The Relative Importance of Parasitic Infections, Ecoimmunological Dynamics and Genetic Factors in Determining Host Condition in a Wild Population of the House Mouse, *Mus musculus domesticus*

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Abstract

The factors which cause changes in condition and immune state are likely to be different in wild animals compared to inbred laboratory strains, as a result of various interacting ecological and environmental pressures not typically found under lab conditions. These include parasitic coinfection, genetic diversity, competition, ageing and seasonal changes in climate and population structure. Over the course of eight trapping sessions, ecological and morphological data from an island population of the house mouse (*Mus musculus domesticus*) were recorded, alongside their parasitic infections, in order to ascertain which factors impact host condition, and to what extent. The immune state of the mice was determined using multiplex ELISA to measure circulating cytokine levels, and qPCR to measure gene expression. Parasites infecting the mice were found to exist in a complex network of positive and negative associations, observed across all recorded groups of parasites. While the nematode parasites *Calodium hepaticum* and *Trichuris muris* are associated with splenic immune expression analogous to laboratory studies, parasitic infection was not a major source of variation in host condition. Seasonality was the key driver of variation in mesenteric immune expression and circulating cytokines, while SNPs within immune genes were shown to influence all measures of immune expression, and parasite community composition. Seasonality, age and immune expression were found to be the main factors associated with measures of host condition, indicating that ecological and environmental factors other than parasitic infection may be the key factors affecting fitness in this population.

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Chapter 1. Introduction

The interplay between immunity and infection is a key factor affecting the condition of any vertebrate, and yet for a number of reasons, the precise relationship between these two factors as it exists in wild populations has been rarely explored in great depth. Wild populations of animals and their parasites are affected in a diversity of ways by ecological factors and extrinsic environmental variation. The vast majority of immunological study has, however, been conducted in laboratory context on inbred animal strains (Babayan *et al.*, 2011; Pedersen and Babayan, 2011). This research has provided a vast wealth of immunological knowledge and a useful toolbox of methodologies which are now sufficient for assessing the immune function of wild animal populations in a comprehensive and informative way, particularly lab model species. Much laboratory-based research has been conducted in mice and rats, and so wild rodent populations provide an opportune candidate for studying the effect of various ecological factors on the immune system of wild populations (Pedersen and Babayan, 2011).

Parasites are an integral component of the ecology of every vertebrate population, and parasitic infection can have profound impacts on the state of the host. These impacts will in turn be affected by changes in the external environment. In this thesis I will be exploring how the ecology of a population of the house mouse (*Mus musculus domesticus*), dynamically interacts with a variety of parasite species, and how differential immune responses to

parasitic infection by different parasites interact with various ecological, environmental and genetic factors to determine an outcome in the host.

1.1 PARASITIC INFECTION IN WILD POPULATIONS

Parasites are organisms which live inside, or adhering to, a host animal, either transiently or chronically, at some fitness cost to the host. All wild animal populations contend with a diversity of parasitic infectious challenges. These parasites can include endoparasites, which live internally to the host, and ectoparasites which adhere to the exterior. They can also be either unicellular organisms (microparasites) or multicellular animals (macroparasites). Fitness costs incurred in host animals can manifest in various ways, including damage to tissues and immunopathology (Garside *et al.*, 2000), modification of behaviour (Poulin, 1994; Lafferty and Kimo Morris, 1996; Libersat, Delago and Gal, 2009), increased use of metabolic resources (Kristan and Hammond, 2000; Hicks *et al.*, 2018), reduction in nutrient uptake (Kyriazakis, Tolkamp and Hutchings, 1998), and inhibition of reproductive capability (Neuhaus, 2003). Hosts typically undergo some level of co-evolution with their parasites, with both species adapting over evolutionary time to maximise their fitness while contending with the other's own adaptive responses (Woolhouse *et al.*, 2002). Over generations of evolution, this can lead to fine-tuned adaptation in life history of both species, and the establishment of complex epidemiological and immunological dynamics between host and parasite.

The level of morbidity caused by a parasite can be highly variable, and will depend on how the host has been able to optimise its immune response and physiology within its ecosystem, according to various ecological factors. As such, the most beneficial response to parasitic infection by the host might not be one which clears it outright, if the required response is metabolically expensive or in some way pathological. Infection outcome in the host can be broadly characterised as either 'tolerance', whereby the infection persists but fitness impacts upon the host are reduced, or 'resistance', in which the host actively prevents or combats infection (Schneider and Ayres, 2008). Models of genetically-determined tolerant and resistant phenotypes show that a diversity of responses can be maintained in host populations through evolution, depending on the fitness benefits and frequency of each phenotype (Roy and Kirchner, 2000; Miller, White and Boots, 2005; Råberg, Graham and Read, 2009).

Similarly, a parasite will usually require its definitive host to survive with some level of mobility in order for its transmission cycle to be perpetuated, and as such many parasites will adapt their virulence in order to maximise the efficiency of their transmission cycles (Anderson and May, 1982; Toft and Karter, 1990). The fitness costs of resistance compared to tolerance on the part of the host, and between levels of virulence on the part of the parasite, will together determine the outcome of an infection. This evolutionary

context is crucial for understanding the relationship between parasite and host in wild populations.

1.1.1 Parasite Taxa

One group of macroparasites which commonly infect vertebrate hosts are the helminth worms, which include an estimated 77,000 parasitic species (Dobson *et al.*, 2008). The term 'helminth' does not describe a specific taxonomic group but rather refers to three clades of worm-like invertebrates commonly involved in parasitism. The most prevalent are nematodes (Phylum: Nematoda), known commonly as roundworms. Nematodes can be free-living, although an estimated 33% are parasitic (Anderson, 1974). Parasitism is estimated to have independently evolved 15 times in the nematodes, indicating its success as an evolutionary strategy (Blaxter and Koutsovoulos, 2015). They are ubiquitous, being found in almost every ecosystem on earth, and affecting every phylum of animal with greater body mass than the parasite itself (Zhang, 2011). The great diversity of this group is accompanied by an equal diversity of hosts across both plant and animal taxa, and exhibiting a range of transmission methods. The other two groups of helminth parasites are the cestodes (Class: Cestoda) and trematodes (Class: Trematoda), which are both members of the platyhelminths, or flatworms (Phylum: Platyhelminthes). Cestodes are hermaphroditic worms, whereas trematodes can be monoecious or dioecious, and typically have more complex

life-cycles than other helminths, requiring transmission through both an intermediate and definitive host.

Levels of pathology resulting from helminth infection are variable. Intestinal worms typically lead to morbidity and do not generally cause extensive fitness costs (Hudson *et al.*, 2002). In human populations, gastrointestinal helminth infections have been linked to malnutrition, anorexia and reduced growth caused by appetite loss, diarrhoea and reduced nutrient uptake (Crompton, 1984; Hlaing, 1993; Stephenson, Latham and Ottesen, 2000). Helminth infections can also cause pathology of infected tissues resulting from host inflammatory immune responses (Pearlman *et al.*, 1997; D'Elia *et al.*, 2009).

The other key group of macroparasites affecting wild rodent populations are arthropod ectoparasites, commonly found in the fur. These parasites can come from a range of taxonomic groups within the arthropods, including insects such as lice (Order: Phthiraptera) and fleas (Order:Siphonaptera), and arachnids such as mites and ticks (Subclass: Acari). Many of these parasites feed directly on the tissue of the vertebrate host- commonly blood, but also skin and fur in some groups. Ectoparasites, as with gastrointestinal helminths, typically cause morbidity rather than mortality (Wall and Shearer, 2008). Infection on the skin is typically linked with inflammation of dermal tissue, and loss of variable amounts of blood and body mass (Lello and De Rosis, 2003).

The greater risk posed by these parasites is often the microparasites which they transmit. By breaking the skin barrier and exposing the host's blood to external infectious agents, ectoparasites can increase infection risk generally (Bandilla *et al.*, 2006). In addition, some ectoparasites act as vectors for specific microparasites, acting as an integral element of their transmission cycle. It is in this way that ectoparasites can be most harmful to their hosts, not as parasites themselves, but as transmission vectors. Notable examples of such parasites include *Babesia* and *Plasmodium*, two genera of apicomplexan parasites transmitted during blood-feeding by ticks and mosquitoes respectively (Kjemtrup and Conrad, 2000; CDC, 2018), and *Trypanosoma brucei,* a flagellate protozoa causing sleeping sickness and transmitted by the tsetse fly (Brun *et al.*, 2010). All three of these transmitted parasites can cause severe pathology and mortality if untreated (Murray *et al.*, 2012; Kennedy, 2013). As such, ectoparasites can indirectly have significant impacts on population health and fitness.

The precise definition of microparasites can vary between sources, with some including bacterial species, while others only including eukaryotic cells like protists and amoebas. For the purposes of this thesis, the term 'microparasite' will refer to both eukaryotic and prokaryotic organisms, and the distinction between the two will be made clear if necessary.

1.1.2 Parasites of British Rodents

Many parasitological surveys of British rodent populations have been conducted, which give an indication as to what parasite taxa might be expected to be present in a wild population of *M.m.domesticus*. Species previously recorded in wild rodent populations in the UK and around Europe include helminths, arthropods, and microparasites, and are summarised in Table 1.1. As can be seen, prevalences of parasites across populations are highly variable across all taxonomic groups.

* = non-UK population	Recorded Prevalences			
	Mus musculus	*Apodemus sylvaticus*	*Myodes glareolus*	*Microtus agrestis*
Nematodes				
Aspiculuris sp.	73.7% (Behnke, 1975) 6.2% (Singleton, 1985)* 12.8% (Kataranovski *et al.*, 2008)*	3% (Fuentes *et al.*, 2004)*	0-23% (Thomas, 1953) 6.4% (Sharpe, 1964)	-
Heligmosomoides sp.	39.2% (Kataranovski *et al.*, 2008)*	60% (Elton *et al.*, 1931) 56.5%-93.5% (Thomas, 1953) 80.6% (Behnke *et al.*, 1999) 29.5% (Sharpe, 1964)	54-75% (Thomas, 1953) 4.5% (Sharpe, 1964)	-
Syphacia sp.	2.3% (Behnke and Wakelin, 1973) 41% (Owen, 1976) 67% (Singleton, 1985)* 4.9-6.1% (Kataranovski *et al.*, 2008)*	32% (Elton *et al.*, 1931) 6.5% (Thomas, 1953) 54.6% (Sharpe, 1964) 56.7% (Behnke *et al.*, 1999)	-	-
Capillaria sp.	33% (Resendes *et al.*, 2009)*	7% (Elton *et al.*, 1931) R-26.5 U-43.5% (Thomas, 1953) 8.2% (Behnke *et al.*, 1999)	15.5-80% (Thomas, 1953)	-
Nematopiroides sp.	-	60% (Elton *et al.*, 1931)	-	-

Table 1.1 Recorded prevalences of commonly occurring helminth and arthropod macroparasites, and microparasites from populations of four - studied small rodent species from across the UK (*=non-UK population). Where percentage ranges are shown, prevalence data was recorded separately for neighbouring subpopulations.

Trichuris sp.	74.4% (Behnke and Wakelin, 1973) 2.6% (Kataranovski *et al.*, 2008)*	19.4% (Behnke *et al.*, 1999)	0-7.5% (Thomas, 1953)	-
Trichostrongylus sp.	-	-	7.5-10% (Thomas, 1953)	-
Longistriata sp.	-	-	2.5-54% (Thomas, 1953)	-
Pelodera sp.	-	26.1% (Behnke *et al.*, 1999)	-	-
Cestodes				
Catenotoenia sp.	5.9% (James, 1954) 7.2% (Kataranovski *et al.*, 2008)*	7% (Elton *et al.*, 1931) 6.8% (Sharpe, 1964)	18.6% (Sharpe, 1964)	-
Taenia sp.	1.4% (Kataranovski *et al.*, 2008)* 5.3% (Singleton, 1985)*	3% (Elton *et al.*, 1931) 3% (Behnke *et al.*, 1999)	-	-
Hymenolepsis sp.	1.8% (Owen, 1976) 7% (Singleton, 1985)*	0.4% (Elton *et al.*, 1931)	-	-
Trematodes				
Lyperosomum sp.	-	0.9% (Elton *et al.*, 1931)	-	-

Table 1.1 Recorded prevalences of commonly occurring helminth and arthropod macroparasites, and microparasites from populations of four -studied small rodent species from across the UK (*=non-UK population). Where percentage ranges are shown, prevalence data was recorded separately for neighbouring subpopulations.

Brachylaemus sp.	-	2% (Elton *et al.*, 1931) 3% (Behnke *et al.*, 1999)	-	-
Plagiorchis sp.	-	0.1% (Elton *et al.*, 1931)	-	-
Arthropods				
Nosopsyllus sp.	36.7% (Singleton, 1985)*	-	-	-
Mites (Trombidiformes)	93.9% (Singleton, 1985)*	-	-	-
Mites (Meostigmata)	39.8% (Singleton, 1985)*	-	-	-
Microparasites				
Babesia sp.	-	18.5% (Healing, 1981)	51.1% (Healing, 1981) 15.9% (Duh *et al.*, 2003)*	33.8% (Healing, 1981)
Bartonella sp.	-	10.5% (Healing, 1981)	8.3% (Healing, 1981)	16.2% (Healing, 1981)
Trypanosoma sp.	-	1% (Healing, 1981)	11.1% (Healing, 1981)	10.3% (Healing, 1981)

Table 1.1 Recorded prevalences of commonly occurring helminth and arthropod macroparasites, and microparasites from populations of four - studied small rodent species from across the UK (*=non-UK population). Where percentage ranges are shown, prevalence data was recorded separately for neighbouring subpopulations.

1.1.3 Parasite Life Cycles

The life cycles of parasites can greatly influence their distribution within a population. Most helminth infections result from ingestion of parasite eggs from the environment (Flynn, 2007b). The whipworm *T.muris* has a direct life cycle; adult worms embedded in the gut epithelium, feeding on syncytial protoplasm, and mate to produce fertilised eggs. Eggs are released and enter the environment with the faeces, after which they will embryonate within two months. After being ingested by the host, the eggs hatch, and first stage larvae become established in the gut mucosa, where they moult and become mature adults (*T.muris* life cycle reviewed in Flynn 2007; Wakelin 1969).

The pinworm *S.obvelata* has a similar, direct life cycle. Adult worms inhabit the lumen of the gut. Following mating, eggs are released, and either enter the environment, or are deposited on the perianal region, where they embryonate within a few hours. Infection occurs when embryonated eggs are ingested from the environment or perianal region (*S.obvelata* life cycle reviewed in Flynn 2007).

The hepatic parasite *Calodium hepaticum* persists in the liver and is typically only transmitted upon the death of the host via necrophagy of infected liver tissue, or through the release of eggs into the soil. Upon exposure to air, eggs embryonate within four to six weeks. The ingested eggs hatch in the intestine,

move through the intestinal wall and migrate to the liver via the hepatic portal vein, where they develop into adults in around 30 days. Adult male worms die off after around 33 days, leaving only female worms full of eggs (*C.hepaticum* life cycle reviewed in Wright 1961; Terrier et al. 1999; Yadav et al. 2016; Flynn 2007; Spratt & Singleton 1986; Lee 2009).

Infections by the apicomplexan parasite *Sarcocystis sp.* require a definitive carnivore host, and an intermediate host. After ingestion of sporocysts from the environment by the intermediate host, the parasite penetrates the gut wall and develops in the epithelium of blood vessels. Mature merozoites eventually penetrate into muscle or other tissue, where they form 'sarcocysts' full of bradyzoites, which are transmitted during predation (*Sarcocystis sp.* life cycle reviews in Buxton 1998; Markus et al. 1974; Flynn 2007).

Bartonella sp. is a vector-borne bacterium, transmitted by fleas and lice during blood-feeding (CDC, 2015). A number of species of *Bartonella* species have been recorded in rodent hosts, with the species most commonly identified in *M.musculus* being *Bartonella grahamii* (Houpikian and Raoult, 2001; Inoue *et al.*, 2009).The bacterium persists intracellularly in a primary niche, potentially incorporating a number of cell types including endothelial cells. The bacterium then migrates to the blood stream where it persists and replicates inside mature erythrocytes, until transmission to an arthropod

vector can occur during blood feeding (*Bartonella spp.* life cycle reviewed in Harms & Dehio 2012; Chomel et al. 2009).

Many species of fleas infecting rodent populations share similar life cycles (Flynn, 2007b). Adult northern rat fleas (*Nosopsyllus fasciatus*) lay eggs on the host, which fall from the host to the external environment, typically the host nest or burrow. Eggs hatch after around 4 days, depending on temperature and humidity, and proceed to moult through three larval stages. Larvae feed on the faeces of the adult fleas, containing blood from parasitized hosts. Upon maturation, an adult flea can sense vibrations from nearby hosts, and will jump on to one if nearby, where they will blood-feed on the host, mate and lay eggs (*N.fasciatus* life cycle reviewed in Flynn 2007; Sharif 1937).

Fur mites are typically transmitted directly between hosts, and as with fleas, life cycles are presumed to be largely similar between species (Flynn, 2007; Pritchett-Corning and Clifford, 2012). *Myobia musculi*, a fur mite of mice, infects juvenile hosts soon after birth, through direct transfer of reproducing females. Eggs are released by females, which hatch after 7-8 days. After around 10 days the hatched larvae will moult to the nymph stage, and after 15 days will develop into mature adults capable of further reproduction. The level of infestation in the fur of the host rapidly increases after host fur growth begins (7-8 days after birth), and infections can be carried for long

periods of time (*M.musculi* life cycle reviewed in Friedman and Weisbroth, 1977; Flynn, 2007; Pritchett-Corning and Clifford, 2012)

1.2 PARASITE IMMUNOLOGY

1.2.1 Vertebrate Immunity and the Role of T helper cells

The vertebrate immune system employs a diversity of cell types of varying origins and roles in order to effectively combat the variety of pathogens and parasites with which a wild animal will have to contend. The most basal division of immune defence is between innate and adaptive immunity. Innate immunity describes the non-specific first line of defence against infection, and includes physical barriers like skin and mucus. In addition, molecular mechanisms such as complement and antimicrobial peptides are produced and secreted (Turvey and Broide, 2010). It is typically characterised by inflammation of the involved tissue, and vasodilation and recruitment of certain cell populations to combat the infection. These cells include monocyte macrophages, neutrophils and basophils, which help to destroy infectious agents by phagocytosis (Beutler, 2004). Dendritic cells, a subpopulation of phagocytic myeloid cells, can present antigens from dead pathogens and induce an adaptive response (Chan *et al.*, 2006; Steinman and Hemmi, 2006).

Adaptive immunity is the means by which the immune system can respond to non-self antigens, and use pathogen-specific effector mechanisms to clear an

infection (Warrington *et al.*, 2011). An adaptive immune response is typically induced following an initial innate response (Bonilla and Oettgen, 2010). Adaptive immunity is largely maintained through the activity of thymus-derived T cells and bone marrow-derived B cells. Both innate and adaptive responses are employed in the defence against parasitic infections.

Crucial for the induction and maintenance of innate inflammatory immune responses, as well as all other branches of the immune system, are small signalling proteins called cytokines (Turner *et al.*, 2014). These proteins are produced and secreted by immune-associated cells, and allow the immune environment within a tissue to be dynamically controlled or altered in response to stimuli (Turner *et al.*, 2014). Different cytokines are associated with different types of immune response, and while some are linked with innate inflammation, they are also an essential element of adaptive immunity (Dinarello, 2007).

A major group of cytokines is the interleukins. The term 'interleukin' was developed as a way of grouping signalling molecules derived from leukocytes which play a role in controlling immunity. The first designated interleukins were interleukin-1 (IL-1, previously termed lymphocyte-activating factor) and interleukin-2 (IL-2, previously termed thymocyte stimulating factor), when it became apparent that the molecules being studied had a multitude of roles which were being attributed to separate factors (Mizel and Farrar, 1979).

Currently, the interleukins describe a large group of cytokines with a range of roles, but which often share little in terms of molecular identity (Brocker *et al.*, 2010).

Other key groups of cytokines include the tumour necrosis factor (TNF) family and the interferon (IFN) family. TNFs were named for their cytotoxic effects upon cancerous cells, and are typically involved in inflammatory responses (Tracey and Cerami, 1994). Interferons are named for their ability to 'interfere' with viruses, and are commonly involved in responses to infection by viruses and microparasites (Isaacs and Lindenman, 1957; Flynn *et al.*, 1993; Silva-Barrios and Stäger, 2017). Cytokines can cause changes in the immune state, but are also involved in complex networks of cross-regulation, mediated through induction of gene expression, interference of signalling cascades, and induction of apoptosis in specific cytokine-expressing cells (Yates *et al.*, 2000; Palucka *et al.*, 2005; Radtke *et al.*, 2010).

1.2.2 Adaptive Responses to Parasites

It is well established that different taxonomic groups of parasites require different types of immune response in order to effectively remove or suppress the activity of a parasite (Romagnani, 1997). While the innate response is a non-specific measure, the adaptive response can respond to previously-encountered pathogens in a specific manner resulting from presentation of

antigens (Zhu, Yamane and Paul, 2010). It is often characterised by the establishment of one of two types of immune environment, involving communication networks with characteristic cytokine profiles. These two main pathways are mediated by T helper cells (Th cells), which differentiate from naïve CD4 cells to either Th1 or Th2 type (Mosmann and Sad, 1996; Sethi *et al.*, 2013). These two responses can be identified by measuring levels of specific groups of cytokines involved in signalling and inducing specific immune-mediated outcomes, which are often involved in complex networks of control and feedback (Romagnani, 1997).

The Th1 response is characterised by expression of cytokines interferon gamma (IFNγ) and interleukin-12 (IL-12), which maintain a Th1 environment through positive feedback loops of cytokine induction (Trinchieri, 1998; Zhu and Paul, 2008). Inflammatory Th1 responses are also associated with expression of tumor necrosis factor alpha (TNFα) (Iwamoto *et al.*, 2007). The transcription factor T-bet, encoded by the gene T-box Transcription Factor 21 (*TBX21*), is a master regulator of the Th1 response, and directs the development of Th1 cells through induction of IFNγ expression (Szabo *et al.*, 2000; O'Connor *et al.*, 2013). Protective Th1 responses typically act against microparasites and intracellular pathogens, mediated through cell-mediated killing, phagocytosis and the oxidative burst (Warren and Vogel, 1985; Livingston *et al.*, 1989; Mosmann and Coffman, 1989; Mills *et al.*, 1993; Spellberg and Edwards, 2001). Th1-mediated protection against

microparasites has been demonstrated in models of infection by the protozoan parasites *Leishmania major* (Zimmermann *et al.*, 1998), *Trypanosoma cruzi* (Kumar and Tarleton, 2001) and *Plasmodium chabaudi* (Jacobs, Radzioch and Stevenson, 1996). If unchecked, excessive Th1 responses can cause immunopathology through the damage of healthy host tissue by inflammation (Liblau, Singer and McDevitt, 1995).

The Th2 response is generally described as an 'allergic response'. Signature Th2 cytokines are interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-9 (IL-9), interleukin-13 (IL-13), and a Th2-type response commonly involves the employment of eosinophils, basophils, proliferation of mast cells and increased production of immunoglobulin E (IgE) antibodies (Miller and Jarrett, 1971; Lundgren *et al.*, 1989; Parsons, Coffman and Grieve, 1993; Lai and Mosmann, 1999). The Th2 response is accompanied by a variety of physiological changes, including sloughing of the gut lining, changes in the mucosal lining of the gut, increased epithelial permeability, goblet cell hyperplasia and the production of reactive oxygen intermediates (Gause, Urban and Stadecker, 2003; Perrigoue, Marshall and Artis, 2008). This response is typically associated with resistance to extracellular pathogens and parasites, and is commonly associated with protective responses against helminths (Grencis, Hültner and Else, 1991; Else, Entwistle and Grencis, 1993; Fallon *et al.*, 2006; Michels *et al.*, 2006; Perrigoue, Marshall and Artis, 2008; Allen and Maizels, 2011; Neill and McKenzie, 2011).

A key characteristic of Th1/2 immune expression is polarisation, whereby if a response of one type is induced, it reduces the immune system's inducibility to the other, thus reinforcing a polarised immune environment. This again is achieved through cytokine expression; IL-12, associated with promoting Th1 expression, will actively suppress expression of the Th2 cytokine IL-4 (Manetti *et al.*, 1993). Conversely, the Th2-linked transcription factor *GATA3* binds to the transcription factor *Runx3*, preventing it from inducing IFNγ expression and thus inhibiting the establishment of a Th1 response (Yagi *et al.*, 2010). This polarisation allows suitable responses to specific infectious challenges to be maintained, but has implications for coinfected populations which can be infected with multiple types of parasite simultaneously (Fenton, Lamb and Graham, 2008).

Both branches of the T helper cell response are prevented from being overexpressed by another form of CD4-derived T lymphocyte called regulatory T cells (Treg cells). The immunosuppressive activity of these cells is dependent on the expression of a transcriptional regulator protein forkhead-box-P3 (*FoxP3*) (Brunkow *et al.*, 2001; Fontenot, Gavin and Rudensky, 2003; Hori, Nomura and Sakaguchi, 2003). These cells are associated with expression of the cytokines transforming growth factor beta (TGF-β) and interleukin-10 (IL-10), and act as a general immunosuppressant of both Th1 and Th2 activity (Liu *et al.*, 2003; O'Garra *et al.*, 2004).

Treg activity is counterbalanced by Th17 cells which generally promote inflammation, and are characterised by production of the cytokines interleukin-17 (IL-17), interleukin-21 (IL-21) and TNFα (Moseley *et al.*, 2003; Witowski, Ksiązek and Jörres, 2004; Iwamoto *et al.*, 2007; Korn *et al.*, 2007). Differentiation of Th17 cells is promoted by the transcription factor RAR-related orphan receptor gamma (RORγ), encoded by the gene RAR-related orphan receptor C (*RORC*). As with Treg cells, TGF-β is also required for their development, although here in the presence of interleukin 6 (IL-6) (Bettelli *et al.*, 2006; L. Zhou *et al.*, 2007). Shifts towards an inflammatory Th17 response can be a major source of autoimmune pathology (Lohr *et al.*, 2006). While Th1 and Th2 responses involve recruitment and proliferation of macrophages and eosinophils (granule-containing white blood cells associated with allergic immunity), respectively, a hallmark of Th17-type inflammation is neutrophilia (Weaver *et al.*, 2013; Sandquist and Kolls, 2018).

Th17 responses are thought to be protective against extracellular microparasites, with IL-17 receptor-deficient mice being more susceptible to the extracellular pathogens *Candida albicans* (Huang *et al.*, 2004) and *Klebseilla pneumoniae* (Ye *et al.*, 2001), but showing no change in susceptibility to the intracellular *Mycobacterium tuberculosis* (Khader *et al.*, 2005; Bettelli *et al.*, 2006; Bettelli, Korn and Kuchroo, 2007). This is in contrast

to Th1 responses, which are typically protective against intracellular pathogens (Bettelli *et al.*, 2006).

The role played by the innate response in helminth expulsion – if any exists- has remained somewhat unclear (Neill *et al.*, 2010; Neill and McKenzie, 2011). Some studies have, however, suggested that as with other adaptive responses, it is activation of the innate immune system by some molecular marker characteristic of helminths that causes downstream activation of the Th2 adaptive response. There are a number of candidate helminth markers that may fulfil this role, as reviewed by Perrigoue and colleagues, including glycans, proteases and chitin (Perrigoue, Marshall and Artis, 2008). It has also been found that specialised innate cells termed nuocytes play a key role in priming a Th2 response, quickly multiplying and spreading, while providing the necessary cytokine environment for Th2 activation- specifically through secretion of IL-5 & IL-13 (Neill *et al.*, 2010; Saenz, Noti and Artis, 2010). This Th2 induction results in a complex, reinforcing network between innate and adaptive immune cells of Th2-associated cytokines, ensuring an anti-helminth response is maintained until the worm is cleared (Anthony *et al.*, 2007). Polarisation of naive T cells and the role of different arms of the T helper cell response are summarised in Fig 1.1.

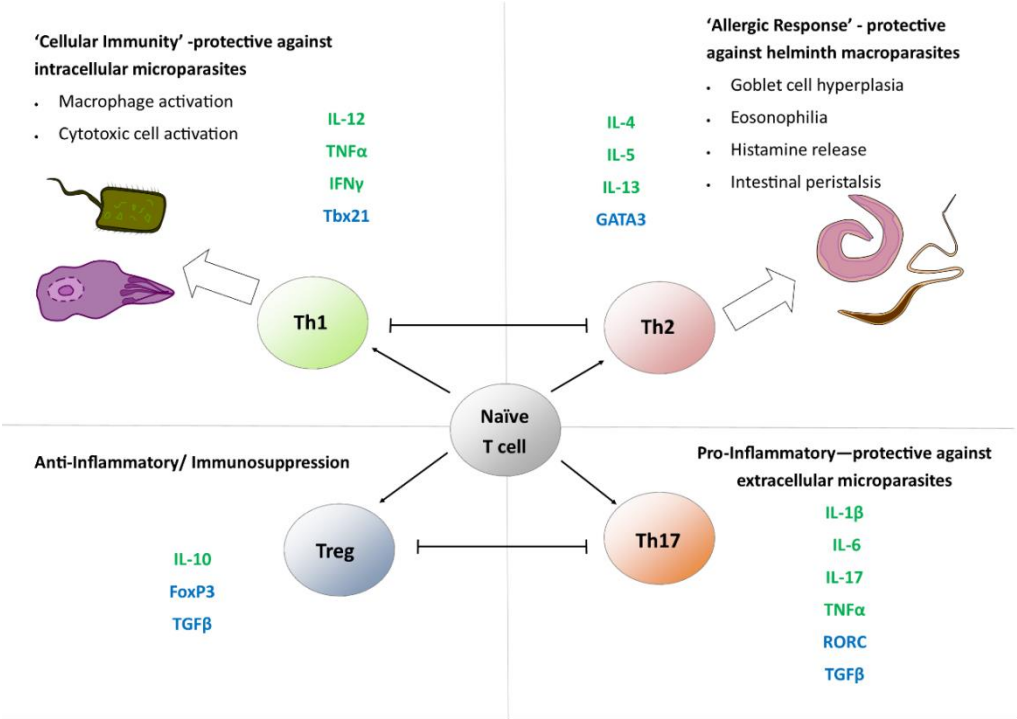


Fig 1.1 Diagram showing the differentiation of naïve CD4 cells into T-cell subsets, with associated cytokine profiles (green), and upstream transcription factors (blue).

1.3 DIFFERENCES BETWEEN LABORATORY ANIMALS AND WILD POPULATIONS

Rodents are frequently used as laboratory models, providing a strong theoretical and methodological foundation for research in the wild. House mice began to be used in biomedical research in the 17th century, and have been used increasingly ever since, being easy to house and breed, and with infection responses comparable to humans (Vandenbergh, 2000). House mice can have an important role in epidemiology on whole-ecosystem scales, as small rodent populations are known to be key reservoirs of pathogens and parasites infectious to other species, including humans (Mills and Childs,

1998). House mice specifically have been implicated as zoonotic sources of *Leptospira sp.* (Matthias and Levett, 2002), *Leishmania sp.* (Helhazar *et al.*, 2013) and *Borrelia sp.* (Gern *et al.*, 1998).The use of inbred strains of mice has helped to control for the confounding element of genetic variation in laboratory studies, while phenotypic differences between strains allows for the role of specific genes to be discovered (Beck *et al.*, 2000). The mouse genome was published in 2002, greatly increasing the potential of the species for studying the genetic underpinnings of different phenotypes, including those involved with immunity and defence against parasites (Waterston *et al.*, 2002). These factors make rodents, and particularly *Mus musculus* an extremely powerful tool for lab-based analyses. Despite the usefulness of laboratory mice studies, they have significant limitations, especially when applying findings to naturally infected populations.

Immunological findings from lab animals might not be directly applicable to wild populations, in part due to a lack of genetic diversity found in lab populations. Typically, lab mice are highly inbred strains (BALB/C etc.), which are used to study or control for the effects of specific genes, without any confounding effects of genetic diversity. While this is useful when establishing the fundamentals of immune function, particularly with regards to genetic control, wild populations *do* often display high levels genetic diversity, and so the behaviour of the immune system to parasitic challenge will be unlikely to precisely mirror that of a lab strain in a given wild individual (Guénet and

Bonhomme, 2003). Indeed, different strains of lab mice show different propensities to different kinds of immune response between each other, and from wild-caught individuals (Reidl, Elliott and Steiner, 1986; Abolins *et al.*, 2011).

This effect is compounded when whole-genome diversity is considered, as epigenetic, epistatic and pleiotropic effects can often hold untold significance in gene expression (Schmid-Hempel, 2003; Turner and Paterson, 2013). Epigenetic effects are likely to be significant in affecting phenotype of populations inhabiting heterogeneous environments, and have been observed in the physiology of wild populations of house mice (Berry, 1963; Berry and Searle, 1963). Epistasis (Caron *et al.*, 2005; Desrosiers *et al.*, 2005) and pleiotropy (Tracey and Cerami, 1994; Neville, Mathiak and Bagasra, 1997; Kaisho and Akira, 2004) have been found to impact phenotype in controlled infection models of inbred mouse strains, and are thus likely to be significant across genetically diverse populations. Using inbred strains in lab-based studies is, however, extremely useful and necessary as a foundation for ecoimmunological research, and insights gained into the complex nature of the immune system from lab models can be applied to a more 'realistic' spread of genetically diverse individuals.

Another issue concerning the use of lab experiments is the difference in conditions compared to wild habitats. There are numerous environmental

factors impacting every stage of an individual's life-cycle in the wild, which may be vastly different in a laboratory (Latham and Mason, 2004). Most modern laboratory conditions are generally less stressful than living in the wild (Pedersen and Babayan, 2011). This is due to a lack of predation, little exposure to adverse weather, minimal chance of non-intended infection by pathogens, an ample supply of food and a reduction in intraspecific competition for resources, territory or dominance (Pedersen and Babayan, 2011). As a result of these conditions, an individual is not experiencing the constant cost-benefit trade-offs between traits required for continued survival and reproduction.

An immune response given in the wild will not necessarily have evolved to be the most efficient way possible of destroying or removing a pathogen, but instead to provide the best response possible when other essential fitness measures are taken into account. Differences observed between resistant and tolerant strains of lab mice do not necessarily indicate that the resistant strain would outcompete the other in the wild, as it may not have the metabolic resources to mount such a response under food competition, or may suffer immunopathology. Although laboratory studies are controlled and convenient to carry out, they are far removed from the environmental conditions in which any trait being observed originally evolved.

Genetic and environmental differences do indeed lead to immunophenotypic differences, as demonstrated by Abolins and colleagues (Abolins *et al.*, 2011). Through measurement of a number of immune parameters, including antibody and cytokine expression, and immune tissue morphology, they found that wild *M.musculus* generally had greater immune responses than C57BL/6 strain lab mice, both in response to antigenic challenge and at a baseline level. Although numbers of B cells (antibody-secreting cells derived from the bone marrow) and splenocytes (cells from the spleen, an organ important in controlling immune state) - both cell types crucial to proper immune function – were significantly lower in the wild mice, they were far more active with higher turnover. This is perhaps indicative of greater levels of metabolic efficiency required when facing the environmental stresses of the wild. Additionally, wild mice had lower growth rates, exemplifying a potential trade-off between different metabolic burdens that may not be manifest to the same extent in laboratory conditions (Abolins *et al.*, 2011).

In wild populations, host immune state and physiology, current infectious status, infection history (the infectious challenges experienced by the host throughout its lifetime), and genetic background all interact with host ecology and seasonal environmental variation to affect an outcome of infection in the host, which may cause changes in condition and fitness. These factors are summarised in Fig 1.2. Accounting for all of these variables, and the possible combinations of interactions between them, can be challenging, as most

studies will be limited in the amount of data that can be collected and evaluated.

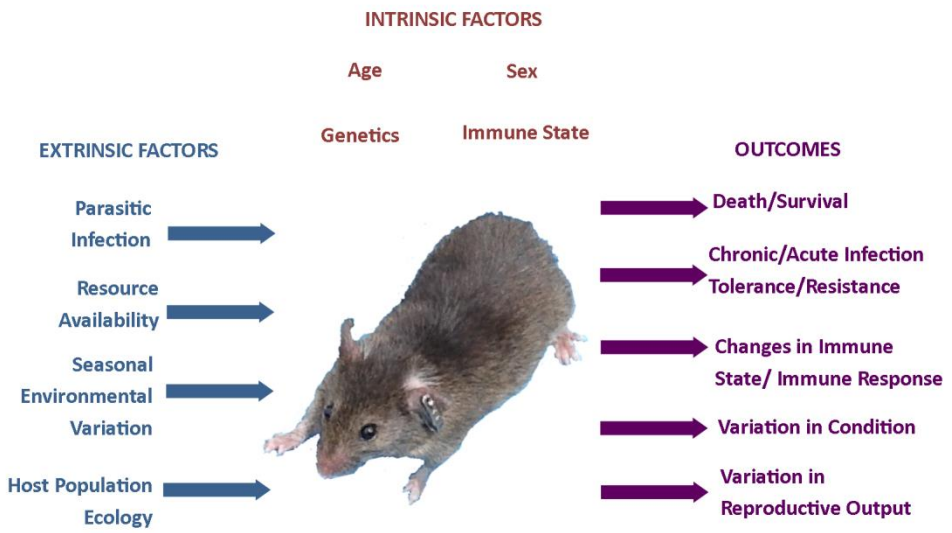


Fig 1.2 Summary of extrinsic and intrinsic factors affecting host fitness and outcomes of infection, that should be considered during ecoimmunological studies of wild populations.

Some studies have, however, attempted to use wild rodent populations to perform comprehensive ecoimmunological studies. qPCR analysis of immune gene expression in cultured splenocytes of a field vole (*Microtus agrestis*) population in Kielder Forest has been used to assess the influence of seasonality, age, reproductive status, host condition, genetic diversity and infection status on splenic cytokine expression levels (Jackson *et al.*, 2011; Turner *et al.*, 2011; Taylor *et al.*, 2018). The observations made from this population have given new insight into the control of immunity in the wild.

Jackson and colleagues performed cross-sectional surveys on this population, and carried out splenocyte cultures, both unstimulated, and stimulated with toll-like receptor (TLR) antagonists and mitogens (Jackson *et al.*, 2011). qPCR analysis of a selection of pro- and anti-inflammatory genes was then performed on these cultured splenocytes, and the results analysed alongside a range of ecological, morphological and environmental variables. Seasonality was found to have the strongest impact on immunity, with pro-inflammatory gene expression reaching a maximum in late winter, and a minimum in the autumn. Pro-inflammatory expression was also associated with poorer condition, while anti-inflammatory immune expression was lower in pregnant than non-pregnant females, and lower in mating males than non-mating males.

Within this same population Turner and colleagues also demonstrated how genetic diversity within immune genes acts as an intrinsic source of variation in immune expression and parasite resistance (Turner *et al.*, 2011). More recently, Taylor and colleagues demonstrated that two bloodborne microparasites, *Babesia microti* and *Bartonella spp.* display antagonistic interactions, with *B.microti* infection causing splenomegaly and increased IL-10 expression (Taylor *et al.*, 2018). Neither of these parasites, however, were found to significantly impact survival or reproductive output. By utilising a range of ecological and immunological methods and analyses within a well-characterised wild population, an increasingly detailed picture of the complex

ecoimmunological dynamics within the Kielder field voles is being established, and serves as a strong foundation for further ecoimmunological research.

A cross-sectional study of wild *M.musculus* populations in south-west England aimed to comprehensively determine the ecological factors contributing to immune state in wild populations (Abolins *et al.*, 2018). By taking different measures of immunity, including concentration of IgG, IgE antibodies and acute phase proteins in serum, faecal IgA levels, and numbers of a range of immune-associated cell types, they found that different aspects of host immune state are affected differently by various ecological variables. Age and day length affected both cellular and immune responses, with the condition of the host only impacted the former. They also observed associations with microbial infections, which differentially affected males and females. This study provides a comprehensive assessment of how host immunology is impacted by ecology and environment, gives a good foundation upon which to explore these dynamics alongside more detailed parasitological data, and provides a point of comparison for determining how generalisable ecoimmunological dynamics in *M.musculus* are across distinct populations.

1.4 FITNESS AND CONDITION EFFECTS OF INFECTION AND IMMUNE RESPONSE

In order to understand the impact of ecoimmunological and parasitological dynamics of wild population in an evolutionary context, their relationship with the condition and fitness of the animals being studied must be considered. The best measures of 'true fitness' are typically obtained through longitudinal study, in which survival and reproductive success of individuals can be monitored over time. For example, such studies have been used to highlight impacts of cowpox infection upon survival and reproduction rates in wild populations of *Apodemus sylvaticus* and *Clethrionomys glareolus* (Telfer *et al.*, 2002, 2005), and impacts of mounting immune responses on host survival in eider, *Somateria mollissima* (Hanssen *et al.*, 2004). Fitness can also be estimated from measures of condition, including visual measures of fat reserves, and condition indices, which are generally calculated from an animal's mass relative to other morphometric measurements, as an indication of its metabolic energy reserves (Jakob *et al.*, 1996; Stevenson and Woods, 2006). There is, however, debate as to what extent condition indices give an accurate representation of energy reserves (Schulte-Hostedde *et al.*, 2005; Schamber, Esler and Flint, 2009), and whether measurements of condition can be taken as a proxy for true fitness (Gaillard *et al.*, 2000; Wilder, Raubenheimer and Simpson, 2016). Biochemical measurements can also be used to estimate fitness, with concentration of the hormone leptin from blood serum commonly used as an indicator of levels of adipose tissue in the host, from which it is primarily produced (Gentry *et al.*, 2002; Sagawa *et al.*, 2002;

Gámez-Vázquez *et al.*, 2008; Spady *et al.*, 2009). Condition indices and fitness estimation are further discussed and explored in Chapter 6.

1.5 ECOIMMUNOLOGY RESEARCH AND METHODOLOGY

Obtaining a clear picture of the internal state, including the immune environment and infection status, of a wild animal has long been methodologically challenging. Studying wild populations in the field often prohibits access to laboratories in which more sophisticated assays can be performed. Advances in methodology in recent decades have been a key element in facilitating ecoimmunological study. The vast array of immunological techniques developed in biomedical research contexts has been combined with traditional ecological and parasitological research practices to provide great insight into the behaviour of the immune system in the wild. Throughout this thesis we use a combination of lab-developed assays and field techniques to provide a dataset rich for ecoimmunological analysis.

Different immunological methods can give qualitatively diverse information regarding the immune state of an animal (Pedersen and Babayan, 2011). Deciding which immune parameters may be relevant in determining the outcome of infection by a given parasite is crucial for understanding specific aspects of immunity. A number of immunological methodologies have been

established in laboratory settings, and some of these have been applied to varying extents to wild populations.

1.5.1 Organ Mass as an Indicator of Immune Function

The mass of organs involved in immune function has been commonly used to assess immune activity in wild animals (Corbin *et al.*, 2008). Spleen mass been used as an indicator of the number and rate of production of lymphocytes in birds (Fänge and Silverin, 1985; Birkhead, Fletcher and Pellatt, 1998; Møller *et al.*, 1998; Møller and Erritzøe, 2000). In rodents, spleen mass has found to be associated with infection by a number of micro- and macroparasites. Laboratory rat strains with greater spleen mass show increased survival after infection with the bacterium *Streptococcus pneumoniae* (Van Wyck *et al.*, 1980), while laboratory house mice display splenomegaly in response to infection by the helminth *Heligmosomoides dubius* (Ali and Behnke, 1985). Similarly, wild populations of the montane vole, *Microtus montanus*, show an association between splenomegaly and *B.microti* infection (Watkins *et al.*, 1991), while laboratory populations show a similar response to *Trypanosoma gambiense* infection (Seed *et al.*, 2006). Despite these results, the usefulness of using spleen mass as a measure of immunity has been questioned, as the spleen has many roles within maintenance of the blood and the immune system, including filtration of the blood for the detection of pathogens, and the development, induction and control of systemic inflammatory response to infections (Bronte and Pittet, 2013). Measurement of spleen mass can at best

give a scalar measure of general immune function, rather than a qualitative idea of the type of immune response being generated (Smith and Hunt, 2004; Corbin *et al.*, 2008).

Increased thymus size has been shown in laboratory rats to be strongly associated with numbers of leukocytes in the blood (Kelley *et al.*, 1986). Studies of human populations have also found strong correlations between having a larger thymus volume in infants with reduced mortality and increased numbers of CD4 cells (Vigano *et al.*, 2000; Aaby *et al.*, 2002). Increases in the mass of the mesenteric lymph nodes, a secondary lymphoid tissue, have also been observed in experimentally infected mice (Ali and Behnke, 1985). Measuring differences and changes in the mass of lymphoid organs can give useful information about the immune function of wild populations when access to more sophisticated assays is limited, though these measurements should be interpreted only as a basic metric of immune state (Demas *et al.*, 2011).

1.5.2 Responses to Mitogens

Immune function can be assessed by inducing a stimulated immune response through the introduction of mitogens, both *in vivo* and *in vitro*. Different types of leukocytes respond in diverse ways to different mitogens, so these techniques can give information about the various arms of the immune

system (Gery, Gershon and Waksman, 1972; Lazda and Baram, 1974). One of the most commonly used mitogens used in ecological studies is phytohaemagglutinin-A (PHA), a plant-derived lectin which stimulates a T cell response (Adler *et al.*, 1970; Stadecker *et al.*, 1977; Goto *et al.*, 1978; Kupper, 1990). PHA can be injected subcutaneously, with the amount of swelling of the affected tissue taken to be representative of the intensity of cell-mediated response (Smits, Bortolotti and Tella, 1999). Many laboratory studies have used PHA stimulation of cultured spleen or other lymphoid cells in order to assess levels of immune response to challenge. It can be used to measure cell proliferation or the induction of cytokines (Tursi *et al.*, 1969; Gery, Gershon and Waksman, 1972; Lindahl-Magnusson, Leary and Gresser, 1972; Ron, Laufer and Gery, 1973; Gillis *et al.*, 1978).

The measurement of swelling of dermal tissue in response to PHA in wild animals was pioneered and has been widely used in bird populations (Lochmiller, Vestey and Boren, 1993; Casto, Nolan and Ketterson, 2001; Ewenson, Zann and Flannery, 2001), and has since been adapted for other animals including wild rodent populations (Shepherd *et al.*, 1986; Sinclair and Lochmiller, 2000; Derting and Compton, 2003; Goüy de Bellocq *et al.*, 2006). Although stimulation of cultured cells can be logistically difficult when studying wild animals, it has been used in a wild population of the field vole *Microtus agrestis* alongside qPCR analysis of immune gene expression,

providing a more detailed picture of response to the stimulation of immune cells (Jackson *et al.*, 2011; Taylor *et al.*, 2018).

Bacterium-derived lipopolysaccharide (LPS) is also commonly used as a mitogen, which binds to toll-like receptors (TLRs), and induces both innate immunity (Poltorak *et al.*, 1998; Prendergast *et al.*, 2004; Creely *et al.*, 2007) and a B cell-derived antibody response (Coffman *et al.*, 1986; Leadbetter *et al.*, 2002). Other antigens commonly used in laboratory research include keyhole limpet protein (KLH) and sheep red blood cells (SRBC), which induce T-cell dependent antibody responses (Herscowitz, Harold and Stavitsky, 1972; Doherty, 1981; Ladics, 2007; Tonomura *et al.*, 2008). Utilisation of such techniques, alongside molecular assays which assess the immune response to stimulation in detail, is a promising method for future ecoimmunological research.

1.5.3 Quantifying Cytokines

Of increasing prevalence and importance to ecoimmunology are molecular techniques, which can be used to measure the expression levels of immune genes, or concentrations of immunity-associated proteins. Quantitative PCR (qPCR) was developed as a sensitive means of measuring concentrations of DNA, and through reverse transcription, mRNA (Heid *et al.*, 1996; Fleige *et al.*, 2006; Nolan, Hands and Bustin, 2006). This method has been used in

laboratory studies of rodents to measure expression levels of cytokines, signalling molecules and transcription factors associated with immune function (Waris *et al.*, 1996; Chen *et al.*, 2005; Nakazawa *et al.*, 2006; Zaiss *et al.*, 2006; Garbett *et al.*, 2008; Li *et al.*, 2010; Shimamoto *et al.*, 2012; Xu *et al.*, 2013). It has also been shown to reveal the activity of the immune system in response to PHA that are not detectable through simple measurement of tissue swelling (Vinkler *et al.*, 2014).

qPCR has been used to a lesser extent in studies immunity in wild populations. It was employed in a study of the population of field voles (*M.agrestis*) in Kielder forest to measure changes in cytokine expression under different ecological and morphological conditions, and under infection with microparasites (Jackson *et al.*, 2011; Taylor *et al.*, 2018). Obtaining a comprehensive suite of qPCR primers for a given species when full genomes are not available can be challenging, and for the Kielder voles, a new set of primers for all genes of interest needed to be developed through *de novo* sequencing of immune genes, which utilised primers designed from alignments of multiple existing rodent sequences (Jackson *et al.*, 2011). Obtaining qPCR primers for model species is generally much more cost effective and efficient, as they will often be already designed and available, or can be more easily designed by accessing widely available genome data.

Concentrations of circulating cytokines, antibodies and other molecules involved in immunity in the blood can also be measured through enzyme-linked immunoabsorbent assays (ELISAs) (Engvall and Perlmann, 1971). Stimulation of splenocytes with a suite of mitogenic agonists, followed by ELISA measurements of cytokine levels, was successfully used to assess the factors impacting levels of TNFα in a wild population of the wood mouse (Jackson *et al.*, 2009). Multiplex ELISAs are able to detect levels of multiple proteins at once, and so can be more efficient during broad- spectrum analyses (Ray *et al.*, 2005). These techniques have been used to detect levels of immune cytokines in various strains of lab rodents, and thus show promise for similar applications in wild populations (Demas *et al.*, 2002; Zysling and Demas, 2007; Farnsworth *et al.*, 2015). Care should be taken when using this approach, however, particularly when measuring responses to infections, as viruses including vaccinia, cowpox and ectromelia have been shown to produce proteins homologous to host proteins involved in immunity, such as interleukin binding and complement proteins (Miller, Shchelkunov and Kotwal, 1997; Smith, Bryant and Alcamí, 2000). As such, in naturally infected populations with potentially unaccounted-for infections, care should be taken to account for any risk of binding to non-target proteins.

1.5.4 Tissue Histology

Observation of the structure of lymphoid tissues is a well-established but destructive method of obtaining a snapshot of an animal's immune effector

mechanisms and the impacts of infection on the affected tissue (Cesta, 2006). Lab infection models have shown that parasites can cause significant changes to tissues. For example, infection with the nematode *Schistosoma mansoni* causes hepatic granuloma formation in susceptible animals (Fallon *et al.*, 2000). Granulomas are also observed during zoonotic infection with *Bartonella spp.*, alongside other histological changes including angiomastosis and lymphoadenopathy (Lamps, Gray and Scott, 1996; Cheuk *et al.*, 2006; Caponetti *et al.*, 2009).

The gastrointestinal helminth *Nippostrongylus brasiliensis* causes mass proliferation of tuft cells in the intestinal epithelium in mice exerting a Th2 resistant phenotype (Gerbe *et al.*, 2016). These changes can be observed by specifically staining sections of tissue, to highlight the cell population of interest. Stains can be linked to antibodies specific to host cell types, such as UCHT1 and DAKO-Pan B, which will stain T-cells and B-cells, respectively (Ghosh, Spriggs and Mason, 1985). The usefulness of histological analysis in wild populations is debatable – while histological analyses have been found to support PHA-based measurements of immunity in wild birds (Martin *et al.*, 2006), in wild frogs, swab qPCRs were found to be a more reliable indicator of infection status (Kriger *et al.*, 2006). As histological methods require either the death of an animal or disruptive tissue biopsy, they are of most use during destructive sampling of wild populations.

1.5.5 Diagnosing Parasitic Infection

While parasitic infection can cause characteristic phenotypes in the host, a number of techniques can be used to identify the specific taxon of parasite present. Arthropod ectoparasites are perhaps the easiest to survey, as they are typically large enough to be easily observable under a microscope, and through manipulation of fur with dissection forceps (Auffray *et al.*, 2011a). Additionally, being external to the host, no dissection or molecular techniques are required. There is, however, a risk of loss of adherent parasites during trapping and handling, as well as difficulties with surveying parasites in animals with wet fur after rain.

For gastrointestinal helminth species, dissections of the gut are commonly used to identify the taxa of parasite present and quantify the infection burden, typically with the use of a microscope (Montgomery and Montgomery, 1990; Pakdel *et al.*, 2013; Sepulveda and Kinsella, 2013; Zarei *et al.*, 2016). Reliable estimates of helminth burden can be made using faecal egg counts, as a non-lethal alternative to dissection (Roberts and Swan, 1981; Sithithaworn *et al.*, 1991). Egg shedding is, however, a dynamic process, which can vary significantly between parasite species and between hosts (Keymer and Hiorns, 1986). Some helminth parasites, such as *Strongyloides sp.*, show non-linear associations between faecal egg counts and adult worms dissected in the gut, as a result of density-dependent impacts on parasite fecundity, mediated by host immunity (Paterson and Viney, 2002; Romeo *et al.*, 2014).

Adult mouse whipworm (*T.muris*) has been shown to alter the rate of success of its own egg hatching through interactions with the host microflora, which may affect the relationship between excreted eggs and adult worm burden (Hayes *et al.*, 2010; White *et al.*, 2018). Faecal egg counts may therefore be more useful as presence/absence indicators of infection, or measures of environmental infection risk (Sargison, 2013).

Microparasite species can be observed and identified through staining and subsequent microscopy for visual identification (Flynn, 2007a). For example, Steiner Silver stain can be used to identity *Bartonella* cells (Caponetti *et al.*, 2009), while Giemsa stain can be used to observe a range of common microparasites, including *Babesia sp.* and *Trypanosoma sp.* (Auffray *et al.*, 2011b). For some larger microparasites, such as *Trypanosoma sp.*, the distinctive morphology of the parasite allows for easy visual identification under a microscope (Auffray *et al.*, 2011b). Although somewhat time consuming, staining and microscopy allows for quantification of the parasite through cell counting.

Microparasites are often detected through PCR of taxon-specific genes from a tissue sample taken from the host, commonly blood. Primers specific to a parasite taxon of interest can be used, with commonly-targeted genes include the 18s ribosomal RNA gene in eukaryotes (Jauregui *et al.*, 2001; Akhavan *et al.*, 2010), and the homologous 16s ribosomal RNA gene in prokaryotes

(Adelson *et al.*, 2004). This technique has been used to detect a range of parasites, including *Leishmania sp.* (Akhavan *et al.*, 2010)*, Babesia sp.*(Persing *et al.*, 1992)*, Trypanosoma sp.* (Noyes *et al.*, 1999) and the prokaryotes *Bartonella sp.* and *Anaplasma sp.* (Adelson *et al.*, 2004). Visualisation of PCR product through gel electrophoresis will give an indication of presence or absence of the parasite of interest (Weiss, 1995).

While traditional PCR is not quantitative, qPCR can be used to measure concentrations of parasite DNA, and thus an indication of infection burden. This technique has been used to survey hepatitis and hokovirus infection in wild boar (Adlhoch *et al.*, 2009, 2010), and *Plasmodium sp.* infection in blue tits (Knowles, Palinauskas and Sheldon, 2010). While PCR is more commonly used for microparasite detection, it can also be used to detect macroparasites. For example, the helminth *Strongyloides stercoralis*, can be detected through PCR of faecal samples (Verweij *et al.*, 2009), though the sensitivity of this approach may be lower than other methods (Knopp *et al.*, 2014). PCR is a powerful and popular method of infection diagnosis, but requires access to laboratories not always available in field studies of wild populations, as well as primers specific to the parasite taxon of interest.

1.5.6 Cross-Sectional vs Longitudinal Studies

Ecological analyses are typically in one of two forms. Longitudinal studies involve taking measurements from individuals at different time points, e.g. throughout the year, between years etc. This allows any temporal patterns in parasite prevalence, immune responses, survival, reproduction rate and any other relevant measures to be observed. These studies are typically limited to using samples obtained in a non-invasive manner; for example, blood samples, faecal samples, small external tissue samples and gross morphometry such as body length and weight. Longitudinal studies are important for ecoimmunology, as they allow individuals to be monitored over time, and the causality and duration of interactions between parasites, host immune function, and environmental variables to be better understood.

Longitudinal studies are also useful for cross-generational research, where specific genetic and phenotypic traits can be analysed in successive generations of offspring. This has been used to assess heritability of antibody responsiveness in Soay sheep (*Ovis aeris*) (Graham *et al.*, 2010), and of survival and fitness measures in wild blue tits (*Cyanistes caeruleus*) (Papaïx *et al.*, 2010).

Cross-sectional studies involve destructive sampling of a proportion of the population at distinct points in time. This technique has limitations, as

changes within individuals in the population through time are not monitored, and any observed effects must be inferred from a 'snapshot' analysis of many individuals. This can mean that any inferences from the data are less reliable; for instance age can often be hard to determine in non-human hosts at any moment in time, and so effects of age may be incorrectly accounted for (Bradley and Jackson, 2008). It does, however, mean that the research is less limited in terms of methodologies, and a broader selection of immunologically relevant tissues can be further analysed. The methods used throughout this thesis are all taken from cross-sectional sampling.

1.5.7 Age Estimation

While basic visual indicators (e.g. bodysize) can give a rough idea of an animal's age through broad distinctions between 'juvenile' and 'mature' animals, cross-sectional studies allow for age to be ascertained through more quantitative means. Eye lens mass is commonly used to estimate age as it grows continuously throughout an animal's lifespan (Augusteyn, 2008). There is, however, some debate as to its reliability; slight sources of error were found to be present in the estimated value in common voles, associated with trapping history and mass changes affected by female reproductive state (Jánová, Havelková and Tkadlec, 2007; Jánová, Nesvadbová and Tkadlec, 2007).

Despite this, continuous growth in eye lens weight with age has been reported in at least 130 species (Augusteyn, 2014). When compared to body mass, body length, tail length, foot length and ear length in corn mice, eye lens mass proved the most reliable at discriminating age, with the addition of other morphometric measurements providing no improvement to the estimations (Carreno *et al.*, 1990). While measuring insoluble lens proteins has shown to improve reliability of age estimation in older laboratory cotton rats (130 days+), the benefits of this method in a wild population, in which individuals will generally die younger, should be weighed against the extra time and resources involved (Birney, Jenness and Baird, 1975).

1.6 *MUS MUSCULUS DOMESTICUS* ON THE ISLE OF MAY – HISTORY AND ECOLOGY

The house mouse (Fig 1.3) is extraordinarily widespread across the globe, as a result of its generally commensal lifestyle with human populations and high adaptability to various climates and habitats (Berry and Jakobson, 1975). Mitochondrial DNA analysis from a global sample of mice has shown that the ancestral *M.musculus*

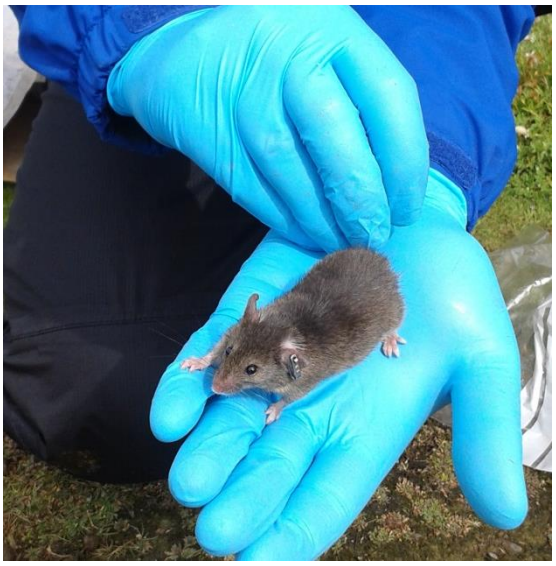


Fig 1.3 The house mouse, *Mus musculus domesticus* on the Isle of May [photograph by author].

population likely originated in southwestern Asia, and spread globally alongside the development of agricultural practices (Suzuki *et al.*, 2013). The close ties with historical human migration have given *Mus musculus sp.* a unique phylogeographical history (Bonhomme and Searle, 2012). While taxonomic divisions have been disputed, it is currently generally accepted that *M.musculus* split into subspecies corresponding to different regions of global expansion, with *M.m.domesticus* spreading across most of western Europe (Rajabi-Maham, Orth and Bonhomme, 2008; Bonhomme and Searle, 2012).

This widespread global distribution is a key feature of house mouse ecology, but can also prove a complicating factor when regarding its study. This is because observation made on any one population may not hold true for mice inhabiting a different ecosystem or habitat. Specifically, for the population of interest in this thesis, the mice are unusual in that they live largely a feral lifestyle on a small island. Island populations, particularly those which are not subject to high amounts of human activity, offer a valuable opportunity to elucidate interactions between well-characterised parasite populations, with minimal risk of disturbance from factors like the introduction of novel pathogens or parasites (Greenhough, 2006). The feral population of *M.m.domesticus* on the Isle of May is an ideal case subject for answering questions about parasite interactions in wild populations.

The Isle of May is a small island, approximately 500m across and 1.8km long, in the Firth of Forth, Scotland (Fig 1.4). The first record of mice on the Isle of May is from 1882, from the account of a resident lighthouse keeper, though as there had been small settlements on the island previously, it is unclear when they were first introduced (Muir, 1885; Triggs, 1991). In 1982, a population of 77 mice from Orkney were released onto the island in an attempt to assess levels of gene flow between isolated populations of a species (Berry *et al.*, 1990; Scriven, 1992). The introduced mice from Orkney carried three Robertsonian fusions i.e. fusion of non-homologous chromosomes (2n=36), whereas the native Isle of May population did not (2n=40). These Robertsonian fusions are characteristic of mouse populations from North-East Scotland, and are thought to be markers of a distinct population, derived from Scandinavian mice which migrated to Scotland on Viking ships (Searle *et al.*, 2009). Hybrids between the native and introduced mice on the Isle of May were observed 6 months after the introduction, and after four years, both allelic and chromosomal frequencies had stabilised across the island, indicating successful introgression of the introduced population (Berry *et al.*, 1990; Scriven, 1992).

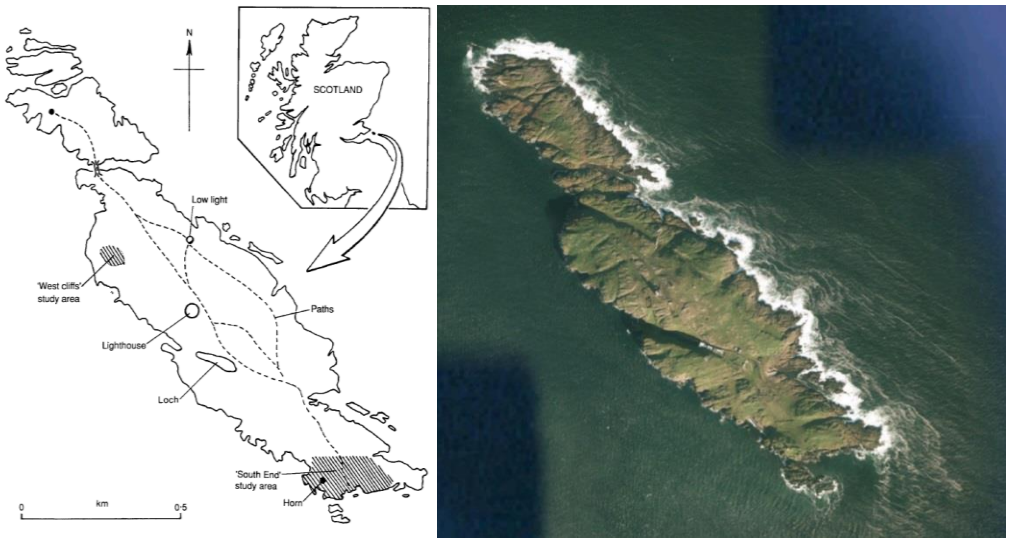


Fig 1.4 Maps showing the layout and geographical position of the Isle of May, Scotland (L) Taken from Triggs (1991) (R) Taken from Google Earth.

While parasitological surveys of this population are largely lacking, an analysis of 12 dead mice from the island made in 1938 identified the presence of the gastrointestinal nematode *T.muris* in the caecum of 6 of the mice (Southern, 1938). Nine of the mice were infected with mites (*Aleurobius farinae*), and 3 with Northern rat fleas (*N.fasciatus*). While this survey was limited, it gives an idea of the types of parasites that may be present in the population, and indicates that coinfection is common, as 5 of the 12 mice were infected with at least 2 of the 3 recorded species.

The ecology of the Isle of May mice has been recorded previously, which provides a useful foundation for this ecoimmunological study. Much of this study is constituted by a 1991 paper by Triggs (Triggs, 1991). Using mark-recapture methods across the whole island, he made a number of observations and estimations of population structure. Notably, the population

numbers were found to dramatically vary both throughout the year, and annually, primarily due to variation in temperature, with estimates ranging from 450 mice in September 1970, to 3250 mice in January 1972. Specifically, numbers increase from a Spring low to a peak in mid-Winter. Mice were found to be distributed throughout the island, but remained within given territories which overlapped each other. These factors could all have implications for parasite transmission. Another crucial element of this study is Triggs' finding that the mice tend to live 'close to their physiological limits' as a result of natural selection. Male-male competition has found to be largely absent in this population, in contrast to mainland populations, due to the infeasibility of maintaining strictly controlled territories within an environment of scattered resources and negligible predation, and reduced aggression exhibited by males in response to other males (Gray and Hurst, 1998).

The mouse population shares the island with another resident mammal, the European rabbit (*Oryctolagus cuniculus*), and a seasonal population of grey seals (*Halichoerus grypus*). Some endoparasite and ectoparasite genera recorded in wild *O.cuniculus* populations are also observed in *M.musculus* populations. While some of these parasites, such as *Trichuris spp*. infect the host in a species specific manner (*T.muris* in mice and *T.laporis* in lagomorphs such as rabbits), others, such as *N.fasciatus* can infect both hosts (Shepherd and Edmonds, 1973; Eira *et al.*, 2007). Levels of cross-infection between

rabbits and mice on the island are currently unknown, although Triggs observed no effect of co-habitation between species on mouse populations (Triggs, 1991).

In addition to these mammal populations, a number of seabird populations nest on the island, including European shags (*Phalacrocorax aristotelis*), guillemots (*Uria aalge*), black-legged kittiwakes (*Rissa tridactyla*), razorbills (*Alca torda*), Northern fulmar (*Fulmarus glacialis*), arctic and common terns (*Sterna paradisaea & Sterna hirundo*), herring and lesser black-backed gulls (*Larus argentatus & Larus fuscus*), common eider (*Somateria mollissima*), Eurasian oystercatchers (*Haematopus ostralegus*) and Atlantic puffins (*Fratercula arctica*) (Hemsley, 2008; CEH, 2016). The puffin population dig burrows close to the island cliff-edges in which to nest, which are used by the mouse population for shelter when vacant.

Although these species annually nest on the island, a large number of birds from a global distribution can be seen on the island throughout the year while stopping along their migratory routes. While the parasite communities of birds on the Isle of May have not been comprehensively surveyed, the kittiwake and puffin populations have been found to host the seabird tick *Ixodes uriae* (Danchin, 1992; McCoy *et al.*, 2003) and the shag population has been shown to suffer from the nematode endoparasite *Contracaecum rudolphii* (Burthe *et al.*, 2013; Hicks *et al.*, 2018), none of which typically infect

rodent populations. It is also likely that migratory birds stopping on the island carry diverse parasitic infections from a global distribution (Møller and Erritzøe, 1998; Waldenström *et al.*, 2002; Pérez-Tris and Bensch, 2005). Due to the general unfeasibility of comprehensively accounting for the parasite communities of populations of other species on the island, they have not been accounted for in any analyses within this thesis.

The population of mice on the Isle of May is uniquely positioned for ecoimmunological study. It is an isolated population with restricted range, allowing for maximal trapping coverage of the whole population, with minimal perturbation from migration or introduction of novel infections. It is a model species, greatly increasing the scope of potential assays analyses which can be employed, with inbred lab strains of mice showing high levels of genetic similarity with the *M.m.domesticus* subspecies in particular (Yonekawa *et al.*, 1980). Finally, it is a truly wild population, with negligible commensal behaviour, a rarity for model rodent species like mice and rats, and thus the hypotheses tested in this thesis will be more generically applicable to wild mammal populations. For instance, the mouse populations studied by Abolins and colleagues, while described as wild, were trapped in a mixture of arable and pastoral farms, as well as one urban environment, and thus are potentially being impacted by human activity (Abolins *et al.*, 2018). These characteristics of the Isle of May mice will be exploited to answer a

range of questions regarding the significance and role of natural parasitic infections in wild populations.

1.7 AIMS & OBJECTIVES

The infectious and immunological factors affecting fitness outcomes in wild populations require greater understanding, and the availability of diverse methodologies, and a unique population of a model species will allow us to achieve this. Throughout this thesis I will aim to:

1) **Understand in detail the relative contributions of parasite burden and ecological parameters on various measures of immune expression in the host.**
2) **Examine the relationship between parasite species in a co-infected population.**
3) **Determine the role of host genetics in determining host immunity and parasite communities, relative to ecological factors.**
4) **Assess the relative influence of parasitology and immunity on the condition of the host.**
5) **Ascertain the relative utility of different lab-derived methodologies in examining immunity in wild populations.**
6) **Contrast the impacts of parasitic infection between lab models of infection and naturally infected wild populations.**

Chapter 2. Methodology

2.1 *M.M.DOMESTICUS ON THE ISLE OF MAY – FIELD METHODS*

Eight trapping sessions were carried out between 2013 and 2017 on the Isle of May, in the Firth of Forth, Scotland, the dates of which are detailed in Table 2.1. A combination of Ugglan and Longworth live-traps (Fig 2.1) were baited with hay and bird seed. While differences in effectiveness between these traps are disputed (Jung, 2016), with some studies finding Ugglans to have reduced trapping efficiency (Jacob, Ylönen and Hodkinson, 2002; Ylönen, Jacob and Kotler, 2003), both types were used in order to maximise the number of traps available, and thus our trapping coverage of the island.

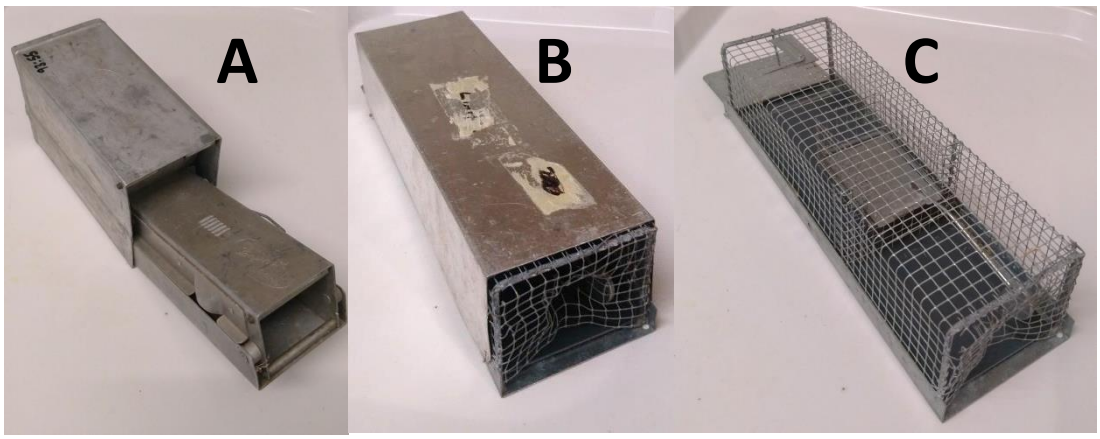


Fig 2.1 Traps used for live-trapping of *M.m.domesticus* on the Isle of May: (A) Longworth trap (B) Ugglan trap with cover on (C) Ugglan trap with cover off (photos by the author).

Session	First Trapping Day	Last Trapping Day
Summer 2013	22/08/2013	23/08/2013
Summer 2014	13/08/2014	14/08/2014
Spring 2015	09/04/2015	15/04/2015
Summer 2015	15/07/2015	17/07/2015
Autumn 2015	09/10/2015	12/10/2015
Spring 2016	06/04/2016	09/04/2016
Autumn 2016	11/10/2016	13/10/2016
Summer 2017	11/07/2017	11/07/2017

Table 2.1. Schedule of trapping sessions conducted on the Isle of May, from 2013 to 2017. Traps were checked twice a day for all dates covered by each session.

Transects of twenty traps each were laid out in different areas across the island (Fig 2.2), with equal proportions of trap types at each transect, in order to maximise the coverage of the island within the limits of the terrain, and allowing clearance for bird breeding sites. Traps were set overnight and checked first thing in the morning and again in the afternoon. Any traps that had been triggered by a mouse were collected and brought to the lab ready for dissection. After dissection, triggered traps were washed and reset with food, and then returned to the transect the same day.



Fig 2.2 Satellite view of the Isle of May, Scotland, showing named areas chosen for trapping transects. Traps were placed in the byres buildings, marked with a star, though not in a directional transect. Satellite imagery was obtained from Google Maps.

Numbers of mice caught showed great differences between sessions, in line with what would be expected from similar seasonal population cycles of small mammals, and what was described in this population by Triggs (Triggs, 1991). As mice were captured along transects over a small but heterogenous area without longitudinal monitoring, it was difficult to create population density estimates by traditional means. As such, population density estimates are here presented as average capture numbers per transect per day.

2.2 *M.M.DOMESTICUS ON THE ISLE OF MAY – DISSECTION & MORPHOMETRICS*

Mice were euthanised by being placed into a sealed chamber, and subject to increasing CO_2 concentrations until breathing was undetectable. Death was confirmed by exsanguination through puncture of the heart. The mouse was then immediately weighed. The snout-vent length and tail length were measured using callipers. Blood extracted during exsanguination was allowed to clot at room temperature for 2 hours, followed by 30 minutes at 4°C. Clots were stored at -20°C and retained for microparasite diagnostics. The remaining serum was divided into 40ul aliquots and stored in liquid nitrogen for use in multiplex ELISA analysis. Livers were extracted and kept at -20°C until return from the field, after which they were stored at -80°C. In male mice, testes and seminal vesicles were extracted and weighed.

The gastrointestinal tract was severed at each end and stored in 80% ethanol for subsequent helminth surveys. The spleen and mesenteric lymph nodes (MLNs) were removed, cut into small sections, and stored in a 0.6ml tube in a RNA stabilisation buffer (RNAlater – Merck, Darmstadt, Germany). All trapping and dissection protocols were carried out according to the University of Nottingham's ethics guidelines.

2.2.1 Condition Indices

Body mass and snout-vent measurements were used to calculate scaled mass index (SMI), a measure of relative body mass which scales against a curve for the population as a whole, to be used as a proxy for animal condition (Peig and Green, 2009 - Condition indices further discussed in Chapter 6). Visual measures of fat levels were taken during dissection. Levels of subcutaneous fat in the thorax and mesenteric fat were semi-quantitatively recorded by giving a fat score from 0-5 (Fig 2.3).

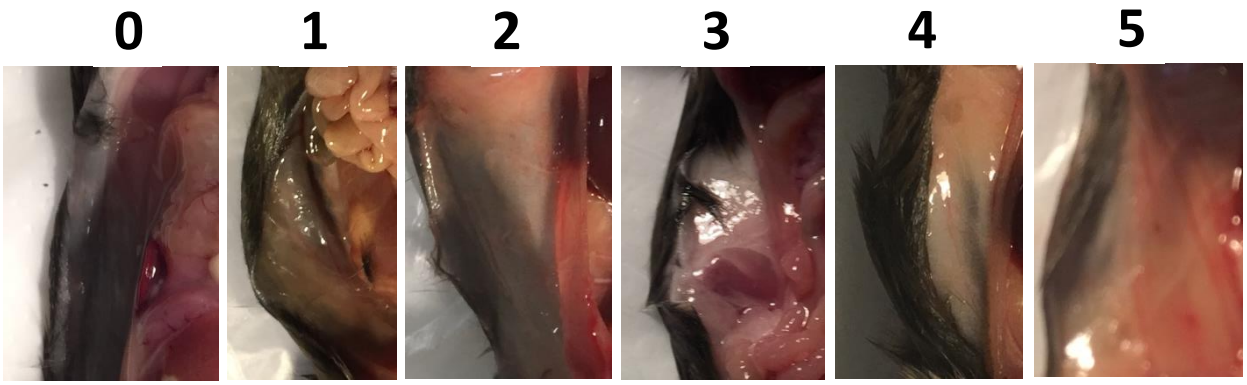


Fig 2.3 Range of visual subcutaneous fat scores observed during *M.m.domesticus* dissection, increasing from '0', where visible fat under the skin is negligible to '5', where a thick layer of opaque fat completely obscures the skin (Photos used courtesy of Ann Lowe & Dr Andrew Wolfenden).

2.2.2 Age Estimation

Prior to dissection, mice were broadly categorised into 'mature' and 'juvenile' categories according to general appearance and size. During dissection, the eyes were removed and stored in 80% formalin for use in more precise age

estimation upon return from the field. To obtain an estimate for age, the methodology laid out by Rowe and colleagues, which uses dry eye lens mass to estimate age, was used (Rowe *et al.*, 1985). As this study was cross-sectional (i.e. no impact of trapping history), and pregnant females were excluded, the use of eye lens mass was deemed sufficient for assessing general associations with age (see Chapter 1.5.7).

Eye lenses were extracted from the eyes using forceps. Each pair of lenses was weighed, then placed in a metal rack and into a heat dryer. A sample of the lenses was weighed each day until their mass stopped decreasing, indicating complete dehydration. A tray of silica crystals was kept at the bottom of the dryer to prevent water reabsorption during periods when the dryer could not be left on (e.g. overnight). The formulae used to estimate age from the final dry lens weight is shown in Fig 2.4.

Age estimate for males:

log10(age in days + 20 days) =

1.019 + 0.175 (mass of eye lenses in grams)

Age estimate for females:

Log10(age in days + 20 days) =

1.004 + 0.182 (mass of eye lenses in grams)

Fig 2.4 Formulae used to estimate the age in days for males and females of *M.musculus*, as developed and described by Rowe et al (1985).

The validity of these estimates was assessed by comparing them between mice than had been visually identified as ‘juvenile’ and ‘mature’. ‘Mature’ mice were found to have significantly higher age estimates, as shown in Fig 2.5.

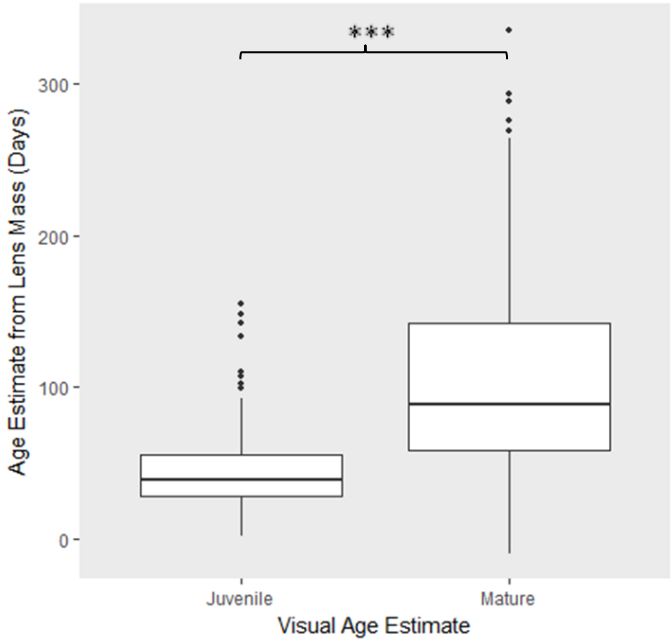


Fig 2.5 Significant difference in estimated age of mice from dry eye lens mass between individuals identified as ‘juvenile’ and ‘mature’ in the field. Linear model $p<2.26x10^{-16}$ (*=p<0.05, **=p<0.01, ***=p<0.001, n=367).

2.3 *MUS MUSCULUS DOMESTICUS* ON THE ISLE OF MAY –

PARASITOLOGICAL SURVEYS

2.3.1 Helminths

During dissection, the liver was visually assessed for infection by the hepatic helminth *Calodium hepaticum,* by scoring the level of decolouration from 0 (no decoloration) to 5 (extensive decoloration), to obtain a measure of infection intensity. An example of an infected liver is shown in Fig 2.6, and the visual scale used to score infection is exemplified in Fig 2.7. During the first trapping session, some mice were scored on a 1-10 scale of infection, which were subsequently adapted to the 1-5 scale by dividing scores by two - as a result, some mice have infection scores between integer values.

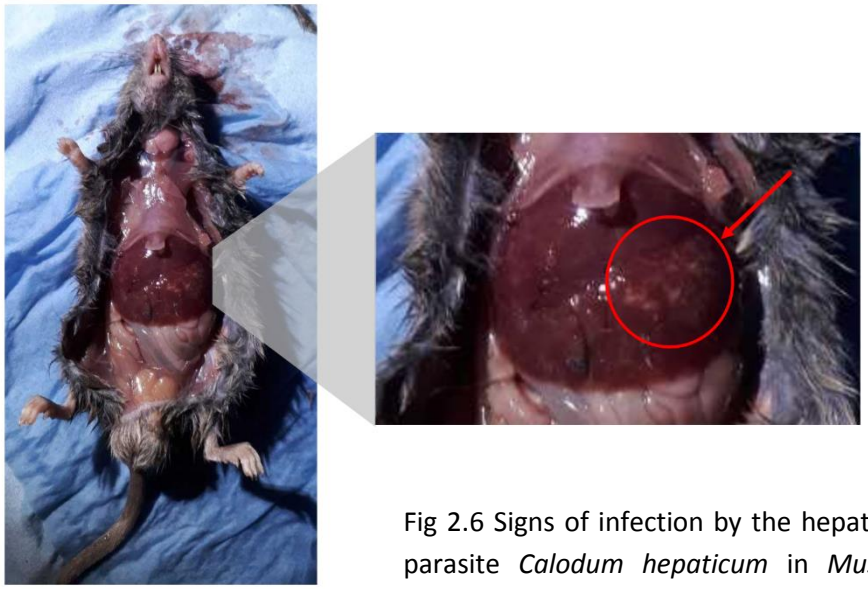


Fig 2.6 Signs of infection by the hepatic helminth parasite *Calodum hepaticum* in *Mus musculus domesticus*, mid-dissection. Infection is observed as white, fibrotic liver tissue, which is indicated by the red circle and arrow (Photo used courtesy of Dr Iris Mair).

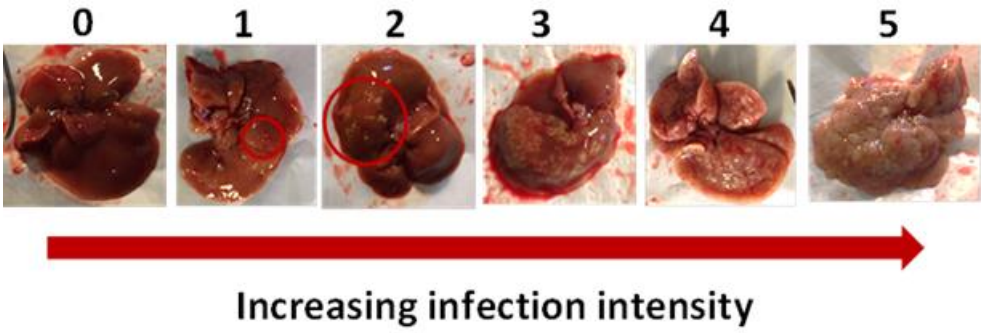


Fig 2.7 Scale for scoring level of infection by *C.hepaticum* in dissected livers of *M.m.domesticus*, increasing from no visible infection (0) to complete discolouration of the liver (5). Red circles on scores 2 and 3 highlight small patches of discolouration, indicating lower levels of infection (Photos used courtesy of Ann Lowe & Dr Andrew Wolfenden).

In order to assess the prevalence and abundance of gastrointestinal helminths, gastrointestinal (GI) tracts were separated by section (stomach, small intestine, caecum and colon), and dissected in turn. Dissection was chosen to assess gastrointestinal helminth infection, as faecal egg counts and PCR detection are not always accurate indicators of adult worm burden (discussed in Chapter 1.4.5). Each GI tract section was opened into a scored petri dish with a small amount of water to allow any helminths in the lumen to wash out. The surfaces of the GI tract section were then gently scraped and rinsed out to dislodge any attached parasites. To identify and count parasites, the petri dish was examined under a dissecting microscope, following the scored lines in the dish. (Surveys performed with assistance from Stuart Young)

2.3.2 Bloodborne Microparasite Assays

For the first three trapping sessions, no blood clot material was available for microparasite PCR, so for these parasites there are only data for 262 individuals, rather than the full dataset of 366. Four common microparasites of rodents were analysed by PCR: The protozoan parasites *Babesia microti* and *Trypanosoma sp.*, and the bacteria *Anaplasma sp.* and *Bartonella sp.* (Norman *et al.*, 1995; Birtles and Raoult, 1996; Krause *et al.*, 1996; Noyes, Belli and Maingon, 1996; Noyes *et al.*, 1999, 2000b; Adelson *et al.*, 2004).

DNA was extracted from the frozen blood clots using a 2-step extraction kit (Extracta DNA Prep, Quanta Bio Massachussetts, USA), and the resulting DNA used for PCR detection according to Table 2.2. Positive controls were obtained from Salford University (*Bartonella sp.*, *Trypanosoma sp.* & *Anaplasma sp.*) and the Sutton Bonnington campus at the University of Nottingham (*B.microti*). Negative controls were also included in each assay to ensure no contamination was occurring. Genes were selected by considering primers that were readily available, and which had been shown to amplify successfully, as well as through trial and error to determine which available primers most successfully amplified positive controls.

Amplified DNA was run on a 2% agarose gel for 30 minutes at 100v, and visualised under ultraviolet light to confirm amplification. Any successful

amplification of DNA was recorded as a positive infection. 3 samples of amplified DNA from the *B.microti* PCR and 2 from the *Bartonella sp.* PCR were sequenced at an external company (Source BioScience, Nottingham, UK) to confirm their identity using the BLAST sequence analysis tool (National Center for Biotechnology Information).

Parasite	Gene Target	Primers	Cycling Conditions	Fragment Length	Reference
Babesia microti	16s-like Gene	**Bab1** 5'CTTAGTATAAGCTTTTATACAGC **Bab4** 5'ATAGGTCAGAAACTTGAATGATACA	95°C - 4 mins [94°C - 1 min, 55°C - 1 min, 72°C - 2 mins]x35 72°C - 7 mins	238bp	(Persing *et al.*, 1992)
Trypanosoma sp.	18S RNA	***1st round*** **TTRY927F** 5'GAAACAAGAAACACGGGAG **TRY927R** 5'CTACTGGGCAGCTTGGA ***2nd round*** **SSU561F** 5'TGGGATAACAAAGGAGCA **SSU561R** 5'CTGAGACTGTAACCTCAAAGC	[94°C-30 s, 55°C-60 s, 72°C-90s]x30 (both rounds)	927bp	(Noyes, Perez Camps and Chance, 1996; Noyes *et al.*, 1999, 2000a)
Anaplasma phagocytilum	16S rDNA	**HGE1F** 5'GGATTATTCTTTATAGCTTGCT **HGE3R** 5'TTCCGTTAAGAAGGATCTAATCTC	95°C - 4 mins [94°C - 30s, 52°C - 30s, 72C - 1 min]x34 72C - 1 min	920bp	(Adelson *et al.*, 2004)
Bartonella sp.	gltA	**CS140f** 5'-TTACTTATGATCCKGGYTTTA **CS1137r** 5'-AATGCAAAAAGAACAGTAAACA	95C - 2 mins [95°C - 1 min, 54°C - 1 min, 72°C - 2 mins] x 40 72°C - 7 mins	1039bp	(Norman *et al.*, 1995; Birtles and Raoult, 1996)

Table 2.2 Gene targets for the identification by PCR of four microparasites surveyed in mice from the Isle of May, including primer sequences and cycling conditions. Parasites were chosen for detection based on common infections in wild rodent populations. Gene targets and primers were selected based on assays with proven efficacy in other rodents, and which effectively amplified positive controls

Sequences from the *B.microti* PCR showed most identity similarity with ribosomal RNA from another apicomplexan parasite group known to infect mammals, *Sarcocystis sp.*, with all three samples sharing 98-99% identity with *Sarcocystis* sequences. The primers used evidently show low specificity within the apicomplexans, and for the purposes of this thesis, this parasite will be taken as *Sarcocystis sp.* Both *Bartonella* sequences shared 100% identity with *Bartonella taylorii* ribosomal spacer region. (Microparasite PCRs were performed by Sarah Goertz)

2.3.3 Ectoparasites

As mice were released from the traps ready for dissection, any ectoparasites observed on the inside of the trap were recorded. After dissection was completed, the fur and skin of each animal was examined under a dissecting microscope. The fur was manipulated along the sides and back, then around the face and the base of the tail. Ectoparasites were identified using a visual key, and counted (Key created at University of Nottingham, based on photos taken by Laura Myhill). As fleas tended to be highly motile, and would typically leap from the fur during dissection and fur inspection, only their presence or absence was recorded, as a reliable count of abundance could not be made. All ectoparasite surveys were carried out on the same day as dissection, and dissected bodies were wrapped in absorbent paper and refrigerated until the ectoparasite survey could be carried out.

2.4 *MUS MUSCULUS DOMESTICUS* ON THE ISLE OF MAY – QPCR ANALYSIS OF GENE EXPRESSION

2.4.1 RNA Purification and Reverse Transcription

RNA was extracted from spleen and MLNs using a column-based purification method (Nucleopsin, Macherey-Negel, Düren). DNAse treatment was included in the extraction method to ensure any genomic DNA was removed. The purified RNA was eluted into 60µl of nuclease-free H_2O. The concentration and purity were assessed by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Massachusetts, USA). Purity was estimated from the ratio of absorbance of 260nm-wavelength light compared to 280nm and 230nm. A value of ~1.8 for the 260/280 ratio and ~2 for the 260/230 ratio indicate purity of nucleic acid (ND-1000 Spectrophotometer V3.5 User's Manual, 2007). Any samples measuring above 200µg/µl were diluted with nuclease-free H_2O to this threshold value. Reverse transcription was performed using a 2-step kit (Precision nanoScript2 Reverse Transcription Kit, PrimerDesign, Southampton, UK) to produce 20µl of cDNA from RNA (Fig 2.8). cDNA was diluted with nuclease-free H_2O for use in qPCR (1 in 10 dilutions for spleen cDNA, 1 in 5 dilutions for MLN cDNA). Mixed reference samples made from 1µl of diluted cDNA from each individual were created for MLN and spleen, to be used in accounting for inter-plate variation during qPCR. Differences between measurements of the mixed reference sample between plates

measuring the same gene allow normalisation between plates during data analysis. cDNA dilutions were stored at -20°C.

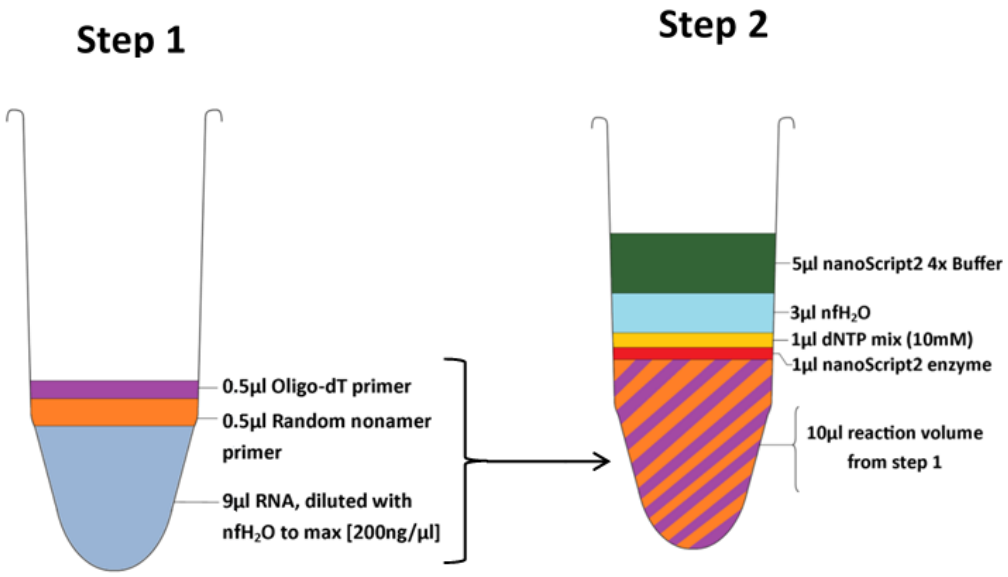


Fig 2.8 Reaction volumes for the two-step process of reverse transcription, for the production of cDNA from RNA purified from spleen and MLN tissue from *M.m.domesticus*. The reaction mixture for step 1 is incubated for 5 minutes at 65°C, during which time primers attach the the RNA and prevent the formation of secondary structures. A mastermix containing buffer, nuclease-free H2O, dNTP mix and reverse transcriptase enzyme (nanoScript2) is then added, providing the reaction volumes for step 2. The mixture is heated to 25°C for 5 minutes, then at 42°C for 20 minutes, allowing the enzyme to reverse transcribe the primed RNA to cDNA.

2.4.2 GeNorm Analysis

Before qPCR assays were carried out, a GeNorm assay was performed. The purpose of this assay is to determine which housekeeping genes show the most stable expression across samples, and should therefore be used as reference genes against which immune gene expression should be measured (Vandesompele *et al.*, 2002). cDNA from 15 individuals was used in a qPCR assay with 6 supplier-recommended candidate reference genes known to

have stable expression in *M.musculus* (Primerdesign, Southampton, UK). These genes are shown in Table 2.3.

Gene	Role
RPL13α	Encodes the 60s ribosomal protein L13.
SDHA	Encodes succinate-ubiquinone oxidoreductase, a subunit of the mitochondrial respiratory enzyme,.
UBC	Encodes the protein polyubiquitin-C, a form of ubiquitin.
ActB	Encodes for the protein β-actin, a cytoskeletal protein.
18S	Encodes for the 18s ribosomal subunit.
GAP-DH	Encodes for the enzyme Glyceraldehyde 3-phosphate dehydrogenase, involved in glycolysis.

Table 2.3 Prospective reference genes for qPCR of *M.musculus* cDNA, used in GeNorm analysis of expression stability.

Variation in expression levels of these 6 genes was compared across these 15 individuals using the GeNorm macro for Microsoft Excel. The analysis found that the two genes with the lowest levels of inter-individual variability were SDHA and UBC, for both MLN and spleen cDNA (Fig 2.9). These two genes were used as reference genes for all subsequent qPCR assays as a value of gene expression.

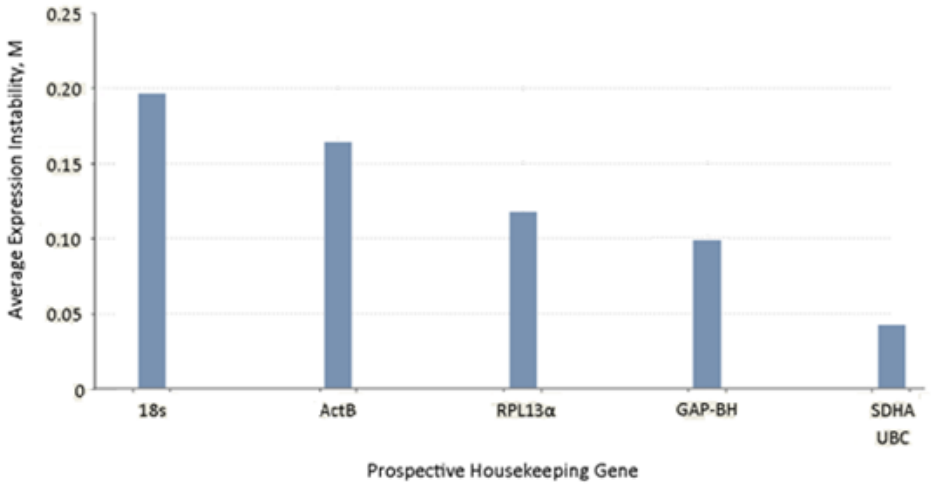


Fig 2.9 Output of geNorm analysis, showing prospective housekeeping genes for qPCR, in order of increasing expression stability. The stability factor 'M' is calculated as the pairwise variation of expression across samples between two prospective genes (Vandesompele et al, 2002). The pairwise SDHA-UBC calculation gives the lowest value of M, indicating the highest level of expression stability between two genes, and thus the best combination for qPCR housekeeping gene expression. All other bars in the output (18s, ActB, RPL13α and GAP-BH) represent average values of variability for each gene across all possible pairwise gene combinations.

2.4.3 qPCR Assays

The immune genes selected for analysis are summarised in Table 2.4. These genes were selected to represent different branches of the immune system involved in responses to parasitic infection, including Th1, Th2, Treg and Th17-type immune expression. For the genes IL-5, IL-12β, FoxP3 and RORC, high levels of failed amplification from MLN-derived cDNA during qPCR meant that only splenic expression values were used for analysis.

Gene	Role	Primer Sequences	References
IL-1β	Produced by phagocytes, involved in innate immunity and inflammation, associated with Th1-type responses and TNF induction.	L 5'-CAACCAACAAGTGATATTCTCCAT R 5'- GGGTGTGCCGTCTTTCATT	(Ikejima *et al.*, 1990; Chizzolini *et al.*, 1997; Sansonetti *et al.*, 2000; Sims and Smith, 2010; Cho *et al.*, 2012)
IL-4	Development Th2-type responses against helminth macroparasites, inhibition of IFN and Th1-type immunity.	L 5'-AGCAACGAAGAACACCACAG R 5'- GCAGCTCCATGAGAACATA	(Swain *et al.*, 1990; Vercelli *et al.*, 1990; Kopf *et al.*, 1993)
IL-5	Th2-associated stimulation of eosinophilia and eosinophil survival.	L 5'-ATGGACGCAGGAGGATCAC R 5'-TTGAAGTTAGATAGGAGCAGGAAG	(Lopez *et al.*, 1988; Yamaguchi *et al.*, 1988; Walsh *et al.*, 1990)
IL-6	Pro-inflammatory cytokine, involved in both Th17-type inflammation and linking innate and adaptive responses.	L 5'-TCCATCCAGTTGCCTTCTTG R 5'-GGTCTGTTGGGAGTGGTATC	(Jones, 2005; L. Zhou *et al.*, 2007)
IL-10	Th2-associated immunoregulatory cytokine involved in limiting tissue damage by inhibiting Th1-type inflammation.	L 5'-GGGAAGAGAAACCAGGGAGAT R 5'-GCCACAGTTTTCAGGGATG	(Mosmann and Moore, 1991; D'Andrea *et al.*, 1993; Yang *et al.*, 1999)

Table 2.4 Immune genes used in qPCR analysis of splenic and mesenteric lymph node gene expression levels. Genes were selected covering a range of roles in the immune system, and include cytokines and transcription factors involved in the regulation of cytokine expression.

Gene	Role	Primer Sequence	References
IL-12β	Induces Th1 responses by inhibiting IL-4 and increasing IFNγ expression.	L 5'-CACGGCAGCAGAATAAATATGAG R 5'-GAGTTCTTCAAAGGCTTCATCTG	(Manetti, 1993; Magram *et al.*, 1996)
IL-13	Development of Th2 cells, and Th2-type protection against helminth macroparasites.	L 5'-GCCAGCCCACAGTTCTACA R 5'-CCACCAAGGCAAGCAAGAG	(Grünig *et al.*, 1998; McKenzie, Bancroft, *et al.*, 1998; McKenzie, Emson, *et al.*, 1998; Wynn, 2003)
IL-17A	Pro-inflammatory cytokine, characteristic of Th17-type inflammatory responses, causes neutrophil recruitment.	L 5'-GTCTGCCCTCCACAATGAAA R 5'-TTAAAGTCCACAGAAAAACAAACA	(Miyamoto *et al.*, 2003; Moseley *et al.*, 2003; Kolls and Lindén, 2004; Lohr *et al.*, 2006)
TNFα	Pro-inflammatory cytokine associated with Th1 and Th17-type inflammation.	L 5'-AGCCAGGAGGGAGAACAGA R 5'-CAGTGAGTGAAAGGGACAGAAC	(Iwamoto *et al.*, 2007; Aravena *et al.*, 2011)
IFNγ	Polarisation and maintenance of Th1-type responses, promotion of IL-12 activity	L 5'-TGATTACTACCTTCTTCAGCAACAG R 5'-CTGGTGGACCACTCGGATG	(Bradley, Dalton and Croft, 1996; Wenner *et al.*, 1996)
RORC	Transcription factor promoting differentiation of Th17 cells and Th17-type inflammation	L 5'-TTTCTGCCTTTCTTCTTCCTAGTT R 5'-CACCCTCATTCTTTACACATCCA	(Ivanov *et al.*, 2006)

Table 2.4 (cont) Immune genes used in qPCR analysis of splenic and mesenteric lymph node gene expression levels. Genes were selected covering a range of roles in the immune system, and include cytokines and transcription factors involved in the regulation of cytokine expression.

Gene	Role	Primer Sequences	References
Tbx21	Transcription factor controlling Th1-type responses and IFN induction	L 5'-AACCAGCACCAGACAGAGAT R 5'-GACCACATCCACAAACATCCT	(Szabo *et al.*, 2000; Mullen *et al.*, 2001)
Irf5	Promotion of Th1 and Th17 inflammatory responses	L 5'-CTTCCAGCCAGCCCCCTA R 5'-TGATCTCTAGGTCCGTCAAAGG	(Krausgruber *et al.*, 2011)
GATA3	Transcription factor promoting expression of Th2-type cytokines and inhibiting IFN expression	L 5'-GAAGACTTTATTGTACCTGGATAGC R 5'-TGGACATCAGACTTAGTGGTTTC	(Zheng and Flavell, 1997; Yagi *et al.*, 2010)
FoxP3	Transcription factor, a master regulator of immunosuppression and characteristic of Treg cells	L 5'-AGGACAGACCACACTTCATGC R 5'-CCAGTGGCAGCAGAAGGTG	(Fontenot, Gavin and Rudensky, 2003; Hori, Nomura and Sakaguchi, 2003; Yuan *et al.*, 2010)

Table 2.4 (cont) Immune genes used in qPCR analysis of splenic and mesenteric lymph node gene expression levels. Genes were selected covering a range of roles in the immune system, and include cytokines and transcription factors involved in the regulation of cytokine expression.

All qPCR assays were performed on 96-well plates. Each sample was analysed in duplicate, with 2µl of each sample used per well. Also included on each plate were duplicate wells of a negative control (nuclease-free H_2O) and the mixed reference sample. The reaction volumes for each sample are shown in Fig 2.10. Cycle conditions for the qPCR assays are shown in Table 2.5. (qPCR assays were performed with assistance from Stuart Young)

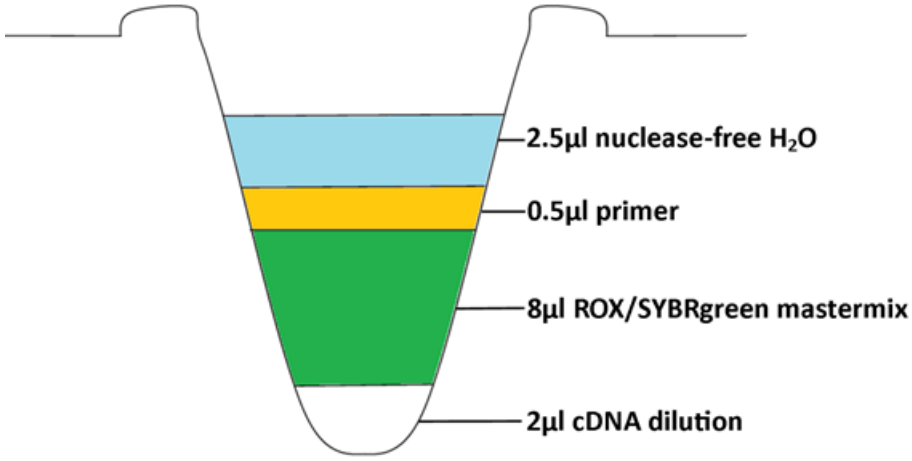


Fig 2.10 Reaction volumes of qPCR of cDNA of *M.musculus*. Reactions were performed on a clear 96-well plate. cDNA (or negative control) was pipetted to the bottom of each well, with each sample pipetted in duplicate. The reaction mixture containing SYBRgreen mastermix, the primer for the gene being analysed, and nuclease-free H_2O was then added before the plate was sealed and spun down prior to the reaction.

Stage	Temperature	Duration
Holding Stage	95°C	20s
Cycling Stage (x40)	95°C	3s
	60°C	30s
Melt Curve Stage	95°C	15s
	60°C	60s
	SLOW TEMPERATURE INCREASE AT MINIMUM RAMP	
	95°C	15s
	60°C	15s

Table 2.5 Cycling conditions for of qPCR assays of *M.musculus* cDNA (for use with Applied Biosystems 7500 real-time PCR system). The holding and cycling stages occur as with traditional PCR, allowing the cDNA to amplify, followed by a melt curve stage to assess purity of the amplified product.

The qPCR assay gives an output CT (Cycle Threshold) value for each well, which represents the qPCR cycling stage at which the fluorescence passes a specified threshold value, i.e. a value between 1 and 40 for an amplification with 40 cycles. The CT value is dependent on double-stranded DNA concentration, as the SYBRgreen dye included in the mastermix (Primerdesign, Southampton, UK) must bind to double-stranded DNA to fluoresce. Lower-concentration samples will therefore reach this threshold value at a later cycling stage, and thus will have a higher CT value. The raw CT value for each sample is averaged across the duplicate wells, then transformed using the method described by Rao and colleagues (Fig 2.11)

(Rao *et al.*, 2013). The resulting values were then used throughout further analysis.

$$\Delta CT = CT(\text{Mixed Reference}) - CT(\text{sample})$$

$$E\Delta CT = 2^{\Delta CT}$$

$$REF = \text{Geometric Mean}(E\Delta CT \text{ of SDHA} + E\Delta CT \text{ of UBC})$$

Expression Level = Ratio (EΔCT:REF)

Fig 2.11 Equation for calculation of gene expression values, taken from raw qPCR CT values. This process normalising expression data between plates, and gives a scaled value for gene expression, relative to the concentration of housekeeping genes from the same sample.

After amplification is complete, the cDNA is subject to slowly increasing temperatures ($60°^{C}$-$95°^{C}$). The amplified qPCR product will melt (separate into single strands) at a temperature specific to the size of the amplified product, causing the dye to dissociate from the DNA, and cause a consequent drop in fluorescence. Rate of change in fluorescence is plotted against increasing temperature as a 'melt curve', causing a peak at the melting point of the amplicon, as fluorescence sharply decreases. If only the target gene has been successfully amplified, a single peak will be present, with double-peaks indicating amplification of unintended gene targets. Melt curves of successful and unsuccessful melt curves are exemplified in Fig 2.12.

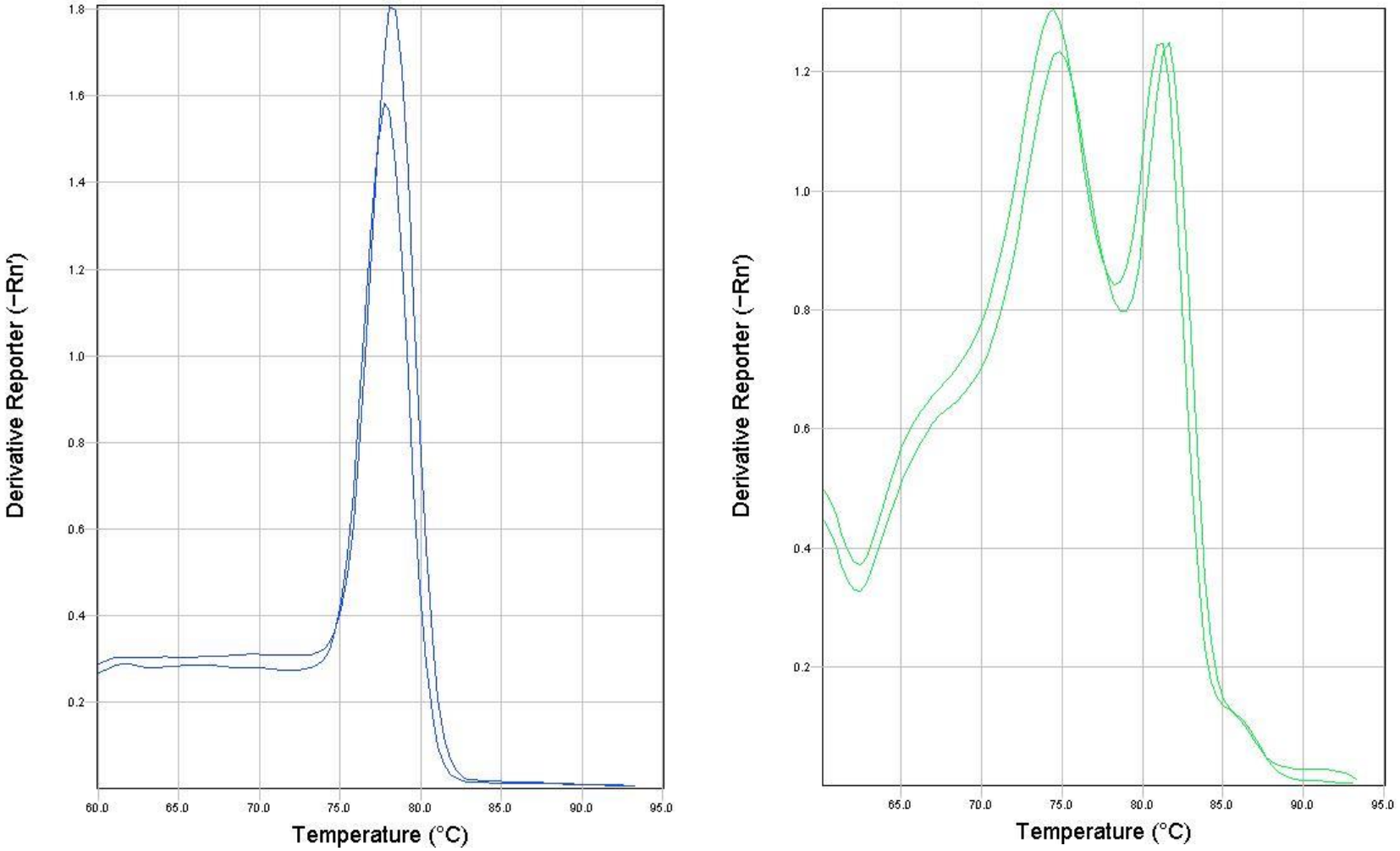


Fig 2.12 Examples of melt curves from qPCR assays: (L) successful specific amplification of the target gene is indicated by a clear melt curve peak, representing a change in fluorescence which corresponds the a uniform melting of the amplicon (R) unsuccessful specific amplification of the target gene is indicated by a double peak surrounded by humps, representing changes in fluorescence at different temperatures, as amplicons of different sizes melt and dissociate from the reporter dye.

2.5 *M.M.DOMESTICUS* ON THE ISLE OF MAY –

MULTIPLEX BEAD ASSAY OF CIRCULATING CYTOKINES

Cytokines selected for multiplex ELISA were IFNγ, IL-1β, IL-5, IL-6, IL-10, IL-12β, IL-13, IL-17a, TNFα and leptin. They were chosen to correspond with genes used in qPCR analysis, again representing different types of immune response known to be important in the outcome of infection by different groups of parasites. Measuring cytokines which correspond to gene expression data allow for the association between gene expression and cytokine concentrations to be assessed. In addition, concentration of the hormone leptin was measured to be included in analyses of host condition (see Chapter 1.4).

Multiplex bead assay was performed using the Bio-Plex Pro Assay procedure (Bio-Rad Laboratories, California, USA). A fourfold dilution series of standards was produced in order to establish the standard curve for the cytokines to be quantified. Blood serum aliquots were thawed and diluted to a 1 in 4 dilution using a provided diluent. Magnetic beads, conjugated to antibodies against the cytokines of interest, were vortexed and diluted to a 1 in 10 dilution with a provided assay buffer. Different coloured beads corresponded to different antibodies, to allow measurements of different cytokines to be distinguished upon fluorescence.

The diluted beads were vortexed, and 50µl was added to each well. The plate was then washed twice with 100µl of wash buffer. Every wash conducted throughout the assay wash performed with the plate clipped to a magnetic base, to hold the beads at the bottoms of the wells and prevent them from being lost. Samples, standards and blanks were vortexed, and 50µl was added to corresponding wells in duplicate. The plate was covered and incubated on a shaker at room temperature at 850rpm for 30 minutes. The plate was then washed three times with 100µl wash buffer.

Detection antibodies conjugated to biotin for each cytokine of interest were vortexed, and 25µl was added to each well. The plate was again covered and incubated on a shaker at room temperature at 850rpm for 30 minutes. The plate was then washed three times with 100µl wash buffer.

Streptavidin, R-Phycoerythrin Conjugate (SA-PE) was diluted to a 1 in 100 dilution in the provided assay buffer, and vortexed. 50µl of diluted SA-PE was added to each well. The plate was again covered and incubated on a shaker at room temperature at 850rpm for 10 minutes. The plate was then washed three times with 100µl wash buffer.

Beads were resuspended by adding 125µl of assay buffer to each well. The plate was covered and incubated on a shaker at room temperature at 850rpm

for 30 seconds. The plate was then measured using the computer-linked Bio-Plex 200 plate reader system (Bio-Rad Laboratories, California, USA), which subjects each sample to lasers of different wavelengths, causing fluorescence which is proportional to levels of SA-PE (and thus cytokine), and coloured according the cytokine-specific bead. Fluorescence readings from each well were compared against the standard curve produced from the dilution series of standards to give a concentration value for each cytokine from each sample. The protocol for the multiplex bead assay is summarised in Fig 2.13. (Cytokine bead assays were carried out by Benoit Poulin & Ann Lowe)

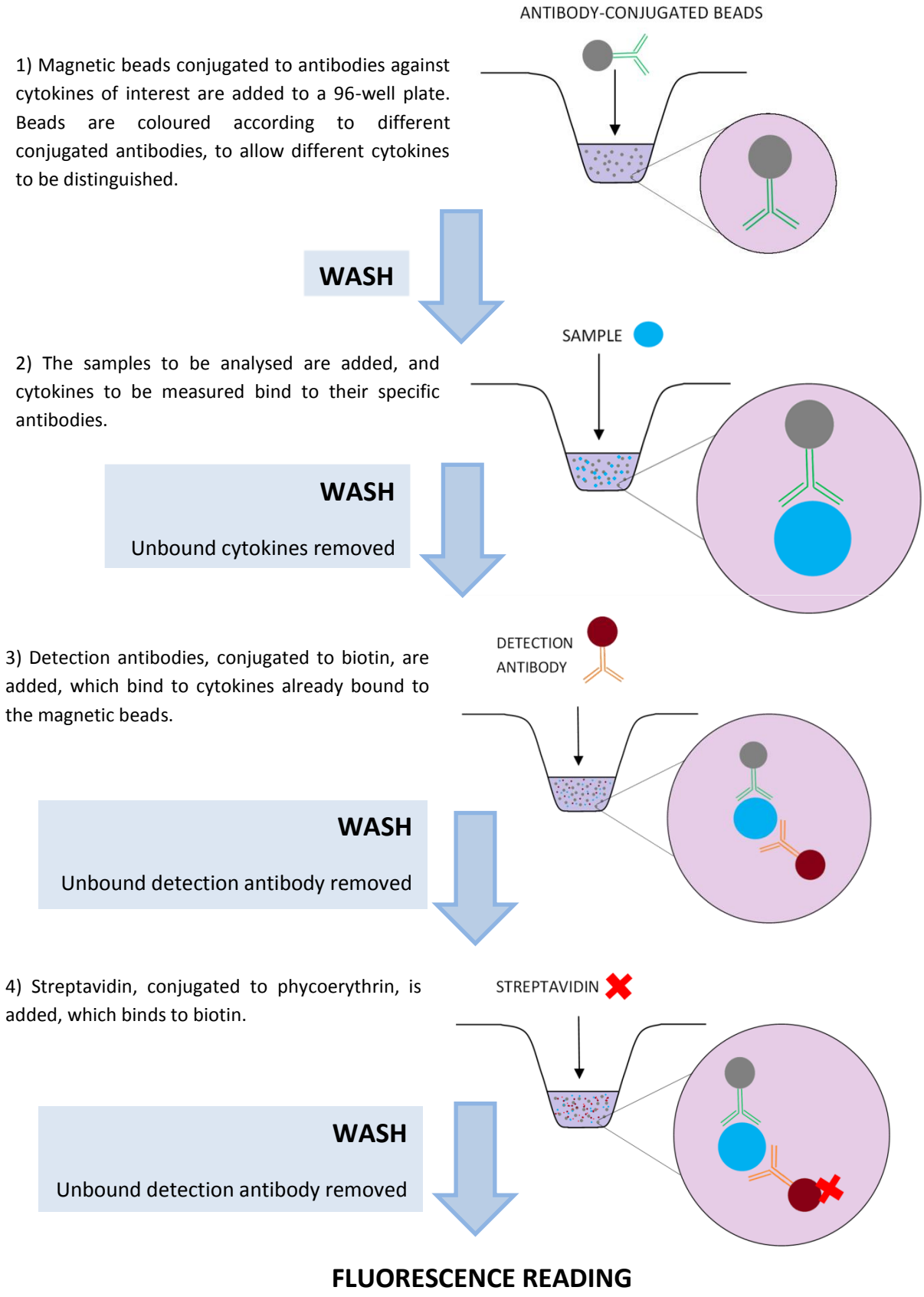


Fig 2.13 Summary of multi-step protocol for multiplex ELISA of cytokines from *M.m.domesticus* blood serum.

Chapter 3. Parasitology & Parasite Interactions

3.1 INTRODUCTION

All wild animal populations are subject to parasitic infection in some form (Knowles *et al.*, 2013). The environmental and ecological factors affecting the prevalence of these parasites in wild populations are manifold, making the prediction of infection patterns uniquely difficult. This complexity is compounded by the fact that parasites may be affected by ecological forces in dynamically different ways, according to their diverse adaptive strategies and transmission cycles (Criscione and Blouin, 2004). This ecological landscape includes other parasites, which may be concurrently infecting the same host.

3.1.1 Parasite Distributions in Wild Rodent Populations

Ectoparasites and helminths in naturally infected populations typically exhibit a negative binomial burden distribution, with most infected individuals having low infection burdens, and rare individuals having high infection burdens (Barger, 1985; Haukisalmi, 1986; Behnke *et al.*, 2000; Krasnov, Khokhlova and Shenbrot, 2002; Šimková *et al.*, 2002; Pullan *et al.*, 2014). These distributions become established through non-random variation in susceptibility between individuals, with differences occurring between age groups, sexes, genotypes, locations, and other ecological factors (Crofton, 1971; Maizels *et al.*, 1993).

Some helminths, like *Heligmosomoides polygyrus, Trichuris muris* and *Calodium hepaticum* will commonly form chronic infections in the host, and thus show greater average prevalence with increasing age (Spratt and Singleton, 1986; Bundy, 1988; Monroy and Enriquez, 1992; Fuehrer, 2014; Walker *et al.*, 2017). In contrast, worms which less commonly form chronic infection in wild-type mice, such as the pinworm *Syphacia sp.* do not exhibit a similar age distribution (Müller-Graf *et al.*, 1999; Michels *et al.*, 2006).

Seasonal impacts upon infection distributions are well reported in wild rodent populations. Haukisalmi and colleagues found that in a cyclic vole population, distribution of six helminth parasites were significantly impacted by seasonality (Haukisalmi, Henttonen and Frantisek, 1988). Common and rare helminth infections were found to respond differently, with common species showing more predictable cycles of prevalence corresponding with host population density, and rarer species showing complete absence at various points in the year, being more dependent on suitable environmental conditions for transmission (Haukisalmi, Henttonen and Frantisek, 1988). This is supported by the fact that *Calodium sp.*, one of the rare reported parasites in this vole population, has highly specific temperature and moisture requirements for environmental embryonation to occur (Spratt and Singleton, 1986). Kataranovski and colleagues found that across seven helminth

parasites of *R.norvegicus*, infection prevalence generally peaked in the summer (Kataranovski *et al.*, 2011).

Seasonal changes in ectoparasite infections have been recorded in several rodent populations, with a variety of seasonal effects observed. In a wild population of Wagner's gerbil (*Gerbillus dasyurus*), abundance of two flea species, *Xenopsylla dipodilli* and *Nosopsyllus iranus*, were found to increase with host density throughout the year, though in curvilinear and linear relationships, respectively (Krasnov, Khokhlova and Shenbrot, 2002). Sharp decreases in prevalence infection by the flea *Leptopsylla segnis* in commensal rat (*Rattus sp.*) populations of rural Egypt during the summer have been attributed to high temperature-dependency by the parasite, and a consequent depression of activity and reproduction during the warmer months (Soliman, Main, *et al.*, 2001). Conversely, other fleas of other Egyptian rat populations have shown positive relationships between temperature and parasite prevalence (Njunwa *et al.*, 1989).

In woodland populations of the bank vole, *Myodes glareolus* and the yellow-necked mouse, *Apodemus flavilcollis*, changes in ectoparasite prevalence were closely linked to seasonal changes in the host populations (Harris *et al.*, 2009). Two flea species showed different changes, with *Hystrichopsylla orientalis* increasing with availability of nesting rodents in late Autumn, while *Ctenophthalmus agyrtes* larvae overwintered with their hosts and emerged in

the early Spring. Seasonal shifts in flea and lice infections affecting male Richardson's ground squirrels were ascribed to a potential trade-off between reproductive effort and effective immune response (Lindsay and Galloway, 1997). Seasonal factors impacting ectoparasite distribution are evidently highly variable between species and host populations.

Natural microparasite infections also commonly display strong seasonality. Seasonal changes in prevalence of different strains of *Bartonella* infecting wild populations of bank voles (*C.glareolus*) and wood mice (*A.sylvaticus*), were found to be variable between strains, with *B.grahamii*, *B.taylorii* and *B.birtlesii* exhibiting peaks in the late Summer and early Autumn, and *B.doshiae* peaking in the Spring (Telfer *et al.*, 2007). These differences were proposed to the result of corresponding seasonal changes in flea vector populations. Prevalence of four microparasites of a population of *C.glareolus* (*Babesia microti, Hepatozoan erhardovae, Trypanosoma evotomys* and *Bartonella sp.*) have been found to reach a maximum transmission in the Summer and late Autumn (Turner, 1986). This pattern was again attributed to seasonal peaks of the relative arthropod vectors.

In cyclic rodent populations, age profiles and immune state will often covary, and so great care should be taken during analysis to ascertain which factors are actually impacting parasite prevalence (Behnke *et al.*, 1999). Populations of all measured parasites on the Isle of May have been shown to be highly

subject to seasonal variation, both intra- and inter-annually (Young, S. – Unpublished observations, thesis pending publication).

3.1.2 Parasitic Coinfection

Commonly, animals facing multiple parasitic challenges will be coinfected with multiple species. These parasites may impact one another through direct competition, or the alteration of the host environment upon infection (Petney and Andrews, 1998; Pedersen and Fenton, 2007; Johnson and Buller, 2011; Knowles *et al.*, 2013; Martin *et al.*, 2013). In order to understand the mechanisms by which these interactions are established, and the forces which led to their evolution, the interactions themselves should first be understood in as much detail as possible.

The relationship between any two parasites will depend on the niche of each species within the host with regards to where they reside, what resources they use, and which immunological conditions are most favourable for their survival. In a meta-analysis of studies of coinfection with helminths and microparasites, Graham (2008) suggests that such interactions should be analysed in an ecological framework, with immune effects and niche competition treated as top-down and bottom-up controls of parasite numbers, respectively (Cattadori, Boag and Hudson, 2008; Graham, 2008). This framework is in contrast to viewing parasitic infections as discrete events

which impact host health, in isolation from the ecology of both host and parasite. Looking at coinfection in this way is helpful to establish the infection dynamics of a wild population within a broader ecological context, as it removes theoretical divides between parasite immunology and ecology, positioning infections as elements within a greater ecological network.

A variety of relationships between different groups of parasites have been examined in lab infection models and in wild populations, with varying outcomes, including mutualistic and competitive coinfection dynamics (Knowles, 2011). Mutualistic interactions occur when infection by one parasite increases the chances of survival by another. These relationships are typically immune-mediated, whereby one parasite will either suppress the host response to increase its own chances of survival, or polarise the immune environment such that other parasites are under reduced immune pressure (Graham *et al.*, 2007; Cattadori, Boag and Hudson, 2008). Competitive relationships between parasite species can arise due to competition over physical niches or resources, or through induction of an immune response protective against both infections (Hellard *et al.*, 2015).

3.1.3 Helminth-Microparasite Interactions

A commonly studied coinfection dynamic is that between parasitic helminths and *Plasmodium sp.*, which have shown both mutualistic and competitive

interactions across strains of lab mice and parasites (Hartgers and Yazdanbakhsh, 2006). In humans, individuals with gastrointestinal helminths suffer greater risk of infection with *Plasmodium sp.* (Nacher *et al.*, 2002). Mice experimentally infected with *Schistosoma mansoni* showed increased parasitaemia with *Plasmodium berghei*, and delayed clearance following antimalarial treatment (Legesse, Erko and Balcha, 2004). *Plasmodium sp.* infection has been shown to reduce Th2 responses to helminths, and positive impacts of coinfection on both parasites are attributed to reductions in TNFα expression and generally impairment of an effective targeted immune response (Helmby, Kullberg and Troye-Blomberg, 1998). Mice infected with certain strains of *Plasmodium sp.*, experienced higher levels of morbidity during coinfection with helminths (Graham *et al.*, 2005; Knowles, 2011).

Rats infected with the protozoan parasite *Trypanosoma brucei* were unable to expel infection by the GI helminth *Nippostrongylus brasiliensis* (Urquhart *et al.*, 1973). This mutualistic effect is attributed to Th2 immune responses to helminths, which polarise the immune system away from a Th1 environment, releasing the *Plasmodium sp.* parasite from a protective immune response (Pearce, 1991; Su *et al.*, 2005).

The inverse of this effect has also been reported, with antagonistic effects observed between helminths and microparasites in a naturally coinfected population of the wood mouse, *Apodemus sylvaticus*, which when treated

with an antihelminthic, showed that removal of helminth infection was accompanied by an increase in the microparasite *Eimeria sp.* (Knowles *et al.*, 2013). In addition, in both BALB/C and A/J lab mice, *S.mansoni* infection was shown to increase resistance to *Plasmodium sp.*, through the induction of Th1 expression (Yoshida *et al.*, 2000; Moriyasu *et al.*, 2018).

Conflicting reports regarding the impact of helminth infection on microparasite resistance may result from differential protective responses that are employed at different stages of the life cycle of both parasites. Th1 expression during advanced malaria can actually be pathological, while Th2 expression becomes protective at various stages of the parasite's life cycle (Langhorne *et al.*, 1998; Torre *et al.*, 2002; Hartgers and Yazdanbakhsh, 2006). These differences, in addition to phenotypic differences between mouse and parasite strains, may explain the different coinfection dynamics observed, and highlight that coinfection interactions in wild populations may be unpredictable.

3.1.4 Helminth-Helminth Interactions

A mutualistic interaction between helminth species coinfecting lab mice (*M.musculus*) was demonstrated by Budischak et al (2015), who showed that the helminth parasite *Nippostrongylus brasiliensis* has increased abundance during coinfection with *Heligmosoides polygyrus*, (Budischak *et al.*, 2015). This

interaction was attributed to Treg-type suppression of immunity being induced by *H.polygyrus* improving the survival of both species.

A similar positive association was recorded by Jackson et al, who observed positive associations between *C.hepaticum* and the intestinal nematode *H.polygyrus* in the wood mouse *Apodemus sylvaticus* (Jackson *et al.*, 2009). Infection with *H.polygyrus* in this population was associated with reduced innate immune response. Moon et al observed a protective immune response to infection by *C.hepaticum* and a similar hepatic nematode *Clonorchis sinensis* in laboratory rats, characterised by eosinophilia and lymphocyte proliferation, alluding to an immunoregulatory role for *H.polygyrus* in positive coinfection associations (Moon *et al.*, 2017).

A review of coinfection studies involving *Schistosoma sp.* in both rats and lab mice showed that *Schistosoma sp.* infection typically has a negative impact on subsequent successful infection by *Fasciola sp.*, another parasitic trematode (Abruzzi and Fried, 2011). In this instance, the timing of infection was found to be highly important, a factor which can be difficult to account for in observation studies of wild populations. A range of both positive and negative associations of various parasitic helminths with *Schistosoma* species were identified, sometimes differing between cases of coinfection with the same species.

Helminth coinfection dynamics were explored by Boag et al, who examined aggregation levels of five species of parasitic helminth in wild rabbits over 22 years (Boag *et al.*, 2001). Higher aggregations of parasite species were recorded in older mice, as well as in males, an effect which was attributed to their larger ranges and consequent increased chances of infection. There were significantly lower levels of helminth aggregation in individuals displaying symptoms of the virus *Myxomatosis*, possibly due to immune effects. Within the helminth infections, cestodes showed lower levels of aggregation than nematodes, an effect which was attributed to the greater size of cestodes, leading to increased competition for space in the GI tract, as well as the fact that nematodes can spread via direct transmission, whereas the cestodes required an orbatid mite to complete their life cycle. Ecology and life-cycle of parasite species is likely to be highly significant in determining outcomes of coinfection.

3.1.5 Microparasite-Microparasite Interactions

Telfer and colleagues performed a longitudinal analysis of four microparasitic infections in a wild population of field voles (*Microtus agrestis*) in order to ascertain how these infections interacted (Telfer *et al.*, 2010). These microparasites included the bacteria *Anaplasma phagocytophilum* and *Bartonella spp.*, the protozoan parasite *Babesia microti* and the cowpox virus (CPXV). A network of associations between parasites was discovered,

including both positive and negative relationships, and involving every pairwise combination of parasite species. Negative associations were observed between *Bartonella sp.* and both *A.phagocytophilum* and *B.microti*, whereas *A.phagocytophilum* and *B.microti* displayed a combination of positive and negative interactions. Negative associations were proposed to be due to alterations to the immune environment and competition for host erythrocytes, whereas positive associations were the result of co-transmission by ectoparasitic vectors. Time and order of infection were found to be highly important to these interactions, with differences between infection stages influencing both the weight and the positive or negative nature of the relationship between two parasite species. *B.microti* has been shown to reduce probability of infection by *Bartonella spp.* in field voles (Taylor *et al.*, 2018), though it is unclear as to whether to mechanism of this interaction is immunological or physiological.

3.1.6 Ectoparasite Interactions

Ectoparasites are often the vectors of microparasites, and so coinfection between these groups are often linked in wild populations. This positive association has been observed in fleas infecting rats with *Bartonella sp.* (Brettschneider *et al.*, 2012). Alternatively, positive correlations between lice and microparasites in willow ptarmigan (*Lagopus lagopus*) have been attributed to immune responses reducing the energy available for preening (Holmstad, Jensen and Skorping, 2008).

Less clear is to what extent ectoparasites interact with helminth parasites. Ferrari et al found that in wild yellow-necked mice (*Apodemus flavicollis*), *H.polygyrus* displayed a negative association with the tick *Ixodes ricinus* (Ferrari *et al.*, 2009). This was counter to their hypothesis that the immunomodulatory effects observed elsewhere in naturally infected populations of *H.polygyrus* would here increase susceptibility to tick infestation.

In a 3-year study of rock sengis (*Elephantulus myurus*), Lutermann et al found through experimental treatment against ectoparasite infection, that reestablishment of ectoparasitic communities was complex and involved both positive and negative interactions (Lutermann, Fagir and Bennett, 2015). Positive interactions occurred between species of the same taxon, attributed to decreased immune response. Associations between taxa, however, were largely antagonistic, due to competition for feeding sites. Mechanisms of ectoparasite coinfection dynamics are often unclear.

3.1.7 Impacts of Coinfection on the Host

Although the diversity of potential immune responses to coinfection has been discussed, the actual impact of the infections and the associated immune response on the fitness or condition of the host is often disregarded. Variation in the outcomes of coinfection are likely to be the result of differences in the

context in which infection occurred, with regards to the environment, the host and the timescale. A study combining trematode parasite assemblage data from over 2,000 amphibian hosts showed that coinfection generally had a negative influence on host fitness, as measured by host survival (Johnson and Hoverman, 2012). This effect was, however, highly dependent on the assemblage of parasites, with some species having disproportionately negative effects on host fitness, and whether new infections were additive or replaced existing infections.

Conversely, mice infected by different strains of the same microparasite species, *Trypanosoma brucei* showed increased survival, as a result of mutual competitive suppression between the strains (Balmer *et al.*, 2009). In this instance, the negative impact of strain coinfection on the more virulent strain caused a positive effect on host fitness. In a population of field voles (*M.agrestis*) coinfected with *B.microti* and *Bartonella spp.*, although *B.microti* infection was associated with splenomegaly, and *Bartonella spp.* infection caused weight loss and elicited and IL-10 immune response, no impacts upon fitness, as measured by host survival and fecundity, were detected (Taylor *et al.*, 2018). These examples demonstrate that as with the progression of individual infections, the impact of parasitic coinfection on the host is likely to be dependent on the species involved, and the manner in which the host is infected.

3.1.8 Hypotheses

The mice on the Isle of May are naturally infected with a range of parasites, including arthropod ectoparasites, helminths and blood-borne microparasites. Here, I describe the characteristics of micro- and macro-parasites identified on the Isle of May, and determine some of the factors affecting their prevalence and abundance within the host. I explore the infection dynamics that exist between parasite species, and gain some ideas about how these relationships are impacted by host ecology. Based on precedent from both wild and laboratory infection studies, I test the following hypotheses:

1) Ageing will increase general levels of parasitism.

2) Chronic helminth infections will be positively associated with other helminth infections.

3) Positive associations will be observed between helminth and microparasite infections.

4) Negative associations will exist between different microparasite taxa.

5) Positive associations will exist between *Bartonella sp.* prevalence and arthropod ectoparasites.

3.2 METHODS

All field, morphological and parasitological methods are detailed in Chapter 2 of this thesis (2.1 – 2.3).

3.2.1 Parasitological Analysis

Microparasites were detected through PCR of blood clot material obtained during dissection. Gastrointestinal helminths were identified and counted via dissection of gastrointestinal tract. *C.hepaticum* infection burden was assessed by scoring levels of decolouration of the liver during dissection. Ectoparasites were identified and counted by surveying the fur under a dissecting microscope (2.3– Parasitological Surveys).

3.2.2 Statistical Analyses

The prevalence of each parasite was used in a corresponding series of binomial general linear mixed models (GLMMs). Ecological variables included in each model were including the age estimated from eye lens mass, sex, trapping location, scaled mass index (SMI) as a measure of condition, captures per transect per day, as a proxy for population density, and the prevalence and abundance of other parasites. Location and trapping session were included as random effects. Although it is likely that trapping session would have a significant bearing on parasite infection dynamics, as both rodent and parasite populations often show significant seasonal changes in population

sizes and activity, the irregular spacing between sessions means that any seasonal or interannual patterns of parasite distribution are unlikely to become evident, and so trapping session was not included as a fixed effect.

For parasite species with measures of abundance (gastrointestinal helminths and fur mites), a series of negative binomial GLMMs were performed using abundance data, and excluding uninfected individuals. As infection levels of *C.hepaticum* were measured using a semi-quantitative 0-5 scale of infection score (0 being uninfected, 5 indicating widespread infection) a cumulative link mixed model was used, taking the infection score as an ordinal factor. For this model, individuals with infection scores of zero were taken to be uninfected, and thus were excluded.

Due to missing data for *Bartonella sp.*, *Sarcosystis sp.* and *C.hepaticum,* infections, models in which these parasites were not the predicted variable were repeated with these species excluded to increase statistical power. Any significant relationships between pairs of parasites with complete datasets that are henceforth reported, are taken from these exclusionary models.

All statistical analyses were performed in R. For all models, model averaging, using the '**dredge**' function in R package '**MuMIn**', was used to provide an

average best model for each parasite species' prevalence. The '**clmm**' function of the '**ordinal**' package was used for cumulative link models.

3.3 RESULTS

3.3.1 Helminths

Two species of helminth worms were commonly found in the GI tracts of the dissected animals: the whipworm *Trichuris muris* and the pinworm *Syphacia obvelata* (Fig 3.1). Also recorded was the tapeworm *Hymenolepsis sp.*, though this was rarely recorded (5 instances over 366 recorded mice) and so has not been included in further analysis.

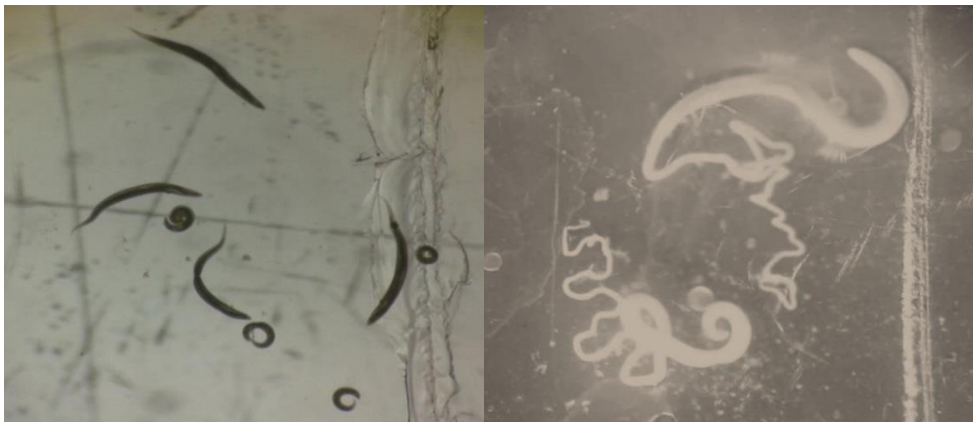


Fig 3.1 Two species of helminth worm commonly recorded in the GI tracts of *M.m.domesticus* on the Isle of May (L) *S.obvelata* (R)*T.muris*.

Overall prevalence of *T.muris* and S*.obvelata*, taken across all trapping sessions, are shown in Fig 3.2. Out of 366 recorded mice, 127 were uninfected with *T.muris* or *Syphacia* (34.70%), 169 were infected with *T.muris* (46.17%) and 112 were infected with *Syphacia* (30.60%). Of these mice, 42 (11.48%)

were coinfected with both parasites (Fig 3.2). Burden distributions for both species were found to favour a negative binomial distribution, with infections greatly shifted towards low infection burdens, with rare individuals having dramatically higher loads (Fig 3.3).

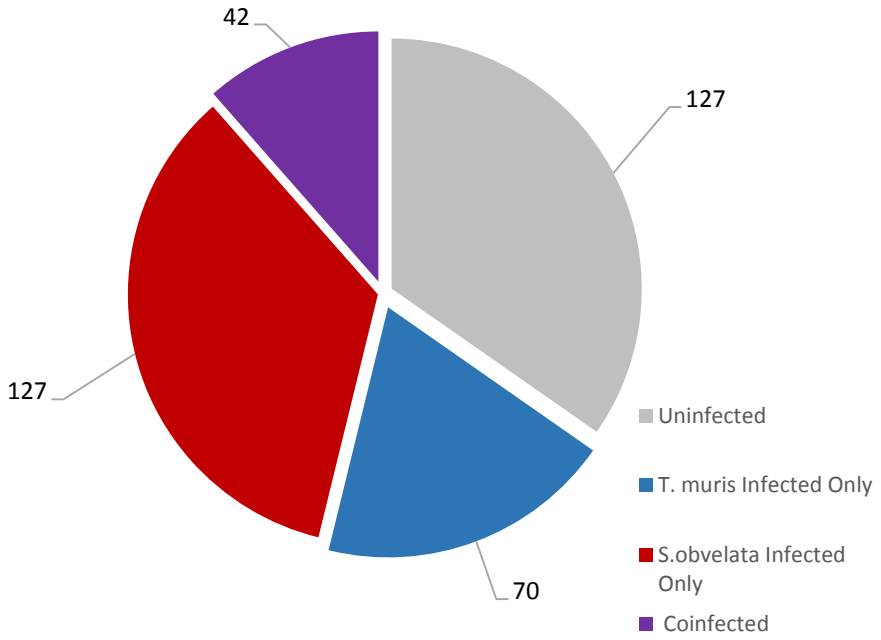


Fig 3.2 Proportions of mice either uninfected by gastrointestinal helminths, infected with *T.muris*, *S.obvelata* or both, taken across all trapping sessions 2013-2016.

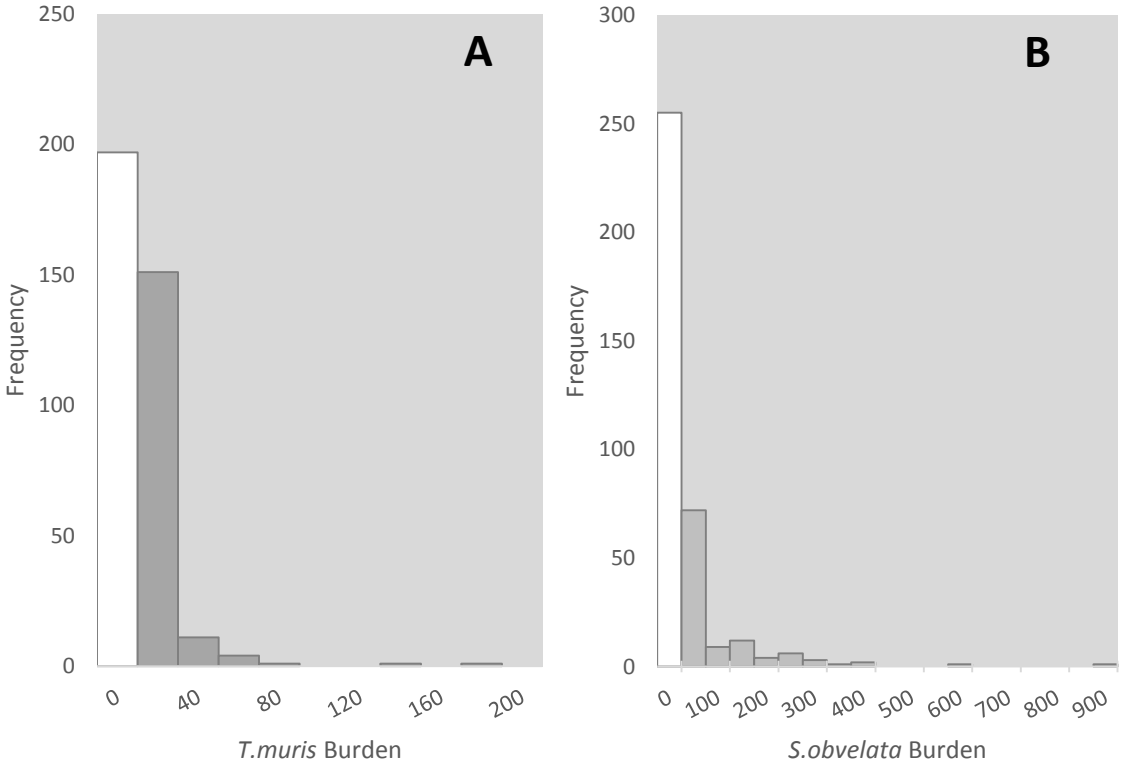


Fig 3.3 Histograms showing negative binomial infection distributions of (A) *Trichuris muris* and (B) *Syphacia obvelata*. White columns indicate zero infection burden.

The *T.muris* binomial mixed model found the prevalence of the parasite increasing significantly with age (Fig 3.4). There was no association observed, however, between age and parasite abundance, suggesting that once infection has established, the number of parasites established in the host remains fairly stable.

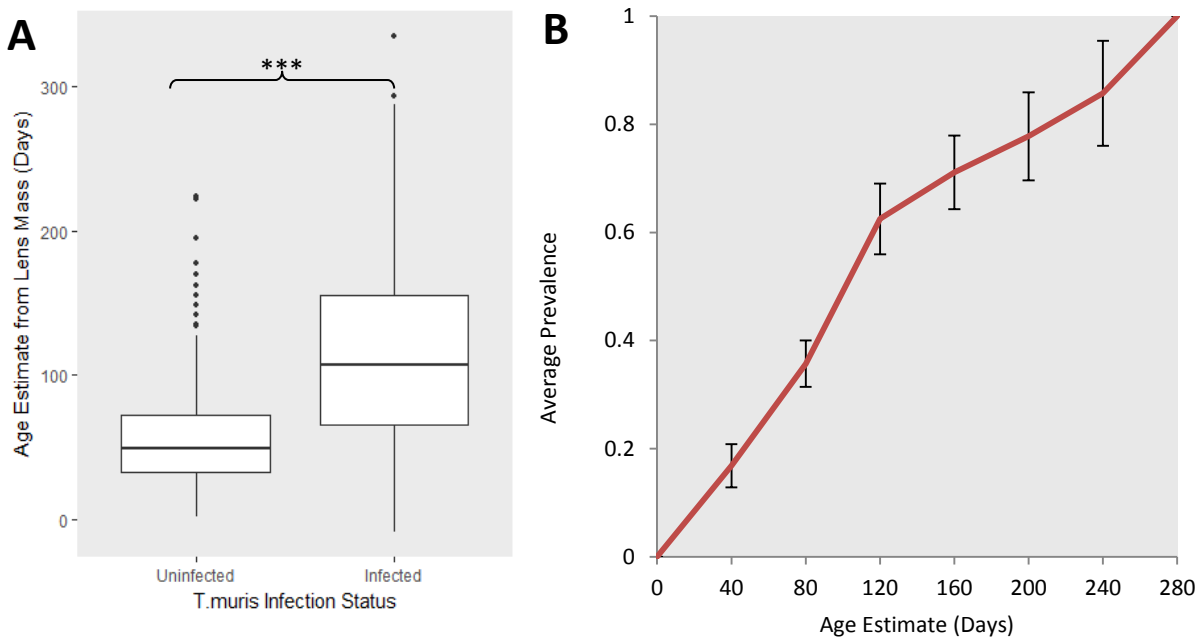


Fig 3.4 (A) Difference in mean estimated age between *T.muris* infected and uninfected mice (n=366,$p<2x10^{-1}$) *=p<0.05,**=p<0.01,***=p<0.001 (B) Mean levels of infection prevalence of *T.muris* with increasing age, showing increase in average prevalence in the population towards 100% infection. Bars= SEM

The prevalence of *S.obvelata* was significantly negatively associated with the number of captures per transect per day, here used as an estimate of population density, indicating that individuals in areas with lower population density are more likely to be infected (Fig 3.5).



Fig 3.5 Differences in population density, as estimated by captures per transect per day, between individuals uninfected and infected with *S.obvelata*. (p=0.0066) *=p<0.05,**=p<0.01,***=p<0.001.

Most captured individuals (152 out of 261) showed some level of infection by *C.hepaticum*. The distribution of infection intensity scores is predominantly uniform (Fig 3.6). The prevalence and infection intensity of the parasite was positively associated with age (Fig 3.7).

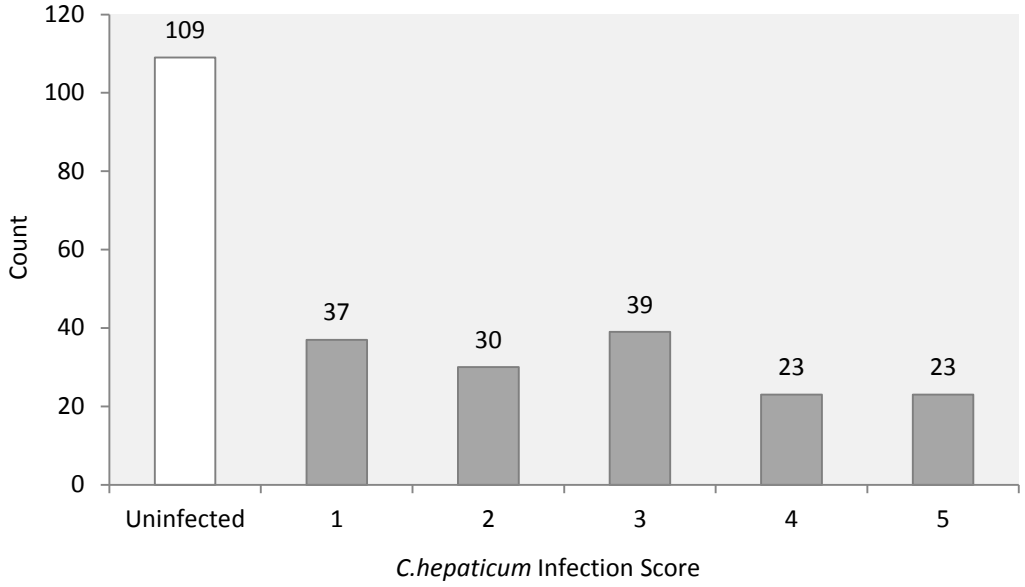


Fig 3.6 Distribution of infection prevalence and infection scores of *C.hepaticum*, indicating that most individuals experience some level of infection, and that distribution of infection scores is predominantly uniform.

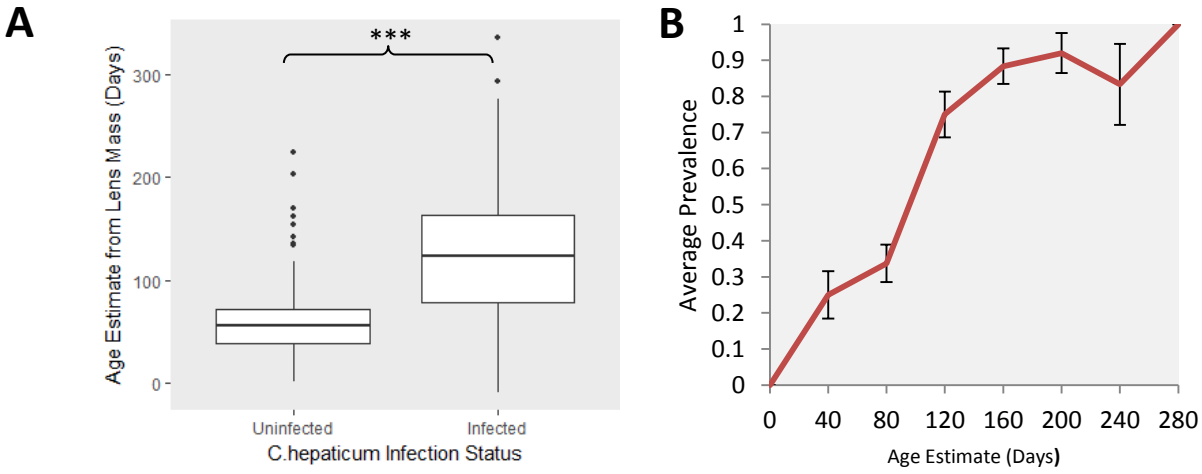


Fig 3.7 (A) Difference in mean estimated age between *C.hepaticum*- infected and uninfected mice ($n=261, p=8.82 \times 10^{-5}$) *=p<0.05,**=p<0.01,***=p<0.001 (B) Mean levels of infection prevalence of *C.hepaticum* with increasing age, showing increase in average prevalence in the population towards 100% infection. Bars=SEM

3.3.2 Microparasites

No *Anaplasma sp.* or *Trypanosoma sp.* were detected by PCR in any of the mice. In 127 mice, no microparasites were detected. 123 mice were found to be infected with *Bartonella sp.*, and 18 were found to be infected with *Sarcocystis sp.*. Of these mice, 7 were coinfected with both parasites (Fig 3.8).

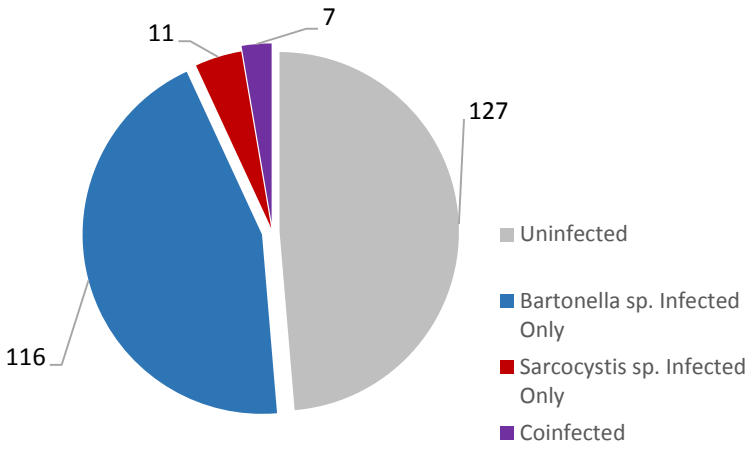


Fig 3.8 Proportion of individuals infected with *Bartonella sp., Sarcosytis sp.,* or coinfected with both, as detected by PCR of blood clots.

Prevalence of *Sarcocystis sp.* was strongly positively associated with increasing age (p=0.0129). No ecological parameters were found to significantly impact *Bartonella sp.* prevalence.

3.3.3 Arthropod Ectoparasites

Four genera of mite (*Myobia*, *Radfordia*, *Myocoptes* & *Trombicula*), and one species of flea (*Nosopsyllus fasciatus*) were found in the fur of the mice. Prevalence of fleas was found to be positively associated with age (Fig 3.9). Abundance of mites also showed a positive relationship with SMI, indicating an increased condition, as well as age.

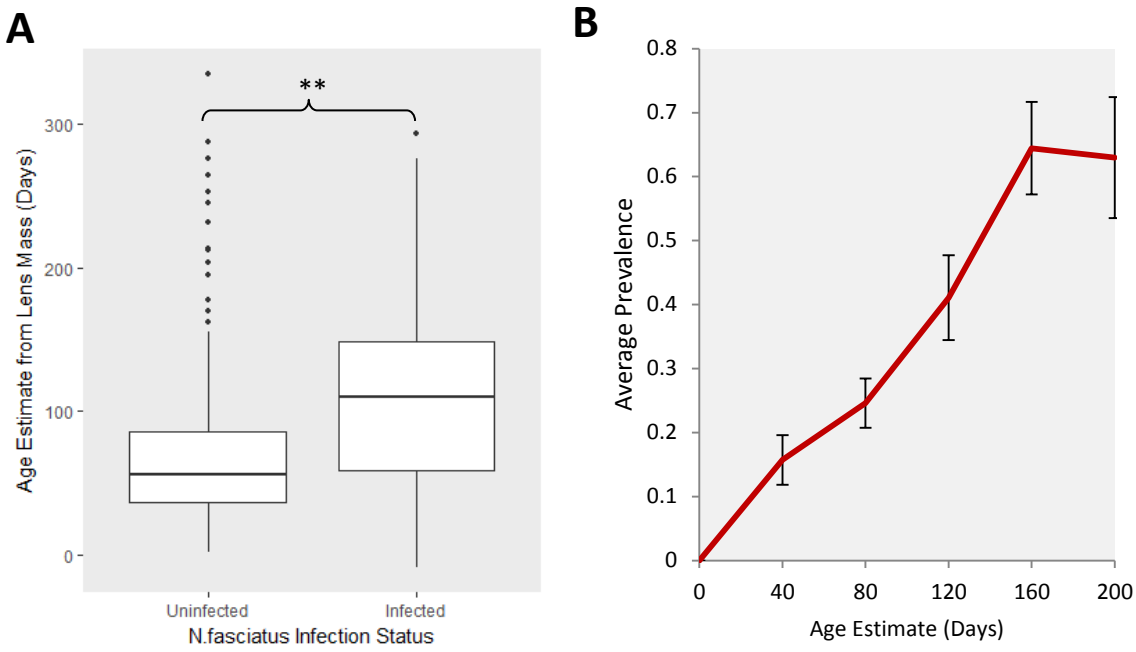


Fig 3.9 (A) Difference in mean estimated age between *N.fasciatus*-infected and uninfected mice (n=366, p=0.0029) *=p<0.05,**=p<0.01,***=p<0.001 (B)Mean levels of infection prevalence of *N.fasciatus* with increasing age. Bars=SEM

3.3.4 Parasite Interactions

A number of significant associations between different parasites, both positive and negative, were observed in the host population. These associations exist across taxonomic groups, and are seen in effects upon both the prevalence and abundance of certain parasites. All parasites measured in this population were involved in some form of inter-parasite relationship.

The two common species of gastrointestinal helminths have a negative association, as evidenced by both the *S.obvelata* prevalence and *T.muris* abundance models. This negative relationship is shown in Fig 3.10.

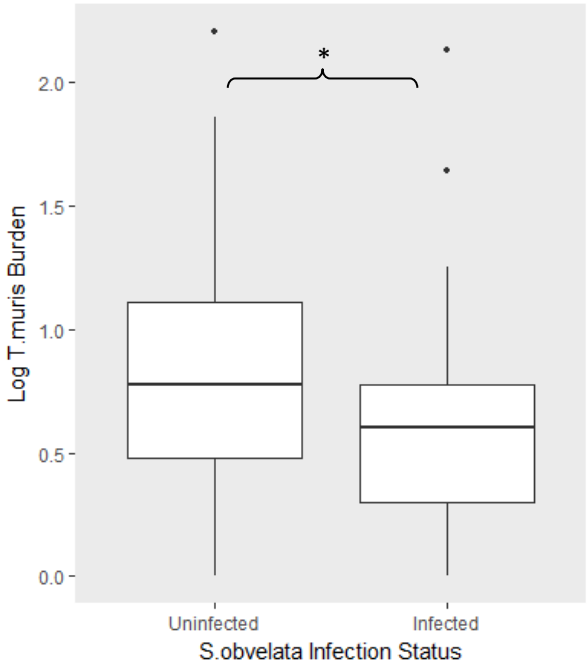


Fig 3.10 Difference in mean infection burden of *T.muris* in infected individuals, between individuals infected and uninfected with *S.obvelata,* with higher average abundance of *T.muris* in mice not infected with *S.obvelata,* p=0.0136. (*=p<0.05,**=p<0.01,***=p<0.001)

There was a strong positive association observed between *C.hepaticum* and *T.muris*, with the prevalence of the former significantly predicting that of the latter (p=0.00311), and with *C.hepaticum* infection score significantly predicting the abundance of *T.muris* (Fig 3.11).

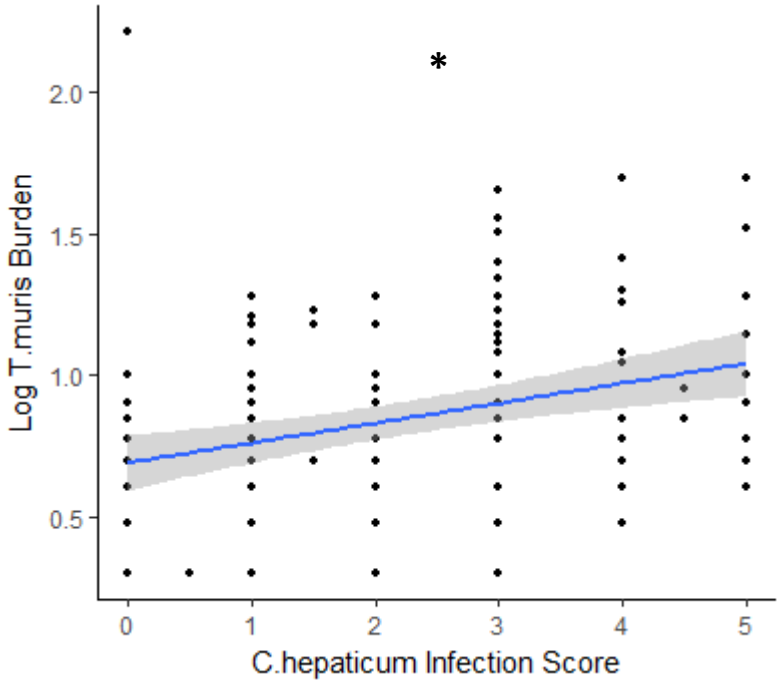


Fig 3.11. Positive association between average abundance of *T.muris* and *C.hepaticum* infection score. (n=261,p=0.0117) *=p<0.05,**=p<0.01,***=p<0.001.

C.hepaticum prevalence also showed a positive association with *Bartonella sp.* prevalence, a relationship that is observed in the models for both species (*C.hepaticum* predicted by *Bartonella sp.*, p=0.0488, *Bartonella sp.* predicted by *C.hepaticum*, p=0.0203). The abundance of mites in the fur showed positive associations with the prevalence of *C.hepaticum* (Fig 3.12) and fleas in the fur (Fig 3.13).

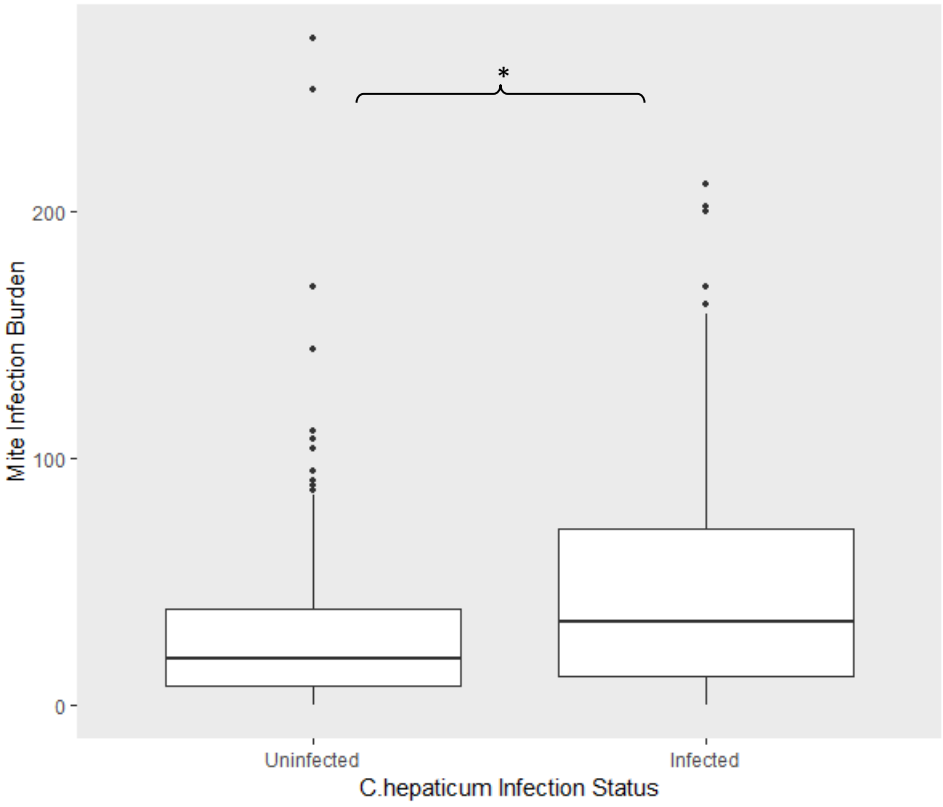


Fig 3.12. Difference in mean abundance of mites in the fur between individuals infected and uninfected with *C.hepaticum* (n=261,p) *=p<0.05,**=p<0.01,***=p<0.001.

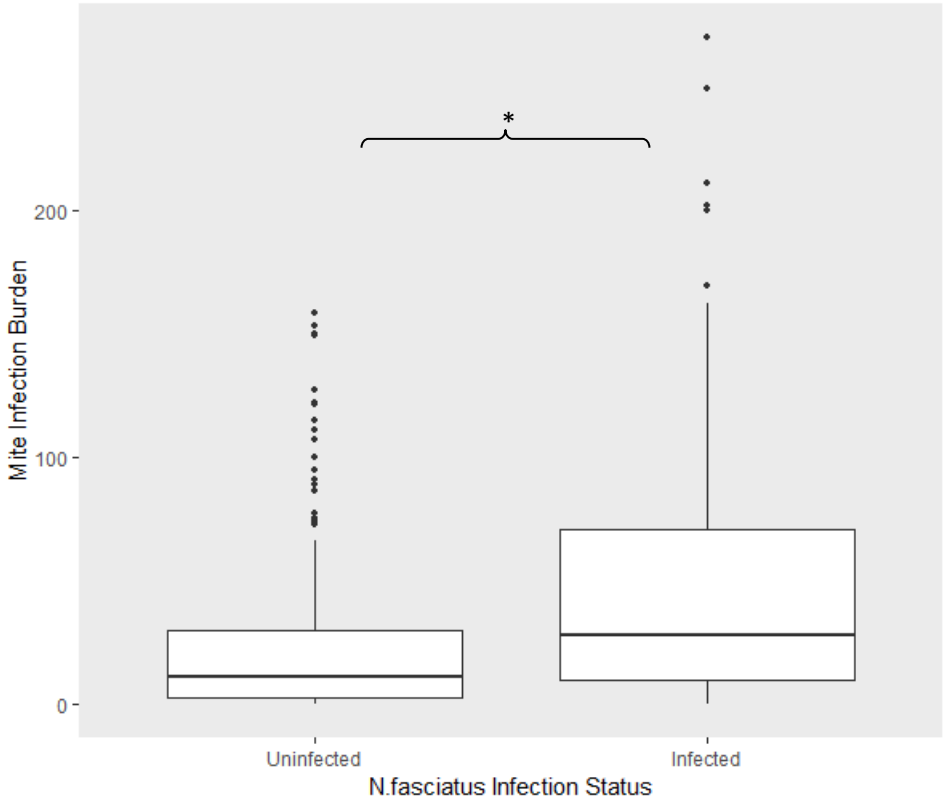


Fig 3.13 Difference in mean abundance of mites in the fur between individuals infected and uninfected with *N.fasciatus* (n=366,p=0.0271) *=p<0.05,**=p<0.01,***=p<0.001.

The network of parasite interactions seen in this population of *M.musculus* is shown in Fig 3.14, and a summary of outputs from all of the models performed is presented in Table 3.1.

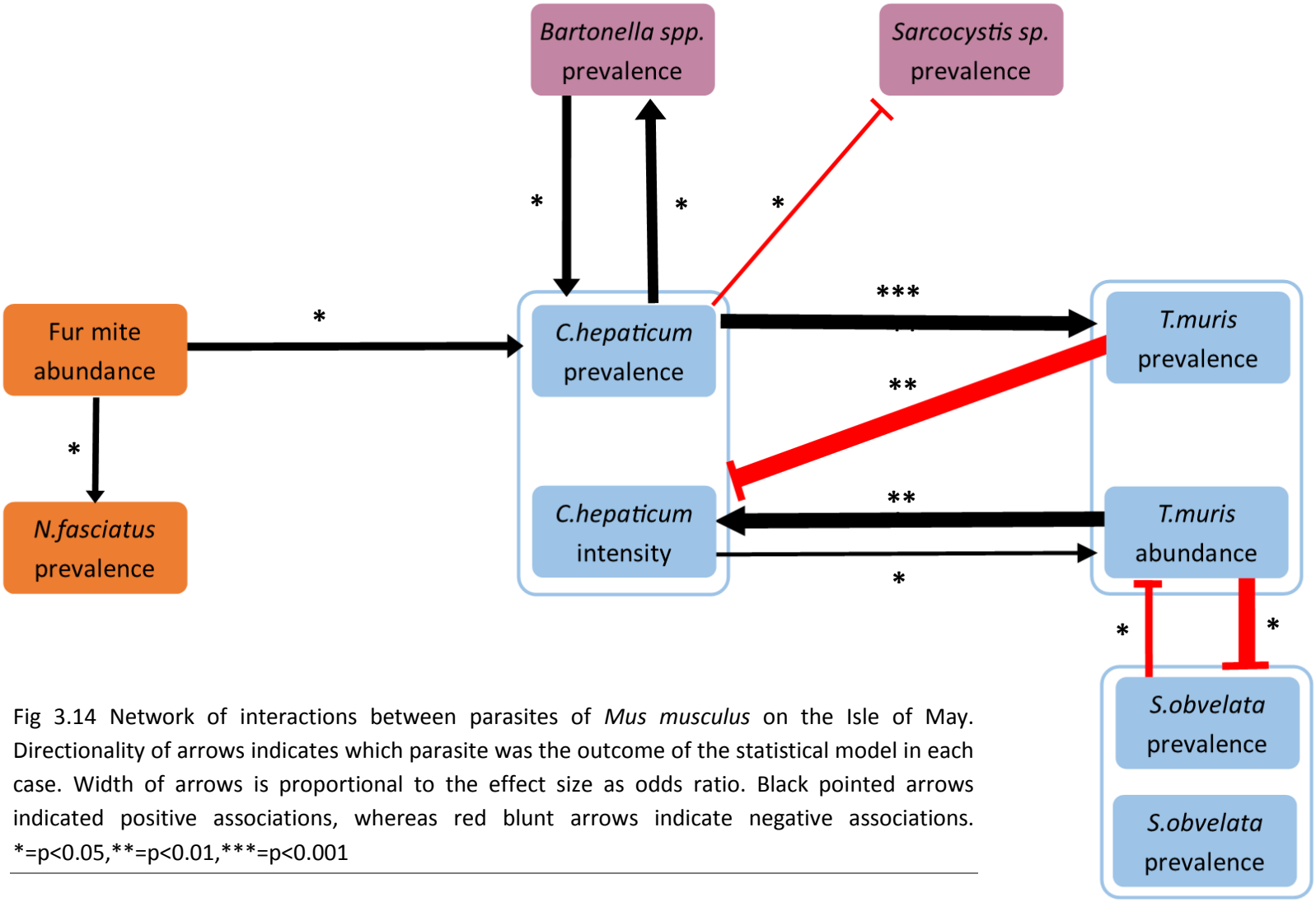


Fig 3.14 Network of interactions between parasites of *Mus musculus* on the Isle of May. Directionality of arrows indicates which parasite was the outcome of the statistical model in each case. Width of arrows is proportional to the effect size as odds ratio. Black pointed arrows indicated positive associations, whereas red blunt arrows indicate negative associations. *=p<0.05,**=p<0.01,***=p<0.001

RESPONSE		*T.muris*		*S.obvelata*		*N.fasciatus*	Fur mites	*C.hepaticum*		*Bartonella sp.*	*Sarcocystis*
		Abundance	Prevalence	Abundance	Prevalence	Prevalence	Abundance	Intensity	Prevalence	Prevalence	Prevalence
PREDICTORS		-	-	-	-	-	-	-	-	-	-
C.hepaticum	Intensity	**1.189***	-	-	-	-	-	-	-	-	**-1.815***
	Prevalence	-	**4.273*****	-	-	-	-	-	-	**3.067***	-
Bartonella sp.	Prevalence	-	-	-	-	-	-	-	**2.648***	-	-
n (individuals)		261	261								
n (models)		21	7								
Average DF		7.90	7.71								
T.muris	Abundance	-	-	-	**-4.839***	-	-	**4.465****	-	-	-
	Prevalence	-	-	-	-	-	-	**-5.397****	-	-	-
S.obvelata	Abundance	-	-	-	-	-	-	-	-	-	-
	Prevalence	**-2.193***	-	-	-	-	-	-	-	-	-
N.fasciatus	Prevalence	-	-	-	-		-	-	-	-	-
Fur mites	Abundance	-	-	-	-	**1.866***	-	-	**2.384***	-	-
SMI		-	-	-	-	-	**1.060***	-	-	-	-
Sex		-	-	-	-	-	-	-	-	-	-
Age estimate		-	**1.026*****	-	-	**1.007***	**1.006*****	**1.01****	**1.020*****	-	**1.017***
Density Estimate		-	-	-	**-1.632****	-	-	-	-	-	-
n (individuals)		366	366		366	366	366	261	261	261	261
n (models)		14	6		10	20	22	5	14	21	40
Average DF		6.93	5.83		7.10	7.50	7.50	8.21	10.20	7.00	7.50

Table 3.1 Model outputs of parasite interaction models. Associations are represented as positive (+) or negative (-) odds ratios, with level of significance (*<0.05,**<0.01,***<0.001). Models with *C.hepaticum* and *Bartonella sp.* as predictors are listed separately, as analyses were re-run without these variables to improve power. For each model, the number of individuals included, the number of models used in the model averaging process, and the averaging number of degrees of freedom of those models are listed.

3.4 DISCUSSION

Here I have identified some of the ecological processes underlying the distribution of parasitic infection in a host population, and highlighted the significance of both positive and negative inter-parasite interactions in coinfected individuals.

3.4.1 Ecological Impacts on Infection

As hypothesised, age exerts a strong influence on the distribution of a number of infections, including an increased infection prevalence of *T.muris, C.hepaticum, Sarcocystis sp.* & *N.fasciatus*, and increased infection intensity by *C.hepaticum* and mites. There are a number of potential causes for this, including changes in host condition or immunocompetence (Goodman and Makinodan, 1975), increases in exploratory behaviour (Barber and Dingemanse, 2010; Boyer *et al.*, 2010) and the lifecycle of the parasite itself.

The negative-binomial distributions of helminths and ectoparasites is typical of what might be expected from natural infections. Chronic *T.muris* infections have been observed in wild populations of *M.musculus* and the wood mouse *Apodemus sylvaticus* (Behnke and Wakelin, 1973). Artificial infection of animals from wild populations found that that the parasite typically persisted at low burdens of less than 20 worms (Behnke and Wakelin, 1973). *T.muris* infections have also been shown in lab models to form chronic infections in

tolerant hosts, facilitating immune evasion and allowing the parasite to persist indefinitely. The steady increase in prevalence towards 100% infection by *T.muris* with increasing age across this population, strong influence of age exhibited from the *T.muris* mixed models, and generally low parasite numbers found in the mice suggest that the chronic infections observed in other populations and lab mice is present here. A similar dynamic may also be occurring with the *C.hepaticum* infections. The fact that infection intensity of *C.hepaticum* also increases with age in this population suggests that this parasite can continually accumulate within a host, and does not maintain a low infection burden as with *T.muris*.

The increased prevalence of fleas, and abundance of mites in older mice may be due to simple accumulation of parasites during ageing. Studies of ectoparasites in other wild rodent populations have found similar increases in ectoparasite prevalence (Soliman, Marzouk, *et al.*, 2001). In wild gerbils, increased presence of ectoparasites in older individuals was not due to accumulation of infection, but rather an increased risk of mortality in highly parasitized juveniles (Hawlena, Abramsky and Krasnov, 2006). While this is also a possibility in *M.musculus* in the Isle of May, the level of pathology caused by, or indicated by ectoparasitic infection is unclear. Increases in mite abundance with age may be attributed to an increase in absolute body size associated with age, providing a larger physical niche for mite infestation. Positive associations between host body size and ectoparasites have been

recorded in populations of rats (*Rattus sp.*) (Mohr, 2006), striped field mice *(Apodemus agrarius*) and yellow-necked mice (*A.flavicollis*) (Stanko *et al.*, 2002), and red squirrels (*Tamiasciurus hudsonicus*) (Gorrell and Schulte-Hostedde, 2008), though these relationships are weakly supported, and distinguishing effects of age from absolute body size can be statistically challenging.

The only parasite showing a significant association with host population density is *S.obvelata*. Helminth infections have been shown to increase in abundance with host density across a variety of host taxa, so it is perhaps surprising that a negative association is observed here (Morand and Poulin, 1998). However, another population of wild rodents, bank voles (*Clethrionomys glareolus*) show decreased prevalence of common helminth parasites during periods of high host density (Haukisalmi, Henttonen and Frantisek, 1988). This effect may be attributed to seasonal changes in habitat use, and increased susceptibility of hosts during periods of lower population size. It is unclear whether something similar is occurring in the Isle of May population, as variation between trapping sessions was accounted for statistically. More robust estimates of population density from longitudinal mark-recapture work would be needed to elucidate the situation.

Although one might predict arthropod ectoparasite infections to flourish with increasing host density, as has been observed in surveys of wild populations

of gerbils (Krasnov, Khokhlova and Shenbrot, 2002), and other small mammals (Stanko *et al.*, 2002), the fact that no such effect is observed on the Isle of May may be due to changes in social behaviour such as increased levels of grooming with increasing host density (Stanko *et al.*, 2002). Alternatively, the measurements here used as proxies for host population density may be unreliable, and thus more robust mark-recapture methods of density estimation may uncover associations with ectoparasite distribution not evident from this cross-sectional dataset.

One significant predictor of mite abundance was host condition, as measured by SMI. The relationship is positive, suggesting mice in better condition have more mites. There are a number of potential causes for this. It may be due to the simple fact that relatively heavier mice are larger, and thus have a greater surface area of skin and fur for mites to become established. It may also be the result of some form of trade-off, whereby individuals devoting more resources to growth and feeding expending fewer resources on combatting parasitic infection.

While most recorded parasite species infect via direct transmission, *Sarcocystis sp.* requires a definitive predator host (see Chapter 1.1.2). Due to the absence of mammalian predators on the Isle of May, this likely to be one or multiple species of predatory bird. A number of birds of prey have been recorded on the island, including sparrowhawks (*Accipiter nisus*), buzzards

(*Buteo buteo*), gyrfalcons (*Falco rusticolus*), merlins (*Falco columbarius*), peregrines (*Falco peregrinus*), hen harriers (*Circus cyaneus*), Montagu's harriers (*Circus pygargus*), marsh harriers (*Circus aeruginosus*), short-eared owls (*Asio flammeus*), long-eared owls (*Asio otus*) and osprey (*Pandion haliaetus*) (Zonfrillo, 1981; Steel, 2018), any of which may be perpetuating infection.

3.4.2 Parasite Interactions

Although the ecological impacts discussed so far are crucial for understanding parasite distribution, the most significant and common variable determining infection prevalence and intensity was coinfection with other parasites. Contrary to our hypothesis, models of the two common gastrointestinal helminths indicate a negative association between *T.muris* and *S.obvelata*. There are various ways in which this relationship could manifest. Both parasites are typically found in the caecum or proximal colon, indicating that there may be some element of niche competition occurring. This could apply to the physical space which the parasites occupy, or resource-based competition over food. The latter scenario is perhaps more likely, as *T.muris* has shown plasticity in its site of establishment in caecectomised mice (Panesar and Croll, 1980), and embed in the gut wall when mature, whereas *S.obvelata* are generally found free in the lumen.

Another possibility is that there is some form of immune antagonism occurring. In laboratory-based infection models of *T.muris*, the parasite tends to establish chronic infections, characterised by low infection burdens, immunosuppression of protective Th2 responses and polarisation to a Th1 response (Else, Hültner and Grencis, 1992; Artis *et al.*, 1999). There are suggestions of this occurring in our wild population, as evidenced by the steady increase in prevalence associated with age, which indicates a lack of protective response on the part of the host, and the fact that the majority of infected individuals experience low burdens. Prevalence of *S.obvelata*, however, shows no significant impact of age, indicating that parasite clearance and possible reinfection could be the norm. Th2-type responses are typically protective against helminths, and so induction of Th2 responses by *S.obvelata* could disrupt the immunosuppressive environment established by *T.muris*, negatively impacting its abundance (Moreau and Chauvin, 2010). If this relationship is indeed immune-mediated, the order of infection is likely to be significant. If a mouse is first infected with *S.obvelata*, the immune environment may be altered such that the immunosuppressive effects of *T.muris* are less successful and the chronic infection cannot be established. If, however, *T.muris* is first able to establish and maintain a favourable Th1 environment, subsequent infection by *S.obvelata* may be insufficient to shift the immune state back to a protective Th2-type response.

Conversely, there is a clear positive association between infection intensity and prevalence of *T.muris* and *C.hepaticum*. Although these two parasites exist in separate organs for most of their time in the host, *C.hepaticum* eggs must migrate through the gut to reach the liver, and so an anti-Th2 influence of *T.muris* infection could positively impact *C.hepaticum* survival in the early stages of infection. The interaction observed in our population may thus be mediated by an immunosuppressive effect on the part of *T.muris* affecting the liver, protecting *C.hepaticum* from a protective immune response. Although protective IL-5-linked Th2 responses to adult *C.hepaticum* have been previously reported, the eggs of the parasite have been recorded as initiating an IFNγ-linked Th1 response (Kim, Joo and Chung, 2007). Depending on which stage of the parasite is most prevalent in the host, *C.hepaticum* may be polarising the internal environment of the host away from a Th2 environment, thus improving survival of *T.muris.* The basis of the apparent negative association between *T.muris* prevalence and *C.hepaticum* infection intensity is unclear, but may be due to a Th2 immune response to adult *C.hepaticum* infection preventing *T.muris* from becoming initially established.

The models presented here also provide evidence of a macro-micro parasite interaction. The prevalence of the microparasite *Sarcocystis sp.* is negatively associated with infection with *C.hepaticum*. Infection with microparasites like *Sarcocystis sp.* are often characterised by protective Th1 responses, so this association could be due to a polarisation to a Th1 immune environment by

C.hepaticum (Kim, Joo and Chung, 2007). Due to the low numbers of *Sarcocystis*-infected individuals in this population, it was not included as a predictor in the models, so this particular interaction could not be observed in both directions. As hypothesised, *Bartonella sp.* showed positive association with *C.hepaticum* prevalence. This may be due to polarisation from a Th1 to a Th2 environment caused by the adult worm, as has been observed in experimentally-infected rats (*Rattus sp.*) (Kim, Joo and Chung, 2007; Moon *et al.*, 2017). Another possibility is that *Bartonella sp.* is released from competition, caused by the negative association between *C.hepaticum* and *Sarcocystis sp.,* whereby reduced prevalence by the former parasite may increase prevalence of the latter.

Contrary to our hypotheses, however, no direct interaction was observed between the two species of microparasite present in this population, or between arthropod ectoparasites and microparasite infection. Ecological effects such as ageing and helminth interactions are more significant for the distribution of *Sarcosystis sp.* and *Bartonella sp.*, respectively.

Both *C.hepaticum* and *N.fasciatus* show positive associations with the abundance of mites in the fur. Being ectoparasites, there is perhaps less clear precedent of immune-mediated antagonism being the cause of these interactions, though inflammation on the skin may well contribute to protection against parasitic arthropods (Moats *et al.*, 2016), and the dermal

tissue is becoming increasingly recognised as a site of important immune activity (Kupper, 1990). Wild white-footed mice (*Peromyscus* leucopus) have shown increased innate immune function with higher abundances of ticks (Rynkiewicz *et al.*, 2013), so it is possible that this association could still be due to alterations in the immune environment.

What has become apparent in this population is that *C.hepaticum* is involved in interactions with most of the other measured parasites, and sits at the centre of the interaction network. Most parasitological studies of wild rodent populations have focussed on microparasites, ectoparasites and gastrointestinal helminths - here I highlight the importance of an often-overlooked parasite which may be crucial to understanding within-host parasite communities in wild rodents. While this network is informative as it includes infections from a diversity of taxa, it is likely to be incomplete, as no viral infections or microbiome communities were accounted for, and microparasite species not tested for may be present on the island. Any unrecorded infections may well add to the network as it is here described, and may help to better understand how coinfection dynamics are related to parasite distributions within the host population.

3.4.3 Conclusion

Here I have characterised the infection levels and distributions of various parasitic infections of the wild mouse population on the Isle of May, and found an assortment of associations between parasites, both within and between parasite groups. We can infer how some of these relationships might be established from cross sectional data, but longitudinal study is needed to confirm their mechanics. In order to elucidate how these infection dynamics actually come to exist in wild populations, it will also be crucial to look at factors such as genotype and immunophenotype, alongside environmental and ecological factors.

As was predicted, the network of relationships between parasites and their host in this population is complex. The sheer number of infectious agents present in a wild environment mean that the infection status of any one individual host is likely to be rare or even unique. As such, it is extremely difficult and perhaps impracticable to ascertain how the introduction of a new infection might affect all others, and the host, in the specific ecological conditions which exist. The models described here, however, give a good idea as to which parasites are significantly impacted by the presence of another and gives hints as to how the relationships between them might be working. This can be used as a foundation from which explore other key elements which determine the courses of infection, such as the strength and type of

immune response being elicited in response to infection, and the genetics of the host.

These analyses are unfortunately limited by a lack of seasonal resolution and a lack of longitudinal data, though when accounting for these limitations there are still a number of notable observations to be made. Due to the cross-sectional nature in which the mice were sampled, it is impossible to infer the direction of causality for some of the interactions between them. Being unable to assess causal relationships in host-parasite and parasite-parasite dynamics is an inherent limitation of cross-sectional ecological studies, and as such would be well-served with an accompanying longitudinal study, potentially assessing helminth parasite infections from faecal egg counts and microparasite levels from non-invasive blood sampling.

Chapter 4. Immune Responses to Parasitic Infection

4.1 INTRODUCTION

Parasites vary significantly in their life cycles and the manner in which they interact with their host. Correspondingly, the way in which a host responds to infection by a parasite also varies, depending on factors such as the condition of the host, the intensity and site of infection, and adaptive strategies for survival and transmission on the part of the parasite (Yetter *et al.*, 1980; Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002; Schmid-Hempel and Ebert, 2003). One key method by which parasitic infection can be combatted is through an induced immune response. Variation in the outcome of an infection can arise from differences in the types of immune responses elicited, as well as the age, sex, health, reproductive status and/or genotype of the host (Gause, Urban and Stadecker, 2003). The optimal response on the part of the host may also vary between tolerance and protective immunity (Petney and Andrews, 1998). As a result, when an individual is coinfected, the eventual effect on the host may be difficult to predict (Martin *et al.*, 2013).

The immune state of wild *Mus musculus* is relatively understudied (Viney, Lazarou and Abolins, 2015). Analysis of wild mouse populations have, however shown that they are more immunologically primed than laboratory mice, having higher baseline concentrations of antibodies and showing

greater increases in antibody and leukocyte concentrations in response to mitogenic stimulation, which implicates an important role for prior infections in immune response (Abolins *et al.*, 2011; Boysen, Eide and Storset, 2011). The fundamental differences between laboratory mice and wild populations are addressed in Chapter 1, with the prevalence of multiple infections being a key cause of differences in immunity (Chapter 1.3 - Differences Between Laboratory Animals and Wild Populations).

Different groups of parasites have been shown to activate and polarise the immune environment of the host in different ways. These differences can generally be associated with the taxonomic group of the parasite. Protective responses to intracellular microparasites and helminth macroparasites will often involve a Th1 and Th2-type responses respectively, though variation between species, life-cycle stages and levels of tolerance on the part of the host mean these responses can be highly variable (Chapter 1.2 – Parasite Immunology).

4.1.1 Immune Response to Helminths

Two species of gastrointestinal helminth were commonly recorded in the Isle of May mice; the whipworm *Trichuris muris* and the pinworm *Syphacia obvelata*. Reinfection studies of *S.obvelata* in lab mice showed that although adult mice show reduced worm burdens, no acquired immunity was induced

(Panter, 1969). In addition, experimental infection caused no increase in goblet cell numbers, a key mechanism of Th2-associated helminth expulsion (Marillier *et al.*, 2008).

In contrast, other studies have show transient Th2-style responses after infection with *Syphacia sp.* in murine models, characterised by eosinophil production and elevated levels of IL-4 and IL-13, after which the parasite is expelled (Bugarski *et al.*, 2006; Michels *et al.*, 2006). Production of *Syphacia* –specific antibodies has also been reported in laboratory mice in response to infection with *S.obvelata* (Sato *et al.*, 1995). Taghipour and colleagues found that in C57BL/6 mice, although numbers of $FoxP3^+$ Treg cells increased upon *S.obvelata* infection, indicating parasite-mediated immunosuppression, levels of pro-inflammatory cytokines like TNFα and IL-17 also increased (Taghipour *et al.*, 2017). The immunogenic role of *S.obvelata* is thus unclear. The fact that no acquired immunity or goblet cell hyperplasia is observed, however, suggests that despite recorded changes in cytokine profiles, immune-mediated expulsion may not be occurring.

The relationship between *Trichuris sp.* infection and host immunity is better characterised, from both lab and natural population studies of humans and rodents, in large part due to its significance in human health (WHO, 2006). Although different species of *Trichuris* infect humans and mice (*Trichuris trichiura* and *T.muris* respectively), both species have demonstrated antigenic

cross-reactivity, suggesting that similar interactions with the mammalian immune system may occur with both species (Roach *et al.*, 1988). In human populations naturally infected with *T.trichuria*, a Th2-type response has been shown to be required for resistance, indicated by increased levels of IL-13 (Jackson *et al.*, 2004). This response is influenced by age; older people with lower worm burdens and chronic infection have cytokine profiles indicating a shift from Th2 to Th1 expression, though also show higher levels of IgE antibodies associated with a Th2 response, which are proposed to keep burden levels low (Faulkner *et al.*, 2002).

Acquired immunity to *T.muris* in a laboratory mouse strain was first demonstrated by Wakelin, who found that mice which experienced an initial immunizing infection were able to clear worms more effectively than those who had never been infected (Wakelin, 1967). When challenged with high burdens of *Trichuris sp.*, resistant laboratory mice strains can clear the infection with a successful Th2-type response (Else and Grencis, 1991; Else, Hültner and Grencis, 1992; Else *et al.*, 1994). When initially faced with low burdens, however, chronic infections can manifest, whereby the parasite will suppress a protective Th2 response by the host and polarise the immune system to maintain a Th1-type environment, in which it can persist in a sensitive burden-dependent manner (Else and Grencis, 1991; Else, Hültner and Grencis, 1992; Grencis, 1993; Bancroft, Else and Grencis, 1994; Else *et al.*, 1994). Such 'trickle' infections are argued to be more indicative of the type of

infections faced in the wild (Bancroft *et al.*, 2001). Different strains of lab mice have shown to have different propensities between these two types of response, with BALB-strain individuals being more resistant than B10-strain mice, suggesting that resistance and tolerance to *T.muris* infection is at least in part genetically determined (Else and Wakelin, 1988; Wassom and Hauda, 1990; Else, Hültner and Grencis, 1992).

Tolerance to *T.muris* and chronic infection have been linked to inhibition of Th2-type immune responses by the parasite (Else *et al.*, 1994). This immunosuppressive effect of *T.muris* infection has been shown in C57BL/6 lab mice to vary between parasite strain, with higher worm burdens being associated with higher numbers of immunoregulatory $FoxP3^{+}$ cells (D'Elia *et al.*, 2009). The immunosuppressive influence of *T.muris* is purported to be adaptive on the part of the parasite, rather than a simple response by the host in order to prevent immunopathology or reserve metabolic resources (Bradley and Jackson, 2004). Excretory/secretory (ES) products produced by both larval and adult worms have shown to be important for polarisation away from a Th2 environment in tolerant strains of lab mice, reducing levels of allergic inflammation and IgE production (Else, 2005; Johnston *et al.*, 2005; Ebner *et al.*, 2014).

Although the mechanisms by which *Trichuris* species modulate host immunity are not well characterised, other helminths which alter immune responses do

so through mimicking host cytokines involved in immunosuppression, such as TGFβ, and the production of non-protein molecules and cystatins, which interfere with the activity of dendritic cells and major histocompatibility complexes (MHCs) respectively (Maizels and Yazdanbakhsh, 2003). A molecule mimicking the activity of IFNγ has been identified in *T.muris*, which may act to polarise the host gut to a Th1-type environment, and impart a chronic, tolerant phenotype (Grencis and Entwistle, 1997). This example of host/parasite coevolution, with mammalian hosts evolving a helminth-specific Th2 response, and the parasite adapting a means of immune evasion, is well established in lab infection models, but its implications for the health of wild animal populations invites further investigation.

Little has been published on immune responses to the hepatic nematode found in the Isle of May mice, *Calodium hepaticum*. Experimental infection studies have shown that *C.hepaticum* infection can induce a protective response in lab rodent models, involving a combination of Th1 and Th2 responses, which may act at different stages of the parasite's life cycle (Kim, Joo and Chung, 2007; Moon *et al.*, 2017). While adult *C.hepaticum* persists primarily within the liver, infection occurs through ingestion of eggs, which migrate through the GI tract and hepatic portal vein rather than the mucosal surface of the gastrointestinal tract. Kim et al found no significant shifts in cytokine expression in the MLNs, but rather increases in both Th1 and Th2 cytokines were detected from splenic expression data, indicating that host

immunity may be primarily responding to the adult parasite, particularly during egg shedding (Kim, Joo and Chung, 2007).

4.1.2 Immune Response to Microparasites

Two microparasites were identified in our study population, the apicomplexan *Sarcocystis sp.* and the bacterial parasite *Bartonella sp.* Mice which were infected with *Sarcocystis sp.* displayed immunity to later infection after parasitaemia had cleared, indicating the development of acquired immunity (Cox and Young, 1969). This acquired immunity was later shown to be mediated by T cells (Ruebush and Hanson, 1980). Infection experiments with C57BL/6 mice detected a protective response to *Sarcocystis neurona* infection, involving general increase in both CD4+ T helper cells and CD8+ killer cells (Witonsky *et al.*, 2003), a response that has also been recorded in naturally-infected horses (Yang *et al.*, 2006).

Evidence for a role of Th1-type responses against *Sarcosystis sp.*, as indicated by increased level of IFNγ and IL-12, have been recorded in sheep (Abdel-Baki *et al.*, 2009). This response may, however, be dependent on the life-stage of the parasite: measurement of different antibody classes in infected rats found that while a Th1-response as associated with acute infection, chronically infected rats elicited a mixed Th1-Th2 response (Jäkel *et al.*, 2001). Additionally, in pigeons (*Columbia livia*), while late stages of infection elicited

a Th1-type response, earlier stages of the disease were associated with reduced levels of Th1 cytokines (Olias *et al.*, 2013). Immunosuppression of Th1 responses has also been recorded in infected horses (Spencer *et al.*, 2004). These studies indicate that while a Th1 response may be protective against *Sarcocystis sp.* infection, suppression of this response early in infection could lead to a lack of parasite clearance and the establishment of chronic infection.

Bartonella sp. induces Th1 responses in C57BL/6 mice, characterised by increased splenic IFNγ expression and cell-mediated killing (Arvand *et al.*, 2001). Karem and colleagues, however, reported differences in response dependent on the method of infection, with mice infected orally or interperitoneally showing a Th1 response characterised by high IFNγ levels, but mice infected subcutaneously also showing an increase in IL-4, implying a role for Th2 responses (Karem *et al.*, 1999). This difference is significant, as transmission of *Bartonella sp.* can occur from blood feeding by arthropod vectors, as well as ingestion of infected faecal matter (Breitschwerdt and Kordick, 2000; Bown, Bennett and Begon, 2004). Consequently, the immune response to *Bartonella sp*. infection may be dependent on the route of infection.

Induction of the immunoregulatory cytokine IL-10 was observed in BALB/c after infection with *Bartonella henselae*, alongside an induced IFNγ response,

which is suggested to be adaptive on the part of the parasite to prevent Th1-mediated killing (Kabeya *et al.*, 2007). An IL-10 response has also been recorded in wild field voles (*Microtus agrestis*), proposed to be either an adaptive response by the parasite to dampen a protective Th1 response, or an adaptive approach by the host to reduce inflammation-associated tissue damage (Taylor *et al.*, 2018). Resistance to *Bartonella sp.* infections has been demonstrated via experimental infection of laboratory-bred cotton rats (*Sigmodon hispidus*), with hosts showing resistance to secondary infection in a species-specific manner (Kosoy *et al.*, 1999). These infections were dose dependent; individuals infected with a lower initial dose eventually developed more persistent and intense levels of bacteraemia, associated with a lack of antibody response, and indicative of a level of immune evasion to aid parasite survival. Transfer of serum antibodies from immunocompetent to immunocompromised lab mice (*M.musculus*) restored the ability of the mice to clear *Bartonella grahamii* infection, implying a crucial role in serum antibodies in protective responses to *Bartonella spp.* (Schulein *et al.*, 2014).

4.1.3 Immune Response to Arthropod Ectoparasites

Ectoparasites commonly recorded on the Isle of May mice include the flea *Nosopsyllus fasciatus* and mites of the genera *Myobia, Radfordia* and *Trombicula.* Immune responses to fleas and mites are less well characterised from lab studies than those of endoparasites, particularly with regards to role of specific genes and cytokines. *N.fasciatus* has been shown to reduce the

ability of the common vole, *Microtus arvalis,* to produce antibodies against artificially introduced keyhole limpet haemocyanin (KLH), an effect attributed to either impaired development of immune organs, immune suppression caused by the parasite, or a trade-off between different types of immune response (Devevey *et al.*, 2008).

The fleas *Xenopsylla conformis mycerini* and *Xenopsylla ramesis* have been shown to increase the leukocyte blast index (a measure of reactivity of mononuclear cells), and reduce phagocytic activity in jirds (*Meriones crassus*) (Khokhlova *et al.*, 2004). Gerbils (*Gerbillus spp.*) infected with *X.ramesis* however showed differences in white cell counts, but no differences antibody levels, leukocyte blast index or phagocytic activity (Khokhlova, 2004). In white-footed mice (*Peromyscus leucopus*) and prairie voles (*Microtus ochrogaster*) no association was observed between infestation with the flea species *Ctenophthalmus pseudagyrtes* and *Orchopeas leucopus*, and bacterial killing ability (Rynkiewicz *et al.*, 2013).

Analysis of parasite communities on immunoregulation in a wild population of wood mice (*Apodemus sylvaticus*) found toll-like receptor responses were modified in animals suffering infection with the louse *Polyplax serrata*, but not mites or fleas (Jackson *et al.*, 2009). Immunity to the scabies mites in rabbits was found to be dependent on having upregulated Th1 and downregulated Th2 responses (Arlian, Rapp and Morgan, 1995). In addition, lab mice have

shown immune response to dust mites to involve Th2-type allergic inflammation, promoted by IL-21 (Coquet *et al.*, 2015). Thus, there is conflicting evidence from experimental and wild studies as to the immunogenic significance of arthropod ectoparasite infections. Despite this, many of the methods used in these studies have given fairly basic measures of immune function, and thus more sophisticated assays may highlight some immune impacts of ectoparasitic infection in more detail.

4.1.4 Localisation of Responses and Concordance Between Measures

When assessing immune responses to infection it is crucial to consider the measures used when interpreting the results. Immune expression is a complex network of induction, inhibition and regulation and the immune environment within an individual is constantly shifting, so it is important to understand exactly what aspect of an individual's immunophenotype is being represented by an assay or measurement. In this thesis, three molecular methodologies were used to assess immunity- qPCR analysis of gene expression from two tissue types, and multiplex bead assay measuring concentration of circulating cytokines. Comparisons of mRNA and protein levels in yeast have found levels of correlation to be variable, with some genes showing high disparity between mRNA and protein measures (Gygi *et al.*, 1999; Maier, Güell and Serrano, 2009). Stimulated spleen cells of inbred ICR-strain mice showed differences in mRNA-protein correlations between

cytokines, with minimal correlation seen in TNFα, but greater levels in IFNγ and IL-4 (Favre, Bordmann and Rudin, 1997).

There are a number of possible causes of variation between the two forms of measurement, including different decay half-lives of proteins and RNA (Cleveland and Yen, 1989; Varshavsky, 1996; Pérez-Ortín *et al.*, 2013), and regulation occurring between transcription and translation of mRNA (Zhao, Roundtree and He, 2017; Boccaletto *et al.*, 2018). In addition to these factors, many of the infections discussed thus far induce localised immune responses, and so measures of immune response obtained from a tissue near the site of infection may demonstrate responses not observed elsewhere (Engwerda and Kaye, 2000). The extent to which these measures correlate in wild animal populations is currently unclear.

4.1.5 Seasonal Immune Variation

Significant seasonal changes in immune function have been observed in rodents, with conflicting results. Lab-based studies have shown increases in immune function when shorter days of winter were simulated (Blom, Gerber and Nelson, 1994; Demas and Nelson, 1998a; Yellon, Fagoaga and Nehlsen-Cannarella, 1999). Changes associated with shorter days in lab studies include increased leukocyte numbers and enhanced cytolytic capacity of natural killer cells in Siberian hamsters (*Phodopus sungorus*) (Yellon, Fagoaga and Nehlsen-

Cannarella, 1999; Bilbo *et al.*, 2002), increased spleen mass in Syrian hamsters (*Mesocricetus auratus*) (Brainard *et al.*, 1987) and increased spleen mass and lymphocyte numbers in deer mice (*Peromyscus maniculatus*) (Blom, Gerber and Nelson, 1994; Demas and Nelson, 1998a).

This dynamic is less clearly demonstrated in wild populations; in wild cotton rats (*Sigmodon hispidus*), although a number of immune measures varied seasonally, these changes were different across measures, and not consistent between years (Lochmiller, Vestey and McMurry, 1994). Systemic patterns of seasonal changes in immunity have, however, been recorded. Across three years, field voles (*M.agrestis*) in Kielder Forest, UK, showed consistent reductions in lymphocytes over the Winter and Spring, indicating a reduction in immune function, and contrasting with a Spring increase in monocytes and lymphocytes associated with increased inflammation (Beldomenico *et al.*, 2008b). Changes in immunity with season are also likely to be dependent on the life-cycle of the population in question- for example, ground squirrels (*Citellus richardsoni*) showed reductions in antibody titres and plaque-forming spleen cells in winter, as a result of yearly hibernation (Sidky, Hayward and Ruth, 1972). The adaptive nature of seasonal immune control in wild populations will depend on the relative fitness costs of infection, immunity, and trade-offs with other seasonal processes like reproduction, and will therefore likely vary between populations and species according to their ecology (Martin *et al.*, 2008).

Control of immune response in relation to seasonality is widely attributed to shorter day lengths causing increased levels of melatonin, a hormone essential for biorhythmicity in animals (Axelrod, Wurtman and Snyder, 1965; Walton, Weil and Nelson, 2011). The mechanism of melatonin on immunity may be imparted through the production of endogenous opioid peptides, which bind to opioid receptors on antigen-activated leukocytes (Maestroni, Conti and Pierpaoli, 1987, 1988).

4.1.6 Ageing and Immunity

The impacts of ageing on immune expression, often termed 'immunosenescence', are well recorded from murine laboratory models (Brubaker, Palmer and Kovacs, 2011). These changes are typically associated with alterations or dysfunction in inflammatory expression; older mice have been shown to have increased levels of proinflammatory cytokines including IL-1β, TNFα and IL-6, and increased levels of autoimmunity (Bruunsgaard, Pedersen and Pedersen, 2001; Brubaker, Palmer and Kovacs, 2011). Williams and colleagues observed increased innate responses and higher levels of pro-inflammatory cytokines in response to infection by the bacterium *Streptococcus pneumoniae* in aged mice relative to younger mice, as well as reduced IL-10 expression (Williams *et al.*, 2015). Despite this, the innate immune response to infection and wound healing has shown to be impaired in aged mice (Swift *et al.*, 2001; Murciano *et al.*, 2008). These findings indicate a general reduction in regulation of inflammation in immunity in older mice.

Prior exposure to infection, leading to a more primed immune state, is an effect that may also complicate the impact of age on immune state in wild populations (Boysen, Eide and Storset, 2011; Abolins *et al.*, 2018).

Jackson and colleagues found that older males from a wild population of field voles (*M.agrestis*) displayed a more tolerant phenotype in response to macroparasite infection, whereby older males accumulated high parasite burdens, associated with increased expression of the Th2-associated transcription factor GATA3, and an increase in body condition (Jackson *et al.*, 2014). While it was proposed that this effect could potentially be the result of increased mortality of poor condition, highly parasitized older males (which would therefore not be sampled), or an increase in infection risk in good-condition animals, longitudinal data implicated GATA3 expression as an actual response to infection in older males, which results in better condition (Jackson *et al.*, 2014).

4.1.7 Sex Differences in Immunity

Differences in immune state between sexes are well recorded in a number of vertebrate species, with immune function generally reduced in males compared to females (Klein, 2000; Klein and Flanagan, 2016). Stimulated dendritic cells from women show increased IFNα expression compared to those from men (Griesbeck *et al.*, 2015). Differences in immunity are thought

to be largely hormone-mediated; levels of IgG and IgM antibodies are reduced in male lab mice relative to females, an effect which is negated upon castration (Eidinger and Garrett, 1972; Tartakovsky *et al.*, 1983).

Female mice show increased IFNγ and reduced IL-10 expression after fungal infection, associated with macrophage fungicidal activity, relative to males (Pinzan *et al.*, 2010). This difference can be reversed through castration of males, or testosterone treatment of females (Pinzan *et al.*, 2010). Treatment of both female and male mice with testosterone caused reduction in splenic natural killer cell activity (Hou and Zheng, 1988). In contrast to this, sexual differences in immune function of deer mice (*Peromyscus sp.*) and meadow voles (*Microtus pennsylvanicus*) were not associated with changes in hormone levels, but in the case of deer mice, larger males showed reduced splenocyte proliferation (Klein and Nelson, 1997, 1999). Mechanisms of immune suppression in males may therefore be a combination of resource allocation trade-offs and hormonal effects. Sex-associated differences in parasite burden and prevalence have been linked to sex hormones in wild and laboratory rodents, an effect which may well be mediated through their effects on the immune system (Reviewed in Klein, 2004).

Mechanisms of sexual differences in immunity in wild populations are less well documented. Wild female wood mice show greater increases in spleen mass in response to increasing parasite diversity, while PHA levels of male

wood mice are negatively associated with increased testes mass (Bordes *et al.*, 2012). While sexual differences in wild populations may in theory also be attributable to allocation of metabolic resources to sexual competition over immunity in males, rather than a purely hormonal effect, this distinction is largely untested in wild mammal populations (Sheldon and Verhulst, 1996; Nunn *et al.*, 2009). In wild field voles (*M.agrestis*), while immune changes were associated with life history stages across sexes, with lower anti-inflammatory expression observed in both pregnant females and mating males, there were no systematic differences in immune expression between sexes, highlighting that sex differences are likely to be population- and context-dependent (Jackson *et al.*, 2011).

4.1.8 Hypotheses

Using lab-based immunological assays, I here attempt to relate the parasitological profile of each individual host animal with an associated immunophenotype. By looking at immune responses to parasites in a wild population of a commonly used lab infection model species, I determine how those responses manifest in a resource-limited, heterogeneous and diverse ecosystem, and the relative significance of infections in determining the immune environment. Working from precedent from lab and field studies, I hypothesise that:

1) **Seasonal variation will cause significant differences in immune state across trapping sessions.**

2) **Older mice will show differences in expression of pro-inflammatory cytokines relative to younger mice.**
3) **Male mice will have reduced levels of cytokine expression relative to females.**
4) **Measures of immune genes mRNA will not closely correlate with concentrations of circulating cytokines.**
5) **High burdens of gastrointestinal helminths will be associated with a protective Th2 response, whereas chronic infections will be associated with a Th1 or Treg-type response.**
6) **Protective Th1 responses will be observed upon infection with microparasites, with potential immunosuppressive IL-10 expression during *Bartonella sp.* infection.**

4.2 METHODS

4.2.1 Parasitological Analysis

All parasitological surveys for helminths, microparasites and ectoparasites were performed as described in Chapter 2.3 - *M.m.domesticus* on the Isle of May – Parasitological Surveys.

4.2.2 Immunological Assays

qPCR of splenic and MLN gene expression and multiplex assays of cytokine concentration were performed as described in Chapter 2.4-2.5.

4.2.3 Concordance Between Immune Measures

Pairwise correlation matrices were drawn between mRNA and cytokine levels between each pair of measures and tested for significant correlations. Correlations were also tested across measures for functional groups of immune genes, constrained into artificial principal components (Th1,Th2,Th17-linked genes) and overall principal components of expression. These analyses were conducted using the '**rcorr.adjust**' function of the '**RcmdrMisc**' package in R, with p-values corrected by the Holm-Bonferroni correction for multiple tests.

4.2.4 Statistical Analysis

For each measure of immune function (MLN gene expression, spleen gene expression and circulating blood cytokine concentration), a scaled redundancy analysis (RDA), which determines redundancy axes explaining the greatest amount of variation within the data, was performed. Redundancy analyses simplify variation in response variables into redundancy axes, analogous to principle components, but with the inclusion of explanatory variables which determine the loadings of the axes. For each measure, an initial analysis was performed including ecological variables including trapping session, age estimate, sex, location and population density estimate. A second analysis was then performed including prevalence and intensity of parasites as explanatory variables, with any significant ecological variables from the initial analysis included as conditions. These analyses were performed to simplify general trends in immune expression and find correlations between levels of expression of different genes within a measure, while also seeing which elements of an individual's ecology might be causing shifts in broader patterns of immune expression.

For the RDAs to be performed, only individuals with gene expression data for every measured gene could be included, thus reducing the power of the analysis. As such, mixed models were also performed for individual genes. Expression data for each individual gene were used as response variables, with environmental predictors that were found to be significant in the

redundancy analyses included as random effects. Each model was then put through a model averaging process. All analyses were performed in R, using the '**lme4'**, '**vegan**' and '**MuMIn**' packages.

4.3 RESULTS

qPCR of genes IL-5, Il-12β, RORC and FoxP3 from MLN-derived cDNA were unsuccessful, as successful amplification of only the gene of interest could not be confirmed from the melt curve. As such, MLN measures for these genes are not included in further analysis (see 2.4 – qPCR Analysis of Gene Expression).

4.3.1 Concordance of Immune Measures

Immune expression within measures tended to positively correlate, whereas no statistically significant correlations between genes were found between immune measures. The outputs from these are shown in Fig 4.1

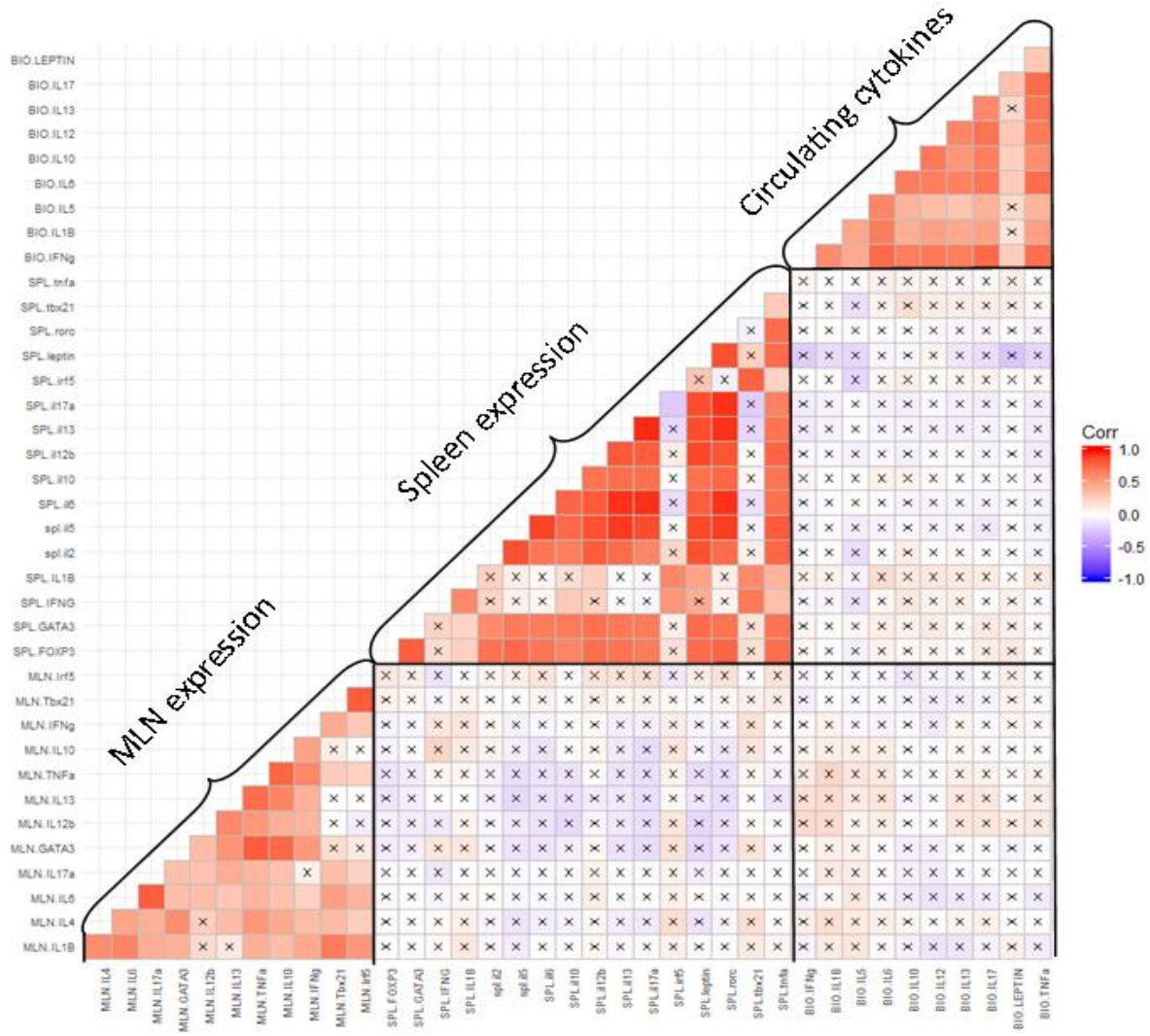


Fig 4.1 Correlation matrix of levels of immune expression across immune measures, comparing values of immune gene expression obtained from splenic and mesenteric lymph node RNA, and circulating cytokines measured from multiplex ELISA. Darkness of coloration indicates correlation coefficient. Statistically insignificant correlations are marked with an 'x'. Red coloration indicates positive correlation, whereas blue coloration indicates negative correlation.

No significant correlations were found between functional groups of cytokines between measures, while strong levels of correlation were recorded within measures. These results are shown in Fig 4.2.

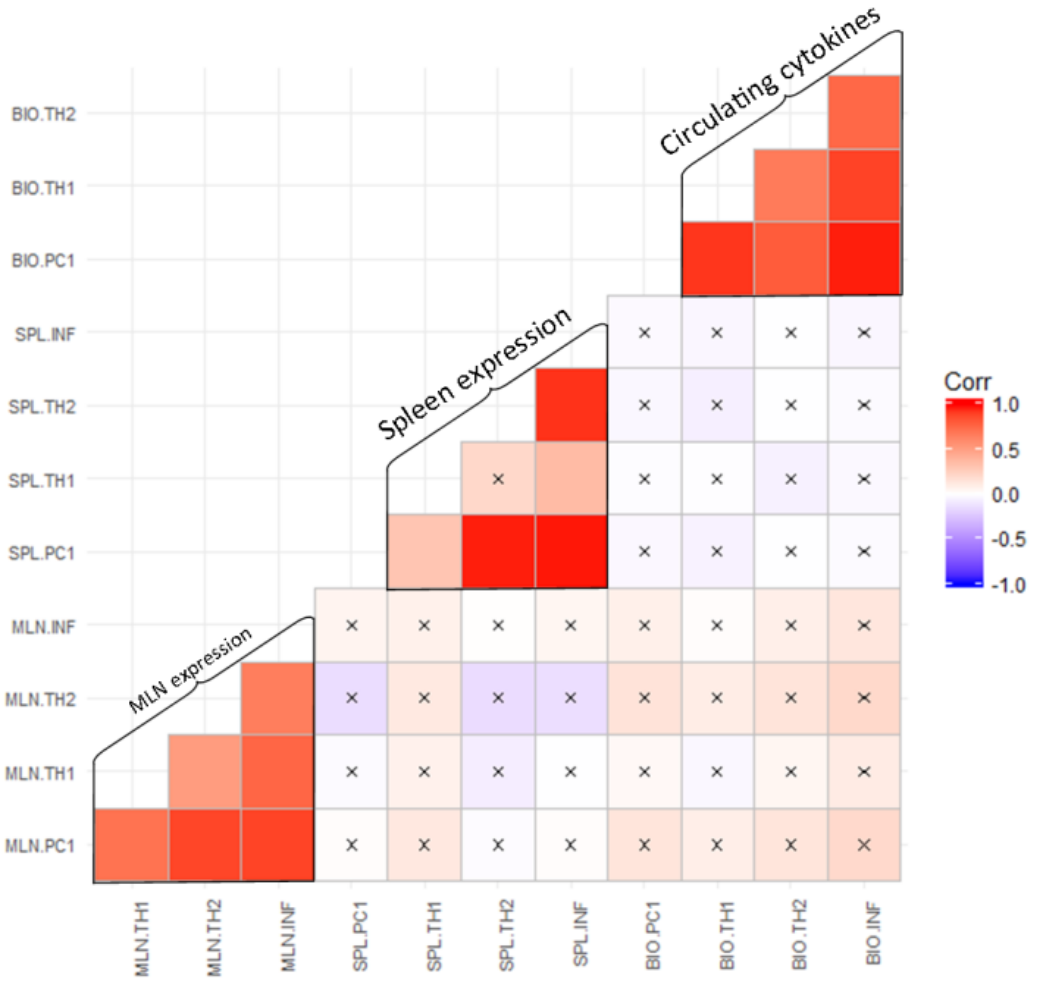


Fig 4.2 Correlation matrix of functional immune groups across three measures: MLN and spleen gene expression measured by qPCR, and multiplex bead assay of circulating proteins. Functional groups include first principal components (PC1), indicating overall immune expression, Th1, Th2 and inflammatory (Th17) expression. Darkness of coloration indicates correlation coefficient. Insignificant correlations are indicated by an 'x'. Red coloration indicates positive correlation whereas blue coloration indicates negative correlation.

4.3.2 Ecological Redundancy Analyses

The ecological redundancy analyses are summarised in Table 4.1. Significant differences in circulating cytokines were observed between sexes (p=0.0047) and trapping sessions (p=0.0059) (Fig 4.3). Significant differences in MLN gene expression were observed between trapping sessions (p<0.0001) (Fig 4.4). The trapping session schedule relating to these differences is listed in Chapter 2 – Methodology (Table 2.1). No significant impacts upon splenic gene expression were observed from ecological explanatory variables.

	Cytokines	MLN Expression	
	RDA1	RDA1	RDA2
IFNγ	0.491	0.241	-0.141
IL1B	0.442	0.0954	-0.354
IL4		0.409	-0.0180
IL5	0.232		
IL6	0.340	0.0808	-0.396
IL10	0.201	0.400	0.245
IL12	0.298		
IL13	0.229	0.492	0.109
IL17	0.351	0.194	-0.413
TNFα	0.298	0.413	-0.0750
GATA3		0.366	0.139
Tbx2		0.0813	-0.403
Irf5		-0.0935	-0.517
p	0.0214	<0.0001	<0.0001
Adj R	0.0788	0.182	
n	235	226	
% variation explained	58.66	41.59	30.56

Table 4.1 Loadings of redundancy axes of circulating cytokine levels and MLN gene expression, in response to ecological variables. Cytokine RDA1 represents universal increases and decreases across all measured cytokines. MLN RDA1 represents universal increases and decreases across expression of all measured genes except Irf5. MLN RDA2 represents increases and decreases in expression of the Th2-linked genes GATA3 and IL13, and the Treg-linked gene IL1-, relative to expression of all other measured genes.

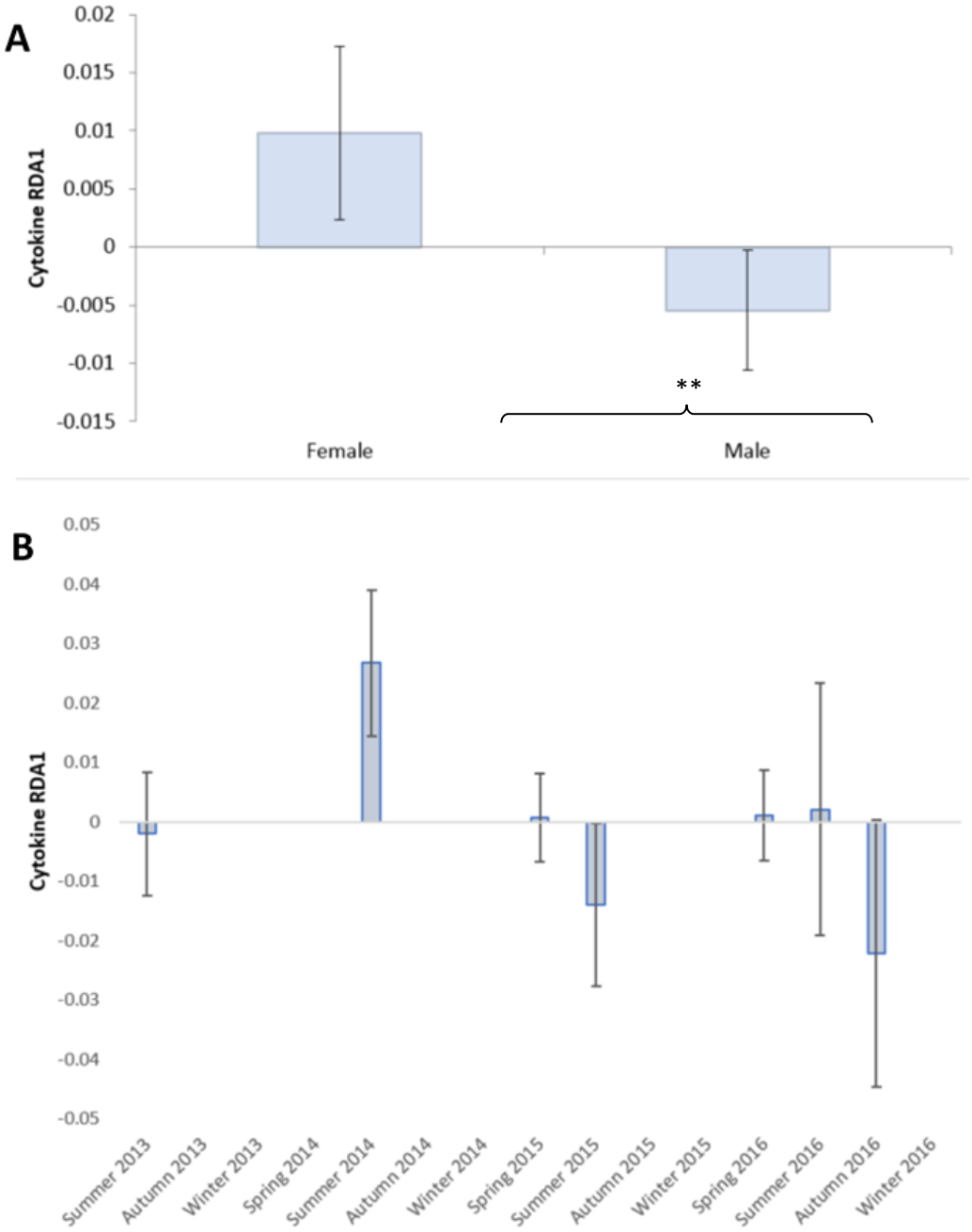


Fig 4.3 Differences in overall levels of cytokine RDA1, as determined by multiplex ELISA, (A) between sexes, with males having lower levels of circulating cytokines on average p=0.0047**, (B) across trapping sessions, with high variability between session, but insufficient data to resolve this variability to seasonal patterns. (Bars=SEM, p=0.0059**. *=p<0.05,**=p<0.01,***=p<0.001).

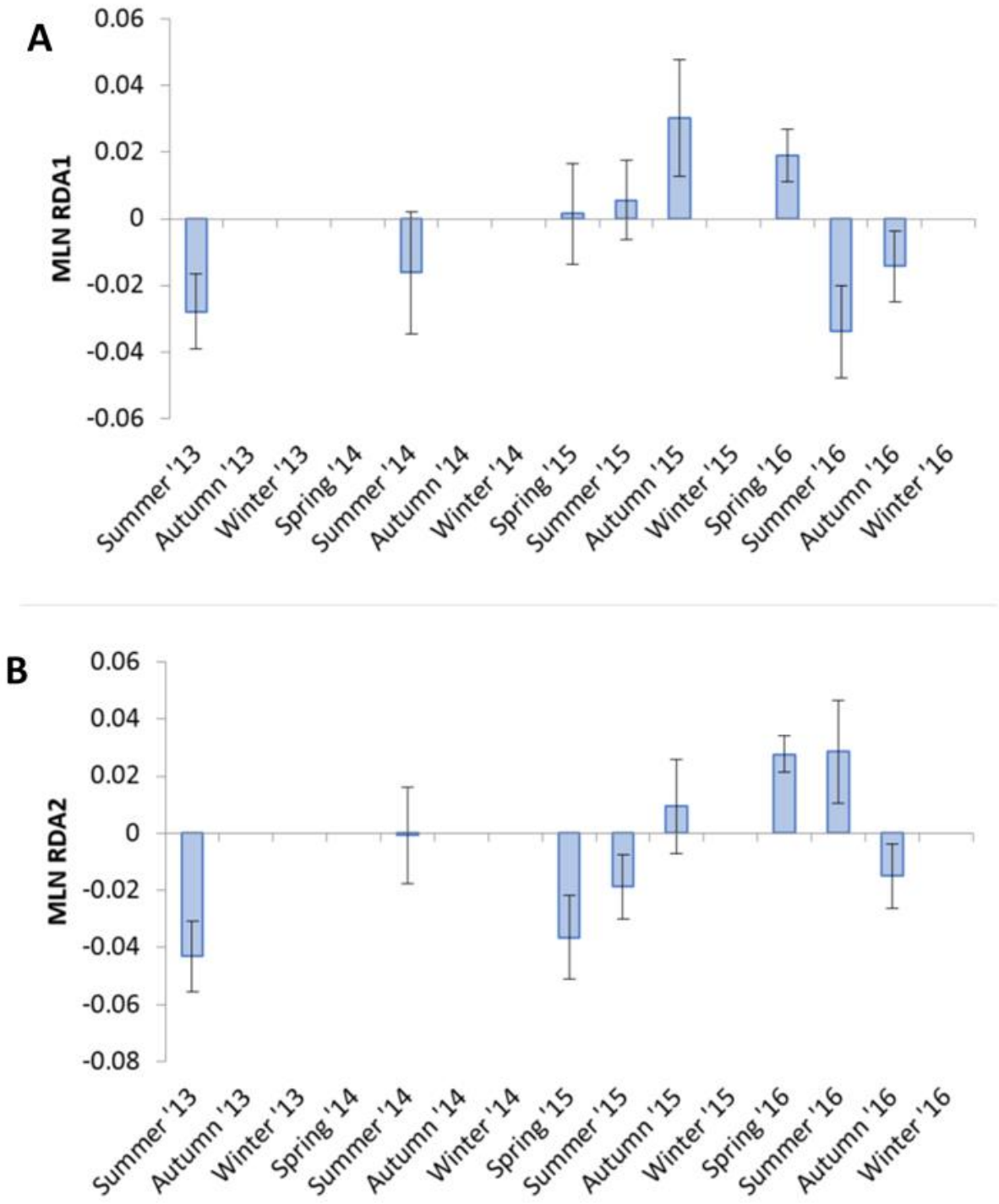


Fig 4.4 Significant variation in levels of MLN immune gene expression between across trapping sessions, showing marked inter-annual variability, p=0.0001***. (A) Increasing RDA1 values indicate increasing expression levels of all cytokines except Irf5. (B) Increasing RDA2 values indicate reduced expression of pro-inflammatory cytokines (Bars=SEM, *=p<0.05,**=p<0.01,***=p<0.001)

4.3.3 Parasite Redundancy Analyses

No significant impacts of infection upon redundant axes of immune expression were observed across any measures.

4.3.4 Mixed Models

No significant impacts of infection were observed across multiplex assay or MLN expression mixed models. Increased infection burden of *T.muris* was associated with reduced splenic expression of FoxP3 (Fig 4.5, p=0.00983). Infection with *C.hepaticum* was associated with reduced expression or IFNγ ($p=3.70x10^{-6}$) and Irf5 ($p=2.89x10^{-5}$) in spleen tissue (Fig 4.6).

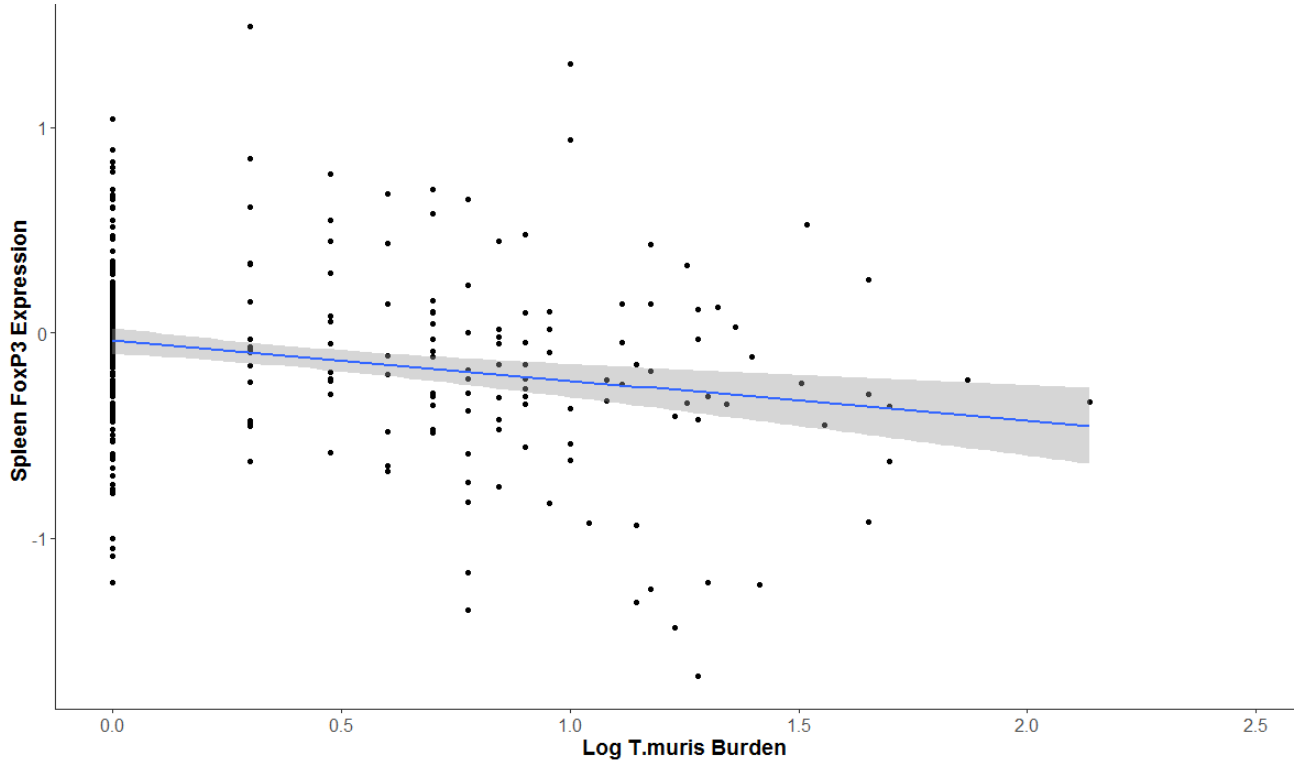


Fig 4.5 Decrease in splenic expression of FoxP3 with increased infection burden of *T.muris*. p=0.00983**, coefficient =-0.278 (*=p<0.05,**=p<0.01,***=p<0.001)

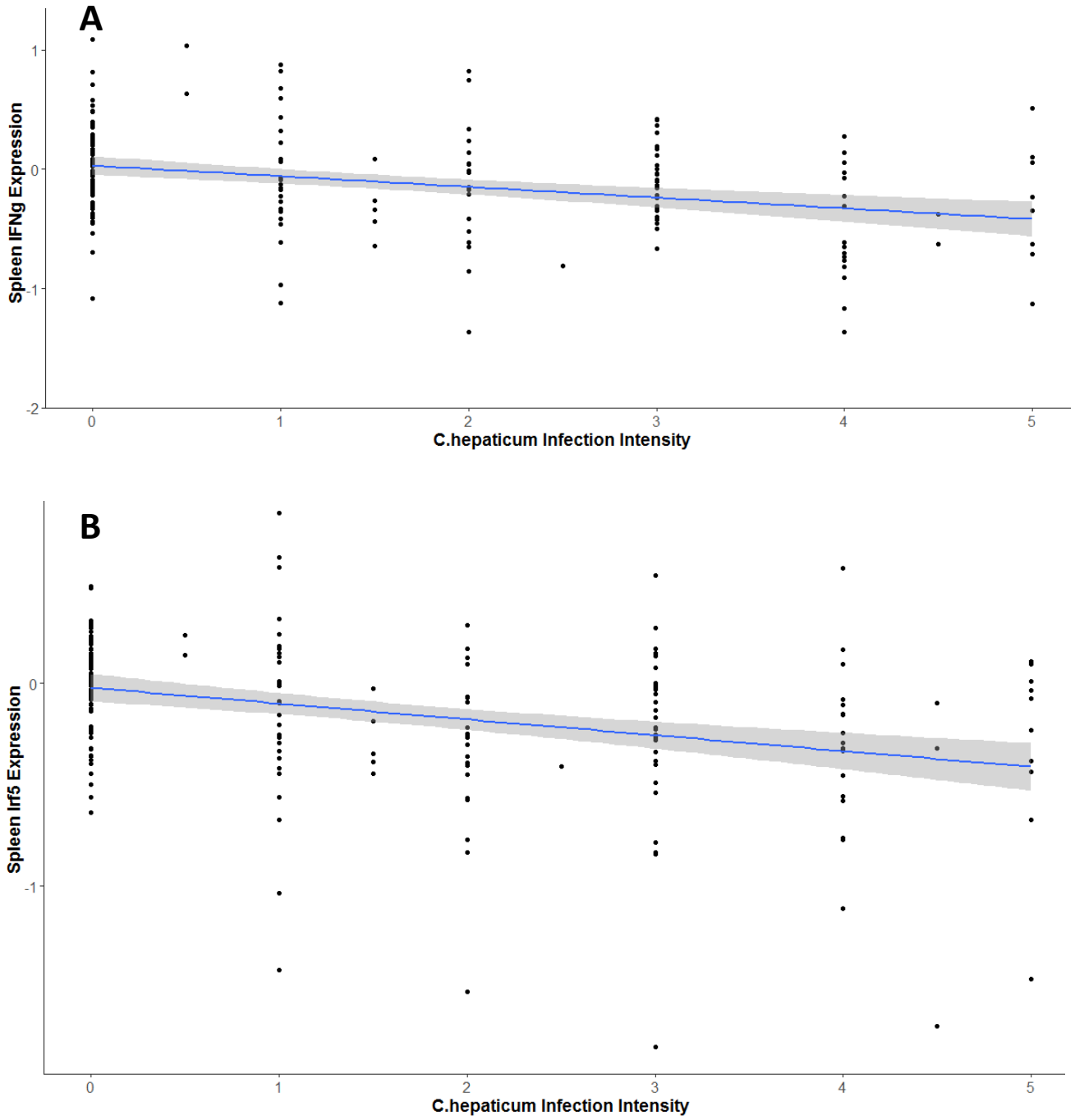


Fig 4.6 (A) Decrease in splenic expression of IFN-γ ($p=2.00x10^{-7}$***) (B) Irf5 ($p=9.16x10^{-5}$***), with increased infection burden of *C.hepaticum*, as explained by mixed model analysis of gene expression. (*=p<0.05,**=p<0.01,***=p<0.001).

4.4 DISCUSSION

Both circulating cytokine levels and MLN gene expression levels showed a strong influence of trapping session. Changes in immune function with season, attributed to changes in resource availability and metabolic needs throughout the year are well documented in rodent populations (Demas and Nelson, 1998b; Nelson, 2004). No clear pattern is observed between seasons, and there appears to be variation between years. This may in part be due to poor resolution resulting from gaps between sessions, and thus longitudinal data of greater resolution throughout the year may help us to better understand this variation. Unpredictable levels of physiological variation both between and within years is a recorded feature of cyclic populations of small mammals, resulting from complex interactions of ecological factors, and this may be being reflected in the immune responses of mice on the Isle of May (Taylor, White and Sherratt, 2013).

No effect of age was observed on any measures of immune state, either through changes in levels of pro-inflammatory cytokines as hypothesised, or through a focussed shift to a tolerant phenotype as observed by Jackson and colleagues (Jackson *et al.*, 2014). While inflammation in older mice has been observed in a number of laboratory settings, mice in these situations often live longer than wild mice – no mice on the Isle of May were recorded as being over one year old as estimated from eye lens mass, where in Williams and colleagues' study on inflammation in ageing BALB/c lab mice, the aged

group were culled at 24 months (Williams *et al.*, 2015). As such, it is possible that mice on the Isle of May are dying before any measurable effects of ageing on immune state can occur. The complex influence of infection history found in wild populations may also be complicating any underlying ageing effects. Alternatively, markers for inflammation which were not included in this analysis may be influenced by age.

There is a significant association of sex with cytokine levels, with males having generally lower levels of circulating cytokines. Reduced immune function in males has been observed in rodent populations, resulting from male-male competition expense of the immune function (Li *et al.*, 2007). This effect may also be due to hormonal impacts upon immunity, whereby hormonal differences between males and females underly persistent differences in immune function (Barnard, Behnke and Sewell, 1994), or cause differential responses to environmental turbulence (Rooney, Matulka and Luebke, 2003).

As hypothesised, there was no significant correlation between measures of immune function found between gene expression in the spleen and the MLNs, and circulating cytokines in the blood. While these differences may be due to factors like localisation of immune effects, discrepancies in decay rates or mRNA and protein, or multi-level immune regulation, it is notable that external factors affecting the immune environment were discovered distinctly

across all three measures. This may be due to different aspects of immune control being affected in different ways, or certain effects being more detectable by different measures. Regardless, these results validate the use of multiple measures when attempting to obtain an optimal, comprehensive picture of an animal's immune function within a heterogeneous environmental context. While analysis over time may help to elucidate any temporal relationships between gene expression and cytokine concentration, collection and analysis of different tissues types in this way typically requires killing of the animal. Care should therefore be taken when selecting which measure(s) are selected for analysis, and when inferring the biological significance of any observed difference in immune state.

There was no effect of any ectoparasitic infection across any immune measures. In addition, contrary to our hypothesis, no impact of *Sarcocystis sp.* or *Bartonella sp.* infection was detected. In rodents, apicomplexan parasites such as *Babesia microti* (Telford and Spielman, 1993; Krause *et al.*, 1998; Taylor *et al.*, 2018) and in some cases, *Bartonella sp.* (Kosoy *et al.*, 1997; Brouqui *et al.*, 1999; Birtles *et al.*, 2001; Chiaraviglio *et al.*, 2010) have been found to establish chronic infections in the host, whereby the parasite evades the host immune system and persists for long periods without being cleared. In a wild environment, the potential metabolic and immunopathological costs of combatting microparasitic infections such as these may outweigh the

benefits, and so these parasites may be establishing chronic infections in the context of a tolerant host phenotype.

The only observed immune impacts of parasitic infection in this population were by helminth worms, and all detected in splenic gene expression. Increase in infection burden of the gastrointestinal nematode *T.muris* was accompanied by a decrease in expression of the regulatory cytokine FoxP3. Chronic *T.muris* infection are characterised in lab models by a Th1-polarised environment, with a protective Th2 response being elicited during acute infection (Else and Grencis, 1991; Bancroft *et al.*, 1997; Hasnain, Thornton and Grencis, 2011). The related *Trichuris suis* has been shown to reduce protective Th2 responses, whereas another gastrointestinal helminth, *Heligmosomoides polygyrus* has been shown to induce FoxP3, both through the release of excretory/secretory products (Grainger *et al.*, 2010; Ebner *et al.*, 2014). This may be the effect observed in this population, as low level chronic infections are accompanied by higher levels of FoxP3 expression, and are able to establish and persist via active suppression of the host's immune system. That no changes were detectable in MLN expression may be due to the influence of microfloral communities in the gut, which are prone to turbulence by outside factors such as diet (Goldin and Gorbach, 1977), and are intimately linked with gastrointestinal immune expression (Tlaskalova-Hogenova *et al.*, 2004).

The other helminth associated with significant shifts in splenic immune expression is the hepatic worm *C.hepaticum*. Increasing infection intensity of this parasite was associated with increased expression of IFNγ and the associated cytokine Irf5, indicating a Th1 response. This is in line with what was observed by Kim et al, who found immune responses to this parasite mediated by splenic rather than MLN expression, and a polarisation toward Th1 expression during egg production (Kim, Joo and Chung, 2007). It is still unclear as to whether this Th1 response is protective against the parasite's eggs, or whether it is an adaptive response on the part of the parasite to prevent expulsion by a Th2 response characterised by IL-5 expression, as purported by Kim and colleagues (Kim, Joo and Chung, 2007). The fact that this polarisation is occurring may, however, explain to positive interaction observed between this parasite and *T.muris*, and described in Chapter 3 of this thesis, as polarisation away from a Th2 environment would prevent expulsion of adult *T.muris* from the gut.

Here I have explored the ecological and parasitological factors affecting immune expression in an island population of the house mouse with well characterised infections. What is clear is that different measures of immune function can give contrasting, but equally informative, information about how an animal's immune environment is reacting to its environment. Most of the parasites recorded in the Isle of May mice do not appear to be having a significant impact on host immunity, and being an isolated population, the

mice may have grown tolerant to many of these infections over time. We have, however, observed effects of two helminth parasites, which agree well with prior laboratory studies. These observations demonstrate that while lab models of infection and immunity are crucial for understanding the mechanisms of immune response, infections in natural populations may act in unexpected ways, or be impacted by other selective pressures of the environment.

Chapter 5 – Genetic Influence on Immune State and Infection Outcome

5.1 INTRODUCTION

A characteristic feature of wild populations is genetic diversity. All wild populations will show some level of between-host variation in how they respond to infections that is not explained by extrinsic factors, but their genotype. Genetic diversity and heterozygosity are essential for the health and survival of any animal population, with reductions in diversity associated with inbreeding depression, and increased susceptibility to pathogens (Reed and Frankham, 2003; Spielman *et al.*, 2004; Ganz and Ebert, 2010). Genetic variation can be maintained in populations when the selective pressures acting on that population change across space and time (Hedrick, 1986; Jump, Marchant and Peñuelas, 2009). As the combination of parasites with which an individual can be infected will vary from host to host, and will in turn be impacted by a heterogeneous environment, it is unlikely that there is any one genotype that will be universally 'fittest'. Exploring how genetic variation interacts with infection dynamics can help us predict how an individual might cope with a new infection, and tell us more about the adaptive evolutionary dynamic between parasite and host.

Theoretical models can explain how diversity of immune genes might be maintained. Roy & Kirchner use such a model to explain how resistant genes do not become fixed in populations (Roy and Kirchner, 2000). If resistance genes become ubiquitous in a population, then transmission will drop drastically and parasite levels with greatly reduce. In this instance, it is favourable to express genes associated with reduced immune response, at levels that would tolerate parasite infection, as likelihood of infection is greatly reduced, and metabolic resources can be saved. The spread of successful tolerance genes would increase infection and transmission rates, making resistance genes more favourable again. This cyclical pattern of genetic fitness will establish an equilibrium within a population, and maintain a diversity of genetically-controlled immune responses through evolutionary time (Roy and Kirchner, 2000).

5.1.1 Genetic Control of Immunity in Lab Models

One way in which the role of specific genes in immunity is studied is through the breeding of 'knockout' mice, which have a 'normal' genotype, with the exception of a specific gene of interest, which is artificially removed or deactivated. Such models have been used to establish the role of various genes in maintaining a specific immune response, and subsequent successful removal of certain parasites. Phenotypes associated with resistance and susceptibility that have been observed from knockout mice models include *STAT2*, which suffer impaired lymphocyte proliferation in response to IFNα

and IFNγ signalling and reduced antiviral capabilities (Park *et al.*, 2000), IL-10, which showed enhanced Th1 expression (Yang *et al.*, 1999), IL-12, which had increased susceptibility to *Leishmania major* (Mattner *et al.*, 1996) and IgE, which showed increased susceptibility to *Schistosoma mansoni* (King *et al.*, 1997).

The use of transgenic mice in murine lab infection models also allows for the precise role of immune genes to be elucidated, and give ideas as to how natural variation of a gene in a natural population might manifest into different phenotypes. Schorle and colleagues introduced a mutated version of the *IL2* gene into developing blastocysts of C57BL/6 mice, which caused IL-2 deficiency (Schorle *et al.*, 1991). These mice demonstrated an inhibited ability of T cells to induce antibody production, though the impact upon infection resistance was not examined.

Having a strictly controlled pedigree of lab mice also allows for differences between alleles of the same gene to be observed, often by comparing phenotypes between established lab strains (Keane *et al.*, 2011). These studies are perhaps more directly comparable to genetic diversity in wild populations, which are more likely to maintain diversity through polymorphisms rather than deletions, especially in genes which are essential to a competent immune system. Such studies have established phenotypic differences between alleles in a number of genes, including the H-2 complex

(a major histocompatibility complex), which affects susceptibility to cytomegalovirus (Allan and Shellam, 1984), and in toll-like receptors, which conferred different levels of IL-10 and IFNγ expression (Dhiman *et al.*, 2008).

5.1.2 Genetic Control of Immunity in Wild Populations

A detailed understanding of the influence of genetic diversity on infection and immunity in wild populations is more difficult to obtain. As with immunology and parasitology, wild rodent populations provide a good opportunity to understand how genetic effects observed in controlled experiments relate to wild populations, due to their widespread use as lab models (Turner and Paterson, 2013).

Genetic variation in response to parasite infection has been shown in the hybrid zone between two geographically-separated subspecies of the house mouse, *Mus musculus musculus* and *Mus musculus domesticus* (Moulia *et al.*, 1993). It was found that mice with hybrid genotypes had increased abundances of cestodes and nematodes compared to non-hybrid mice. Mice from the two populations, and hybrids between them, were given anti-helminth treatment until free of parasites, then artificially infected with 500 eggs of the pinworm *Aspiculuris tetraptera*. As was observed from the original populations, the hybrid mice showed higher abundances of infection, confirming that the reduced ability to keep parasite abundances low is at least

in part a genetic effect, rather than purely ecological. This was attributed to a loss of 'genetic harmony' between genes responsible for resistance, resulting from the mixing of genes during meiosis and hybridisation.

As part of their investigation into the ecological factors impacting immune state in wild mice, Abolins and colleagues found that although the populations being observed were genetically diverse, genetic distance between individuals, calculated from 1,183 autosomal loci, was not related to immunological distance, as calculated from antibody concentrations and immune cell counts (Abolins *et al.*, 2018). This may indicate a reduced role of genotyping in determining immune state in wild populations, although the authors concede that key loci with strong links to immunity may have been neglected by the analysis.

Polymorphisms in the *SLAM/CD2* gene family, which are known to be involved in autoimmunity in lab mice, were identified in wild *M.musculus* taken from a global distribution (Limaye *et al.*, 2008). These polymorphisms are derived from ancestral populations of *Mus,* indicating that diversity at these loci has been maintained in the face of a changing infectious landscape. Stabilising selection of immune genetic diversity, as observed in this study, is likely to occur whenever different forms of immune diversity are differentially advantageous under different circumstances.

Clusters of polymorphisms in the TLR2 (toll-like receptor 2) gene in wild bank voles (*Myodes glareolus*) showed significant differences in susceptibility to *Borrelia azfelii* (Tschirren *et al.*, 2013). Polymorphisms in TLR genes have proven significant in relation to infection resistance in lab models and human populations (Schröder and Schumann, 2005; Netea, Wijmenga and O'Neill, 2012), but the discovery of a similar role in resistance to natural parasites of a wild rodent population was novel.

Variation in genes encoding cytokines have been found to have significant impacts on both immune gene expression and parasite communities in wild field voles (*Microtus agrestis*) (Turner et al. 2011). Polymorphisms in genes encoding the cytokines IL-1β, IL-2 and IL-12β were found to be significantly associated with communities of both ecto- and endo-parasites, and influenced the expression of GATA3, IFNγ, Tgfb1 and IL-10. Significantly, the changes in immune expression did not manifest as direct links between genes and the proteins encoded by those genes, with the exception of IL-1β. In wild populations, polymorphisms are thus potentially likely to influence immunity by altering regulation networks and downstream signalling, rather than through isolated changes to the roles of single cytokines or transcription factors.

Another technique which has been used to explore genetic control of parasite is quantitative trait loci (QTL) mapping. QTL has been used in murine models

to expose the genetic underpinnings of resistance to infections of human clinical importance, including *Mycobacterium tuberculosis* (Sánchez *et al.*, 2003; Eruslanov *et al.*, 2005) and *Leishamania major* (Roberts *et al.*, 1997). Although primarily employed in lab models, QTL was used to highlight the significance of the gene *Nramp1* in resistance to *Salmonella typhimurium* in a wild-derived population of mice (Sebastiani *et al.*, 1998). In wild, non-rodent populations, QTL detection in host populations has been used to find heritable genetic differences in parasite communities including helminth communities of Soay sheep, (Beraldi *et al.*, 2007), and trypanosome parasites of cattle (Hanotte *et al.*, 2003).

5.1.3 Hypotheses

Mice on the Isle of May experience a host of parasitic infections (Chapter 3) and demonstrate significant variation in immune responses, which are only partially explained by infection and other ecological factors (Chapter 4). We here hypothesise that:

1) Genetic diversity explains a significant amount of variation across measures of immune expression.

2) Discrete changes in expression of specific genes will be linked to polymorphisms within those genes.

3) Parasite communities will vary between mice of different genotypes.

5.2 METHODS

5.2.1 DNA Extraction

In order to investigate a potential genetic basis for immune control, DNA was extracted from liver tissue from the mice. DNA was extracted from small sections of liver using a two-step extraction kit (Extracta DNA Prep, Quanta Bio, Massachussetts, USA). Yield and purity of DNA was assessed on a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Massachusetts, USA). These DNA extractions were then kept at -20°C.

5.2.2 SNP Selection

A list of candidate single nucleotide polymorphisms (SNPs) from 816 immune genes was drawn up from initial group of 12 trial mice. These genes were taken from the 'Immunome' reference database of genes associated with immune function, as established from human studies (Ortutay and Vihinen, 2006). From the genes in this list, 43 SNPs were selected for further genotyping assays. Suitability for genotyping as determined by preliminary quality control checks run by the external genotyping company (LGC Genomics, Hoddesdon, UK).

From those SNPs suitable for genotyping, SNPs were selected with regards to chromosomal coverage, potential associations with genes being expressed in immunological assays, and known impacts on immune expression from

knockout studies and polymorphism association with disease in human populations. SNPs in genes relating to specific measures of immunity to be measured, such as the interleukin genes, were prioritised, in order to ascertain whether genetic diversity within those genes is associated with differences in the associated expression levels or cytokine concentrations. The final SNP selection, and further detail on their chromosomal location and phenotypes associated with polymorphisms and gene knockouts are detailed in Appendix A. Selected SNPs conferred a range of silent (n=22) and missense (n=20) mutations in the associated genes, which are detailed in Appendix B. Extracted DNA from 150 mice, selected according to DNA yield and purity from across all trapping sessions, was sent in 96-well plates to an external company (LGC Genomics, Hoddesdon, UK) for genotyping at the selected SNP loci.

5.2.3 Equilibrium Testing

All SNPs were tested to ascertain whether or not they were in Hardy-Weinberg equilibrium, and thus whether evolutionary forces like natural selection might be in effect. This was performed using the '**HWChisq**' function, from the '**HardyWeinberg**' package in **R.**

5.2.4 SNP Clustering

In order to ascertain levels of clustering and shared heritability between the selected immune SNPs, an unsupervised k-means clustering method was used to estimate hierarchical pairwise *Fst* between individuals using the biallelic SNP data. Discriminant function analysis was then performed to predict the relationship between the clusters. This process groups mice from the population according to the number of shared SNPs within the analysed genes, without the need for prior information on the population structure or any pedigree data, making it useful for cross-sectional studies. Analyses were performed in R using the '**find.clusters**' and '**dapc**' functions in the '**adegenet**' package.

5.2.5 Redundancy Analyses

Redundancy analyses were performed using MLN and spleen expression values and circulating cytokine concentrations as response variables in three separate analyses, each using the genotypes of mice across all SNPs as explanatory variables. Secondly, two sets of analyses were performed using parasite data; one including the parasites *Sarcocystis sp., Bartonella sp.* and *Calodium hepaticum*, and one without. There is a reduced dataset for these parasites, so the second analysis was performed to increase the power of the analysis.

5.2.6 Mixed Effect Models

Mixed effect linear models were used to establish whether variance between these same response variables could be attributed to groups of inherited polymorphisms as defined by the k-means clustering method described in 5.2.4. Principal components of immune measures were used as the response variables. This method allows for individuals to be grouped even where there is some missing SNP data, and thus increases the number of individuals included in the analysis. Trapping session was used as a random effect, as it has by far the strongest influence on both parasite communities and immune expression (see Chapters 3 & 4).

5.3 RESULTS

5.3.1 Hardy-Weinberg Analysis

Only one SNP was found not exist in HW equilibrium from the selection analysed- an **A/T** polymorphism in the gene *Gzma*, which encodes the protein Granzyme A ($p=5.552x10^{-10}$). No individuals were found to be **TT** homozygous at this locus.

5.3.2 SNP Clustering

The analysed SNPs, as shown in Fig 5.1, fall into four distinct groups, which were used to group individuals by immunogenotype. Clusters 1 & 2 show some overlap, though enough distinction between groups to warrant separate classification.

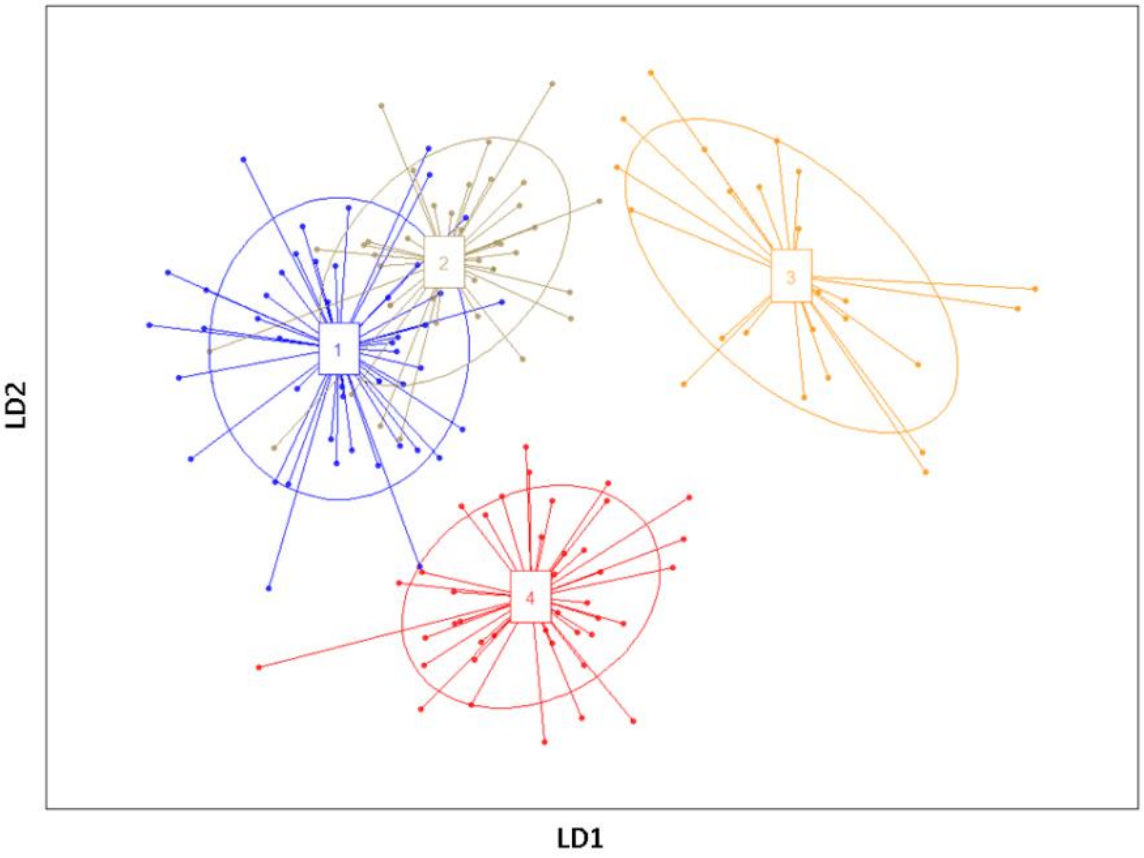


Fig 5.1 Unsupervised K-means clustering of immune SNPs into four distinct groups in *M.m.domesticus* in the Isle of May (n=150). Axes represent eigenvalues determined by the DFA analysis which best separate individuals based on their genotypes at the analysed SNPs.

5.3.3 Redundancy Analyses

The redundancy analyses highlighted the importance of certain SNPs in first redundancy axes of immune expression, which represent overall cytokine levels. No effects on the expression of specific genes were observed from these analyses. The significant effects observed across all immune measures are shown in Table 5.1.

Spleen Expression		MLN Expression		Circulating Cytokines		Parasitology	
FoxP3	0.307	IL1B	0.407	IFNγ	0.331	*S.obvelata* prevalence	-0.323
GATA3	0.300	IL4	0.329	IL1B	0.271	*S.obvelata* burden	-0.405
IFNγ	0.027	IL6	0.406	IL5	0.256	*N.fasciatus* prevalence	-0.074
IL1B	0.089	IL17A	0.314	IL6	0.395	Fur Mite burden	-0.010
IL5	0.310	GATA3	0.215	IL10	0.328	*T.muris* prevalence	0.548
IL6	0.329	IL13	0.190	IL12	0.335	*T.muris* burden	0.653
IL10	0.303	TNFα	0.279	IL13	0.330		
IL12B	0.320	IL10	0.216	IL17	0.379		
IL13	0.333	IFNγ	0.228	Leptin	0.094		
IL17A	0.321	Tbx21	0.330	TNFα	0.338		
Irf5	-0.010	Irf5	0.308				
RORC	0.333						
Tbx21	-0.047						
TNFα	0.278						

Table 5.1 Significant influence of SNPs in immune genes across measures of immune gene expression, concentrations of circulating cytokines and parasite communities.

The redundancy axis of parasite community structure affected by SNPs describes an axis moving from high prevalence of fleas, high abundance of mites, and high prevalence and abundance of the pinworm *Syphacia obvelata*, to a high prevalence and infection burden of the whipworm *Trichuris muris* relative to the other recorded parasites. The loadings of the axes created by the redundancy analyses are summarised in Table 5.2.

Measure	SNP	Sequence	p	r^2
MLN qPCR	*IL17a*	CACTTTCAGGGTC **[A/G]** AGAAGATGCTGGTGG	0.0249	0.471
Spleen qPCR	*Gzma*	GTCASCTCCCTCTGA **[T/A]** ACTCTGAGAGAAGTC	0.0274	0.557
	IL9	GCAACCAGACCATGG **[T/C]** AGGCAACACACTGTC	0.0424	
	Socs5	CGTGCCAGATTTGCT **[T/A]** CAGATCACAGGGAAT	0.0212	
	Tirap	GTCGTAGTCACTGCC **[G/A]** TGTGCTGCTCATCAC	0.0052	
Multiplex Bead Assay (Circulating Cytokines)	*Mr1*	CTTCAATAAAGACAC **[C/T]** CTCTCCTGGTTGGCC	0.0156	0.567
	Tlr4	TCTCTCTTTAGACAT **[G/A]** TCTTTGAACCCAATT	0.0264	
Parasite Communities	*Irf9*	GAAGAGGAGAAAGC **[T/C]** GCCTTGTTCCTGTTA	0.0058	0.458
	Myd88	CTGCTACTGCCCCAA **[C/T]** GATATCGAGTTTGTG	0.0102	

Table 5.2 Loadings of first redundancy axes established by redundancy analysis, with immune SNP data explaining variation across three immune measures, and parasite community structure (MLN qPCR n=93, spleen qPCR n=102, multiplex bead assay n=102, parasite communities n=130).

5.3.4 Mixed Effect Models

Mixed models performed using clusters from the k-clustering process found no significant differences between groups in principal components across any of the measures of immune function, or across parasite communities.

5.4 DISCUSSION

The Hardy-Weinberg analysis performed on the selected SNPs indicates that for most immune-linked genes in this population, there is a reduced impact of evolutionary forces, and the immunogenotype is generally stable. This might be expected from a population which is largely isolated and may therefore not face as many infectious challenges as a comparable mainland population, particularly from migrant mice. The one exception was the gene *Gzma*, which codes for a protein thought to be involved in the lysis of foreign cells by host natural killer cells. It is unclear why this particular gene is not under equilibrium. While it is possible that this gene is under selection, the fact that one class of homozygotes at this locus was not detected suggests that either it is a recessive lethal genotype, or perhaps more likely, that there was an error when genotyping at this locus.

The redundancy analyses on immune expression found differing results across the three immune measures. All observed effects were upon primary principal components, which describe universal increase or decrease in expression of

all immune genes, or concentration of all circulating cytokines. The genes involved in these differences range in their function, including cytokines (*IL9*, *IL17a*), proteins involved in regulating cytokines (Socs5, Irf9), proteins involved in cell killing (*Gzma*), genes relating to the function of recognition of pathogens by toll-like receptors (*Tlr4*, *Tirap*, *Myd88*) and proteins involved in presentation of pathogen antigens (*Mr1*).

The only SNP found to affect MLN expression was *IL17a*. Polymorphisms in the *IL17a* gene in humans have been implicated in inflammatory gastrointestinal pathologies such as Crohn's disease (Kim *et al.*, 2011) and ulcerative colitis (Arisawa *et al.*, 2008). IL-17a is crucial for establishing Th17-linked inflammatory responses, and so its significance in mesenteric expression indicates that polymorphisms in this population may explain variation in levels of gastrointestinal inflammation (Kolls and Lindén, 2004).

SNPs implicated in affecting splenic expression were found in genes *Gzma*, *IL-9*, *SOCS-5* and *Tirap*. *Gzma* is a pro-inflammatory gene involved in death of infected or cancerous host cells (Hochegger, Eller and Rosenkranz, 2004; Lieberman, 2010). *IL-9* is a cytokine linked with Th2-type inflammation, and polymorphisms in this gene in human populations have been linked to atopic dermatitis, characterised by allergic-style inflammation (Namkung *et al.*, 2011) and increased susceptibility to respiratory syncytial virus (Schuurhof *et al.*, 2010). The *SOCS* (Suppressor of Cytokine Signalling) family of proteins are

generally involved with suppression of immune signalling and expression (Yoshimura, Naka and Kubo, 2007). *SOCS-5* specifically is involved with repressing IL-4-associated Th2 polarisation (Ozaki *et al.*, 2005), and polymorphisms in this gene have been linked to impaired differentiation of dendritic cells (Toniolo *et al.*, 2016) and asthma (Saltykova *et al.*, 2013). *Tirap* is a gene involved with toll-like receptor signalling, and induction of innate immunity. Polymorphism in this gene has been linked to susceptibility to pulmonary tuberculosis in human populations (Selvaraj *et al.*, 2010). As with MLN expression, the SNPs associated with changes in spleen immune expression are largely involved with regulation and induction of inflammation.

Two SNPs were associated with alterations in levels of circulating cytokines. *Tlr4* encodes a toll like receptor, which is important for induction of innate responses (Beutler, 2009). Polymorphisms in *Tlr4* have widely been shown to reduce successful induction of protective responses against bacteria and viruses (Arbour *et al.*, 2000; Kurt-Jones *et al.*, 2000; Fagerås Böttcher *et al.*, 2004; Rezazadeh *et al.*, 2006). *Mr1* is associated with major histocompatibility proteins, and the presentation of antigenic material to T cells, in order to induce protective responses (Huang *et al.*, 2009). As with *Tlr4*, polymorphisms in this gene are associated with increased susceptibility to bacterial infection (Georgel *et al.*, 2011; Seshadri *et al.*, 2017).

Across all three measures of immune function, it is evident that polymorphic genes affecting immune state are primarily those involved with control of inflammation, innate responses, and susceptibility to microparasites (*IL17a*, *Gzma,Tlr4, Tirap, Mr1)* . This is informative, as it suggests that immune genetic diversity in this population may be maintained more through responses to microparasites than macroparasites. The concept of pathogen-mediated selection (PMS) being a driver of genetic diversity in has been explored in human populations, with microparasitic infections being implicated in diversity within major histocompatibility complex (MHC) genes, which are involved in recognition of infections by the host (reviewed in Spurgin & Richardson 2010; Jeffery & Bangham 2000).

Potential mechanisms of PMS maintenance of diversity include heterozygote advantage, whereby MHCs of heterozygotes are able to present a greater range of antigens and thus respond to a greater diversity of pathogens, and rare allele selection, by which rare alleles are maintained by the occurrence of sporadic infections for which having a rare allele is advantageous, and subsequently increase in frequency in the population (Jeffery and Bangham, 2000; Spurgin and Richardson, 2010). While evidence of PMS leading to genetic diversity in wild populations is not common, diversity in MHC genes has been found to be associated with parasite infection in populations of water voles (*Arvicola* terrrestris) (Oliver, Telfer and Piertney, 2009) and three-spined stickleback (*Gasterosteus aculeatus*) (Wegner, Reusch and Kalbe,

2003), though not specifically microparasites. Diversity at these loci may also maintained by trade-offs between the relative fitness costs of inflammation and microparasitic infection.

Despite the range of polymorphic genes found to be impacting immune expression, we have not been able to resolve any effects upon expression levels of specific cytokines. Contrary to our hypotheses, no SNPs could be associated with changes in expression in their corresponding mRNA or proteins. This may be an indication that individuals with specific polymorphisms have a generally more activated immune state, rather than, for example, higher propensity towards Th1 or Th2-style responses. It may also be due to the relatively low number of SNPs included in the analysis. Here, SNPs were selected from across the genome which were in genes known to be important to different aspects of the immune response. By using high-throughput methods to analyse thousands of immune-linked SNPs, or indeed SNPs from across the genome generally in a less discriminative manner, we may be able to ascertain in more fine detail how genetic diversity affects immunity (Kumar *et al.*, 2014).

The effect of genetic diversity on parasite communities in this population is more specifically defined. Rather than an overall increase or decrease in burden and prevalence of all parasites, there is a shift between one group of parasites – ectoparasites and the pinworm *S.obvelata* - to another, the

whipworm *T.muris*. The negative association between the two gastrointestinal helminth parasites has been established in this population, in Chapter 3 of this thesis, though the directionality and mechanism of this relationship is unclear. The fact that differences in parasitology are at least in part explained by diversity in immune genes provides evidence that immune responses underpin this relationship.

It is important to note when interpreting the effects of SNPs on phenotype that each polymorphism may not directly be impacting the expression of a protein. Any of the SNPs here measured could be indicative of different haplotypes, with the observed polymorphism in linkage with the another which is causing a change in phenotype. This is observed in human populations, in which different haplotypes of genes involved in T-cell regulation are associated with susceptibility to autoimmune diseases such as type-1 diabetes and Grave's disease (Ueda *et al.*, 2003). Despite this, six of the nine SNPs associated with significant changes in immunity or parasitology are missense mutations (*IL17a*, *IL9*, *Gzma*, *Tlr4*, *Socs5* and *Tirap* – see Appendix B), compared with less than half of the SNPs as a whole, suggesting that in these cases the polymorphisms could be having a direct effect on phenotype. Silent polymorphisms can also affect phenotype by altering the splicing and stability of transcribed mRNA (Hunt *et al.*, 2009). More detailed genetic analysis of this population will help to better understand the role of haplotypes in controlling immune state and maintaining genetic diversity.

Genetic diversity is a key feature of wild animal populations and is a crucial distinguishing feature between lab model experiments and natural populations. With a limited amount of genetic data, we have here shown that genetic diversity is having a measurable influence on how mice on the Isle of May respond to infection and regulate their immune state. Parasites and pathogens can be a key driver of genetic diversity in host populations, which may explain how genetic diversity of immune genes is maintained in this population (Spurgin and Richardson, 2010). A more detailed study of the genes involved with immunity, looking at a greater number of polymorphisms, would help to better understand how diversity in this population interacts with infection and other ecological processes. It is, however, apparent that genetic control of immunity should be considered when looking at infections in wild populations as a significant source of variation.

Chapter 6: Influence of Infection and Immunity on Host Condition

6.1 INTRODUCTION

Wild immunology and parasitology help us to understand how host immunity and a shifting infectious landscape fit within a natural ecosystem. The immune system of mammals is derived through evolution to operate within their broader ecology, and thus should be considered in the context in which it evolved (Pedersen and Babayan, 2011). Integral to this understanding of immunity and infection are their effects on host fitness, as impacts upon fitness can drive evolution by natural selection within a population or species. Parasites are classically defined by their ability to negatively impact upon the host's fitness and utilise metabolic resources from the host, and so it is vital to understand the severity of infections on populations relative to other ecological forces on host health (Leung and Poulin, 2008).

It can be challenging to obtain a robust assessment of a wild animal's true fitness without detailed monitoring over extended periods and measures of fecundity and offspring survival (Schulte-Hostedde, Millar and Hickling, 2001). As such, it can be more useful to use proxies for fitness based on an animal's morphology. No single measurement can be made during cross-sectional

analysis which can give an exact measure of fitness, and there are multiple ways in which fitness can be estimated, through various condition indices.

6.1.1 Fitness and Condition

One key principle in fitness estimation is the concept that healthier animals will experience more general growth, and will have increased survival. This is based on the idea that an individual which is subject to selective pressures such as infection, competition and escape from predators will have fewer resources to use for growth and reproduction and will therefore be subject to trade-offs between aspects of survival (Werner and Anholt, 1993; Zera and Harshman, 2001; Robinson *et al.*, 2006). Evolutionary and ecological trade-offs are well characterised in small mammal populations (Schulte-Hostedde, Millar and Hickling, 2005). There may also be metabolic associations between growth rates and reproductive output, as in some small mammals which show increases in muscle mass of males, due to higher levels of testosterone associated with larger testes (Schulte-Hostedde, Millar and Hickling, 2003). Larger testes have been associated with increased numbers of offspring in a number of species (Sachser *et al.*, 1999; Preston *et al.*, 2003; Soulsbury, 2010). Condition indices are therefore often based on morphometric measurements of size and weight.

There are, however, limitations to such indices, as although the animal in question may indeed be a greater mass and thus 'healthier', increased growth could be caused by reduced investment in reproductive organs, or foregoing mating behaviour in order to feed. Examples of such trade-offs have been demonstrated in guppies (*Poecilia reticulata*) (Reznick, 1983), bison (*Bison bison*) (Green and Rothstein, 1991) and amphipods (Robinson and Doyle, 1985). For females, the metabolic costs of reproduction can be great, both before and after birth. This is particularly true in mammals which wean their young, and is evident in a number of small mammals including the vole species *Clethrionomys glareolus*, *Clethrionomys gapperi* and *Microtus pennsylvanicus* (Innes and Millar, 1981; Koivula *et al.*, 2003), cotton rats (*Sigmodon hispidus*) (Mattingly and Mcclure, 1982), degus (*Octodon degus*) (Naya *et al.*, 2008), grasshopper mice (*Onychomys leucogaster*) (Sikes, 1995), deer mice (*Peromyscus maniculatus*) (Millar, 1979), and lab populations of house mice (*Mus musculus*) (Kounig, Riester and Markl, 1988).

Males can also experience significant metabolic costs due to reproduction, due to maintenance of territories and harems (Yoccoz *et al.*, 2002), physical competition and confrontation with other males (Marler and Moore, 1988), and through investment into reproductive organs and sperm (Olsson, Madsen and Shine, 1997; Schulte-Hostedde, Millar and Hickling, 2003). As a result of the complex interaction between metabolic expenditure and reproductive

success, a higher condition index based on whole-body mass could in fact indicate a reduction in reproductive fitness, and vice versa.

Van Noordwijk and De Jong theorised that trade-offs between somatic indices and reproductive indices would occur when variation between the resource allocation capabilities of individuals was greater than that of resource acquisition, and that they would be more common in laboratory studies when variation in resource acquisition was reduced (Noordwijk and Jong, 1986). This theory was supported in a review by Glazier, who analysed 38 studies, finding only 6 which reported negative correlations between somatic index and reproductive output – 5 of which were laboratory studies (Glazier, 1999). Of the studies performed in rodent species, only one laboratory study found a negative correlation between somatic index and reproductive investment (Randolph *et al.*, 1977), while all studies of wild rodent populations found either no association or a positive correlation (Kiell and Millar, 1980; Dobson and Michener, 1995; Wauters, Dhondt and Dhondt, 1995; Humphries and Boutin, 1996; Dobson, Risch and Murie, 1999).

White-footed mice (*Peromyscus leucopus*) in oak forests show increases in overwinter survival and reproductive output following larger acorn masts, with most variability in population size explained by variation in acorn availability (McShea, 2000; Schnurr, Ostfeld and Canham, 2002; Clotfelter *et al.*, 2007). *P.leucopus* survival while infected with intestinal helminths has

been shown to be worsened when host condition is negatively impacted following a low acorn mast (Pedersen and Greives, 2008). This supports the use of somatic indices in the Isle of May mice as an indicator of true fitness, as being a wild population, they would experience higher variation in resource acquisition than allocation i.e. the condition of the animals is more dependent in the amount of food available, than how metabolic resources are utilised.

A number of condition indices have been developed, each using various mathematical means to scale an animal's body mass relative to its body length. These include widely-used Body Mass Index (BMI) (Keys *et al.*, 1972), Fulton's index (Fulton, 1904), the Residual Index (Jakob *et al.*, 1996), the Relative Condition Index 'K_n' (LeCren, 1951) and the Scaled Mass Index (SMI) (Peig and Green, 2009). A comparison of condition indices found that SMI best accounted for variation in relative body mass between sexes and ages, and is the more reliable measure of an animal's condition (Peig and Green, 2010).

Estimating reproductive potential without having longitudinal data on animal pedigrees can be achieved in cross-sectional analyses by measuring the size or mass of reproductive organs like testes and seminal vesicles in males, and has been carried out across multiple taxa, including frogs (Byrne, Roberts and Simmons, 2002). Testes size has also been shown to be associated with increased reproductive success in yellow-pine chipmunks (*Tamias namoenus*) (Schulte-Hostedde and Millar, 2004) and Soay sheep (*Ovis aeris*) (Preston *et*

al., 2003). Assessing reproductive potential based on simple physiological measurements is not as simple or methodologically established for females, although reproductive output has been shown to be affected by hormone levels (Sinervo and Licht, 1991) and genotype (Rothschild *et al.*, 1996), both of which could potentially be assessed in cross-sectional analysis.

A commonly used measure of condition is leptin, a hormone secreted by adipose tissue, which regulates food uptake and energy expenditure. Being produced by fatty tissue, levels of leptin can be used to estimate levels of fat deposition (Li and Wang, 2005; Wang, Zhang and Wang, 2006; Spady *et al.*, 2009). This relationship is somewhat complicated, however, as leptin levels are involved in a negative feedback loop, whereby increased leptin levels cause a reduction in food intake and an increase in energy expenditure (Campfield *et al.*, 1995; Friedman, 1998). There are also situations in which leptin levels do not show uniform increases with fat levels, but rather seasonal decreases during periods of high food availability to allow for maximum fat uptake, as observed in striped mice (*Rhabdomys pumilio*) (Schradin *et al.*, 2014) and Mongolian gerbils (*Meriones unguiculatus*) (Zhang and Wang, 2007). Differences in dietary fat types have also been found to influence leptin levels in rats (Cha and Jones, 1998). The role of leptin is, therefore, complicated, and may potentially vary between species and populations with different ecological contexts. This ecological variation,

however, makes it important to consider and attempt to understand for its key role in both determining, and indicating, wild animal condition.

6.1.2 Fitness Impacts of Infection

Haemoparasitic infections have been shown to both reduce survival in blue tits (*Cyanistes caeruleus*) (Lachish *et al.*, 2011), and reduce clutch sizes during periods of low food abundance in Tengmalm's owls (*Aegolius funereus*) (Korpimaki, Hakkarainen and Bennett, 1993). Fitness impacts of parasites have also been observed in a number of small rodent populations, including reduced body mass of parents and offspring caused by infection with *Eimeria spp*. in bank voles (*C.glareolus*) (Hakkarainen *et al.*, 2007), reduction of reproductive output and survival in bank voles and wood mice (*Apodemus sylvaticus*) artificially infected with cowpox virus (Feore *et al.*, 1997; Telfer *et al.*, 2005; Burthe *et al.*, 2008), and reduced erythrocyte and lymphocyte counts in field voles (*Microtus agrestis*) (Beldomenico *et al.*, 2008a). Additionally, in white-footed mice (*P.maniculatus*), population crashes were prevented via the removal of intestinal parasites (Pedersen and Greives, 2008). In contrast to these studies, however, infection with cowpox virus has also been associated with seasonal increased survival rates in bank voles (Telfer *et al.*, 2002).

6.1.3 Immunological Impacts on Fitness and Condition

Although immune responses are greatly important for an animal's health and survival in the face of infection, they can come at a significant metabolic costs to an animal, and thus their ultimate effect on host fitness can be unclear (Lochmiller and Deerenberg, 2000). Eurasian kestrels (*Falco tinnunculus*) displayed different impacts upon fitness between different types of immune expression (Parejo and Silva, 2009), whereby adult males with higher levels of natural antibodies, showed greater levels of reproductive success. Offspring survival was also positively associated complement and antibody levels, and negatively associated with levels of leukocytes and heterophils in the blood. Complement-linked fitness effects in this population were attributed to defence against the haemoparasite *Haemoproteus spp.,* though no other infections were monitored (Parejo and Silva, 2009).

Levels of certain immune parameters have also shown to be associated with fitness in Soay sheep (*O.aries*). Individuals with higher levels of anti-nuclear antibodies, taken as a proxy for proliferation of immune cells, showed reduced reproductive ability, but increased survivability of females over harsh winters, and increased offspring survival (Graham *et al.*, 2010). This trade-off between immune function and reproduction was interpreted as a possible source of genetic diversity in the host population, with heterogeneity of phenotypes being maintained through variation in harshness of winters. In contrast, lactating Brandt's voles (*Lasiopodomys brandtii*) showed no

differences in measures of adaptive immunity between small and large litter sizes when accounting for the mass of offspring, indicating that acquired immunity may have a less prominent impact on fitness in rodent populations (Xu, Yang and Wang, 2012). These systems demonstrate complex relationships between immunity, reproduction, survival and climate, but do not account for the variety of pathogens and parasites which could potentially be invoking immune responses and fitness costs.

Jackson and colleagues found that the relationship between immunity and body condition in male field voles (*M.agrestis*) was dependent on age, with older males exhibiting higher GATA3 expression, associated with tolerance to parasite infection and an increase in body condition (Jackson *et al.*, 2014). This indicates that the costs and benefits of immunity, and any associations with the condition and fitness of the animal in question, may well be dependent on its life history stage.

6.1.4 Ecological Impacts on Fitness and Condition

Knowledge of energetic and metabolic resources availability and allocation are fundamental for understanding the trade-off dynamics between aspects of an animal's ecology. Seasonal changes in temperature and food availability can profoundly impact upon an individual's condition, an effect which has been demonstrated in a number of small mammal populations (Dark, Zucker

and Wade, 1983; Boswell, Woods and Kenagy, 1994; Nagy, Gower and Stetson, 1995; Atsalis, 1999; Nelson, 2004; Schradin and Pillay, 2006).

6.1.5 Hypotheses

The limitation of many studies is that the variables impacted by, or impacting upon fitness or condition, are typically examined in isolation, without full consideration of an animal's ecology. Here I attempt to explore the condition of mice on the Isle of May, taking fitness and condition as cornerstones of the mouse's ecology, and explore its relationship with shifting ecosystems, multiple infections, immune expression and genotype. By incorporating a more holistic range of variables, I aim to obtain a uniquely comprehensive overview of the factors interacting with fitness in a wild population, including a broad taxonomic range of parasites and immunological assays, and better understand how phenotypic diversity in the population is maintained. I here hypothesise that:

1) Measures of condition will be greatly impacted by seasonality, and will show high variability between trapping sessions.

2) Parasitic infection will cause a reduction in all measures of host condition.

3) A trade-off will exist between reproductive condition of males, and other measures of condition.

6.2 METHODS

Measurements taken to assess condition include SMI and subcutaneous and mesenteric fat scores. In males, paired mass of seminal vesicles and paired mass of testes were included as an estimate of reproductive potential (2.2 - Dissection Methods & Morphometrics). As pregnant females were excluded from cross-sectional analysis, effects of pregnancy upon condition indices are removed.

For inclusion in analyses of condition, infection prevalence and intensity of a number of parasites (2.3 – Parasitological Surveys), immune expression from the spleen and mesenteric lymph nodes (2.4 - qPCR Analysis of Gene Expression), and concentrations of circulating cytokines (2.5 – Multiplex Bead Assay of Circulating Cytokines) were used.

Measures of condition were grouped and used as response variables in redundancy analyses. Measures were grouped in this way in order to obtain an overall measure of an animal's condition, with the intention of more easily ascertaining associations between explanatory variables and a general measure of animal health, while consolidating data from less stringent measures such as visual fat scores into a more robust measure. Condition measures used include subcutaneous and mesenteric fat scores, SMI, and in

males, weight of testes and seminal vesicles. Each analysis was performed twice; once including male reproductive measures, and once without, to increase statistical power. An initial analysis used ecological factors as predictors. Four sets of conditional redundancy analyses were then performed, using ecological factors with significant effects as conditions, one using parasite data, and three using the different measures of immune function, as explanatory variables. All statistical analyses were performed in R, using the '**vegan**' package.

6.3 RESULTS

6.3.1 Ecological Effects

Summaries of the ecological redundancy analyses are shown in Table 6.1. For the first analysis incorporating both sexes, loadings from the first redundancy axis represent negative correlation between fat scores and SMI. The first redundancy axis from the male-only analysis shows a negative relationship between SMI and fat scores with mass of testes and seminal vesicles. The redundancy analyses showed a significant association between trapping session and measures of condition ($p<0.0001$), and when measures of male reproductive organs were included, both trapping session ($p<0.0001$) and age ($p=0.0007$). These effects are shown in Figs 6.1-6.3.

	All Mice		Males Only
	RDA1	RDA2	RDA1
Leptin	0.157	0.054	0.0445
SC Fat	-0.938	0.180	0.673
MS Fat	-0.233	0.156	0.123
SMI	0.203	0.967	0.243
SV Paired Mass			-0.531
TS Paired Mass			-0.433
p	<0.001	0.0048	<0.001
Adj. R^2	0.145		0.224
n	263		108
% variation explained	51.12	26.59	58.41

Table 6.1 Summary of redundancy analyses of ecological variables on measures of condition, including whole-population and male-only analyses.

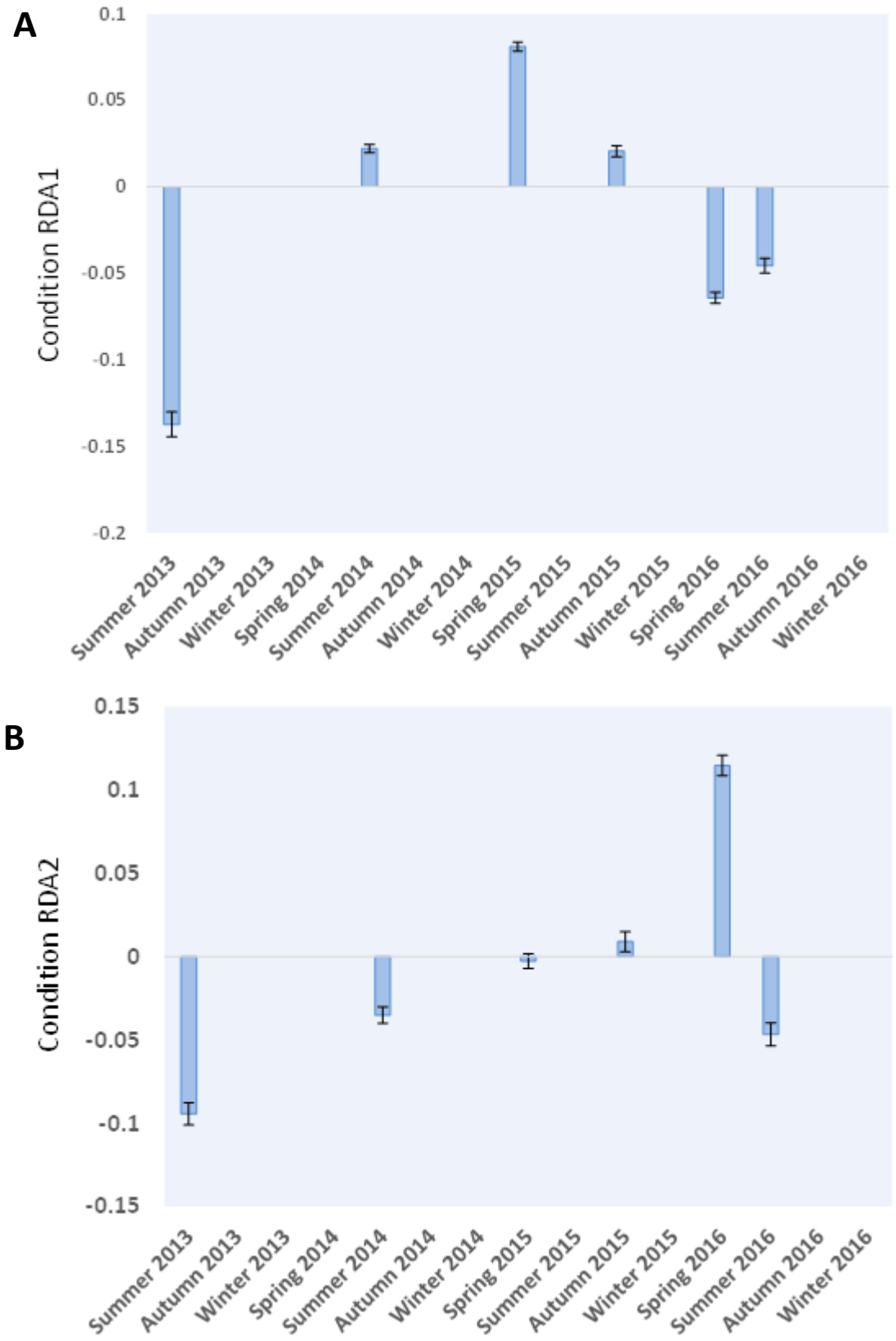


Fig 6.1 Impact of changes in season upon first (p=0.0001***) and second (p=0.0048**) redundancy axes of measures of condition, taken across the whole population. (A) Higher RDA1 values increased values of SMI and leptin concentration and decreased values of subcutaneous and mesenteric fat. (B) Higher RDA2 values indicate increase across all included measures of condition. Bars=SEM (*=p<0.05,**=p<0.01,***=p<0.001)

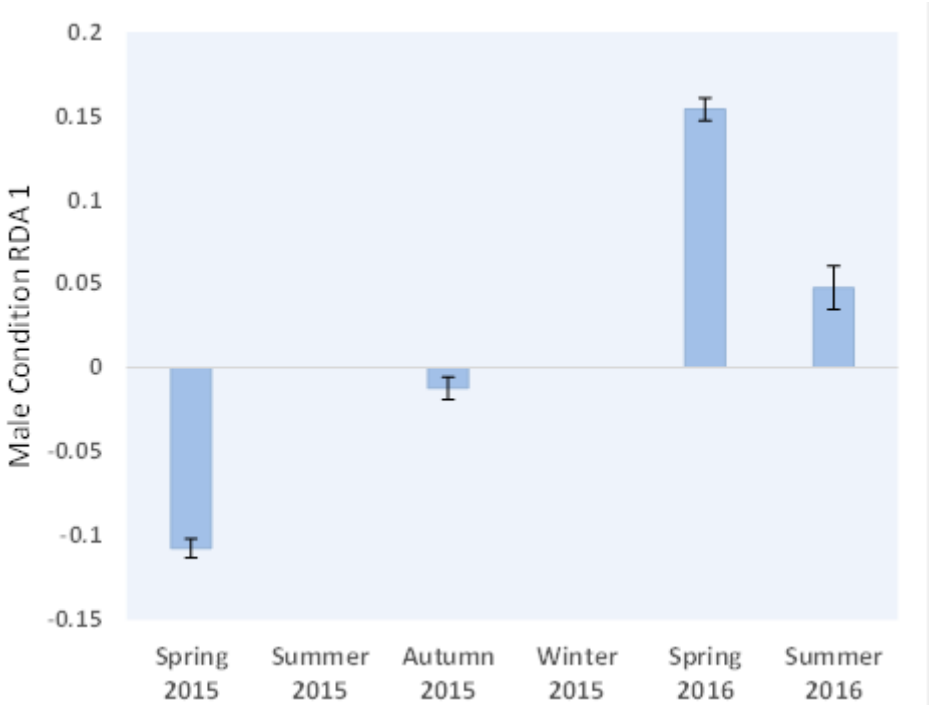


Fig 6.2 Impact of changes in season upon measures of condition including male-only measures of reproductive output. Increases in RDA1 indicate increased measures of leptin expression, SMI and fat scores relative to mass of seminal vesicles and testes, p=0.0001*** (Bars=SEM, *=p<0.05,**=p<0.01,***=p<0.001)

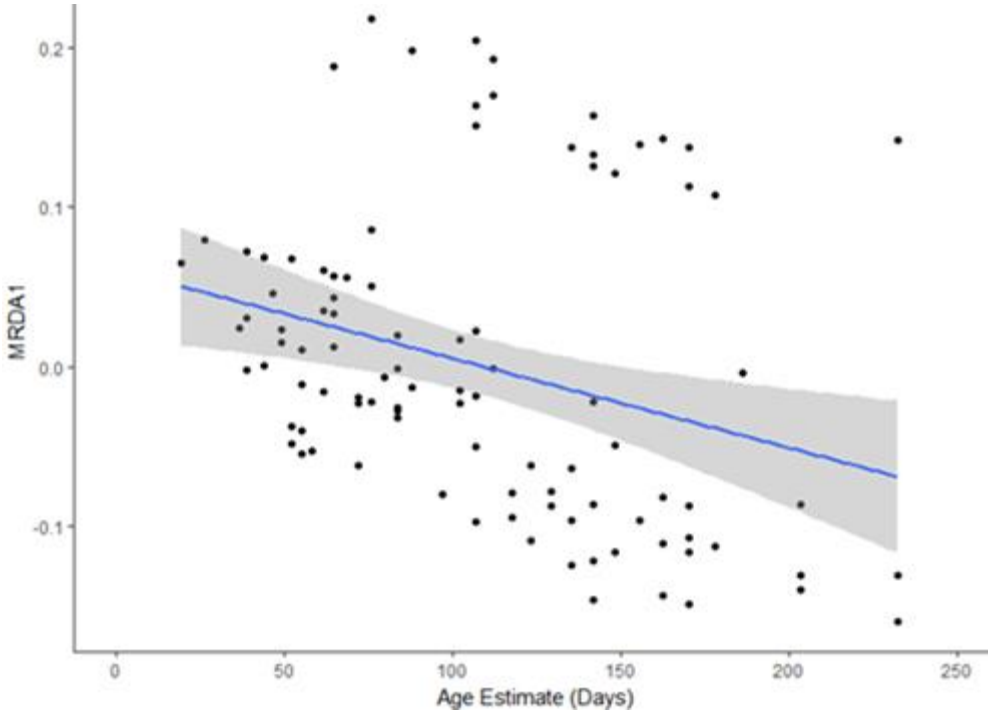


Fig 6.3 Change in condition including male-only measures of reproduction with age. Decreasing RDA values indicate increased mass of seminal vesicles and testes relative to leptin expression, SMI and fat scores, p=0.0007*** (*=p<0.05,**=p<0.01,***=p<0.001)

6.3.2 Parasite Analyses

No measures of prevalence or infection intensity showed any significant impact on condition measures when accounting for ecological variation.

6.3.3 Immunology Analyses

No measures of expression levels of immune genes from the mesenteric lymph nodes or spleen showed any significant relationship with condition measures when accounting for ecological variation. Levels of circulating cytokines were significantly associated with general measures of condition; IL-1β (p=0.0289), IL-10 (p=0.0321) and TNFα (p=0.0311). Condition of males, including reproductive measures, were not significantly impacted by immune measures. Loadings and summaries of this redundancy analyses are shown in Table 6.2, and significant associations with cytokines are shown in Fig 6.4.

	RDA1
Leptin	0.0852
SC Fat	0.230
MS Fat	-0.160
SMI	-0.956
p	0.0373
R^2	0.0549
n	256
% variation explained	50.23

Table 6.2 Summary of redundancy analyses of circulating cytokine levels on measures of condition, including whole-population and male-only analyses.

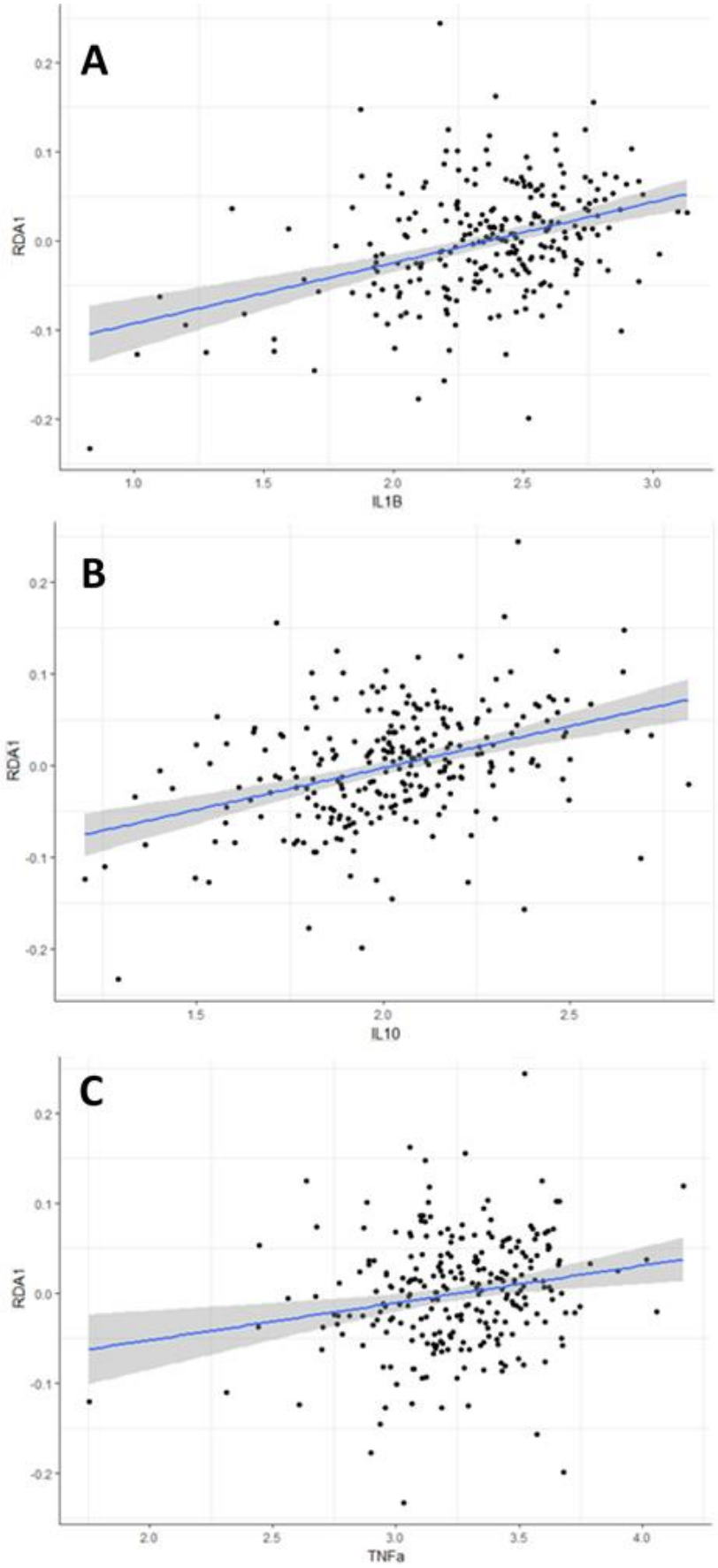


Fig 6.4 Significant effects in redundancy analyses of levels of circulating (A) IL1B (p=0.0298), (B) IL10 (p=0.0321) and (C) TNFα (o=0.0311), on measures of condition. Increase in RDA1 indicates an increase in leptin levels and subcutaneous fat scores, and a relative decrease in SMI and mesenteric fat score. (*=p<0.05,**=p<0.01,***=p<0.001)

6.4 DISCUSSION

The main factor affecting measures of condition in this population is the trapping session in which they are studied. This finding is in concordance with variation observed in a number of characteristics of the population including age profile, population density (Chapter 2), parasitology (Chapter 3) and immune expression (Chapter 4), providing evidence that seasonal and interannual changes in the environment are the main environmental factors influencing the life history of this population.

Notably, measures of condition do not uniformly positively correlate in the first redundancy axis of condition as explained by ecological variables. Rather, in the cross-sex analysis, SMI and leptin are negatively associated with visual fat scores. A negative association between fat and SMI may seem counterintuitive, though fat deposition is not the only way in which an animal's mass can increase. Growth in other tissues, such as muscle to help with escape from predators or physical intraspecific competition, or thicker fur to improve survival over colder periods, would increase mass relative to body length without increasing, or even at the expense of, fat reserves. Variation in the percentage of fatty tissues represented by body mass indices are well documented in humans, and this analysis highlights that mass indices can be limited in what they represent with regards to growth of different tissues (Deurenberg, Deurenberg-Yap and Guricci, 2002; Shah and Braverman, 2012).

The negative association between leptin and fat is also unexpected, as leptin is produced by adipose tissue. This may be the result of season-specific effects as observed in other rodents and small mammals (Wang, Zhang and Wang, 2006; Schradin *et al.*, 2014). Lower fat scores may be indicative of periods of low food availability, which are accompanied by increases in leptin to allow for increased food uptake and reduced energy expenditure. The second redundancy axis describes a uniform increase across all condition measures, which similarly significantly varies with trapping session.

The analysis including measures of male reproductive potential show a negative association between mass of seminal vesicles and testes, and the other measures of condition. This suggests a trade-off occurring between growth and reproduction in the mice, as might be predicted by classical models of life history trade-offs. This axis showed significant variation between trapping session. The minimum male-specific RDA value, indicating increased reproductive investment, was observed in Spring 2015, with a maximum value in Spring of the following year. The cross-sex analysis indicated that SMI and fat storage relative to leptin levels were also highest in Spring 2016. Therefore, this difference may indicate an increase in fat due to increased food availability relative to the previous year, rather than changes in the reproduction/growth trade-off between years. Male mice showed increased investment in reproductive organs relative to other measures of

condition with increasing age, which is to be expected as the mice reach sexual maturity.

Due to interannual variation observed, and the sporadic distribution of trapping sessions, it is difficult to highlight any clear seasonal patterns that would explain these shifts in condition. Two separate spring trapping sessions, in 2015 and 2016, showed increases in condition indicating increased SMI. Mice on the Isle of May reproduce in late winter or early spring, typically before April, when these spring sessions were carried out (Triggs, 1991). These spring increases may, therefore, be reflecting an increased level of growth post-reproduction. It should be noted that no pregnant females were killed during this cross-sectional study, and as such, any increases in mass highlighted by these analyses should not simply be indicating pregnancy, but rather a reallocation of metabolic resources. This is shown in the males-only analysis, whereby the secondary principal component shows a reduced relative mass of testes in the Spring sessions, which then gradually increases throughout the year.

Leptin has been found to have a suppressive impact on various aspects of male reproductive development in rats (Tena-Sempere *et al.*, 1999, 2001; Tena-Sempere and Barreiro, 2002), so it is also possible that the shift from reproductive growth to body fat is a *result* of leptin expression, rather than leptin being an indicator of resource trade-offs between reproduction and

growth. The opposite effect has, however, been reported in mice, whereby leptin-treated males have increased testicle and seminal vesicle mass (Barash *et al.*, 1996).

There were no significant associations observed between parasitic infection and any measure of condition. There are several possible reasons as to why costs of infection on host condition may not be detected. There may simply be minimal fitness costs associated with infection, i.e. the pathology caused by successful infection is negligible. There were few changes in immune state resulting from infection in this population (Chapter 4), so it may be the case that this population has becoming largely tolerant to infection, and the metabolic and immunopathological costs of immune-mediated removal of parasites are too great to be worthwhile. Most of the parasites recorded in this population show generally high levels of prevalence (Chapter 3), suggesting that for most of the population, condition costs of infection are low enough to tolerate. A lack of association between parasitic infection and host fitness is observed in other populations, such as *M.agrestis* infected with *Bartonella sp.* and *Babesia microti* (Taylor *et al.*, 2018), indicating that established parasites in wild populations may not be as significant as is often assumed, and may exist in a more commensal relationship with their hosts.

Alternatively, parasite species with lower recorded prevalence such as *Sarcocystis sp.* (6.9% infection prevalence) may be impacting host condition,

but are too scarce for any associations to be detected statistically. Although a range of infections were included in the analysis, it is still possible that there are unaccounted-for infections which are negatively influencing condition.

In many studies concerned with impacts of infection on condition and fitness, parasites are generally considered as the cause of changes in health in the host. It should be remembered, however, that the direction of causality between changes in condition and establishment of infection are not uniform, and that there is evidence from longitudinal studies that pre-existing variation in condition affects probability of successful infection (Beldomenico *et al.*, 2009). The data here used is cross-sectional and taken from trapping sessions often far apart. As such discerning the directionality of any associations is extremely difficult. Consequently, this population would benefit from future longitudinal studies to ascertain the mechanisms underlying the observed effects.

Although levels of gene expression did not show any significant associations with condition, levels of circulating cytokines did. Although leptin is here used an indicator of condition, it also has a role in immune expression, as it is linked with inflammation in fatty tissues. As such, disentangling its roles in the immune system from its metabolic functions can be difficult, particularly in terms of causality, i.e. whether leptin levels are an indicator of condition, an

indicator of immune environment which is impacting upon condition, or a combination of both.

Increased levels of IL-1β, IL-10 and TNFα are associated with increases in levels of leptin and subcutaneous fat, relative to SMI and mesenteric fat scores. Adipose tissue is well established as an inflammatory tissue in both mice models (Kim *et al.*, 2012) and humans (Xu *et al.*, 2003). IL-1β and TNFα are pro-inflammatory cytokines and would therefore be associated with adipose tissue and increased leptin levels. IL-10, however, is typically an immunoregulatory cytokine, and so its role is less clear. Inflammation can be detrimental to the condition of an animal, both in terms of metabolic resources and autoinflammation (Ashley, Weil and Nelson, 2012), and so it may indicate an adaptive response on the part of the host to limit inflammation-associated damage in fatty tissues.

The differences between fat types as described by the redundancy analyses may indicate that changes in immune expression are associated with localised differences in fat metabolism. In humans, subcutaneous and mesenteric adipose show clear metabolic differences, whereby subcutaneous fat is the major source of leptin, but mesenteric fat is more metabolically active (Ibrahim, 2010). The effect observed here may indicate that at times of low energy expenditure, mesenteric fat levels are high, as a result of lower leptin levels, which are associated with reduced levels of inflammatory cytokines.

These effects highlight the complications of using traditional measures of condition as responses to other changes in the host, as 'fatter' mice, which would generally be considered in good health, may be experiencing higher levels of inflammatory damage to tissues, which could be equally relevant to an individual's chances of survival. Additionally, differences in types of fat, and the complex relationship between fat, condition and inflammation create a complicated picture of animal health.

Here, I have highlighted some of the main ecoimmunological factors affecting the condition, and by extension, the fitness of mice on the Isle of May. Interestingly, parasites do not have a detectable impact on the health of the animals, and the population as a whole appears to tolerate a range of infections with minimal or negligible impact on fitness. Far more significant are changes between season, causing dramatic shifts in condition which are likely to be explained at least in part by reproductive cycles. Variation observed between years, however, shows that other environmental factors, such as changing climate, may be influential upon the health of this population. The inflammatory environment within the animals is linked to their health, and better understanding how resource availability varies throughout the year may help to ascertain how these associations arise.

Chapter 7. General Discussion

Assessing the impact of parasitic infections and immune expression and control upon wild populations in a heterogeneous landscape has previously proven difficult, but through the combination of molecular assays developed in laboratory models, and traditional ecological methods, a comprehensive analysis of the impact of the factors affecting the condition of animals in wild populations can be made. In this thesis I have used an isolated population of a model organism to better understand the role and significance of parasites and immunity in wild rodent ecology. While this population is unique in many aspects, and will be no means representative of universally applicable rules regarding wild animals, it provides an opportunity to examine how measures of infection and immunity in changing ecosystems can be obtained and interpreted. Throughout this thesis, different techniques and analyses were performed to answer different questions about infection and immunity in house mice on the Isle of May.

7.2 Summary of Findings

A diversity of parasite species were identified in this population, including protozoan and bacterial microparasites, gastrointestinal and hepatic helminths, and arthropod ectoparasites. These parasites each typically infected a significant proportion of the population, and coinfection with at least two parasite species was the norm. The interactions between these

species have shown to be highly significant to their distribution, and a network of interactions involving every analysed parasite was identified. Helminth infections proved highly important to these interactions, and central to the network was the hepatic helminth *Calodium hepaticum*. The significance of helminths was notable, particularly in contrast to microparasites, which have shown to have marked influences on host health in laboratory infection models. The interconnectedness of these species lends credence to the idea that parasites should be considered as a continuation of the ecosystem of their host, with dynamics occurring between parasites in a host being as significant as classical ecological concepts like predation and competition.

While it is possible that some of these interactions are mediated through processes like niche competition, the fact that every studied species is implicated suggests that immune changes may also play a significant role. By using molecular assays to ascertain levels of circulating cytokines and expression levels of genes linked to immune responses, we were able to assess associations between infection and changes in immune profile. Notably, markedly different results were observed across different measures of immune function. Cytokine levels and mesenteric gene expression were primarily influenced by seasonality, and in the case of cytokine levels, sex. No impact of parasites on these two measures was observed. Splenic expression, however, was altered by infection with *C.hepaticum* and *Trichuris muris*. This

finding, alongside the central role that these two species play in interactions with other parasites, suggests that the parasite communities found within mice in this population are associated with changes in splenic immune expression in response to specific helminth infections.

T.muris infection showed a burden-dependent negative correlation with the immunoregulatory gene FoxP3, which is in line with lab-based observations of low-level chronic infections maintaining an immunosuppressed environment, and higher-burden acute infections eliciting a protective Th2 response. While the chronicity of infections could not be ascertained in this cross-sectional study, a burden-dependent shift in immunity response could explain the combination of positive and negative associations observed between this parasite and *C.hepaticum*, as acute *T.muris* infections create an unfavourable immune environment for other helminths. Induction of a protective Th2 environment is also indicated by reduced levels of Th1 cytokines with increasing infection intensity of *C.hepaticum*. It seems highly likely that changes between immune environments caused by these parasites are implicated in associations between parasite species, but future longitudinal analyses of infection and immune state must be carried out to fully understand the causality of these dynamics.

While immune state and infection are clearly closely linked, and similarly impacted by external environmental and ecological factors, we attempted to

understand to what extent these differences were genetically determined. A number of SNPs in immune genes generally linked to control of inflammation were found to be associated with changes in all measures of immune state. Despite this, all observed changes were seen in overall levels of gene expression or circulating cytokine levels, rather than specific genes or cytokines. In addition, while other immune SNPs were found to alter parasite communities, none of the SNPs were the same as those implicated in changes in immunity. Despite this, the fact that SNPs in immune genes delineate populations of *T.muris* from other parasite populations may indicate that tolerance to this parasite is in part genetically determined.

In particular, the fact that burden of *T.muris* is the parasitological variable most affected by differences in genotype, suggests that differences between chronic and acute infections observed between different strains of lab mice may also be apparent in this wild population. Reduced immunoregulation with increasing *T.muris* burden, as indicated from the spleen qPCR data, may be genetically influenced, while a protective Th2 response caused by high *T.muris* burdens may negatively impact other parasites. The genetic study conducted on this population was limited to a relatively low number of SNPs, and did not include background population genetics from which to highlight a specific role for immune genes. This would be a prime area for further study in this population, as a dramatically increased number of analysed SNPs may expose functional groups of genes and haplotypes implicated in genetic

control of immune response to infection. The effects observed from this limited genetic study, however, clearly show that considering the genetic diversity of wild populations is a crucial element of ecoimmunology.

The interplay between host immunity, parasite communities, environmental change and host ecology is clearly complex and trying to understand the causality of relationships between these areas can be difficult. Crucial to interpreting any of these effects, though, is understanding the impact on the host. Assessing true fitness in wild animals is challenging, but by using various measures of condition, we can understand how different aspects of infection are associated with physiological changes in the host. Unexpectedly, parasitic infections were not found to influence any measure of host condition in this study. This was surprising, as parasites have classically been defined by their negative influence on host fitness.

Far more influential were seasonality and, in males, trade-offs with reproductive outputs. This paints the picture of a parasite community that is primarily commensal, regularly infecting hosts but with negligible impact on the host population. This may be due the fact that this population has been mostly isolated and undisturbed for a long time, allowing host and parasite to coevolve to the point where host responses are optimally adapted to minimise fitness costs. As with many of the findings of this thesis, longitudinal study of this population, to estimate survival rates and obtain a more accurate

measure of fitness, will more clearly illustrate the role of parasites, and determine whether the health of isolated island population is uniquely unaffected by parasitism relative to other populations.

Laboratory experiments which explore the many phenomena involving changes in the immune system have given us a solid framework to understand how wild animals are responding to infection. For some parasites, such as *T.muris*, there is a wealth of controlled, detailed parasitological and immunological study from controlled experiments that can be directly compared to natural infections and help to better understand how the parasite is interacting with the host. Conversely, performing ecoimmunological studies in wild populations can highlight how lab experiments can be unrepresentative of real world infections, and how multiple elements of a wild animal's life history can intersect to give surprising results.

By incorporating a range of analyses and techniques, I have in this thesis characterised key elements associated with changes in immune state, distribution of parasites, and ultimate impact on the host. While different parasite species show some limited associations with observed changes in immune state, their ultimate impact on host health is surprisingly minimal in this population. While associations between infection and host health have not been detected through the measurements and observations made in this

thesis, further investigation may reveal thus far unseen roles for parasites in this population. Despite this, the robust network of interactions seen between species highlights their significance within the broader ecosystem of the host. The findings of this thesis illustrate novel ecoimmunological dynamics in a unique, wild population of a model species, highlight the limitations of conclusions drawn from laboratory studies on the impacts of infection, and provide a foundation of information for deeper research questions to be explored in the Isle of May mice.

7.2 Potential for Further Investigation

While this cross-sectional study has highlighted a number of interesting associations and potential interactions between a wild population and its environment, the limitations inherent in the collected data restrict what can be gathered about the precise mechanisms and causal relationships of these effects. Despite this, they also offer opportunities for expanding the work described here in a number of ways.

One of the more prominent drawbacks of this study was the fact that immunity, infection and condition of the host population could not be monitored over time. Conducting a longitudinal study of this population, building upon what has been established in this cross-sectional analysis is the most effective means by which the role of infection dynamics in the mice can

be better understood. Conducted over longer periods, it will also allow seasonal variation in these factors to be resolved, both throughout the year, and inter-annually. Seasonality is an important factor affecting population cycles of both parasite and host species, and better characterisation of seasonal shifts in host condition and infection status will not only allow for more rigorous analyses of the interactions explored in this thesis, but also provide a much more comprehensive picture of the role of environmental variation in affecting the impact of infection. This would be complimented by detailed meteorological data, which would help to more precisely discern how seasonal changes occur, for instance showing how a host population is more severely impacted by infection during harsher winters.

Longitudinal studies also provide an opportunity to better assess host survival and reproductive success, and thus gain a much clearer picture of the importance of various ecological and infectious factors in determining true fitness. Monitoring multiple generations of the host population would in addition allow for better characterisation of the influence of genetic diversity on the host condition and outcome of infection. By following known pedigrees within the host population, the success of specific genotypes and haplotypes under different conditions can be observed, and the underlying factors maintaining genetic diversity within the population better understood.

High-resolution longitudinal analysis would greatly improve our understanding of this population, but any further studies would also greatly benefit from a more systematic and widespread trapping layout across the island. The mice on the island exist in a highly contained area, and while for this study, trapping was conducted according to where transects could most practically be established, a systematic trapping grid would allow for coverage of almost the entire range of the population.

While the parasitological surveys conducted in this study have attempted to cover a broad range of potential infections, the breadth of parasites recorded across rodent populations is vast (Flynn, 2007b). While ectoparasites and gastrointestinal helminths were surveyed thoroughly, microparasites were selected for detection by PCR beforehand, and as such, species not selected for analysis may have been overlooked. The prevalence of *C.hepaticum* in the liver also alludes that parasites may be detectable in other tissue types that were not surveyed in this study and are not commonly examined, such as muscle tissue (Flynn, 2007b). Increasing the coverage of parasitological surveys in this way, as well as accounting for viral infections and the underlying microbiome, will give a more comprehensive picture of the many organisms existing within the mammal host, and thus a more comprehensive idea as to their role in the host's ecology.

Parasitological analyses of this population could also be improved by better characterisation of the infectious cycles of the parasites. Combined with robust longitudinal seasonal data, the factors controlling infection dynamics, be they shifts in climate, or changes in the density or behaviour of the host population, could be established. Understanding the underlying factors controlling infection cycles will in turn provide a better framework for analysing their impact on host immune state. While migration of mice carrying new infections to the island is highly unlikely, the parasites of the birds and other mammals on the island should not be overlooked. While cataloguing the parasites of rabbits (*Oryctolagus cuniculus*), seals (*Halichoerus grypus*), and both resident and migratory birds inhabiting the island may pose great difficulty, the potential for cross-species infection may be crucial to understanding how parasites are infecting the mice.

The immunological techniques used in this study took advantage of a wealth of prior lab-based study and assay development, and as such were of uncommon value within studies of wild populations. These techniques could, however, be expanded to cover a greater range of genes and cytokines, such as other members of the interleukin family. Assays which quantify other facets of the immune system like complement and antibodies might help to better establish some of the actual effector mechanisms being employed by the immune system, rather than just the network of cytokines which regulate these mechanisms.

The forms of genetic analysis used for the purposes of this thesis are an area with great potential for more sophisticated analyses. Within the scope of this study, only a limited number of SNPs in immune genes could be examined. Relative to the rest of the genome, and even the immune-associated genes, the analysis was very restricted. Analysis of much higher numbers of SNPs via high-throughput methods will give a much more comprehensive overview of the level of diversity within immune genes on the island, and whether polymorphisms within specific genes or groups of genes are significant to particular aspects of immune expression. Better understanding of the population genetics of the mice would provide an informative background for any future genetic study, as diversity in immune genes could be compared with underlying genetic diversity. Whether immune genetic diversity is more shaped more by the infectious challenges the mice face, or the pedigree or ancestral population of the mice (see Chapter 1.6) is an area of great potential future research.

7.3 Conclusion

The burgeoning area of ecoimmunology has massive potential for increasing our understanding of how the complex networks of interactions that control host physiology and immunity and infection dynamics relate to each other against a background of constantly shifting biotic and abiotic factors. Better characterising these relationships allows us in turn to answer some profound

questions regarding the evolutionary origin of the immune system and its position as an adaptive system within populations and ecosystems. Molecular methodologies now allow us to generate large amounts of detailed transcriptomic and proteomic data for many individuals in a population, which can be used to elucidate large-scale patterns across time, across individuals, and under different types and levels of infection. In *M.m.domesticus* on the Isle of May, I have used these techniques to interrogate many aspects of ecoimmunology in a well-characterised population, and thus illustrated how parasites and control of infection can be integrated into field studies of wild populations facing multiple infections.

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Appendix A: Phenotypes Associated with Polymorphism and Knockout of Genes Used in SNP Genotyping (Chapter 5)

Gene		Gene Type	Location	SNP/KO Phenotypes
Ccr9	Chemokine (C-C motif) receptor 9	Receptor	Chromosome 9: 123,678,439-123,783,457 FS	Human SNPs - Prevalence of inflammatory disease (Inamoto *et al.*, 2010) Mouse KO - Impaired settling of progenitor cells in the thymus (Zlotoff *et al.*, 2010)
Cd27	CD27 antigen	Antigen	Chromosome 6: 125,232,622-125,237,010 RS	Novel human mutation - Hyperproliferation of lymphocytes (Alkhairy *et al.*, 2015) Mouse KO - Delayed formation of B cell germinal centres (Xiao *et al.*, 2004)
Ctla4	Cytotoxic T-lymphocyte-associated protein 4	Receptor	Chromosome 1: 60,887,000-60,915,832 FS	Human SNPs - Predisposition to autoimmune disease (Djilali-Saiah *et al.*, 1998; Ueda *et al.*, 2003; Yang *et al.*, 2012) Mouse KO - Lymphocyte proliferation, autoimmunity, splenomegaly, increased Th2 expression (Khattri *et al.*, 1999; Wing *et al.*, 2008).
Gata3	GATA binding protein 3	Transcription Factor	Chromosome 2: 9,857,078-9,890,034 RS.	Human SNPs – Predisposition to Th1/2 type immunity, changes in IgE expression and prevalence of asthma (Pykäläinen *et al.*, 2005)
Gzma	Granzyme A	Effector Protein	Chromosome 13: 113,093,825-113,100,981 RS	Mouse KO – Impaired apoptosis of infected or damaged host cells (Müllbacher *et al.*, 1996, 1999; Simon *et al.*, 1997)
Ifnar2	Interferon (alpha and beta) receptor 2	Receptor	Chromosome 16: 91,372,783-91,405,589 FS	Human SNPs – Susceptibility to autoimmune diseases and chronic viral infection (Leyva *et al.*, 2005; J. Zhou *et al.*, 2007)
Ifngr1	Interferon gamma receptor 1	Receptor	Chromosome 10: 19,591,949-19,610,229 FS	Human SNPs – Susceptibility to *Leishmania* infection, tuberculosis and *Helicobacter pylori* – associated gastric carcinoma (Bulat-Kardum *et al.*, 2006; Salih *et al.*, 2007; Canedo *et al.*, 2008)
Il10	Interleukin 10	Cytokine	Chromosome 1: 131,019,845-131,024,974 FS	Mouse KO – Levels of Th1-type inflammation, increased inflammatory and necrotic pathology during *Toxoplasma gondii* infection (Gazzinelli *et al.*, 1996; Leon, Kozak and Kluger, 1998; Yang *et al.*, 1999)
Il10rb	Interleukin 10 receptor, beta	Receptor	Chromosome 16: 91,406,164-	Human SNPs – Susceptibility to early-life colitis, and levels of intestinal inflammation (Moran *et al.*, 2013; Shouval *et al.*, 2014)

			91,425,834 FS	Mouse KO – Chronic colitis and splenomegaly (Spencer *et al.*, 1998)
Il12a	Interleukin 12a	Cytokine	Chromosome 3: 68,690,644-68,698,547 FS	Human SNPs – Susceptibility to dermatitis and asthma (Namkung *et al.*, 2010; Chen *et al.*, 2011) Mouse KO - Increased susceptibility to *H.pylori* infection, and increased levels of IL-17 expression (Aggarwal *et al.*, 2003; Panthel, Faller and Haas, 2003)
Il12b	Interleukin 12b	Cytokine	Chromosome 11: 44,400,063-44,414,033 FS	Human SNPs- Susceptibility to Tuberculosis, severity of asthma symptoms and recover from hepatitis infection (Morahan *et al.*, 2002; Mueller *et al.*, 2004; Tso *et al.*, 2004) Mouse KO - Increased susceptibility to *H.pylori* infection, and increased levels of IL-17 expression (Aggarwal *et al.*, 2003; Panthel, Faller and Haas, 2003)
Il12rb2	Interleukin 12 receptor, beta 2	Receptor	Chromosome 6: 67,291,318-67,376,188 RS	Human SNPs - Susceptibility to dermatitis and cirrhosis (Isomura *et al.*, 2008; Hirschfield *et al.*, 2009) Mouse KO – Increased levels of autoimmunity and malignancy (Airoldi *et al.*, 2005)
Il13ra1	Interleukin 13 receptor, alpha 1	Receptor	Chromosome X: 36,112,110-36,171,259 FS	Human SNPs – Levels of IgE production (He *et al.*, 2003) Mouse KO – Impaired Th2-associated IgE production (Munitz *et al.*, 2008)
Il16	Interleukin 16	Cytokine	Chromosome 7: 83,642,848-83,745,726 RS	Human SNPs – Susceptibility to dermatitis and autoimmune diseases including lupus, Crohn's disease and Grave's disease (Glas *et al.*, 2003; Reich *et al.*, 2003; Gu *et al.*, 2008; Xue *et al.*, 2009) Mouse KO – reduced renal ischemia-reperfusion and inflammatory injury (Wang *et al.*, 2008)
Il17a	Interleukin 17A	Cytokine	Chromosome 1: 20,730,905-20,734,496 FS	Human SNPs – Susceptibility to asthma, tuberculosis and bronchiolitis (Chen *et al.*, 2010; Ocejo-Vinyals *et al.*, 2013) Mouse KO – Reduced antigen-specific T cell sensitisation, reduced allergic responses (Nakae *et al.*, 2002)
Il17ra	Interleukin 17 receptor A	Receptor	Chromosome 6: 120,463,247-120,487,559 FS	Humans SNPs – Susceptibility to cerebral malaria, and autoimmune disease including alopecia and psoriasis (Lew *et al.*, 2012; Batalla *et al.*, 2015; Marquet *et al.*, 2015) Mouse KO – Reduced expression of Th17-associated cytokines and decreased infiltration of myeloid cells into tumours (Wang *et al.*, 2014)
Il18bp	Interleukin 18 binding protein	Inhibitor	Chromosome 7: 102,015,079-102,018,155 RS	Human SNPs – Levels of proinflammatory IL-18 expression (Thompson *et al.*, 2007) Mouse KO – Increased severity of colitis, depletion of mature goblet cells (Nowarski *et al.*, 2015)

Il1a	Interleukin 1 alpha	Cytokine	Chromosome 2: 129,299,610-129,309,972 RS	Human SNPs – Susceptibility to systemic sclerosis and multiple sclerosis (Kawaguchi *et al.*, 2003; Mirowska-Guzel *et al.*, 2011) Mouse KO – Reduced neutrophil recruitment during inflammation (Rider *et al.*, 2011)
Il1r1	Interleukin 1 receptor, type I	Receptor	Chromosome 1: 40,225,080-40,317,257 FS	Human SNPs – Susceptibility to Crohn's disease and (Latiano *et al.*, 2013) Mouse KO – Reduction in pro-inflammatory cytokine expression (Torres *et al.*, 2009)
Il20ra	Interleukin 20 receptor, alpha	Receptor	Chromosome 10: 19,712,587-19,760,053 FS	Human SNPs -Susceptibility to psoriasis (Kingo *et al.*, 2008, 2010)
Il25	Interleukin 25	Cytokine	Chromosome 14: 54,932,695-54,935,837 FS	Human SNPs – Susceptibility to multiple sclerosis (Javan *et al.*, 2014) Mouse KO – Impaired Th2-type inflammation and IL-9 expression (Fort *et al.*, 2001; Angkasekwinai *et al.*, 2010)
Il2ra	Interleukin 2 receptor, alpha chain	Receptor	Chromosome 2: 11,642,807-11,693,193 FS	Human SNPs – susceptibility to multiple sclerosis and type 1 diabetes (Matesanz *et al.*, 2007; Maier *et al.*, 2009; Cavanillas *et al.*, 2010; Hartmann *et al.*, 2014) Levels of Treg cells in response to cholangitis in the live (Sebode *et al.*, 2014)
Il6	Interleukin 6	Cytokine	Chromosome 5: 30,013,114-30,019,981 FS	Human SNPs – susceptibility to chronic arthritis, colorectal cancer and graft-versus-host disease (Fishman *et al.*, 1998; Cavet *et al.*, 2001; Landi *et al.*, 2003) Mouse KO – Reduced intestinal permeability and increased expression of IL-10 (Wang, Fang and Hasselgren, 2001)
Il7r	Interleukin 7 receptor	Receptor	Chromosome 15: 9,506,161-9,529,876 RS	Human SNPs – Susceptibility to a range of autoimmune and inflammatory diseases, and allergies (Teutsch *et al.*, 2003; Shamim *et al.*, 2007; Mazzucchelli, Riva and Durum, 2012) Mouse KO – Impaired development of T cells and B cells (Candéias *et al.*, 1997)
Il9	Interleukin 9	Cytokine	Chromosome 13: 56,479,277-56,482,246 RS	Human SNPs -Susceptibility to atopic dermatitis and asthma (Rosenwasser, 1999; Namkung *et al.*, 2011)
Irf7	Interferon regulatory factor 7	Transcription Factor	Chromosome 7: 141,262,706-141,266,481 RS	Human SNPs – Levels of IFNα production in response to HIV and during lupus (Salloum *et al.*, 2010; Chang *et al.*, 2011) Mouse KO – Impaired IFN expression and Th1 immunity (Honda *et al.*, 2005)
Irf9	Interferon regulatory factor 9	Transcription Factor	Chromosome 14: 55,603,571-55,610,030 FS	Mouse KO – Impaired TLR7-mediated B cell activation and antibody class switching (Thibault *et al.*, 2008)

Mr1	Major histocompatibility complex, class I-related	MHC	Chromosome 1: 155,127,277-155,146,814 RS	Human SNPs – Susceptibility to tuberculosis (Seshadri *et al.*, 2017) Mouse KO – Impaired early production of proinflammatory cytokines and recruitment of Th1 cells during pulmonary bacterial infection (Meierovics, Yankelevich and Cowley, 2013)
Myd88	Myeloid differentiation primary response gene 88	Adaptor Protein	Chromosome 9: 119,335,934-119,341,411 RS	Human SNPs – Susceptibility to ulcerative colitis (Matsunaga *et al.*, 2014) Mouse KO – Impaired B cell proliferation and macrophage cytokine secretion in response to LPS (Kawai *et al.*, 1999)
Nod2	Nucleotide-binding oligomerization domain-containing 2	Pattern Recognition Receptor	Chromosome 8: 88,647,315-88,688,474 FS.	Human SNPs – Susceptibility to Crohn's disease (Hampe *et al.*, 2002) Mouse KO - Impaired inflammation in response to bacterial peptidoglycan (Rosenzweig *et al.*, 2008)
Rorc	RAR-related orphan receptor gamma	Receptor	Chromosome 3: 94,372,794-94,398,276 FS	Mouse KO – Lack of secondary lymphoid organs, dysregulated T cells, lymphoma development (Sun *et al.*, 2000; Liljevald *et al.*, 2016)
Socs5	Suppressor of cytokine signalling 5	Inhibitor	Chromosome 17: 87,107,679-87,137,583 FS	Human SNPs – Association with bronchial asthma (Saltykova *et al.*, 2013)
Socs7	Suppressor of cytokine signalling 7	Inhibitor	Chromosome 11: 97,362,551-97,398,542 FS	Human SNPs – Associations with obesity and insulin levels (Tellechea *et al.*, 2013) Mouse KO – Increased insulin function and enlargement of islets of Langerhans (Banks *et al.*, 2005)
Tirap	Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein	Adaptor Protein	Chromosome 9: 35,184,551-35,200,291 RS	Humans SNPs – Susceptibility to pneumococcal disease, bacteraemia and tuberculosis (Khor *et al.*, 2007; Castiblanco *et al.*, 2008; Selvaraj *et al.*, 2010) Mouse KO – Impaired cytokine production and TLR signalling in innate immunity (Horng *et al.*, 2002; Yamamoto *et al.*, 2002)
Tlr3	Toll-like receptor 3	Receptor	Chromosome 8: 45,395,665-45,410,608 RS	Human SNPs – Susceptibility to HIV (Sironi *et al.*, 2012), hepatitis C (Medhi S *et al.*, 2011), nasopharyngeal carcinoma (He *et al.*, 2007) Mouse KO – Impaired TNFα and iNOS expression in Schwann cells (Lee *et al.*, 2006) Reduced inflammation in response to rhinovirus infection (Wang *et al.*, 2011)
Tlr4	Toll-like receptor 4	Receptor	Chromosome 4: 66,827,584-66,930,284 FS	Human SNPs – Susceptibility to transplant rejection (Hwang *et al.*, 2009), Crohn's disease, ulcerative colitis (Franchimont *et al.*, 2004), atopic asthma and levels of LPS-induced IL-12 and IL-10 expression (Fagerås Böttcher *et al.*, 2004)

				Mouse KO – Impaired B cell function (Pasare and Medzhitov, 2005), impaired response to LPS (Hoshino *et al.*, 1999)
Tlr5	Toll-like receptor 5	Receptor	Chromosome 1: 182,954,788-182,976,044 FS	Human SNPs – Susceptibility to Crohn's disease Mouse KO – Impaired IL-10 expression by regulatory dendritic cells in response to ovalbumin (Shim *et al.*, 2016)
Tnf	Tumor necrosis factor	Cytokine	Chromosome 17: 35,199,381-35,202,007 RS	Human SNPs – Susceptibility to inflammatory bowel disease (van Heel *et al.*, 2002), arthritis, lupus and tuberculosis (Correa *et al.*, 2005) Mouse KO – Impaired development of splenic macrophage and B cell populations populations (Pasparakis *et al.*, 1996, 2000)
Tnfrsf1b	Tumor necrosis factor receptor superfamily member 1B	Receptor	Chromosome 4: 145,213,463-145,246,870 RS	Human SNPs – Susceptibility to Crohn's disease(Sashio *et al.*, 2002) Mouse KO - Increased resistance to TNF-induced cell death (Erickson *et al.*, 1994)
Traf3	TNF receptor-associated factor 3	Signalling Protein	Chromosome 12: 111,166,370-111,267,153 FS	Human SNPs – Multiple myeloma and myeloid leukemia susceptibility (Du *et al.*, 2011; Rybka *et al.*, 2016) Mouse KO – Impaired development of T cells and responses to T cell antigens (Xu, Cheng and Baltimore, 1996)
Tyk2	Tyrosine kinase 2	Enzyme	Chromosome 9: 21,104,069-21,127,346 RS	Human SNPs – Susceptibility to lupus (Sigurdsson *et al.*, 2005; Graham, Akil and Vyse, 2007) and multiple sclerosis (Mero *et al.*, 2010) Mouse KO 0 Reduced response to IL-12 IFNα and IFNβ, impaired production of nitric oxide in response to LPS, reduced T cell response and infection clearance in response to viral infection (Karaghiosoff *et al.*, 2000; Shimoda *et al.*, 2000)

Appendix B: Polymorphisms Genotyped in the Isle of May Mice (Chapter 5)

Gene	Mutation Type	Sequence
Ccr9	Missense: Alanine -> Serine	GCCATTGTACAGGCCATGAAG**[T/G]**CTCAGGTCTGGAGGCAGAAAA
	Silent	AGTTCTCTATGTTTTTGTTGG**[T/C]**GAGAGATTCCGAAGGGATC
Cd27	Silent	ACCTTGGTAGGACTCTCAGCTAC**[T/C]**CTAGCCCCAAACAGCTGT
Ctla4	Silent	CAGCTTTCCATGTGAATATTCACC**[G/A]**TCACACAACACTGATGAGGTCCGG
Gata3	Silent	TCCAGCCACATGCTGACCACACC**[G/A]**ACGCCCATGCATCCGCCCTCCGGCC
Gzma	Missense: Glutamine -> Aspartic Acid	TGGCAATAAGTCASCTCCCTCTGA**[T/A]**ACTCTGAGAGAAGTCAACATCACTGTC
Ifnar2	Silent	AGAACTGTTCAGATACCAC**[G/A]**AAGTCATCATGTGACGTGACAGATAAGT
Ifngr1	Missense: Proline -> Leucine	GGAGTGGAGCTTTGACGAGCAC**[T/C]**GAGGATCCTGAGCCTCCCTCGGTGCCT
Il10	Silent	TGACTGGCATGAGGATCAGCAG**[A/G]**GGCCAGTACAGCCGGGAAGACAATA
Il10rb	Missense: Leucine -> Phenylalanine	GGGCTGCTTTGTCGTGCTGTGG**[T/C]**TCATTTATAAGAAGACCAAGCA
Il12a	Missense: Arginine -> Glutamine	CCTGCCAGGTGTCTTAGCCAGTCCC**[G/A]**AAACCTGCTGAAGACCACAGA
Il12b	Missense: Methionine -> Threonine	CTGACTCTCGGGCAGTGACATGTGGAA**[T/C]**GGCGTCTCTGTCTGCAGAGA
Il12rb2	Missense: Threonine -> Methionine	ATCAAGCATTTGCATCGCTATCATCA**[C/T]**GGTGGGCACGTTCTCAATTCGTTACTT
Il13ra1	Silent	TATTCCTTTTGTTACTTCTTTG**[C/T]**CTTATTGTGGAGAAGGAAGAA
Il16	Silent	AACCCCTACAGTGCTGGAGAAAT**[C/T]**GATTTTCCAATGACCAAGAAATCTGC
Il17a	Missense: Glutamic Acid -> Lysine	GCCCCTTCACTTTCAGGGTC**[G/A]**AGAAGATGCTGGTGGGTGTGGGC
Il17ra	Silent	CAGAACTTCTCAAGGCTA**[C/T]**GCAGAGCATGTCAGTGAAGAAACG
Il18bp	Missense: Threonine -> Isoleucine	CACATCTGCACCTCAGACAA**[C/T]**TGCCACTGTCTTAACTGGAA
Il1a	Silent	TCACAACTGTTCGTGAGCGCTCA**[G/A]**GGAGAAGACCAGCCCGTG
Il1r1	Missense: Alanine -> Valine	TGACAGCAAGACCCCCATATCAG**[C/T]**GGACCGGGACTCC
Il20ra	Silent	AATCCACCAGAGAGTCTACA**[C/T]**GGAGTTGAAGTCACATACACTGTGCA

Il25	Silent	TTCTACCGGCGGCCATGCCATGG**[T/C]**GAGGAAGGTACCCATCGCCG
Il2ra	Missense: Serine -> Leucine	CAATGACGGAGACATTTGTGCT**[C/T]**ACAATGGAGTATAAGGTAGCAG
Il6	Missense: Threonine -> Lysine	ACCCCAATTTCCAATGCTCTCCTAA**[C/A]**RGATAAGCTGGAGTCACAGAAGGA
Il7r	Silent	GCTGTTTCTGGAGAAAGTGG**[A/C]**AATGCCCAGGATGGAGACCTAGAA
Il9	Missense: Alanine -> Valine	AAAAGCCATGCAACCAGACCATGG**[C/T]**AGGCAACACACTGTCATTTCTGA
Irf7	Missense: Proline -> Serine	GAATCCGAGTCTGGGGCAGAC[C/T]CCGTCCCACCACAGGCTCCTGGCCA
Irf9	Silent	GAAGATTCCAGAAGAGGAGAAAGC[T/C]GCCTTGTTCCTGTTACAGCACAC
Mr1	Silent	TCATCATCTTCAATAAAGACAC[C/T]CTCTCCTGGTTGGCCATGGATTAT
Myd88	Silent	CTTTATCTGCTACTGCCCCAA[C/T]GATATCGAGTTTGTGCAGGAGA
Nod2	Silent	TCAGCGCTCCTCAGGAAGTT[C/T]GTCCGTACAGAGTGCCAACTGAAGG
Rorc	Silent	TCCTAGCCAAGCTGCCACCCAAAGG[G/A]AAACTCCGGAGCCTGTGCAGC
Socs5	Missense: Phenylalanine -> Leucine	ACTGCCTCGTGCCAGATTTGCT[T/A]CAGATCACAGGGAATCCCTGT
Socs7	Missense: Arginine -> Glutamine	AGCATCTTTGCAGATTCCGGATCC[G/A]GCAGCTTGTCCGAATAGACC
Tirap	Missense: Arginine -> Histidine	GGCACTGAGTCGTAGTCACTGCC[G/A]TGTGCTGCTCATCACTCCAGGC
Tlr3	Missense: Threonine -> Methionine	TATCATCTACAAAGTTGGGAA[C/T]GGGGGTCCAACTGGAGAACCTCC
Tlr4	Missense: Isoleucine -> Methionine	CCACAAGTCAATCTCTCTTTAGACAT[A/G]TCTTTGAACCCAATTGACTTCAT
Tlr5	Silent	CTATCTGGCAACCAGATTCACAG[C/T]CTCCGCCTCCATTCTTCATTCCGG
Tnf	Silent	CTACGTGCTCCTCACCCACAC[C/T]GTCAGCCGATTTGCTATCTCATA
Tnfrsf1b	Silent	TTCTGCAACAAGACCTCGGACAC[C/T]GTGTGTGCGGACTGTGAGGCA
Traf3	Missense: Proline -> Leucine	GGCGCAGGGTCCGTGCTCGTGC[C/T]GGAGCAAGGAGGCTACAAGGAGAAG
Tyk2	Silent	TAAGGTCTGGCTGCCCCCAAACCATATT[C/T]TGGATACATCCCAAGACATGAA

Appendix C: PIPS Reflective Statement

Note to examiners: This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIPS Reflective Statement

My PIPS placement was completed at the National Biodiversity Network (NBN), a Nottingham-based charity from May to July, 2018. The goal of the charity is to improve public access and engagement with biodiversity recording, and collate national biodiversity data from a range of sources.

My role was primarily to produce multimedia to assist with navigating and using the NBN Atlas, a new service designed to explore, manipulate and export biodiversity record data in a variety of ways. This involved the production of written guides to be posted online, and videos giving step-by-step instructions on using all aspects of the service. In order to create these videos, I taught myself how to use video-editing software, and recorded spoken instructions for each guide. In addition to producing these materials, I also assisted with answering specific queries from via phone and email from people using the service.

PIPS Reflective Statement

My secondary role was to assist with publicity, promotion and outreach for the charity, and particularly to encourage member organisations to put forward nominees for the NBN's annual awards for biological recording. This was achieved through contact over phone and email. I created a short promotional video outlining the work of the charity for presentational purposes, and created flyers and posters to promote the annual awards, which were sent out to member organisations. Occasionally I assisted with promotion over social media.

Outside of my main roles, I developed articles using the data held by the NBN to be included in regular emails to members of the charity, each showing how visualising biodiversity data can show the history of species, such as in the invasive grey squirrel, in the UK. I also wrote a profile of myself and my work at the NBN which was published on the website when I finished my placement.

The experience I gained while at the NBN was extremely valuable. I was able to develop skills related to science and research, but which were completely different to those used in my PhD research. These include the communication of scientific ideas and the use of scientific data to a wider audience, including the general public and amateur naturalists. I learned how to develop a variety of multimedia to clearly illustrate scientific concepts. I also gained great insight into the workings of a charity, including how to engage people with your organisation's activities. As my colleagues at the NBN were not from a research background, I learned a great deal about working with people with entirely different professional strengths and expertise to achieve a common goal.

PIPS Reflective Statement

Having completed this placement, I would now consider a more diverse range of options for my future career. While I greatly enjoy participating in active research, informing the public about scientific concepts, and engaging with scientific data in a completely different context was intellectually stimulating in a completely different way. As such, I would consider working for charities or organisations which seek to engage people with important scientific ideas, and engage them with important issues related to science such as biodiversity or conservation.