was further enhanced in increasing flow rate as also previously found by Barbin and Krueger (1994) in American eels (*Anguilla rostrata*). This indicates that measures of time-averaged velocity for the study of fitness may not be sufficient given a mean difference of as much as 7.0 cm/s between two regions of a flume, fish clearly having a preference for the lower velocity region. Fish infected with *A. foliaceus* demonstrated an even greater preference for this same low velocity region than their uninfected counterparts. In addition to the energy saving behaviours observed around the boundary layer, infected fish also spent a greater proportion of their time swimming in a static position in the flume and not swimming up or down its 1 m length. With the

combined preference for low velocity, low energy swimming infected sticklebacks appear to be demonstrating heightened energy saving behaviours in order to offset the negative impacts of infection on swimming performance. Such a response could be comparable to fish or other animals that become less active when infected with certain parasite taxa (Brassard *et al.*, 1982; Poulin, 1994).

Sticklebacks perform two sorts of locomotion: labriform (pectoral fin oscillations) and subcarangiform (beating of the caudal fin) (Lindsey, 1979; Garenc *et al.*,1998). Of these, labriform swimming may be considered less energetic; when a fish is able to employ both forms of swimming: the labriform is used at lower flow rates and the subcarangiform at higher flow rates (Archer & Johnston, 1989). In particular labriform locomotion seems to be particularly efficient at keeping fish stationary in flowing water (Gordon *et al.*, 2000; Kato, 2000) and the efficiency of this swimming is related to the size of the pectoral fins (Walker & Westneat, 2002). In the current study, we found that fish with larger pectoral fins spent more time holding station, which typically involved more labriform locomotion, indicating larger finned fish may be using this form of locomotion as a more energy efficient swimming technique. It is also the case that larger fins allow the fish to generate greater forward momentum (Walker & Westneat, 2002) allowing them to continue labriform swimming at higher velocities before they are forced backwards by the flow and need use subcarangiform locomotion.

In summary, this study has revealed a major impact of parasite-induced pathology on the swimming performance of fish but a perhaps surprising lack of any hydrodynamic effect caused by increased drag or instability as a result of infection with *A. foliaceus*. Sticklebacks showed a strong preference for low velocity regions of the flume and for energy saving behaviours particularly at higher flow rates or when infected. Lastly, fish with larger pectoral fins spend more time performing stationary swimming using labriform locomotion, also attributed to energy saving and the fact that at higher velocities larger fins will give greater thrust.

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Chapter 9 - General Discussion

The main objective of this thesis was to study the effects of temperature, climate change and infection on three-spined stickleback (*Gasterosteus aculeatus*) immunity, and the parasitic oomycete *Saprolegnia parasitica* and monogenean *Gyrodactylus gasterostei*. To this end a series of experiments were conducted looking into the effects of: a 2°C increase in mean temperature, winter length, short term temperature shock and circannual rhythms (Chapters 3-6). In addition, two experiments focused on infection with a generalist parasite, *Argulus foliaceus*, with a focus on its immunomodulatory impact on co-infection and pathological consequences for the fish (Chapters 7-8). Over the course of these experiments multiple culture techniques were collated leading to the production of Chapter 2 for the benefit of research colleagues using the stickleback model.

Temperature, *Saprolegnia* and Immunity

With climate change and increasing global temperature (see IPCC, 2013a, b) the current study set out to understand the impacts of mean temperature variation and variability on the host responses of sticklebacks to *Saprolegnia parasitica*. To this end three studies were conducted utilising *S. parasitica* infections to understand temperature impacts on the outcomes and dynamics of the host immune response.

The temperature at time of *S. parasitica* infection was found to be the dominant force in determining the likelihood and intensity of infection rather than historical winter or the residual effect of a mean increase of 2°C (Chapters 3 and 4). In warmer conditions, there was a higher likelihood of infection and a corresponding higher intensity of infection (Chapters 4 and 5). Such a relationship was considered unlikely given the history of *S. parasitica* as a winter infection (Bly & Clem, 1991; Bly *et al.*, 1992), however, the cause of winter *Saprolegnia*sis has more often been attributed to rapid drops in temperature rather than the temperature at infection itself (Bly & Clem, 1991; Bly *et al.*, 1992). The current study did not find a higher likelihood of infection when pre-exposing fish to an 8°C drop in temperature, however, an increase of 8°C did reduce the likelihood of infection (Chapter 5). Analysis of immune genes may explain the reduced incidence and intensity of infection at colder temperatures. Across Chapters 4 and 5 that analysed immunity, *tbk1* was highly expressed in individual fish at colder temperatures. Similarly *il-17* had higher levels of expression in colder temperatures

(Chapter 5) and a related increase in expression if exposed to winter temperatures along with *il-1rlike* (Chapter 4). Given that these three genes belong to the pro-inflammatory response, known to be upregulated in *S. parasitica* infection (see Bruijn *et al.,* 2012; Belmonte *et al.,* 2014), at constant colder temperatures, the immune system could therefore be protecting fish from infection. The current study found no effects of rapid temperature change that could explain higher incidences of infection in fish that experience a rapid temperature decrease; the likely cause being that the interval between infection and sampling (72 h) may have been sufficient time for the immune system to have stabilised.

The current studies (Chapters 4 and 5) found that *S. parasitica* is immunomodulatory, causing up-regulation the pro-inflammation responses and suppression of adaptive genes, supporting Belmonte *et al.* (2014). In accordance with Belmonte *et al.* (2014) this study identified upregulation of pro-inflammatory genes *tbk1* (Abe & Barber, 2014) and *β-def* (Kim *et al.,* 2010) and decreases in the expression of the adaptive genes *cd8* and *foxp3* (Chapters 4 and 5). In addition lysozyme, a gene whose increased expression is attributed to reduced mortality in fish infected with *S. parasitica* (see Das *et al.,* 2013), was supressed in the current study in infected fish (Chapter 4), suggesting immunomodulation by the parasite to improve its survival or transmission.

It is clear that our results do not suggest that winter is the main period of transmission for *S. parasitica*. To further our understanding of this system additional studies should be conducted into the disease progression (i.e. the change in immune response over 72 h) and how that is affected by temperature stochasticity.

The Foothold Hypothesis

Three-spined sticklebacks were exposed to variable lengths of winter (0-3 months at 7°C) and a year-long thermal cycle in order to assess the impact of temperature variability on *Gyrodactylus gasterostei* infections. Previous studies have often found that lower temperatures have a negative impact on gyrodactylid reproduction (see Chappell, 1969; Lester & Adams, 1974; Harris, 1982; Jansen & Bakke, 1991). Until the current study the circannual effects of temperature had only been explored in field studies (Chappell, 1969; Mo, 1997; Winger *et al.,* 2007; You *et al.,* 2008) where multiple environmental variables might have influenced infection outcomes and where repeated measures of infection were either not possible or extremely difficult. Here, across two studies, sticklebacks were exposed to either 1) variable lengths of winter and

then a temperature rise to simulate the effects of spring on infection (Chapter 4 or 2) were kept in mesocosms at ambient or 2°C above ambient to simulate a circannual thermal regime under current environmental conditions and the increase in mean temperature under predicted climatic changes (see IPCC, 2013a, b; Chapter 3).

Sticklebacks infected with *G. gasterostei* at 15°C displayed a reduction in the intensity of infection from Day 15-20 (Chapter 4; see also Chapter 7). In contrast, fish exposed to infections at colder temperatures and then a warming period, developed higher intensities of infection in terms of both peak intensity and total infection over time; these fish were also less likely to clear their infections (Chapter 4). Similarly, sticklebacks exposed to infections in November or February, which then experienced a decline or increase in temperature respectively, also reached higher infection totals than those fish infected during the summer (Chapter 3). Such results would normally be attributed to temperature. This does not however explain the higher levels of peak infection for the November samples when parasite reproduction should be suppressed (see Chappell, 1969; Lester & Adams, 1974; Harris, 1982; Jansen & Bakke, 1991). Instead the current results may be explained by low levels of established infection providing a foothold for when conditions become more variable and thus granting the parasite a secure position from which from which expansion of the population can occur. The foothold hypothesis would also go some way to explaining why fish infected in May or August at a mean temp of 14-15°C did not experience the associated rapid decrease in infection after 15-20 days (Chapter 3). In these May and August samples, although the mean temperature was similar to the laboratory experiment (Chapter 4), daily changes in temperature, which even in summer could drop below 10°C at night, would also offer the parasite an advantage under the foothold hypothesis. In addition, this hypothesis is supported by field sampling, which may find higher levels of infection 1-2 months after spring warming (Chappell, 1969; Mo, 1997; Winger *et al.*, 2007; You *et al.*, 2008), consistent with the increases we see in both experiments. A caveat on the foothold hypothesis and its correlation with wild studies is that transmission dynamics and demographics may play an important part in infection varying from the within-host effects of the experiments (Chapters 3 and 4). Although it is not possible to confirm from the current results, this may be the result of a more plastic or rapidly adaptive genotype in *G. gasterostei* than the stickleback; allowing the parasite to gain an advantage over the stickleback immune response in changeable conditions. In order to confirm the foothold hypothesis one further experiment is required holding fish at

greater than one constant temperature and then exposing subsets of these fish to temperature change; the expected results being that at constant temperatures resistance while in the variable conditions no matter the direction of temperature change infection intensities are greater compared to controls. Further to this research it would be useful to assess the cause of the foothold hypothesis looking at the parasite transcriptome and potential epigenome to understand how *G. gasterostei* is able to adapt to temperature variability faster than the host. An analogous example of this phenotypic change occurs in parasites that host switch between insects and warm-blooded animals, this host switching requires the parasite to rapidly adapt to its new environment. In particular heat shock proteins expression rapidly changes in response to thermal shock (Tirard *et al.*, 1995; Giambiagi-Demarval *et al.*, 1996; Syin & Goldman, 1996) taking as little as 1 h to respond to heak shock in the case of *Perkinsus marinus* (Tirard *et al.*, 1995).

Body Condition: Fuelling the Fires of Infection

Two of the studies within this thesis have highlighted a third variable that dramatically increases the prevalence and intensity of infection; body condition (Chapters 3 and 5). The increase in *Saprolegnia parasitica* prevalence with high body condition is attributed to a trade-off in fish between high body condition and immunity. This being the case the pro-inflammatory associated and anti-microbial gene *β-def* was supressed in higher condition individuals, although the also pro-inflammatory gene *tbk1* was upregulated in high condition fish it was not to the same extent as downregulation of *β-def*. Such a relationship has previously been found in common toads (*Bufo bufo*) infected with *Batrachochytrium dendrobatidis*, where toads in a higher condition, also had a higher prevalence of infection (Garner *et al.*, 2011). The higher intensity of *S. parasitica* in high condition individuals is a more common phenomenon. In other parasites species, measures of greater host fitness often benefit the parasites causing: larger parasites, faster rate of growth or a greater number of infective transmission stages (Bean & Winfield, 1991; Ebert *et al.*, 2000; Bedhomme *et al.*, 2004; Barber, 2005; Seppälä *et al.*, 2008; Blanchet *et al.*, 2009). Such a relationship likely occurs as the result of more abundant resources available to the parasite causing growth to be either more rapid or reach a higher peak before the host is overcome.

The Pathology and Co-infection of Argulids

Sticklebacks infected with *Argulus foliaceus* were prone to higher peaks of co-infecting *Gyrodactylus gasterostei* infection (Chapter 7). In the first study of its kind two co-infecting eukaryote ectoparasites were used that could be competing directly on the

surface of the host or indirectly via the immune system. Such interspecific competition between gyrodactylids can alter parasite distribution on the host or cause exclusion of one species from the host entirely (Paperna, 1964; Harris, 1982). Likewise, immune manipulation is often the cause of increased infection intensity in co-infected individuals (e.g. Su *et al.,* 2005). In Chapter 7 there was no evidence for interspecific competition but higher *G. gasterostei* intensities on co-infected sticklebacks suggests immune manipulation by *A. foliaceus* (see Saurabh *et al.,* 2010; Kar *et al.,* 2013). This occurs because certain immune responses such as increased complement activity are associated with gyrodactylid resistance (Buchmann, 1998; Harris *et al.,* 1998); suppression of these factors by argulids, as is the case with complement, allows the viviparous infrapopulation growth of *G. gasterostei* to continue unchecked by the immune system. Research on argulids could be expanded to assess the impact of argulids on co-infecting parasites other than *G. gasterostei.* In addition a study on another immunomodulating generalist, sea lice, and their relationship with *Gyrodactylus* spp. could be examined to see if the same co-infection relationship exists. The immunomodulation and pathology caused by *A. foliaceus* also has potential implications for the host.

A study of the ability of *A. foliaceus* to inhibit the swimming ability of stickleback, found that reductions in long and short distance swimming ability were the result of pathology and not mechanical drag (Chapter 8). Additionally, sticklebacks displayed a major preference for low velocity areas of the flume and less energetic behavioural activities. This highlights the need for other studies using flumes to also address the preference of fish for these low velocity boundary layers which, at higher flow rates, velocities diverged by as much as 57% from other areas of the flume. A further experiment has also been suggested that utilises 3D printing technology to print fish and their parasites in order to study hydrodynamic flow changes and drag caused by the parasites. This experiment was going to use micro-CT scanning to build a computer frame of both the stickleback and the parasite, which could then be printed. Load cells and particle image velocity could then be used to measure increased drag and water flow over the infected fish; such an experiment would produce an estimate at which number or size of a parasite might begin to affect drag. Further parasites, such as the sea louse or fish leech, could also be scanned and printed to assess their effect on fish.

Summary

This thesis has highlighted the effects of temperature on *Saprolegnia parasitica* infections demonstrating that cold conditions are not sufficient to cause infection, indeed warmer temperatures were actually found to increase infection success in sticklebacks. This result has implication for aquaculture as we now look towards a multifactorial cause of infection. Of particular importance is the detrimental effect of host body condition, increasing the likelihood and intensity of infection. Such a result is of concern for aquaculture where fish diet is optimized for growth, which may subsequently increase disease incidence.

A further concern for the aquaculturist is the effect *Argulus foliaceus* on increasing *Gyrodactylus gasterostei* infections, being a generalist and common parasite, co-infection could increase the abundance of multiple *Gyrodactylus* spp. leading to greater host morbidity and mortality. An interesting component of the current study has been the multicomponent foothold hypothesis, which seeks to explain high circannual variation in *G. gasterostei* populations. Employing this knowledge treatment of fish during the establishment phase of infection could reduce the peak burdens of infection seen on fish.

Overall, this thesis has uncovered previously unknown relationships between host, infection, temperature and immunity. Also highlighted has been the cause of circannual variation in immune gene expression in sticklebacks, the consequences of co-infection and the effect of a large parasite on fish swimming performance. Findings of this research may ultimately impact on aquaculture and our understanding of how temperature affects host-parasite interactions.

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Appendix 1

Appendix 1: Recipes for three-spined stickleback (*Gasterosteus aculeatus*) husbandry or parasite culture.

Name	Use	Solvent	Recipe
0.02 % MS222	Fish anaesthesia	Dechlorinated water	20 mg/L^{-1}, make fresh on demand
Hanks' stock – without phenol red or sodium bicarbonate	*in vitro* breeding	Distilled water	9.7 g/L^{-1} of Hanks' power without phenol red or sodium bicarbonate
Sodium Bicarbonate solution	*in vitro* breeding	Distilled water	0.35 g sodium bicarbonate in 10 ml
Hanks' without phenol red	*in vitro* breeding	-	9.9 ml hank's stock and 0.1 ml – sodium bicarbonate solution
Hatchery water	Breeding	Dechlorinated water	0.001-0.005 g/L^{-1} methylene blue and 5-8 ppt (0.5-0.8%) aquarium or marine salt
PDA	*Saprolegnia parasitica* culture	Distilled water	39 g/L^{-1}, microwaved into solution, autoclaved, pour into Petri dishes
Pea Broth	*Saprolegnia parasitica* culture	Distilled water	125 g garden peas per litre, autoclaved, strained through muslin to remove peas, volume brought up to 1 L, autoclaved again.
Schistocephalus solidus culture media	*Schistocephalus solidus* culture	-	250 ml Minimum Essential Medium with Earle's salts and L-glutamine, 25mM HEPES-buffer, 1 g penicillin/streptomycin 6.5 g D-glucose, buffered to pH 7.5 with sodium hydroxide
Spirulina feed†	Feed for copepods and *Daphnia* spp.	Dechlorinated water	14 g of baker's yeast and 25 g of *Spirulina* to 250 ml of dechlorinated water; shake well and then add a further 250 ml of dechlorinated water. This mixture will keep for several months at 4°C; shake before use.

†*Chlorella*, which has a smaller cell size, may also be used for species with small feeding parts.

Appendix 2

Appendix 2: Primers used for quantitative real-time Q-PCR measurements of three-spined stickleback (*Gasterosteus aculeatus*) genes.

Gene	Ensembl gene number (or other source of sequence)	Primers
cd8a	ENSGACG00000008945	F - CCACCCTGTACTGCAATCGA
		R - CCGCCTGCTGTTTTCTTTTG
IgM	ENSGACG00000012799	F - GGAGGCAAAGGACGCTACTTT
		R - AACCACATTTGGCCTTTGGA
IgZ	Gambón-Deza et al., 2010	F - TCAACAAAGGAAATGAACCAAAA
		R - TCTTCCTCTGGGAGGACGTG
foxp3b	ENSGACG00000012777	F - TCTGAACACAGTCATGGGGAGA
		R - CCAGGATGAGCTGACTTTCCA
il1r-like	ENSGACG00000001328	F - GAACGCGAGAACTGCAAGAAC
		R - GGGACGCTGGTGAAGTTGAA
il-4	Ohtani *et al.*, 2008	F - CCAAAATCAAACCTGTGCAGTGT
		R - CGAGAAGTCGCGGAATCTGT
il-17	ENSGACG00000001921	F - GGGCCTACAGGATCTCCTACG
		R - GCCCCTGCACAGGCAGTA
il-12ba	ENSGACG00000018453	F - TTCATCAAAGCTTGGCGTT
		R - CCGCCGTCCACAGAACAC
β-def	ENSGACG00000020700	F - TGCAGACGGTTCTGCTATGC
		R - GGCACAGCACCTGTATCGTC
tbk1	ENSGACG00000000607	F - AGACGGAGCAGCTGTTCGA
		R - GCATATCTCATCATATCTGACGACAT
lyso	ENSGACG00000018290	F - TGTCAGAGTGGCAATCAATTGTG
		R - CCCACCCAGGCTCTCATG
tirap	ENSGACG00000006557	F - GGGGCGCCATTTCTACAGA
		R - TGCATCATGTACTGGCACC
gpx4a	ENSGACG00000013272	F - CCAGGAACCCGGCAATG
		R - GAACCGAGCGTTGTAAGGAC
orai1	ENSGACG00000011865	F - GCACCTCGGCTCTGTTGTC
		R - CCATGAGGGCGAAGAGGTGTA

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