THE UNIVERSITY
of EDINBURGH

The causes and consequences of immune variation in a wild mammal

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Thesis submitted for the degree of Doctor of Philosophy

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2016

Abstract

The immune system provides protection against parasites and is crucial for survival, but mounting, maintaining and controlling an immune response is expensive. Under limited resources these costs can lead to investment trade-offs between life history traits in order to maximize an individual's fitness. Understanding how these trade-offs relate to immunity can be important in understanding individual variation in fitness and the broader ecological implications that this may have in a population. In the wild there is evidence of trade-offs between life history traits and immunity, but there are relatively few studies which have measured specific aspects of the immune system under natural parasitic exposure. Using reagents developed in domestic sheep, I measured an unusually broad range of immune markers in a wild population of Soay sheep on the island of Hirta, St Kilda, Scotland. These include: T cell subsets (CD4+, CD8+, CD4+ & CD8+ naïve, gamma delta and Foxp3), anti-*T.circumcincta (T. circ)* antibody isotopes, (IgA, IgE, IgG), leukocyte subtypes (neutrophil:lymphocyte ratio & eosinophils), and leukocyte telomere length (LTL).

I found that, in a year under high selection pressure for survival, anti-*T. circ* IgG positively predicted survival across all ages and for both sexes. Additionally, females had higher proportions of naïve T cells than males; a previously unreported sex difference in a wild mammal. In chapter 2, analysis of lambs in early life found higher growth rates associated with low antibody measures, while lower growth rates related to low antibody measures and high levels of inflammatory marker. I also found that male lambs with high anti-*T. circ* IgE and IgG were less likely to survive over-winter, contrary to the findings across all ages in chapter 1. In chapter 3, I detected an increase in LTL attrition with age in males >3 years, but this was not significant in females or in younger animals. In male lambs, high investment in horn growth was related to reduced LTL. Changes in LTL were independent of variation in leukocyte cell populations. The data in this thesis demonstrate the complexity of immune variation in the wild, and highlight the value of multiple ecologically relevant markers to understanding the evolutionary implications of resource trade-offs.

Lay summary

The immune system provides important protection against parasites, which can otherwise cause damage and reduce survival. However, an immune response uses valuable resources, which can limit the amount left to spend on other activities, such as growth or reproduction. In the wild, resources can be hard to come by, so how they are used is important to give an individual the best chance to survive and reproduce. This leads to resource trade-offs between investing resources in processes such as growth and reproduction, and investing in immunity. We know that these trade-offs occur in the wild, but there are relatively few studies which have measured specific markers of the immune system under natural parasitic infection. Using tools developed for domestic sheep, I measured an unusually broad range of immune markers in a wild population of Soay sheep living on the island of Hirta, St Kilda, Scotland. This thesis explores evidence of resource trade-offs with these immune markers, and whether this has an impact on survival.

I found immune markers that respond to a particular parasite were important for survival, but that the way they affected survival can depended on a range of things, including the age and sex of the individual. Age and sex are important drivers of how these immune marker vary in indivudals, and in lambs there is lot of pressure on resources to grow and resist parasites, but this can come at a cost. Lambs that grew fast had lower amounts of the parasite-specific immune markers, but those that grew slowly had high measures of an immune marker linked to chronic infection. Additionally, male lambs which invested heavily to grow large horns, had reduced markers of cellular ageing, but not any of the immune markers. In older males, this marker of cellular ageing was found to decrease with age, but not in females or in younger animals. This thesis demonstrates how age, sex and resource trade-offs can influence variation in immunity and highlights the importance of parasite-specific markers to understanding the impact that this can have on survival.

Declaration

I declare that I have written this thesis under the guidance of my supervisors. I conducted all of the analyses presented here and wrote the thesis.

The long-term data set used in this thesis was collected as part of the Soay Sheep Project, St Kilda.

In the field I collected and processed the blood samples used in the thesis with the assisstance of the Soay Sheep annual catch teams 2011-2015. I performed the lab work for the T cell analysis myself, with the expecption of the 2011 samples which were processed by honours student Daniel Cooney. The antibody EILSA's were carried out by Kathryn Watt. I had assisstance in analysing the slide data from Sarah Underwood and honours student Pheobe Hooper. The telomere data was generated by Jennifer Fairlie, Racheal Adams and Sarah Underwood. Immune reagents for the antibody and T cell analysis were provided by Tom McNeilly, Moredun Research Institute, Edinburgh.

All other work was my own.

This work has not been submitted for any other degree or professional qualification.

Rebecca L Watson

Acknowledgements

This thesis would not have been possible without the hard work of many people, all of whom I am very grateful to and I would like to thank them whole-heartedly for their support. I sincerely thank my superviors, Dr Dan Nussey, Prof. Rose Zamoyska and Dr Tom Mc Neilly, for their guidance and encouragement during that last four years. Thank-you to all members of the Nussey lab group for their enthusiastic and helpful discussions, and of course the many, many cakes! A special thanks to Kathryn Watt for her positive influence both in and out of the lab.

This project would not have been possible without the existence of the Soay Sheep Project and the many people behind it, particularly the work of Josephine Pemberton, and Jill Pilkington. My fieldwork was made an absolute pleasure by all of the many wonderful people I met along the way. Special thanks to Sheepie 1, for making my experiences in the field some of my very best, and for the many life lessons I will always carry with me. Importantly, this work would not have happened without the hard work and enthusiasm of all members of the August catch teams 2011-2015 (Felix, honourary member, 2013-14).

Without the infinite wisdom and patience of Martin Waterfall I would not have achieved successful flow, and my skills in cell culture are all thanks to David Wright; thank-you both for your advice and guidance, it was always appreciated. I'd also like to thank the many members of both the Zamoyska group and the McNeilly group, who all made me feel at home in their labs and were always so very helpful to me.

A great big thank-you to my PhD cohort: Liam Brierley, Sinclair Cooper, Mizuho Fukushige, Andrea Kemter, Josianne Lachapelle, Nick Lowery, Maarit Mäenpää, Lindsey MacDonald, Ele Sylvester, Christine Tansey, and James Westrip. Special mentions to Caroline Thompson and Aly Paavit, for seemingly endless amounts of tea and wisdom, and to Josh Moatt for the many coffee chats. To all those

I've been lucky enough to share an office with, thank-you for putting up with me! IEB has been a wonderful departement to work in and I thank everyone I've met along the way for contributing the supportive and friendly place I've enjoyed being a part of.

A huge thank-you to my friends keeping for always keeping my spirits high, and my mind happy - I'm lucky to have you guys! Special mentions to Jenny Iles and Harry French for their constant supply of moral support, cheese and crap jokes, to Sarah-Jayne Forster for all the much needed surprise catch ups, to Gillian Simpson for keeping me focused on liiiiffee, and to Louise 'Lizard' Christensen for keeping me happy and away from onsbols.

My family have always been there to guide, help, and encourage me through everything I have sought to acheive, and to them I will always be extremely grateful. Their unwavering support and endless care packages over the years have kept me going in body and spirit, and without them this would simply not have been possible. Finally, I thank Phil Ellis, for keeping me going when things got tough, for his encouragement, strength, and support in all that I do, but most importantly for the adventures.

TEAM!

Contents

Chapter 1

General Introduction

"The game is afoot!"

Sir Arthur Conan Doyle

The Adventure of the Abbey Grange

1.1 Summary

Providing protection against pathogenic infection is a vital role of the immune system, but this comes at a cost of resources and a risk of autoimmunity. Balancing the benefits and costs of immune investment results in trade-offs with life history traits, such as growth, reproduction, and survival, but how these trade-offs occur in the wild is still being understood. By evaluating a range of key immune markers in a population of wild Soay sheep this work will relate patterns in immune variation to life history traits. Specifically, this study will measure patterns in humoral and cellular immune markers which will be compared to individual variation in age, sex and survival, and other life history components, including parasite burden, growth, and biomarkers of ageing. This data will address evolutionary and ecologically relevant questions concerning the causes and consequences of immune variation in a wild mammal population.

1.2 Eco-Immunology

The interdisciplinary field of eco-immunology combines immunology, ecology, evolutionary biology and physiology to understand variation in the immune response and ultimately the causes behind this variation. Initiating an immune response is beneficial to an individual through the avoidance of parasites and the pathological damage that they can inflict upon the host. However, these benefits need to be balanced against the potential negative impacts of immune investment, such as resource expenditure, metabolic costs and immunopathology resulting in host damage (Belloni et al., 2010; Guerreiro et al., 2012). This balance between allocating resources to immunity and other life history components leads to trade-offs between these processes, especially in the wild when resources are limited (Schmid-Hempel, 2003; Stearns, 1992). For the immune system to be influential in optimal life history strategies it must incur a cost to the individual and so be limited by available

resources (Norris and Evans, 2000). In birds, there is evidence that investment in life history components can result in reduced immuncompetence, indicating trade-offs with immunity (Lochmiller and Deerenberg, 2000; Norris and Evans, 2000). In common eiders (*Somateria mollissima*), initiating a humoral immune response following immunisation was shown to considerably impair long term survival (Hanssen et al., 2004). Similarly, initiating an immune response after immunization was shown to reduce weight gain in both chickens (*Gallus gallus domesticus*) (Klasing et al., 1987) and zebra finches (*Taeniopygia guttata*) (Deerenberg et al., 1997). There is a broad range of evidence for immune trade-offs with reproduction, growth and survival, although these trade-offs can be difficult to determine as immune costs are condition dependant and are sometimes only detected under limited resources (Hõrak et al., 1999; Ilmonen et al., 2003; Merila and Andersson, 1999; Moret and Schmid-Hempel, 2000; Råberg et al., 2000; Schmid-Hempel, 2003).

Research into immunity has predominantly focused on experimental laboratory studies to determine the mechanisms of such a complex system. In controlled environments experimental manipulation makes it possible to test specific relationships, in genetically similar individuals, and look at the causal relationships between immune cells. However, this is not representative of a natural response to infection and recently studies have begun to compare lab-bred mice to wild or pet shop derived mice and found distinct differences in immune phenotype between lab strains and mice obtained from other sources (Beura et al., 2016; Japp et al., 2016). Lab mice tend to have immune systems more comparable to those of immature humans (Reese et al., 2016). To investigate the fitness costs of immune variation requires a system where resources are restricted, conspecifics compete for mates and resources, infections are multiple and simultaneous, and individuals reproduce under natural selection (Sheldon and Verhulst, 1996). In order to fully understand the costs, benefits and overall fitness consequences of variation in immunity it is necessary to measure parasite density, host fitness and the relevant immune response (Graham et al., 2011). Reviews of ecological immunity suggest a lack of ecological evidence to support the link between immune function and fitness (Demas et al., 2011; Norris and Evans, 2000), and that there is much to be gained in bringing immunological,

evolutionary and ecological techniques together into the field to better understand immune mechanisms in the natural system (Pedersen and Babayan, 2011).

In the wild, immunological studies are particularly difficult due to the complexity of the systems themselves, and limitations in obtaining data, particularly due to a very restricted range of immunological reagents available for most non-model species (Boughton et al., 2011; Demas et al., 2011). As a result of this, studies have relied upon a small number of broader stimulation assays that measure the functional immune response to an artificial stimulation. These techniques can be readily applied across species and are a good tool to measure the immune response independent of the host's pathology (Zuk and Stoehr, 2002). However, they do not reflect the capacity of the host to respond to an ecologically relevant pathogen under natural conditions. In humans, there are a wide range of biomarkers to assess immune variation, used in disease diagnosis (Amatya et al., 2004) and predicting autoimmunity (Boren and Gershwin, 2004). The development of the immune phenotype is becoming increasingly popular in animal studies, including as a selection criteria in multi-trait selection programmes to improve livestock production and health (Flori et al., 2011). Despite the potential advantages to measuring specific immune responses in the wild, evidence from natural populations experiencing ecologically-relevant parasite challenges remains relatively scarce (Møller and Saino, 2004; Schimd-Hempel, 2011; Seppälä, 2015; Viney et al., 2005). A recent review suggests that ungulates are well suited as a model system for wild immunology (Jolles et al., 2015). Among other benefits, these large, long-lived herbivores are relatively well studied due to the importance of domestic livestock, which means there is a springboard of immunological, genetic and biometric data from which to begin the complex analysis of immunity in the wild.

1.3 The immune system

The immune system provides vital protection against pathogens and the detrimental effects of infection by performing four main tasks; immunological recognition, immune effector functions, immune regulation and immunological memory (Murphy et al., 2012). Leukocytes, white blood cells, are the fundamental cells of the immune system and can be separated into two categories; phagocytes, cells of the innate immune system, and lymphocytes, cells of the adaptive immune system. The innate immune system consists of both physical barriers and biochemical defences, and is less specific than the adaptive immune response. The vertebrate immune system is a highly complicated system, which is illustrated in a simplified schematic in figure 1.1 (Demas et al., 2011).

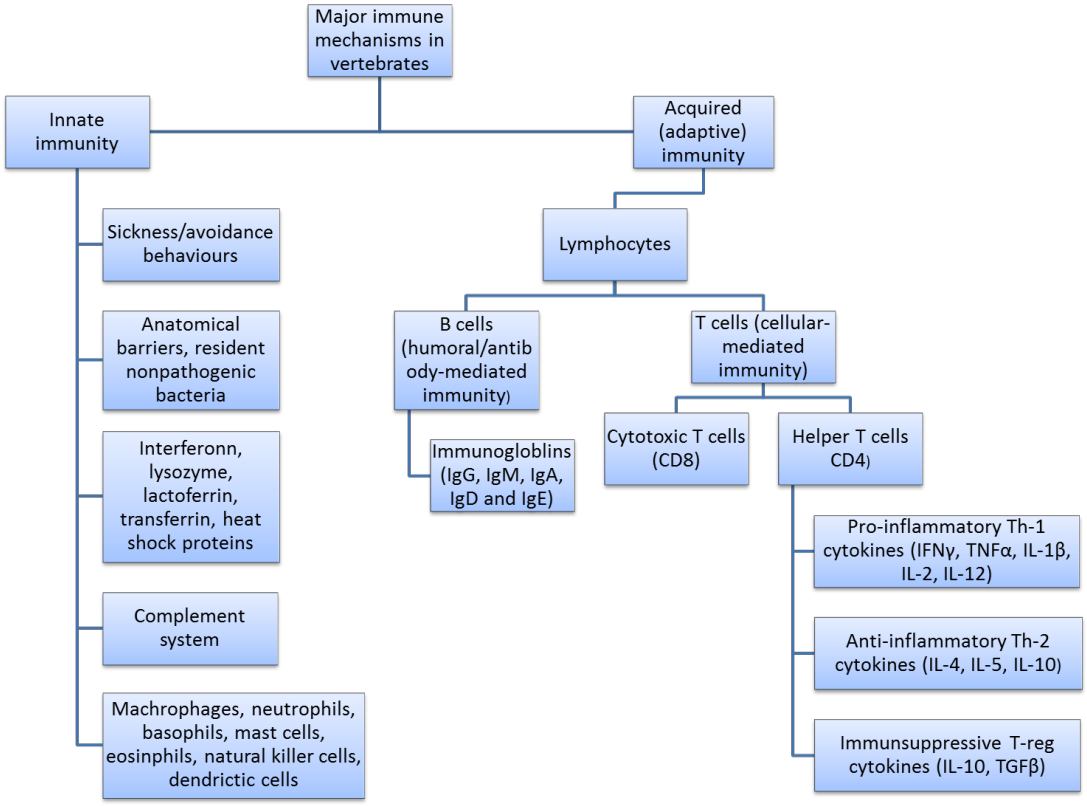


Figure 1.1: This schematic from Demas et al. (2011) demonstrates the complexity of the vertebrate immune system in a simplified summary. This details the range of cell types and responses involved in both the innate and adaptive immune systems.

The immune response involves a series of steps starting with recognition of the infectious agent, the marking of it as non-self and then the killing and removing it from the host (Eckert et al., 2002). Through a range of immune effector functions the infection can be contained or completely eradicated in some cases. In order to prevent self-damage, the risk of allergies and autoimmune immune disease the immune system must correctly identify self antigens from non-self antigens. There can also be damage to the host caused by the immune response itself, so regulation of the response by the action of regulatory immune cells is also important. Once exposure to an infectious agent has occurred immunological memory is established which reduces the risk of immediate reinfection and initiates a faster, stronger response should a secondary infection occur in the future.

1.3.1 *Innate immunity*

The innate immune system provides an immediate response to infection by detecting common features of pathogens and destroying them, through the functions of phagocytic cells. Phagocytes detect bacterial infection and use pathogen recognition receptors (PRRs) to recognise pathogen-associated molecular patterns (PAMPs) characteristic of microbial surfaces (BSI). Monocytes, natural killer (NK) cells and dendritic cells all contribute to this response, as do macrophages which play a vital role in instigating an inflammatory response. The innate response is immediate and is the first line of defence against pathogen invasion; however, this response can be overcome by pathogens and does not contribute to the accumulation of immune memory (Murphy et al., 2012).

1.3.2 *Adaptive immunity*

The adaptive immune system consists of a cellular response effected by T cells, which mature in the thymus, and a humoral response effected by B cells, which mature in

the bone marrow. Although the adaptive immune response takes days rather than hours to initiate it is able to provide an efficient response through the use of a vast repertoire of antigen specific receptors (Murphy et al., 2012). Activation of the adaptive immune response is initiated by antigen-presenting cells (APCs) which cause lymphocytes to proliferate and then differentiate, in the case of B cells, into plasma cells, and in the case of T cells, functional effector cells (Eckert et al., 2002). On activation B cells proliferate and mature into antibody producing cells and endogenous antibodies produced act on the cell surface as specific antigen receptors. Functional effector cells form three categories of cell types; cytotoxic cells (T_c) which kill infectious cells, helper T cells (T_H) which influence other cells types to provide a more effective response, and regulatory T cells (T_{reg}) which control the immune response through suppression of other lymphocytes (Murphy et al., 2012). A number of these activated lymphocytes differentiate into memory cells, which upon secondary exposure to the same pathogen quickly differentiate into specific effector cells. This provides long lasting immunity through immunological memory which is built up throughout the life span of an individual.

1.4 *Sex differences*

Trade-offs with immunity and life history traits can be relatively complex in the wild, especially when considering the differences between the sexes. Sexual dimorphism is positively related to male-biased mortality (Andersson, 1994; Promislow, 1992) and it is thought that parasites play a role in this relationship, as a cost of sexual selection (Hudson, 2002; Moore and Wilson, 2002; Nunn et al., 2000; Zuk, 1990). The mating system of a species can influence the extent of sex biased parasitism, and in cases when one sex is larger, especially in polygynous systems, there are more instances of male-biased parasitism (Klein, 2000; Moore and Wilson, 2002; Zuk, 1990). An explanation for this male biased increase in parasitism and reduction in immunity is a trade-off with high growth leading to larger males having lower immune function due to the somatic costs of growth (Rolff, 2002; Sheldon and Verhulst, 1996).

Sex-specific somatic costs of increased parasitic infection have been shown in a study of Tawny pipits (*Anthus campestris*) during early life; male nestlings that were infected with avian malaria parasites had a lower daily mass gain than females with the same infection status (Calero-Riestra and García, 2016). Additionally, sex hormones are also known to effect immunity, and in males high testosterone, which can result in increased size, is known to cause immunosuppression (Folstad and Karter, 1992). The dominant female sex hormone, oestrogen is known to decrease cell-mediated immunity, but can have a positive effect on humoral immunity (Grossman, 1985). Given the differences between males and females in rates of parasitism, sexual selection, reproductive costs, and mortality, we would expect that immunity would also vary between the sexes. There are a number of studies which suggest that, in vertebrates, males tend to have a lower immune response in addition to reduced resistance to parasites than females (Møller et al., 1999, 1998).

1.5 Immuno-senescence

In later life, there are age-related changes in immunity, when an individual undergoes senescence (table 1.1). The immune system declines in its functional capacity and the ability to effectively respond to a pathogen is reduced. This can result in increased pathogen susceptibility and an increased risk of autoimmune disease, as the immune system is less able to self-regulate the responses (Müller and Pawelec, 2015), which can have negative impacts on morbidity and mortality (Belloni et al., 2010). In later life, there are age-related changes in the immune system, such as a reduction in cell numbers or function, and these are more distinct in the adaptive than the innate immune system (Franceschi et al., 1999). However, due to the close interactions of these two arms of the immune system, a decline in function, or immunosenescence, would reflect both aspects of the immune response (Gomez et al., 2008; Lord et al., 2001; Müller et al., 2013; Weiskopf et al., 2009). These changes in immune capacity with age could influence life-history trade-offs and be driving the observed differences in these traits between ages.

Table 1.1: General summary of changes within the innate and adaptive immune response in later life adapted from Weiskopf et al. (2009)

Age related changes		
Innate Immunity	**Adaptive Immunity**	
	T cells	*B cells*
Increased inflammatory background	Thymic involution leading to a decreased naïve T cells output	Reduced B cell production
Functional defects of APC impair uptake and presentation of antigens	Accumulation of effector T cells	Defects in isotype switching
Impaired phagotoxic capacity and oxidative burst of macrophages	Restricted T cell repertoire	Weak and low affinity antibody responses
Elevated natural killer (NK) cells numbers and reduced cytotoxic function		

Research in immune ageing has predominantly focused on human studies and laboratory work with mice, however, later life declines in function are also a key interest in the evolutionary ecology of wild animals. A review by Nussey et al. (2013) demonstrates an abundance of studies which provide evidence of senescence in the wild, and expresses the benefits of an integrative approach between laboratory experiments and ecological research. A study in free-living Tree Swallows *Tachycineta bicolor* uses field experiments to support laboratory models, and demonstrates a higher cost of immune defence in older individuals, which are also dependent on environmental factors such as weather and food availability (Palacios et al., 2011). Variation due to environmental changes and individual characteristics has also been demonstrated in a longitudinal study of free-living Common Lizards, *Lacerta vivipara*, (Massot et al., 2011). This integrative study demonstrated age related immune variation in a wild population, including a trade-off between early and later-life performances, and a sex difference in immune variation; older females had higher metabolic rate and an increased T cell-mediated immune response. The study of a free-living population can evaluate the effects of multiple and simultaneous infections over time and assess relationships between immune decline and other physiological processes of ageing.

Telomeres are repeat sequences (TTAGGG) of non-coding DNA on the ends of chromosomes, which are important for chromosomal stability and successful replication (Blackburn, 1994, 2005). Telomeres shorten with each cell replication and when telomere length (TL) reaches a critical threshold this initiates cellular senescence, which suggests that there may be close links between telomere attrition and cellular ageing (Allsopp et al., 1992; Gomes et al., 2011). Average TL declines with age in numerous species and is a biomarker of ageing of considerable current interest in human epidemiology and evolutionary ecology (Blackburn et al., 2015; Monaghan, 2014). In mammals, TL is measured in DNA extracted from blood samples and reflects the average leukocyte telomere length (LTL). LTL is a measurement which encompasses a range of different white blood cell types, which are known to vary with age (Cichoń et al., 2003; Jego et al., 2014; Nussey et al., 2012; Palacios et al., 2011), and have different TL depending on cell type (Aubert et al., 2012; Baerlocher et al., 2006; Kimura et al., 2010). Therefore, we might expect that changes in leukocyte population composition could be driving LTL variation (Sanders and Newman, 2013). Whether changes in LTL reflect changes in the composition of different leukocyte cell types, or are an independent response to ageing is still relatively unknown. There is clear evidence of senescence in the wild and of age related declines in immunity, but more research needed to understand the physiological processes underpinning this variation and the consequences they have on fitness.

1.6 Gastro-intestinal nematodes

Nematodes, roundworms, are a diverse parasite group infecting almost every part of the body, but the principal site of infection is the alimentary canal (Wharton 1986). Gastro-intestinal nematodes (GIN) infect a range of different sites within the intestine and there is a breadth of pathology associated with the different species, including direct damage from the parasite and indirect pathology caused by the hosts own immune response (Kennedy and Harnett, 2011). The life cycles of GIN are also

diverse, but generally the infective larval stage is ingested by the sheep from contaminated pasture. The parasite then matures to adulthood in the host and for all species of GIN the adults reside in the intestine. Eggs produced by the adult worms are shed in faeces back onto the pasture, where they moult and develop into larva, eventually reaching the infective larval stage(Croll, 1966; Wakelin, 1966).

The diseases caused by GIN typically occur over a long period and are of great economic concern in domestic livestock production (Wakelin, 1966). Infection with GIN can have damaging consequences to the host, and is associated with anorexia, weight loss, and reduced growth (Greer, 2008). Acquired immunity is the major form of host defence against parasites and can prevent the establishment of larvae, supress parasite growth and expel adult worms from the host (Stear et al., 1996, 1995). As with other types of immune response, resistance against GIN is condition dependant and increases with higher resource availability (Brunsdon, 1964; Coop et al., 1995; Moret and Schmid-Hempel, 2000). Therefore, we might expect there to be trade-offs and costs associated with investment in GIN resistance and indeed studies have reported reduced growth is linked to infection status (Calero-Riestra and García, 2016; Greer, 2008; Moreno et al., 2008). A reduction in growth can be problematic in livestock production, with maintenance of GIN thought to result in 15% loss of production in sheep (Greer, 2008). In domestic sheep, current anthelminthic treatments against GIN are becoming less effective, and new strategies to target parasites using the host's own immune response could be a future solution (McRae et al., 2015; Papadopoulos, 2008; Sargison et al., 2010; Wilson and Sargison, 2001).

1.7 Study system

The free-living population of Soay sheep (*Ovis aries*) inhabiting the Village Bay area on the island of Hirta, St.Kilda, has been the focus of a long term evolutionary study since 1985. This population is a unique opportunity to study immunology in a wild system, where we can apply specific immune techniques and test the ecological and

evolutionary causes and consequences of immune variation. The sheep do not coexist on Hirta with any predators and there is little to no direct interspecies competition for resources. This population is unmanaged, reproduces under natural selection and experiences a harsh natural environment with limited resources. Population density fluctuates in a distinctive, cyclical dynamic characterised by low and rising sheep numbers followed by high mortality ('crash') winters which have death rates above 50% (figure 1.2) (Clutton-Brock and Pemberton, 2004). These population dynamics are driven by harsh winters coupled with high competition for resources at high density (Catchpole and Freeman, 2000; Coulson, 2001; Coulson et al., 1999).

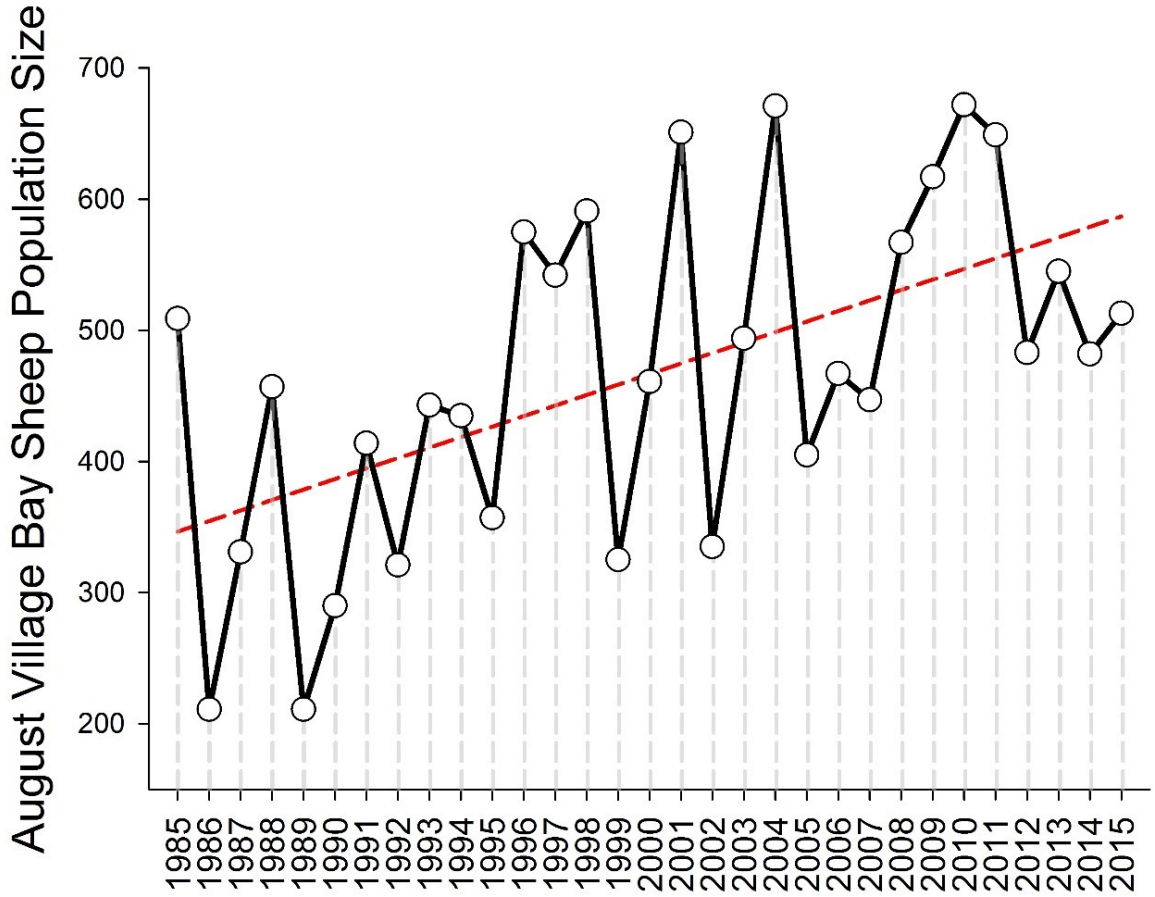


Figure 1.2: Population density of the Village Bay field site over the course of the Soay Sheep Project study on the island of Hirta, St Kilda, Scotland. The open circles represent the total population numbers counted each August, which fluctuate almost cyclically. There is a general increase in population density represented by the dotted line

There has been much research conducted on this unique population of wild mammals including population dynamics, genetic variation and many other evolutionary questions at both an individual and population level. As part of routine fieldwork, individuals are tagged with a unique identifier at birth during the spring, and then re-caught annually in August. Upon capture, each individual is weighed, measured, and blood and faecal samples are taken for various analyses, including immunity and parasite measures. All immune measures in this study were collected during the August catch field season, as were the associated biometric measures. The exception to this is in Chapter 3, which uses data collected at birth during the Spring field season. However, this includes only biometric measures of weight and no immune measures were collected at this time in the life history of individuals. In terms of senescence, research in this population has shown declines with age in reproductive performance (Hayward et al., 2015)and likelihood of survival (Catchpole and Freeman, 2000). Body mass is associated with increased fitness in both sexes, and males are larger than females with double the mortality rate of females (Coulson, 2001; Milner et al., 1999). There are three horn types in Soay sheep; normal, scurred and polled (figure 1.3). This is determined by a genetic polymorphism, and larger horns are linked to increased reproduction and reduced survival in normal horned males (Johnston et al., 2013; Robinson et al., 2006).

Parasites associated with the Soay sheep are predominantly nematodes and protozoa, but also include two species of tapeworm, and single species each of fly, lice and bacteria (Clutton-Brock and Pemberton, 2004). Of the nematode species, *Teladorsagia circumncincta (T. circ)* is the most prevalent and pathogenic GIN in this population (Clutton-Brock and Pemberton, 2004; Gulland, 1992). There is a notable absence of *Haemonchous contortus (H. cont)* from the St Kilda Soay sheep, which is severely pathogenic and common in the warmer Southern regions of the UK (Morgan and van Dijk, 2012). The assessment of parasite load has predominantly involved using an indirect measure of Strongyle faecal egg count (FEC) using the McMaster technique (Ministry of Agriculture Fisheries and Food, 1971), to determine the density of parasite eggs per gram of (wet weight) faeces (Clutton-Brock and Pemberton, 2004). This measure of Strongyle FEC encompasses the following

Figure 1.3: Examples of the horn type morphology of Soay sheep (photo credit Arpat Ozgul (St Kilda Soay Sheep Project, zgul))

species: *Teladorsagia* spp., *Chabertia ovina*, *Bunostomum trigonocephalus*, *Strongyloides papillosus*. Strongyles are roundworms, which are ingested by the host during the third larval stage, and then develop into adult worms in the abomasum or intestine. They then reproduce in the host and the parasite eggs are shed in the host's faeces. These remain on the pasture where they develop through to the third larval stage and are re-ingested by another host.

In this population, parasite burden is predominantly highest in lambs, particularly males,which also tend to have higher parasite burdens than females more generally (figure 1.4). In relation to ageing previous work has shown an increase in parasite burden with older ages, which is emphasised if the individual has encountered a high amount of environmental stress (Hayward et al., 2009). Parasite burden is also a predictor of first winter survival, but it is not yet known how this might associate with immunity (Hayward et al., 2011). A study by Gulland (1992)

carried out post mortem analysis of nematode worm load on Soay sheep which were found dead between February to May 1989. During this period the population had undergone a 'crash' due to high density dependant mortality resulting in a dramatic reduction in population size. The study found that it was malnutrition which led to immunosuppression and increased parasite prevalence, and it was due to the cumulative effect of infection and a food shortage which resulted in mortality. Two parasite taxa, Stronglyes and Coccidia, demonstrate independant selection on Soay sheep and the effect of these parasites depends on the age of the individual; both taxa had independent negative correlation with host weight in adults, but only Strongyle eggs had a significant negative correlation in early life (Craig et al., 2008). There is a clear impact of parasite worm burden on the fitness of an individual, and also the population dynamics as a whole, but how this relates to immunity is still to be explored.

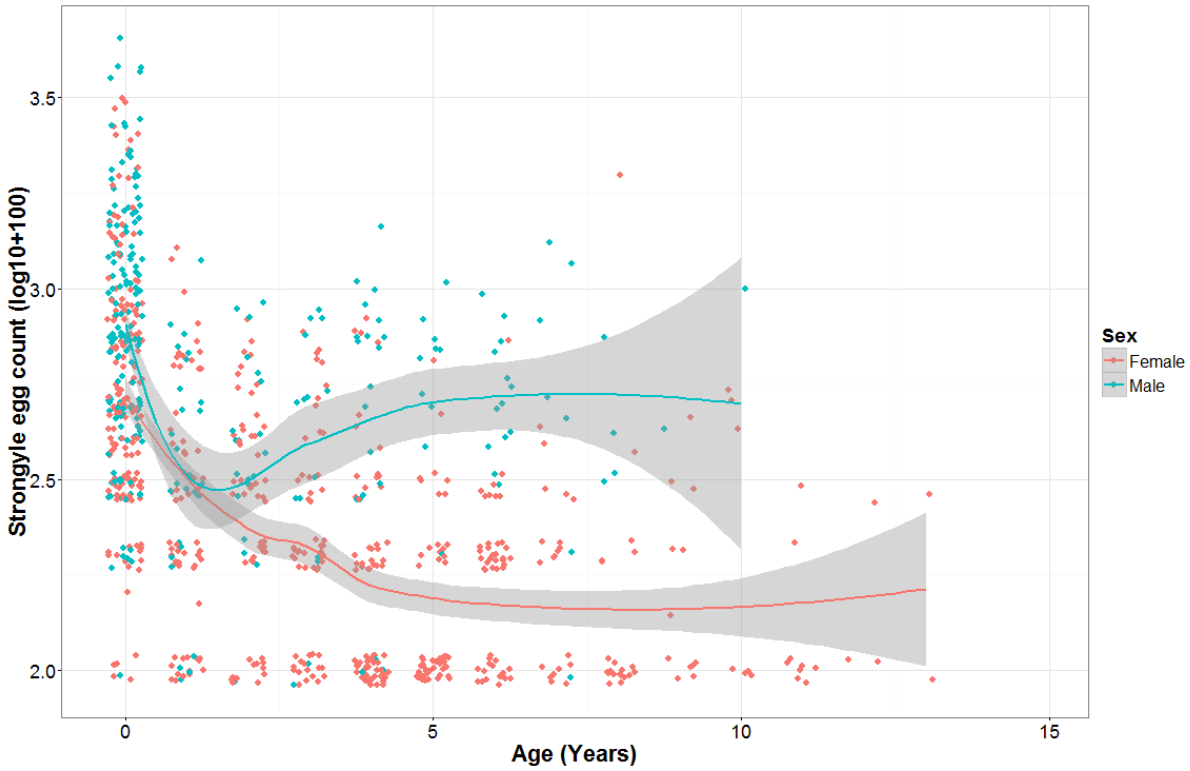


Figure 1.4: The variation in Strongyle faecal egg counts between different ages and seperated into males (blue) and females (red). This data includes all individuals sampled during August between 2011-2014. The lines fitted are smoothers with the standard error shaded in grey.

Previous studies in this system have found interesting associations between immunity and other life history traits.In both males and females, higher reproductive success has been assocaited with lower measures of natural antibodies, and in females inparticular, lower natural antibody measures were identified in individuals which produced the heaviest 25% of lambs (Graham et al., 2010). In females, higher measures of both natural and parasite-specific antibodies have been shown to be positively correlated with over-winter survival during crash years, although it is not yet known whether this is also true in males. (Graham et al., 2010; Nussey et al., 2014). There is evidence of negative relationships between immune markers, particularly antibody measures, and faecal egg counts (FEC), suggestting a protective function of these antibodies against parasite (Coltman et al., 2001; Graham et al., 2011; Hayward et al., 2014). Investigation into variation of inflammatory markers in this population has shown similarities to research in humans, specifically with increases in two acute phase proteins, haptoglobin and serum amyloid A, but there was no age related variation found with the anti-inflammatory cytokine interleukin-10 (IL-10) or pro-inflammatory cytokine interleukin-6 (IL-6) (Nussey et al., 2012). Analysis of T cell variation identified strong age trends in T cell subset, including a marked decrease in naïve T helper cell populations with age as well as a decrease in the proportion of gamma-delta T cells two years post birth (Nussey et al., 2012). The work in this thesis will expand upon this previous study to include multiple years of data and both sexes, as previously only females were measured.

1.8 Immune markers

To cover a broad range of the immune system, I selected three types of immune markers; leukocyte markers primarily of the innate immune system, T cells and B cells. Within these three immune marker types, I measured various immune markers which have known importance relative to individual variation from immunology literature and research on domestic livestock in particular. The availability of reagents to measure these cell types, due to their importance in domestic research, is a great

advantage to the study of specific immune variation in a wild system. All field protocols are included in full in the appendix, and laboratory methods are described within each chapter.

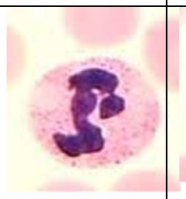
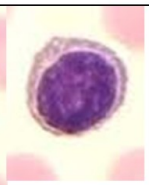
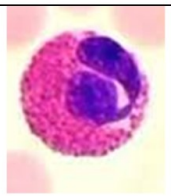
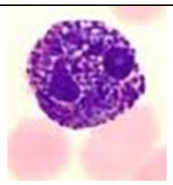
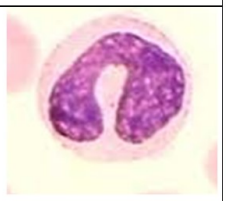
1.8.1 *Leukocyte subtypes*

Leukocytes are the main cell types of the immune system and are crucial to the detection and elimination of parasites. Details of the leukocyte subtypes counted are summarised in table 1.2, although only neutrophils, eosinophils and lymphocytes were included in analysis due to the rarity of monocytes and basophils. It is due to such a low presence of basophils and monocytes that identification of these is very infrequent and more prone to error (Pastoret et al., 1998). These three cell types have been found to compose the majority of white blood cells in healthy sheep (Egbe-Nwiyi et al., 2000), figure 1.5 shows these three cell types as viewed during analysis. These counts are performed under a compound microscope using blood smears of fresh samples stained using a Reastain©staining kit, detailed staining protocol available in the appendix (S1). The blood smears were carried out in the field on St Kilda as soon as possible after the sample was collected, the protocol for this is also detailed in the appendix (S1), and the cell counts were performed back in Edinburgh using the Battlement protocol (see Chapter 2).

Granulocytes represent 60-70% of blood leukocytes and include neutrophils, eosinophils and basophils (Riott et al., 2006). The predominant granulocytes are neutrophils which represent around 90% in the circulation. The primary function of neutrophils is destruction of microorganisms and phagocytosis of foreign antigens and they are present at high numbers at the site of infection, a character of the inflammatory response (Wakelin, 1966). In this study the number of neutrophils was measured in relation to the number of lymphocytes as the Neutrophil:Lymphocyte ratio (NLR), which is commonly used as an indicator of chronic inflammation or stress (Davis et al., 2008; Wakelin, 1966). Eosinophils account for around 2-5% of

blood leukocytes and play an important role against helminth parasites through the process of degranulation(Riott et al., 2006). In sheep in particular, eosinophils are responsible for damaging larval stages of the parasite to prevent establishment (Meeusen and Balic, 2000; Stear et al., 1995) and are influential in nematode resistance (Stear et al., 2002).

Table 1.2: Description and images of five leukocyte subsets. * indicates the cells are not functionally active until antigen stimulation

Leukocyte subset	Neutrophil	Lymphocyte	Eosinophil	Basophil	Monocyte
Microscope image					
Activated function	Phagocytosis and activation of bacterial mechanisms	*Mediate humoral (B cells) and cellular (T cells) adaptive immunity	Killing of anti-body coated parasites	Promotion of allergic responses and augmentation of anti-parasitic immunity	*Precursor of tissue macrophages

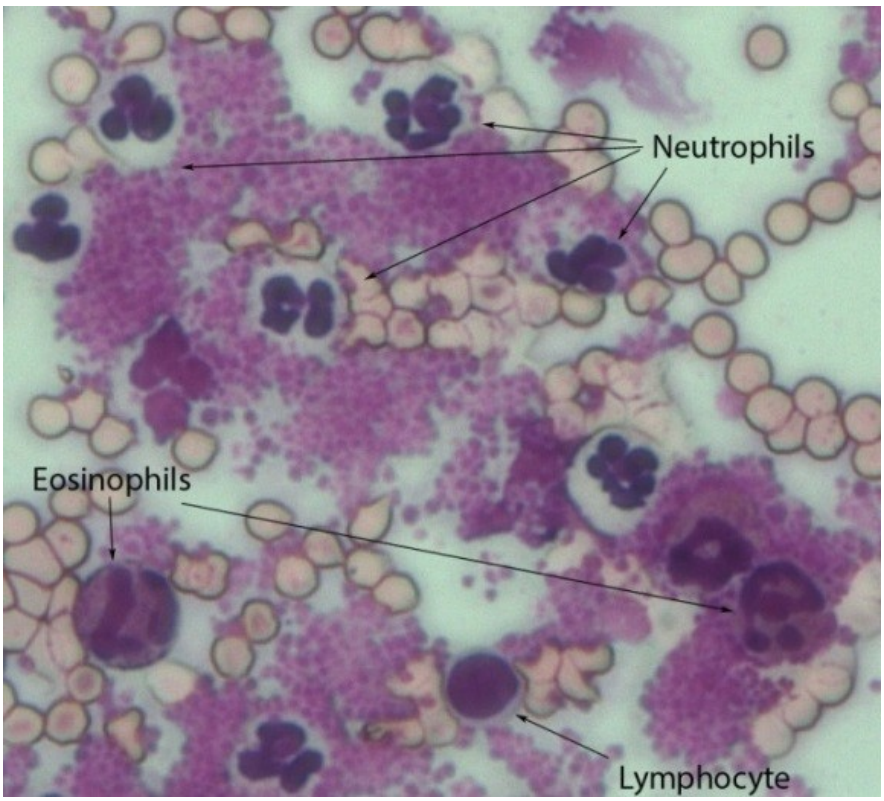


Figure 1.5: Microscope image showing examples of the subtypes of leukocytes measured; eosinophils, neutrophils and lymphocytes. Original photograph by Michael Edwards, edited by P Hopper using Adobe Photoshop CS5.1 12.1 2011

The methodology for cell counts was optimised as part of an honours project with Pheobe Hopper. This involved the direct comparison of each two techniques testing the reliability and repeatability of each one. The primary method, which had originally been used to count slides from samples collected in 2011, was tested against an alternative battlement method, which proved to have higher repeatability and so was ultimately used for analysis of all slides (2011 and 2012). Spearman Rank correlation analysis was performed for all cell counts via both methods and the battlement method showed higher correlation coefficients in all occasions.

1.8.2 *T cells*

Using flow cytometry I measured proportions of T cell sub populations including gamma-delta+, CD4+, CD8+, CD45RA+ and FoxP3+, details of cell function and age variation are outlined in table 1.3. T cells are functional effector cells of the adaptive immune system and as mentioned previously, are characterised into three general cell types. CD8+ T cells are cytotoxic cells (T_c) and are involved in killing infectious agents. CD4+ T cells are helper cells (T_H) which co-ordinate and influence other cell types to provide a response to infection. Both T_c and T_H cells require antigen activation to become active, and exist in naïve forms, CD8+CD54RA+ and CD4+CD45RA+, until then they encounter a pathogen. The Foxp3+ T cell subset are regulatory T cells (T_{reg}), which control the immune response and maintain immune tolerance through the suppression of other lymphocytes (Muehlenbein, 2010).

Flow cytometry performs single cell counting based on the specific fluorescent markers used. These markers are labelled antibodies which are specific to cell surface markers allowing differentiation between subpopulations of cell types. For this technique the sample is processed, which in this case involves red blood cell lysis and leukocyte fixation, the cells are then suspended in solution and stained with the appropriate labelled antibodies. The full protocols for these cell fixation procedures are detailed in the appendix 3. Once stained the cells are then sorted via the flow

cytometer through which they travel past a laser in single file to allow single cell sorting. If the selected marker is present on the cell as it passes past the laser, the flourophore absorbs the laser energy and releases a specific wavelength which is detected by the flow cytometer. The cells are also sorted based on their charge which is measured as forward scatter (FSC), which indicates the cell size, and side scatter (SSC), which indicates the granularity of the cell. The flow cytometer provides a lot of information for each sample, which needs to be refined to provide the relevant data required. figure 1.6 demonstrates a typical read out of the raw data and explains how the required information is extracted via gating techniques; this analysis is carried out using FlowJo software.

Table 1.3 Description of function and predicted changes in proportion with ageing of T cell subsets which will be measured using flowcytometry. Predictions made in accordance with preliminary study (Nussey et al., 2012).

Subset	Function	Predicted change with ageing
CD4+	*Helper cells* Release cytokines and growth factor that regulate other immune cells Activate macrophages and help induce B cell Ab production	Increase with age
Gamma-delta (γδ+)	*Assembled in* γ:δ *heterodimer* Highly abundant in ruminants Broad functional plasticity involved in inflammation, tumour surveillance, infectious disease and autoimmunity	Decrease with age
CD8+	*Cytotoxic cells* Defend against intracellular pathogens including viruses, bacteria and tumour structure	Increase with age
FoxP3+	*Regulatory* Transcription factor critical in maintaining immune tolerance and suppression of the immune system	Decline with age
CD45RA+	*Naïve* Responds to pathogens not yet encountered by the immune system	Decrease with age

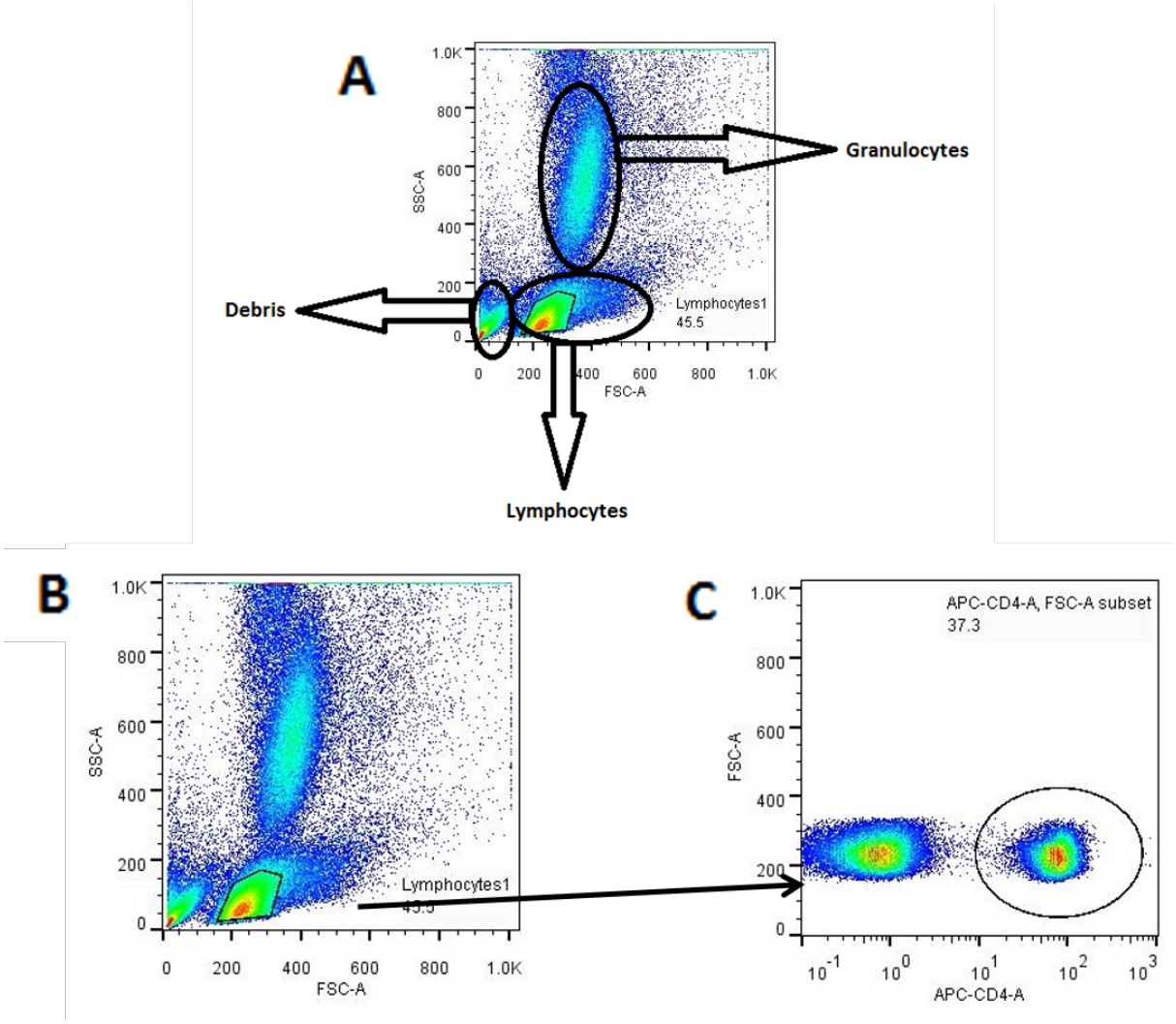


Figure 1.6: A: Example of a density plot of cell counts sorted by FSC and SSC. The lymphocytes, granulocytes and dead matter (debris) are each labelled. B: This image shows how a gate to isolate the lymphocyte counts is drawn around the area containing the highest number of lymphocytes. C: Showing the gated population of lymphocytes from B in isolation. Note that here the cells are plotted according to FSC and the amount of CD4+ staining according to the fluorescent marker APC (647). The cluster of cells on the left represents unstained cells and the cluster on the right indicates positive staining for CD4+

In the original protocols for the flow cytometry analysis, each cell type was stained and assayed separately, partly as four of the five antibodies were produced in-house and were not directly conjugated. As a result, the same secondary antibody was used to detect more than one cell type and so they could not be analysed comparatively in the same assay. While this method was able to capture cell proportions well, the running of multiple assays was time consuming, required a higher volume of fixed cells, and allowed fewer combinations of cell types to be analysed. As well as increasing the amount of information and accuracy of the assay, a multi-panel stain can facilitate fluorescence minus one (FMO) controls. FMO controls include all stains except one, to check for any non-specific binding occurring and are considered a more accurate replacement of previously used isotype controls, which use an antibody (Baumgarth and Roederer, 2000). Therefore, a large part of this project was to develop a multi-stain protocol, which measured all cell types in one assay using directly conjugated antibodies. This involved producing specific antibodies using cell culture, purifying the antibody product and then conjugating each cell type to a different fluorophore to develop a compatible multi-panel stain. Examples of staining with this method, inclding single stains, FMO controls and an example sample, are shown in FACS plots of the raw data (appendix 5).

1.8.3 *Parasite specific antibodies*

Antibodies, which are produced by B cells, constitute humoral immunity, and act in three ways to protect the host from infection; neutralisation (attaching to the pathogen and preventing replication), opsonisation (binds to surface of pathogen promoting recognition phagocytosis by other cells), and complement activation (leading to recognition of bacteria which would not otherwise be identified) (Murphy et al., 2012). There are five main antibody isotypes , immunoglobin (Ig) A, IgD, IgE, IgM and IgG, distinguished by the heavy-chain regions, and each carry out a particular function. Self-antibodies, or ‘natural’ antibodies, bind with low affinity to either novel non-self antigens or self antigens, whereas induced antibodies bind to specific target

antigens (Murphy et al., 2012). In this study, we measured induced antibodies against larval stage 3 (L3) of the dominant GIN in this population, *T.circ*. We ran ELISA's to measure three different antibody isotypes, IgA, IgE and IgG, which have known relationships with parasites and survival in this population(Coltman et al., 2001; Hayward et al., 2014; Nussey et al., 2014). IgG is the dominant antibody present in circulation, whereas IgA is are more centrally involved in mucosal immunity, being the dominant antibody on mucosal surface in the gut (Pastoret et al., 1998).

1.9 Thesis aims and objectives

The overall aim of this thesis is to test the relevance of specific measures of immunity in understanding the causes and consequences of immune variation in a wild mammal. Within this thesis there are abreviations used for some of the immune markers measured, which are summarised in table 2.4. Due to the different questions addressed in each chapter there is variation in the precise data set and immune markers used, this is summarised in table 1.4.

1.9.1 *Chapter 2*

In this chapter I used a data set collected in August 2011 to take a cross-sectional snap shot of the full panel of immune measures across all ages in the population. This data set preceded a population crash and as a result the individuals in this analysis are under strong selection pressure for survival. The analysis explores age and sex trends in a panel of 11 immune markers, which included differential white blood cell counts, T cell subpopulations, and *T. circumcincta* specific antibodies. We expected immune markers to vary with respect to the age , as research undertaken in lab mice and humans indicates that the proportions of different cell types vary between old and young individuals. Proportions of naive T cells are known to decrease with age and in contrast the proportions of mature T cells are found to be higher in older individuals with a developed immune system. Antibody measures are also known to develop with age, so would be expected to be higher in older individuals. We expected to find a female biased sex difference in immunity due to predcitions from evolutionary theory and some evidence from wild and lab studies. We expected males to have lower measures of immunity as evidence suggests males are more susceptible to infection and often carry higher parasite burdens. I also tested for a relationship between immune markers and survival across all ages and in both sexes. Robust immune respsonse are important for survival and may indicate a high quality indiviudal. Therefore individuals with higher immune measures may show increased survival.

1.9.2 *Chapter 3*

Here, I focused on early life using a four-year longitudinal data set collected betweeen 2011-14 to test for trade-offs with immune investment in lambs. The analysis included white blood cell counts and parasite-specific antibody markers. I tested for trade-offs between immunity and growth rate during the first four months of life, with th expectation that high growth in early life would come at a cost of developing immunity. I examined whether antibody measures were related to Strongyle faecal egg counts (FEC) to assess whether these were markers of exposure or immune protection. If these antibodies are providing protection against parasites, then we would expect individuals with high antibody measures to have low parasite burdens. In this data set I tested for sex-specific trade-offs with immunity, particularly in male secondary sexual characteristics. We might expect that if there is a cost of growth to the development of immunity then investment in secondary sexual characteristics in males would lead to an increased cost to immunity and sex-specific trade-off between these two traits. Whether there are immediate fitness consequences of immune variation to first over-winter survival is largely unknown, but we might expect that as shown in previous analyses, higher immune responses would be indicative of increases survival.

1.9.3 *Chapter 4*

The data in this chapter is a two-year longitudinal data set collected in 2014-15, this analysis included all ages of individuals accross the population. I addressed whether changes in leukocyte telomere length (LTL) are independent of age-related variation in leukocyte population composition, and whether these differed between the sexes. Leukocyte population compostion is known to change with age, and leukocytes also exhibit variation in telomere lengths, with naïve cell types having typically longer telomeres. We might expect that as the propotion of naïve leukocytes declines with age, then so does the overall LTL. As we found naïve T cells to differ between the

Table 1.3: Description of abreviations used for immune markers within the thesis chapters

Abbreviations	Immune maker or equivalent labels
CD4+	Helper T cells
CD8+	Cytotoxic T cells
GD	Gamma Delta
Foxp3+	Treg
CD45RA+	Naive T cells
CD4CD45RA+	Naive helper T cells
CD8CD45RA+	Naive cytotoxic T cells
NLR	Neutrophil:Lymphocyte ratio
Anti-*T.circ* IgA/G/E	Anti-*Teladorsagia circumcinta* IgA/G/E

sexes, I tested for sex-specific variation in LTL, as we might also predict that males have shorter LTL due to also having lower naïve T cells. I focused on male secondary sexual characteristics, as telomeres are known to shorten with each cell replication, so we could expect that males which have the additional growth pressures of secondary sexual characteristics have a cost of reduced telomere length.

Table 1.4: Samples sizes for each chapter in this thesis, broken down by sex, age and immune parameters measured.

			Sex	Age					Immune Measures		
	Year	Total number of individuals	Males	Females	Lambs	Yearlings (1yr)	Adults (2-6yrs)	Geriatric (>6yr)	Differential white blood cells	T cells	*T.circ* antibodies
Chapter 2	2011	287	99	188	9	23	133	32	X	X	X
Chapter 3	2011	99	50	49	99				X		X
	2012	55	23	32	55				X		X
	2013	116	56	60	116				X		X
	2014	102	55	47	102				X		X
	All years	372	184	102	372						
Chapter 4	2014	260	83	177	101	24	101	28		X	
	2015	541	66	175	103	22	75	36		X	
	All years	501	149	352	204	46	176	64			

Chapter 2

Cellular and humoral immunity in a wild mammal: Variation with age & sex and association with over-winter survival

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This chapter has been accepted for publication as:

Watson, R.L., McNeilly, T.N., Watt, K.A., Pemberton, J.M., Pilkington, J.G., Waterfall, M., Hopper, P.R.T., Cooney, D., Zamoyska, R., & Nussey, D.H. (*2016*) "Cellular and humoral immunity in a wild mammal: Variation with age & sex and association with over-winter survival" *Ecology & Evolution.*

2.1 Abstract

Immune defences are expected to be crucial for survival under the considerable parasite pressures experienced by wild animals. However, our understanding of the association between immunity and fitness in nature remains limited due to both the complexity of the vertebrate immune system and the often-limited availability of immune reagents in non-model organisms. Here, we use methods and reagents developed by veterinary researchers for domestic ungulates on blood samples collected from a wild Soay sheep population, to evaluate an unusually broad panel of immune parameters. Our evaluation included different innate and acquired immune cell types as well as nematode parasite-specific antibodies of different isotypes. We test how these markers correlate with one another, how they vary with age group and sex, and, crucially, whether they predict over-winter survival either within or among demographic groups. We found anticipated patterns of variation in markers with age, associated with immune development, and once these age trends were accounted for, correlations among our 11 immune markers were generally weak. We found that females had higher proportions of naïve T cells and gamma-delta T cells than males, independent of age, whilst our other markers did not differ between sexes. Only one of our 11 markers predicted over-winter survival: sheep with higher plasma levels of anti-nematode IgG antibodies were significantly more likely to survive the subsequent high mortality winter, independent of age, sex or weight. This supports a previous finding from this study system using a different set of samples, and shows that circulating antibody levels against ecologically-relevant parasites in natural systems represent an important parameter of immune function and may be under strong natural selection. Our data provide rare insights into patterns of variation among age and sex groups in different T cell subsets and antibody levels in the wild, and suggest that certain types of immune response, notably those likely to be repeatable within individuals and linked to resistance to ecologically-relevant parasites, may be most informative for research into the links between immunity and fitness under natural conditions.

2.2 Introduction

Parasites and pathogens represent a major selective force on their hosts, and the development and maintenance of immune defences are expected to be important for survival and fitness under natural conditions (Schmid-Hempel, 2003; Seppälä, 2015; Sheldon and Verhulst, 1996). Although positive associations between markers of immunity and survival have been observed in laboratory and domestic settings, evidence from natural populations experiencing ecologically-relevant parasite challenges remains relatively scarce(Møller and Saino, 2004; Schimd-Hempel, 2011; Seppälä, 2015; Viney et al., 2005). Immunological studies in wild vertebrates are made particularly challenging by the staggering complexity of the vertebrate immune system, coupled with the limited range of immunological reagents available for most non-model species (Boughton et al., 2011; Demas et al., 2011). For this reason, research to date within the field of ecological immunology has tended to rely on a relatively small number of functional immune assays that can readily be applied across species (Demas et al., 2011). However, key questions, such as how different aspects of immunity are related, and which immune parameters are most important in predicting fitness under natural conditions, can only be answered through the application of a wider range of immunological methods in the field (Boughton et al., 2011; Demas et al., 2011; Pedersen and Babayan, 2011). Here, we measure a range of immune cell types and parasite-specific antibodies from blood samples collected from a free-living population of mammals. We test how these immune parameters relate to one another, whether they differ among age classes and sexes, and whether they predict subsequent over-winter survival within and across these demographic groups.

The vertebrate immune system is typically regarded as consisting of the innate and adaptive arms, although interactions between these two systems are essential for an effective immune response (Murphy et al., 2012). The innate immune system, which is the older in evolutionary terms, refers to nonspecific defence mechanisms which become activated immediately or within hours of antigen encounter. These defence mechanisms include anatomical barriers and behaviours to avoid infection,

humoral factors such as complement and acute phase proteins, and a variety of functionally diverse white blood cells including macrophages, neutrophils and eosinophils (Demas et al., 2011). The adaptive immune system refers to antigen-specific immunity, and is more complex than innate immunity and slower to develop. Adaptive immune responses are, however, capable of producing long-lasting immunological protection against particular parasites as a result of immunological 'memory'. The key cells are lymphocytes, composed principally of B and T cells, which are capable of discriminating host and non-host molecular patterns and co-ordinating a directed immune response against parasites and pathogens (Parkin and Cohen, 2001). B cells produce antibodies which play a critical role in recognising and neutralising infectious agents (Alberts et al. 2002). A variety of functionally distinct antibody isotypes are involved in antibody-mediated immunity (Murphy et al., 2012). In mammals, IgG is the predominant circulatory antibody isotype, whilst IgA and IgE are detected at lower concentrations in circulation, but predominate at mucosal surfaces where they play an important role in immune functions (Manz et al., 2004). Most mature alpha-beta ($\alpha\beta$) T cells are produced in the thymus, emerging in a "naïve" state and activating to become effector or memory T cells when they recognise a peptide expressed in conjunction with an MHC molecule at the surface of a host cell and receive appropriate stimulatory signals from other immune cells (Murphy et al., 2012). Gamma-delta ($\gamma\delta$) T cells, which are rare in the circulation in humans but more common in ruminants and poultry, emerge from the thymus in an effector state, are not restricted to recognising antigen presented with MHC, and can produce rapid, localised responses (Bonneville et al., 2010). A wide range of $\alpha\beta$ T cell sub-types with distinct functions have been described based on specific expression of intra- and extra-cellular markers and quantitatively evaluated in using techniques, such as flow cytometry (Murphy et al., 2012). Very broadly, these sub-types include cytotoxic T cells which recognise and destroy cells with intracellular pathogens, helper T cells which co-ordinate immune response to infection, and regulatory T cells (Treg) which play a key role in maintaining peripheral immunological tolerance by actively suppressing the immune response (Muehlenbein, 2010). To our knowledge, no study of a wild vertebrate has simultaneously measured variation in different T cell sub-types, antibody levels and

innate immune cell numbers and related these different aspects of the immune phenotype to survival.

Profound changes in immune phenotype and function are observed over the course of an organism's lifespan, with resistance to infection typically developing through early life into adulthood and certain aspects of immune function becoming compromised in old age (Simon et al., 2015). Variation in markers associated with immune function with age have been widely reported in wild vertebrates (Cichoń et al., 2003; Jego et al., 2014; Nussey et al., 2012; Palacios et al., 2011). Notably, the structure and function of the thymus in mammals and in birds deteriorates remarkably early in life in many vertebrates and the output of naïve T cells may be greatly reduced by the time an individual reaches sexual maturity (Cockburn, 1992; Møller and Erritzøe, 2001). Although age-dependent declines in circulating naïve T cells have been widely observed in laboratory and domestic mammals and humans, the fitness consequences of variation in the availability of naïve T cells at different ages under natural conditions are completely unknown (Shanley et al., 2009). Sex differences in immune function are also predicted by evolutionary theory and there is some evidence to support these predictions from natural and laboratory systems (Zuk and McKean, 1996). A major challenge for our understanding of how variation in immunity impacts evolutionary and population dynamics of natural systems, given the expectation and observation of age and sex differences in immunity, is therefore to test whether the associations between measures of immunity and fitness differ among demographic groups.

In this study, we measured differential white blood cell counts, used ELISA to assay parasite-specific antibody levels, and used flow cytometry to differentiate T cell sub-types to generate a broad panel of 11 immune markers in a cross-sectional sample spanning all ages and both sexes in a population of free-living Soay sheep (*Ovis aries*). Our work builds on a previous study which documented patterns of age-related change in T cell sub-types consistent with those observed in the human, laboratory rodent and domestic ruminant literature, but had insufficient sample sizes to meaningfully test for associations with fitness measures independently of age (Nussey

et al., 2012). We have also previously shown that antibodies against a highly prevalent gastrointestinal nematode parasite in our study population, *Teladorsagia circumcincta (T. circ)*, are negatively associated with parasite egg counts and positively associated with over-winter survival in adult Soay sheep (Coltman et al., 2001; Hayward et al., 2014; Nussey et al., 2014). Here, we test how our immune markers, which encompass a range of different innate and immune cell types, are correlated with one another, how they vary with age and sex, and to what extent the markers predict over-winter survival and whether associations with survival are age- or sex-dependent.

2.3 Materials and methods

2.3.1 *Study system & field data collection*

Soay sheep are a primitive breed of domestic sheep, which has dwelt unpredated and unmanaged in the remote St Kilda archipelago for several millennia. The animals resident to the Village Bay area of the main island of Hirta within the archipelago have been the focus of a long-term individual-based study since 1985 (Clutton-Brock & Pemberton 2004). These individuals are caught and marked at birth, and their life histories are closely monitored from birth to death. Most are caught once a year during summer for sampling and measurement. The population exhibits a distinctive, unstable dynamic, characterised by low and rising sheep numbers followed by high mortality ('crash') winters in which more than half of the population may perish (Clutton-Brock & Pemberton 2004). High mortality winters are associated with strong selection on a range of phenotypic traits, and are thought to result from a combination of low food availability due to competition, harsh winter climate conditions and parasite pressure, predominantly from Strongyle gastro-intestinal nematodes (GINs) (Gulland & Fox 1992, Gulland 1992, Coulson 2001). Age-related variation is well understood in this population, with differences evident between lambs, yearlings, prime age adults (2-6 years) and geriatrics (>6 years) in

demographic rates and phenotypic traits both within and among the sexes (Coulson 2001).

Samples and data for this study were collected in August 2011, during the Soay sheep research project's annual summer catch. During a two week period, 287 marked individual sheep, were rounded up, caught and processed in a series of corral traps set up in the Village Bay area. Our sample comprised 50 male and 49 female lambs (approximately four months old), 5 male and 18 female yearlings (1 year and four months), 40 male and 93 female adults (2-6 years) and 4 male and 28 female geriatrics (7 or more years). Upon capture each individual was weighed, measured and then blood and faecal samples were collected. Faecal samples were used to measure Strongyle and Strongyloides parasite faecal egg counts (FEC), using a modification of the McMaster technique (Ministry1971, Gulland & Fox 1992). Two 9ml Li-heparin Vacutainer tubes of whole blood were taken from each individual and stored at 4°C until processing. One Vacutainer was used for plasma extraction for antibody analysis, and the other was used for differential cell counts and leukocyte fixation for subsequent staining for flow cytometry. The winter of 2011/2012 was a high mortality ‘crash’ winter on Hirta. Winter censuses and mortality searchers in the study area allowed us to ascertain which of the animal's sampled in August 2011 died over the following winter. 139 of our 287 sampled individuals (48%) had died of natural causes by 1st May 2012.

2.3.2 *Laboratory methods*

2.3.2.1 *Differential white blood cell counts*

Within 12 hours of collection 5µl of whole blood was applied on to one end of a standard glass microscope slide. The drop of blood was then spread at a 45° angle and drawn across the slide to produce an even film. Slides were air dried overnight and stained using a Quick-Diff Kit stain (Gentaur) the following day, as per

manufacturer's instructions. 100 cells were counted at 40x magnification using the Battlement Track method and based on staining and morphology, identified as either lymphocytes, eosinophils or neutrophils (Bain 2008). Basophils and monocytes were observed too rarely to analyse. Only slides with a clear regular monolayer of cells were counted. Slides with uneven cell density or unclear staining were omitted from analysis (20%). From our counts, we calculated a total eosinophil count and the neutrophil to lymphocyte ratio (NLR).

2.3.2.2 *Antibody measures*

Within 24 h of collection, one Vacutainer of whole blood was centrifuged at 1008g for 10 min and the plasma layer removed and stored at -20°C. We then followed previously published methods to measure levels of IgE, IgA and IgG antibodies binding larval stage 3 antigens from *T. circ*, a highly prevalent gastrointestinal Strongyle nematode parasite of Soay sheep on Hirta (Nussey et al., 2014). Samples were analysed using the same protocol and procedure as in (Nussey et al., 2014) with minor adaptations. We used *T. circ* L3 somatic antigen, diluted to 2µg per ml of 0.06M Carbonate buffer at pH 9.6. L3 somatic antigen was prepared by re-suspending *T. circ* L3 in PBS (5 x 105 larvae per ml) in Lysing Matrix D tubes (MP Biomedicals) and homogenising in a Precellys©24 tissue homogeniser. Debris was pelleted by centrifugation at 16, 000 x g at 4°C and the somatic antigen containing supernatant stored at -80°C prior to use. Total protein concentration of the L3 antigen preparation was estimated using a Pierce™ BCA Protein Assay Kit (Thermo Scientific).

In each assay, 50µl of appropriately diluted antigen solution was added to each well of a Nunc immuno 96-microwell plate, which was subsequently covered and incubated overnight at 4°C. The wells were then washed three times in Tris-buffered saline-Tween (TBST) using a plate washer. Then 50µl of an appropriately diluted Soay sheep plasma sample was added to each well. Sample dilutions used (adapted from optimisation procedure described by Nussey et al. (2014)) were as follows: anti-

T. circ IgA: 1:50; anti- *T. circ* IgG: 1:12800; anti -*T. circ* IgE: 1:50.

The plates were then covered and incubated at 37°C for 1 hour and then washed five times with TBST. For the anti-*T. circ* IgA & anti-*T. circ* IgG assays 50µl per well of the appropriate rabbit anti-sheep detection antibody conjugated to horseradish peroxidise (HRP) was added (anti-ovine IgA-HRP diluted 1 µl in 8mls in TBST, anti-ovine IgG-HRP diluted 0.5 µl in 8mls in TBST: all AbD Serotec, cataologue numbers: AHP949P and 5184-2504, respectively). For the anti-Tc IgE assay50µl of anti-ovine IgE (mouse monoclonal IgG1, clone 2F1) diluted 1:100 in TBST was added to each well, followed by 1 hour incubation at 37°C, five washes with TBST and then the addition of 50µl of goat anti-mouse IgG1-HRP detection antibody (AbD Serotec catalogue number: STAR132P), diluted to1µg in 8000µl of TBST to each well. All plates were then covered and incubated at 37°C for 1 hour. They were then washed five times with TBST and 100µl of SureBlue TMB 1-Component microwell peroxidase substrate (KPL) was added per well and then left to incubate for 5 minutes in the dark, in a cardboard box, at 37°C. Reactions were then stopped by adding 100µl 1M HCl and optical densities (ODs) were read immediately at 450nm using a Thermo Scientific Multiskan GO Spectrophotometer.

Each assay on each selected Soay sheep plasma sample was performed twice on separate ELISA plates. On each plate we also included four sample-free wells as a duplicate negative control (TBST: 200ml of 10xTrisBuffered Saline in 1800mls distilled water with 1ml Tween 20) and a duplicate positive control (Moredun Research Institute (MRI) positive sample: purified lymph from *T. circ* infected sheep). We excluded samples across all assays for which there was obviously poor correspondence across duplicate OD scores, presumably due to human error (n=2 Anti-*T. circ* IgA, n=1 Anti-*T. circ* IgG). We then checked the correlation of ODs across duplicate plates and re-ran both plates if $r < 0.80$. For subsequent analyses, we took the average OD across the duplicate runs minus the average of the two negative control well ODs across the two plates as our assay measure.

2.3.2.3 *T cell subsets*

Lymphocytes were preserved in fixative after collection on St Kilda and later analysed, following antibody labelling, by flow cytometry in Edinburgh (following Nussey et al. 2012). Briefly, red cells were removed from 1ml of whole blood by addition of 5ml of ammonium chloride lysing solution (1.5M NH4Cl, 100mM NAHCO3, 10mM Na2EDTA) was added to 1ml of whole blood and mixed gently before centrifuging at 1200rpm for 10 minutes. The supernatant was removed and the cell pellet re-suspended in 9ml phosphate buffered saline (PBS) to wash. The sample was then spun, the supernatant removed and the pellet re-suspended in 2.5ml 1% paraformaldehyde (PFA) in PBS at room temperature for 10 minutes. After the cells were spun at 1008g for 1 minute the supernatant was removed and the cell pellet re-suspended in 9ml phosphate buffered saline (PBS) to wash. The cells were then spun again at 1008g for 1 minute and finally resuspended in PBS + 0.02

Flow cytometry protocols broadly followed those described in Nussey et al. (2012) with some modifications as described below. Fluorescently labelled monoclonal antibodies were used to identify the proportions of T helper cells (CD4+), cytotoxic T cells (CD8+) and gamma-delta T cells within the total lymphocyte population within each sample and the proportion of T helper cells which were Treg (FoxP3+). Naïve helper and cytotoxic T cells, were identified by their co-expression of the CD45RA marker, and the sub-population of regulatory T helper cells which were Treg (FoxP3+) were identified by co-expression of CD4+ and FoxP3+ antibodies.

Using a 96-well plate 100µl of each sample of fixed white blood cells was spun at 2000rpm for 1 minute at 4°C and the supernatant discarded. The cells were re-suspended in 200µl of 20% Normal Goat Serum (NGS) and the plate was incubated at 4°C for 30 minutes before spinning at 2000rpm for 1 minute. For the single colour stains cells were re-suspended and incubated at 4°C for 30 minutes with 100µl of anti-ovine monoclonal antibodies (mAb) to either gamma-delta (clone 86D,

mouse IgG1, (Mackay et al., 1989), CD4 (clone 17D, mouse IgG1, (Mackay et al. 1988)) or CD8 (clone 7C2, mouse IgG2a, (Young et al., 1997)) or the appropriate isotype control antibody (mouse IgG2a and mouse IgG1k). The plate was washed by adding 100µl FACS buffer, spinning at 2000rpm for 1 minute, adding 200µl FACS buffer and a final spin at 2000rpm for 1 minute. Cells were then re-suspended in 100µl of secondary antibody (goat anti-mouse IgG-Alexa 647 [H+L], Invitrogen, Carlsbad, CA) and the plate incubated for 30 minutes at 4°C. The plate was then washed as described previously and the cells suspended in 200µl PBS solutions before spinning at 2000rpm for 1 minute. Cells were fixed by adding 200µl 1% PFA in PBS and incubating the plate at room temperature for 10 minutes before spinning and re-suspension in 200µl PBS solution. At this point the plate is covered and stored at 4°C until being read on a BD FACSCanto II©flowcytometer (BD Biosciences, San Jose, California, USA) within 48 hours.

For the triple colour stains cells were re-suspended and incubated at 4°C for 30 minutes with 100µl of anti-ovine mAb to either CD8 (clone 7C2, mouse IgG2a, (Young et al., 1997)), CD45RA (clone 73B, mouse IgG1 (Mackay et al., 1990)) or the appropriate isotype control (mouse IgG1).The plate was then incubated at 4°C for 30 minutes and then washed as described previously and spun at 2000rpm for 1 minute. The cells were re-suspended in 100µl of secondary antibody (goat anti-mouse IgG-Alexa 647 [H+L], Invitrogen, Carlsbad, CA or rat anti-mouse IgG2a-PE). The plate was then incubated for 30 minutes at 4°C and then washed as described previously. Cells were re-suspended in 200µl of 10% normal rat serum in FACS buffer and incubated at 4°C for 30 minutes. After spinning the cells were re-suspended in 100µl of either anti-ovine CD4 mAb conjugated to FITC (clone 44.38, mouse IgG2a, AbDserotec) or the mouse IgG2a-FITC isotype control. The plate was incubated at 4°C for 30 minutes before washing as described previously and cells were re- suspended in 200ul PBS solution and spun again at 2000rpm for 1 minute. Cells were fixed by adding 200µl 1% PFA in PBS and incubating the plate at room temperature for 10 minutes before spinning at 2000rpm for 1 minute and re-suspension in 200µl PBS solution. At this point the plate was covered and stored at 4°C until being read on a BD FACSCanto II©flowcytometer (BD Biosciences, San

Jose, California, USA) within 48 hours.

For the Treg (CD4+CD45RA+Foxp3) stain cells were re-suspended and incubated at 4°C for 30 minutes with 100µl of either anti-ovine CD4 mAb conjugated to FITC (mouse IgG2a), anti-ovine mAb to CD45RA (clone 73B, mouse IgG1, (Mackay et al., 1990)) or the appropriate isotype control (mouse IgG1 or mouse IgG2a-FITC). The plate was then incubated at 4°C for 30 minutes and washed as described previously. Cells were re-suspended in 100µl of the secondary antibody (Goat anti-mouse IgG1-PE). The plate was incubated for 30 minutes at 4°C and washed as described previously before cells were re-suspended in 200µl PBS solutions and spun at 2000rpm for 1 minute. Cells were fixed by adding 200µl 1% PFA in PBS and incubating the plate at room temperature for 10 minutes before spinning at 2000rpm for 1 minute and re-suspension in 200µl PBS solution. After spinning at 2000rpm for 1 minute cells were re-suspended in 200µl permeabilisation solution (PBS + 0.2% saponin + 20% NRS) and incubated 4°C for 14-18 hours and spun at 2000rpm for 1 minute before resuspension goat anti-rat Foxp3 mAb conjugated to Alexaflour647 (goat anti-rat IgG2a-647). The plate then incubated for 1 hour at 4°C before washing as described previously. Cells were re-suspended in 200µl PBS solutions and spun at 2000rpm for 1 minute. Cells were fixed by adding 200µl 1% PFA in PBS and incubating the plate at room temperature for 10 minutes before spinning at 2000rpm for 1 minute and re-suspension in 200µl PBS solution. At this point the plate is covered and stored at 4°C until being read on a BD FACSCanto II©flowcytometer (BD Biosciences, San Jose, California, USA) within 48 hours.

Our single stain assays were performed within two months of returning from the field in late August 2011, but due to time taken optimising our multi-stain assays these were run seven months after fixation. Both sets of assays produce estimates of the proportion of CD4+ and CD8+, and to check that samples had not degraded by the time we ran our multi-stain assays we examined the correlation between these proportions. These were reasonably high, suggesting no meaningful degradation of the fixed samples had occurred: $r = 0.56$ for CD4+ and $r = 0.71$ for CD8+).

Flow cytometry data was analysed using FlowJo version X.0.7 analysis software. Proportions of T cells were measured by firstly placing a gate encompassing the entire lymphocyte population. T cell populations were then gated using specific CD4, CD8 or gamma-delta fluorescence, followed by appropriate sub-type gating based on CD45RA and Foxp3 fluorescence. Only samples with sufficient numbers of positive cells (>100) were included in analysis. Those with poor staining, poor cell profiles or low cell numbers were omitted from analysis (see table 2.1 for data available for each T cell subset).

2.3.3 *Data Analysis*

Available sample sizes varied among immune assays for various reasons, discussed above. A small number of extreme outliers were identified in the raw data, and because these lay outside what seemed a biologically plausible range based on previous studies (Egbe-Nwiyi et al., 2000; Holman, 1944; Nussey et al., 2012; Pisek et al., 2008), we excluded them from further analyses. This included 2 data points for NLR (both >6), 2 for eosinophil counts (>30%), 2 for CD8+ (>25% of lymphocytes) and one for CD4+Foxp3+ (>65% of CD4+ T cells). The final available sample sizes for each marker are presented in table 2.1.

The immune measures generally approximated a normal distribution and, in the few cases where skew was evident, our results were unchanged when we used log10 transformations and we therefore present results from untransformed data. All analyses were conducted in R version 3.1.3 (R Development Core Team, 2008). We estimated the Pearson's correlation coefficient for all pairs of immune markers, as well as exploring the dimensionality of the data using principal components analysis (PCA). Our further analyses, as well as previous studies (Nussey et al., 2012) suggested many of the markers differ among age groups and our PCA suggested a main axis of variation associated with age (see Results). Following previous demographic and immunological work on this population, we elected to consider

Table 2.1: Summary of total individuals sampled and numbers of samples passing quality control for each of the 11 immune parameters measured in this study.

Immune Marker	Total number sampled	Number of samples passing quality control		
		Male	Female	Total
Neutrophil:Lymphocyte	282	76	145	221
Eosinophil	282	76	146	222
CD4+	200	49	139	188
CD8+	200	48	129	177
CD4+ naïve	200	49	130	179
CD8+ naïve	200	49	130	179
γδ+ TcR	200	47	130	177
Treg	200	49	139	188
Anti-Tc IgA	287	94	187	281
Anti-Tc IgE	287	96	187	283
Anti-Tc IgG	287	96	185	281

discrete age groups of animals in our analyses (Coulson, 2001; Nussey et al., 2012). We examined variation among lambs, yearlings, adults (2-6 years) and geriatrics (>6 years). To capture covariation among markers within age groups, we re-ran our correlation analyses using residuals from a model of each marker including age group as a factor. We went on to test how each marker varied with age group and sex using separate linear models (LMs) including an age group-by-sex interaction. This interaction was removed from the model if found to be non-significant based on a likelihood ratio test, and the significance of the main effects of age group and sex were then tested.

We tested whether our immune measures predicted over-winter survival by fitting generalised linear models (GLMs) of survival as a binary variable (coded one for survivors, zero for animals that died) with a binomial error distribution and a logit link function. As previous studies have suggested that over-winter survival varies with age, sex, August weight and FEC, we initially examined the effects of these terms in our sample (Coulson, 2001; Gulland, 1992). We found evidence for a significant age-by-sex interaction ($X^2 = 9.84$, $P = 0.02$) with highest survival in female and adult

groups, as expected. We also found a significant positive association with August weight (X^2= 10.98, p = 0.001), but did not find a significant association with August FEC (X^2= 2.746, p=0.098) independent of weight. We therefore tested for associations among our immune markers and survival by adding them to a GLM including an age group-by-sex interaction and August weight, either separately, in groups (differential cell count data, FACS data, antibody data) or all together. When more than one immune variable was added to the model, the variable with the lowest likelihood ratio test (LRT) statistic upon removal from the model was sequentially deleted from the model until only terms significant at $p < 0.05$ remained. Each removed variables were then added back into the final model sequentially and the LRT was used to confirm that the markers omitted did not significantly increase the explanatory power of the model.

2.4 Results

There was evidence for some moderate correlations ($r > 0.4$) among the 11 immune markers, but these were predominantly driven by age-dependent variation in those markers (figure 2.1). The strongest positive associations were found among the two naïve T cell types (CD4+ and CD8+) and gamma-delta T cells on the one hand, and eosinophil counts and the three anti-*T. circ* antibody measures on the other, with negative correlations present between these two groups of markers (figure 2.1). The first axis of our PCA explained 35% of the overall variation, but subsequent axes all explained only 12% or less (table 2.2). The first PCA axis had heavy positive loadings from gamma-delta T cells, the two naïve T cell types, and negative loadings from eosinophil counts and the *T. circ* antibody measures (table 2.2). We found that the markers with positive loadings on PC1 declined strongly with age, whilst the markers with negative loadings increased with age (figure 2.2 and below), suggesting most of the observed correlation structure among the measures was associated with age. Supporting this, correlations among immune measures corrected for age differences (using residuals from models including age group as a factor) were considerably

reduced, although associations among the naïve T cells and gamma delta cells remained moderately high and positive within age groups ($r > 0.4$, figure 2.1).

All immune parameters except regulatory T cells varied significantly with age group, and three (naïve T helper,naïve T cytotoxic and gamma-delta T cells) differed between the sexes (figure 2.3 & 4, table 2.3). We found no evidence for age-by-sex interactions in any of the immune parameters (table 2.3). Eosinophil counts and anti-*T. circ* antibodies increased progressively from the lamb to adult age groups, whilst NLR and the proportion of CD8+ (cytotoxic) and CD4+ (helper) T cells were higher in adults and geriatrics than in lambs (table 2.3,figure 2.2). The proportion of naïve T cells in both helper and cytotoxic T cell subsets and the proportion of gamma-delta T cells showed a progressive decline across the age groups, and were also higher in females than males (figure 2.2 & figure 2.3, table 2.3).

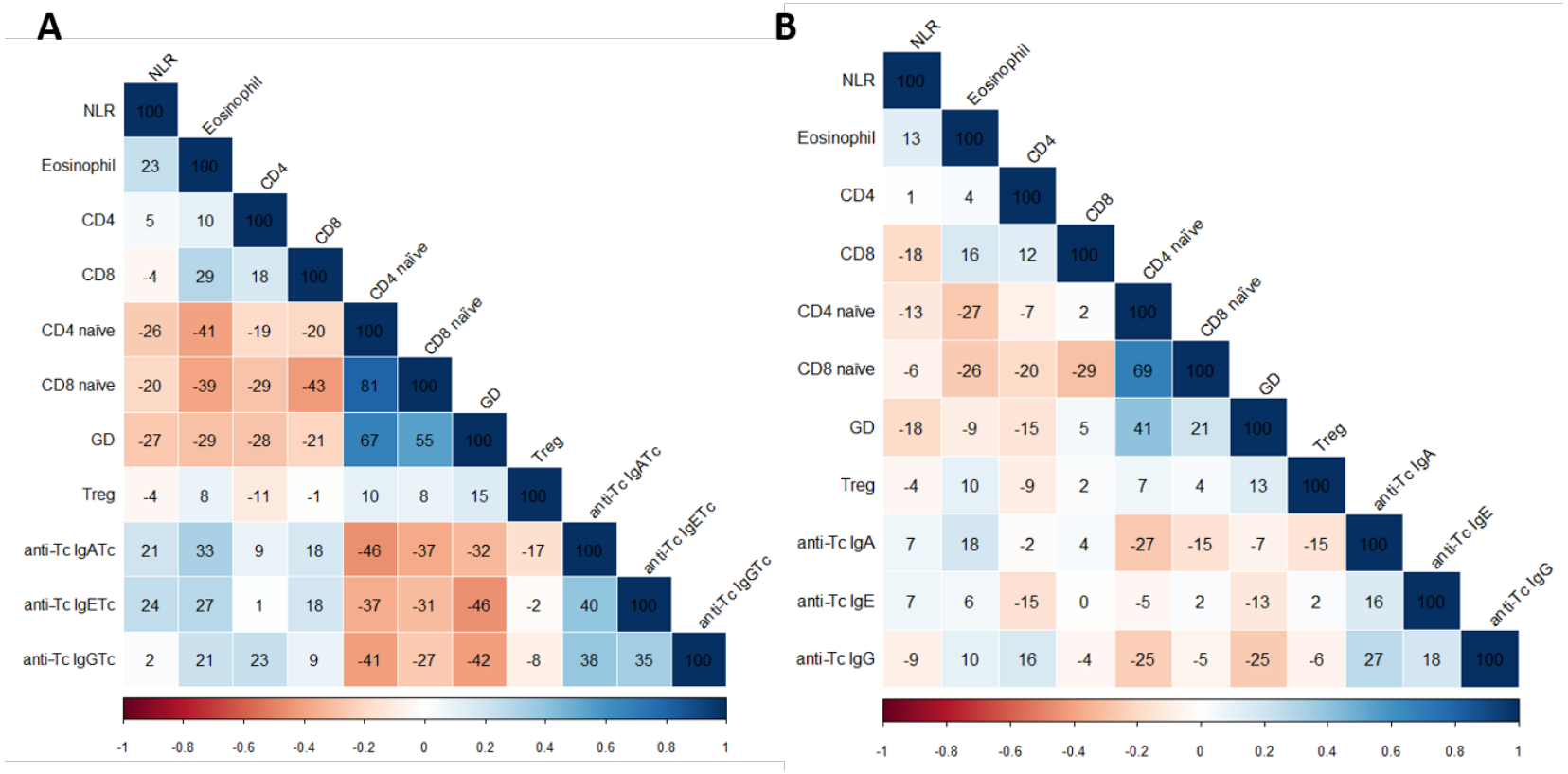

Figure 2.1: Correlation matrices showing pairwise Pearson's correlation coefficients among all 11 immune parameters. (A): raw data, (B): correlations among residuals from a model of the immune parameter including age group as a factor. The strength of each pairwise correlation coefficient is represented in the strength of the colour, which is blue for positive correlations and red for negative correlations. For ease of visualisation the number in the boxes have been *100. Abbreviations refer to neutrophil: lymphocyte ratio (NLR) and gamma delta T cells (GD).

Table 2.2: Principal component analysis of the 11 immune parameters measured in this study. The standard deviation and proportion of variance explained by each axis is shown in the upper table, and the loadings of each variable on each axis are displayed in the lower part of the table.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11
Standard deviation	1.962	1.1572	1.065	0.983	0.908	0.893	0.850	1.743	0.622	0.560	0.335
Proportion of variance	0.350	0.122	0.103	0.088	0.075	0.073	0.066	0.050	0.035	0.028	0.010
Cumulative proportion of variance	0.350	0.472	0.575	0.663	0.738	0.810	0.846	0.926	0.961	0.990	1.000
	Loadings										
Immune Marker	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**	**PC9**	**PC10**	**PC11**
γδ+ TcR	0.393	-0.118	0.182	-0.013	-0.080	0.178	-0.356	-0.014	-0.706	0.316	-0.187
CD4+	-0.145	0.329	-0.287	-0.684	-0.420	0.169	0.219	0.082	-0.223	-0.061	-0.083
CD4+naïve	0.447	0.041	0.088	-0.128	-0.297	0.030	-0.095	-0.309	0.152	-0.027	0.746
CD8+	-0.232	-0.371	0.206	-0.432	-0.206	-0.479	-0.931	-0.105	0.247	0.255	-0.136
CD8+ naïve	0.415	0.228	0.142	0.046	0.218	0.070	0.037	-0.426	0.352	-0.155	-0.608
Treg	0.061	-0.648	0.007	0.118	-0.461	0.412	0.221	0.306	0.171	-0.035	-0.065
Neutrophil:Lymphocyte	-0.175	0.071	-0.680	0.301	-0.306	0.034	-0.298	-0.237	0.061	0.336	-0.013
Eosinophil	-0.282	-0.326	-0.067	-0.222	0.335	0.413	-0.100	-0.599	-0.66	-0.204	0.016
Anti-Tc IgA	-0.314	0.236	0.275	0.178	-0.294	0.179	-0.566	0.176	-0.026	-0.256	0.036
Anti-Tc IgE	-0.306	-0.075	0.250	0.290	-0.365	-0.321	0.425	-0.405	-0.400	-0.100	0.040
Anti-Tc IgG	-0.305	0.309	0.456	0.047	-0.037	0.407	0.109	-0.033	0.204	0.612	0.070

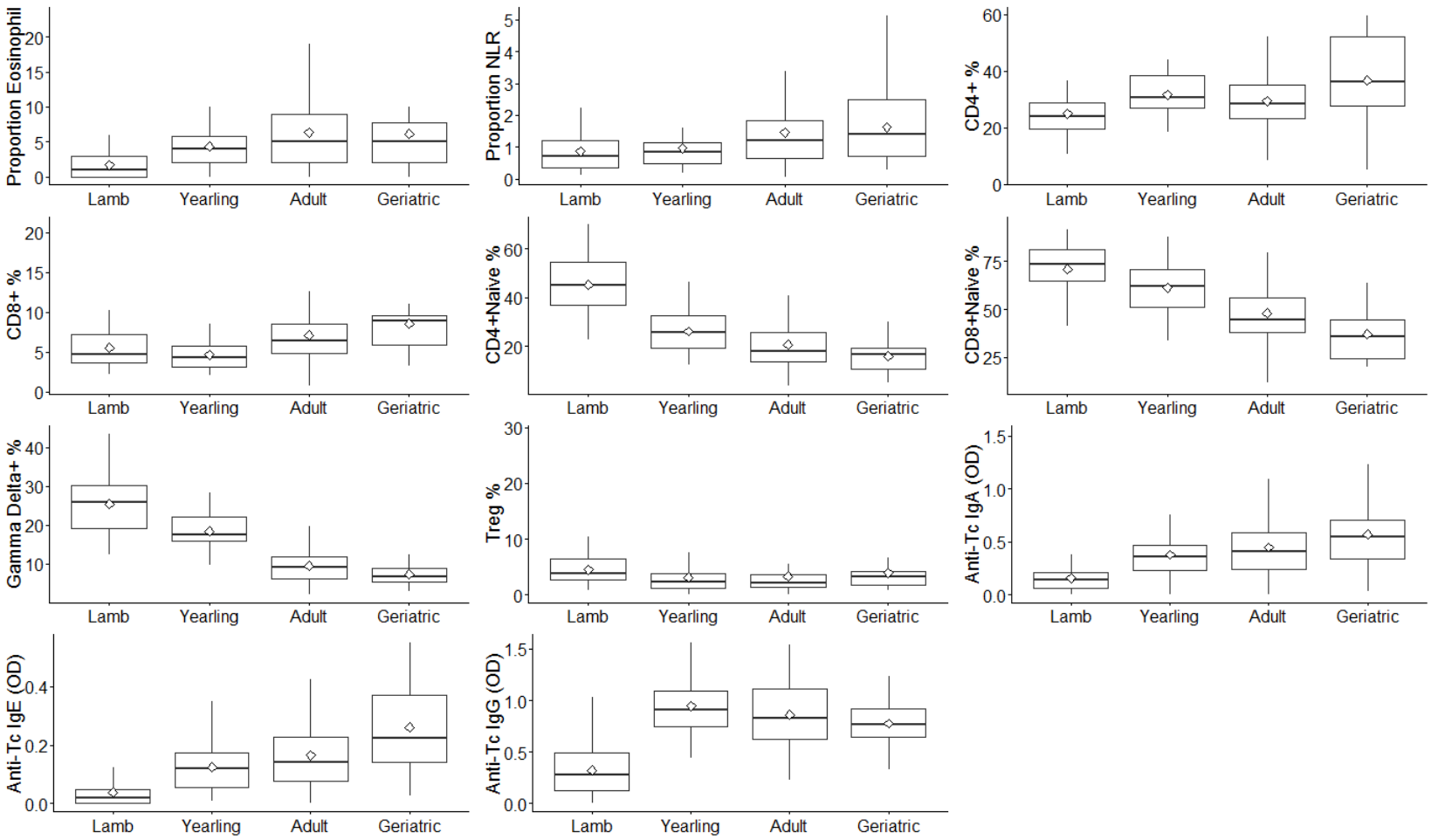

Figure 2.2: Variation in immune parameters among age groups. The mean for each age group is represented by a diamond shape within each box and the median by a black horizontal line. The box represents the inter-quartile range (IQR) and the whiskers show the highest and lowest values within 1.5*IQR.

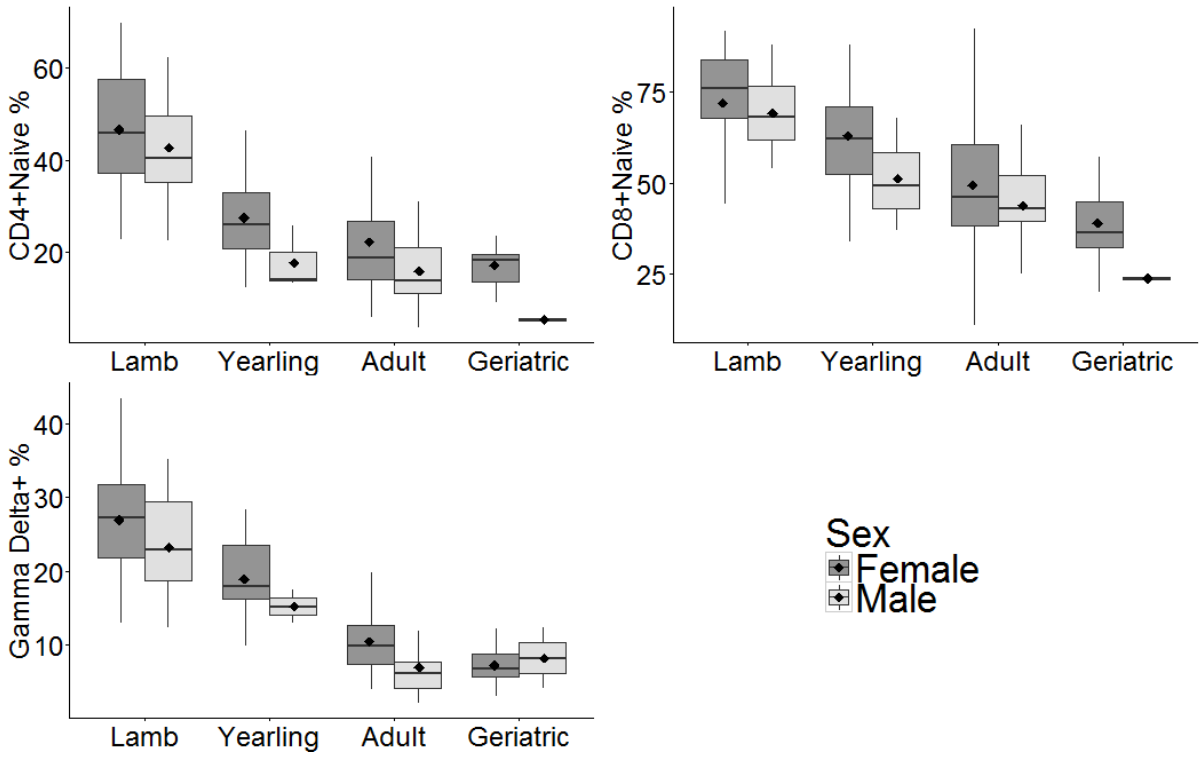

Figure 2.3: Sex differences observed in three of the 11 immune parameters: naïve T cytotoxic (CD8+), naïve T helper (CD4+) and gamma-delta T cells. In all three parameters shown females have higher proportions of each cell type than males, and this difference was found to be independent of age (see table 3). The mean for each age group is represented by a diamond shape within each box and the median by a black horizontal line. The box represents the inter-quartile range (IQR) and the whiskers show the highest and lowest values within 1.5*IQR.

Table 2.3: Results of linear models testing age and sex effects on each of the 11 immune parameters measured in this study. Global F tests of the significance of the age-by-sex interaction, and the age group and sex main effects are reported. Interactions between sex and age group were tested but were not significant in any case, so main effects of sex and age group are reported with the interaction dropped from the model. Estimated differences in the mean among age and sex groups are reported along with standard errors of differences in brackets, expressed as difference from the female lamb group. The R-squared value refers to the model with age and sex included as main effects only.

	Global tests			Post-hoc comparisons					
Immune Marker	**Age x Sex**	**Age**	**Sex**	**Intercept (female lambs)**	**vs. males**	**vs. yearlings**	**vs. adults**	**vs. geriatrics**	**r^2**
Neutrophil: Lymphocyte	2.213	8.343***	1.159	0.798 (0.126)	0.147 (0.137)	0.140 (0.228)	0.613 (0.144)	0.819 (0.217)	0.087
Eosinophil	0.461	17.346***	1.324	2.085 (0.560)	-0.699 (0.608)	2.392 (1.015)	4.512 (0.642)	4.111 (0.968)	0.201
CD4+	0.854	7.424***	0.088	25.185 (1.464)	-0.489 (1.647)	6.539 (2.563)	4.287 (1.671)	11.510 (2.535)	0.097
CD8+	0.117	7.806***	0.217	5.617 (0.464)	-0.246 (0.528)	-0.918 (0.811)	1.593 (0.535)	2.987 (0.882)	0.103
CD4+ Naive	0.440	72.022***	10.707**	47.392 (1.606)	-5.936 (1.814)	-20.426 (2.808)	-25.229 (1.847)	-30.845 (2.988)	0.544
CD8+ Naive	0.525	32.524***	4.462*	73.020 (2.296)	-5.480 (2.594)	-10.953 (4.016)	-23.673 (2.641)	-35.205 (4.274)	0.373
$\gamma\delta$+ TcR	0.401	112.87***	12.42***	26.796 (0.856)	-3.353 (0.951)	-7.928 (1.450)	-16.404 (0.970)	-19.180 (1.435)	0.655
Treg	0.742	1.221	0.004	4.0504 (0.588)	-0.042 (0.662)	-1.410 (1.013)	-1.170 (0.672)	-0.532 (1.035)	-0.002
Anti-Tc IgA	0.763	28.970***	0.508	0.168 (0.031)	-0.024 (0.034)	0.215 (0.0 60)	0.286 (0.035)	0.404 (0.054)	0.248
Anti-Tc IgE	2.311	44.195***	0.178	0.041 (0.012)	-0.006 (0.013)	0.085 (0.024)	0.127 (0.014)	0.221 (0022)	0.332
Anti-Tc IgG	0.664	82.609***	0.010	0.325 (0.032)	-0.003 (0.035)	0.625 (0.062)	0.537 (0.036)	0.452 (0.056)	0.480

The only immune measure which significantly predicted subsequent over-winter survival was anti-*T. circ* IgG levels. Individuals with higher levels of these antibodies were more likely to survive, independent of age group, sex and weight (table **??**, figure 2.4). There was no evidence of sex-by-immune measure interactions and none of the other immune parameters were significant when fitted alone in the survival GLMs (table 2.4). This result was unaffected when we included multiple immune parameters as either groups or altogether and simplified the GLMs: the only measure remaining in our simplified model was anti-*T. circ* IgG antibody level.

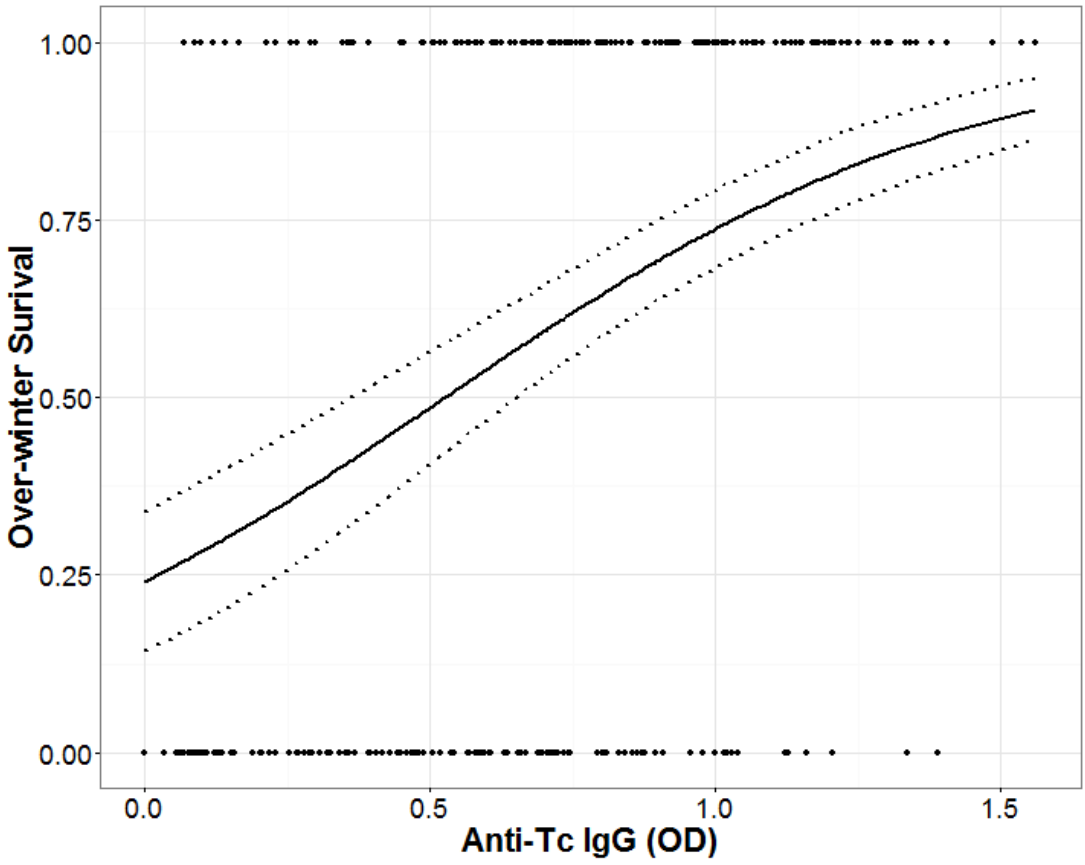

Figure 2.4: A logistic regression plot of the relationship between August anti-*T.circumcintca* IgG antibody levels and subsequent over winter survival (1 = survived, 0 = died). The points in the plots are the raw data and include all age groups and sexes combined. The black line is of the predicted values for adult females from the final model. The dotted lines show the standard error of these values.

Table 2.4: Generalised linear models of over-winter survival including an age-by-sex interaction and August weight (see Results section for details) with each immune parameter separated included and tested. A sex-by-immune parameter interaction was tested and dropped where non-significant, and the slope (with standard error in brackets) and likelihood ratio test statistic is reported for each immune parameter fitted separately.

Immune maker	Sex*Immune Marker X2	Immune b(s.e)	Marker X2
Neutrophil:Lymphocyte	0.244	-0.291 (0.172)	2.854
Eosinophil	0.319	-0.063 (0.037)	0.956
CD4+	1.775	-0.013 (0.018)	0.565
CD8+	0.927	-0.007 (0.055)	0.017
CD4 naive	0.690	0.015 (0.017)	0.848
CD8 naive	3.603	0.018 (0.012)	2.288
Gamma Delta	0.303	0.052 (0.035)	2.275
Foxp3 (Treg)	0.172	0.030 (0.044)	0.488
Anti-*T.circ* IgA	0.494	0.648 (0.550)	1.428
Anti-*T.circ* IgE	0.002	0.723 (1.354)	0.287
Anti-*T.circ* IgG	1.844	2.179 (0.577)	15.505***

2.5 Discussion

We have measured an unusually broad range of immune cell types and antibody isotypes in a wild vertebrate population, facilitated by the availability of reagents from veterinary immunology for our study species. Once the strong and expected age related changes in our immune measures had been accounted for, correlations amongst our 11 markers were weak. Other vertebrate studies measuring many immune markers have reported generally weak correlations and complex patterns of association between these measures and resistance or health outcomes (Banos et al., 2013; Buehler et al., 2011; Flori et al., 2011; Keil et al., 2001; Matson et al., 2006). This suggests that one or a few measures of immunity are unlikely to capture broad-scale variation in immune responsiveness and the ability to resist parasites, and raises the important question of which of the potentially huge range of immune markers are likely to be most relevant for ecological and evolutionary studies (Boughton et al., 2011; Demas et al., 2011). We found that the only immune marker to predict survival over the subsequent winter on St Kilda was the abundance of IgG antibodies against *T. circ*. The strong, positive association of this marker with survival affirms an identical result from a study of adult female Soays sampled across three previous crash years (Nussey et al., 2014). Below, we discuss the implications of the observed age and sex trends in our immune measures and offer potential reasons why this particular marker, and not others, was found to be predictive of survival.

The strong increases in antibody measures and declines in naïve T cell and gamma-delta T cell subsets with age observed are all consistent with patterns of normal immunological development. The development of immunity to GIN parasites including T. circ is thought to be antibody-mediated in sheep (Stear et al., 1996), and experimental studies show that antibody responses increase with age as the immune response develops (Nguyen, 1984; Smith et al., 1985; Watson et al., 1994). All individuals are exposed to these parasites from very early life on St Kilda (Clutton-Brock and Pemberton, 2004), so it is likely that the population-level increase in antibody levels reflects the steady development of the immune response to these

worms over the animal's first few years. Clear and progressive declines in the proportions of naïve T cells have previously been reported in this population from a smaller sample of animals (Nussey et al., 2012). We expected and observed that circulating proportions of naïve T cells declined with age as the supply of naïve cells reduces with thymic involution, and as the pool of naïve cells activate to become mature effector or memory T cells over time (Aspinall and Andrew, 2000; Cunningham et al., 2001). In young domestic ruminants the proportions of circulating gamma-delta T cells are relatively high, up to an order of magnitude greater than those seen in mice and humans, but decline sharply with age (Hein and Mackay, 1991), a pattern also previously observed in Soay sheep (Nussey et al., 2012). This is presumably the result of a drop in thymic output of these cells related to thymic involution, and their continuous removal from the circulatory system over time. Although within-individual immune development seems the most likely explanation for these patterns, our data are cross-sectional and so we cannot exclude the role of among-individual processes, such as annual variation in exposure to parasites or selective appearance or disappearance.

This is the first study to report sex differences in naïve and gamma-delta T cell sub-populations in a wild mammal. We found that females had higher proportions of these T cell sub-types than males, independent of age. Similar patterns have been observed in laboratory rodents and in humans (Caccamo et al., 2006; Pido-Lopez et al., 2001; Scotland et al., 2011). Human females have higher circulating levels of V$\gamma\delta$/Vδ2+ T cells, which are the major component of human peripheral gamma-delta T cells (Caccamo et al., 2006). When aged between 20- 62 years, women also have a higher thymic output than age-matched men, although the absolute number of T cells did not differ between the sexes (Pido-Lopez et al., 2001). Sex hormones have been shown to affect the rate and maintenance of thymic output in humans (Ansar Ahmed et al., 1985; Dumont-Lagace et al., 2015), resulting in higher thymic output in adult females than males (Gui et al., 2012), and could be behind the sex difference in naïve and gamma-delta T cells in Soay sheep. It is interesting to note that, as in humans and many other polygynous mammals, Soay sheep females have lower annual mortality and longer lifespan than males (Clutton-Brock and Pemberton, 2004). Conceivably,

reduced thymic output in males could reflect weaker selection to maintain certain aspects of immune function into later adulthood compared to longer-lived females. Whether there is any fitness cost of reduced thymic output and whether the cost differs between the sexes remain interesting questions for further study, although in our cross-sectional data there was no association during adulthood between naïve or gamma-delta T cell proportions and survival in either sex.

Given the prediction that robust immune defences are essential for survival in parasite-rich natural environments, why did only one of our eleven immune parameters significantly predicted over-winter survival? What do levels of circulating anti-*T. circ* IgG antibodies tell us about host immunity in this population that our other immune parameters do not? In our study population, gastro-intestinal nematodes appear to represent the major parasite pressure (Craig et al., 2006; Graham et al., 2016). Anti-*T. circ* antibodies measure levels of circulating immunoglobulins with known effector function against a specific ecologically-important parasite, as opposed to our other measures which characterise the relative proportions of functionally distinct immune cell types. These latter measures do not differentiate which, if any, parasites these different cells are reacting to, nor how well they are functioning in that capacity. Furthermore, infection is associated with the sequestration of immune cells from circulation to sites of infection. While nematode-specific antibodies in blood correlate with mucosal antibody activity at the site of infection in sheep ((Prada Jiménez de Cisneros et al., 2014), it is not clear whether the proportions of different leukocytes measured here reflect only the available immune cell selection pool rather than the mucosal effector population.

Finally, we have previously found that plasma levels of anti-*T. circ* IgG, but not IgA or IgE, predicted over-winter survival in adult female Soays (Nussey et al., 2014), and the present study confirms this finding, showing it to be age- and sex-independent. Despite IgA and IgE isotypes having well-documented roles in the development of resistance to GIN parasites in young domestic sheep (Schallig, 2000), they have short half-lives in circulation relative to IgG and may be present in plasma at high levels only during acute infections (Manz et al., 2004). IgG may therefore

represent a more temporally-stable measure of anti-GIN immunity in blood than other isotypes, a suggestion supported by the presence of significant repeatability within individuals across years of a pan-isotype anti-*T. circ* antibody measure, which would have predominantly measured IgG due to its abundance in plasma (Hayward et al., 2014). These findings add weight to our previous suggestion that anti-*T. circ* IgG levels in summer may provide a repeatable indicator of an individual's ability to cope with established GIN infections, and therefore predict their ability to survive the interacting nutritional, thermoregulatory and parasitological challenges of a 'crash' winter on St Kilda (Nussey et al., 2014). Overall, our data suggest that immune measures which capture among-individual variation in responses to ecologically-relevant parasites and are repeatable within individuals over time may offer the clearest insights into the relationship between immunity and fitness under natural conditions.

Our findings provide novel insight into patterns of variation across immune cell types in a wild vertebrate. Although we could find no association between any T cell sub-populations and over-winter survival, ours is a relatively small and cross-sectional data set and much further work is required to determine if and how measures of T cell phenotype and function relate to fitness under natural conditions. It may be that the T cell phenotypes used in this study were too broad or inappropriate to detect ecologically important relationships. For example, it may be that total numbers of each T cell subset or their antigen-specificity, are more relevant to survival and fitness than the proportion of the T cell subsets within the peripheral lymphocyte pool. Studies of T cell function in response to ecologically-relevant stimulus ex vivo have been conducted in domestic ruminants (McNeilly et al., 2009) and wild mammals (Beirne et al., 2016), and could be an important indicator of immune function in natural systems. It is also worth noting that survival is only one aspect of lifetime fitness and our measures were taken several months before the onset of winter when the animals actually die. Although a longitudinal study in cattle suggests that many of the T cell measures we identified are highly repeatable within individuals (Banos et al., 2013), this remains to be established under natural conditions. Our data is cross-sectional and it may be that longitudinal changes in some of these parameters

(i.e. year-on-year decline within the lifetime of an individual), rather than measures at a single point in time, are most informative in terms of the individual's immune health and fitness. Longitudinal studies incorporating a wide range of immune parameters and linking them to health or fitness remain rare (although see (Banos et al., 2013; Keil et al., 2001)), but are very important for developing our understanding of how immunity and fitness relate to one another over entire lifetimes.

Chapter 3

Associations among immunity, growth, parasite burden and over-winter survival during early life in a wild mammal

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3.1 Abstract

Early life is an important time for growth and immune development, when juveniles face high resource demands and are under strong selection for survival. The development of immunity against prevalent parasites is expected to be crucial for survival under natural conditions. However, since resources are limited, investment in an immune response could come at a cost to growth or survival. Trade-offs between growth and immune function have been shown in experimental studies, but are less well understood in natural conditions. This study will explore how immune variation during early life is associated with key life history traits, including growth, parasite load, male-specific sexual investment and survival. We use data collected from a long-term study of free-living Soay sheep on St.Kilda to test whether there is evidence of trade-offs between immunity and growth, parasite load and survival in the wild. We measured leukocyte subtypes and parasite specific antibodies (IgA, IgE, and IgG) against a prevalent gastrointestinal nematode, *Teladorsagia circumcincta (T. circ)*, in samples collected over a 4-year period on St Kilda. Our data suggest that the costs of immunity on growth rate in early life are dependent on the immune marker applied under natural conditions. Anti- *T. circ* IgE and anti- *T. circ* IgG measures were highest at moderate growth rates, whereas the inflammatory marker NLR was highest in lambs with the slowest growth rates. Anti- *T. circ* IgA and anti- *T. circ* IgG were negatively associated with Strongyle faecal egg counts, suggesting a protective role of these specific antibody-mediated immune measures. Despite no significant relationship between immunity and secondary sexual characteristics, we report a sex specific association with immunity and survival; males with higher anti- *T. circ* IgE had reduced first year survival. This study highlights both the complexity of juvenile immunity and the variation in associations between life history traits and different measures of immunity in the wild.

3.2 Introduction

Early life is an important time for growth and immune development (Lindström, 1999), when selection via survival is high. While the immune response provides important protection against parasites, mounting this response can be costly and may damage the host. Similarly, due to competition for finite resources, investment in other life history components can reduce immunocompetence (Belloni et al., 2010; Guerreiro et al., 2012; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Schmid-Hempel, 2003). Therefore, in resource limited wild systems, we would expect trade-offs to occur, and that immunity might come at a cost to growth (Møller et al., 2009). Due to variation in optimal life history investment strategies, the rate and pattern of growth and immunity varies between individuals, populations and species (Calero-Riestra and García, 2016). In bird species in particular, studies have shown that growth tends to be prioritised over immune investment under limited resources and that there is reduced weight gain in poultry (Demas et al., 1997; Van Der Most et al., 2011) and in zebra finches (*Taeniopygia guttata*) (Deerenberg et al., 1997), indicating the costs of initiating an immune response and its impact on growth. In barn swallows (*Hirundo rustica*), increased parasite burden led to an increased immune response, which was related to a reduction in condition, but an increase in feather growth (Saino et al., 1998), demonstrating the complex relationship between parasites, immunity and growth, especially in early life. In amphibian species that have faster growth rates, there is also a higher rate of infection and increased pathology compared to slow growing species (Johnson et al., 2012). In addition, a number of studies in invertebrates have shown that an artificially elevated immune response can have a negative effect on development, growth and survival (Schmid-Hempel, 2003). Correlative studies in domestic sheep have shown that high parasite burden is related to reduced weight, and that this relationship might have a genetic component (Bishop and Stear, 2001; Greer, 2008; Morris et al., 2005). Despite evidence for links between growth, immunity and survival in laboratory experiments and studies of livestock, understanding of associations in natural conditions remains limited.

Due to condition dependant costs and compensatory resource acquisition, it can be difficult to detect the costs of immunity in the wild (Schmid-Hempel, 2003). Environmental and seasonal variation in resource availability, as well as individual variation in resource acquisition, can result in positive correlations between traits, when we might expect to see negative correlations indicative of trade-offs (van Noordwijk and de Jong, 1986). Where the costs of a trait are condition dependant, it is possible that individuals are able to compensate under good conditions, which would make fitness trade-offs difficult to identify (Merilä and Svensson, 1997; Råberg et al., 2000; Schmid-Hempel, 2003). This is supported by research, which shows that a relationship between traits, such as growth or survival, and immunity is only detected under restricted resources (Alonso-Alvarez and Tella, 2001; Moret and Schmid-Hempel, 2000). In a lab environment, it is much more feasible to control and measure resource availability and acquisition at an individual level, which can make specific trade-offs easier to identify. However, these studies occur in a controlled environment, using individuals with restricted genetic variance, under single infections, and with supplemented resources. In order to measure fitness costs of trade-offs, it is important to test host-parasite theory in natural environment with genetic variability (Bradley and Jackson, 2008; Hayward et al., 2014; Pedersen and Babayan, 2011). There is generally a lack of evidence to support the link between immune function and fitness in ecological contexts with natural infection and genetic variability (Demas et al., 2011; Norris and Evans, 2000; Sheldon and Verhulst, 1996). Longitudinal studies of wild vertebrates that can link immunity, infection and life history traits are therefore important to better understand the fitness consequences of immune variation (Bradley and Jackson, 2008; Hayward et al., 2014; Pedersen and Babayan, 2011).

Sexual dimorphism is common in species with polygynous mating systems, where males are generally under greater sexual selection, and are usually the larger, more aggressive sex that competes for mating opportunities (Moore and Wilson, 2002; Zuk, 1990). Larger male size has been shown to correlate with reproductive success (Andersson, 1994), although there are also costs, including higher rates of parasitism and reduced survival in comparison to females conspecifics (Moore and

Wilson, 2002; Promislow, 1992; Zuk, 1990). There are sex differences in parasitic prevalence, with males tending to have higher burdens than females, although this is not universal across species (McCurdy et al., 1998; Schalk et al., 1997). In vertebrates, males tend to have a lower immune response and a reduced resistance to parasites than females (Møller et al., 1999, 1998). These sex-specific costs could be due to both a direct impact of higher growth using more resources and leaving less for immune function, or to the physiological actions of sex specific hormones (Folstad and Karter, 1992; Moore and Wilson, 2002; Zuk, 1990). Sex-specific somatic costs of increased parasitic infection have been shown in a study of Tawny pipits *Anthus campestris* during early life; male nestlings that were infected with avian malaria parasites had a lower daily mass gain than females with the same infection status (Calero-Riestra and García, 2016). Hormones can be both immunosuppressive and beneficial to successful reproductiom, which could be due to the influence of the mating system on the relationship between immunity, sexual traits and hormones (Klein, 2000). Testosterone can supress both humoral and cell-mediated immunity, whereas oestrogen, whilst also supressing cell-mediated immunity, has been shown to increase humoral immunity (Grossman, 1985). Therefore, due to sex-specific costs driven by sexual selection, we might expect that types of immune response might vary between the sexes.

Secondary sexual characteristics are important to reproductive success in many species, but they are expected to be costly (Grafen, 1990; Nur and Hasson, 1984; Zahavi, 1977). The “immunocompetence handicap” hypothesis proposes that sex specific traits, for instance male weaponry, are an indicator of quality due to the incurred cost of investment, which must be absorbed by the individual. For example, increased testosterone production leads to good quality sexual characteristics, but comes at a cost of immunosuppression (Folstad and Karter, 1992; O’Neal and M, 2013; Roberts et al., 2004). On the other hand, high immunity supresses testosterone production which can result in reduced sexual characteristics (Kilpimaa et al., 2004; Peters et al., 2004). So we could expect to see a trade-off between investment in sexual characteristics and immune protection against parasites. However, there is evidence to the contrary in birds in cases where males with higher testosterone have

increased dominance and greater access to resources, which enables them to afford adequate immune investment to resist parasites (Poiani et al., 2000). Within the immune system there is also variation in relation to sexual characteristics; higher testosterone is associated with an increase in the inflammatory response and a reduction in humoral immunity (Kilpimaa et al., 2004; Peters et al., 2004), which suggests that there could be a cost of adaptive immunity. If this is the case then high immune function in early life could have consequences on life history traits in later life, which makes the trade-offs occurring within the initial months of life important for long term fitness.

A number of studies have shown that high investment in immunity can be beneficial to survival (Hegemann et al., 2013; Hõrak et al., 1999; Nussey et al., 2014). Individuals, which can mount a strong immune response are better able to protect themselves from parasites and so are more likely to survive. However, this positive relationship between immunity and survival is not universal, and other studies have found a survival cost of immunity. In common eiders (*Somateria mollissima*) the cost of initiating a humoral immune response following immunisation was shown to considerably impair long-term survival (Hanssen et al. 2004). Similarly, in invertebrates, an immune challenge reduced survival under conditions when resources were limited (Moret and Schmid-Hempel, 2000). While an immune response can be beneficial to survival, there may also be circumstances where a maximal response can be detrimental, due to immunopathology or limited resource availability (Råberg et al., 1998; Sheldon and Verhulst, 1996; Westneat and Birkhead, 1998). In blue tits (*Parsus major*), individuals with an intermediate parasite burden had the greatest fitness benefit, whereas survival was lower in those with high or low parasite loads (Råberg and Stjernman, 2003). In another study, Råberg et al. (2003) found that individuals with intermediate immune responses to diptheria were more likely to survive, as immune responsiveness was under stabilising selection. However, this study also showed that secondary immune responsiveness to tetanus was under positive selection, so that high antibody production had a negative effect on selection. Thus, there is contrasting evidence on the impact of immunity on survival in the wild, with relationships being dependent upon condition or resource availability.

In domestic sheep, resistance against gastrointestinal nematodes (GIN) causes an estimated loss of 15% productivity due to demands on metabolism and resources (Greer, 2008; McRae et al., 2015). Repairing damage and mounting an immune response against GIN places a strain on host resources, which in addition to pathologically induced anorexia, can lead to a loss of weight (Greer, 2008; Stear et al., 2003). Acquired immunity is a major form of defence against GIN, although this takes time to develop. In particular resistance to *Teladorsagia circumcintca (T. circ)*, an extremely prevalent GIN is slower to develop than that against other GIN species in lambs (Bishop et al., 1996). Age determines capacity to resist infection (Merino, 2010), and in sheep, it is during their first grazing season that immune naïve individuals are particularly at risk of parasitic infection (Stear et al., 1999). Production is an important focus of domestic farming and when worms are removed, through the use of anthelminthics, growth is found to significantly increase (Coop et al., 1982; Sykes and Greer, 2003).

Isotypes IgA and IgG are both associated with supressing GIN development and egg production, but IgG is the primary antibody isotype in circulation, whereas IgA is the main mucosal antibody isotype (Halliday et al., 2007; Stear et al., 2004; Strain et al., 2002). The IgE antibody isotype is also a mucosal antibody, but is a more aggressive response associated with parasite expulsion (Huntley et al., 2001; Murphy et al., 2010). Parasite-specific antibody mediated immunity provides an important element of defence against GIN, but there are also cells of the innate immune system which are active in resisting GIN. Neutrophils have been shown to prevent establishment of some nematode species (Bowdridge et al., 2015), and eosinophils are thought to be responsible for damaging larval stages of the parasite to prevent establishment (Meeusen and Balic, 2000; Stear et al., 1995). High numbers of neutrophils relative to the number of lymphocytes is an indicator of chronic tissue inflammation (Wakelin 1996; Saino et al. 2000) and has been linked to elevated glucocorticoids and stress across a wide range of taxa (Davis et al., 2008; Maxwell, 1993; Puppe et al., 1997). Eosinophils are also markers of tissue inflammation and have been shown to be influential in nematode resistance, specifically against *T. circ* in blackface lambs at three months (Stear et al., 2002). While much is known about

immunity to GIN parasites in domestic sheep that are fed and regularly treated with anthelmintic drugs, relatively little is known about relationships among growth, immune markers and parasite burden in ruminants experiencing natural infection conditions.

Here, data was collected as part of a longitudinal long-term study on a population of free-living Soay sheep (*Ovis aries*) on the island on Hirta within the remote St Kilda archipelago. Specifically, this project monitors sheep residing in the Village Bay area of Hirta, which have been the focus of long-term individual-based study since 1985 (Clutton-Brock and Pemberton, 2004). In Soay sheep, males are shorter lived and larger in size than females, and body mass is associated with increased fitness in both sexes (Clutton-Brock and Pemberton, 2004; Milner et al., 1999). In addition, two male sexual characteristics, horn length and testes diameter, have been shown to correlate with testosterone in this population (Preston et al., 2011). Horns are an important sexual trait in males in this population, particularly as they are grown incrementally each year and not shed seasonally. Horn type is determined by a genetic polymorphism, which has been identified and linked to increased reproduction and reduced survival in the case of males with the normal horn type (Johnston et al., 2013). In males, larger horns are associated with increased reproductive success, but reduced longevity (Robinson et al., 2006). High mortality winters are associated with strong selection on a range of phenotypic traits, and are thought to result from a combination of low food availability due to competition, harsh winter climate conditions and parasite pressure, predominantly from Strongyle GINs (Coulson, 2001; Gulland, 1992). *T. circ* is a prevalent GIN in sheep, and in this study population has been shown to have an impact on reproduction and survival (Coulson, 2001; Craig et al., 2006; Gulland, 1992; Hayward et al., 2011; Wilson et al., 2004). A genetic link has been demonstrated in a relationship between faecal egg counts (FEC) and a microsatellite polymorphism, as well as a relationship between reduced FEC and *T. circ* -specific IgA, which illustrates a link between measures of parasite burden and cell-mediated immune response (Coltman et al., 2001). There are also differences in selection pressures between the sexes; in males, weight is an important predictor of reproductive success, whereas in females age is a

key predictor (Hayward, 2013; Preston et al., 2003). We might expect any relationships between immunity and other life history traits to vary between the sexes, due to these sex-specific differences in selection pressure. Previous research has shown that GIN parasite burden is predominantly highest in lambs, particularly males (Hayward et al., 2011), and is a predictor of first winter survival, but it is not yet known whether immunity against GIN differs between the sexes.

We measured two different types of immune marker representing both innate and adaptive immunity, and including parasite specific markers. Our study will capture immune measures at 3-4 months of age, once immunity has begun to develop, at around 2-3 months (McRae et al 2015). These included white blood cells; Neutrophil:Lymphocyte ratio (NLR) and eosinophils, and three antibody isotypes specific to larval stage 3 (L3) of anti-*T. circ* antibodies; IgA, IgE and IgG. Previously these antibodies have been linked to other life history traits and parasite burden in this population (Graham et al., 2010; Hayward et al., 2014), particularly anti- *T. circ* IgG which has been a positive predictor of survival across multiple years and age groups, demonstrated in Chapter 1 and by Nussey et al. (2014). However, how immune measures are related to early life traits is not yet fully understood in this system. Using a four-year data set and the aforementioned five immune markers we tested whether immunity varies in relation to (i) growth rate, (ii) parasite burden, (iii) secondary sexual characteristics, and (iv) survival, and whether these relationships were consistent between the sexes.

3.3 Materials and methods

3.3.1 *Study system & field data collection*

As part of the long-term study of Soay sheep resident to Village Bay, each spring, lambs are caught and marked shortly after birth. Every summer during a two-week

period in August, sheep are rounded up, caught and processed in a series of corral traps set up in the Village Bay area. Upon capture each individual was weighed, horn length and testes diameter was measured and then blood and faecal samples were collected. The survival of individuals is also monitored throughout the winter period. Horns are an important sexual trait in males in this population, of which there are two male horn types determined by a genetic polymorphism (Johnston et al. 2013). Roughly 85% of males have the normal horn type, which grow in a spiral shape and have a strong bony core (Clutton-Brock and Pemberton, 2004). The remaining 15% of males have 'scurred' horns, which do not grow in the usual spiral shape, are often irregular, non-symmetrical and lack a bony core (Clutton-Brock and Pemberton, 2004). The horn length data used in this study is restricted to males of normal horn type. This Village Bay population exhibits a distinctive, cyclical dynamic characterised by low and rising sheep numbers followed by high mortality ('crash') winters in which more than half of the population may die (Clutton-Brock and Pemberton, 2004).

The data for this study were collected in August every year from 2011 to 2014 during the Soay sheep research project's annual summer catch. Sample sizes for each year were as follows: 2011 n=99 (males=50, females=49), 2012 n=55 (males=23, females=32), 2013 n=116 (males=56, females=60) , 2014 n=102 (males=55, females=47). A 9ml Li-heparin vacutainer tube of whole blood was taken for each individual and stored at 4°C after collection. The whole blood was then processed to extract plasma for antibody analysis in the field within 24 hours. Faecal samples were taken on capture for Strongyle parasite faecal egg counts (FEC) performed using a modification of the McMaster technique (Ministry of Agriculture Fisheries and Food, 1971). From observational and census data taken between October-November and February-May we were able to identify which individuals survived the over-winter period. Across the four-year study period a total of 231 (62.1%) of the sampled lambs died over their first winter (before May of the year after birth). The survival rates over-winter were highly variable between years, especially as our data set included a 'crash' year during winter 2011-12, and our data set exclusively focuses on lambs, which are particularly vulnerable to reduced over-winter survival. For lambs in this

study, the over-winter death rates were as follows: 2011 (93.9%), 2012 (7.3%), 2013 (66.9%), 2014 (53.9%).

3.3.2 *Laboratory methods*

3.3.2.1 *Differential white blood cell counts*

Within 12 hours of collection 5µl of whole blood was applied on to one end of a standard glass microscope slide from 345 of the sampled lambs (144 males, 147 females). The drop of blood was then spread at a 45° angle and smeared evenly across the slide. Slides were air dried overnight and stained using a Quick-Diff Kit stain (Gentaur) the following day as per manufacturer's instructions. Lymphocytes, eosinophils and neutrophils were counted at 40x magnification using the Battlement Track method (Bain, 2008). Only slides with a clear regular monolayer of cells were counted. Slides with uneven cell density or unclear staining were omitted from analysis: 291 out of the 345 slides were deemed of acceptable quality for analysis. In wild populations high proportions of neutrophils have been used as a measure of infection and low lymphocyte counts to indicate reduced immune investment or poor condition (Bennett et al., 2013). In this study we have combined the two measures as a neutrophil/lymphocyte ratio (NLR). High NLR is used in human research as a marker of systemic inflammation, which can precede a number of prevalent chronic conditions (Imtiaz et al., 2012). The correlation coefficients for a sample of 25 slides which were recounted twice and analysed using Spearman rank correlation where r=0.70 for NLR and r=0.65 for Eosinophils.

3.3.2.2 *Anti-T.circumcintca Antibodies*

Within 24 h of collection, one Vacutainer of whole blood was centrifuged at 1008g for 10 min and the plasma layer removed and stored at -20°C. We then followed

previously published methods to measure levels of IgE, IgA and IgG antibodies binding larval stage 3 antigens from (*T. circ*), a highly prevalent gastrointestinal Strongyle nematode parasite of Soay sheep on Hirta (Nussey et al., 2014).Samples were analysed using the same protocol and procedure as in (Nussey et al., 2014) with minor adaptations. We used Tc L3 somatic antigen, diluted to 2µg per ml of 0.06M Carbonate buffer at pH 9.6. L3 somatic antigen was prepared by re-suspending *T. circ* L3 in PBS (5 x 105 larvae per ml) in Lysing Matrix D tubes (MP Biomedicals) and homogenising in a Precellys©24 tissue homogeniser. Debris was pelleted by centrifugation at 16, 000 x g at 4°C and the somatic antigen containing supernatant stored at -80°C prior to use. Total protein concentration of the L3 antigen preparation was estimated using a Pierce™ BCA Protein Assay Kit (Thermo Scientific).

In each assay, 50µl of appropriately diluted antigen solution was added to each well of a Nunc immuno 96-microwell plate, which was subsequently covered and incubated overnight at 4°C. The wells were then washed three times in Tris-buffered saline-Tween (TBST) using a plate washer. Then 50µl of an appropriately diluted Soay sheep plasma sample was added to each well. Sample dilutions used (adapted from optimisation procedure described by (Nussey et al., 2014)) were as follows: anti- *T. circ* IgA: 1:50; anti- *T. circ* IgG: 1:12800; anti -*T. circ* IgE: 1:50.

The plates were then covered and incubated at 37°C for 1 hour and then washed five times with TBST. For the anti-*T. circ* IgA & anti-*T. circ* IgG assays 50µl per well of the appropriate rabbit anti-sheep detection antibody conjugated to horseradish peroxidise (HRP) was added (anti-ovine IgA-HRP diluted 1 µl in 8mls in TBST, anti-ovine IgG-HRP diluted 0.5 µl in 8mls in TBST: all AbD Serotec, cataologue numbers: AHP949P and 5184-2504, respectively). For the anti-Tc IgE assay50µl of anti-ovine IgE (mouse monoclonal IgG1, clone 2F1, [2]) diluted 1:100 in TBST was added to each well, followed by 1 hour incubation at 37°C, five washes with TBST and then the addition of 50µl of goat anti-mouse IgG1-HRP detection antibody (AbD Serotec catalogue number: STAR132P), diluted to1µg in 8000µl of TBST to each well. All plates were then covered and incubated at 37°C for 1 hour. They were then washed five times with TBST and 100µl of SureBlue TMB 1-Component microwell

peroxidase substrate (KPL) was added per well and then left to incubate for 5 minutes in the dark, in a cardboard box, at 37°C. Reactions were then stopped by adding 100µl 1M HCl and optical densities (ODs) were read immediately at 450nm using a Thermo Scientific Multiskan GO Spectrophotometer.

Each assay on each selected Soay sheep plasma sample was performed twice on separate ELISA plates. On each plate we also included four sample-free wells as a duplicate negative control (TBST: 200ml of 10xTrisBuffered Saline in 1800mls distilled water with 1ml Tween 20) and a duplicate positive control (Moredun Research Institute (MRI) positive sample: purified lymph from Tc infected sheep). We excluded samples across all assays for which there was obviously poor correspondence across duplicate OD scores, presumably due to human error (n=2 Anti-*T. circ* IgA, n=1 Anti-*T. circ* IgG). We then checked the correlation of ODs across duplicate plates and re-ran both plates if $r < 0.80$. For subsequent analyses, we took the average OD across the duplicate runs minus the average of the two negative control well ODs across the two plates as our assay measure.

3.3.3 *Data Analysis*

NLR, anti- *T. circ* IgA and anti- *T. circ* IgG all showed a left skewed distribution which was normalised using a log10 + 0.1 transformation. Anti- *T. circ* IgE and eosinophil counts were more severely left-skewed and could not be readily transformed to normal. All analyses were conducted in R version 3.2.3 (R Core Team 2015).

To test how growth rate was associated with our markers of immunity we ran separate linear mixed-effects models (LMMs) for each individual measure of immunity. We began by starting with a base fixed effects model containing age in days at August capture, sex (two-level factor), year (four-level factor), twin or singleton (two-level factor) and birthweight (calculated by subtracting a growth rate

of 108g/day multiplied by capture age (Clutton-Brock and Pemberton, 2004; Robertson et al., 1992)). To this base model we added a two-way growth rate-by-sex interaction, with growth rate included as a quadratic term. We then performed model simplification by removing non-significant terms, based on a likelihood ratio test, starting with the two way interactions first, then growth rate as a quadratic term and finally the main effect of growth rate. Growth rate was measured as the change in weight per day of each individual, by subtracting birthweight from August weight and dividing by age in days. We included maternal identity as a random effect to account for repeated measures of offspring from the same mother (201 mothers gave birth to the 241 lambs in our data set). Log-transformed anti- *T. circ* IgA and IgG and NLR were modelled as normal within a linear mixed-effects model using the package lme4 (Bates et al., 2015). For anti- *T. circ* IgE and eosinophil count, we compared the fit of generalised linear mixed models (GLMMs) including the base model fixed terms and mother as random incorporating a Gaussian, Poisson, zero-inflated Poisson or negative binomial error structure in the package glmmADMB (Skaug et al., 2016) using Akaike's information criteria (AIC). To run the Poisson and negative binomial models, we first had to convert anti- *T. circ* IgE to an integer, by multiplying by 100 and then rounding to the nearest whole number. The negative binomial models fitted the data considerably better in both cases (Δ AIC greater than 10 units lower than any other model), so we fitted this error structure in the following analyses of those markers.

We then investigated whether our immune markers were predictive of FEC and two male specific secondary characteristics; horn length and testes circumference. These three measures were fitted as response variables in separate LMMs with Gaussian distributions. FEC was left skewed and was normalised using a log10+100 transformation. For each model we used the same initial method as above, starting with a base model which included sex, age, year, twin, growth rate, and birth weight as fixed effects and maternal identity as a random effect. The exception being that the models of male secondary characteristics did not include sex as a fixed effect as the data only comprised of males (n=184). Additionally, in the horn length models the data was further reduced to include only males with normal horns (n=167). For each

of the three traits, we added each immune marker in turn, firstly in two way marker-by-sex interaction, with the immune term included as a quadratic term. Then we simplified the model, as above, by removing non-significant terms, based on a likelihood ratio test, starting with the two-way interaction with sex, then the quadratic term and finally the main effect. After running separate models with a single immune marker in each model we compiled a final model containing all immune markers which were significant when fitted alone, to test whether they independently explained variation in the three traits.

To assess whether August immunity is predictive of over winter survival we used GLMMs with a binomial distribution, with survival as a binary variable (coded one for survival, zero for animals that died). As there were very few individuals that survived in 2011 (n=6) and very few that died in 2012 (n=4) the sample sizes for comparison between those that survived and those that did not in those two years are too small. Therefore, we conducted the survival analysis using only the years that were informative in terms of survival; 2013 and 2014. For the initial base model we included the following fixed effects year, twin, birth weight and growth rate, and maternal ID as a random effect. To account for the pervasive effects of age at August capture on our immune measures (see Results), we used residuals from a linear model of each immune marker including age at capture as a linear covariate as explanatory variables in our survival models. In the first instance, each immune marker was included in a separate model of survival. We began by including each immune measure as a quadratic term in a two-way interaction with sex and then removed terms, most complex first, which were not significant based on a likelihood ratio test. After testing each immune marker separately, we built a final model that included all significant immune terms together to test whether or not they remained significant.

3.4 Results

Three of the immune markers, anti- *T. circ* IgA, IgG and NLR, had a skewed distribution, which was normalised using a log10 transformation (figure 3.1). Between the antibody markers, moderate positive correlations were present ($r<0.4$), and the correlations between NLR and eosinophils were even weaker ($r=0.23$) (figure 3.1). Between the cellular markers and the antibody markers the correlations were very weak ($r<0.1$) (figure 3.1).

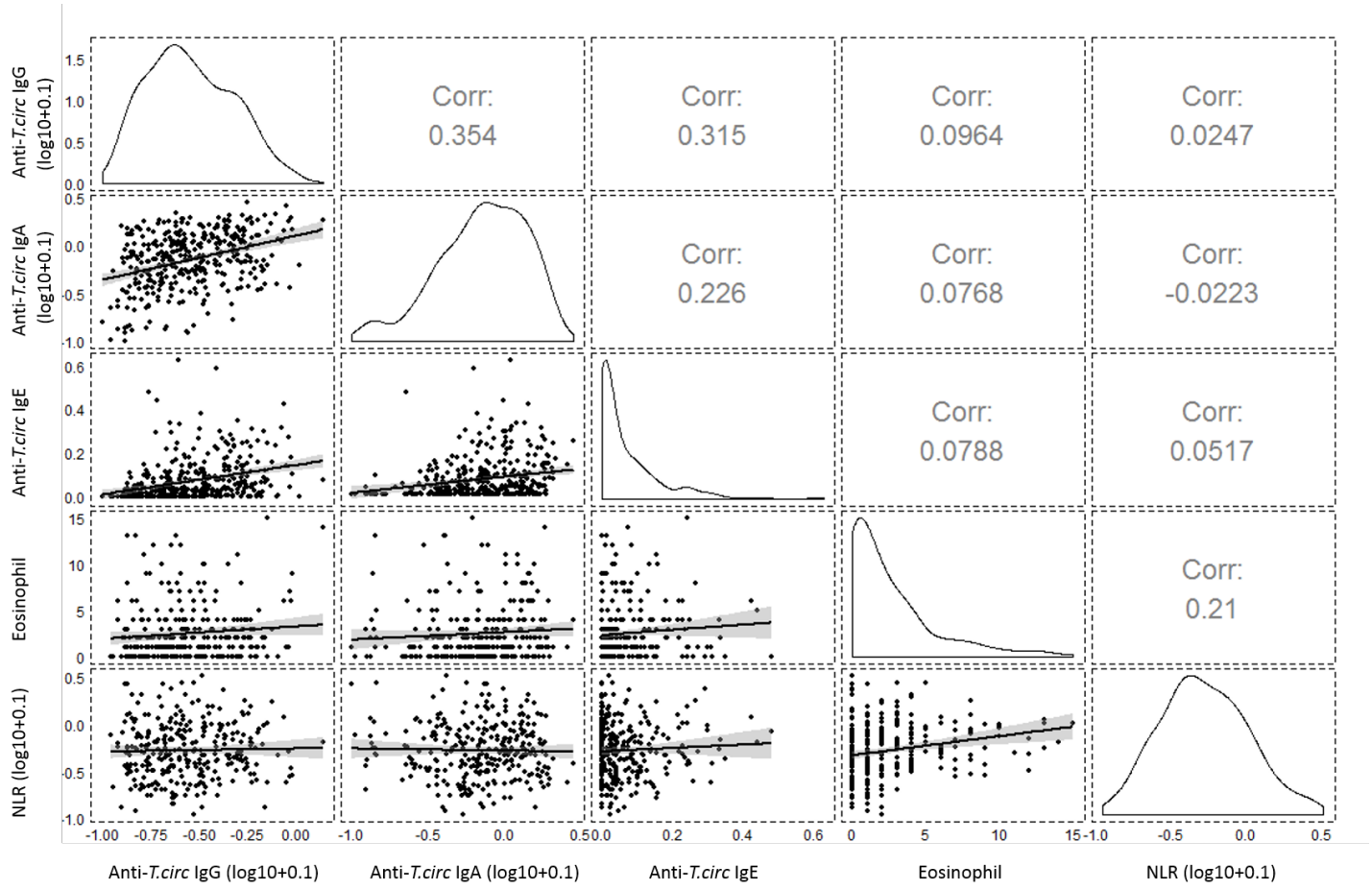

Figure 3.1: Scatter plot matrix of immune markers showing the raw data in the lower left area of the plot (with smoother lines and shaded standard error), the distribution of each immune marker on the diagonal and the correlation coefficients from a Pearson's pairwise correlation in the upper right area. The data for anti-*T. circ* IgA, IgG and NLR has been log10 transformed. For anti-*T. circ* IgE and eosinophils, we were not able to normalise the distribution of the data by transformation, so we present the raw data.

Our models did not support sex-by-growth rate interactions for any of the immune markers (table 3.1, figure 3.2). The models of NLR, anti-*T. circ* IgE and anti-*T. circ* IgG showed significant quadratic relationships between growth rate (table 3.1, figure 3.22). To further investigate these curvilinear relationships, we split the data at the inflection point of the quadratic function from the LMMs, and tested for effects of growth either side of this point in separate models. NLR had a decelerating negative relationship with growth rate, which was stronger in the low growth rates (b=-4.837, s.e=1.798, X^2=7.473 , p=0.006), becoming weaker at the faster growth rates (b=-4.567 , s.e=3.660 , X^2=1.778 , p=0.182) (table 3.1, figure 3.2 A). Anti- *T. circ* IgG and anti- *T. circ* IgE models showed more obviously curvilinear relationships with growth rate, with highest antibody measures at the mid-range of growth (table 3.1, figure 3.2 A). We found a positive relationship in the lower growth rates (IgG: b=0.992, s.e=1.788 , X^2=0.331 , p=0.565 – IgE: b=0.267, s.e=10.953 , X^2=0.000 , p=1) and a negative relationship in the higher growth rates (IgG:(b=-3.224 , s.e=1.901 , X^2=3.046 , p=0.081– IgE: b=50.225, s.e=16.674 , X^2=8.726 , p=0.003). With respect to the base model variables, all markers had a significant positive relationship with age, but only NLR had significant relationship with sex, in that males had slightly higher measures of NLR than females. In both the NLR and anti-*T. circ* IgG models, year was a significant explanatory marker and the NLR measures were higher before the population crash in 2011 than in any other year, but anti- *T. circ* IgG was highest in 2012 after the population crash (table 3.1). With the exception of anti- *T. circ* IgA, we found no significant effect of twin or birth weight (table 3.1). However, twins had significantly higher measures of anti- *T. circ* IgA than singletons, and lambs born heavier had lower measures of anti-*T. circ* IgA in August.

Table 3.1: Summary of model outputs for each immune marker testing the significance of growth rate, including as a quadratic term, main effect and both of these terms in an interaction with sex. Model simplification removed the higher terms if non-significant based on a likelihood ratio test in a stepwise method. The reference factor levels are females for sex and 2011 for year. All significant variables remaining in the final model are reported in bold. All of the base model variables are reported from the final model, which included remaining significant test variables.

		Immune Markers									
		NLR		**Eosinophil**		**Anti-*T. circ* IgA**		**Anti-*T. circ* IgE**		**Anti-*T. circ* IgG**	
	Effects	b(S.E)	X^2 (p)	b(S.E)	X^2 (p)	b(S.E)	X^2 (p)	b(S.E)	X^2 (p)	b(S.E)	X^2 (p)
Two-way interactions	Growth Rate^2: Sex		3.478 (0.062)		2.510 (0.113)		1.171 (0.279)		1.054 (0.305)		0.658 (0.417)
	Growth Rate: Sex		0.190 (0.663)		0.404 (0.525)		0.017 (0.898)		0.452 (0.501)		0.460 (0.497)
Main effects	Growth Rate^2	**84.342 (28.776)**	**8.748 (0.003)**		2.924 (0.087)		1.712 (0.191)	**-456.284 (173.300)**	**6.708 (0.010)**	**-56.807 (19.721)**	**8.463 (0.004)**
	Growth Rate	**-17.975 (5.618)**			0.034 (0.854)		0.753 (0.386)	**98.471 (32.813)**		**9.494 (3.645)**	
Base variables in final model	Sex	**0.085 (0.037)**	**5.555 (0.018)**	**0.499 (0.978)**	**0.584 (0.445)**	**-0.048 (0.032)**	**2.340 (0.126)**	**-0.190 (0.201)**	**0.896 (0.344)**	**-0.001 (0.026)**	**0.002 (0.965)**
	Age (days)	**0.011 (0.003)**	**14.782 (<0.001)**	**0.033 (0.016)**	**4.360 (0.037)**	**0.013 (0.003)**	**18.922 (<0.001)**	**0.071 (0.018)**	**13.666 (<0.001)**	**0.010 (0.002)**	**21.674 (<0.001)**
	Year (2012)	**-0.135 (0.067)**	**29.599 (<0.001)**	**0.452 (0.328)**	**2.402 (0.493)**	**-0.045 (0.052)**	**0.823 (0.844)**	**0.615 (0.358)**	**4.832 (0.185)**	**0.130 (0.044)**	**29.731 (<0.001)**
	Year (2013)	**-0.276 (0.051)**		**0.240 (0.250)**		**-0.017 (0.044)**		**0.095 (0.278)**		**-0.096 (0.036)**	
	Year (2014)	**-0.162 (0.049)**		**0.297 (0.241)**		**-0.010 (0.043)**		**-0.012 (0.282)**		**-0.022 (0.035)**	
	Twin	**-0.037 (0.050)**	**0.574 (0.449)**	**-0.373 (0.258)**	**2.108 (0.147)**	**0.103 (0.047)**	**4.882 (0.027)**	**0.448 (0.280)**	**2.634 (0.105)**	**0.012 (0.036)**	**0.121 (0.728)**
	Birth Weight	**-0.008 (0.045)**	**0.034 (0.854)**	**0.059 (0.214)**	**0.052 (0.820)**	**-0.086 (0.042)**	**4.381 (0.036)**	**-0.029 (0.246)**	**0.014 (0.906)**	**0.018 (0.033)**	**0.300 (0.584)**

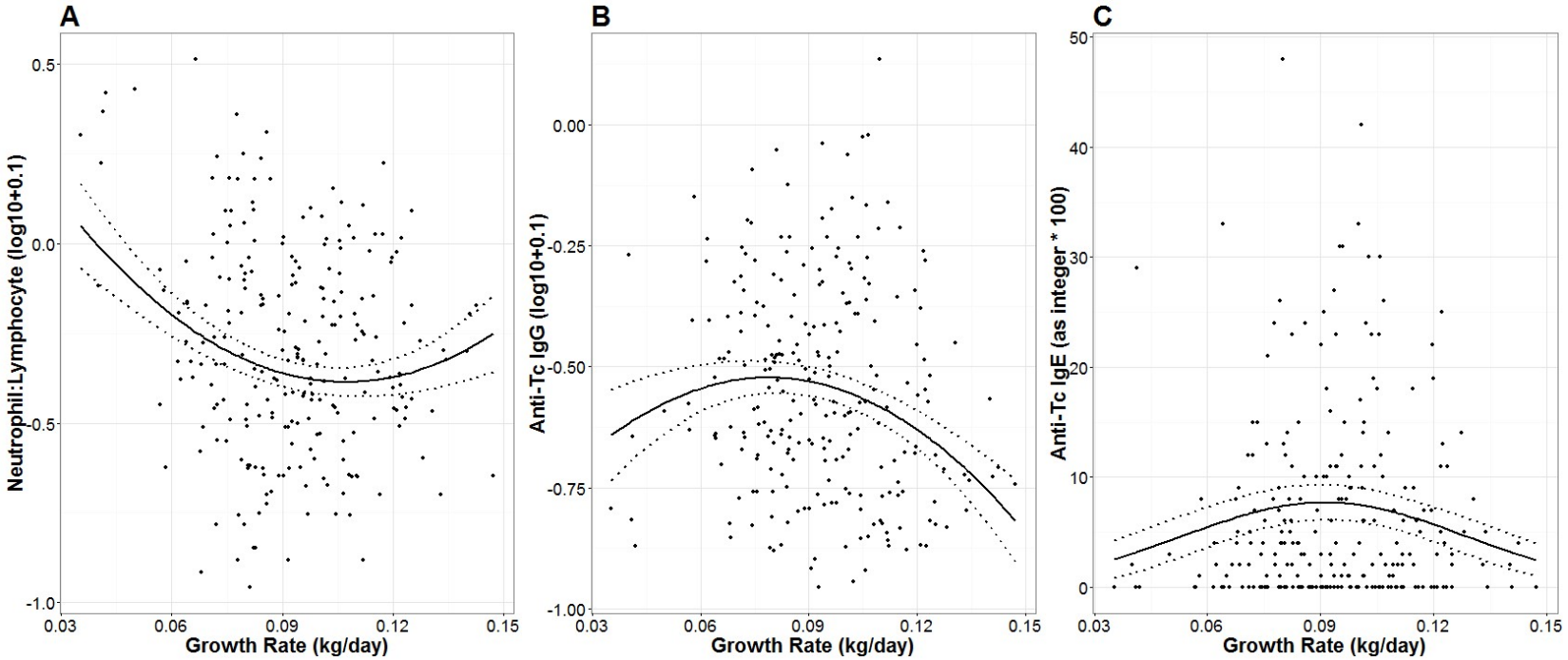

Figure 3.2: Growth rate was found to significantly explain variation in three immune markers; NLR (A), anti- *T. circ* IgG (B) and anti- *T. circ* IgE (C). All immune measures were taken during August and growth rate is calculated as the mass (kg) increase per day. The results are independent of sex, birth weight, twin status, year and age. Lambs with growth rates which are nearer the average show both lower NLR and increased measures of both anti- *T. circ* antibodies. The black points are of the raw data and the black lines are the model predictions with the standard error shown by the dotted lines. The model prediction and standard error in plot C of anti- *T. circ* IgE is calculated using a generalised linear model with a negative binomial distribution rather than a generalised linear mixed model used in the analysis. The predicted lines are based on the mean values of age in days and birth weight and factors held constant at 2011, females and non-twins.

In the FEC models we initially included quadratic immune terms and two-way interactions between sex and each immune marker as a quadratic term, but these were not significant in any case (table 3.2) and were therefore removed from further models. We found that FEC was negatively associated with two of the immune markers, anti- *T. circ* IgG and anti- *T. circ* IgA (table 3.2, figure 3.3). However, these antibodies were not independent of each other, as when we included both terms in the final model, anti- *T. circ* IgA became marginally non-significant (b=-0.066, se=0.038, X^2=3.169, p=0.075), but anti-T.circ IgG remained significant (b=-0.199, se=0.095, X^2=4.496, p=0.034).

In the male secondary sexual characteristic models we again included quadratic immune terms and two-way interactions between sex and each immune marker as a quadratic term, but these were not significant in any case (table 3.2) and so were removed from further models. We found no association between immunity and either of the male secondary sexual characteristics of testis diameter or horn length (table 3.2).

Table 3.2: Summary of model outputs for FEC, survival, horn length and testicle circumference. The significance of each immune marker was tested as both a main effect and in an interaction with sex. Model simplification removed the higher terms if non-significant based on a likelihood ratio test. Quadratic terms for each marker as a main effect and within a two-way interaction with sex were tested, but are not reported here as none were significant in any case. The reference factor levels were females for sex and 2011 for year, except in the case of the survival model for which the reference factor level for year was 2013. All significant variables remaining in the final model are reported in bold. The base model variables are reported from the base model of each response variable, which included none of the test variables.

		Immune Markers							
		FEC		Survival (2013-2014)		Horn length		Bol circ	
	Effects	b(S.E)	X^2 (p)	b(S.E)	X^2 (p)	b(S.E)	X^2 (p)	b(S.E)	X^2 (p)
Two-way interactions	NLR: Sex		0.303 (0.582)		0.233 (0.629)	NA	NA	NA	NA
	Eosinophil :Sex		2.655 (0.103)		0.068 (0.794)	NA	NA	NA	NA
	Anti-*T. circ* IgA:Sex		2.434 (0.119)		0.704 (0.401)	NA	NA	NA	NA
	Anti-*T. circ* IgE:Sex		0.031 (0.861)	**-15.003 (5.489)**	**9.568 (0.002)**	NA	NA	NA	NA
	Anti-*T. circ* IgG:Sex		0.025 (0.873)	**-4.509 (2.329)**	**4.106 (0.043)**	NA	NA	NA	NA
Main effects	NLR		0.064 (0.804)		0.155 (0.694)	-0.270 (6.069)	0.001 (0.975)	-0.278 (3.322)	0.008 (0.931)
	Eosinophil		1.227 (0.268)		0.019 (0.892)	-0.396 (0.916)	0.212 (0.645)	0.297 (0.520)	0.347 (0.556)
	Anti-*T. circ* IgA	**-0.095 (0.035)**	**7.398 (0.007)**		0.632 (0.427)	-1.789 (5.780)	0.102 (0.750)	2.306 (3.050)	0.451
	Anti-*T. circ* IgE		1.733 (0.188)		1.463 (0.227)	-49.778 (26.657)	3.655 (0.056)	14.597 (13.957)	1.138 (0.286)
	Anti-*T. circ* IgG	**-0.261 (0.089)**	**8.725 (0.003)**		0.723 (0.395)	-26.507 (17.075)	2.429 (0.119)	7.958 (8.837)	0.836 (0.361)
Base variables in base model (no test variables included)	Sex	0.234 (0.036)	39.932 (<0.001)	-0.787 (0.384)	4.342 (0.037)	NA	NA	NA	NA
	Growth rate (kg/day)	-4.530 (1.291)	12.439 (<0.001)	17.599 (11.813)	2.280 (0.131)	324.686 (184.699)	3.188 (0.074)	1044.022 (97.568)	88.703 (<0.001)
	Birth weight (kg)	-0.034 (0.047)	0.549 (0.459)	0.331 (0.430)	0.595 (0.440)	24.222 (6.954)	12.193 (<0.001)	14.373 (3.591)	15.931 (<0.001)
	Age (days)	-0.008 (0.003)	5.622 (0.018)	NA	NA	0.686 (0.446)	2.466 (0.116)	1.476 (0.240)	35.116 (<0.001)
	Year (2012)	-0.328 (0.062)	41.799 (<0.001)	NA	NA	31.431 (9.837)	15.305 (0.002)	6.530 (5.097)	2.313 (0.51)
	Year (2013)	-0.018 (0.051)		NA	NA	17.088 (7.265)		1.038 (3.705)	
	Year (2014)	0.020 (0.049)		0.998 (0.359)	8.041 (0.005)	2.895 (7.578)		0.054 (3.850)	
	Twin	0.099 (0.054)	3.702 (0.062)	-0.428 (0.472)	0.831 (0.362)	NA	NA	NA	NA

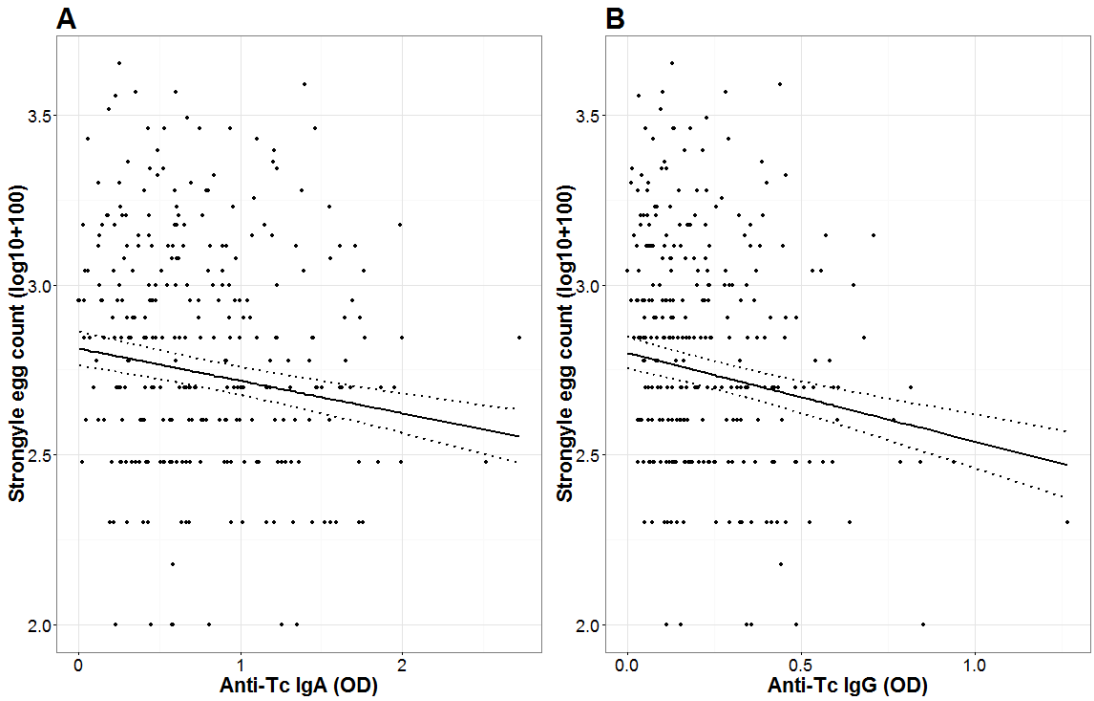

Figure 3.3: Strongyle faecal egg count (FEC) was found to be significantly lower in lambs which had higher measures of anti-*T. circ* IgA (A) and IgG (B). All parasite and immune measures were taken during a two week period in August for each year. The results are independent of growth rate, sex, birth weight, twin status, year and age. The black points are of the raw data and the black lines are the model predictions with the standard error shown by the dotted lines. The predicted lines are based on the mean values of age in days, birth weight and growth rate and factors held constant at 2011, females and non-twins.

None of the quadratic immune terms in the survival models were significant in any case, and were therefore removed from further models. The results show that for only two of the immune markers, anti-*T. circ* IgG and anti- *T. circ* IgE, there was a significant sex-dependant relationship with over-winter survival (table 3.2, figure 3.4). There were no significant relationships with survival in any of the other immune markers. For the two models with a significant sex-by-antibody interaction, we ran each model separately for males and females, which showed that the relationship was only significant for males and not females. The results were negative and significant in males: anti- *T. circ* IgG (b= -3.716, se= 1.961, X^2=4.338, p=0.037) and anti- *T. circ* IgE (b= -12.944, se= 4.835, X^2= 10.938, p=0.001). Where as in females the relationship was positive, but not significant: anti- *T. circ* IgG (b= 1.062, se= 1.437, X^2=0.551, p=0.458) and anti- *T. circ* IgE (b= 2.855, se= 2.804, X^2= 1.056, p=0.304). When we ran a final model of survival, which included both significant interactions, the sex-by-anti- *T. circ* IgE interaction remained significant (b= -13.186, se= 5.695, X^2= 6.459, p=0.011), but the sex-by-anti- *T. circ* IgG was no longer significant (b= -2.271 , se= 2.392, X^2= 0.928, p=0.336). These results suggest that the sex-by-anti- *T. circ* IgE interaction (table 3.2) is explaining more of the variation in survival.

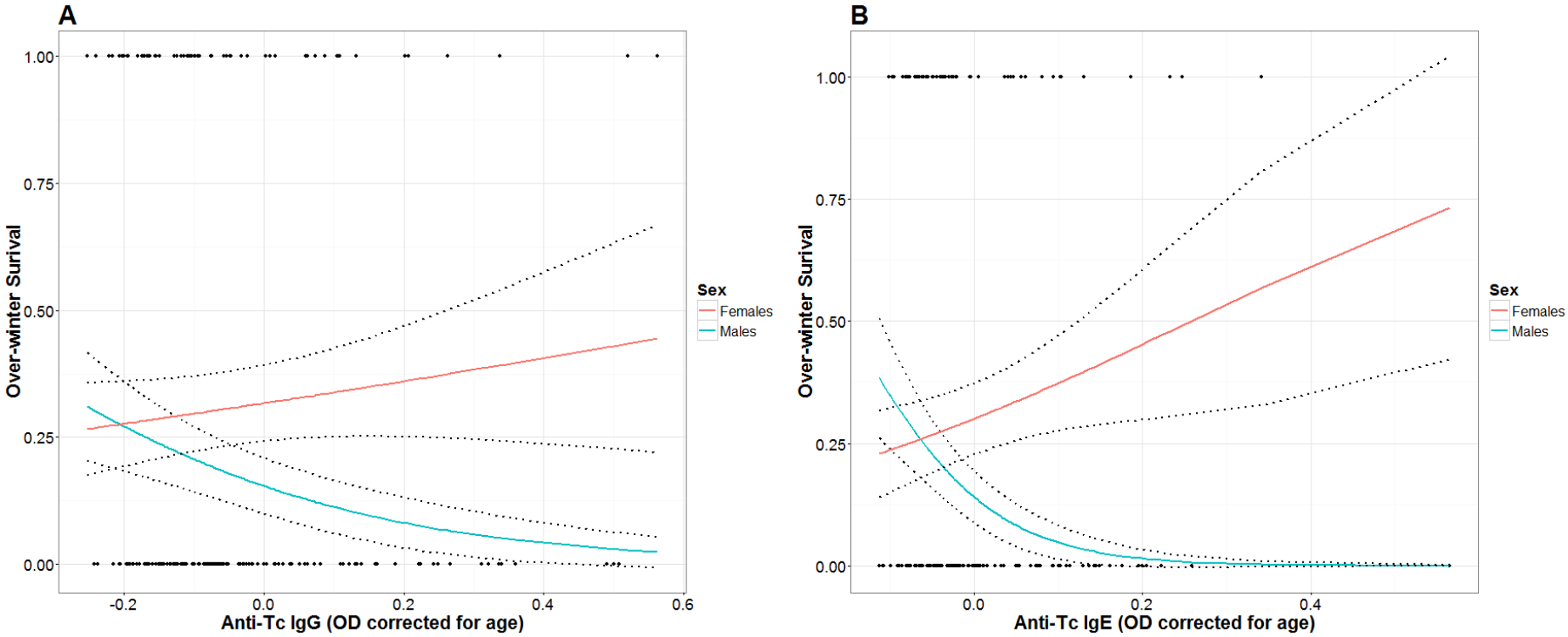

Figure 3.4: Overwinter survival was shown to be dependent on both sex and two subtypes of anti-*T. circ* antibody, IgE (A) and IgG (B). Immune measures were taken during a two-week period in August for each year. The results are independent of growth rate, birth weight, twin status, year and age. Female lambs with higher anti-*T. circ* IgE or IgG in August are more likely to survive over winter. However, the opposite is true for males. The black points are of the raw data and the coloured lines are the model predictions with the standard error shown by the dotted lines. The predicted lines are based on the mean values of birth weight and growth rate as factors.

3.5 Discussion

Our study has identified complicated associations between immunity and growth rate in early life, suggesting that the optimal immune response is not a maximal one. The negative relationships with FEC and anti-*T. circ* IgA, and anti-*T. circ* IgG, provide evidence for a protective role of antibody mediated immunity against GIN in early life. There are sex-dependant relationships between anti-*T. circ* IgE, and anti-*T. circ* IgG, which demonstrate the variation in selection pressure acting on males and females in trade-offs with immunity.

Three of the immune markers, anti-*T. circ* IgE, anti-*T. circ* IgG and NLR, had significant non-linear relationships with growth rate, which did not differ between the sexes. These associations between immunity and extreme rates of growth, particularly in the case of slow growers where antibody measures were low and inflammatory markers high, could indicate a resource constraint in early life between growth and immunity. For the NLR measure, there was an overall negative relationship with growth rate, which was strongest in the slowest growers. NLR is an indicator of chronic inflammation, and so individuals with slower growth rates, could be suffering slower growth due to the increased demand on resources from high inflammatory responses (Venturina et al., 2013; Wakelin, 1996). These individuals could be investing more heavily in innate immunity rather than adaptive responses, supported by evidence that the slowest growers also had reduced measures of adaptive immunity, anti-*T. circ* IgE, and anti-*T. circ* IgG, perhaps an indication of chronic infection (Venturina et al., 2013). Therefore, slow growing individuals with low immune capabilities, may be displaying the effects of either poor condition or the effects of low acquisition of resources. We also found there were reduced measures of anti-*T. circ* IgE, and anti-*T. circ* IgG in the fastest growing individuals. One explanation for this could be that fast growers are allocating such a high amount of resources into growth, that there is less available to invest in immunity, resulting in this relative cost to antibody measures and a trade-off between these two traits. This is supported by other studies, which also show an associated cost to immunity in fast

growing individuals (Brommer, 2004; Greer, 2008; Mauck et al., 2005; Soler et al., 2003). We did not find any significant relationship between growth rate and the third antibody mediated immune marker, anti-*T. circ* IgA. This marker is primarily present on mucosal surfaces with <1ug/ml in the blood serum (Cripps et al., 1985; Pastoret et al., 1998). However, anti-*T. circ* IgE and anti-*T. circ* IgG are present at higher concentration in the blood, particularly anti-*T. circ* IgG which is the dominant antibody isotype in the blood stream (Pastoret et al., 1998). Therefore, due to the presence of anti-*T. circ* IgA in such low quantities in circulation, it may not be possible to detect variation with growth rate in this marker. We also found that twins had significantly higher measures of anti-*T. circ* IgA than singletons, and lambs born heavier had lower measures of anti-*T. circ* IgA in August, but this was not the case for anti-*T. circ* IgG or anti-*T. circ* IgE. This suggests that anti-*T. circ* IgA measured in August may be more strongly related to conditions around birth, and less sensitive to growth over the first four months of life, than the other isotypes, although the reasons this might be the case are unclear.

Measures of anti-*T. circ* IgG and anti-*T. circ* IgA were lowest in individuals that also had the highest parasite burdens (table 3.2, figure **??**). This negative relationship suggests that these antibodies are providing protection against these prevalent GIN parasites, rather than being a consequence of exposure. This is supported by previous studies, including work in this system, showing similar negative relationships (Beraldi et al., 2008; Bisset and Morris, 1996; Coltman et al., 2001; Douch et al., 1984; Hayward et al., 2014; Shaw et al., 1999). It is interesting that this negative relationship is only significant for anti-*T. circ* IgA and anti-*T. circ* IgG, and not for the third isotype, anti-*T. circ* IgE. We know from previous studies in domestic sheep that anti-*T. circ* IgA is strongly associated with reducing worm reproduction and development (Halliday et al., 2007; Stear et al., 2004; Strain et al., 2002). There is also evidence that anti-*T. circ* IgG is involved in reducing worm growth and reproduction, although this is primarily documented in mice (Wakelin, 1996). Therefore, we might expect these two markers to be particularly relevant to our measure of parasite burden, which specifically counts the numbers of eggs shed by the parasite, and is perhaps more of an indicator of parasite reproduction. In contrast,

the role of anti-*T. circ* IgE has been linked to worm expulsion and the reduction in the number of worms, rather than limiting growth or reproduction of the parasite. The elimination of worms will no doubt have a negative impact on FEC, but it is also possible that the remaining worms could actually increase reproduction in the absence of competition from other parasites. This could result in no overall change in the number of eggs shed, and make the protective effects of anti-*T. circ* IgE antibodies difficult to detect with FEC alone.

While it was expected that in this polygynous species, there would be sex specific immune variation, we did not find a significant effect of immune investment on sexual characteristics in male lambs (table 3.2), in either horn growth or testicular circumference. A study, in Bighorn sheep (*Ovis canadensis*), found that the relationship between horn growth and survival was age-dependant, with a strong relationship being present in yearlings only (Bonenfant et al., 2009). It could be that the greatest investment in horn growth occurs in older yearlings rather than lambs, and so the trade-off between horn growth and immunity might only be detected in older age classes. However, we did find a sex-dependant relationship between survival and immunity, as males with high anti-*T. circ* IgE or anti-*T. circ* IgG were less likely to survive the first winter. As expected in this dimorphic polygynous system, where there is evidence of male-biased mortality, the costs of immune investment and the pressure of sexual selection differ between the sexes (Clutton-Brock and Pemberton, 2004; Moore and Wilson, 2002; Promislow, 1992; Zuk, 1990). Males tend to have higher parasite burdens and lower immune measures than females (McCurdy et al., 1998; Møller et al., 1999, 1998; Schalk et al., 1997), as a result of higher growth or the effects of sex specific hormones (Folstad and Karter, 1992; Moore and Wilson, 2002; Zuk, 1990). Therefore, due to sex-specific costs driven by sexual selection, we might expect that types of immune response might vary between the sexes. The findings in this study, that high anti-*T. circ* IgE or anti-*T. circ* IgG comes at cost of reduced survival in males only, supports the suggestion that the costs of immune investment are different for males and females, and that there may be different selection pressures acting on each sex.

A previous study of this population found a positive relationship between anti-*T. circ* IgG and survival in adult females across three crash winters between 1997 and 2005 (Nussey et al., 2014). In Chapter 2, I found a similar relationship for survival of the 2011/2012 winter crash that was independent of age and sex. In the present survival analyses, data from 2011 was excluded because lamb mortality was so high. It seems probable that, given the extremely low number of lambs surviving the 2011/2012 crash, there may have been insufficient power to detect sex-dependent associations in lambs and the signal we detected came from older animals where a lower proportion of individuals died. This could explain why the significant sex-dependant relationship between anti-*T. circ* IgE and survival observed in this chapter, is absent in chapter 2. It is possible that anti-*T. circ* IgE is only significantly related to survival in lambs, however, further analysis would be needed to confirm whether this relationship is reflected in adults within this same data set. We found that anti-*T. circ* IgG was the only isotype to vary significantly among years in lambs, but the pattern of annual variation was not consistent with annual variation in survival. Anti-*T. circ* IgG levels were highest in 2012 when lamb survival was highest, but were lowest in 2013 when survival was intermediate (table 2). This implies that variation in immunity is not the main driver of annual variation in lamb survival, which is unsurprising since density and winter climate are known to play a major role in annual demographic variation in this system (Albon, A et al., 1987; Coulson, 2001; Coulson et al., 1999; Milner et al., 1999). However, in years of intermediate lamb mortality (2013 and 2014), we were able to pick up more subtle within year associations between anti-*T. circ* IgG and lamb survival and show that these differed between the sexes. It would be interesting more generally to expand this research further and evaluate whether this sex difference is robust across all age classes in non-crash years.

We did not find that any significant relationship between anti-*T. circ* IgA and lamb survival over the first winter. As mentioned previously, anti-*T. circ* IgA, is the main mucosal antibody isotype and is relatively low in circulation compared to other isotypes (Pastoret et al., 1998). This could explain why we cannot detect a relationship with survival from our anti-*T. circ* IgA measures taken from peripheral blood samples. We also found that there was no significant relationship between

survival and the two leukocyte markers, NLR and eosinophil. The analysis measured survival during the first year of life, but did not test for later life consequences of early life investment in immunity, although there is much evidence to suggest that early life conditions or traits have fitness consequences in later life (Hayward et al., 2009; Lemaître et al., 2015; Lindström, 1999; Metcalfe and Monaghan, 2001; Nussey et al., 2007). In zebra finch (*Taeniopygia guttata*) nestlings, reduced immunity and growth due to poor diet resulted in reduced survival in later life, despite no effect on other life traits as adults (Birkhead et al., 1999). Although this study was unable to address this question, due to an insufficient number of longitudinal samples, future work in this system might go on to test whether early life immune investment is beneficial survival in later life.

Chapter 4

Sex differences in leukocyte telomere length in a wild mammal: independent of leukocyte population composition

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This chapter has been submitted to Molecular Ecology as:

Watson, R.L., Underwood, S., Adams, R., Fairlie, J., Bird, E. , Salvo-Chirnside, E., Pilkington, J.G., Pemberton, J.M., Zamoyska, R., McNeilly, T.M. , Froy, H. & Nussey, D.H.

"Sex differences in leukocyte telomere length in a free-living mammal"

4.1 Abstract

Telomeres are repeat G-rich sequences (TTAGGG) of non-coding DNA on the ends of chromosomes, which shorten with each cell replication. Telomere attrition is closely linked to cellular ageing and average telomere length (TL) is a biomarker of considerable current interest not only in humans, where it appears predictive of subsequent late-onset diseases mortality and chronic stress, but also in wild studies through associations with reduced survival. TL in humans varies between the sexes, potentially related to differences in lifespan, but studies in wild mammals are few, especially regarding associations with reproductive investment in secondary sexual traits. In contrast to other vertebrate groups, mammals have enucleated red blood cells, and so TL measured in DNA extracted from blood samples reflects the average leukocyte telomere length (LTL). This represents a challenge for mammalian studies, as these cell types have different immune functions, which result in differences in proliferative capacity, telomerase expression and ultimately telomere length. In addition, leukocyte proportions vary with age and sex, and so variation in LTL could be reflecting changes in underlying cell population structure. How LTL varies with age and sex, and whether this is associated with variation in leukocyte cell structure in this population, has yet to be tested in a wild mammal. In a wild population of sheep, resident on the island of St Kilda, Scotland, this study addresses this question, and also tests for a cost of reduced LTL through investment in secondary sexual characteristics. This is the first study of this kind to identify a clear sex difference in LTL dynamics and a distinct decline in adult male LTL with age in a wild mammal. The results indicate that these differences in LTL are not driven by leukocyte population composition, and instead are related to systemic changes in telomere length, and is reflective of telomere length in the stem cell population.

4.2 Introduction

Telomeres are repeat G-rich sequences (TTAGGG) of non-coding DNA on the ends of chromosomes, which are important for chromosomal stability and successful replication (Blackburn, 1994; de Lange, 2004). During cell replication DNA is at risk of fusion or degradation due to the 'end replication problem' (Olovnikov, 1996), which results in the loss of small regions of terminal sequence that cannot be synthesised, and telomeres provide protection from this (McEachern et al., 2000). Telomeres shorten with each cell replication, and are also highly sensitive to damage by reactive oxygen species (Von Zglinicki, 2002), but can be replenished through the action of the enzyme telomerase (Armanios and Blackburn, 2012; Counter et al., 1992). In some species, when telomere length drops below a critical threshold it triggers the onset of cellular senescence and thus there may be close links between telomere attrition and cellular ageing (Allsopp et al., 1992; Gomes et al., 2011). Average telomere length (TL), which is typically measured from DNA obtained from whole blood samples, has been shown to decline with age in numerous species and is a biomarker of considerable current interest in human epidemiology and evolutionary ecology (Blackburn et al., 2015; Monaghan and Haussmann, 2006). In humans, TL in adulthood appears predictive of subsequent late-onset diseases and, in some studies, mortality risk (Blackburn et al., 2015; Cawthon et al., 2003; Epel et al., 2004; Rode et al., 2015), whilst prior experience of chronic stress or trauma is associated with shorter TL (Choi et al., 2008; Epel et al., 2004; Monaghan, 2014; Shalev, 2012). Studies of wild birds and mammals have also recently found associations between short TL and reduced subsequent survival or recapture rates (e.g.(Barrett et al., 2013; Bize et al., 2009; Fairlie et al., 2016; Salomons et al., 2009)).

Evidence of sex differences in TL is strongest for humans, with a recent meta-analysis of forty data sets from adults concluding that there was a significant trend for females to have longer telomeres than males (Gardner et al., 2014). More recent, large-scale studies have confirmed this pattern (Berglund et al., 2016; Lapham et al., 2015), which mirrors the pervasive gender difference in longevity observed in

humans and other polygynous mammals, leading to speculation that sex differences in TL may be causally related to differences in lifespan (Aviv et al., 2005; Barrett and Richardson, 2011; Stindl, 2004). However, it remains unclear whether sex differences in TL are present in early life or emerge in later adulthood due to differences in telomere attrition rates in humans. Several studies have failed to document significant sex differences in TL measured in cord blood (Akkad et al., 2006; Aubert et al., 2012; Okuda et al., 2002; Shi-Ni et al., 2013), although a recent larger-scale study did find significantly longer telomeres in new born females than males (Factor-Litvak et al., 2016). Additionally, a recent meta-analysis demonstrated that TL is shorter in males among studies of adults (Gardner et al., 2014). Studies of captive primates have yielded equivocal results: no sex differences in TL were found in cynomolgus macaques (*Macaca fascicularis*; (Gardner et al., 2007)), whilst female rhesus macaques (*Macaca mulatta*) had significantly longer TL than males in leukocytes but not in other tissue types (Smith et al., 2011). Longer telomeres in females than males have been reported in laboratory populations of rats (*Rattus rattus*; (Cherif et al., 2003; Tarry-Adkins et al., 2006) and Algerian mice (*Mus spretus*; (Coviello-McLaughlin and Prowse, 1997)). There are few studies comparing telomere dynamics between the sexes in mammals other than humans and laboratory rodents, despite issues with extrapolating results of laboratory studies of short lived mice to humans (Barrett and Richardson, 2011; Davis and Kipling, 2005; Hodes et al., 2002). Two recent studies report no consistent evidence for a sex difference in TL (Beirne et al., 2014; Lewin et al., 2015)). While sex differences in TL and body size have been reported in birds and reptiles (Barrett and Richardson, 2011; Olsson et al., 2011), such studies are rare in non-human mammals. Furthermore, polygyny and the expression of secondary sexual traits in males are common in mammals but, to our knowledge, no studies have tested whether hypothesised costs of such traits may be reflected by shortened TL.

Several non-mutually exclusive explanations for gender differences in TL have been proposed, including heterogametic disadvantage and differences in the effects of sex hormones on oxidative stress and telomerase function (Barrett and Richardson, 2011; Gardner et al., 2014). It has also been hypothesized that, in species with sexual

size dimorphism, increased cell proliferation rates and oxidative stress associated with increased growth in the larger sex could reduce TL (Barrett and Richardson, 2011; Stindl, 2004). Increased parasite burdens in males relative to females have been documented in polygynous mammals and proposed as a driver of male-biased mortality (Moore and Wilson, 2002). Experiments in laboratory-kept house mice (*Mus musculus*) demonstrated faster TL loss in males than females following repeated bacterial infection, raising the possibility that sex differences in LTL could reflect increased infection pressure in males (Ilmonen et al., 2008). Polygynous males also typically exhibit secondary sexual characteristics other than increased body size (e.g. seasonal colouration, vocalisations, ornaments and weapons) and highly energetically expensive intra-sexual competition for access to mates (Andersson, 1994). Male investment in these reproductive traits may be associated with increased cellular proliferation or oxidative stress, resulting in faster TL attrition in males relative to females (Barrett and Richardson, 2011; Monaghan, 2010). A recent review found little consistent evidence that sex differences in TL were associated with heterogamy, the degree of body size dimorphism, or mating system (Barrett and Richardson, 2011). However, direct tests for sex-specific associations between TL and either weight, reproductive investment or parasite burden within polygynous species remain rare (Beirne et al., 2014; Olsson et al., 2011).

In mammals, which have enucleated red blood cells, TL measured in DNA extracted from blood samples reflects the average leukocyte telomere length (LTL). This is in contrast to other vertebrate groups with nucleated erythrocytes, in which erythrocyte telomere length (ETL) is measured. This represents a challenge for mammalian studies, as LTL is a measurement which encompasses a range of different white blood cell types. These cell types have different functions and roles within the immune system, which result in differences in proliferative capacity and telomerase expression and ultimately telomere length (Hodes et al., 2002; Weng, 2001). For instance, in humans and baboons granulocytes have longer telomeres than lymphocytes in adulthood, most likely due to the fact that granulocytes are terminally differentiated cells whilst lymphocytes have the capacity to rapidly replicate and differentiate (Aubert et al., 2012; Baerlocher et al., 2006; Kimura et al., 2010).

Lymphocytes also vary in telomere length, with naïve T cells having longer telomere lengths in comparison to memory T cells, again due to greater proliferative history of the latter (Aubert et al., 2012; Weng, 2001). The composition of circulating leukocyte cell types can change profoundly with age and vary between sexes (Giefing-Kroll et al., 2015; Linton and Dorshkind, 2004; Pawelec et al., 2010), and changes found in average LTL in relation to age and sex could therefore reflect changes in underlying cell population structure (Weng, 2001). Studies in humans and primates have reported very strong within-individual correlations in TL measured in different leukocyte sub-populations and among different tissues, despite differences in mean TL among individuals and tissue types (Baerlocher et al., 2006; Daniali et al., 2013; Gardner et al., 2007; Kimura et al., 2010; Okuda et al., 2002). Based on this apparent 'synchrony' in TL across tissue and cell types, it has been argued that among-individual variation in LTL reflects differences in the TL of the haematopoietic stem cell pool, which is primary determined genetically and by early life environment (Daniali et al., 2013). However, beyond a handful of small, cross-sectional studies in humans and laboratory primates, efforts to investigate the dependence of LTL and its associations with age, sex and other traits on variation in leukocyte population structure remain very limited.

Soay sheep have a polygynous breeding system: males compete for mating opportunities with oestrous females during the autumn rut and have highly skewed reproductive success, with a handful of males obtaining the majority of paternities each year (Clutton-Brock and Pemberton, 2004). In the population in this study, body mass is associated with increased fitness in both sexes, although males are shorter lived and larger in size than females, (Clutton-Brock and Pemberton, 2004; Milner et al., 1999). Therefore, we might expect to see a sex difference in LTL as the costs of investment in growth differ between males and females. Although both sexes may grow horns in Soay sheep, horns represent an important secondary sexual character in males and horn length is positively associated with plasma testosterone levels and subsequent male annual reproductive success (Johnston et al., 2013; Preston et al., 2012; Robinson et al., 2006). This population is infected by a variety of Strongyle gastrointestinal nematode parasites, and parasite burdens – as measured by faecal egg

counts (FEC) – are predictive of over-winter survival and greater in males than females at all ages (Hayward et al., 2009; Wilson et al., 2004). A previous study of LTL in this population examined longitudinal variation in LTL and showed that short LTL predicted mortality in early life in females (Fairlie et al., 2016). Furthermore, measures of variation in leukocyte population structure from samples collected in this population suggest that there is age and sex related variation in the leukocyte proportions (Nussey et al., 2012; Chapter 2). However, whether there are sex differences in LTL and how LTL may be associated with variation in leukocyte cell structure in this population, has yet to be tested. In this study, we test for a sex difference in LTL in wild Soay sheep and if there is a cost of reduced LTL through investment in secondary sexual characteristics. We also examine whether variation in LTL can be explained by changes in proportions of different leukocytes, and test whether observed sex differences and associations with secondary sexual traits are independent of underlying variation in leukocyte population composition.

4.3 Materials and methods

4.3.1 *Study system & field data collection*

Soay sheep are a primitive breed of domestic sheep that have been living on the remote St Kilda archipelago with minimal human management for the last few millennia (Clutton-Brock and Pemberton, 2004). Soay sheep have a polygynous breeding system: males compete for mating opportunities with oestrous females during the autumn rut and have highly skewed reproductive success, with a handful of males obtaining the majority of paternities each year (Clutton-Brock and Pemberton, 2004). As is typical in polygynous systems, males are larger and shorter lived than females: by five years of age, males average around 38kg and females around 24kg in summer with maximum recorded lifespan of ten years in males and sixteen years in females (Clutton-Brock and Pemberton, 2004). Horns, which are grown

incrementally each year, are an important secondary sexual trait in males in Soay sheep, and polymorphism in horn type is present in both sexes. Horn types are defined as follows: normal horns are sturdy and consist of a bony core covered in a keratin sheath, whilst scurred horns consist of keratin but lack a bony core. A polled phenotype is present only in females and involves a complete absence of visible horn growth (Clutton-Brock and Pemberton, 2004). The genes underpinning this polymorphism have recently been identified and shown to be under balancing selection (Johnston et al., 2013).

This study uses data and samples from animals caught during Augusts 2014 and 2015. In August 2014, we caught a total of 260 individuals, including 83 males and 177 females. In August 2015, 241 individuals were caught, including 66 males and 175 females. Within the available sample of 149 males, 134 had normal horns and 15 were scurs and of the 352 females, 78 were normal horned, 128 were scurs, and 146 were polled. Two 9ml vacutainers of blood were taken from each individual and kept in a cool box or fridge from the point of sampling until further processing. Blood samples were processed in the field, to allow subsequent flow cytometry, differential cell counting and quantitative PCR analyses in the laboratory in Edinburgh, as follows. The first vacutainer of blood was spun at 3,000 rpm for 10 minutes and the plasma layer was then drawn off and replaced by the same quantity of 0.9% w/v NaCl solution and spun again at 3,000 rpm for 10 minutes. The intermediate buffy coat layer, comprising mainly white blood cells, is then drawn off into a 1.5 ml Eppendorf tube and stored at -20°C until used to assay leukocyte telomere length. Within 12 hours of collection, 5µl of whole blood from the second vacutainer was applied on to one end of a standard glass microscope slide. The drop of blood was then spread at a 45° angle and drawn across the slide to produce an even film. Slides were air dried overnight and stained using a Quick-Diff Kit stain (Gentaur), the following day as per manufacturer's instructions. A further 1ml of whole blood from the second vacutainer was used to prepare a fixed sample of lymphocytes for later flow cytometry analyses, following (Nussey et al., 2012). Red cells were lysed by adding 5ml of ammonium chloride lysing solution (1.5M NH4Cl, 100mM NAHCO3, 10mM Na2EDTA) to 1ml of whole blood and, mixing gently and centrifuging at 1008g for 10 minutes. The

supernatant was removed and the cell pellet re-suspended in 9ml of phosphate buffered saline (PBS) to wash. The sample was then centrifuged (as above), the supernatant removed and the pellet re-suspended in 2.5ml 1% paraformaldehyde (PFA) in PBS and incubated at room temperature for 10 minutes. The sample was then centrifuged at 1008g for 10 minutes, the supernatant was removed and the cell pellet re-suspended in 9ml phosphate buffered saline (PBS) to wash. The cells were then spun again at 1008g for 10 minutes and finally re-suspended in PBS + 0.02

4.3.2 *Laboratory methods*

4.3.2.1 *Differential white blood cell counts*

Within 12 hours of collection 5µl of whole blood was applied on to one end of a standard glass microscope slide. The drop of blood was then spread at a 45° angle and smeared evenly across the slide. 100 cells were counted at 40x magnification using the Battlement Track method and based on staining and morphology, identified as either lymphocytes, eosinophils or neutrophils (Bain 2008). Basophils and monocytes were observed too rarely to analyse. Only slides with a clear regular monolayer of cells were counted. Slides with uneven cell density or unclear staining were omitted from analysis leaving an overall number of 465 samples from 501 animals sampled. From our counts, we calculated the neutrophil to lymphocyte ratio (NLR) and number of eosinophils.

4.3.2.2 *Flowcytometry*

Fluorescently labelled monoclonal antibodies were used to identify the proportions of T helper cells (CD4+), cytotoxic T cells (CD8+) the proportion of naïve helper and naïve cytotoxic T cells, were identified by their co-expression of the CD45RA marker (see table 4.1). A regulatory cell (Treg) Foxp3 was also measured in this protocol, but

as no replicative senescence of Tregs has been observed, this measure was not included in further analysis (Fessler et al., 2013). We used a multi-panel stain which identified all cell types using only one sample per individual, over a two-day protocol. On the first day cells were stained for CD4+, CD8+, and naïve cell types and the overnight incubation was set up to make the cell walls permeable in preparation for the intracellular stain. 100µl of fixed cells was added to each well; one individual per well in sample wells and an aliquot of a generic 'golden sample' made up of multiple individuals to control wells. The plates were spun at 2000rpm for 1 minute at 4°C, before removing the supernatant by flicking plate downwards into sink, taking care not to lose the pellet. Non-specific binding sites were blocked by re-suspending cells in 200ul of a 20% solution of Normal Goat Serum diluted in FACS buffer (PBS + 5% heat-inactivated foetal calf serum + 0.02% sodium azide) to each well and incubating at 4°C for 30 minutes. Plates were spun as before and then re-suspended in 100µl of antibody mix in each sample well, 100µl of each antibody in the corresponding control well and 100µl of a specific antibody control mix in the negative control wells (fluorescence minus one (FMO)). Remaining wells were re-suspended in 100µl FACS buffer and plates were incubated at 4°C for 30 minutes before spinning as before. Plates were washed by adding 100ul FACS buffer to each well, before spinning and re-suspending in 200µl FACS buffer for the final wash step. Plates were spun as before and the re-suspended in 200µl permabilisation solution (PBS + 0.2% saponin + 20% normal rat serum) in all wells. Plates were incubated at 4°C overnight. On day two of the protocol, plates were spun as before re-suspended in either were re-suspended in 100µl anti-FoxP3 647 (1:80) for sample and corresponding control wells or permabilisation buffer without rat serum (PBS + 0.2% saponin) for the remaining wells. Plates were incubated at 4°C for one hour and then washed in a two-step process as before. Cells in all wells were re-suspend in 200ul PBS solution and then plates were covered and stored at 4°C for less than 24 hours before reading on the MACSQuant.

Flow cytometry data was analysed using FlowJo version X.0.7 analysis software (TreeStar, San Carlos, CA). Proportions of T cells were measured by firstly placing a gate encompassing the entire lymphocyte population. T cell populations were then

Table 4.1: Details of the reagents used in the flow cytometry analysis of T cell subsets. All antibodies were used directly conjugated and were either sourced as direct conjugates commercially or conjugated using commercial kits with antibodies produced in-house.

Antibody	Host/isotype*	Clone	Conjugate	Labelling**	Dilution (ug/ml)	FACSCanto Excitation (nm)	sEmission (nm)
Anti-CD4	Mouse IgG2a	17D	Alexa Fluor® 488	Innova: nlightening system	0.2	488	530/30
Anti-CD8	Mouse anti Sheep IgG2a	LT8	PE	Commercial	0.1	488	575/26
Anti-CD45RA	Mouse IgG1	73B	PerCp-Cy5.5	Lightning-Link®	0.02	488	695/40
Anti-Foxp3	Anti-Mouse/Rat IgG2a	FJK-16s	660	Commercial	0.125	633	660/20

gated using specific CD4 or CD8 fluorescence, followed by appropriate sub-type gating based on CD45RA fluorescence. Those with poor staining, poor cell profiles or low cell numbers were omitted from analysis leaving an overall number of 449 samples for CD4:CD8 and 446 samples for both naïve cell subtypes from 501 animals sampled.

4.3.2.3 *Telomere length measurement*

Genomic DNA was extracted from buffy coat using the Qiagen DNeasy Blood and Tissue Kit following manufacturer's guidelines for animal blood (Cat No. 69581, Manchester, UK). The protocol was modified slightly to facilitate sample flow through the spin columns which subsequently improved DNA yield and purity (see SOM). These are outlined below;

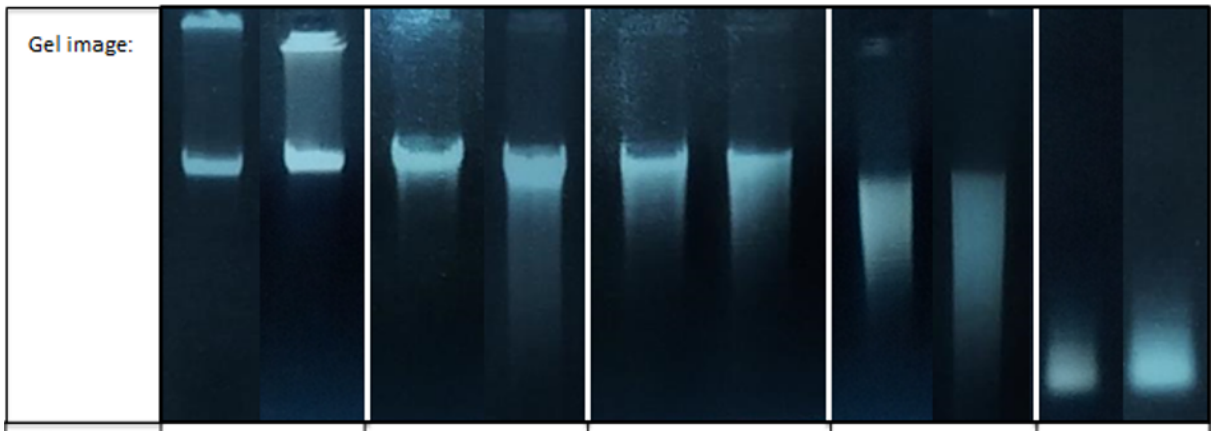
- Prior to step 1a, 50 µl of buffy coat was mixed thoroughly with 300 µl Qiagen Red Blood Cell (RBC) lysis solution (Cat No. 158902, Manchester, UK) and then centrifuged for 30 s at 14000 x g to produce a white blood cell (WBC) pellet. The supernatant was then discarded leaving approximately 10 µl residual

liquid. 100 µl PBS was added to the sample and the WBC pellet re-suspended by vortexing. This step removes all remaining red blood cells.

- The volume of proteinase K added in step 1a was increased from 20 µl to 30 µl to ensure complete WBC digestion.
- Incubation with buffer AL at 56°C in step 2 was increased from 10 min to 1 hour, vortexing the sample at 30 min mid- incubation, to optimise WBC lysis.

Following DNA extraction and elution in buffer AE (10mM TrisCl, 0.5 EDTA, pH 9.0), a strict quality control protocol was implemented to determine DNA quality and integrity. First, each sample was individually tested for DNA yield and purity using a Nanodrop ND-1000 9 spectrophotometer (Thermo Scientific, Wilmington DE, USA). Samples yielding < 20 ng/µl were immediately rejected. Samples yielding $\leq$ 20 ng/µl were checked for DNA purity; acceptable ranges for absorption were 1.7 - 2.0 for 260/280 nm ratio and 1.8 - 2.2 for 260/230 nm ratio. Once samples passed Nanodrop QC, they were standardized to 10 ng/µl and their DNA integrity assessed by running 20 µl (200 ng total DNA) on a 0.5% agarose gel. Samples were scored for integrity on a scale of 1 to 5 by visual examination of their DNA crowns, with samples scoring higher than 2 being excluded from further analyses (see figure 4.1). Samples which failed one or more of the above QC measures were re-extracted and if they failed QC a second time they were excluded from the study.

Relative leukocyte telomere length (LTL) was measured using real-time quantitative PCR (QPCR; (Cawthon, 2002)), using protocols previously developed and validated in sheep and cattle blood samples (Fairlie et al., 2016; Seeker et al., 2016)). The QPCR method estimates the total amount of telomeric sequence present in a sample relative to the amount of a non-variable copy number reference gene. In this study we used the beta-2-microglobulin (B2M) as our reference gene using primers supplied by Primer Design (Southampton, UK). For telomeric amplification tel1b (5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3') and tel 2b (5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3') primers were used (Epel et al., 2004). Telomere primers were manufactured, HPLC purified and supplied by Integrated DNA Technologies (IDT, Glasgow, UK).



Gel score:	1	2	3	4	5
Description:	1. Clear DNA band 2. All DNA bands on the same level 3. no smear	1. Clear DNA band 2. All DNA bands on the same level 3. Smear visible but clearly distinct from DNA band	1. DNA band is not clearly distinct from smear 2. Majority of DNA on same level as DNA bands of samples with gel scores 1-2	1. No DNA band visible 2. All DNA further down the gel than DNA bands of samples with gel scores 1-2	1. DNA migrated very far along the gel 2. No DNA band visible

Figure 4.1: Numerical DNA integrity gel score (Seeker et al., 2016)

Telomere and reference gene reactions were run in separate wells of the same qPCR plate at a concentration of 300 nM and 900 nM respectively. Samples were diluted to 1ng/µl with buffer AE just prior to qPCR analysis. Each reaction was prepared using 5ul of LightCycler 480SYBR Green I Master Mix (Cat No. 04887352001, Roche, West Sussex, UK) and 1ng of sample DNA in a total reaction volume of 10µl. We used 384 well plates which were loaded with sample DNA and master mix using an automated liquid handling robot (Freedom Evo-2 150; Tecan).

Each plate included two calibrator samples (1 ng/µl) to account for plate to plate variation and a non-template control (NTC) consisting of nuclease free water. The calibrator sample was extracted from a large quantity of buffy coat prepared from blood supplied from a single domestic sheep (Cat No. SHP-BUFCT-LIHP, Sera Laboratories International LTD, West Sussex, UK). We ran a large number of extractions from this sample, applied the same quality control as above and then pooled the extracts and aliquoted them for subsequent use. Samples, calibrators and NTCs were all run in triplicate. All qPCRs were performed using a Roche LC480

instrument using the following reaction protocol; 10 min at 95°C (enzyme activation), followed by 50 cycles of 15 s at 95°C (denaturation) and 30 s at 58°C (primer annealing), then 30 s at 72°C (signal acquisition). Melting curve protocol was 1 min at 95°C, 30s at 58°C, then 0.11°C/s to 95°C followed by 10 s at 40°C (cool down).

We used the LinRegPCR software package (version 2016.0; (Ruijter et al., 2009)) to correct our amplification curves for baseline fluorescence, and to calculate well-specific reaction efficiencies and Cq values. A constant fluorescent threshold was set within the window of linearity for each amplicon group, calculated using the average Cq across the first 6 plates. The threshold values used were 0.193 and 0.222, and the average efficiency across all plates were 1.88 and 1.91 for B2M and telomeres, respectively. Samples were excluded from further analysis if the coefficient of variation (CV) across triplicate Cq values for either amplicon was > 5%, or if at least one of their triplicate reactions had an efficiency that was 5% higher or lower than the mean efficiency across all wells on that plate for the respective amplicon. Overall, nine samples were excluded based on quality control failure at either extraction or QPCR stages, leaving 492 samples available for use in further analyses.

Relative LTL for each sample were calculated, following (Pfaffl, 2001), using average reaction efficiencies for each plate and Cq for each sample determined by LinRegPCR as follows:

$$LTL = (ETEL(CqTEL[Calibrator] - -CqTEL[Sample]))/(EB2M(CqB2M[Calibrator] - -CqB2M[Sample]))$$

Where ETEL and EB2M are the mean reaction efficiencies for the respective amplicon group across all samples on a given plate; CqTEL[Calibrator] and CqB2M[Calibrator] are the average Cqs for the relevant amplicon across all calibrator samples on the plate; and CqTEL[Sample] and CqB2M[Sample] are the average of the triplicate Cqs for the sample for each amplicon.

4.3.3 *Data Analysis*

We began by checking the distribution of our telomere, FEC and immune cell measures (figure 4.2). LTL was normally distributed but our FEC and immune measures showed left skew. Log transformation yielded approximately normally distributions, and so log transformed FEC and immune cell measures were used in all analyses that followed. 11 samples came from animals that were not caught at birth and so had uncertain ages and were excluded from further analyses. We went on to calculate the Pearson's correlation coefficient among LTL and the immune cell measures. To test how LTL varied with age and sex, we ran linear mixed effect models (LMMs) of LTL including individual identity (91 animals were caught in both years) and qPCR plate as random effects and year, sex and age (as a linear covariate) and an age-by-sex interaction as fixed effects. We subsequently tested whether independent effects of August weight and FEC on LTL were present, having accounted for age effects, by adding weight or FEC and its interaction with sex to the model and testing whether their addition improved model fit using LRTs and removing them if non-significant. We further tested whether horn type was associated with LTL by adding an age-by-sex-by horn type interaction and sequentially deleting non-significant terms associated with horn type until only significant terms remained in the model. Finally, to test for associations between LTL and leukocyte cell proportions, we tested the significance of adding all immune cell measures to the LMM and also of adding each measure separately to the LMM using LRTs. We went on to run LMMs of each immune cell measurement in a similar fashion to that described above for LTL except that we did not include a random effect of qPCR plate in these models. Firstly, in model 1, we tested for an age-by-sex interaction and sequentially removed non-significant terms based on LRT until only significant terms remained. Secondly, we tested a weight by sex interaction in the reduced model 1, containing only significant terms, and removed non-significant terms sequentially, but conserved any previously significant terms. Thirdly, we tested a horn type by sex interaction in the final model, which contained only the remaining significant terms from models 1 and 2.

To test our hypothesis that costs of investment in a key secondary sexual trait in males, horn length, might be reflected in either LTL or leukocyte cell proportions, we ran a separate set of LMMs restricting our data set to only normal horned males (N = 134). As horns are grown incrementally each year in sheep, horn length is very strongly determined by an animal's age. To avoid the potentially confounding association between age and horn length in our models (as LTL is age-dependent in males, see Results), we ran a separate models for lambs and adults. In lambs, horn length reflects horn growth over the first four months of life, and we tested their significance by adding a horn length term to a LMM with plate as random effect (there was only one observation per individual in this data set) and year as a fixed effect. In older males, horn growth continues to increase with age although in a decelerating fashion as the size of horn increments decreases with age (Robinson et al., 2006). We tested the effect of horn length on LTL in adults in a similar fashion to lambs, but included age as a fixed effect to account for the age-related change in both LTL and horn length. We assessed effects of horn length on immune cell measures in an identical way within LMMs of these measures. All analyses were conducted in R version 3.2.3 (R Development Core Team, 2008).

4.4 Results

We found a significant age by sex interaction in the models of LTL ($X^2_{(d.f.=1)} = 5.61$, $P < 0.05$): males, but not females, showed a decline in telomere length with age (figure 4.3). There was no significant difference among years ($X^2_{(1)} = 1.58$, $P = 0.21$). If we separated the data set by age and re-ran our LMMs (without individual as a random effect, because there were no or very few repeat measures in each data subset), we found that this sex difference was only significant in adults aged three or above (males vs. females: b =-0.089, se=0.042, $X^2_{(1)} = 4.58$, $P < 0.05$). There was no significant sex difference among lambs (b=0.004, se=0.023, p=0.855) or individuals aged 0-2 years (b=0.016, se=0.020, p=0.432). Neither August weight nor FEC predicted LTL independent of age and sex: addition of main effects and their

interactions with sex did not improve model fit ($X^2_{(2)}$= 1.29, P = 0.53 and $X^2_{(2)}$ = 0.36, P = 0.84, respectively). There was also no evidence that LTL varied with horn type (comparison of model including age by sex interaction with model including a horn type by age by sex three-way interaction and all associated lower order terms ($X^2_{(6)}$ = 3.15, P < 0.79)).

LTL and the leukocyte cell measures were only very weakly correlated (figure 4.2). Among the immune markers the strongest correlation was between the two most similar cell types, naïve CD8+ and naïve CD4+ T cells. There are were also moderate correlations between the two white blood cell markers eosinophils and NLR, and these two markers were also moderately correlated with the two naïve T cell subtypes, particularly NLR, which had a slightly stronger correlation with the naïve cells. None of the leukocyte cell proportion measures were significant predictors of LTL when fitted altogether (addition of all immune cell measures to model including age-by-sex interaction: $X^2_{(5)}$= 4.14, P = 0.53) and the age-by-sex interaction remained significant when all immune terms were included ($X^2_{(1)}$ = 4.32, P < 0.05). None of the leukocyte cell proportion measures were significant predictors of LTL when fitted individually to the model including the age-by-sex interaction (table 4.2).

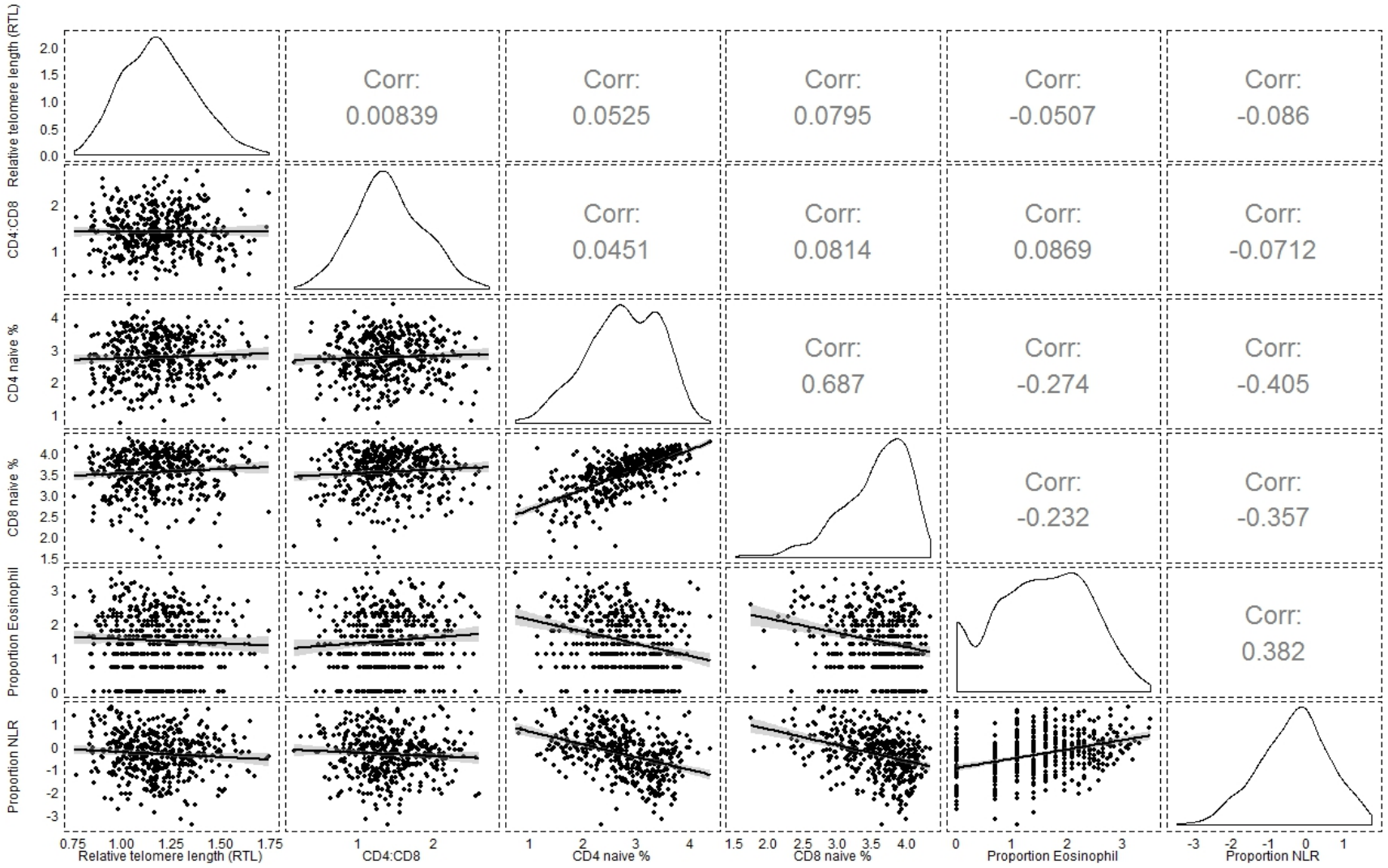


Figure 4.2: Scatter plot matrix of LTL and leukocyte subsets indicating the correlations between these measures and their individual distributions. The raw data is shown in the lower left area of the plot with smoother lines and shaded standard error indicating the relationship between the x and y variables. The distribution of each biomarker runs through along the diagonal of the plot. The correlation coefficients

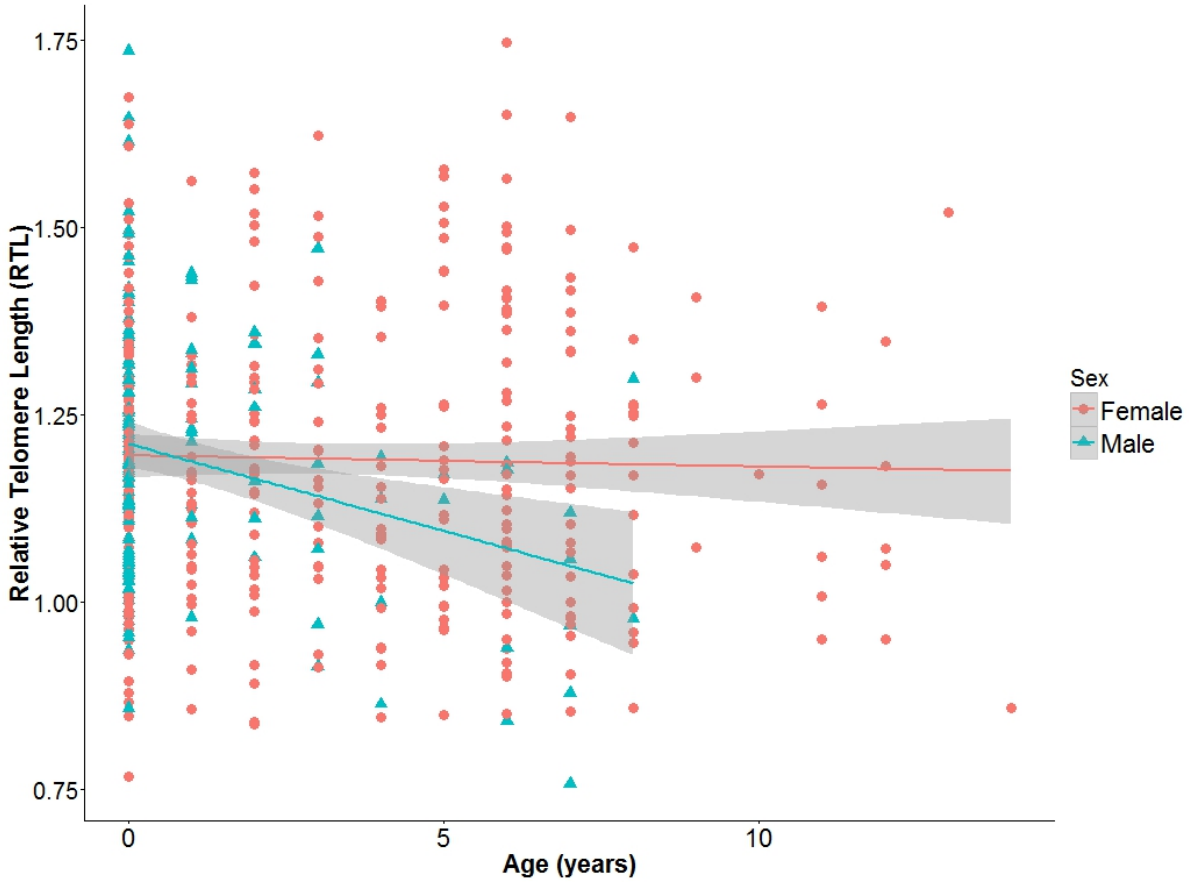


Figure 4.3: The raw data plotted to show the relationship between LTL and an age by sex interaction. Males (blue triangles) and females (red circles) are differentiated by shape and colour. There is a significant decrease in males, but not in females, although this is only significant in males 3 years and older. The lines represent the model output, and the standard error is shown by the grey area.

Table 4.2: Summary of LMM models of LTL testing the significance of each immune marker independently of the significant age by sex interaction. Each immune marker was independently added into the final model of LTL, which included an age by sex interaction. The reference factor levels for sex is females and 2014 for year.

Immune Marker	CD4:CD8		CD4+ naive		CD8+ naive		Eosinophil		NLR	
	b (se)	X^2 (p)	*b* (se)	X^2 (p)	*b* (se)	X^2 (p)	*b* (se)	X^2 (p)	*b* (se)	X^2 (p)
Year (2015 vs. 2014)	-0.017 (0.020)	0.786 (0.375)	-0.019 (0.017)	1.209 (0.272)	-0.013 (0.018)	0.568 (0.451)	-0.017 (0.017)	1.084 (0.298)	-0.017 (0.016)	1.112 (0.292)
Age	-0.002 (0.003)		-0.004 (0.004)		-0.001 (0.004)		-0.000 (0.003)		0.001 (0.003)	
Sex (M vs F)	0.009 (0.023)		0.013 (0.024)		0.010 (0.023)		0.018 (0.023)		0.019 (0.023)	
Age:Sex	-0.020 (0.008)	5.851 (0.156)	-0.021 (0.009)	6.307 (0.012)	-0.021 (0.008)	5.944 (0.015)	-0.017 (0.009)	3.748 (0.053)	-0.016 (0.009)	3.667 (0.056)
Immune marker	-0.002 (0.023)	0.011 (0.916)	-0.001 (0.018)	0.397 (0.529)	0.016 (0.023)	0.488 (0.485)	-0.005 (0.010)	0.211 (0.646)	-0.008 (0.010)	0.539 (0.463)

The models of each immune marker showed various associations with age, sex and weight, but none showed a significant relationship with horn type or evidence of horn type-by-sex interactions (table 4.3). The CD4:CD8 immune measure showed a decrease with age, but was not significantly related to sex or weight, either as main effects or within any of the interactions (table 4.3; figure 4.4). There was a significant age-by-sex interaction in the CD4+ naïve model, showing that these cells decrease with age, and that decrease is stronger in males than females (table 4.3; figure 4.4). CD8+ naïve T cells also decline with age, but as a main effect, not in an age-by-sex interaction, although we did find a significant sex-by-weight interaction, which showed a negative relationship between CD8+ naïve cells and weight in males, but not in females. Eosinophils increase slightly more with age in males than in females, shown in the significant age-by-sex interaction, and eosinophils decrease with weight in females, but not males, shown in the significant weight-by-sex interaction (table 4.3; figure 4.4). Finally, the model of NLR had a significant age-by-sex interaction, showing that the increase in NLR with age is slightly stronger in males, but this result is no longer significant ($X^2_{(1)} = 0.086$, $P = 0.842$) when we include a weight as a main effect (table 4.3; figure 4.4).

Table 4.3: Summary of LMM models of immune variables and relationships with age (model 1), weight (model 2) and horn type (model 3), including an interaction with sex for each of these variables. Firstly, in model 1, I tested an age by sex interaction and then if non- significant, based on test statistic (X^2), I tested the main effects of age and sex. Secondly, I tested a weight by sex interaction in the reduced model 1, containing only significant terms (indicated in bold), and then weight as a main effect if the interaction was non-significant. Thirdly, I tested a horn type by sex interaction in the final model, containing remaining significant terms form models 1 and 2. The reference factor levels are females for sex and scurs for horn type. Although not reported, year was included in all models to account for annual variation.

Immune Marker	Model 1						Model 2				Model 3
	Age		Sex (M Vs F)		Age:Sex		Weight		Weight:Sex		Horn Type:Sex
	b (se)	X^2 (p)	*b* (se)	X^2 (p)	*b* (se)	X^2 (p)	*b* (se)	X^2 (p)	*b* (se)	X^2 (p)	X^2 (p)
CD4:CD8	**-0.041 (0.006)**	**41.94 (<0.001)**		2.401 (0.121)		0.136 (0.712)		2.199 (0.138)		1.196 (0.274)	0.212 (0.642)
CD4+ naïve	-0.144 (0.009)		0.156 (0.063)		**-0.057 (0.023)**	**6.355 (0.012)**				1.383 (0.240)	0.489 (0.783)
CD8+ naïve	**-0.095 (0.006)**	**<0.001**		0.692 (0.405)		0.069 (0.792)	-0.022 (0.006)		**0.013 (0.006)**	**4.855 (0.028)**	0.074 (0.786)
Eosinophil	0.160 (0.040)		-0.341 (0.106)		**0.106 (0.040)**	**7.174 (0.007)**	0.069 (0.014)		**-0.060 (0.023)**	**6.923 (0.009)**	0.019 (0.892)
NLR	0.144 (0.014)		-0.089 (0.100)		**0.091 (0.038)**	**5.839 (0.016)**	**-0.002 (0.046)**	**11.686 (0.001)**		0.076 (0.783)	0.012 (0.913)

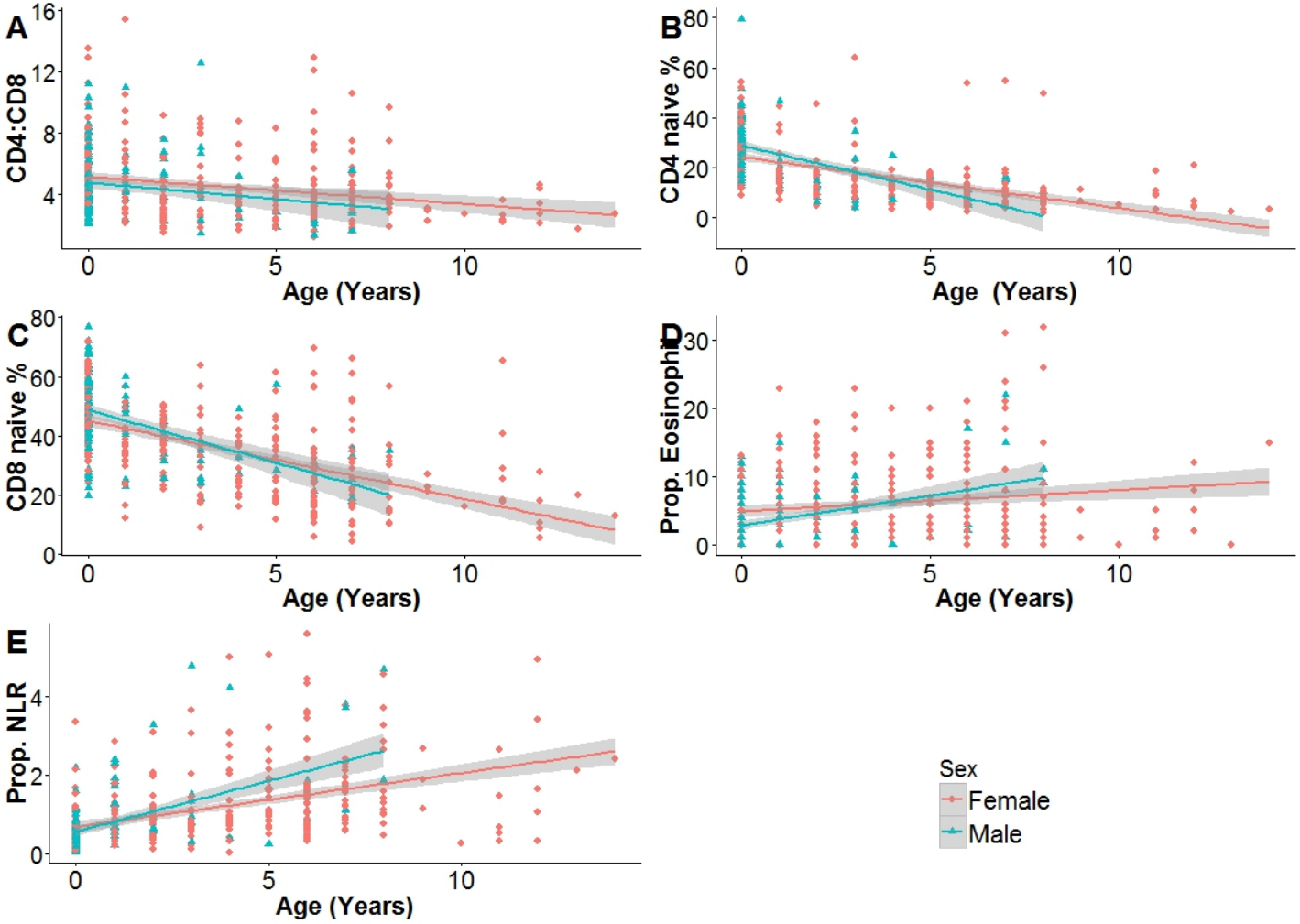


Figure 4.4: Each of the five plots shows the relationship between each immune marker and age, (A) CD4CD8, (B) CD4 naïve, (C) CD8 naïve, (D) Eosinophil, (E) NLR. Males (blue triangles) and females (red circles) are differentiated by shape and colour. The lines represent the model output and the standard error is shown by the grey area.

Horn length was negatively associated with LTL in normal horned male lambs, but not in older individuals once their age was accounted for (figure 4.5). In a model of LTL including all normal horned males, there was a significant decline in LTL with age (b= -0.023 +/- 0.008 SE, $X^2_{(1)} = 9.28$, $P < 0.01$). In a model restricted to lambs only, horn length significantly negatively predicted LTL (b= -0.0010 +/- 0.0004 SE, $X^2_{(1)} = 5.43$, $P < 0.05$; figure 4.5A) and remained significant when lamb weight was included in the model ($X^2_{(1)}) = 11.56$, $P < 0.01$). In models including older normal horned males, horn length was significant when age was not included in the model (b= -0.0007 +/- 0.0002 SE, $X^2_{(1)} = 10.06$, $P < 0.01$; figure 4.5B) but was non-significant when the age-related decline in LTL was accounted for (b= -0.0002 +/- 0.0003 SE, $X^2_{(1)} = 0.59$, $P = 0.44$; figure 4.5C). Horn length in normal horned males was not associated with any of the immune markers, either in the models restricted to lambs or those including only older individuals (1 year old and above) once age was accounted for (table 4.4).

Table 4.4: Summary of LLM models testing the effect of horn length for each immune marker independently. I ran separate models for lambs and adults 1 year and older, and the adult models also included age as an additional main effect. Although not reported, year was included in all models to account for annual variation.

	Lambs		Adults			
	Horn Length		Age		Horn Length	
Immune Marker	*b* (se)	X^2 p	*b* (se)	X^2 p	*b* (se)	X^2 p
CD4:CD8	-0.001 (0.001)	0.124 (0.303)	-0.016 (0.056)	0.079 (0.780)	-0.002 (0.001)	3.854 (0.058)
CD4+ naïve	0.001 (0.001)	0.837 (0.363)	-0.131 (0.068)	3.696 (0.063)	0.000 (0.001)	0.057 (0.812)
CD8+ naive	-0.000 (0.001)	0.062 (0.805)	-0.063 (0.040)	2.437 (0.128)	0.000 (0.001)	0.181 (0.673)
Eosinophil	0.008 (0.002)	0.698 (0.406)	0.235 (0.119)	3.937 (0.056)	-0.004 (0.002)	2.971 (0.095)
NLR	0.001 (0.002)	0.457 (0.501)	0.106 (0.132)	0.647 (0.428)	0.001 (0.003)	0.225 (0.639)

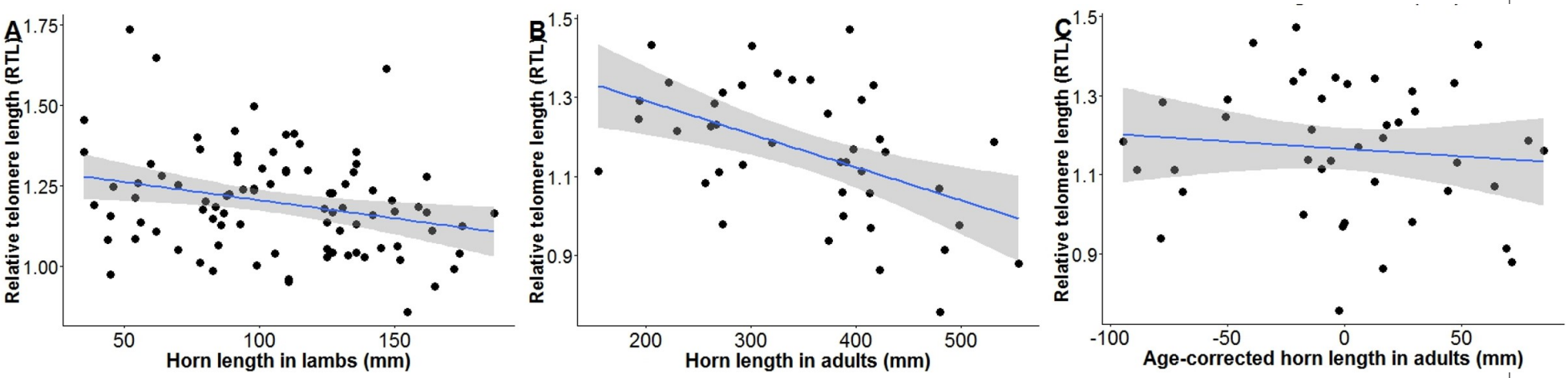


Figure 4.5: The relationship between horn length and LTL in (A) Lambs, (B) Older adults, and (C) Older adults using age-corrected horn length. The line represents the model output, and the standard error is shown by the grey area.

4.5 Discussion

In this analysis we found an as yet unreported age-dependant sex difference in LTL in a wild mammal. Our results show that males aged three or older have significantly lower LTL than females of the same age, but that this is not true in early life. This pattern mirrors studies of humans and laboratory rodents. Shorter LTL in adulthood in males appears a general trend in humans (Gardner et al., 2014), but whether this pattern is also generally present in early life remains controversial. Several small-scale studies measuring TL in cord blood could not identify significant sex differences (all N < 200; (Akkad et al., 2006; Aubert et al., 2012; Okuda et al., 2002; Shi-Ni et al., 2013), but a more recent study with a much larger sample size did find significantly shorter TL in males (N = 490; (Factor-Litvak et al., 2016)). A study measuring LTL by QPCR on a massive scale in cross-sectional samples from subjects aged 20 years and older (N > 100,000) found that significantly shorter telomeres in males were only observed after 50 years of age (Lapham et al., 2015). Studies in lab rats, which measured TL in a range of organs, also found that sex differences became more pronounced in older animals (Cherif et al., 2003; Tarry-Adkins et al., 2006). In wild Soay sheep, the presence of shorter LTL in older males could be due to sex differences in telomere attrition rate or to selective mortality associated with telomere length. Sex differences in selection on ETL have been documented in wild sand lizards (*Lacerta agilis*; (Olsson et al., 2011)), and winter mortality in Soay sheep on St Kilda is male-biased at all ages (Clutton-Brock and Pemberton, 2004). However, current evidence from wild vertebrates points to positive associations among LTL or ELT and either annual survival or longevity (Barrett et al., 2013; Beirne et al., 2014; Bize et al., 2009; Fairlie et al., 2016; Olsson et al., 2011; Salomons et al., 2009). Thus, male-biased selective disappearance would be expected to increase mean LTL with age at the population in males relative to females, which is opposite to our observations. Sex differences in telomere attrition rate therefore seem the most plausible explanation for our results, although longitudinal telomere data spanning the period from birth to later adulthood are required to demonstrate this conclusively. That males are subject to a faster rate of telomere attrition than females, could be

driven by differences in reproductive effort between the sexes. Male-male competition is vital for access to females and mating opportunities, but can cause a great deal of physical damage to males, including increased cellular damage, increased metabolic rate during fights, and increased costs of repair to damage caused during the rut period. This could be contributing to telomere attrition. According to the disposable soma theory of ageing, either a reduction in investment towards cellular maintenance or an increased demand on finite resources towards repair, would lead to faster rate of accumulation of cellular damage, which in turn would contribute to faster telomere attrition in males. However, as mentioned previously, there is a need to analyse within individual variation in LTL to determine conclusively that males have a faster rate of attrition than females.

We have presented rare evidence of reduced telomere length associated with investment in a reproductive trait under natural conditions. This is consistent with the hypothesis that investment in growth and reproduction should be reflected by telomere shortening, due to increased cell proliferation requirements and oxidative stress (Monaghan, 2014; Monaghan and Haussmann, 2006). Studies of birds in both the laboratory and the wild have found that experimentally increasing reproductive effort decreased ETL in parents, in the short term at least ((Heidinger et al., 2012; Reichert et al., 2014; Sudyka et al., 2014), but see (Beaulieu et al., 2011; Voillemot et al., 2012)), whilst a non-manipulative field study found negative associations between ETL and arrival date and the number of nestlings (Bauch et al., 2013). We found a significant negative association between LTL and horn length in males at around four months of age, but not in older males. This is consistent with LTL shortening reflecting some physiological cost of horn growth, but raises the question of why it was only detected in lambs. Lambs aged four months are growing rapidly and contending with their first exposure to prevalent nematode parasites on St Kilda (Clutton-Brock and Pemberton, 2004), and this may mean that the costs of investment in secondary sexual traits such as horn growth are most pronounced at this age. This stage also captures the relationship between LTL and horn growth prior to potentially confounding effects of over-winter viability selection and subsequent rutting effort and incremental horn growth, which could make the cost easier to detect. A study of

wild sand lizards documented disruptive selection on ETL in females but not in males (Olsson et al., 2011), and previous work on Soay sheep showed that the alleles associated with horn growth improve breeding success come at a cost to longevity in males but not females (Johnston et al., 2013). These studies and our present results suggest that the degree to which sex differences in LTL are generated and maintained by sexual differences in selection on telomere length is an important area for future study.

We found no sign of any relationship between LTL and either weight or Strongyle FEC in either sex, suggesting sex differences in growth or body size and in infection with gastrointestinal parasites could not explain observed sex differences in LTL in later life. This adds to the general lack of support for the sexual size dimorphism hypothesis from both among- and within-species studies (Barrett and Richardson, 2011; Beirne et al., 2014; Olsson et al., 2011). Although previous studies have found associations between micro-parasite infection status and telomere length (Asghar et al., 2015; Ilmonen et al., 2008), this study measured a proxy of burden with chronically infecting nematode parasites. It seems plausible that a larger longitudinal study may be required to detect the immune consequences of such long lasting infections for LTL. Studies in laboratory rats have suggested sex differences in TL emerge around puberty as a direct result of the differential effects of sex hormones on telomere dynamics (Cherif et al., 2003; Tarry-Adkins et al., 2006). Soay sheep are sexually mature in their first year, so our data imply that sex differences in LTL emerge several years after puberty, but could still be the result of cumulative telomere eroding effects of testosterone relative to oestrogen. There may also be indirect effects of testosterone on LTL, such as increased aggression, which might increase interactions with other males and lead to a greater demand on resources, higher levels of oxidative damage and increased need for cellular repair, resulting in reduced lifespan. Over and above hormonal causes, males surviving to later adulthood will have experienced the cumulative physiological demands associated with years of rutting which could generate further differences in the rate of telomere attrition compared to females.

In our models of immune measures we found that all of the markers showed clear increases or declines with age. These findings are supported by our previous work (see Chapter 1), and by research on immune phenotypes more generally. We also found various associations relating to sex and weight, but none of the immune markers were significantly related to either horn type or length. The CD4:CD8 immune measure was the only marker to have a significant relationship with age alone, and no associations with any of the other variables. We found a decrease in CD4:CD8 with age, however, there are contradictory findings in humans, with some studies showing a decrease with age (Lu et al., 2015), and others an increase in CD4:CD8 with age (Yan et al., 2010). There was a significant age-by-sex interaction in the CD4+ naïve model, showing that these cells decrease with age, and that decrease is stronger in males than females. This sex difference has not been identified in this population before, however, previous work has shown females to have higher CD4+ naïve proportions than males, which is supported by literature in humans and mice (Caccamo et al., 2006; Pido-Lopez et al., 2001; Scotland et al., 2011). Furthermore, the presence of a sex by age interaction for CD4+ naïve cells, driven by males that have more rapid rates of declines compared to females, could have been responsible for the observed sex difference in LTL with age. CD8+ naïve T cells also declined with age, but this did not differ with sex. The decline in the proportion of naïve helper and cytotoxic T cells could generate population level declines in mean LTL because naïve T cells have longer telomeres than effector and memory T cells (Aubert et al., 2012; Weng, 2001). Both eosinophils and NLR increase slightly more with age in males than in females, which could be expected to drive an increase in average LTL with age, as combined they represent granulocytes which tend to have longer TL than lymphocytes (Aubert et al., 2012; Weng, 2001).

We also found interesting relationships with immune cells and weight; eosinophils decrease with weight in females but not males, but CD8+ naïve cells decrease with weight in males but not in females. This could be indicative of variation between the sexes of investment within the immune system. It is not possible to determine the reasons for this from this analysis, but a potential cause of sexual variation in immunity could be related to sex hormones, known to both suppress and

increase the immune response (Grossman, 1985; Klein, 2000), or variation in selection pressures between the sexes; reproductive success is driven by weight in males, but age in females (Hayward, 2013; Preston et al., 2003).

Despite this variation with age and sex in the immune markers, the results show indirect evidence that the observed relationships among LTL, age and sex are not driven by variation in the proportions of particular leukocyte cell types, which are known have different telomere lengths during adulthood in humans (Aubert et al., 2012; Kimura et al., 2010; Weng, 2001). As in chapter 1, we found age-related changes in the proportions of different leukocyte cell types that were consistent with observations in humans and laboratory mice, and in our own previous studies of this system (Linton and Dorshkind, 2004; Nussey et al., 2012; Pawelec et al., 2010; Watson et al., 2016). However, we found little evidence that LTL was correlated with any of the leukocyte proportion measurements and we showed that the main results of our LTL models were not influenced by the inclusion of these measurements. Although our approach is crude in comparison to human studies which have used cell sorting or flow-FISH techniques to directly measure TL in different cell types, our findings are consistent with the mounting evidence that within individual TL is highly correlated across leukocyte cell types and across tissues more generally (Aubert et al., 2012; Daniali et al., 2013; Kimura et al., 2010; Reichert et al., 2013). This suggests LTL or ETL could reflect variation that exists in the individual's haematopoietic stem cell compartment, and even more general organism-wide variation in telomere length (Daniali et al., 2013; Kimura et al., 2010; Reichert et al., 2013).

Chapter 5

General Discussion

5.1 Thesis overview

Ungulates have been proposed as a good study system for wild immunology, particularly with the wide range of reagents available from domestic research (Jolles et al., 2015). In addition, ungulates are also a reasonably long-lived group which reproduce across multiple breeding seasons. This thesis took advantage of these benefits and explored how immune variation is related to life history traits in a wild system. The results highlight both the complexity of studying immunity in the wild and the variety of information to be gained in doing so, particularly with regard to the impact of immune investment on survival. I measured a broad panel of specific markers, initially attempting to characterise the individual immune variation in chapter 2, supporting evidence of strong age trends across markers and some sex differences found in experimental lab studies. In chapter 3 the analysis testing for trade-offs between early life growth and development of immunity in lambs, suggested that the optimal strategy was one of moderation for both growth and immunity. In both chapters 2 and 3, the question of whether immunity was associated with survival pointed to the importance of parasite specific antibodies. However, it is also clear that even in this relatively simple system, without the interactions between micro- and macro-parasites or immigration from other populations, these associations with survival are complex due to the sex and age dependency of the relationships. The results of chapter 4 add to this overall complexity with an age-dependant sex difference in leukocyte telomere length (LTL) attrition, but no relationship between T cell populations and LTL. The work in this thesis identifies diverse age and sex dependent relationships with immunity and highlights the advantages of using available reagents to measure immune markers specific to ecologically relevant parasites. The associations with immunity and early life development, particularly with survival, demonstrate the importance of immune variation and with the addition of longitudinal analysis, we can aim to further our understanding of the fitness consequences of immune investment under natural conditions.

5.2 Key findings

5.2.1 Chapter 2

This analysis exposed strong age trends among immune markers, which were in line with intial predictions and with previous work in this population and experimental lab studies of immune variation with age. The decline in naïve CD4+ and CD8+ T cells demonstrates evidence of immunosensence in the wild, and the increases in anti-*Teladorsagia circumcincta (T. circ)* antibodies shows the development of specific immunity with age, as it would be expected with acquired immunity. The ability to confirm trends previously identified in experimental work demonstrates that when reagents are available, it is possible to apply immune techniques and measure specific cell types in a wild system. In addition to age trends, I also found a sex difference in gamma delta and naïve CD4+ and CD8+ T cells, with females having higher proportions of these T cell subtypes than males. This was in line with the predictions made and is supported by evidence that females have higher immunity than males, especially in human literature, and is the first time this sex difference in T cell subtypes has been shown in the wild. It was expected that higher immunity would be related to increased survival, and while we did identify this relationship in one immune marker, it was not true of immunity overall. Survival was positively related to high measures of anti-*T. circ* IgG independent of age and sex, suggesting a survival benefit of investment into parasite specific immunity. This result supports a previous study in this population by Nussey et al. (2014), and confirms the robustness of this relationship across an additional population crash, including more individuals and for both sexes.

5.2.2 Chapter 3

In the analysis of trade-offs between growth and immunity in early life, faster growers had reduced measures of anti-*T. circ* IgG and IgE antibodies, which suggests that optimal immunity is not necessarily the best strategy for fitness and that, equally maximal growth comes at a cost. This result met our prediciton that high growth in early life would negatively impact immune development, and in addition highlights that low immunity may also be indicative of poor quality. The data show that slow growers have reduced anti-*T. circ* IgG and IgE antibodies and high measures of Neutrophil:Lymphcyte ratio (NLR), possibly as these individuals are experiencing chronic inflammation and are unable to maintain resources for growth and antibody production. Individuals with low faecal egg count (FEC) had significantly higher measures of anti-*T. circ* IgG and IgA, which provides evidence of the protective role these parasite specific immune markers play against gastro-intestinal (GIN), as predicted. Contrary to the findings in Chapter2, I found that in lambs, there is a sex-specific trade-off between anti-*T. circ* IgG and IgE and survival; males with higher antibody measures were less likely to survive the first winter, but in females there was no significant relationship. This is evidence of sex-specific selection pressures on survival in early life and the association with sex-specific variation in immunity. As previous analyses did not identify a sex difference in survival, or a relationship with anti-*T. circ* IgE, this result was not expected and highlights the importance of age in not only immune variation but also in the relationships between immuntiy and life history traits.

5.2.3 Chapter 4

I found an age-dependent sex difference in LTL, as males aged three years or above had significantly higher LTL attrition with age. This is the first time this sex difference in LTL attrition with age has been documented in a wild system. It was anticipated that males may exhibit shorter telomeres, an expectation which was

supported in the analysis, but this was not due to the sex differences in leukocyte proportions and initially predicted. Despite strong age trends among leukocyte subpopulations, I found that the decline in LTL with age was independent of variation in leukocyte populations composition with age, suggesting that immune decline and LTL attrition biomarkers of ageing are distinct from one another. The analysis in Chapter 4 also found evidence of an age-dependent trade-off between investment in secondary sexual characteristics and LTL. Among normal-horned male lambs there is a negative relationship between horn growth and LTL, with male lambs with longer horns having shorter LTL, however this relationship was not significant in older males. It was anticipated that there would be a negative relationship between LTL and horn length, but it was unexpected that this was only significant in the youngest age class. Here, and in chapter 3 the results are directing a focus towards early life and highlight this life stage as an important time for interactions between immunity and life history traits.

5.3 Study limitations

5.3.1 Flow cytometry

The constraints on tools available for use directly on samples in the field required the cells to be fixed and transported back to Edinburgh for analysis by flow cytometry. This needed additional steps involved in fixing and processing samples, which ideally would be avoided, as multiple manipulations of a sample undoubtedly increases the risk of error in measurement. Also, in relation to analysis by flow cytometry, fixation can cause cells to become ‘sticky’ and the sample to clump together. While care was taken to avoid this, and most samples were analysed without too much issue, it is an unavoidable consequence of fixation, and some samples were unable to be used due to cell clumping. Advances in the equipment used to perform these techniques are producing machines which are more compact and porTable, so in the future, it may be

possible to analyse cells immediately in the field, removing the need for cell fixation and the associated issues this brings.

Across a five-year period we fixed cells and analysed them by flow cytometry, but due to issues that arose with cell fixation and processing, samples from two of the years (2012 and 2013) were not able to be measured. The requirement to fix leukocytes and transport them to Edinburgh for analysis via flow cytometry at a later date became more of an issue when switching from the original indirect stains to the multi-panel stain. In the initial analysis in 2011, cells were fixed in August and then analysed via flow cytometry up to seven months after fixation. This time period under fixation did not appear to have any negative effect on cell proportions, and indeed when the same cell types were compared from analysis at two and seven months, the proportions were highly correlated. Therefore, we accepted that cells could be analysed up to seven months post fixation. Development of the multi-stain assay required time intensive cell culture techniques and antibody purification steps. In addition, the antibody conjugations and dilution optimization took a great deal of time to validate, given the small-scale in-house techniques available. As result of this, and given the success of the 2011 staining, cells in 2012 and 2013 were ran within six months of fixation. However, it appears retrospectively that this was not soon enough, and I found that as time since fixation increased the number of cells measured successfully with the multi-stain assay decreased. Unfortunately, this trend was only possible to identify after multiple years had been analysed, which resulted in T cell data using the multi-stain assay being successfully collected in 2014 and 2015 only when samples were run within two months of fixation. While it is possible to carry out immunological techniques in the wild, caution is required regarding the wide range of issues that may arise given the unknown effects of the adaptations needed to apply sophisticated immune techniques under field conditions.

5.3.2 Infection status

In this study I used FEC as a measure of parasite burden during analyses, however, FEC is an indirect measure of parasite load, as it measures the reproductive output of the worms rather than the actual number of worms present in the host. Therefore, in this study individual infection status was not directly addressed and so it could be argued that the relationships with immune markers were not explicitly related to the hosts infection status. While it is true that measuring parasite burden using FEC can be quite variable, the non-invasive nature of this study system makes it difficult to identify exact worm numbers in a living host. In addition, it is possible to sample individuals post-mortem, and studies in this Soay sheep population show that during crash years FEC and worm burden are highly correlated, suggesting that FEC is a good indicator of GIN worm burden (Grenfell et al., 1995). However, there was no measure of parasite exposure included in this study, and so we do not know if parasite exposure varied between individuals, which could have an influence on the immune response.

The analysis in chapter 2 demonstrate that parasite-specific antibodies, anti-*T. circ* IgA and IgG, were negatively associated with FEC, suggesting that there is a protective role of anti-*T. circ* antibodies against GIN. While parasitic exposure would not adequately explain the negative associations we found between FEC and anti-*T. circ* IgG and anti-*T. circ* IgA antibodies, it could still be influencing these relationships. Therefore, we may be finding a much weaker negative relationship than if variation in exposure was properly accounted for. In the chapter 2 analysis, we accounted for temporal variation and variation in population density, which is also known to influence FEC (Wilson et al., 2004), by fitting year as a fixed effect, but this only accounts for annual variation rather than seasonal changes. It has previously been reported in this system that parasite exposure varies both spatially across the pasture and seasonally throughout the year (Gulland, 1992; Wilson et al., 2004). As such, we might not be capturing the whole picture with our single measures of parasite burden and immunity. Additionally, this relationship between FEC and

anti-Tc antibodies in chapter 2 was in lambs only, which generally have very high FEC (Clutton-Brock and Pemberton, 2004). Therefore, it would be interesting to explore this further and test whether this relationship varies with age.

Additionally, the Soay sheep on St Kilda are a relatively simple system, with a great deal of knowledge relating to individual life histories, ecologically relevant parasites and importantly the reagents to measure the antibodies against them. However, while this simplicity can be helpful in understanding the relationships between parasites and immunity, it is not possible to examine more complex associations which might occur with macro- and micro-parasites for example. Therefore, while this study highlights parasite-specific markers as important immune measures, we have only used a marker for one type of parasite, and a more complicated system would be needed to address the effects of parasite interactions on the host immune response.

5.4 Future directions

5.4.1 Immune markers

This study set out to test whether more specific immune measures, as a response to natural infection, could be significant in identifying the costs of immune investment, but there are many approaches to studying immune variation in the wild. Artificial stimulation of the immune system in vivo is useful in experimental manipulation and to separate immune response from parasite pathology (Zuk and Stoehr, 2002). Other studies have taken a different approach, such as measuring pro- and anti-inflammatory responses (transcription factors and cytokines) in vitro using Q-PCR to measure a broad panel of functional immune markers, including cytokine expression in splenocyte cultures (Jackson et al., 2011), although this requires invasive sampling of the animal, which is not always feasible. Other work in wild rodents has focus on

immune genes, identifying a biomarker of immune tolerance (Gata3) and highlighting the importance of longitudinal sampling in understanding how immunity and trade-offs with immunity can change with age within an individual (Jackson et al., 2014).

The analysis in this study highlighted some immune markers, particularly the parasite-specific antibodies, to be significantly related to life history traits, suggesting there is importance and value in exploring trade-offs in wild systems. However, we did not find any of the T cell subtypes to have any significant associations with life history traits. Through the availability of domestic reagents that can be applied in this system, there is a distinct advantage to examining these specific immune markers, in order to justify whether the information gained warrants the expenditure, time, and energy to validate them in non-model systems. Therefore, given the importance of the parasite-specific markers measured in this study, I would argue that targeting measures which are directly related to the parasite ecology of the study system would be the most effective approach to identifying immune markers which are associated with life history trade-offs and survival.

This study had access to a wide range of reagents to measure a novel panel of immune markers, but there are of course many other markers, which could potentially be important indicators of fitness. In particular, this study did not incorporate a measure of immune function, which would provide information about how well the cells protect the host in the response rather than just the quantity of cell types. During the course of this work, I collected and cryopreserved samples for such functional assays, although there was insufficient time to take this further. These stored samples would be an interesting focus of future work to identify a broader immune phenotype. Following successful resuscitation, stimulation assays could be used to measure the functional capacity of specific immune cells to respond to parasitic exposure. Additional immune markers of interest to eco-immunologist could be identified in other types of parasite specific markers. This study measured antibodies specific to *T. circ* specific, and although these ELISAs have high cross-reactivity between related parasite species, this has not been tested for unrelated parasite species (Watt et al.

unpublished). GIN are not the only parasites present in the Soay sheep, and identifying the relationship of immune markers specific to other parasites might prove to be interesting. Protazoan parasites could be the next target group, especially *Eimeria* species, such as coccidia, which have been associated with weight in adults (Craig et al., 2006).

5.4.2 Reproductive fitness

Evolutionary fitness is a combination of both survival and reproductive success. While this study examined the costs of immunity on survival, I did not test whether there were any reproductive costs to immune investment. There is evidence that there is negative relationship between immune investment and reproductive success, especially under restricted resources (Adamo et al., 2001; Folstad and Karter, 1992; French et al., 2007). In this population, individuals with high natural antibodies also had increased over-winter survival, but at a cost of reduced reproduction (Graham et al., 2010). In addition, adults with high measures of pan-isotype anti-*T. circ* antibodies were found to have reduced reproductive success (Hayward et al., 2014). This thesis did not explore the relationship between reproduction and immunity, largely due to the mostly cross-sectional nature of the data. However, it could be possible to test for an immune cost to the age at first reproductive event in a small number of individuals, which might be related to immune development in early life. A full examination of whether immune investment is related to reproductive fitness would require a larger, longitudinal data set. In particular, it would be interesting to explore the relationship between lifetime reproductive success and the observed decline in immunity with age. As we know that males and females differ in lifespan, amongst other traits, this future analysis might develop upon the sex-dependent relationships I found between survival and immunity, testing whether there are trade-offs with reproduction occurring. The hypothesis being that males invest less in immunity and somatic maintenance during early life to attribute more resources to reproductive traits which comes at a cost to longevity, but an increase in reproductive

success. On the other hand, females invest more resources towards immunity and somatic maintenance in order to increase both longevity and their reproductive potential.

5.4.3 Sex differences

The mechanisms driving sex-biases in immunity and parasitism are linked to the costs of high somatic growth due to sexual dimorphism and the immunosuppressive effect of endocrine hormones (Folstad and Karter, 1992; Owens and Wilson, 1999; Rolff, 2002; Sheldon and Verhulst, 1996). In chapter 3, I found a survival cost of immune investment in male lambs; those that did not survive over-winter had higher measures of anti-*T. circ* IgE and IgG. Additionally, in chapter 4, I found a negative relationship in male normal-horned lambs between LTL and horn growth, indicating a trade-off between investment in secondary sexual characteristics and somatic maintenance. These two sex-specific trade-offs could be due to some individuals having higher testosterone, which as a result of immunosuppression reduces resource investment in immunity and allows for greater investment in horn growth or survival. Further work could explore the effects of endocrine hormones, particularly testosterone, on the immune response and whether this is related to the occurrence of sex-specific variation in immunity and survival.

Interestingly, these sex-specific relationships were only significant in lambs, and were not present in older individuals. A potential explanation for this could be the selective disappearance of males which invest heavily in immunity as this is associated with reduced survival, and so they are removed from the population. Equally males which invest in high somatic maintenance may also be selected against as this is related to reduced horn growth, which could affect mating success and reproductive fitness.

5.4.4 Longitudinal measures

In this thesis, despite taking samples over a number of years, there is still a lack of sufficient longitudinal data to look thoroughly at within individual variation across lifespan. Using the data available I was able find a great deal of variation in immunity between individuals of different ages, but how these markers vary within an individual over time is still unknown. Information concerning within individual variation would be valuable to understanding how trade-offs with immunity might vary over time, given changes in environmental conditions and population density. This information would be useful in a number of ways, for example, in chapter 2, females had higher proportions of naïve T cells, but I was unable to ascertain whether this was due to males having a lower pool of naïve T cells than females to begin with or whether males are less conservative than females in activating these cells.

In addition, as we know that parasite density and resource demands for traits such as reproduction fluctuate throughout the year, we could expect that immune markers might also vary temporally within a given year. Using the largely cross-sectional data in this study, I have found interesting variation in immunity, particularly regarding age, sex and survival. However, these relationships could be explored further by taking multiple measures throughout the year, particularly during periods of high reproductive investment, such as the rut or lambing seasons. This would provide information during a time of year when demand for resources for reproduction is high, and when we might expect trade-offs between immunity and reproductive investment to occur. However, as we are unable to obtain blood samples outside of the annual catch in August, transferring to using non-invasive methods of measuring immunity would be required to allow a seasonal analysis of immune variation within a year to be acquired. A recent study in this population has tested the potential to use faecal samples to measure antibodies, which would allow any seasonal variation in these immune measures to be captured (Watt et al., 2015).

5.4.5 Heritability

GIN are becoming a growing concern in livestock production, as current methods of control, primarily anthelminthic treatments, are becoming less effective as parasites develop resistance (Kaplan and Vidyashankar, 2012; Nieuwhof and Bishop, 2005; Papadopoulos, 2008; Torres-Acosta and Hoste, 2008). There are a number of alternative strategies, including grazing management, vaccination, nutritional supplementation, targeted selective treatments and genetics. Studies have shown resistance in GIN to be a moderately heriTable trait, which has fuelled further interest in developing selective breeding programmes to increase GIN resistance and reduce anthelminthic resistance (Moore et al., 2016; Sweeney et al., 2016; Woolaston et al., 1996). Selection for resistance against GIN and the integration of immunogenomics in ruminant breeding programmes, predominantly focusing on FEC, and have been effective in reducing FEC through selective breeding (Davies et al., 2006; Windon, 1990; Woolaston et al., 1996). In addition to GIN and FEC, there is interest in the heritability of immune markers as good candidates for a panel of phenotypic biomarkers to be used in future selective breeding programmes. A study in pigs measured 54 immune traits and showed that many were heriTable, including eosinophil and neutrophil counts, and a number of inflammatory markers, and it was serum inflammatory proteins levels and specific IgGs which demonstrated the greatest phenotypic variance (Flori et al., 2011).

Given the importance of the parasite specific immune markers in this study and the influence they appear to have on survival and growth in early life, they could make good candidates for future breeding programmes, especially given evidence of their heritability; IgA, IgG, FEC and eosinophils (Coltman et al., 2001; Flori et al., 2011; Henderson and Stear, 2006; Stear et al., 1999; Strain et al., 2002). Key factors to both ecological research and domestic breeders are the trade-offs that occur with immune investment and the impact of immune variation on survival. Wild systems could make a good starting point for determining useful biomarkers in selective breeding programmes, as individuals are under resource limitations resulting in investment

trade-offs, and are often under strong survival pressures. Despite the ability to measure these types of immune parameters, this study was unable to estimate heritability of immune measures, as this type of analyses would require a much larger data set to do so. However, due to the importance in domestic livestock of the consequences that some immune markers seem to have on fitness and growth, this would be an interesting focus for further work.

5.5 Concluding remarks

The outcomes of this thesis demonstrate the complexity of immune variation and the importance of studying immunity under natural conditions to determine the causes and consequences of this variation. The Soay sheep population are a useful system to test the informative value of particular immune markers to ecological questions under natural conditions, given the amount of life-history data available, and the potential to apply reagents developed for domestic ruminants. The work in this thesis highlights parasite specific markers to be a key player effecting survival, early life development, and parasite resistance, which demonstrates the ecology of the system to be an important factor in determining trade-offs and associations with immunity. The overall lack of correlation between immune markers, and indeed other biomarkers (LTL), suggests that there is much to be gained from a broad approach to immune phenotyping, to capture the breadth of variation across age and sex. However, despite this, ecologically relevant parasite specific markers are consistently the most influential marker in understanding the fitness consequences of immune variation in the wild. The age and sex specific variation found throughout this thesis point to interesting areas for future work, to examine the reproductive and long-term effects of immune investment, with the addition of longitudinal, within-individual analysis.

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St Kilda Soay Sheep Project

Photo credit: Arpat Ozgul. St Kilda Soay Sheep Project, Meet the Sheep. `http://soaysheep.biology.ed.ac.uk/meet-sheep`.

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Appendix

Supplementary methods of field protocols

1 Plasma and buffy coat preparation

- Aim to spin / aliquot samples the same day as catch (note down time of capture and time of spinning for each sample)
- *For plasma:*
  - Whole blood (the whole of one 9ml Vacutainer used) spun in unchilled centrifuge at 3,000 rpm for 10 mins
  - Plasma layer drawn off (with 3ml Pastette) and split into 5 roughly equal aliquots into 1.5 ml Eppendorfs (1 x red, 1 x yellow, 1 x green, 2 x clear)
- *For white blood cells:*
  - Plasma replaced by 0.9% NaCl up to 9ml mark (using squeezy bottle) on vacuette and mixed
  - Centrifuge at 3,000 rpm for 10 mins
  - White cells, plus many red cells, removed from top of cell layer (with 3ml Pastette) into a 1.5ml Eppendorf (1 x clear).
- Each set of the six aliquots placed in separate sealable bags and kept at -20°C

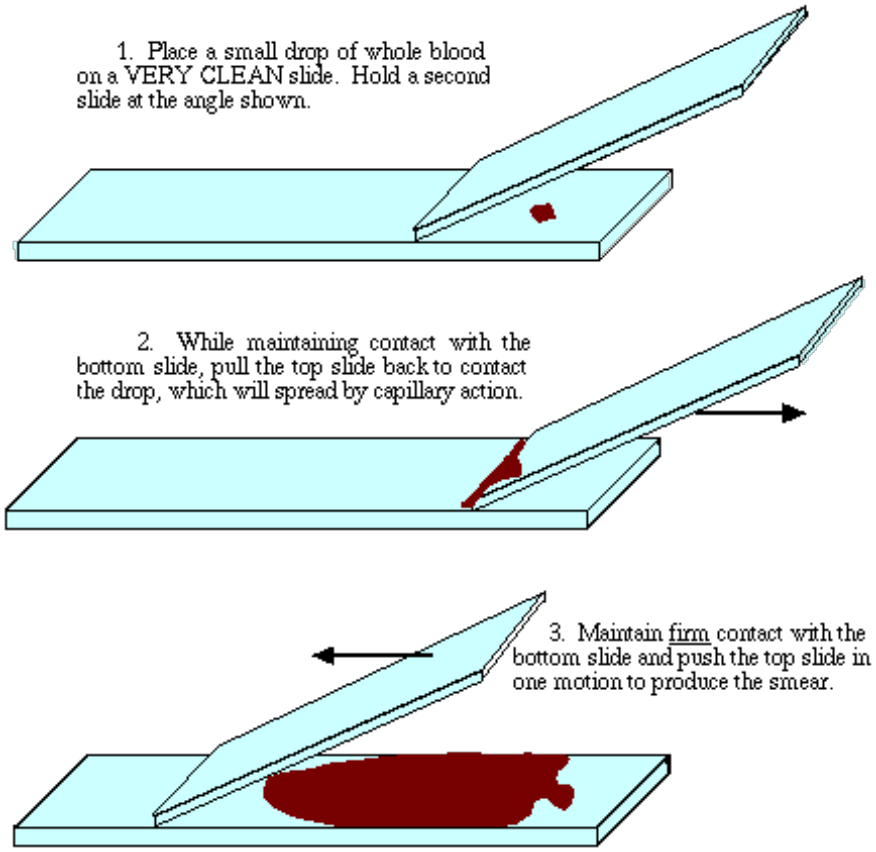
2 Whole blood smear stain for cell differential count

- Put 5ul whole blood (20ul Gilson+racked unsterile white tips) on to the sample slide (near frosted end)
- Put the edge of a second clean slide (spreader slide) at a 45° angle to the drop of blood, and “back” it into the drop of blood.
- Allow the blood to spread along edge of the spreader slide (holding a steep angle for a short smear or a shallow angle for a long smear)
- Push the spreader slide along the sample slide to other end, forming blood smear by pulling the blood drop
- Wipe the edge of the spreader slide with paper towel+clean water and use it as the next sample slide
- Air dry sample slides in cardboard trays overnight

Following day: stain the next day using Quick-Diff Kit stain

- 6 x 1 second dips in FIX solution
- 9 x 1 second dips in RED solution
- 9 x 1 second dips in BLUE solution
- Rinse (1-2 second dip) in 1:20 diluted 1x PBS
- Wipe back of slide and place in cardboard tray

Leave to dry overnight AND PUT CLINGFILM OVER THE BEAKERS Store in slide boxes



Appendix, figure S1: Slide preparation protocol for blood smear preparation (Caprette, 1996)

3 Leukocyte fixing for FACS analysis

Preparation:

- Select samples for fixing (same as Protocol 4 plus as many others as possible?)
- Print labels for two 1.5 ml ependorfs (one with PFA in already)
- Heat 20% PFA aliquot to 70°C (until it changes from cloudy to clear) + then dilute to 1% with 1x PBS
  - Dilute PFA to 1% (Put 12.5ml (10ml pipette+Orange Pipettor) 20% PFA (after heating) into an empty bottle, add 237.5mls (measuring cylinder) x1 PBS solution – will be enough for 100 samples). Can be done a few hours (or even the morning) before the samples need to be fixed
  - Dilute the 10x lysing solution to 1x
  - Use 5ml of the 1x lysing solution for each sample –put 50ml (measuring cylinder) of cold 10X solution into an empty bottle, and add 450mls (measuring cylinder) distilled water – will be enough for 100 samples. DO NOT STORE

Procedure:

- Into a 15mL Falcon tube add (with a 1ml Pastette) 1mL of Li-Heparin blood together with 5mL (10ml pipette+Orange Pipettor) 1X lysing solution (1mL Blood + 5mL 1X lysing solution).
- Mix gently (inverting gently only) until lysis is observed (blood turns a light slightly see through red colour, usually <30s and definitely <2 min), THEN centrifuge at 1200rpm for 10min
- Pour off supernatant from cell pellet and flick the base of the tube until pellet is re-suspended in remaining solution. Add 1ml PBS (with a 3ml Pastette) and do a rough volume check of what is in the tube (eg 1.1, 1.2, 1.4 mls), by sucking up into the Pastette, and note it on datasheets.
- Put 50ul (200µl Gilson and racked unsterile yellow tips) of the cell suspension

in a labelled eppendorf containing 0.5ml of 1% paraformaldehyde (filled in Edinburgh) and store at 4°C for immediate cell counting on return to Edinburgh.

- Add 9ml PBS (10ml pipette+Orange Pipettor) to the falcon tube to make up to around 10ml and mix, AND centrifuge at 1200rpm for 10min
- Discard supernatant and flick base of tube until pellet is re-suspended in remaining solution
- Add 2.5mL 1% Paraformaldehyde (PFA) in PBS (3ml Pastette) and mix
- Incubate at room temperature for 10 minutes, THEN centrifuge at 1200rpm for 10min
- Discard supernatant and flick base of tube until pellet is re-suspended in remaining solution
- Add 10mL PBS (10ml pipette+Orange Pipettor) and mix AND centrifuge at 1200rpm for 10 min
- Discard supernatant and flick base of tube until pellet is re-suspended in remaining solution
- Add 1mL PBS (1ml Pastette) containing 0.02% NaN3, mix, and transfer with 1ml Pastette to labelled 1.5ml Eppendorf. Store at 4°C prior to Flow Cytometry staining at MRI.

Leave to dry overnight AND PUT CLINGFILM OVER THE BEAKERS Store in slide boxes

4 Leukocyte cryo-preservation

Preparation:

- Get FCS out of freezer at time of catch
- Select samples for cryo-preservation [based on: (i) whether FACS assayed in August 2010; (ii) proportion of previous years alive caught and weight in August, (iii) birth weight available, (iv) mum's birth weight available]
- Prepare a Freezing Solution of 10
- Label 15ml falcons and print labels for 2x 1.8 ml Nunc cryo-tubes.

Procedure:

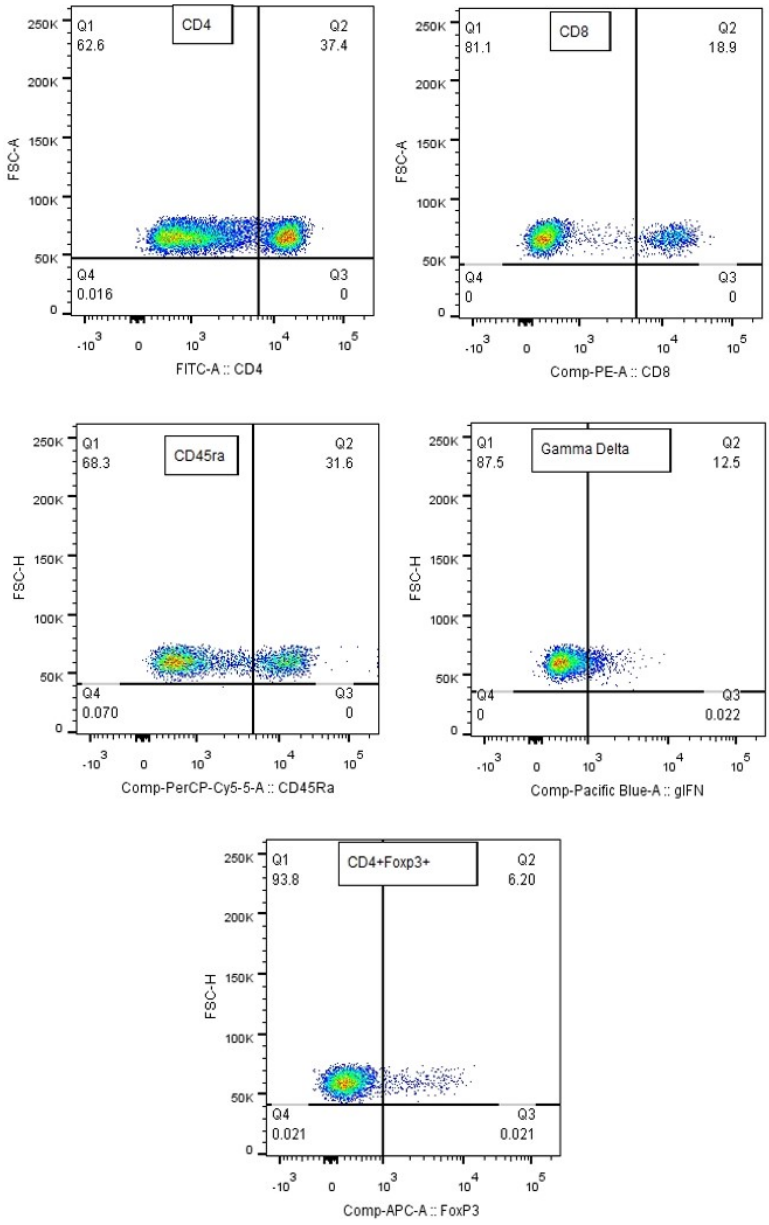
- In a 15mL Falcon tube add 2mL (which should contain 5-7x106 WBC per ml = 1.0-1.4x107 cells in 2mls) of Li-Heparin blood (3ml Pastette) together with 10mL 1X lysing solution (10ml pipette+Orange Pipettor).
- Mix gently (inverting gently not shaking) until lysis is observed (blood will turn to a light slightly see through red colour, usually <30s and certainly within 2 min).
- Centrifuge cells at 1200rpm for 10 minutes
- Discard supernatant and flick base of tube until pellet is re-suspended in remaining solution
- Add 10ml PBS and mix (10ml pipette+Orange pipettor)
- Centrifuge cells at 1200rpm for 10 minutes
- Discard supernatant and flick base of tube until pellet is re-suspended in remaining solution
- Add 10ml PBS and mix (10ml pipette+Orange pipettor)
- Centrifuge cells at 1200rpm for 10 minutes
- Discard supernatant and flick base of tube until pellet is re-suspended in remaining solution
- Add 2ml Freezing Solution using a 3ml pastette very slowly and carefully over

a time period of at least 30 seconds to give a cell concentration of 5-7x106 cells per ml

- Aliquot the cell suspension into the pre-labeled cryo-vials using a 3ml pastette; 1 ml into each 1.8 ml vial. Pipette gently and slowly to minimize shear forces.
- Place samples into CoolCell, and place in -80°C freezer for 2 hours
- Transfer the samples in to tights and keep in -80°C freezer
- Transfer to liquid nitrogen as soon as possible after return home for indefinite storage

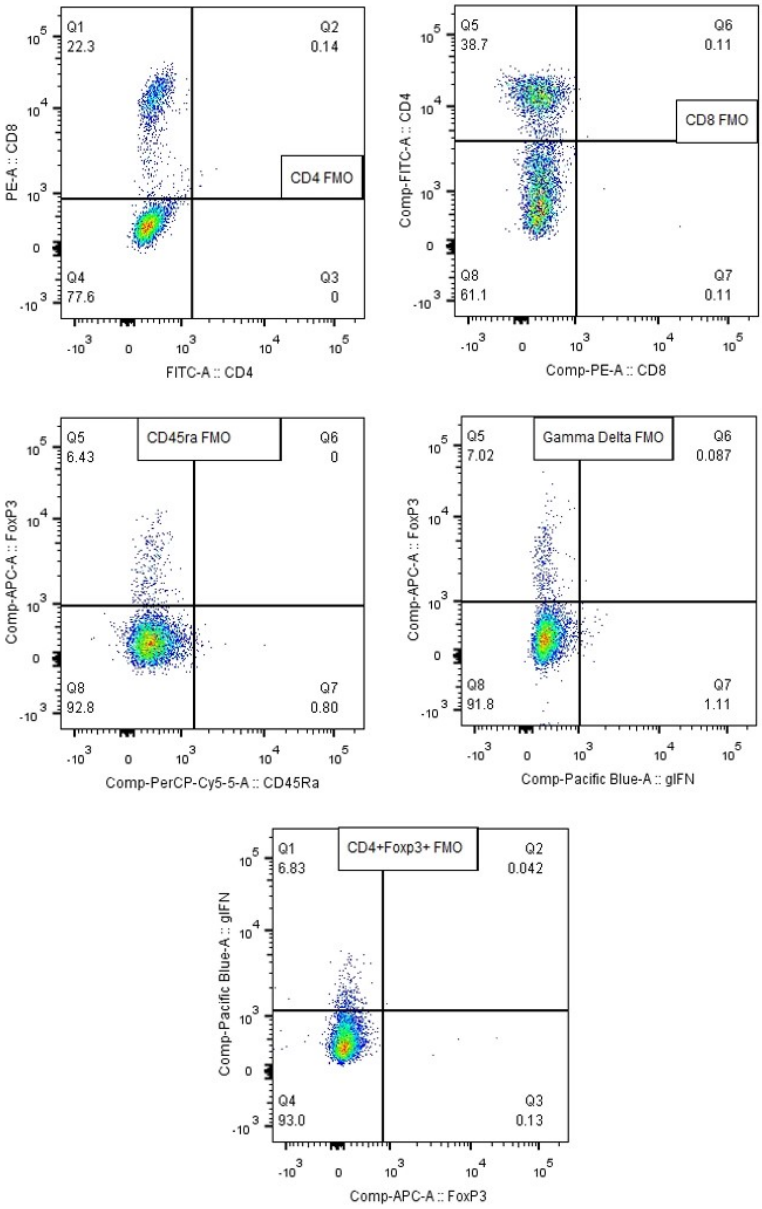
5 Flowcytometry raw data flowjo outputs

5.1 Single stains



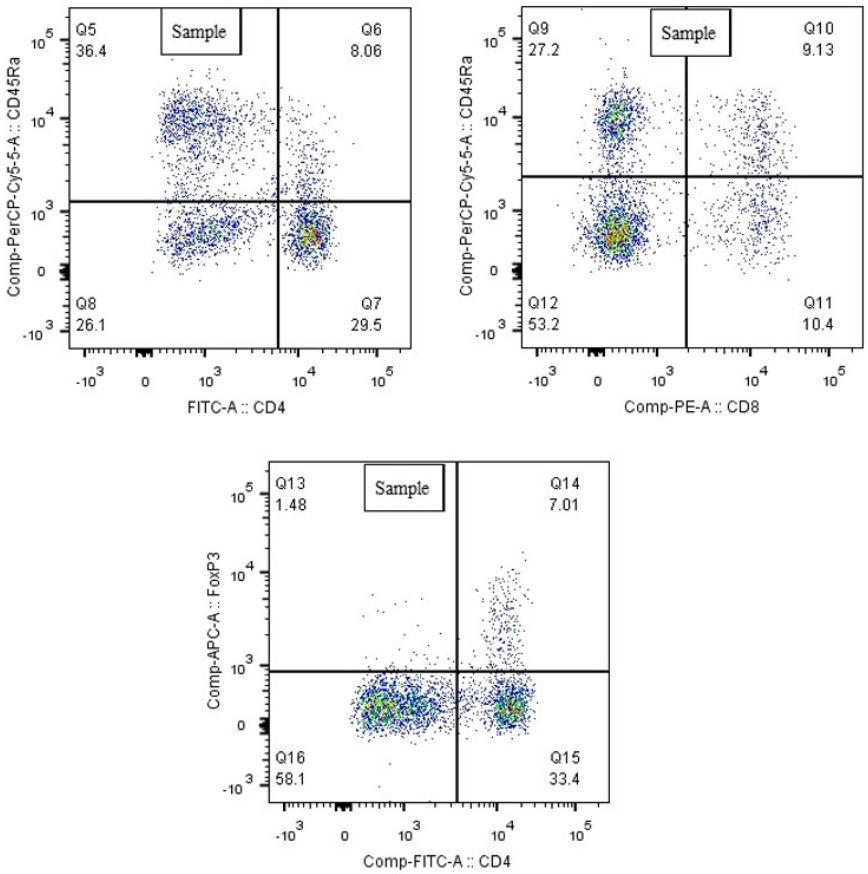
Appendix, figure S2: Examples of each of the single stain controls used in the multi-colour FACS assay. Each plot is broken into four sections; cells on the left are negative and cells on the right are postive for the flourophore and associated cell type on the x-axis. The y-axis is the forward scatter of cells proportional to cell size.

5.2 Fluorescent Minus One (FMO) controls



Appendix, figure S3: Examples of each of the flourescent minus one (FMO) controls used in the multi-colour FACS assay. Each plot is broken into four sections; the cells in the lower left are negative for both flourophores and associated cell types, the cells in the upper left are positive for the flourophore and associated cell type on the y-axis, the cells in the upper right are positive for the flourophore and associated cell type on the x-axis and y-axis,the cells in the lower right are positive for the flourophore and associated cell type on the x-axis. Staining in the upper or lower sections of the x-axis indicates non-specific staining.

5.3 Staining example sample



Appendix, figure S4: Examples of each of the staining in a sample analsyed using in the multi-colour FACS assay. Each plot is broken into four sections; the cells in the lower left are negative for both flourophores and associated cell types, the cells in the upper left are positive for the flourophore and associated cell type on the y-axis, the cells in the upper right are positive for the flourophore and associated cell type on the x-axis and y-axis,the cells in the lower right are positive for the flourophore and associated cell type on the x-axis. In the top two plots, staining in the upper right section indicates CD4+CD45ra+ (left plot) and CD8+CD45ra+ (right plot), i.e naive T cells. In the lower plot, staining in the upper right section indicates CD4+Foxp3+, i.e T_{reg} cells.