

The ecology of immune variation in wild house mice (*Mus musculus* *domesticus*)

Stuart Young *BSc (Hons), MSc*

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Chapter 2: Richard Glennie (University of St Andrews) developed the SECR model used to estimate population sizes.

Chapter 3: Angela Lamb (British Geological Survey) performed the mass spectrometry, advised on sample collection and preparation, and provided comments on drafts. René van der Wal (University of Aberdeen) provided distribution maps of plant species on the Isle of May.

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Chapter 1

General introduction

1.1 Introduction

In this thesis, I examine the causes of individual variation in the immune phenotype of a population of wild house mice (*Mus musculus domesticus*) on the Isle of May, Scotland. Here, I outline our current understanding of the variation in immune response, and the trade-offs associated with mounting a tolerant or resistant response. This is placed in an ecological and evolutionary context, and I provide a summary of the nascent field of 'ecoimmunology' – the study of the immune system in a natural setting using wild populations. I then introduce the house mouse, highlighting its importance in immunological studies, as well as introducing the macroparasites found in the Isle of May mouse system. Finally, I outline the main aims and objectives of the remaining data chapters that make up this thesis.

1.2 Immunology in the wild

The mammalian immune system has been the subject of extensive study for many years, however this has mainly focussed on laboratory reared rodents that are often genetically modified, maintained in controlled conditions and infected with just a single strain of parasite (Abolins *et al.* 2011). By minimising variation, these studies have

provided a valuable insight into the molecular and cellular mechanisms of the immune system, however they fail to address the fact that animals living in the wild are exposed to infection by multiple pathogens, all while coping with various environmental stresses (Babayan *et al.* 2011; Pedersen & Babayan 2011; Maizels & Nussey 2013).

In the past two decades there has been a move towards studying the immune response in an ecological and evolutionary context, giving rise to the field of ecological immunology (Sheldon & Verhulst 1996), or 'ecoimmunology' (Boughton *et al.* 2011; Pedersen & Babayan 2011). Ecoimmunology aims to understand the variation in immune function among individuals and to determine effect of this variation on fitness (Norris & Evans 2000; Rolff & Siva-Jothy 2003; Schmid-Hempel 2003; Pedersen & Babayan 2011).

1.3 The vertebrate immune system

1.3.1 Cytokines

Cytokines are small, secreted immune-related proteins, and are the major signalling molecules of the immune system (Graham *et al.* 2007; Zimmerman *et al.* 2014). Measuring cytokines is particularly informative as different subclasses of T helper cells secrete different cytokines, representing the functional arms of the adaptive immune response (Zimmerman *et al.* 2014).

Secreted cytokines circulating in the blood typically represent those not consumed at local sites of infection or rapidly removed from

circulation (Aziz *et al.* 2015). In humans, accurate measurement of circulating cytokines can be used in disease diagnosis (Aziz *et al.* 2015), such as in the detection of HIV infection (Nakajima *et al.* 1989; Fahey *et al.* 1990; Salazar-Gonzalez *et al.* 1997; Aziz *et al.* 1998).

1.3.2 Immunologically important tissues

The immune response may be localised and tissue specific (Hu & Pasare 2013). Two tissues involved in the immune response are the spleen and mesenteric lymph nodes (MLNs). In vertebrates, the spleen and lymph nodes form part of the network of secondary lymphoid tissues. In these tissues, foreign antigens can be presented to immune helper and effector cells. Further, secondary lymphoid tissues can help regulate the immunopathological effects of autoreactive T cells (van de Pavert & Mebius 2010).

The spleen acts as the main filter for blood-borne pathogens and antigens (Bronte & Pittet 2013) and is able to efficiently mount both innate and adaptive immune responses (Mebius & Kraal 2005). The spleen is composed of regions of red pulp and white pulp, separated by a marginal zone (Bronte & Pittet 2013). The red pulp contains macrophages that filter the blood as well as initiating the innate and adaptive immune responses (Mebius & Kraal 2005; Bronte & Pittet 2013). The white pulp is similar in structure to the lymph nodes and contains T cell and B cell rich regions. White pulp regions can generate antigen-specific immune responses against blood-borne pathogens (Bronte & Pittet 2013).

The gastrointestinal (GI) tract is continually exposed to dietary antigens, commensal bacteria and occasionally macroparasites (Houston *et al.* 2016), and the mesenteric lymph nodes (MLNs) are the 'first pass' for nutrients and microbes entering the lymph fluid in the lamina propria of the GI tract (Henao-Meija *et al.* 2013). Indeed, MLNs are key to inducing tolerance to food proteins, as well as preventing commensal GI bacteria from entering the immune system (Macpherson & Smith 2006). The MLNs are the primary immune tissues draining the GI tract and are important sites for T cell activation (Houston *et al.* 2016). As such, one might anticipate a specific, localised response to infectious challenges in the gut.

1.3.4 Immune response and associated trade-offs

The host's defence against invasive organisms is the immune system. This vast network of cells, signals and molecules allows mammals to respond generically to any antigen considered foreign (innate), and specifically to individual epitopes (adaptive).

The adaptive immune system is comprised of distinct, cross-regulated effector arms, each of which uses a subset of anti-pathogen mechanisms for use against certain types of infection (Bradley & Jackson 2008). The activities of each arm are co-ordinated by distinct subsets of T-helper (Th) cells (Reiner 2007). For example, cell-mediated defences against intracellular microparasites are activated by Th1 cells (Reiner 2007). Antibody-reliant defences against extracellular macroparasites

are activated by Th2 cells (Maizels *et al.* 2009), and Th17 cells stimulate neutrophilia and activate epithelial defences against extracellular microbes (Bradley & Jackson 2008). These responses can be inhibitory, so at any one time the immune system may be better able to defend against certain types of infection than others. For example, the release of Interferon Gamma (IFN γ) by Th1 cells inhibits the Th2 response (Long & Nanthakumar 2004). Likewise, the release of Interleukin-4 (IL-4) inhibits the Th1 response (MacDonald *et al.* 2002; Pearce & MacDonald 2002).

An immune response is typically associated with inflammation. While this can clear an infection, it can also lead to immunopathology – the self-damage of surrounding host tissue (Graham 2002). For example, the clinical symptoms of Schistosomiasis are the result of IL-13 being released in response to *Shistosoma* infection, which leads to the formation of granulomas and fibrosis in the infected tissues (Graham *et al.* 2005). Further, the release of Tumour Necrosis Factor Alpha (TNF- α) in response to a malaria infection has been shown to degrade haemocytes, causing anaemia and a loss of vigour in mice (Graham *et al.* 2005). Indeed, there is evidence that the release of inflammation-inducing cytokines in response to parasitic infection is associated with an increased risk of developing cancer (Lin & Karin 2007). Inflammation, however, can be down-regulated by a further regulatory subset of Th cells (Treg), which helps to provide an environment suitable for wound healing (Ortega-Gómez *et al.* 2013).

Further, the impact of immunopathology, inflammation and wound healing varies with Th subset. For example, Th1 and Th17 are associated with rapid inflammation and subsequently significant immunopathology, however they are able to control the lethal risk posed by rapidly increasing microbes (Bradley & Jackson 2008). Macroparasites are slower growing yet continually acquired from the environment, so Th2 responses are typically less violent but do include some mediators in wound healing and repair (Allen & Wynn 2011).

1.3.5 Parasite resistance versus parasite tolerance

Variation in the immune response can be broadly described as expressing either a 'resistant' or 'tolerant' phenotype. By mounting a resistant response, the infection is cleared rapidly, however this is associated with inflammation and immunopathology – both of which reduce host health (Råberg *et al.* 2007; Råberg *et al.* 2009). As well as causing damage to host tissue (Clatworthy *et al.* 2007), resistance to parasites can also deplete the energetic reserves of the host (Allen & Little 2011). Alternatively, a tolerant response accepts infection but maintains host health (Schneider & Ayres 2008). However, through effects on both the individual hosts and on parasite transmission (Best *et al.* 2008; Boots 2008), there is evidence that adopting a tolerance strategy could impact the epidemiology and co-evolutionary dynamics of infectious disease (Jackson *et al.* 2014). This can be by tolerant individuals becoming infectious 'super spreaders' (Lloyd-Smith *et al.*

2005), or by the evolution of higher rates of transmission in tolerant populations (Miller *et al.* 2005).

1.3.6 Resistance and tolerance: drivers of variation

It would seem reasonable to predict that an individual would adopt a resistant or tolerant strategy dependent on being exposed to either an acute or chronic infection. However, individuals living in seemingly similar circumstances in the natural environment still differ in their immune response, and some are apparently better at either resisting or tolerating infection (Jackson *et al.* 2014). Turner *et al.* (2011) have shown that polymorphism in cytokine genes is associated with variation in immunity and resistance to pathogens, and the effect of such genetic factors was as significant as that of other intrinsic factors such as sex or mass. Further, it was shown that these associations were consistent across a subset of cytokine genes (IL-1 β , IL-2 and IL-12 β), and these showed evidence of diversity maintained by natural selection (Turner *et al.* 2012). This indicates that variation in immune response has a genetic basis, and individuals may be born to follow a more resistant or a more tolerant strategy.

Individuals also vary in their gut microbiota, and it is becoming increasingly apparent that this gut flora plays an important role in modulating immune response (Molloy *et al.* 2012; Lively *et al.* 2014). Indeed, the gut microbiota of bumblebees was shown to have a greater effect on susceptibility to infection by the parasite *Crithidia bombi* than host genotype did (Koch & Schmid-Hempel 2012).

Wild living individuals are exposed to a variety of pathogens throughout their lifetime. Historic exposure by a pathogen may have an effect on subsequent immune responses. For example, Telfer *et al.* (2010) showed that following exposure by one pathogen species, the probability of subsequent infection by a second species is much greater. There is increasing evidence that prolonged exposure to parasite infection over time leads to a shift towards a tolerant phenotype. For example, Jackson *et al.* (2014) demonstrated a shift away from resistance in young male field voles, *Microtus agrestis*, towards a tolerant response in older males.

Further, wild animals are unable to expend unlimited resources on immunity: food may be limited, the climate may be harsh and reproduction may draw on reserves. So, if a host is in poor condition it may make a reduced response or a different type of response. For example, when protein is limited, laboratory mice will suppress their protective Th2 response (Ing *et al.* 2000). Indeed, Beldomenico *et al.* (2008) have demonstrated a 'vicious circle' involving condition and infection: those individuals in the worst condition are more likely to become and remain infected; infected individuals suffer a reduction in condition (Beldomenico & Begon 2010).

1.4 The study species: wild house mice (*Mus musculus*)

1.4.1 Natural history of *Mus musculus*

The house mouse (*Mus (Mus) musculus*) species complex is widely spread and consists of multiple subspecies including *M. m. bactrianus* on the Indian subcontinent, *M. m. castaneus* in Southeast Asia, *M. m. gentilulus* in the Arabian Peninsula and *M. m. musculus* from northern Eurasia (Suzuki *et al.* 2013; Viney *et al.* 2015). The most widely spread subspecies, however, is *M. m. domesticus*, which originated in the Middle East and the Mediterranean basin (Singleton & Krebs 2007; Suzuki *et al.* 2013). Here, *M. m. domesticus* became commensal with people, and spread into Europe with increasing human trade (Cucchi *et al.* 2005; Pocock *et al.* 2005). Due to its close association with people, *M. m. domesticus* is now found across western Europe, the Americas, Africa, Australasia and various oceanic islands (Pocock *et al.* 2005). Indeed, the global distribution and ubiquity of *M. m. domesticus* has led to it being described as the “ultimate ‘mammalian weed’” (Singleton & Krebs 2007).

Across their range, house mice are primarily commensal, with competition from other small mammals often excluding them from non-commensal niches (Singleton & Krebs 2007). Indeed, interspecific competition from other small mammals can lead to the extinction of island populations of house mice, largely by the prevention of breeding and a

lack of recruitment (Berry *et al.* 1982). On Brooks Island in San Francisco Bay, the previously healthy mouse population went extinct within 18 months of the introduction of California voles (*Microtus californicus*) (Lidicker 1966), and mice on the Faroe island of Bordøy went extinct following the colonisation of the island by brown rat (*Rattus norvegicus*) (Berry *et al.* 1978a). On the St Kilda archipelago in the Outer Hebrides, the endemic subspecies of house mouse (*M. m. muralis*) became extinct shortly after the evacuation of the human population from the islands in 1930 (Harrisson & Moy-Thomas 1933). This was associated with the subsequent invasion by St Kilda field mice (*Apodemus sylvaticus hirtensis*) into the village areas, and an increase in interspecific competition (Berry & Tricker 1969). This poor competitive ability means that populations of house mice living on small islands, non-commensally with humans, can only persist in the absence of other small mammals (Berry & Tricker 1969; Berry & Jackson 1979; Dueser & Porter 1986).

Interestingly, the islands on which house mice do survive non-commensally are typically associated with significant seabird colonies, indicating the potential importance of seabird colonies as a source of food to enable populations to persist (see Chapter 3). Such islands include a number of subantarctic islands (Berry *et al.* 1978b; see Chapter 3), several Faroe islands (Evans & Vevers 1938; Berry *et al.* 1978a), the Orkney island of Faray (Berry & Jakobson 1975; Berry *et al.* 1992), Lunga in the Treshnish Isles (Darling 1938; Higgins 1971), the Shetland island of Foula (Berry & Tricker 1969), Skokholm off the southwestern coast of Wales (Berry 1964) and the Isle of May (Southern 1938).

Further information about the population biology and behavioural ecology of house mice, particularly regarding the Isle of May population, can be found in Chapter 2. For further information about the dietary ecology of wild house mice, see Chapter 3.

1.4.2 Parasites of wild house mice

Wild house mice play host to a diverse macroscopic endoparasite (Table 1.1) and ectoparasite (Table 1.2) fauna (Singleton & Krebs 2007), and this can vary across their global range (Phifer-Rixey & Nachman 2015). The exact nature of the geographic variation in infection is understudied (Phifer-Rixey & Nachman 2015), despite infection by pathogens and parasites being an important driver of evolution (Anderson & May 1979; Phifer-Rixey & Nachman 2015).

Serological surveys have revealed that wild house mice are infected by a range of viruses at varying seroprevalences, including Sendai virus, mouse hepatitis virus and mouse parovirus (Smith *et al.* 1993; Becker *et al.* 2007; Parker *et al.* 2009; Viney *et al.* 2015; Weldon *et al.* 2015; Masopust *et al.* 2017). In addition to this, wild mice are often exposed to microbial pathogens (Masopust *et al.* 2017), including *Mycoplasma pulmonis* (Parker *et al.* 2009; Weldon *et al.* 2015; Masopust *et al.* 2017), *Helicobacter* sp. (Parker *et al.* 2009), *Giardia muris* and *Eimeria* sp. (Feliu *et al.* 2012). Wild mouse populations can act as reservoirs of infection for laboratory mice (Parker *et al.* 2009), as well as harbouring various zoonoses (Singleton & Krebs 2007) including

cryptosporidiosis (Morgan *et al.* 1999), leptospirosis (Yager *et al.* 1953) and salmonellosis (Guard-Petter *et al.* 1997).

Importantly, wild mice are frequently infected by multiple pathogens. Indeed, Viney *et al.* (2015) found that that wild mice in Wales and Southern England were commonly affected by up to four different infections by the time they reached weaning age (approximately four weeks old), and by five weeks of age no mice were free from infection.

While serological and microbiological surveys were beyond the scope of this project, a number of macroparasites have been identified on the Isle of May mice (Southern 1938; Triggs 1991; Robertson 2014; Stewart *et al.* 2017; see Tables 1.1 and 1.2, and Chapter 4). Annual variation in the macroparasite infection dynamics of Isle of May mice, as well as the interactions with host body condition, are explored in detail in Chapter 4. The relationship between these infections and the mouse immune response is explored in Chapter 6. Here, however, I provide a brief overview of the general biology of these parasites.

Table 1.1: Summary of macroscopic endoparasites recorded in wild populations of *Mus musculus*. Adapted from Tattersall *et al.* (1994) and Singleton and Krebs (2007).

Parasite species	Locations recorded
Phylum: Nematoda (roundworms)	
<i>Aonchotheca annulosa</i>	Corsica ¹
<i>Aspicularis tetraptera</i>	Australia ² ; Corsica ¹ ; Egypt ² ; Formentera ¹ ; Iran ² ; Italy ² ; Poland ⁴ ; Serbia ³ ; Sicily ¹ ; UK ² ; USA ²
<i>Calodium hepaticum</i> *	Australia ² ; Azores ⁵ ; Corsica ¹ ; Egypt ⁶ ; New Zealand ¹ ; Sicily ¹ ; UK (Isle of May) ²⁰ ; USA ²
<i>Eucoleus bacillatus</i>	Corsica ¹
<i>Gallegostrongylus australis</i>	Australia ¹
<i>Gallegostrongylus ibicensis</i>	Ibiza ¹
<i>Gongylonema aegypti</i>	Egypt ²
<i>Gongylonema musculi</i>	Egypt ² ; Sicily ¹
<i>Gongylonema neoplasticum</i>	Brazil ² ; Tunisia ⁷
<i>Gongylonema</i> sp.	Australia ² ; Formentera ⁸ ; Menorca ⁸ ; Serbia ³ ; USA ⁹
<i>Heligmosomoides polygyrus</i>	Australia ¹ ; Corsica ⁸ ; Ireland ² ; Korea ¹¹ ; New Zealand ² ; Serbia ³ ; Sicily ¹⁰ ; UK ² ; USA ²
<i>Heterakis spumosa</i>	Australia ^{1,2} ; Canary Islands ¹² ; Korea ¹¹ ; Serbia ³ ; Sicily ¹⁰
<i>Mastophorus muris</i>	Canary Islands ¹² ; Corsica ⁸ ; Menorca ⁸ ; New Zealand ¹ ; Serbia ³ ; Sicily ⁸
<i>Muspicea borreli</i>	Australia ¹ ; UK ¹
<i>Nippostrongylus brasiliensis</i>	Mexico ¹³ ; Korea ¹¹ ; New Zealand ¹ ; Sicily ¹⁰
<i>Physaloptera getula</i>	Chile ¹⁴ ; Formentera ⁸ ; New Zealand ¹
<i>Protopirura marsupialis</i>	Egypt ⁶
<i>Protopirura muris</i>	Australia ¹ ; Canary Islands ¹² ; Egypt ⁶ ; Italy ¹ ; New Zealand; Pakistan ¹⁵ ; USA ¹
<i>Ritularia</i> sp.	Canary Islands ¹²
<i>Strongyloides ratti</i>	Egypt ⁶
<i>Syphacia muris</i>	Egypt ⁶ ; Iran ¹⁶
<i>Syphacia obvelata</i> *	Australia ² ; Belgium ² ; Canary Islands ¹² ; Corsica ⁸ ; Egypt ² ; Formentera ⁸ ; Guillo Island ¹ ; Ibiza ⁸ ; Iran ¹⁶ ; Italy ² ; Korea ¹¹ ; Mallorca ⁸ ; Menorca ⁸ ; New Zealand ² ; Poland ⁴ ; Serbia ³ ; Sicily ⁸ ; UK ² (Isle of May) ^{20,21} ; USA ²
<i>Syphacia stoma</i>	Ireland ²
<i>Trichinella spiralis</i> *	UK (Isle of May) ²⁰ ; USA ²
<i>Trichosomoides crassicauda</i>	Australia ² ; Egypt ¹
<i>Trichuris muris</i> *	Australia ² ; Belgium ² ; Canary Islands ¹² ; Corsica ⁸ ; Mexico ¹³ ; Formentera ⁸ ; Egypt ² ; Korea ¹¹ ; Pakistan ¹⁵ ; Serbia ³ ; Sicily ⁸ ; UK ² (Isle of May) ¹⁷
Class: Trematoda (flukes)	
<i>Brachylaima</i> sp.	Corsica ⁸ ; Formentera ⁸ ; Hyères Islands ⁸ ; Ibiza ⁸ ; Menorca ⁸ ; Sicily ¹⁰
<i>Brachylaima cribbi</i>	Australia ¹
<i>Corrigia</i> sp.	Corsica ⁸ ; Hyères Islands ⁸
<i>Echinostoma indoense</i>	Spain ²
<i>Fasciola hepatica</i>	Corsica ⁸
<i>Mesostephanus aegypticus</i>	Egypt ⁶
<i>Prosthodendrium ascidia</i>	Egypt ⁶
<i>Stictodora tridactyla</i>	Egypt ⁶
<i>Zonorchis</i> sp.	Corsica ¹

Table 1.1 continued (overleaf)

Table 1.1 (continued): Summary of macroscopic ectoparasites recorded in wild populations of *Mus musculus*. Adapted from Tattersall *et al.* (1994) and Singleton & Krebs (2007).

Ectoparasite species	Locations recorded
Phylum: Acanthocephala (thorny-headed worms)	
<i>Moniliformis moniliformis</i>	Colombia ² ; Iran ¹⁹
<i>Plagiorhynchus cylindraceus</i>	Canary Islands ¹²
Class: Cestoda (tapeworms)	
<i>Catenotaenia pusilla</i>	Corsica ⁸ ; Egypt ² ; Formentera ⁸ ; Israel ² ; Italy ² ; Majorca ⁸ ; Serbia ³ ; Sicily ⁸ ; UK ²
<i>Echinococcus multilocularis</i>	France ² ; USA ²
<i>Hymenolepis diminuta</i> (= <i>Rodentolepis diminuta</i>)	Australia ² ; Colombia ² ; Corsica ⁸ ; Egypt ² ; Iran ¹⁶ ; Iraq ² ; New Zealand ² ; Pakistan ¹⁵ ; Sicily ⁸ ; UK ² ; USA ²
<i>Hymenolepis nana</i> (= <i>Rodentolepis fraterna</i>)	Australia ¹ ; Egypt ⁶ ; Italy ² ; Pakistan ¹⁵ ; Serbia ³
<i>Hymenolepis straminea</i> (= <i>Rodentolepis microstoma</i>)	Australia ¹ ; Canary Islands ¹² ; Corsica ⁸ ; New Zealand ² ; Sicily ⁸ ; UK ²
<i>Mesocestoides</i> sp.	Formentera ⁸
<i>Paranoplocephala</i> sp.	Corsica ⁸
<i>Raillientina</i> sp.	Egypt ²
<i>Taenia taeniaeformis</i> (= <i>Cysticereus fasciolaris</i>)	Australia ¹ ; Canary Islands ¹² ; Colombia ¹ ; Corsica ⁸ ; Egypt ¹ ; Formentera ⁸ ; Ibiza ⁸ ; Iran ¹⁶ ; Iraq ¹ ; Italy ¹ ; Majorca ⁸ ; Mexico ¹⁸ ; New Zealand ¹ ; Pakistan ¹⁵ ; Serbia ³ ; UK ¹ ; USA ¹

*Recorded on the Isle of May (Southern 1938; Triggs 1991; Robertson 2014; Stewart *et al.* 2017).

References: 1 Singleton & Krebs (2007); 2 Tattersall *et al.* (1994); 3 Kataranovski *et al.* (2008); 4 Izdebska and Rolbiecki (2013); 5 Resendes *et al.* (2009); 6 Elshazly *et al.* (2008); 7 Jrijer *et al.* (2015); 8 Milazzo *et al.* (2003); 9 Parker *et al.* (2009); 10 Milazzo *et al.* (2010); 11 Kim *et al.* (2015); 12 Feliu *et al.* (2012); 13 Panti-May *et al.* (2015); 14 Landaeta-Aqueveque *et al.* (2007); 15 Rafique *et al.* (2009); 16 Allymehr *et al.* (2012); 17 Southern (1938); 18 Rodriguez-Vivas *et al.* (2011); 19 Zarei *et al.* (2016); 20 Robertson (2014); 21 (Stewart *et al.* 2017).

Table 1.2: Summary of macroscopic ectoparasites recorded in wild populations of *Mus musculus*. Adapted from Singleton & Krebs (2007).

Ectoparasite species	Locations recorded
Subclass: Acari (mites and ticks)	
<i>Acarus siro</i> *	UK (Isle of May) ¹
<i>Androlaelaps fahrenheitsi</i>	USA ²
<i>Dermacarus hypadaei</i>	USA ²
<i>Dermanyssus gallinae</i>	Iran ³
<i>Echinonyssus butantanensis</i>	Australia ²
<i>Eulaelaps</i> sp.	Australia ² ; New Zealand ² ; USA ²
<i>Kleemania lumosa</i>	Australia ²
<i>Haemaphysalis longicornis</i>	New Zealand ²
<i>Haemaphysalis punctata</i>	Iran ⁴
<i>Haemogamasus longitarus</i>	USA ²
<i>Haemogamasus pontiger</i>	New Zealand ²
<i>Haemolaelaps</i> sp.	Iran ⁴
<i>Hirstionyssus laticutatus</i>	New Zealand ²
<i>Laelaps algericus</i>	Iran ⁴
<i>Listrophorus leuckarti</i>	USA ²
<i>Mesolaelaps australiensis</i>	Australia ²
<i>Microtrombicula</i> sp.	Iran ⁴
<i>Myobia musculli</i> *	Australia ² ; New Zealand ² ; USA ² ; UK (Isle of May) ⁵
<i>Myocoptes musculus</i> *	Australia ² ; Iran ³ ; New Zealand ² ; UK ⁶ (Isle of May) ⁵ ; USA ²
<i>Myonyssus decumani</i>	New Zealand ²
<i>Ornithonyssus bacoti</i>	Australia ² ; Iran ³ ; USA ²
<i>Paraspeleognathopsis bakeri</i>	Australia ²
<i>Proctolaelaps hypundaei</i>	New Zealand ²
<i>Proctolaelaps pygmaeus</i>	New Zealand ²
<i>Radfordia affinis</i> *	Australia ² ; New Zealand ² ; USA ² ; UK (Isle of May) ⁵
<i>Trichoecius rombousti</i>	Australia ²
<i>Trombicula autumnalis</i> *	UK (Isle of May) ⁷
Order: Siphonaptera (fleas)	
<i>Ctenophthalmus agyrtes</i>	Finland ⁸ ; Iran ⁴ ; Poland ⁸
<i>Ctenophthalmus pseudagyrtes</i>	USA ²
<i>Echidnophaga murina</i>	Canary Islands ⁹
<i>Epitedia wenmanni</i>	USA ²
<i>Leptopsylla segnis</i>	Canary Islands ⁹ ; New Zealand ² ; Poland ⁸ ; Sicily ¹⁰
<i>Nosopsyllus barbarus</i>	Canary Islands ⁹
<i>Nosopsyllus fasciatus</i> *	Australia ² ; Finland ¹² ; Iran ⁴ ; New Zealand ² ; UK (Isle of May) ¹
<i>Nosopsyllus londiniensis</i>	Australia ² ; New Zealand ²
<i>Orchopeas leucopus</i>	USA ²
<i>Peromyscopsylla silvatica</i>	Finland ¹²
<i>Pulex simulans</i>	Guatemala ¹²
<i>Stenoponia tripectinata</i>	Canary Islands ⁹
<i>Xenopsylla cheopis</i>	Canary Islands ⁹
Order: Phthiraptera (lice)	
<i>Hoplopleura acanthopus</i>	Poland ⁸
<i>Hoplopleura captiosa</i>	Iran ⁴ ; Poland ⁸ ; USA ²
<i>Polyplax serrata</i>	Iran ⁴ ; New Zealand ² ; Poland ⁸

*Recorded on the Isle of May (Southern 1938; Triggs 1991; Robertson 2014)

References: 1 Southern (1938); 2 Singleton & Krebs (2007); 3 Allymehr *et al.* (2012); 4 Moravvej *et al.* (2016); 5 Robertson (2014); 6 Weldon *et al.* (2015); 7 Triggs (1991); 8 Izdebska and Rolbiecki (2013); 9 Feliu *et al.* (2012); 10 Milazzo *et al.* (2003); 11 Laakkonen *et al.* (2007); 12 Escobar *et al.* (2011).

1.4.2.1 Endoparasites of the Isle of May house mice

The Isle of May mice can be infected by a range of endoparasites, including pinworms (*Syphacia obvelata*), whipworms (*Trichuris muris*), tapeworms (*Hymenolepis* sp.) and the hepatic nematode *Calodium hepaticum* (see Table 1.1 and Chapter 4). Robertson (2014) noted the presence of *Trichinella spiralis* in mice trapped in 2013, however this had not been observed in previous studies, or in the present study (see Chapter 4). Further, *Hymenolepis* sp. was only identified in 1.4% of mice in the present study (Chapter 4), and so was not included in any analyses (see Chapters 4 and 6). Therefore, *T. spiralis* and *Hymenolepis* sp. are not discussed here.

1.4.2.2 *Calodium hepaticum*

Calodium hepaticum (syn. *Capillaria hepatica*) is a globally distributed nematode that infects the livers of mammals (Spratt & Singleton 2008). The primary hosts are rodents and lagomorphs, but it will occasionally infect other species including humans (Redrobe & Patterson-Kane 2005; Reperant & Deplazes 2005; Spratt & Singleton 2008).

C. hepaticum has a unique life cycle among helminth parasites of mammals in that its transmission requires the death of the host (Spratt & Singleton 1986). Adult female worms invade the liver of the host and deposit hundreds of eggs in the hepatic parenchyma. The unembryonated eggs remain here until they are exposed to the

environment by decomposition, predation or cannibalism (Spratt & Singleton 2008; Figure 1.1). Embryonation occurs over a period of weeks in the soil (Spratt & Singleton 2008), however this can be accelerated if the unembryonated eggs have passed through the intestinal tract of a predator, scavenger or cannibal (Redrobe & Patterson-Kane 2005).

Time to embryonation is largely dependent on temperature, with development occurring more rapidly at higher temperatures (Spratt & Singleton 1986). Development can occur at temperatures up to 37°C, and eggs can survive exposure to temperatures as low as -15°C for as long as 60 days (Wright 1961). Temperatures on the Isle of May rarely reach these extremes (Berry *et al.* 1991), so eggs here are likely to embryonate and survive year-round.

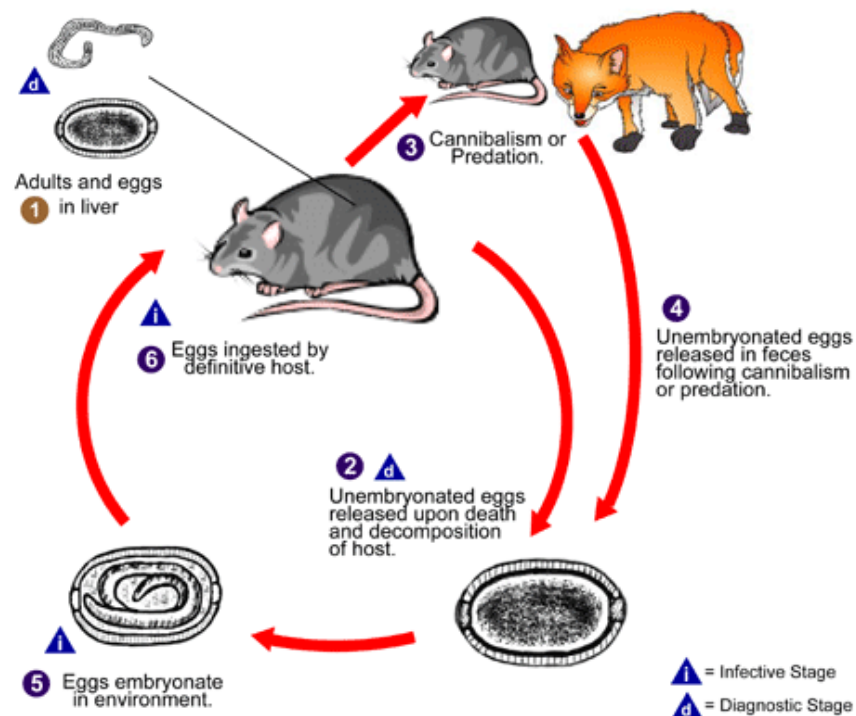


Figure 1.1: Life cycle of *Calodium hepaticum*. Figure modified from the Center for Disease Control and Prevention (www.cdc.gov; accessed November 2017).

In a single yellow-necked mouse (*Apodemus flavicollis*) from Switzerland, up to 938,000 eggs were reported (Reperant & Deplazes 2005). Egg antigens trigger a granulomatous response, and cause the eggs to be trapped in a fibrous reaction in the liver (Spratt & Singleton 1986; Sinniah *et al.* 2014) leading to hepatomegaly (Ceruti *et al.* 2001). Indeed, mice experimentally infected with high doses of *C. hepaticum* (1,000 eggs) all exhibited hepatomegaly and splenomegaly (Spratt & Singleton 1986). Infection by *C. hepaticum*, however, was not associated with variation in the innate immune response in wild wood mice (*Apodemus sylvaticus*), indicating a lack of immunomodulatory properties (Jackson *et al.* 2009). *C. hepaticum* infection can be fatal to mouse hosts (Luttermoser 1938) and has been identified as a potential tool for population control (Spratt & Singleton 1986).

1.4.2.3 Pinworm (*Syphacia obvelata*)

The Oxyurid nematode *Syphacia obvelata* is commonly found in the caecum of wild house mice from populations around the world (Tattersall *et al.* 1994; Table 1.1). Indeed, in some populations it is the only helminth species present (Pisanu *et al.* 2001).

S. obvelata has a direct life cycle, and is complete in as little as 12 days (Lewis & D'Silva 1986). Adult worms inhabit the caecum, where females mature in 11 to 15 days (Michels *et al.* 2006). Females migrate to the anus and deposit an average of 350 eggs in the perianal region (Chan 1952). The eggs embryonate within 24 hours, at which point they

become infective (Chan 1952). Infection occurs either by direct ingestion of embryonated eggs, ingestion of contaminated food or water, or by retroinfection (Chan 1952; Meade & Watson 2014), in which eggs cling to the fur, hatch and then enter the animal. While the eggs of other *Syphacia* species may survive in the environment for up to five weeks (Clifford & Watson 2008), *S. obvelata* eggs have been reported to survive for only 42 hours under ideal conditions (Chan 1952; Grice & Prociv 1993).

S. obvelata infection was initially thought to be non-pathogenic (Hoag 1961; Michels *et al.* 2006), but it has since been linked with mucoid enteritis, rectal prolapse and intestinal impaction (Hoag 1961; Michels *et al.* 2006). Further, McNair and Timmons (1977) noted decreased exploratory behaviour in laboratory mice infected with *S. obvelata*.

Infection with *S. obvelata* was shown to induce a Th2-type response in laboratory mice (Agersborg *et al.* 2001; Michels *et al.* 2006), with elevated levels of IL-4, IL-5 and IL-13 cytokine production (Michels *et al.* 2006). Further, *S. obvelata* infection is associated with elevated IgG1 levels (Agersborg *et al.* 2001; Michels *et al.* 2006), while a positive relationship between IgE and *Syphacia* sp. infection was observed in wild mice (Abolins *et al.* 2011).

Abundance of *S. obvelata* in the wild mouse population inhabiting the subantarctic Guillou Island was found to have no relationship with host population dynamics (Pisanu *et al.* 2002). This indicates that infection by *S. obvelata* in natural populations is modulated by intrinsic

factors, such as host immunity, rather than host abundance (Pisanu *et al.* 2002).

1.4.2.4 Whipworm (*Trichuris muris*)

Trichuris muris is a cosmopolitan (Table 1.1) gastrointestinal nematode of murine rodents (Feliu *et al.* 2000). It has a direct life cycle, and is transmitted by the ingestion of infective, embryonated eggs (Cliffe & Grencis 2004). Eggs accumulate and hatch in the caecum, and the larvae penetrate the mucosa of the large intestine. The worms reach adulthood in around 32 days, and the head of the worm remains buried in the mucosa while the body extends into the lumen (Panesar & Croll 1980). Following mating, the female releases eggs which are shed in the faeces. Over a period of around two months, the eggs embryonate and become infective (Cliffe & Grencis 2004).

In order to successfully establish, *T. muris* depends on the gut microbiota of the host modulating the immune response (Hayes *et al.* 2010). Indeed, whether an infection becomes acute or chronic depends largely on the nature of the adaptive immune response (Demiri *et al.* 2017). Chronic infections, in which worms are present 35 days post-infection, are associated with Th1 responses, while a Th2 response results in the clearance of infection prior to worms reaching fecundity (Bancroft *et al.* 1994; Cliffe & Grencis 2004; Demiri *et al.* 2017). The balance between response and outcome of infection is dependent upon host genetics, gender and infection dose (Hurst & Else 2013; Demiri *et*

al. 2017). For example, a high-dose infection in C57BL/6 mice stimulates a Th2 response and clearance, while a low-dose results in a Th1 response and chronic infection (Bancroft *et al.* 1994; Demiri *et al.* 2017). Indeed, Behnke and Wakelin (1973) found that *T. muris* was only able to reach sexual maturity in wild wood (*Apodemus sylvaticus*) and house mice when experimentally infected at single or low-doses. Further, naturally infected house mice showed a similar pattern of low worm burdens (Behnke & Wakelin 1973).k

1.4.2.5 Ectoparasites of the Isle of May house mice

A range of ectoparasites have been found on the Isle of May mice, including the northern rat flea (*Nosopsyllus fasciatus*), harvest mite (*Trombicula autumnalis*) and the fur mites *Myobia musculi*, *Myocoptes musculus* and *Radfordia affinis* (see Table 1.2 and Chapter 4). Southern (1938) noted the presence of the flour mite (*Acarus siro*) on mice during a 1936 survey, however this has not been observed since. This can be considered a ‘pseudoparasite’ (Clemmons & Taylor 2016) and was likely only present due to historic food storage practices, so is not discussed here.

1.4.2.6 Northern rat flea (*Nosopsyllus fasciatus*)

N. fasciatus is the most widely spread member of its genus (Wall & Shearer 1997), and is found in temperate regions around the world

(Wall & Shearer 1997; Durden 2009). While the primary host of *N. fasciatus* is the brown rat (*Rattus norvegicus*) (Durden 2009), it will parasitise other rodent species including wild house mice (Wall & Shearer 1997). *N. fasciatus* has been observed feeding on humans, and can act as a vector for several zoonotic pathogens (Wall & Shearer 1997; Durden 2009) including *Yersinia pestis* (the agent of plague), *Salmonella enteritidis* and murine typhus (Durden 2009). Further, *N. fasciatus* is a known intermediate host of the rat tapeworm (*Hymenolepis diminuta*) and the dwarf tapeworm (*Hymenolepis nana*) (Wall & Shearer 1997; Durden 2009).

Infestation by *N. fasciatus* has been shown to negatively influence the growth of common voles (*Microtus arvalis*), as well as inducing anaemia and depressing the host immune response (Devevey *et al.* 2008). Further, the mean life span of voles parasitised by *N. fasciatus* was decreased by 36% (Devevey & Christe 2009).

1.4.2.7 Harvest mite (*Trombicula autumnalis*)

The harvest mite (*Trombicula (Neotrombicula) autumnalis*) is the most widely spread trombiculid mite in central Europe and the British Isles (Schöler *et al.* 2006). Nymphal and adult stages inhabit soil and predate on other arthropods and their eggs (Minter 1957; Varma 1993; Schöler *et al.* 2006). They inhabit a range of habitats, and soil morphology, pH or moisture content appear to have no effect on distribution (Schöler *et al.* 2006). The larval stages (chiggers) parasitise

birds and mammals, including humans (Varma 1993; Schöler *et al.* 2006; Figure 1.2). Larvae grow up to 0.4 mm and feed for up to ten days, and are typically active from July to October (Garben *et al.* 1977; Schöler *et al.* 2006).

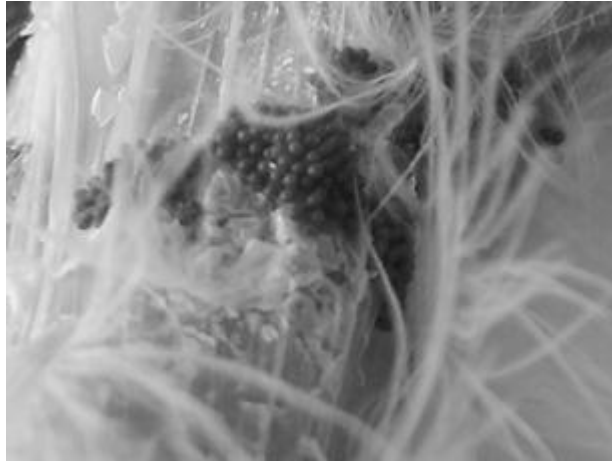


Figure 1.2: Larval colony of harvest mites (*Trombicula autumnalis*) on the leg of a little bittern (*Ixobrychus minutus*). Image taken from Cakiroglu *et al.* (2008).

Due to lytic enzymes in excreted saliva, infestation by larval *T. autumnalis* can lead to pruritic dermatitis (Varma 1993; Schöler *et al.* 2006; Cakiroglu *et al.* 2008). Heavy infestation in birds is associated with droopiness and a refusal to eat, and can lead to death from starvation and exhaustion (Literák *et al.* 2001; Cakiroglu *et al.* 2008). Further, *T. autumnalis* act as a vector for a number of pathogens, including *Anaplasma phagocytophilum*, *Ehrlichia phagocitophila* (Fernández-Soto *et al.* 2001) and *Borrelia* sp. (Kampen *et al.* 2004; Literák *et al.* 2008).

1.4.2.8 Fur mites (*Myobia musculi*, *Myocoptes musculus* and *Radfordia affinis*)

The Murine fur mites *Myobia musculi*, *Myocoptes musculus* and *Radfordia affinis* are non-invasive ectoparasites that frequently parasitise laboratory mice (Pate *et al.* 2011). Indeed, up to 40% of rodent facilities have reported the presence of these mites (Carty 2008).

Fur mites are typically transmitted through direct mouse-to-mouse contact (Pate *et al.* 2011), although it is possible for animals to become infected by mobile adults in the environment or through contact with shed fur containing eggs (Pritchett-Corning & Clifford 2012). Adult mites will infest neonates within four to five days of birth, and may live for up to nine days on dead hosts (Pritchett-Corning & Clifford 2012).

M. musculus can complete a life cycle of egg to reproductively mature adult in eight days (Watson 1961), while *M. musculi* can take up to 23 days (Pate *et al.* 2011). The life cycle of *R. affinis* is little known, but it is assumed to be similar to that of *M. musculi* (Baker 2006).

Infestation of laboratory mice by fur mites can lead to weight loss, pruritus, alopecia, ulcerative dermatitis and lymphadenopathy (Watson 1961; Whiteley & Horton 1965; Arbona *et al.* 2010). Indeed, infestation with *M. musculi* in C57BL/6 mice was associated with increased incidence of ulcerative dermatitis and a decrease in reproductive effort (Weisbroth *et al.* 1976). Further, skin lesions caused by fur mites can lead to secondary bacterial infections (Barthold *et al.* 2016).

Fur mite infestations have been shown to invoke a Th2 response in laboratory mice (Jungmann *et al.* 1996). This is associated with elevated levels of serum IgE (Laltoo *et al.* 1979; Morita *et al.* 1999) and IgG (Pochanke *et al.* 2006), as well as eosinophilia, lymphocytopenia and higher levels of inflammatory cytokines (Johnston *et al.* 2009). Further, infestation with *M. musculus* leads to increased susceptibility to other pathogens such as *Toxoplasma gondii* (Welter *et al.* 2007).

1.4.3 Immunology of wild house mice (*Mus musculus*)

The global ubiquity of house mice and their long established use as a model organism in laboratory studies (Guénet & Bonhomme 2003) makes them an obvious candidate for wild immunology research. Despite this, wild house mouse populations have typically been “immunologically understudied” (Abolins *et al.* 2017).

1.4.3.1 Immune response in wild mice

To date, the few studies that have directly addressed immune function in wild *M. musculus* have highlighted the differences in the immune response of wild and laboratory mice (Viney *et al.* 2015). By immunising with sheep red blood cells (SRBC), Lochmiller *et al.* (1991) were initially able to show that wild caught mice developed a significantly greater anti-SRBC antibody response than laboratory reared mice. Similarly, immunisation with keyhole limpet haemocyanin (KLH) showed that wild mice developed higher concentrations and avidity of anti-KLH

antibodies than the “immunologically high responding” C57BL/6 strain mice, as well as higher concentrations of total IgG and IgE (Abolins *et al.* 2011). Further, flow cytometry revealed that wild mice had a greater overall activation of splenic leucocytes (Abolins *et al.* 2011) as well as higher resting levels of natural killer cell activation (Boysen *et al.* 2011). Further, Beura *et al.* (2016) found that mice living in unsterile conditions, in this instance wild caught and pet store sourced mice, had higher levels of CD8⁺ T cells than laboratory mice reared in a sterile environment. In a recent comprehensive study, Abolins *et al.* (2017) found that the high levels of pathogen exposure faced by wild mice leads to the increased levels of activated immune cells. Additionally, in wild mice they identified a novel population of highly activated hypergranulocytic myeloid cells that are not found in laboratory mice (Abolins *et al.* 2017).

As well as highlighting the increased immune response in wild mice compared to laboratory strains, each of these studies also found the among-individual variation in immune measures to be greater in wild mice (Lochmiller *et al.* 1991; Abolins *et al.* 2011; Boysen *et al.* 2011; Beura *et al.* 2016; Abolins *et al.* 2017). Despite this increased variation, it is important to note that immunological reagents developed for use in laboratory mice consistently work successfully in wild mice (Abolins *et al.* 2017).

Many of these ‘wild’ mice were sourced from unnatural, anthropogenic environments such as farms (Abolins *et al.* 2011; Beura *et al.* 2016; Abolins *et al.* 2017), petting zoos (Beura *et al.* 2016) and the London Underground (Abolins *et al.* 2017). Some mice, however, were

sourced from natural, undisturbed populations such as those living in Oklahoma grasslands (Lochmiller *et al.* 1991) and the Welsh island of Skokholm (Abolins *et al.* 2017).

The gut microbiota plays a key role in the maturation and development of the host immune system (Hooper & Gordon 2001; Round & Mazmanian 2009; Cerf-Bensussan & Gaboriau-Routhiau 2010). Recent work has demonstrated that wild house mice and laboratory mice have significantly different gut microbial communities (Kreisinger *et al.* 2014; Wang *et al.* 2015; Rosshart *et al.* 2017), and that the diversity of the intestinal microbiota in wild mice varies geographically (Linnenbrink *et al.* 2013; Weldon *et al.* 2015), spatially along the gastrointestinal tract (Suzuki & Nachman 2016) and with diet (Wang *et al.* 2014), age and body condition (Weldon *et al.* 2015).

While viral infection is positively correlated with the alpha-diversity of intestinal microbiota, macroparasite infection is negatively correlated (Weldon *et al.* 2015). Further, wild *M. m. domesticus* and C57BL/6 mice inoculated with a wild-type microbiome had higher rates of survival than sterile laboratory mice when exposed to lethal doses of influenza and colorectal tumours (Rosshart *et al.* 2017). Those mice with a natural microbiota had a more balanced immune response, with increased levels of anti-inflammatory cytokines but decreased levels of pro-inflammatory cytokines (Rosshart *et al.* 2017). Further, hybrids between two subspecies of house mouse with different gut microbial communities (*M. m. musculus* and *M. m. domesticus*) had aberrant immune gene

expression leading to excessive inflammation and immunopathology (Wang *et al.* 2015).

1.5 Thesis outline

1.5.1 Chapter 2 – The study system: population ecology and morphometrics of Isle of May mice

In this chapter, I introduce the study system by providing an overview of the Isle of May and the ecology of its mice. I also introduce the methodologies for trapping and processing the mice used throughout this thesis. I then explore the demographics and morphology of the Isle of May mice, and how these vary seasonally. I also examine the relationships between morphometric measures and mouse age, sex and reproductive status. Finally, I provide estimates for the population size across several sessions and explore the temporal variation in population.

1.5.2 Chapter 3 – Mice are what they eat: investigating the diet of wild house mice using stable isotope analysis

Here, I use stable isotope analysis to identify the diet of the Isle of May mice. I explore the ecological drivers of this diet, as well as the interactions between diet and body condition.

1.5.3 Chapter 4 – Macroparasites of the Isle of May mice: seasonal variation and interactions with body condition

In this chapter, I identify the macroparasites infecting the Isle of May mice. I explore how this varies seasonally, as well as the interactions between parasite load and host body condition.

1.5.4 Chapter 5 – Relationships between immune gene expression and circulating cytokine levels in wild house mice

Here, I explore the relationships between circulating cytokine levels and the corresponding immune gene expression. Further, I examine the relationships between immune response types and tissue types.

1.5.5 Chapter 6 – Factors affecting immune response in wild house mice

Finally, I explore how various extrinsic and intrinsic factors affect the immune response of this wild house mouse population. I investigate the relationships between various measures of immune response and host age, sex, body condition and parasitology.

1.5.6 Chapter 7 – General discussion

Finally, I collate my findings across chapters, and discuss how the results from each chapter link together. I then discuss future avenues, and the next steps for this research.

Chapter 2

The study system: population ecology and morphometrics of the Isle of May mice

2.1 Introduction

2.1.1 The field site: the Isle of May

The Isle of May (56°11'11.6"N, 2°33'24.1"W) is located approximately 8 km off the south-east coast of Scotland in the Firth of Forth (Figure 2.1). The island is approximately 1.6 km long and 0.5 km wide with an area of around 57 ha, with two islets in the north (Rona and North Ness) and one in the south (South Ness), separated from the main island at high tide. The climate is temperate with an annual rainfall of 56 cm, mean temperatures of 4°C in January and 14°C in July (Berry *et al.* 1991) and summer averages of 80% humidity and 4 ms⁻¹ wind speed (Riddick *et al.* 2016).

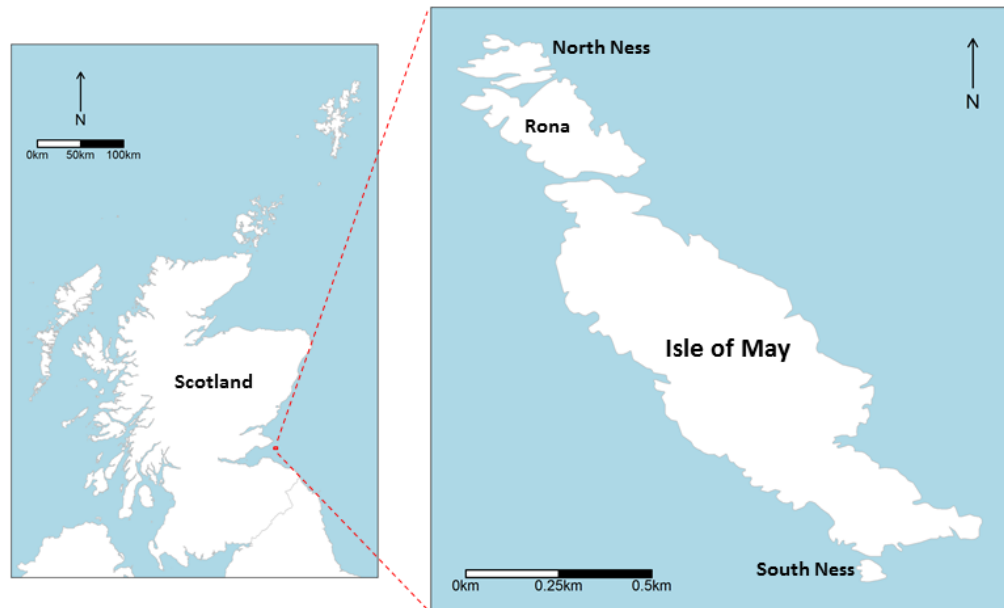


Figure 2.1: The Isle of May and its associated islets (Rona, North Ness and South Ness), and their location within Scotland. Maps were generated using the “Island Groups 2011” datafile provided by National Records of Scotland (www.nrscotland.gov.uk) and plotted using “ggmap” (Kahle & Wickham 2013).

Apart from a few stands of hazel (*Corylus avellana*), elder (*Sambucus nigra*) and sycamore (*Acer psuedoplatanus*) – planted to attract migratory birds – the island is largely treeless and is mostly covered by different types of bird-modified maritime grassland (Eggeling 1960; Sobey & Kenworthy 1979; Wright *et al.* 2010). Those areas affected by salt spray are typically covered by sea-spurrey (*Sparganium marina*), sea milkwort (*Lysimachia maritima*) and scurvy grass (*Cochlearia officinalis*) (Sobey & Kenworthy 1979). Those areas further from the shore are dominated by sea campion (*Silene uniflora*) and grasses such as Yorkshire fog (*Holcus lanatus*), red fescue (*Festuca rubra*), meadow grass (*Poa annua*) and bentgrass (*Agrostis* sp.) (Sobey & Kenworthy 1979; Wright *et al.* 2010). Sea thrift (*Armeria maritima*) and

numerous lichen species dominate the many areas of exposed rock (Eggeling 1960; Sobey & Kenworthy 1979). The enclosed gardens still present on the island contain large stands of nettles (*Urtica dioica*) and ragwort (*Jacobaea vulgaris*).

Archaeological evidence suggests that the Isle of May has been inhabited by people since the Bronze Age, possibly since as early as 2000 BCE (Rees 2003). The island likely had Celtic wooden churches as early as the 5th century (Willows 2015) and was used as a retreat by monks in the 6th or 7th century, with records showing that all of these monks were killed during a Viking raid in the late 9th century (Butler 1866). A Benedictine priory was founded in 1140 and occupied for 150 years (Duncan 1956; Eggeling 1960; James & Yeoman 2008; Willows 2017), with farming continuing for a further 200 years (Willows 2015). The Isle of May remained a popular pilgrimage destination throughout the medieval period (Duncan 1956; Eggeling 1960; Yeoman & James 1999; James & Yeoman 2008; Willows 2017). Between the 16th and 18th centuries there was a small village (Eggeling 1960), and lighthouse keepers occupied the island from the construction of the first lighthouse in 1636 until automation in 1988 (Berry *et al.* 1991). During both the First and Second World Wars the island was occupied by the Royal Navy (Eggeling 1960). Currently, the only human inhabitants on the island are the Scottish Natural Heritage (SNH) wardens and research staff from various institutions living in Fluke Street, as well as members of the birding community in the Low Light Bird Observatory, in varying numbers from March to December each year.

The Isle of May falls within the Forth Islands Special Protection Area (SPA) and is managed as a National Nature Reserve (NNR), Special Area of Conservation (SAC) and Site of Special Scientific Interest (SSSI) by SNH. It is an important nesting site for eleven species of seabird, forming one of four 'Key Site' colonies in the UK's Seabird Monitoring Programme (Mavor *et al.* 2008). The breeding seabird assemblage includes the Atlantic puffin (*Fratercula arctica*), kittiwake (*Rissa tridactyla*) and the European shag (*Phalacrocorax aristotelis*) – all on the Royal Society for the Protection of Birds (RSPB) 'Red list' due to severe declines in their UK breeding populations and range over the last 25 years, with puffins also listed as being vulnerable to extinction (IUCN 2017). The overall numbers of seabirds on the Isle of May have significantly increased since the start of the 20th Century, with the colony of herring gulls (*Larus argentatus*) and lesser black-backed gulls (*Larus fuscus*) increasing from a single pair in 1907 to 39,000 pairs in 1972 (Sobey 1976) and an increase in puffin numbers of 10.9% per annum between 1970 and 2003 (Harris *et al.* 2003). With around 40,000 pairs (Harris *et al.* 2009; Harris & Wanless 2013; Newell *et al.* 2017a), the Isle of May puffin colony is the third largest in the UK and of international importance.

The island is also home to one of the largest breeding colonies of grey seal (*Halichoerus grypus*) in the UK, with up to 4,000 adult animals present in the breeding season between September and January producing over 2,000 pups each year (Brownlow *et al.* 2016). Other than rabbits (*Oryctolagus cuniculus*) – which have been present on the island

since at least 1329 (Eggeling 1985) – the only other wild living terrestrial mammals are house mice (Southern 1938).

2.1.2 The study system: Isle of May mice

It is unknown exactly when mice were first introduced to the Isle of May, and this could have happened at any point during human inhabitation. The earliest record of mice on the island is in an undated letter from a resident lighthouse keeper, quoted by Muir (1885): “We have no rats, but legions of mice, and most impudent mice they are, for they sit and look at your face, and even gnaw at the legs of your trousers with a composure that is quite amusing”.

Mice are present on all parts of the island, including on the islets of Rona and North Ness, with most mice living in the rockier, more peripheral areas. The grassy central areas are occupied mainly when the population is at high density (Triggs 1991). Mice do live in and around human occupied buildings, but at densities no higher than undeveloped areas of the island (Triggs 1991; Gray & Hurst 1998).

Population estimates from trapping sessions in the 1970s suggest that the population fluctuates between a low of 450 in the spring up to 3250 at the end of the year, with a density of between 58 and 140 mice ha⁻¹ (Triggs 1991). Home ranges were found to be between 100 and 400 m², with a significant degree of overlap between individual territories (Triggs 1991; Beynon *et al.* 2002). The size of the home range is largely dependent on habitat – those mice living in more open areas typically

have larger territories than mice living in areas with more vegetation and cover (Triggs 1991).

Isle of May mice are noted for their lack of aggressive and defensive behaviour (Mackintosh 1981; Gray & Hurst 1998), a feature which can explain their ability to survive at such high densities and with overlapping territories. Gray and Hurst (1998) suggest that this lack of aggression is due to the infeasibility of defending large home ranges with limited resources, as well as the low predation risk on the island.

Kestrels (*Falco tinnunculus*) and short-eared owls (*Asio flammeus*) are common visitors to the island and will predate on mice (D. Steel and M. Newell, pers. comms). Mice may also be predated on by gulls (*Larus* sp.), but this occurs at low levels (Harris 1965). The Isle of May mice have a more positive relationship with the islands other burrowing species, however. Mice use the burrows of rabbits and puffins as runs and nesting sites, with nest chambers often leading directly from burrows (Triggs 1991; Gray & Hurst 1998). With around 45,000 breeding pairs of puffins (Harris *et al.* 2009; Newell *et al.* 2017a), burrows are extensive across the island.

Mice on the Isle of May show a seasonal breeding pattern, with incidences of pregnancy peaking in mid-summer and little reproduction occurring through the winter. The post-weaning life expectancy for May mice is around 150 days, with a maximum life span of around 18 months (Triggs 1991). As with most wild house mice, females live longer than males (Triggs 1991). Overwinter (October to April) survival ranges from 34% to 57%, with rates of reproduction and survival being lowest in

colder, wetter years (Triggs 1991). The probability of mice surviving the winter is dependent on numerous factors such as age, genotype and physiological and behavioural adaptations to cold weather. Some mice will enter a period of torpidity (Triggs 1977), and others show lowered heat production, reduced weight loss, increased amounts of brown fat and higher haematocrit and haemoglobin levels in response to lowered ambient temperatures (Triggs 1977; Jakobson 1978). The lack of aggressive behaviour displayed by Isle of May mice (Mackintosh 1981; Gray & Hurst 1998) is linked to their ability to 'huddle' together in winter; a further adaptation to cold and wet conditions (Triggs 1977).

Up to 17% of overwintered mice have been observed to move from the territory they occupied in the autumn to a new area on the island (Triggs 1991). This, combined with the mosaic of overlapping territories (Triggs 1991; Beynon *et al.* 2002), means that there is considerable genetic 'churning', such that the population can be considered a panmictic unit (Triggs 1991).

2.1.3 Isle of May mice: population genetics

In April 1982, 77 mice were introduced to the Isle of May from the Orkney island of Eday (59°11'1.7"N, 2°46'15.6"W) (Berry *et al.* 1990). The release of these 42 male and 35 female mice to the southern end of the island was conducted with the intention of investigating hybridisation and gene flow (Berry *et al.* 1990; Berry *et al.* 1991). Mice on the Isle of May prior to this introduction had the common European karyotype ($2n = 40$) and were effectively homozygous with little genetic variation at over

70 loci (Berry & Peters 1977; Berry *et al.* 1991), including some loci with no variation at all (Figueroa *et al.* 1986). Conversely, Eday mice have a karyotype with three pairs of Robertsonian translocations ($2n = 34$) (Scriven 1992) and are highly heterozygous at many allozyme loci (Berry 1981). Eday mice represent a unique 'Orkney' lineage of *M. musculus domesticus* (Searle *et al.* 2009), characterised by having *M. m. domesticus* mitochondrial DNA but the nuclear DNA of *M. musculus musculus* (Prager *et al.* 1993).

Hybrids between native and introduced animals were found across all parts of the island six months after the introduction (Berry *et al.* 1991), and three years after the introduction the introduced alleles and Robertsonian chromosomes had reached equilibrium (Figueroa *et al.* 1986; Scriven 1992; Jones *et al.* 1995). The hybrid mice were significantly larger than the pre-introduction Isle of May mice or Eday mice and had mandibles of intermediate shape (Scriven & Bauchau 1992). There is also evidence of sexual selection for the introduced Eday genes, either by female mate choice or male competitive behaviour – Y-chromosome linked genes had spread more quickly and extensively through the population than any matrilineally inherited genes (Berry *et al.* 1990; Jones *et al.* 1995). Indeed, other island populations of house mice appear more susceptible to male introgression (Jones & Searle 2015).

2.1.4 Aims and objectives

Here, I detail the fieldwork and trapping regime employed for sampling the Isle of May mice. I provide a summary of the dataset

generated by this sampling – a dataset that will be utilised throughout this thesis. I use this dataset to explore seasonal trends in mouse size and age, and the relationships between these morphometric measures. Finally, I provide updated population estimates using data from capture-recapture experiments.

2.2 Methods

2.2.1 Trapping regime

Fieldwork was undertaken from 2014 to 2016, with trapping sessions in August 2014, then April, July and October in 2015 and 2016. Traps were placed in up to 11 locations across the island (Figure 2.2). Trapping locations were determined following the advice of SNH staff and were selected to maximise the coverage of the island while avoiding disturbance to breeding birds. In April 2015, traps were also placed on the islet of Rona, to the north of the Isle of May. Rona is separated from the main island at high tide but is accessible over rocks at low tide. This site, however, was abandoned for further trapping due to health and safety concerns regarding access. The July 2016 session was focussed solely on marking animals for capture-recapture analysis (detailed in section 2.2.5), and no destructive sampling occurred.

At each location, 15 – 20 traps were placed in pairs, approximately every two metres along a transect. Longworth traps (Longworth Scientific Instrument Co.) were primarily used, along with small numbers of Ugglan

(Granhab) and homemade 'Jordan' traps (Perrow & Jowitt 1995). The location of each pair of traps was recorded using GPS.

All traps were baited with a commercially available wild bird seed mix, and hay was provided as insulation. Traps were set in the late afternoon (at approximately 16.00) and checked the following morning (at approximately 08.00). Any triggered traps were taken back to be processed in the laboratory based in the island's main building complex. Traps were checked again in the afternoon (at approximately 13.00), and triggered traps were again taken for processing.

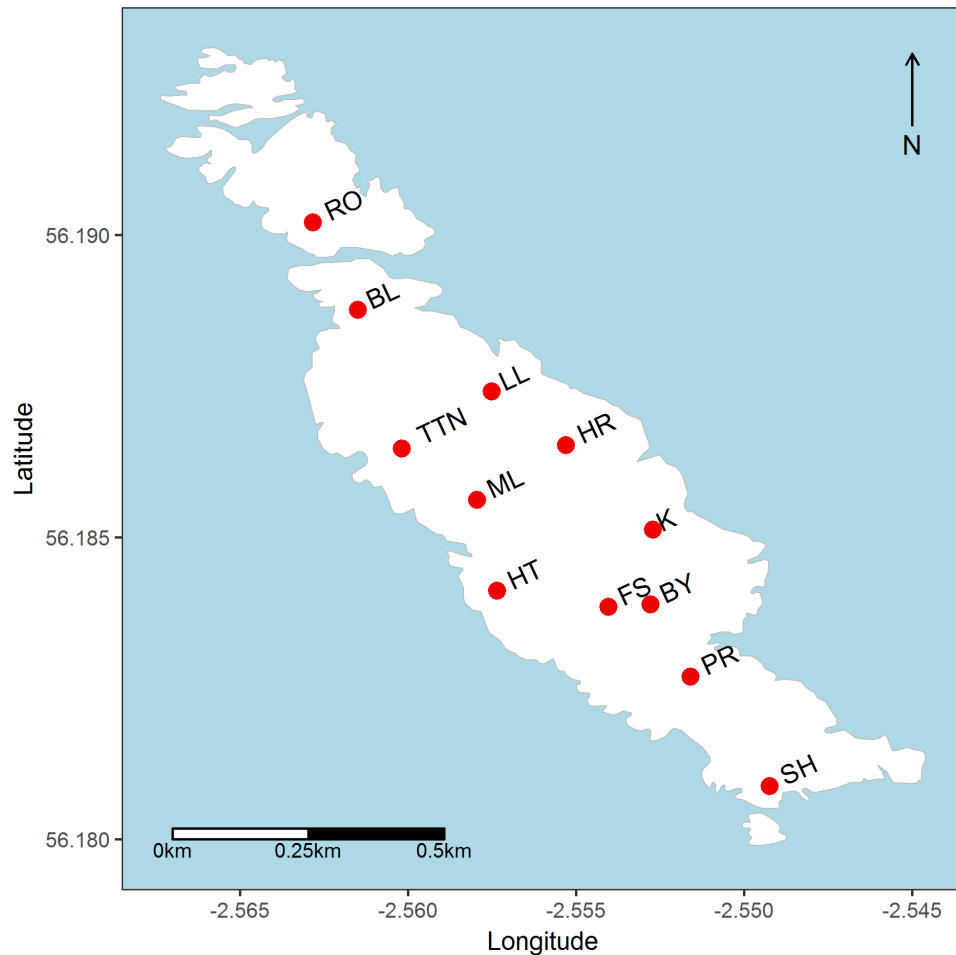


Figure 2.2: Approximate trapping locations (●) on the Isle of May. RO = Rona; BL = Burnett's Leap; LL = Low Light; TTN = Three Tarn Nick; ML = Main Light; HR =

Holyman's Road; K = Kettle; HT = High Tarn; FS = Fluke Street; BY = Byres; PR = Priory ruins; SH = South Horn. Note that Rona was used only in April 2015 and was subsequently abandoned due to health and safety concerns. Map generated using the "Island Groups 2011" datafile provided by National Records of Scotland (www.nrscotland.gov.uk) and plotted using "ggmap" (Kahle & Wickham 2013).

2.2.2 Culling and dissection

Mice were euthanized in a CO₂ chamber with a rising gradient of CO₂. The eye and foot reflexes were tested, and death was confirmed through exsanguination by cardiac puncture. All procedures were conducted in accordance with the UK Home Office code of practice on the humane killing of animals, according to Schedule 1 of the Animals (Scientific Procedures) Act 1986. The total body mass (g) and snout-vent length (SVL, mm) of each individual were recorded. Further dissection and collection of specific samples and data is detailed in the relevant chapters.

2.2.3 Body condition (BMI)

As a measure of individual condition, the body mass index (BMI) was calculated using the following formula:

$$\text{BMI} = \left(\frac{\text{Body mass (g)}}{\text{SVL (cm)}^2} \right) \times 100$$

BMI is positively correlated with body fat in humans (Maffei *et al.* 1995) as well as laboratory rodents (Maffei *et al.* 1995; Sjögren *et al.* 2001), and has been used as an indicator of individual body condition in

wild animals (Graves *et al.* 2012), including wild rodents (Schwensow *et al.* 2011; Winternitz *et al.* 2012).

Scaled Mass Index (SMI) is a measure of relative body mass scaled against a curve of the population as a whole, which can be used as a proxy for body condition (Peig & Green 2009). In these mice, SMI significantly positively correlated with BMI ($r_s=0.921$, $p<0.001$), so BMI is used throughout.

2.2.4 Mouse age

The eyes were removed and fixed in 10% formalin. The lenses were then removed from each eye, and dried at 60°C for 48 hours or until they showed no further weight loss (Rowe *et al.* 1985). The final dry weights of the paired lenses (g) were then used to estimate mouse age in days, using the following equations (Rowe *et al.* 1985):

$$\text{Males: } \log_{10}(\text{age} + 20 \text{ days}) = 1.019 + 0.175 (\text{paired lens weight (g)})$$

$$\text{Females: } \log_{10}(\text{age} + 20 \text{ days}) = 1.004 + 0.182 (\text{paired lens weight (g)})$$

2.2.5 Reproductive status

Following Jackson *et al.* (2011), each destructively sampled individual was visually sexed and then assigned a reproductive status based on its life history stage category. If males had undescended testes and were non-scrotal, they were classified as 'non-reproductive'. Scrotal

males with large testes and seminal vesicles were classified as 'reproductive'. Live-caught males used in capture-mark-recapture experiments (see Section 2.2.5) were similarly visually assessed; the presence or absence of descended testes was used to determine whether a male was 'scrotal' or 'non-scrotal' (Jackson *et al.* 2014).

Females that were non-lactating, non-pregnant or had an imperforate vagina were classified as 'non-reproductive'. Lactating females and those with a perforate vagina were classified as 'reproductive' (Jackson *et al.* 2011; Jackson *et al.* 2014). A large degree of abdominal distension indicated advanced pregnancy, and the development of the nipples or the absence of fur around the nipples indicated whether an individual was lactating or nursing (Jackson *et al.* 2014). Where possible when processing animals, females that were visibly pregnant or lactating were tagged and released in an effort to minimise any long-term population impacts. However, a small number ($n = 28$) of pregnant females were processed; these were categorised as 'reproductive'.

2.2.6 Capture-mark-recapture

Live trapping and tagging of animals for capture-recapture analysis took place in the 2015 and 2016 field seasons. The trapping regime was as described previously (see section 2.2.1).

Following first capture, each animal was marked. In the 2015 field season, animals were marked using individually numbered small animal

ear tags (VetTech Solutions Ltd.). In 2016, animals were marked with Passive Integrated Transponder (PIT) tags (AVID FriendChip Mini; 8 mm long and 0.03 g in weight), injected subcutaneously. To enable easy identification of previously marked animals, and to allow detection of failed or missing PIT tags, an ear punch was also taken from the left ear of each individual.

On first capture within a trapping session, each individual was weighed using a spring balance and its body length was recorded. The sex and life history stage were determined as described in Section 2.2.4 (Jackson *et al.* 2011; Jackson *et al.* 2014).

2.2.7 Data analysis

Data from live capture sessions were analysed using spatially explicit capture-recapture models (SECRs) in the package “secr” (Efford 2016). SECRs were used to calculate recapture probability (g_0), the spatial scale of the detection function (σ) and to estimate population size and survival between sessions (ϕ).

Differences in age, body length, mass and condition between seasons were analysed by one-way ANOVA, using the “aov()” function, followed by a post-hoc Tukey’s HSD test, using the “TukeyHSD()” function. All data analysis was conducted in R version 3.4.0 (R Core Team 2017).

2.3 Results

2.3.1 Dataset

Across all field sessions described here (2014 to 2016), data was obtained from a total of 315 destructively sampled mice (Table 2.1). This includes 54 from summer 2014, 49 from spring 2015, 16 from summer 2015, 100 from autumn 2015, 42 from spring 2016 and 54 from autumn 2016. Details and samples from an additional 51 mice trapped in August 2013 were also available (Robertson 2014).

2.3.2 Mouse age

The mean age of sampled mice ranged from 50.16 days in summer 2014 to 168.90 days in spring 2015 (Table 2.2). There were significant differences in the mean age of mice between sessions ($F_{6,359}=42.09$, $p<0.001$). Post-hoc analysis revealed that the mean age of mice in spring 2015 and spring 2016 were significantly higher than in all other sessions ($p<0.001$). There was no significant difference between the age of mice in spring 2015 or spring 2016 ($p=0.076$). There were no significant differences between the ages of mice in any other sessions ($p>0.326$).

Table 2.1: Numbers of destructively sampled house mice (*Mus musculus domesticus*) used in this study. Mice were trapped on the Isle of May, Scotland, across several sessions. Showing total numbers of mice, as well as numbers by sex and reproductive status. Reproductive status was assessed visually following Jackson *et al.* (2011).

	Summer 2013	Summer 2014	Spring 2015	Summer 2015	Autumn 2015	Spring 2016	Autumn 2016	Total
Total mice	51	54	49	16	100	42	54	366
Male	25	31	41	12	73	27	33	242
Female	26	23	8	4	27	15	21	124
Reproductive	41	28	49	7	55	29	29	238
Non-reproductive	10	26	0	9	45	13	25	128

Table 2.2: Estimated ages of destructively sampled house mice (*Mus musculus domesticus*) used in this study. Mice were trapped on the Isle of May, Scotland, across several sessions. Ages in days were estimated using dry eye lens weights Rowe *et al.* (1985).

	Summer 2013	Summer 2014	Spring 2015	Summer 2015	Autumn 2015	Spring 2016	Autumn 2016	Overall mean
Minimum age (days)	17.00	10.01	83.87	7.48	19.49	64.92	17.00	31.40
Mean age (days)	69.93	50.16	168.90	53.36	71.91	137.41	66.33	88.29
Maximum age (days)	288.32	276.14	276.14	123.38	335.63	268.40	293.62	265.95

2.3.3 Sex

The numbers of male and female mice processed in each field season are summarised in Table 2.1. The percentage of males processed ranged from 49.0% in summer 2013 to 83.7% in spring 2015 (Figure 2.3). The proportion of males was significantly greater in spring 2015 (83.7%) than summer 2013 (49.0%; $p=0.007$), summer 2014 (57.4%; $p=0.035$), spring 2016 (64.3%; $p<0.001$) or autumn 2016 (61.1%; $p=0.015$). The proportion of males was significantly lower in summer 2013 (49.0%) compared to summer 2015 (75.0%; $p=0.002$) and autumn 2015 (73.0%; $p=0.035$). There was no significant difference in the proportion of males between any other sessions ($p>0.081$). The percentage of females varied from 16.3% in spring 2015 to 51.0% in summer 2013 (Figure 2.3). The summer 2013 session was the only one in which more females than males were processed.

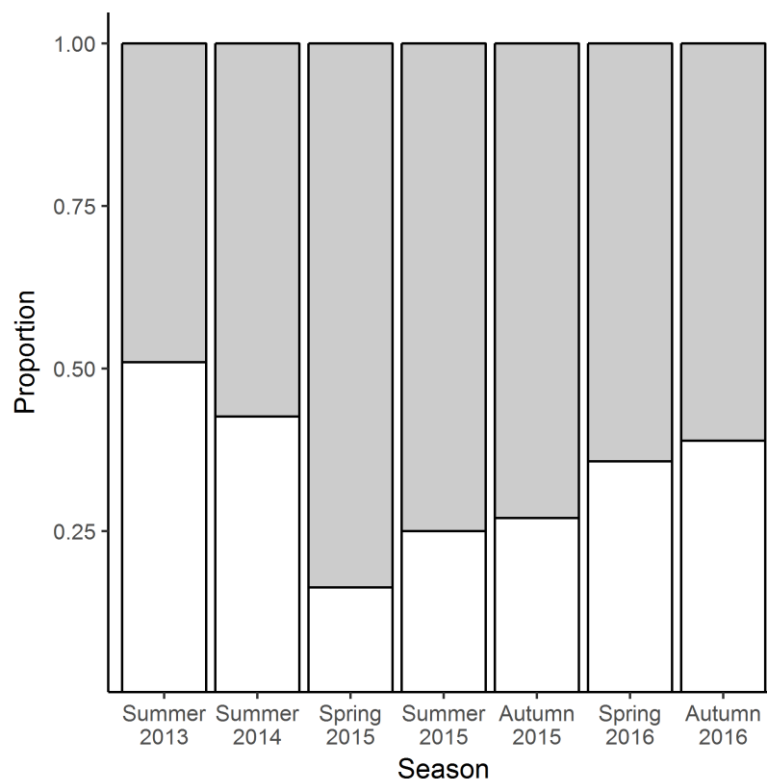


Figure 2.3: Seasonal variation in the proportions of male and female mice (*Mus musculus domesticus*) processed on the Isle of May, Scotland. Grey (■) represents the proportion of males, white (□) is the proportion of females.

2.3.4 Reproductive status

The proportion of reproductively active animals processed varied from 43.8% in summer 2015 to 100% in spring 2015 (Table 2.1) – indeed, the proportion of reproductively active animals was significantly greater in spring 2015 than any other session ($p < 0.001$). Further, the proportion of reproductively active animals was higher in summer 2013 (80.4%) than in summer 2014 (51.9%; $p = 0.010$), summer 2015 (43.8%; $p = 0.019$), autumn 2015 (55.0%; $p = 0.007$) and autumn 2016 (53.7%; $p = 0.010$).

There was no significant difference in the proportion of reproductively active animals between other sessions ($p>0.189$). Summer 2015 was the only session in which the majority of processed animals were non-reproductive (Figure 2.4).

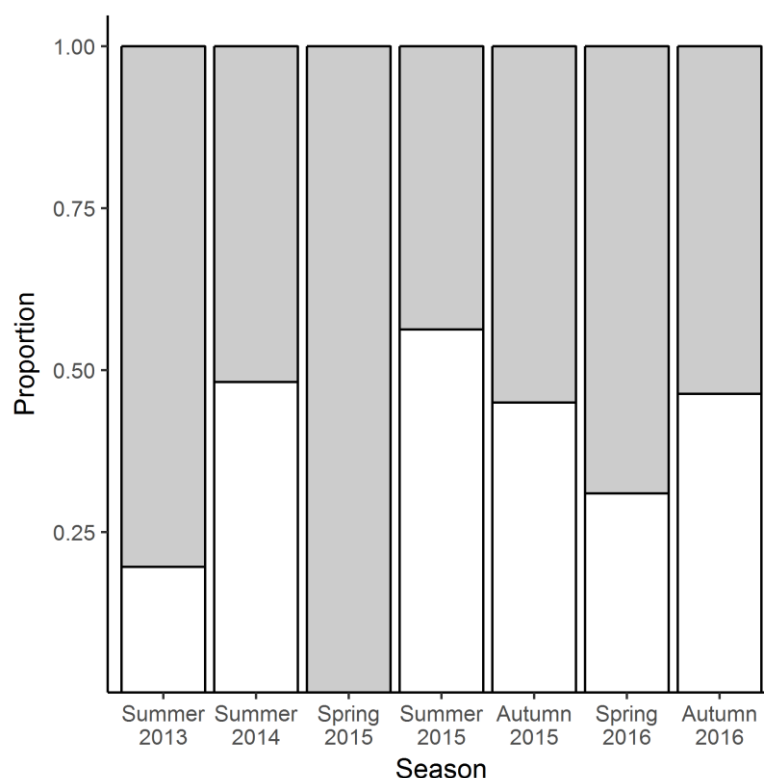


Figure 2.4: Seasonal variation in the proportions of reproductively active mice (*Mus musculus domesticus*) processed on the Isle of May, Scotland. Grey (■) represents the proportion of reproductively active individuals, white (□) is the proportion of non-reproductive individuals.

Pregnant females were present in all field sessions apart from autumn 2016. These, however, were present at low numbers ($n=10$ in summer 2013, $n=7$ in summer 2014, $n=5$ in autumn 2015, $n=4$ in spring 2015, $n=1$ in summer 2015 and $n=1$ in spring 2016). It is important to

note, however, that visibly pregnant females were not culled to prevent any long-term population impacts.

2.3.5 Body size and condition

2.3.5.1 Relationships between body size and life history

Body length ($r=0.585$), total body mass ($r=0.704$) and BMI ($r=0.607$) were all significantly ($p<0.001$) positively correlated with mouse age. Indeed, body length ($t_{231.53}=12.717$, $p<0.001$), total body mass ($t_{275.9}=14.533$, $p<0.001$) and BMI ($t_{268.22}=9.063$, $p<0.001$) are all significantly higher in reproductively active mice compared to immature, non-reproductive mice (Table 2.3; Figure 2.5).

In mature, reproductively active individuals, there were no significant differences in body length ($t_{77.986}=0.732$, $p=0.467$), total body mass ($t_{79.096}=-0.087$, $p=0.931$) or BMI ($t_{85.502}=-1.476$, $p=0.144$) between sexes. Further, there were no significant differences between sexes in body length ($t_{121.94}=0.550$, $p=0.583$), total body mass ($t_{125.53}=0.338$, $p=0.736$) or BMI ($t_{113.76}=0.009$, $p=0.993$) of immature, non-reproductively active individuals.

Within reproductively active females, there were no significant differences in total body mass ($t_{58.926}=-2.484$, $p=0.059$), body length ($t_{65.86}=-1.558$, $p=0.124$) and BMI ($F_{52.01}=-2.276$, $p=0.072$) between pregnant or non-pregnant individuals.

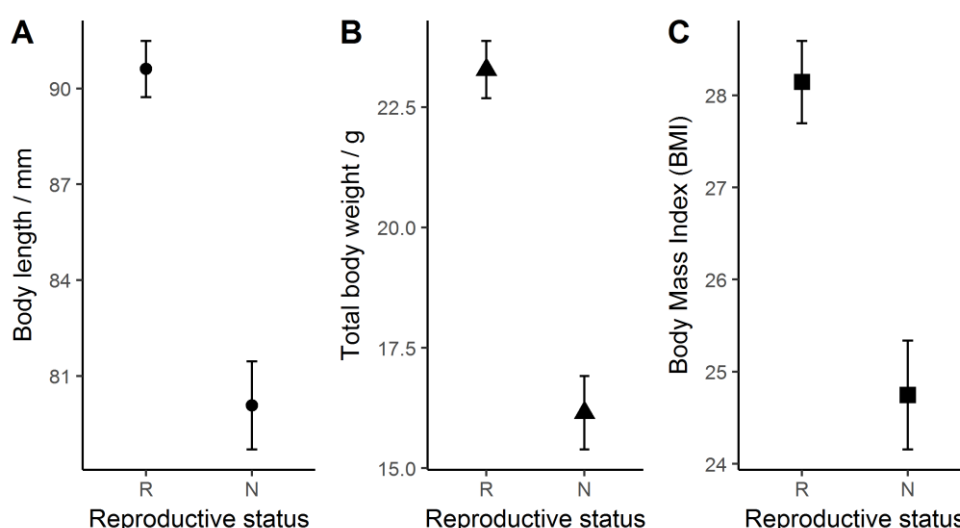


Figure 2.5: Mean body length (A), total body mass (B) and BMI (C) in wild *Mus musculus domesticus* from the Isle of May, Scotland. Shown here is the variation in size between reproductively active (R) and non-reproductively active (N) individuals. Bars represent $\pm$ Standard Error.

2.3.5.2 Seasonal variation in body length

There were significant differences in body length between sessions ($F_{6, 359}=10.12$, $p<0.001$; Table 2.4; Figure 2.6). Post-hoc analysis revealed that mice trapped in spring 2015 were significantly larger than those trapped in summer 2014 ($p<0.001$), summer 2015 ($p=0.002$), autumn 2015 ($p<0.001$), spring 2016 ($p<0.001$) and autumn 2016 ($p<0.001$). Mice trapped in summer 2013 were significantly smaller than those trapped in summer 2014 ($p=0.051$), autumn 2015 ($p=0.011$) and spring 2016 ($p<0.001$). The size difference between mice caught in summer 2013 and summer 2015 was marginally insignificant ($p=0.064$). There was no significant difference in size between any other sessions ($p>0.491$).

2.3.5.3 Seasonal variation in total body mass

There were significant differences in total body mass between sessions ($F_{6, 359}=11.70$, $p<0.001$, Figure 2.6). Mice trapped in spring 2015 were significantly larger than those trapped in summer 2013 ($p<0.001$), summer 2014 ($p<0.001$), summer 2015 ($p<0.001$), autumn 2015 ($p<0.001$), spring 2016 ($p<0.001$) and autumn 2016 ($p<0.001$). There were no significant differences in mass between any other sessions ($p>0.114$).

2.3.5.4 Seasonal variation in Body Mass Index (BMI)

There were significant differences in BMI between sessions ($F_{6,359}=18.24$, $p<0.001$, Figure 2.6). Mice trapped in spring 2015 had a significantly higher BMI than those trapped in summer 2013 ($p<0.001$), summer 2014 ($p<0.001$), summer 2015 ($p<0.001$), autumn 2015 ($p<0.001$) and autumn 2016 ($p<0.001$). Mice trapped in spring 2016 had a significantly higher BMI than those trapped in summer 2013 ($p<0.001$), summer 2014 ($p=0.009$) and summer 2015 ($p=0.053$). Those trapped in autumn 2015 had a significantly higher BMI than those trapped in summer 2013 ($p=0.001$) and autumn 2016 ($p<0.001$). There were no significant differences in BMI between any other sessions ($p>0.206$).

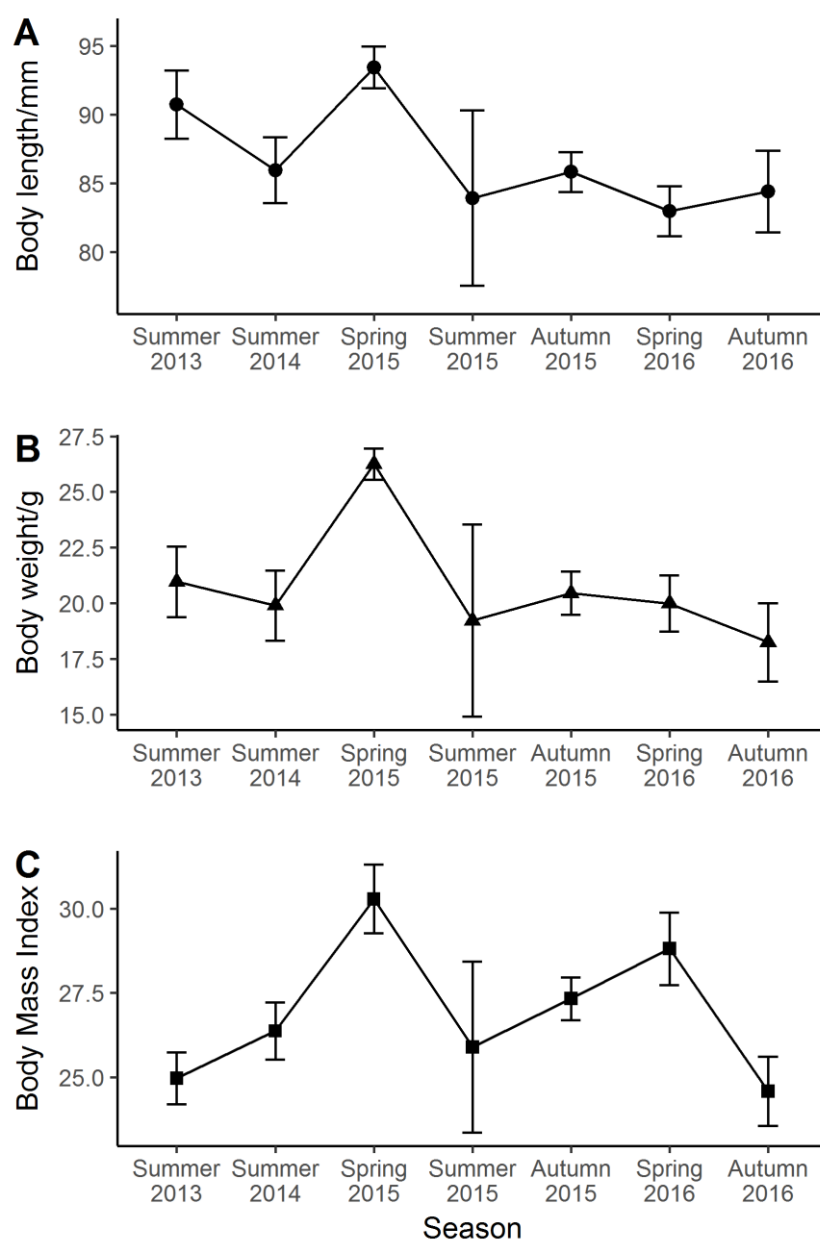


Figure 2.6: Seasonal variation in the mean body length (A), body mass (B) and body mass index (C) of wild house mice (*Mus musculus domesticus*) from the Isle of May, Scotland. Bars represent $\pm$ Standard Error.

Table 2.3: Variation in body size between mature, reproductively active and immature, non-reproductive wild mice (*Mus musculus domesticus*) from the Isle of May, Scotland. Shown here is mean ($\pm$ SE) body length (mm), mass (g) and Body Mass Index (BMI). Reproductive status was assessed visually following Jackson *et al.* (2011).

	Reproductive	Non-reproductive
Mean body length/mm	90.61 ($\pm$ 0.45)	80.08 ($\pm$ 0.70)
Mean total body mass/g	23.28 ($\pm$ 0.30)	16.15 ($\pm$ 0.39)
Mean BMI	28.14 ($\pm$ 0.23)	24.75 ($\pm$ 0.30)

Table 2.4: Seasonal variation in body size of wild mice (*Mus musculus domesticus*) from the Isle of May, Scotland. Shown here is mean ($\pm$ SE) body length (mm), mass (g) and Body Mass Index (BMI).

	Summer 2013	Summer 2014	Spring 2015	Summer 2015	Autumn 2015	Spring 2016	Autumn 2016
Mean body length/mm	90.72 ($\pm$ 1.24)	85.96 ($\pm$ 1.20)	93.42 ($\pm$ 0.75)	83.94 ($\pm$ 2.98)	85.83 ($\pm$ 0.73)	82.98 ($\pm$ 0.89)	84.49 ($\pm$ 1.50)
Mean total body mass/g	20.97 ($\pm$ 0.79)	19.90 ($\pm$ 0.79)	26.26 ($\pm$ 0.35)	19.22 ($\pm$ 2.03)	20.45 ($\pm$ 0.49)	19.99 ($\pm$ 0.62)	18.34 ($\pm$ 0.89)
Mean BMI	24.97 ($\pm$ 0.38)	26.37 ($\pm$ 0.42)	30.28 ($\pm$ 0.51)	25.89 ($\pm$ 1.19)	27.33 ($\pm$ 0.32)	28.81 ($\pm$ 0.53)	24.65 ($\pm$ 0.52)

2.3.6 Capture-recapture

Estimates of population size obtained from SECR models ranged from 459 in April 2016 to 2269 in October 2015 (Table 2.5). Across the 2015 and 2016 field sessions, population estimates were lowest in spring and highest in autumn (Table 2.5).

Table 2.5: Results of spatially explicit capture-recapture (SECR) models for house mice (*Mus musculus domesticus*) on the Isle of May. Shown here are the trapping session, number of nights of trapping, total number of individual mice (total captures), probability of detection (g_0), spatial scale of detection function (σ) and population estimate (N).

Session	Nights	Mice (n captures)	g_0	σ (m)	N
April 2015	7	11 (30)	0.06	38.64	493.27
July 2015	7	34 (61)	0.08	13.52	716.89
October 2015	4	143 (180)	0.08	15.47	2268.81
April 2016	5	56 (111)	0.08	36.42	458.72
July 2016	4	45 (66)	0.09	11.61	795.20
October 2016	4	178 (254)	0.07	13.35	1992.75

Estimates of survival were high from spring to summer (71% in 2015 and 66% in 2016), and lower from summer to autumn (44% in 2015 and 42% in 2016). Overwinter (October 2015 to April 2016) survival was estimated to be 31% (Table 2.6).

All overwintered animals (those recaptured in April 2016 that had previously been marked in 2015) were mature, scrotal males, with a mean total body mass of 23.5 g and a mean body length of 77.75 mm.

Table 2.6: Results of spatially explicit capture-recapture (SECR) models for house mice (*Mus musculus domesticus*) on the Isle of May. Shown here is the probability of an individual surviving (ϕ) between field sessions.

Session	Survival probability (ϕ)
April 2015 – July 2015	0.71
July 2015 – October 2015	0.44
October 2016 – April 2016	0.31
April 2016 – July 2016	0.66
July 2016 – October 2016	0.42

2.4 Discussion

During this study, the estimated population of Isle of May mice showed a four to five-fold variation in size, from a low in spring to a peak at the end of the year. Triggs (1991) observed a similar seasonal pattern in the Isle of May population, however the degree of variation was greater (seven-fold). Similar seasonal variation has been observed in the house mice populations on Skokholm (Berry 1968) and Marion Island (Matthewson 1994), but again the degree of variation was much higher (12 and 13-fold, respectively). Comparable to the present study, house mice on Gough Island in the South Atlantic also show a four to five-fold change in population size across seasons (Cuthbert *et al.* 2016).

While the winter peak in population size observed here matches previous observations of mice on Skokholm (Berry 1968) and the Isle of May (Triggs 1991), Triggs (1991) found the timing of the peak to fall in January/February. On Skokholm, however, the peak occurred in

October/November (Berry 1968). During this study, however, trapping did not occur between October and April due to access issues, so a detailed estimation of the timing of the peak cannot be made.

To estimate population sizes in this study, a variety of small mammal traps were used. While Jacob *et al.* (2002) found that Ugglan traps were significantly less effective than Longworth traps at capturing house mice in Australian grain fields, Lambin and MacKinnon (1997) and Jung (2016) found no difference in the efficacy of the two trap types when capturing field voles (*Microtus agrestis*) or deer mice (*Peromyscus* spp.), respectively. Further, Kettel *et al.* (2016) found that Longworth traps were significantly more successful at capturing harvest mice (*Micromys minutus*) than homemade traps, however this could be due to the small size of the harvest mice (average adult mass of 6-7 g). House mice are significantly larger (average mass in this study of 21 g), so here the lack of sensitivity is less of an issue. The most frequently used in this study was the Longworth trap, so any differences in trapping efficiency should have a negligible impact.

Survival estimates here were lowest overwinter (October 2015 to April 2016). The 31% rate of survival observed here is similar to the previous estimates of 34% to 57% for Isle of May mice (Triggs 1991). Indeed, high mortality in winter is an important factor in regulating island populations of house mice (Berry 1968; Triggs 1991; Ferreira *et al.* 2006; Cuthbert *et al.* 2016).

All recaptured overwintered animals were large, mature males. Indeed, mice captured in spring 2015 were significantly larger than in any other season, highlighting the importance of body size in the winter survival of these mice. A similar pattern has been observed in a range of temperate and polar small mammals, with body mass positively correlating with probability of survival (Murie & Boag 1984; Pucek *et al.* 1993; Karels *et al.* 2000; Korslund & Steen 2006; Schorr *et al.* 2009; Cuthbert *et al.* 2016).

While the temperatures and rainfall experienced in the winter of 2014/2015 were close to average (Met Office 2016), the winter of 2015/2016 was unusually warm, with mean temperatures in December 4.1°C above average (Met Office 2016). These milder temperatures could have enabled smaller individuals to survive, explaining the lower body sizes present in spring 2016 compared to spring 2015.

This dataset presents a unique opportunity for ecoimmunological studies. These mice are non-commensal and readily trapped, and inhabit an island with limited effects of immigration, predation or pest control (Triggs 1991). Further, they represent a wild population of a model species, allowing us to answer complex ecoimmunological questions using readily available reagents (Viney *et al.* 2015). Indeed, the hybrid population of the Isle of May could make the ideal wild analogue to laboratory-reared mice. The ancestral Isle of May mice likely represented Western European *M. musculus domesticus* (Suzuki *et al.* 2013), while

the introduced Eday mice had *M. m. domesticus* mitochondrial DNA but the nuclear DNA of *M. musculus musculus* (Prager *et al.* 1993). Male linked genes – exclusively nuclear – spread rapidly and extensively through the population following introduction (Berry *et al.* 1991; Jones *et al.* 1995). Therefore, the resulting hybrids likely resemble the genome of the laboratory mouse – a mosaic of the *M. musculus musculus* and the *M. musculus domesticus* genomes (Wade *et al.* 2002).

The dataset presented here consists of individuals of varying age, size, sex and reproductive status, allowing us to address a range of intrinsic drivers of variation in the immune response. In addition to this, the data spans numerous sessions over several years and is spread across the island, allowing us to investigate the role of these extrinsic factors.

Chapter 3

Mice are what they eat: Investigating the diet of wild *Mus musculus domesticus* using stable isotope analysis

3.1 Introduction

Wild house mice (*Mus musculus*) are largely omnivorous and are able to survive on a variety of diets (Singleton & Krebs 2007), however they were long assumed to feed primarily on plant material (Anderson & Condry 1974; Berry *et al.* 1978a). Indeed, stomach content analyses showed that mice inhabiting cereal farms in south-eastern Australia were mainly granivorous (Bomford 1987). Additional stomach content analyses of mice on Australian cereal farms revealed that while these mice do show a preference for wheat grain, they will eat invertebrates when grain is unavailable or of poor nutritional content (Tann *et al.* 1991). Further, while plant material was shown to be the most important food source for mice in agricultural land in Indiana, USA, invertebrate sources comprised up to 24.2% of the diet (Whitaker 1966; Houtcooper 1978).

Wild mice living in uncultivated areas may show a greater preference for animal-based food sources (Bomford 1987). Indeed,

stomach content analyses have revealed that invertebrate prey are the main food source for mice living in coastal heathland in Australia (Cockburn 1980), broadleaf-podocarp and beech forests (Fitzgerald *et al.* 1996) and sand dune habitats (Miller & Webb 2001) in New Zealand and the mesic forests of Hawaii (Shiels *et al.* 2013).

This preference for terrestrial invertebrates has also been observed on several subantarctic islands including Antipodes (Marris 2000), Guillou (Le Roux *et al.* 2002), Macquarie (Copson 1986) and Marion (Gleeson & Van Rensburg 1982). Indeed, predation by mice has led to the extinction of the flightless moth *Pringleophaga kerguelensis* on Marion Island (Vári 1971) and the local extinction and decline in abundance of several invertebrate species on Antipodes Island (Marris 2000).

The relative importance of different food sources can vary seasonally (Angel *et al.* 2009). Studies on various subantarctic islands (Marion, Gough and Guillou) show that the proportion of plant material in the diet of mice will increase in mid to late-summer (Avenant 1999; Le Roux *et al.* 2002; Smith *et al.* 2002; Jones *et al.* 2003). This change could be a response to decreasing invertebrate densities, or due to increasing seed loads (Avenant & Smith 2004). Similarly, faecal analyses show that the littoral amphipod *Talitrus saltator* was a major component of the winter diet of mice living on Skokholm Island, off the south west coast of Wales (Berry & Jakobson 1974).

House mice are one of the most widely spread mammal species and have been introduced to many oceanic islands (Cuthbert & Hilton

2004). Introduced mammalian predators can have significant impacts on island ecosystems, causing changes in species composition and ecosystem structure (Roemer *et al.* 2002; Towns *et al.* 2009), and can lead to species extinctions (Moors 1984). Many seabirds breed on isolated islands in the absence of terrestrial mammals (Schlaepfer *et al.* 2002), so will typically exhibit behaviours and life-history characteristics that leave them vulnerable to predation by introduced mammalian predators (Croxall & Rothery 1991; Croxall *et al.* 2012). Indeed, invasive rodents could be the leading cause of the extinction of bird species from islands (Howald *et al.* 2007; Jones *et al.* 2008).

While it was known that house mice preyed on small seabirds and ground nesting passerines on several islands (Fugler *et al.* 1987; Burger 1994; Campos & Granadeiro 1999; Jones & Ryan 2010), it has been assumed that the impact of feral mice on bird populations was negligible (Moors 1984; Atkinson 2001; Cuthbert & Hilton 2004; Witmer *et al.* 2007). However, on Gough Island in the South Atlantic mouse predation has been identified as the most likely cause of high chick mortality in the Tristan albatross (*Diomedea dabbenena*) (Cuthbert & Hilton 2004), leading to this species being listed as Critically Endangered (BirdLife International 2017). Further, Wanless *et al.* (2007) showed that mice on Gough were preying upon and killing healthy Atlantic petrel (*Pterodroma incerta*) chicks. Indeed, more than one million seabird chicks are predated by house mice on Gough each year (Wanless *et al.* 2012), and groups of mice have been observed attacking chicks (Wanless *et al.*

2007; Cuthbert *et al.* 2013a), with chicks dying up to five days later (Davies *et al.* 2015).

Angel *et al.* (2009) note that the only recorded instances of mouse predation on seabird eggs or chicks have been on islands on which mice are the only introduced mammal. Indeed, the first evidence of mouse attacks on wandering albatross (*Diomedea exulans*) chicks on Marion Island was in 2003 (Jones & Ryan 2010), following the eradication of feral cats in the early 1990s (Bester *et al.* 2002). In the absence of predation or competition from other invasive mammals, mice can grow to greater sizes and reach higher population densities which may trigger predatory behaviour (Courchamp *et al.* 1999; Harris & Macdonald 2007; Wanless *et al.* 2007; Cuthbert *et al.* 2016).

Cuthbert *et al.* (2016) found that mice on Gough Island inhabiting areas of high chick predation had stable or increasing body condition, and this contributed to over-winter survival. Indeed, diet is one of the most important drivers of body condition (Pothoven *et al.* 2001; Sorensen *et al.* 2009; Harrison *et al.* 2011; Resano-Mayor *et al.* 2016). For example, Bonelli's eagle (*Aquila fasciata*) nestlings with a greater proportion of preferred prey (rabbits) in their diet typically had increased body condition, while increased diet diversity was negatively associated with body condition (Resano-Mayor *et al.* 2016).

Mice on the Isle of May are subject to low-levels of predation and no competition from other rodent species (Triggs 1991; see Chapter 2). Here, mice have been observed scavenging on seabird, rabbit and grey seal (*Halichoerus grypus*) carcasses, however no direct predation has

been observed (D. Steel & M. Newell, pers. comms.). Indeed, direct predation by mice on seabirds has only been observed on the islands of Gough (Wanless *et al.* 2007) and Marion (Dilley *et al.* 2015; Dilley *et al.* 2016). Direct food consumption is rarely observed in wild rodents, as these are often nocturnal, secretive and burrow underground (Lindsey *et al.* 1999; Shiels *et al.* 2013). Further to this, gut and faecal content analyses can underestimate consumption of highly digestible food items, including bird eggs and nestlings (Stapp 2002).

Stable isotope analysis, however, has been an important tool in ecology since the 1970s, when it was first used to investigate the foraging ecology of animals (Boutton *et al.* 1978; DeNiro & Epstein 1978b, a; Fry *et al.* 1978; Vogel 1978). Since then, it has been utilised in a wide range of ecological studies, including paleoecology (Garrett *et al.* 2015), migration studies (Hobson 1999) and different aspects of trophic ecology (Kelly 2000). In stable isotope analysis, ratios of stable carbon ($^{13}\text{C}/^{12}\text{C}$) and nitrogen ($^{15}\text{N}/^{14}\text{N}$) isotopes can be used to identify dietary sources (Stapp 2002). Differences in carbon isotope ratios can identify whether the diet is derived from a marine or terrestrial source (Peterson & Fry 1987), and differences in nitrogen isotope ratios can be used to determine dietary source and trophic position (Schoeninger *et al.* 1983; Minagawa & Wada 1984). Recently, stable isotope mixing models have been used to estimate the contribution of different sources to an animal's diet (Phillips & Koch 2002; Phillips *et al.* 2005; Moreno *et al.* 2010; Parnell *et al.* 2010; Hopkins & Ferguson 2012; Phillips *et al.* 2014). Indeed, stable isotope analysis has been used to provide evidence of predation by

Keen's mice (*Peromyscus keeni*) on seabird eggs on Triangle Island in British Columbia (Drever *et al.* 2000), and predation by black rats (*Rattus rattus*) on seabirds on the Shiant Islands in the Outer Hebrides (Stapp 2002).

Here, we aim to use stable isotope analysis to provide a direct assessment of the diet of Isle of May mice. The Isle of May is an important nesting site for 11 species of seabird, including the globally threatened Atlantic puffin (*Fratercula arctica*) and Black-legged kittiwake (*Rissa tridactyla*) (see Chapter 2). Therefore, to inform future conservation management of the island, we wanted to determine the proportion of the diet of these mice that was contributed by seabird sources. Further, we aimed to assess the proportion contributed by other potential dietary sources, such as invertebrates, vegetation and terrestrial mammals. We explore how this diet varies spatially across the island, in relationship to habitat type and breeding seabird densities. We predict that seabird sources will contribute a higher proportion of diet in areas of higher seabird density (Jones & Ryan 2010; Cuthbert *et al.* 2016). As isotopic signatures can vary with age (Ben-David & Flaherty 2012) and sex (Graves *et al.* 2012), we also explore their relationship with $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. In addition to this, we wanted to determine the effect diet had on the fitness of an individual. Here, we explored the relationship between diet and body condition – an indicator of fitness – with the prediction that animals with higher proportions of vertebrate-sourced protein in their diet would be better able to maintain higher body condition (Cuthbert *et al.* 2016).

To our knowledge, there are currently only three published examples of stable isotope analysis being used to determine the diet of wild house mice. The first of these was a study of introduced mammals on New Island in the Falkland Islands. Here, Quillfeldt *et al.* (2008) found that house mice fed on a predominantly terrestrial-based diet. In a study of invasive rodents in Hawaiian mesic forests, Shiels *et al.* (2013) found that invertebrates made up the majority of the diet of house mice. Recently, Wang *et al.* (2014) used stable isotope analysis to show that wild mice with a mainly vertebrate-based diet had a significantly different intestinal microbiota than mice with a primarily plant-based diet. The present study, therefore, represents a novel approach to investigating the diet of an island population of house mice.

3.2 Methods

3.2.1 Sample collection

Mice used in this study were trapped on the Isle of May between 09/10/2015 and 12/10/2015 in ten locations across the island. 16 traps were set in each location, placed in pairs approximately every 2 metres along a transect. Mice trapped around the inhabited buildings at Fluke Street (see Chapter 2) were excluded from this study, as they were likely to have been feeding on anthropogenic food sources. Mice were euthanized according to UK Home Office regulations using an appropriate Schedule I technique, as described in Chapter 2.

3.2.2 Mouse life history, body condition and physiology

3.2.2.1 Life history and morphology

Mice were sexed and aged as described in Chapter 2. Body mass index (BMI) was used as a measure of individual body condition (Chapter 2).

3.2.2.2 Somatic indices

Somatic indices, the relative size of internal organs, are widely used measures of individual health and physiological condition in small mammals (Tête *et al.* 2013). Indeed, liver size is related to forage quality in herbivorous mammals (Norrdahl *et al.* 2004), and spleen size can indicate reduced immunocompetence or prevailing infection (Norrdahl *et al.* 2004). Here, the spleen and liver were removed and weighed. Relative organ mass was calculated as a percentage ratio of total body mass, following Sánchez-Chardi *et al.* (2007):

$$\text{Relative organ mass} = \left(\frac{\text{Organ mass (g)}}{\text{Total body mass (g)}} \right) \times 100$$

3.2.2.3 Haematocrit

Haematocrit, the estimated proportion of red blood cells in whole blood, was used as a further measure of individual health and

physiological condition (Milenkaya *et al.* 2013). To obtain this, blood was collected using micro-capillary tubes during dissection. The capillary tubes were sealed at one end with clay, and then centrifuged at 14, 000 rpm for 10 min in a micro-haematocrit centrifuge. Haematocrit was measured using a micro-haematocrit scale (Christe *et al.* 2002).

3.2.2.3 Serum leptin levels

Circulating leptin levels were used as a further biomarker of physiological status and body condition (Abolins *et al.* 2011; Abolins *et al.* 2018). Leptin is produced by adipose cells, and concentrations are significantly correlated with percentage body fat in various species including humans (Pasquali & Casimirri 1993; Fors *et al.* 1999; Wang *et al.* 2003), laboratory mice (Maffei *et al.* 1995; Sjögren *et al.* 2001) and dairy cows (Garnsworthy *et al.* 2008), as well as several wild species including American black bear (*Ursus americanus*) (Spady *et al.* 2009) and baboons (*Papio* sp.) (Banks *et al.* 2001). In addition to this, leptin concentrations positively correlate with body condition scores in various species including domestic horses (Suagee *et al.* 2013), dogs (Ishioka *et al.* 2002; Sagawa *et al.* 2002) and captive African elephants (*Loxodonta africana*) (Morfeld & Brown 2016). Further, leptin levels can be used as a proximate indicator of energy available to the immune system in wild populations (French *et al.* 2011), and leptin can regulate the proinflammatory immune response (Loffreda *et al.* 1998; Lam & Lu 2007;

Conde *et al.* 2010). Here, serum leptin levels were measured by multiplex bead assay, as described in Chapter 5 (Section 5.2.5).

3.2.3 Plant communities

The vegetative community for each site was defined using the most recent plant species distribution maps available for the Isle of May (van der Wal, unpublished). Two communities were categorised: those areas where the perennial grass *Holcus lanatus* is dominant and those areas where sea campion (*Silene uniflora*) is dominant (see Appendix 2.1).

3.2.4 Breeding bird density

Using a whole-island count of occupied puffin burrows (Newell *et al.* 2017b), a 'breeding bird density' was assigned to each site. Sites were categorised based on whether they were in an area of 'high' (>1.5 occupied puffin burrows ha⁻¹), 'medium' (0.5 – 1.49 occupied burrows ha⁻¹) or 'low' (<0.49 occupied burrows ha⁻¹) breeding bird density (see Appendix 2.2).

3.2.5 Tissue sampling and preparation

Leg muscle tissue was taken from euthanised mice to determine consumer isotope values. Muscle tissue was used as this will accurately

reflect the isotopic composition of an animal's diet over several months (Bearhop *et al.* 2003; Dalerum & Angerbjörn 2005), while tissues with a higher metabolic rate (e.g. hair or liver) will reflect a shorted dietary period (Tieszen *et al.* 1983; MacNeil *et al.* 2005).

To represent the potential seabird dietary source, great black-backed gull (*Larus marinus*) pectoral muscle tissue was obtained from an adult bird culled on the Isle of May in July 2016. Rabbit leg muscle tissue was obtained from a freshly deceased adult animal on the Isle of May in July 2016. Samples of earwig (*Forficula auricularia*), commonly found in the traps used to catch mice, were used to represent the potential terrestrial arthropod dietary source. A mixed sample of *S. uniflora* and *H. lanatus* was used to represent the potential vegetative component of the diet, as these are the dominant plant species across much of the island (van der Wal, unpublished).

All samples were kept frozen prior to drying, and were then freeze-dried at -50°C for approximately 12 hours before being ground to a fine powder with a mortar and pestle. All muscle tissues had lipids removed by soaking in a 2:1 chloroform:methanol solution (Folch *et al.* 1957; Cherry *et al.* 2011). Lipids typically have less ^{13}C (DeNiro & Epstein 1977), so lipid extraction reduces the risk of significant bias in $\delta^{13}\text{C}$ values (Tieszen *et al.* 1983; Post *et al.* 2007).

3.2.6 Stable Isotope Analysis

Approximately 1.6 mg of plant sample and 0.6 mg of animal tissue samples were used in stable isotope analysis. The isotope ratio mass spectrometry took place at the NERC Isotope Geosciences Facilities (Keyworth, UK), measured on a continuous flow-elemental analyser (Flash/EA) coupled to a ThermoFinnigan Delta Plus XL via a ConFlo III interface. Isotopic results are expressed as delta (δ) values, reported in per mil (‰) relative to international standards for $\delta^{13}\text{C}$ (Vienna Pee Dee Belemnite (VPDB)) and $\delta^{15}\text{N}$ (atmospheric nitrogen (AIR)), according to the following equation:

$$\delta X = [(R_{\text{sample}} - R_{\text{standard}}) - 1] \times 1000$$

Where X is either ^{13}C or ^{15}N and R_{sample} and R_{standard} are the $^{13}\text{C}:^{12}\text{C}$ or $^{15}\text{N}:^{14}\text{N}$ ratios of the sample or standard, respectively. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ ratios were calibrated using an in-house reference material M1360p (powdered gelatine from British Drug Houses) with expected delta values of -20.32‰ (calibrated against CH7, IAEA) and $+8.12\text{‰}$ (calibrated against N-1 and N-2, IAEA) for C and N respectively. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analyses were undertaken in duplicate and the average standard deviation of these pairs was $\delta^{15}\text{N} = \pm 0.11\text{‰}$ and $\delta^{13}\text{C} = \pm 0.22\text{‰}$. The 1σ reproducibility for mass spectrometry controls for these analyses was better than $\pm 0.2\text{‰}$ for both isotopes.

During this study, we were unable to obtain any seabird egg from the Isle of May, so previously published $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values for

rhinoceros auklet (*Cerorhinca monocerata*) and Cassin's auklet (*Ptychoramphus aleuticus*) egg contents were used (Drever *et al.* 2000).

A further potential dietary source for Isle of May mice is grey seal. Mice on the island have been observed consuming seal carcasses, however grey seals are typically only onshore in significant numbers between October and January (Bonner 1972; Brownlow *et al.* 2016). Stable isotope analysis of muscle tissue typically provides an estimate of the diet for up to the previous six months (Bearhop *et al.* 2003; Dalerum & Angerbjörn 2005), and so October-caught mice are unlikely to have had the opportunity to forage seal in this period. Therefore, seal was omitted from this study.

3.2.7 Stable isotope mixing model

The contribution of each potential dietary source to the mouse diets was estimated with stable isotope mixing models, using the package 'simmr' (Parnell 2016) in R version 3.4.0 (R Core Team 2017). Simmr uses a Markov chain Monte Carlo (MCMC) model fitting algorithm, and this was run for 10,000 iterations with a burn-in of 1,000.

Using a Bayesian approach, such as simmr, can provide more robust estimates of dietary proportion than linear mixing models (Moreno *et al.* 2010). This is largely because Bayesian mixing models incorporate the inherent variability associated with source data, as well as discrimination factors (Shaner & Macko 2011).

Due to discrimination during assimilation and excretion processes, an animal's tissues will typically have higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic values than its diet (Olive *et al.* 2003). So, before applying a mixing model, a trophic discrimination factor (TDF) must be applied to the food source isotope values (Phillips *et al.* 2014). Here, a mouse muscle tissue specific TDF of +1.6‰ for $\delta^{13}\text{C}$ (MacAvoy *et al.* 2005; Caut *et al.* 2009) and +3‰ for $\delta^{15}\text{N}$ (Arneson & MacAvoy 2005; Caut *et al.* 2009) was applied.

3.2.8 Statistical analysis

Pearson's correlation coefficients were used to investigate the relationship between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and eye lens weight (a proxy for age). Two sample Student's *t* tests were used to compare the mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of males and females. One-way ANOVA was used to investigate the relationship between isotope signatures ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and habitat type, bird breeding density and location. To explore the relationship between diet and body condition, general linear models (GLMs) were fitted with stable isotope value ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$ or C/N) as the variable. Measures of body condition and physiology (BMI, leptin concentration, haematocrit, liver and spleen somatic indices) were included as continuous variables. A minimum adequate model was generated by sequentially dropping non-significant terms. Wald F-tests were used to determine the significance of remaining terms. All statistical analyses were conducted in R version 3.4.0 (R Core Team 2017), and

data were visualised spatially using the package 'ggmap' (Kahle & Wickham 2016).

3.3 Results

3.3.1 Stable isotope values

The 69 mice analysed had a mean $\delta^{13}\text{C}$ value of -25.6‰ , ranging from -27.8‰ to -23.8‰ (Figure 3.2). There was no difference between males and females in $\delta^{13}\text{C}$ values ($t = 0.639$, $p = 0.525$), and $\delta^{13}\text{C}$ did not correlate with age ($r = 0.093$, $p = 0.429$).

There was a mean $\delta^{15}\text{N}$ value of 14.5‰ , ranging from 10.0‰ to 19.8‰ (Figure 3.2). There was no difference in $\delta^{15}\text{N}$ values between male and female mice ($t = 1.328$, $p = 0.239$), and $\delta^{15}\text{N}$ values were uncorrelated with age ($r = 0.166$, $p = 0.157$). The lack of relationship between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values and age or sex means that the population can be pooled in further analyses.

Gull tissue and seabird egg contents occupied a similar isospace (Figure 3.2) and were estimated to contribute a similar dietary proportion, so were combined *a priori* into a single 'Egg + Gull' source category representing potential seabird dietary sources (Phillips *et al.* 2005).

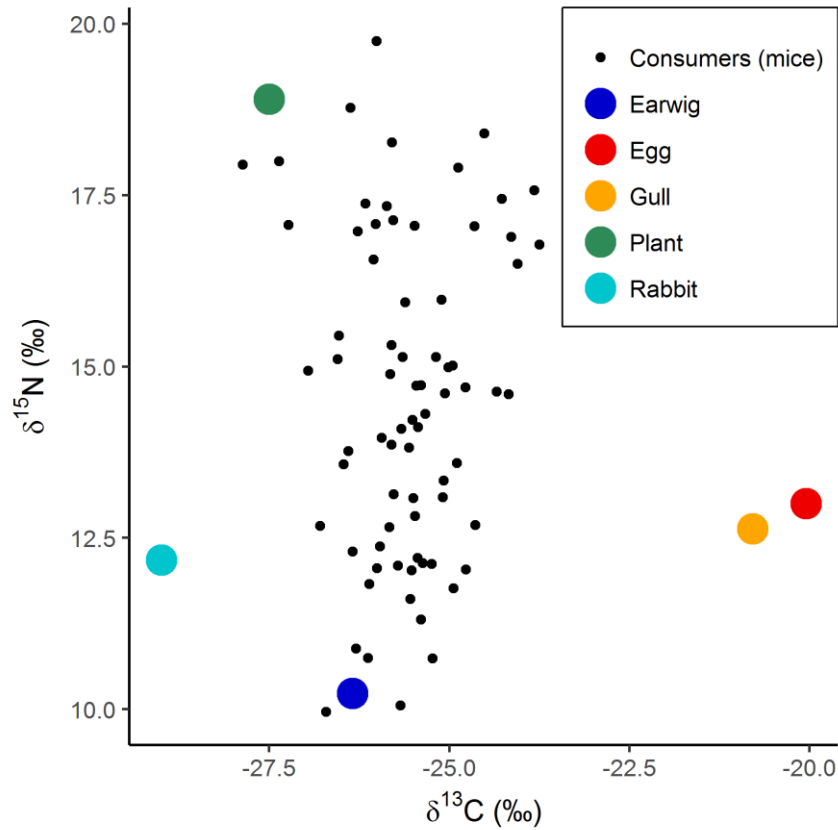


Figure 3.2: Isospace plot showing values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in muscle tissue of house mice (*Mus musculus domesticus*) from the Isle of May, as well as potential dietary sources. Potential dietary sources include; terrestrial arthropods represented here by earwig (*Forficula auricularia*); mammal tissue represented here by rabbit (*Oryctolagus cuniculus*); seabird, represented here by great black-backed gull (*Larus marinus*) tissue and combined Cassin's auklet (*Ptychoramphus aleuticus*) and rhinoceros auklet (*Cerorhinca monocerata*) egg; and terrestrial plants, represented here by combined *Holcus lanatus* and *Silene uniflora*. Egg content data are taken from Drever *et al.* (2000).

3.3.2 Spatial variation in isotope values

The $\delta^{15}\text{N}$ isotope signature from mice was significantly higher in the south of the island compared to the north ($F_{1,72} = 7.474$, $p = 0.008$,

Figure 3.3a). There was no significant difference in $\delta^{13}\text{C}$ between the north and south of the island ($F_{1,72} = 1.383$, $p = 0.244$, Figure 3.3b).

Neither $\delta^{13}\text{C}$ ($F_{2,71} = 0.276$, $p = 0.759$) or $\delta^{15}\text{N}$ ($F_{2,71} = 1.662$, $p = 0.197$) varied significantly with breeding bird densities. $\delta^{13}\text{C}$ showed no relationship with vegetation type ($F_{1,72} = 0.387$, $p = 0.536$), but $\delta^{15}\text{N}$ was higher in mice from *S. uniflora* dominant areas compared to those from *H. lanatus* dominant areas ($F_{1,72} = 6.372$, $p = 0.014$) (Figure 3.4).

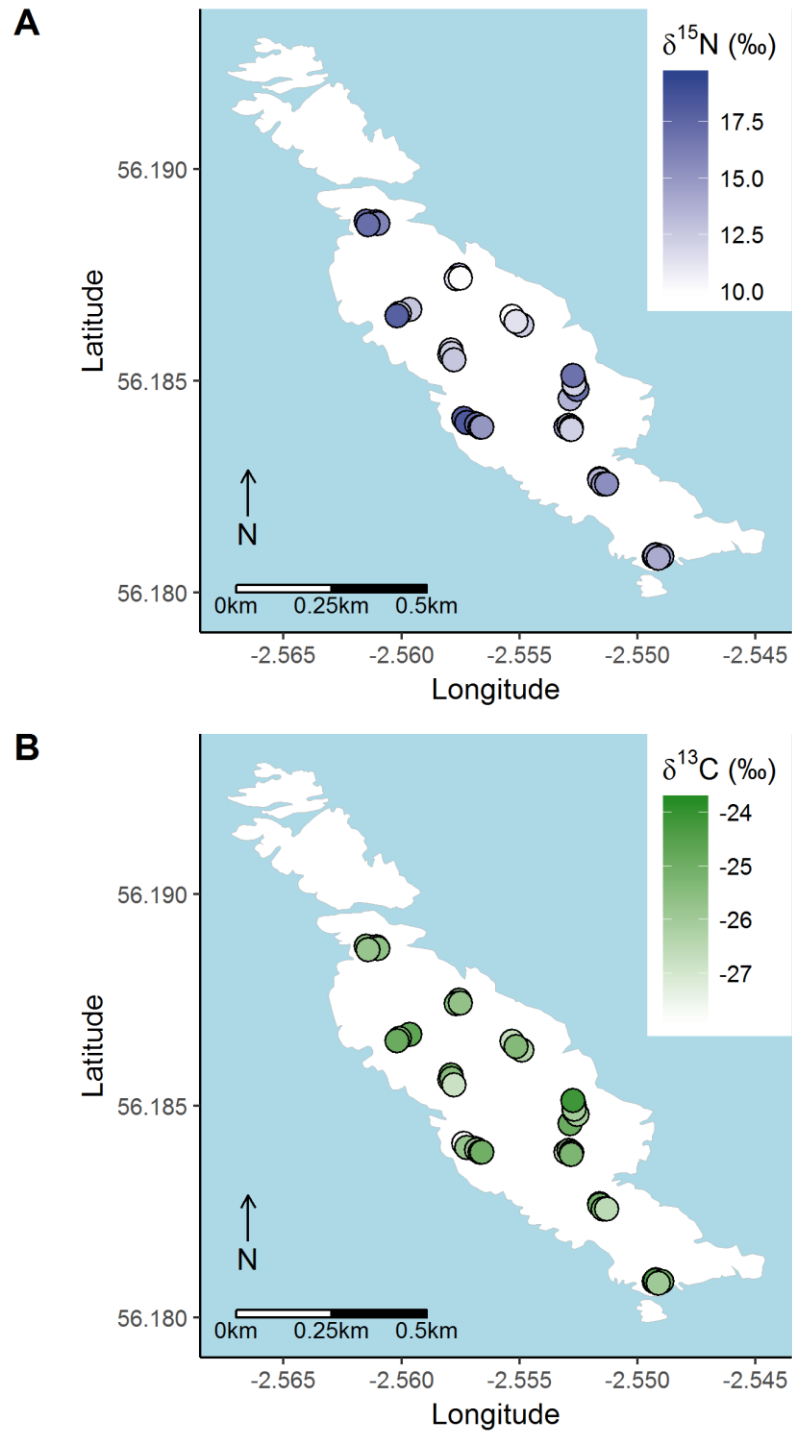


Figure 3.3: Map showing spatial variation in $\delta^{15}\text{N}$ (A) and $\delta^{13}\text{C}$ (B) stable isotopes from wild mouse (*Mus musculus domesticus*) muscle tissue across the Isle of May. Maps were generated using the "Island Groups 2011" datafile provided by National Records of Scotland (www.nrscotland.gov.uk). Maps were plotted, and data visualised, using "ggmap" (Kahle & Wickham 2013).

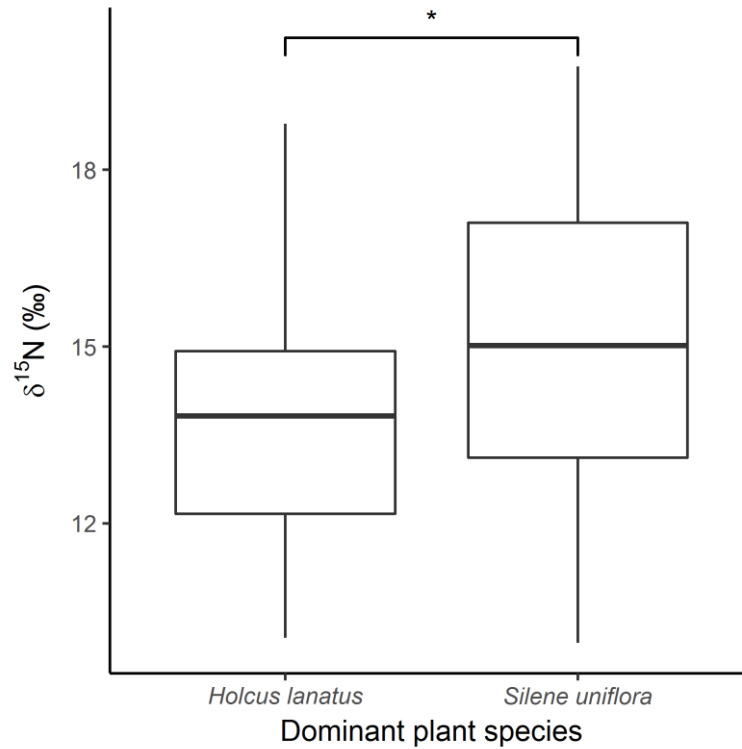


Figure 3.4: Wild Mice (*Mus musculus domesticus*) caught in *Silene uniflora* dominant habitat on the Isle of May, Scotland, had higher $\delta^{15}\text{N}$ stable isotope ratios than those living in *Holcus lanatus* dominant habitats (* denotes significance at $p < 0.05$).

3.3.3 Stable isotope mixing model

The stable isotope mixing model estimated the mean (Bayesian 95% credibility intervals) proportion of diet contributed by plant material was 9.3% (3.1-17.4%). The proportion contributed by terrestrial arthropod sources (represented by earwig) was 59.7% (43.4-78.8%), and the proportion contributed by terrestrial mammals (represented by rabbit) was 27.7% (2.0-46.9%). Seabird sources were estimated to contribute 4.0% (0.7-9.8%) (Figure 3.5).

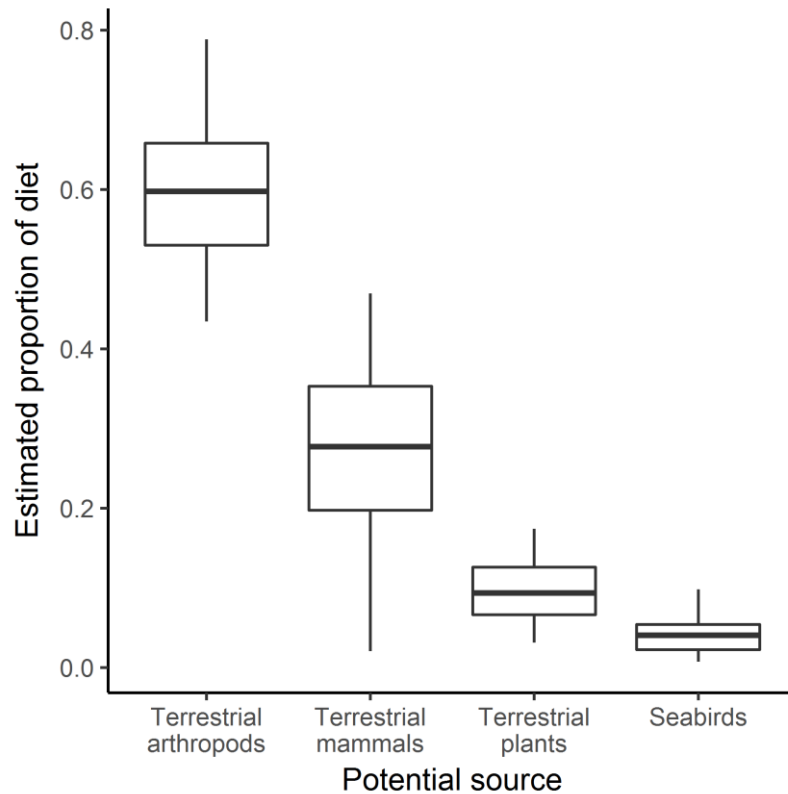


Figure 3.5. Proportions of four potential food groups in Isle of May mice, estimated from stable isotope Bayesian mixing models (simmr in R). Horizontal lines represent the mean, while boxes indicate 50% Bayesian credibility intervals (C.I.) and vertical lines represent 95% Bayesian C.I.

3.3.4 Dietary differences with vegetation type

The mixing model was run again with vegetation type as a group. The proportion of diet estimated from terrestrial arthropods was higher in *H. lanatus* dominant areas (51.7% (31.2-70.3%)) than in *S. uniflora* dominant areas (47.9% (32.6-68.2%)), with an estimated probability of 0.89. The proportion of diet estimated to be contributed by terrestrial mammals was also higher in *H. lanatus* dominant areas (41.1% (13.6-60.7%)) than in *S. uniflora* dominant areas (28.6% (11.9-55.5%)), with an estimated probability of 0.71 (Figure 3.6).

The proportion of diet estimated to be contributed by terrestrial plant material was higher in *S. uniflora* dominant areas (15.7% (7.0-22.6%)) than in *H. lanatus* dominant areas (6.0% (1.0-11.5%)), with an estimated probability of higher plant consumption in *S. uniflora* dominant areas of 0.89. Seabird sources were estimated to contribute 7.8% (2.2-17.7%) in *S. uniflora* dominant areas and 3.2% (0.6-6.3%) in *H. lanatus* dominant areas, with a 0.77 probability of the dietary proportion being higher where *S. uniflora* is dominant. (Figure 3.6).

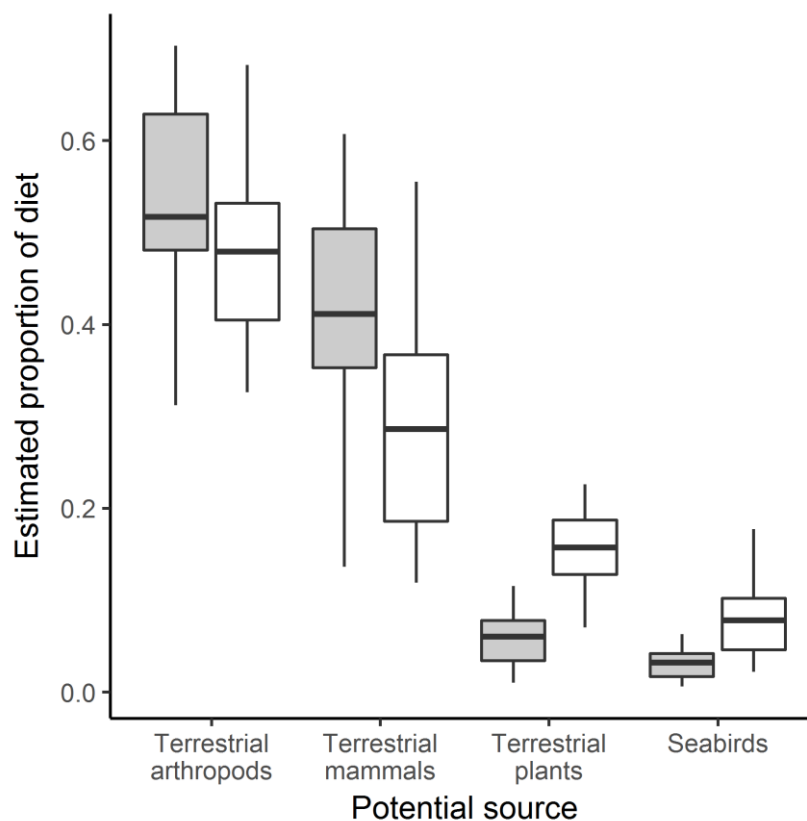


Figure 3.6. Comparison of the proportions of four potential food groups in Isle of May mice across habitat types (■ = *Holcus lanatus* dominant, □ = *Silene uniflora* dominant), estimated from stable isotope Bayesian mixing models (simmr in R). Horizontal lines represent the mean, while boxes indicate 50% Bayesian credibility intervals (C.I.) and vertical lines represent 95% Bayesian C.I.

3.3.5 Dietary differences with breeding bird densities

The mixing model was run again, including breeding bird density as groups. The estimated proportion that each potential dietary source contributed to the diet of mice living in areas of differing breeding bird density is shown in Table 3.1.

Table 3.1: The estimated proportion of the diet of house mice (*Mus musculus domesticus*) on the Isle of May, from areas of differing breeding bird density, made up by various potential sources.

Breeding bird density	Dietary source	Estimated contribution	95% C.I.
High	Arthropod	60.7%	37.2 – 76.4%
	Mammal	18.1%	1.8 – 45.3%
	Plant	13.9%	5.3 – 20.9%
	Seabird	7.3%	1.1 – 12.5%
Medium	Arthropod	53.1%	30.5 – 74.2%
	Mammal	30.9%	4.4 – 58.9%
	Plant	11.3%	3.4 – 20.0%
	Seabird	4.7%	0.8 – 9.5%
Low	Arthropod	43.6%	18.5 – 70.9%
	Mammal	41.7%	5.8 – 72.3%
	Plant	10.0%	2.6 – 21.0%
	Seabird	4.7%	0.7 – 9.5%

The proportion of diet contributed by terrestrial mammal material was greater in areas of low breeding bird density than medium (Probability 0.69) or high (0.83) density areas. The proportion of diet contributed by terrestrial mammal material was greater in areas of medium compared to high breeding bird density, with a probability of 0.75 (Figure 3.7).

The proportion of diet contributed by all other potential sources was greater in areas of high breeding bird density than in areas of medium (arthropod = 0.69, plant = 0.67, seabird = 0.67) or low (arthropod = 0.75, plant = 0.72, seabird = 0.68) density. The estimated contribution of these dietary sources was also greater in areas of medium breeding bird density compared to low density areas (arthropod = 0.86, plant = 0.59, seabird = 0.51; Figure 3.7).

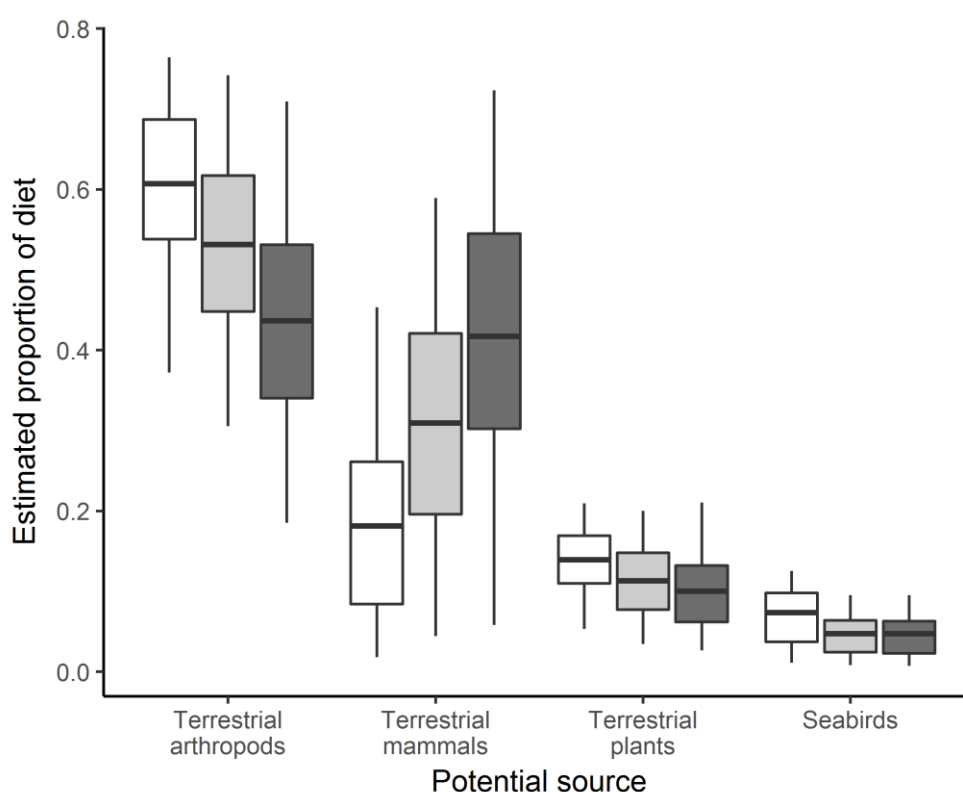


Figure 3.7: Comparison of the proportions of four potential food groups in Isle of May mice across areas of different breeding bird density (□ = high density, ■ = medium density, ■ = low density), estimated from stable isotope Bayesian mixing models (simmr in R). Horizontal lines represent the mean, while boxes indicate 50% Bayesian credibility intervals (C.I.) and vertical lines represent 95% Bayesian C.I.

3.3.6 Diet and body condition

The C/N ratio was significantly positively correlated with circulating leptin levels ($F_{1,69}=14.471$, $p<0.001$, Figure 3.8a), while $\delta^{13}\text{C}$ was significantly negatively correlated ($F_{1,69}=6.184$, $p=0.015$, Figure 3.8b).

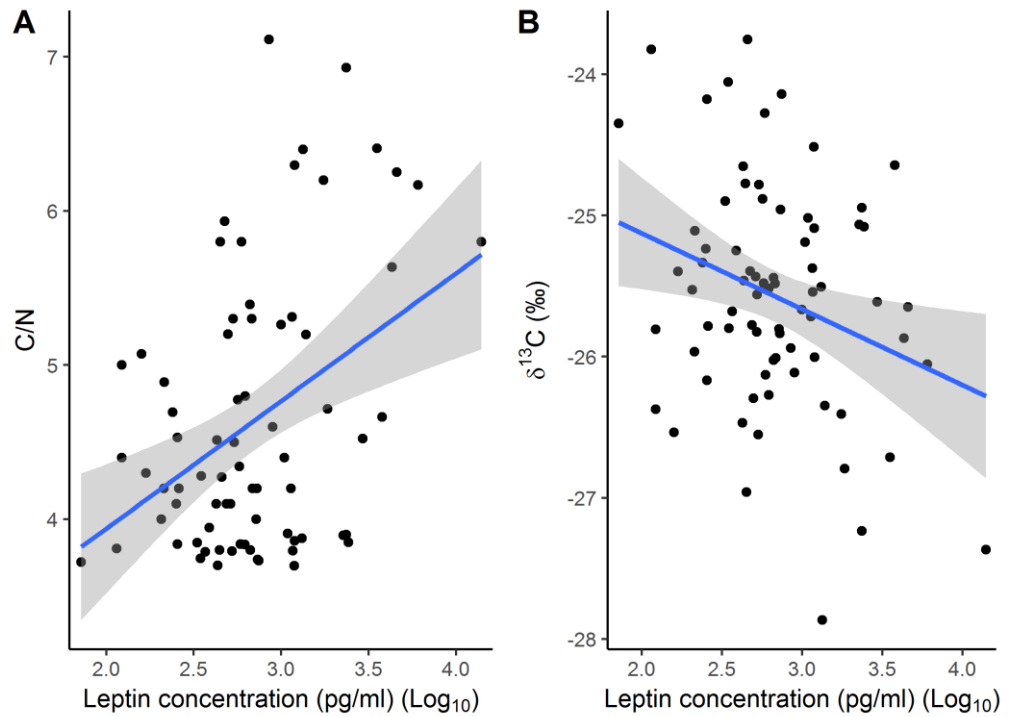


Figure 3.8: The relationships between the C/N ratio (A) and $\delta^{13}\text{C}$ (B) and circulating levels of leptin in wild *Mus musculus domesticus* from the Isle of May.

The only significant relationships between C/N or $\delta^{13}\text{C}$ and any measure of physiology or body condition came with circulating leptin levels (Table 3.2). There were no significant relationships between $\delta^{15}\text{N}$ and any measure of physiology or body condition (Table 3.2).

Table 3.2: The relationship between diet and body condition in wild house mice (*Mus musculus domesticus*). Shown here are summaries of minimum adequate GLMs fitted to stable isotope data ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$, C/N) from wild house mice inhabiting the Isle of May, Scotland. Measures of body condition and physiology (BMI, circulating leptin levels, haematocrit, proportional spleen and liver size) were included as factors.

Stable isotope value	Condition measure	<i>F</i>	df	<i>p</i>
$\delta^{15}\text{N}$	No significant factors	-	-	-
$\delta^{13}\text{C}$	Circulating leptin concentration	6.184	1,69	0.015
C/N	Circulating leptin concentration	14.471	1,69	<0.001

3.4 Discussion

In this study, we used stable isotope analysis to identify the diet of wild house mice on the Isle of May, and the proportion of diet contributed by potential sources (terrestrial arthropods, terrestrial mammals, terrestrial plants and seabirds). We explored how this diet varied spatially across the Isle of May, and the relationships between diet and habitat type and breeding seabird density. Finally, we explored the relationships between the isotopic ratios of mouse muscle tissue and various measures of mouse body condition and physiology.

This study shows that the majority of the diet of house mice on the Isle of May is derived from animal sources (90.7%), with invertebrate prey (represented by earwigs) making up 59.7% of the total diet. A similar preference for invertebrate prey has been observed in other non-commensal house mouse populations (Cockburn 1980; Fitzgerald *et al.* 1996; Miller & Webb 2001; Shiels *et al.* 2013), including populations on

several subantarctic islands (Gleeson & Van Rensburg 1982; Copson 1986; Le Roux *et al.* 2002).

Seabird sources provided an estimated 4.0% of the diet. It is important to note that while mice on the Isle of May have been observed scavenging on seabird carcasses, no direct predation has been recorded. Indeed, direct predation by mice on seabirds has only been observed on the islands of Gough (Wanless *et al.* 2007) and Marion (Dilley *et al.* 2015; Dilley *et al.* 2016); evidence of predation on other islands is based on the presence of wounded chicks, gnawed egg shells or observations of mice eating dead chicks (Angel *et al.* 2009). Therefore, the dietary component of Isle of May mice coming from seabird sources likely represents scavenging on dead birds or non-viable eggs, or possibly predation at low, stable levels that have no significant impact on population sizes.

Indeed, the overall numbers of seabirds on the Isle of May have significantly increased since the start of the 20th Century (see Chapter 2). Importantly, any noted declines in local seabird populations have been linked to factors other than mouse predation; declines in Arctic tern (*Sterna paradisaea*) numbers were linked to increased predation by the growing gull colony (Sobey 1976), puffin declines in 2008/2009 were linked to climatic conditions (Harris *et al.* 2009) and declines in the successful breeding of Isle of May kittiwakes were associated with migratory behaviour (Bogdanova *et al.* 2017).

Further to this, mice and seabirds have coexisted on the Isle of May for up to 4,000 years (see Chapter 2). Quillfeldt *et al.* (2008) suggest

that the regulation of rodent populations (*M. musculus* and *R. rattus*) by over-winter survival has helped facilitate their 100-years of coexistence with thin-billed prions (*Pachyptila belcheri*) on New Island. Over-winter survival of rodents on New Island is largely driven by a scarcity of food, including the absence on breeding birds (Quillfeldt *et al.* 2008). Similarly, the Isle of May mouse population is regulated by over-winter survival driven by food availability (Triggs 1991; see Chapter 2), and there are no winter-breeding birds on the island (see Chapter 2).

Winter-breeding seabirds are present on both Gough and Marion islands – the only islands on which direct predation by mice has been observed (Wanless *et al.* 2007; Dilley *et al.* 2015; Dilley *et al.* 2016). Mice on Gough seasonally shift to a predominantly bird-sourced diet in the winter as the availability of plant and arthropod material decreases (Cuthbert & Hilton 2004). Similarly, there is a shift back to a plant dominated diet in the summer period when the availability and nutritional quality of vegetation increases (Jones *et al.* 2003). Indeed, the presence of winter-breeding seabirds on Gough provides an energetically rich prey source for mice during the critical winter period (Cuthbert *et al.* 2016). As a result, mice on Gough experience low seasonal variation in population size compared to other islands (Cuthbert *et al.* 2016; see Chapter 2). Further, the unusually high densities of seabirds in the winter has allowed for positive selection for predatory behaviour and extreme body size in Gough mice (Cuthbert *et al.* 2016). Indeed, it is only on Gough that the levels of predation have led to population declines across several bird

species (Wanless *et al.* 2007; Ryan & Cuthbert 2008; Cuthbert *et al.* 2013b; Cuthbert *et al.* 2016).

The density of seabirds is an important factor in mouse predation, with predation more likely on islands with higher bird densities (Jones & Ryan 2010; Cuthbert *et al.* 2016). Further, levels of predation on Gough are higher in areas with greater densities of nests (Cuthbert *et al.* 2016). In this present study, seabird sources are estimated to contribute a higher proportion of the diet in areas of high puffin burrow density compared to low or medium density areas. This would be expected, as there will be an increased availability of seabird carcasses and eggs to scavenge in high density areas.

Terrestrial mammals (rabbits) made up a greater proportion of the diet in areas of low breeding bird density than in medium or high-density areas. This possibly reflects the availability of mammal material; rabbits can compete with puffins for burrows (Warham 1967), so numbers of rabbits are likely higher in areas of low bird density. Further, areas of low-bird density are likely to be low in nitrogen (Sjögersten *et al.* 2010; Andriuzzi *et al.* 2013), so mice may seek additional nitrogen from animal-based sources.

The $\delta^{15}\text{N}$ values for plant material shown here are higher than are typical of terrestrial habitats (Drever *et al.* 2000; Sayle *et al.* 2016), and indicates a high-input of marine sourced nitrogen, as seen in other seabird colonies (Mizutani & Wada 1988; Cocks *et al.* 1998; Wainright *et al.* 1998; Drever *et al.* 2000; Sjögersten *et al.* 2010). Indeed, the environment on Isle of May is enriched in nitrogen by its seabirds

(Andriuzzi *et al.* 2013), with levels of nitrogen deposition increasing with proximity to the cliff-nesting colonies (Wright *et al.* 2010). The spatial variation in values of $\delta^{15}\text{N}$ seen here, with mice from the north of the island having higher values than mice from the south, is likely linked to this variation in nitrogen deposition. This is reflected in the increased levels of $\delta^{15}\text{N}$ in mice inhabiting *S. uniflora* dominant areas compared to those in *H. lanatus* dominant areas. Plant species with shorter root systems that acquire nitrogen close to the soil surface, such as *S. uniflora*, will access more nitrogen from seabird guano than those species with deeper root systems, e.g. grasses such as *H. lanatus* (Sjögersten *et al.* 2010). Further, on the Isle of May, soil beneath *S. uniflora* typically has a higher organic matter content than soil beneath grass (Wright *et al.* 2010).

Terrestrial mammal and arthropod-based proteins make up a larger proportion of the diet of Isle of May mice caught in *H. lanatus* dominated areas compared to *S. uniflora* dominated areas. Mice typically show a preference for an animal-based diet, particularly for invertebrates when the opportunity arises (Gleeson & Van Rensburg 1982), however preferences can change dependent on food availability (Bomford 1987; Tann *et al.* 1991; Miller & Webb 2001; Avenant & Smith 2004; Kaboodvandpour & Leung 2010). Therefore, this variation could be due to the relative abundances and availability of animal sources in different habitat types. Also, the lower nitrogen content of *H. lanatus* could require the mice to obtain additional nitrogen from alternative, animal-based sources.

In these mice, circulating leptin levels, an indicator of body condition, were positively correlated with the C/N ratio. A similar relationship was observed in migratory great grey owls (*Strix nebulosi*), where C/N of muscle was positively correlated with BMI (Graves *et al.* 2012). A higher C/N ratio is linked to greater lipid reserves (Okumara *et al.* 2002; Post *et al.* 2007; Ehrich *et al.* 2011; Graves *et al.* 2012), so mice with lower C/N (and therefore lower levels of circulating leptin) are likely to be experiencing nutritional stress (Graves *et al.* 2012).

Further, $\delta^{13}\text{C}$ was negatively correlated with circulating leptin levels in these mice. Higher levels of $\delta^{13}\text{C}$ are related to a more marine-based diet (Peterson & Fry 1987), indicating that mice feeding on marine sourced materials (i.e. seabirds) are typically in worse body condition. This may be due to mice on the Isle of May seeking seabird sources as something of a “last resort” when under nutritional stress, in a similar way to the mice on Gough shifting to a seabird-based diet when other foods are limited (Jones *et al.* 2003; Cuthbert & Hilton 2004; Cuthbert *et al.* 2016).

This study did not include mice trapped near the human habitation on the Isle of May (see Chapter 2). These mice would potentially have fed on anthropogenic food sources (e.g. processed food), which are depleted in ^{15}N compared to natural terrestrial and marine prey (Jahren & Kraft 2008). Indeed, ring-billed gulls (*Larus delawarensis*) that had been feeding on anthropogenic food sources had depleted liver $\delta^{15}\text{N}$ values compared to those that had fed on a natural diet (Aponte *et al.* 2014).

The mice used in this study were all trapped in a single season (October 2015), and so no inferences can be made regarding temporal variation in the diet. Populations of wild house mice that typically show a preference for arthropods have been shown to exhibit seasonal shifts to more herbivorous diets in response to plant availability (Bomford 1987; Tann *et al.* 1991; Avenant 1999; Le Roux *et al.* 2002; Smith *et al.* 2002; Jones *et al.* 2003). Further, small mammals are more likely to predate on seabird eggs in the spring, when they first come available (Drever *et al.* 2000). In addition to this, the winter-breeding grey seal colony (Brownlow *et al.* 2016) could provide an important source of animal-based protein for the Isle of May mice during the critical winter period. However, it was not possible to make inferences about the winter diet of these mice.

Chapter 4

Macroparasites of the Isle of May mice: interactions with body condition

4.1 Introduction

Parasites and infectious diseases are a constant challenge to individuals and populations (Brown *et al.* 2013). These form a major selective pressure (Anderson & May 1979; Acevedo-Whitehouse & Cunningham 2006; Paterson & Pieltney 2011), with studies suggesting that host-parasite interactions may help to explain the evolution of sexual reproduction (Hamilton 1980), female mate choice (Hamilton & Zuk 1982) and the maintenance of genetic diversity (Haldane 1949). Further, infection can affect individual health, the stability of populations (Anderson & May 1979; May & Anderson 1979) and the functioning of entire ecosystems (Hudson *et al.* 1998). Parasites and other infectious pathogens typically elicit an immune response, and this can vary widely both between and within species (Schmid-Hempel 2003). Understanding why variation in such an ecologically important trait exists, and how it is maintained, is a key goal in evolutionary biology (Brown *et al.* 2013).

Parasitism can directly affect an individual's health, with animals in poorer body condition often being more heavily parasitized than those in better condition (Tompkins *et al.* 2011; Rafalinirina *et al.* 2015). Indeed, there can be a “vicious circle” involving condition and infection, in which individuals in poor body condition are more likely to become and remain infected, but infected individuals will suffer a reduction in condition (Beldomenico *et al.* 2008; Beldomenico & Begon 2010).

Diet can be an important driver of body condition (Resano-Mayor *et al.* 2016), and individuals with a better quality diet can typically mount a stronger immune response (Wiehn & Korpimäki 1998; Lochmiller & Deerenberg 2000; Cressler *et al.* 2014). Indeed, high-quality diet and increased resource availability is typically associated with reduced parasite loads in vertebrates (Ezenwa 2004; Cressler *et al.* 2014; Forbes *et al.* 2014), and increased resource availability can mediate parasite-driven population changes (Pedersen & Greives 2008).

While negative relationships between measures of parasitism and host body condition have been observed across a range of vertebrate species (Debeffe *et al.* 2016), it is possible that animals in better body condition may shift towards tolerance of parasitic infections and thus have higher parasite burdens (Råberg 2014; Kutzer & Armitage 2016). In adult male field voles (*Microtus agrestis*), parasite load increases with body condition, while there is no such pattern in juveniles (Jackson *et al.* 2014). In Soay sheep (*Ovis aries*), Hayward *et al.* (2014) found that more tolerant individuals had higher lifetime reproductive success, indicating that tolerance is under positive selection.

In addition to this, a lack of relationship between body condition and parasite load appears common in nature (Debeffe *et al.* 2016), and has been observed in wild populations of a range of species including moose (*Alces alces*) (Davidson *et al.* 2015), Cururu toad (*Rhinella icterica*) (Moretti *et al.* 2014) and grey squirrel (*Sciurus carolinensis*) (Scantlebury *et al.* 2010). This, therefore, highlights the need for further research into this relationship (Debeffe *et al.* 2016).

The house mice of the Isle of May present the ideal system in which to further elucidate the complex relationship between parasitism and host body condition. Islands can be considered “natural laboratories” (Whittaker *et al.* 2017), and allow the study of parasite dynamics with limited influence of migration or the introduction of novel pathogens. Further, the Isle of May mice are subject to no competition from other small mammals and have limited predation pressures (Triggs 1991).

4.1.1 Macroparasites of Isle of May mice: previous observations

In a survey of 12 Isle of May mice in July 1936, the only endoparasite recorded was the nematode *Trichuris muris* at a prevalence of 50% (Southern 1938). A more recent analysis of the intestinal tracts of 14 mice trapped on the island in January 2013 again found *T. muris* at a prevalence of 50% (Robertson 2014). Here, the nematodes *Syphacia obvelata* and *Trichinella spiralis* were also recorded, each with a prevalence of 43% (Robertson 2014). Liver cysts have also been observed, likely representing infection by an unidentified trematode or

cestode species (Triggs 1991; Robertson 2014). Presence of the hepatic nematode *Calodium hepaticum* (syn. *Capillaria hepatica*) was also noted, although no prevalence data were recorded (Robertson 2014).

The most frequently encountered ectoparasite during the 1936 survey was the flour mite (*Acarus siro*), present on 75% of observed mice (Southern 1938). This has not been observed since, probably due to grain and flour no longer being stored on the island in the same way (Robertson 2014). Indeed, *A. siro* is a stored-product pest, and is often regarded as a ‘pseudoparasite’ (Clemmons & Taylor 2016). Southern (1938) also recorded the northern rat flea (*Nosopsyllus fasciatus*) at a prevalence of 16.7%. Harvest mites (*Trombicula autumnalis*) were “commonly seen” on mice caught on the island during late summer in 1970 – 1972 (Triggs 1991), however no observations were noted regarding any other ectoparasites at this time.

These previous observations of the macroparasites of Isle of May mice consist of small sample sizes and are limited in the timescale they cover. Triggs (1991) processed a large number of mice over an extended time frame, however no detailed parasitological observations were made.

4.1.2 Aims and objectives

Here, we identify and quantify the macroparasite species infecting Isle of May mice over several seasons. We explore how this parasite community varies seasonally, and the relationship with host population dynamics as well as any interspecific interactions. We also examine the

relationship between parasite burden and various host life history and body condition measures, including nutritional status. We hypothesised that measures of parasitism will vary with host age and sex, with older animals (Hayward *et al.* 2009; Burlet *et al.* 2011) and males (Poulin 1996; Zuk & McKean 1996; Moore & Wilson 2002) having increased parasite burdens. Based on previous observations of other small mammal species, we hypothesise that older animals are likely to adopt a more tolerant strategy, where individuals will have increasing parasite loads with body condition (Jackson *et al.* 2014). Younger animals would be expected to be more resistant to infection (Jackson *et al.* 2014), so those individuals in poorer body condition will have higher parasite richness, abundance and prevalence.

4.2 Methods

4.2.1 Sample collection

Live-trapping of mice took place on the Isle of May between August 2013 and October 2016, as described in Chapter 2. The culling and processing of mice is also described in detail in Chapter 2.

4.2.3 Life history, body condition and physiology

Mice were sexed, aged and a reproductive stage was assigned as described in Chapter 2. Body mass index (BMI) was used as a measure of individual body condition (Chapter 2). The haematocrit and somatic

indices of liver and spleen were measured as described in Chapter 3. Serum leptin levels were measured by multiplex bead assay, as described in Chapter 5.

4.2.4 Nutritional status: stable isotope analysis

Stable isotope ratios can be used as indicators of body condition, with elevated $\delta^{15}\text{N}$ levels indicating nutritional stress (Hobson *et al.* 1993; Cherel *et al.* 2005; Mekota *et al.* 2006; Graves *et al.* 2012). Further, the ratio of carbon to nitrogen (C/N) can be used as an additional proxy for nutritional stress (Graves *et al.* 2012). Here, stable carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) ratios were calculated from the muscle tissue of 74 mice trapped in October 2015, as described in Chapter 3.

4.2.5 Host population size

Mouse population estimates were generated using spatially explicit capture-recapture (SECR) models, based on recapture data obtained from individually marked mice in 2015 and 2016. This is described in detail in Chapter 2.

4.2.6 Parasitological measurements

4.2.6.1 Gastrointestinal helminths

The digestive tract was removed from each animal and stored individually in plastic universal vials containing 70% ethanol. The digestive tract was then divided into four sections: stomach, small intestine, caecum and colon. These individual sections were examined under a dissecting microscope in a glass Petri dish half filled with distilled water. The contents were washed and checked, and then the mucosa was scraped and checked. Any gastrointestinal helminths (GIH) found were counted and recorded – classified by species based on morphology and categorised as male, female or larvae. Any unidentifiable parasites were noted and stored in 70% ethanol for future identification.

4.2.6.2 Hepatic nematodes – *Calodium hepaticum*

The livers were removed and visually assessed for infection by the nematode *Calodium hepaticum*. Deposited *C. hepaticum* eggs are associated with fibrotic reactions (Redrobe & Patterson-Kane 2005), so infection severity was visually assessed according to the extent of fibrotic burrows in the liver (Jackson *et al.* 2009). Here, a score of 0 meant that fibrotic burrows were absent, while a score of 5 indicated extensive masses of fibrotic burrows were present in multiple lobes.

4.2.6.3 Ectoparasites

Following dissection, the carcass was examined under a dissecting microscope. Using forceps, all body surfaces were thoroughly examined. Any ectoparasites found were noted, categorised by species and individually counted. Any unidentifiable parasites were noted and stored in 70% ethanol for future identification.

Present here were the fur mites *Myobia musculi*, *Radfordia affinis* and *Myocoptes musculus*. These fur mites are primarily detritivorous and do not feed directly on host tissues, so could be classed as commensal rather than parasitic (Jackson *et al.* 2014). Therefore, these were grouped together as 'fur mites' for analyses (Mather & Lausen 1990; Jackson *et al.* 2014).

4.2.7 Data analysis

Due to low prevalence and abundance, *Hymenolepis* sp. and *Trombicula autumnalis* were excluded from species-level analyses. These were included, however, in measures of total GIH abundance/species richness and total ectoparasite abundance/species richness, respectively. Due to the skewed distribution of the data, all parasite abundances, age and serum leptin levels were log-transformed ($\log_{10} [x + 1]$) prior to analyses.

Seasonal variation in prevalence (the proportion of sampled hosts infected by a parasite) was analysed using Fisher's exact test in the

package 'rcompanion' (Mangiafico 2017). A Bonferroni correction was applied to account for multiple testing.

Seasonal variation in intensity (the mean number of parasites found on infected individuals) was analysed by one-way ANOVA, using the 'aov()' function, followed by a post-hoc Tukey's HSD test using the 'TukeyHSD()' function.

To test for interactions between parasite species, zero-inflated regression models were used along with binomial generalised linear models (GLMs) using the package 'pscl' (Jackman 2017). This allowed both count and prevalence data to be considered in a single model. Insignificant terms were dropped using the package 'mpath' (Wang 2017) to generate a minimum adequate model.

To test whether macroparasite infections are related to population size or host diet, parasite abundance, species richness and prevalence were correlated (Spearman's rank correlation coefficient (r_s)) with population size, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and C/N. Spearman's rank correlation coefficients and associated significance levels were generated using the *rcorr()* function in the 'Hmisc' package (Harrell Jr 2017).

To examine the relationship between body condition indices and parasite burden, linear mixed models (LMMs) were fitted with parasite burden as the response. 'Session' was included as a random term to control for seasonal variation. Host age, sex, BMI, relative spleen and liver mass, haematocrit, and serum leptin levels were fitted as explanatory variables. An interaction between age and BMI was also included. LMMs were fitted using the 'nlme' package (Pinheiro *et al.*

2017), and significance levels were determined using Wald's F test. All data analysis was conducted in R version 3.4.0 (R Core Team 2017).

4.3 Results

4.3.1 Macroparasites present

4.3.1.1 Hepatic nematodes – *Calodium hepaticum*

No measure of *C. hepaticum* infection was made in the 2013 or 2014 sessions; livers were only assessed for infection in 2015 and 2016. Of all mice assessed for hepatic infection ($n=261$), 58.2% ($n=152$) were infected by *C. hepaticum*. As *C. hepaticum* infection was given a visual severity score rather than an actual parasite count (Jackson *et al.* 2009), intensity of infection was not recorded.

4.3.1.2 Gastrointestinal helminths

Two nematode species (*Syphacia obvelata* and *Trichuris muris*) were found to infect the Isle of May mice (Figure 4.1). *S. obvelata* had a total prevalence of 30.6% ($n=112$) and intensity of 79.6 while *T. muris* had a prevalence of 46.2% ($n=169$) and intensity of 10.2. The cestode species *Hymenolpis* sp. was detected in just 1.4% ($n=5$) of all mice, with an intensity of 1.4.

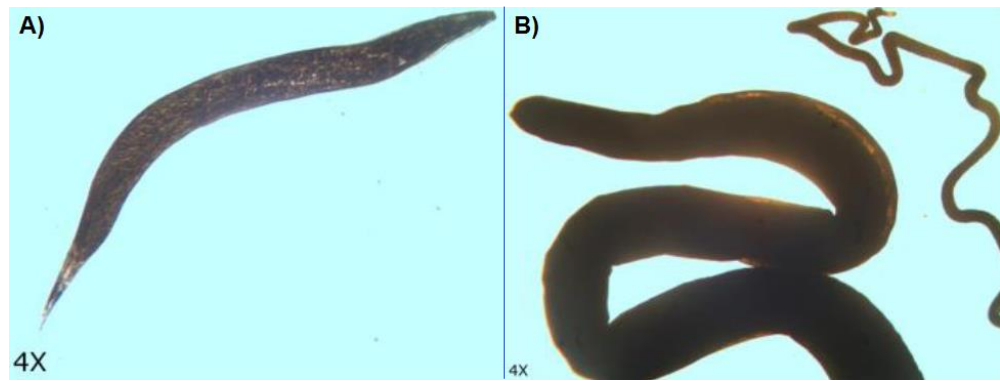


Figure 4.1: Gastrointestinal nematodes found in Isle of May mice: *Syphacia obvelata* (A) and *Trichuris muris* (B). Picture credit: J. Robertson.

4.3.1.3 Ectoparasites

Fur mites (*M. musculi*, *R. affinis* and *M. musculus*) were found on 83.6% ($n=306$) of all mice, with an intensity of 38.6 (Figure 4.2).

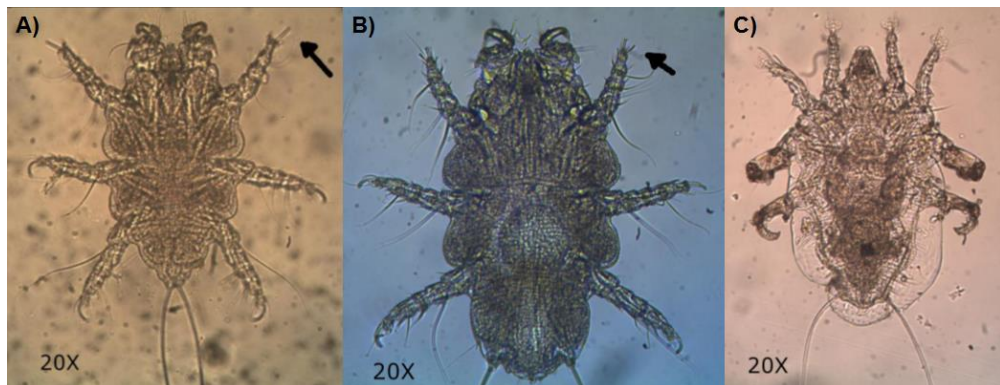


Figure 4.2: Fur mites found on Isle of May mice: *Myobia musculi* (A); *Radfordia affinis* (B) and *Myocoptes musculus* (C). Picture credit: J. Robertson.

The northern rat flea (*Nosopsyllus fasciatus*) was found on 34.2% ($n=125$) of all mice with an intensity of 3.4, and harvest mites (*Trombicula autumnalis*) infected 6.8% ($n=25$) of all mice with an intensity of 10.2 (Figure 4.3).

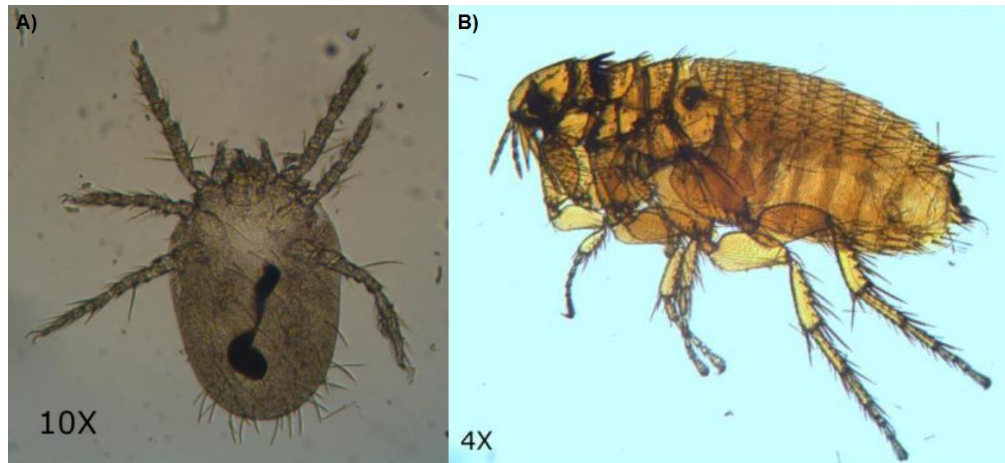


Figure 4.3: Other ectoparasites found on Isle of May mice: harvest mite (*Trombicula autumnalis*) (A) and northern rat flea (*Nosopsyllus fasciatus*) (B). Picture credit: J. Robertson.

4.3.2 Interspecific interactions

S. obvelata burden was significantly negatively associated with *T. muris* burden ($z=-9.627$, $p<0.001$, Figure 4.4). Similarly, *T. muris* burden was significantly negatively associated with *S. obvelata* burden ($z=-7.285$, $p<0.001$, Figure 4.4). Mice infected with *N. fasciatus* had higher burdens of fur mites compared to uninfected individuals ($z=2.813$, $p=0.005$, Figure 4.4), and there was a significant positive relationship between *N. fasciatus* burden and fur mite burden ($z=5.115$, $p<0.001$, Figure 4.4). There were no significant interactions between *C. hepaticum* and any other macroparasite species ($p>0.05$).

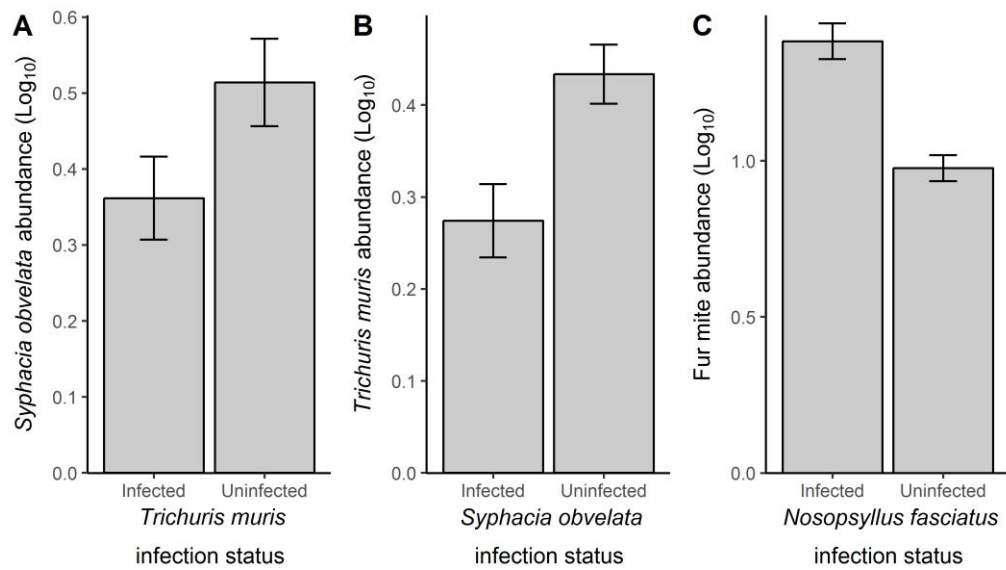


Figure 4.4: Interactions between macroparasite infections of wild *Mus musculus domesticus* on the Isle of May, Scotland. Mice infected with *Trichuris muris* had a significantly higher mean burden of *Syphacia obvelata* (A). Similarly mice infected with *S. obvelata* had a significantly higher mean *T. muris* burden (B). Mice infected with *Nosopsyllus fasciatus* typically had higher fur mite (*Myobia musculi*, *Radfordia affinis* and *Myocoptes musculus*) abundances (C). Bars represent $\pm$ standard error.

4.3.3 Seasonal variation in parasite prevalence

S. obvelata prevalence (Figure 4.5, Table 4.1) was significantly higher in summer 2013 (47.1%) than in summer 2014 (16.7%; $p=0.003$), spring 2015 (10.2%; $p<0.001$) or spring 2016 (0%; $p<0.001$). Prevalence was also significantly higher in autumn 2015 (55.0%) than in summer 2014 (16.7%; $p<0.001$), spring 2015 (10.2%; $p<0.001$), spring 2016 (0%; $p<0.001$) or autumn 2016 (25.9%; $p=0.002$). With no recorded *S. obvelata* infections, spring 2016 had a significantly lower prevalence than summer 2014 (16.7%; $p=0.009$), summer 2015 (31.3%; $p=0.003$) or

autumn 2016 (25.9%; $p<0.001$). There was no significant difference in prevalence between any other seasons ($p>0.08$, Table 4.1).

T. muris prevalence (Figure 4.5, Table 4.1) was significantly higher in spring 2015 (89.8%) than in summer 2013 (47.1%; $p<0.001$), summer 2014 (14.8%; $p<0.001$), summer 2015 (50.0%; $p=0.004$), autumn 2015 (36.0%; $p<0.001$), spring 2016 (45.2%; $p<0.001$) or autumn 2016 (55.6%; $p<0.001$). Prevalence was also higher in autumn 2016 (55.6%) than in summer 2014 (14.8%; $p<0.001$) or autumn 2015 (36.0%; $p=0.046$). Prevalence was lower, however, in summer 2014 (14.8%) than summer 2013 (47.1%; $p=0.002$), summer 2015 (50.0%; $p=0.012$), autumn 2015 (36.0%; $p=0.011$) or spring 2016 (45.2%; $p=0.004$). There was no significant difference in prevalence between any other seasons ($p>0.355$, Table 4.1).

N. fasciatus prevalence (Figure 4.5, Table 4.1) was significantly higher in spring 2015 (57.1%) than in summer 2013 (5.9%; $p<0.001$), summer 2014 (16.7%; $p<0.001$) or autumn 2015 (25.0%; $p<0.001$). Prevalence in spring 2016 (78.6%) was also significantly higher than in summer 2013 (5.9%; $p<0.001$), summer 2014 (16.7%; $p<0.001$), summer 2015 (43.8%; $p=0.045$), autumn 2015 (25.0%; $p<0.001$) or autumn 2016 (37.0%; $p<0.001$). Further, summer 2013 had a significantly lower prevalence of *N. fasciatus* (5.9%) than summer 2015 (43.8%; $p=0.002$), autumn 2015 (25.0%; $p=0.008$), or autumn 2016 (37.0%; $p<0.001$). There was no significant difference in prevalence between any other seasons ($p>0.06$, Table 4.1).

Prevalence of fur mites (Figure 4.5, Table 4.1) was significantly lower in summer 2014 (16.7%) than in summer 2013 (94.1%; $p<0.001$), spring 2015 (93.6%; $p<0.001$), summer 2015 (75.0%; $p<0.001$), autumn 2015 (99.0%; $p<0.001$), spring 2016 (97.6%; $p<0.001$) or autumn 2016 (94.4%; $p<0.001$). Prevalence was also significantly lower in summer 2015 (75.0%) than in autumn 2015 (99%; $p=0.004$) or spring 2016 (97.6%; $p=0.046$). There was no significant difference in prevalence between any other seasons ($p>0.101$).

T. autumnalis prevalence (Figure 4.5, Table 4.1) was significantly higher in summer 2013 (29.4%) than summer 2014 (7.4%; $p=0.025$), autumn 2015 (1.0%; $p<0.001$), spring 2016 (0%; $p=0.001$) or autumn 2016 (0%; $p<0.001$). There was no significant difference in prevalence between any other seasons ($p>0.005$, Table 4.1).

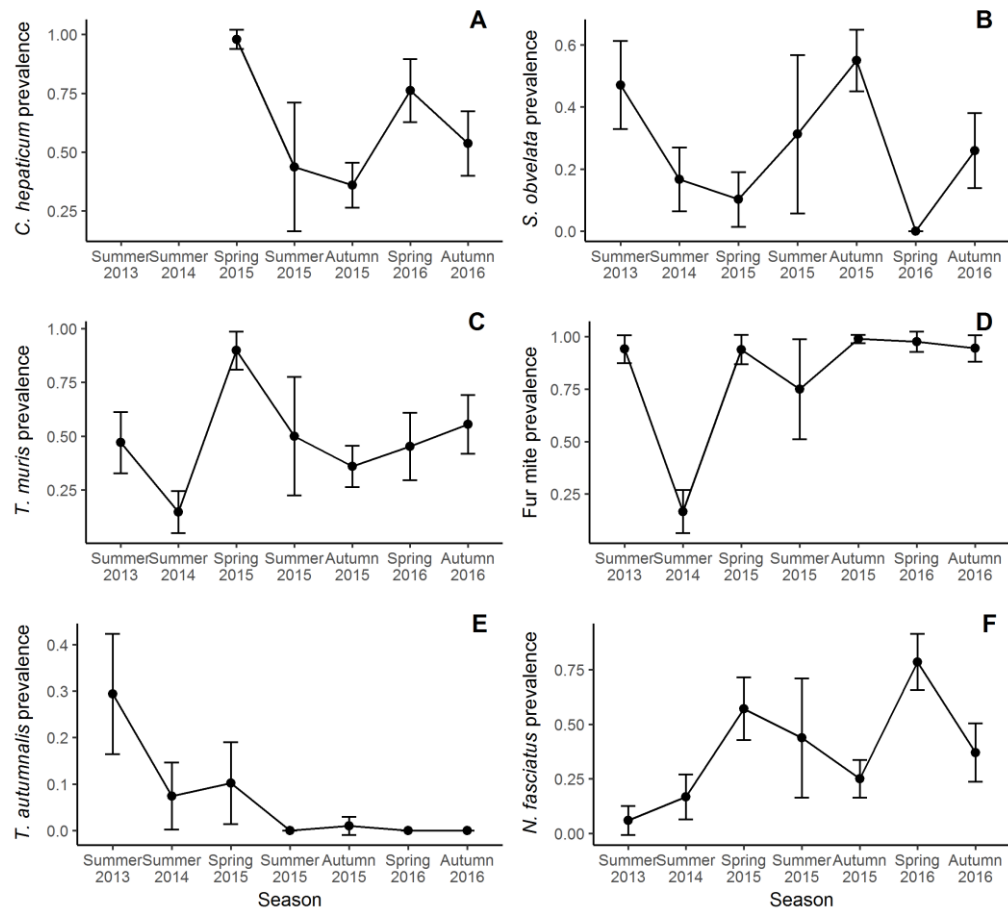


Figure 4.5: Significant seasonal variation in the prevalence of endoparasites (*C. hepaticum* (A), *S. obvelata* (B) and *T. muris* (C)) and ectoparasites (fur mites (D), *T. autumnalis* (E) and *N. fasciatus* (F)) infecting wild *Mus musculus domesticus* from the Isle of May, Scotland. 'Fur mites' combines *Myobia musculi*, *Radfordia affinis* and *Myocoptes musculus* (Mather & Lausen 1990; Jackson *et al.* 2014). Error bars represent 95% confidence intervals.

There were no statistically significant differences in *Hymenolepis* sp. prevalence between seasons ($p=1$). Infection by *Hymenolepis* sp. was not observed in any mice sampled in summer 2013, summer 2015 or autumn 2015 (Table 4.1).

Table 4.1: Prevalence (%) and 95% binomial confidence intervals (C.I.) of macroparasites infecting *Mus musculus domesticus* on the Isle of May across seasons. Includes number of mice sampled (*N*) from each season.

	Summer 2013 <i>N</i> = 51	Summer 2014 <i>N</i> = 54	Spring 2015 <i>N</i> = 49	Summer 2015 <i>N</i> = 16	Autumn 2015 <i>N</i> = 100	Spring 2016 <i>N</i> = 42	Autumn 2016 <i>N</i> = 54	Total <i>N</i> = 366
Nematodes								
<i>Calodium hepaticum</i> ¹	-	-	98.0 (93.9-100.0)	43.8 (16.4-71.1)	36.0 (26.4-45.6)	76.2 (62.8-89.6)	53.7 (40.0-67.4)	58.2 (52.2-64.3) ²
<i>Syphacia obvelata</i>	47.1 (32.9-61.2)	16.7 (6.4-26.9)	10.2 (1.4-19.0)	31.3 (5.7-56.8)	55.0 (45.1-64.9)	0	25.9 (13.9-38.0)	30.6 (25.9-35.3)
<i>Trichuris muris</i>	47.1 (32.9-61.2)	14.8 (5.0-24.6)	89.8 (81.0-98.6)	50.0 (22.5-77.5)	36.0 (26.4-45.6)	45.2 (29.5-60.9)	55.6 (41.9-69.2)	46.2 (41.0-51.3)
Cestodes								
<i>Hymenolepis</i> sp.	0	1.9 (0-9.6)	4.1 (0-9.8)	0	0	2.4 (0-7.2)	1.9 (0-5.6)	1.4 (0.2-2.6)
Arachnids								
Fur mites ³	94.1 (87.4-100.0)	16.7 (6.4-26.9)	93.9 (86.9-100.0)	75.0 (51.2-98.8)	99.0 (97.0-100.0)	97.6 (92.8-100.0)	94.4 (88.1-100.0)	83.6 (79.8-87.4)
<i>Trombicula autumnalis</i>	29.4 (16.5-42.4)	7.4 (0.2-14.6)	10.2 (1.4-19.0)	0	1.0 (0-3.0)	0	0	6.8 (4.2-9.4)
Insects								
<i>Nosopsyllus fasciatus</i>	5.9 (0-12.6)	16.7 (6.4-26.9)	57.1 (42.8-71.5)	43.8 (16.4-71.1)	25.0 (16.4-33.6)	78.6 (65.6-91.5)	37.0 (23.7-50.3)	34.2 (29.3-39.0)

¹No measures of *C. hepaticum* infection were taken in 2013 or 2014.

²The total sample size (*N*) of mice assessed for *C. hepaticum* infection is 261.

³Fur mites' combines *Myobia musculi*, *Radfordia affinis* and *Myocoptes musculinus* (Mather & Lausen 1990; Jackson *et al.* 2014).

4.3.4 Seasonal variation in infection intensity

There was no statistically significant seasonal variation in the intensity of infection (Table 4.2) by *T. autumnalis* ($F_{3,22}=0.18$, $p=0.909$), *T. muris* ($F_{6,162}=0.71$, $p=0.646$), *Hymenolepis* sp. ($F_{3,1}=0.47$, $p=0.761$) or *S. obvelata* ($F_{5,106}=1.95$, $p=0.093$).

The intensity of infection by *N. fasciatus* varied significantly between seasons ($F_{6,118}=2.90$, $p=0.011$, Figure 4.6). Post-hoc analysis revealed that the intensity of *N. fasciatus* infection was significantly higher in both spring 2015 (4.4) and spring 2016 (4.9) than in summer 2014 (3.0; $p<0.001$), autumn 2015 (2.0; $p<0.001$) or autumn 2016 (1.9; $p<0.001$). There was no significant difference in infection intensity between any other seasons ($p>0.071$).

Fur mite infection intensity also varied significantly between seasons ($F_{6,299}=17.71$, $p<0.001$, Figure 4.6). Post-hoc analysis showed that the intensity of fur mite infection was significantly greater in spring 2016 (86.6) than in any other season ($p<0.001$). Intensity was significantly higher in autumn 2015 (37.8) than in summer 2013 (13.9; $p=0.012$) summer 2014 (2.1; $p<0.001$) or summer 2015 (6.8; $p<0.001$). Fur mite infection intensity was also significantly higher in autumn 2016 (51.5) than in summer 2013 ($p<0.001$), summer 2014 ($p=0.012$), spring 2015 (24.5; $p=0.015$) or summer 2015 ($p=0.009$). There was no significant difference in the intensity of fur mite infection between any other seasons ($p>0.132$).

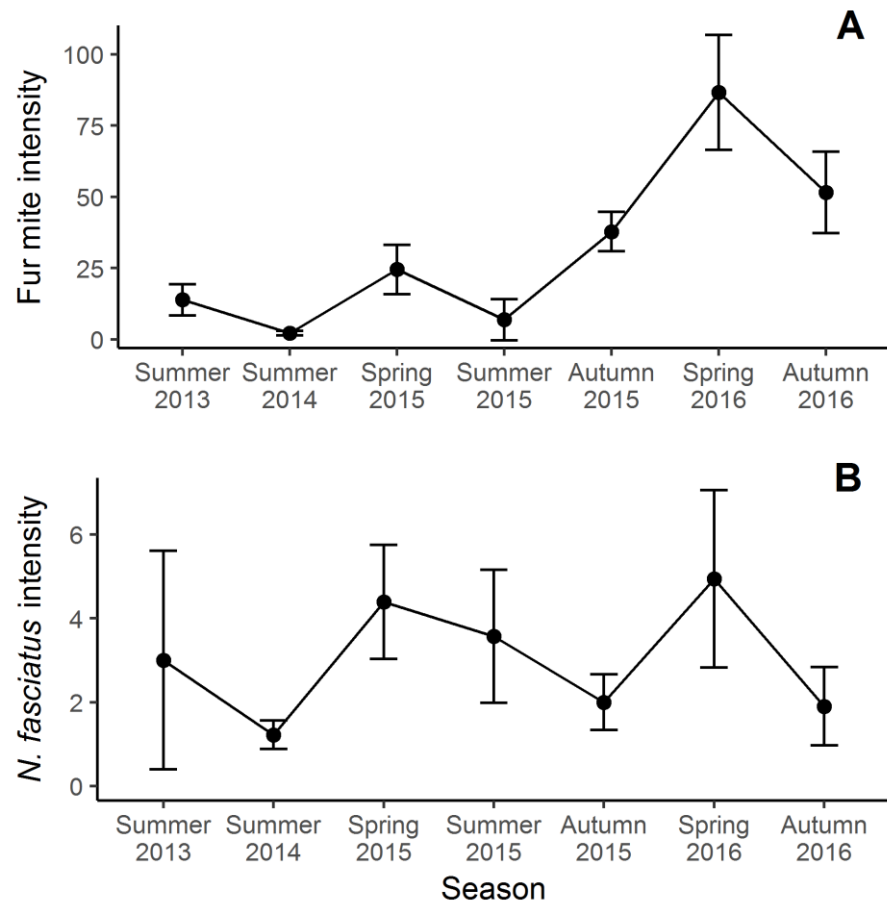


Figure 4.6: Significant seasonal variation in the intensity (mean number of parasites on infected individuals) of fur mite (A) and northern rat flea (*Nosopsyllus fasciatus*) (B) infection on wild *Mus musculus domesticus* on the Isle of May, Scotland. 'Fur mites' combines *Myobia musculi*, *Radfordia affinis* and *Myocoptes musculus* (Mather & Lausen 1990; Jackson *et al.* 2014). Error bars represent standard error.

Table 4.2: Intensity and standard error of macroparasites infecting *Mus musculus domesticus* on the Isle of May across seasons. Intensity is the mean number of parasites on infected individuals. Includes number of mice sampled (*N*) from each season.

	Summer 2013 <i>N</i> = 51	Summer 2014 <i>N</i> = 54	Spring 2015 <i>N</i> = 49	Summer 2015 <i>N</i> = 16	Autumn 2015 <i>N</i> = 100	Spring 2016 <i>N</i> = 42	Autumn 2016 <i>N</i> = 54	Total <i>N</i> = 366
Nematodes								
<i>Syphacia obvelata</i>	136.9 (50.4-223.4)	21.0 (12.9-29.1)	75.4 (0-176.2)	41.4 (0-116.2)	80.2 (52.0-108.3)	0	31.6 (1.7-61.4)	79.6 (55.9-103.2)
<i>Trichuris muris</i>	14.9 (2.8-27.0)	12.6 (0-33.1)	10.3 (7.7-13.0)	11.0 (0-33.1)	5.4 (3.7-7.1)	9.9 (5.3-14.5)	11.3 (0.5-22.2)	10.2 (7.4-13.0)
Cestodes								
<i>Hymenolepis</i> sp.	0	1.0 (0-9.6)	2.0 (0-7.9)	0	0	1.0 (0-7.2)	1.0 (0-5.6)	1.4 (0.7-2.1)
Arachnids								
Fur mites ¹	13.9 (8.3-19.4)	2.1 (1.3-2.9)	24.5 (15.7-33.2)	6.8 (0-14.0)	37.8 (30.8-44.7)	86.6 (66.5-106.7)	51.5 (37.3-65.8)	38.6 (33.5-43.7)
<i>Trombicula autumnalis</i>	13.6 (0-31.2)	7.5 (5.9-9.1)	6.4 (4.0-8.8)	0	4.0 (0-42.1)	0	0	10.5 (0.8-20.3)
Insects								
<i>Nosopsyllus fasciatus</i>	3.0 (0.4-5.6)	1.2 (0.9-1.6)	4.4 (3.0-5.7)	3.6 (2.0-5.2)	2.0 (1.3-2.7)	4.9 (2.8-7.1)	1.9 (1.0-2.8)	3.4 (2.7-4.0)

¹Fur mites¹ combines *Myobia musculi*, *Radfordia affinis* and *Myocoptes musculus* (Mather & Lausen 1990; Jackson *et al.* 2014).

4.3.5 Relationship between host population size and parasites

Prevalence of *S. obvelata* was significantly positively correlated with estimated mouse population size ($r_s=0.899$, $p<0.001$). Ectoparasite species richness ($r_s=-0.342$, $p<0.001$), *C. hepaticum* prevalence ($r_s=-0.811$, $p<0.001$), *T. muris* prevalence ($r_s=-0.277$, $p<0.001$), *N. fasciatus* prevalence ($r_s=-0.912$, $p<0.001$) and abundance ($r_s=-0.446$, $p<0.001$) were all significantly negatively correlated with estimated population size. There were no other significant correlations between parasite measures and estimated population size ($p>0.096$, Table 4.3).

Table 4.3: Spearman's rank correlation coefficients (r_s) for parasite abundance (*N. fasciatus*, *S. obvelata*, *T. muris*, *T. autumnalis*, *Hymenolepis* sp. and fur mites), infection severity (*C. hepaticum*), prevalence and species richness with estimated population size of *Mus musculus domesticus* on the Isle of May, Scotland. Significant relationships ($p<0.05$) are highlighted in bold.

Parasite measure	r_s	p adj
<i>Nosopsyllus fasciatus</i> prevalence	-0.912	<0.001
<i>Syphacia obvelata</i> prevalence	0.899	<0.001
<i>Nosopsyllus fasciatus</i> abundance	-0.446	<0.001
Ectoparasite species richness	-0.342	<0.001
<i>Calodium hepaticum</i> prevalence	-0.811	<0.001
<i>Trichuris muris</i> prevalence	-0.277	<0.001
Endoparasite species richness	0.134	0.096
<i>Syphacia obvelata</i> abundance	0.462	0.104
<i>Calodium hepaticum</i> severity	-0.300	0.260
Fur mite abundance	-0.087	0.358
<i>Hymenolepis</i> sp. prevalence	-0.718	0.358
<i>Trichuris muris</i> abundance	-0.300	0.533
Fur mite prevalence	0.300	0.533
<i>Trombicula autumnalis</i> prevalence	0.112	0.533
<i>Hymenolepis</i> sp. abundance	-0.136	0.624
<i>Trombicula autumnalis</i> abundance	0.051	0.858

4.3.6 Parasite burden and life history

For most macroparasite species included in the body condition LMMs, as well as the combined GIH and ectoparasite variables, burden significantly increased with host age, estimated from eye lens weights ($p < 0.030$, Table 4.4, Figure 4.7). The exception was with *S. obvelata* abundance, in which there was no significant relationship with host age ($F_{1,357} = 2.00$, $p = 0.159$, Table 4.4). There were no significant relationships between any parasite measure and host sex ($p > 0.130$, Table 4.4).

4.3.7 Parasite burden and somatic indices

Mice with a proportionally larger liver had significantly higher burdens of *T. muris* ($F_{1,300} = 7.25$, $p = 0.001$, Figure 4.8), *C. hepaticum* ($F_{1,246} = 63.88$, $p < 0.001$, Figure 4.8), *N. fasciatus* ($F_{1,300} = 7.36$, $p = 0.007$, Figure 4.7) and total ectoparasites ($F_{1,300} = 4.12$, $p = 0.043$, Figure 4.8). Conversely, *S. obvelata* burden decreased with increasing relative liver size ($F_{1,300} = 9.83$, $p = 0.002$, Figure 4.8). There were no significant relationships between any parasite measure and relative spleen size ($p > 0.095$, Table 4.4).

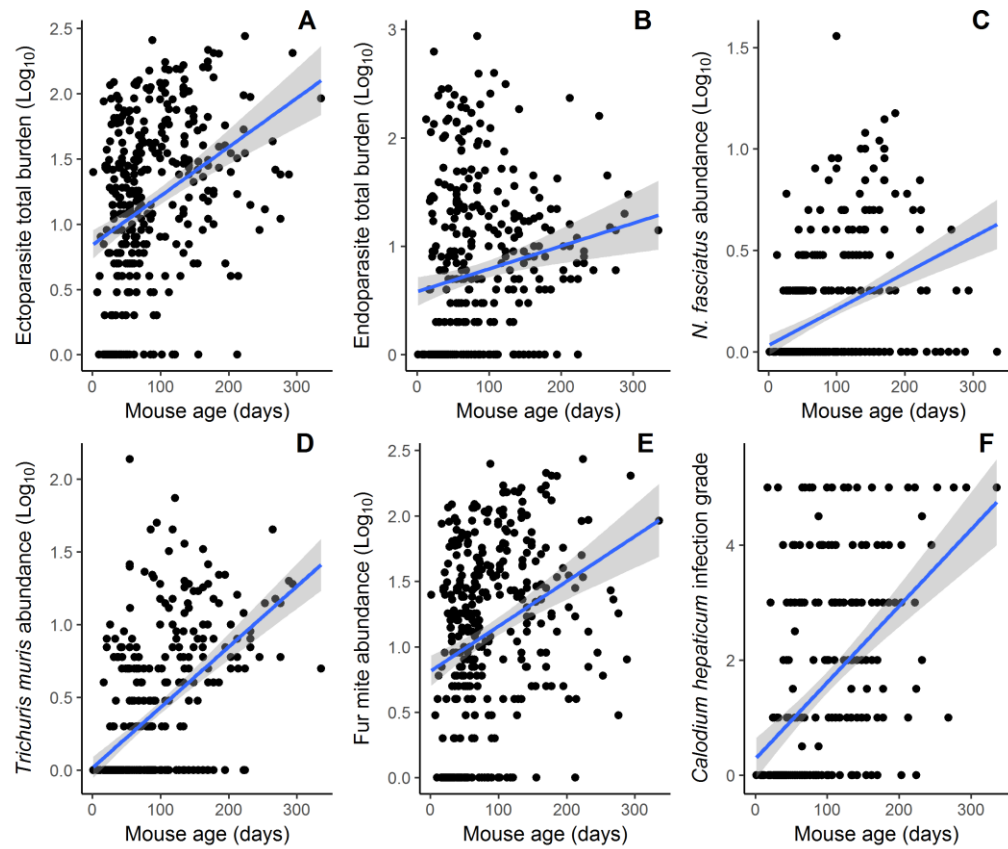


Figure 4.7: Relationship between host age and total ectoparasite (A) and endoparasite (B) abundance in wild *Mus musculus domesticus* from the Isle of May, Scotland. The abundance of *N. fasciatus* (C), *T. muris* (D), fur mites (E) and *C. hepaticum* (F) were significantly positively correlated with host age.

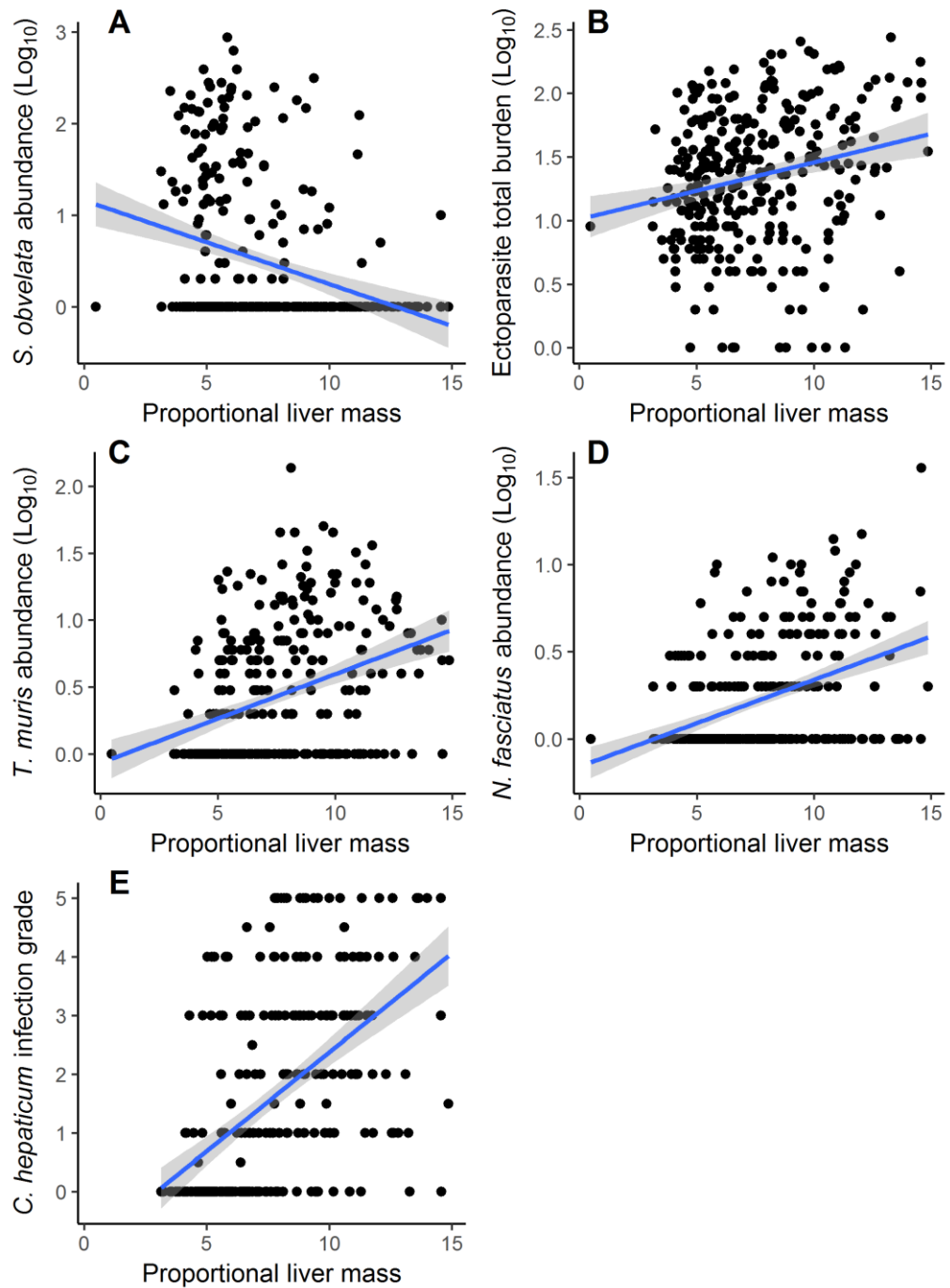


Figure 4.8: Relationship between proportional liver mass (% of total body mass) and macroparasite abundance in wild *Mus musculus domesticus* from the Isle of May, Scotland. *S. obvelata* abundance has a significant negative relationship with proportional liver mass (A), while the abundances of ectoparasites (B), *T. muris* (C), *N. fasciatus* (D) and *C. hepaticum* (E) are significantly positively correlated with proportional liver mass.

4.3.8 Parasite burden and circulating leptin concentrations

Animals with higher concentrations of leptin – indicative of increased body fat – typically had lower abundances of *S. obvelata* and overall endoparasites (Figure 4.9). Increasing levels of circulating leptin showed a significant relationship with decreasing *S. obvelata* burdens ($F_{1,286}=4.88$, $p=0.028$), as well as the total burdens of GIH ($F_{1,290}=6.73$, $p=0.015$).

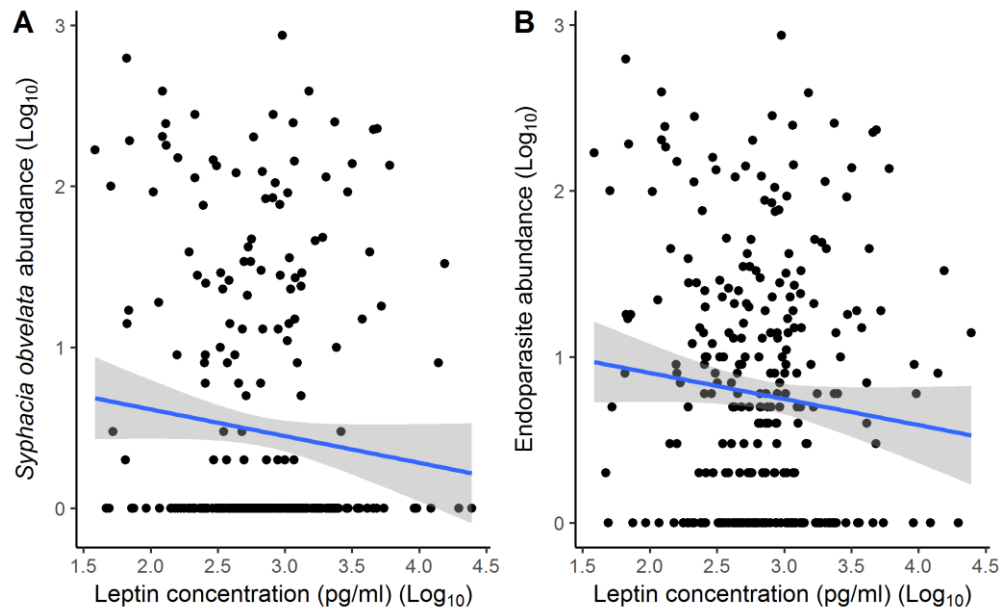


Figure 4.9: Relationship between circulating leptin levels and macroparasite abundance in wild *Mus musculus domesticus* from the Isle of May, Scotland. *S. obvelata* abundance (A) and total endoparasite abundance (B) were significantly negatively correlated with circulating leptin levels.

4.3.9 Parasite burden and heamatocrit

Increased haematocrit levels were associated with decreased *C. hepaticum* infection ($F_{1,138}=4.54$, $p=0.035$, Figure 4.10) and *T. muris* burden ($F_{1,135}=5.21$, $p=0.024$, Figure 4.10).

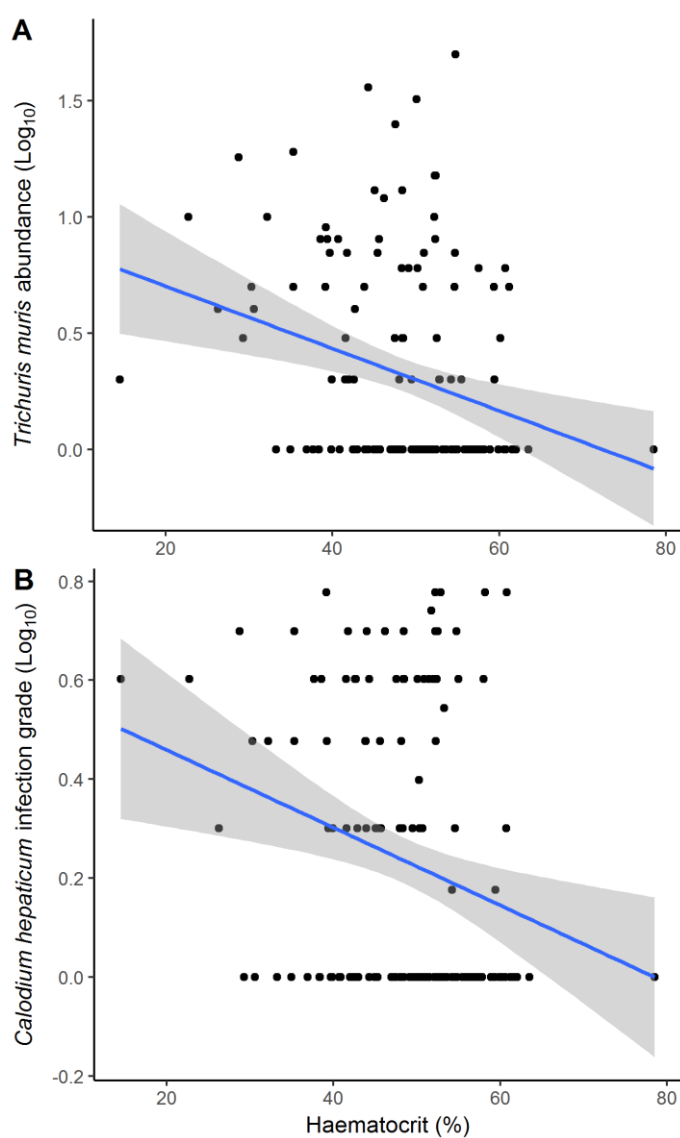


Figure 4.10: Relationship between heamatocrit (estimated proportion of red blood cells in whole blood) and *Trichuris muris* abundance (A) and *Calodium hepaticum* infection grade (B) in wild *Mus musculus domesticus* from the Isle of May, Scotland.

4.3.10 Parasite burden and BMI

Individuals with a higher BMI typically had increased *T. muris* burdens ($F_{1,357}=10.49$, $p=0.001$, Table 4.4). This relationship was only present in older, reproductively active animals ($F_{1,357}=12.33$, $p<0.001$, Figure 4.11). In young, non-breeding individuals, those animals with a higher BMI typically had a lower *T. muris* burden (Figure 4.11).

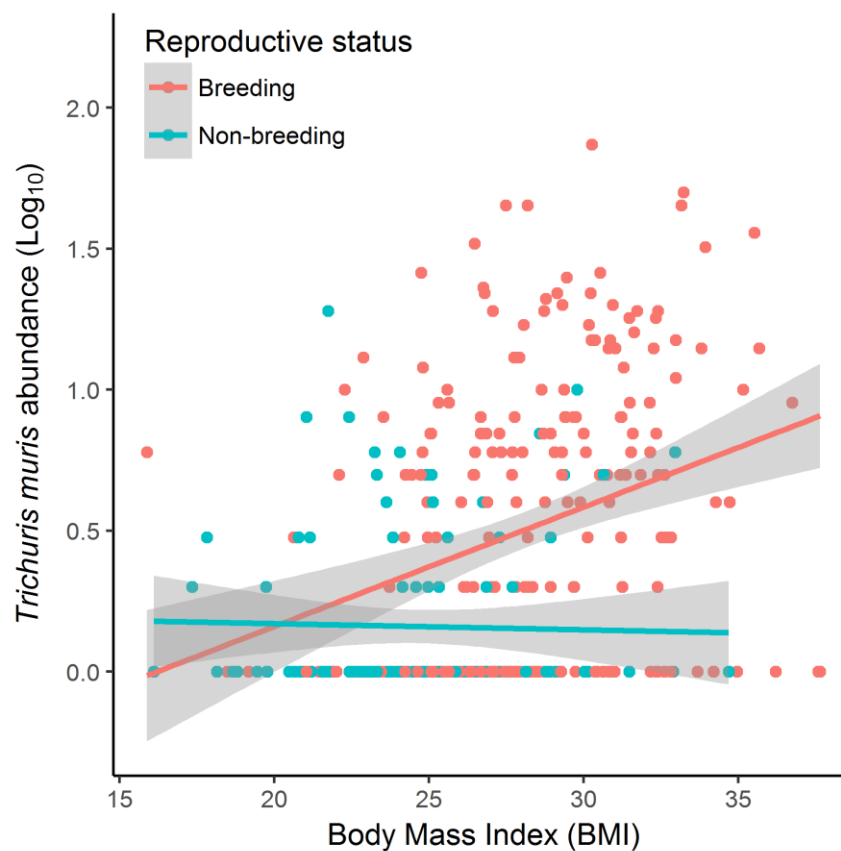


Figure 4.11: Relationship between BMI and *Trichuris muris* abundance in wild *Mus musculus domesticus* from the Isle of May, Scotland. Trend lines indicate the different relationships for breeding, i.e. mature, mice and non-breeding, i.e. juvenile, mice.

Table 4.4: Association of measures of body condition and life history with parasitic infection in wild house mice (*Mus musculus domesticus*) from the Isle of May, as measured by linear mixed models (LMMs). Infection data were parasite abundance (Log₁₀), apart from *C. hepaticum* in which infection was visually scored according to severity (Jackson *et al.* 2009). 'Fur mites' combines *Myobia musculi*, *Radfordia affinis* and *Myocoptes musculinus* (Mather & Lausen 1990; Jackson *et al.* 2014). 'Ectoparasites' is the total abundance of fur mites, *N. fasciatus* and *Trombicula autumnalis*. 'GI helminths' is the total abundance of *S. obvelata*, *T. muris* and *Hymenolepis* sp. Spleen and liver size are organ mass as a proportion of total body mass. In these LMMs, 'Season' was included as a random effect. Significant ($p < 0.05$) factors are marked in bold.

	<i>Calodium hepaticum</i>		<i>Syphacia obvelata</i>		<i>Trichuris muris</i>		Fur mites		<i>Nosopsyllus fasciatus</i>		GI helminths		Ectoparasites	
Sex	$F_{1,246}=0.15$	0.699	$F_{1,357}=1.24$	0.266	$F_{1,357}=1.92$	0.167	$F_{1,357}=0.02$	0.904	$F_{1,357}=0.40$	0.529	$F_{1,357}=2.31$	0.130	$F_{1,357}=0.14$	0.707
Age	$F_{1,246}=59.91$	<0.001	$F_{1,357}=2.00$	0.159	$F_{1,357}=60.01$	<0.001	$F_{1,357}=6.84$	0.009	$F_{1,357}=4.78$	0.030	$F_{1,357}=27.03$	<0.001	$F_{1,357}=14.72$	<0.001
BMI	$F_{1,246}=2.44$	0.120	$F_{1,357}=0.53$	0.467	$F_{1,357}=10.49$	0.001	$F_{1,357}=0.07$	0.792	$F_{1,357}=2.29$	0.131	$F_{1,357}=1.16$	0.283	$F_{1,357}=0.31$	0.578
Age*BMI	$F_{1,246}=0.09$	0.768	$F_{1,357}=0.21$	0.644	$F_{1,357}=12.33$	<0.001	$F_{1,357}=0.41$	0.523	$F_{1,357}=0.16$	0.693	$F_{1,357}=1.48$	0.225	$F_{1,357}=0.91$	0.340
Liver	$F_{1,246}=63.88$	<0.001	$F_{1,300}=9.83$	0.002	$F_{1,300}=7.25$	0.001	$F_{1,300}=3.14$	0.077	$F_{1,300}=7.36$	0.007	$F_{1,300}=1.69$	0.195	$F_{1,300}=4.12$	0.043
Spleen	$F_{1,246}=0.01$	0.945	$F_{1,246}=2.34$	0.128	$F_{1,250}=2.26$	0.134	$F_{1,250}=2.80$	0.095	$F_{1,250}=0.62$	0.431	$F_{1,250}=0.60$	0.440	$F_{1,250}=2.48$	0.117
Leptin	$F_{1,189}=0.59$	0.440	$F_{1,286}=4.88$	0.028	$F_{1,286}=1.74$	0.188	$F_{1,290}=2.61$	0.107	$F_{1,290}=2.07$	0.151	$F_{1,290}=6.73$	0.015	$F_{1,290}=4.14$	0.083
PCV	$F_{1,138}=4.54$	0.035	$F_{1,135}=1.51$	0.222	$F_{1,135}=5.21$	0.024	$F_{1,135}=0.08$	0.275	$F_{1,135}=2.44$	0.121	$F_{1,135}=0.19$	0.662	$F_{1,135}=1.56$	0.215

4.3.11 Relationship between diet and parasites

Both endoparasite species richness ($r_s=0.240$, $p=0.040$, Figure 4.12) and total species richness ($r_s=0.280$, $p=0.016$) were significantly positively correlated with levels of $\delta^{15}\text{N}$. Further, *S. obvelata* abundance also had a significant positive correlation with levels of $\delta^{15}\text{N}$ ($r_s=0.356$, $p=0.002$, Figure 4.12). There was no significant correlation between levels of $\delta^{15}\text{N}$ and abundances of *C. hepaticum* ($r_s=0.015$, $p=0.900$), *N. fasciatus* ($r_s=0.060$, $p=0.613$), *T. muris* ($r_s=0.024$, $p=0.839$), fur mites ($r_s=-0.040$, $p=0.737$) or ectoparasite species richness ($r_s=0.148$, $p=0.210$).

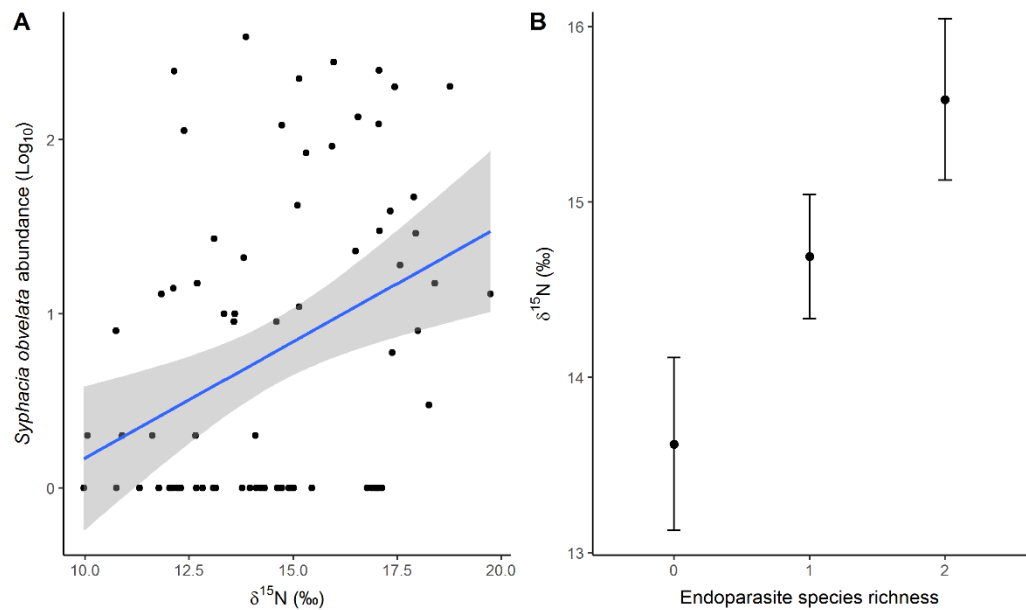


Figure 4.12: Relationship between nitrogen isotope values ($\delta^{15}\text{N}$) and *Syphacia obvelata* abundance (A) and endoparasite species richness (B) in wild *Mus musculus domesticus* from the Isle of May, Scotland.

There were no significant correlations between C/N ($r_s < 0.139$, $p > 0.239$) or levels of $\delta^{13}\text{C}$ ($r_s < 0.102$, $p > 0.380$) and any measures of parasite abundance or species richness (Table 4.5).

Table 4.5: Spearman's rank correlation coefficients (r_s) for parasite abundance (*N. fasciatus*, *S. obvelata*, *T. muris*, fur mites), infection severity (*C. hepaticum*) and species richness with carbon and nitrogen isotope values for muscle from *Mus musculus domesticus* on the Isle of May, Scotland. Significant relationships ($p < 0.05$) are highlighted in bold.

Stable isotope/parasite measure		r_s	p
$\delta^{13}\text{C}$	<i>Calodium hepaticum</i>	0.102	0.388
	<i>Nosophyllus fasciatus</i>	-0.050	0.671
	<i>Syphacia obvelata</i>	-0.041	0.726
	<i>Trichuris muris</i>	0.039	0.739
	Fur mites	-0.097	0.409
	Ectoparasite species richness	-0.104	0.380
	Endoparasite species richness	0.002	0.986
$\delta^{15}\text{N}$	<i>Calodium hepaticum</i>	0.015	0.900
	<i>Nosophyllus fasciatus</i>	0.060	0.613
	<i>Syphacia obvelata</i>	0.356	0.002
	<i>Trichuris muris</i>	0.024	0.839
	Fur mites	-0.040	0.737
	Ectoparasite species richness	0.148	0.210
	Endoparasite species richness	0.240	0.040
C/N	<i>Calodium hepaticum</i>	-0.046	0.698
	<i>Nosophyllus fasciatus</i>	0.124	0.297
	<i>Syphacia obvelata</i>	0.103	0.388
	<i>Trichuris muris</i>	0.051	0.671
	Fur mites	-0.025	0.831
	Ectoparasite species richness	0.139	0.239
	Endoparasite species richness	0.073	0.540

4.4 Discussion

This study represents the first detailed, multi-seasonal record of the macroparasites infecting wild house mice on the Isle of May. Observed here were the GIHs *S. obvelata*, *Hymenolepis* sp. and *T. muris*, the hepatic nematode *C. hepaticum*, the flea *N. fasciatus* and the mites *M. musculi*, *M. musculus*, *R. affinis* and *T. autumnalis*.

T. spiralis was present in mice trapped on the Isle of May in January 2013 (Robertson 2014), but wasn't recorded in 1936 (Southern 1938) or in this present study. It is possible that this infection was transmitted to these mice by eating infected meat, either from the food stores of the island staff or by scavenging seal or bird carcasses (Robertson 2014). However, the lack of location data or samples suitable for stable isotope analysis for these individuals means it is not possible to determine this. It is also possible that *T. spiralis* was misidentified.

4.4.1 Relationships with host population size

The prevalence of *C. hepaticum*, *T. muris* and *N. fasciatus*, as well as ectoparasite species richness and *N. fasciatus* abundance were significantly negatively correlated with estimated population size. As observed in bank and field voles, animals captured at the end of a population crash (in this instance individuals captured in spring) are more likely to be infected and suffering heavy disease burdens (Soveri *et al.*

2000). Here, however, prevalence of *S. obvelata* is highest during the estimated population peak. This is likely related to the short generation time of *S. obvelata* (Chan 1952) and the increased likelihood of transmission in high population densities (Anderson & May 1978, 1979; Rifkin *et al.* 2012).

In both years it was recorded, the prevalence of *C. hepaticum* was significantly higher in spring than any other season. Previous studies of seasonality in *C. hepaticum* infection in rodents have produced varied results; in Norway rats (*Rattus norvegicus*), black rats (*R. rattus*) and Natal multimammate mice (*Mastomys natalensis*) from South Africa there was no seasonal variation in prevalence (Archer *et al.* 2017), while *R. norvegicus* in Canada have a higher prevalence in autumn (Rothenburger *et al.* 2014) and the prevalence in *M. musculus* and *R. rattus* from Pakistan peaked in the summer (Ahmad *et al.* 2011).

4.4.2 Relationship with host sex

Here, no relationship with sex was found with any parasite measure. Typically, male animals are more parasitised than females (Poulin 1996; Zuk & McKean 1996; Moore & Wilson 2002). This can be due to the immunosuppressive effects of testosterone (Klein 2004; Trigunaite *et al.* 2015) increasing susceptibility to infection, as well as male competitive behaviours and dispersal increasing the likelihood of

contact with parasites (Zuk & McKean 1996; Klein 2000). A recent study of feral horses (*Equus caballus*), however, found that females tended to have increased parasite burdens (Debeffe *et al.* 2016). The lack of relationship observed here could be due to the extensive overlap in individual territory and the communal living exhibited by Isle of May mice (Triggs 1991; Gray & Hurst 1998). This could lead to increased exposure to parasites regardless of host gender. Indeed, a survey of *M. musculus* and *R. rattus* in Sicily found no differences in levels of parasitism by helminths and ectoparasites between sexes (Milazzo *et al.* 2003).

4.4.3 Parasite burdens increase with host age

All parasite burdens, with the exception of *S. obvelata*, increased with mouse age. Increasing parasite burdens with age are common among wild mammals (Santín-Durán *et al.* 2008; Hayward *et al.* 2009; Body *et al.* 2011; Burlet *et al.* 2011), and can be related to immunosenescence increasing susceptibility to infection (Hayward *et al.* 2009) or the accumulation of parasites over time (Burlet *et al.* 2011). Alternatively, increased parasite loads in older animals can be an indicator of tolerance (Jackson *et al.* 2014).

4.4.4 Patterns of tolerance

The increasing parasite burden in individuals of higher body condition, as measured by various markers, provides further evidence of tolerance. For example, animals with proportionally larger livers – an indicator of high body condition in wild rodents (Norrdahl *et al.* 2004; Jackson *et al.* 2014) – had increased abundances of *T. muris*, *N. fasciatus* and total ectoparasites, as well as more severe *C. hepaticum* infections. It is important to note, however, that *C. hepaticum* infection can cause granulomatous inflammation (Sinniah *et al.* 2014), and is typically associated with hepatomegaly (Ceruti *et al.* 2001). This could, therefore, explain the relationship observed between increasing proportional liver mass and higher levels of infection by *C. hepaticum*, but does not explain the relationship seen with GIHs and *N. fasciatus*.

The increasing *T. muris* burden with BMI in adult animals, but not in immature animals, could be a further indicator of a shift towards a tolerance (Jackson *et al.* 2014). A similar pattern has been observed in field voles, where mature animals in better body condition tend to have increased macroparasite burdens and are able to mount an elevated Th2 response (Jackson *et al.* 2014).

T. muris burden and *C. hepaticum* infection severity were highest in animals with lower haematocrit levels. While haematocrit can be used as a measure of individual health and condition (Milenkaya *et al.* 2013), high haematocrit levels can result from dehydration (Hammond *et al.*

2000; Fair *et al.* 2007). Further, increased haematocrit levels can increase blood the viscosity of blood (Birchard 1997) causing oxygen transport to be reduced (Birchard 1997; Schuler *et al.* 2010). Indeed, in wild-type laboratory mice, exhaustion during exercise occurs faster at higher than optimal haematocrit values (Schuler *et al.* 2010). Therefore, high haematocrit levels may be indicative of poor body condition, further supporting the idea that mice in better condition are better able to tolerate *T. muris* and *C. hepaticum* infection.

4.4.5 *Syphacia obvelata* infection and body condition

Increased $\delta^{15}\text{N}$ enrichment was associated with increased *S. obvelata* abundances and endoparasite species richness. Enrichment of $\delta^{15}\text{N}$ can be an indicator of nutritional stress, as has been observed in a range of taxa including humans (Mekota *et al.* 2006), American black bears (*Ursus americanus*) (Lohius *et al.* 2007), Japanese quail (*Coturnix japonica*) (Hobson *et al.* 1993) and Arctic ground squirrels (*Spermophilus parryi*) (Ben-David *et al.* 1999). Indeed, $\delta^{15}\text{N}$ negatively correlates with body condition in captive guppies (*Poecilia reticulata*) and a range of wild freshwater fish species (Bowes *et al.* 2014).

The increasing *S. obvelata* burden and ectoparasite species richness in animals with higher $\delta^{15}\text{N}$ levels could, therefore, be linked to their poor body condition. Indeed, both *S. obvelata* and total endoparasite

abundance, as well as total ectoparasite abundance, are negatively correlated with leptin levels. Further, *S. obvelata* abundance decreases with increasing proportional liver mass.

S. obvelata infection stimulates a vigorous Th2 response, associated with tolerance, in laboratory mice (Michels *et al.* 2006). The lack of any apparent tolerance to *S. obvelata* here, however, could be due to its short generation time and adult lifespan (Chan 1952), meaning that instantaneous population counts may not reflect the recent history of infection (Jackson *et al.* 2009). Further, *S. obvelata* feeds in the gut lumen at all stages of the life-cycle, and so would have less contact with the immune system than nematodes that actively feed on tissues, such as *T. muris* (Jackson *et al.* 2009).

4.4.6 Interactions between *S. obvelata* and *T. muris*

It is important to note that there is a negative association between *S. obvelata* and *T. muris* infection in these mice; individuals with a higher *T. muris* burden are more likely to have a lower *S. obvelata* burden, and vice versa. This is likely related to the fact that the Th2-type response is suppressed by *T. muris* (Cliffe & Grencis 2004), while it is induced by *S. obvelata* (Michels *et al.* 2006). Therefore, in mice already infected with *S. obvelata* a *T. muris* burden may fail to establish or abundances may be suppressed due to this Th2 induction. Indeed, a Th2 response has been

shown to clear *T. muris* infection prior to worms reaching fecundity in laboratory mice (Bancroft *et al.* 1994; Cliffe & Grencis 2004; Demiri *et al.* 2017). Further, *S. obvelata* and *T. muris* both inhabit the caecum (Panesar & Croll 1980; Michels *et al.* 2006), so interspecific competition for space and resources could contribute to this negative relationship.

4.4.7 Relationship between immune response and parasitism

While splenomegaly is associated with increased parasite infection in a range of taxa (Morand & Poulin 2000; Brown & Brown 2002; Lefebvre *et al.* 2004; Stenkewitz *et al.* 2015), no relationship between spleen size and levels of infection was observed here. Therefore it is likely that spleen size in these mice is more closely related to body condition and the ability for those animals in better condition to mount a stronger immune response rather than any measure of parasitism – a pattern that has been observed in various bird species (Møller *et al.* 1998b), American mink (*Neovison vison*) (Schulte-Hostedde & Elsasser 2011) and wild-derived house mice (Goüy de Bellocq *et al.* 2007). Indeed, wild mice have proportionally smaller spleens than laboratory mice (Abolins *et al.* 2017), despite these spleens containing proportionally more T helper and phagocytic cells as a response to systemic infection (Abolins *et al.* 2017). Further, Tian *et al.* (2015) found no relationship between spleen size and circulating lymphocytes in wild-derived house mice. Therefore, relative spleen size may not be an accurate measure of

immunocompetence in wild mice (Tian *et al.* 2015), so further, detailed immunological studies are required (see Chapter 6).

4.4.8 Future steps

The interactions between micro- and macroparasite infection can have profound effects on host body condition and population dynamics (Cizauskas *et al.* 2015). While recording microparasites was beyond the scope of this study, the haemoparasites *Babesia microti* and *Bartonella* sp. have been identified in these mice (S. Goertz, unpublished). *B. microti* is tick-borne, and is likely transmitted by the seabird tick (*Ixodes uriae*), a species commonly found on the islands nesting seabirds (Nunn *et al.* 2005). However, no tick species have been observed on Isle of May mice. Investigating the coinfection dynamics between micro- and macroparasites, and their relationship with body condition, is key for future research in this system.

Chapter 5

Relationships between immune gene expression and circulating cytokine levels in wild mice

5.1 Introduction

Since the field of ecoimmunology emerged over two decades ago (Sheldon & Verhulst 1996), measuring the immune function of wild animals has remained a challenging undertaking (Pedersen & Babayan 2011). To date, the vast majority of ecoimmunological studies have used non-model species (Jackson 2015), and so have been limited in their scope or by the availability of appropriate assays or reagents (Fassbinder-Orth 2014; Zimmerman *et al.* 2014). Because of this, many early studies relied on less specific immunological assays, such as leukocyte counts, haemagglutination, phytohaemagglutinin and bactericidal assays (Demas *et al.* 2011; Pedersen & Babayan 2011; Zimmerman *et al.* 2014).

As modern sequencing technology has become more accessible and affordable, it has become possible to measure immune responses in non-model organisms using more specific assays (Jackson 2015). For example, quantitative real-time PCR (qPCR) can be used to measure the

expression of immune genes, such as cytokines, via mRNA concentrations. This gene expression-based approach has been widely used to investigate immune function in wild populations of a range of taxa (Fassbinder-Orth 2014).

Gene expression is regularly used as a proxy for measuring functional protein products (Fassbinder-Orth 2014), and it is often assumed that the levels of mRNA expression will correlate with the levels of the corresponding proteins (Maier *et al* 2009; Vogel & Marcotte 2012; Fassbinder-Orth 2014). Indeed, many researchers have focussed solely on qPCR and measuring levels of gene expression (Koussounadis *et al* 2015). While protein concentration can be predicted from mRNA concentration with some accuracy in yeast (Csardi *et al* 2015), the correlation in mammalian cells is often much lower and dependent on cell type and state (Silva & Vogel 2016) – indeed, only around 40% of variation in cellular protein levels can be predicted by mRNA concentrations (Kendrick 2014; Vogel & Marcotte 2012). Therefore, mRNA concentrations may not always accurately represent the expression levels of the bioactive protein (Pradet-Balade *et al.* 2001; Jackson 2015)

Further, the immune response may be localised and tissue specific (Hu & Pasare 2013). For example, the genes expressed by spleen tissue may reflect a systemic response while genes expressed by MLNs may be a localised GI response (see Chapter 1). The localisation of immune environments is not, however, dictated by clear-cut divisions.

Activated proteins from local tissues may ‘spill-over’ – so the local response from MLN may, for example, be detectable in serum cytokine concentrations.

Secreted cytokines circulating in the blood typically represent those not consumed at local sites of infection or rapidly removed from circulation (Aziz *et al.* 2015). In humans, accurate measurement of circulating cytokines can be used in disease diagnosis (Aziz *et al.* 2015), such as in the detection of HIV infection (Nakajima *et al.* 1989; Fahey *et al.* 1990; Salazar-Gonzalez *et al.* 1997; Aziz *et al.* 1998).

Although these studies may indicate an induced immune response in the host (Jackson 2015), it should not necessarily be assumed that levels of gene expression completely align with their corresponding effector proteins. In addition to localisation of responses, there may be levels of regulation occurring between genes, or delays between transcription and translation and protein modification (Pradet-Balade *et al.* 2001; Payne 2015). Indeed, studies have found that the extent to which mRNA expression correlates with the final gene products can vary with transcriptional and post-translational processes, and that the rates of decay between the two can significantly differ (Schwanhäusser *et al.* 2011; Munsky *et al.* 2012).

Therefore, making robust measures of molecular phenotype requires the measurement of active proteins using antibody reagents against key immunological biomolecules (Jackson 2015). This is particularly important when dealing with natural populations in which

variation is intrinsically higher (Abolins *et al.* 2011; Abolins *et al.* 2017). Protein levels can only reliably be analysed using sophisticated assays that are typically only available for model species, such as house mice (*Mus musculus*). For these species, commercial off-the-shelf reagents are readily available. Due to structural variation in immune molecules such as cytokines, however, the transferability and cross-reactivity of these assays to non-model species is extremely limited (Jackson 2015). Further, the discovery of any *M. musculus* antibodies that will successfully cross-react with cytokines from other rodent species can involve lengthy and costly 'trial and error' processes (e.g. Oko *et al.* 2006; Jackson *et al.* 2009; Friberg *et al.* 2011). Developing specific antibodies is technically possible, however this too is expensive and time-consuming (Oko *et al.* 2006; Bradley & Jackson 2008; Friberg *et al.* 2010; Pederson & Babayan 2011; Jackson 2015). Therefore, it is informative to understand the precise relationships between measured gene expression and the effector proteins that will actually interact at the immune interface with a pathogen or parasite, particularly in wild populations.

Recently, however, a number of studies have investigated the immune function of wild populations of model species, primarily house mice. These have measured immune function by measuring antibody concentrations and avidity (Lochmiller *et al.* 1991; Abolins *et al.* 2011), or circulating cytokine concentrations (Abolins *et al.* 2017).

Here, using the natural population of *M. m. domesticus* on the Isle of May, we used qPCR to measure the expression levels of key cytokine genes representing four arms of the adaptive immune response (Th1, Th2, Treg and Th17), as well as the innate immune response. These were measured from two key immunological tissues; the spleen and MLN. The circulating levels of the corresponding secreted cytokines were measured from serum by multiplex bead assay. Further, the relative mRNA expression of several important immunity-related transcription factors was measured using qPCR from spleen tissue.

We aim to assess the relationship between the expression of these key cytokine genes and the corresponding circulating protein levels in a natural population of *M. m. domesticus*. Further, by using both MLN and spleen tissue, we aim to explore the relationship between immune gene expression from two immunologically important tissue types.

Due to post-translational processes and protein degradation, we hypothesised that there would not be widespread correlation between gene expression and circulating cytokine levels. Where correlation may exist, we would expect this between gene expression levels from spleen tissue and circulating cytokines as these both represent the systemic immune response.

The expression level of genes from MLN tissue, however, are unlikely to relate to the expression level of genes from spleen tissue or concentrations of circulating cytokines as these represents a localised, gastrointestinal response. Further, we hypothesised that there may be

antagonistic relationships between some response types. For example, the Th1-type response would not be expected to correlate with the Th2-type response due to their antagonistic relationship (Kaiko *et al.* 2008).

5.2 Methods

5.2.1 Sample collection

The trapping, processing and euthanasia of mice on the Isle of May was as described in Chapter 2. Immediately following the confirmation of death, a sample of blood was removed to a 1.5 mL microcentrifuge tube. This was stored at room temperature for ~60 minutes, then stored at 4°C for a further ~60 minutes. The resulting clot was carefully removed, and the remaining plasma was spun at 7,000 rpm for ten minutes. The serum was removed and snap frozen using liquid nitrogen, before being stored at -80°C until its use in multiplex bead assays.

The spleen and MLN were removed and placed in RNAlater solution (Life Technologies). Following the manufacturer's recommendations, tissues were sectioned into smaller pieces (≤ 5 mm) prior to immersion in the solution. Samples were kept at 4°C for 24 hours, then the supernatant was removed, and samples were stored at -80°C until RNA extraction.

5.2.2 RNA extraction & cDNA synthesis

RNA was extracted from up to 30 mg spleen and MLN tissue stored in RNAlater using the NucleoSpin RNA kit (Machery-Nagel) following the manufacturers' protocol (see Appendix 3.1). A DNase treatment step is included in this protocol, and RNA was eluted in 60 μ L of nuclease free water. The purity and concentration of RNA was assessed on a NanoDrop 1000 spectrophotometer (Thermo Scientific), with a desired 260/280 absorbance ratio of >1.80 (Robertson *et al.* 2016). To check for contamination, and to assess the purity of RNA, 6 μ L of each sample was mixed with 4 μ L of Orange G gel loading dye (Sigma Aldrich). This was incubated at 65°C for 10 minutes, followed by visualisation on a 2% agarose gel stained with ethidium bromide run at 90V for 30 minutes.

Synthesis of cDNA was performed on up to 2 μ g of total RNA using the nanoScript2 Reverse Transcription kit (Primerdesign), using a combination of oligo-dT and random nonamer primers (0.5 μ L of each). Nuclease free water was used to make a total reaction volume of 7 μ L. This was incubated at 65°C for five minutes, before being cooled on ice. To this, 10 μ L of extension-mix (5.0 μ L of nanoScript2 4X Buffer, 1.0 μ L of 10 mM dNTP mix, 1.0 μ L of nanoScript2 enzyme and 3.0 μ L of nuclease free water) was added. This was incubated at 25°C for five minutes then 42°C for 20 minutes, before being heat inactivated at 75°C for ten minutes. No-enzyme controls and intron-spanning PCR were used to monitor samples for contamination by genomic DNA. All cDNA

samples were diluted 1:10 with nuclease free water and then stored at -20°C for future analysis by qPCR. For full details, see Appendix 3.2.

5.2.3 qPCR Conditions

qPCR was carried out to measure normalised mRNA expression in spleen and MLN tissue of a suite of genes reflecting different functional arms of the immune system. mRNA levels of seven cytokine genes of interest were measured from both spleen and MLN tissue including: Interferon- γ (IFN- γ), Interleukin (IL)-1 β , IL-6, IL-10, IL-12 β , IL-13 and IL-17. Expression levels of IL-5 were also measured from spleen tissue, but not MLN (Table 5.1).

Further, the relative mRNA expression of several important immunity-related transcription factors was measured using qPCR from spleen tissue. These included: Interferon regulatory factor 5 (Irf5), retinoic acid receptor-related orphan receptor- γ (ROR- γ), forkhead box p3 (FoxP3), T-box 21/Tbet (Tbx21) and GATA binding protein 3 (Gata3). Expression levels of tumour necrosis factor- α (TNF- α) were measured from both spleen and MLN tissue (Table 5.1).

All primers used were designed and validated by Primerdesign (Southampton). The genes analysed, along with their corresponding GenBank accession numbers and primer sequence information, are summarised in Table 1. Each qPCR reaction contained 0.5 μ L of the relevant primer mix (Table 5.1), 2.5 μ L of template cDNA, 2.0 μ L of

nuclease free H₂O and 5 µL PrecisionFAST qPCR Mastermix with LOW ROX (Primerdesign), using SYBR Green chemistry, to make a total reaction volume of 10 µL. All samples were run in duplicate, with each plate also containing negative controls and a pooled reference sample.

All assays were run using 96-well optical PCR plates with optical seals (StarLab) in an ABI 7500 FAST real-time thermocycler (Applied Biosystems). Each reaction included an initial enzyme activation step of 95°C for 20 seconds, followed by 40 cycles of a further denaturation step of 95°C for 3 seconds, then an annealing step of 60°C for 30 seconds. All runs included a post-PCR melt curve stage consisting of 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds and finally 60°C for 15 seconds.

5.2.4 Quantification of Gene Expression

To obtain reliable data from qPCR experiments, accurate normalisation against reference genes with stable expression is essential. Often, the optimal reference genes are tissue specific or specific to certain experimental conditions (Dheda *et al.* 2005). The immunological target genes analysed here (Table 5.1) were normalised to the endogenous control genes *Sdha* (Accession number: NM_023281.1; Amplicon size: 181bp) and *Ubc* (Accession Number: NM_019639.4; Amplicon size: 178bp). For cDNA from both spleen and MLN tissue, these were selected as the most stable combination of

reference genes from a panel of six candidate endogenous genes (also including *Actb*, *Gapdh*, *Rn18s* and *Rpl13a*) using the geNorm algorithm (Vandesompele *et al.* 2002). The stability of expression of these six genes was analysed using qbase+ (Biogazelle). In this geNorm assay, 15 randomly selected spleen or MLN cDNA samples were included (the same 15 samples were used with each of the six candidate genes).

The expression of each target in each sample relative to expression in the reference cDNA was calculated using the $2^{-\Delta\Delta C_T}$ method (Winer *et al.* 1999; Livak & Schmittgen 2001; Pfaffl 2001), standardised against the geometric mean C_T of the two reference genes for each sample (Vandesompele *et al.* 2002).

Table 5.1: Primer details for qPCR assays of a suite of cytokines and immunity-related transcription factors in wild house mice (*Mus musculus domesticus*) from the Isle of May. ‘Gene’ gives the gene name and official gene symbol (in italics). ‘Accession number’ gives the gene on which primers were based. ‘Amplicon size’ indicates the size of the product in base pairs. ‘Single exon’ shows where the primers cover a single exon (‘Yes’), span an intron (‘No’), or where this data is not determined (‘ND’). ‘Function’ summarises the immunological role of the target gene. Gene expression was measured from either spleen or mesenteric lymph node (MLN) tissue.

Gene	Accession number	Primer sequence	Amplicon size (bp)	Single exon	Function	Molecule type	Tissue
IFN- γ <i>Ifng</i>	NM_008337	L 5'-TGATTACTACCTTCTTCAGCAACAG R 5'-CTGGTGGACCACTCGGATG	128	No	Upregulation of Th1 response (Turner <i>et al.</i> 2011)	Cytokine	MLN; Spleen
IL-1 β <i>Il1b</i>	NM_008361	L 5'-CAACCAACAAGTGATATTCTCCAT R 5'-GGGTGTGCCGTCTTTCATTA	127	Yes	Innate inflammation (Jackson <i>et al.</i> 2011)	Cytokine	MLN; Spleen
IL-5 <i>Il5</i>	NM_010558	L 5'-ATGGACGCAGGAGGATCAC R 5'-TTGAAGTTAGATAGGAGCAGGAAG	128	No	Upregulation of Th2 response (Turner <i>et al.</i> 2011)	Cytokine	Spleen
IL-6 <i>Il6</i>	NM_031168	L 5'-TCCATCCAGTTGCCCTTCTTG R 5'-GGTCTGTTGGGAGTGGTATC	106	Yes	Pro-inflammatory Th17 response (Ambrosi <i>et al.</i> 2012)	Cytokine	MLN; Spleen
IL-10 <i>Il10</i>	NM_010548	L 5'-GGGAAGAGAAACCAGGGAGAT R 5'-GCCACAGTTTTCAGGGATGA	102	Yes	Anti-inflammatory Treg response (Jackson <i>et al.</i> 2011)	Cytokine	MLN; Spleen
IL-12 β <i>Il12b</i>	NM_008352	L 5'-CACGGCAGCAGAATAAATATGAG R 5'-GAGTTCTTCAAAGGCTTCATCTG	106	No	Pro-inflammatory Th1 (Turner <i>et al.</i> 2011)	Cytokine	MLN; Spleen
IL-13 <i>Il13</i>	NM_008355	L 5'-GCCAGCCCACAGTTCTACA R 5'-CCACCAAGGCAAGCAAGAG	98	ND	Upregulation of Th2 response (McKenzie <i>et al.</i> 1998)	Cytokine	MLN; Spleen
IL-17 <i>Il17a</i>	NM_010552	L 5'-GTCTGCCCTCCACAATGAAA R 5'-TTAAAGTCCACAGAAAAACAAACAC	75	Yes	Pro-inflammatory Th17 response (Ambrosi <i>et al.</i> 2012)	Cytokine	MLN; Spleen
TNF- α <i>Tnf</i>	NM_013693	L 5'-AGCCAGGAGGGAGAACAGA R 5'-CAGTGAGTGAAAGGGACAGAAC	96	Yes	Innate inflammation (Turner <i>et al.</i> 2011)	Cytokine	MLN; Spleen
FoxP3 <i>Foxp3</i>	NM_054039	L 5'-AGGACAGACCACACTTCATGC R 5'-CCAGTGGCAGCAGAAGGTG	127	No	Promotes development of Treg cells (Hori <i>et al.</i> 2003)	Transcription factor	Spleen
Gata3 <i>Gata3</i>	NM_008091	L 5'-GAAGACTTTATTGTACCTGGATAGC R 5'-TGGACATCAGACTTAGTGGTTTC	118	ND	Th2 effector responses (Zhu <i>et al.</i> 2006)	Transcription factor	MLN; Spleen
Irf5 <i>Irf5</i>	NM_001252382	L 5'-CTTCAGCCAGCCCCCTA R 5'-TGATCTCTAGGTCCGTCAAAGG	84	ND	Pro-inflammatory Th17 response (Ambrosi <i>et al.</i> 2012)	Transcription factor	Spleen
ROR- γ <i>Rorc</i>	NM_011281	L 5'-TTTCTGCCTTTCTTCTCTAGTT R 5'-CACCTCATTCTTTACACATCCA	96	ND	Promotes Th17 cell differentiation (Ivanov <i>et al.</i> 2006)	Transcription factor	Spleen
Tbx21 <i>Tbx21</i>	NM_019507	L 5'-AACCAGCACCAGACAGAGAT R 5'-GACCACATCCACAAACATCCT	120	ND	Th1 inflammation (Mullen <i>et al.</i> 2001)	Transcription factor	Spleen

5.2.5 Multiplex bead assay

To determine serum cytokine levels, serum samples were processed in duplicate with a custom Bio-Rad Bio-Plex mouse cytokine reagent kit according to manufacturer's protocol (Bio-Rad). Included here were detection antibodies for the following cytokines: IFN- γ , IL-1 β , IL-5, IL-6, IL-10, IL-12 β , IL-13, IL-17 and TNF- α (see Table 5.1 for functions). Detection antibodies for leptin – a biomarker for physiological condition – were also included (see Chapter 3).

Following incubation, the reaction mixture was analysed using a Bio-Plex 200 Luminex-based multiplex analysis system (Bio-Rad). Unknown cytokine concentrations were calculated by Bio-Plex Manager Software using standard curves derived from recombinant cytokine standards – data are expressed as fold change from control (Al Gadban *et al.* 2012). Data that were below the assay's range of detection were assigned values of 0.001 (Abolins *et al.* 2017).

5.2.6 Data analysis

All data were corrected to account for interplate variation by equalising means and normalised by log-transformation ($\log_{10} [x + 1]$). All analyses were conducted in R version 3.4.0 (R Core Team 2017).

To test for correlation between gene expression levels of all genes expressed by spleen and MLN tissue, and the circulating serum cytokine levels, the non-parametric Spearman's correlation coefficient was used.

Spearman's rank correlation coefficients and associated significance levels were generated using the *rcorr()* function in the 'Hmisc' package (Harrell Jr 2017). Correlation matrices were plotted using the 'corrplot' package (Wei & Simko 2017). To control for multiple testing, adjusted *p*-values were obtained using the Benjamini-Hochberg procedure. Correlations were deemed to be significant when the adjusted *p*-value <0.05.

Following Jackson *et al.* (2011), genes and proteins were then grouped into 'pro-inflammatory' (IFN- γ , IL-12 β , TNF- α , IL-1 β , IL-6, IL-17, Irf-5, Tbx21 and ROR- γ) or 'anti-inflammatory' (IL-5, IL-10, IL-13, FoxP3 and Gata3) response types by principal components analysis (PCA) using the *prcomp()* function. PCA aims to extract important information from a series of variables to be expressed in new, orthogonal variables (Abdi & Williams 2010; Schwensow *et al.* 2011). Across tissue types and circulating cytokine levels, the dominant first principal component (PC1) explained a high proportion of variance in the data (>62.30%). Using these PC1 scores, Spearman's correlation coefficient was again used to test for correlations between the pro- and anti-inflammatory gene and circulating protein groupings.

Following Robertson *et al.* (2016), genes and proteins were then further divided into more specific response types (Table 5.2). Where appropriate, PCA was again used to group relevant gene expression or circulating cytokine levels. For all response types in all tissues or circulating protein levels, PC1 explained a high proportion of variance (>67.93%). The exception was in the Th1-type response as measured

from spleen tissue, where PC1 explained 49.80% of variance. Here, however, IL-12 β did not correlate with other measures; when IL-12 β was omitted, PC1 explained 74.80% of the variance.

Table 5.2: Levels of immune gene expression from spleen or mesenteric lymph node (MLN) tissue and circulating cytokine levels, measured in wild *Mus musculus domesticus*, were subdivided into immune response type. Measures of immune response were combined using the PC1 scores from PCA.

	Tissue measured	Circulating cytokine	Spleen	MLN
Response type				
Innate		TNF- α	TNF- α	TNF- α
		IL-1 β	IL-1 β	IL-1 β
Th1		IFN- γ	IFN- γ	IFN- γ
		IL-12 β	IL-12 β * Tbx21	IL-12 β
Th2		IL-5	IL-5	IL-13
		IL-13	IL-13 Gata3	Gata3
Th17		IL-6	IL-6	IL-6
		IL-17	IL-17 ROR- γ Irf5	IL-17
Treg		IL-10	IL-10 FoxP3	IL-10

*IL-12 β was omitted due to lack of correlation with other measures. When IL-12 β was included, PC1 explained 49.80%. When removed, PC1 explained 74.80% of variance.

5.3 Results

5.3.1 Sample sizes

The total sample size (i.e. number of individual mice) available varied across the genes and circulating cytokines investigated (Table

5.3). This was due to repeated failed assays, missing samples or other technical issues (see Appendix 4).

Table 5.3: The sample size (n) of expression levels of immune genes expressed by spleen or mesenteric lymph node (MLN) tissue, or the circulating levels of cytokines as measured by multiplex bead assay. Samples were obtained from wild *Mus musculus domesticus* on the Isle of May.

Target	Source	n	Gene expression ($\Delta\Delta C_T$)	Cytokine concentration (pg ml ⁻¹)
FoxP3	Spleen	300	1.86 (± 5.98)	
Gata3	Spleen	304	3.01 (± 14.45)	
	MLN	350	5.10 (± 35.91)	
IFN- γ	Circulating cytokines	323		116.64 (± 272.18)
	Spleen	290	1.50 (± 3.45)	
	MLN	305	6.87 (± 23.21)	
IL-1 β	Circulating cytokines	323		397.09 (± 728.81)
	Spleen	303	1.52 (± 2.82)	
	MLN	345	1.87 (± 6.46)	
IL-5	Circulating cytokines	323		15.46 (± 17.98)
	Spleen	300	2.35 (± 6.84)	
IL-6	Circulating cytokines	323		126.43 (± 789.52)
	Spleen	302	2.67 (± 8.67)	
	MLN	341	2.73 (± 12.49)	
IL-10	Circulating cytokines	323		138.66 (± 143.80)
	Spleen	304	1.94 (± 7.09)	
	MLN	343	8.91 (± 26.76)	
IL-12 β	Circulating cytokines	323		1073.02 (± 1456.16)
	Spleen	285	3.13 (± 8.31)	
	MLN	241	8.44 (± 21.63)	
IL-13	Circulating cytokines	323		1088.39 (± 3278.70)
	Spleen	291	2.49 (± 9.23)	
	MLN	334	9.07 (± 29.86)	
IL-17	Circulating cytokines	323		354.39 (± 428.94)
	Spleen	296	3.14 (± 13.16)	
	MLN	342	3.78 (± 18.79)	
Irf5	Spleen	306	1.72 (± 2.66)	
ROR- γ	Spleen	306	3.82 (± 19.18)	
Tbx21	Spleen	299	1.79 (± 3.13)	
TNF- α	Circulating cytokines	323		3226.26 (± 5008.21)
	Spleen	303	2.56 (± 6.49)	
	MLN	337	6.33 (± 26.37)	

5.3.2 Correlations between all genes and circulating cytokines

The strongest correlations came within tissue types for gene expression or when comparing the relationship between levels of circulating cytokines (Figure 5.1). The concentrations of circulating cytokines were all significantly positively correlated (Table 5.4), as were the expression levels of most genes expressed by MLN tissue (Table 5.5). The exceptions here were the insignificant relationships between IL-13 and IL-1 β ($r_s=0.133$, $p=0.072$), and between IFN- γ and IL-17 ($r_s=-0.054$, $p=0.572$).

There were significant correlations between most (72.53%) genes expressed by spleen tissue (Table 5.6). Most significant relationships were positively correlated, except for the significant negative relationships between Irf5 and IL-17 ($r_s=-0.202$, $p=0.002$), Irf5 and IL-6 ($r_s=-0.151$, $p=0.029$), Tbx21 and IL-13 ($r_s=-0.154$, $p=0.029$), and Tbx21 and IL-17 ($r_s=-0.184$, $p=0.006$). 27.47% of the relationships between expression levels of genes measured from spleen tissue were insignificant (Table 5.7).

Where significant correlations existed between genes expressed in different tissue types, or between gene expression and circulating cytokine levels, these were weak (r_s ranging from -0.227 to 0.248; Table 5.8). The nonsignificant correlations between genes expressed in different tissues, or between gene expression and circulating cytokines, are presented in Appendix 5.1.

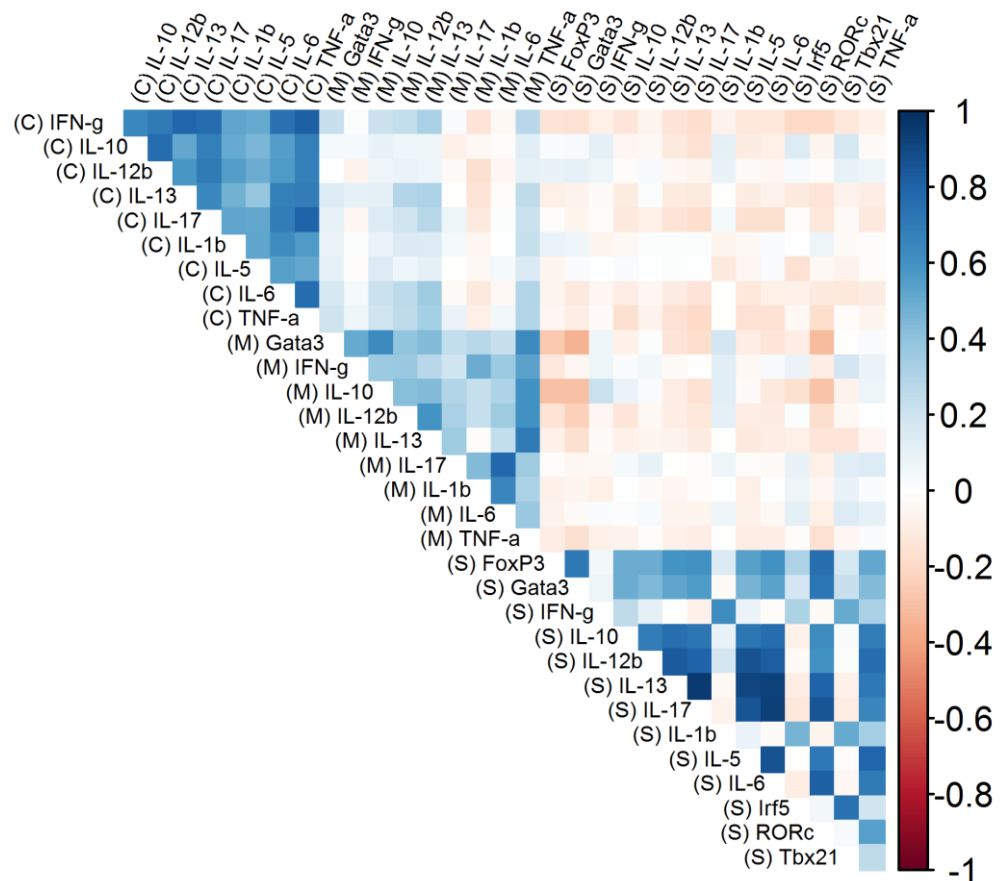


Figure 5.1: Correlation matrix showing the relationships between immune genes expressed by spleen tissue (S), immune genes expressed by mesenteric lymph node (M) tissue and circulating cytokine levels measured by multiplex bead assay (C) in wild house mice (*Mus musculus domesticus*) from the Isle of May, Scotland.

Table 5.4: Spearman's rank correlation coefficients (r_s) and associated p -values between circulating cytokines, measured by multiplex bead assay from serum of wild house mice (*Mus musculus domesticus*) on the Isle of May, Scotland. To control for multiple testing, p -values were adjusted using the Benjamani-Hochberg procedure. Concentrations of all measured cytokines were significantly positively correlated ($p < 0.001$).

Circulating cytokines		r_s	p adj.
IFN- γ	TNF- α	0.826	<0.001
IL-13	IFN- γ	0.811	<0.001
IL-17	TNF- α	0.750	<0.001
IL-13	TNF- α	0.731	<0.001
IL-17	IFN- γ	0.727	<0.001
IL-12 β	IL-17	0.719	<0.001
IL-10	IL-12 β	0.702	<0.001
IL-12 β	IFN- γ	0.688	<0.001
IL-6	IFN- γ	0.678	<0.001
IL-6	TNF- α	0.672	<0.001
IL-12 β	TNF- α	0.659	<0.001
IL-10	IL-17	0.646	<0.001
IL-10	IFN- γ	0.644	<0.001
IL-10	TNF- α	0.639	<0.001
IL-6	IL-13	0.637	<0.001
IL-1 β	IL-6	0.629	<0.001
IL-13	IL-17	0.613	<0.001
IL-1 β	IFN- γ	0.597	<0.001
IL-1 β	TNF- α	0.589	<0.001
IL-1 β	IL-13	0.578	<0.001
IL-6	IL-17	0.562	<0.001
IL-12 β	IL-13	0.562	<0.001
IL-10	IL-13	0.547	<0.001
IL-1 β	IL-12 β	0.526	<0.001
IL-1 β	IL-17	0.524	<0.001
IL-1 β	IL-10	0.515	<0.001
IL-1 β	IL-5	0.498	<0.001
IL-6	IL-10	0.491	<0.001
IL-5	IL-6	0.480	<0.001
IL-6	IL-12 β	0.466	<0.001
IL-5	TNF- α	0.425	<0.001
IL-5	IFN- γ	0.415	<0.001
IL-5	IL-10	0.403	<0.001
IL-5	IL-13	0.377	<0.001
IL-5	IL-12 β	0.377	<0.001
IL-5	IL-17	0.373	<0.001

Table 5.5: Spearman's rank correlation coefficients (r_s) and associated p -values between immune gene expression, measured by qPCR of mesenteric lymph node tissue from wild house mice (*Mus musculus domesticus*) on the Isle of May, Scotland. To control for multiple testing, p -values were adjusted using the Benjamini-Hochberg procedure. Significant correlations are highlighted in bold, and all significant relationships were positively correlated.

Genes expressed		r_s	p adj.
TNF- α	Gata3	0.776	<0.001
IL-10	TNF- α	0.748	<0.001
IL-6	IL-17	0.730	<0.001
IL-10	Gata3	0.725	<0.001
IL-13	TNF- α	0.720	<0.001
IL-12 β	IL-13	0.647	<0.001
IL-1 β	IL-6	0.638	<0.001
IL-10	IL-13	0.616	<0.001
IFN- γ	TNF- α	0.575	<0.001
IL-12 β	TNF- α	0.544	<0.001
IL-13	Gata3	0.529	<0.001
IFN- γ	Gata3	0.514	<0.001
IFN- γ	IL-1 β	0.481	<0.001
IFN- γ	IL-10	0.428	<0.001
IL-1 β	IL-17	0.425	<0.001
TNF- α	IL-6	0.425	<0.001
IL-10	IL-12 β	0.409	<0.001
TNF- α	IL-1 β	0.403	<0.001
IL-1 β	Gata3	0.385	<0.001
IFN- γ	IL-12 β	0.373	<0.001
IFN- γ	IL-13	0.363	<0.001
IL-6	Gata3	0.351	<0.001
IL-13	IL-6	0.347	<0.001
IL-12 β	Gata3	0.347	<0.001
IL-12 β	IL-6	0.341	<0.001
IL-10	IL-1 β	0.333	<0.001
IL-10	IL-6	0.326	<0.001
IL-17	Gata3	0.301	<0.001
TNF- α	IL-17	0.294	<0.001
IFN- γ	IL-6	0.291	<0.001
IL-13	IL-17	0.235	<0.001
IL-10	IL-17	0.211	0.001
IL-12 β	IL-17	0.196	0.012
IL-12 β	IL-1 β	0.187	0.019
IL-13	IL-1 β	0.133	0.072
IFN- γ	IL-17	-0.054	0.572

Table 5.6: Spearman's rank correlation coefficients (r_s) and associated p -values of significant ($p < 0.05$) relationships between immune genes expressed by spleen tissue in wild house mice (*Mus musculus domesticus*) on the Isle of May, Scotland. To control for multiple testing, p -values were adjusted using the Benjamini-Hochberg procedure.

Genes expressed		r_s	p adj.
IL-17	Irf5	-0.202	0.002
IL-17	Tbx21	-0.184	0.006
IL-17	Gata3	0.537	<0.001
IL-17	FoxP3	0.587	<0.001
IL-17	TNF- α	0.640	<0.001
IL-17	ROR- γ	0.846	<0.001
IL-1 β	FoxP3	0.152	0.028
IL-1 β	IL-10	0.171	0.010
IL-1 β	Tbx21	0.361	<0.001
IL-1 β	Irf5	0.376	<0.001
IL-1 β	TNF- α	0.391	<0.001
IL-5	Gata3	0.491	<0.001
IL-5	FoxP3	0.574	<0.001
IL-5	IL-10	0.735	<0.001
IL-5	ROR- γ	0.746	<0.001
IL-5	TNF- α	0.798	<0.001
IL-5	IL-17	0.853	<0.001
IL-5	IL-6	0.881	<0.001
IL-5	IL-13	0.909	<0.001
IL-6	Irf5	-0.151	0.029
IL-6	Gata3	0.494	<0.001
IL-6	Foxp3	0.588	<0.001
IL-6	TNF- α	0.714	<0.001
IL-6	IL-10	0.758	<0.001
IL-6	ROR- γ	0.816	<0.001
IL-6	IL-13	0.934	<0.001
IL-6	IL-17	0.935	<0.001
Irf5	Foxp3	0.243	<0.001
Irf5	Tbx21	0.801	<0.001
ROR- γ	Foxp3	0.709	<0.001
TNF- α	Gata3	0.397	<0.001
TNF- α	ROR- γ	0.525	<0.001
TNF- α	Foxp3	0.546	<0.001

Genes expressed		r_s	p adj.
FoxP3	Tbx21	0.141	0.046
Gata3	Irf5	0.221	<0.001
Gata3	Tbx21	0.256	<0.001
Gata3	FoxP3	0.652	<0.001
Gata3	ROR- γ	0.698	<0.001
IFN- γ	IL-10	0.279	<0.001
IFN- γ	Irf5	0.298	<0.001
IFN- γ	TNF- α	0.326	<0.001
IFN- γ	Tbx21	0.435	<0.001
IFN- γ	IL-1 β	0.600	<0.001
IL-10	Gata3	0.408	<0.001
IL-10	FoxP3	0.468	<0.001
IL-10	ROR- γ	0.595	<0.001
IL-10	TNF- α	0.692	<0.001
IL-10	IL-17	0.714	<0.001
IL-10	IL-13	0.723	<0.001
IL-12 β	IFN- γ	0.238	<0.001
IL-12 β	IL-1 β	0.244	<0.001
IL-12 β	Gata3	0.427	<0.001
IL-12 β	FoxP3	0.496	<0.001
IL-12 β	ROR- γ	0.594	<0.001
IL-12 β	IL-10	0.696	<0.001
IL-12 β	TNF- α	0.762	<0.001
IL-12 β	IL-17	0.787	<0.001
IL-12 β	IL-13	0.806	<0.001
IL-12 β	IL-6	0.806	<0.001
IL-12 β	IL-5	0.833	<0.001
IL-13	Tbx21	-0.154	0.029
IL-13	Gata3	0.523	<0.001
IL-13	Foxp3	0.580	<0.001
IL-13	TNF- α	0.699	<0.001
IL-13	ROR- γ	0.817	<0.001
IL-13	IL-17	0.949	<0.001

Table 5.7: Spearman's rank correlation coefficients (r_s) and associated p -values of nonsignificant ($p>0.05$) relationships between immune genes expressed by spleen tissue in wild house mice (*Mus musculus domesticus*) on the Isle of May, Scotland. To control for multiple testing, p -values were adjusted using the Benjamini-Hochberg procedure.

Genes expressed		r_s	p adj.
TNF- α	Tbx21	0.136	0.056
IL-13	Irf5	-0.137	0.060
IL-10	Irf5	-0.133	0.061
IL-6	Tbx21	-0.133	0.066
TNF- α	Irf5	0.100	0.188
IL-10	Tbx21	-0.085	0.299
IL-1 β	IL-5	0.087	0.314
IFN- γ	FoxP3	0.071	0.408
IFN- γ	IL-5	0.069	0.447
IFN- γ	IL-6	0.049	0.592
IL-12 β	Irf5	-0.052	0.592
Irf5	ROR- γ	0.045	0.627
IL-1 β	Gata3	-0.044	0.638
IL-1 β	IL-6	0.044	0.638
IL-5	Tbx21	-0.042	0.666
IL-5	Irf5	0.040	0.671
IL-12 β	Tbx21	-0.041	0.679
IFN- γ	Gata3	0.037	0.695
IFN- γ	IL-17	-0.036	0.708
IL-1 β	ROR- γ	-0.035	0.708
IL-1 β	IL-17	-0.026	0.788
IFN- γ	ROR- γ	-0.016	0.882
ROR- γ	Tbx21	-0.013	0.893
IFN- γ	IL-13	0.012	0.899
IL-1 β	IL-13	-0.012	0.903

Table 5.8: Spearman's rank correlation coefficients (r_s) and associated p -values of significant relationships ($p < 0.05$) between immune gene expression, measured by qPCR of mesenteric lymph node (MLN) or spleen tissue, and circulating cytokine concentrations from wild house mice (*Mus musculus domesticus*) on the Isle of May, Scotland. To control for multiple testing, p -values were adjusted using the Benjamini-Hochberg procedure.

Gene expression or circulating cytokine concentration		r_s	p adj.
IFN- γ (cytokines)	IL-12 β (MLN)	0.207	0.008
IFN- γ (cytokines)	IL-13 (MLN)	0.177	0.010
IL-12 β (cytokines)	IL-1 β (MLN)	-0.143	0.045
IL-13 (cytokines)	IL-12 β (MLN)	0.177	0.029
IL-13 (cytokines)	IL-13 (MLN)	0.169	0.015
IL-17 (cytokines)	IL-1 β (spleen)	0.141	0.046
IL-1 β (cytokines)	TNF- α (MLN)	0.155	0.029
IL-5 (cytokines)	Irf5 (spleen)	-0.162	0.017
IL-6 (cytokines)	IL-12 β (MLN)	0.212	0.006
IL-6 (cytokines)	IL-13 (MLN)	0.227	<0.001
IL-6 (cytokines)	TNF- α (MLN)	0.160	0.023
IL-6 (cytokines)	IL-1 β (spleen)	0.172	0.010
TNF- α (cytokines)	IL-1 β (spleen)	0.142	0.045
Foxp3 (spleen)	Gata3 (MLN)	-0.188	0.005
Foxp3 (spleen)	IL-10 (MLN)	-0.206	0.002
Gata3 (spleen)	Gata3 (MLN)	-0.197	0.003
Gata3 (spleen)	IL-10 (MLN)	-0.148	0.038
IFN- γ (spleen)	IL-10 (MLN)	0.221	0.001
IL-17 (spleen)	Gata3 (MLN)	-0.145	0.047
IL-5 (spleen)	IL-13 (MLN)	-0.159	0.035
ROR- γ (spleen)	Gata3 (MLN)	-0.227	<0.001
ROR- γ (spleen)	IL-10 (MLN)	-0.212	0.001
ROR- γ (spleen)	TNF- α (MLN)	-0.158	0.026
Tbx21 (spleen)	IFN- γ (MLN)	0.248	<0.001

5.3.3 Correlations between inflammation responses

When grouped into 'inflammatory' or 'anti-inflammatory' genes and proteins, the strongest relationships are again within tissue types (Table 5.9, Figure 5.2). Spleen inflammatory and anti-inflammatory

($r_s=0.899$, $p<0.001$) and MLN inflammatory and anti-inflammatory ($r_s=0.744$, $p<0.001$) were both significantly positively correlated. Circulating inflammatory protein levels had a significant negative correlation with circulating anti-inflammatory protein levels ($r_s -0.857$, $p<0.001$). No other relationships were significant or strongly correlated ($r_s<0.208$, $p>0.057$; Table 5.9).

Table 5.9: Spearman's rank correlation coefficients (r_s) and associated p -values of relationships between inflammatory and anti-inflammatory immune responses in wild house mice (*Mus musculus domesticus*) from the Isle of May, Scotland. Immune response was measured by the expression of immune genes from spleen or mesenteric lymph node (MLN) tissue, or by concentrations of circulating cytokines. To control for multiple testing, p -values were adjusted using the Benjamani-Hochberg procedure. Significant relationships ($p<0.05$) are in bold.

Response type (source)		r_s	p adj.
Inflammatory (spleen)	Anti-inflammatory (spleen)	0.899	<0.001
Inflammatory (MLN)	Anti-inflammatory (MLN)	-0.744	<0.001
Inflammatory (cytokines)	Anti-inflammatory (cytokines)	0.859	<0.001
Inflammatory (cytokines)	Anti-inflammatory (MLN)	0.130	0.171
Anti-inflammatory (cytokines)	Inflammatory (MLN)	-0.150	0.197
Anti-inflammatory (spleen)	Anti-inflammatory (MLN)	-0.126	0.205
Inflammatory (cytokines)	Inflammatory (MLN)	-0.130	0.233
Anti-inflammatory (cytokines)	Anti-inflammatory (MLN)	0.106	0.233
Anti-inflammatory (spleen)	Inflammatory (MLN)	0.074	0.341
Anti-inflammatory (spleen)	Inflammatory (cytokines)	-0.053	0.392
Inflammatory (spleen)	Anti-inflammatory (MLN)	-0.097	0.410
Inflammatory (spleen)	Inflammatory (cytokines)	-0.049	0.442
Anti-inflammatory (spleen)	Anti-inflammatory (cytokines)	-0.074	0.533
Inflammatory (spleen)	Anti-inflammatory (cytokines)	-0.038	0.548
Inflammatory (spleen)	Inflammatory (MLN)	-0.007	0.934

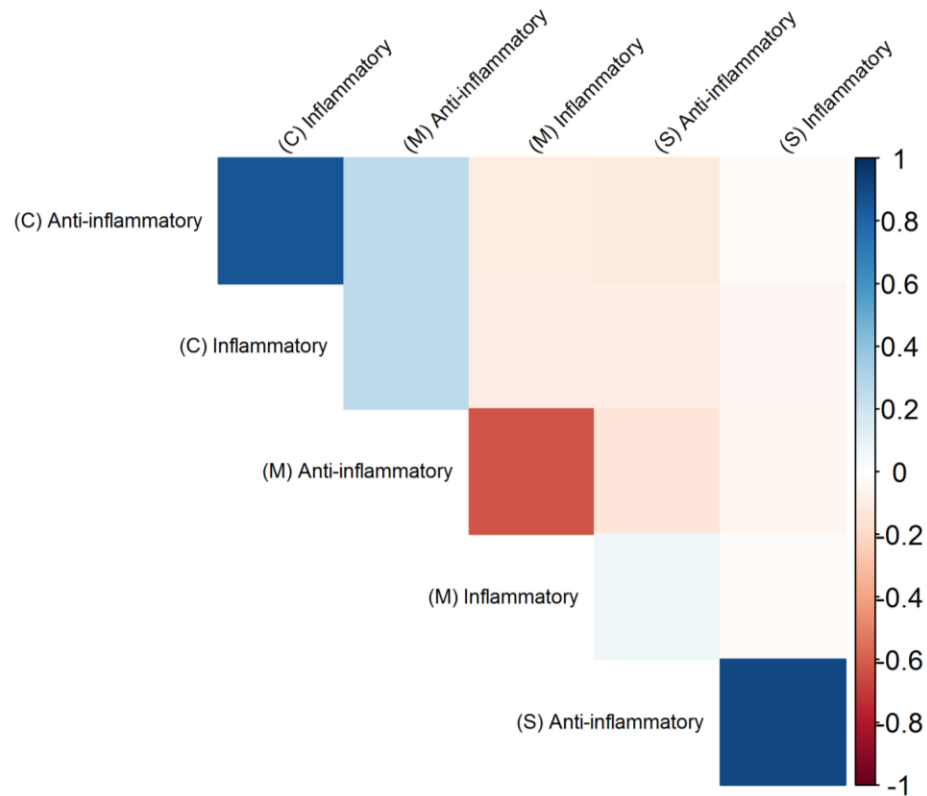


Figure 5.2: Correlation matrix showing the relationship between the inflammatory and anti-inflammatory immune response in wild *Mus musculus domesticus* from the Isle of May. Immune response was measured using genes expression from spleen (S) or mesenteric lymph node (M) tissue and circulating levels of inflammatory and anti-inflammatory cytokines measured by multiplex bead assay (C).

5.3.4 Correlations between response type

The gene expression and circulating cytokine levels were then further split into specific response types, following Jackson *et al.* (2011) and Robertson *et al.* (2016). Again, the strongest relationships came within the tissue type from which gene expression was measured, or within circulating cytokine levels (Figure 5.3, Table 5.10). The exception here was the weakly negative correlation between the Th1-type response

and Treg-type response as measured by gene expression from spleen tissue ($r_s=-0.138$, $p=0.019$).

While there were significant correlations between response type as measured by gene expression from different tissue types, or between gene expression and circulating cytokine levels, these were often weak relationships (r_s ranging from -0.216 to 0.193; Table 5.10).

Table 5.10. Significant relationships between immune response type in wild *Mus musculus domesticus* from the Isle of May, as measured by gene expression from spleen or mesenteric lymph node (MLN) tissue or circulating cytokine levels. Shown here is the response type and tissue from which it was measured for gene expression levels, or circulating cytokines, Spearman's rank correlation coefficient (r_s) and level of significance (p) for all significant ($p > 0.05$) relationships.

Response and tissue type		r_s	p adj.
Innate (circulating cytokines)	Th1 (circulating cytokines)	-0.716	<0.001
Innate (circulating cytokines)	Th2 (circulating cytokines)	-0.711	<0.001
Th1 (circulating cytokines)	Th2 (circulating cytokines)	0.759	<0.001
Innate (circulating cytokines)	Th17 (circulating cytokines)	-0.733	<0.001
Th1 (circulating cytokines)	Th17 (circulating cytokines)	0.753	<0.001
Th2 (circulating cytokines)	Th17 (circulating cytokines)	0.720	<0.001
Innate (circulating cytokines)	Treg (circulating cytokines)	0.588	<0.001
Th1 (circulating cytokines)	Treg (circulating cytokines)	-0.723	<0.001
Th2 (circulating cytokines)	Treg (circulating cytokines)	-0.569	<0.001
Th17 (circulating cytokines)	Treg (circulating cytokines)	-0.594	<0.001
Treg (MLN)	Th2 (MLN)	0.733	<0.001
Th1 (MLN)	Innate (MLN)	-0.646	<0.001
Treg (MLN)	Innate (MLN)	0.706	<0.001
Th2 (MLN)	Innate (MLN)	0.702	<0.001
Th1 (MLN)	Th2 (MLN)	-0.576	<0.001
Th2 (MLN)	Th17 (MLN)	0.397	<0.001
Th1 (MLN)	Treg (MLN)	-0.459	<0.001
Innate (MLN)	Th17 (MLN)	0.488	<0.001
Th1 (MLN)	Th17 (MLN)	-0.340	<0.001
Treg (MLN)	Th17 (MLN)	0.316	<0.001
Innate (spleen)	Th2 (spleen)	-0.608	<0.001
Innate (spleen)	Th17 (spleen)	0.500	<0.001
Th2 (spleen)	Th17 (spleen)	-0.940	<0.001
Innate (spleen)	Treg (spleen)	0.626	<0.001
Th2 (spleen)	Treg (spleen)	-0.798	<0.001
Th17 (spleen)	Treg (spleen)	0.771	<0.001
Innate (spleen)	Th1 (spleen)	-0.455	<0.001
Th17 (circulating cytokines)	Th2 (MLN)	-0.216	0.001
Treg (circulating cytokines)	Th1 (spleen)	0.178	0.009
Th2 (circulating cytokines)	Th2 (MLN)	-0.174	0.010
Th17 (spleen)	Th2 (MLN)	0.173	0.016
Innate (circulating cytokines)	Innate (MLN)	0.165	0.017
Th2 (circulating cytokines)	Th1 (MLN)	0.193	0.019
Th17 (spleen)	Treg (MLN)	0.160	0.025
Th2 (spleen)	Th2 (MLN)	-0.163	0.039
Th17 (circulating cytokines)	Innate (MLN)	-0.152	0.048

All other correlations were insignificant ($p>0.051$) and weakly related (r_s ranging from -0.138 to 0.152). The majority of insignificant correlations were between gene expression levels from different tissues, or between circulating cytokine levels and gene expression. However, the Th1-type response measured by gene expression from spleen tissue showed no significant relationship with either a Th17-type response ($r_s=0.106$, $p=0.074$) or Th2-type response ($r_s=0.042$, $p=0.489$), both as measured by gene expression from spleen tissue (see Appendix 5.2).

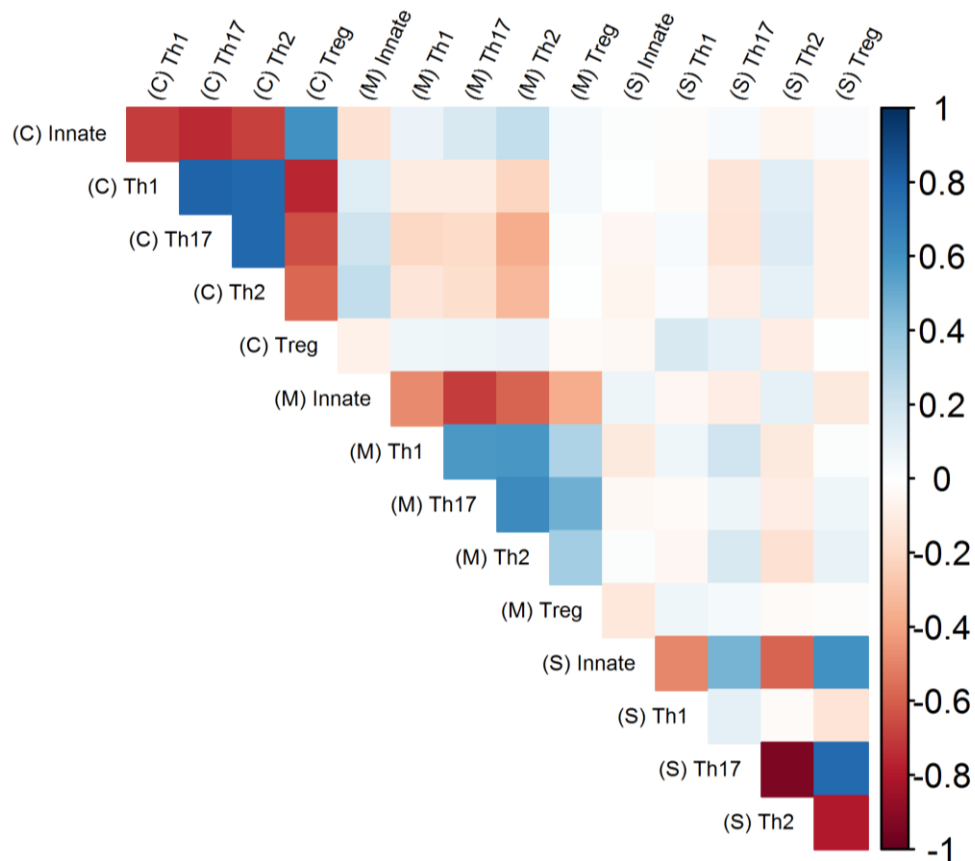


Figure 5.3: Correlation matrix showing the relationship between immune response type in wild *Mus musculus domesticus* from the Isle of May. Immune response was measured by gene expression from spleen (S) or mesenteric lymph node (M) tissue, or by circulating cytokine (C) levels measured by multiplex bead assay.

5.4 Discussion

Here, we measured the expression levels of key immune genes from two immunologically important tissues (spleen and MLN) in wild house mice. In addition to this, we also measured the circulating levels of key cytokines. We found a lack of correlation between the expression levels of immune genes from different tissues, and between gene expression and circulating cytokine levels. Further, when levels of gene expression and circulating cytokines were grouped by response type, the strongest correlations came among tissue types.

The level of variation in the correlations seen between the measures of immune response made in this study are indicative of the difficulties of interpreting measures of immune response, particularly in wild animals. While weak correlations are observed in functional groups of cytokines across gene expression and protein levels within a tissue type, there is negligible correlation between genes and their corresponding proteins across tissues. There are a number of potential causes for these discrepancies, both biological and methodological which should be considered when using molecular techniques to assess immune function.

5.4.1 Temporal effects

Here, qPCR was measured from tissues obtained from destructively sampled animals, so provided a snapshot of an individual's

immune response. Such cross-sectional sampling techniques are not ideal when considering delays between transcription and translation, and differences in degradation rates between proteins and RNA. However, by utilising cell culturing and stimulatory cellular assays it is possible to measure the potential immune response to future challenges (Bradley & Jackson 2008; Robertson *et al.* 2016). We were unable to utilise cell culturing techniques here due to logistical constraints, but establishing reliable field protocols for cell-culture across tissues might give a clearer idea of differences between genes expression across tissues and resultant protein concentrations.

5.4.2 Downstream effects

The fact that gene expression does not linearly align with protein levels in this study may be indicative of the complexities of the molecular pathways involved. Many of the cytokines analysed have key roles in inducing or suppressing the expression of different cytokines. The lack of correlation between immune measures seen in this study has also been noted in previous human studies, where it was largely explained by post-transcriptional and post-translational regulation (Kozak 2007; Mehra *et al* 2003; Nie *et al* 2006; Shebl *et al* 2010; Shen & Pili 2008).

Expressed genes can play regulatory roles, whereby the translated protein may primarily be involved in inducing transcription of other genes downstream, rather than acting as effector proteins at the actual site of infection. In addition, there may be immunomodulation on

the part of the infectious agent, such as suppression of Th2 immune function in lab models of *Trichuris* infection. These typically involve altering gene expression in the host, rather than the degradation of the cytokine proteins themselves. Due to these dynamics of regulation and modulation of gene expression occurring throughout the course of infection, delays between expression and translation and protein modification, and differences in degradation rate between protein and RNA, it is perhaps not surprising that there is little correlation between the immune measures that were taken.

Edfors *et al* (2016) used an RNA-to-protein conversion factor index to increase predictability of protein copy numbers from RNA levels. This, however, is calculated using a whole transcriptome analysis, and so is not appropriate for targeted qPCR studies such as this one. In addition, it is highly unlikely that the weak correlations between gene expression and protein levels seen here would become strong, even with post-translational or post-transcriptional correction. Further, this study was investigating the correlation of genes expressed by two different tissue types with secreted proteins, so universally strong correlations were not necessarily expected.

5.4.3 Localisation of responses

An important element which is likely very important for explaining the lack of correlation between tissue types is the level of localisation between immune environments in different parts of the body. The gut and

the spleen are both very significant immunological tissues, but they are, however, subject to wholly different immunological challenges. The spleen, being a key site of leukocyte maturation, is key for responding to infections in the blood, such as microparasite infections. This means that the spleen can respond to infections throughout the body, if immunoactive elements enter the blood and are filtered through the spleen. The primary role of the MLNs, on the other hand, is to drain the gastrointestinal tract, and respond to any pathogenic or parasitic threat. The GI tract is a unique immune environment generally, due to the fact that a complex and careful balance of bacterial communities must be maintained in order to facilitate normal digestion, while preventing systemic infection of pathology. In addition to this, the parasites which can dwell in the GI tract, often reside in the lumen, either wholly or in part. They are therefore external to the hosts body, and will require unique immune measures in order to deal with infection. The dramatically different manner in which splenic and MLN immune cells must respond to differently localised infections would make an equally localised level of expression of immune genes an expected observation.

There are many examples of such localised responses from laboratory infection models. Laboratory mice experimentally infected with the gastrointestinal nematode *Heligmosomoides polygrus* showed a decrease in levels of spleen IL-4 after 21 days of infection, while MLN levels remain elevated up to 70 days after infection (Finney *et al.* 2007). Indeed, increased expression of MHC genes in response to helminth infection might not be detectable in the spleen, but would be detected in

tissues local to the infection, i.e. intestinal tissues or MLNs (Schwensow *et al.* 2011).

Further, when comparing the transcriptome of spleen, thymus and lymph node tissue in wild crab-eating macaques (*Macaca fascicularis*), gtEefd Uli *et al* (2017) found that spleen and lymph node tissue had the greatest overlap of expressed genes (6,476 genes shared).

5.4.4 Conclusions

Most studies of wild immunology so far have focussed on non-model species and so have been restricted to measuring immune gene expression (Jackson *et al.* 2011; 2014). Protein levels can only be reliably analysed using sophisticated assays that are generally only available for model species such as laboratory mice, *M. musculus*. Although bioplex assays can be developed for non-model species, it can be a costly and time-consuming process, and so is often not practical. Regardless, the discrepancy between RNA levels and corresponding proteins should be a consideration that is addressed when reporting results. Other means of comprehensively assessing immune expression do exist and have potential for applications in wild populations. RNA-seq provides a whole transcriptome approach, and so any relevant changes in gene expression outside of those target genes can be identified. The qPCR approach used here, however, has the benefit of being comparatively cheap, thus allowing a higher number of replicates and greater statistical power. Further, the genes of interest selected were based on *a priori*

knowledge of immune function, providing a much more targeted approach.

What is evident from these results is that the true extent of variation in immune response can only be examined by taking into account both gene expression and secreted protein levels. Dependent on the precise questions being asked during an experiment, it might be more appropriate to focus on one measure or tissue type than other. For instance, one might be interested in the immune response to parasite or pathogen localised to a specific tissue, or want to ascertain how immune responses are regulated by upstream gene expression rather than looking at downstream effector proteins. Selecting the measure of immune function should, however, be handled with great care, particular in wild populations, as coinfection effects and peripheral changes to immune expression mean significant effects could be missed. As such, it is advisable to obtain the most comprehensive picture of immunity as is possible in most circumstances.

Chapter 6

Factors affecting immune response in wild mice

(Mus musculus domesticus)

6.1 Introduction

The immune system defends the body against infection and plays a key role in the survival of vertebrates (Barnard & Behnke 2006). The strength and effectiveness of the immune response, however, can vary widely both between and within species (Schmid-Hempel 2003). Understanding why variation in such an ecologically important trait exists, and how it is maintained, is a key goal in evolutionary biology (Lee 2006; Brown *et al.* 2013).

Studies using laboratory mice have been instrumental in establishing our understanding of the mechanisms of the mammalian immune system (Abolins *et al.* 2011). These experiments, however, have typically been carried out in sterile laboratories with genetically homogenous animals, thus eliminating many of the intrinsic and extrinsic drivers of variation (Babayan *et al.* 2011; Pedersen & Babayan 2011; Maizels & Nussey 2013; Abolins *et al.* 2017). Indeed, wild house mice are immunophenotypically different to laboratory strains (Abolins *et al.* 2011; Boysen *et al.* 2011; Viney *et al.* 2015; Abolins *et al.* 2017), and

have been shown to develop higher antibody responses (Lochmiller *et al.* 1991; Abolins *et al.* 2011). Further, mice raised in sterile conditions lack the antigen-experienced CD8⁺ T cells present in wild mice (Beura *et al.* 2016). Housing laboratory-reared C57BL/6 mice with wild caught mice, however, induces these T cells (Beura *et al.* 2016) and C57BL/6 mice exposed to outdoor environments are significantly more susceptible to infection by *Trichuris muris* than those reared in laboratory conditions (Leung *et al.* 2018). Natural populations are characterised by genetic variation, as well as variation in parasite loads and the abiotic environment. These studies highlight the importance of this variation in the natural functioning of the immune system (Lazzaro & Little 2009; Martin *et al.* 2011; Robertson *et al.* 2016).

The availability of food and resources can be limited in wild populations, and this can constrain immune function (Wiehn & Korpimäki 1998; Lochmiller & Deerenberg 2000; Abolins *et al.* 2011; Cressler *et al.* 2014). Indeed, fasting in captive mallards (*Anas platyrhynchos*) led to a reduction in the adaptive and innate immune responses (Bourgeon *et al.* 2010), while laboratory mice on a protein restricted diet will suppress their protective Th2 response (Ing *et al.* 2000). Further, supplementary feeding in wild sagebrush lizards (*Sceloporus graciosus*) leads to an increased innate immune response (Ruiz *et al.* 2010).

In the wild, resource availability can vary seasonally. Outside of the tropics, food sources are typically more scarce in the winter, and this is associated with a decreased immune function in wild animals (Nelson & Demas 1996; Nelson 2004; Altizer *et al.* 2006). Further, seasonal

variation in photoperiod or the reproductive status of an individual can contribute to these changes in immunity (Nelson & Demas 2004).

Diet is an important driver of body condition (Resano-Mayor *et al.* 2016), and individuals in poor body condition typically have a reduced immune response compared to those in better condition (Lochmiller & Deerenberg 2000; MacColl *et al.* 2017). Indeed, in pallid Atlantic Forest rats (*Delomys sublineatus*), expression levels of inflammatory cytokines were positively correlated with body condition (Schwensow *et al.* 2011). Further, individuals in better body condition may be more likely to exhibit a tolerant immunophenotype (Råberg *et al.* 2009). Similarly, in mature male field voles (*Microtus agrestis*), parasite load was positively correlated with body condition (Jackson *et al.* 2014). In addition to this, expression levels of Gata3, a mediator of the Th2 response and associated with tolerance (Allen & Wynn 2011), were also positively correlated with parasite load and body condition (Jackson *et al.* 2014). This relationship was not observed in immature voles, indicating that there is a shift from resistance to tolerance with age (Jackson *et al.* 2014).

Immunosenescence – the decline in immune function with advancing age – is well documented in humans and laboratory rodents (Montecino-Rodriguez *et al.* 2013). This is typically associated with declines in circulating naïve T lymphocytes and increases in inflammatory markers (Linton & Dorshkind 2004; Singh & Newman 2011). Similar patterns of change and decline in immune function have been observed in wild populations of various mammal (Nussey *et al.* 2012; Schneeberger *et al.* 2014; Watson *et al.* 2016) and bird (Palacios

et al. 2011) species. Thus, there is increasing evidence that the process of immunosenescence is common across most, if not all, vertebrates (Shanley *et al.* 2009).

There can also be variation in immune function between sexes. In sexually dimorphic species where one sex is larger than the other, particularly polygynous species, there can be a trade-off with increased growth leading to reduced immune function (Sheldon & Verhulst 1996; Rolff 2002). In addition to this, testosterone can have immunosuppressive effects (Klein 2004; Triglinaite *et al.* 2015) leading to a lower immune response in males (Zuk & McKean 1996; Møller *et al.* 1998a; Møller *et al.* 1999).

Here, we use the house mice on the Isle of May to explore the extrinsic and intrinsic factors affecting immune response in a wild population. Immune response was measured by expression levels of key immune genes, as well as concentrations of circulating cytokines. We examined the relationship between the immune response and microparasite infection, host age, sex, nutritional status and various body condition measures. We also explored how the immune profile of Isle of May mice varied seasonally, and the relationships between immune response and macroparasite load.

We hypothesised that females would show a stronger immune response and increased resistance to parasites compared to males (Zuk & McKean 1996; Møller *et al.* 1998a; Møller *et al.* 1999). Further, individuals in better body condition would be expected to be more immunocompetent and therefore exhibit a stronger immune response

(Schwensow *et al.* 2011; Graham *et al.* 2015). We also hypothesised that older animals with higher body condition will exhibit a tolerant immunophenotype, and there would be a shift from resistance to tolerance with age (Jackson *et al.* 2014). Indeed, *T. muris* burden increases with body condition in mature, but not immature, Isle of May mice (see Chapter 4). Jackson *et al.* (2014) identified expression of Gata3 – a Th2-associated transcription factor – as a marker of tolerance in wild field voles. Therefore, we expect positive relationships between the Th2 response and the age, body condition and parasite load of Isle of May house mice (Schwensow *et al.* 2011; Jackson *et al.* 2014).

6.2 Methods

6.2.1 Sample collection

Live-trapping of mice took place on the Isle of May between August 2013 and October 2016, as described in Chapter 2. The culling and processing of mice was also as described in Chapter 2.

6.2.2 Body condition and life history

Mice were sexed, aged and a reproductive stage was assigned as described in Chapter 2. The following body condition and physiological measures were recorded:

- Body Mass Index (BMI) (for detailed methods, see Chapter 2)
- Somatic indices of liver and spleen (see Chapter 4)
- Haematocrit (see Chapter 4)
- Circulating leptin levels (see Chapter 5)

6.2.3 Parasitological measurements

All macroparasites were identified and quantified as described in Chapter 4. Endoparasites identified here include the gastrointestinal helminths *S. obvelata* and *T. muris*, the cestode *Hymenolepis* sp. and the hepatic nematode *C. hepaticum*. Ectoparasites identified include the fur mites *M. musculi*, *R. affinis* and *M. musculus*, the harvest mite *T. autumnalis* and the northern rat flea *N. fasciatus* (see Chapter 4 for details).

Due to low overall prevalence, *Hymenolepis* sp. and *T. autumnalis* were omitted from analyses (1.4% and 6.8% respectively, see Chapter 4). *M. musculi*, *R. affinis* and *M. musculus* were grouped together as 'fur mites' for analyses (Mather & Lausen 1990; Jackson *et al.* 2014).

6.2.4 Host diet and nutritional status

The ratios of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) isotopes were calculated from the muscle tissue of 74 mice trapped in October 2015 by Stable Isotope Analysis, as described in Chapter 3.

6.2.5 Immune measures

Expression levels of a suite of immune genes were measured by qPCR from spleen and MLN tissue, as described in Chapter 5. Further, the circulating levels of a suite of cytokines were measured by multiplex bead assay, as described in Chapter 5. Gene expression and circulating cytokine levels were grouped into immune response types using PCA, as described in Chapter 5.

6.2.5 Statistical analysis

In order to examine which factors related to the immune response in wild mice, general linear models (GLMs) were fitted with response type as the variable. Response type was the PC1 score of the relevant immune gene expressions or circulating cytokine concentrations (see Chapter 5). The Treg response from MLN tissue and circulating cytokines was measured only by IL-10 gene expression and cytokine concentration, respectively. Therefore, the individual measures were used here.

Maximal models contained sex (2 levels) and session (6 or 7 levels) as factors. Age, BMI, liver and spleen somatic indices, haematocrit, leptin concentration and parasite measures were included as continuous variables. Biologically relevant interaction terms (age x BMI and age x parasites) were also fitted. A minimum adequate model

was generated by sequentially dropping non-significant terms. Wald F-tests were used to determine the significance of remaining terms. Where season remained a significant term in the minimum adequate model, a post-hoc Tukey's HSD test was used to identify significant differences in immune response between seasons.

To examine the relationship between diet and immune response, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ levels and the C/N ratio were correlated (Spearman's rank correlation coefficient (r_s)) with response type. These were excluded from the GLMs due to small sample size ($n=74$). Further, to determine the relationships between the expression levels of specific immune genes and the concentration of specific cytokines, these were correlated (r_s) with body condition and parasite measures. To correct for multiple testing, the Benjamini-Hochberg False Discovery Rate was applied. Spearman's rank correlation coefficients and associated significance levels were generated using the *rcorr()* function in the 'Hmisc' package (Harrell Jr 2017). All data analysis was conducted in R version 3.4.0 (R Core Team 2017).

6.3 Results

A summary of the significant components in minimal adequate models representing different arms of the immune response measured by qPCR from spleen and MLN tissue and concentrations of circulating cytokines is shown in Table 6.1.

Table 6.1: Summaries of minimum adequate GLMs fitted to immune response data from wild *Mus musculus domesticus* on the Isle of May, Scotland. Immune response was measured by expression levels of key genes from spleen or mesenteric lymph node (MLN) tissue, or circulating concentrations of corresponding cytokines. The response variables used here were the PC1 scores of appropriate gene/cytokine groupings. Significant relationships ($p < 0.05$) are indicated in bold.

Response	Tissue	Factor	df	F	p
Innate	Spleen	<i>Calodium hepaticum</i> severity	1, 297	6.101	0.014
		Season	6, 297	6.735	0.001
		BMI	1, 297	6.249	0.042
	MLN (TNF α)	<i>Syphacia obvelata</i> abundance	1, 251	3.804	0.047
	Circulating Cytokines	Sex	1, 322	7.779	0.006
		Leptin concentration	1, 322	4.748	0.030
		<i>Trichuris muris</i> abundance	1, 322	3.941	0.048
Th1	Spleen	BMI	1, 257	10.816	0.001
		<i>Calodium hepaticum</i> severity	1, 257	6.218	0.013
		Proportional liver mass	1, 204	5.833	0.017
	MLN	Age	1, 183	6.993	0.009
		Season	6, 183	12.941	<0.001
	Circulating Cytokines	Leptin concentration	1, 320	10.593	0.001
		Age	1, 320	3.930	0.048
		<i>Trichuris muris</i> abundance	1, 320	5.767	0.017
		Season	7, 320	6.684	0.002
Th2	Spleen	Proportional spleen mass	1, 271	5.673	0.018
	MLN	<i>Syphacia obvelata</i> abundance	1, 245	12.281	<0.001
		Age	1, 245	9.830	0.014
		Season	6, 245	7.105	0.001
	Circulating Cytokines	Sex	1, 319	18.229	<0.001
		Leptin concentration	1, 319	15.002	<0.001
		Age	1, 319	3.540	0.061
		<i>Trichuris muris</i> abundance	1, 319	0.606	0.437
		Age : <i>T. muris</i> abundance	1, 319	8.414	0.004
Th17	Spleen	Proportional spleen mass	1, 188	7.387	0.007
	MLN	No significant factors	-	-	-
	Circulating Cytokines	Leptin concentration	1, 323	21.330	<0.001
		Age	1, 323	5.025	0.026
		Spleen mass	1, 214	5.091	0.025
		Proportional liver mass	1, 267	7.669	0.006
		<i>Calodium hepaticum</i> severity	1, 323	7.316	0.007
Treg	Spleen	No significant factors	-	-	-
	MLN (IL10)	Age	1, 256	9.310	0.003
		BMI	1, 256	4.782	0.030
		<i>Syphacia obvelata</i> abundance	1, 256	10.225	0.002
	Circulating cytokines (IL10)	Leptin concentration	1, 323	6.656	0.010

6.3.1 Seasonal variation in immune response

There was significant seasonal variation in both the expression levels of Th1 genes measured from MLN tissue ($F_{6,320}=12.941$, $p<0.001$) and the concentration of circulating Th1 cytokines ($F_{6,277}=6.684$, $p=0.002$, Figure 6.1). Further, there was significant seasonal variation in the expression levels of innate immune genes measured from spleen tissue ($F_{6,297}=6.735$, $p=0.001$) and Th2 genes measured from MLN tissue ($F_{6,245}=7.105$, $p=0.001$, Figure 6.1).

Post-hoc analysis revealed that levels of circulating Th1 cytokines were significantly higher in spring 2015 than all other seasons (a *posteriori* Tukey's HSD test; $p<0.001$), with the exception of summer 2015 where there was no significant difference ($p=0.168$). There were no significant differences in levels of circulating Th1 cytokines between any other seasons ($p>0.304$). Similarly, the expression levels of Th1 genes measured from MLN tissue were higher in spring 2015 than in autumn 2015, although this was not significant ($p=0.057$). There were no significant differences in Th1 gene expression levels between any other seasons ($p>0.140$).

The expression levels of innate immune genes measured from spleen tissue were significantly higher in spring 2015 than in summer 2013 ($p<0.001$) and autumn 2015 ($p=0.002$). There were no significant differences in innate immune gene expression levels between any other seasons ($p>0.094$).

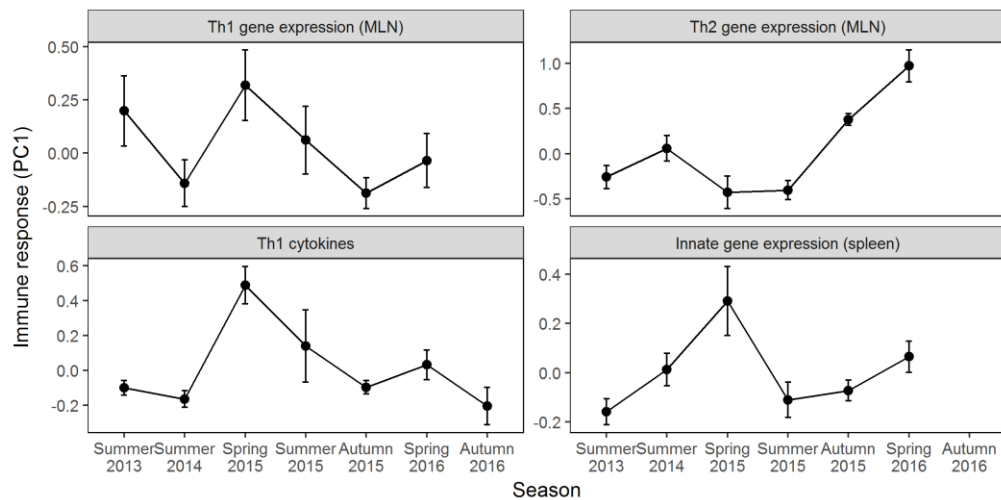


Figure 6.1: Seasonal variation in the immune response of wild *Mus musculus domesticus* from the Isle of May, Scotland. Immune response was the PC1 score of expression levels of key immune genes expressed by spleen (innate) or MLN (Th1, Th2) tissue, or concentrations of circulating cytokines (Th1).

The expression levels of Th2 genes measured from MLN tissue were significantly higher in spring 2016 than in any other season ($p < 0.045$), other than autumn 2015 ($p = 0.339$). MLN Th2 gene expression levels were significantly higher in autumn 2015 than in summer 2013 ($p < 0.001$), spring 2015 ($p < 0.001$) or summer 2015 ($p = 0.010$). There were no significant differences in Th2 gene expression levels between any other seasons ($p > 0.089$).

6.3.2 Differences in immune response between sexes

Circulating protein levels of Th2 ($F_{1,319} = 18.229$, $p < 0.001$) cytokines were elevated in male mice compared to females (Figure 6.2). Conversely, the concentration of circulating innate cytokines

($F_{1,320}=7.779$, $p=0.006$) was higher in female mice compared to males (Figure 6.2).

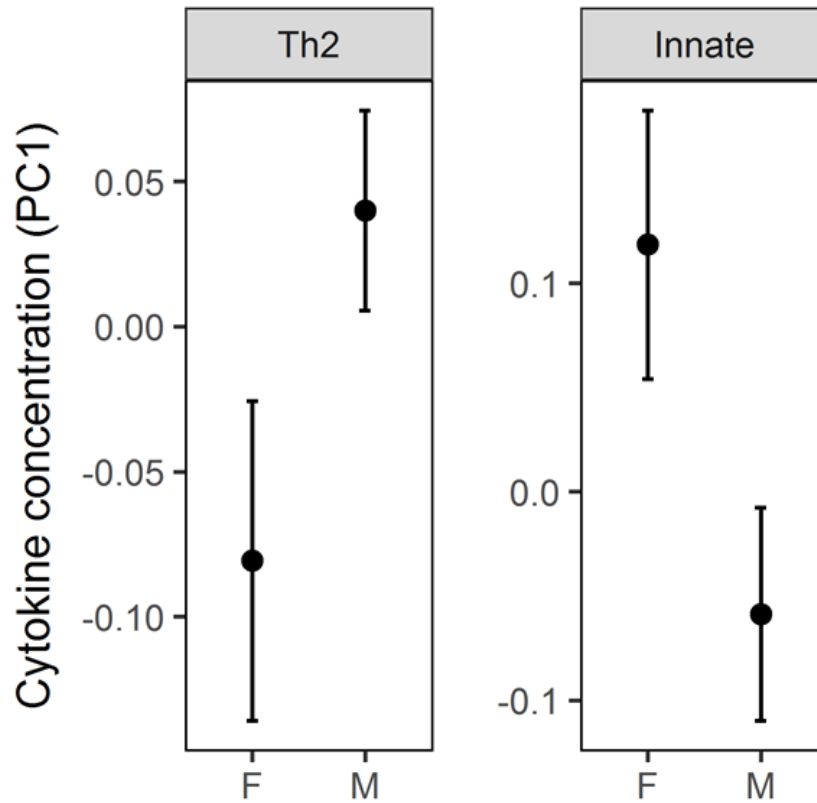


Figure 6.2: Sex difference in the immune response of wild *Mus musculus domesticus* from the Isle of May, Scotland. Immune response was the PC1 score of circulating cytokines.

6.3.3 Variation in immune response with age

Concentrations of circulating Th1 ($F_{1,320}=3.930$, $p=0.048$) and Th17 ($F_{1,323}=5.025$, $p=0.026$) cytokines as well as expression levels of Th1 ($F_{1,183}=6.993$, $p=0.009$) and IL10 (i.e. Treg) ($F_{1,256}=9.310$, $p=0.003$) genes in MLN tissues significantly increased with mouse age. However,

the expression levels of Th2 ($F_{1,245}=8.9.830$, $p=0.014$) genes in MLN tissue significantly decreased with mouse age (Figure 6.3).

6.3.4 Interactions between host age, parasite load and immune response

Further, concentrations of circulating Th2 cytokines were significantly positively correlated with *T. muris* abundance in mature mice but were significantly negatively correlated in immature mice ($F_{1,319}=8.414$, $p=0.004$, Figure 6.4).

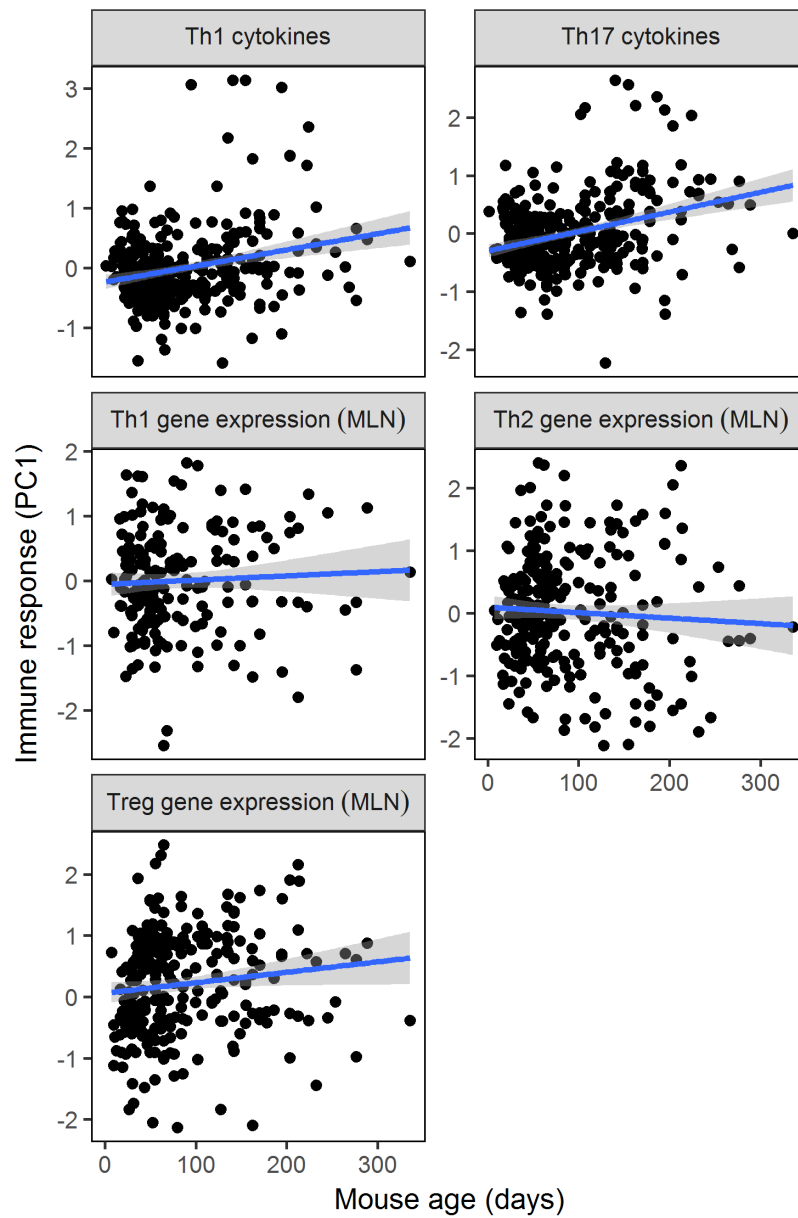


Figure 6.3: Relationship between age and immune response in wild *Mus musculus domesticus* from the Isle of May, Scotland. Immune response was the PC1 score of circulating cytokines or immune genes expressed by MLN tissue.

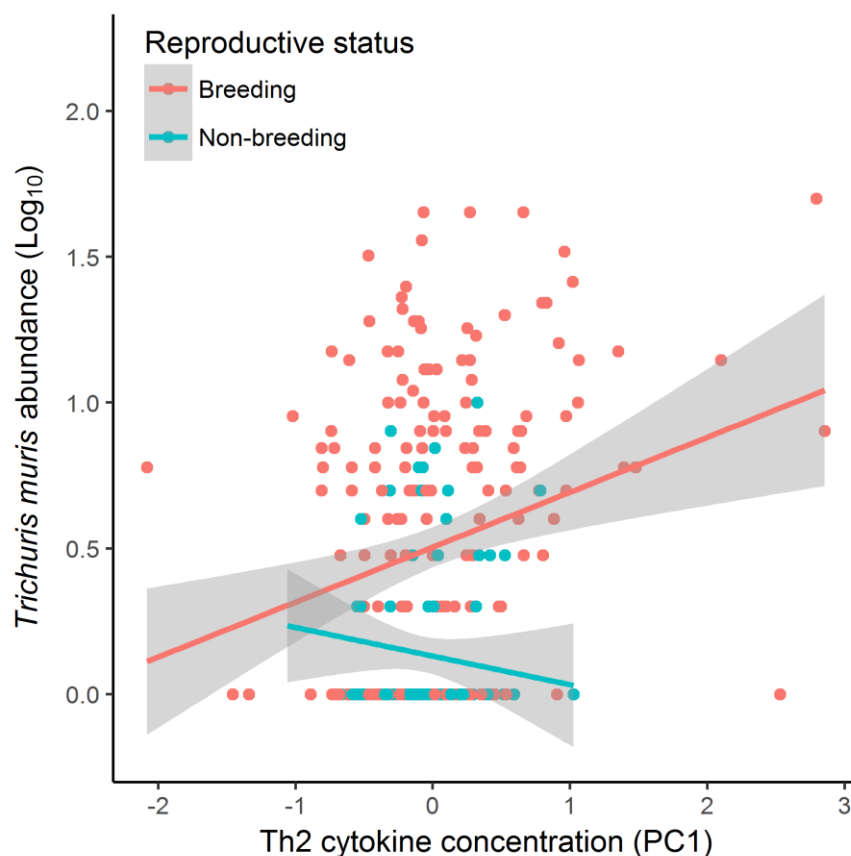


Figure 6.4: Relationship between *Trichuris muris* abundance and the Th2 immune response (measured by PC1 of relevant circulating cytokines) in wild *Mus musculus domesticus* from the Isle of May, Scotland. Trend lines indicate the different relationships for breeding, i.e. mature, mice and non-breeding, i.e. juvenile, mice.

6.3.5 Macroparasite infection and immune response

Mice with more severe *C. hepaticum* infection typically had significantly increased levels of innate gene expression from spleen tissue ($F_{1,297}=6.101$, $p=0.014$), as well as significantly higher levels of circulating Th17 cytokines ($F_{1,323}=7.316$, $p=0.007$). Conversely, those mice with more severe *C. hepaticum* infection had significantly lower levels of Th1 gene expression from spleen tissue ($F_{1,257}=6.218$, $p=0.013$, Figure 6.5a).

Expression levels of TNF- α (representing the innate response) ($F_{1,319}=8.414$, $p=0.004$), IL-10 (representing the Treg response) ($F_{1,319}=8.414$, $p=0.004$) and Th2 genes ($F_{1,319}=8.414$, $p=0.004$) from MLN tissue were all significantly positively correlated with *S. obvelata* abundance (Figure 6.5b). *T. muris* abundance was significantly positively correlated with circulating levels of Th1 cytokines ($F_{1,320}=5.767$, $p=0.017$), however circulating levels of innate cytokines ($F_{1,22}=3.941$, $p=0.048$) were significantly negatively correlated (Figure 6.5c).

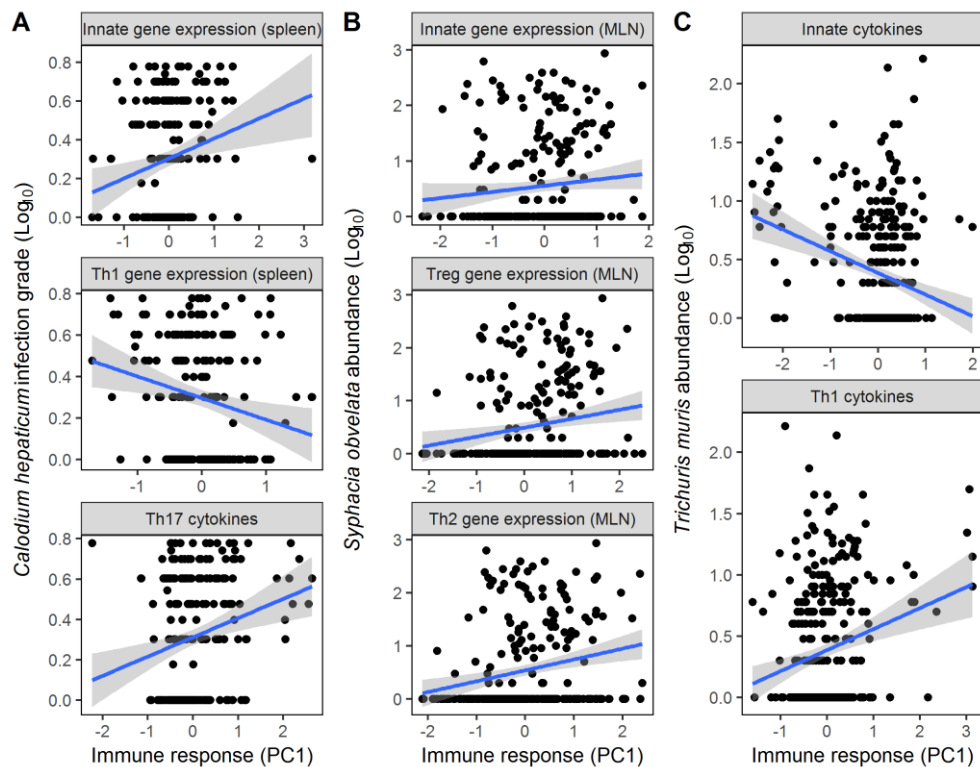


Figure 6.5: Relationship between macroparasite infection and immune response in wild *Mus musculus domesticus* from the Isle of May, Scotland. Immune response was the PC1 score of circulating cytokines or immune genes expressed by MLN or spleen tissue. Shown here is infection severity ($\log_{10} + 1$) of *C. hepaticum* (A) and abundances ($\log_{10} + 1$) of *S. obvelata* (B) and *T. muris* (C).

6.3.6 Proportional organ size and immune response

Mice with proportionally larger spleens typically had elevated expression levels of splenic Th2 genes ($F_{1,271}=5.673$, $p=0.018$), but spleen size was significantly negatively correlated with Th17 gene expression levels from spleen tissue ($F_{1,188}=7.387$, $p=0.007$, Figure 6.6a). Concentrations of circulating Th17 cytokines, however, were significantly positively correlated with proportional spleen mass ($F_{1,214}=5.091$, $p=0.025$, Figure 6.6a).

Th17 cytokine concentrations were also positively correlated with proportional liver size ($F_{1,267}=7.669$, $p=0.006$, Figure 6.6b), however mice with proportionally larger livers typically had decreased expression levels of splenic Th1 genes ($F_{1,204}=5.833$, $p=0.017$, Figure 6.6b).

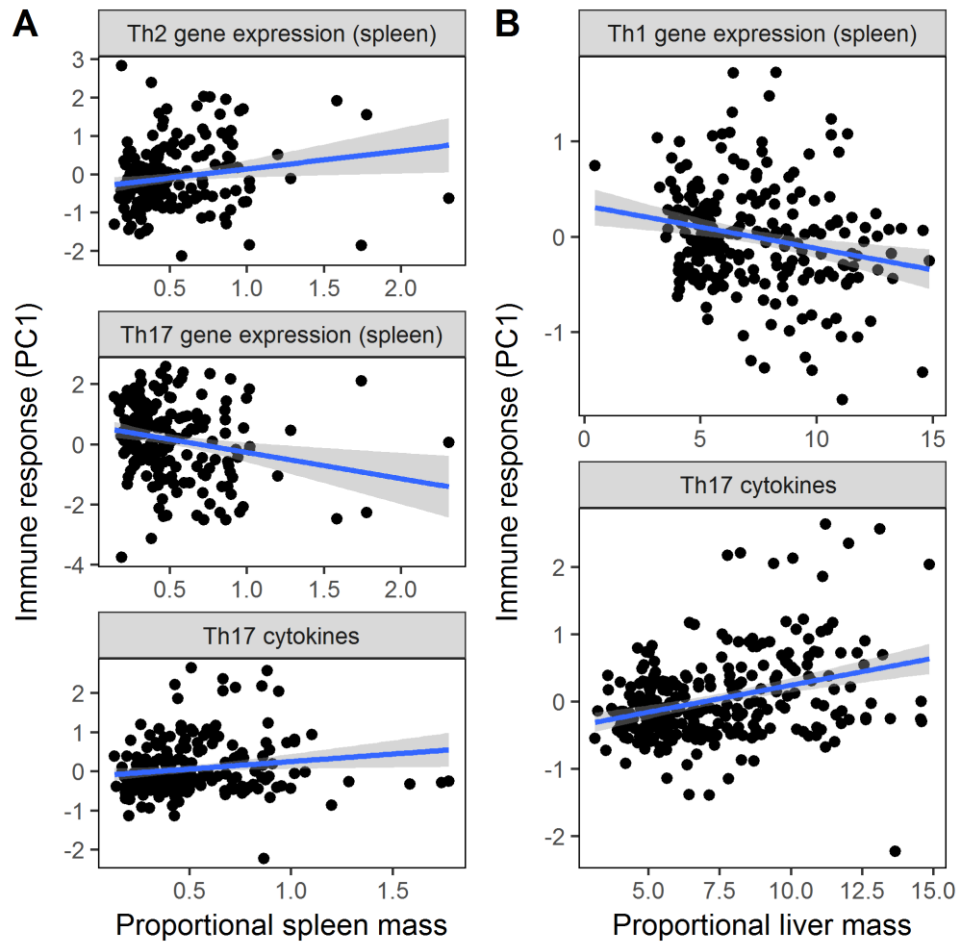


Figure 6.6: Relationship between proportional organ mass of spleen (A) or liver (B) and immune response in wild *Mus musculus domesticus* from the Isle of May, Scotland. Immune response was the PC1 score of circulating cytokines or immune genes expressed by spleen tissue.

6.3.7 Effect of body condition on immune response

Mice in better body condition typically had an elevated innate immune response; innate immune gene expression from spleen tissue was significantly positively correlated with BMI ($F_{1,297}=6.249$, $p=0.042$, Figure 6.7a), and circulating levels of innate cytokines were significantly positively correlated with serum leptin concentration ($F_{1,322}=4.748$, $p=0.030$, Figure 6.7b). Similarly, IL-10 gene expression (representing a

Treg response) in MLN tissue was significantly positively correlated with BMI ($F_{1,256}=4.782$, $p=0.030$, Figure 6.7a), and circulating levels of IL10 cytokines were significantly positively correlated with leptin concentration ($F_{1,323}=6.656$, $p=0.010$, Figure 6.7b).

Mice with a higher BMI had significantly lower expression levels of Th1 genes from spleen tissue ($F_{1,257}=10.816$, $p=0.001$, Figure 6.7a), and those with increased levels of circulating leptin had significantly lower concentrations of circulating Th1 cytokines ($F_{1,320}=10.593$, $p=0.001$, Figure 6.7b). Leptin concentrations were also significantly negatively correlated with circulating levels of Th2 ($F_{1,319}=3.540$, $p<0.001$, Figure 6.7b) and Th17 ($F_{1,323}=21.330$, $p<0.001$, Figure 6.7b) cytokines.

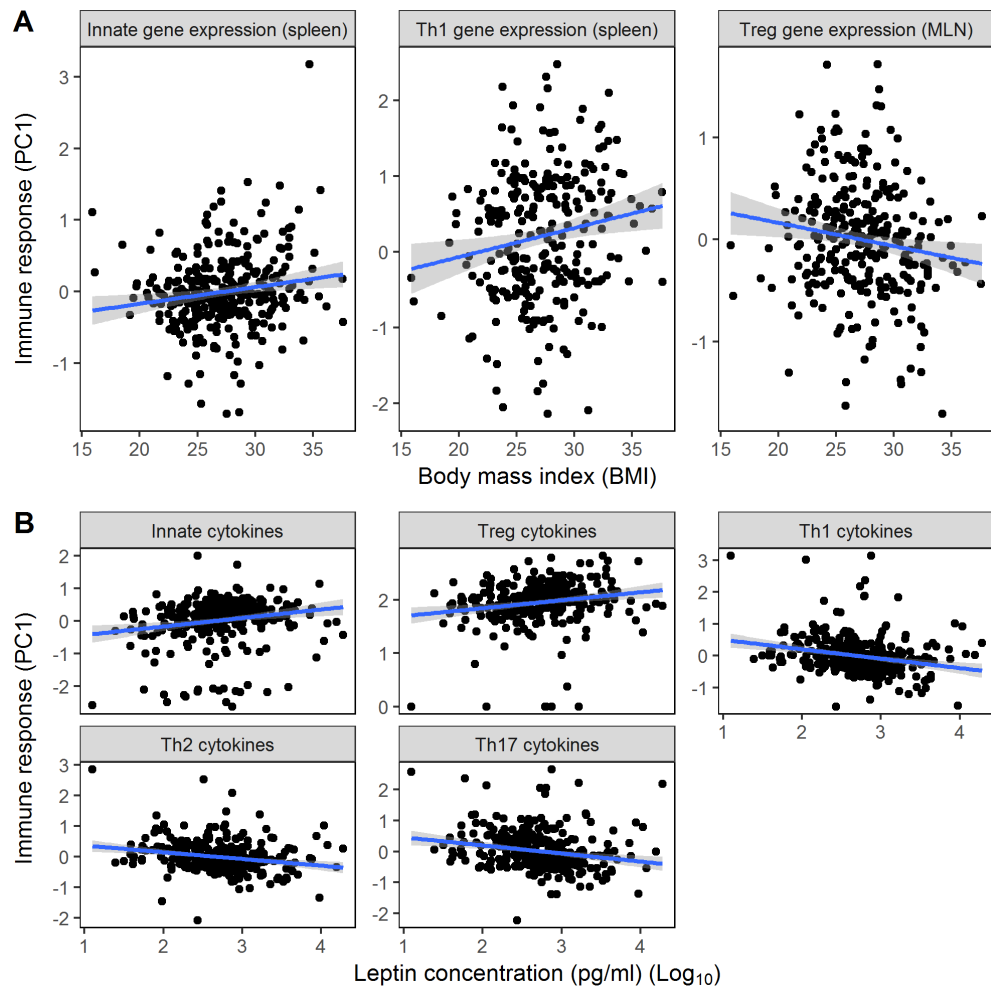


Figure 6.7: Relationship between BMI (A) and circulating leptin levels (B), measures of body condition, and immune response in wild *Mus musculus domesticus* from the Isle of May, Scotland. Immune response was the PC1 score of circulating cytokines or immune genes expressed by spleen and MLN tissue.

6.3.8 Immune response and diet

There were no significant correlations between any stable isotope values and any immune response type ($p > 0.057$).

6.3.9 Factors relating to individual gene expression and cytokine levels

Those continuous variables that were significantly correlated with individual expression levels and circulating levels of genes and cytokines, respectively, involved in the innate immune response are summarised in Table 6.2. For Th1 genes and cytokines they are summarised in Table 6.3, Th2 are summarised in Table 6.4, Th17 in Table 6.5 and Treg genes and cytokines are summarised in Table 6.6.

Table 6.2: Significant Spearman's rank correlation coefficients (r_s) between individual measures of the innate immune response and various host body condition, life history and parasite load variables from wild house mice (*Mus musculus domesticus*) on the isle of May, Scotland. Immune measures were concentrations of circulating cytokines, or expression levels of immune genes from spleen or mesenteric lymph node (MLN) tissue.

Innate immune measure	Host variable	r_s	p adj
IL-1 β (cytokine)	Age	-0.157	0.035
	<i>Calodium hepaticum</i> infection severity	0.243	0.004
	Circulating leptin concentration	0.194	0.005
	<i>Trichuris muris</i> abundance	-0.279	<0.001
TNF- α (cytokine)	Age	-0.334	<0.001
	<i>Nosopsyllus fasciatus</i> abundance	-0.190	0.007
	Circulating leptin concentration	0.186	0.008
	<i>Trichuris muris</i> abundance	-0.237	<0.001
IL-1 β (spleen)	Age	-0.219	0.002
	Body mass index (BMI)	0.213	0.003
	<i>Calodium hepaticum</i> infection severity	0.319	<0.001
TNF- α (spleen)	Age	-0.163	0.029
	C/N ratio	0.395	0.006

Table 6.3: Significant Spearman's rank correlation coefficients (r_s) between individual measures of the Th1 immune response and various host body condition, life history and parasite load variables from wild house mice (*Mus musculus domesticus*) on the isle of May, Scotland. Immune measures were concentrations of circulating cytokines, or expression levels of immune genes from spleen or mesenteric lymph node (MLN) tissue.

Th1 immune measure	Host variable	r_s	p adj
IFN- γ (cytokine)	Age	0.315	<0.001
	<i>Calodium hepaticum</i> infection severity	-0.212	0.013
	Circulating leptin concentration	-0.185	0.008
	Proportional liver mass	-0.304	<0.001
	<i>Trichuris muris</i> abundance	0.272	<0.001
IL-12 β (cytokine)	Circulating leptin concentration	-0.196	0.005
IFN- γ (spleen)	<i>Calodium hepaticum</i> infection severity	-0.318	<0.001
	Proportional liver mass	-0.227	0.005
IL-12 β (spleen)	C/N ratio	-0.399	0.010
Tbx21 (spleen)	Body mass index (BMI)	-0.156	0.042
	Fur mite abundance	-0.283	<0.001

Table 6.4: Significant Spearman's rank correlation coefficients (r_s) between individual measures of the Th2 immune response and various host body condition, life history and parasite load variables from wild house mice (*Mus musculus domesticus*) on the isle of May, Scotland. Immune measures were concentrations of circulating cytokines, or expression levels of immune genes from spleen or mesenteric lymph node (MLN) tissue.

Th2 immune measure	Host variable	r_s	p adj
IL-13 (cytokine)	Age	-0.369	<0.001
	Body mass index (BMI)	-0.173	0.014
	Circulating leptin concentration	-0.165	0.022
IL-5 (cytokine)	Circulating leptin concentration	-0.225	0.001
IL-13 (MLN)	<i>Syphacia obvelata</i> abundance	0.226	0.004
Gata3 (spleen)	<i>Calodium hepaticum</i> infection severity	0.187	0.044
	C/N ratio	-0.331	0.029
IL-13 (spleen)	C/N ratio	-0.340	0.024
IL-5 (spleen)	C/N ratio	-0.334	0.029

Table 6.5: Significant Spearman's rank correlation coefficients (r_s) between individual measures of the Th17 immune response and various host body condition, life history and parasite load variables from wild house mice (*Mus musculus domesticus*) on the isle of May, Scotland. Immune measures were concentrations of circulating cytokines, or expression levels of immune genes from spleen or mesenteric lymph node (MLN) tissue.

Th17 immune measure	Host variable	r_s	p adj
IL-17 α (cytokine)	Age	0.218	0.001
	Proportional liver mass	0.286	<0.001
IL-6 (cytokine)	Age	0.336	<0.001
	Calodium	0.220	0.010
	Proportional liver mass	0.281	<0.001
IL-17 α (MLN)	<i>Syphacia obvelata</i> abundance	0.180	0.041
IL-17 α (spleen)	C/N ratio	-0.310	0.049
	Proportional spleen mass	-0.222	0.016
IL-6 (spleen)	C/N ratio	-0.333	0.027
Irf5 (spleen)	Age	0.181	0.012
	Body mass index (BMI)	-0.187	0.010
	Ectoparasite species richness	-0.180	0.013
	Fur mite abundance	-0.299	0.000
	Proportional liver mass	0.207	0.009
	Proportional spleen mass	-0.286	0.001
Rorc (spleen)	C/N ratio	-0.372	0.011

Table 6.6: Significant Spearman's rank correlation coefficients (r_s) between individual measures of the Treg immune response and various host body condition, life history and parasite load variables from wild house mice (*Mus musculus domesticus*) on the isle of May, Scotland. Immune measures were concentrations of circulating cytokines, or expression levels of immune genes from spleen or mesenteric lymph node (MLN) tissue.

Treg immune measure	Host variable	r_s	p adj
IL-10 (cytokine)	Circulating leptin concentration	0.150	0.042
IL-10 (MLN)	Body mass index (BMI)	0.167	0.041
	Endoparasite species richness	0.252	0.001
	<i>Syphacia obvelata</i> abundance	0.177	0.028
Foxp3 (spleen)	C/N ratio	0.352	0.017
	Ectoparasite species richness	-0.197	0.007
	<i>Trichuris muris</i> abundance	-0.156	0.041

6.4 Discussion

Much of our current understanding of immune function comes from studies on laboratory mice (Abolins *et al.* 2011). Wild house mice, however, are immunophenotypically different to laboratory strains (Abolins *et al.* 2011; Boysen *et al.* 2011; Viney *et al.* 2015; Abolins *et al.* 2017). In this study, we aimed to investigate the factors driving the immune response in wild mice. We explored seasonal variation in the immune response, as well as the relationships with macroparasite infection, host age, sex, body condition and nutritional status. These mice show evidence of tolerance to parasites (see Chapter 4), so we also aimed to determine whether there is a biomarker present for this trait.

6.4.1 Seasonal variation in immune response

Th1 gene expression and circulating cytokine levels, as well as innate gene expression, were elevated in animals caught in spring 2015 compared to those in other seasons. While it might be expected that these individuals would have a reduced immune response due to limited resource availability through the winter (Nelson & Demas 1996; Nelson 2004; Altizer *et al.* 2006), these animals are typically in better body condition than those from other seasons (see Chapter 2). Therefore, this higher body condition could explain the observed increased immune response (Lochmiller & Deerenberg 2000; MacColl *et al.* 2017). Th2 gene expression, however, is lower in spring 2015 compared to other seasons;

this is likely due to the antagonistic relationship between the Th1 and Th2 responses (Kaiko *et al.* 2008).

6.4.2 Immune response and sex

Here, we observed that female mice have higher concentrations of circulating innate cytokines than males. Indeed, an elevated innate immune response in females has typically observed in humans and laboratory rodents (Klein & Flanagan 2016; Doyle & Murphy 2017), with females generally producing higher levels of pro-inflammatory cytokines (Aulock *et al.* 2006; Calippe *et al.* 2010; Drew and Chavis 2000; Engler *et al.* 2016; Loram *et al.* 2012). Here, males had elevated levels of anti-inflammatory Th2 cytokines compared to females.

6.4.3 Immune response and age

Here, there was an age related increase in Th1 cytokines and gene expression, as well as Th17 cytokines. This pattern of increased pro-inflammatory responses is expected due to immunosenescence, and has been observed in several wild vertebrate populations (Palacios *et al.* 2011; Nussey *et al.* 2012; Schneeberger *et al.* 2014; Watson *et al.* 2016). In these mice, increasing age is also associated with a decreasing anti-inflammatory Th2 response.

Conversely, there was an age-related decrease in the circulating concentrations and the gene expression levels of the innate cytokines IL-

1 β and TNF- α . While no relationship has been observed between age and TNF- α cytokines in olive baboons (*Papio hamadryas anubis*) (McFarlane *et al.* 2011) or with innate gene expression in stickleback (Robertson *et al.* 2016), the pattern observed here is likely due to the increasing importance of adaptive immune defences as animals age.

6.4.4 Immune response and body condition – a marker of tolerance?

In these mice, the concentration of circulating Th2 cytokines increased with *T. muris* infection in mature, but not immature, individuals. This indicates a possible shift from a resistant to a tolerant immunophenotype with age, similar to the pattern observed in wild field voles (Jackson *et al.* 2014). In field voles, Jackson *et al.* (2014) identified Gata3 as a biomarker for tolerance. Here, Gata3 gene expression is positively correlated with *C. hepaticum* infection levels, indicating a possible pattern of tolerance to this hepatic nematode.

Here, the circulating levels of Th2 cytokines have a negative relationship with leptin, a marker of body condition. Further to this, individual measures of the Th2 response (IL-3 and IL-5 circulating cytokines and gene expression from spleen tissue; Gata3 gene expression from spleen tissue) are negatively correlated with various measures of body condition, including BMI, C/N ration and leptin. This indicates that while tolerance may be present in this population (Chapter 4), we have been unable to identify an obvious biomarker for this trait.

Both IL-1 β gene expression from spleen tissue and circulating cytokines are positively correlated with *Calodium* infection, as well as BMI and leptin levels, respectively. Due to the pro-inflammatory nature of IL-1 β , this is unlikely to be related to tolerance. This relationship, however, could indicate that those animals in better body condition are more likely to mount an inflammatory response, possibly due to being better able to cope with the associated energetic costs (Schwensow *et al.* 2011).

6.4.5 Immune response to *S. obvelata* infection

Here, the expression levels of Th2-related genes from MLN tissue were positively correlated with *S. obvelata* infection, specifically with higher levels of IL-13 expression. A similar response has been observed in laboratory mice, with elevated IL-4, IL-5 and IL-13 cytokine production (Michels *et al.* 2006). Indeed, the pattern observed here provides further evidence that IL-13 plays a dominant role in the control of *S. obvelata* (Michels *et al.* 2006).

6.4.6 Conclusions

This study highlights that individual immune profiles in wild house mice are shaped by multiple intrinsic and extrinsic factors. A similar pattern of complex factors influencing an individual's immune response has been observed in wild populations of other vertebrate species, including three-spined stickleback (*Gasterosteus aculeatus*) (Robertson

et al. 2016), field voles (Jackson *et al.* 2011) and Soay sheep (Nussey *et al.* 2014).

Despite tolerance to infection by macroparasites being present in this population (Chapter 4), we were unable to identify a biomarker for this. However, here we were able to show that mice with better body condition are more likely to mount an inflammatory response. This is likely due to the fact that these mice are better able to manage the energetic costs and potentially negative health impacts of such an aggressive immune response (Schwensow *et al.* 2011).

Microparasites have been identified in these mice (S. Goertz, unpublished; see Chapter 4 for more detail), however their inclusion was beyond the scope of these analyses. Microparasites, and their interactions with macroparasites, can play an important role in driving host body condition and population dynamics (Cizauskas *et al.* 2015). The Th1 response is active against intracellular parasites, such as *Babesia* sp. and *Bartonella* sp. Indeed, increased expression of Th1 antibodies in response to *B. microti* infection has been observed in laboratory mice (Chen *et al.* 2000). A recent study, however, identified increased expression of IL-10 in wild field voles infected with *Bartonella* (Taylor *et al.* 2018). This may reduce the Th1 response and minimise the negative impacts of inflammation on the host (Taylor *et al.* 2018). More work is needed to understand the relationships between micro- and microparasite infection and host immune response in the Isle of May mice.

Chapter 7

General discussion

7.1 Thesis overview

Due to the availability of a wide range of reagents, and a solid understanding of immune function in their laboratory counterparts, wild house mice have been proposed as an ideal study system for ecoimmunology (Abolins *et al.* 2017). In this thesis, I utilised the island population of house mice on the Isle of May to ultimately explore the intrinsic and extrinsic drivers of variation in the immune response of a wild mammal.

In **Chapter 2**, I was able to provide estimates for the population sizes of mice on the Isle of May. I then explored the seasonal variation in population size, as well as variation in the demographics and morphometrics of these mice. In **Chapter 3**, I used stable isotope analysis to identify the diet of Isle of May mice. Using these isotopic indicators, I then explored the relationship between diet and body condition. In **Chapter 4**, I identified the macro-parasites infecting Isle of May mice and explored how parasite load and prevalence varied seasonally. I also examined the relationships between these parasite indices and a variety of measures of host life history, body condition, physiology and nutrition. In **Chapter 5**, I introduced the molecular measures I would be using to explore the immune response of Isle of

May mice. I explored how these measures – expression levels of key immune genes from spleen and MLN tissues and circulating cytokine concentrations – correlated with each other. The lack of correlation between these measures highlights the importance of using various indices to fully capture the immune profile of an individual. Finally, in **Chapter 6** I examined the relationships between these measures of immune function and various measures of life history, physiology, body condition, nutritional status and parasite load. This highlights the complexity of studying immune response in a wild population, and that the immune profile of an individual is shaped by multiple intrinsic and extrinsic factors.

7.2 Key findings

7.2.1 Chapter 2

Using spatially explicit capture-recapture (SECR) models, I was able to estimate the population sizes of mice on the Isle of May. The SECR models predicted a low in the spring with a peak in the winter, which aligned with previous observations of this population (Triggs 1991). The degree of variation observed here was less than previously observed on the Isle of May (Triggs 1991).

7.2.2 Chapter 3

I used stable isotope analysis to reveal that the majority of the diet of Isle of May mice is derived from animal sources, with invertebrate prey contributing over half of the total diet. Seabird sources contribute just 4% of the diet, indicating that mice are likely scavenging on seabird materials at low levels. Therefore, the mice on the Isle of May do not represent an immediate conservation concern. Levels of $\delta^{13}\text{C}$ were negatively correlated with body condition, indicating that mice feeding on marine sourced materials (i.e. seabirds) were in worse body condition. This could be a result of mice seeking out seabird sourced food as a last resort when under nutritional stress.

7.2.3 Chapter 4

As well as significant seasonal variation in macroparasite infection, I was also able to show a pattern of tolerance in this population. Here, *T. muris* burden increased with increasing body condition in adult animals, but not in immature animals, possibly indicating a shift towards a tolerance (Jackson *et al.* 2014).

7.2.4 Chapter 5

Here, I investigated the relationships between immune genes expressed by two immunologically important tissues (spleen and MLN), and circulating cytokine levels. The lack of correlation observed here

highlights that using a limited number of measures of immunity (e.g. gene expression from only one tissue type) is unlikely to capture the wide variation in immune response present in wild populations (Watson *et al.* 2016). Indeed, when working with non-model species in which using extensive genomic or proteomic immunological measures may be costly and time consuming (Jackson 2015), it is important to consider which available immune measures are most informative for ecoimmunological studies (Boughton *et al.* 2011; Demas *et al.* 2011; Watson *et al.* 2016).

7.2.5 Chapter 6

Here, I investigated the relationship between various extrinsic and intrinsic factors and host immune response. While I was unable to identify a biomarker for tolerance, I was able to highlight that animals in better body condition typically mounted an increased Th1 response. This is likely due to their ability to better moderate the negative effects of this aggressive, pro-inflammatory response (Schwensow *et al.* 2011).

7.3 Concluding remarks

Despite their wide use as a model species in immunology, and biological sciences as a whole, house mice are ‘immunologically understudied’ in the wild (Abolins *et al.* 2017). This is largely due to the fact that wild mouse populations live commensally with humans and are treated as pests, and so are subject to population control measures. By

utilising the non-commensal mice of the Isle of May, as we have done here, the sophisticated and sensitive techniques that have been developed to measure immune response in the lab can be easily transferred to a wild population, further closing the gap between traditional and eco-immunology.

This study highlights the vast array of extrinsic and intrinsic factors that affect an individual's immune response, including diet, age, sex, body condition and parasite infection. Much as Dobzhansky (1973) famously said that “nothing in biology makes sense except in the light of evolution”, it is becoming increasingly apparent that “nothing makes immunological sense in light of the wild” (Viney & Riley 2014).

8 References

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Appendix 1.1: DTP statement for examiners



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

Research Training Structure

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

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

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

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

from a portfolio of relevant Master Courses which they can attend alongside their lab rotation projects.

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

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

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

Appendix 1.2: Professional Internships for PhD Students reflection form

Name of Organization

Chester Zoo (The North of England Zoological Society)

Details of Placement

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

Working for Chester Zoos Field Programmes team, my main task was to provide a review of all available camera trap data management systems. The aim of this was to provide recommendations for software to be used by Chester Zoo. This would help to streamline experimental design, data handling, processing and analysis across all zoo staff conducting camera trap studies. This included *in situ* behavioural analyses being conducted by the Applied Science team to *ex situ* population and biodiversity monitoring surveys conducted by the Conservation Science teams.

As well as this, I assisted with general administrative duties related to conservation, including reviewing grant proposals. I also worked closely with members of the IUCN SSC Asian Wild Cattle Specialist Group (AWCSG), helping to develop a bi-annual newsletter. This involved designing a template, outlining the scope of the newsletter and developing author guidelines.

In addition to this, I was able to spend time working hands-on with some of the zoos animals. I shadowed keepers on the 'Twilight' section, which includes fruit bats, red pandas, aardvarks and other small mammals. I was also able to assist with a zoo-funded field conservation project, radio-tracking reintroduced pine marten in Wales.

Placement Achievements

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

The findings of my review into camera trap data management programs were presented in a poster session at the Future Directions in Conservation Science symposium, held in Chester in April 2017. Further, this review (Software to facilitate and streamline camera trap data management: a review) has been accepted for publication by Ecology & Evolution.

The recommendation from my review was that the science teams at Chester Zoo should use the 'Camera Trap Analysis Tool' (CTAT), a program in development by the Zoological Society of London (ZSL). Further, I played a key role in the formation of a partnership and signing of a Memorandum of Understanding between Chester Zoo and ZSL. This means that Chester Zoo will now assist with the future development of CTAT, and the finished version will be marketed as a joint venture. In order to facilitate this partnership and

the future development of CTAT, I helped write a grant proposal for 'Conservation and Research Support' from Chester Zoos conservation budget. This grant application was successful and awarded the project £5,000.

Skill development

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

During this placement, I had to work closely with members from the various science teams within Chester Zoo as well as staff from other institutions. This greatly developed my team working skills, as well as my negotiating skills. Presenting my work to various staff, with varying levels of background knowledge, as well as presenting it to a much wider audience at the symposium, meant that my presentation and public speaking skills were enhanced.

Future Work

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

This placement provided a valuable insight into the workings of a conservation non-governmental organisation, particularly allowing me to see how conservation research (both *in situ* and *ex situ*) is conducted within a leading zoo.

I feel that the various networking opportunities presented to me during this placement will prove invaluable when forging a future career. As well as working closely with both the Applied and Conservation Science teams at Chester Zoo, I was also able to forge links with staff at ZSL as well as members of the IUCN AWCSG. Presenting my findings at the symposium enabled me to make contacts with research institute-based academics as well as researchers and conservation practitioners from other zoos and NGOs.

As a direct result of this placement, I was offered my current position of Programme Officer for the IUCN AWCSG, based at Chester Zoo.

Appendix 2.1: RNA extraction protocol

Extracting RNA from tissue (stored in RNAlater®)
Machery-Nagel NucleoSpin® RNA

Prepare **DNase reaction mixture** – for each reaction 10µL **rDNase** + 90µL **reaction buffer for rDNase**, keep on ice.

Use up to **30mg** tissue.

1. Add 350µL **Buffer RA1** and 3.5µL **β-mercaptoethanol** to tissue (600µL and 6µL if using >20mg tissue), homogenise.
2. Place filter (**violet ring**) in a 2mL collection tube. Apply the lysate and centrifuge for 1 min @ 11,000 x g.
3. Discard the filter and add 350µL (600µL if using >20mg tissue) **70% ethanol** – mix by pipetting up and down (five times).
4. Pipette lysate up and down 2-3 times, and load to a column (**light blue ring**) (max 750µL, discard flow-through and repeat if larger volumes are to be processed). Centrifuge for 30s @ 11,000 x g.
Place the column in a new Collection Tube (**2mL**).
5. Add 350µL **MDB** – centrifuge for 1 min @ 11,000 x g to dry the membrane.
6. Apply 95µL **DNase reaction mixture** onto the centre of the membrane of the column.
Incubate at room temperature for 15 min.
7. Add 200µL **Buffer RAW2**. Centrifuge for 30s @ 11,000 x g. Place the column in a new Collection Tube (**2mL**).
8. Add 600µL **Buffer RA3**. Centrifuge for 30s @ 11,000 x g. Discard flow-through and place the column back in the Collection Tube.
9. Add 250µL **Buffer RA3**. Centrifuge for 2 min @ 11,000 x g to completely dry the membrane. Place the column into a nuclease free Collection Tube (**1.5mL**).
10. Elute the RNA in 60µL **RNase-free H₂O** and centrifuge for 1 min @ 11,000 x g.

NanoDrop can now be used to check quantity and quality of RNA.

To check for contamination by genomic DNA, mix 6µL sample with 4µL Orange Dye. Run this alongside a small amount of 100bp ladder on a 2% Agarose gel @ 90V for ~30 min.

There should be two bands for RNA, a third band present indicates contamination.

The extracted RNA can be stored @ -80°C, or used for cDNA synthesis.

Appendix 2.2: cDNA synthesis protocol

cDNA Synthesis

Primerdesign *Precision nanoScript™ 2* Reverse Transcription Kit

Annealing Step

1. Add up to 2µg RNA template to a mastermix containing (x2 for each sample, -RT and +RT):

Oligo-dT primer	0.5µL
Random nonamer primer	0.5µL
RNAse free H ₂ O	XµL
Total volume (including water and template)	10µL

2. Heat to 65°C for 5 mins. IMMEDIATELY cool on ice – do not cool prior to placing on ice.

Extension Step

3. Make up a +RT mastermix containing:

nanoScript2 4X Buffer	5.0µL
dNTP mix 10mM	1.0µL
RNAse free H ₂ O	3.0µL
nanoScript2 enzyme	1.0µL
Total volume	10.0µL
4. Make up a -RT mastermix containing:

nanoScript2 4X Buffer	5.0µL
dNTP mix 10mM	1.0µL
RNAse free H ₂ O	4.0µL
Total volume	10.0µL

5. Add 10µL of the relevant mix to each of the appropriate samples on ice.
6. Mix tubes by briefly vortexing followed by a pulse spin.
7. Incubate at 25°C for 5 mins, then at 42°C for 20 mins. Heat inactivate by incubation at 75°C for 10 mins.
8. NanoDrop samples and record cDNA concentrations.
9. Store cDNA samples at -20°C until further use, RNA samples to be stored at -80°C.

Appendix 3: qPCR

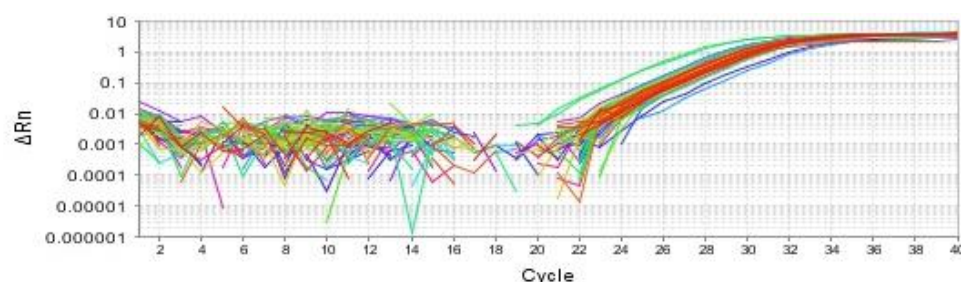


Figure A3.1: qPCR amplification plot of *I15* expressed by spleen tissue from wild house mice (*Mus musculus domesticus*) on the Isle of May. This illustrates the 'bunching' characteristic of primer-dimers, possibly as a result of low template concentration.

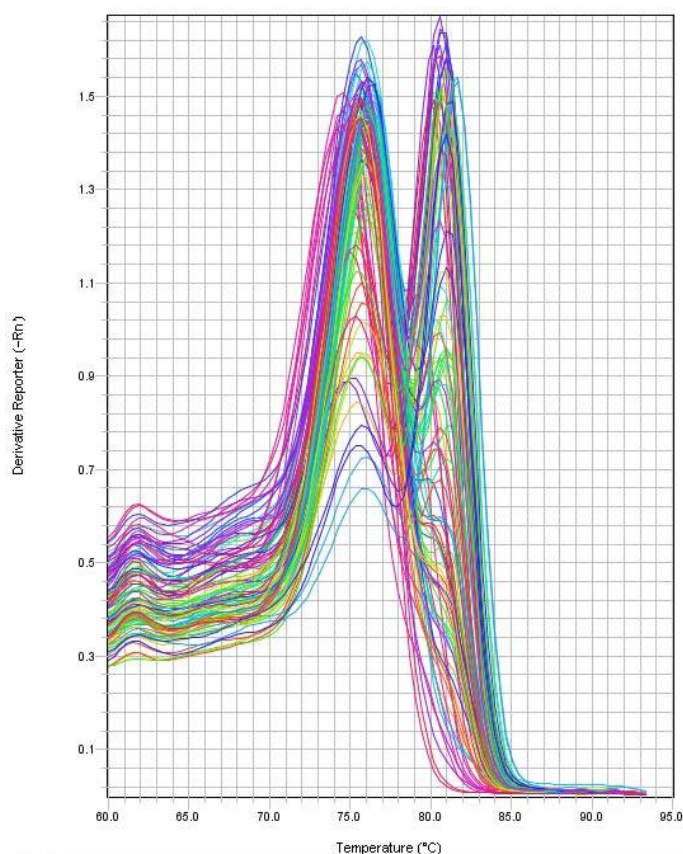


Figure A3.2: qPCR melt curve plot of *I15* expressed by spleen tissue from wild house mice (*Mus musculus domesticus*) on the Isle of May. Multiple peaks indicate possible gDNA contamination or primer dimerization, possibly as a result of low template concentration.

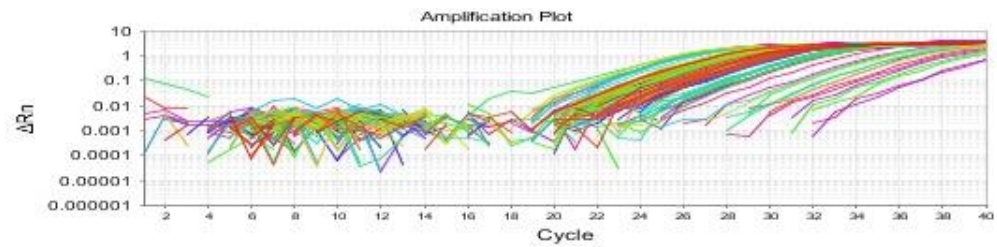


Figure A3.3: qPCR amplification plot of *Irf5* expressed by spleen tissue from wild house mice (*Mus musculus domesticus*) on the Isle of May. This is an example of a 'good' run, in which products of different concentrations amplify at different points. There is no clear evidence of primer-dimerisation.

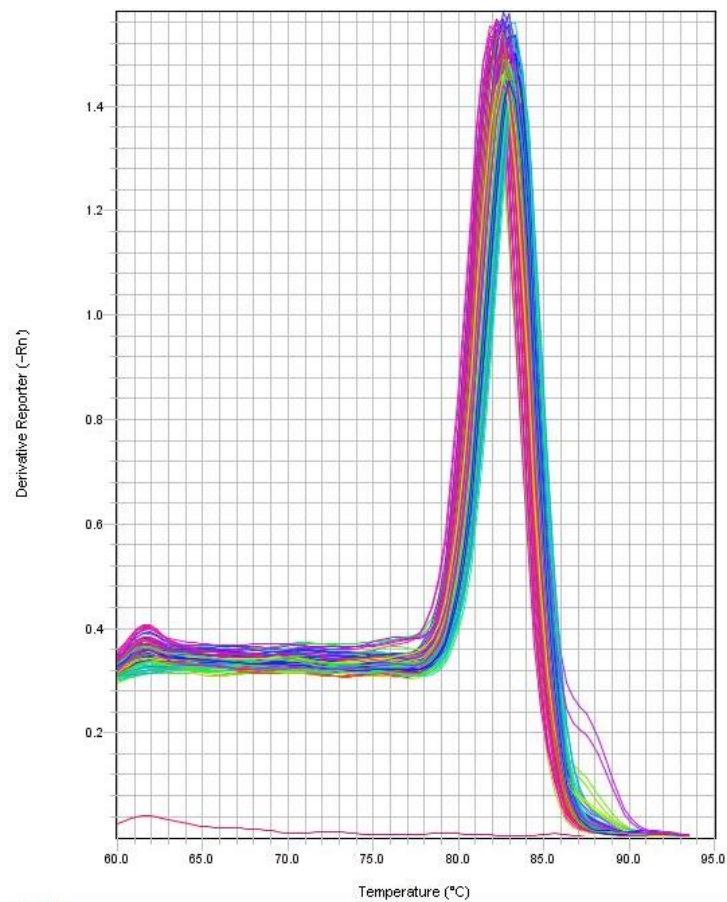


Figure A3.4: qPCR melt curve plot of *Irf5* expressed by spleen tissue from wild house mice (*Mus musculus domesticus*) on the Isle of May. This is an example of a 'good' run, in which melt occurs at a single phase. There is no clear evidence of primer-dimerisation or gDNA contamination.

Appendix 4: Correlations

Table A4.1: Spearman's rank correlation coefficients (r_s) and associated p -values of nonsignificant relationships ($p>0.05$) between immune gene expression, measured by qPCR of mesenteric lymph node (MLN) or spleen tissue, and circulating cytokine concentrations from wild house mice (*Mus musculus domesticus*) on the Isle of May, Scotland. To control for multiple testing, p -values were adjusted using the Benjamini-Hochberg procedure.

Gene expressed/ circulating cytokine		r_s	p adj.
IL-17 (spleen)	IL-10 (MLN)	-0.144	0.053
Gata3 (spleen)	IL-12 β (MLN)	-0.160	0.059
IL-10 (spleen)	IL-12 β (MLN)	-0.159	0.060
IL-10 (cytokines)	Irf5 (spleen)	0.132	0.066
TNF- α (cytokines)	IL-12 β (MLN)	0.154	0.067
IL-6 (cytokines)	IL-17 (MLN)	-0.134	0.067
IL-13 (cytokines)	TNF- α (MLN)	0.135	0.067
IL-10 (cytokines)	IL-1 β (spleen)	0.131	0.070
IL-13 (cytokines)	IL-1 β (spleen)	0.131	0.070
IFN- γ (spleen)	Gata3 (MLN)	0.133	0.072
IL-1 β (spleen)	Gata3 (MLN)	0.130	0.072
TNF- α (cytokines)	IL-13 (MLN)	0.133	0.072
IL-10 (spleen)	IL-13 (MLN)	-0.134	0.072
IL-1 β (cytokines)	IL-13 (MLN)	0.133	0.072
IL-5 (spleen)	TNF- α (MLN)	-0.139	0.072
IL-17 (spleen)	IL-13 (MLN)	-0.136	0.073
IL-1 β (cytokines)	IL-12 β (MLN)	0.149	0.076
IFN- γ (cytokines)	Tbx21 (spleen)	-0.128	0.077
IL-5 (cytokines)	TNF- α (MLN)	0.130	0.077
IL-17 (spleen)	TNF- α (MLN)	-0.130	0.089
IL-5 (cytokines)	IL-13 (MLN)	0.127	0.089
ROR- γ (spleen)	IL-13 (MLN)	-0.127	0.092
IFN- γ (cytokines)	TNF- α (MLN)	0.125	0.092
ROR- γ (spleen)	IL-12 β (MLN)	-0.143	0.097
IL-1 β (spleen)	IL-1 β (MLN)	0.123	0.097
IL-12 β (cytokines)	IL-6 (MLN)	-0.123	0.097
IL-10 (spleen)	TNF- α (MLN)	-0.124	0.097
IL-10 (cytokines)	Tbx21 (spleen)	0.121	0.098
IL-13 (spleen)	Gata3 (MLN)	-0.123	0.100
IL-1 β (cytokines)	IL-1 β (spleen)	0.119	0.102
IL-12 β (cytokines)	IL-1 β (spleen)	0.118	0.107
IL-6 (spleen)	TNF- α (MLN)	-0.121	0.113
IL-17 (cytokines)	IL-13 (MLN)	0.119	0.115
Irf5 (spleen)	IFN- γ (MLN)	0.124	0.117
IL-10 (cytokines)	IFN- γ (spleen)	0.118	0.117
IL-1 β (spleen)	IFN- γ (MLN)	0.123	0.117
IL-17 (spleen)	IL-12 β (MLN)	-0.138	0.118
IL-17 (cytokines)	IL-12 β (MLN)	0.134	0.119
IL-6 (cytokines)	IL-10 (MLN)	0.115	0.122
IL-5 (spleen)	Gata3 (MLN)	-0.120	0.123
IL-6 (spleen)	IL-13 (MLN)	-0.117	0.127
IL-13 (spleen)	TNF- α (MLN)	-0.118	0.128
IL-6 (spleen)	IL-10 (MLN)	-0.115	0.129
IL-5 (cytokines)	Tbx21 (spleen)	-0.113	0.129
ROR- γ (spleen)	IFN- γ (MLN)	-0.118	0.137
IL-6 (cytokines)	Tbx21 (spleen)	-0.111	0.139
IL-6 (spleen)	Gata3 (MLN)	-0.110	0.142
TNF- α (spleen)	IL-13 (MLN)	-0.112	0.149
Gata3 (spleen)	IL-1 β (MLN)	-0.109	0.149
IL-1 β (cytokines)	FoxP3 (spleen)	0.107	0.157
IFN- γ (spleen)	IFN- γ (MLN)	0.115	0.157
IL-1 β (spleen)	IL-10 (MLN)	0.108	0.157
IFN- γ (cytokines)	IL-1 β (spleen)	0.103	0.178
TNF- α (cytokines)	TNF- α (MLN)	0.103	0.187
Irf5 (spleen)	IL-10 (MLN)	-0.101	0.194
IL-6 (spleen)	IL-12 β (MLN)	-0.116	0.208
IL-17 (cytokines)	IL-6 (MLN)	-0.099	0.210
IL-6 (cytokines)	IFN- γ (MLN)	0.103	0.213
IL-12 β (cytokines)	IL-17 (MLN)	-0.098	0.213
IFN- γ (cytokines)	Irf5 (spleen)	-0.096	0.213
FoxP3 (spleen)	TNF- α (MLN)	-0.098	0.228
Irf5 (spleen)	Gata3 (MLN)	-0.094	0.231
TNF- α (cytokines)	FoxP3 (spleen)	0.094	0.232
IFN- γ (spleen)	IL-17 (MLN)	-0.097	0.239
IL-5 (cytokines)	IFN- γ (spleen)	-0.092	0.260
Gata3 (spleen)	TNF- α (MLN)	-0.092	0.263
IL-17 (cytokines)	IL-5 (spleen)	-0.093	0.268
IL-5 (cytokines)	IL-17 (MLN)	0.090	0.274
IL-13 (spleen)	IL-13 (MLN)	-0.093	0.278
IL-13 (spleen)	IL-10 (MLN)	-0.089	0.296

Table A4.1 continued overleaf

Table A4.1: Continued.

Gene expressed/ circulating cytokine		r_s	p adj.
IL-13 (cytokines)	IL-13 (spleen)	-0.087	0.297
IL-10 (cytokines)	IL-17 (MLN)	-0.086	0.301
ROR- γ (spleen)	IL-1 β (MLN)	-0.086	0.302
FoxP3 (spleen)	IFN- γ (MLN)	-0.089	0.314
IL-17 (cytokines)	FoxP3 (spleen)	0.083	0.318
IFN- γ (spleen)	IL-12 β (MLN)	-0.099	0.318
IL-5 (cytokines)	IL-10 (MLN)	0.083	0.320
IL-12 β (spleen)	IL-6 (MLN)	0.090	0.323
IL-13 (spleen)	IL-12 β (MLN)	-0.098	0.324
TNF- α (cytokines)	IL-1 β (MLN)	-0.081	0.331
IL-17 (cytokines)	IL-1 β (MLN)	-0.081	0.338
FoxP3 (spleen)	IL-12 β (MLN)	-0.095	0.339
ROR- γ (spleen)	IL-17 (MLN)	-0.080	0.348
IL-5 (cytokines)	Gata3 (MLN)	0.079	0.350
IFN- γ (cytokines)	Gata3 (spleen)	-0.078	0.351
IL-6 (cytokines)	IL-10 (spleen)	0.078	0.352
IFN- γ (cytokines)	IL-1 β (MLN)	-0.078	0.356
IFN- γ (cytokines)	IL-10 (spleen)	0.076	0.366
IL-10 (cytokines)	IL-6 (MLN)	-0.077	0.369
IL-12 β (cytokines)	FoxP3 (spleen)	0.075	0.385
IFN- γ (cytokines)	IL-10 (MLN)	0.076	0.385
FoxP3 (spleen)	IL-13 (MLN)	-0.078	0.385
IL-12 β (cytokines)	Gata3 (MLN)	-0.073	0.391
IL-12 β (spleen)	IL-13 (MLN)	-0.083	0.391
IL-13 (cytokines)	IL-17 (MLN)	-0.075	0.391
IL-17 (cytokines)	IL-17 (spleen)	-0.075	0.391
IL-5 (spleen)	IL-1 β (MLN)	-0.078	0.391
IL-5 (cytokines)	IL-5 (spleen)	-0.077	0.391
IL-5 (spleen)	IL-12 β (MLN)	-0.089	0.396
IL-10 (cytokines)	IL-10 (spleen)	0.071	0.406
IL-10 (cytokines)	IL-12 β (spleen)	-0.077	0.406
IL-17 (cytokines)	IL-17 (MLN)	-0.072	0.406
IL-17 (cytokines)	TNF- α (MLN)	0.073	0.406
IL-10 (cytokines)	FoxP3 (spleen)	0.071	0.407
IL-10 (spleen)	Gata3 (MLN)	-0.071	0.408
IL-10 (cytokines)	IL-13 (spleen)	-0.072	0.408
TNF- α (cytokines)	IL-6 (MLN)	-0.071	0.412
Gata3 (spleen)	IL-17 (MLN)	-0.071	0.420
IL-13 (cytokines)	IL-1 β (MLN)	-0.070	0.421
IL-5 (cytokines)	IL-6 (MLN)	0.069	0.427
TNF- α (cytokines)	Tbx21 (spleen)	-0.069	0.427

Gene expressed/ circulating cytokine		r_s	p adj.
IFN- γ (cytokines)	IL-13 (spleen)	-0.070	0.427
IL-12 β (cytokines)	Gata3 (spleen)	0.068	0.433
IFN- γ (cytokines)	IL-6 (MLN)	-0.068	0.433
FoxP3 (spleen)	IL-17 (MLN)	-0.068	0.441
IL-6 (cytokines)	IL-6 (MLN)	-0.067	0.447
IL-13 (cytokines)	IFN- γ (MLN)	0.069	0.455
IL-5 (spleen)	IL-10 (MLN)	-0.069	0.461
ROR- γ (spleen)	IL-6 (MLN)	-0.065	0.464
IL-1 β (cytokines)	TNF- α (spleen)	0.064	0.464
IL-13 (spleen)	IFN- γ (MLN)	-0.069	0.470
IL-17 (cytokines)	IL-13 (spleen)	-0.064	0.476
IL-17 (cytokines)	IL-6 (spleen)	-0.063	0.476
Tbx21 (spleen)	IL-17 (MLN)	-0.064	0.477
IL-13 (cytokines)	IL-5 (spleen)	-0.064	0.494
IL-5 (cytokines)	IL-12 β (spleen)	-0.065	0.500
IL-13 (cytokines)	Tbx21 (spleen)	-0.061	0.502
IL-17 (spleen)	IFN- γ (MLN)	-0.065	0.508
IL-6 (cytokines)	FoxP3 (spleen)	0.060	0.510
Gata3 (spleen)	IL-13 (MLN)	-0.061	0.510
TNF- α (cytokines)	IL-17 (MLN)	-0.060	0.510
Irf5 (spleen)	IL-17 (MLN)	-0.060	0.510
Tbx21 (spleen)	IL-1 β (MLN)	0.060	0.510
IL-10 (cytokines)	Gata3 (MLN)	-0.058	0.518
Tbx21 (spleen)	IL-6 (MLN)	0.059	0.525
IL-1 β (cytokines)	IL-10 (spleen)	0.057	0.526
IL-5 (cytokines)	IL-1 β (spleen)	-0.057	0.527
IL-5 (cytokines)	IL-1 β (MLN)	0.057	0.531
IFN- γ (cytokines)	TNF- α (spleen)	0.057	0.531
IL-5 (cytokines)	IL-12 β (MLN)	0.066	0.534
IL-10 (cytokines)	IL-17 (spleen)	-0.057	0.545
IL-13 (cytokines)	IL-6 (MLN)	-0.056	0.546
IL-5 (spleen)	IFN- γ (MLN)	-0.061	0.549
IL-17 (cytokines)	Irf5 (spleen)	0.054	0.549
IL-13 (cytokines)	ROR- γ (spleen)	-0.054	0.549
IL-10 (cytokines)	IL-1 β (MLN)	-0.055	0.551
IL-5 (spleen)	IL-17 (MLN)	-0.058	0.556
IL-13 (cytokines)	IL-17 (spleen)	-0.055	0.559
IL-1 β (spleen)	IL-12 β (MLN)	0.063	0.560
IL-6 (cytokines)	TNF- α (spleen)	0.053	0.560
IL-13 (cytokines)	Gata3 (spleen)	-0.053	0.561
IL-1 β (cytokines)	IL-1 β (MLN)	0.053	0.564

Table A4.1 continued overleaf

Table A4.1: Continued.

Gene expressed/ circulating cytokine		r_s	p adj.
TNF- α (cytokines)	IL-17 (spleen)	-0.053	0.567
IL-6 (cytokines)	IL-17 (spleen)	-0.053	0.567
IL-1 β (cytokines)	Tbx21 (spleen)	-0.053	0.567
TNF- α (cytokines)	TNF- α (spleen)	0.052	0.567
TNF- α (spleen)	TNF- α (MLN)	-0.053	0.570
IL-6 (cytokines)	Gata3 (MLN)	0.051	0.572
IL-5 (cytokines)	FoxP3 (spleen)	-0.051	0.573
IL-6 (cytokines)	IL-12 β (spleen)	-0.055	0.573
IL-17 (cytokines)	IL-12 β (spleen)	-0.054	0.576
IL-1 β (cytokines)	IL-17 (MLN)	0.051	0.582
IFN- γ (cytokines)	Gata3 (MLN)	0.050	0.587
IFN- γ (cytokines)	IL-17 (spleen)	-0.050	0.592
TNF- α (cytokines)	IL-13 (spleen)	-0.048	0.617
IL-13 (spleen)	IL-17 (MLN)	-0.048	0.627
IL-12 β (cytokines)	IFN- γ (MLN)	-0.049	0.627
TNF- α (spleen)	IL-1 β (MLN)	0.047	0.627
IL-12 β (cytokines)	Irf5 (spleen)	0.045	0.632
IL-12 β (cytokines)	IL-10 (spleen)	0.044	0.638
IL-6 (cytokines)	IL-13 (spleen)	-0.045	0.638
IFN- γ (cytokines)	IL-17 (MLN)	-0.045	0.638
Irf5 (spleen)	IL-6 (MLN)	0.045	0.638
IL-12 β (cytokines)	IL-6 (spleen)	-0.044	0.638
IFN- γ (spleen)	TNF- α (MLN)	0.046	0.638
IL-1 β (spleen)	TNF- α (MLN)	0.045	0.638
IL-12 β (cytokines)	IL-12 β (MLN)	0.051	0.641
IFN- γ (cytokines)	IFN- γ (spleen)	-0.044	0.646
IL-6 (cytokines)	Gata3 (spleen)	-0.043	0.646
IL-6 (spleen)	IL-17 (MLN)	-0.044	0.646
IFN- γ (cytokines)	IL-5 (spleen)	-0.045	0.646
IL-1 β (cytokines)	IL-10 (MLN)	0.043	0.650
Tbx21 (spleen)	TNF- α (MLN)	0.043	0.652
IL-12 β (cytokines)	IFN- γ (spleen)	0.043	0.652
IL-12 β (spleen)	IL-10 (MLN)	0.046	0.653
IL-1 β (cytokines)	Gata3 (MLN)	0.041	0.659
IL-10 (cytokines)	Gata3 (spleen)	0.041	0.663
IL-12 β (spleen)	Gata3 (MLN)	0.044	0.664
TNF- α (cytokines)	IFN- γ (MLN)	0.042	0.668
IL-1 β (spleen)	IL-6 (MLN)	0.041	0.668
Tbx21 (spleen)	Gata3 (MLN)	0.040	0.671
TNF- α (spleen)	Gata3 (MLN)	-0.040	0.671
Irf5 (spleen)	IL-12 β (MLN)	0.046	0.671

Gene expressed/ circulating cytokine		r_s	p adj.
IL-10 (cytokines)	IL-5 (spleen)	-0.041	0.671
IFN- γ (cytokines)	ROR- γ (spleen)	-0.039	0.671
IL-1 β (cytokines)	Gata3 (spleen)	-0.038	0.675
IL-17 (cytokines)	IL-10 (MLN)	0.038	0.687
IFN- γ (spleen)	IL-6 (MLN)	-0.039	0.687
IL-12 β (cytokines)	IL-13 (spleen)	-0.038	0.691
TNF- α (cytokines)	IL-12 β (spleen)	-0.039	0.703
FoxP3 (spleen)	IL-1 β (MLN)	-0.036	0.708
IL-1 β (spleen)	ROR- γ (spleen)	-0.035	0.708
IL-12 β (cytokines)	TNF- α (spleen)	0.035	0.708
IL-5 (cytokines)	IFN- γ (MLN)	-0.037	0.716
IL-12 β (spleen)	IL-1 β (MLN)	0.037	0.717
IL-10 (cytokines)	IL-6 (spleen)	-0.034	0.717
IL-6 (spleen)	IFN- γ (MLN)	-0.036	0.724
IL-10 (spleen)	IL-6 (MLN)	-0.034	0.724
IL-6 (cytokines)	IL-5 (spleen)	-0.034	0.726
IL-5 (spleen)	IL-6 (MLN)	-0.035	0.726
IL-10 (cytokines)	TNF- α (spleen)	0.033	0.726
IL-13 (cytokines)	Gata3 (MLN)	0.032	0.727
IL-1 β (cytokines)	IFN- γ (MLN)	0.034	0.727
IL-1 β (cytokines)	IL-13 (spleen)	-0.033	0.727
IL-12 β (cytokines)	Tbx21 (spleen)	0.032	0.727
IL-10 (cytokines)	IFN- γ (MLN)	0.033	0.737
IL-12 β (spleen)	IL-12 β (MLN)	-0.039	0.743
IL-17 (cytokines)	Gata3 (MLN)	-0.030	0.750
IL-13 (cytokines)	Irf5 (spleen)	-0.030	0.750
IL-13 (spleen)	IL-1 β (MLN)	-0.031	0.752
TNF- α (cytokines)	Gata3 (spleen)	-0.029	0.755
IL-10 (spleen)	IL-17 (MLN)	-0.030	0.755
TNF- α (cytokines)	IL-10 (MLN)	0.030	0.757
IL-12 β (spleen)	IL-17 (MLN)	0.031	0.768
IL-6 (spleen)	IL-6 (MLN)	-0.029	0.773
TNF- α (cytokines)	IFN- γ (spleen)	-0.028	0.773
IL-12 β (cytokines)	IL-5 (spleen)	-0.029	0.773
IL-1 β (cytokines)	IL-5 (spleen)	-0.028	0.780
IFN- γ (spleen)	IL-1 β (MLN)	0.028	0.780
TNF- α (cytokines)	IL-5 (spleen)	-0.028	0.780
IL-17 (cytokines)	ROR- γ (spleen)	-0.026	0.785
IL-1 β (cytokines)	ROR- γ (spleen)	0.026	0.787
IL-12 β (cytokines)	TNF- α (MLN)	0.026	0.790
IL-12 β (cytokines)	IL-17 (spleen)	-0.026	0.795

Table A4.1 continued overleaf

Table A4.1: Continued.

Gene expressed or circulating cytokine		r_s	p adj.
TNF- α (cytokines)	Gata3 (MLN)	0.024	0.801
TNF- α (spleen)	IFN- γ (MLN)	0.026	0.801
TNF- α (spleen)	IL-10 (MLN)	-0.025	0.801
IL-13 (cytokines)	IL-10 (spleen)	0.024	0.801
IL-5 (cytokines)	IL-10 (spleen)	0.024	0.801
IL-17 (spleen)	IL-17 (MLN)	-0.025	0.801
IL-1 β (cytokines)	IL-6 (spleen)	0.024	0.801
IL-5 (cytokines)	IL-6 (spleen)	0.024	0.801
IL-10 (cytokines)	ROR- γ (spleen)	0.024	0.801
IL-10 (cytokines)	IL-12 β (MLN)	0.027	0.812
Irf5 (spleen)	IL-1 β (MLN)	0.023	0.815
FoxP3 (spleen)	IL-6 (MLN)	-0.022	0.826
IL-12 β (cytokines)	IL-13 (MLN)	-0.021	0.835
Irf5 (spleen)	IL-13 (MLN)	-0.021	0.840
IL-13 (cytokines)	IL-10 (MLN)	0.020	0.840
IFN- γ (cytokines)	IL-12 β (spleen)	-0.021	0.841
IL-1 β (cytokines)	IFN- γ (spleen)	-0.020	0.849
IL-10 (cytokines)	IL-10 (MLN)	0.019	0.849
IL-10 (spleen)	IL-10 (MLN)	0.019	0.854
IL-13 (cytokines)	FoxP3 (spleen)	0.018	0.861
IL-6 (cytokines)	Irf5 (spleen)	-0.018	0.861
IL-13 (cytokines)	IL-6 (spleen)	-0.017	0.869
TNF- α (spleen)	IL-17 (MLN)	-0.017	0.881
IL-5 (cytokines)	TNF- α (spleen)	-0.016	0.881
IFN- γ (cytokines)	FoxP3 (spleen)	0.016	0.882
IL-13 (cytokines)	IFN- γ (spleen)	0.016	0.882
TNF- α (spleen)	IL-12 β (MLN)	-0.018	0.882
IL-1 β (spleen)	IL-17 (MLN)	-0.016	0.882
TNF- α (cytokines)	ROR- γ (spleen)	-0.015	0.882
IL-13 (cytokines)	TNF- α (spleen)	0.016	0.882
TNF- α (cytokines)	IL-10 (spleen)	0.015	0.882
IL-10 (cytokines)	IL-13 (MLN)	-0.015	0.882
IL-13 (spleen)	IL-6 (MLN)	-0.016	0.882
IL-12 β (spleen)	IFN- γ (MLN)	0.016	0.884
TNF- α (cytokines)	IL-6 (spleen)	-0.014	0.884
TNF- α (spleen)	IL-6 (MLN)	0.014	0.889
IL-1 β (cytokines)	IL-12 β (spleen)	-0.014	0.893
IL-17 (cytokines)	Tbx21 (spleen)	-0.013	0.893

Gene expressed or circulating cytokine		r_s	p adj.
IL-10 (cytokines)	TNF- α (MLN)	0.013	0.893
IL-17 (cytokines)	Gata3 (spleen)	0.012	0.899
IL-6 (cytokines)	ROR- γ (spleen)	-0.012	0.899
Irf5 (spleen)	TNF- α (MLN)	-0.013	0.899
Tbx21 (spleen)	IL-13 (MLN)	-0.012	0.905
IL-5 (cytokines)	IL-13 (spleen)	0.012	0.905
IFN- γ (cytokines)	IFN- γ (MLN)	0.012	0.908
IL-10 (spleen)	IFN- γ (MLN)	-0.011	0.910
Tbx21 (spleen)	IL-10 (MLN)	0.010	0.910
IL-17 (cytokines)	IL-10 (spleen)	0.009	0.910
IL-13 (cytokines)	IL-12 β (spleen)	-0.010	0.910
IL-1 β (cytokines)	IL-17 (spleen)	-0.010	0.910
IL-5 (cytokines)	IL-17 (spleen)	0.010	0.910
IL-6 (cytokines)	IL-1 β (MLN)	0.010	0.910
IL-17 (spleen)	IL-1 β (MLN)	-0.010	0.910
IL-1 β (cytokines)	Irf5 (spleen)	0.010	0.910
TNF- α (cytokines)	Irf5 (spleen)	0.009	0.910
Gata3 (spleen)	IFN- γ (MLN)	-0.010	0.912
IFN- γ (spleen)	IL-13 (MLN)	0.010	0.912
IL-5 (cytokines)	Gata3 (spleen)	0.008	0.924
IL-17 (cytokines)	IFN- γ (spleen)	0.008	0.924
IL-10 (spleen)	IL-1 β (MLN)	0.008	0.924
IL-17 (cytokines)	TNF- α (spleen)	-0.008	0.924
IL-6 (spleen)	IL-1 β (MLN)	-0.006	0.944
IL-12 β (cytokines)	ROR- γ (spleen)	0.005	0.949
IL-5 (cytokines)	ROR- γ (spleen)	-0.005	0.949
IL-12 β (spleen)	TNF- α (MLN)	-0.006	0.949
IL-12 β (cytokines)	IL-12 β (spleen)	0.004	0.963
IL-1 β (cytokines)	IL-6 (MLN)	0.004	0.963
IL-6 (cytokines)	IL-6 (spleen)	0.003	0.979
Tbx21 (spleen)	IL-12 β (MLN)	-0.003	0.980
IL-1 β (spleen)	IL-13 (MLN)	0.002	0.980
IFN- γ (cytokines)	IL-6 (spleen)	-0.002	0.982
IL-12 β (cytokines)	IL-10 (MLN)	0.001	0.995
IL-17 (spleen)	IL-6 (MLN)	0.001	0.995
IL-6 (cytokines)	IFN- γ (spleen)	0.000	0.998
Gata3 (spleen)	IL-6 (MLN)	0.000	0.998
IL-17 (cytokines)	IFN- γ (MLN)	0.000	0.999

Table A4.2: Nonsignificant ($p < 0.05$) relationships between immune response type in wild *Mus musculus domesticus* from the Isle of May, as measured by gene expression from spleen or mesenteric lymph node (MLN) tissue or circulating cytokine levels. Shown here is the response type and tissue from which it was measured for gene expression levels, or circulating cytokines, Spearman's rank correlation coefficient (r_s) and level of significance (p). To control for multiple testing, p -values were adjusted using the Benjamani-Hochberg procedure.

Response and tissue type		r_s	p adj.
Innate (cytokines)	Th1 (MLN)	-0.182	0.051
Th2 (cytokines)	Innate (MLN)	0.155	0.065
Th1 (spleen)	Treg (spleen)	-0.138	0.071
Th17 (spleen)	Innate (MLN)	0.151	0.082
Th1 (cytokines)	Th2 (MLN)	-0.136	0.090
Th2 (spleen)	Innate (MLN)	-0.139	0.094
Th1 (cytokines)	Innate (MLN)	-0.133	0.094
Treg (spleen)	Th2 (MLN)	0.130	0.105
Treg (spleen)	Innate (MLN)	0.122	0.131
Th2 (cytokines)	Treg (MLN)	0.121	0.145
Th2 (spleen)	Treg (MLN)	-0.122	0.145
Th17 (cytokines)	Treg (MLN)	0.116	0.150
Th1 (spleen)	Th17 (spleen)	0.106	0.159
Th2 (spleen)	Th1 (MLN)	0.134	0.169
Treg (cytokines)	Th17 (MLN)	-0.101	0.196
Innate (spleen)	Th2 (MLN)	0.106	0.198
Th2 (cytokines)	Th1 (MLN)	-0.126	0.199
Th1 (cytokines)	Th2 (spleen)	0.100	0.222
Treg (cytokines)	Innate (spleen)	-0.093	0.229
Th1 (spleen)	Th17 (MLN)	-0.094	0.229
Th17 (cytokines)	Innate (spleen)	-0.091	0.236
Th2 (cytokines)	Th1 (spleen)	-0.094	0.237
Treg (spleen)	Th1 (MLN)	-0.110	0.237
Th17 (spleen)	Th1 (MLN)	-0.109	0.250
Innate (cytokines)	Treg (MLN)	0.092	0.272
Th1 (cytokines)	Th17 (MLN)	0.079	0.329
Th1 (spleen)	Treg (MLN)	0.079	0.361
Treg (cytokines)	Treg (MLN)	0.077	0.378
Th1 (cytokines)	Treg (MLN)	-0.075	0.385
Th1 (cytokines)	Th1 (MLN)	0.088	0.385
Th1 (cytokines)	Th17 (spleen)	-0.070	0.392
Th17 (cytokines)	Th1 (MLN)	-0.086	0.392
Innate (cytokines)	Innate (spleen)	-0.067	0.413
Th17 (cytokines)	Th17 (spleen)	0.067	0.413
Th1 (cytokines)	Th2 (spleen)	-0.068	0.413

Table A4.2 continued overleaf

Table A4.2: Continued.

Response and tissue type		r_s	p adj.
Th1 (cytokines)	Innate (spleen)	0.064	0.415
Innate (spleen)	Th1 (MLN)	0.076	0.429
Innate (cytokines)	Th17 (spleen)	0.063	0.438
Th2 (spleen)	Th17 (MLN)	-0.062	0.451
Th17 (spleen)	Th17 (MLN)	0.059	0.456
Treg (cytokines)	Treg (spleen)	-0.058	0.456
Th2 (cytokines)	Th17 (MLN)	0.057	0.480
Treg (cytokines)	Th17 (spleen)	0.055	0.491
Th17 (cytokines)	Th17 (MLN)	-0.053	0.510
Th1 (spleen)	Th1 (MLN)	-0.060	0.546
Th1 (spleen)	Th2 (MLN)	-0.052	0.546
Treg (cytokines)	Th2 (spleen)	-0.049	0.546
Treg (spleen)	Th17 (MLN)	0.042	0.600
Th1 (spleen)	Th2 (spleen)	-0.042	0.603
Innate (cytokines)	Th2 (spleen)	-0.044	0.603
Innate (cytokines)	Th1 (spleen)	-0.037	0.668
Th1 (cytokines)	Th1 (spleen)	0.034	0.688
Treg (cytokines)	Innate (MLN)	0.035	0.696
Th17 (cytokines)	Treg (spleen)	-0.032	0.699
Th2 (cytokines)	Treg (spleen)	0.025	0.791
Innate (spleen)	Treg (MLN)	-0.023	0.799
Innate (cytokines)	Th17 (MLN)	0.022	0.799
Treg (cytokines)	Th1 (MLN)	-0.026	0.799
Th2 (cytokines)	Innate (spleen)	0.021	0.799
Innate (cytokines)	Treg (spleen)	0.021	0.799
Th1 (cytokines)	Treg (spleen)	0.020	0.807
Th2 (cytokines)	Th2 (spleen)	-0.020	0.812
Th1 (spleen)	Innate (MLN)	0.019	0.812
Treg (spleen)	Treg (MLN)	0.018	0.818
Innate (spleen)	Innate (MLN)	0.017	0.819
Th17 (cytokines)	Th1 (spleen)	0.013	0.849
Th2 (cytokines)	Th17 (spleen)	0.013	0.849
Innate (spleen)	Th17 (MLN)	-0.010	0.872
Treg (cytokines)	Th2 (MLN)	0.002	0.971

Appendix 5: The CTLA4 polymorphism in the Isle of May mice

A5.1 Introduction

The introduction of Eday mice to the Isle of May (Berry *et al.* 1990) likely increased heterozygosity of the Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) gene (Robertson 2014). CTLA4 is a candidate gene in the helminth immune response (Behnke *et al.* 2006) and has a SNP (Ueda *et al.* 2003) – individuals with a ‘G’ allele appear to show greater resistance to helminthic infection than those with an ‘A’ allele (Noyes *et al.* 2015). However, the ‘G’ allele is associated with autoimmune disorders such as diabetes (Vijayakrishnan *et al.* 2004). The ‘G’ allele was likely introduced to the Isle of May with the Eday mice (Robertson 2014), and has since been found to have spread across the island and be at Hardy-Weinberg Equilibrium (Robertson 2014).

A5.2 Methods

A5.2.1 DNA extraction

To assess the **CTLA4** genotype, DNA was extracted from ~2 mm² of tail tissue, using the Extracta DNA Prep for PCR – Tissue kit (Quanta BioSciences). Tail tissue was submerged in 100 µL of Extraction Reagent and incubated at 95°C for 30 minutes. The sample was then allowed to cool to room temperature, and 100 µL of Stabilisation Buffer was added. For use as positive controls, DNA was also extracted from the tail tissue

of BALB/c and C57BLK/6 strain laboratory mice (representing 'GG' and 'AA' homozygotes, respectively). Extracted DNA was stored at -20°C until used in genotyping.

A5.2.2 Genotyping

PCR was performed using the AccuStart II GelTrack PCR SuperMix (2X) (Quanta BioSciences) and the following primer pair (Sigma-Aldrich), at a concentration on 10 µM:

- CTLA4 (Forward): 5'-GAGCTTGCAGGAGTTCATCC-3'
- CTLA4 (Reverse): 5'-GCAGAGGTACAGTCCCGTGT-3'

Each PCR reaction was run in a 25 µL total volume, containing 12.5 µL SuperMix, 2.5 µL of each primer, 2.5 µL of nuclease-free water and 5 µL of DNA template.

PCR was run at 94°C for 3 minutes, followed by 36 cycles at 94°C for 15 seconds, 60°C for 15 seconds and 72°C for 60 seconds. Secondary PCR products were visualised by electrophoresis on a 1.5% agarose gel with a 100 bp DNA ladder. A band at 357 bp confirmed that PCR had been successful.

The PCR product was restriction digested with Bccl enzyme and CutSmart Buffer (New England Biolabs), following the manufacturers' protocol. Each reaction contained 8.5 µL of PCR product, 1 µL of buffer and 0.5 µL of restriction enzyme at a concentration of 10,000 units/mL. Following incubation at 37°C for 45 minutes and 65°C for 20 minutes, the

product was visualised on 2% agarose gel with a 100 bp DNA ladder. Bands at 357 bp represented the 'G' allele and bands at 160 bp and 197 bp represented the 'A' allele – all three bands were present in heterozygotes. The BALB/c and C57BLK/6 positive controls were used to confirm the reaction had been successful.

A5.2.3 Data analysis

To test for Hardy-Weinberg equilibrium (HWE), the exact test for HWE was used in the “HardyWeinberg” package (Graffelman 2017). Temporal variation in allele frequencies was tested using Fisher’s exact test. All analyses were conducted in R version 3.4.0 (R Core Team 2017).

A5.3 Results

Across all sampling sessions, the mice on the Isle of May were at HWE for the CTLA4 polymorphism (Table A5.1).

Table A5.1: Genotype and allele frequencies for the polymorphic CTLA4 gene in *Mus musculus domesticus* on the Isle of May. Using the exact test for HWE, the population was found to be at equilibrium across all sessions.

	Sampling session					
	Summer 2014	Spring 2015	Summer 2015	Autumn 2015	Spring 2016	Autumn 2016
Genotype						
AA	0	2	1	2	0	2
AG	15	7	5	19	5	11
GG	39	40	10	79	37	41
Allele frequency						
A	0.14	0.11	0.22	0.12	0.06	0.14
G	0.86	0.89	0.78	0.89	0.94	0.86
HWE						
P-value	0.575	0.092	1	0.612	1	0.276
D	1.042	-1.383	-0.234	-0.678	0.149	-0.958

There were no statistically significant differences in allele frequency across field seasons (Table A5.2).

Table A5.2: Comparison of allele frequencies for the polymorphic CTLA4 gene in Isle of May mice (*Mus musculus domesticus*) across field seasons. Shown here are *p*-values from Fisher's exact test, used to compare frequencies between sessions.

	Summer 2014	Spring 2015	Summer 2015	Autumn 2015	Spring 2016
Spring 2015	0.856				
Summer 2015	0.268	0.351			
Autumn 2015	0.773	0.817	0.483		
Spring 2016	0.115	0.241	0.186	0.227	
Autumn 2016	0.842	0.716	0.439	0.912	0.365

A5.4 Discussion

The CTLA4 gene was at HWE across all field sessions, and there were no significant differences in allele frequencies over time. This is consistent with previous findings (Robertson 2014). It is likely that the “G” allele was introduced to the Isle of May with the Eday mice in 1982 (Robertson 2014) – the finding here that this polymorphic gene is at equilibrium supports previous observations that hybrids between the introduced and native mice were widespread across the island (Berry *et al.* 1991) and that other introduced alleles and the introduced Robertsonian chromosomes had also reached equilibrium (Scriven 1992). Therefore the Isle of May mice represent a recombinant, introgressed population – ideal for quantifying genotype-phenotype associations (Berry *et al.* 1991).

A4.5 References

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