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Spatiotemporal and Individual Drivers of Variation in Parasitism and Immunity in Wild Red Deer

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Authorship Declaration

I composed this thesis. All analyses and writing are my own work, conducted with the help of my supervisors and coauthors. Josephine Pemberton, Dan Nussey, Fiona Kenyon, and Dan Becker provided comments on writing, analyses, and methodology for the data chapters. Alison Morris and Sean Morris collected a substantial number of the analysed faecal samples, as did several field helpers that I recruited and worked with, particularly Olly Gibb. Ali and Sean and their predecessors collected all the life history data contained in my analyses, along with dozens of field helpers over the years. Kathryn Watt designed and helped out with the antibody assays used in chapters III-V, helped by Rosie Keith as part of her honours project. Sam Ebdon carried out some parasite identification and sequencing as part of his honours project. I performed all the parasite counts and have full understanding of all the analyses carried out here. I use the word "we" throughout the data chapters because they were written as papers.

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Publications arising from this thesis

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Now let's get on with it.

Lay summary

Parasites are present in all natural populations, but are hard to study in the wild. Because parasites – and the immune system that combat them – are complex and hard to observe, it can be very hard to draw conclusions about disease processes occurring in wild animals. A lot of this difficulty originates from the many different interacting factors in the system under study, and difficulty obtaining samples. This is particularly true of studies of large wild mammals, which are few and far between, and which often rely on convenient, one-off samples obtained through culling or incidental sampling. As a result, large wild mammal systems with a lot of available individual information can be extremely useful for disease ecology analyses, allowing researchers to gain large sample sizes and to untangle complex sets of factors.

In this thesis, I describe the first study of the immune systems and parasites of the wild red deer (*Cervus elaphus*) on the Isle of Rum, Scotland. This population has been studied continuously for four decades, with unrivalled data on the behaviour, reproduction, survival, and genetics of thousands of individually-known deer. By collecting faecal samples from identified individuals, I tied their reproduction, behaviour, and fitness to the parasites living in their digestive systems, and the immune responses used to combat them. I found that almost all deer were infected with parasitic helminths. Parasite counts varied heavily across seasons, and were generally highest in the warmer months and lowest in the winter. However, different deer had different seasonal patterns of infection.

I showed that in a given year, females that gave birth to calves had lower antibody levels, and those that raised their calf to the winter also gained higher parasite levels. This showed that giving birth to a calf had a cost for the immune system, whereas the cost of lactation and associated behavioural changes resulted in increased parasite exposure. I then showed that this increased parasitism likely resulted in reduced reproduction and survival in the following year. This explains a longstanding tradeoff in the deer, where individuals that gave birth were less likely to survive, but the mechanism was previously unknown. Finally, using high-resolution behavioural observations, I examined how individuals' distributions in space related to their immunity and parasites. I found that individuals living in different regions of the study area had very different levels of antibodies and parasites, and that the spatial distributions of these measures differed. These spatial patterns also varied seasonally. As a general takeaway, this thesis demonstrates that observational studies of known individuals can be highly informative for individual- and environment-level processes in disease ecology, even over small geographic areas and short time periods.

Thesis Abstract

Parasites are a ubiquitous presence in nature that can profoundly impact the evolution and ecology of their hosts. Despite their longstanding interest for many branches of mammalian biology, there are relatively few large-scale longitudinal investigations of immunity and parasitism in large wild mammals. Furthermore, very few studies take full advantage of longitudinal studies' ability to examine spatiotemporal variation, life history correlates, and fitness consequences of immunity and parasitism simultaneously.

This thesis comprises the first parasitological and immunological investigation in the individually-monitored study population of red deer (*Cervus elaphus*) on the Isle of Rum in the Inner Hebrides, Scotland. Over the course of nine field seasons spanning 2016-2018, colleagues and I collected 2091 faecal samples from 447 identified individuals. I examined these faecal samples for eggs and larvae of gastrointestinal helminth parasites and protozoa. I particularly focussed on counts of three highly prevalent helminth taxa: strongyle nematodes, the common liver fluke *Fasciola hepatica*, and the tissue nematode *Elaphostrongylus cervi*. In addition, I adapted and employed a method of faecal antibody quantification originally developed for use in sheep. Samples were analysed for total immunoglobulin A (IgA) and anti-*Teladorsagia circumcincta*-specific IgA, giving measures of both general and specific immune allocation.

I used these immune and parasite measures in several analyses, making use of the high-resolution life history, fitness, and behavioural data available for the Rum red deer population, focussing mainly on samples collected from adult females. The principal findings were:

1. The red deer were infected with multiple species of helminths and protozoa, present at high prevalence but relatively low intensity. These parasites showed seasonal patterns of infection with strong age and sex biases, all of which varied between parasite taxa. Generally, younger individuals had higher helminth intensities, and autumn and winter seasons featured the lowest parasite intensities.

2. Parasite counts were repeatable within individuals. However, repeatability varied according to the sampling timescale, with strongyle counts being more similar within sampling trips than between trips. This implied contrasting seasonal patterns in different individuals, so that sampling at different times of year would give different impressions of patterns of parasitism across the population.
3. Females that reproduced had lower antibody levels and higher parasite intensities in the following year. However, different components of reproduction had different costs for different immune and parasite measures: gestation was associated with lower total IgA levels, while only lactation resulted in increased parasite counts, implying an important role of exposure in mediating reproduction-parasitism tradeoffs in this system.
4. I investigated the impact of reproduction, immunity, and parasitism on fitness-related traits using path analysis. Parasite count in a given year was found to correlate negatively with reproduction the following year, indicating a possible cost of parasitism for multiple fitness-related traits, above and beyond that accounted for by current reproductive status itself. Increased anti-*Teladorsagia circumcincta* IgA was also found to be associated with increased probability of reproduction, beyond any association with strongyles themselves.
5. I quantified and controlled for spatial patterns of immunity and parasitism using Integrated Nested Laplace Approximation (INLA) models. These analyses revealed stark differences in the range and patterns of spatial heterogeneity for different immune and parasite measures. However, fixed effects remained largely unchanged by the incorporation of spatial effects, indicating that spatial variation was unlikely to be confounding my earlier findings.

I discuss these findings and their implications for longitudinal studies of immunity and parasitism in wild animals and the further integration of spatiotemporal, life history, immune, and parasite data.

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Chapter I: General Introduction

Part 1: Overview

Parasites are a ubiquitous force in nature. Every extant animal species that has been examined plays host to a diverse array of parasites, and the vast majority of organisms are parasites (Windsor, 1998; Hudson *et al.*, 2002; Poulin, 2007). As a result, animals have evolved to deal with the fitness consequences of parasite infection using resistance and damage amelioration mechanisms performed by the immune system. In combination with the rest of their phenotype, this immunity is integral to hosts' survival and reproductive success. In the past few decades, a significant movement in ecology and evolutionary biology has pushed to understand parasites and immunity in wild contexts (Sheldon and Verhulst, 1996; Babayan *et al.*, 2011). An initial interest in the costs and consequences of immune and parasite variation has led to a deepening understanding of correlations with a growing list of environmental factors and intrinsic traits. More recently, an emerging interest has concerned the immune and non-immune processes that influence fitness outcomes for both host and parasite (Sheldon and Verhulst, 1996; Viney *et al.*, 2005; Graham *et al.*, 2011). Despite considerable progress, there remain many unanswered questions concerning immunity and parasitism in wild vertebrates, particularly when it comes to the intersection of spatiotemporal and individual-level variation.

Ecoimmunology and disease ecology are two distinct but complementary fields investigating disease processes in the wild, experiencing increased integration over time (Graham *et al.*, 2011; Hawley and Altizer, 2011; Brock *et al.*, 2014). Ecoimmunology generally aims to understand within-individual immune and disease processes in a wild, ecological context. Meanwhile, disease ecology focusses more on parasites and between-individual disease processes and the way they scale up to ecological and

evolutionary consequences. Much work has been done on connecting these layers of processes (e.g. Hawley and Altizer, 2011; Brock *et al.*, 2014), and many studies fall on a continuum between the two. This thesis contains elements of both, and ultimately aims to integrate analysis of immune phenotypes and parasite intensities to strengthen inferences about their fitness consequences (Graham *et al.*, 2011).

Most studies of immunity and parasitism in wild animals are restricted to certain time windows and spatial environments, and very few are able to study the same individuals at high enough resolution (or for long enough) to track fitness consequences. For these reasons, long-term longitudinal studies of wild individuals are extremely valuable for ecoimmunology and disease ecology (Wilson *et al.*, 2004; Clutton-Brock and Sheldon, 2010; Festa-Bianchet *et al.*, 2017). To name just a few advantages, these studies gain power through repeated sampling of host individuals, which reduces extraneous variation; host genetic information is commonly available to untangle genetic and environmental sources of variation; and high-resolution behavioural data allow examination of hosts' spatial and social behaviour (Clutton-Brock and Sheldon, 2010; Festa-Bianchet *et al.*, 2017). Findings from these studies can often be used to inform conservation and disease control strategies, as well as informing researchers about fundamental processes in ecology and evolution and elaborating on the ecology of the system as a whole (Gehman *et al.*, 2019).

In this thesis, I outline the first parasitological and immunological investigation of a long-term study of a wild population of Scottish red deer (*Cervus elaphus*). This species is a wide-ranging forestry pest, an ecological engineer of natural plant communities, a livestock animal, an ecological model organism, and an iconic cultural symbol. Using noninvasive faecal samples from known individuals paired with the wealth of behavioural and life history data in the study population, I examine

how spatiotemporal variation, individual life histories, and fitness outcomes relate to both immunity and parasitism. In this chapter I describe the ecology of helminth parasites, and the immunity that combats them; I describe the general factors that we expect to play a role in determining immunity and parasitism in wild mammals; I outline some of the common difficulties and hurdles encountered in studies of disease in wild animals; and I provide background on the Isle of Rum red deer population and prior knowledge of parasitism in wild deer.

Part 2: Helminth parasitism and contributing factors

The mammalian immune response to gastrointestinal helminths

Internal metazoan parasites of wild mammals are generally worm-shaped, and are lumped into a paraphyletic group known as helminths. These animals live within an animal's tissues or cavities, appropriating its resources and/or directly feeding on host tissues. The host generally fights to prevent damage caused by these processes; however, the immune response to gastrointestinal (GI) helminths is complex, widely varied, and unfortunately poorly understood relative to our understanding of microparasites (Maizels *et al.*, 2012). As with any other parasite or pathogen, in order to defend against helminths the host must be able to detect infection; to respond to infection by killing, containing, or expelling the parasite; and then to repair the damage caused. Generally, immunity to helminths can act through three main mechanisms: expulsion of adult nematodes, reduction of size, and reduction in fecundity (Balic *et al.*, 2000). Importantly, a substantial proportion of the damage incurred during infection originates from the host immune response itself; consequently, hosts do not necessarily resist infection entirely, but may exhibit varying degrees of tolerance to infection, repairing and ameliorating damage rather than combatting the parasite directly (Graham *et al.*, 2005; Råberg *et al.*, 2009a; Medzhitov *et al.*, 2012). Tolerance is defined in ecological contexts as a host's ability

to experience increasing parasitism without necessarily suffering worsened symptoms (Graham *et al.*, 2005; Hayward *et al.*, 2014a; b). This is particularly relevant for helminth infections, as helminths have often evolved to evade and downregulate the immune response and share many antigenic targets with their animal hosts, so that they offer a relatively limited set of viable targets compared to e.g. bacteria (Maizels and Yazdanbakhsh, 2003; Maizels *et al.*, 2012). Bacterial lipopolysaccharides (LPS) comprise a good example: LPS commonly evokes a strong immune response in mammal hosts because it is never found in mammals in the absence of bacterial infection.

Most of our knowledge of immunity to GI helminths in ruminants comes from the immune response in sheep and cattle (Barrell, 1997; Balic *et al.*, 2000; McRae *et al.*, 2015). Following invasion, innate receptor molecules such as Toll-like receptors (TLRs) identify pathogen-associated and damage-associated molecular patterns, inducing cytokine signalling cascades which then activate other arms of the immune system, including dendritic cells, macrophages, and the adaptive immune system (Balic *et al.*, 2000; Meeusen *et al.*, 2005; McRae *et al.*, 2015). Macrophages and neutrophils initially prevent establishment of larvae, e.g. in *Haemonchus contortus* infection in sheep (Bowdridge *et al.*, 2015), and produce chitinases to degrade helminth exoskeletons (Nair *et al.*, 2005).

Once infection has been recognised, cytokines are produced and inflammatory responses are induced to attract and activate effector cells. In particular, mast cells in the mucosa and underlying connective tissue degranulate upon activation, producing inflammatory mediators including histamine and a series of proteases (Stone *et al.*, 2010; Abraham and St. John, 2010). In addition, these cells produce cytokines that encourage a T helper-2 (Th2) response, attracting natural killer cells, neutrophils, and

eosinophils, all of which help in combatting infection and/or damage repair (Balic *et al.*, 2000).

Ruminant immunity to gut nematodes generally involves an important humoural (antibody-based) adaptive component (Barrell, 1997). This generally takes the form of a Th2 response, with self-reinforcing expression of IL-3, IL-4, IL-5, and IL-13 cytokines (Meeusen *et al.*, 2005). IL-3 promotes mast cell expansion (Lantz *et al.*, 1998), and IgE production induced by these cytokines contributes further to mast cell degranulation (Gilfillan and Tkaczyk, 2006; Stone *et al.*, 2010), ultimately inhibiting helminth growth and fecundity (Stear *et al.*, 1995). Eosinophils are similarly important effector cells, with receptors for multiple antibody subtypes which can induce degranulation (Lamkhioued *et al.*, 1995; Meeusen *et al.*, 2005). Although induced in primary infection, it is generally thought that their influence in this case is minimal compared to repeat infections, likely because of the lack of specific antibodies in primary infection (Balic *et al.*, 2000; Meeusen and Balic, 2000). Antibody levels are frequently found to correlate negatively with nematode infection both at the phenotypic level (Smith *et al.*, 1987; Hayward *et al.*, 2014b; Watt *et al.*, 2016) and genetically (Morris *et al.*, 1995). As such, breeding sheep for increased helminth resistance results in higher antibody levels (Bisset *et al.*, 1996). Immunoglobulin A (IgA) production is stimulated by IL-5-dependent activation of B cells associated with the Th2 response (Harriman *et al.*, 1988). IgA is a particularly important molecule for preventing the establishment of larvae in the gut, thus reducing parasite intensity (Harrison *et al.*, 2008; Shaw *et al.*, 2013), and also reduces adult helminth size and egg production as a result (Stear *et al.*, 1995). In addition, Secretory IgA can activate eosinophils, effectively stimulating degranulation (Lamkhioued *et al.*, 1995), thereby encouraging killing of parasite larvae. Primary exposure to nematodes in sheep results in waning IgA expression over time, which is only maintained if exposure is sustained

(Smith *et al.*, 1987).This waning implies that continuous exposure to helminths is needed to encourage continuous IgA expression.

It is also clear that cell-mediated immunity plays a role; for example, greater cell-mediated immune expression is associated with resistance to *Haemonchus contortus* in breeds of sheep (Bowdridge *et al.*, 2015), and transferring lymphocytes can convey resistance (Smith *et al.*, 1984), demonstrating that these cells are necessary for defence against these parasites . Expulsion of the nematodes generally requires a strong hypersensitivity response which can harm the host digestive system (Stewart, 1955; Jones and Emery, 1991; Meeusen, 1999); therefore, immune control and reduction of parasite growth and fecundity are more common, particularly in ruminants (Maizels and Holland, 1998; Balic *et al.*, 2000). Limiting growth is advantageous to the host partly because larger worms tend to be more pathogenic (Stear *et al.*, 2003), and produce more eggs (Stear *et al.*, 1995).

Because of helminths' weak immunogenicity and the fitness advantages of tolerance, the adaptive response to helminths rarely conveys absolute resistance, and reinfection is extremely common. Reinfection is therefore an extremely important component of the life cycle of soil-transmitted helminths, both in humans (Jia *et al.*, 2012) and in ruminants (Barrell, 1997; Hayward *et al.*, 2014b). The environment represents an important compartment for infectious stages, and the relative advantage of tolerance versus resistance may depend heavily on the rate of environmental exposure (Hayward *et al.*, 2014b). Sheep are commonly selectively bred for parasite resistance, demonstrating that resistance is under strong genetic control, particularly at quantitative trait loci and Major Histocompatibility Complex (MHC) regions (McManus *et al.*, 2014). Although the literature on red deer immunity is minimal compared to that of sheep and cattle, it is reasonable to assert that immune

mechanisms comprising the response to GI helminths will be qualitatively similar for deer.

GI helminths reside as adults in the digestive system, where food is plentiful and immune responses are relatively easy to evade. Many species undertake tissue migration for periods of their life cycle, potentially harming the host considerably in the process (Read and Skorping, 1995; Mulcahy *et al.*, 2005). They copulate, then lay eggs into the gut lumen rather than multiplying within the host (Taylor *et al.*, 2016a). Because autoinfection is uncommon, infection with gut helminths is generally acquired through regular reinfection via the external environment (Hoberg *et al.*, 2001). Thus, an animal's helminth burden is determined by two fundamental elements: its rate of exposure to infectious stages, and its susceptibility to infection with said parasites. Each of exposure and susceptibility will be influenced by a range of intrinsic and extrinsic factors, some of which I outline below.

Intrinsic factors affecting helminth parasitism

Intrinsic drivers of helminth infection arise as the result of individual-level traits such as physiology or genetics, and include sex, age, reproduction, stress, and sociality, among others. Different sexes often show different immune profiles, immune strengths, parasite burdens, and consequences of infection: in particular, males often have a faster pace of life and invest less in immunity, rendering them the "sicker sex" (Zuk, 2009). Sex biases are often linked to hormonal differences (Foo *et al.*, 2017), but stress and social behaviour may also contribute (Grear *et al.*, 2009; Takahashi *et al.*, 2018). Ageing processes such as ontogeny and senescence have a profound impact on the expression and efficacy of the immune response, as well as determining the environment that an organism encounters. Particularly in mammals, young animals exhibit immature, developing immune systems, suffering heavy parasite burdens as a

result (Ashby and Bruns, 2018). However, older individuals have had more time to accumulate parasites, so that younger individuals do not necessarily exhibit the highest burdens (e.g. Lutermann *et al.*, 2012). Additionally, different age classes may exhibit qualitatively different immune phenotypes (Soulsbury *et al.*, 2017), and senescence often causes immunity to peak in middle age and wane in late life (Hayward *et al.*, 2009). This immunosenescence can be confounded with demography, as high-quality individuals (or those with specific immune phenotypes [Graham *et al.*, 2010]) may be the most likely to survive to old age. For these reasons, longitudinal analyses are commonly required to discern ageing patterns in immunity and parasitism in the wild (Nussey *et al.*, 2008; Hayward *et al.*, 2015). Age and sex can also interact in determining parasitism, for instance where different behaviour between males and females results in varying levels of exposure at different life stages (e.g. Grear *et al.*, 2009). Age varies with a wide range of other factors besides sex (e.g. ranging behaviour [Froy *et al.*, 2018]), so should be controlled for where possible, but this is often hard in wild populations if individuals' identities are not known or tracked (e.g. Rödel *et al.*, 2016).

Immunity and parasites consume host resources that are also necessary for host processes such as reproduction or locomotion (Sheldon and Verhulst, 1996; Cressler *et al.*, 2014; Van Dijk and Matson, 2016). By definition, under resource-limited conditions, investment in any of these costly processes will result in within-host competition for resources, creating tradeoffs that prevent the organism from maximising all traits at once (Stearns, 1989). Allocation of resources to costly intrinsic traits is therefore commonly associated with decreased immunity and/or with increased parasitism (French *et al.*, 2009; Knowles *et al.*, 2009). Reproduction is a particularly costly activity, and is critical to an organism's fitness (Williams, 1966). We therefore expect that reproductive individuals will have weaker immunity and

higher parasite burdens than non-reproductive counterparts, all else being equal (Sheldon and Verhulst, 1996; Harshman and Zera, 2007; Speakman, 2008; Knowles *et al.*, 2009). The male-biased immunodeficiency and parasitism described above (Zuk, 2009) can be viewed as an example of such a tradeoff where males invest proportionally more into reproduction, redirecting more resources away from immunity as a result.

Individual identity

Individual animals may show consistent immune phenotypes and/or parasite burdens through time (e.g. Arriero *et al.*, 2017), and the same has been demonstrated in humans (Carr *et al.*, 2016). The ultimate causes of these "immune personalities" are inevitably intrinsic or extrinsic factors, but these factors may be impossible to examine and/or account for directly. Therefore, in studies of wild animals, the influence of individual identity is commonly controlled for and quantified using the variance component of a linear mixed model (Paterson and Lello, 2003; Nakagawa and Schielzeth, 2010). Individuals often vary in their quality, which complicates wild studies that investigate fitness and life history components (van Noordwijk and de Jong, 1986; Moyes *et al.*, 2009). Condition is an expression of this individual quality that varies over time and with life history investment. Body condition can be quantified using a range of physiological and morphological measures such as body mass (Irvine *et al.*, 2006), fat scores (Schoenle *et al.*, 2017), or cell counts (Milenkaya *et al.*, 2015). As a general indicator of health which should have knock-on implications for immunity, poor condition is often associated with weaker immunity and/or increased parasitism (Sánchez *et al.*, 2018). There is no universally accepted definition for "condition" (or even "health"), and in some circumstances immune parameters themselves are used to approximate condition (e.g. Milenkaya *et al.*, 2015).

Longitudinal sampling increases studies' ability to detect and control for these quality effects (Clutton-Brock and Sheldon, 2010, and references within).

Generally, within-individual variation is important to quantify because repeatability is useful when developing new methods. If an assay is highly repeatable at the within-individual level, this is indicative of long-term immune and parasite phenotypes. This is particularly important for immune assays given the high plasticity and rapid induction of many components of the immune response (Boughton *et al.*, 2011). Individual repeatability is also important because the individual is often the unit on which selection acts; repeatability incorporates genetic differences between hosts (Falconer and Mackay, 1996), which are another important determinant of the individual component of immunity. Quantitative genetics analyses can reveal heritable variation in immunity and parasitism as a component of between-individual variance (e.g. Graham *et al.*, 2010; Hayward *et al.*, 2014b).

Extrinsic factors influencing immunity and helminth parasitism

Extrinsic factors are determined by an organism's environment, and mainly comprise environmental or biotic variables. Environmental gradients in climatic conditions generally determine parasites' ability to transmit between hosts. For example, sunshine causes certain *Salmonella* strains to desiccate, preventing their transmission between sleepy lizards (*Tiliqua rugosa*) in sunnier areas (Parsons *et al.*, 2015), and low temperatures constrain the transmission of avian blood parasites at higher elevations (Zamora-Vilchis *et al.*, 2012). However, environmental gradients can also impact immunity and susceptibility, often in opposing directions to their effects on exposure.

Environmental gradients of host nutrition and resource availability offer a good example of factors that can differentially determine patterns of immunity and parasite exposure. Simplistically, intentional or accidental resource provisioning provides

hosts with more energy and resources to divert to immunity, and parasite countsometimes decreases as a result (Becker and Hall, 2014). However, animals may respond to increased resource availability by reproducing at a higher rate and aggregating on resource provisioning sites, thereby increasing exposure and producing positive effects of resource availability on parasitism (Hines *et al.*, 2007; Becker and Hall, 2014). Further complicating matters, parasites compete for host resources, so low levels of resources can be detrimental to the parasite; occasionally, parasite countmay even peak at intermediate (rather than maximum or minimum) resource availability (Cressler *et al.*, 2014). Resource provisioning illustrates the potential complexity of environmental factors affecting immunity and parasitism in wild animals, and the need to consider multiple potential causal mechanisms where possible.

Environmental factors often vary in space and in time, and where they impact susceptibility and exposure, spatiotemporal patterns of infection will emerge. Parasite infection is spatially heterogeneous (Poulin *et al.*, 2011; Dallas *et al.*, 2018), and seasonality of parasite infection is near-ubiquitous (Altizer *et al.*, 2006). It has been argued that seasonal patterns of immunity may be responsible for seasonal fluctuations of parasitism (Martin *et al.*, 2008), and the same may be true of variation in space: that is, spatial variation in immunity may produce spatial variation in parasitism. Many studies compensate for differences between areas and time periods by incorporating fixed effects of region or time in their model. Clustering in time and space can inflate the type I error rate, and so some studies use autocorrelation methods to correct for this inflation in order to strengthen inference (Zuur *et al.*, 2017; Pawley and McArdle, 2018). These methods effectively account for and quantify the effect of space and/or time on the mean value of a response variable. However, the

proportion of ecoimmunology studies that actually fit autocorrelation structures in their models is on the order of <5% (Becker *et al.*, in revision).

Complex interactions among space, time, life history, immunity, and parasitism

The patterns I have described so far serve to illustrate that parasitism and immunity can be affected additively by changes in multiple intrinsic and extrinsic factors. Many studies successfully identify changes in these factors that contribute to immune and parasite variation – however, it is rarely the case that only one variable (e.g. season, location, life history allocation) is impacting immunity and parasitism at a time. Individuals' intrinsic phenotypes themselves often vary over space and time, potentially in response to environmental cues. For example, seasonally reproducing animals may invest more in reproduction at certain times of the year (Altizer *et al.*, 2006; Martin *et al.*, 2008); individuals inhabiting certain geographic areas will be able to achieve higher fitness than others (Stopher *et al.*, 2012b); and individuals with more extroverted personalities may inhabit or explore different areas, affecting their exposure to parasites (Ezenwa *et al.*, 2016a). Because of these individual-by-environment interactions, individuals may vary in their response to spatiotemporal cues, and/or the environment may alter associations among phenotypic variables, creating variation in slopes rather than in mean effects. Very few studies account for such variation in slopes, and this is a knowledge gap that I aim to address in this thesis.

Many studies include concurrent measures of space, time, and life history to account for differences in the mean effects, yet it is rarer that studies examine whether spatiotemporal and individual-level variation interact. For example, studies that investigate reproductive traits across the annual period must fit seasonality as an effect in their analysis (e.g. Lutermann *et al.*, 2012). However, fitting both life history

traits and spatiotemporal factors in the same model is not always possible: for example, season and reproduction/sex can be entirely confounded either biologically (e.g. Krams *et al.*, 2017) or operationally (e.g. Irvine *et al.*, 2006). Many studies have nevertheless achieved successful integration of aspects of spatiotemporal and life history variation in ecoimmunology and disease ecology by sampling from multiple life history classes at different times (e.g. Schoepf *et al.*, 2017) or in different locations (e.g. Ardia, 2005), and fitting their interactions. For example, a study in tree swallows (*Tachycineta bicolor*) demonstrated that reproduction-immunity tradeoffs vary in strength across their geographic range, with stronger reproductive costs for the immune systems of swallows living at higher latitudes (Ardia, 2005). Similarly, intensity of sickness responses varies latitudinally in American song sparrows, with stronger sickness responses in California than in Washington or Alaska (Adelman *et al.*, 2010). If the intensity of reproductive tradeoffs vary spatially (across populations), it is also likely that different reproductive classes will exhibit different seasonal patterns of immunity and parasitism, yet this prediction has rarely been tested (Martin *et al.*, 2008).

The cost of immunity

Immunity, like any other physiological function, requires resources to function. These resources can come in many different forms, including energetic costs, molecules, nutrients, time, and more. Importantly, any organism has a finite resource pool from which to draw; were this not the case, organisms could potentially maximise all their traits, creating so-called "Darwinian Demons" (Gustafsson *et al.*, 1994). As such, immunity is often characterised as a costly trait, where allocation of resources to immunity necessitates their diversion from an organism's other traits, creating tradeoffs. The identification of immunity's costliness was fundamental to the establishment of ecoimmunology as a field (Gustafsson *et al.*, 1994; Sheldon and

Verhulst, 1996; Lochmiller and Deerenberg, 2000), and continues to produce interesting revelations concerning the evolution and ecology of the immune system (e.g. Best *et al.*, 2011; Cressler *et al.*, 2015; Metcalf and Graham, 2018).

The costliness of immunity has been demonstrated in many contexts, using one of three main methods. First, researchers can add resources and observe changes in immunity, usually finding that resource supplementation increases the immune response as more energy and molecules are available for immunity (French *et al.*, 2007). Second, researchers can experimentally alter organisms' allocation to other processes and observe changes in immunity that occur as a result. This usually comes in the format of increasing allocation to these other traits: e.g., in stressing the animal (Svensson *et al.*, 1998), increasing reproductive investment by experimentally increasing birds' clutch sizes (Knowles *et al.*, 2009), or reducing reproductive allocation by sterilising the animal (Cox *et al.*, 2010). A third method of investigating the cost of immunity takes an alternative causal route: researchers can induce an immune response in the absence of infection and then observe downstream changes in other traits such as locomotion or fitness (Graham *et al.*, 2011; Van Dijk and Matson, 2016; Finnerty *et al.*, 2018). Early investigations into the immune systems of wild birds demonstrated that invoking an inflammatory response resulted in reduced survival (Hanssen *et al.*, 2004) and reproduction (Ilmonen *et al.*, 2000; Raberg *et al.*, 2000), and increased energetic output (Svensson *et al.*, 1998; Eraud *et al.*, 2005).

All the above methods are regularly employed to demonstrate that immunity has costs in terms of resources. However, resource-linked mechanisms do not always reveal costs of or for immunity: for example, altering resource availability had no impact on the expression of reproduction-immunity tradeoffs in Gryllus texensis crickets (Stahlschmidt *et al.*, 2013). Similarly, early studies of the energetics of

immunity in birds demonstrated that input and output costs of immunity can differ (Svensson *et al.*, 1998): that is, immunity's mechanistic links have important effects on the ways that life history affects immunity and parasitism, and on their downstream effects on energetics and fitness. Additionally, rather than identifying tradeoffs, observational systems can reveal positive correlations between costly traits because the same individuals of high quality, with high resource intake rates, are more able to allocate more resources to several traits at once (the so-called "big house, big car" problem); as such, observational studies must be careful to consider this individual-level quality (van Noordwijk and de Jong, 1986). This integrative perspective has contributed to the ongoing development of a holistic perspective of immunity as it relates to a suite of host characteristics, behaviours, physiological functions, and environmental contexts (e.g. Lochmiller and Deerenberg, 2000; Van Dijk and Matson, 2016; Tieleman, 2018).

Part 3: Common difficulties in ecoimmunology and disease ecology

Studies of parasitology and immunology in the wild commonly encounter a set of practical and analytical difficulties, particularly where the study organism is a large mammal. I outline a few of these obstacles below.

Immunological and parasitological sampling

There are several practical difficulties with sampling wild animals to measure immunity or parasitism. First, some methods for counting parasites (e.g. counting adult gastrointestinal helminths in wild ungulates) require that the animals be dead. This includes counting adult helminths in the gut of wild ungulates to ascertain burden (Budischak *et al.*, 2015). Dead animals are also often the easiest source of phenotypic information such as body weight. Consequently, studies of wildlife disease may be forced to rely on opportunistic convenience sampling when animals

die naturally or are being culled, in which case researchers may have little to no choice over the time and location of samples (Nusser *et al.*, 2008). Studies which rely on necropsied individuals that died naturally only receive large influxes of samples during periods of high mortality such as epizootics or winter periods of low nutrition, and the sampled individuals are therefore a biased group (e.g. Coltman *et al.*, 1999; Ross-Gillespie *et al.*, 2007). As a result, these studies can give an unclear picture of the epidemiology of the system. Similarly, animals that are culled provide an ideal source of cadavers for study, but availability of these samples will commonly be restricted to seasonal culling windows or to specific demographic categories. In some cases, demography and seasonality may be confounded: for example, red deer males are culled earlier in the year than are females, so seasonal patterns are inscrutable (Irvine *et al.*, 2006).

In addition to these confounding factors and uneven distributions in time and space, studies which rely on any combination of necropsy, culling, or opportunistic *post mortem* samples are restricted by their cross-sectional nature: each individual is present only once in the dataset. Cross-sectional studies are fundamentally unable to make inferences as strong as longitudinal analyses (Clutton-Brock and Sheldon, 2010; Fenton *et al.*, 2014; Sánchez *et al.*, 2018). Causality is hard to discern in wild disease ecology studies, and often necessitates experimental manipulations (particularly treatments) and longitudinal analysis (Fenton *et al.*, 2014). Even when experiments can be successfully carried out, care must be taken to ensure that the correct variables are being manipulated and controlled for. Fortunately, observational approaches can be extremely revealing when the natural variation on which they rely is sufficient, and when sample sizes are large enough, particularly when the study is longitudinal in nature. Furthermore, experiments are impractical in many cases, particularly where

the study organism is a wild mammal, so that observation is the only available recourse (Rödel *et al.*, 2016; Sánchez *et al.*, 2018).

The diverse data required to answer certain questions in wildlife disease can be at odds with the variety of samples needed for immune or parasite assays. Movement analyses of disease processes provide a good example, as an area of research which is often facilitated by Global Positioning System (GPS) tracking (Dougherty *et al.*, 2018). Although GPS provides high-resolution data on individuals' movements, the sample size is restricted by the high cost of the methods (although this is decreasing; Kie *et al.*, 2010), and collecting samples from the tracked individuals presents difficulties. Even in systems where individuals can be caught and sampled directly, the effort involved can be extremely high, so that these studies are likewise often operationally restricted to one or two capture seasons in few locations, again presenting difficulties with detecting seasonal and spatial variation.

Finally, ecoimmunologists have historically struggled to find immunological tools that are applicable, interpretable, and reliable in wild contexts (Boughton *et al.*, 2011; Garnier *et al.*, 2017). For this reason, developing and validating assays for use in wild, non-model systems is a priority, and many studies leverage tools from domestic species to facilitate measure selection and development (Garnier and Graham, 2014; Taylor *et al.*, 2016a). Where the validity of assays is limited or uncertain, repeated testing of individuals can increase their reliability (e.g. Lamberton *et al.*, 2014), and/or reveal hidden variation in disease or immune phenotypes (e.g. Plowright *et al.*, 2017). Similarly, many immunological measures – particularly those that come from white blood cells – rely on serological samples. Blood sampling is difficult, invasive, and requires special training to carry out, applying logistical restrictions to sampling regimes. Parasites will be most affected by local immune responses, so if the focal

parasite resides in locations other than the blood, serological measures may be only transitively indicative of the relevant immune effectors. Sampling from the tissues in which parasites reside can be extremely difficult, but where possible, methods that allow measurement of immunity and parasitism in the same within-host location may be more powerful for detecting immune-parasite interactions.

Analytical hurdles

Observational studies of disease processes in wild animals also encounter a number of theoretical and statistical difficulties, some of which I introduce below.

Parasites are highly aggregated both within- and between-hosts, creating difficult-to-handle distributions. These distributions often follow an 80:20 rule, where ~20% of individuals harbour ~80% of the burden or intensity of parasitism (Woolhouse *et al.*, 1997). To deal with this problem statistically, models can assume overdispersed distributions (Grenfell *et al.*, 1995; Morrill and Forbes, 2012) and/or model zero-inflation to deal with excess zeros (Zuur *et al.*, 2009; Chipeta *et al.*, 2013). Spatial and temporal dependence structures can be fitted in the model in order to control for clustering in time and space (Zuur *et al.*, 2017; Pawley and McArdle, 2018), and random effects can be fitted to control for pseudoreplication when sampling the same individuals or groups at multiple time points (Paterson and Lello, 2003). While these complex data structures are commonly perceived as an inconvenience, they are also often of interest to researchers, and thus quantifying them – rather than merely controlling for them – can be highly revealing.

Immune and disease processes can be difficult to understand and predict in the wild due to the vast complexity of interacting factors across multiple scales. The immune system is highly complex at a microscopic scale, while the ecology of wild animals involves a suite of interacting individuals and populations, both within and between

trophic levels. These different scales are hard to resolve because small changes at the microscopic level can extend to have wide-ranging consequences, and environmental changes can have unpredictable implications for disease. For example, in 2015, two thirds of the world's Saiga antelope (*Saiga tatarica tatarica*; roughly 200,000 animals) mysteriously died in Kazakhstan in the course of only 3 weeks (Kock *et al.*, 2018). It was later discovered that a combination of weather conditions had provoked the *en masse* migration of commensal bacteria across the nasal epithelia into the bloodstream, resulting in widespread deaths from septicaemia without the necessity for any between-individual transmission (Kock *et al.*, 2018). It is difficult to imagine how this epizootic could have been predicted, and given that a huge proportion of current disease ecology research effort is directed towards increasing predictive capacity (e.g. Carroll *et al.*, 2018) and buffering for incipient global change (Carlson *et al.*, 2017), this environment-host-pathogen nexus has far-reaching consequences.

Cause and effect in ecoimmunology and disease ecology

Parasites often play a role as both response and explanatory variables, whether on proximate (mechanistic) or ultimate (ecological or evolutionary) timescales. Mechanistically, the immune system comprises molecules that function as markers or effectors, some of which (most notably antibodies) can function as both, so that parasites both elicit changes in these immune responses and are combatted by them (Bradley and Jackson, 2008; Garnier *et al.*, 2017). That is, infection induces increased antibody levels, which then combat the parasite, reducing subsequent parasite counts. As a result, a common issue of causality in ecoimmunology revolves around the interpretation of high antibody levels: does increased antibody expression reflect high levels of exposure, or strong resistance (Bradley and Jackson, 2008; Gilbert *et al.*, 2013; Garnier *et al.*, 2017)?

Ultimately, parasites both are influenced by host health and can determine host survival and reproduction. For example, it has long been theorised that parasitism is a fundamental process linking reproduction with fitness costs (Williams, 1966; Harshman and Zera, 2007), yet this has rarely been demonstrated in a wild population. Reproduction should increase parasite burden, which should then reduce survival. Given correlations between fitness parameters and variation in individual quality, survival and reproduction probability may themselves correlate (van Noordwijk and de Jong, 1986; Pekkala *et al.*, 2011). At a given time point, pregnant individuals should be suffering from higher parasitism as they are investing more in reproduction – however, the females in the best condition (and with the strongest immunity) are most likely to become pregnant in the first place. It is hard to know *a priori* which of these to expect, and their relationship likely varies in time. Longitudinal analyses can help to extricate these confounding issues of individual quality (Clutton-Brock and Sheldon, 2010). Very similar arguments revolve around body condition (Sánchez *et al.*, 2018). Fortunately, the ability to study both immunity and parasites in tandem can substantially increase the strength of inference in these scenarios (Bradley and Jackson, 2008; Graham *et al.*, 2011).

Most of the problems described above have the same problem at their root: difficulties collecting large numbers of samples with associated data which have either few confounding factors, or potential statistical routes for controlling for these factors. Experimental approaches can commonly address many of these problems, but fall prey to many logistical limitations (described above). As a solution to this problem, I used longitudinal immunological and parasitological sampling of known individuals with known life histories and ranging behaviour due to regular censusing. The Isle of Rum red deer system offers an unprecedented opportunity for this endeavour, for reasons that I outline below.

Part 4: Red deer and the Isle of Rum study system

The European red deer (*Cervus elaphus*) is a large, wide-ranging ungulate that is closely related to the North American wapiti (*Cervus canadensis*). Inhabiting most of Europe, red deer are ecologically important, acting as influential herbivores in many ecosystems – a role for which they are often culled. Their ecological importance and cultural significance has also encouraged a broad literature concerning, for example, their behaviour (Rivrud *et al.*, 2010), seasonality (Ryder, 1977), and population dynamics (Langvatn and Loison, 1999). A wild, unmanaged population of red deer inhabits the North Block of the Isle of Rum: a small island in the Inner Hebrides, off the west coast of Scotland. A scientific investigation into this population was established in the 1970s, and has continued uninterrupted since then, based on individual recognition of all deer in the study population (Clutton-Brock *et al.*, 1982). In this time, the population has been used for studies in population ecology (Clutton-Brock *et al.*, 2002), molecular ecology (Walling *et al.*, 2011; Huisman *et al.*, 2016), and quantitative genetics (Kruuk *et al.*, 2003), among many others. A comprehensive summary of the inner workings of the study system can be found in Clutton-Brock *et al.*, (1982).

The Isle of Rum study system has well-defined data collection methods that have been employed consistently since its establishment in the 1970s, most of which revolve around tracking reproduction patterns and familial interrelationships (Clutton-Brock *et al.*, 1982). During the calving season, considerable effort is expended to catch and mark as many calves as possible. Field workers watch the hillside for lone females suspected of having a new-born calf so that they can observe when a calf is picked up to suckle. When the calf lays down again, another field worker is guided to it by radio, catches it, marks it and takes phenotypic data (e.g. sex, weight) and samples (ear

punch, hair, and blood). The ear samples are used for genotyping on a Single Nucleotide Polymorphism (SNP) array, enabling paternity inference and accurate estimation of relatedness in the population (Huisman *et al.*, 2016). The calves are marked with a unique combination of collar, ear tags, and ear punches. These markings remain for years, often identifying the deer as individuals for the rest of their lives. Because females (and to a lesser extent males) are followed for such extended periods, cumulative life history information is available for all individuals and can be summarised in terms of both short-term (annual) and ultimate (lifetime) fitness measures (Clutton-Brock *et al.*, 1989; Froy *et al.*, 2016; Huisman *et al.*, 2016).

Female deer on Rum begin breeding at 3 or 4 years of age and give birth to one calf at a time, mostly in May and June (Clutton-Brock *et al.*, 1982). The considerable cost of raising a calf commonly results in females taking a year out from reproduction to regain condition, so that females give birth on average once every two years or so (Clutton-Brock *et al.*, 1989). The calf remains at foot for most of its first year, and females often associate with their mothers for many years longer. Because female deer can be remarkably long-lived (up to 24 years old; median 12), these interrelationships form the basis of most social structuring in the population (Clutton-Brock *et al.*, 1982).

In contrast to the females, most male deer disperse in the course of their second and third years to form all-male herds; on Rum, this results in males migrating out of the study area to inhabit other areas of the island (Clutton-Brock *et al.*, 1982). These males (and others not born within the study area) migrate back into the study area during the rut to mate, regularly using areas near their birthplace to rut (Clutton-Brock *et al.*, 1982; Stopher *et al.*, 2012a). This and other aspects of the Rum study system have contributed to regular inbreeding in the population, with debilitating fitness consequences for inbred individuals of all ages and sexes (Stopher *et al.*, 2012a;

Huisman *et al.*, 2016). Males exhibit much higher reproductive skew than females, and they tend to live much shorter lives (up to 17 years; median 9). For the first 5-6 years of life males are generally poor competitors for mates, so peak rutting years are between 7-11 years, with a sharp decrease in breeding success following this period (Clutton-Brock and Albon, 1979; Clutton-Brock *et al.*, 1982). Females that live longer tend to have more calves; however, in a given year, females that raise a calf to the winter successfully are more likely to die overwinter as a result of the considerable resource expenditure (Clutton-Brock *et al.*, 1982, 1989; Froy *et al.*, 2016).

External to these life history measures, the deer are censused five times a month for eight months a year (total 40 censuses/year) to keep track of their movements, and to identify when individuals are alive and dead, and the population are censused intensively during the mating period to provide measures of male reproductive success (Clutton-Brock *et al.*, 1982). Censuses give an accurate depiction of an individual's ranging behaviour which can be used to untangle environmental and genetic sources of variation (Stopher *et al.*, 2012b) and to identify how ranging behaviour relates to life history and fitness (Froy *et al.*, 2018). Red deer are highly seasonal, with annual cycles in moulting (Ryder, 1977), ranging (Rivrud *et al.*, 2010), mating (Lincoln, 1971), calving, and social behaviour (Lincoln *et al.*, 1972). They likewise experience considerable changes in condition which are reflected in their physiology (Mitchell *et al.*, 1976). Males grow and shed large antlers each year as part of their breeding cycle, exacting a substantial toll in terms of energy and resources (Kruuk *et al.*, 2003; Clements *et al.*, 2010). This cost is hypothesised to have considerable negative ramifications for immune function, potentially acting through immunogenetics (Buczek *et al.*, 2016). It is unknown how the strong seasonality impacts immunity and parasitism in the population.

Despite the many high-resolution behavioural and life history/fitness measures, there have been few instances of physiological measurements from the Isle of Rum deer. Although direct measurements are often precluded by the observational nature of the study (and the practical difficulty of capturing wild large mammals [see above]), a previous PhD project used hormone concentration data from noninvasive faecal samples collected from known individuals (Pavitt *et al.*, 2014a; b). The ability to collect these samples implied that regular large-scale collection of faecal samples could be used to gain immunological and parasitological insights.

Part 5: Parasites in red deer

Despite its forty-year study history, the population of red deer inhabiting the North block of the Isle of Rum has never undergone formal parasitological investigation. One study examined culled deer outside the study area in Rum, quantifying associations between condition, reproductive status, and gastrointestinal helminths using a combination of abomasal sampling and faecal propagule counts (Irvine *et al.*, 2006). However, this study did not benefit from the wealth of individual-level information available for deer living within the study area, on which this thesis focuses.

Red deer and disease ecology

Red deer, and the Isle of Rum system specifically, are ideal for an individual-based parasitology study for many reasons (Jolles and Ezenwa, 2015). First, the wealth of information on the ecology of red deer provides a good background on which to base an investigation into immunity and disease. Deer culling has formed the basis for multiple disease ecology studies, providing important context, background information, and parasite species to investigate (Irvine *et al.*, 2006; Vicente *et al.*, 2007a; Buczek *et al.*, 2016). In addition, red deer are farmed in New Zealand, which

has contributed somewhat to the parasitological literature and immunological tools available for use in red deer (Gajadhar *et al.*, 1994; Mason, 1994; Mackintosh *et al.*, 2014). Finally, red deer are closely related and broadly similar to multiple other well-studied ungulates, which offer further template investigations on which to build (Jolles and Ezenwa, 2015). A close relative of the red deer is the North American wapiti (*Cervus canadensis*), the disease ecology of which has likewise been well-studied in North America (Hines *et al.*, 2007; Downs *et al.*, 2015), and which I treat as conspecific to red deer in this thesis.

Sample collection in a noninvasive context

The study system is not without its challenges for investigation of immunity and parasitism. Foremost among these challenges is the noninvasive nature of the Rum red deer project: the deer are only rarely handled beyond the first few days after birth, and as such few direct measures of condition, immunity or parasitism can be collected (Clutton-Brock *et al.*, 1982). This restricts the choice of measures to those which can be taken noninvasively. Such a lack of direct sampling is a problem common to many longitudinal studies of ungulates (Festa-Bianchet *et al.*, 2017).

Secondly, and related, the study is entirely observational and precludes any experimental manipulations which might allow teasing out of causal relationships. However, the noninvasive and observational nature of the project also stands as a positive: any pattern revealed can be understood as fully "natural" and untouched by human intervention. Avoiding capturing the deer minimises risk to the animals, and increases the probability that the deer will remain habituated to human presence. The deer inhabit a very heterogeneous environment and there is no shortage of between-individual variation to leverage for making inferences, as evidenced by the wealth of previous work conducted on the population.

Parasites and disease ecology of related ungulates

Longitudinal studies of ungulates have been uniquely informative (Festa-Bianchet *et al.*, 2017), particularly in the field of disease ecology (Jolles and Ezenwa, 2015). There is a range of parasitological literature encompassing a wide variety of relevant ungulates, including cattle (e.g. Butler, 1969); sheep (e.g. Kenyon *et al.*, 2013); horses (e.g. Nielsen *et al.*, 2010); buffalo (e.g. Budischak *et al.*, 2015); and red deer themselves (e.g. Gajadhar *et al.*, 1994; Mason, 1994; Mackintosh *et al.*, 2014). As a result, a great many immunological tools exist for use in ungulates, including antibody ELISAs for deer and related animals (Mackintosh *et al.*, 2014; Watt *et al.*, 2016) and methods for detecting parasites noninvasively (Kenyon *et al.*, 2013; Taylor *et al.*, 2016b). One such popular parasitological method is the faecal egg count (FEC). FECs rely on the reproductive biology of gastrointestinal helminths, wherein adult helminths residing in the host lay eggs into the gut lumen to be passed in the faeces. Faecal samples can be collected noninvasively, and propagules counted in order to approximate the burden of parasites within the host (Sargison, 2013; Taylor *et al.*, 2016c). The FEC method encounters difficulties where propagule output fluctuates in time, through seasons, with changes in faecal consistency, and/or where the reproductive biology of different worms differs (Villanúa *et al.*, 2006; Turner *et al.*, 2010; Sargison, 2013; Budischak *et al.*, 2015). However, faecal propagule counts are often found to correlate well with actual burden (Budischak *et al.*, 2015; French *et al.*, 2016). In some cases, detecting adult helminths is near-impossible as a result of their life cycle or tissue location, and so propagule counts are the only feasible method of quantification (Gajadhar *et al.*, 1994).

Because faecal propagule counts are an emergent phenomenon which are determined by complex chains of causality, where they are used to approximate parasitism,

researchers must bear in mind that many different processes could be causing changes in the observed counts. Most notably, although propagule count commonly correlates with adult burden, there is some variation in this relationship (McKenna, 1981; Sargison, 2013; Budischak *et al.*, 2015), for example where bigger worms produce different more eggs (Stear *et al.*, 1995). As described above, some of the main immune mechanisms combatting helminth infection involve reductions of worm growth and fecundity (Balic *et al.*, 2000). As such, changes in propagule count may reflect a reduction in adult helminth count, or an immune-mediated reduction in worm fecundity, or both. The two will likely occur over different timescales, and may have different implications for the ecological mechanisms at hand. For example, a reduction in exposure should over time reduce the intake of infective stages, leading to a reduction in adult helminths; in contrast, a short-term increase in resource intake may increase the host's allocation to immunity, allowing rapid improvement of the immune response, with associated short-term reductions in worm fecundity.

Parasites investigated in this thesis

The wealth of parasitological literature on red deer parasites has been well summarised elsewhere (Haigh *et al.*, 2002; Bohm *et al.*, 2007). This thesis focuses on three parasites types found to have the highest prevalence in the study population. Strongyle nematodes (order: Strongylida) are an ancient lineage of nematodes which are common ungulate parasites, together forming a ubiquitous threat to farming productivity (Balic *et al.*, 2000; Hoberg *et al.*, 2001). On Rum this group includes *Trichostrongylus askivalli*, a species of strongyle that has only been reported from the Rum red deer (Dunn, 1964).

Despite their diversity, strongyle parasites are commonly counted at order-level. This methodology is practised in veterinary contexts, in sheep (Kenyon *et al.*, 2013; Melville

et al., 2016), cattle (Callaby *et al.*, 2014), and goats (Rinaldi *et al.*, 2009) – among others – as well as in disease ecological studies of horses (Wood *et al.*, 2013; Debeffe *et al.*, 2016), sheep (Hayward *et al.*, 2014a, 2019), and a range of African bovids (Ezenwa, 2004; Turner and Getz, 2010). Part of the reason for this lumping is its practicality: strongyle eggs are hard to differentiate, particularly at the species level (Hoberg *et al.*, 2001; Taylor *et al.*, 2016b); in horses, they have been described as having "virtually no diagnostically useful features" (Bredtmann *et al.*, 2017). Even where a researcher is determined to identify the strongyles past the order level, this differentiation relies on the prior identification of the strongyle species present and the construction of a morphological key. A select few strongyle species can be easily differentiated where their egg morphology differs substantially from the order-level average; notably, *Nematodirus* sp. is a cold-adapted strongyle which commonly infects young sheep, and which produces very large eggs (~200 microns). These eggs are generally counted separately from the strongyle-order counts, allowing them to be analysed separately (e.g., Kenyon *et al.*, 2013).

Another reason for the order-level lumping is its generality: in particular, worms in the strongyle order have relatively simple, similar life cycles. In all species the adult strongyle resides within the gut. After reproducing, the adult lays eggs into the gut lumen, and they are then excreted. In the environment, eggs develop into infectious larvae which are then ingested, beginning the cycle again (Hoberg *et al.*, 2001; Taylor *et al.*, 2016a). Finally, lumping together strongyle species provides a larger sample size to test, reducing the problems of data sparsity and overdispersion described above: counting all strongyles together increases power, thereby increasing models' ability to identify meaningful variation. If strongyle species are expected to react to biological changes in qualitatively similar ways – e.g., if all are expected to increase in response to allocation to reproduction – it is more likely that testing several species

as one will identify these effects compared to several independent tests of each species, and the latter risks type I errors.

Regardless, strongyle counts must be interpreted in light of their cryptic diversity. Despite sharing egg morphology and transmission modes, strongyles may differ in their interactions with the immune system, which can have important differences for the organism as a whole (Balic *et al.*, 2000; see above). Additionally, different strongyles exhibit different feeding modes and within-host niches: some species (e.g. *Trichostrongylus*) feed on mucosa, while some (e.g. *Haemonchus*) feed on the host's blood supply. Both the diversity of immune interactions and feeding strategies will affect the pathology and fitness consequences that result from infection; as such, these effects will vary heavily across species (Balic *et al.*, 2000; Hoberg *et al.*, 2001; Taylor *et al.*, 2016b). Most notably, blood-feeders such as *Haemonchus* are expected to be much more pathogenic than their non-bloodsucking counterparts (Budischak *et al.*, 2018; Lello *et al.*, 2018a). Therefore, when linking immunity with parasitism, and then when linking parasitism with its fitness consequences, the lumping together of a diverse array of strongyle species may risk introducing considerable variation which is hard to account for. African buffalo (*Syncerus caffer*) offer a good recent example: repeated sampling of the same individuals revealed that infection with different strongyle species and differential host immune responses determined the fitness consequences of infection itself (Budischak *et al.*, 2018). In summary, the order-level strongyle counts in this thesis prioritise methodological and statistical simplicity and tractability, at the expense of potentially important biological complexity and realism, and this must be kept in mind when interpreting the results presented.

Fasciola hepatica, or the common liver fluke, is a trematode parasite commonly found in cattle and sheep (Taylor *et al.*, 2016a; Beesley *et al.*, 2018). The adult liver fluke

resides in the liver, laying eggs which exit into the gastrointestinal system (Taylor *et al.*, 2016b). After excretion, these eggs develop and hatch over several weeks into miracidia, which then seek out *Galba truncatula* water snails. After infecting the snail, the fluke develops into cercariae which encyst on water vegetation as metacercariae. This vegetation is later eaten by ruminants. A recent study examined *F. hepatica* in culled deer in the Scottish highlands, finding a relatively high prevalence which varied considerably between sites and age and sex classes (French *et al.*, 2016).

Elaphostrongylus cervi is a highly prevalent red deer-specific nematode parasite (Mason, 1989; Gajadhar *et al.*, 1994; Vicente *et al.*, 2006). Residing within the central nervous system and skeletal musculature, adult *E. cervi* lay eggs into the bloodstream which develop into larvae, migrate to the lungs and are coughed up and swallowed. These L1 larvae can be extracted from the faeces via Baermannisation and then counted (Gajadhar *et al.*, 1994). Despite living in the deer's tissues, these parasites are generally thought to be relatively asymptomatic in red deer (Mason, 1989, 1994), and several wild studies support this (Irvine *et al.*, 2006; Alberti *et al.*, 2011).

Strongyles, *F. hepatica*, and *E. cervi* are investigated in parallel in all the following chapters. A few other parasites were discovered during this research, but were not analysed in detail, prevalences of which are described in chapter II. *Dictyocaulus* lungworms are another common livestock parasites which is found in wild deer populations (Irvine *et al.*, 2006; Buczek *et al.*, 2016); *Moniezia expansa* tapeworms are generally found in young sheep (Taylor *et al.*, 2016a); and coccidian protozoa have been reported in red deer but rarely analysed (Hines *et al.*, 2007). *Eimeria* sp. are common hit-and-run protozoan parasites which often especially infect young individuals (Taylor *et al.*, 2016d). This is not an exhaustive list of the parasites that have been studied in *C. elaphus*: for example, studies have previously examined

tuberculosis infections (*Mycobacterium bovis*; Queirós *et al.*, 2016) and blood parasites such as *Bartonella* (Dehio *et al.*, 2004). However, given the noninvasive nature of the project I could not examine these parasites.

As mentioned above, red deer culling regimes have led to a wealth of information on red deer parasites (and to a lesser extent their immunity), and on parasites' relationship with intrinsic and extrinsic factors affecting the deer. Extrinsic factors include geographic variation in prevalence and intensity of various parasites (Vicente *et al.*, 2006; Hines *et al.*, 2007; French *et al.*, 2016) and of immunity (Downs *et al.*, 2015), and seasonal variation in parasitism (Vicente *et al.*, 2007b; French *et al.*, 2016). Red deer and wapiti parasites increase in populations with higher density (Hines *et al.*, 2007) which may increase investment in constitutive immunity (Downs *et al.*, 2015). Deer outside the study area in Rum are culled annually, as they are through much of Scotland.

One study used culled specimens to examine the parasites present, and discovered multiple species of strongyles as well as *Elaphostrongylus cervi* (Irvine *et al.*, 2006). There were multiple associations with intrinsic factors: in particular, lower body condition was associated with increased parasitism, younger individuals had higher parasite intensities and burdens, and females exhibited higher parasite burdens (Irvine *et al.*, 2006). The findings of this paper, including our knowledge on the strongyle species present, are further reviewed in Chapter II.

Part 6: Aims

The aims of this thesis were as follows:

1. To examine the gut parasite fauna of the Isle of Rum red deer using noninvasive faecal samples, identifying parasite taxa and host groups of importance and developing parasitological and statistical methods with which to analyse them (Chapter II).

2. To quantify the importance of intrinsic and extrinsic factors determining infection prevalence and intensity in individual deer, particularly with regards to within-individual repeatability (Chapter II).

3. To examine the reproductive traits of adult female deer and their interactions with immunity, identifying life history tradeoffs and reproductive costs for immunity and parasitism (Chapter III).

4. To place immunity and parasites in the context of a pre-existing life history tradeoff, examining and comparing the roles of reproduction, immunity, and parasitism in determining individual fitness (Chapter IV).

5. To describe and contrast the spatial patterns of immunity and parasitism in the deer, investigating whether environmental variation might obscure or produce life history-immunity associations (Chapter V).

I discuss these findings in the context of the ecoimmunology and disease ecology literature and the statistical analysis of parasitism in wild populations. In particular, I will outline future avenues for the Isle of Rum red deer project (Chapter VI).

Chapter II: Temporal variation in helminth infection in wild red deer varies between individuals and between parasite taxa

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Summary

Parasitism in wild mammals can vary according to myriad intrinsic and extrinsic factors, many of which vary seasonally. This seasonal variation in parasitism is rarely studied using repeated samples from known individuals, so it is unclear whether individuals vary in their seasonal patterns of infection. Here we used a wild population of individually recognised red deer (*Cervus elaphus*) on the Isle of Rum to quantify seasonality and intrinsic factors affecting gastrointestinal helminth parasitism over the course of a year. We collected 1020 noninvasive faecal samples from 328 known individuals which we then analysed for propagules of three helminth taxa: strongyle nematodes, the common liver fluke *Fasciola hepatica,* and the tissue nematode *Elaphostrongylus cervi*. Zero-inflated Poisson models were used to investigate how season, age, and sex were associated with parasite prevalence and intensity, while Poisson models were used to quantify individual repeatability within and between sampling seasons. Parasite intensity and prevalence varied according to all investigated factors, with opposing seasonality, age profiles, and sex biases between parasite taxa. Repeatability was moderate, decreased between seasons, and varied between parasites; both *F. hepatica* and *E. cervi* showed significant between-season repeatability. Strongyle nematode counts were only repeatable within-season and showed no repeatability within individuals across the year, implying that different individuals exhibit different seasonal patterns of strongyle parasitism.

Introduction

Gastrointestinal helminths include a range of nematode, trematode, and cestode species and are an important selective force in wild vertebrate populations (Poulin, 2007). Although they can cause severe pathology in some cases, adult helminths can survive for years within a host; infections are commonly chronic and associated with immunosuppression and minimal overt pathology (Maizels and Yazdanbakhsh,

2003). The life cycles of helminth parasites vary, but broadly involve the parasite living and feeding within the host and producing propagules (eggs or larvae) into the gut lumen which are shed into the environment in the faeces, from which the parasite spreads to other hosts – sometimes via an intermediate host (Bohm *et al.*, 2007). Helminth infection is commonly quantified by counting these propagules in noninvasively collected faecal samples. Faecal egg counts (FECs) are often found to correlate well with burden (McKenna, 1981; Budischak *et al.*, 2015), despite egg output being a complex product of both host and parasite biology (Sargison, 2013) which can fluctuate over time (Villanúa *et al.*, 2006; Turner *et al.*, 2010). In both livestock and wild mammals one striking feature of FECs is their distribution, which is typically strongly right-skewed with a small number of individuals with relatively high counts (Woolhouse *et al.*, 1997; Wilson *et al.*, 2004). A major challenge for helminth epidemiology is to determine the degree to which variation in FEC is driven by factors intrinsic to the host (e.g. age, sex, genotype) versus extrinsic factors (e.g. season, climate, host density). Although there is good cross-sectional evidence that FEC varies with season, age, and sex in the wild, longitudinal data are more able to separate within- and among-individual processes, and to determine the repeatability of helminth infection within the same host (Clutton-Brock and Sheldon, 2010). Longitudinal FEC data remain relatively scarce in wild or free-living mammals, although prior studies suggest counts are to some degree repeatable within individuals over years and seasons (Wilson *et al.*, 2004; Wood *et al.*, 2013; Debeffe *et al.*, 2016). Importantly, as seasonal changes can affect hosts through changes in e.g. nutrition, immunity, and reproductive allocation (Martin *et al.*, 2008), parasitism may differ seasonally across the host population, so that different individuals peak in burden or transmission at different times of the year. Demonstrating this requires comparing and contrasting individual repeatability of FEC across seasons and parasite

groups. We aimed to use repeated counts at multiple timescales to separate real between-season differences in parasitism from baseline variation arising from experimental error or small-scale temporal fluctuations.

Parasites are commonly influenced by seasonal factors: weather patterns can impact parasites by affecting survival and movement of transmission stages, and regular changes in host biology such as parturition or mating seasons can affect resource availability (Altizer *et al.*, 2006). In temperate regions, helminths usually show a peak in egg output during the spring and summer, with lower or arrested transmission in the winter as a result of i) reduced survival and mobility of parasites (Stromberg, 1997) and ii) adaptive coincidence between infective parasites and immunologically naïve young in the spring (Wilson *et al.*, 2004). However, different species do show different adaptive seasonality: for example, in Svalbard reindeer the nematode *Marshallagia marshalli* transmits throughout the winter months despite extreme cold and different host feeding patterns (Carlsson *et al.*, 2012). Few comparisons of seasonality of egg counts across helminth groups have been made in wild vertebrate systems to date. Studies that rely on post-mortem sampling are often seasonally restricted to windows of mortality (e.g. Craig *et al.*, 2006) or culling seasons (e.g. Irvine *et al.*, 2006), and are therefore unable to investigate seasonal trends. Two important host factors associated with parasitism are age and sex. Age-dependent parasitism occurs across the animal kingdom, with higher intensities typically observed in the young and/or the elderly (Hayward *et al.*, 2009). Considering gastrointestinal helminths in wild vertebrates, there is strong evidence for declining parasitism from birth to adulthood, which is typically ascribed to two processes: first, animals gain adaptive immunity as they age (Woolhouse, 1992; Stear and Murray, 1994; Turner and Getz, 2010); second, animals that are most susceptible to parasites and therefore show the highest egg counts are more likely to die, meaning the animals that survive to old age are those

with lower parasite infections (Wilson *et al.*, 2004). Increasing helminth parasitism in elderly animals has also been ascribed to immunosenescence, although selective loss of highly infected individuals can also confound estimates of within-individual change in later life (van de Pol and Verhulst, 2006). A particular advantage of longitudinal studies is that they allow differentiation of the within- and among-individual processes responsible for age related variation (Clutton-Brock and Sheldon, 2010). Sex differences in parasitism are another common phenomenon in wild mammal species (Poulin, 1996), with higher burdens in males than females commonly observed in polygynous mammals in particular (Moore and Wilson, 2002). This is typically linked to sexual dimorphism and increased investment in short-term reproduction rather than immunity, rendering males the "sicker sex" (Zuk, 2009); however, a recent meta-analysis revealed that this effect is generally small and not robust to controlling for phylogenetic effects, calling into question the generality of these sex-related biases (Kelly *et al.*, 2018).

Wild ungulates are commonly infected with a range of helminth parasite species, and have formed the basis of many important individual-based studies in disease ecology (Hoberg *et al.*, 2001; Jolles and Ezenwa, 2015). Red deer have been the subject of multiple parasitological studies, due partly to their abundance in the wild where population management often requires culling (Bohm *et al.*, 2007), and as farm animals, principally in New Zealand (Mason, 1994). They are of particular interest due to their ability to act as reservoirs and vectors of parasites that commonly infect livestock (Alberti *et al.*, 2011; Chintoan-Uta *et al.*, 2014; Davidson *et al.*, 2014), and cross-sectional culling studies have formed a useful knowledge base for red deer parasitology. Previous studies of helminth parasites in wild red deer have demonstrated through cross-sectional post-mortem sampling that parasite burdens vary with age (Vicente *et al.*, 2006), sex (Irvine *et al.*, 2006; Vicente *et al.*, 2007b;

French *et al.*, 2016), and condition (Irvine *et al.*, 2006; Vicente *et al.*, 2007a; b). Although such cross-sectional approaches provide detailed data on the parasite community and adult parasite burden, they cannot separate within- and among-host processes involved in helminth epidemiology and host-parasite interactions. Furthermore, male deer are culled earlier in the year than females, therefore confounding sex and seasonal effects (e.g. Irvine *et al.*, 2006; French *et al.*, 2016). Longitudinal studies monitoring and comparing the within-host repeatability and temporal variation in counts are currently lacking for helminth parasites of wild red deer, although seasonality of *E. cervi* and strongyles has been demonstrated using the collection of fresh faecal samples (though without the full benefits of individual-level data; Vicente *et al.*, 2005; Hines *et al.*, 2007), and strongyles in red deer are known to undergo a winter season of arrested development (Connan, 1997). An earlier study examining gastrointestinal parasites of red deer living across the Isle on Rum in Scotland (Irvine *et al.*, 2006) used animals culled for management purposes in the late summer-autumn (males) or autumn-winter (females). It found a high prevalence, but low burden, of strongyle nematodes *Ostertagia* spp. and *Oesophagostomum venulosum*, as well as the nematodes *Nematodirus* sp., *Capillaria* sp., *Trichuris ovis*, *Elaphostrongylus cervi*, *Dictyocaulus* sp. and coccidian *Eimeria* sp.. Importantly, *Nematodirus* is in the strongyle clade, but has large (~200 microns) distinguishable eggs, so would not be included in strongyle faecal egg counts. In contrast, *Ostertagia*, *Oesophagostomum*, and *Trichostrongylus* would all be included among strongyle counts because of their difficult-to-discern egg morphology. In addition, *Ostertagia* nematodes are occasionally identified as *Teladorsagia*, and *vice versa*, being closely related (Lichtenfels and Hoberg, 1993). Hence, it is possible that *Teladorsagia circumcincta*, a common sheep nematode, was (and is) also present in the Rum deer. The tissue nematode *Elaphostrongylus cervi* and the generalist liver fluke *Fasciola*

hepatica, both of which have life cycles that involve an intermediate snail host, have been documented using egg counts in wild red deer (Vicente *et al.*, 2007b; French *et al.*, 2016). However, strongyle nematodes in wild red deer have rarely been studied using noninvasive methods, which is surprising given their detailed study in related livestock hosts including cattle and sheep (Hoberg *et al.*, 2001). In the present study, we repeatedly collected faeces from known study individuals of different ages and sexes on Rum within and across seasons in 2016. We analysed propagule counts of the three most prevalent helminth species groups: strongyle nematodes, *E. cervi*, and *F. hepatica*. Our aims were to: (i) examine the parasite fauna of the Rum red deer and identify taxa of high prevalence for statistical analysis; (ii) assess the repeatability of noninvasive parasitological measures in the deer at multiple timescales, particularly between seasons; (iii) investigate how prevalence and intensity of infection with the abundant taxa are associated with season, host age and host sex.

Methods

Study area and sample collection

The study was conducted in the North block of the Isle of Rum National Nature Reserve in the Inner Hebrides, Scotland (57°N 6°20'W). The island has a mild, wet climate and the vegetation consists of a mosaic of high-quality grassland and lower quality dry and wet heath and blanket bog. The study population comprises ~350 animals at any one time. Neonates are caught during the calving period May-July and individually marked with collars, ear tags, coloured flashes and ear punches, enabling life-long individual identification. Censuses are carried out five times a month for nine months of the year with more frequent informal monitoring between censuses allowing compilation of individual life histories. The study area population has not been culled since 1973 and runs at the carrying capacity determined by the ground and prevailing weather conditions (Clutton-Brock *et al.*, 1982). Faecal sampling was

conducted on a seasonal basis, with two-week trips carried out in winter (January), spring (April), summer (August) and autumn (November); each trip was considered to be representative of the three-month season in which it occurred. Data for this study were all collected in 2016; as red deer are born in May-June this resulted in the study sampling two different cohorts of calves, born 2015 (sampled in winter and spring before their first birthday) and 2016 (sampled in summer and autumn). Groups of individually recognised deer were observed for defecation events from 15-250m using binoculars and telescopes, with the samples recovered as quickly as possible without unduly disturbing the deer. Samples were stored in resealable plastic bags until processing. Efforts were made to sample as many different individuals in a trip as possible, with a subset of individuals deliberately sampled more than once in each season in order to examine the within-season repeatability of parasitological measures. Faecal analysis can be affected by the hatching, development and death of parasite propagules, influenced by temperature and oxygen availability (Nielsen *et al.*, 2010). For this reason, time of defecation, time of collection and date of count were all recorded. Following return to the field station, samples were weighed and homogenised by hand in their resealable bags to minimise oxygen exposure. These bags were then put inside date-specific larger bags to keep them as anaerobic as possible. All samples were kept refrigerated at 4°C until parasitological analysis. Upon return to the lab, analysis was carried out within the next 8-10 weeks (see details below). Over the course of four seasons in 2016, Ns=1020 faecal samples were collected from Ni=328 individuals, equating to 783 different individual-season combinations with 237 within-season repeats. The sampled (and resampled) individuals were a mixture of calves, yearlings, two-year-olds and adults of both sexes – although adult males were sampled much less frequently (Ns=43, Ni=20) than adult females (Ns=522,

Ni=137) because relatively few adult males live in the study area. The age range of animals sampled was 0-21 years old (median 3 years old).

Parasitology

Parasitological terms will be used as defined in Margolis *et al.* (1982), with intensity based on propagule counts. We use "burden" to refer to the number of worms of a species infecting an individual, which could not be measured directly. Strongyle FECs were carried out within three weeks of collection using a modified sedimentation-salt flotation method (Taylor *et al.*, 2016c) with an accuracy of 1 egg per gram (EPG). The method was modified from Kenyon *et al.* (2013). Briefly, 2-15g of faecal matter were mixed with 10ml water per gram of faeces and the mixture thoroughly homogenised to suspend the eggs. 10ml of this suspension was filtered through a tea strainer and washed through with 5ml water. The resulting liquid was decanted into a 15ml polyacrylate test tube, which was centrifuged at 1500 rpm for 2 minutes and the supernatant removed. The resulting pellet was mixed with saturated salt solution and resuspended, then centrifuged again, leaving the eggs and light debris at the surface of the liquid. Using medical forceps to clamp below the meniscus, this surface layer was poured off into a cuvette which was topped up with saturated salt and then a lid was added to seal the contents. The entire surface area of the cuvette was counted at 4X magnification to give a count of eggs in 1g faeces, revealing strongyle nematode eggs (excluding *Nematodirus*) and a selection of other species the eggs of which are less dense than the salt solution. This included *Nematodirus* sp. and *Capillaria* sp. The assay also revealed oocysts of the coccidian parasite *Eimeria* sp., which came in two varieties ("large" and "small"), and segments of the cestode *Moniezia expansa*. 730 strongyle faecal egg counts were repeated to estimate the technical repeatability of this method in our hands. These counts were averaged to give an EPG value for the sample.

Fasciola hepatica eggs were detected through a sedimentation method (French *et al.*, 2016; Taylor *et al.*, 2016c) conducted on 0.5-2g of faecal matter, from the same homogenate as the strongyle FEC, within ten weeks of collection. In the interim all samples were kept refrigerated and oxygen-deprived, both of which prevent hatching (Hurtrez-Boussès *et al.*, 2001). After filtering the faecal matter through a tea strainer, the sample was left to sediment in a conical beaker for three minutes. During this time the heavier debris (including the eggs) settles to the bottom of the beaker, and the lighter material can then be removed to leave the eggs with as little debris as possible. The remaining filtrate was pipetted onto a petri dish and stained with methylene blue (1% w/v). The petri dish was then examined microscopically at 4X magnification and the eggs, which are yellow against blue debris background, were counted, with the counts divided by the weight in grams of sample used.

Elaphostrongylus cervi and *Dictyocaulus* sp. larvae were isolated via a modified Baermannisation assay (Gajadhar *et al.*, 1994) within four weeks of collection. 1-14g of faecal matter was wrapped in muslin cloth and submerged fully in a 50ml falcon tube filled with water. This was left at room temperature for 20-24h for the L1 larvae to emerge from the faeces and fall to the bottom of the tube. The supernatant was then carefully removed to leave <2ml containing the larvae. This fraction was preserved with Lugol's iodine and kept refrigerated at 4°C until counting. Counts were performed on a subsample under 4x magnification and divided by the weight of faeces used to give a measure of larvae per gram.

Some samples were not large enough to be analysed for all parasite types – hence final sample sizes were 1014 (strongyles), 991 (*F. hepatica*) and 1003 (*E. cervi*). Fluctuations in faecal water content can lead to variation in per-gram faecal egg count, particularly across host sex and age classes and across seasons (Turner *et al.*, 2010). For this reason,

the proportion faecal dry matter (FDM) per gram of collected faeces was calculated for each sample by drying a known weight of faecal matter in an oven at 60°C for 48 hours and then weighing the resulting solid. FDM was mean-centred around 1 to prevent changing the distribution of non-zero counts relative to the zero counts. Counts were divided by FDM to give a measure of eggs or larvae per gram of dry matter, which was rounded to the nearest whole number to allow the use of integer count-based models.

Statistical analysis

Correlations of repeated counts. Since many of the parasite groups identified were present at low prevalence (<30%, see Table 1), we restricted further analyses to the three most prevalent parasite egg groups: strongyle nematodes, the liver fluke *Fasciola hepatica*, and the tissue worm *Elaphostronylus cervi*. Initially, we estimated the repeatability of propagule counts at different temporal scales by calculating Spearman's rank correlation coefficients (r) among: (1) repeated counts from the same sample ("technical repeatability"); (2) repeated counts from the same individual within a season ("within-season repeatability"); and (3) averaged within-season measures from the same individual in different seasons ("between-season repeatability"). We also investigated whether factors related to the collection and processing of the samples influenced egg counts, with simple linear models for each parasite taxon. We investigated the influence of a) time of collection, b) time to processing and c) time to counting. However, these factors had no effect and so were not included in further analysis.

Intrinsic and extrinsic factors influencing parasite prevalence and intensity. To test how season, age and sex were associated with parasite egg counts and to decompose the within- and among-individual variation in these counts, we used Generalised

Linear Mixed Models (GLMMs). Parasite egg count distributions are often non-normal and strongly overdispersed (Alexander, 2012) and can feature significantly more zero counts than would be expected given their distribution (Chipeta *et al.*, 2014). Analysis was carried out in R version 3.4.0 (R Core Team, 2018), with the Bayesian statistical package MCMCglmm (Hadfield, 2010), which is flexible with respect to error structures. In this set of models, we used averages of any repeat measures from the same individual within a season. We fitted zero-inflated Poisson (ZIP) GLMMs, with which we generated two estimates for each fixed or random effect within the model. The first estimate (Poisson) calculated the effect a factor has on the data assuming an overdispersed Poisson distribution, including an expected number of zero counts, while the second (zero-inflation) estimated the effect that a factor has on the number of zeros in the data. We consider these two estimates as reflecting variation in parasite intensity and prevalence, respectively. We follow convention by presenting results for prevalence before intensity for each parasite taxon. This method was chosen as factors can have contrasting effects on parasite prevalence and intensity (Chipeta *et al.*, 2013). The explanatory variables fitted in the models include age category (with 4 levels: Calf, Yearling, 2-year-old and Adult), sex (Female and Male) and season (Winter, Spring, Summer and Autumn), with individual identity as a random effect to control for variation between individuals (Paterson and Lello, 2003). Models were run for 2.6 million iterations (thinning interval 2000, burnin 600,000). Significance of differences among factor level means was calculated by comparing the proportion overlap of the posterior distributions of the MCMC estimates for each level and then doubled to give P_{MCMC} following Palmer, Hadfield and Obbard (2017).

Model-derived repeatability. The variance component associated with the individual random effect within mixed effects models is often used to calculate within-individual

repeatabilities (Falconer and Mackay, 1996). This method accounts for variation between individuals that occurs as a result of the model's fixed effects (i.e. originating from differences between age, sex and seasonal categories) to estimate the proportion of variation which is explained by differences between individuals. However, there is currently no accepted method of extracting repeatability from the random effects structure of zero-inflated models. We therefore re-ran our models of each parasite count on a non-zero-inflated subset of the data using a standard Poisson model featuring additive overdispersion and applied the method described by Nakagawa and Schielzeth (2010) to calculate repeatability. We included an individual identity random effect to estimate among-season variation between hosts and an individual-by-season interaction as a second random term to estimate within-season variation within hosts. In the absence of between-season repeatability, significance of this latter term would demonstrate consistency within individuals of those repeat samples collected within seasons. Repeatabilities were calculated on the count scale rather than the latent scale. The data analysed with this model included all samples including 237 within-season repeat samples. Prior to analysis we removed the winter season data (when counts were very low for all parasite taxa) and a subset of mainly prepatent individuals (calves in the summer for F. *hepatica* and calves in the summer and autumn for *E. cervi*), as the repeatability of these counts would be of little biological interest and they were the major source of zero-inflation.

Results

Correlations of repeated counts

The seasonal prevalence, intensity and maximum count of each of the parasite taxa is displayed in Table 1. All parasites were found throughout the year except *Dictyocaulus* sp. and *M. expansa*, which were not found in the winter. Repeated strongyle counts of the same sample were strongly correlated (Spearman's R=0.95, Figure 1). Within-

season resampling of the same individuals revealed strong correlations for all three parasites (Spearman's R>0.6, Figure 2). Between-season averaged counts from the same individuals correlated less, and varied more between parasites (see Figure 3 for coefficients). Strongyle nematodes showed the lowest between-season repeatability, with *E. cervi* and *F. hepatica* higher. These results were qualitatively similar to the model variance-derived repeatability estimates (see below).

	Strongyles			***Fasciola hepatica***			***Elaphostrongylus cervi***			***Dictyocaulus sp.***			***Nematodirus sp.***			***Capillaria sp.***			***Eimeria* (large)**			***Eimeria* (small)**			***Moniezia expansa***		
	Mean	Max	Prevalence	Mean	Max	Prevalence	Mean	Max	Prevalence	Mean	Max	Prevalence	Mean	Max	Prevalence	Mean	Max	Prevalence	Mean	Max	Prevalence	Mean	Max	Prevalence	Mean	Max	Prevalence
Winter	1	7	37%	3	47	52%	9	118	93%	0	0	0%	0	3	10%	0	4	13%	0	7	15%	0	8	18%	0	0	0%
Spring	20	157	97%	9	155	57%	69	299	94%	0	7	15%	0	4	4%	0	11	11%	0	18	11%	1	34	12%	0	44	1%
Summer	17	167	97%	4	50	44%	37	337	74%	4	193	32%	0	8	11%	0	11	4%	16	930	14%	3	145	11%	1	65	2%
Autumn	8	85	64%	8	147	62%	67	645	76%	3	65	36%	1	37	18%	0	20	4%	1	37	8%	1	28	9%	10	322	7%

Table 1: Seasonal prevalence and mean and maximum count in propagules/g faeces of each parasite found. Minimum count of every season-parasite combination was zero.

		Strongyles		*F. hepatica*		*E. cervi*	
Factor	Statistic	Estimate	Pmcmc	Estimate	Pmcmc	Estimate	Pmcmc
Intercept	Prevalence	3.36 (1.14,5.59)	<0.001***	0.97 (-0.18,2.06)	0.082	1.2 (-0.05,2.54)	0.06
	Intensity	0.24 (-0.18,0.59)	0.232	1.34 (0.98,1.72)	<0.001***	1.77 (1.39,2.12)	<0.001***
AgeCat: Y	Prevalence	1.61 (-1.86,5.26)	0.396	1.22 (0.04,2.23)	0.018*	4.03 (2.76,5.32)	<0.001***
	Intensity	-0.79 (-1.02,-0.53)	<0.001***	-0.19 (-0.46,0.1)	0.186	0.62 (0.35,0.92)	<0.001***
AgeCat: 2Y	Prevalence	-3.52 (-6.43,-0.77)	0.038*	0.57 (-0.87,2.01)	0.466	4.57 (2.79,6.69)	<0.001***
	Intensity	-1.37 (-1.78,-1)	<0.001***	-0.52 (-1,-0.08)	0.024*	0.44 (0.06,0.87)	0.022*
AgeCat: A	Prevalence	-4.96 (-7.13,-2.98)	<0.001***	-1.58 (-2.61,-0.56)	0.002**	5.73 (3.98,7.31)	<0.001***
	Intensity	-1.45 (-1.71,-1.18)	<0.001***	-1.31 (-1.81,-0.85)	<0.001***	-0.03 (-0.36,0.38)	0.842
Sex: Male	Prevalence	1.06 (-0.59,3.12)	0.248	0.35 (-0.6,1.46)	0.496	-0.8 (-1.87,0.31)	0.15
	Intensity	0.27 (0.05,0.52)	0.024*	0.16 (-0.19,0.51)	0.386	-0.33 (-0.61,0.01)	0.04*
Season: Spring	Prevalence	5.2 (3.12,7.35)	<0.001***	0.15 (-0.91,1.07)	0.782	0.97 (-0.78,2.18)	0.174
	Intensity	2.98 (2.6,3.37)	<0.001***	1.06 (0.79,1.34)	<0.001***	2.02 (1.82,2.22)	<0.001***
Season: Summer	Prevalence	5.18 (3.07,7.16)	<0.001***	-1.33 (-2.4,-0.31)	0.008**	-2.26 (-3.73,-1.02)	<0.001***
	Intensity	2.81 (2.42,3.21)	<0.001***	0.75 (0.41,1.07)	<0.001***	1.06 (0.84,1.27)	<0.001***
Season: Autumn	Prevalence	1.11 (-0.32,2.5)	0.114	1.12 (0.02,2.17)	0.036*	-1.7 (-3.04,-0.42)	0.012*
	Intensity	1.98 (1.58,2.42)	<0.001***	0.81 (0.5,1.16)	<0.001***	1.78 (1.58,1.99)	<0.001***

Table 2: MCMCglmm model estimates for zero-inflated Poisson GLMMs for each analysed parasite taxon. The estimates represent the posterior mean, with 95% credibility intervals in brackets. Prevalence (zero-inflation) values have been multiplied by -1 to aid interpretation: a positive value in the model corresponds to an increase in zero-inflation - i.e., a decrease in infection probability. Asterisks represent significance intervals:*, ** and *** refer to P<0.05, P<0.01 and P<0.001 respectively.

Intrinsic and seasonal correlates of counts

Model results revealed contrasting trends for all three parasites according to all investigated factors (Table 2; Figure 4). Strongyle prevalence and intensity peaked in the spring and summer (Figure 5, $P_{MCMC}<0.001$) and decreased in the autumn, with intensity remaining higher than winter levels ($P_{MCMC}<0.001$) but not prevalence ($P_{MCMC}=0.114$). Spring and summer were not significantly different in prevalence ($P_{MCMC}=0.988$) or intensity ($P_{MCMC}=0.086$). There was a persistent age effect in that older individuals tended to be less often infected and at lower intensities (Figure 5). Calves showed a higher prevalence of infection than two-year-olds ($P_{MCMC}=0.038$) and adults ($P_{MCMC}<0.001$) and had a higher intensity than all age categories ($P_{MCMC}<0.001$). Yearlings also had higher prevalence than adults ($P_{MCMC}<0.001$), and higher intensity than both 2-year-olds and adults ($P_{MCMC}=0.01$; $P_{MCMC}<0.001$ respectively). Sex also had an effect in the strongyle model (Figure 6), with males showing higher intensity infections ($P_{MCMC}=0.024$) but no difference in prevalence ($P_{MCMC}=0.436$).

F. hepatica increased in intensity after the winter ($P_{MCMC}<0.001$) as did strongyles, but decreased in prevalence in the summer ($P_{MCMC}<0.001$) and prevalence was highest in the autumn compared to the winter ($P_{MCMC}=0.036$), spring ($P_{MCMC}=0.042$), and summer ($P_{MCMC}<0.001$). Unlike strongyles, *F. hepatica* prevalence peaked in yearlings ($P_{MCMC}=0.01$) rather than in calves, and decreased in prevalence and intensity thereafter. Intensity of infection was lower in 2-year-olds than in calves ($P_{MCMC}=0.024$), and adults had lower prevalence and intensity of infection compared to all other age classes ($P_{MCMC}=0.002$ for prevalence; $P_{MCMC}<0.001$ for intensity). There was no evidence of a sex bias in either prevalence ($P_{MCMC}=0.496$) or intensity ($P_{MCMC}=0.386$) of *F. hepatica* infection, in contrast to the results of the strongyle model.

Prevalence of *E. cervi* was lower in the summer and autumn than in the winter and spring (P_{MCMC}<0.001, Figure 5), differing again from the patterns shown by either strongyles or *F. hepatica*. However, like the other parasites *E. cervi* intensity was highest in the spring (P_{MCMC}<0.001). The *E. cervi* age trend differed from the other parasites in that older age classes had higher prevalence but lower intensity of infection. Calves showed a lower *E. cervi* prevalence than all age classes (P_{MCMC}<0.001) and lower intensity compared to yearlings (P_{MCMC}<0.001) and 2-year-olds (P_{MCMC}=0.022). Adults had a higher prevalence than yearlings (P_{MCMC}=0.044) but a lower intensity than yearlings and 2-year-olds (Both P_{MCMC}<0.001). Unlike both strongyles and *F. hepatica, E. cervi* showed a weak female sex bias, with increased intensity in females (P_{MCMC}=0.04). Prevalence was also higher in females, although this was not significant (P_{MCMC}=0.15).

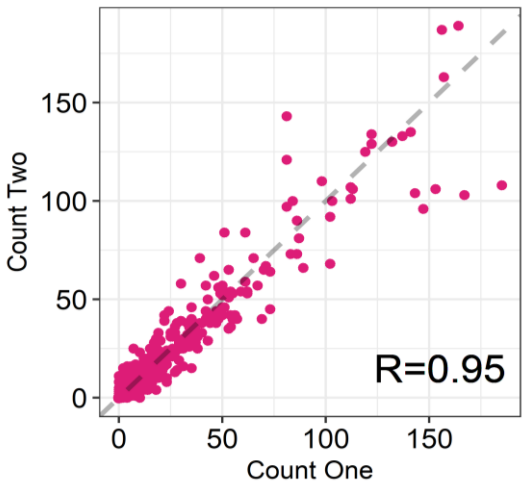

Figure 1: Correlation between first and second strongyle faecal egg count of the same sample (Ns=730). R is the Spearman's rank correlation.

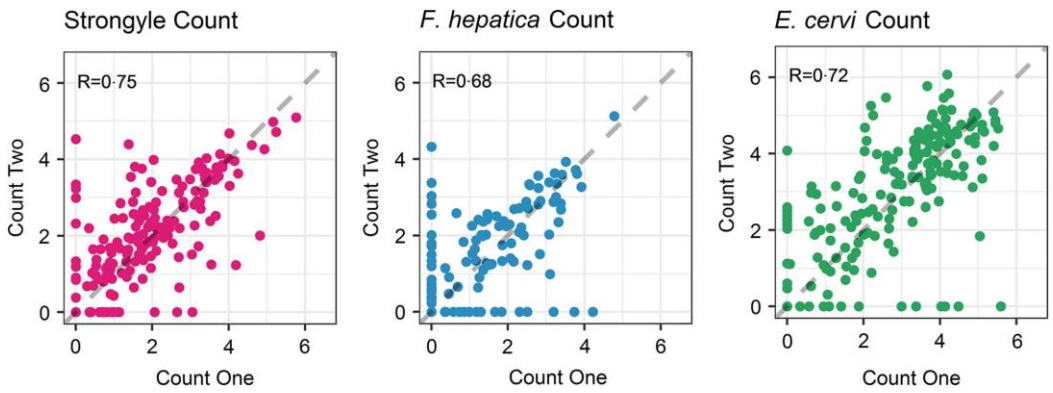

Figure 2: Within-season correlations of individuals' counts from different faecal samples (A: Strongyles, B: *F. hepatica*, C: *E. cervi*). Axes have been log(x+1) transformed for display purposes. The dashed line represents equal counts, y=x. R is the Spearman's rank correlation.

Model-derived repeatability estimates

After accounting for differences in age, sex and season using overdispersed Poisson models, the individual component of variation in count differed between all parasites (P_{MCMC}=0.014 for *F. hepatica* and *E. cervi*; P_{MCMC}<0.001 otherwise). The direction and significance of the fixed effects from these models closely followed the Poisson component of the ZIP models. Strongyle counts had very low repeatability between-seasons with posterior mode R^2=0 (95% credibility intervals 0-0.07). *F. hepatica* and *E. cervi* repeatabilities were higher: R^2=0.19 (0.12-0.27) and 0.30 (0.25-0.38) respectively. However, strongyle counts were repeatable within-season, as demonstrated by variance accounted for by the ID:Season interaction term, R^2=0.38 (0.24-0.49). This term was low for *F. hepatica* and *E. cervi* (R^2=0 for both), showing that while both were repeatable at all levels this was expressed in the between-season individual identity term, with no additional repeatability within seasons. These results differ markedly from the raw correlations (Figure 2; 3) as they take into account variation that is attributable to age, sex and season, thereby estimating the within-individual correlations given these factors.

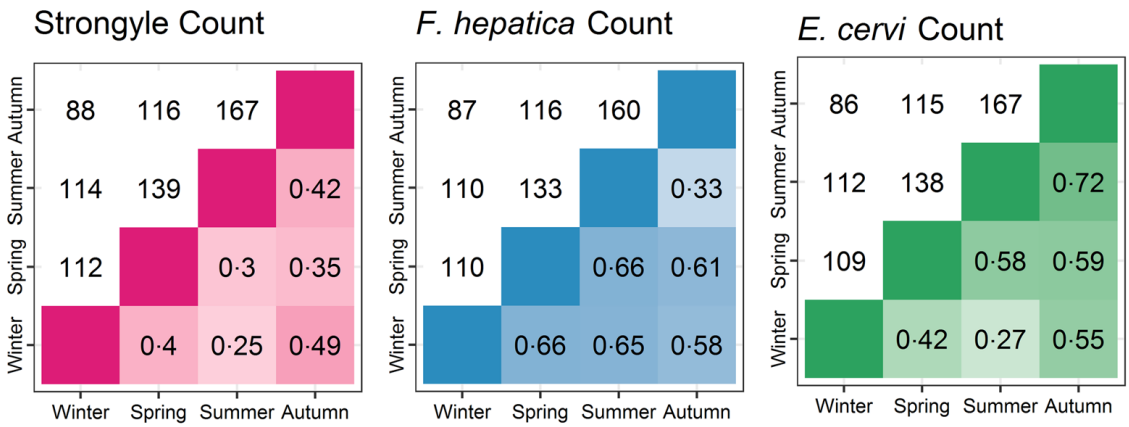

Figure 3: Between-season correlations of parasite counts (A: Strongyles, B: *F. hepatica*, C: *E. cervi*). Values above the diagonal represent the number of pairs of individual samples the comparisons are based on; values below the diagonal are Spearman's Rank correlations.

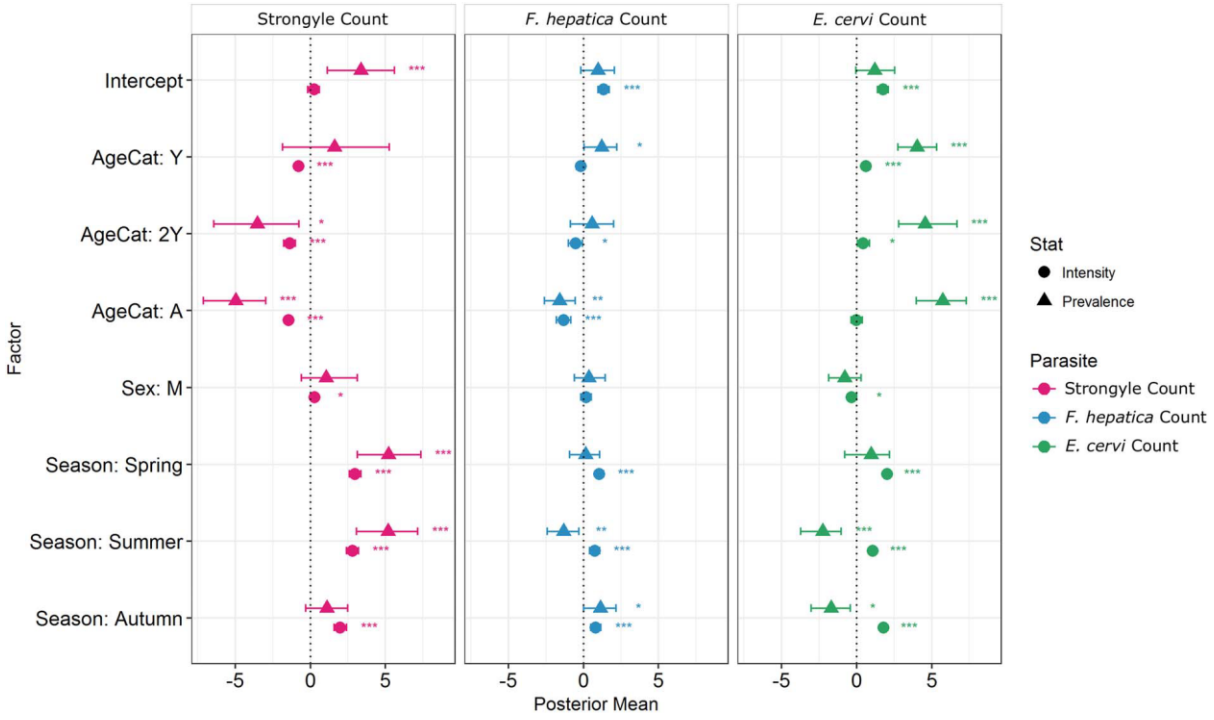

Figure 4: MCMCglmm zero-inflated Poisson model output for each of the three analysed parasite taxa. Points represent posterior estimates for mean effect sizes; error bars represent the 95% credibility intervals of the mean. Symbol corresponds to the statistic being estimated - zero-inflation (Prevalence) or Poisson (intensity). Zero-inflation coefficients have been multiplied by -1 to aid interpretation; that is, a positive value represents a decrease in zero-inflation and therefore an increase in prevalence.

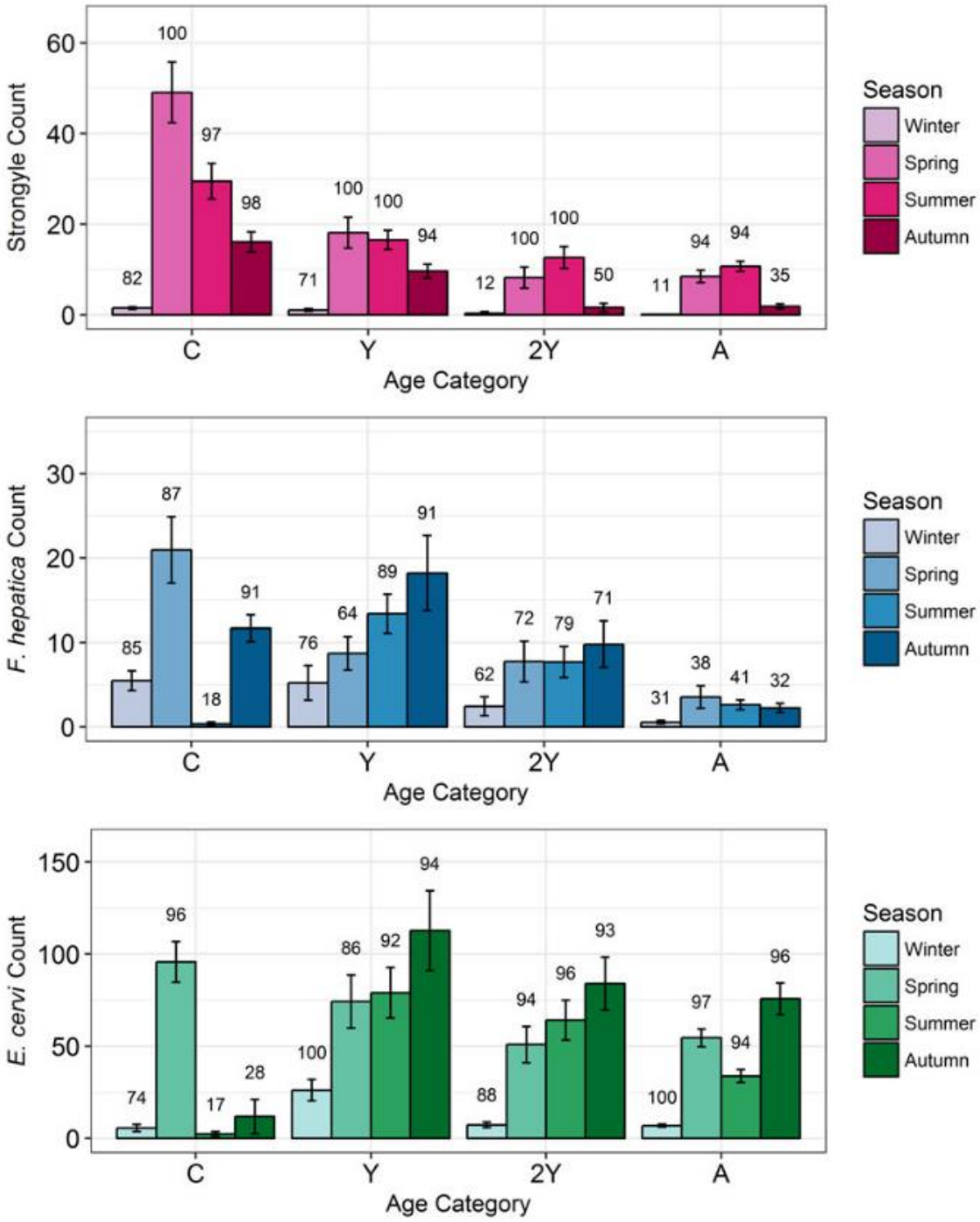

Figure 5: Seasonal mean counts in eggs or larvae per gram (+/- SE) for each parasite (A: Strongyles, B: *F. hepatica*, C: *E. cervi*) in each age category. Numbers correspond to per cent prevalence. Groups on the x axis are calves, yearlings, two-year-olds and adults in order. Figures were created using raw faecal dry matter-transformed data. The calf category represents two different cohorts: those born in 2015 (winter and spring) and those born in 2016 (summer and autumn).

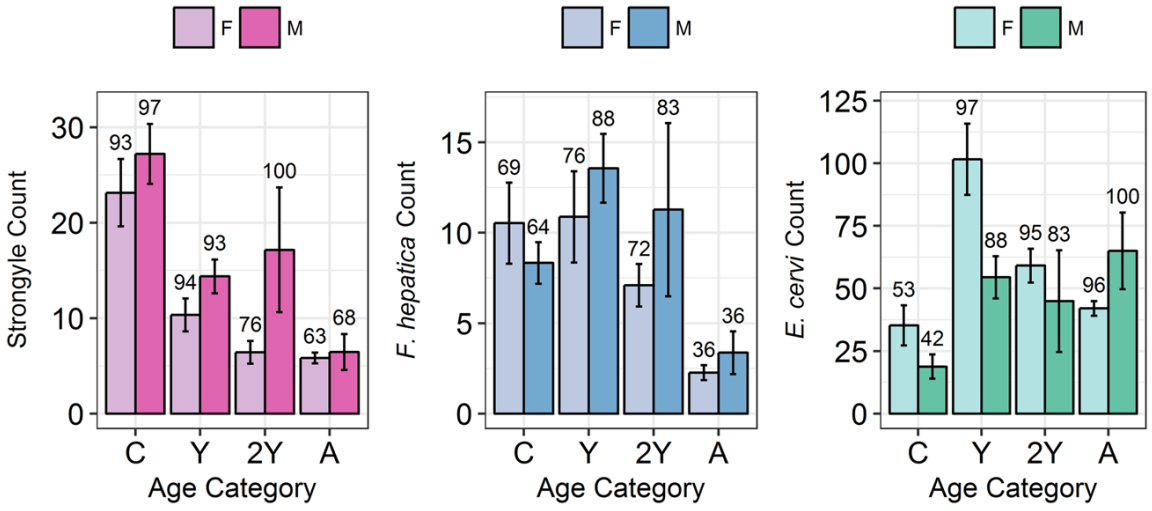

Figure 6: Mean count in eggs per gram or larvae per gram (+/- SE) for each parasite (A: Strongyles, B: *F. hepatica*, C: *E. cervi*) according to sex and age category, calculated as in Figure 5. Numbers above the bars correspond to per cent prevalence. Groups on the x axis are calves, yearlings, two-year-olds and adults in order. Figures were created using raw faecal dry matter-transformed data.

Discussion

We discovered a significant individual component of parasitism despite extensive temporal variation in parasite counts across the year. Both *F. hepatica* and *E. cervi* were repeatable between seasons once age and sex category were accounted for using Poisson models, while strongyle counts were only repeatable within seasons. Seasonal trends varied, with strongyles showing the most extensive seasonality, though counts of all parasites were lowest in winter. Younger individuals tended to have higher-intensity infections than adults as expected, but this was not mirrored in increased prevalence except in strongyles. Interestingly, sex biases differed between parasites, with higher strongyle intensities in males and marginally higher *E. cervi* prevalence in females. An overriding feature of this study is the contrasting and asynchronous effects shown by the different parasite taxa: despite all being helminths and having relatively similar life cycles, they exhibited substantially different seasonality and intrinsic trends as well as showing different levels of repeatability which are not attributable to their different detection assays. Studies in wild mammals which

investigate multiple pathogens often examine distantly related microparasites and macroparasites with very different life cycles (e.g. Vicente *et al.*, 2007). Here we demonstrate the value of investigating multiple high-prevalence parasite taxa; even where life cycles are ostensibly similar, the parasites likely vary in e.g. their life history characteristics, interactions with immunity, and exposure patterns in ways that impact their dynamics considerably. In accordance with previous studies (e.g. Chipeta *et al.*, 2013), zero-inflated Poisson models successfully revealed that some factors affected prevalence and intensity in different directions. While factors that increased prevalence of infections also tended to increase intensity whether their effects were significant or not (Figure 4), there were notable exceptions in the season categories: the summer (and autumn for *E. cervi*) featured increased intensity but reduced prevalence of *F. hepatica* and *E. cervi* compared to winter. Similarly, the models distinguished between increased prevalence but reduced intensities of *E. cervi* in adults compared to younger age classes. We advocate the use of these models where sample sizes are sufficient, particularly in situations where differences between seasons and classes will result in divergent processes affecting helminth prevalence and intensity (e.g. age-related infection prepatency).

Propagule output is a function of both host and parasite biology (Sargison, 2013), representing a combination of adult worm burden and host health as well as fluctuations in worm reproduction; hence faecal propagule counts are subject to fluctuations through time. We have shown that despite this, helminth counts can be repeatable among wild individuals within and across seasonal time frames. In the repeated counts, strongyle egg count had a Spearman's rank correlation of R=0.95, demonstrating high technical repeatability of this count method. Within-season correlations were high for all parasites, showing that each sample taken was largely representative of an individual's parasite count regardless of when the sample was

taken in the day or within the sampling trip. This repeatability also demonstrates a high reliability of the assays used and low importance for potential nuisance factors such as time to processing or time of sampling. This is in accordance with helminth studies in other ruminants (e.g. Rinaldi *et al.*, 2009) – however, there may still be effects of time to collection and analysis that were not detected in this dataset and may reduce repeatability. In addition, it is noteworthy that a number of individuals switched between zero and non-zero counts for all parasites (Figure 2) and therefore a negative propagule count is not necessarily indicative of an uninfected individual, demonstrating the potential value of repeated sampling (Lamberton *et al.*, 2014; Buzdugan *et al.*, 2017) and the difficulty of diagnosing helminth infection using noninvasive faecal sampling. Similar high repeatability for different parasite taxa is nevertheless surprising, given that propagule counts were performed using three different assays which may differ in their reliability; the low intensity and possible low burden of infection; and the fact that propagule shedding of all three taxa is intermittent (Gajadhar *et al.*, 1994; Vercruysse and Claerebout, 2001; Schär *et al.*, 2014).

Between-season correlations were lower than within-season correlations due to differences that emerged between individuals between seasons. These correlations decreased further when repeatability was derived from Poisson models, thereby accounting for variance due to season, age category and sex. This shows that while our counts were accurate and repeatable, much of the variation was due to certain classes (e.g. calves) showing higher prevalence and intensity than others, and individuals' counts were less consistent when looking within-category. Nevertheless, model-derived R^2 estimates associated with individual identity did not overlap with zero for either *F. hepatica* or *E. cervi*, demonstrating consistent differences between individuals throughout the year. Strongyle counts were not as repeatable between

seasons, but did show individual consistency within seasons (R^2=0.38), demonstrating that individual repeatability decreased between seasons rather than being absent at all levels. This R^2 value is intermediate compared to other studies investigating strongyle FEC of wild horses within and between seasons (Wood *et al.*, 2013; Debeffe *et al.*, 2016) but low compared to between-season repeatability in farmed horses (Scheuerle *et al.*, 2016) and goats (Hoste *et al.*, 2002). Low repeatability compared to farmed animals is unsurprising given the large range of different conditions that wild individuals experience. High variability of parasite counts within individuals has implications for their quantification in wildlife. For example, an individual that shows high strongyle FEC in the spring may not do so in the summer. This demonstrates the value of multiple sampling seasons as well as reflecting the evolutionary ecology of the deer and their helminths. Different seasonal peaks may be associated with between-individual variation in seasonal trade-offs with immunity through e.g. reproduction (Martin *et al.*, 2008). Similarly, asynchronous peaks of egg output across the host population may be adaptive for the helminths in encouraging year-round transmission and bet-hedging to buffer for unfavourable climatic conditions.

All parasites showed some transmission in each sampling trip, although as expected intensity of infection was lowest in the winter for all three parasites, with peaks in the spring and summer which continued into the autumn for *F. hepatica* and *E. cervi*. This low transmission in colder seasons likely reflects a reduction in egg production rather than solely burden, as strongyle burden in Spanish red deer stays constant or increases in the winter (Santín-Durán *et al.*, 2008) and *F. hepatica* is found regularly in necropsies of the Rum deer throughout the mortality period (Sean Morris, pers. obs). Freezing, which regularly occurs on the ground in the winter on Rum, is known to damage strongyle eggs and larvae (Foreyt, 1986; Wharton and Allan, 1989) and *F. hepatica* eggs (French *et al.*, 2016). However, overwinter transmission can occur in

some parasite species (e.g. Carlsson *et al.*, 2012); future work identifying the strongyle species present may be able to identify whether winter and autumn strongyle transmission involves a few frost-resistant species. As well as increasing the survival of environmental stages, high helminth transmission in the spring is a possible adaptive strategy resulting in coincidence between vulnerable calves and maximum infective parasites in the environment, similarly to the periparturient rise in sheep strongyle FEC (Armour, 1980). *F. hepatica* and *E. cervi* showed less variation between seasons than did strongyles, which may be linked to their reduced reliance on the influx of young naïve individuals, as well as reflecting a longer lifespan of individual parasites and/or hardier propagules more capable of year-round transmission. Reduced intra-annual variation in counts of these parasites is surprising given that both go through intermediate snail hosts, their infection of which would be expected to rely on weather conditions (Olsen *et al.*, 2015; Kim *et al.*, 2016), and given that temperatures above 10°C are required for *F. hepatica* egg development (Ollerenshaw and Smith, 1969). *E. cervi* output varies according to monthly rainfall patterns in Spanish red deer (Vicente *et al.*, 2005), so larval output may be dependent on environmental cues on a shorter timescale rather than fluctuating annually as do strongyles. It is important to clarify that verifying seasonal dynamics such as this would require continuous sampling rather than employing discrete seasons as we do here for practical reasons. We therefore cannot confirm exactly during which period each parasite peaks at an individual- or population-level; however, the high repeatability of *F. hepatica* and *E. cervi* between seasons supports higher population-level synchrony, with more variable strongyle transmission. Asynchrony in seasonal peaks between parasites will affect host-parasite community interactions by necessitating different immune responses at different times of the year. In this case, immunity to strongyles is likely to rise in the warmer months; lower seasonality of *F.*

hepatica and *E. cervi* transmission will necessitate year-round immunity to these parasites, possibly interacting with seasonal costs of variation in nutrition, mating, reproduction and maternal care.

Importantly, this study included only one year of investigation, with only one replicate per season. As such, we were limited in our ability to conclusively identify seasonal trends. For example, it is difficult to identify whether the summer decrease in *E. cervi* count was a real seasonal change – i.e., a change that would occur in following years – or merely the result of a fluctuation acting uniquely in this year. Continuing sampling of the population will allow more thorough statistical testing of population-level variation in parasitism, and potentially inter-annual variation in this seasonality.

Young individuals tended to experience higher-intensity and a higher prevalence of infections than adults, likely playing an important role in maintaining and transmitting helminth infections in the population. Strongyles were particularly age-biased, with calves showing a higher prevalence and intensity that decreased with each successive life stage, in a similar pattern to that seen in other studies of ungulate strongyles (e.g. Wilson *et al.*, 2004). *F. hepatica* and *E. cervi* also closely followed previously-seen age profiles, increasing in prevalence at the yearling stage (Vicente *et al.*, 2007b; French *et al.*, 2016). However, *F. hepatica* prevalence and intensity decreased in adults while *E. cervi* increased in prevalence but decreased in intensity past the yearling stage. This age-biased infection implies that studies based on selective culling regimes that focus on, for example, adults, may indeed be missing relevant season-group categories which are important in determining the extent of parasitism within a population. For example, the high numbers of calves and yearlings sampled in our study contributed to the high prevalence of *F. hepatica* seen here

(>50%) compared to that in a study of Scottish deer which largely used culled adults (French *et al.*, 2016, mean 26% prevalence). The stronger age bias in strongyles may result from strongyle infection causing higher mortality or more effective adaptive immunity than *F. hepatica* and especially *E. cervi*. This concurs with the view of some strongyle species as highly pathogenic (Hoberg *et al.*, 2001) while *E. cervi* is often asymptomatic in red deer (Mason, 1989; Irvine *et al.*, 2006; Alberti *et al.*, 2011). Despite having high-intensity infections in spring, and in contrast to strongyles, calves exhibited very low prevalence of *F. hepatica* and *E. cervi* in the summer and autumn, as revealed by the zero-inflation term (Figure 4). This was a likely result of infection prepatency: *F. hepatica* has an 8 week prepatent period in cattle (de León *et al.*, 1981), while *E. cervi* can take 80-200 days to develop in red deer depending on dose (Gajadhar *et al.*, 1994). This influence of prepatency on prevalence patterns reinforces the need to understand seasonality when investigating helminths using noninvasive methods: it is likely that many calves with zero or low counts in the summer were in fact heavily infected with both parasites, but this was not yet detectable using faecal examination. Thus, quantifying the repeatability of infection with these parasites in individual calves was not possible until the autumn (for *F. hepatica*) or the spring (for *E. cervi*).

We expected to see a male bias in all three parasites (Moore and Wilson, 2002; Zuk, 2009); however, all three parasites differed in their distribution between the sexes, with a male bias in strongyle intensity and a female bias in *E. cervi* intensity, both evident in the first year, and with no effect evident in *F. hepatica*. The male bias in strongyle infection arose late in life compared to that in Soay sheep, in which male counts greatly increase relative to female counts within months of birth (Wilson *et al.*, 2004). The difference is also small (Figure 4), and *F. hepatica* showed no sex difference, despite a previous (seasonally confounded) study showing higher

prevalence in males than females (French *et al.*, 2016). On Rum most adult males live outside the study area, in areas which are at lower density as a result of culling (Clutton-Brock *et al.*, 2002) and with different geography and grazing. This may reduce exposure levels while influencing susceptibility through e.g. differences in diet (Becker and Hall, 2014) which may begin in early life. The weak female bias in *E. cervi* infection is the opposite of the expected pattern, and is particularly surprising as other red deer studies have shown male biases in *E. cervi* (Vicente *et al.*, 2006, 2007b); opposing sex effects between parasites have been reported, e.g. in ectoparasites and endoparasites of African ground squirrels (Hillegass *et al.*, 2008) and in stickleback helminths (Reimchen and Nosil, 2001), but due to a dearth of studies investigating multiple similar parasites we are not aware of any in ungulates. A possible explanation for this female bias is lower maternal care: it has been shown that hinds invest slightly less in female than male calves (Froy *et al.*, 2016). Earlier weaning may expose female calves to *E. cervi*, leading to earlier patent infections, and/or they may be weaker and therefore more susceptible to helminth infection. This disparity in sex bias disagrees with the expected male bias in helminth infection, and implies that (young) male deer may in fact feature a difference in the community rather than the extent of parasitism. Verifying whether these differences are important in mature males may require more extensive sampling of adult males in the future.

Chapter III: Reproduction has different costs for immunity and parasitism in a wild mammal

This chapter has been published as:

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Summary

1. Life history theory predicts that reproductive investment draws resources away from immunity, resulting in increased parasitism. Studies of reproductive tradeoffs in wild mammals rarely examine multiple measures of reproduction, immunity, and parasitism. It is therefore unclear whether the immune costs of reproductive traits correlate with their resource costs, and whether increased parasitism emerges from weaker immunity.
2. We examined these relationships in wild female red deer (*Cervus elaphus*) with variable reproductive investment and longitudinal data on mucosal antibody levels and helminth parasitism. We noninvasively collected faecal samples, counting propagules of strongyle nematodes (order: Strongylida), the common liver fluke *Fasciola hepatica*, and the red deer tissue nematode *Elaphostrongylus cervi*. We also quantified both total and anti-strongyle mucosal IgA to measure general and specific immune investment.
3. Contrary to our predictions, we found that gestation was associated with decreased total IgA but with no increase in parasitism. Meanwhile, the considerable resource demand of lactation had no further immune cost but was associated with higher counts of strongyle nematodes and *Elaphostrongylus cervi*. These contrasting costs arose despite a negative correlation between antibodies and strongyle count, which implied that IgA was indicative of protective immunity.
4. Our findings suggest that processes other than classical resource allocation tradeoffs are involved in mediating observed relationships among reproduction, immunity, and parasitism in wild mammals. In particular, reproduction-immunity tradeoffs may result from hormonal regulation or maternal antibody transfer, with parasitism increasing via increased exposure arising from resource acquisition constraints. We advocate careful consideration of resource-independent mechanistic links and measurement of both immunity and parasitism when investigating reproductive costs.

Introduction

Costly traits are central to the fields of life history theory and ecoimmunology. Tradeoffs arising between reproductive investment and other aspects of life history are a fundamental prediction of the former (Williams, 1966; Stearns, 1989; Harshman and Zera, 2007), while the latter examines the ecology of costly immune responses (Sheldon and Verhulst, 1996; Graham *et al.*, 2011). Because reproduction and immunity compete for host resources, in resource-limited environments, animals that invest in reproduction should have fewer resources to allocate to immune defences (Sheldon and Verhulst, 1996; Deerenberg *et al.*, 1997; French *et al.*, 2007). If immunity is protective, these individuals will experience higher parasitism as a result. Intuitively, traits with higher resource demands should result in the diversion of more resources away from immunity, leading to higher parasite burdens. However, recent advances have demonstrated that interrelationships among host resources, immunity, and parasitism can be unexpectedly complex (Cressler *et al.*, 2014). Few studies in wild mammals have examined tradeoffs with multiple reproductive traits, so it is unclear whether different components of reproduction have different costs for immune defence, and whether their costs are proportional to their resource demand. Furthermore, studies of reproductive tradeoffs rarely quantify both immunity and parasitism to examine the importance of susceptibility versus exposure in driving higher parasite intensities in reproductive females (Bradley and Jackson, 2008; Knowles *et al.*, 2009). Here, we examine the partitioning of reproductive costs for multiple measures of immunity and parasitism to investigate the possible mechanisms governing a reproduction-immunity-parasitism tradeoff in a wild mammal.

Mammalian reproduction is a complex, multi-stage process, featuring extensive maternal investment which varies in intensity through the reproductive period

(Langer, 2008; Maestripieri and Mateo, 2009). As such, different components of reproduction vary substantially in their resource and fitness costs. In particular, lactation is a highly energetically demanding process which carries costs for immunity, parasitism or fitness in a range of mammals (Clutton-Brock *et al.*, 1989; Woodroffe and Macdonald, 1995; Christe *et al.*, 2000; Beasley *et al.*, 2010; Jones *et al.*, 2012; Froy *et al.*, 2016; Rödel *et al.*, 2016). Meanwhile, only one of these studies uncovered an immunological cost of gestation (Christe *et al.*, 2000), which generally requires fewer resources than does lactation (Clutton-Brock *et al.*, 1989). However, although experimentally modifying resource availability can affect the severity of reproduction-immunity tradeoffs (French *et al.*, 2007; Jones *et al.*, 2012), this is not always the case (Stahlschmidt *et al.*, 2013). Similarly, studies in birds have questioned whether the energetic costs of immunity are sufficient to drive tradeoffs (Svensson *et al.*, 1998; Eraud *et al.*, 2005). Such findings imply that reproduction-immunity tradeoffs can be linked mechanistically as well as through resource reallocation. Potential such links include production of reactive oxygen species, reduction in immunologically active fat stores, or resource-independent hormonal regulation (Svensson *et al.*, 1998; Speakman, 2008).

Different components of mammalian reproduction can have qualitatively different effects on host immunity as well as varying quantitatively in terms of their resource demand. For example, pregnancy necessitates modulation of the immune system to avoid mounting an immune response to the developing foetus, which will directly affect anti-parasite defence (Weinberg, 1984, 1987). Similarly, lactation draws immune molecules away from the mother for transfer to offspring, reducing their availability for the mother's own defence (Grindstaff *et al.*, 2003; Hasselquist and Nilsson, 2009). Reproduction also induces a suite of physiological and behavioural changes which will affect susceptibility and exposure to parasites indirectly: for

example, it has been suggested that bats compensate for the energetic demand of lactation by reducing costly grooming behaviour, with ectoparasite burden increasing as a result (Speakman, 2008). It is unclear how such mechanistic links between components of reproduction and immunity interact with resource allocation to influence immune defence and parasite intensity in wild mammals.

The wild red deer (*Cervus elaphus*) in the North block of the Isle of Rum exhibit a well-studied life history tradeoff, in which reproduction substantially decreases the mother's probability of overwinter survival and reproduction the following year (Clutton-Brock *et al.*, 1989; Froy *et al.*, 2016). However, not all components of reproduction are equally costly: gestation has no detectable fitness cost compared to that of lactation (Clutton-Brock *et al.*, 1989). Moreover, while giving birth late and caring for a male calf compared to a female calf are associated with decreased maternal fitness, their effects are small compared to the cost of lactation itself (Froy *et al.*, 2016). The study population has a high prevalence of gastrointestinal helminths, and parasite burdens can be quantified noninvasively through faecal propagule counts (Albery *et al.*, 2018). Mucosal antibodies, and especially the IgA isotype, are important effectors of ruminant adaptive immunity to gut helminths (Butler, 1969; Balic *et al.*, 2000; McRae *et al.*, 2015). Mucosal IgA can be quantified in wild ruminant faeces, correlating positively with the same isotype measured in plasma or serum and negatively with helminth faecal egg counts (Watt *et al.*, 2016).

In this study, we measured both total and helminth-specific mucosal IgA and propagule counts of multiple helminth species, using faecal samples collected from the Isle of Rum study population. We quantified the associations between several reproductive traits of known fitness cost and subsequent measures of immunity and parasitism. We also examined covariance between IgA and parasites to discern

whether increased IgA was associated with decreased parasite count independently of shared reproductive and seasonal effects, implicating IgA as an indicator of protective immunity. We predicted that reproductive investment would be associated with reduced antibody levels and increased parasite counts, and that aspects of reproduction previously found to be more costly for fitness, especially lactation, should likewise be more costly in terms of both immunity and parasitism. Furthermore, providing that parasitism is mediated by immune susceptibility, aspects of reproduction that are costly for immunity should have similar costs in terms of parasitism.

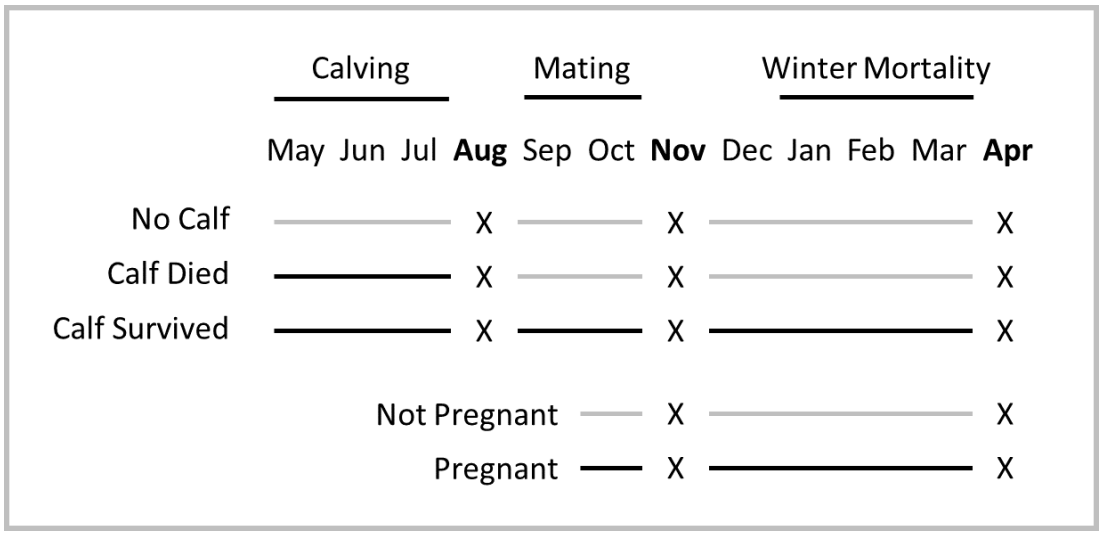

Figure 1: The faecal sampling regime in the context of a deer reproductive cycle ("deer year"). A cross (X) represents a two-week sampling trip. The deer year begins on May 1st when calving begins; individuals are assigned to one of three reproductive status categories (top three lines) according to the birth and survival of their calf over the course of the following year. Individuals are also assigned a pregnancy status in November and April based on reproduction in the following calving season (bottom two lines). Black lines represent periods in which the calf is living or the female is pregnant; grey lines represent periods in which the calf is dead or non-existent or the female is not pregnant.

Methods

Study system, sampling and parasitology

The study population is located in the North block of the Isle of Rum National Nature Reserve in the Inner Hebrides, Scotland (57°N 6°20'W). The resident population comprises ~350 animals at any one time, and is regularly censused to keep track of each individual and its life history. See Clutton-Brock *et al.* (1982) for a full summary of the project and the deer reproductive cycle. Briefly, the deer mate in September and October and give birth in May-June, after an approximately 235 day gestation. Females do not reproduce every year, and produce a maximum of one calf per year. During the calving season, daily monitoring of pregnant females enables the recording of precise birth dates. Neonates are caught, sexed, weighed and individually marked, enabling life-long individual identification. Calves are dependent on their mothers for much of their first year. Regular population censusing throughout the year and winter mortality searches allow dates of death to be reliably assigned to the nearest month for the vast majority of individuals. Most calf deaths occur either within the first few weeks of life, or in the following winter ~6-9 months later. Females that successfully raise a calf to the age of one, or that lose the calf in its first winter, have lower rates of overwinter survival and reproduction the following year compared to those that do not reproduce that year or that lose their calf in the summer (Clutton-Brock *et al.*, 1989; Froy *et al.*, 2016). Many calves die over the winter, but the mothers of these calves have paid the cost of lactation associated with feeding them until the winter, whether or not the calf then survives. Therefore these females are treated as a single category here (Clutton-Brock *et al.*, 1989; Froy *et al.*, 2016).

We collected faecal samples from female deer across the annual reproductive cycle. As a "deer year" runs from May to April, this study examines the effects of reproduction over a year, beginning in May, on egg counts and antibody levels until

the following April. A description of the sample collection procedure can be found in Albery *et al.* (2018). Sampling occurred over seven two-week sampling trips spanning April 2016-April 2018, in August ("summer"), November ("autumn") and April ("spring"). Note that our dataset included an April sampling trip from the deer reproductive cycle starting May 2015, without an accompanying August and November trip from this reproductive cycle. Figure 1 illustrates how sampling relates to different aspects of reproductive investment by female deer across the annual cycle. We classify a female's reproductive status for a given year as "No Calf", "Calf Died" and "Calf Survived" (see Figure 1). "No Calf" samples were collected from females that did not reproduce in the calving season preceding the sampling trip; "Calf Died" samples were collected from females that gave birth to a calf in the preceding calving season which died before October 1st of that year; and "Calf Survived" samples were collected from females that gave birth to a calf in the preceding calving season which survived past October 1st of that year. We excluded females that were reproducing for the first time from our analyses, as their reproductive success is heavily confounded with their young age (mean age 4.21 years). In addition, females may or may not become pregnant during the autumn rut. Samples were therefore assigned a pregnancy status, beginning in November, based on whether or not the female gave birth to a calf in the following spring (Figure 1).

In total 837 faecal samples were collected noninvasively from 140 mature females. All samples were collected by observing known females from a distance, marking the spot in which defecation happened, and promptly collecting the pellets. In the evening after collection, samples were homogenised by hand and subsampled, with 1-15g frozen at -20°C for antibody quantification and the remainder refrigerated at 4°C for parasitological analysis. Subsamples were transferred to Edinburgh at these temperatures. Parasite propagule counts were carried out as previously described,

without correcting for dry weight, and included counts of strongyle nematodes, the common liver fluke *Fasciola hepatica* and the tissue nematode *Elaphostrongylus cervi* (Albery *et al.*, 2018). Final sample sizes for each variable are displayed in Table SI1.

Antibody extraction and quantification

Faecal antibodies were quantified using a protocol modified from Watt *et al.* (2016). Faecal matter was stored at -20°C until extraction. Extractions occurred either in January-March 2017 (session "A", samples collected April-November 2016; N=132), January 2018 ("B", samples collected April-November 2016; N=212) or within the sampling trip ("C", samples collected April 2017-April 2018, N=460). 0.6g (+/- 0.005g) of the homogenate was weighed out into an Eppendorf tube and mixed thoroughly with 0.9ml of protease inhibitor solution (cOmplete™ Mini Protease Inhibitor Cocktail tablets, Roche, Basel, Switzerland; 1 tablet mixed with 7ml Phosphate Buffered Saline). The mixture was left to stand for a minimum of 5 minutes to allow the protease inhibitor to act and then centrifuged at 10,000g for 5 minutes. The supernatant was removed using a micropipette and stored in a separate Eppendorf tube at -20°C until ELISA.

We measured two antibodies by ELISA: total IgA and anti-*Teladorsagia circumcincta* third larval stage IgA (anti-Tc IgA). *T. circumcincta* is an abundant and important sheep strongyle, and is also present in the Rum deer (unpublished data). This method for detecting anti-*T. circumcincta* antibodies shows high cross-reactivity with other strongyle species (Froy *et al.*, in review). Anti-Tc IgA therefore correlates negatively with order-level strongyle faecal egg count and with species-level counts of other strongyle species in wild Soay sheep (Watt *et al.*, 2016; Froy *et al.*, in review). We therefore interpret this assay as representing a general anti-strongyle response rather than a response to *T. circumcincta* specifically. ELISA plates were coated the night

before using sheep-derived capture antibodies (Bethyl Laboratories, Montgomery, TX) for total IgA and with third larval stage antigen for anti-Tc IgA (Moredun Research Institute, Penicuik, Scotland).

After this stage, the ELISA protocol was carried out as described in Watt *et al.* (2016). Briefly: The following day, we defrosted the extracted faecal supernatant at room temperature and homogenised them by vortexing, and then we diluted a subsample of each. For total IgA the samples were diluted in the ratio 1:64; due to lower antibody concentrations, undiluted supernatant was used for the anti-Tc IgA assay. The total IgA dilution was selected by carrying out serial dilutions on a set of samples and selecting the dilution at which different concentrations of antibodies were deemed to have the widest spread of optical densities. ELISA readings diluted linearly as expected. We washed wells three times using tris-buffered saline mixed with Tween-20 surfactant (TBS-Tween). We added samples to their wells and then incubated the plates at 37'C for 1 hour for the coating antibodies to bind the deer IgA. After this, we washed the wells with TBS-Tween five times, we added 50 microlitres of rabbit-derived anti-sheep-IgA detection antibodies to each well, and incubated the plates again at 37'C for 1 hour, allowing the antibodies to bind. We washed the plates once more with TBS-Tween, then added 100 microlitres of SureBlue TMB substrate to each well, and incubated the plates once more for 5 minutes. After this incubation step, we added 100 microlitres of hydrochloric acid as quickly as possible to arrest the reaction, quickly inserting the plates into the plate reader to calculate optical density. The optical density of the resulting solutions is proportional to the concentration of antibodies in the sample. Samples were corrected using controls containing only TBS-Tween (negative) and sheep plasma (positive), according to the calculation: Final OD=(sample OD-mean plate negative OD)/(mean plate positive OD-mean plate negative OD). All samples were run on duplicate plates, which were highly correlated

(R=0.98 across all duplicates). The mean value for the two duplicates was taken for each sample and used for analysis.

Statistical analysis

We used four sets of Generalised Linear Mixed Models (GLMMs) to test how reproductive traits were associated with antibody levels and parasite count. Analyses were carried out in R version 3.5.0 (R Core Team 2018) with the package MCMCglmm (Hadfield, 2010). All models were run for $2.6x10^6$ iterations with a 2000 iteration thinning interval and a $6x10^5$ iteration burn in period. P_{MCMC} values for differences between factor categories were calculated using the proportional overlap of estimates' posterior distributions, divided by half the number of iterations.

Full models

We first constructed five univariate GLMMs using the full dataset (837 samples from 140 individuals). Three models used an overdispersed Poisson distribution, with strongyle, *F. hepatica* and *E. cervi* count as response variables. Models initially included the following fixed effects, without interactions: Year (factor with three levels representative of the deer reproductive cycle beginning in 2015, 2016 and 2017); Season (factor with three levels: Summer, Autumn and Spring); Age in years (continuous); and Reproductive Status (factor with three levels: No Calf, Calf Died and Calf Survived). Individual identity was fitted as a random effect. All continuous variables except parasite counts were scaled to have a mean of 0 and a standard deviation of 1 before analysis.

The two remaining models examined antibodies as response variables. As mucosal antibodies are vulnerable to degradation by temperature-dependent faecal proteases, we had to account for the extraction session and time to freezing and extraction (Appendix A, Figure SI5-6). There was an uneven distribution of year, season, and

status categories across different extraction sessions, so that these variables could not all be fitted in the same model. Therefore, to control for collection factors and quantify reproductive status effects conservatively we first transformed antibody levels to approximate normality (square-root transform for total IgA and cube-root transform for anti-Tc IgA), and fitted a linear model with fixed effects including extraction sessions performed at different times (factor with three levels); day of collection within a sampling trip (continuous integers, range 0-11); time elapsed from sample collection until freezing (continuous, in hours). The scaled residual values from these models (mean=0, SD=1) were used as the response variables in two Gaussian GLMMs with the same fixed and random effects as the parasite GLMMs. This conservative method risks losing information, thereby reducing the risk of Type I errors. Raw distributions of total IgA and anti-Tc IgA (in optical density units) are evident in Appendix A, Figure SI5-6, 8. Total IgA was somewhat right-skewed, and anti-Tc IgA more so, necessitating the square root- and cube root-transformations that we used to approximate normality.

Previous work on the Rum deer revealed extensive seasonal fluctuations in parasite count (Albery *et al.*, 2018). We therefore followed up the above five models by fitting a season by reproductive status interaction in order to investigate whether the effects of reproductive status varied by season. Each model was compared with and without this interaction to investigate whether it affected Deviation Information Criterion (DIC) values as a measure of model fit (threshold values for distinguishing between models $\Delta DIC=2$) or changed model estimates.

Pregnancy models

Pregnancy may directly affect immunity, and effects attributed to reproductive status could be due to correlated variation in pregnancy status over the sampling year. To

check this we ran a second set of models investigating the role of pregnancy status. This used a subset of samples collected in November and April (518 samples from 122 individuals), as mating occurs in the early autumn and females could not be pregnant in August. These five models featured the same explanatory variables as the full status models, with only two levels in the season category (Autumn and Spring), and with Pregnancy included as a binary variable. We compared these models with and without the pregnancy term as a fixed effect to investigate whether its inclusion changed reproductive status effect sizes or affected model fit.

Calving trait models

We next used a restricted dataset consisting of individuals that had given birth in the year of sampling (571 samples from 116 individuals) to investigate whether specific traits associated with a calving event influenced antibody levels and parasitism. We fitted the same fixed and random effects as the full model set, but with only two factor levels in the reproductive status category (Calf Died and Calf Survived), and including several variables relating to each birth: Parturition Date (continuous, centred around median birth date that year); Birth Weight (continuous, in kilograms, calculated from a projection using capture weight and age in hours, slope 0.01696 kg/h); Calf Sex (Female or Male).

Multivariate model

Multivariate mixed-effects models can be used to investigate covariance between measures of immunity and parasitism, while accounting for fixed effects. To test whether antibodies and parasites were correlated we fitted a model with strongyles, *E. cervi*, total IgA and anti-Tc IgA as response variables, with the same fixed effects as the full univariate models. We specified Poisson and Gaussian distributions for the parasites and antibodies respectively, as in the univariate models. Unstructured

variance/covariance matrices were fitted for random and error terms, allowing estimation of the phenotypic correlations between the response variables. Phenotypic covariance between two response variables A and B ($Cov_{phenotypicA,B}$) is calculated using the random (G) and residual (R) variance structure of the model, with the formula $Cov_{phenotypicA,B}=Cov_{IndividualA,B}+Cov_{residualA,B}$. Phenotypic correlation between two response variables ($r_{phenotypicA,B}$) was calculated by dividing the phenotypic covariance by the square root of the sum of the variance in both response variables: $r_{phenotypicA,B}=Cov_{phenotypicA,B}/(V_{phenotypeA}+V_{phenotypeB})^{0.5}$. P_{MCMC} values for correlations were calculated using the posterior distributions, dividing the number of iterations overlapping with zero by half the total number of iterations.

Results

Reproductive investment was strongly associated with both lower antibody levels and increased parasite counts, but patterns differed considerably between different response variables (Figure 2,5). Compared to "No Calf" individuals, "Calf Survived" status was associated with higher strongyle counts ($P_{MCMC}<0.001$) and *E. cervi* count ($P_{MCMC}=0.01$), and with lower total IgA ($P_{MCMC}=0.016$) and anti-Tc IgA levels ($P_{MCMC}<0.001$). "Calf Survived" females also had higher parasite counts than "Calf Died" individuals ($P_{MCMC}<0.001$ for strongyles and *E. cervi*), but these reproductive status categories did not differ in total IgA ($P_{MCMC}=0.502$) or anti-Tc IgA ($P_{MCMC}=0.336$; Figure 2-3). "Calf Died" individuals did not differ from "No Calf" females in strongyle, *E. cervi* or anti-Tc IgA levels (Figure 2) but had lower total IgA levels ($P_{MCMC}=0.018$; Figure 5). That is, "Calf Died" individuals had lower total IgA than "No Calf" females, but with similar parasite intensities, while "Calf Survived" individuals had the same antibody levels as "Calf Died" individuals, but with increased parasite intensities. *F. hepatica* was not associated with reproductive status, but decreased with age ($P_{MCMC}=0.004$; Figure 5) as did *E. cervi* ($P_{MCMC}<0.001$; Figure 5; Appendix A, SI7).

Strongyles and both antibodies all exhibited the same seasonal patterns, peaking in the spring and being lowest in the autumn, with the summer intermediate (Figure 3, all differences $P_{MCMC}<0.001$; see Figure 5 for all effect estimates). *F. hepatica* was higher in the spring than in the summer or autumn ($P_{MCMC}<0.034$), and *E. cervi* was lowest in the summer, with the autumn intermediate ($P_{MCMC}<0.001$). There was also some between-year variation: strongyle levels increased between 2015 and 2016, and again in 2017 (all $P_{MCMC}<0.001$), while total IgA levels decreased in 2017 compared to 2015 and 2016 ($P_{MCMC}<0.024$). Anti-Tc IgA was also lower in 2017 than 2016 ($P_{MCMC}<0.001$). Inclusion of season-by-status interactions improved strongyle model fit ($\Delta DIC=-3.79$), but did not improve the fit of any other models ($\Delta DIC<2$). Fixed status effects remained largely unchanged in magnitude or significance (Appendix A, Figure SI2), suggesting that the observed associations with reproductive status were relatively consistent across seasons in these models (Figure 3). All interaction terms implied an attenuation of reproductive status effects from summer through winter to spring, rather than any major qualitative change in this association (Figure 3; Appendix A, Figure SI2). Both "Calf Died" and "Calf Survived" females had increased antibody levels and decreased parasite intensities relative to "No Calf" females over this period. See Appendix A, Figure SI2 for a comparison of the full model estimates and DIC changes when a season-by-status interaction was included.

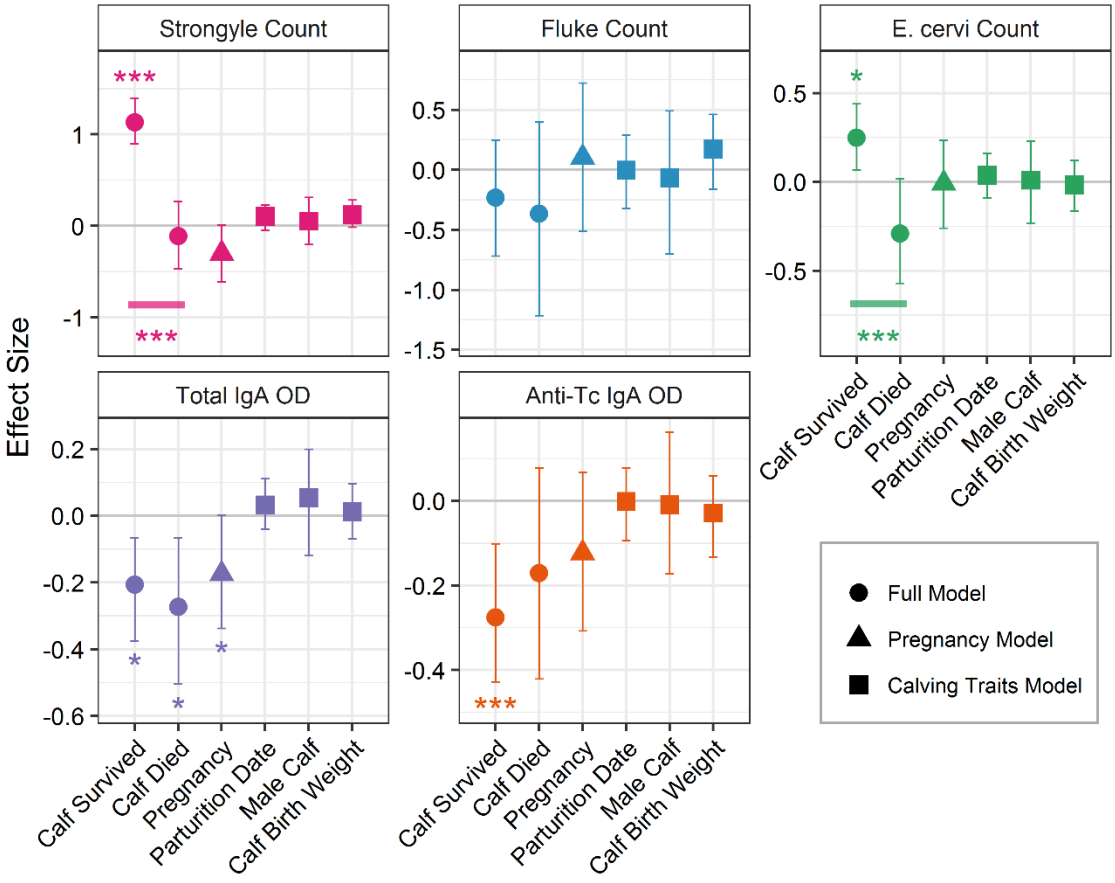

Figure 2: Model outputs depicting the effects (the slope derived from the GLMMs) of reproductive traits, derived from all three univariate model sets. Points and error bars represent model estimates and 95% credibility estimates. Effect sizes for categorical variables (status, pregnancy and calf sex) denote differences from the first (absent) category of each, contained in the intercept ("No Calf", "Not Pregnant" and "Female Calf" respectively). Effect sizes for continuous variables (parturition date and calf birth weight) represent the change in the response variable associated with a change of one standard deviation of the explanatory variable. Asterisks represent significant differences derived from MCMCglmm posterior distribution overlaps: ***, ** and * denote P<0.001, P<0.01 and P<0.05 respectively. Bars denote differences between status categories.

Pregnancy models examining April and November samples revealed marginally lower total IgA in pregnant females (P_{MCMC}=0.034, Figure 2, 5, Appendix A, Figure SI3), but with no other notable differences. Including pregnancy status in our models did not alter the direction or significance of reproductive status effects; in fact, in the case of total IgA and anti-Tc IgA it increased the significance of the "Calf Survived" category's effect (Appendix A, Figure SI3). It also slightly improved the fit of the total IgA model

(ΔDIC=-3.00). No other models were impacted notably by the inclusion of the pregnancy term, although it slightly reduced the effect size of the "Calf Survived" category in influencing strongyle count (Appendix A, Figure SI3). Although the "Calf Died" category was not significant in the total IgA pregnancy model as it was in the full model, the fact that adding and removing pregnancy as a variable had very little effect on the model estimate (Appendix A, Figure SI3) implies that this did not arise from confounding effects of pregnancy.

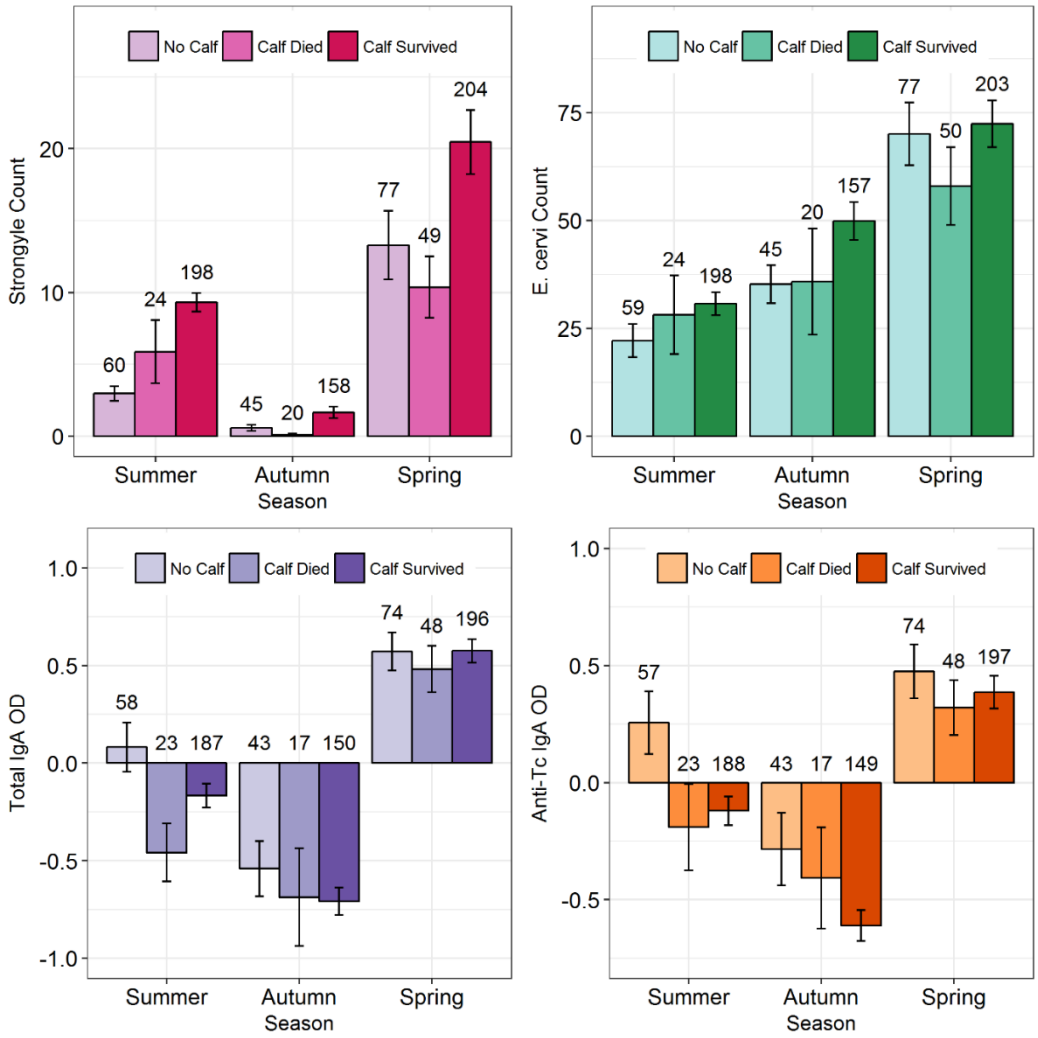

Figure 3: Bar charts displaying mean (+/- SE) parasite counts and antibody levels of each reproductive status category in each season. Antibody measures were taken from the residuals of a model with square root-transformed (total IgA) or cube root-transformed (anti-Tc IgA) antibody OD as the response variable and including collection variables as fixed effects, and scaled to have a mean of 0 and a standard deviation of 1. Numbers above bars denote sample sizes.

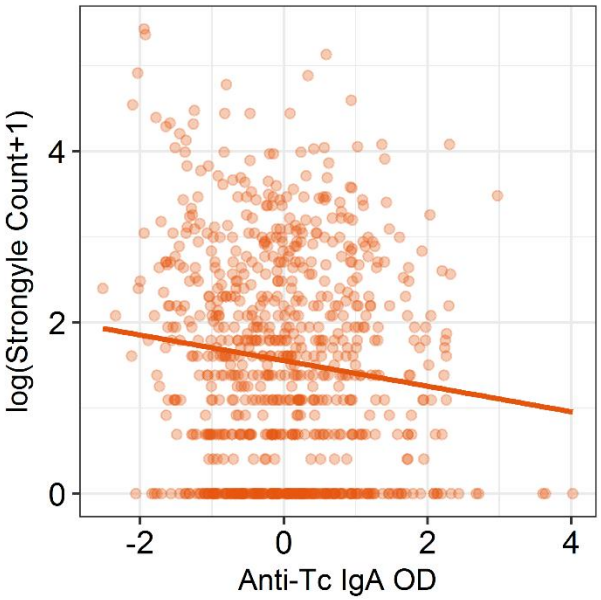

Figure 4: Correlation between anti-*Teladorsagia circumcincta* IgA levels and strongyle count. Individuals with higher anti-Tc IgA had lower strongyle counts (multivariate model phenotypic correlation R_p=-0.142, P_{MCMC}<0.001). The y axis is on the $\log_e$(count+1) scale to aid interpretation; the x axis data were taken from the residuals of a model with cube root-transformed anti-Tc IgA as the response variable and including collection variables as fixed effects. For this figure, these residuals were centred within sampling trips to have a mean of 0 and a standard deviation of 1 to avoid a positive correlation arising from shared seasonal and annual effects.

None of the calving traits modelled (parturition date, calf birth weight or calf sex) were associated with maternal parasite or antibody levels (Figure 2, 5).The fixed effects of the multivariate model were very similar to those of the full models (Appendix A, Figure SI4). The raw correlations between the response variables of the model are displayed in Figures 4 and SI8. Phenotypic correlations (R_p) derived from the variance structure of the multivariate model are as follows. There were strong positive correlations between strongyles and *E. cervi* (R_p=0.26, P_{MCMC}<0.001) and between total and anti-Tc IgA (R_p=0.424, P_{MCMC}<0.001). Strongyle count was also weakly negatively correlated with total IgA (R_p=-0.074, P_{MCMC}=0.016, Appendix A, Figure SI8) and more strongly with anti-Tc IgA (R_p=-0.142, P_{MCMC}<0.001, Figure 4).

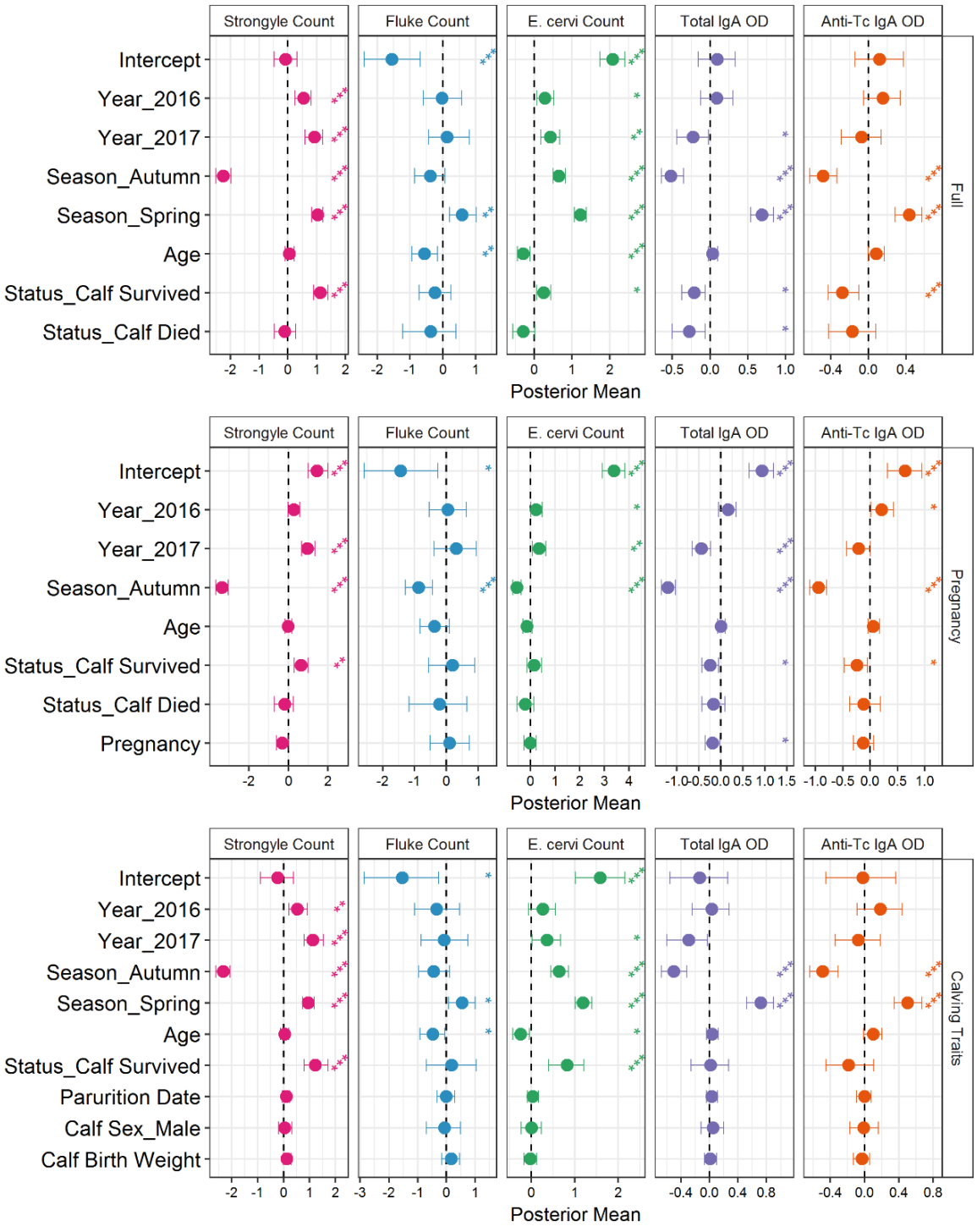

Figure 5: Effect size estimates from the three model sets (full dataset, pregnancy models and calf traits models). Effect sizes for categorical variables denote differences from the first (absent) category of each, contained in the intercept. Effect sizes for continuous variables represent the change associated with a change of one standard deviation of the variable in question. Points and error bars represent model estimates and 95% credibility estimates for each of the five full models. Asterisks indicate the significance of variables: ***, ** and * indicate P<0.001, P<0.01 and P<0.05 respectively.

Discussion

Lactation is associated with weaker immunity or increased parasitism in a range of mammals (Festa-Bianchet, 1989; Woodroffe and Macdonald, 1995; Jones *et al.*, 2012; Rödel *et al.*, 2016). In accordance with these studies, we found that lactating females had both decreased antibody levels and increased parasite counts relative to non-reproductive females. In contrast, gestation is rarely found to be costly for immunity or parasitism in mammals (Woodroffe and Macdonald, 1995; Irvine *et al.*, 2006; Rödel *et al.*, 2016), and carries no detectable fitness cost in the Rum red deer (Clutton-Brock *et al.*, 1989). Here, deer that gave birth to a calf that died as a neonate, thereby incurring a limited lactation cost, had lower total IgA levels than non-reproducing females. Gestation therefore carried an unexpected immune cost in this study. We predicted that resource depletion incurred through investment in a given reproductive trait would lead to reduced immune investment, and that this would lead to increased parasite count (Sheldon and Verhulst, 1996; Knowles *et al.*, 2009). Our results deviated from our expectations in two ways: first, gestation's long-lasting immune cost was not accompanied by increased parasite count. Second, the considerable additional resource investment of prolonged lactation was not associated with additional immune costs relative to gestation, but was instead associated with an increase in parasite count. These results have two implications: reproduction-immunity tradeoffs were unlikely to be mediated by simple resource reallocation, and reproduction-parasitism tradeoffs were unlikely to be mediated solely by immunity – despite our observation that higher immune investment was associated with lower parasite counts between individuals (Figure 4, SI8).

If gestation's lack of detectable fitness cost in our study population (Clutton-Brock *et al.*, 1989) demonstrates a small resource cost, why was gestation associated with reduced total IgA levels, and why did the additional resource cost of lactation not decrease antibody levels further? First, it is possible that reproductive hormones

suppress the immune system without being sensitive to resource availability, comprising an obligate reproductive tradeoff rather than a facultative one (Svensson *et al.*, 1998; French *et al.*, 2007; Foo *et al.*, 2017). Similarly, gestation may lead to alterations in immune investment and antibody production, so that lower IgA resulted from selective investment in alternative immune cells or functions rather than from lower absolute resource investment in immunity. Reproductive mammals are commonly found to exhibit different (rather than weaker) immunity, but specific patterns of immune prioritisation are unpredictable. For example, reproductive vampire bats (*Desmodus rotundus*) prioritise innate over adaptive immunity (Becker *et al.*, 2018), while reproductive rabbits (*Oryctolagus cuniculus*) exhibit reduced white blood cell counts but stronger humoral immunity (Rödel *et al.*, 2016). Assessing whether reproductive deer invest preferentially in aspects of immunity other than mucosal antibodies would therefore necessitate examining numerous additional immune measures – however, in this study we were restricted to quantifying mucosal antibodies using noninvasive faecal samples as the deer are rarely handled as adults (Clutton-Brock *et al.*, 1982).

Alternatively, gestation and early lactation may necessitate export of IgA from the gut to the blood for transfer to offspring (Sheldrake *et al.*, 1984; Jeffcoate *et al.*, 1992). In ungulates a substantial proportion of maternal antibody transfer occurs via the colostrum in the first few days of life (Hurley and Theil, 2011). It is feasible that this diversion of IgA from the gut occurs around parturition and is detectable for an extended period of time without an underlying resource allocation tradeoff, creating lower IgA levels in all reproductive females regardless of their calf's survival. The necessity of transferring immune effectors to offspring may therefore be an important obligate mechanism contributing to reduced antibody levels in reproductive wild mammals (Rödel *et al.*, 2016). In a proposed mechanism for the periparturient rise in helminth egg count in domestic sheep, exportation of IgA from the gut around

parturition releases helminths from immune control (Jeffcoate *et al.*, 1992). However, in this study, the lower total IgA and intermediate anti-Tc IgA levels in female deer that only paid the cost of gestation were not accompanied by any change in parasitism. This is surprising, given that the results of our multivariate model implied that both IgA measures are representative of increased resistance to strongyles (Figure 4, SI8).

If antibody levels were indicative of investment in protective immunity, how were the deer that paid the immune cost of gestation able to maintain low strongyle and *E. cervi* intensities? Or, what produced the higher parasite counts in lactating individuals? Lactating females' anti-Tc IgA levels were significantly lower than non-reproductive females', which could explain their increased parasitism in the absence of a contrast with any other reproductive categories. However, levels of total and anti-Tc IgA in lactating females were not detectably lower than those exhibited by females that paid the cost of gestation (Figure 2). This disparity suggests that additional processes such as exposure were important in driving the high parasite intensities in lactating females (Sheldon and Verhulst, 1996; Knowles *et al.*, 2009). The energetic and resource demand of milk production necessitates substantially increased forage intake and grazing time (Hamel and Côté, 2008, 2009), and may reduce feeding selectivity or the ability to exhibit parasite avoidance behaviours (Hutchings *et al.*, 2006; Speakman, 2008). Thus, lactating females may suffer increased exposure to infective larvae, resulting in higher parasite burdens. This mechanism offers an explanation for our observation that lactation was associated with increased parasite counts, while gestation was not, as individuals that lost their calf as a neonate were not then saddled with a necessity for such high resource acquisition. Based on our results, we suggest that severe effects of mammalian reproduction on parasite infection are partly mediated by exposure as a result of constraints on resource

acquisition, foraging selectivity, and antiparasite behaviours, in addition to increased immune susceptibility.

Effects of foraging on exposure can profoundly affect epidemiological dynamics: for example, in the water flea *Daphnia dentifera*, temperature-induced increases in food intake can increase the magnitude of fungal pathogen epidemics (Shocket *et al.*, 2018). Similar processes may act in the deer, if spatiotemporal variation in climatic conditions, deer density, or food abundance modify feeding behaviour or the threat of exposure. In particular, strongyle and *E. cervi* parasitism will be further exacerbated in years and areas of the study system where deer density is high and food availability is low (Wilson *et al.*, 2004). It is possible that higher parasitism in reproductive individuals will reduce their fitness, thereby producing lactation's fitness cost – and, by extension, gestation's lack of fitness cost – in this system (Williams, 1966; Clutton-Brock *et al.*, 1989; Harshman and Zera, 2007; Froy *et al.*, 2016). If exposure is determining parasitism and parasitism is reducing fitness, we would expect that parasite-mediated life history tradeoffs would be exacerbated in years and areas of high deer density, as more deer will translate to higher levels of pasture contamination (Wilson *et al.*, 2004). Future investigations in this system could address the hypothesized role of parasite exposure and foraging behaviour in reproductive tradeoffs, using available census data (Clutton-Brock *et al.*, 1982; Froy *et al.*, 2018) to examine how annual, seasonal, and spatial variation in habitat use and deer density correlate with environmental larval counts, faecal parasite counts, and the severity of reproductive tradeoffs.

Reproductive tradeoffs are a potential driver of seasonal dynamics of immunity and parasitism, in which periodic reproduction-associated relaxation of immunity leads to increased parasitism (Martin *et al.*, 2008). Our results do not support this mechanism for several reasons: all status categories exhibited seasonality of

antibodies, strongyles, and *E. cervi* rather than only reproductive individuals; reproductive increases in parasitism were not linked to lower immunity; and immunity did not correlate with resource availability, being highest in April, when the deer are in poor condition, having just survived the winter. In fact, antibody levels and strongyle counts correlated positively across seasons despite their negative correlation among individuals. This suggests that seasonality of propagule output is adaptive for helminths, facilitating highest transmission when environmental conditions are favourable and immunologically naïve calves are present, and leading to seasonal upregulation of immunity in warmer months to combat increased exposure (Møller *et al.*, 2003; Wilson *et al.*, 2004).

This study describes unexpected and complex interrelationships between different components of mammalian reproduction, immunity, and parasitism in the wild. We suggest that classical resource allocation mechanisms which are often hypothesised to underlie tradeoffs with immunity (e.g. Sheldon & Verhulst 1996; Deerenberg *et al.* 1997; French *et al.* 2007) are insufficient to explain many of the patterns seen in wild mammals, corroborating findings in other taxa (Svensson *et al.*, 1998; Stahlschmidt *et al.*, 2013). As such, studies examining such tradeoffs in mammals should consider mechanistic links between reproduction and immunity, resource acquisition limitations, and exposure components of parasitism, particularly by quantifying both immunity and parasitism simultaneously (Bradley and Jackson, 2008; Graham *et al.*, 2011). The potential complexity of such interrelationships may contribute to the relative rarity of conclusive evidence for reproduction-immunity-parasitism tradeoffs in mammals.

Chapter IV: Fitness costs of parasites explain multiple life history tradeoffs in a wild mammal

This chapter exists as an online preprint:

Albery, G. F., Nussey, D. H. and Pemberton, J. M. (2019). Fitness costs of parasites explain multiple life history tradeoffs in a wild mammal. *bioRxiv* 683094. doi:10.1101/683094

Summary

A longstanding prediction of life history theory states that parasitism can provide a mechanistic link between current and future fitness, thereby mediating life history tradeoffs. Demonstrating this mechanism in the wild involves linking increased parasitism in reproductive individuals with a cost of this parasitism for subsequent fitness-related traits (e.g., annual survival). This endeavour is facilitated by high-resolution longitudinal monitoring of individuals. Here, we examined associations among reproductive allocation, immunity, parasitism, and subsequent fitness-related traits in a wild population of individually identified red deer (*Cervus elaphus*). Using path analysis, we constructed and analysed possible links among these traits, particularly investigating whether costs of lactation for downstream survival and fecundity were likely to act through increased parasitism. Lactation was associated with increased strongyle parasitism, which was then associated with decreased subsequent fitness for all four fitness-related traits we examined. In addition, lactation was directly associated with later parturition date the following year, and increased helminth-specific antibody levels were associated with increased probability of reproduction. This study offers observational evidence for parasite mediation of multiple life history tradeoffs in a wild mammal, and supports the value of considering life history allocation, immunity, and parasitism simultaneously when examining fitness consequences in disease ecology.

Introduction

A fundamental tenet of life history theory states that reproduction should be costly for subsequent fitness traits, yet the mechanisms behind such tradeoffs are poorly understood (Williams, 1966; Stearns, 1989). It is possible that reproductive costs may act through parasites, where increased reproductive investment diverts resources away from immunity, resulting in increased parasite burdens, which reduce subsequent fitness (Sheldon and Verhulst, 1996; Harshman and Zera, 2007). Parasite mediation of life history tradeoffs involves two necessary components: that reproduction increases parasitism, and that these parasites cause harm or require resources to combat them, reducing subsequent fitness. There is abundant evidence for each component of this theory across a range of taxa: firstly, life history investment is often associated with weaker or altered immune allocation (Neggazi *et al.*, 2016; Rödel *et al.*, 2016; Krams *et al.*, 2017) or with increased parasitism (Festa-Bianchet, 1989; Cizauskas *et al.*, 2015; Debeffe *et al.*, 2016). Secondly, parasitism often decreases the probability of survival (Coltman *et al.*, 1999; Leivesley *et al.*, 2019) or reproduction (Albon *et al.*, 2002; Vandegrift *et al.*, 2008; Hughes *et al.*, 2009). Despite evidence for both in isolation, reproduction-associated increases in parasitism have rarely been linked to downstream fitness consequences in the same study to provide evidence for a parasite-mediated life history tradeoff.

Parasites can reduce host fitness by directly harming the host and sapping its resources - however, immunity is also costly, in that immune responses use resources and cause immunopathology which can likewise reduce fitness (Ilmonen *et al.*, 2000; Raberg *et al.*, 2000). The cost of parasites must therefore be weighed against the cost of immunity itself: it is difficult to predict *a priori* whether increased fitness should result from higher, lower, or intermediate immune responses, yet it is still relatively rare that both immunity and parasitism are quantified in studies of fitness

consequences (Viney *et al.*, 2005; Graham *et al.*, 2011). Furthermore, while it is true that reproduction, immunity, and parasites all compete for host resources, mechanisms governing life history tradeoffs are time-staggered rather than occurring simultaneously. First, reproduction reduces allocation to immunity (Sheldon and Verhulst, 1996). This weaker immunity, plus potentially increased exposure associated with altered behaviour in reproductive individuals, results in higher parasitism (Knowles *et al.*, 2009; Albery *et al.*, 2019b). Finally, future fitness is reduced by damage from parasites (Harshman and Zera, 2007; Graham *et al.*, 2011). This combination of mechanism comprises an "indirect" cost of reproduction acting through parasites. Additional (direct) costs of reproduction can simultaneously act through other mechanisms such as reduced condition, hormonal or phenological regulation, or damage caused by oxidative stress (Stjernman *et al.*, 2004; Harshman and Zera, 2007; Speakman, 2008). Thus, all three of reproductive investment, immunity, and parasitism can have independent effects on fitness. In order to consider parasites' place in tradeoffs between life history components, a distinction must be made between "input" costs of life history investment for immunity/parasitism, and "output" costs of immunity/parasitism for fitness. This distinction is rarely made explicitly, so direct reproductive costs have rarely been compared directly with the costs of immunity or parasitism in mediating life history tradeoffs.

General life history tradeoffs can be hard to demonstrate observationally due to the confounding effects of individual quality or resource acquisition rate (van Noordwijk and de Jong, 1986). Specifically concerning immunity, it is possible that individuals in poor condition both exhibit weaker immunity and do not reproduce, resulting in a reversed pattern at the population level in which reproductive individuals actually suffer decreased burdens or lower parasite-induced mortality rates (e.g. Lynsdale *et*

al., 2017). Similarly, causality may be reversed: for instance, pregnancy can negatively affect immunity (Weinberg, 1984), but more highly parasitized individuals may be less likely to become pregnant in the first place (e.g. Hughes *et al.* 2009). Experimental approaches are therefore preferred when examining life history tradeoffs and their immune correlates, and clutch size manipulations have commonly been employed in birds to investigate these questions (Knowles *et al.*, 2009). However, reproductive effort is hard to manipulate in wild mammals (Rödel *et al.*, 2016), and in any case we are not aware of any published experiment in either birds or mammals in which both reproductive effort and parasite burdens were manipulated in wild animals to investigate causal links.

The wild red deer (*Cervus elaphus*) on the Isle of Rum exhibit a life history tradeoff wherein lactating to a calf until its first winter reduces a female's future reproduction and survival probability (Clutton-Brock *et al.*, 1989; Froy *et al.*, 2016). This reproductive cost has long-lasting fitness consequences (Moyes *et al.*, 2006). Females that give birth to a calf that dies within the first few months of its life have similar fitness outcomes to those that do not give birth, implying that gestation has a minimal cost relative to lactation (Clutton-Brock *et al.*, 1989). The deer are infected with several helminth parasites, with decreased mucosal IgA levels and increased parasite count in reproductive females (Albery *et al.*, 2018, 2019b). Lactation but not gestation results in substantially increased parasite counts, partially reflecting the cost of reproduction for fitness; however, investment in gestation does result in decreased mucosal IgA levels (Albery *et al.*, 2019b). Increased mucosal IgA levels correspond to reduced parasite burden, implying that they are indicative of protective immune responses (Albery *et al.*, 2019b). The deer are not amenable to experimental manipulation, but they are censused regularly, providing detailed information on their survival and reproductive success (Clutton-Brock *et al.*, 1982). In such

observational situations, or in concert with experiments, path analysis can be used to infer links between parasites and their fitness consequences (Pacejka *et al.*, 1998; Stjernman *et al.*, 2004; Brambilla *et al.*, 2015; Leivesley *et al.*, 2019). Notably, a recent analysis in a wild population of Soay sheep used path analysis to demonstrate observationally that reproduction reduced survival through increased parasite count and reduced body weight (Leivesley *et al.*, 2019). Path analysis generally allows inference of likely causal links given one or more *a priori* graphs of interconnected variables (Shipley, 2009). However, it is worth noting that path analysis does not confirm causality any more than other types of statistical analysis: for example, links among variables may belie more complex associations and/or may be confounded with hidden (latent) variables. Path analysis must therefore be used carefully, and in conjunction with specific *a priori* hypotheses and feasible causal pathways (e.g. see Figure 1).

Here, we use path analysis to link reproduction-immunity-parasitism tradeoffs in the Isle of Rum red deer with survival and reproduction in the following year, investigating whether immunity and parasitism are capable of mediating life history tradeoffs, and attempting to separate immune and parasite mediation from direct effects of reproduction acting through alternative mechanisms.

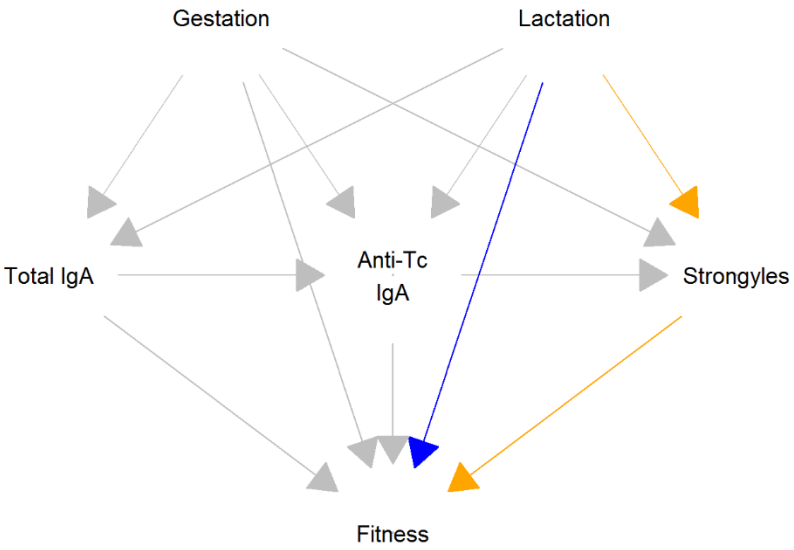

Figure 1: The base Directed Acyclic Graph (DAG) used for all four fitness trait path analyses. The variables are organised into three layers of causality: reproductive allocation (beginning of deer year *t*, top row); immune and parasite variation (deer year t, middle row); and fitness outcomes in the following year (beginning of deer year *t+1*, bottom row). The exception to these timings is survival, for which we investigated effects on samples in both spring in year *t-1* and summer in year *t* on survival to the beginning of year *t+1*. The blue line displays an example direct effect of lactation on fitness; the orange lines display an example indirect effect of lactation acting through strongyles. Anti-Tc IgA=anti-*Teladorsagia circumcincta* larval antigen IgA. Not shown: additional links of year, season, and age in years.

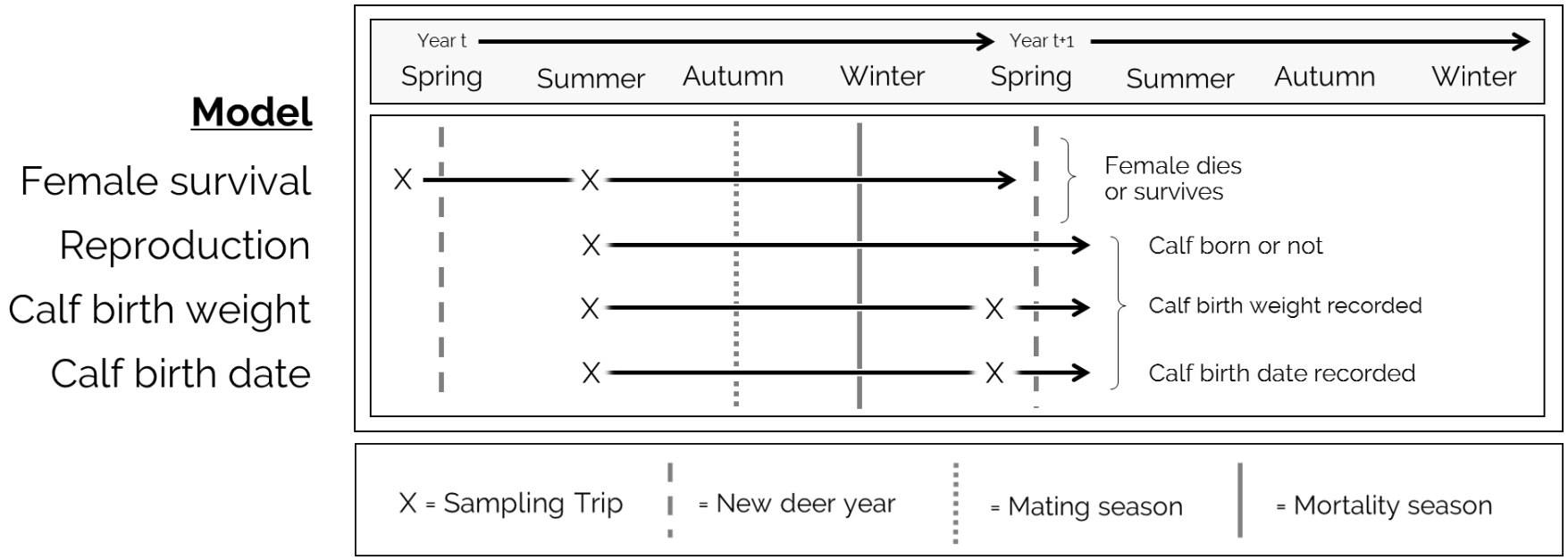

Figure 2: The four models in the context of the red deer reproductive cycle and sampling regime, over an example two-year period. Reproduction begins in spring and summer, at the start of each deer year, and one sampling trip was undertaken each summer (August), after the calving season had finished. Mating occurs in the autumn, and the mortality season begins in winter and lasts until early spring. A second sampling trip occurred each spring (April), after mating and mortality, but before the beginning of the subsequent calving season. The fitness variables investigated were quantified at the start of the subsequent deer year: if a female survived to May 1 the following year she was counted as 1 in the survival analysis, 0 if not, and the presence, weight, and birth date of her calf in the following spring were used as response variables in the remaining three models. The sampling trips included in each model were selected according to feasibility of causal links. For example, females become pregnant in the autumn, so we did not include the spring sampling season in the reproduction model as they would already be pregnant at this point, making it unlikely that parasite counts in April have a direct effect on their probability of having a calf 1-2 months later.

Methods

Study system, sampling, and labwork

The study population is situated in the north block of the Isle of Rum National Nature Reserve (57°N 6°20′ W). The deer are entirely wild and unmanaged, and have been monitored continuously since the 1970s (see Clutton-Brock *et al.*, 1982 for an overview of the project). The life history data collected on the population provide high-resolution estimates of individuals' dates of birth and death, reproduction, and familial relationships. The "deer year" begins on May 1st, and the deer begin giving birth ("calving") in May-June, having conceived in the previous autumn (Figure 2). Deer on Rum give birth to a single calf, and do not reproduce every year. During the calving season, we aim to capture and mark as many of the calves born as possible soon after birth, so that they can be monitored for the rest of their lives. Sex and capture weight (to the nearest 100g) are recorded. A proportion of calves (~20%) die within the first few weeks of life, and giving birth to a calf that dies within this period has little cost to the mother in terms of her survival and reproduction probability the following year (Clutton-Brock *et al.*, 1989). In contrast, if a calf survives into the winter, the mother has spent ~6 months lactating to it, expending considerable resources in doing so, and this cost is associated with substantially decreased survival and reproduction probability the following year (Clutton-Brock *et al.*, 1989; Froy *et al.*, 2016).

The sampling regime (Figure 2) has been described previously (Albery *et al.*, 2019b). During early spring (April) and late summer (August), either side of the calving season, we conducted two-week field trips to collect faecal samples from the deer noninvasively. We watched known individuals for defaecation, marked the spot where the droppings landed, and then collected them while minimising disturbance to the deer, generally within an hour. In the evenings, samples were processed and

put into Ziploc bags for storage. A subsample was extracted by centrifugation and kept frozen for faecal antibody analysis (Watt *et al.*, 2016; Albery *et al.*, 2019b). Another subsample was kept as anaerobic as possible in a Ziploc bag at 4°C to avoid egg hatching.

In the lab, faecal samples were counted for strongyle nematodes eggs using a salt flotation-centrifugation faecal egg count (FEC), accurate to 1 egg per gram (EPG) within 3 weeks of collection. Strongyles are ubiquitous ruminant parasites that are present at high prevalence in this population and which increase in parasitism in lactating individuals (Albery *et al.*, 2019b). Previous studies in this population have also examined the helminths *Elaphostrongylus cervi* and *Fasciola hepatica* (Albery *et al.*, 2018, 2019b). We chose to examine strongyles but not *E. cervi* or *F. hepatica* for several reasons: we did not want to add too many links to the DAG for reasons of interpretability; strongyles are most expected to have strong fitness costs (Hoberg *et al.*, 2001) and exhibited the most profound reproductive tradeoff in terms of significance and magnitude (Albery *et al.*, 2019b); we did not expect *E. cervi* to have strong fitness effects (Irvine *et al.*, 2006); and *F. hepatica* is present at relatively low prevalence in adult females, preventing it from being fitted easily as an explanatory variable (Albery *et al.*, 2018). We also carried out antibody detection ELISAs designed to quantify mucosal IgA in sheep (Watt *et al.*, 2016; Albery *et al.*, 2019b). This protocol quantifies both total IgA levels as a measure of general immune investment, and anti-*Teladorsagia circumcincta* IgA levels (anti-Tc IgA) as a specific anti-strongyle measure. *T. circumcincta* is primarily a sheep strongyle, but the anti-Tc IgA assay shows high cross-reactivity with a range of strongyle nematodes including the mouse helminth *Heligmosomoides polygyrus* and thus can be considered to measure general anti-strongyle responses (Froy *et al.*, in review). The deer are infected with a selection of strongyle nematodes (Irvine *et al.*, 2006), including *Teladorsagia circumcincta*

(unpublished data); thus, this measure is used to approximate anti-strongyle humoural immune responses in the deer (Albery *et al.*, 2019b). To control for collection factors which introduce confounding variation in antibody levels we used the residuals from a model including extraction session, time to freezing, and collection day, as in previous studies (Albery *et al.*, 2019a; b). We also assayed faecal samples collected in November (e.g. Albery *et al.*, 2018a). However, females exhibited very low strongyle prevalence in the autumn compared with spring and summer, preventing our FEC data from approximating normality and providing little variation to test when fitted as an explanatory variable. Hence, autumn data were excluded from our analyses.

Statistical analysis

To investigate links among our variables we used path analysis using the D-sep method, in which a set of linear models are fitted to the data, with some variables appearing as both response and explanatory variables (Shipley, 2009). Combining the linear models in this way allows identification of potential causal links and mediating variables: namely, does lactation itself have a cost for fitness, or does it act through parasite count?

We created four Directed Acyclic Graphs (DAGs), with four ultimate response variables, each representing a different fitness trait or proxy in grown females in the following year (hereafter referred to as "fitness-related traits"; see Figure 2). These measures included two direct fitness measures: the female's overwinter survival (0/1, where 1=survived to May 1 the following year) and reproduction the following year (0/1, where 1=gave birth in the following calving season). We also examined two fitness-associated proxies: the birth weight of a female's calf the following year (continuous, Gaussian distributed, based on a regression of capture weight on capture

age in days) and parturition date the following year (continuous, Gaussian distributed, based on Julian date that year).

Our analyses used three immune and parasite measures, which were: Total IgA level; Anti-Tc IgA level; Strongyle count per gram of faeces (continuous, log(count+1)-transformed to approximate normality). We included two mutually exclusive binary reproductive categories representing the reproductive cost paid that year (Clutton-Brock *et al.*, 1989): Gestation (gave birth to a calf which died before 1st October that year) and Gestation + Lactation (gave birth to a calf which survived to 1st October; henceforth referred to simply as "lactation"). We also included variables to control for extraneous annual, seasonal, and age-related variation: Year (categorical, with three levels: 2015, 2016, 2017); Season (two levels: Summer, Spring); and Age (continuous, in years).

Each of the four DAGs was composed of four similar models, fitted using the INLA package (Rue and Martino, 2009) in R version 3.5 (R Core Team, 2018). All measures included female identity as a random effect to control for pseudoreplication. First, we composed a set of three "input models", where the response variable was an antibody or parasite measure. The aim of these models was to quantify the association between reproduction and the immune/parasite measures, and to quantify links between these measures themselves.

The models were specified as follows for each of our analyses, with immune/parasite measures in bold and reproductive traits in italics. These classifications correspond to the middle row and top row of the DAG (Figure 1), respectively. Variables in brackets were included in the models, but are not displayed in the DAGs for clarity.

1. **Total IgA** ~ *Gestation* + *Lactation* (+ Age + Season + Year)
2. **Anti-Tc IgA** ~ **Total IgA** + *Gestation* + *Lactation* (+ Age + Season + Year)

3. **Strongyles** ~ **Anti-Tc IgA** + *Gestation* + *Lactation* (+ Age + Season + Year)

Following construction of these input cost models for each DAG, we fitted one "output model" for each DAG (bottom row of Figure 1), featuring the fitness-related trait as a response variable, and with reproductive, immune, and parasite measures as explanatory variables:

4. Fitness trait ~ **Strongyles** + **Anti-Tc IgA** + **Total IgA** + *Gestation* + *Lactation* (+ Age + Year)

Combining these two model sets allowed comparison of the significance and magnitude of different traits' costs for fitness in the following year (Figure 1). Combining the estimates from models 1-3 with the estimates from model 4 allows calculation of the direct and indirect (parasite- or immune-mediated) effects of lactation and gestation on subsequent fitness traits (Figure 1, coloured lines). Specifically, we compared the magnitude and credibility intervals of direct lactation effects (effect of lactation in the fitness model [model 4]) with indirect effects (lactation effects on strongyle count [model 3] multiplied by the effects of strongyle count on fitness [model 4]). We took 1000 posterior draws from each of the lactation-strongyle link and the strongyle-fitness link and multiplied them together, and then derived the 95% credibility intervals for this link. We compared these estimates with those for the direct lactation-fitness link to investigate whether effects of lactation were likely to act independently and/or through strongyle count. The models, fitness measures, and datasets used in each analysis are described in Table 1.

Model Set	Fitness Measure	Definition	Dataset	Samples	Individuals
1	Survival	Female survival the following winter (0/1)	All females (Spring year t-1 and Summer year t)	485	134
2	Reproduction	Female reproduction the following deer year (0/1)	All females (Summer)	223	107
3	Calf birth weight	Calf weight the following deer year (Kg)	Females that reproduced the following May-June (Summer year t and Spring year t)	300	94
4	Parturition date	Date of parturition the following deer year (Days from 1st January)		336	106

Table 1: Descriptions of path analyses and the datasets used.

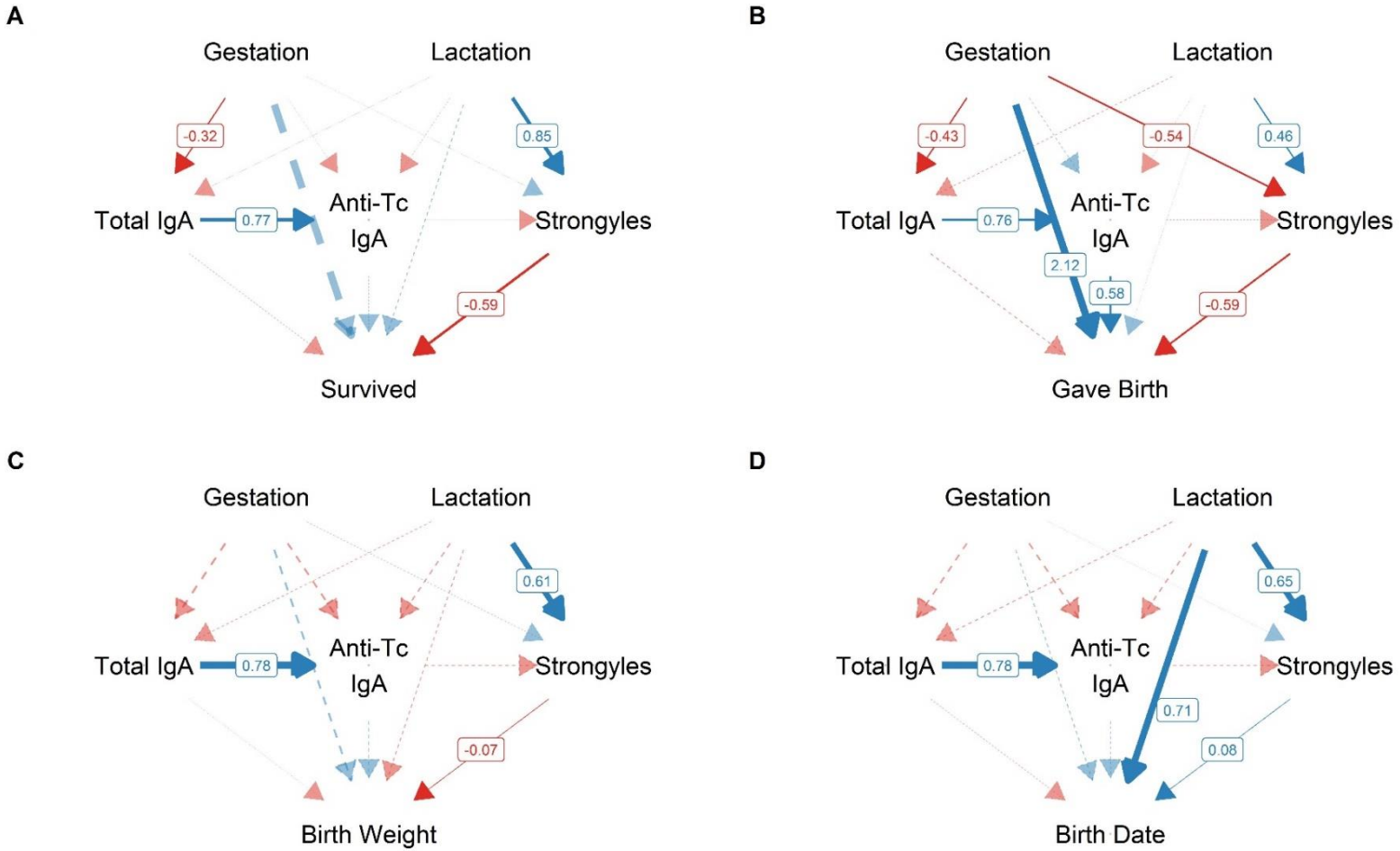

Figure 3: Directed Acyclic Graphs (DAGs). Results are displayed for all four investigated fitness response variables: overwinter survival (A); reproduction (B); subsequent calf birth weight (C); subsequent parturition date (D). Link colour depends on the direction of the effect (blue=positive, red=negative); link width indicates the magnitude of the effect; and only solid, opaque links are significant (estimates did not overlap with zero). Labels denote the link-scale effect sizes (slopes) for the significant effects, derived from GLMMs.

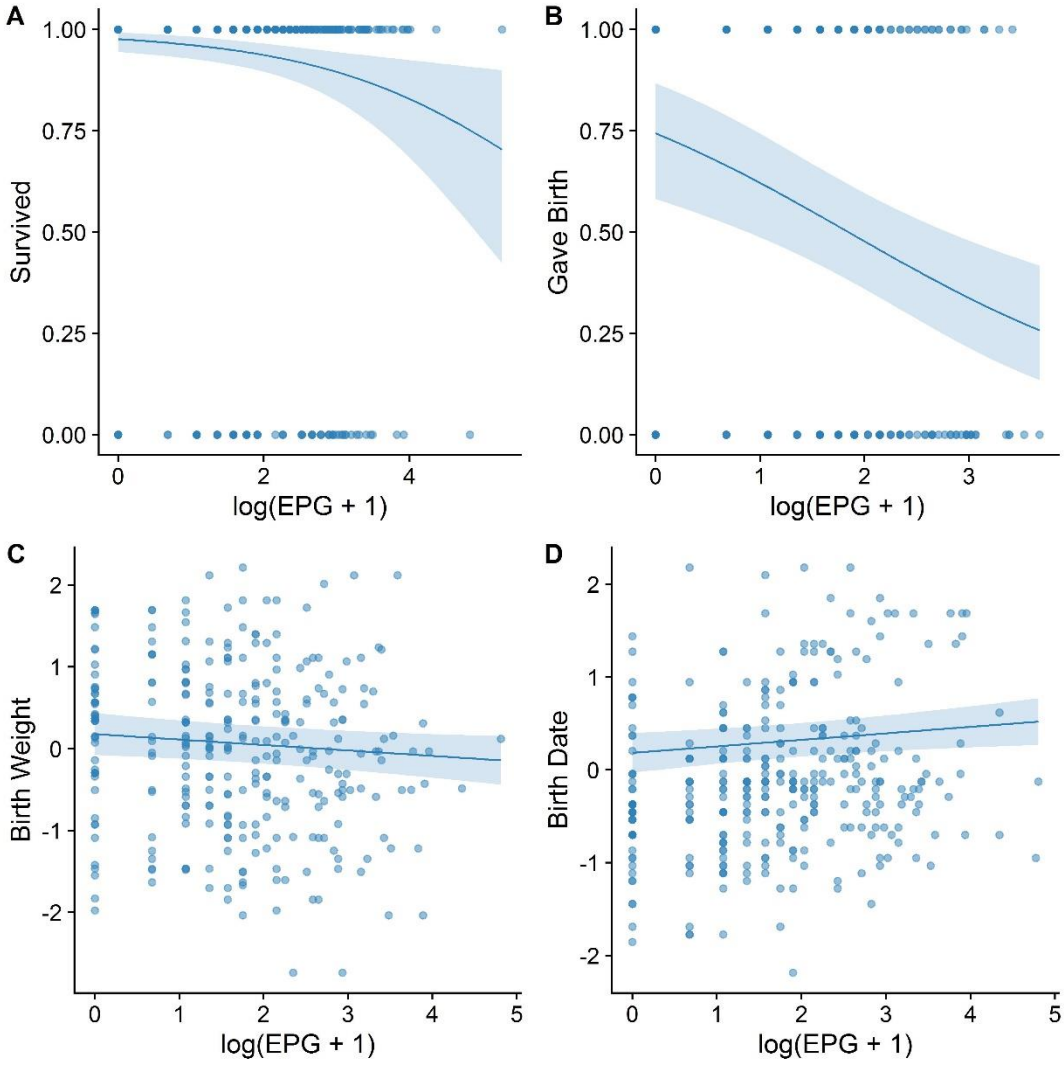

Figure 4: Relationships between strongyle parasite count and fitness measures in the following year. Results are displayed for all four investigated fitness response variables: overwinter survival (A); reproduction (B); subsequent calf birth weight (C); subsequent parturition date (D). The lines denote the fitted slope of parasitism on the response variable, with associated 95% credibility intervals. All four relationships were significant, with effect estimate intervals that did not overlap with zero. Strongyle count was log(x+1)-transformed for analysis.

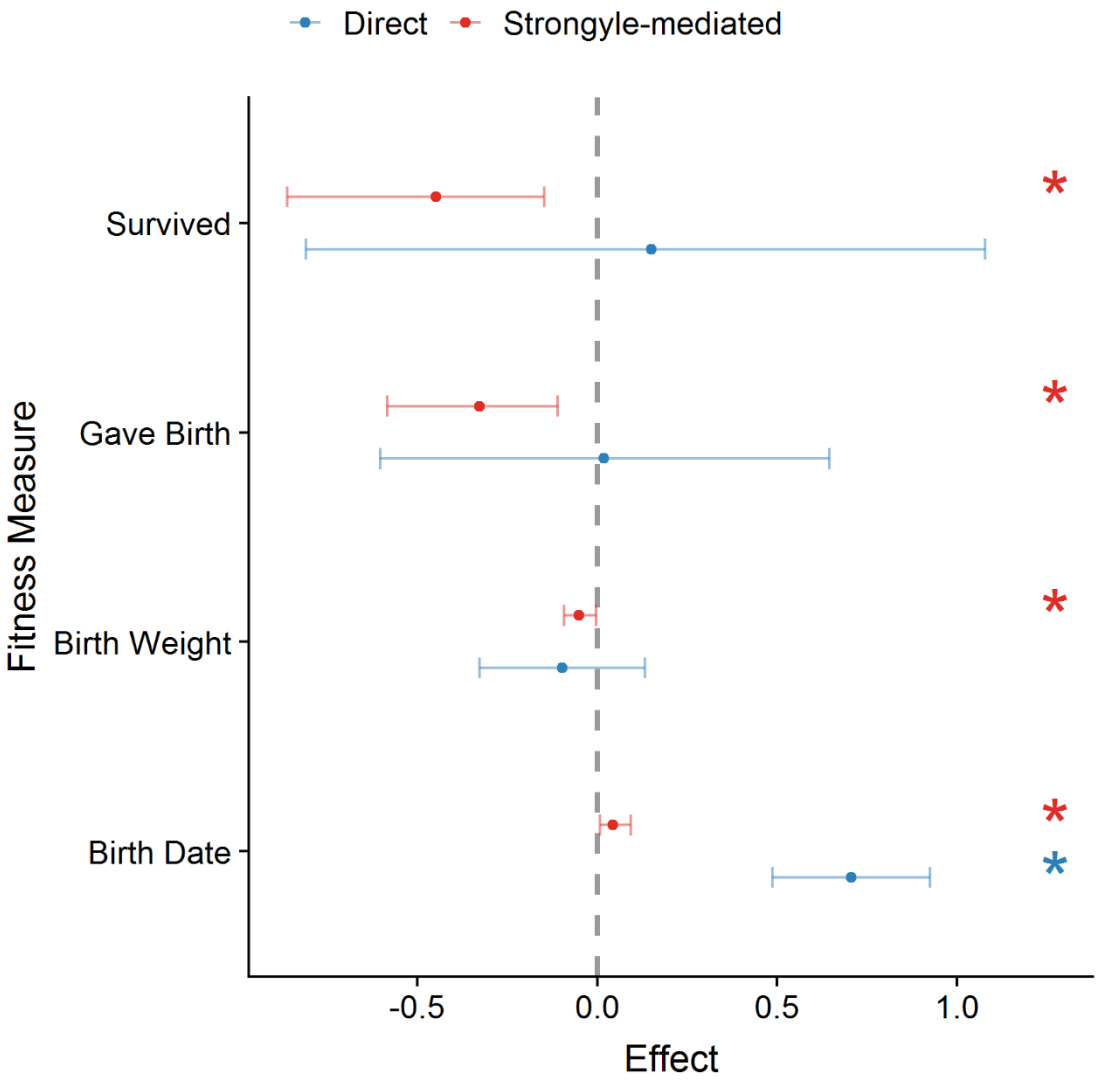

Figure 5: Comparison of direct and indirect (parasite-mediated) effects of lactation on fitness-related traits on the link scale (logistic for survival and reproduction; Gaussian for birth weight and birth date). Points represent mean effect estimates derived from the model posterior distributions, and error bars give 95% credibility intervals; blue corresponds to direct effects, and red corresponds to indirect effects. Parameters with asterisks were significant: i.e., their credibility intervals did not overlap with zero.

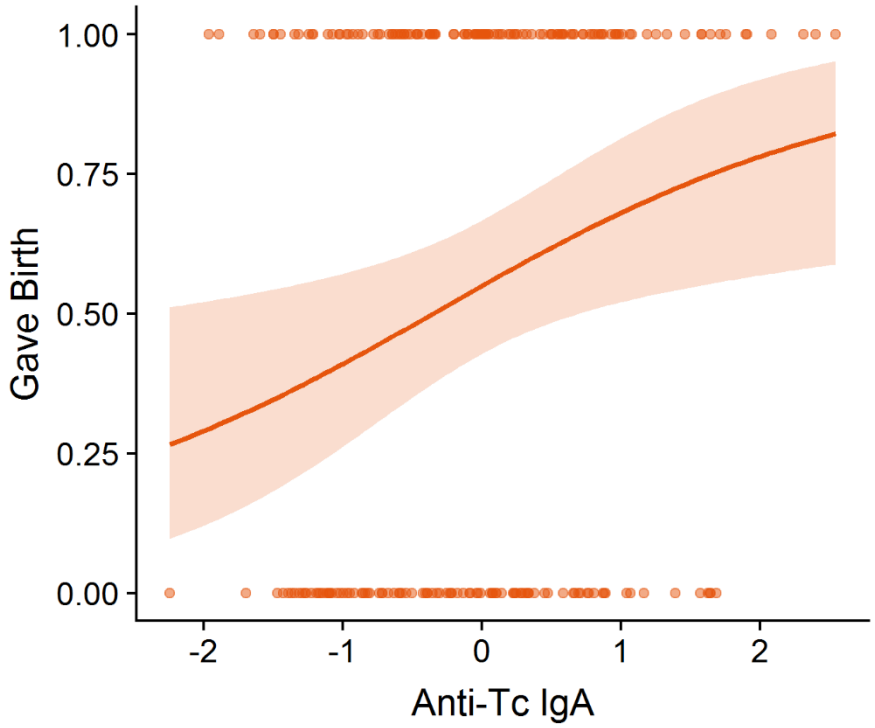

Figure 6: Higher anti-*Teladorsagia circumcincta* IgA was associated with an increased probability of reproducing the following year. Anti-Tc IgA was cube root-transformed and calculated from the residuals of a linear model including collection variables, and was then scaled to have a mean of 0 and a standard deviation of 1. The line represents the output of the reproduction probability model which includes lactation and strongyles as explanatory factors, with associated 95% credibility intervals.

Results

Path analyses consistently revealed strong positive associations between lactation and parasitism, and negative associations between parasitism and subsequent fitness-related traits (Figures 3-5). In contrast, estimates for lactation's association with subsequent fitness itself overlapped with zero for all response variables except parturition date, strongly supporting parasite-mediated reproductive costs for fitness (Figures 3-5). Below, for each of the four fitness-related response variables, we describe the magnitude of the direct association of parasitism with fitness, the direct association of lactation with fitness, and lactation's association with parasitism multiplied by parasitism's association with fitness. The latter gives an estimate of the

indirect effect of lactation on the fitness-related trait acting through strongyle count. For effect sizes we give the mean and 95% credibility intervals (CI). 1 log(EPG+1) increase corresponds to a ~3x increase in strongyle count. Full model effect sizes are displayed in the supplementary information (Appendix B, Figure SI1; Table SI1).

Parasitism had a strong association with survival probability despite high survival rates in the population (Figure 3A,4A). Females with the lowest counts (0 EPG, 10% of samples) had a survival probability of ~100%, while those with the highest (>25EPG, 7% of samples) had a survival probability of <90% (Figure 4A). Combining this effect of parasitism with the strong positive association between lactation and strongyle count (+0.85, CI 0.64, 0.99) created an indirect cost of lactation acting through strongyle count (Figure 5). Although this effect was highly significant on the link (logistic) scale (Appendix B, Figure SI1, Table SI1), given the high survival rates in the population, at the mean EPG value this lactation-associated increased strongyle parasitism would correspond to only a ~2% decrease in survival probability. In contrast, estimates for the direct effect of lactation on survival overlapped widely with zero, and the point estimate was greater than zero, implying that individuals that lactate were slightly more likely to survive when the effects of parasitism were accounted for (Figure 3A,5).

The magnitude of strongyles' association with subsequent reproduction had a similar effect size to its association with overwinter survival (Figure 3B,4B,5; Appendix B, Table SI1; Figure SI1). An increase of 1 log(EPG+1) was associated with a decrease of ~15% probability of reproducing. 0 EPG (17% of samples) corresponded to a ~77% chance of reproducing the following year, and those with >20 EPG (6% of samples) had a reproduction probability of <36% (Figure 4B). The direct effect of lactation on subsequent reproduction was negligible and had very large credibility intervals, as

with survival (Figure 5). In addition to the association with parasitism, individuals with higher levels of anti-Tc IgA were considerably more likely to reproduce the following year (Figure 3B; Figure 6). An increase of 1 standard deviation of anti-Tc IgA levels corresponded to an increase of ~10% in the probability of reproducing. Individuals with the lowest anti-Tc IgA levels (less than -1 SD units) had a reproduction probability of <50%, compared to >75% for those with the highest levels (>1 SD units; Figure 6). Finally, individuals that paid the cost of gestation were much more likely to reproduce the following year, independently of the effects of antibodies and parasites (Figure 3B).

Calving traits exhibited weaker associations with parasitism than did survival and reproduction, although the results still implied an indirect cost of lactation acting through strongyle count (Figures 3-5). The DAG for calf birth weight was similar to that for survival (Figure 3C). An increase of 1 log(EPG+1) corresponded to a slight decrease in calf birth weight the following year (0.07 SD units, or about 86g; Figure 4C). Females with the highest strongyle intensities (>25 EPG) gave birth to calves which were ~400g lighter than those with the lowest intensities (0 EPG), or around 6.24 kg compared to 6.65 kg. As with survival, there was poor support for a direct association between lactation and birth weight (Figure 5). The estimates for this direct effect were close to zero, and credibility intervals overlapped substantially with zero (Figure 5). Lactation's positive effect on strongyle count once again resulted in a significant negative indirect effect of lactation on subsequent calf birth weight acting through strongyles, but the estimates were very small and nearly overlapped with zero (-0.0438kg, CI -0.111.6, -0.005.6).

In contrast to all other metrics we investigated, there was very strong support for a positive and direct effect of lactation on parturition date the following year: that is,

females whose calf survived until the winter were likely to calve later in the following year (~8.5 days later, CI: 5.9, 11.2; Figure 3D; Figure 5), regardless of parasite count. There was a much weaker association between strongyle count and parturition date: an increase of 1 log(EPG+1) produced a delay in calving of ~0.93 days (CI: 0.12, 1.75; Figure 3D,4D; Figure 5). Lactation resulted in an increase of 0.7 log(EPG+1). Combining this estimate with the effect of parasitism on birth date gives an estimate for an indirect effect of lactation acting on birth date totalling 0.58 days' delay (CI 0.06, 1.31; Figure 5). Parturition date was thus the only metric examined here for which lactation's direct effect was definitively larger than its indirect effect acting through strongyle count (Figures 3-5).

There was a strong positive association between total IgA and anti-Tc IgA, as expected given our previous findings (Albery *et al.*, 2018b; Appendix B, Table SI1, Figure SI1). However, lactation had no significant effect on anti-Tc IgA in our DAGs, showing some discordance with these findings (Figure 3, Appendix B, Table SI1, Figure SI1). This disagreement likely originated from a markedly reduced sample size in our path analysis compared to these previous analyses (Table 1).

Discussion

This study offers strong observational evidence for parasite-dependent mediation of life history tradeoffs in a wild mammal (Williams, 1966; Sheldon and Verhulst, 1996), and for broader fitness effects of parasitism, immunity, and reproductive resource allocation. Lactation was associated with higher parasite intensities which translated to reduced survival and reproduction probability in the subsequent year, and among individuals that did reproduce the following year, those with high strongyle counts gave birth later in the year and to smaller calves. Beyond these parasite-mediated effects, lactation had a direct detrimental effect on subsequent parturition date, and

increased anti-strongyle IgA levels were associated with increased reproduction probability. Our observation of a direct (parasite-independent) association between lactation and subsequent parturition date suggests that costs of reproduction for subsequent fitness traits can also arise through a combination of direct and parasite-dependent mechanisms. It is likely that the life history tradeoffs exhibited by the Rum red deer population (Clutton-Brock *et al.*, 1989; Froy *et al.*, 2016) are at least partially mediated by parasites as previously suggested (Albery *et al.*, 2019b), or that parasites and immunity closely correspond to latent condition variables that are responsible for mediating fitness.

Fitness effects of parasites

Lactation's negative association with fitness acted largely through strongyle count for all fitness metrics except parturition date. This may represent a strong parasite-mediated cost, in which pathology and resource allocation associated with increased parasitism are the primary cause of increased overwinter mortality and reduced reproductive fitness in lactating individuals (Clutton-Brock *et al.*, 1989). Allocation of resources to lactation and associated physiological changes may compromise damage repair and resistance mechanisms, leaving lactating females more susceptible to strongyles (Sheldon and Verhulst, 1996; Speakman, 2008). As lactation results in considerably increased forage intake in ungulates (Hamel and Côté, 2008), it is also possible that increased grazing brings resource benefits which fully counteract the direct resource costs of lactation, while intensifying strongyle parasitism (and thereby parasite-associated pathology) through increased exposure (Albery *et al.*, 2019b). Parasitism likely causes gut pathology, interfering with nutrient absorption and thereby exacerbating the nutritional scarcity of the winter period, leading to mortality (Gulland, 1992; Pedersen and Greives, 2008; Maublanc *et al.*, 2009), as well as reducing

females' ability to achieve the body condition necessary to conceive and carry a calf to term (Albon *et al.*, 1986).

Such fitness costs will exert strong selection pressures on the deer, particularly given the low absolute strongyle counts in the population (median count ~= 6 EPG, mean ~= 12 EPG). The relationship between parasitism and reproduction was especially steep (Figure 3b), and the deer will be selected to resist or tolerate infection to avoid such fitness consequences (Graham *et al.*, 2011). Combined with the considerable spatiotemporal variation in parasite exposure in this population (Albery *et al.*, 2018, 2019a), parasite-mediated reproductive costs could create spatial and temporal variation in the fitness consequences of reproduction. In this case, females that give birth and lactate but then avoid areas with high parasite intensities may avoid the fitness reduction associated with reproduction, which could select for behavioural avoidance of infection (Hutchings *et al.*, 2006; Weinstein *et al.*, 2017). It can be hard to detect survival costs of reproduction in long-lived mammals given their low between-individual variance in survival probability (Hamel *et al.*, 2010). Here we discovered a detectable indirect cost of reproduction for survival – in fact, the link-scale effect sizes of the parasite-mediated lactation effect was similar for both survival and reproduction (Figure 5). In contrast, subsequent calf birth weight and parturition date (both fitness-related proxies rather than absolute fitness traits) had much weaker relationships with parasitism (Figure 4). Traits that are more tightly linked to fitness such as annual survival and reproduction may be more quickly compromised by parasitism than less-related fitness proxies such as calf birth weight or date, which may have stronger genetic components (Stopher *et al.*, 2012b). Previous work in ungulates has demonstrated that immune variation and parasite pressure can maintain variation in life history strategies by affecting reproduction and survival differently, which may likewise be the case in this population (Graham *et al.*, 2010;

Garnier *et al.*, 2016). Future investigations could use larger sample sizes collected from more reproductive cycles to determine how the relationships demonstrated here impact calf survival and breeding success.

Alternatively, any latent variable mediating reproduction-fitness tradeoffs may be very well correlated with strongyle count, so that strongyle count assumed the majority of lactation's effect but was not causally responsible for the reduced fitness. For example, hormones can weaken immunity, leading to increased parasitism (Foo *et al.*, 2017), as well as mediating reproductive fitness (Harshman and Zera, 2007). It is possible that fitness-mediating hormones produce a concurrent increase in parasitism in the deer, which then absorbed the variation in fitness attributable to hormonal regulation of reproductive investment in our analysis. Similar arguments could be made for a mediating role of females' body weight, which is a trait generally used to represent broad-strokes condition, and which mediates a life history tradeoff alongside parasite count in Soay sheep (Leivesley *et al.*, 2019). The inability to measure such mediating variables highlights another limitation of the entirely noninvasive nature of the red deer system.

While path analysis has been very useful in revealing interrelationships between the variables studied here, these findings do not prove causality. However, strongyles are an important ungulate parasite which cause considerable morbidity and mortality in wild ungulates (Coltman *et al.*, 1999; Balic *et al.*, 2000; Hoberg *et al.*, 2001) and which are associated with decreased body condition in this population (Irvine *et al.*, 2006). We therefore have strong reasons to believe that strongyles will impact the health of the deer negatively, and the results of our path analysis conform to this expectation. In addition, the conformation of our findings to the proposed sequence of causality (lactation increases parasitism, which decreases fitness) strengthens our confidence

in the inferred importance of strongyles as the mediating factor. Whether or not strongyles are the effectors, our findings nevertheless support the use of these parasites as a proxy for an individual's health.

The importance of temporal context in reproduction-parasite-fitness relationships

Observational disease ecology studies commonly implicitly assume causal relationships which may in fact be unsupported by the data. In particular, parasitism can act as a response variable reacting to variation in exposure and susceptibility, and/or as an explanatory variable influencing health, resource investment, and ultimately fitness, yet parasitism is commonly considered from only one of these perspectives. Here, the observed relationship between parasitism and reproduction depended on whether we examined reproduction in the focal year (lactation year t) or in the following year (giving birth in year t+1). Similarly, although we previously reported no relationship of any immune and parasite measures with calf birth date or birth weight in the year of sampling (Albery *et al.*, 2019b), here both these measures correlated negatively with strongyle count the previous year. These results demonstrate that fitness-related traits quantified at different timepoints can exhibit qualitatively different relationships with parasitism. Therefore, using cross-sectional measures of reproductive investment such as pregnancy in culled animals may be highly misleading, as pregnant individuals may either have become pregnant because they had fewer parasites (Albon *et al.*, 2002; Vandegrift *et al.*, 2008), or may be experiencing higher parasitism due to their increased investment in reproduction (Sheldon and Verhulst, 1996; Knowles *et al.*, 2009; Albery *et al.*, 2019b). This finding supports the virtues of longitudinal studies of known individuals in distinguishing cause from consequence (Clutton-Brock and Sheldon, 2010; Festa-Bianchet *et al.*, 2017).

Among our response variables, only subsequent parturition date was directly associated with lactation the previous year: females that lactated to a surviving calf and then became pregnant gave birth later the following year, regardless of strongyle count. Having raised a calf past its first few months, it is likely that females take longer to regain condition, so they mate later in the year whether or not they are highly parasitized (Albon *et al.*, 1986). As a result, these individuals will calve later the following year (Clements *et al.*, 2011). As lactation and strongyle count are positively correlated, neglecting to include lactation as an explanatory variable in this analysis would substantially exaggerate the perceived importance of parasitism in influencing calving date. When investigating fitness effects of parasitism, it may be advantageous for studies to investigate and control for the influence of life history traits themselves. Future studies in this population could elaborate on these findings by investigating how maternal and calf parasitism correlate and correspond to maternal and calf fitness to investigate transgenerational immunity-parasitism-fitness correlations: a topic that is largely understudied and likely exerts a considerable influence on epidemiological dynamics (Roth *et al.*, 2018).

Interpreting antibody-fitness correlations

Higher anti-Tc IgA levels were associated with substantially increased probability of reproduction the following year (Figure 6). This association is surprising, as it acted independently of any associations between 1) strongyles and probability of reproduction and 2) anti-Tc IgA and strongyles themselves. That is, higher anti-strongyle antibody titres were independently associated with increased reproduction probability rather than acting via reducing parasitism. Moreover, the association between anti-Tc IgA and reproduction probability received considerably stronger support than any connection between total IgA and any fitness-related traits, implying that the driving mechanisms were specific to anti-strongyle IgA rather than

to antibody levels in general. There are several possible explanations for this observation: First, and most probably, anti-Tc IgA may in fact be more strongly correlated with another unmeasured component of individual quality than are strongyles or total IgA. For example, individuals with larger fat reserves may be able to sustain higher anti-Tc IgA levels (Demas *et al.*, 2003) as well as being fitter (Milenkaya *et al.*, 2015). This possibility may reflect the confounding effects of individual quality in observational studies (van Noordwijk and de Jong, 1986). However, it is surprising that this trend was evident for anti-Tc IgA rather than total IgA, as we expected the latter to be a better general indicator of health/condition than parasite-specific IgA. Alternatively, anti-Tc IgA may be protective against other, unmeasured parasites, reducing their impact on fitness, or it may reduce or repair damage from strongyles rather than acting through reducing parasitism. That is, anti-Tc IgA may be an indicator of tolerance as well as of resistance (Råberg *et al.*, 2009b; Graham *et al.*, 2011). Regardless of the cause, this observation supports the value of examining the fitness consequences of immune variation itself, rather than examining parasitism alone (Graham *et al.*, 2011), and of studying parasite-specific antibodies in general (Garnier and Graham, 2014). In particular, path analysis presents a convenient method for contrasting the relative importance of immunity and parasites and their interrelationships in influencing fitness, particularly when allocation to reproduction itself may have a cost.

A previous analysis demonstrated costs of reproduction for immunity as well as parasitism, where investment in lactation was associated with lower total IgA and anti-Tc IgA levels, and gestation with lower total IgA levels (Albery *et al.*, 2019b). Although our estimates fell within the estimates for this previous analysis (Appendix B, Figure SI1), our estimates of lactation's association with total IgA and anti-Tc IgA were weaker, and overlapped marginally with zero in all cases. This weaker

association likely emerged from the reduced sample size in the present analysis compared to our previous analysis, particularly due to our exclusion of the autumn sampling season. As a result, although anti-Tc IgA was associated with increased probability of reproduction the following year, we were unable to identify this as part of a life history tradeoff. It was not possible to transform the autumn parasite counts to conform to normality, and the range of strongyle counts gave very little variation to test, precluding our ability to include this season in the analysis. The detection of tradeoffs was thus dependent on the power of the analysis used, and apparently on the time of year at which the tradeoff is analysed; it is possible that further sampling of the population will allow us to examine these tradeoff mechanisms using analyses with increased power. As we did not uncover any other fitness benefits of immunity, parasites themselves may be a more reliable general indicator of a deer's health than the immune responses to these parasites.

This study offers long-awaited evidence for parasite-dependent mediation of life history tradeoffs in a wild animal population (Williams, 1966). We demonstrate that reproductive costs for fitness metrics may originate largely from parasites, and potentially through alternative mechanisms for the case of parturition date. We suggest that more studies should simultaneously examine the fitness consequences of reproduction, parasites, and immunity in tandem.

Chapter V: The fine-scale landscape of immunity and parasitism in a wild ungulate population

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Summary

Spatial heterogeneity in parasite susceptibility and exposure is a common source of confounding variation in disease ecology studies. Although spatial variation in parasitism is well-established, it is not known whether spatial autocorrelation acts on immunity at small scales, within wild animal populations, and whether this predicts spatial patterns in infection. Here we used a well-mixed wild population of individually-recognised red deer (*Cervus elaphus*) inhabiting a heterogeneous landscape to investigate fine-scale spatial patterns of immunity and parasitism. We noninvasively collected 842 faecal samples from 141 females with known ranging behaviour over two years. We quantified total and helminth-specific mucosal antibodies and counted propagules of three gastrointestinal helminth taxa. These data were analysed with linear mixed models using the Integrated Nested Laplace Approximation (INLA), using a Stochastic Partial Differentiation Equation approach (SPDE) to control for and quantify spatial autocorrelation. We also investigated whether spatial patterns of immunity and parasitism changed seasonally. We discovered substantial spatial heterogeneity in general and helminth-specific antibody levels and parasitism with two helminth taxa, all of which exhibited contrasting seasonal variation in their spatial patterns. Notably, *Fasciola hepatica* count appeared to be strongly influenced by the presence of wet grazing areas, and antibody hotspots did not correlate with distributions of any parasites. Our results suggest spatial heterogeneity as an important factor affecting immunity and parasitism in a wide range of study systems. We discuss these findings with regards to the design of sampling regimes and public health interventions, and suggest that disease ecology studies investigate spatial heterogeneity more regularly to enhance their results, even when examining small geographic areas.

Introduction

Parasite infection in the wild is extremely spatially heterogeneous. The scale at which spatial variation acts depends on the host and parasite being studied, and even fine-scale environmental heterogeneity may influence the spatial epidemiology of human diseases (Murdock *et al.*, 2017). However, the spatial ecology of disease is most often considered in terms of large-scale patterns (e.g. Murray *et al.*, 2018) and using occurrence or prevalence data, which is less informative than intensity, particularly for macroparasites. In addition, due to practical considerations, many studies investigating spatial variation in wild animals compare several discrete populations rather than sampling across a continuous, mixing population (e.g. Downs *et al.* 2015; Cheynel *et al.* 2017). Alternatively, some studies rely on opportunistic convenience sampling, which can produce an inaccurate representation of disease processes and bias estimates of infection prevalence due to their non-random sampling in space (Nusser *et al.*, 2008). As a result, little is known about fine-scale patterns of susceptibility and exposure, and how they influence spatial patterns of infection, in wild animals.

Identifying the relevant spatial scale for disease processes such as susceptibility and exposure is important, as quantifying spatial trends at different scales can introduce uncertainty at best, and can profoundly affect the conclusions drawn at worst (Gilligan *et al.*, 2007; Vidal-Martínez *et al.*, 2010; Lachish and Murray, 2018). For example, Lyme disease risk correlates positively with biodiversity at the within-forest level, but the reverse is true between forests (Wood and Lafferty, 2013). An understanding of spatial processes is therefore helpful for designing public health interventions (Caprarelli and Fletcher, 2014) and sampling regimes (Nusser *et al.*, 2008; Vidal-Martínez *et al.*, 2010; Lachish and Murray, 2018). A deeper understanding of fine-scale spatial variation in disease processes could also inform patterns seen over

wider distances (Murdock *et al.*, 2017; Pawley and McArdle, 2018). In addition, if immunity and parasitism vary over short distances, infection-oriented studies of wild populations could be affected by greater degrees of spatial dependence than previously considered, which can affect inference. When spatial autocorrelation is not considered, the type I error rate may be inflated due to inflated covariance of explanatory and/or response variables emerging from geographic proximity (Pawley and McArdle, 2018).

Spatial variation in immunity can originate from gradients in abiotic conditions such as temperature (Laughton *et al.*, 2017) or in biotic factors such as prey availability (Becker *et al.*, 2018). Spatial variation in parasitism will arise in part as a result of this immune heterogeneity owing to variation in susceptibility, clearance, and tolerance (Jolles *et al.*, 2015), as well as from abiotic factors affecting parasite transmission (e.g. sunlight; Parsons *et al.* 2015) or from variation in abundance of secondary hosts or vectors (Sol *et al.*, 2011; Olsen *et al.*, 2015). In addition, conspecific density can influence resource availability, immune investment, and parasite exposure (Wilson *et al.*, 2004; Hines *et al.*, 2007; Downs *et al.*, 2015; Ezenwa *et al.*, 2016b). We therefore expect to see considerable spatial variation in both immunity and parasitism in heterogeneous environments (Becker *et al.*, 2019); where gradients are steep and mixing is minimal, this variation should occur over short distances. A recent study in wild mice (*Mus musculus*) demonstrated high between-site immune heterogeneity, but with extensive variation in the degree of within-site differentiation, suggesting short-range spatial dependence (Abolins *et al.*, 2018). However, few studies have examined how both immunity and parasitism vary continuously across space within wild animal populations, so it is unclear to what degree spatial variation in parasitism in the wild originates from immune-mediated processes rather than from environmental factors affecting exposure. Finally, spatial patterns are rarely static,

and may change over time (Hawkins, 2012), yet seasonal or annual changes in these spatial patterns are rarely examined.

The red deer (*Cervus elaphus*) is a large land mammal closely related to the American wapiti (*Cervus canadensis*) whose distribution covers much of Europe. The relationship between red deer disease and their spatial behaviour is important for pathogen sharing, as this species carries a plethora of parasites that can infect humans and livestock (Bohm *et al.*, 2007; Brites-Neto *et al.*, 2015), which they can vector between farms and distribute through the landscape (Chintoan-Uta *et al.*, 2014; Qviller *et al.*, 2016). The wild red deer living in the North Block of the Isle of Rum in Scotland are individually recognised and regularly censused, providing detailed information on each individual's life history and ranging behaviour (Clutton-Brock *et al.*, 1982). These censuses have previously been used to uncover important roles of the environment and spatial behaviour in influencing individuals' phenotypes (Stopher *et al.*, 2012b; Froy *et al.*, 2018). Longitudinal noninvasive faecal sampling of the population has revealed a high prevalence of several gastrointestinal helminth parasites including strongyle nematodes, the liver fluke *Fasciola hepatica*, and the tissue nematode *Elaphostrongylus cervi* (Albery *et al.*, 2018). The life cycle of strongyle nematodes is direct (i.e., with a ruminant as their only host), whereas in order to complete their life cycles *F. hepatica* must infect *Galba truncatula* water snails (Taylor *et al.*, 2016b), and *E. cervi* infects a range of land snails (Mason, 1989). Their mucosal antibodies (IgA) have also been quantified by faecal ELISA, offering a measure of immune investment (Albery *et al.*, 2019b). Both helminth faecal egg count and IgA concentrations are affected by deer reproductive investment and fluctuate seasonally (Albery *et al.*, 2019b). The spatial distributions of these immune and parasite measures have yet to be investigated.

In this study, we used regular census data and noninvasive faecal samples from the deer population to investigate how individuals' spatial behaviour was associated with immunity and parasitism at fine spatial scales. We incorporated spatial autocorrelation structures to investigate how this affected model fit, to identify hotspots of immunity and infection, and to quantify the spatial scale at which our data were autocorrelated. We also allowed spatial autocorrelation structures to vary seasonally. We expected that accounting for spatial autocorrelation would improve model fit, and that this would be a more effective way of investigating spatial trends than separating the population into discrete arbitrary subpopulations as was previously done to control for spatial variation (Huisman *et al.*, 2016). We also predicted that individuals living in different areas of the study system would exhibit notably different antibody levels and parasite intensities. Finally, we predicted that *F. hepatica* and *E. cervi* count would be influenced by the habitats of their secondary hosts – particularly that *F. hepatica* would be more common in wetter areas (Olsen *et al.*, 2015).

Methods

Study system and sampling regime

The study population is located in the north block of the Isle of Rum, Scotland (57°N, 6°20′W; Figure 1). The sampling area measures ~4 km north-south and ~3 km west-east (total area ~12.7km^2). The most intensely sampled area consists of a river running from south to north along a valley, flanked by hills on either side, and an extended ranging area around the coast to the east, close to the sea. Peat bogs and *Juncus* marshland comprise much of the southern and central areas of the valley, while the hills are dominated by wet and dry heath and *Molinia* grassland. In the north, moving seaward, the landscape is dominated by *Agrostis* and *Festuca* grassland, followed by sandy dunes and beaches. The study population is wild and unmanaged, and is

censused five times a month for eight months of the year (see Clutton-Brock *et al.*, 1982). During censusing, one of two predetermined routes is walked or driven through the study area and individuals' locations (to the nearest 100 metres) are noted. The northern part of the study area hosts the highest population density, with most deer centred around the high-quality grazing near the mouth of the river and the land around the coast to the east (Figure 1). Annual home ranges are highly repeatable from year to year (Stopher *et al.*, 2012b).

The deer reproductive cycle ("deer year") spans from the start of the calving season, May 1st, until April 30th the following year. Samples were collected as previously described (Albery *et al.*, 2018), on a seasonal basis during 7 two-week trips in August ("summer"), November ("autumn") and April ("spring") between April 2016 and April 2018 inclusive. Note that our dataset included a sampling trip from April 2016, which was part of the deer year beginning in May 2015, with no accompanying summer and autumn trips from this reproductive cycle. In the study period, 842 faecal samples were collected noninvasively from 141 individually known adult females aged 3 and above. Parasite propagule counts and antibody ELISA quantification were carried out on these samples as previously described (Albery *et al.*, 2018, 2019b). Parasites included strongyle nematodes (order: Strongylida), the common liver fluke *Fasciola hepatica* and the red deer tissue nematode *Elaphostrongylus cervi*. Our two antibody measures were total mucosal IgA levels ("total IgA") and anti-*Teladorsagia circumcincta* L3 larval antigen IgA ("anti-Tc IgA"). The former is taken as an indicator of general investment in mucosal immunity, while the latter gives a measure of specific anti-strongyle IgA response which is thought to be more indicative of protective immunity against strongyles (Watt *et al.*, 2016; Albery *et al.*, 2019b). There was not enough faecal matter in all samples to quantify all variables; final sample sizes are displayed in Table 1. Using the census data, each individual's mean easting and

northing over the deer year was taken as their average location. This was taken to be a better indication of an individual's spatial behaviour than the location at which the faecal sample itself was collected. We subdivided the study area into six approximate subpopulations based on each individual's average location, as in a previous analysis (Huisman *et al.*, 2016). These locations and subpopulations are displayed in Figure 1.

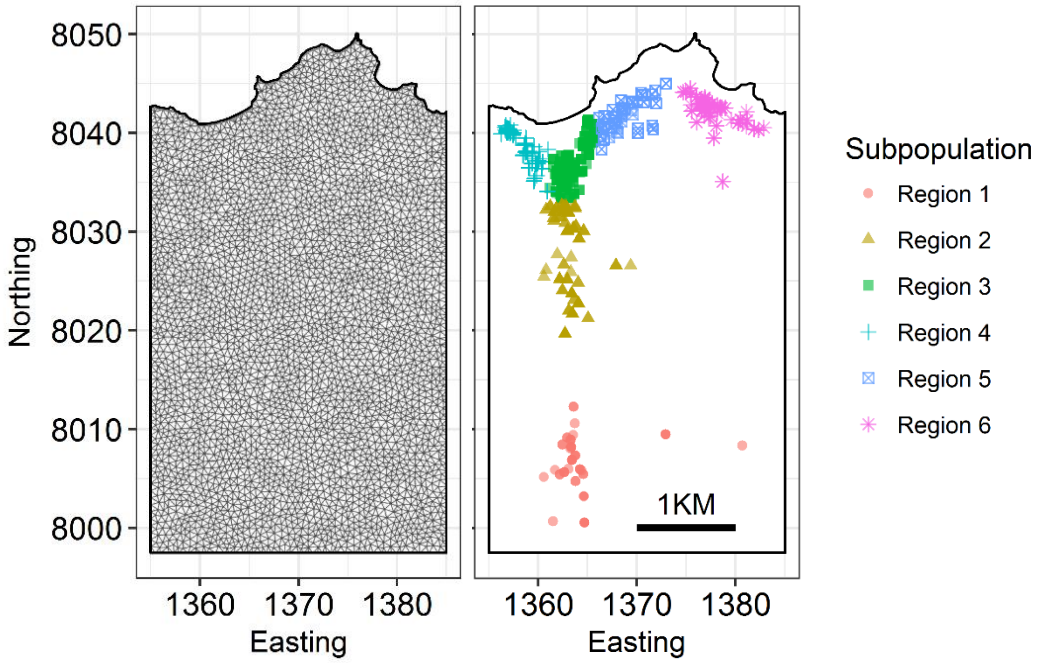


Figure 1: Map of the Isle of Rum red deer study area, depicting the mesh used for the INLA SPDE random effect (left) and the sampling locations and their subpopulations (right). Easting and Northing are in units of 100m grid squares, so that 10 units corresponds to 1km, per the scale bar. The river runs south-north along the 1363 Easting, flanked by hills. Subpopulations are organised and named running from south to north, then west to east.

Statistical analysis

Statistical analysis was carried out using the Integrated Nested Laplace Approximation (INLA). INLA is a deterministic Bayesian approach which is increasingly being used for analysis of spatial data in ecology (Zuur *et al.*, 2017).

Models were fitted in R version 3.5 (R Core Team, 2018) using the linear modelling package R-INLA (Rue and Martino, 2009; Martins *et al.*, 2013). We constructed five generalised linear mixed models (GLMMs) for each response variable, each featuring different combinations of fixed and spatial random effects. The distinguishing components of these model sets are outlined below and displayed in Table 1.

Our five response variables included integer counts per gram of three parasite propagules following a negative binomial distribution (strongyles, *F. hepatica* and *E. cervi*) and Gaussian-distributed optical densities of two mucosal antibodies (total IgA and anti-Tc IgA). Antibody levels were corrected for collection effects as previously described, by taking the residuals from a linear model including raw antibody OD as a response variable and including day of collection, time of collection and extraction session as explanatory variables (Albery *et al.*, 2019b). In our main GLMMs, explanatory variables included: Deer year (categorical with three levels: 2015, 2016, and 2017); Season (categorical with three levels: Summer, Autumn, and Spring); Age (continuous, in years); Reproductive status (categorical variable with three levels: No Calf, Calf Died, and Calf Survived; see Albery, Watt, *et al.* (2018) for definitions); Subpopulation (categorical, six levels). All models included individual ID as a random effect.

INLA allows incorporation of a spatially distributed random effect to account for spatial autocorrelation (Lindgren *et al.*, 2011). This uses a stochastic partial differentiation equation (SPDE) approach to approximate the continuous random field using a triangulated mesh of connected discrete locations (Lindgren and Rue, 2015). The mesh we used for the spatial random effect is displayed in Figure 1. The random effect can be plotted in 2D (giving the "spatial field" of variation) to investigate hot- and coldspots of the response variable, and the kappa/range

parameters can be extracted to investigate the distance at which autocorrelation fades in space. It is also possible to allow multiple spatial fields within a single model, by assigning separate fields to different categories or by linking fields with correlation structures to investigate spatiotemporal variation. The underlying mathematics of INLA and associated spatial/spatiotemporal models have been extensively discussed elsewhere, and such models are increasingly being used to examine spatiotemporal trends (e.g. fisheries ecology; Cosandey-Godin *et al.*, 2015); see http://www.r-inla.org for more examples.

We constructed one set of competing models for each response variable. Each model set contained five models, resulting in 25 models total. Our base model set (model set 1) included year, season, age, and reproductive status as fixed effects, similar to models previously used to investigate associations between reproduction, immunity, and parasitism (Albery *et al.*, 2019b). Model set 2 added subpopulation as a fixed effect to investigate whether this explained any variation and to examine the value of analysing continuous populations using discrete subdivisions (Figure 1). Model set 3 added a spatially distributed SPDE random effect, rather than the subpopulation fixed effect, to control for and quantify spatial autocorrelation. In model set 4, this spatial field was allowed to vary between seasons (summer, autumn, and spring), and model set 5 allowed correlation between these seasonal fields. To allow spatial fields to correlate, we used an "exchangeable" model, where all fields in the model were correlated by the same value (ρ) rather than e.g. following an autoregressive process through time. We elected not to fit different spatial fields across years as our number of replicates was small for detecting annual variation. We also had no *a priori* hypotheses concerning spatial differences between years; splitting up the spatial field into individual sampling trips (field:season:year) would cut down the sample size considerably for each field, reducing the likelihood of picking up spatial patterns; and

we have only one season (Spring) from the first year of collection, so the years are unlikely to be comparable.

For each response variable, the five fitted models were compared using the Deviance Information Criterion (DIC). A change in 2 DIC was selected to distinguish between models and select the most parsimonious model. When the best-fitting models included spatial autocorrelation, we extracted the range parameters to estimate the range of autocorrelation and ρ parameters to estimate correlation between seasonal fields. For the range of autocorrelation, we report the distance at which spatial autocorrelation decayed to 0.5 (henceforth "halving range"; Brooker *et al.*, 2006). Finally, we compared effect sizes from each model to investigate whether incorporating spatial autocorrelation altered any conclusions about the fixed effects. We particularly focussed on whether accounting for spatial autocorrelation altered the estimates for reproductive status effects, which have previously been demonstrated to impact both immunity and parasitism, and vary spatially across the population.

Model Set	Description	Str	Fh	Ec	TotA	TcA
		ΔDIC				
1	Base set	130.13	8.27	**0**	39.27	22.81
2	+ Subpopulation fixed effect	132.13	6.49	3.78	37.8	26.78
3	+ Spatial field random effect	102.42	4.25	**1.36**	32.33	19.37
4	+ Field varying seasonally	**0**	2.29	6.11	**0**	2.02
5	+ correlation between fields	4.08	**0**	2.26	**1.08**	**0**
		Autocorrelation halving ranges (metres)				
3	Static spatial field	59.62	1323.58		150.06	92.58
4-5	Seasonally varying spatial field	32.58	1124.74		640.07	415.82
		ρ (correlation between seasonal fields)				
5	Seasonally varying spatial field	0.09 (-0.24,0.45)	0.67 (-0.02,0.98)		-0.1 (-0.44,0.48)	-0.31 (-0.5,0.3)
		Sample Size				
1-5	Number of samples	834	823	832	799	795
1-5	Number of individuals	139	139	138	137	137

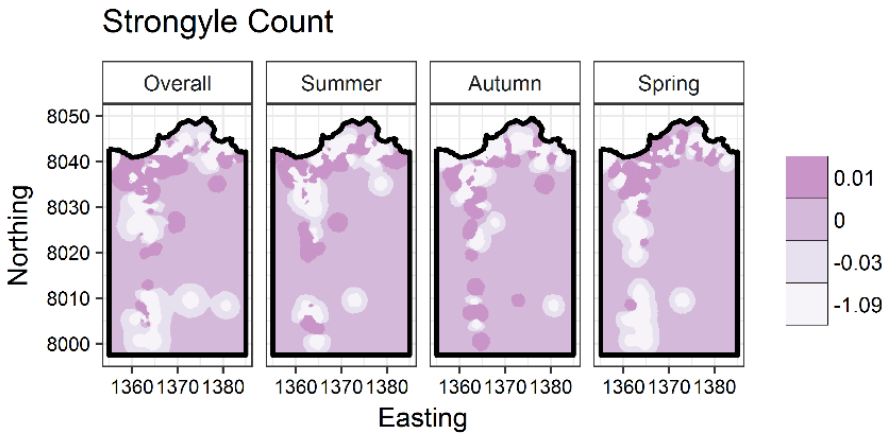
Table 1: Model set descriptions and extracted values (ΔDIC, halving range, ρ and sample size) for each response variable (Str = strongyles; Fh = *F. hepatica*; Ec = *E. cervi*; TotA = Total IgA; TcA = anti-Tc IgA). The model with the lowest DIC for each response variable (ΔDIC=0) is highlighted in bold and underlined; where the best-fitting models are not distinguishable (ΔDIC<2), both are highlighted and underlined. The halving range of each variable represents the distance in metres at which spatial autocorrelation reduces to 0.5, either for model set 3 or for the best-fitting seasonally varying model (from model set 5, or model set 4 for total IgA). ρ value estimates are given with their 0.025 and 0.975 quantiles in brackets.

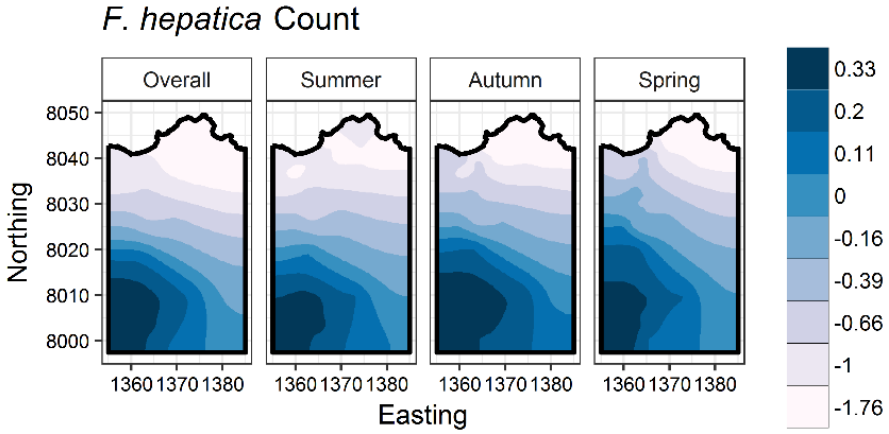
Results

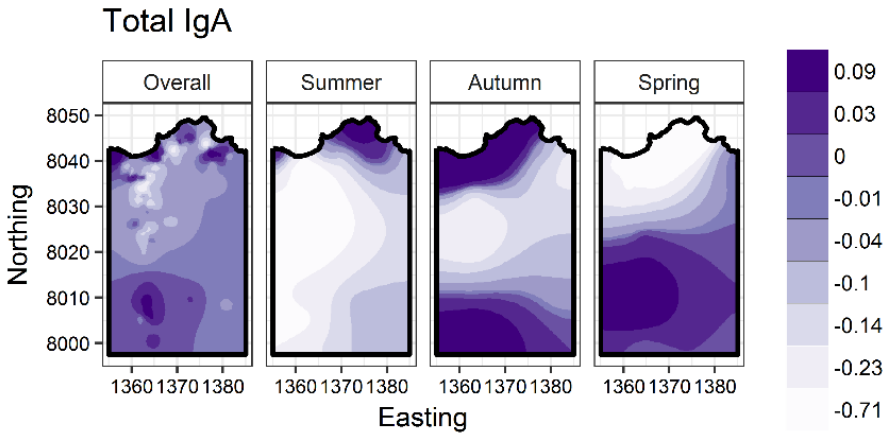
Our models revealed strong and contrasting spatial trends in all but one of our response variables. All models but *E. cervi* were incrementally improved by first incorporating a spatial random effect and then by allowing it to vary between seasons (DIC values in Table 1; all secondary models had ΔDIC≥3.44). In all cases, including spatially distributed random effects improved model fit compared to fitting a subpopulation fixed effect (Table 1; ΔDIC≥2.4). The spatial fields of the random

effects, taken from model sets 3-5, are displayed in Figure 2. For each response variable, we report the spatial field and results from both model set 3 (spatial field constant across the study period) and model set 4 (spatial seasons varying seasonally, with no correlation between fields). *F. hepatica* and anti-Tc IgA are exceptions, for which allowing the seasonal fields to correlate in model set 5 slightly improved model fit (ΔDIC>2.02, Table 1); therefore, for both *F. hepatica* and anti-Tc IgA, we display the fields and results from model sets 3 and 5. Response variables differed considerably in terms of both their spatial fields (Figure 2) and the range at which they varied (Figure 3). Table 1 also displays the distance at which spatial autocorrelation reduced to 0.5 ("halving ranges") and ρ values; as *E. cervi* models were never improved by the inclusion of the subpopulation fixed effect or by SPDE random effects (ΔDIC>1.36), we do not report these results further.

Strongyle nematode count exhibited weak spatial patterns, with a very short range of autocorrelation; this did not increase when spatial fields were allowed to vary seasonally (Figure 2-3, halving range<59.62M). Allowing the spatial field to vary between seasons resulted in similar patchy distributions which are hard to distinguish (Figure 2) but nevertheless improved model fit compared to all other models (Table 1, ΔDIC=4.25). *F. hepatica* demonstrated a strong spatial pattern, with high intensities in the mid- and south-valley decreasing to the north and northeast (Figure 2). This gradual, unidirectional trend was reflected in the long range of autocorrelation (Figure 3, halving range=1323M). Allowing the spatial field to vary between seasons improved *F. hepatica* model fit, but resulted in similar seasonal fields (Figure 2). This was reflected in the positive ρ parameter (ρ=0.67) derived from model 5, which was the best-fitting model for *F. hepatica*, demonstrating that seasonal spatial fields were substantially positively correlated.


Strongyle Count
Overall
Summer
Autumn
Spring
Northing
8050
8040
8030
8020
8010
8000
1360 1370 1380
Easting
0.01
0
-0.03
-1.09


F. hepatica Count
Overall
Summer
Autumn
Spring
Northing
8050
8040
8030
8020
8010
8000
1360 1370 1380
Easting
0.33
0.2
0.11
0
-0.16
-0.39
-0.66
-1
-1.76


Total IgA
Overall
Summer
Autumn
Spring
Northing
8050
8040
8030
8020
8010
8000
1360 1370 1380
Easting
0.09
0.03
0
-0.01
-0.04
-0.1
-0.14
-0.23
-0.71

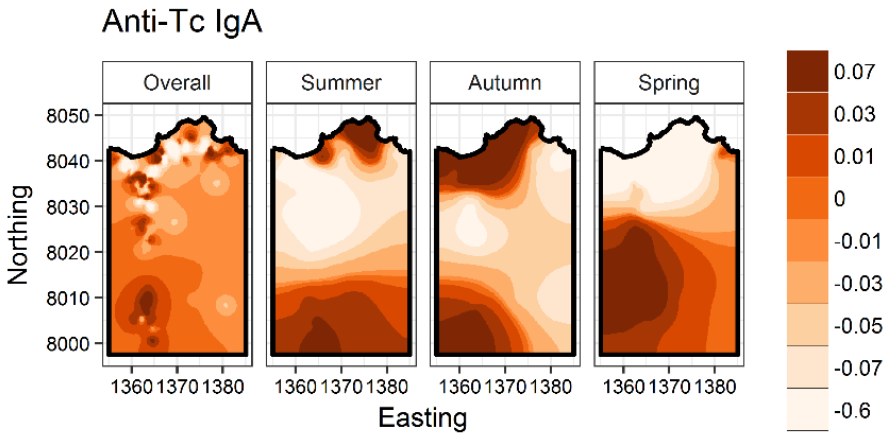

Anti-Tc IgA
Overall
Summer
Autumn
Spring
Northing
8050
8040
8030
8020
8010
8000
1360 1370 1380
Easting
0.07
0.03
0.01
0
-0.01
-0.03
-0.05
-0.07
-0.6

Figure 2 (previous page): Projections of the spatially distributed SPDE random effect (spatial fields) for the models that were improved by its inclusion (one row for each variable, from top row to bottom: strongyles; *F. hepatica*; total IgA; anti-Tc IgA). Spatial fields were taken from model sets 3 (constant spatial field, far left column) and 4-5 (spatial fields varying seasonally, remaining three columns). Colours denote the lower bounds of 9 quantiles of the spatial effects on the link scale, rounded to 2 decimal places, with darker colours representing higher parasite counts (rows 1-2) or antibody levels (rows 3-4). Where there are fewer than 9 colours, this is because rounding the values to 2 decimal places created identical quantile values, and demonstrates that spatial autocorrelation accounted for a smaller proportion of the variation. Easting and Northing are in units of 100m grid squares, so that 10 units corresponds to 1km. the river at the base of the valley runs along the 1363 Easting.

When the spatial field was kept constant across the study period, total IgA and anti-Tc IgA both demonstrated a very short range of spatial autocorrelation (Figure 3, Table 1; halving range<150.06M). Both antibody distributions were similar and negatively correlated with that of strongyles, being lower in the central north and higher in the south and edges of the study area (Figure 2). However, allowing both antibodies' spatial fields to vary between seasons improved model fit substantially compared to all other models (Table 1, ΔDIC<18.58), increased the range of autocorrelation (Figure 3, halving range>415.82M), and resulted in very different seasonal patterns (Figure 2). These patterns were similar between total IgA and anti-Tc IgA, although total IgA had a slightly larger range of autocorrelation (Figure 3, halving range=640.07M and 415.82M for total IgA and anti-Tc IgA respectively). The best-fitting model for total IgA and anti-Tc IgA was either model 4 or 5 for total IgA (Table 1, ΔDIC<2), while model 5 fit slightly better for anti-Tc IgA (ΔDIC=2.02). Hence model 4 is presented for total IgA as the model with fewer degrees of freedom, and model 5 is presented for anti-Tc IgA.

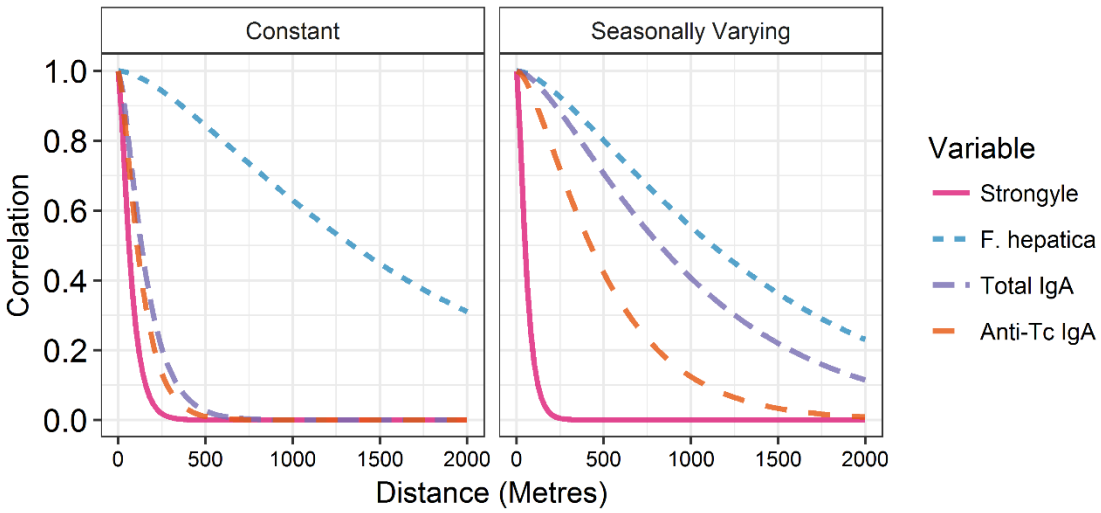


Figure 3: the range of spatial autocorrelation acting on each response variable, in metres, for INLA models with constant spatial fields (model set 3, left) and those with spatial fields that varied between seasons (model sets 4-5, right). In the right panel, values were taken from the best-fitting seasonally varying model: model 4 for total IgA, and model 5 for the remaining response variables (Table 1). See Table 1 for the halving ranges for each variable when the field was kept constant or allowed to vary seasonally.

The subpopulation fixed effects broadly followed the spatial fields of the SPDE random effects (Appendix C, Figure SI1). Briefly, strongyles showed little difference across different regions, although estimates for the two northern regions (regions 3 and 5) did not overlap with zero when compared to the southern region 1. For *F. hepatica* intensities decreased moving northeast from region 1 to region 6, and all regions exhibited significantly decreased levels below the far south region 1. The reverse was true for *E. cervi* intensities. Patterns for total IgA and anti-Tc IgA are harder to interpret and weaker, but broadly the far south region 1 subpopulation featured higher antibody levels than northern regions (regions 2, 3, and 5 for total IgA and region 5 for anti-Tc IgA).

Most fixed effect estimates were only slightly modified by incorporating spatial autocorrelation structures in our models (Appendix C, Figure SI1). No estimates were reduced in significance except the seasonal effects in models 4 and 5 for *F. hepatica* and *E. cervi* (Appendix C, Figure SI1). Examining the spatial fields (Figure 2), this

reduction in seasonal effect probably originated from competition between the seasonally varying spatial random effect and the season variable itself. Otherwise, effect estimates remained unchanged when spatial autocorrelation was included. This was particularly true for reproductive status effects, many of which actually increased slightly in magnitude when we accounted for spatial autocorrelation (Appendix C, Figure SI1). The models therefore replicated our previous study by demonstrating that reproductive investment was associated with lower antibody levels and higher strongyle intensities (Albery *et al.*, 2019b).

Discussion

This study has revealed fine-scale spatial variation in immunity and parasitism at an individual level in a large wild mammal population. Spatial heterogeneity contributed considerably to between-individual differences in immunity and parasitism despite a total sampling area of only ~12.7 km^2. The scale of spatial dependence was therefore extremely short, and well within the scale of the study area. These findings are in accordance with a previous study demonstrating fine-scale immune variation in a discrete spatial context (within-site versus between-site) in wild mice (Abolins *et al.*, 2018). We demonstrate similar spatial variation in a continuous context, and in both antibody levels and parasite counts, despite considerable mixing within the population. Furthermore, the response variables differed in terms of their spatial fields, the distances at which autocorrelation decayed in space, and their interactions with seasonality. Finally, spatial distributions of antibodies were not similar to any parasite distributions, implying that fine-scale environmental factors acting on exposure are more important than host immune susceptibility in driving spatial heterogeneity of parasite infection.

The scale of dependence and its importance for disease ecology studies

Understanding the spatiotemporal scale of disease processes is important for designing sampling regimes and disease control strategies (Caprarelli and Fletcher, 2014; Lachish and Murray, 2018). In this context, our results have several important general implications. Firstly, fine-scale trends like those exhibited here may scale up quickly where environments vary across larger distances, contributing to larger-scale geographic patterns of disease that are more commonly studied (Ostfeld *et al.*, 2005; Murdock *et al.*, 2017; Murray *et al.*, 2018). Second, disease ecology and ecoimmunology studies that do not consider spatial autocorrelation, even over short distances, may be missing important sources of variation in immunity and exposure and risk reporting biased effect estimates. The persistent spatial trend seen in *F. hepatica* (Figure 2) demonstrates that different areas of a given study system can be consistently associated with either higher or lower parasitism, so that uneven sampling in space could introduce confounding variation and bias. In contrast, where the range of autocorrelation is extremely short, as in strongyles, sampling regimes that do not consider spatial dependence may incidentally sample areas of both high and low parasitism, reducing the risk of spatial biasing. The range of autocorrelation was well within the range of an individual deer's home range (Froy *et al.*, 2018), implying that individuals may experience considerable variation in parasitism depending on their movement choices within this range. Trends are not necessarily similar across variables, complicating matters: most notably, spatial gradients of *F. hepatica* and strongyle count differed considerably both in range and patterns, and antibody hotspots did not align with parasite hotspots (Figure 2). Therefore, information on the spatial distribution of one immune or parasite measure could not be used to infer the distribution of another, and appropriate sampling regimes will

differ between response variables. Finally, all models except *E. cervi* were further improved when the spatial field was allowed to vary seasonally, and spatial patterns of antibody levels changed considerably between seasons (Figure 2). This confirmed our expectations that spatial fields would not be static in time (Hawkins, 2012). Therefore, in some cases, even sampling from a wide, contiguous area may only capture a cross-sectional snapshot of the spatial dynamics of a given study system, necessitating longitudinal analysis.

Spatial heterogeneity has the potential to obscure or produce artefactual associations with other variables, modifying conclusions drawn from models without spatial dependence structures – in particular by inflating the type I error rate (Beale *et al.*, 2010; Pawley and McArdle, 2018). However, in this study, fixed effects remained largely unchanged when incorporating spatial dependence structures despite the importance of spatial heterogeneity (Appendix C, Figure SI1). In particular, previously reported reproductive status effects (Albery *et al.*, 2019b) persisted or increased slightly in size, despite the fact that reproductive success varies across the study area (McLoughlin *et al.*, 2006; Stopher *et al.*, 2012b). This demonstrates that spatial variation can contribute to ecological patterns of disease without necessarily obscuring other findings (Pawley and McArdle, 2018). We suggest that disease ecology studies that examine wild populations attempt to investigate spatial variation to enrich their results, rather than viewing spatial autocorrelation as a nuisance (Pawley and McArdle, 2018). In addition, although the spatial fields were broadly reflected by the subpopulation fixed effect results (Appendix C, Figure SI1), the spatial fields were more easily interpretable and increased model fit, and therefore incorporating spatial autocorrelation was advantageous. While integrating spatial dependence did not have severe impacts on effect sizes in our study, we lastly

encourage researchers to consider accounting for spatial dependence even at the fine scales here to improve statistical inference and account for this variation.

Interpreting the spatial fields

The spatial fields derived from our models can help to indicate the factors determining immunity and parasite infection. Spatial trends of *F. hepatica* were especially stark, being much higher in the south of the study area and decreasing to the north and northeast (Figure 2). Given that the parasite distributions were not explained through differences in immune susceptibility, particularly considering minimal overlap with antibody level distributions (Figure 2), spatial patterns in parasite count likely instead resulted from spatial variation in exposure. This heterogeneity likely originated from the drier environment in the north compared to the wet, marshy ground in the south of the valley, the latter of which could be conducive to parasite persistence in the environment. After being excreted, *F. hepatica* eggs develop to form infectious miracidia, which seek out and infect *Galba truncatula* water snails (Taylor *et al.*, 2016b; Beesley *et al.*, 2018). After a period within the snail, cercariae are produced which encyst on vegetation as metacercariae to be consumed by deer. Wet areas are likely to host higher *G. truncatula* abundance, and warmer, wetter environments are conducive to fluke development and host seeking behaviour, both of which will produce higher exposure (Ollerenshaw and Smith, 1969). The observed fluke distribution agrees with a number of studies in livestock demonstrating high fluke risk where grazing and wet areas intersect (e.g. Olsen *et al.*, 2015). Similar relationships with water sources are displayed by the human trematode *Schistosoma mansoni*, which shows a similar range of autocorrelation (Brooker *et al.*, 2006). Our corroboration of these findings in a wild mammal implies that similar environmental risk factors may be influencing trematode infection in wild animals, humans, and livestock.

In contrast to *F. hepatica*, the spatial field of strongyle count is difficult to interpret: spatial autocorrelation introduced important variation, yet the range of autocorrelation was small, similar to that reported for human hookworm infection (Brooker *et al.*, 2006), and displayed no discernible pattern either across the study period nor within seasons (Figure 2). Strongyles may be less impacted by environmental factors than is *F. hepatica* due to their direct life cycle, which does not involve a secondary host, such that spatial autocorrelation in intrinsic factors affecting susceptibility is more important than environmental effects on exposure and transmission. Host genetic similarity is a possible intrinsic factor producing the spatial autocorrelation seen in both strongyle counts and antibody levels: both are heritable in ungulates (Bisset *et al.*, 1992; Callaby *et al.*, 2014; Hayward *et al.*, 2014b), and genetic relatedness is correlated with spatial distance in this system (Stopher *et al.*, 2012b). Alternatively, as social behaviours commonly covary with spatial behaviour (e.g. Sanchez and Hudgens 2015), the spatial patterns established here may be partially explicable through social metrics such as conspecific density. Future studies in this population could examine whether local population density and/or other social factors affect individuals' immunity and parasitism in ways that the INLA SPDE effect was unable to detect, potentially by using individual-level behavioural metrics derived from census data (Coulson *et al.*, 1997; Froy *et al.*, 2018).

Ecological and epidemiological implications

The fine-scale spatial heterogeneity demonstrated here has implications for the ecology and control of infectious disease in wild ungulate populations. For example, localised transmission hotspots may maintain parasite diversity, preventing competitive exclusion of parasites through geographic niche differentiation and contributing to the considerable genetic differentiation seen in liver fluke populations (Beesley *et al.*, 2016). Additionally, when combined with sex-specific deer ranging

patterns, spatial trends could contribute to previously observed sex biases of infection (Albery *et al.*, 2018). Finally, it is possible that the strong seasonality in ranging behaviour (Stopher *et al.*, 2012b) interacts with seasonal patterns of parasitism and immunity (Albery *et al.*, 2018). With more data, future investigations in this system may be able to examine these associations.

As *F. hepatica* is an important livestock parasite, fluke control initiatives should consider the presence of high-risk wet areas of grazing that may be used by deer populations. However, it is worth noting that the fluke hotspots here were observed at the per-capita count level, rather than as an absolute number of parasites in the environment. Given the higher deer density in the north, taking *F. hepatica* as an example, it is likely that the absolute number of fluke eggs being excreted in the north is higher than the south, but these parasites are less likely to complete their life cycle due to unsuitable environmental conditions. In the future, it may be possible to compare the excretion and movement patterns of the deer with pasture larval counts and snail sampling across the study area to examine the rate at which successful infection occurs, and to investigate whether deer living in the high-risk southern area of the valley may indeed be vectoring *F. hepatica* to the north (Chintoan-Uta *et al.*, 2014; French *et al.*, 2016; Qviller *et al.*, 2016).

Chapter VI: General Discussion

Part 1: Thesis overview

The overarching aim of this thesis was to examine the parasite fauna of the Isle of Rum red deer and its correlates with individual life history, behaviour, and fitness. In particular, I leveraged the statistical power of individual-based sampling to demonstrate that life history variation, seasonal fluctuations, and spatial patterns interact to determine immunity and parasitism.

In chapter II, I uncovered substantial seasonal fluctuations in helminth infection which were different for different helminth species, but with generally lower intensities in the colder months. Helminth taxa differed in their age biases, although older individuals generally had lower intensities beyond prepatent periods at the beginning of a calf's life. In addition, helminths differed in their sex biases, with males hosting higher strongyle intensities and females hosting higher *Elaphostrongylus cervi* intensities. Helminth count was highly repeatable, yet strongyles exhibited a strong individual-by-sampling trip interaction effect, demonstrating that injudicious sampling across the year could give a false impression of trends in parasitism.

In chapter III, I examined adult females' reproductive allocation, revealing that gestation was associated with lower mucosal IgA levels but did not affect parasite counts, while lactation was associated with increased parasitism. This pattern implied that this reproduction-immunity tradeoff was partly determined by hormones or mechanistic constraints, while parasitism increased as a result of lactation-associated increased exposure. In addition, mucosal antibodies correlated negatively with strongyle counts, suggesting they are indicative protective immunity.

I investigated the fitness consequences of these reproduction-immunity-parasitism tradeoffs in chapter IV. The lactation-associated increase in strongyle count had

substantial fitness costs, above and beyond any direct costs of lactation itself, providing a potential mechanism linking present and future fitness. In addition, anti-*Teladorsagia circumcincta* antibodies were positively correlated with probability of reproduction the following year, demonstrating a benefit of immunity that did not act through reduced strongyle count.

Finally, in chapter V, analysis of antibody measures and parasite counts alongside behavioural census data revealed strong, contrasting, seasonally varying spatial clines in immunity and parasitism in the population. Despite strong importance of spatial autocorrelation for both IgA isotypes and two parasites, *Fasciola hepatica*'s strong south-north gradient was the only immediately identifiable spatial trend. Spatial variation did not obscure the reproductive costs examined in chapter III, and hotspots differed considerably between antibodies and parasites.

In this chapter, I will discuss the implications of the work in this thesis for disease ecology/ecoimmunology studies, and for the Isle of Rum red deer system itself. I extol the virtues of longitudinal, observational studies of wild animals, and the viability of noninvasive sample collection methods. I will discuss the limitations of the methods used in this thesis and some workarounds, and I will finish by detailing some potential future directions for the parasitological and immunological wing of the Isle of Rum red deer study system.

Part 2: The value of longitudinal, observational work

Ungulates are highly promising but underused subjects for disease ecology studies, and red deer have specifically been highlighted as a potential model species (Jolles and Ezenwa, 2015). This assertion is supported by the findings in this thesis, which demonstrated a large number of well-known or commonly-theorised effects in ecoimmunology and disease ecology. Most notably: Immunity and parasitism varied

spatially; all three parasite taxa that I analysed demonstrated seasonal fluctuations; there were strong age and sex biases in parasitism, and reproductive status was associated with both decreased immunity and increased parasitism. The confirmation of these patterns is promising for the future of ecoimmunological and parasitological investigations in the study system: their establishment in the population will allow a wide range of further, more detailed analyses, some of which are detailed below.

In particular, the availability of multiple parasite species which differ in the presence (and occasionally direction) of effects will allow considerably increased strength of inference in the red deer. For example, although both *Fasciola hepatica* and *Elaphostrongylus cervi* are transmitted by snails, only *F. hepatica* showed spatial dependence. Large sample sizes and the high prevalence of *E. cervi* suggest this is not likely due to lower power; instead, it may be due to *E. cervi*'s low pathogenicity in Scottish red deer (Mason, 1994), encouraging deer to tolerate infection and reducing the incentive to avoid them in space; more widespread distributions of their land snail vectors; or higher importance of frequency of rare exposures for *F. hepatica*. The ability to contrast the species in this way informs the processes driving infection with each, and strengthens our faith in the statistical methods used. This supports the intuitive idea that studying multiple parasites can strongly benefit disease ecology studies.

Chapter IV demonstrated a substantial fitness cost of parasites which is surprisingly rarely documented in wild mammals. In particular, I identified a survival cost which can be especially hard to identify when the study organism is long-lived and has a relatively slow pace of life (Hamel *et al.*, 2010). Demonstrating that parasites are costly (or at least that they reflect an organism's health, given the observational methods)

opens up a set of potential research avenues that will contribute to the red deer as an interesting study system in the future.

In addition to the expected effects of intrinsic and extrinsic factors detailed above, we identified multiple interactions among these factors in influencing immunity and parasitism. For example, individuals of different sex and age classes exhibited different seasonal patterns of strongyle count in chapter II; different reproductive classes exhibited different seasonal patterns of antibody levels and helminth count in chapter III; and spatial distributions of immunity and parasitism varied seasonally in chapter V. Most notably, different individuals exhibited different seasonal patterns of strongyle count in chapter II. As a general take home message, given large enough sample sizes and a stable enough study system, it is possible to detect complex interactions among individual-level and spatiotemporal factors. I hope that future individual-based studies will make use of similar tools (INLA, zero-inflated models, and complex variance components) to investigate these interactions. Analyses identifying reaction norms of disease processes across spatiotemporal environments and life history backgrounds will further understanding of immunity and parasitism as context-dependent processes, rather than e.g. expecting the same fitness optima for immune expression in most situations (as critiqued in Viney *et al.*, 2005; Graham *et al.*, 2011). Longitudinal analyses of wild animals are likely to be particularly well-suited to such questions.

The work in this thesis is also notable for its purely observational nature. Experimental approaches are often preferred when examining certain disease processes such as coinfection (Fenton *et al.*, 2014; Pedersen and Fenton, 2015), and findings in the wild can be obscured by variation in exposure, encouraging researchers to replicate findings in the lab where possible (Graham *et al.*, 2011). Yet,

using no experimental manipulation, and relying entirely on natural variation in disease processes, I have revealed much about red deer antibodies, their helminth parasites, and their relationships with life history and behaviour. This success was likely due to several factors: first, the simultaneous measurement of both immunity and parasitism, which allowed stronger inference about the driving processes; second, the high accuracy of the life history and behavioural measures examined; and third, the large sample sizes taken from a relatively stable population. These latter two factors substantially reduced measurement error and increased the power of our analyses as a result.

Part 3: Methodological successes, challenges and implications

Notably, this thesis made use of noninvasive faecal samples for all immunological and parasitological data. Besides the advantages of longitudinal, observational methods described above, there were several principle merits of this approach. Here I will detail these merits, along with potential improvements that could be incorporated, and the inevitable limitations of this approach that are unlikely to be circumvented.

Noninvasive faecal egg counts (FECs) are a relatively low-tech method of ascertaining helminth prevalence and intensity which are commonly scrutinised for their potentially low reliability and utility (Sargison, 2013; Budischak *et al.*, 2015). Despite being low-tech, the highly sensitive centrifugation method used here proved repeatable and informative. The ability to demonstrate so many patterns in a relatively short time frame was likely the result of high sensitivity and reliability compared to other methods – for example, the method used for the Soay sheep of St Kilda, which has historically been accurate to 50 eggs per gram compared to our 1 egg per gram (Wilson *et al.*, 2004). Beginning with a thorough investigation into the

repeatability of the individual-level assays in chapter II lent credence to the validity of the methods, and I recommend that more pilot studies should carry out similar verification steps. The ability to verify a method's repeatability in this way adds to the benefits of longitudinal sampling for ecoimmunological studies, e.g. by increasing sensitivity and revealing hidden phenotypes (Lamberton *et al.*, 2014; Buzdugan *et al.*, 2017; Plowright *et al.*, 2017).

Chapter III describes one of the first applications of a veterinary faecal antibody test to a wild animal. This assay was successful in detecting multiple associations of antibody levels with life history, behavioural, and fitness traits, despite the fact the assay was developed in sheep (Watt *et al.*, 2016). Our ability to co-opt this veterinary tool for use in a related species with minimal effort is a highly promising achievement from the perspective of ecoimmunologists on the lookout for tools in wild, non-model systems (Garnier and Graham, 2014). I hope that more studies will make use of veterinary tools, of antibody levels as measures of resistance to common parasites rather than exposure to novel ones, and more specifically of faecal antibody levels in wild, non-model ungulate systems (Jolles and Ezenwa, 2015).

Notably, despite the use of an antibody assay that was developed in sheep, the antibody-FEC correlations we report in Chapter III were in fact stronger and more statistically significant than their equivalents in sheep themselves (Watt *et al.*, 2016). In addition, the correlation was considerably stronger than any correlation between plasma antibodies and FEC in the same study (Watt *et al.*, 2016). A possible reason for this high correlation is the high sensitivity of the FEC method detailed above. An additional explanation for the significant correlation is the site of quantification: we measured the immune response against gut helminths in the gut, where they were most likely to be taking effect (Smith *et al.*, 1987; Barrell, 1997). Discordance in the

site of sampling can create serious problems for the interpretation of life history-immunity-parasitism relationships in animals. For example, in farmed sheep, it is thought that the movement of IgA from the gut to the blood in preparation for transfer to offspring in the milk is an important component of the periparturient rise in faecal egg count (Jeffcoate *et al.*, 1992). In this situation, it is possible that measuring IgA in the blood over the lambing period would in fact detect an increase in IgA with a concurrent increase in gut helminth count, producing a highly misleading impression of the mechanisms involved (Smith *et al.*, 1987; Jeffcoate *et al.*, 1992). Similar patterns could be seen in the deer. As many ecoimmunological and disease ecology studies use plasma samples for convenience, this has important implications for the design of studies looking to optimise congruence and relevance of their immune and parasite measures. Ideally, and intuitively, the two should be taken from the same place, from the same time, and from the main site of infection, where possible.

High-tech improvements

Because the analyses in this thesis relied on relatively low-tech methods, they produced relatively limited parasitological information compared to more sophisticated methods that are currently in the parasitologist's toolbox (Taylor *et al.*, 2016c). Most notably, the strongyle counts in this thesis were conducted at the order level as the easiest and fastest method of summarising strongyle parasitism given the morphological similarity of many strongyle eggs (Sargison, 2013; Budischak *et al.*, 2015; Taylor *et al.*, 2016c). Coprocultured larvae can be identified to species level, but this is time-consuming and requires expert knowledge and a predetermined guide to the species present and their distinguishing features (Hoberg *et al.*, 2001; Budischak *et al.*, 2015; Taylor *et al.*, 2016b). As a result, strongyle egg counts are used as a practical alternative. However, lumping together many different species in this way risks losing

considerable amounts of information (Budischak *et al.*, 2015). Different strongyle species have different life cycles and pathologies, with potentially important fitness implications: for example, the sheep strongyle *Haemonchus contortus* is a prolific bloodfeeder, and as a result can have severe effects on sheep lifespan and productivity (Besier *et al.*, 2016). Including counts of *H. contortus* among less pathological species risks obscuring their fitness effects, as infection with very few *H. contortus* worms may be worse for the health of a given individual than many specimens of a more benign species (e.g. *Cooperia*; Budischak *et al.*, 2018). *H. contortus* has not been identified in red deer, but the strongyle species present likely exhibit a similar degree of variation in pathogenicity (Hoberg *et al.*, 2001). This limitation could be overcome using high-resolution genetic differentiation of strongyle species, either by sequencing (a subset of) worm larvae (Budischak *et al.*, 2015), or by using abundance-corrected metabarcoding approaches (Avramenko *et al.*, 2015). This approach would provide more detail on the parasite community, allowing the potential investigation of coinfection interactions and within-host community-level processes.

For example, a recent study in rabbits (*Orcytolagus cuniculus*) demonstrated complex asymmetrical interactions between blood-feeding *Haemonchus contortus* nematodes and less pathogenic *Trichostrongylus colubriformis* nematodes (Lello *et al.*, 2018b). In this system, *H. contortus* infection altered the host environment, reducing jejunal immune responses such that *T. colubriformis* was able to maintain higher numbers; in contrast, *T. colubriformis* had a negative effect on *H. contortus* infection, with fewer arrested larvae in the coinfection treatment. Intriguingly, this effect reflected the mechanisms seen in sheep, and implies that strongyle species interactions and phylogenetic specificities may be conserved across host species. This example both offers a specific hypothesis that could be tested in the red deer system, and serves to illustrate the potential complexities of parasite interactions and the downsides of

order-level lumping. However, investigating coinfection interactions generally requires experimental treatments (Fenton *et al.*, 2014), and it is likely that even species-level resolution of parasite counts in this system would be insufficient for these sorts of inferences. While this system is well-suited to detection of life history costs, spatiotemporal variation, and fitness consequences, it is by no means suited to all questions.

Additional immune and parasite measures

Further data collection will also allow statistical analysis of more parasite taxa. The principle helminths analysed in this thesis (strongyles, *F. hepatica* and *E. cervi*) are only a subset of the parasite biota that can infect red deer in Europe (Bohm *et al.*, 2007). These parasites were selected because of their relatively high (>30%) prevalence on Rum, enabling meaningful statistical analysis. Low-prevalence infections can be hard to analyse statistically due to the abundance of zeroes, even when zero-inflated models are available (Zuur *et al.*, 2009; Chipeta *et al.*, 2014), and the lack of non-zero data makes such analyses particularly uninformative when sample numbers are low. However, it is also not necessarily true that the most prevalent parasites are the most important, and less common infections may in fact be more deadly, warranting eventual investigation into the factors driving infection with these parasites. Given larger sample sizes (which are currently being accrued under further grant funding), the correlates and consequences of infection with *Eimeria*, *Moniezia expansa*, *Capillaria*, and other parasites may become testable in Rum red deer – particularly in young individuals, given the higher prevalence of these taxa in calves and yearlings (Chapter II). In addition, existing frozen faecal samples could yet be analysed for microbiota, for bacterial gut pathogens, or for enteroviruses, to identify whether these very different non-eukaryotic organisms correlate with individual and spatiotemporal variation as do the helminths studied here.

Similarly, I focussed on mucosal IgA as a specific arm of the immune system which I expected to be instrumental in combatting infection with the studied helminths (Smith *et al.*, 1987; Jeffcoate *et al.*, 1992; Watt *et al.*, 2016), yet the mammalian immune system is incredibly complex and multivariate, offering a selection of potentially informative metrics that may be possible to collect in this population. Collecting additional immune measures could be particularly helpful if alternative parasites are quantified: for example, if bacterial pathogens were studied, alternative (non-antibody) immune measures such as cell-mediated immune responses may be best to pair with them to quantify protective immunity. Similarly, if a study investigates coinfection interactions such as those between Th2-stimulating helminths and Th1-stimulating bacteria, it may be helpful to quantify regulatory T cells (Ezenwa and Jolles, 2011). The regulatory arm of the immune system is increasingly appreciated for its important role in mediating immunopathology and the associated costs, and regulatory processes may be particularly relevant to the findings of chapters III and IV given their potentially high resource requirements and interactions with nutrition and fitness (Long and Nanthakumar, 2004). Unfortunately, the impracticality of collecting large numbers of blood samples in this population is an obstacle to obtaining information on many immune components such as cell-mediated immunity or regulatory elements, as these are most easily quantified from white blood cells or serum. Unless proposed immune measures can be taken from faecal samples, it is unlikely that they will be available in this population, and studies going forward will have to plan around this hurdle.

Part 4: Future prospects for parasitological and immunological research in the Rum red deer

Apart from the methodological restrictions described above, two more fundamental limitations of this work were the limited samples and time available for analysis.

Given more samples and/or more time to analyse them, a greatly increased variety of questions will be answerable in the Isle of Rum red deer system, and some of the patterns described in this thesis may be further unpicked. Below, I will discuss a few such questions.

Temporal variation

Chapters II, III, and V were very successful in detecting seasonal trends, partially due to their large, important effects. There was also substantial evidence for inter-annual variation in parasitism and immunity, but I did not have enough annual replicates to properly address whether this inter-annual variation occurred due to extrinsic factors such as density fluctuations or weather conditions. In addition, individual-level repeatability was not overwhelmingly high at the within-sampling-trip level, implying that fluctuations may occur over shorter time periods (i.e., circadially). Circadian infection biology is a growing field, with many exciting developments in recent years (Westwood *et al.*, 2019), and it is possible that more intensive sampling of individual deer would reveal cryptic rhythms that we could not study at the sampling resolution used in this thesis (e.g. Villanúa *et al.*, 2006). Thus, over time, more years of sample collection will allow the detection of both shorter- (hours and days) and longer-scale (inter-annual) temporal patterns of immunity and parasitism.

One potential inter-annual source of variation is population density. Despite the apparent absence of spatial density effects in Chapter V, it is possible that with more data high-density years will be associated with weaker immunity and/or higher parasitism, producing temporal fluctuations in both. Similarly, tradeoffs should be steeper in higher-density areas and years due to increased competition for resources and exposure to parasites (Wilson *et al.*, 2004). Although a previous study revealed no effect of annual density on the severity of life history tradeoffs (Froy *et al.*, 2016),

annual variation in density may in fact alter the slope of reproduction-immunity tradeoffs demonstrated in chapter III.

Fluctuating climatic conditions may provide an alternative source of temporal variation. Weather stations on Rum regularly collect climatic data which have previously provided information on the population's response to climate change (Stopher *et al.*, 2014), and these data will allow testing of the effects of climate on immunity and parasitism. For example, are higher fluke intensities associated with wetter and warmer years that facilitate fluke development (Olsen *et al.*, 2015), or perhaps even with wetter days? Moving windows of climatic variation at various scales can be used to identify the relevant scale at which weather impacts phenotypic traits (e.g. Stopher *et al.*, 2014). It is likely that such an investigation could reveal much about the transmission dynamics of red deer helminths.

Long-term life history associations

More years of data collection, combined with longitudinal data on each individual's life history, will also provide a rich source of information on immunity and parasitism's relationship with growth, development, and life history allocation. I collected >1000 samples from calves, yearlings, and 2-year-olds that were not analysed beyond the variables tested in Chapter II. Do these age classes experience e.g. fitness costs of parasitism that mirror the trends seen in adults in chapter IV? Parasites commonly hit young individuals hardest (Ashby and Bruns, 2018), and helminths have high fitness costs in young individuals of other ungulate species such as Soay sheep (Coltman *et al.*, 1999). Parasitism in early life may influence success in later life, yet these effects are impossible to detect in long-lived animals without extensive longitudinal data for individuals (Clutton-Brock and Sheldon, 2010). Therefore the question of whether early-life parasitism has long-term effects has remained largely

untouched by wild mammal studies, and a recent study failed to provide evidence for this phenomenon in humans (Hayward *et al.*, 2016). In this thesis I was only able to follow one cohort (the 2015 calves) through to maturity, but given larger sample sizes and the high-resolution life history and fitness data available, in future years the influence of annual and individual variation in early-life parasitism on adult development and success will become testable. Similarly, chapter III of this thesis revealed long-lasting (sub-annual) effects of reproduction on immunity and parasitism. As previous work on the deer has demonstrated that reproduction can potentially carry fitness costs multiple years in the future (Moyes *et al.*, 2006), it is likewise possible that these costs will manifest in variation in immunity and parasitism, and/or that immune and parasite phenotypes will correlate with lifelong fitness measures. Finally, relationships between calf traits and maternal traits – particularly in terms of correlations between calf immunity/parasitism and those of the mother – will become testable, potentially allowing identification of more mechanisms by which mothers trade off personal and offspring health.

Social behaviour

One of the foremost advantages of longitudinal studies is their ability to quantify social structuring (Clutton-Brock and Sheldon, 2010), and there are still many open questions concerning the epidemiological implications of social behaviour in wild mammals (Ezenwa *et al.*, 2016b). Centrality in social networks is commonly found to correlate positively with the intensity and prevalence of parasitism, possibly as a result of higher exposure, although this has mainly been demonstrated in primates (MacIntosh *et al.*, 2012; Rimbach *et al.*, 2015; Duboscq *et al.*, 2016). It has been suggested that higher density and contact rates increase exposure to helminths in elk, *Cervus canadensis* (Hines *et al.*, 2007), yet it is becoming increasingly clear that sociality can have both costs and benefits in terms of immunity and parasitism

(Ezenwa *et al.*, 2016b; Ezenwa and Worsley-Tonks, 2018). For example, higher-density populations of *C. canadensis* exhibit stronger rather than weaker immunity (Downs *et al.*, 2015). Studies such as these often study different social groups in different locations. In many wild animal systems, spatial and social behaviours covary strongly (e.g. Sanchez and Hudgens, 2015). This spatial structuring is problematic because as a result, many studies which investigate social correlates of immunity and parasitism must contend with confounding spatial variation like that seen in chapter V. Yet, very few studies have investigated these sources of variation simultaneously. The unravelling of the spatial distributions of immunity and parasitism in chapter V has demonstrated that the census records for red deer are a robust source of behavioural information for disease ecology in this system. The census records include group membership which could be used to identify deer social patterns. Thus, the Rum red deer could be used to investigate the effects of network centrality and local density on immunity, disease, and fitness, revealing whether sociality confers an immunological or parasitological cost in this population, while controlling for spatial variation.

Identifying environmental sources of variation using individual metrics

Finally, further studies could make use of behavioural metrics that have been the subject of previous analyses such as home range size (Froy *et al.*, 2018). Home range size is predicted to be associated with increased exposure, resulting in increased parasite species richness in species with larger home ranges (Bordes *et al.*, 2009). Additionally, home range size is commonly found to covary with ungulates' life history at the individual level (e.g. Saïd *et al.*, 2005), and is affected by environmental variation (e.g. Rivrud *et al.*, 2010), yet few studies have investigated whether individual home ranges correlate with immune or parasite variation. Aalvik *et al.*

(2015) found larger home range size correlated with higher parasitism in individually-tracked Atlantic cod, and exploratory behaviour is sometimes found to correlate with increased parasite exposure (Barber and Dingemanse, 2010), so wide-ranging red deer may exhibit similarly increased parasitism. Fitting specific individual-level spatial behaviour and environmental variables may allow researchers to expand on the spatial patterns of immunity and parasitism described in chapter V and to explain them in terms of intrinsic and extrinsic factors. For example, does the mean elevation of an individual's home range determine its exposure to *F. hepatica*? A previous analysis on the Isle of Rum deer used home range overlap matrices in combination with genetic relatedness matrices to disentangle environmental and genetic sources of variation for phenotypic and behavioural traits, and it was suggested that social proximity matrices could be used in the same analyses (Stopher *et al.*, 2012b). Indeed, a similar analysis in bottlenose dolphins used genetic and social matrices to quantify the importance of sociality in determining fitness (Frere *et al.*, 2010). Multi-matrix analyses are becoming increasingly popular in animal behaviour, ecology and evolution (Thomson *et al.*, 2018), and it is likewise possible to fit multiple similarity matrices as variance components in INLA (Holand *et al.*, 2013). Hence future investigations in the deer could make use of these model structures, in combination with SPDE autocorrelation models, to examine the relative importance of spatial, social, and genetic sources of variation in determining immunity and parasitism (Stopher *et al.*, 2012b; Thomson *et al.*, 2018).

Part 5: Concluding remarks

This thesis strongly extols the virtues of long-term longitudinal studies in disease ecology. Individual-based sampling regimes were highly useful for detecting complex life history correlates and fitness effects of immunity and parasitism, as expected. Furthermore, these results demonstrate that this individual-based sampling can be

used to examine fine-scale spatiotemporal variation of parasitism and immunity, and its interactions with individual variation, even over smaller distances and timespans which are rarely used for these purposes. Above all, we have demonstrated that, given careful selection of relevant immune and parasite measures, observational approaches can be newly applied to well-established study systems to gain revealing insights into the ecology of disease in the focal system. This supports the general viewpoint that ecological studies should measure parasites where possible (Gehman *et al.*, 2019). The ability to piggy-back onto the methodological foundations of a well-understood system represents an exciting opportunity, and more long-term individual-based studies may be calling out for similar treatment.

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Appendix A: supplementary information for Chapter III

Section One: Table SI1

Factor	Prevalence (%)	Model Sample Sizes			Repeatability
		Full	Pregnancy	Calving Traits	
Strongyles	76	835	518	571	0.21 (0.16-0.28)
F. hepatica	33	824	417	507	0.11 (0.06-0.17)
E. cervi	95	833	518	571	0.39 (0.34-0.45)
Total IgA		796	499	550	0 (0-0.08)
Anti-Tc IgA		796	497	547	0.25 (0.17-0.32)

Table SI1: model sample sizes and repeatabilities (95% credibility intervals in brackets).

Section Two: Model output

This section includes model outputs for the fixed effects of all models we ran. We first include the main models reported in the study (Figure SI1). We then compare these results with a set of modifications that we investigated.

The next (Figure SI2) displays the full models including a season-by-status interaction to display the way this affected the estimates, and to demonstrate the seasonal effects. Generally, including a season by status interaction did not improve the model fit or change our findings. The exception for this is the strongyle model (ΔDIC = -3.79). There was, however, a general trend for the differences between status categories to decrease over the autumn and spring seasons as can be seen in Figure 3 in the main text.

Figure SI3 displays the effects from pregnancy models when we either included or removed pregnancy as a fixed effect, to investigate whether this affected the estimates of each status category's effect. Inclusion of the pregnancy term slightly reduced the significance of the "Calf Survived" effect in the strongyle model, and increased the effects of "Calf Survived" in both the total IgA and anti-Tc IgA models. It also improved the fit of the total IgA model (ΔDIC = -3.71). Otherwise, pregnancy had little effect.

Figure SI4 displays the results from the full models again, compared with the results from the multivariate models. The models were extremely similar, with only small changes in effect sizes and significance. This supports our use of this model to derive phenotypic correlations.

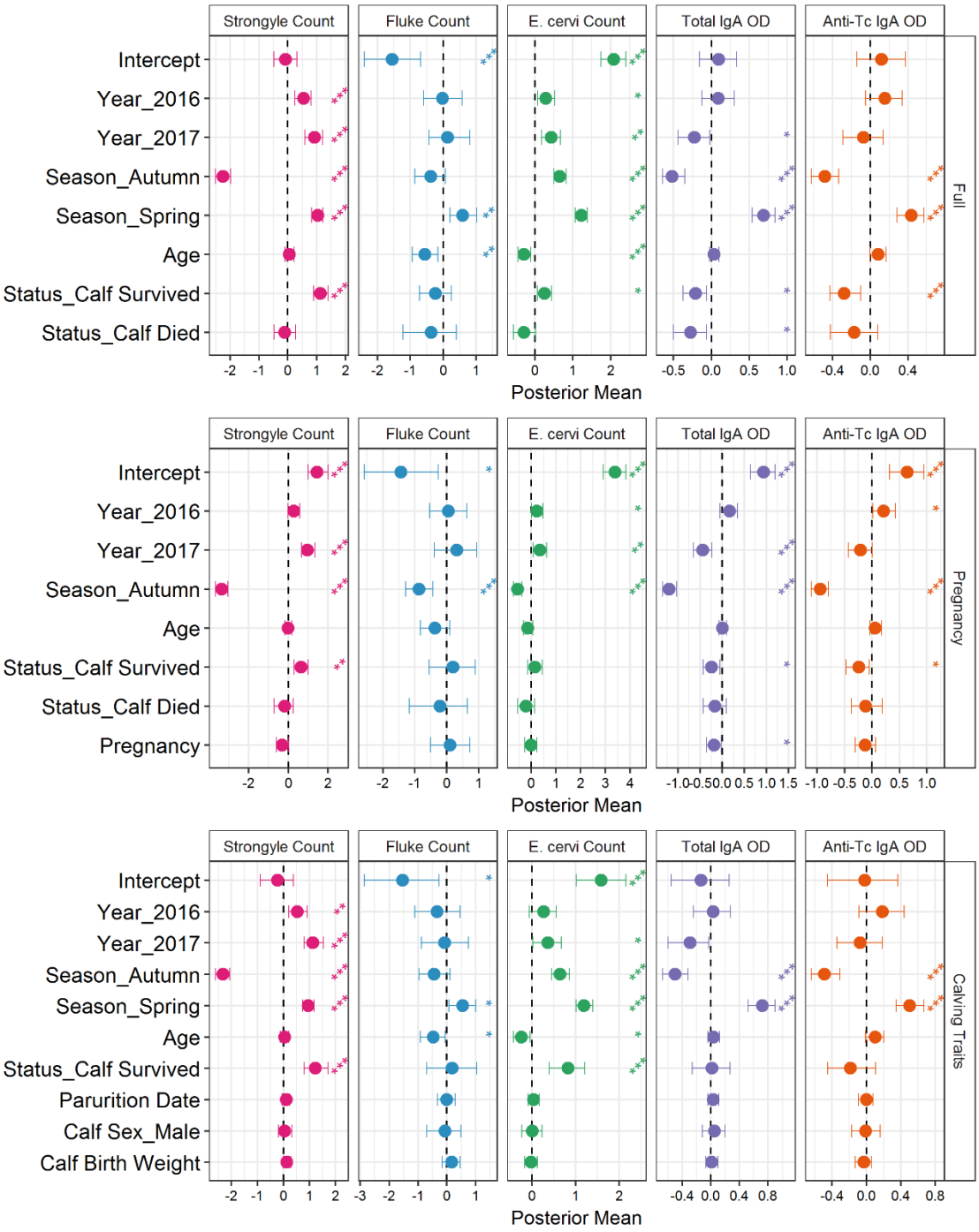

Figure SI1: Effect size estimates from the three model sets (full dataset, pregnancy models and calf traits models). Effect sizes for categorical variables denote differences from the first (absent) category of each, contained in the intercept. Effect sizes for continuous variables represent the change associated with a change of one standard deviation of the variable in question. Points and error bars represent model estimates and 95% credibility estimates for each of the five full models. Asterisks indicate the significance of variables: ***, ** and * indicate P<0.001, P<0.01 and P<0.05 respectively.

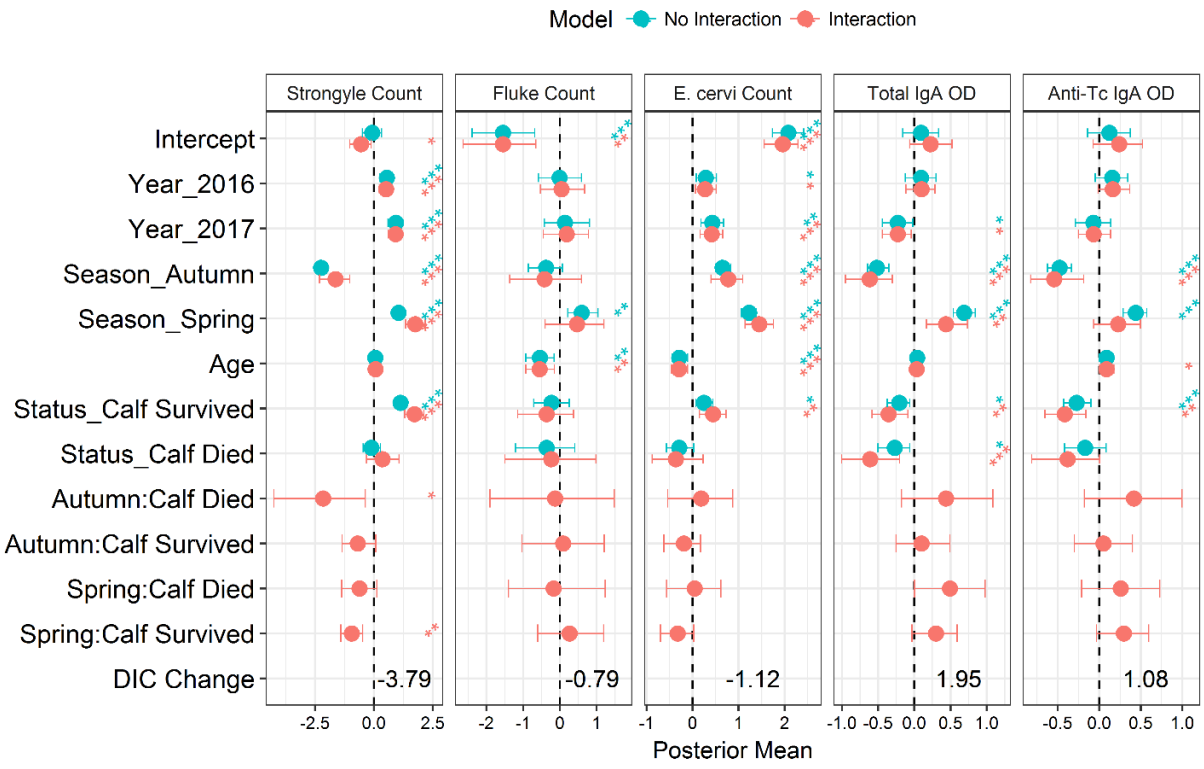

Figure SI2: Comparison between outputs from full models excluding and including a season by status interaction. Points and error bars represent model estimates and 95% credibility estimates for each of the five full models, with and without interactions. Effect sizes for categorical variables denote differences from the first (absent) category of each, contained in the intercept. Effect sizes for continuous variables represent the change associated with a change of one standard deviation of the variable in question. Asterisks indicate the significance of variables: ***, ** and * indicate P<0.001, P<0.01 and P<0.05 respectively. DIC Change represents the change in DIC that occurred when an interaction was included. Including an interaction did not have a substantial effect on most of the original estimates, and only the fit of the strongyle model was significantly improved by its inclusion.

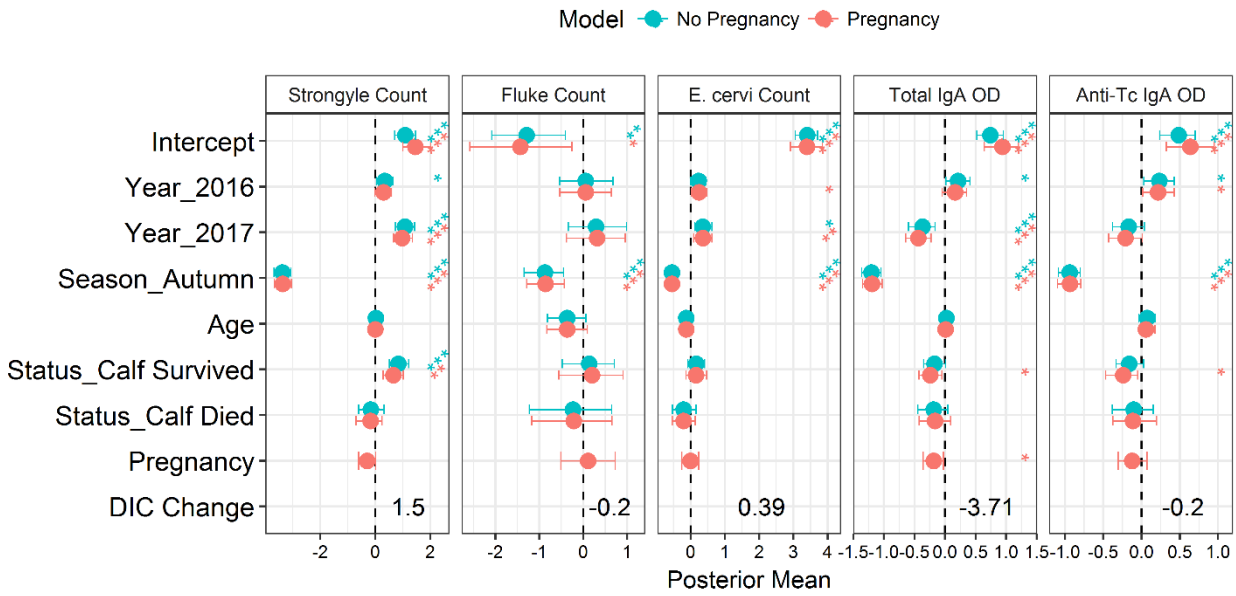

Figure SI3: Comparison between outputs from pregnancy models, excluding and including pregnancy as a covariate to investigate whether this changed the model estimates for the reproductive status categories. Points and error bars represent model estimates and 95% credibility estimates for each of the five full models, without and with pregnancy as a covariate. Effect sizes for categorical variables denote differences from the first (absent) category of each, contained in the intercept. Effect sizes for continuous variables represent the change associated with a change of one standard deviation of the variable in question. Asterisks indicate the significance of variables: ***, ** and * indicate P<0.001, P<0.01 and P<0.05 respectively. DIC Change represents the change in DIC that occurred when pregnancy was included. Including pregnancy as a covariate slightly decreased the effect size of "Calf Survived" for strongyles and increased it for total IgA and anti-Tc IgA, but otherwise had little effect on the estimates. Pregnancy also significantly reduced total IgA levels and improved the total IgA model fit.

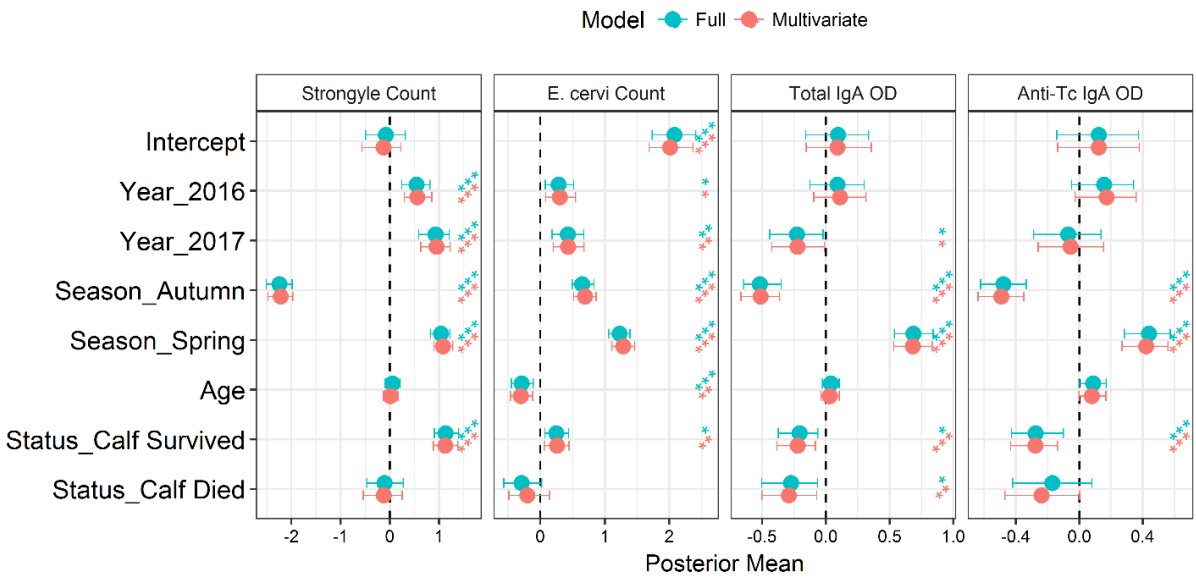

Figure SI4: Comparison of the fixed effect estimates from the full models and the multivariate model. Points and error bars represent model estimates and 95% credibility estimates for each of the five full models and the equivalent fixed effects in the multivariate model. Effect sizes for categorical variables denote differences from the first (absent) category of each, contained in the intercept. Effect sizes for continuous variables represent the change associated with a change of one standard deviation of the variable in question. Asterisks indicate the significance of variables: ***, ** and * indicate P<0.001, P<0.01 and P<0.05 respectively.

Section Three: Additional Figures

This section contains some figures displaying patterns in the data which are of interest. This includes the effects of faecal collection variables on antibody levels, age effects, and correlations between response variables.

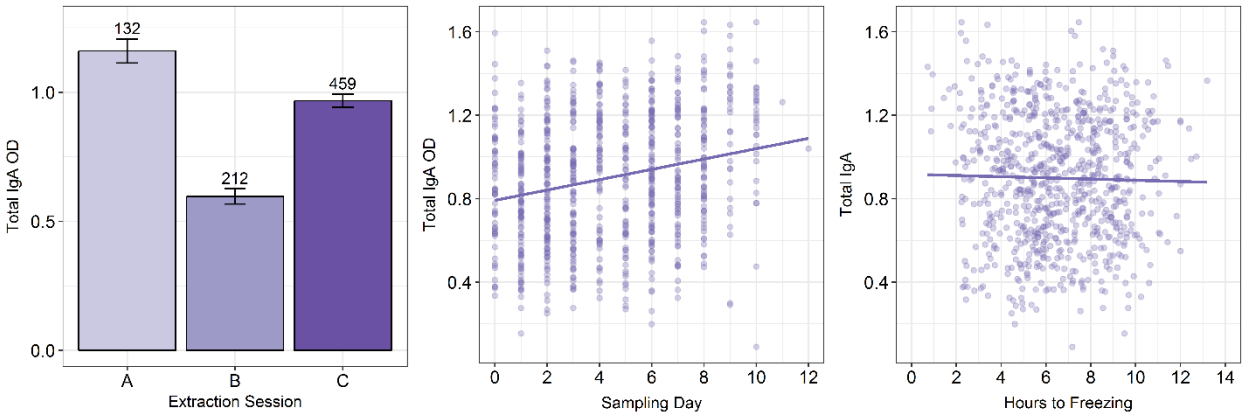

Figure SI5: Impact of faecal collection factors on total IgA level (extraction session, day of collection and hours to freezing). Y axes for figures B and C have been square root transformed.

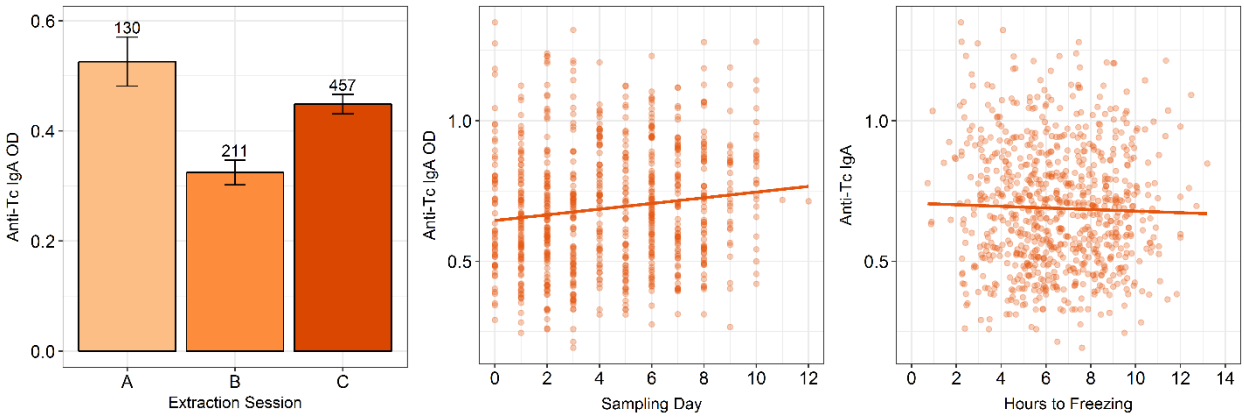

Figure SI6: Impact of faecal collection factors on anti-Tc IgA level (extraction session, day of collection and hours to freezing). Y axes for figures B and C have been cube root transformed.

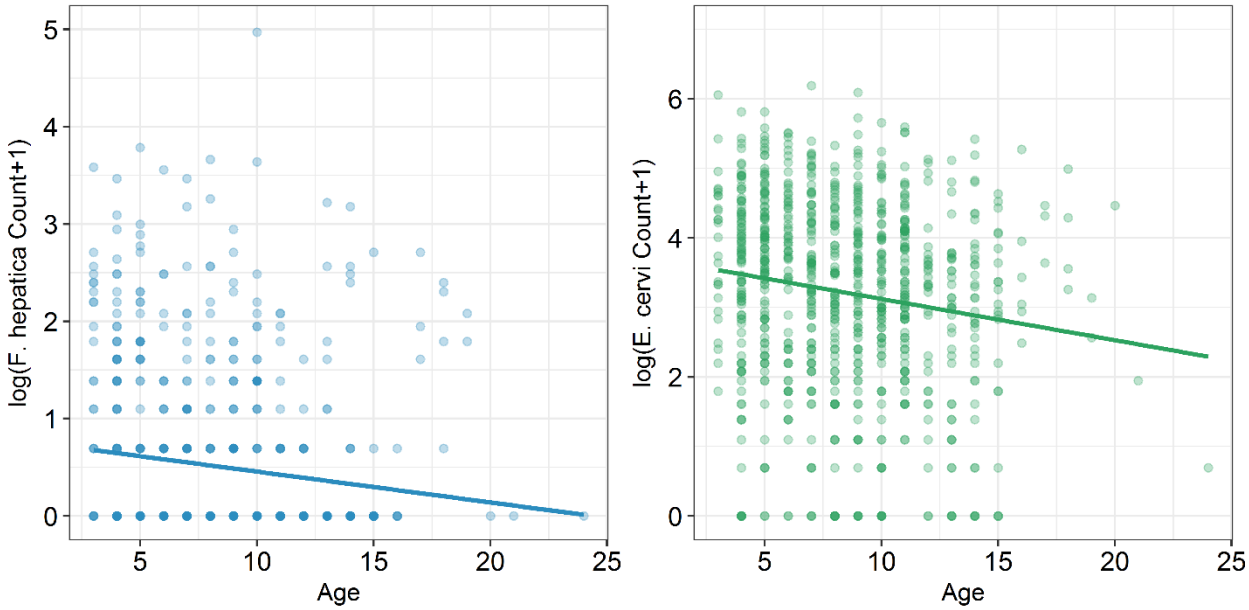

Figure SI7: Age trends in parasite counts using the raw correlations (*F. hepatica* and *E. cervi*). Y axes have been log(count+1)-transformed.

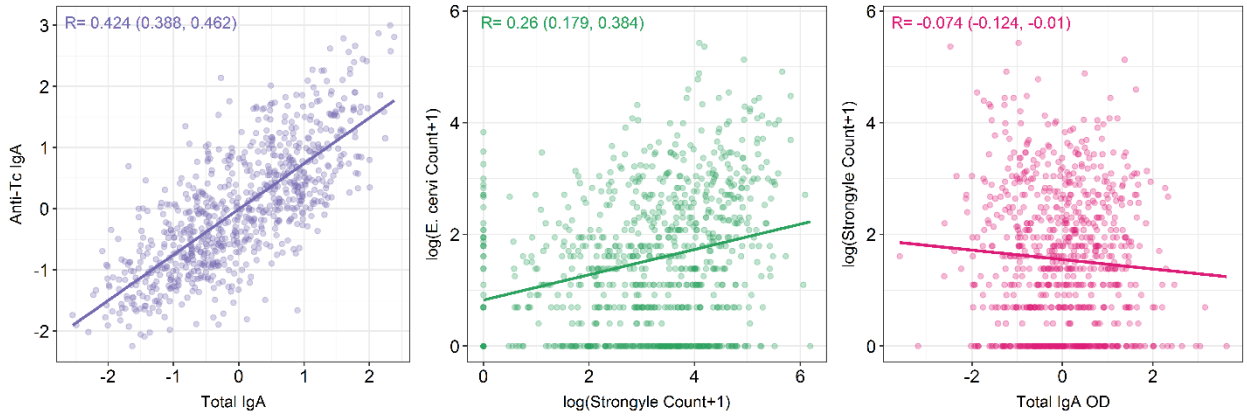

Figure SI8: Correlations between response variables (Total IgA and anti-Tc IgA; Strongyles and E. cervi; Total IgA and Strongyles). Model-derived phenotypic correlations (R_p) are included, with 95% credibility intervals. Both antibody measures are based on the residuals from a model including extraction session, day of collection and hours to freezing, with transformed antibody OD as response variable(square root for the total IgA and cube root for anti-Tc IgA). In the strongyle figure total IgA was scaled within each sampling trip to have a mean of 0 and a standard deviation of 1 to avoid a positive correlation arising from shared seasonal effects.

Appendix B: supplementary information for Chapter IV

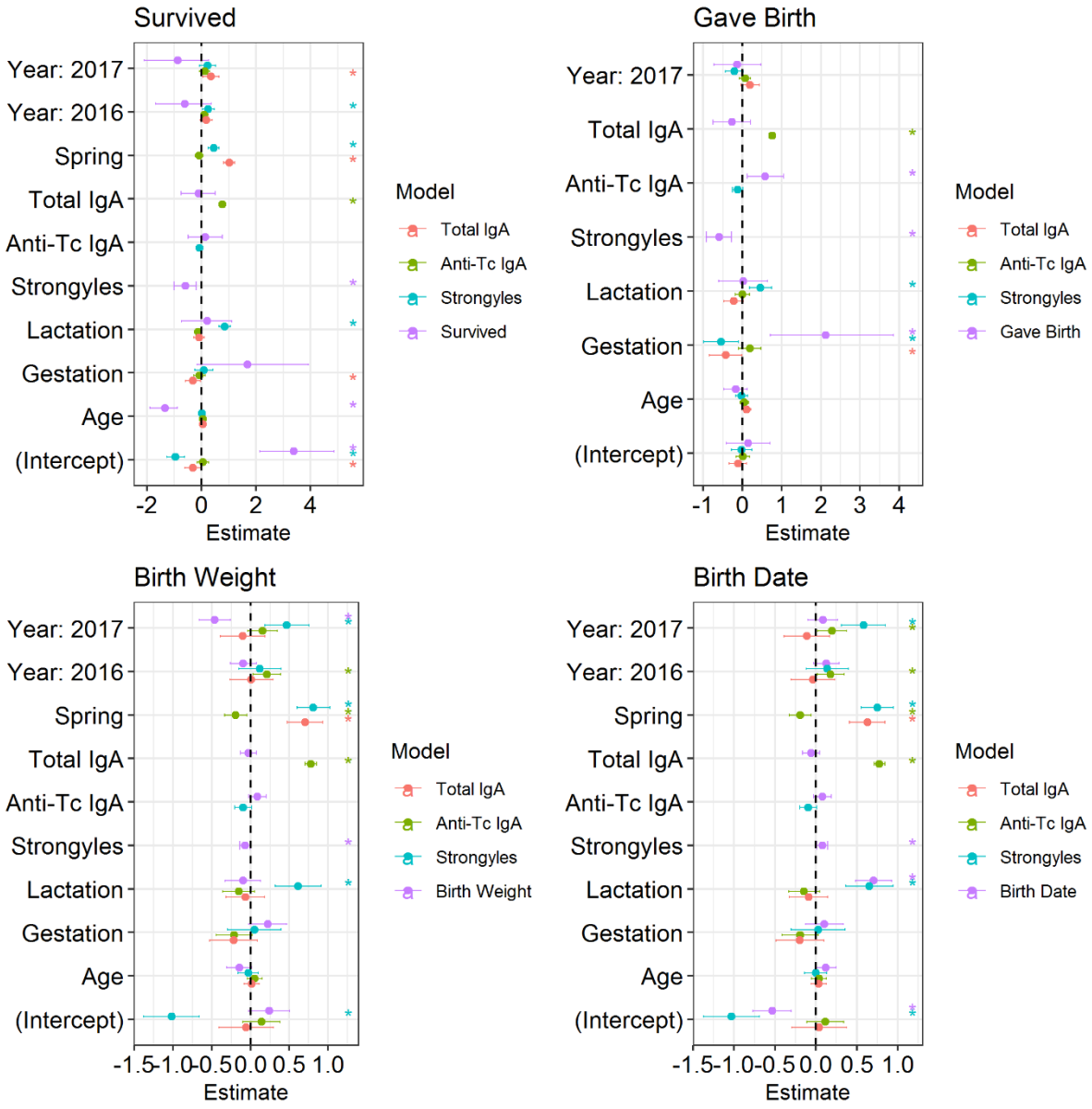

Figure SI1: Model outputs for the component GLMMs of all four path analyses. Colours correspond to different response variables; variables on the y axis correspond to explanatory variables in each component model. Points denote mean estimates from the posterior distribution of effect sizes from the component INLA model. Error bars denote 95% credibility intervals for the distribution. Where these intervals did not overlap with 0, the effect was taken to be significant. These effects are marked with an asterisk. Notably, lactation was positively associated with strongyles (blue estimates) and strongyles were negatively associated with all fitness-related traits (purple estimates).

Survival Path Analysis				
Model	Variable	Estimate	Lower CI	Upper CI
Total IgA	Intercept	-0.3081	-0.609	-0.0075
	Gestation	-0.3182	-0.5999	-0.0367
	Lactation	-0.0912	-0.2842	0.1017
	Age	0.047	-0.0273	0.1212
	Spring	1.0171	0.8138	1.2202
	Year: 2016	0.1765	-0.0441	0.397
	Year: 2017	0.34	0.0436	0.6363
Anti-Tc IgA	Intercept	0.0571	-0.149	0.263
	Total IgA	0.767	0.7095	0.8243
	Gestation	-0.0705	-0.2829	0.1417
	Lactation	-0.1298	-0.2625	0.0027
	Age	0.0538	-0.0285	0.1362
	Spring	-0.0872	-0.2234	0.0491
	Year: 2016	0.109	-0.0265	0.2443
	Year: 2017	0.1151	-0.0715	0.3016
Strongyle Count	Intercept	-0.9499	-1.2653	-0.6347
	Gestation	0.083	-0.2426	0.4084
	Lactation	0.8496	0.6416	1.0569
	Anti-Tc IgA	-0.0785	-0.1675	0.0105
	Age	0.0192	-0.1007	0.1382
	Spring	0.4485	0.2474	0.6495
	Year: 2016	0.2491	0.0375	0.4607
	Year: 2017	0.2247	-0.0654	0.5148
Survival	Intercept	3.3842	2.1424	4.8575
	Gestation	1.6766	-0.1589	3.9344
	Lactation	0.2061	-0.7305	1.1135
	Anti-Tc IgA	0.1306	-0.4932	0.7636
	Total IgA	-0.1087	-0.7467	0.5113
	Strongyles	-0.5896	-1.0085	-0.1937
	Age	-1.3464	-1.8883	-0.8885
	Year: 2016	-0.6102	-1.6816	0.364
	Year: 2017	-0.8665	-2.1049	0.2666
Reproduction Path Analysis				
Model	Variable	Estimate	Lower CI	Upper CI
Total IgA	Intercept	-0.1121	-0.3346	0.1102
	Gestation	-0.4279	-0.8417	-0.0145

	Lactation	-0.2216	-0.471	0.0276
	Age	0.1051	-0.0108	0.2209
	Year: 2017	0.1972	-0.0388	0.4329
Anti-Tc IgA	Intercept	0.0148	-0.1561	0.1853
	Total IgA	0.755	0.6747	0.8351
	Gestation	0.1878	-0.0955	0.47
	Lactation	-0.0021	-0.1816	0.1771
	Age	0.047	-0.0583	0.1522
	Year: 2017	0.0692	-0.07	0.208
Strongyle Count	Intercept	-0.0191	-0.2778	0.2403
	Gestation	-0.5432	-0.9927	-0.0939
	Lactation	0.4618	0.1793	0.7444
	Anti-Tc IgA	-0.1244	-0.2597	0.0106
	Age	-0.0228	-0.1747	0.1275
	Year: 2017	-0.2038	-0.4353	0.0286
Gave Birth	Intercept	0.1424	-0.4085	0.6949
	Gestation	2.122	0.7159	3.8491
	Lactation	0.0188	-0.6041	0.6448
	Anti-Tc IgA	0.5761	0.1144	1.0549
	Total IgA	-0.2661	-0.7449	0.2034
	Strongyles	-0.5885	-0.9166	-0.2737
	Age	-0.1704	-0.4716	0.1247
	Year: 2017	-0.1285	-0.7295	0.4681
Calf Birth Weight Path Analysis				
Model	Variable	Estimate	Lower CI	Upper CI
Total IgA	Intercept	-0.0559	-0.4059	0.2937
	Gestation	-0.2193	-0.5256	0.0866
	Lactation	-0.0667	-0.3142	0.1807
	Age	0.0129	-0.0855	0.1113
	Spring	0.7025	0.4721	0.9326
	Year: 2016	0.0116	-0.2658	0.2888
	Year: 2017	-0.1004	-0.387	0.186
Anti-Tc IgA	Intercept	0.1405	-0.0992	0.3799
	Total IgA	0.7789	0.7074	0.8506
	Gestation	-0.2126	-0.4438	0.018
	Lactation	-0.1524	-0.3547	0.0498
	Age	0.0525	-0.043	0.148
	Spring	-0.1912	-0.3348	-0.0479
	Year: 2016	0.2109	0.0333	0.388

	Year: 2017	0.154	-0.0338	0.3416
Strongyle Count	Intercept	-1.0168	-1.3738	-0.6603
	Gestation	0.0489	-0.2936	0.3925
	Lactation	0.6138	0.3177	0.9118
	Anti-Tc IgA	-0.0962	-0.2046	0.0121
	Age	-0.0306	-0.162	0.1018
	Spring	0.8109	0.5991	1.0222
	Year: 2016	0.1204	-0.15	0.3911
	Year: 2017	0.4678	0.1848	0.7503
Calf Birth Weight	Intercept	0.2411	-0.0236	0.5057
	Gestation	0.2213	-0.023	0.4654
	Lactation	-0.0972	-0.3267	0.1319
	Anti-Tc IgA	0.0909	-0.0243	0.2057
	Total IgA	-0.0278	-0.1342	0.0788
	Strongyles	-0.0715	-0.1404	-0.0028
	Age	-0.1467	-0.3092	0.0156
	Year: 2016	-0.0935	-0.2613	0.0742
	Year: 2017	-0.461	-0.6596	-0.2627
Calf Birth Date Path Analysis				
Model	Variable	Estimate	Lower CI	Upper CI
Total IgA	Intercept	0.0381	-0.2974	0.3734
	Gestation	-0.1961	-0.4888	0.0963
	Lactation	-0.0889	-0.3236	0.1457
	Age	0.0342	-0.0598	0.1282
	Spring	0.6269	0.409	0.8447
	Year: 2016	-0.0365	-0.302	0.2289
	Year: 2017	-0.1125	-0.3907	0.1654
Anti-Tc IgA	Intercept	0.1135	-0.112	0.3389
	Total IgA	0.7769	0.7097	0.8441
	Gestation	-0.1909	-0.4103	0.028
	Lactation	-0.1438	-0.3309	0.0431
	Age	0.0369	-0.0546	0.1286
	Spring	-0.1948	-0.3278	-0.0619
	Year: 2016	0.1799	0.0144	0.3452
	Year: 2017	0.1978	0.0202	0.3753
Strongyle Count	Intercept	-1.0305	-1.3734	-0.6883
	Gestation	0.0273	-0.3045	0.3598
	Lactation	0.6512	0.3645	0.9407
	Anti-Tc IgA	-0.0935	-0.197	0.0098

	Age	-0.0031	-0.1375	0.1319
	Spring	0.7492	0.5533	0.9449
	Year: 2016	0.14	-0.1144	0.3951
	Year: 2017	0.5809	0.3106	0.8508
Calf Birth Date	Intercept	-0.5344	-0.7683	-0.3033
	Gestation	0.1028	-0.1294	0.3341
	Lactation	0.7067	0.4869	0.9255
	Anti-Tc IgA	0.08	-0.0294	0.1894
	Total IgA	-0.0593	-0.1619	0.0429
	Strongyles	0.0772	0.0102	0.1444
	Age	0.1201	-0.008	0.249
	Year: 2016	0.1251	-0.0301	0.2797
	Year: 2017	0.085	-0.0971	0.2668

Table SI1: effect sizes and credibility intervals for the component GLMMs of each path analysis.

Appendix C: supplementary information for Chapter V

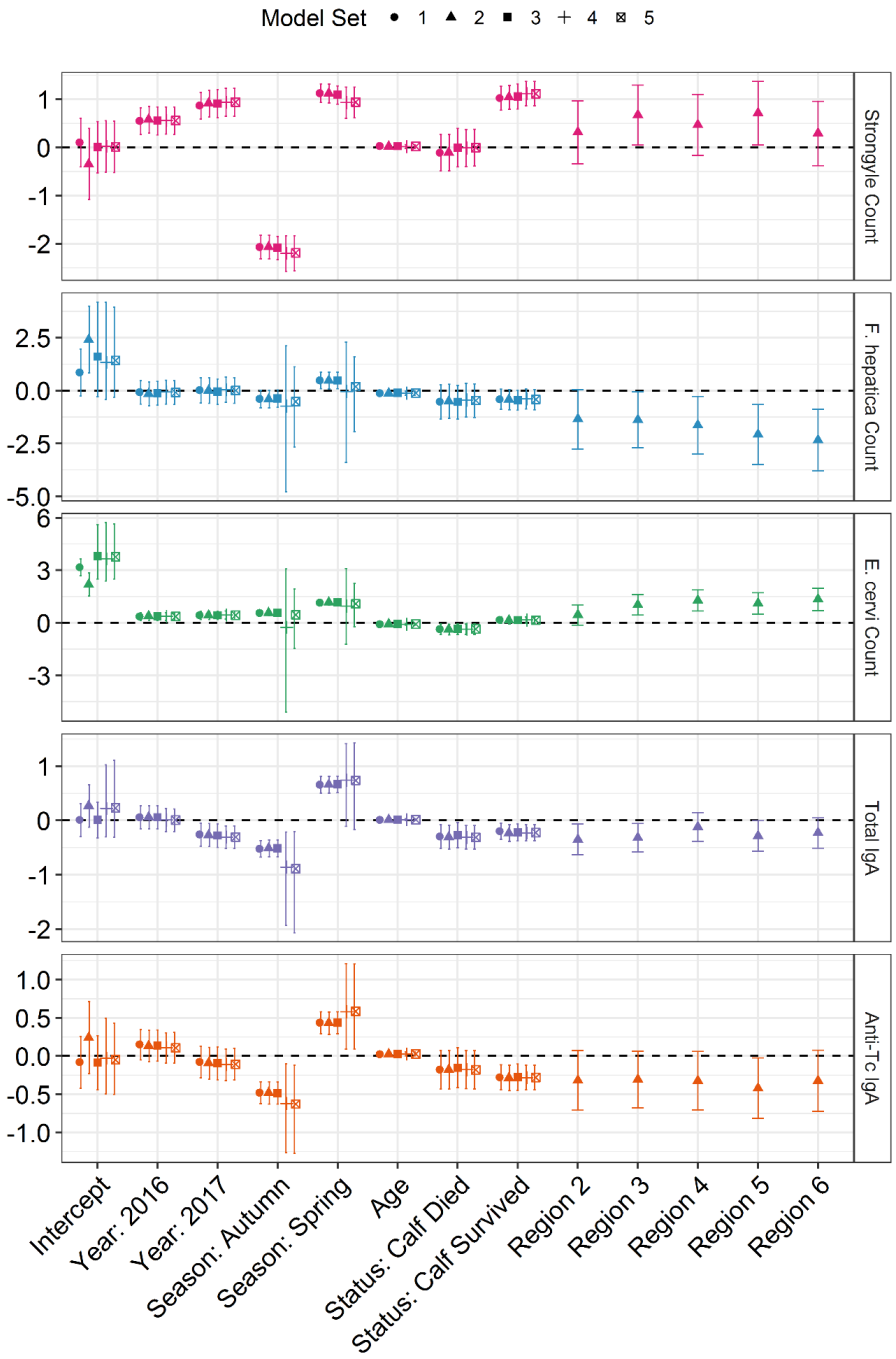

Figure SI1: Comparison of the fixed effect estimates from each model for each response variable. Points denote the mean effect estimate, and error bars represent the 0.025 and 0.975 quantiles. Estimates for categorical variables represent departures from the missing category of each variable (year: 2015, season: summer, status: no calf, subpopulation: region 1), on the link scale. Points of different shapes denote the results from different model sets. Model set 1: base model. Model set 2: base model + subpopulation fixed effect. Model set 3: base model + spatial random effect. Model set 4: spatial random effect allowed to vary between seasons. Model set 5: spatial random effect allowed to correlate between seasons.