Immunity, life history and conservation in the Galapagos sea lion

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Declaration

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors of this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given in the thesis where reference has been made to the work of others.

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In both cases the research was conceived by PMB, KAW, SG and AH. PB and MC carried out fieldwork and laboratory work. PB analysed the data and wrote the first draft of manuscripts. KAW, SG and AH, commented on manuscripts and PB produced the final manuscripts.

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Abstract

Infectious disease threatens health and biodiversity across the globe, and disease emergence may become more common as humans further encroach on habitats and modify environments. To assess the risks of disease emergence in natural populations accurately, we require an understanding of the workings of immunity in the wild. This thesis is about immunity in the context of life history in the endangered Galapagos sea lion, and aims to contribute to understanding of immune dynamics in wild populations, and to evidence-based conservation management.

The introduction reviews the development of the discipline of ecological immunology, and discusses the application of its methodological tools to little-known species such as the Galapagos sea lion. The first data chapter uses these tools to describe the ontogeny of Galapagos sea lion immunity in two contrasting ecological contexts. The second data chapter contextualises the immune variation described in the first by assessing the relationship between immune activity and condition. The third data chapter introduces a genetic dimension through the analysis of inbreeding estimates with immune measures. The fourth and final data chapter uses epidemiological models to assess the risk of Galapagos sea lion exposure to canine distemper virus under different management and environmental scenarios. The discussion brings together the results of the data chapters and evaluates emergent themes and limitations in the context of suggestions for future work.

The results show that the study of immune variation in species such as the Galapagos sea lion can provide useful insight into the dynamics of immunity in the wild, and information that can have practical application to conservation. They also lay a foundation for integrated epidemiological analyses of disease risk that incorporate physiological and immunological variation, and that have potential for constructive development beyond the Galapagos sea lion.

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AIC	Akaike's information criterion
ALB	albumin concentration (relative peak intensity)
ALP	alpha globulin concentration (relative peak intensity)
ANOVA	analysis of variance
BET	beta globulin concentration (relative peak intensity)
CC	control colony
CDV	canine distemper virus
CIMEI	Comité Interinstitucional para Manejo y Control de Especies Introducidas
DDT	dichlorodiphenyltrichloroethane
DML	dyadic likelihood estimator of inbreeding
Δ	change in
EDTA	ethylenediaminetetraacetic acid
EN	El Niño
EOS	eosinophil concentration (10^9 L^{-1})
GAM	gamma globulin concentration (relative peak intensity)
GPS	global positioning system
GSL	Galapagos sea lion
HFC	heterozygosity-fitness correlation
HIC	human-impacted colony
HL	homozygosity by locus
HWE	Hardy-Weinberg equilibrium
IgG	immunoglobulin G
IP	infectiousness period
IR	internal relatedness
IUCN	International Union for Conservation of Nature
LME	linear mixed effects
Ln	natural logarithm
LP	latency period
LRT	likelihood ratio test
LYM	lymphocyte concentration (10^9 L^{-1})
LYRT	Lynch and Ritland's moment estimator of inbreeding
MHC	major histocompatibility complex
MLH	multi-locus heterozygosity
MLR	mass per unit length (kg for pups, Ln kg for juveniles)
MP	mortality probability
NEU	neutrophil concentration (10^9 L^{-1})
OD	optical density
PAMP	pathogen associated molecular pattern

PBDE	polybrominated diphenyl ether
PBM	Puerto Bazquerizo Moreno
PBS	phosphate-buffered saline
PCB	polychlorinated biphenyl
PCV	packed cell volume
PDV	phocine distemper virus
PHA	phytohemagglutinin
PI	peak intensity
RIT	Ritland's moment estimator of inbreeding
SD	standard deviation
SE	standard error
SFT	skinfold thickness (mm)
SH	standardised heterozygosity
SSD	sum of squares difference
TML	triadic likelihood estimator of inbreeding
WBC	total leukocyte concentration (10^9 L^{-1})

Chapter 1

General introduction

Two central questions about immunity in an eco-evolutionary context that remain to be answered are: how are the costs of immunity expressed in natural populations, and how do these costs contribute to the maintenance of phenotypic and genetic immunoheterogeneity (Graham et al. 2010)? This thesis aims to address these and related questions using the study system of the Galapagos sea lion (*Zalophus wollebakei*), a species with a unique ecology that facilitates the testing of hypotheses on immune dynamics in the wild, and in which data on immunity may have value to applied conservation. Specifically, it aims to address the hypotheses that human activity influences immune system ontogeny, that investment in immunity incurs physiological costs and that inbreeding has an impact on immunity. In addition, it aims to address the epidemiological question of how domestic dog management on the Galapagos Islands could most effectively reduce disease risk to the endangered Galapagos sea lion.

In this introduction, I first review some relevant aspects of immunity to provide background for the broader discussion that follows. Then I summarise the conceptual integration of immunity into ecology, and introduce the study system. Finally, I expand on the specific aims of the thesis. Throughout the thesis I use the words ‘immunity’ and ‘immune’ in their general sense to refer to the immune system as a whole and to anything pertaining to it, rather than to a technical physiological state.

Immunity

Animal immune systems comprise dynamic webs of interacting parts, from simple innate barriers to complex acquired responses, which provide protection against pathogens. Innate immunity is taxonomically widespread and has its origins in basal animal groups (Mims et al. 2004). Innate immune

responses are effective over short time periods and may involve inflammation, antimicrobial peptides (e.g. complement, lysozyme, defensins) and cells with roles in antigen presentation (e.g. dendritic cells), phagocytosis (e.g. neutrophils, macrophages) and cytotoxicity (e.g. natural killer cells; Tizard 2009). Innate immunity is non-specific, as it recognises pathogens using common microbial structures, or pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide and double stranded RNA (Schmid-Hempel 2011a). Components of innate immunity can be either constitutive or induced: constitutive components are produced at a constant rate regardless of PAMP exposure (e.g. some defensins), while induced components are produced in response to PAMP exposure (e.g. mannose-binding lectin).

If a pathogen invades a host beyond its anatomical barriers and persists despite innate immunity, acquired immune responses may be triggered. Acquired immunity, although slower to respond than innate, has the advantages of specificity and memory. Specificity is achieved by the random generation of huge numbers of structurally unique receptors, elimination of those that are self-reactive and selection for those that bind to antigens expressed by pathogens (Schmid-Hempel 2011a). Following selection, memory cells preserve functional receptors so that responses to secondary antigen exposure are fast and efficient. There are two broad types of acquired immune response: cell-mediated and humoral. Acquired cell-mediated responses mostly involve cytotoxic T-lymphocytes and macrophages, whereas humoral ones mostly involve B-lymphocytes and antibodies (Tizard 2009). Note, though, that antibodies also have a role in innate immunity, as a subset known as ‘natural antibodies’ bind to PAMPs (Ochsenbein & Zinkernagel 2000).

Although immune components and responses may be grouped into the above categories conceptually, in practice, most immune processes involve a complex mixture of components and modes of action. Immune components interact with one another directly and via the action of immunomodulatory molecules such as cytokines, and are linked to other aspects of physiology, such as the neuroendocrine system, through mediators such as hormones

(Demas et al. 2011a). Due to the regulatory nature of the interactions between immune components and cascades, immune responses are characterised by feedback loops, which makes their definition with reference to conceptual continua and categories not only complex, but also dynamic.

The development of acquired immunity necessitates a period of immunological naivety during early ontogeny while B- and T-lymphocyte repertoires mature. The provision of maternal antibodies to offspring is thought to compensate for this period of vulnerability, which coincides with a period of high mortality risk (McDade 2003). Mammalian offspring with hemochorial and endotheliochorial placentation receive a single class of immunoglobulin (IgG) through the placenta (Day & Schultz 2011). This accounts for only a small proportion of the total IgG transferred from mother to offspring, as the rest is transferred in colostrum, which contains the immunoglobulin classes IgM and IgA in addition to IgG (Tizard 2009). Colostrum also contains cytokines, which are thought to stimulate immune system development in this context, and trypsin inhibitors to prevent digestion of the maternally derived antibody (Tizard 2009). As the majority of antibodies present in colostrum originate in the mother's blood, it is likely that offspring are provided with an antibody repertoire that is matched to the antigenic environment their mother has experienced (Boulinier & Staszewski 2008).

Immunogenetics is a fast-developing field, and there are an increasing number of immunogenetic studies being carried out in ecological contexts (Hill 2001, 2012; Trowsdale & Parham 2004). These studies show that there is considerable genetic influence on many of the aspects of immunity described above, and on eco-evolutionary processes that involve immunity (Acevedo-Whitehouse & Cunningham 2006; Lazzaro & Little 2009). Many studies on the genetics of immunity in the wild have focussed on the hyper-variable region of the major histocompatibility complex (MHC), which has roles in antigen recognition and presentation, and has been associated with both mate choice and kin recognition (Kubinak et al. 2012). However, many other genes play important roles in determining resistance and there is complex temporal variation in the expression of immune genes during the course of infection

(Acevedo-Whitehouse & Cunningham 2006). In addition, selection experiments (van der Most et al. 2011) and heritability studies (Bonneaud et al. 2009; Graham et al. 2010) suggest that there are still many genetic influences on immune variation that we do not fully understand.

Significant advances in the study of immunity in an ecological context have been driven by work on invertebrates (Schmid-Hempel 2003; Rolff & Siva-Jothy 2004; Palmer & Traylor-Knowles 2012). Phagocytic cells, which undertake chemotaxis, adherence, ingestion and digestion, and in these respects are similar to mammalian phagocytes, are present in many invertebrates (Tizard 2009). These cells may also aggregate to plug wounds or isolate invading pathogens through encapsulation. A large number of diverse antimicrobial peptides have been described in invertebrates and many are associated with the production of melanin through the prophenoloxidase pathway, which is induced by the recognition of PAMPs. In addition, although invertebrates do not produce antibodies, there is evidence for an acquired aspect of invertebrate immunity (Sadd & Schmid-Hempel 2006; McTaggart et al. 2012).

Marine mammal immune systems are likely to be adapted to the spectrum of marine pathogens to which they have been exposed over evolutionary time, and to the homeostatic limitations imposed by an aquatic lifestyle. However, the relatively small amount of marine mammal immunology research that has been conducted to date suggests that marine mammal immunity is broadly similar to that of terrestrial mammals (e.g. Gray et al. 2005; Hall et al. 2007; Keogh et al. 2010). There are some notable exceptions: pinnipeds, for example, have substantially higher immunoglobulin G concentrations than terrestrial carnivores (King et al. 2001). However, immunology in marine mammals is still a developing field and more studies would be required to investigate the significance of such broad taxonomic differences. Until more is known about the idiosyncrasies of marine mammal immunity, the generalised mammalian immune system serves as a useful model for studies of marine mammal immunity (King et al. 2001), and that of dogs as one for studies of sea lion immunity (Day 2007; Mancia et al. 2011).

Ecological immunology

Driven by growing interest (Martin et al. 2011) and facilitated by advances in molecular techniques (Pedersen & Babayan 2011), 'ecological immunology' (Sheldon & Verhulst 1996) has become a label for a diversity of research, from comparative studies on the evolution of immunity (e.g. Nunn 2002) to those on the short-term physiological dynamics of immunity within individuals (e.g. Buehler et al. 2011). The unifying similarity of these studies is that they aim to integrate immunity into a broad organismal context in order to address ecological and evolutionary questions about immunity as part of life history, such as how immunoheterogeneity is maintained in natural populations. Immunity has long been considered part of ecology, but ecological immunology aims to explicitly test and define how organisms interact with their biotic and abiotic environment through immunity not as a 'black box' but as a complex and dynamic system (Schulenburg et al. 2009; Martin et al. 2011). To achieve this goal and to enable tests of hypotheses about the role of immunity in eco-evolutionary processes, specifically relevant aspects of immunity need to be carefully defined and explicitly matched to apposite ecological influences, which could range from decadal stochasticity in climate to instantaneous changes in hormone concentration, depending on the question under investigation and the study system.

Ecological immunology research has described immune variation in the context of a huge number of ecological variables. It has been shown, for example, that immunity can vary with ancestral habitat, as we would expect if species have adapted to different pathogen communities (Nunn et al. 2000; Semple et al. 2001; Nunn et al. 2003; Matson 2006). Immunity has also been shown to vary with season (Nelson 2004; Martin et al. 2008), life history strategy (Tieleman et al. 2005; Martin et al. 2006a, 2007b; Sparkman & Palacios 2009) and life history stage (Love et al. 2008). Through interaction with the neuroendocrine system (Demas 2004; Ashley & Wingfield 2012), immunity has been linked to stress responses (Casto et al. 2001; Berger et al. 2005; Martin 2009), reproductive physiology (Evans et al. 2000; McKean &

Nunney 2007; Nunn et al. 2009) and social behaviour (Wilson et al. 2002; Archie et al. 2012). The complexity of the relationship between the neuroendocrine system and immunity is an area of active investigation and one that has driven the development of ecological immunology since its inception (Hamilton & Zuk 1982; Folstad & Karter 1992; Westneat & Birkhead 1998; Roberts et al. 2004; Demas et al. 2011a).

The development of theory and the accumulation of data have precipitated the emergence of several other areas of current interest in ecological immunology. These include trade-offs within the immune system itself, the mechanisms by which they are mediated (Lee 2006; Schmid-Hempel 2011b), and the role of constraints in limiting the plasticity of immune responses and investment strategies (Ardia et al. 2011). The effects of a mother's experience, nutritional status and provisioning behaviour on offspring immunity is another area of growing interest and one that is yielding insights relevant to behavioural ecology, immunology and disease ecology (Pihlaja et al. 2006; Hasselquist & Nilsson 2009; Addison et al. 2009; Garnier et al. 2012; Hasselquist et al. 2012).

Conceptual advances in ecological immunology have highlighted problematic aspects of its practice. Extrapolation between taxa is risky (Owens & Wilson 1999), as the paradigms of immunology are built on research conducted on a small number of model species in controlled laboratory settings (Pedersen & Babayan 2011). The comparability of wild study species to the most appropriate laboratory model is likely to vary due to differences in ancestry and pathogen exposure history over evolutionary time. Another conceptual problem that has received relatively little empirical attention is immunoredistribution, which is the spatial reorganisation of immune components within an individual. This could occur quickly following a stimulus such as an acute stress response, and has the potential to confound the significance of measures of immune activity taken from a single location such as the blood stream (Braude et al. 1999; Martin et al. 2006b, 2008). The generalisation of the relationship between immunity and functional resistance is inadvisable, as incautious assumptions can lead to spurious interpretations

of the significance of immune variation (Adamo 2004; Viney et al. 2005; Goüy de Bellocq et al. 2007). This last issue is at the forefront of ecological immunology research, and research effort is increasingly focussed on defining and quantifying the 'protective immune phenotype' (Pedersen & Babayan 2011).

Trade-offs

A key step in the development of ecological immunology has been the conceptualisation of immunity as a life history trait, one that can interact with other life history traits and that can be integrated holistically into schemes of organismal biology. For this to be possible, immunity must be costly. Ecological immunology applies an optimality approach to the consideration of this cost, by assuming that immunity is regulated to maximise net fitness in the context of costs, ecological influences and constraints (van Boven and Weissing 2004). The pragmatic oversimplification of immunity as a single trait has created difficulties for ecological immunology by widening the gap between theoretical and empirical work, an issue that is discussed in the *Quantifying immunity* section below. However, it has led to significant advances in our understanding of how immunity is related to other life history traits and how it is regulated in response to ecological variation.

The cost of immunity may be expressed in different ways across taxa, arms of the immune system and time scales. An evolutionary cost of immunity, for example, can arise through pleiotropy or negative genetic covariance between immunity and other traits (McKean et al. 2008), and has been demonstrated by selection experiments (Verhulst et al. 1999; van der Most et al. 2011). Over shorter time scales, the maintenance (e.g. Valtonen et al. 2009) and deployment (e.g. Derting & Compton 2003) of immune responses have been shown to exact physiological costs, both in terms of the energy (Lochmiller & Deerenberg 2000; Martin et al. 2003; Ardia et al. 2012) and the materials these processes require (Gasparini et al. 2009; Cotter et al. 2011). Although harder to measure, it is also thought that immunopathology, which refers to collateral damage caused directly by immunity, exacts significant

physiological costs (Råberg et al. 1998; Zuk & Stoehr 2002; Graham et al. 2005; Ashley & Wingfield 2012).

Experimental manipulation has shown that food limitation can lead to the down-regulation of immunity (Martin et al. 2007a, 2008), that the stimulation of the immune system can lead to decreased investment in other fitness components (Bonneaud et al. 2003; Schmid-Hempel 2003; Mallon et al. 2003; Garamszegi et al. 2004; Sanz et al. 2004; Jacot et al. 2005; Uller et al. 2006; Eraud et al. 2005, 2009), and that experimentally induced increases in energy expenditure on activities such as rearing, begging, foraging and sexual behaviour can decrease immune activity (Deerenberg et al. 1997; McKean & Nunney 2001; Hasselquist et al. 2001; Ahtiainen et al. 2005; Verhulst et al. 2005; Moreno-Rueda 2010). Therefore, there is good evidence to suggest that immunity is costly enough to have biologically meaningful impacts on traits that are related to fitness, and researchers are beginning to investigate the heterogeneities of these relationships across different genetic and ecological contexts, and the mechanistic complexities that underlie them.

One such complexity under investigation is how trade-offs within the immune system are mediated. For example, the cost of developing an acquired immune system is presumed to be high given the amount of protein synthesis and gluconeogenesis required to build up B and T-lymphocyte repertoires during early ontogeny (Lochmiller & Deerenberg 2000; Martin et al. 2008). This developmental cost may be offset by the metabolic cheapness of the deployment of acquired relative to innate immune responses later in life (Råberg et al. 2002), a trade-off that is made possible by the down-regulation of innate inflammatory responses by cytokines released during the activation of acquired responses (Lee 2006). Mechanisms regulating tolerance, which refers to host investment in the alleviation of the negative fitness consequences of infection instead of the reduction of parasite numbers, are also receiving increased attention (Råberg et al. 2009; Baucom & de Roode 2011), as selective immunosuppression could be highly advantageous in certain pathological and ecological circumstances.

The ability to detect, as well as the nature of, immune costs may be affected by ecological factors (Sandland 2003; Sadd & Schmid-Hempel 2009). For example, though there may be no detectable difference in the outcome of infection, experimental increases in energy expenditure on other activities may increase the metabolic costs of clearing infection (e.g. Zala et al. 2008). Food limitation or supplementation may only have an effect on immunity in certain age classes (e.g. Birkhead et al. 1999) and may even affect different arms of the immune system in opposite directions (e.g. Gonzalez et al. 1999). In addition, the interactions of environmental factors with one another (e.g. Triggs & Knell 2011) and with genotype (Lazzaro & Little 2009; Doeschl-Wilson et al. 2009) are likely to influence the relative costs and benefits of immune investments. The developing analysis framework for immune variation as a form of phenotypic plasticity aims to incorporate this emergent understanding into research into how immunity participates in life history trade-offs (Graham et al. 2011).

The investigation of mechanisms by which trade-offs between immunity and other life history traits are mediated, is also a growing area of research interest. For differential allocation to two resource-demanding functions to be possible within an individual, they must have a degree of molecular independence. In other words, they cannot be products of the same metabolic cascade, otherwise the ratio of their allocation will be fixed by constraint, as is thought to be in the case with melanin distribution between insect immunity and coloration (Siva-Jothy 2000). However, if resource-demanding functions are products of different cascades, individuals may invest more or less in each. It has been proposed that such differential allocation is mediated by competition between physiological cascades for particular resources, such as molecules involved in lipid transport and metabolism (Adamo et al. 2008; Trotter et al. 2011), micronutrients (Long & Nanthakumar 2004) and hormones that regulate growth and metabolism (Demas et al. 2012). The further investigation of these mechanisms and of the nature of resource allocation to immunity is key to our understanding of immunity as an integrated life history trait.

Quantifying immunity

An issue that has been central to the development of ecological immunology is that of how to best quantify immunity (Siva-Jothy 1995). As noted earlier, the conceptualisation of immunity as a life history trait has created difficulties in the relationship between the theory and practice of ecological immunology. Ecologists seeking to test newly formulated hypotheses on immunity in the context of life history are limited by the relatively small number of measures of immune variation that are possible in the field. Although many field measures can be informative about particular aspects of immune variation (Martin et al. 2006b), they have often been broadly interpreted as measures of 'immunocompetence' (Owens & Wilson 1999), i.e. as empirical measures of the theoretical construct of immunity as a life history trait. When inconsistencies emerged from early ecological immunology studies (Kennedy & Nager 2006; Owen & Clayton 2007), it became clear that there was not a straightforward relationship between measures of 'immunocompetence' and fitness (Norris & Evans 2000) and that more care was needed in the interpretation of single measures of immune variation. The challenge of quantifying complex immunity using simple measures is discussed below in the context of three measures of immune variation commonly used in ecological immunology.

Total leukocyte concentration is a fundamental measure of immunity. However, baseline leukocyte concentrations vary between species (Kerr 2002; Nunn et al. 2003) and total leukocyte concentration can increase or decrease in response to infection (Bossart et al. 2001). In order to understand the significance of leukocyte variation it is important to complement total concentration with the concentrations of specific cell types. Neutrophils and macrophages, for example, are phagocytic cells that respond quickly and non-specifically to invading pathogens as part of the innate immune system, while lymphocytes are involved in both innate and adaptive responses and eosinophils are associated with immune responses to macroparasites and allergens (Wakelin 1996). Summarising leukocyte variation into a single total measure may therefore mask underlying effects, and it is important to

measure the concentrations of functionally distinct types of leukocytes to understand the immunological significance of total leukocyte variation.

Serum immunoglobulin G (IgG) is the most common antibody class found in mammalian blood and IgG concentration can be informative about levels of humoral immune defence. However, IgG concentration can be influenced by many factors: IgG is passed from mother to offspring via the placenta (Chucri et al. 2010) and colostrum (Day 2007); it is produced non-specifically as a form of constitutive innate immune defence (Ochsenbein & Zinkernagel 2000; Mauck et al. 2005; Whiteman et al. 2006; Ujvari et al. 2011); specifically in response to primary antigen exposure early in immune system ontogeny (Freitas et al. 1991); and in response to secondary antigen exposure as part of acquired humoural immune responses (Tizard 2009). Therefore, it is difficult to determine from a single sample why an individual might have a high IgG concentration relative to the rest of the population (Hall et al. 2002). However, studies can allow for a detailed interpretation of IgG concentration, by controlling as many of the above sources of variation as possible, and taking repeated measurements from known individuals across well-defined life history stages.

The phytohemagglutinin (PHA) response is one of the most commonly used measures of immune activity in ecological immunology (Demas et al. 2011b). PHA is used as a mitogen to stimulate the proliferation of lymphocytes *in vitro* and inflammation *in vivo*. When injected into bird wing patagia, PHA induces the infiltration of heterophils, lymphocytes, macrophages, basophils, eosinophils and thrombocytes into perivascular tissue (Martin et al. 2006c; Turmelle et al. 2010). The PHA response has also been measured in pinnipeds, (Hall et al. 1999; Drago et al. 2011), bats (Turmelle et al. 2010), deer (Fernandez-de-Mera et al. 2008) and toads (Brown et al. 2011), and induces comparable inflammatory responses in all of these taxa. *In vivo* PHA-induced inflammation has been associated with survival (Møller & Saino 2004) and shown to be costly: the increase in resting metabolic rate stimulated by PHA in the house sparrow was proportional to the energy required to produce half an egg (Martin et al. 2003). An advantage of this kind

of challenge technique over experimental infections is that they eliminate the possibility of immune evasion by the pathogens, which could confound the interpretation of immune dynamics (Graham et al. 2011). An objection to the use of PHA *in vivo* is that host responses to PHA may be too different from those to pathogens to be informative (Kennedy & Nager 2006), as PHA directly and indiscriminately activates lymphocytes, bypassing antigen processing, antigen presentation and other parts of the cell-mediated immune response cascade. However, if interpreted as an indicator of the "inducibility of pro-inflammatory signalling" at the time of challenge, the PHA response can provide valuable immunological insight (Vinkler et al. 2010). *In vivo*, many physiological processes are likely to influence this degree of inducibility, and the degree to which it is related to fitness-enhancing effects on pathogens is likely to vary amongst pathogens, hosts and stages of infection, which may explain why PHA studies have produced inconsistent results (Owen & Clayton 2007). Therefore, the key to the successful use of the PHA response as a methodological tool of ecological immunology is its careful interpretation in the context of other measures and what is known about the immune physiology of the response (Martin et al. 2006c; Vinkler et al. 2010).

The above three examples demonstrate how the choice of measures, sampling design and the consideration of underlying immune physiology can all increase the reliability of immune variation interpretation in ecological contexts. In addition to these considerations, there is consensus that the more measures that are used to quantify immunity (Martin et al. 2006b; Matson et al. 2006; Millet et al. 2007; Bradley & Jackson 2008; Boughton et al. 2011; Demas et al. 2011b), the better these measures represent different arms of the immune system (Schmid-Hempel & Ebert 2003; Martin et al. 2008), and the better these measures are suited to particular study systems (Graham et al. 2011), the more informative the results of ecological immunology studies will be. However, it is also acknowledged that striking the balance between relevance and feasibility remains a challenge for ecological immunologists (Graham et al. 2011).

Wild immunology

In contrast to traditional laboratory-based immunology, which aims to minimise natural variation as much as possible, ecological immunology explicitly targets it (Babayan et al. 2011). Despite this, many ecological immunology studies are conducted in controlled settings, such as those on wild-caught birds in temporary captivity. However, ecological immunology studies conducted in truly wild contexts have the potential to work in a constructively cyclical manner with laboratory-based immunology: immunological techniques developed in the laboratory can be used to describe natural variation in the wild, and laboratory experiments can be designed in the light of observations made in natural settings. This cycle drives the development of methods for quantifying immunity in wild organisms and the development of design and analysis frameworks for immunity data in ecological contexts (Graham et al. 2011). In turn, these developments make it possible to investigate the complex immune dynamics of wild systems in a holistic way (e.g. Graham et al. 2010), through the integration of genetics, behaviour, environmental stochasticity, pathogen diversity, co-infection dynamics and community ecology (Pedersen & Fenton 2007; Tompkins et al. 2010; Pedersen & Babayan 2011; Babayan et al. 2011). Such studies are recasting the relationship between immunity and ecology from a one-way scheme of ecological inputs and immunological outputs (Ottaviani et al. 2008; Schulenburg et al. 2009) into a cycle that allows feedback in both directions. This has brought ecological immunology closer to the field of disease ecology (Hawley & Altizer 2011) and allows for the investigation of the effects of immune variation on population-level ecological variation (e.g. Pedersen & Greives 2008).

Studies of immune dynamics in the wild are best carried out in species that have well described ecologies, have similar immune systems to laboratory model species and can be manipulated (Pedersen & Babayan 2011). These are the conditions under which such studies are most likely to yield insight into the underlying biological processes that link immunity to ecological variation and life history. However, there are two reasons why it may be worth stretching the methodological tools and analysis frameworks developed in

well-known systems such as mice (Pedersen & Greives 2008) and sheep (Graham et al. 2010) for use in lesser-known species. First, lesser-known species may have ecologies that facilitate the testing of particular hypotheses about immunity in the wild. For example, to test the effect of parasite prevalence on two aspects of humoural immunity in Ground finches (*Geospiza fuliginosa*), researchers made use of the isolation of the Galapagos archipelago and the categorical differences between Galapagos islands as a form of natural experiment (Lindström et al. 2004). Second, in endemic species that are threatened by disease, insight into immunity in an ecological context may have practical application to conservation. It has been shown, for example, also through the opportunistic use of the biogeographic variation across the Galapagos archipelago, that host genetic diversity has a significant effect on natural antibody concentration and parasite load in the endangered Galapagos hawk (*Buteo galapagoensis*; Whiteman et al. 2006). In other endangered species, such ecological immunology studies could be used to identify particularly vulnerable populations, times of year or idiosyncrasies of immune protection that could make a species or age class more vulnerable to a particular pathogen. These kinds of results could be used to increase the efficiency of disease surveillance and control programs and lower the chance that disease contributes to population declines and extinction. In these cases, it is important to ask whether the inherent costs of applying ecological immunology to little-known species, such as the lack of species-specific reagents (Pedersen & Babayan 2011), are worth the potential returns. This is an over-arching question addressed by this thesis, as through the first three data chapters it applies the tools and frameworks of ecological immunology to the Galapagos sea lion (*Zalophus wollebaeki*), a little-known species currently endangered by disease (Alava & Salazar 2006; Aurioles & Trillmich 2008; Levy et al. 2008).

The Galapagos sea lion

The Galapagos sea lion (*Zalophus wollebaeki*) is endemic to the Galapagos archipelago and has a small population (20,000-40,000 animals; Aurioles & Trillmich 2008) that is spatially and genetically structured amongst small

colonies (20-500 animals; Alava & Salazar 2006; Wolf et al. 2008). It has a polygynous mating system (Wolf et al. 2005; Pörschmann et al. 2011), is philopatric (Wolf & Trillmich 2007) and parental care is provided exclusively by the mother (Bowen et al. 2002). Its closest living relative and only extant congener is the California sea lion (*Zalophus californianus*), and their most recent common ancestor is estimated to have lived 2.3 ± 0.5 million years ago (Wolf et al. 2007a).

Galapagos sea lion ecology is defined by sensitivity to the unpredictable variation in ocean productivity that characterises the Galapagos marine ecosystem (Trillmich & Limberger 1985; Trillmich & Dellinger 1991). This variation causes the small Galapagos sea lion population to undergo stochastic decreases in size, through effects on the pupping probability of females and the survival of pups (Mueller et al. 2011; Mueller 2011). This sensitivity was one of the two reasons cited by the International Union for Conservation of Nature (IUCN) as justification for the classification of the Galapagos sea lion as endangered in 2008 (Aurioles & Trillmich 2008).

Female Galapagos sea lions reach sexual maturity at approximately six years old and produce a pup every 2.7 years on average (Mueller 2011). As income breeders, Galapagos sea lion mothers follow a cyclical attendance pattern: after remaining with their pup for 4 to 10 days following birth, they alternate 1-2 day foraging trips with 1-2 day periods of nursing (Boness & Bowen 1996; Trillmich & Wolf 2008). Galapagos sea lions are thought to wean at two to three years of age (Mueller et al. 2011), which is unusually late for an otariid. The prolongation of maturation in the Galapagos sea lion is thought to result from an interaction between the development of physiological diving capability and seasonal variation in food availability (Jeglinski et al. 2012).

As antibodies may be passed from mother to offspring via colostrum in mammals (Chucri et al. 2010), the mode and duration of nursing are important considerations relating to immunity in the Galapagos sea lion. There is evidence that antibodies are transferred in the colostrum of phocids (Ross et al. 1994), but there is little evidence that this occurs in otariids (Cavagnolo &

Vedros 1979; Castinel et al. 2008). However, given that the period during which bitches provide colostrum to their whelps and during which whelp digestive physiology alters to allow antibody absorption is short (Day 2007), it is unlikely that young Galapagos sea lions receive antibody-containing colostrum throughout nursing, especially when they begin to supplement their milk intake with prey that requires digestion.

Despite their geographical isolation, the Galapagos Islands are not exempt from the dangers of introduced diseases. As the number of tourists, residents and domesticated animals arriving on the archipelago each year increases, so does the threat of disease to resident wildlife (Kilpatrick et al. 2006). The endemism and small population sizes typical on the Galapagos mean that infectious disease could significantly increase the extinction risk of Galapagos species (Wikelski et al. 2004; Whiteman et al. 2006). Disease from domestic animals is a global threat to wild carnivores (Deem et al. 2000; Cunningham et al. 2003; Smith et al. 2009), including pinnipeds (e.g. McCarthy et al. 2007). A pathogen of particular concern in the case of the Galapagos sea lion is canine distemper virus (CDV) from dogs (Alava & Salazar 2006; Wolf et al. 2007a), but other pathogens such as *Leptospira* spp. (Lloyd-Smith et al. 2007; Norman et al. 2008; Zuerner et al. 2009), *Brucella* spp. (Foster et al. 2002), *Toxoplasma* spp. (Dubey et al. 2003; Miller et al. 2008) and *Streptococcus* spp. (Nico de Bruyn et al. 2008) may also pose risks. The threat of infectious disease from domesticated animals was the second reason cited by IUCN as justification for the classification of the Galapagos sea lion as endangered in 2008 (Aurioles & Trillmich 2008). Therefore, as immunity may play an important role in protection against emergent pathogens (Woolhouse et al. 2005), ecological immunology findings could support conservation efforts in the Galapagos sea lion by contributing to our understanding of how immune dynamics influence disease risk in this species and how policy makers and conservation practitioners can best reduce it.

The threat of infectious disease from domestic animals is especially high in a Galapagos sea lion colony on the island of San Cristobal, which is unique in its location in the centre of a rapidly growing town, Puerto Bazquerizo Moreno

(PBM; see Appendix 1.1 for photographs and satellite images). Galapagos sea lions are unusually fearless and in PBM they breed on a beach adjacent to the main street. This means that they come into contact with domestic dogs, cats, rats and mice, the populations of which in PBM are growing rapidly with the human population. Due to the geographical isolation of the Galapagos archipelago and the spatial aggregation of pinnipeds into colonies, the comparison of this singular colony at PBM with other colonies that are located in the protected zone of the Galapagos National Park provides an opportunity akin to a microcosmic natural experiment on the effects of anthropogenic influence on immunity in a wild mammal. In addition to their exposure to domesticated animals, the sea lions resident in the human-impacted colony of PBM on San Cristobal are exposed to pollution (Alava 2011). Despite the agriculture and tourism on San Cristobal, there is no evidence that the levels of chemical pollutants such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), dichlorodiphenyltrichloroethane (DDT) and hydrocarbons are present in the bay at higher than background levels (Alava et al. 2009, 2011). However, sewage from the town water system, which is contaminated with faecal coliform bacteria (Cordoba et al. 2008), is deposited in the bay, and higher than background concentrations of faecal coliform bacteria have been recorded there (Rodriguez & Valencia 2000; Alava 2011).

In contrast to findings in California and New Zealand sea lions, in which hookworm is a significant cause of pup mortality (Lyons et al. 1997; Lyons et al. 2005; Castinel et al. 2007), relatively little Galapagos sea lion mortality is currently associated with disease. Galapagos sea lion pups have been found to be infected with hookworms that have similar morphology to *Uncinaria* (Paras et al. 2003), a genus that infects other otariid species (Lyons et al. 2000, 2001; Nadler et al. 2000; Berón-Vera et al. 2004; Castinel et al. 2007; Spraker et al. 2007; Acevedo-Whitehouse et al. 2009), but they have not been described in the Galapagos sea lion and infections are rare (Paras et al. 2003). The only formally described helminth parasite of Galapagos sea lions is an eye fluke (*Philophthalmus zalophi*), which is prevalent in pups but has not been recorded in juveniles (Dailey et al. 2005). In general, pinnipeds

acquire lungworm (*Parafilaroides decorus*) infections when they begin feeding independently (Dailey 2001), and lungworms have been observed in a single dead 24-month-old Galapagos sea lion (Acevedo-Whitehouse, unpublished data). In addition, antibodies to Group A rotavirus have been detected in Galapagos sea lion pups, but whether this evidence of exposure has any clinical significance is unknown (Coria-Galindo et al. 2009). Lastly, Galapagos sea lions have been shown to host a species of mite (*Orthohalarachne diminuata*) and a species of louse (*Antarctophthirus microchir*), but there is no suggestion that these infections cause significant pathology (Dailey et al. 2005). Therefore, current Galapagos sea lion mortality due to disease appears to be low, and the disease threat cited by IUCN is founded on the possibility that new diseases emerge (Aurioles & Trillmich 2008).

Thesis aims

This thesis approaches the application of ecological immunology to the Galapagos sea lion in three stages, which correspond to the first three data chapters, and then addresses related epidemiological hypotheses in the fourth and final data chapter.

Chapter 2 aims to address the hypothesis that human activity influences immune system ontogeny in the Galapagos sea lion. To make this possible, Chapter 2 first describes immune system ontogeny over the first two years of life, and then compares the process in two colonies: PBM on San Cristobal where the human impacts described above are present, and a comparison colony where they are not. Chapter 2 predicts that immune activity and investment will be higher in the human-impacted colony, as sea lions are likely to be exposed to a greater number and diversity of pathogens where domestic animals and sewage are present than where they are not.

The aim of Chapter 3 is to address one of the major tenets of ecological immunology: the hypothesis that investment in immunity is costly because of trade-offs with other life history traits (Schmid-Hempel 2011b). Chapter 3 tests for a physiological cost associated with immune investment by assessing

correlations between changes in key measures of immunity and changes in measures of condition over time in known individuals. Chapter 3 predicts that individuals that invest more in immunity will have less energy and resources available for investment in growth and energy storage, and therefore that changes in immune measures will be negatively correlated with changes in condition.

Chapter 4 aims to put the immune variation described and analysed in the preceding data chapters into a genetic context by testing the hypothesis that inbreeding has a negative impact on immunity. Given the small population size and polygynous mating system of the Galapagos sea lion (Wolf et al. 2005; Aurioles & Trillmich 2008; Pörschmann et al. 2011), Chapter 4 predicts that inbreeding estimates will suggest that inbreeding is present in the Galapagos sea lion, and that inbreeding estimates will be negatively correlated with immune measures, as there is evidence from other systems that inbreeding increases infection risk in the wild (Coltman et al. 1999; Acevedo-Whitehouse et al. 2003a; Townsend et al. 2010).

Chapter 5 takes a different but related approach to that of ecological immunology by applying the tools of epidemiology to assess how domestic dog management could most effectively reduce disease risk to the Galapagos sea lion. In addition, Chapter 5 tests whether variation in ocean productivity could affect disease risk to the Galapagos sea lion through an effect on immunity, as food availability is known to affect immune function, which can have an impact on epidemiological dynamics. This analysis, therefore, represents a step towards a holistic assessment of disease risk in an endangered species that is informed by ecological immunology.

Chapter 6, the discussion, brings together the results of the data chapters and, in the context of the findings and limitations of the analyses, addresses the question of whether the Galapagos sea lion can contribute to ecological immunology by developing our understanding of the dynamics of immunity in the wild, and whether ecological immunology can contribute to the

conservation of the Galapagos sea lion by generating data that can be put to practical use by policy makers and conservation practitioners.

Chapter 2

Applying the tools of ecological immunology to the Galapagos sea lion

Abstract

This chapter applies the generalised techniques of ecological immunology to describe immune activity and dynamics in the endangered Galapagos sea lion (*Zalophus wollebaeki*), which is threatened simultaneously by disease from domestic animals and fluctuations in food supply driven by unpredictable environmental variation. It address the hypothesis that human activity influences immune system ontogeny by comparing immune activity from shortly after birth until 2 years of age between two Galapagos sea lion colonies, one heavily influenced by humans and the other on an uninhabited island. Controlling for ontogeny, immune activity was higher in the human-impacted colony, as assessed with both humoral and cellular immune components and cumulative and snapshot measures of immune activity. I discuss the possibility that sea lions in the human-impacted colony are under greater immunostimulatory pressure from their environment during development than those in the comparison colony, which could have implications for individual fitness, colony stability and disease risk. This chapter demonstrates the utility of a generalised and widely applicable approach to quantifying immune activity in a wild mammal and highlights important aspects of the system for targeted analysis and further study.

Introduction

Infectious disease threatens health, welfare and biodiversity around the world (Daszak et al. 2000; Lafferty & Gerber 2002; de Castro & Bolker 2005; Jones et al. 2008; Acevedo-Whitehouse & Duffus 2009). Immunity is the major line of defence against disease, and understanding the dynamics of immunity in the wild is key to predicting infectious disease emergence. As discussed in Chapter 1, the discipline of ecological immunology (Martin et al. 2011) is strengthening the link between laboratory and field studies on immunity (Pedersen & Babayan 2011), and developing design and analysis frameworks for dealing with immunity data in an ecological context (Graham et al. 2011). These developments pave the way for investigations of poorly-studied species in which immune variation may play a role in the design of conservation management programmes, and accurately direct future sampling and analyses. In this chapter I aimed to address the hypothesis that human activity influences immune system ontogeny in the Galapagos sea lion through the application of the methods of ecological immunology to describe immune variation in the context of development.

The analysis presented in this chapter quantified immune activity in two contrasting ecological contexts: a sea lion colony located in the town of Puerto Bazquerizo Moreno, San Cristobal, which is described in Chapter 1, and a sea lion colony on the island of Santa Fe, where there are no resident humans or domestic animals. Although Santa Fe is uninhabited, tourists visit it on day-trips and research scientists are permitted to camp there. Guides and National Park guards accompany tourists and scientists respectively, and ensure the protocols in place to minimise ecosystem damage are followed. The distance between the two study colonies is 49 km (Fig. 2.1), and it is likely that adult sea lions can move between them, given the genetic homogeneity amongst colonies in the central part of the archipelago (Wolf et al. 2008), and that satellite-tracked sea lions from another central colony (Caamaño) have been observed to haul out on multiple islands and at locations more than 49 km apart (Villegas-Amtmann et al. 2008, 2011).

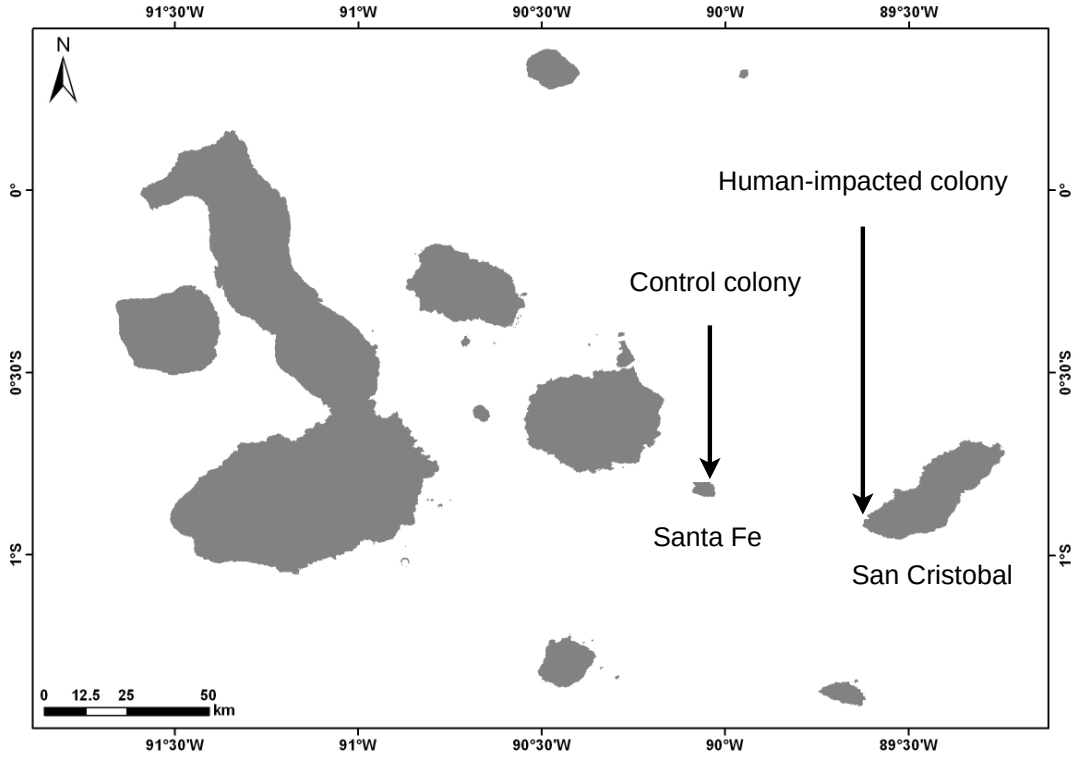

Figure 2.1 Map of the Galapagos Islands, showing the location of the study colonies at Puerto Bazquerizo Moreno, San Cristobal (human-impacted colony; 0°54'07" S, 89°36'44" W) and Bahia Paraiso, Santa Fe (control colony; 0°48'15" S, 90°02'28" W). See Appendix 1.1 for photographs and satellite images of the study colonies.

Following previous studies, this analysis aimed to incorporate the variation between developmental stages in young Galapagos sea lions into the sampling design (Trillmich & Wolf 2008; Mueller et al. 2011; Jeglinski et al. 2012). I sampled pups (defined here as younger than 6-months-old) shortly after birth and at 3-months-old, between which two ages they undergo a growth spurt (Mueller et al. 2011) and begin swimming for the first time. I sampled juveniles (defined here as 6-months-old or older) at: 6-months-old, when they are entirely dependent on their mothers for nutrition; 12-months-old, when they begin independent foraging but still receive milk from their mothers; 18-months-old, when they forage more independently but still receive some milk from their mothers; and at 24-months-old, when they are likely to be undergoing the transition to full independence (Jeglinski et al. 2012). Given that the majority of Galapagos sea lions are born between October and December, these age classes are linked to seasonal as well as ontogenetic differences: 6 and 18-month-olds are present during the warm season when food is relative scarce, while 12 and 24-month-olds are present during the cold season when food is relatively abundant (Jeglinski et al. 2012).

Therefore, the first two years of development in the Galapagos sea lion include various changes in physiology and environmental exposure that could have implications for disease and immunity. In this chapter, I aimed to describe immune activity in the context of this development in two colonies with different levels of human-impact, and to assess the evidence for, and implications of, any effect of human impact on Galapagos sea lion immunity. I predicted that immune activity and investment would be higher in the human-impacted colony, as sea lions are likely to be exposed to a greater number and diversity of pathogens and antigens where domestic animals and sewage are present than where they are not.

Methods

Sampling

I collected data in the sea lion colony and town of Puerto Bazquerizo Moreno, San Cristobal (human-impacted colony; 0°54'07" S, 89°36'44" W) and the sea lion colony of Bahia Paraiso, Santa Fe (control colony; 0°48'15" S, 90°02'28" W). As part of a Galapagos National Park team, I captured sea lions using hoop nets (Fuhrman Diversified, Texas, USA) and briefly restrained them in a prone position without the use of chemical immobilization, following Jeglinski et al. (2010). I sampled 30 6-month-old juveniles in each colony during April 2009 and marked them with the tagging method of a long-running Galapagos sea lion study (Wolf & Trillmich 2007). I re-sampled these juveniles at 12, 18 and 24 months of age. To compensate for disappearances, I sampled an additional 30 juveniles at 12 months of age, marked them by shaving and re-sampled them at 18 and 24 months. I sampled 30 pups shortly after birth in each colony during November 2009, marked them by shaving and re-sampled them 2 months later. In addition, I sampled a second cohort of 20 pups in each colony shortly after birth in November 2010, marked them by shaving and re-sampled them 2 months later. During the sampling period the mean census colony size was 518 individuals (n = 8) in the human-impacted colony and 325 individuals (n = 16) in the control colony (Actis 2012). A summary of the twelve physiological immune-related measures used to describe immune activity is presented in Table 2.1.

Table 2.1 Physiological immune-related measures used to describe immune activity in this study; see Boughton et al. (2011) and Demas et al. (2011b) for reviews on ecological immunology techniques.

	Description	References and examples
Immunoglobulin G (IgG)	Most common circulating antibody class in mammals; provides information on levels of humoural immune defence, including non-specific constitutively-produced natural antibody and specific antibody produced in response to antigen exposure	Avrameas 1991; Bowen et al. 1993; Ochsenbein & Zinkernagel 2000; Hall et al. 2002; Hall et al. 2003; Ferreira et al. 2005; Mauck et al. 2005; Ujvari et al. 2011; Apanius & Nisbet 2006; Day 2007; Castinel et al. 2008; Mills et al. 2009
Phytohemagglutinin (PHA) response *in vivo*	Inflammatory response to intradermal injection of lectin; PHA binds directly to lymphocytes, causing lymphocyte proliferation and a complex array of inter-linked physiological responses	Hall et al. 1999; Berger et al. 2005; Martin et al. 2006c; Fernandez-de-Mera et al. 2008; Turmelle et al. 2010; Brown et al. 2011; Drago et al. 2011
Total leukocytes	Many and varied roles in immunity; baseline levels vary between species; high levels can be associated with acute infection, low levels can be associated with chronic infection	Hall et al. 1997; Bossart et al. 2001; Kerr 2002; Nunn 2003; Matson et al. 2006; Hall et al. 2007; Buehler et al. 2008a; Keogh et al. 2010
Lymphocytes	Include natural killer (NK) cells, T-lymphocytes and B-lymphocytes; multiple roles in both acquired and innate immunity; high levels are associated with inflammation, acute bacterial infection and chronic viral infection	
Neutrophils	Short-lived cells that migrate quickly to sites of inflammation and engulf and phagocytize pathogens; high levels are generally associated with acute responses to bacterial infection; low levels are generally associated with viral infection	

Eosinophils	Role in defence against parasites by degranulation; high levels associated with macro-parasitic infection and allergic responses	
Total protein	Low levels associated with malnutrition and chronic disease; used in combination with hematocrit to diagnose clinical disorders in marine mammals	Kaymaz et al. 1999; Bossart et al. 2001; Nordøy & Thoresen 2002; Gray et al. 2005; Tella et al. 2008; Greig et al. 2010; Forsman et al. 2010
Albumin	Most common serum protein; low levels are associated with malnutrition and parasitic infection	
Alpha globulins	Include acute phase proteins (e.g. haptoglobin); high levels are associated with acute inflammatory disease	
Beta globulins	Include acute phase and immunomodulatory proteins (e.g. C-reactive protein, complement, transferrin, fibrinogen); high levels are associated with liver disease and parasitic infection	
Gamma globulins	Includes all circulating antibody classes; provides information on levels of humoural immune defence, including non-specific constitutively-produced natural antibody and specific antibody produced in response to antigen exposure	
Hematocrit	Packed cell volume (PCV); used in combination with total protein to diagnose clinical disorders in marine mammals	Bossart et al. 2001; Fair & Ricklefs 2002; Buehler et al. 2008b

Haematology

I took a single 7.5 mL blood sample from the caudal gluteal vein of each sea lion during each capture (Bossart et al. 2001). I stored 1.5 mL of each sample in ethylenediaminetetraacetic acid (EDTA) for the determination of total leukocyte concentration and hematocrit, and for blood smears. I allowed the remaining 6 mL to clot and then centrifuged it at 3000 rpm for 15 minutes to extract serum, which I stored at -80 °C. I fixed blood smears in 90 % methanol and stained them using May-Grunwald and Giemsa. I counted total leukocyte numbers using a hemocytometer (Neubauer, Philip Harris, UK) after diluting 20 µL of blood in 380 µL of Rees-Ecker solution (sodium citrate 3.8 g, formalin 40 % 0.2 mL, brilliant cresyl blue 0.1 g; Fisher Scientific, UK). I measured hematocrit using a portable centrifuge and microhematocrit reader (Hawksley, UK).

Serum proteins

I carried out serum protein electrophoresis using serum protein kits (SAS-MX, Helena Biosciences, UK) according to the manufacturer's instructions, except that I ran gels for 50 instead of 30 minutes to ensure full band separation, and diluted the barbital buffer 1:2 further than recommended to prevent over-heating. I scanned stained gels using a digital flatbed scanner (5590, Hewlett Packard, UK), imported images to *Quantity One* (BIO-RAD) for analysis, created lane and peak density profiles using the *Band Analysis Toolkit* and manually checked all peak designations and ranges. I identified serum protein fractions using migration distances relative to that of the albumin fraction following Gray et al. (2005) and Barsanti et al. (1977), and report peak intensities of serum protein fractions as proportions of the sum of all peak intensities in a sample. I quantified total serum protein using the biuret method (TP245, Randox, UK) according to the manufacturer's instructions and report total protein optical density as a proportion of the bovine serum positive control, which had a total protein concentration of 60.8 g L^{-1}.

Immunoglobulin G

I measured total immunoglobulin G (IgG) concentrations with a protein A ELISA as described in Hall et al. (2002) and report IgG optical density as a proportion of the dog reference serum positive control (RS10-105, Bethyl Laboratories, USA), which had a total immunoglobulin G concentration of 31 g L^{-1}. I assayed all samples in duplicate. The mean inter-assay coefficient of variation was 7.62 % (n = 24); the mean intra-assay coefficient of variation was 2.84 % (n = 24); and the mean duplicate coefficient of variation was 2.34 % (n = 587).

Phytohemagglutinin

During each sea lion capture I measured the thickness of the webbing that separates the second and third digits of the hind flippers at the point equidistant from both digits and 1 cm from the posterior edge of the flipper. I then administered 0.05 mL intra-dermal injections of 100 μg μL^{-1} phytohemagglutinin (PHA) solution (L8754, Sigma-Aldridge, UK) and phosphate buffered saline (PBS; P3813, Sigma-Aldridge, UK) at these points in the left and right hind flippers respectively. I recaptured sea lions as close to 24 hours later as possible and re-measured webbing thicknesses. I took all measurements to the nearest 0.01 mm using a thickness gauge (7/7309, Mitutoyo, UK) and repeated each measure three times. I calculated PHA response as the difference between the PBS-induced change in median thickness of the right flipper and the PHA-induced change in median thickness of the left flipper. To test whether prior exposure affected the magnitude of the PHA response in the Galapagos sea lion as it may do in birds (Tella et al. 2008), I also administered PHA to previously unchallenged control groups (n = 18, n = 20 and n = 10) alongside 3, 12 and 24-month-olds, each time judging age by visual comparison with the marked cohorts.

Statistical analysis

To test for differences in immune measures between colonies, I fitted linear mixed effects (LME) models to each of the 12 measures listed in Table 2.1, with colony, sex, age and the interaction between colony and age as

explanatory variables. I analysed pups and juveniles separately, and in order to account for the pseudoreplication implicit in repeated measures of the same individuals, I included individual identity as a random effect in all models (Crawley 2007). In addition, I entered cohort identity as a random effect into models of juvenile data to account for the additional juveniles sampled at 12 months of age, and year as a random effect into models of pup data.

I omitted data on albumin, alpha globulin, beta globulin and gamma globulin concentrations from 24-month-old juveniles, as sample sizes for these measures were too small in 24-month-olds to generate stable parameter estimates. All ages included in final models were represented by at least eight data points per colony. I fitted models with maximum likelihood and compared those with and without interactions with likelihood ratio tests (Crawley 2007). I checked all models for signs of heteroscedasticity, heterogeneity of variance, non-normality of error and the disproportionate influence of outliers (Zuur et al. 2009), log-transformed response variables when necessary to normalize model residuals, and carried out all analyses in R 2.11.1 (R Development Core Team 2010).

I fitted time to recapture and an interaction between time to recapture and colony as additional fixed effects in the PHA models, since it was not always possible to capture sea lions 24 hours after administering the PHA challenge. Time to recapture may affect the magnitude of the inflammation response as different immune components are recruited to the site of challenge at different times following injection (Martin et al. 2006c; Turmelle et al. 2010). In addition, time to recapture could influence the effect of any stress response to capture on the inflammation induced by the PHA challenge (Fernandez-de-Mera et al. 2009). I tested for an effect of prior exposure on the PHA response by fitting analysis of variance (ANOVA) models to the subset of relevant data with previous exposure as a two-level factor, sex, colony and time to recapture as explanatory variables. Year was not included as an explanatory variable in the pup model, as this comparison was only conducted in 2010.

Selective disappearance can mask or masquerade as an effect of age in datasets that follow cohorts from which there is drop-out (Graham et al. 2010). I tested for the potentially confounding influence of selective disappearance in juveniles with t-tests comparing mean levels of immune measures at 6 months between sea lions that were still present at 24 months and those that were absent. I performed a similar analysis in pups, testing for an association between immune activity shortly after birth and pup presence at 3 months, but with ANOVA so that year could be included as an additional explanatory variable.

As little is known about maternal antibody transfer in the Galapagos sea lion (Coria-Galindo et al. 2009), I investigated the effect of suckling on pup IgG concentrations. I took additional 1 mL blood samples 24 hours after the first sample from 30 pups in 2010, 18 of which were suckling during the intervening 24-hour period and 12 of which were not. I tested for an effect of suckling as a two-level factor on the increase in IgG over 24 hours with an ANOVA, including colony and sex as additional explanatory variables.

In order to gain insight into within-individual variation in antibody diversity, I analysed the shape of gamma globulin serum protein electrophoresis traces in juveniles. Tall, sharp gamma globulin peaks suggest low diversity gamma globulin populations (Tizard 2009). First, I fitted an LME model with area under the gamma globulin curve as the response, with gamma globulin curve peak intensity, colony, sex, age and the interaction between peak intensity and colony as fixed effects, and cohort and individual identity as random effects. I included the interaction to test whether there was a colony difference in the area under the curve per unit of intensity, i.e. whether there was a difference in gamma globulin diversity given concentration. I tested the significance of the interaction by comparing models with and without it using a likelihood ratio test. Second, to approach the problem of testing for different curve shapes between colonies in another way, I calculated the length of the base of a triangular approximation of each gamma globulin curve by doubling the area under the curve and dividing by peak intensity. Then I fitted an LME model with base length as the response and colony, sex and age as fixed

effects, including cohort and individual as random effects. I tested the significance of colony as an explanatory variable by comparing models with and without it using a likelihood ratio test.

Immune measure covariation

The relationships amongst immune measures of the kind used in this study are complex and these kinds of measures may describe variation at too coarse a resolution to detect evolutionary trade-offs within the immune system (Matson et al. 2006). However, comparing the dynamic relationships amongst these measures using datasets in which cohorts are followed through time can offer insight into the physiological activity of different parts of the immune system in relation to one another over short timescales (Buehler et al. 2011).

The aim of this analysis of covariation was to identify the relationships between immune measures that were most different between colonies, without calculating an unjustifiably large number of *p*-values. First, where necessary, I transformed variables so they were normally distributed (Shapiro-Wilk, *p*-value > 0.5). Six and three out of 12 variables could not be transformed in this way in pups and juveniles respectively, so I conducted this analysis using nine variables in juveniles only. I calculated Pearson product-moment correlation coefficients for all pairwise relationships between the nine variables for each colony separately and took the difference in correlation coefficient between colonies. Then I tested for a statistical difference between the slopes of the colonies for the five pairwise relationships with the largest correlation coefficient differences. I did this by fitting linear mixed effect models in which one immune measure was fitted as the response and the other as a fixed effect as part of an interaction with colony, and tested the significance of interactions using likelihood ratio tests. I fitted age and sex as additional fixed effects and individual identity and cohort as random effects. For the calculation of the correlation coefficients, I reduced the data set to include only those records that were complete for all nine measures, but I fitted the targeted linear mixed effects models to more complete datasets, from which I only removed records missing data for the two immune

measures being compared in each model. Therefore, this approach somewhat ameliorates the problem of missing data.

Results

PHA response and the concentrations of IgG, eosinophils and albumin varied between colonies in pups, and with the exception of albumin, these differences were only evident in older pups (Fig. 2.2 and Table 2.2). Pups from the human-impacted colony had higher levels of all of these measures. In juveniles, there were colony differences in PHA response and concentrations of total leukocytes, eosinophils, albumin, alpha globulins, beta globulins and gamma globulins (Fig. 2.2). Juvenile colony differences were not consistent across ages, but when they were present, juveniles from the human-impacted colony had higher values of all measures except albumin concentration (Table 2.3).

Pup age influenced PHA response and the concentrations of IgG, total leukocytes, neutrophils, eosinophils, total protein and beta globulins (Fig. 2.2). These age-related changes were positive for serum proteins and negative for white blood cells. Pup PHA response increased with age in the human-impacted colony but did not change with age in the control colony (Fig. 2.2b). The only measures related to pup sex were IgG and PHA: male pups had higher PHA responses while female pups had higher IgG concentrations (Table 2.2).

In juveniles, total leukocyte, eosinophil, albumin, beta and gamma globulin concentrations changed with age (Fig. 2.2). Total leukocyte and eosinophil concentrations peaked at 12 and 24 months in the human-impacted colony but only at 24 months in the control colony (Figs 2.2c and f, Table 2.3). The concentrations of beta and gamma globulins increased from 6 to 18 months (Figs 2.2j-k), and juvenile males had lower total leukocyte, neutrophil and albumin concentrations than juvenile females (Table 2.3). Time to recapture had a negative effect on pup PHA response in both colonies (Table 2.2) and in juveniles from the human-impacted colony, but no effect in juveniles from the control colony (Table 2.3).

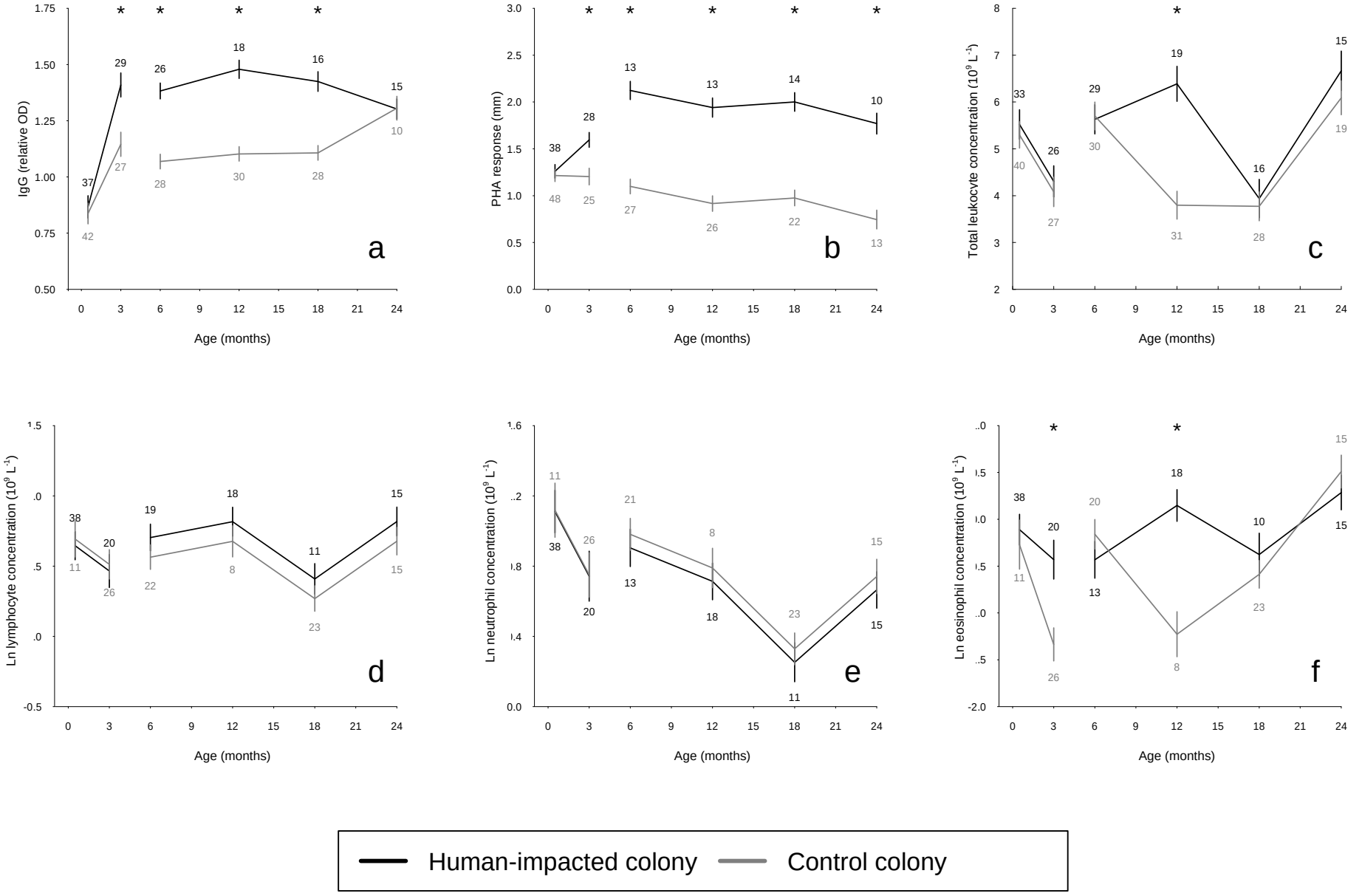
IgG (relative OD)
Age (months)
a
PHA response (mm)
b
Total leukocyte concentration (10^9 L^{-1})
c
Ln lymphocyte concentration (10^9 L^{-1})
d
Ln neutrophil concentration (10^9 L^{-1})
e
Ln eosinophil concentration (10^9 L^{-1})
f
Human-impacted colony
Control colony

Figure 2.2

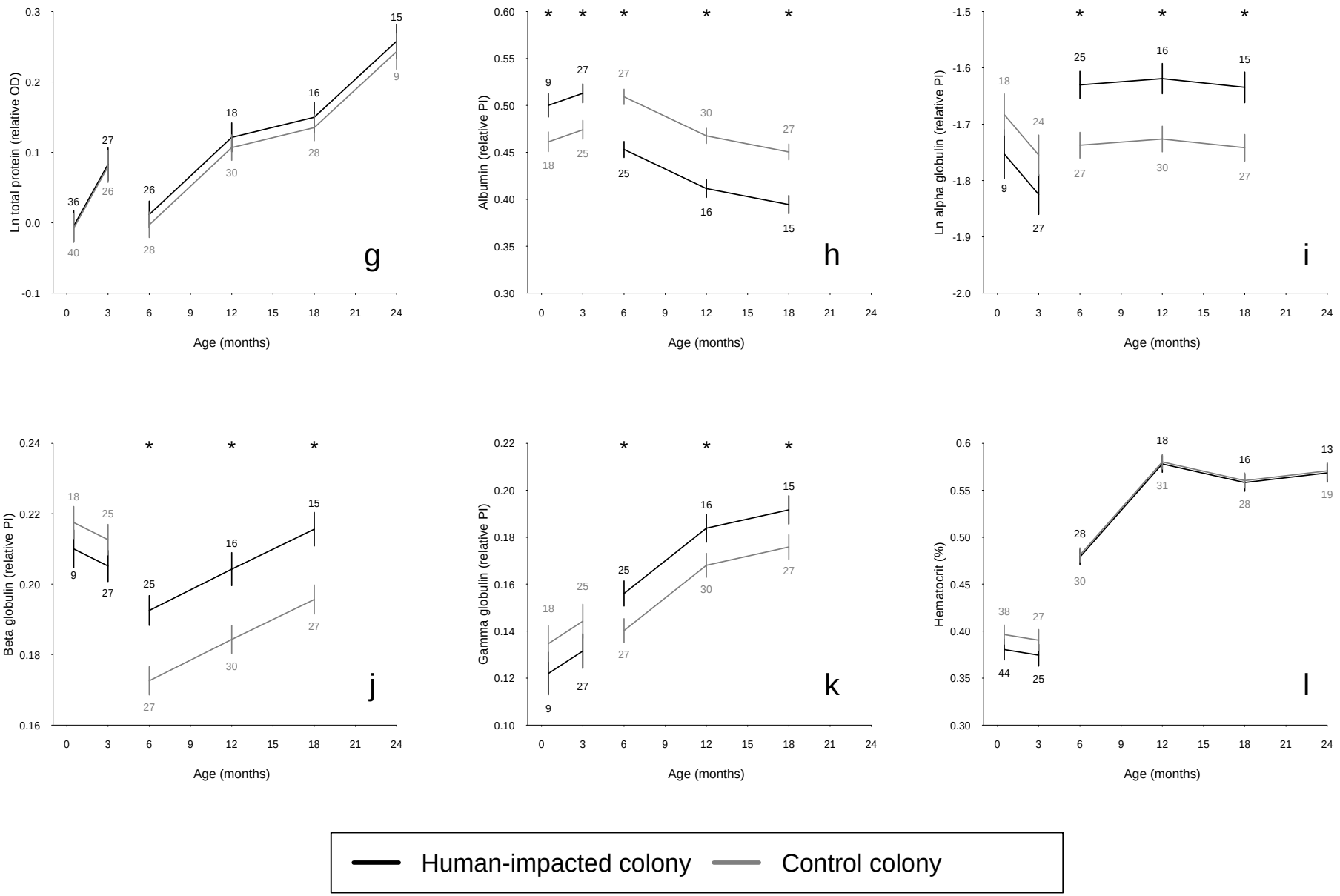

Figure 2.2 (cont.)

Figure 2.2 (legend) Predictions from LME models of 12 immune-related physiological measures in two Galapagos sea lion colonies over the first 2 years of life. Black lines represent the human-impacted colony, grey lines represent the control colony; a) immunoglobulin G concentration (relative OD); b) PHA response (mm); c) total leukocyte concentration (10^9 L^{-1}); d) lymphocyte concentration (10^9 L^{-1}); e) neutrophil concentration (10^9 L^{-1}); f) eosinophil concentration (10^9 L^{-1}); g) total protein concentration (relative OD); h) albumin concentration (relative PI); i) alpha globulin concentration (relative PI); j) beta globulin concentration (relative PI); k) gamma globulin concentration (relative PI); l) hematocrit (%). Plotted estimates are for females and for mean time to recapture in the case of PHA; 'relative OD' = optical density relative to control; 'relative PI' = relative peak intensity; 'Ln' = natural logarithm. Data on serum proteins in 24-month-olds were omitted. All included time points were represented by at least eight data points per colony. See Tables 2.2 and 2.3 for model details.

Table 2.2 Full linear mixed effects models describing variation in 12 immune-related physiological measures from shortly after birth until 3 months of age; 'relative OD' = optical density relative to control; 'relative PI' = relative peak intensity; 'Ln' = natural logarithm. Likelihood ratio tests (LRTs) compared models that included the interaction between colony and age with those that did not.

Immunoglobulin G (relative OD)	Estimate	SE	*t*	*p*
(Intercept)	0.867	0.050	17.451	<0.0001
Colony (control)	-0.029	0.059	-0.503	0.617
Age (3 months)	0.542	0.064	8.420	<0.0001
Sex (male)	-0.134	0.045	-2.988	0.004
Colony (control) * Age (3 months)	-0.234	0.091	-2.581	0.012
N (total) = 135; N (individuals) = 83				
Random: year SD <0.0001, individual SD <0.0001, residual SD = 0.254				
LRT: $\chi^2 = 6.748$, $p = 0.009$				

PHA response (mm)	Estimate	SE	*t*	*p*
(Intercept)	1.260	0.135	9.345	<0.0001
Colony (control)	-0.045	0.084	-0.537	0.593
Age (3 months)	0.336	0.096	3.505	0.001
Sex (male)	0.152	0.065	2.327	0.022
Time to recapture (days)	-0.264	0.106	-2.504	0.016
Colony (control) * Age (3 months)	-0.346	0.139	-2.483	0.016
N (total) = 139; N (individuals) = 86				
Random: year SD < 0.0001, individual SD <0.0001, residual SD = 0.373				
LRT: $\chi^2 = 6.299$, $p = 0.012$				

Total leukocytes (10^9 L^{-1})	Estimate	SE	*t*	*p*
(Intercept)	5.520	0.330	16.749	<0.0001
Colony (control)	-0.224	0.336	-0.665	0.507
Age (3 months)	-1.215	0.282	-4.312	<0.0001
Sex (male)	0.081	0.336	0.240	0.811
N (total) = 126; N (individuals) = 79				
Random: year SD <0.0001, individual SD = 0.828, residual SD = 1.489				
LRT: $\chi^2 = 0.138$, $p = 0.710$				

Ln lymphocytes (10^9 L^{-1})	Estimate	SE	*t*	*p*
(Intercept)	0.699	0.207	3.373	0.001
Colony (control)	-0.149	0.117	-1.268	0.208
Age (3 months)	-0.212	0.114	-1.867	0.077
Sex (male)	-0.045	0.104	-0.433	0.666
N (total) = 95; N (individuals) = 75				
Random: year SD = 0.251, individual SD = 0.224, residual SD = 0.414				
LRT: $\chi^2 = 0.943$, $p = 0.331$				

Ln neutrophils (10^9 L^{-1})	Estimate	SE	*t*	*p*
(Intercept)	1.111	0.129	8.614	<0.0001
Colony (control)	0.007	0.147	0.051	0.959
Age (3 months)	-0.368	0.117	-3.144	0.005
Sex (male)	-0.083	0.139	-0.598	0.552

N (total) = 95; N (individuals) = 75

Random: year SD <0.0001, individual SD = 0.415, residual SD = 0.453

LRT: $\chi^2 = 0.011$, $p = 0.917$

Ln eosinophils (10^9 L^{-1})	Estimate	SE	*t*	*p*
(Intercept)	-0.111	0.168	-0.660	0.511
Colony (control)	-0.160	0.278	-0.576	0.567
Age (3 months)	-0.320	0.181	-1.769	0.093
Sex (male)	-0.120	0.180	-0.667	0.507
Colony (control) * Age (3 months)	-0.745	0.343	-2.167	0.033

N (total) = 95; N (individuals) = 75

Random: year SD <0.0001, individual SD = 0.515, residual SD = 0.598

LRT: $\chi^2 = 4.254$, $p = 0.039$

Ln total protein (relative OD)	Estimate	SE	*t*	*p*
(Intercept)	-0.005	0.022	-0.212	0.833
Colony (control)	-0.003	0.021	-0.141	0.887
Age (3 months)	0.088	0.022	3.998	<0.0001
Sex (male)	-0.009	0.022	-0.401	0.689

N (total) = 129; N (individuals) = 83

Random: year SD <0.0001, individual SD = 0.008, residual SD = 0.120

LRT: $\chi^2 = 1.112$, $p = 0.291$

Albumin (relative PI)	Estimate	SE	*t*	*p*
(Intercept)	0.500	0.012	40.333	<0.0001
Colony (control)	-0.038	0.011	-3.580	0.007
Age (3 months)	0.013	0.011	1.122	0.281
Sex (male)	0.006	0.011	0.522	0.604

N (total) = 78; N (individuals) = 64

Random, year SD <0.0001, individual SD <0.0001, residual SD = 0.045

LRT: $\chi^2 = 0.072$, $p = 0.788$

Ln alpha globulin (relative PI)	Estimate	SE	*t*	*p*
(Intercept)	-1.753	0.044	-40.184	<0.0001
Colony (control)	0.070	0.038	1.811	0.075
Age (3 months)	-0.072	0.039	-1.834	0.090
Sex (male)	0.049	0.038	1.268	0.210

N (total) = 78; N (individuals) = 64

Random: year SD <0.0001, individual SD = 0.052, residual SD = 0.151

LRT: $\chi^2 = 0.606$, $p = 0.436$

Beta globulin (relative PI)	Estimate	SE	*t*	*p*
(Intercept)	0.210	0.012	17.224	<0.0001
Colony (control)	0.007	0.004	1.779	0.080
Age (3 months)	-0.005	0.005	-1.002	0.333
Sex (male)	-0.003	0.004	-0.799	0.428

N (total) = 78; N (individuals) = 64

Random: year SD = 0.015, individual SD <0.0001, residual SD = 0.017

LRT: $\chi^2 = 0.029$, $p = 0.865$

Gamma globulin (relative PI)	Estimate	SE	*t*	*p*
(Intercept)	0.122	0.009	13.315	<0.0001
Colony (control)	0.012	0.008	1.562	0.123
Age (3 months)	0.010	0.008	1.194	0.252
Sex (male)	-0.008	0.008	-0.960	0.341

N (total) = 78; N (individuals) = 64

Random: year SD <0.0001, individual SD = 0.013, residual SD = 0.031

LRT: $\chi^2 = 2.521$, $p = 0.112$

Hematocrit (%)	Estimate	SE	*t*	*p*
(Intercept)	0.380	0.016	23.256	<0.0001
Colony (control)	0.016	0.012	1.342	0.183
Age (3 months)	-0.006	0.010	-0.614	0.542
Sex (male)	0.021	0.012	1.778	0.079

N (total) = 134; N (individuals) = 83

Random, year SD = 0.015, individual SD = 0.033, residual SD =0.051

LRT: $\chi^2 = 0.0009$, $p = 0.975$

Table 2.3 Full linear mixed effects models describing variation in 12 immune-related physiological measures from 6 to 24 months of age; 'relative OD' = optical density relative to control; 'relative PI' = relative peak intensity; 'Ln' = natural logarithm. Likelihood ratio tests (LRTs) compared models that included the interaction between colony and age with those that did not; the additional likelihood ratio test for PHA response compared a model that included an interaction between colony and time to recapture with one that did not.

Immunoglobulin G (relative OD)	Estimate	SE	*t*	*p*
(Intercept)	1.382	0.036	38.627	<0.0001
Colony	-0.313	0.044	-7.128	<0.0001
Age (12 months)	0.096	0.042	2.296	0.024
Age (18 months)	0.042	0.043	0.974	0.333
Age (24 months)	-0.080	0.045	-1.787	0.078
Sex (male)	0.005	0.031	0.173	0.863
Colony (control) * Age (12 months)	-0.062	0.057	-1.098	0.275
Colony (control) * Age (18 months)	-0.004	0.058	-0.061	0.951
Colony (control) * Age (24 months)	0.317	0.068	4.659	<0.0001
N (total) = 171; n (individuals) = 85				
Random: cohort SD <0.0001, individual SD = 0.096, residual SD = 0.126				
LRT: χ^2 = 30.016, *p* = <0.0001				

PHA response (mm)	Estimate	SE	*t*	*p*
(Intercept)	2.052	0.185	11.106	<0.0001
Colony	-0.926	0.214	-4.312	0.0001
Age (12 months)	-0.140	0.090	-1.564	0.123
Age (18 months)	-0.098	0.092	-1.056	0.295
Age (24 months)	-0.133	0.104	-1.281	0.205
Sex (male)	0.086	0.089	0.966	0.337
Time to recapture (days)	-0.391	0.086	-4.564	<0.0001
Colony (control) * time to recapture (days)	0.459	0.118	3.872	<0.0001
N (total) = 138; n (individuals) = 72				
Random: cohort SD <0.0001, individual SD = 0.227, residual SD = 0.357				
LRT (1): χ^2 = 4.108, *p* = 0.25; LRT (2): χ^2 = 14.810, *p* = <0.0001				

Total leukocytes (10^9 L^{-1})	Estimate	SE	*t*	*p*
(Intercept)	5.631	0.312	18.024	<0.0001
Colony	0.071	0.406	0.174	0.862
Age (12 months)	0.757	0.458	1.652	0.102
Age (18 months)	-1.692	0.484	-3.498	0.001
Age (24 months)	1.036	0.494	2.097	0.039
Sex (male)	-0.549	0.232	-2.363	0.020
Colony (control) * Age (12 months)	-2.660	0.607	-4.381	<0.0001
Colony (control) * Age (18 months)	-0.236	0.633	-0.374	0.710
Colony (control) * Age (24 months)	-0.648	0.672	-0.964	0.338
N (total) = 187; n (individuals) = 88				
Random: cohort SD <0.0001, individual SD <0.0001, residual SD = 1.514				
LRT: χ^2 = 21.874, *p* = <0.0001				

Ln lymphocytes (10^9 L^{-1})	Estimate	SE	*t*	*p*
(Intercept)	0.700	0.100	6.993	<0.0001
Colony	-0.132	0.092	-1.429	0.157
Age (12 months)	0.061	0.112	0.548	0.586
Age (18 months)	-0.290	0.102	-2.838	0.006
Age (24 months)	0.200	0.105	1.909	0.061
Sex (male)	0.031	0.091	0.337	0.737

N (total) = 131; n (individuals) = 72

Random: cohort SD <0.0001, individual SD = 0.201, residual SD = 0.404

LRT: $\chi^2 = 2.456$, $p = 0.483$

Ln neutrophils (10^9 L^{-1})	Estimate	SE	*t*	*p*
(Intercept)	0.904	0.185	4.895	<0.0001
Colony	0.076	0.098	0.776	0.440
Age (12 months)	-0.191	0.129	-1.483	0.144
Age (18 months)	-0.652	0.115	-5.658	<0.0001
Age (24 months)	-0.240	0.116	-2.076	0.043
Sex (male)	-0.190	0.082	-2.311	0.024

N (total) = 124; n (individuals) = 72

Random: cohort SD = 0.173, individual SD <0.0001, residual SD = 0.433

LRT: $\chi^2 = 1.498$, $p = 0.682$

Ln eosinophils (10^9 L^{-1})	Estimate	SE	*t*	*p*
(Intercept)	-0.432	0.197	-2.195	0.032
Colony	0.272	0.238	1.143	0.257
Age (12 months)	0.579	0.240	2.409	0.020
Age (18 months)	0.056	0.279	0.200	0.843
Age (24 months)	0.717	0.250	2.866	0.006
Sex (male)	-0.175	0.127	-1.376	0.173
Colony (control) * Age (12 months)	-1.645	0.367	-4.475	0.0001
Colony (control) * Age (18 months)	-0.483	0.345	-1.403	0.168
Colony (control) * Age (24 months)	-0.047	0.337	-0.139	0.890

N (total) = 122; n (individuals) = 71

Random: cohort SD <0.0001, individual SD = 0.127, residual SD = 0.628

LRT: $\chi^2 = 23.683$, $p = <0.0001$

Ln total protein (relative OD)	Estimate	SE	*t*	*p*
(Intercept)	0.012	0.020	0.595	0.554
Colony	-0.014	0.018	-0.803	0.424
Age (12 months)	0.110	0.021	5.265	<0.0001
Age (18 months)	0.138	0.021	6.471	<0.0001
Age (24 months)	0.246	0.026	9.576	<0.0001
Sex (male)	-0.013	0.018	-0.747	0.457

N (total) = 170; n (individuals) = 85

Random: cohort SD <0.0001, individual SD = 0.032, residual SD = 0.101

LRT: $\chi^2 = 2.385$, $p = 0.496$

Albumin (relative PI)	Estimate	SE	*t*	*p*
(Intercept)	0.453	0.008	53.040	<0.0001
Colony	0.056	0.008	6.919	<0.0001
Age (12 months)	-0.041	0.009	-4.399	0.0001
Age (18 months)	-0.058	0.009	-6.062	<0.0001
Sex (male)	0.022	0.007	2.765	0.007

N (total) = 140; n (individuals) = 83

Random: cohort SD <0.0001, individual SD <0.0001, residual SD = 0.045
LRT: $\chi^2 = 0.169$, $p = 0.918$

Ln alpha globulin (relative PI)	Estimate	SE	*t*	*p*
(Intercept)	-1.630	0.026	-62.431	<0.0001
Colony	-0.107	0.025	-4.143	0.0001
Age (12 months)	0.011	0.024	0.454	0.651
Age (18 months)	-0.004	0.024	-0.181	0.856
Sex (male)	-0.024	0.025	-0.976	0.332

N (total) = 140; n (individuals) = 83

Random: cohort SD <0.0001, individual SD = 0.067, residual SD = 0.109
LRT: $\chi^2 = 0.032$, $p = 0.984$

Beta globulin (relative PI)	Estimate	SE	*t*	*p*
(Intercept)	0.192	0.004	45.609	<0.0001
Colony	-0.019	0.004	-4.983	<0.0001
Age (12 months)	0.011	0.004	2.513	0.014
Age (18 months)	0.023	0.004	4.827	<0.0001
Sex (male)	-0.006	0.003	-1.747	0.084

N (total) = 140; n (individuals) = 83

Random: cohort SD <0.0001, individual SD <0.0001, residual SD = 0.022
LRT: $\chi^2 = 2.917$, $p = 0.232$

Gamma globulin (relative PI)	Estimate	SE	*t*	*p*
(Intercept)	0.156	0.005	29.118	<0.0001
Colony	-0.015	0.005	-3.114	0.002
Age (12 months)	0.027	0.005	4.692	<0.0001
Age (18 months)	0.035	0.006	5.868	<0.0001
Sex (male)	-0.008	0.004	-1.783	0.078

N (total) = 140; n (individuals) = 83

Random: cohort SD <0.0001, individual SD <0.0001, residual SD = 0.028
LRT: $\chi^2 = 1.701$, $p = 0.4273$

Hematocrit (%)	Estimate	SE	*t*	*p*
(Intercept)	0.479	0.008	60.155	<0.0001
Colony	0.002	0.007	0.301	0.763
Age (12 months)	0.099	0.009	10.879	<0.0001
Age (18 months)	0.079	0.009	8.442	<0.0001
Age (24 months)	0.090	0.010	8.684	<0.0001
Sex (male)	0.010	0.007	1.356	0.179

N (total) = 183; n (individuals) = 88
Random: cohort SD <0.0001, individual SD <0.0001, residual SD = 0.045
LRT: $\chi^2 = 1.443$, $p = 0.695$

In the control colony, mean values of immune measures at 6 months did not vary ($p > 0.05$) between juveniles that were present at 24 months (n = 10) and those that were absent (n = 20). However, in the human-impacted colony, hematocrit ($t_{1,27} = 2.44$, $p = 0.02$), total protein ($t_{1,24} = 2.63$, $p = 0.01$) and PHA response ($t_{1,15} = -2.62$, $p = 0.01$) differed between the juveniles present (n = 15) and those absent at 24 months (n = 15). In the cases of total protein and hematocrit, sea lions that were present at 24 months had relatively low values when 6-months-old. However, sea lions that were present at 24 months had relatively high PHA responses at 6 months. In pups, immune measures shortly after birth did not vary between those that were present and those that were absent at 3 months in either colony ($p > 0.05$). However, drop-out from the pup cohort was relatively low compared with that from the juvenile cohort: only nine of the 67 pups sampled in the human-impacted colony and 22 of the 76 sampled in the control colony shortly after birth were not present at 3 months of age.

Prior exposure to PHA did not influence PHA response in any of the age classes tested (3 months: $t_{1,32} = -0.07$, $p = 0.93$; 12 months: $t_{1,21} = 1.74$, $p = 0.09$; 24 months: $t_{1,11} = -0.11$, $p = 0.92$). The mean increase in IgG over 24 hours was equivalent to 0.233 g L^{-1} when converted with the dog reference serum positive control, and suckling had no effect on change in IgG ($t_{1,26} = 0.51$, $p = 0.61$). There was an interactive effect of gamma globulin peak intensity with colony on juvenile gamma globulin peak area. In the control colony, gamma globulin curves had higher areas per unit of intensity ($N_{total} = 155$, $N_{individuals} = 84$; estimated human-impacted colony slope = 4.67, SE = 0.29; estimated colony difference in slope = 1.42, SE = 0.47; $\chi^2 = 9.12$, $p = 0.0025$). There was also a colony difference in base length: triangles approximating gamma globulin curves in the control colony had significantly longer bases than those in the human-impacted colony ($N_{total} = 155$, $N_{individuals} = 84$; estimate = 0.72, SE = 0.31; $\chi^2 = 5.29$, $p = 0.0214$).

The differences in correlation coefficient between colonies for each pairwise immune measure relationship in juveniles are shown in Table 2.4. Two of these colony differences were estimated as significant by the targeted LME

model analyses (Table 2.5). These analyses showed that the slope of the relationship between IgG and lymphocyte concentration was negative in the human-impacted colony but positive in the control colony, and that the slope of the relationship between IgG and alpha globulin concentration was positive in the human-impacted colony but negative in the control colony (Fig. 2.3 and Table 2.6).

Table 2.4 Colony differences in correlation coefficient for all pairwise relationships between 9 immune measures in juveniles. IgG = total immunoglobulin G (relative OD); PHA = PHA response (mm); LYM = Lymphocytes (10^9 L^{-1}); NEU = Neutrophils (10^9 L^{-1}); EOS = Eosinophils (10^9 L^{-1}); ALB = Albumin (relative PI); ALP = Alpha globulin (relative PI); BET = Beta globulin (relative PI); GAM = Gamma globulin (relative PI); "relative OD" denotes optical density relative to control; "relative PI" denotes relative peak intensity. The five largest differences are in bold.

	IgG	PHA	LYM	NEU	EOS	ALB	ALP	BET	GAM
IgG	-	-	-	-	-	-	-	-	-
PHA	-0.30	-	-	-	-	-	-	-	-
LYM	**-0.59**	**0.34**	-	-	-	-	-	-	-
NEU	-0.31	0.26	-0.06	-	-	-	-	-	-
EOS	-0.23	-0.11	0.16	0.05	-	-	-	-	-
ALB	-0.22	0.03	0.13	-0.07	0.24	-	-	-	-
ALP	**0.44**	-0.14	-0.27	-0.28	-0.13	-0.31	-	-	-
BET	0.08	**0.35**	0.25	0.05	-0.17	0.21	0.15	-	-
GAM	-0.04	-0.19	-0.19	0.28	-0.15	0.13	0.20	**-0.44**	-

Table 2.5 Linear mixed effect models testing for colony differences in the five relationships highlighted by the correlation analysis (see Table 2.4). IgG = total immunoglobulin G (relative OD); PHA = PHA response (mm); LYM = Lymphocytes (10^9 L^{-1}); ALP = Alpha globulin (relative PI); BET = Beta globulin (relative PI); GAM = Gamma globulin (relative PI); "relative OD" denotes optical density relative to control; "relative PI" denotes relative peak intensity; "Ln" denotes natural logarithm. Sample sizes are given as the number of data points followed by the number of individuals. See Table 2.6 for full models.

Model	*r* difference	N	Slope difference	SE	*t*	*p*
IgG ~ LYM * Colony	-0.59	122, 68	0.132	0.037	3.545	0.009**
IgG ~ ALP * Colony	0.44	137, 73	-3.196	0.949	-3.366	0.001**
GAM ~ Ln (BET) * Colony	-0.44	156, 84	0.059	0.034	1.701	0.093
PHA ~ Ln (BET) * Colony	0.35	117, 70	-0.639	0.687	-0.931	0.357
PHA ~ LYM * Colony	0.34	95, 56	-0.179	0.111	-1.626	0.113

Figure 2.3

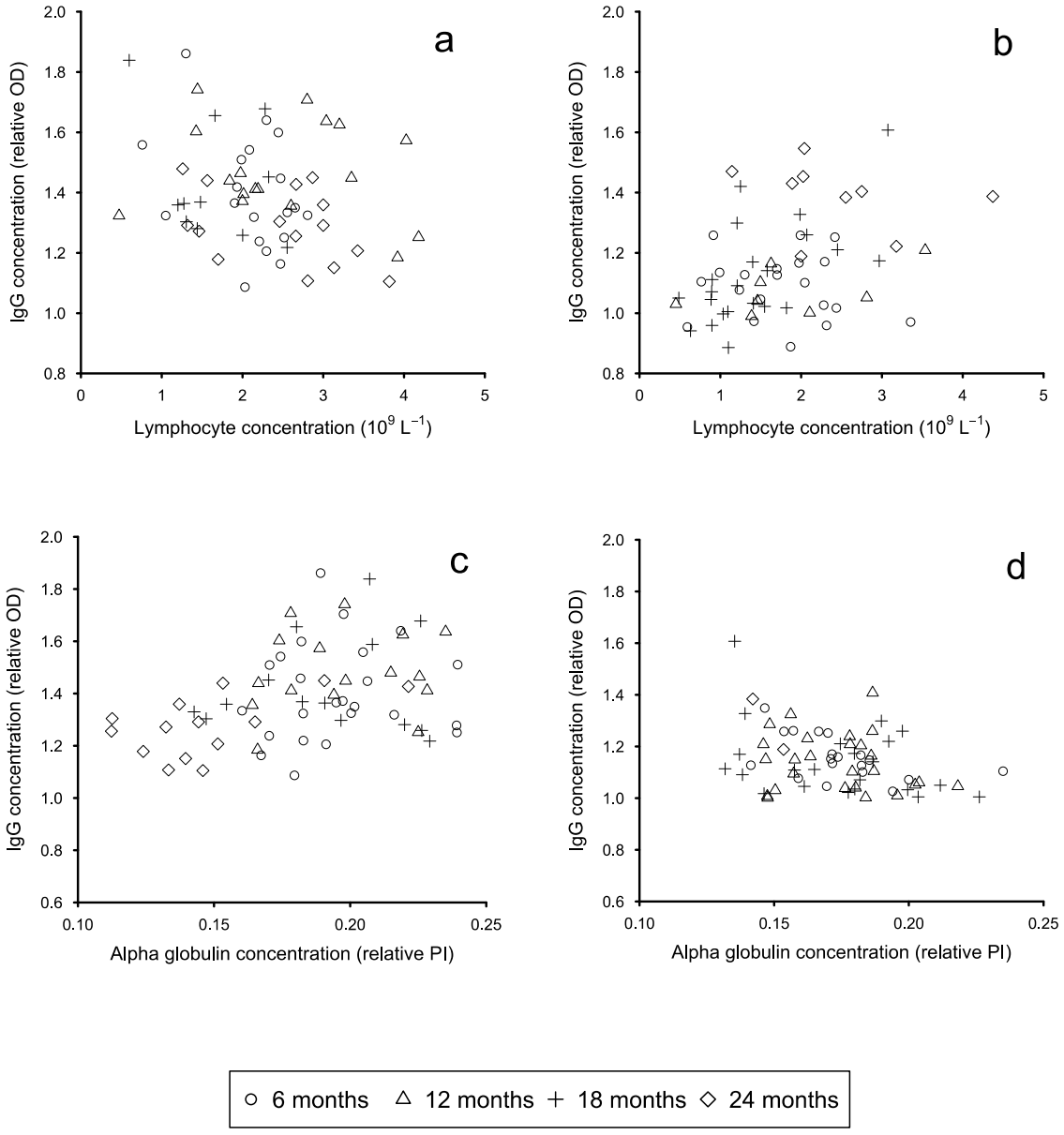

Figure 2.3 (a, b) Relationships between IgG concentration (relative OD) and lymphocyte concentration (10^9 L^{-1}); and (c, d) IgG concentration (relative OD) and alpha globulin concentration (relative PI); in juveniles from the human-impacted colony (a, c), and the control colony (b, d). 'OD' = optical density, 'PI' peak intensity.

Table 2.6 Full linear mixed effects models that tested for colony differences in the five immune measure covariation relationships in juveniles highlighted by the correlation analysis. Units: lymphocytes (10^9 L^{-1}); alpha globulin (relative PI); beta globulin (relative PI); gamma globulin (relative PI). 'relative OD' = optical density relative to control; 'relative PI' = relative peak intensity; 'Ln' denotes natural logarithm; likelihood ratio tests (LRTs) compared models with and without interactions between immune measures and colony.

IgG ~ lymphocytes * colony + age + sex	Estimate	SE	*t*	*p*
(Intercept)	1.456	0.085	17.029	<0.0001
Lymphocytes	-0.079	0.026	-3.052	0.004
Colony	-0.488	0.086	-5.679	<0.0001
Age (12 months)	0.133	0.042	3.202	0.002
Age (18 months)	0.061	0.037	1.629	0.110
Age (24 months)	0.060	0.039	1.530	0.133
Sex (male)	0.020	0.035	0.572	0.570
Lymphocytes * colony	0.132	0.037	3.545	0.009

N (total) = 122; n (individuals) = 68
Random: cohort SD = 0.056, individual SD = 0.092, residual SD = 0.131
LRT: $\chi^2 = 12.370$, $p = 0.0004$

IgG ~ alpha globulin * colony + age + sex	Estimate	SE	*t*	*p*
(Intercept)	1.052	0.142	7.415	<0.0001
Alpha globulin	1.562	0.667	2.340	0.023
Colony	0.332	0.176	1.882	0.064
Age (12 months)	0.060	0.029	2.086	0.041
Age (18 months)	0.047	0.030	1.590	0.117
Age (24 months)	-0.028	0.048	-0.579	0.565
Sex (male)	-0.003	0.028	-0.099	0.921
Alpha globulin * colony	-3.196	0.949	-3.366	0.001

N (total) = 137; n (individuals) = 73
Random: cohort SD = 0.051, individual SD = 0.068, residual SD = 0.115
LRT: $\chi^2 = 11.491$, $p = 0.0007$

IgG ~ Ln beta globulin * colony + age + sex	Estimate	SE	*t*	*p*
(Intercept)	0.150	0.043	3.466	0.001
Ln beta globulin	-0.003	0.026	-0.126	0.900
Colony	0.087	0.058	1.505	0.136
Age (12 months)	0.027	0.006	4.520	<0.0001
Age (18 months)	0.032	0.006	5.018	<0.0001
Age (24 months)	0.016	0.009	1.739	0.087
Sex (male)	-0.008	0.005	-1.652	0.103
Ln beta globulin * colony	0.059	0.034	1.701	0.093

N (total) = 156; n (individuals) = 84
Random: cohort SD <0.0001, individual SD <0.0001, residual SD = 0.028
LRT: $\chi^2 = 1.109$, $p = 0.292$

PHA ~ Ln beta globulin * colony + age + sex	Estimate	SE	*t*	*p*
(Intercept)	1.853	0.904	2.051	0.044
Ln beta globulin	0.333	0.556	0.598	0.553
Colony	-1.223	1.131	-1.081	0.284
Age (12 months)	-0.109	0.113	-0.964	0.341
Age (18 months)	-0.028	0.119	-0.233	0.817
Age (24 months)	-0.181	0.185	-0.977	0.334
Sex (male)	0.139	0.092	1.510	0.136
Ln beta globulin * colony	-0.639	0.687	-0.931	0.357

N (total) = 117; n (individuals) = 70
Random: cohort SD <0.0001, individual SD = 0.032, residual SD = 0.454
LRT: $\chi^2 = 0.899$, $p = 0.343$

PHA ~ lymphocytes * colony + age + sex	Estimate	SE	*t*	*p*
(Intercept)	0.998	0.218	4.582	<0.0001
Lymphocytes	0.166	0.080	2.065	0.047
Colony	0.227	0.248	0.916	0.364
Age (12 months)	-0.249	0.133	-1.866	0.071
Age (18 months)	-0.004	0.123	-0.029	0.977
Age (24 months)	-0.162	0.135	-1.198	0.239
Sex (male)	0.121	0.099	1.221	0.228
Lymphocytes * colony	-0.179	0.111	-1.626	0.113

N (total) = 95; n (individuals) = 56
Random effects - cohort SD <0.0001, individual SD = 0.127, residual SD = 0.409
LRT: $\chi^2 = 2.806$, $p = 0.094$

Discussion

This chapter demonstrates the utility of a generalised approach to describing immune variation in a species for which there is little baseline information available. A combination of humoral with cellular immune components, and cumulative with snapshot measures supplied an overview of immune activity during the first two years of life in the Galapagos sea lion. In addition, the longitudinal sampling design allowed immune system development to be taken into consideration, and targeted sampling in the context of the unique ecology of the Galapagos sea lion allowed for the investigation of habitat differences in immune physiology associated with human-impacts. Although the colony differences in immune activity were heterogeneous across measures and ages, the results generally supported the prediction that immune activity would be higher in the human-impacted colony than the control colony, and provided suggestive evidence in support of the hypothesis that human activity has an influence on Galapagos sea lion immune development.

Immune variation with colony and age

IgG concentration was substantially higher in the human-impacted colony than in the control in older pups and younger juveniles. The intercept estimated for human-impacted colony juveniles between the ages of 6 and 18 months was higher than that for the control colony juveniles by the equivalent of 9.7 g L^{-1}. A difference in IgG concentration of 9.7 g L^{-1} is likely to be biologically meaningful, given that in grey seals an equivalent increase was associated with a decrease in survival probability of 4.9% (recalculated from Hall et al. 2002). In addition, the colony differences in gamma globulin curve shape suggest that sea lions from the human-impacted colony had relatively low diversity gamma globulin fractions compared with those from the control colony, even when overall concentration was taken into account. This may mean that the high concentrations of IgG and gamma globulin observed in the human-impacted colony arose due to relatively strong stimulation of the humoural immune system by relatively few antigens in the human-impacted colony compared with the control colony.

IgG concentrations are thought to reach adult levels in dogs at 12 months of age (Day 2007) and these data reveal a similar pattern in Galapagos sea lions. IgG synthesis is stimulated by antigen exposure during the early post-natal development of young mammals (Freitas et al. 1991; Hall et al. 2002), but IgG can also be passed from mother to offspring via colostrum (Chucri et al. 2010) and be produced independently of antigen exposure as natural antibody (Avrameas 1991; Ochsenbein & Zinkernagel 2000; Mauck et al. 2005; Ujvari et al. 2011). Suckling had no effect on serum IgG concentrations in the youngest pups included in the study. Therefore, observed changes in IgG concentration in the Galapagos sea lion are likely to be caused by IgG production in pups themselves. However, this does not preclude the possibility of vertical IgG transmission via colostrum in the Galapagos sea lion; I did not test for vertical IgG transmission, as I sampled pups only after their mothers had left for their first post-partum foraging trip to avoid negative impacts on welfare.

Therefore, there are two pairs of possible explanations for the higher plateau of IgG concentration in the human-impacted colony relative to the control colony, two of which invoke selection and two of which do not. First, higher pathogen exposure over evolutionary time may have selected sea lions in the human-impacted colony for higher constitutive production of natural antibody (Ochsenbein & Zinkernagel 2000), an effect similar to that proposed to explain natural antibody variation in ground finches (Lindström et al. 2004). Second, higher pathogen exposure over evolutionary time may have selected sea lions in the human-impacted colony for sensitive primary antibody responses during early development, which is plausible given the evidence for heritability of antibody responsiveness (Verhulst et al. 1999; Graham et al. 2010; van der Most et al. 2011). Third, sea lions in the human-impacted colony may have been exposed to a higher diversity and concentration of PAMPs during development than sea lions in the control colony, which could increase their natural antibody production. This explanation is plausible because a recent study suggested that “disease environment” could effect natural antibody production (Palacios et al. 2010), even though it is generally thought to be

constitutive (Mauck et al. 2005; Whiteman et al. 2006). Fourth, sea lions in the human-impacted colony may have been exposed to a higher diversity and concentration of antigens during development than sea lions in the control colony, which could increase their primary antibody response (Freitas et al. 1991; Hall et al. 2002).

The first two explanations, which invoke selection on immunity, are less well supported by the data from this study system than the second two explanations, which invoke only environmental effects. This is because there is evidence for high gene flow amongst the sea lion colonies in the central part of the Galapagos archipelago, which include Santa Fe and San Cristobal (Villegas-Amtmann et al. 2008; Wolf et al. 2008). In addition, differences in human-impact between the colonies are likely to have arisen only during the last 100 years (Epler 2007). As for distinguishing between the third and fourth propositions, the measurement of IgG concentration does not allow for the distinction of specific from natural antibody. However, the majority of natural antibody in humans is in the form of IgM rather than IgG (Ochsenbein & Zinkernagel 2000), which is not measured by the IgG quantification assay used here. If the same is true in sea lions, then the most likely explanation of the difference in IgG concentration plateau between the colonies is a difference in antigen exposure during early development that leads to differences in primary antibody response (Freitas 1991; Hall et al. 2002). This explanation is similar to that proposed to explain variation in antibody concentration that is independent of genetic and nutritional differences in humans (McDade 2003).

The above explanation is congruent with the gamma globulin curve shape findings. The colony differences in gamma globulin curve shape are most likely to be explained by colony differences in the proportions of circulating immunoglobulin classes. If antigen exposure during early development stimulated IgG production more than it did IgM production, sea lions from the human-impacted colony would have proportionally more IgG, leading to the relatively tall gamma globulin peaks that were observed. However, if PAMP exposure during early development stimulated natural antibody production,

we would not expect to see colony differences in gamma globulin peak shape, as IgM and IgG natural antibody populations would be expected to vary in magnitude proportionally.

The changes in IgG concentration between 18 and 24 months are curious, and could be driven by ontogenetic behaviour changes. Juveniles at this stage of development spend less time in the colony, consume more prey and visit more haul out sites (Mueller et al. 2011). As prey items are likely to contain parasites (e.g. Moles & Heintz 2007) and antigen exposure is likely to increase as juveniles expand their movement ranges. For example, pinnipeds acquire lungworm (*Parafilaroides decorus*) infections when they begin feeding independently (Dailey 2001), and lungworm have been observed in dead 24-month-old Galapagos sea lions (Acevedo-Whitehouse, unpublished data). This behavioural transition could increase net antigen exposure and could have led to the increase in IgG concentration observed in the control colony. On the other hand, if antigen exposure in the human-impacted colony is high, this ontogenetic transition may lead to a net decrease in antigen exposure, which could explain the decrease in IgG concentration in the human-impacted colony. However, it also possible that this pattern was driven by an undetected effect of selective disappearance, as the power of the t-tests that were used to test for selective disappearance was low.

The PHA response is best interpreted as an indicator of the “inducibility of pro-inflammatory signalling” at the time of challenge (Vinkler et al. 2010). The colony differences in PHA response could be the results of greater levels of antigen exposure or infection having activated pro-inflammatory pathways in the sea lions of the human-impacted colony (e.g. Saks et al. 2006). This is consistent with the relatively high concentrations of inflammation-associated alpha globulins in the human-impacted colony, and is not confounded by the difference in PHA response at 6 months between those still present at 24 months and those absent, as there was no change in PHA response with age in juveniles. Although there is evidence for heritability of the PHA response (Gleeson et al. 2005; Bonneaud et al. 2009; Drobniak et al. 2010), given that selected differences between colonies are unlikely to exists for the reasons

given above, it is more likely that the colony difference in PHA is driven by differences in antigen exposure or infection between the colonies.

In line with findings in Steller sea lions (Keogh et al. 2010) and dogs (Blount et al. 2005), leukocyte concentrations decreased with age in pups. The high leukocyte concentrations observed shortly after birth are likely to be caused by high levels of leukocyte production and distribution that are typical of early immune system development in mammals (Day 2007). It is noteworthy that the decrease in pup eosinophils is greater in the control colony, and that juvenile eosinophil concentration in the human-impacted colony increases between 6 and 12 months, while it decreases in the control colony. This shows that variation in pup eosinophils is masked by variation in other white blood cell types when all are combined into the total leukocyte measure, which demonstrates the utility of complementing total leukocyte concentration with the concentrations of particular leukocyte cell types.

Eosinophilia is associated with macroparasite infections in humans (Quinnell et al. 2005) and sea lions (Spraker et al. 2007). As discussed in Chapter 1, Galapagos sea lion pups can be infected with a species of hookworm (*Uncinaria* spp.; Paras et al. 2003) and a species of eye fluke (*Philophthalmus zalophi*; Dailey et al. 2005). In the California sea lion (*Zalophus californianus*) hookworm infection intensity is highest during the breeding season (Lyons et al. 2005) and all infections are cleared by the age of 6-8 months (Lyons et al. 2000). The transmission of hookworm from mother to pup in otariids is transmammary (Lyons et al. 2003; Castinel et al. 2007), and given the long suckling period of the Galapagos sea lion, variation in eosinophils may be due to the as yet unknown dynamics of hookworm in this species. If this is the case, the data suggest that the dynamics of hookworm infection differ between the two sampled colonies up to 18 months of age. A role for macroparasites as drivers of colony differences in eosinophil and total leukocyte concentrations is supported by the absence of such differences in lymphocyte and neutrophil concentrations, which we might expect if there were different levels of micro-parasite infection between colonies (Bossart et al. 2001). It is interesting that eosinophil concentrations are elevated in both

colonies in 24-month-olds. This could be due to increased exposure to particular macroparasites in prey (e.g. Moles & Heintz 2007), pathogens or antigens, as juveniles forage more independently at this age (Mueller et al. 2011; Jeglinski et al 2012), as discussed above in the context of IgG variation.

As the concentrations of albumin and the globulins are calculated relatively, they should be considered together. The variation in juvenile gamma globulin concentration corresponds to that of IgG, which is to be expected, as IgG makes up the majority of the gamma globulin fraction. It is possible that the low albumin concentrations of juveniles from the human-impacted colony were caused by elevated gamma globulin concentrations skewing the relative calculation of albumin concentrations. However, low albumin concentration could also indicate a trade-off between investment in production of different serum protein fractions: juveniles in the human-impacted colony that were stimulated to produce high concentrations of IgG when they were pups may have had low albumin concentrations as a consequence.

Why pups of the human-impacted colony had relatively high albumin concentrations is an interesting question. As albumin is related to nutritional status (Bossart et al. 2001; McDade 2003), this might indicate that mothers in the human-impacted colony invested more energy in their pups than mothers from the control colony. This is consistent with recent findings in California sea lions that suggest that anthropogenic disturbance increases pup growth rate while decreasing female reproductive output (French et al. 2011). Total protein and hematocrit may not have varied between colonies in this way as they are constrained by links to key physiological functions: the osmotic balance of the blood in the case of total protein (Tella et al. 2008), and the storage and transport of oxygen during diving in the case of hematocrit (Trillmich et al. 2008). This suggestion raises the possibility that pups and juveniles in the human-impacted colony have higher levels of immune activity not because of extrinsic pressure on their immune systems, but because they receive more nutrition from their mothers, and therefore have more resources to invest in all aspects of life history, including immunity, than sea lions in the control colony. The distinction between the outcomes of condition-dependent

investment in immunity and trade-offs between immunity and other life history traits is a central consideration of ecological immunology (van Noordwijk & de Jong 1986; McDade 2003), which I address explicitly in Chapter 3.

Although sex was included in the statistical models in order to control for a potentially confounding source of variation, it is interesting to note that the sex differences observed were in line with generalised ecological immunology expectations. This was because juvenile males had lower measures of immune activity than juveniles females, and because there was a suggestion in pup data that the sexes may pursue different immune investment strategies, with females investing relatively more in what may represent preventative protective immunity (i.e. IgG) and males investing relatively more in costly induced responses to immune challenge (i.e. PHA response). However, as neither the pups nor the juveniles sampled in this study were sexually mature, it is difficult to infer the significance of these sex differences.

Immune measure covariation

Given that antibodies have relatively stable half-lives compared with leukocytes, IgG and lymphocyte concentration are likely to vary over different timescales, and colony differences in their correlation may have arisen through variation in the timing of events. However, the even distribution of the age classes across the ranges of IgG and lymphocyte concentration suggests this is not the case in the human-impacted colony (Fig. 2.3a). The negative relationship between IgG and lymphocyte concentration in the human-impacted colony may instead have arisen because, above threshold concentrations, IgG has a suppressive regulatory effect on B-lymphocytes (Mims et al. 2004). If individuals in the human-impacted colony were exposed to the same antigen environment, they may have varied in the strength of their humoral immune response, some producing levels of IgG sufficient to regulate lymphocytes, others mounting responses more biased towards cell-mediated immunity. If, on the other hand, they were exposed to different antigen environments, those with high lymphocyte and low IgG concentrations may have done so because they were exposed to pathogens that elicit

predominantly cell-mediated responses. Either way, the relatively large random effects of individual identity on IgG and lymphocyte concentration (Table 2.6) suggest that consistent individual variation across ages was important in shaping this relationship in the human-impacted colony. This consistency may have arisen either because there are consistent individual differences in response to similar exposure across ages, or because there are consistent individual differences in exposure across ages.

In the control colony, the positive relationship observed between IgG and lymphocyte concentration was driven by variation at 18 months of age (Fig. 2.3b). The positive relationship at 18 months may have arisen because this age preceded an increase in IgG in the control colony (Fig. 2.2a) and therefore a time when IgG was being produced. In this case, we might have expected to see a different relationship following 18 months, when B-lymphocyte numbers had returned to lower levels after a surge of production, but IgG molecules had accumulated. Although the sample size is small, there is a suggestion of this, as the relationship at 24 months appears to be negative (Fig. 2.3b).

There is a clear age effect on the relationship between IgG and alpha globulin concentration in the human-impacted colony, as the left side of the distribution consists mainly of data points from 24-month-olds (Fig. 2.3c). However, samples from 6 to 18-month-olds contribute homogenously to the hump-shaped right side of the distribution. Alpha globulins perform many functions, from binding free haemoglobin to transporting copper ions (Tella et al. 2008). For the purposes of this study the most important alpha globulins are the acute phase proteins because of their association with inflammation. It is not clear why intermediate concentrations of alpha globulins were associated with the highest IgG concentrations in the human-impacted colony. This pattern could be a result of methodological constraints, such as the low resolution of serum protein electrophoresis data, or the non-independence of IgG and gamma globulin concentrations, and may even have arisen due to an interaction between such methodological artefacts and biologically meaningful immune variation. However, it is clear that whatever drives this variation is not

consistent between the colonies, as the relationship between IgG and alpha globulins is straightforwardly negative in the control colony (Fig. 2.3d).

The ecological context

Variation in immune activity in the wild can be driven by many factors (Pedersen & Babayan 2011), so the colony differences described here should be assessed in as broad an ecological context as possible. For example, there are more sea lions resident in the human-impacted colony than in the control colony. Dense populations are expected to have high pathogen prevalence due to elevated horizontal transmission rates (Alexander 1974) and immune variation can be density-dependent (Wilson et al. 2002). Although I did not collect fine-scale spatial data, I observed that sea lions were distributed at similar densities in both colonies and that the control colony had a smaller area of suitable habitat (see Appendix 1.1 for satellite images). In addition, a recent study quantified the PHA response in five South American sea lion (*Otaria flavescens*) colonies of different sizes and found that colony size had no effect on the response in pups (Drago et al. 2011). As Galapagos sea lion density in particular microhabitats varies between seasons (Wolf et al. 2005), seasonal variation offers an insight into the effect of changes in fine-scale spatial distribution on immune variation within colonies. All sea lions sampled in this study were born between October and December, so 6 and 18-month-olds were always sampled during the non-reproductive season, while 12 and 24-month-old sea lions were always sampled during the reproductive season. IgG and PHA levels showed little seasonal variation, yet were consistently higher in the human-impacted colony. There were interesting fluctuations in concentrations of leukocytes that allow for the possibility of seasonal effects, and interactions between seasonal and colony effects. The low concentrations of total leukocytes, lymphocytes and neutrophils observed in both colonies at 18 months, for example, may be because this is the period when juveniles are most food-limited (Jeglinski et al. 2012), and thus when they may be most prone to physiological stress-induced immunosuppression.

Stress can suppress immune function (Padgett & Glaser 2003), and given the disturbance that sea lions of the human-impacted colony experience (e.g. industrial machinery, marching bands, fireworks, car horns, amplified music and harassment by dogs) we might expect them to be relatively immunosuppressed compared with the sea lions from the control colony. However, it is possible that sea lions resident in the human-impacted colony are attenuated to stress (Martin et al. 2005; French et al. 2010). This might be particularly important for the PHA response, which is mounted during the 24 hours following capture. If sea lions from the control colony are less attenuated to anthropogenic disturbance, they may mount greater stress responses to capture, which may lead to acute stress-induced immunosuppression that could lower their PHA responses (Fernandez-de-Mera et al. 2009). Stress is also associated with changes in leukocyte populations and antibody production (Webster Marketon & Glaser 2008; Müller et al. 2011), but these effects are likely to occur over longer periods than sea lion capture (Buehler et al. 2008b), so colony variation in these immune measures is unlikely to be affected by capture stress.

Immune function in marine mammals is also affected by contaminants, particularly PCBs (polychlorinated biphenyls; Schwacke et al. 2011). However, PCBs, PBDEs (polybrominated diphenyl ethers) and DDT (dichlorodiphenyltrichloroethane) have been shown to be below toxic thresholds in Galapagos sea lions and to be unrelated to proximity to human settlements (Alava et al. 2009, 2011). Inbreeding is another important consideration with respect to immune function in the wild (Reid et al. 2007). Given the site fidelity and polygamy of otariids, the small population size (Aurioles & Trillmich 2008) and its low overall genetic diversity relative to the California sea lion (Wolf et al. 2007a), inbreeding depression may occur in the Galapagos sea lion. However, given the evidence for movement (Villegas-Amtmann et al. 2008) and gene flow (Wolf et al. 2008), levels of inbreeding are unlikely to be different between the two colonies sampled here, and are therefore unlikely to solely account for the colony differences in immunity. Chapter 4 explicitly addresses this issue by testing for colony differences in

inbreeding value estimates, and for the effects of inbreeding on immune variation in the Galapagos sea lion.

The distinction between immune responses to exposure as opposed to infection is important, but I have not discussed it above because of lack of data. I attempted to quantify parasite density by carrying out external examinations of all the sea lions sampled, screening faecal samples and blood smears, and conducting necropsies. Except for a small number of ectoparasites and a single dead pup heavily infected with hookworm, there were very few signs of infection. Therefore, it was not possible to distinguish between immune variation driven by exposure to PAMPs, antigens, allergens or pathogens (including parasites) using the tools available to the project. However, heightened investment in immunity has fitness consequences, even when it is not associated with disease risk, due to its energetic costs (Lochmiller & Deerenberg 2000) and to immunopathology (Zuk & Stoehr 2002; Colditz 2008).

The energetic cost of immunity may be a particularly relevant life history consideration in the Galapagos sea lion, as during warm years they are food-limited and during El Niño events many starve (Trillmich & Limberger 1985; Trillmich & Dellinger 1991; Mueller et al. 2011). If the relatively high immune activity observed in the human-impacted colony is not plastic (i.e. can not be regulated in response to environmental changes that affect food availability), sea lions of the human-impacted colony may be more sensitive to climate-driven decreases in food supply, regardless of the disease risk associated with the immune stimulus they appear to experience. This may increase the chance of starvation or reduce the energy available for investment in reproduction, which could have impacts on individual fitness and the long-term stability of the population. On the other hand, if investment in immunity is plastic and the observed immune activity in the human-impacted colony is protective against potentially harmful pathogens, climate-driven decreases in food supply and the consequent down-regulation of immunity could increase

the risk of disease emergence in this endangered species. This topic is further explored in Chapter 3.

Chapter 3

Negative correlation between changes in immune activity and body condition under human influence

Abstract

The flow of energy through individuals in natural populations can be finely tuned to the relative benefits of investment in different aspects of life history. Immunity may compete with other life history traits for resources such as energy and protein, and the damage caused by immunopathology can sometimes outweigh the protective benefits that immune responses confer. However, our understanding of how the costs of immunity are expressed in the wild and how they relate to the myriad energetic demands on free-ranging organisms is limited. This chapter addresses two topics of growing general interest: the degree to which immunity participates in life history trade-offs within individuals, and the extent to which human disturbance challenges wild organisms energetically. The analysis makes use of the unique ecology of the Galapagos sea lion (*Zalophus wollebaeki*) to test for a physiological cost associated with immunity in the wild. The results show that during the first three months of life, changes in immunoglobulin G (IgG) concentration were negatively related to changes in mass per unit length, skinfold thickness and serum albumin concentration, but only in a sea lion colony exposed to anthropogenic environmental impacts. This suggests that the relatively high IgG concentrations found in sea lions from the human-impacted colony reported in Chapter 2 may be associated with negative impacts on fitness, through a trade-off between investment in immunity and resistance to starvation. The relative benefits of these investments may change quickly and unpredictably in the wild, which allows for the possibility that individuals fine-tune their investment strategies in response to changes in environmental conditions. In addition, the results suggest that anthropogenic environmental impacts may impose subtle energetic costs on individuals, which could contribute to population declines, especially in times of energy shortage.

Introduction

As discussed in Chapter 1, immunity is costly, and because of these costs, maximal immune responses are unlikely to be beneficial in most circumstances (van Boven & Weissing 2004). Ecological immunology aims to disentangle how organisms manage the allocation problem of immune investment in a variable environment (Martin et al. 2011; Graham et al. 2011; Pedersen & Babayan 2011). Despite the evidence for a cost of immunity in wild-caught and captive birds (e.g. Verhulst et al. 2005), invertebrates (e.g. Jacot et al. 2005), reptiles (e.g. Uller et al. 2006) and mammals (e.g. Derting & Compton 2003), there are relatively few studies on the cost of immunity in free-ranging mammals (but see Hall et al. 2002 and Graham et al. 2010). In this chapter I address the issue of the physiological cost of immunity in the Galapagos sea lion, by testing for correlations between changes in immune measures and changes in condition in known individuals over time.

Quantifying immunity in the wild is a challenge, especially in a species for which no specific laboratory reagents have been developed (Pedersen & Babayan 2011). Chapter 2 described variation in 12 immune-related physiological measures during the first two years of life in the Galapagos sea lion. Its results showed that Galapagos sea lions from the human-impacted colony on San Cristobal had relatively higher levels of immune activity – quantified using cell-mediated and humoral immune components, and snapshot and cumulative measures – than sea lions from a colony on an uninhabited island. This chapter used three of the previously described immune measures: immunoglobulin G (IgG) concentration, the *in vivo* inflammation response to phytohemagglutinin (PHA), and total leukocyte concentration, as these measures were possible in the greatest number of individuals at the greatest number of time points, and therefore allowed for the greatest statistical power, and because they varied with both colony and age.

I used three measures of condition to assess nutritional status: mass per unit length, serum albumin concentration and skinfold thickness. I do not consider these to be indicators of the underlying and immeasurable ‘quality’ of an individual, but rather as different aspects of dynamic physiological state

associated with resource availability (Wilson & Nussey 2010). The relationship between body mass and body length is used as a measure of condition in many vertebrate taxa, and there are a number of ways in which it can be expressed (Hall et al. 2002; Ujvari & Madsen 2006; Townsend et al. 2010). Variation in mass per unit length is most often calculated for adults, and it is assumed that higher values are indicative of better nutritional status. If measured in immature animals at multiple time points, mass per unit length can also serve as an indicator of relative investment in the growth of different tissues, as it describes how skeletal size changes with overall tissue mass (Field et al. 2007; Hewison et al. 2011). Despite debate over the details of the calculation and interpretation of mass per unit length (Peig & Green 2010), it has been correlated with fitness-related traits in many species, including pinnipeds (Hall et al. 2002; Hall et al. 2001; Schulte-Hostedde et al. 2005). Albumin is a transporter molecule and protein reservoir, and its concentration in serum is commonly used to diagnose malnutrition in marine mammals (Bossart et al. 2001). Skinfold thickness is a measure of how much fat is stored under mammalian skin (Luque & Aurioles-Gamboa 2001; Hall & McConnell 2007) and is likely to be better correlated with total body fat in pinnipeds than in other taxa, as the majority of pinniped fat is stored subcutaneously and relatively little is stored in internal deposits (Pond 1998). Total body fat is an important determinant of fitness in marine mammals as it is correlated with their ability to resist starvation (Iverson 2002).

Given the complexity of immune dynamics in natural populations, a physiological cost associated with immunity may only be observable under certain ecological conditions (Sandland 2003; Schmid-Hempel 2011b). The unique ecology of the Galapagos sea lion makes it a suitable system in which to test for such an association in the wild, as the species is sensitive to unpredictable food limitation, exposed to the human impacts of disease threat and pollution and is geographical aggregated into distinct colonies (Chapter 1). In addition to insight into the link between immune variation and fitness in wild populations, evidence for a cost of immunity in the Galapagos sea lion could have important ramifications for its conservation. This chapter tested two hypotheses: 1) that changes in immune measures over time were

negatively associated with changes in condition, and 2) that any such negative associations were more pronounced in the human-impacted colony than in the control colony.

Methods

The details of sea lion capture, sampling and the quantification of IgG concentration, PHA response and total leukocyte concentration are described in Chapter 2. During captures, I measured body mass to the nearest 0.5 kg using a spring balance (Pesola, Switzerland) and curved body length to the nearest 0.5 cm with a tape measure. I re-measured body length 24 hours after initial capture during re-captures and used the average of the two values for analysis. In pups, I took three repeated measurements of dorsal axial skinfold thickness (Luque & Aurioles-Gamboa 2001) to the nearest 0.01 mm with callipers (Wiha, USA). I calculated mass per unit length as the residuals of a linear regression between body mass and mean body length. In juveniles, mass and length needed to be log-transformed prior to regression to normalise residuals (Shapiro-Wilk, $p > 0.05$). I calculated pup skinfold thickness as the median of the three repeated measures, and excluded sets of repeated measurements with coefficients of variation greater than 25 %.

Prior to analysis, I calculated absolute changes in immune and condition variables between consecutive time points for each individual. Correlations amongst changes in immune and condition variables were non-significant in both pups and juveniles ($p > 0.05$). I tested for colony and sex differences in condition changes by fitting analysis of variance models (ANOVAs) to pup data, and linear mixed effect (LME) models that included period of change and individual identity as random effects to juvenile data. Next, I fitted the nine possible linear models to test the effect of change in a single immune variable on change in a single condition variable in pups. I fitted change in condition as the response and change in the immune measure, sex, colony and their interactions as explanatory terms. In juveniles, I could fit six such models, as I did not collect data on juvenile skinfold thickness; I fitted these as LME models including individual identity and period of change as random effects. As the first step in model selection I compared these 15 maximal models with null models, using F-tests for pup data and likelihood ratio tests for juvenile data. I further considered only those relationships for which maximal models performed significantly better than null models.

I was principally interested in the relationship between immunity and condition and how this varied between colonies, but it was also important to consider the role that sex could have played in shaping or obscuring any relationship. Sex differences in the way condition changes with age can arise through sex-specific modes of growth, development and maternal investment (Hall & McConnell 2007; Hewison et al. 2011). In addition, changes in food availability have been shown to have different effects on male and female immune activity (Dubiec et al. 2006; Martin et al. 2008), and immune challenge has been shown to differentially affect male and female condition (Romano et al. 2011). In order to control for possible sex differences and to avoid over-complicating models, I split data for further analysis by colony. For each relationship selected by the null model comparison, and for each colony, I fitted change in condition as the response and change in the immune measure, sex and their interaction as explanatory terms. Then I compared these models to models without the interaction using F-tests in pups and likelihood ratio tests in juveniles. As before I fitted linear models to pup data and LME models that included period of change and individual identity as random effects to juvenile data. I checked all models for signs of heteroscedasticity, heterogeneity of variance, non-normality of error and the disproportionate influence of outliers (Zuur et al. 2009), and carried out all analyses in R 2.11.1 (R Development Core Team 2011).

A possible alternative to this two-stage statistical approach would have been to apply an information-theoretic model selection approach based on Akaike's information criterion (AIC; Symonds & Moussalli 2011). The full models described above test multiple, non-exclusive hypotheses on the form of the relationship between immunity and condition, and all of their explanatory terms represent biologically realistic hypotheses (Burnham et al. 2011). For example, a positive relationship between immunity and condition can arise through the condition-dependence of immunity, and a negative one can arise through a cost of immunity (Schmid-Hempel 2003). As noted above, sex differences in the way condition changes with age can arise through sex-specific modes of growth, development and maternal investment (Hall & McConnell 2007; Hewison et al. 2011); differences in condition between seal

colonies have been described in the wild (Hall et al. 2002); and immune challenge can differentially affect male and female condition (Romano et al. 2011). Finally, the relationship between condition and immune activity may differ between environments, especially if environments vary in the pathogen pressure that they exert on hosts. Therefore, information-theoretic model selection based on AIC could have been appropriate, as it simultaneously evaluates hypotheses, balancing complexity against goodness of fit, and does not assume a single best model (Garamszegi 2011). However, the question this analysis aimed to answer was not which variables predicted changes in condition best, but specifically whether changes in immune activity predicted changes in condition when other potential influences had been taken into account. The information-theoretic approach was therefore less appropriate than the two-stage statistical process described above, which addressed the main hypothesis of this analysis more explicitly.

Results

Across the sample of 55 pups, change in mass per unit length was higher in the control colony than in the human-impacted colony (contrast estimate = 0.85 kg, SE = 0.41 kg, $t_{2,52}$ = 2.08, p = 0.042), meaning that these pups gained more mass per unit length of skeletal growth, and there was no sex difference ($t_{2,52}$ = 1.52, p = 0.132). Change in skinfold thickness was higher in males than in females (contrast estimate = 0.094 cm, SE = 0.045 cm, $t_{2,52}$ = 2.07, p = 0.042), suggesting that males invested relatively more in stores of body fat than females, and there was no difference between colonies ($t_{2,52}$ = -0.16, p = 0.873). There was neither a colony difference ($t_{2,39}$ = -1.34, p = 0.185) nor a sex difference ($t_{2,39}$ = -1.56, p = 0.125) in change in albumin concentration in pups. In juveniles there were neither colony differences (N_{total} = 73, $N_{individuals}$ = 38, t_{68} = 1.11, p = 0.271) nor sex differences (N_{total} = 73, $N_{individuals}$ = 38, t_{68} = -0.85, p = 0.396) in change in mass per unit length or albumin concentration (colony, N_{total} = 60, $N_{individuals}$ = 36, t_{55} = -0.86, p = 0.389; sex, N_{total} = 60, $N_{individuals}$ = 36, t_{55} = 0.26, p = 0.792).

Six of the 15 maximal models of the relationship between change in a single immune variable and change in a single condition variable explained significantly more variation than equivalent null models (Table 3.1). In pups, there was a negative relationship between changes in all 3 measures of condition and changes in IgG concentration in the human-impacted colony (Table 3.2 and Fig. 3.1a, c, e), so that animals that increased most in IgG concentration increased least or decreased most in condition measures. In the control colony there was a positive relationship between change in skinfold thickness and change in IgG concentration, and between change in mass per unit length and change in total leukocyte concentration (Table 3.2 and Fig. 3.1d, f), so that animals that increased most in IgG concentration, increased most in subcutaneous fat stores and animals that increased most in total leukocyte concentration increased most in mass per unit length. In addition, there was a positive relationship between change in mass per unit length and change in IgG concentration in females of the control colony (Table 3.2 and Fig. 3.1b). In juveniles there was a negative relationship

between change in albumin concentration and change in IgG concentration in the males of the human-impacted colony (Table 3.2).

Table 3.1 Comparison of full and null models of relationships between changes in immune measures and changes in condition; F-tests in pups, likelihood ratio tests in juveniles. 'Δ' = change in, 'MLR' = mass per unit length (kg in pups; Ln kg in juveniles), 'SFT' = skinfold thickness (cm), 'ALB' = albumin concentration (relative peak intensity), 'IgG' = immunoglobulin G concentration (mg mL^{-1}), 'WBC' = total leukocyte concentration (10^9 L^{-1}) and 'PHA' = response to phytohemagglutinin (mm). 'Ln' denotes natural logarithm.

		Pups			Juveniles			
Condition Variable	Immune Variable	N	F	*p*	N_{total}	$N_{individuals}$	χ^2	*p*
Δ MLR	Δ IgG	51	3.452	0.005 **	73	38	4.991	0.661
Δ MLR	Δ PHA	55	1.205	0.319	61	36	12.860	0.075
Δ MLR	Δ WBC	51	2.431	0.034 *	84	45	2.687	0.912
Δ SFT	Δ IgG	47	2.578	0.028 *	-	-	-	-
Δ SFT	Δ PHA	55	1.036	0.419	-	-	-	-
Δ SFT	Δ WBC	51	2.294	0.044 *	-	-	-	-
Δ ALB	Δ IgG	39	2.342	0.046 *	58	35	15.740	0.027 *
Δ ALB	Δ PHA	42	0.912	0.509	42	27	7.594	0.369
Δ ALB	Δ WBC	39	0.603	0.749	61	36	5.952	0.545

Table 3.2 Relationships between changes in immune measures and changes in condition; see Tables 3.3 and 3.4 for full model details. 'Δ' = change in, 'MLR' = mass per unit length (kg), 'SFT' = skinfold thickness (cm), 'ALB' = albumin concentration (relative peak intensity), 'IgG' = immunoglobulin G concentration (mg mL $^{-1}$), 'WBC' = total leukocyte concentration (10^9 L^{-1}) and 'PHA' = response to phytohemagglutinin (mm), 'HIC' = human-impacted colony, 'CC' = control colony. Juvenile sample sizes are shown as the total number of data points followed by the number of individuals.

	Condition Variable	Colony	Immune Variable	N	Slope	SE	*t*	*p*
Pups	Δ MLR (kg)	HIC	Δ IgG	27	-0.0511	0.0214	-2.385	0.025 *
		CC (Females)	Δ IgG	24	0.1134	0.0353	3.208	0.004 **
	Δ MLR (kg)	HIC	Δ WBC	25	0.1604	0.1458	1.100	0.283
		CC	Δ WBC	26	0.3613	0.1228	2.941	0.007 **
	Δ SFT (cm)	HIC	Δ IgG	24	-0.0042	0.0019	-2.262	0.034 *
		CC	Δ IgG	23	0.0100	0.0035	2.855	0.010 *
	Δ SFT (cm)	HIC	Δ WBC	25	0.0231	0.0124	1.864	0.076
		CC	Δ WBC	26	0.0266	0.0175	1.514	0.144
	Δ ALB (relative PI)	HIC	Δ IgG	22	-0.0018	0.0008	-2.235	0.038 *
		CC	Δ IgG	17	-0.0003	0.002	-0.176	0.863
Juveniles	Δ ALB (relative PI)	HIC (Males)	Δ IgG	30, 17	-0.0073	0.0033	-2.244	0.034 *
		CC	Δ IgG	28, 18	0.0003	0.0032	0.101	0.920

Table 3.3 Full models of the five selected relationships between change in an immune measure and change in a condition variable in pups. The effects of sex are reported as contrasts and females were used as the reference sex. 'Δ' = 'change in', 'MLR' = mass per unit length (kg), 'SFT' = skinfold thickness (cm), 'ALB' = albumin concentration (relative peak intensity), 'IgG' = immunoglobulin G concentration (mg mL^{-1}) and 'WBC' = total leukocyte concentration (10^9 L^{-1}).

	Colony	N	R^2	Model term	Estimate	SE	*t*	*p*
Δ MLR ~ Δ IgG + Sex	HIC	27	0.27	Intercept	0.2315	0.4749	0.488	0.630
				Δ IgG	-0.0511	0.0214	-2.385	0.025 *
				Sex (male)	1.2590	0.5453	2.309	0.030 *
Δ MLR ~ Δ IgG * Sex	CC	24	0.34	Intercept	0.0029	0.4545	0.007	0.994
				Δ IgG	0.1134	0.0353	3.208	0.004 **
				Sex (male)	1.0054	0.7334	1.371	0.186
				Δ IgG * Sex (male)	-0.1291	0.0511	-2.528	0.020 *
Δ MLR ~ Δ WBC + Sex	HIC	25	0.18	Intercept	-0.3067	0.4936	-0.621	0.540
				Δ WBC	0.1604	0.1458	1.100	0.283
				Sex (male)	1.1441	0.6159	1.858	0.077
Δ MLR ~ Δ WBC + Sex	CC	26	0.27	Intercept	0.9027	0.3686	2.449	0.022 *
				Δ WBC	0.3613	0.1228	2.941	0.007 **
				Sex (male)	1.2114	0.6292	1.925	0.067
Δ SFT ~ Δ IgG + Sex	HIC	24	0.20	Intercept	0.1207	0.0449	2.688	0.013 *
				Δ IgG	-0.0042	0.0019	-2.262	0.034 *
				Sex (male)	0.0314	0.0462	0.681	0.503

Δ SFT ~ Δ IgG + Sex	CC	23	0.34	Intercept	-0.0972	0.0552	-1.759	0.093
				Δ IgG	0.0100	0.0035	2.855	0.010 **
				Sex (male)	0.0895	0.0735	1.218	0.237
Δ SFT ~ Δ WBC + Sex	HIC	25	0.26	Intercept	0.0320	0.0419	0.764	0.453
				Δ WBC	0.0231	0.0124	1.864	0.076
				Sex (male)	0.1003	0.0523	1.919	0.068
Δ SFT ~ Δ WBC + Sex	CC	26	0.15	Intercept	0.0097	0.0526	0.185	0.854
				Δ WBC	0.0266	0.0175	1.514	0.144
				Sex (male)	0.1738	0.0899	1.933	0.066
Δ ALB ~ Δ IgG + Sex	HIC	22	0.30	Intercept	0.0481	0.0188	2.559	0.019 *
				Δ IgG	-0.0018	0.0008	-2.235	0.038 *
				Sex (male)	-0.0252	0.0193	-1.304	0.208
Δ ALB ~ Δ IgG + Sex	CC	17	0.01	Intercept	-0.0111	0.0289	-0.385	0.706
				Δ IgG	-0.0003	0.0018	-0.176	0.863
				Sex (male)	-0.0119	0.0419	-0.283	0.781

Table 3.4 Full models of the selected relationship between change in IgG concentration and change in albumin concentration in juveniles. The effects of sex are reported as contrasts and males were used as the reference sex. 'Δ' = 'change in', 'ALB' = albumin concentration (relative peak intensity), 'IgG' = immunoglobulin G concentration (mg mL $^{-1}$), 'HIC' = human-impacted colony, 'CC' = control colony.

	Colony	N_{total}	$N_{inidividuals}$	Model term	Estimate	SE	*t*	*p*
Δ ALB ~ Δ IgG * Sex	HIC	30	17	Intercept	0.0086	0.0255	0.337	0.739
Random, time-point SD = 0.028				Δ IgG	-0.0073	0.0033	-2.244	0.034 *
Random, individual SD = 0.059				Sex (female)	0.0124	0.0251	0.494	0.626
Random, residual SD = 0.022				Δ IgG * Sex (female)	0.0118	0.0042	2.819	0.010 *
Δ ALB ~ Δ IgG * Sex	CC	28	18	Intercept	-0.0201	0.0193	-1.037	0.310
Random, time-point SD < 0.0001				Δ IgG	0.0003	0.0032	0.101	0.920
Random, individual SD = 0.0600				Sex (female)	-0.0016	0.0250	-0.064	0.950
Random, residual SD = 0.0003								

Figure 3.1

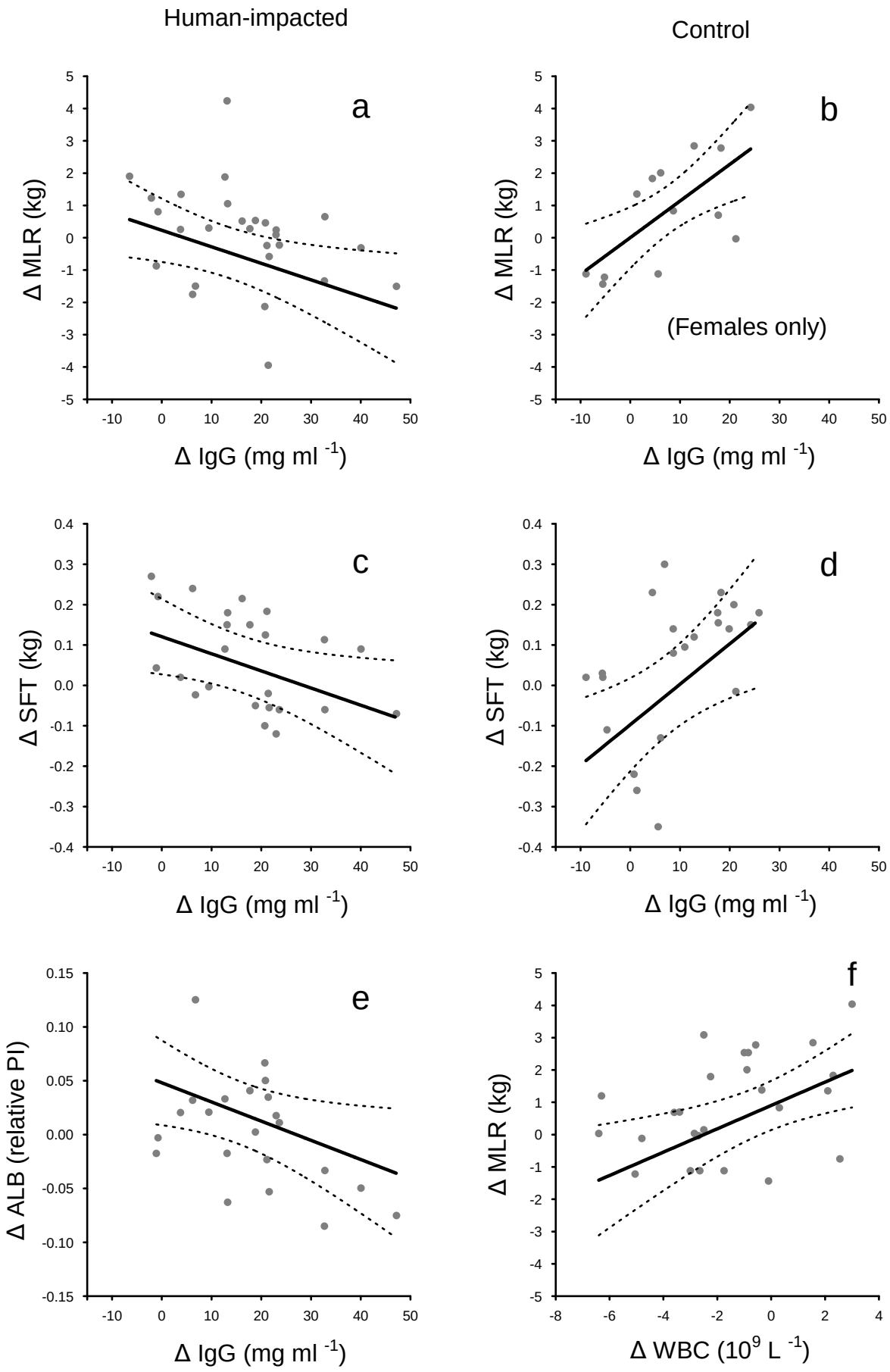
Human-impacted
Control
a
b
c
d
e
f
Δ MLR (kg)
Δ SFT (kg)
Δ ALB (relative PI)
Δ IgG (mg ml -1)
Δ WBC (10^9 L -1)
(Females only)

Figure 3.1 (legend) Predicted relationships between changes in immune measures and changes in condition in pups from the human-impacted colony (a, c, e) and the control colony (b, d, f). Dotted lines represent 95% confidence intervals. 'Δ' = change in, 'MLR' = mass per unit length (kg), 'SFT' = skinfold thickness (cm), 'ALB' = albumin concentration (relative peak intensity), 'IgG' = immunoglobulin G concentration (mg mL^{-1}), 'WBC' = total leukocyte concentration (10^9 L^{-1}) and 'PHA' = response to phytohemagglutinin (mm). Note that the relationship shown in (b) is for females only.

Discussion

The analysis presented in this chapter has two main findings that directly relate to the two hypotheses proposed in the introduction. First, as predicted, there was a negative correlation between changes in an immune measure and changes in condition. This result suggests that changes in IgG concentration may have had a negative effect on physiological condition in the Galapagos sea lion. Although the correlation that underlies this suggestion does not allow inference of causation or direction, such evidence from the wild is rare, and study systems such as the Galapagos sea lion are rarely manipulable. Second, the negative association was only evident in the colony where sea lions were exposed to anthropogenic environmental impacts (Rodriguez & Valencia 2000; Cordoba et al. 2008; Alava 2011) and the presence of domestic animals (Alava & Salazar 2006; Aurioles & Trillmich 2008). Together, these results are consistent with the hypothesis that investment in immunity is costly because of trade-offs with other life history traits, and suggest that human influence may have a negative impact on Galapagos sea lion fitness through an effect on immunity. However, these results are correlative, and consistency of the results with the hypothesis of a cost of immunity is not the same as evidence for a causative link; alternative explanations for the correlative results that do not invoke a direct cost of immunity are considered below.

In pups from the control colony the results showed a positive relationship between changes in immune measures and condition, such as we would expect under condition-dependent investment in immunity (McDade 2003). Given that the opposite pattern was observed in the human-impacted colony, this positive relationship is unlikely to be fixed by constraint. Therefore, it may have arisen because Galapagos sea lions invest in immunity and growth according to the resources they have available, under circumstances not strongly influenced by humans (i.e. in the control colony). Pups that have better access to resources, because they were born to more experienced mothers, for example, may invest more in both immunity and condition. This phenomenon is known as ‘phenotypic correlation’ (McDade 2003) and suggests that the variation in the acquisition of resources is greater than the

variation in their allocation (van Noordwijk & de Jong 1986; Schmid-Hempel 2011b), in this case, in the control colony.

The negative relationships between change in IgG concentration and all three measures of condition in the human-impacted colony may have been caused by a trade-off between the energy and resources consumed by IgG production and those available for growth and development (Eraud et al. 2005). This explanation may be particularly plausible in the case of skinfold thickness, as there is evidence to suggest that trade-offs between investment in immunity and growth are mediated by competition between physiological cascades for molecules involved in lipid transport and metabolism (Adamo et al. 2008; Trotter et al. 2011). As discussed in Chapter 2, IgG concentration is likely to have increased more in pups from the human-impacted colony than the control colony because those in the human-impacted colony experienced a richer post-natal antigenic environment. It is possible – although speculative given the nature of the evidence presented here – that the reason that these larger increases in IgG concentration in the human-impacted colony, but not the smaller increases observed in the control colony, were negatively associated with changes in condition because Galapagos sea lion immune system ontogeny has adapted to an environment free from human influence. In this case, Galapagos sea lion immune systems would have been selected to respond to their antigenic environment during early immune system ontogeny with sensitivity appropriate to human-free conditions. This would confer an advantage in the control colony, where present conditions are relatively unchanged from historical ones. However, such sensitivity may be disadvantageous in the newly antigen-rich environment of the human-impacted colony, where sea lions come into close contact with domestic animals (Alava & Salazar 2006; Aurioles & Trillmich 2008) and where the bay in which the sea lions live is contaminated with faecal coliform bacteria by sewage from the town water system (Rodriguez & Valencia 2000; Cordoba et al. 2008; Alava 2011).

In the context of the phenotypic correlation proposed to explain the positive correlation in the control colony, the negative relationship in the human-

impacted colony could be interpreted as a contrasting effect based on the same underlying trade-off between investment in immunity and condition. In other words, the immune system stimulation associated with human-impact may cause a functional inversion of the ratio between variation in resource acquisition and allocation (van Noordwijk & de Jong 1986; Schmid-Hempel 2011b). Such an explanation assumes that the variation in resource acquisition is equivalent between the two sampled colonies but that variation in the response to the relatively high level of antigen exposure experienced by pups in the human-impacted colony supersedes the influence of variation in acquisition. This possibility is supported by the wider range of changes in IgG concentration that were observed in the human-impacted colony compared with the control colony. However, it does not preclude the possibility that variation in antigen exposure between individuals within the human-impacted colony, rather than variation in their response to equal exposure, played a role in shaping this relationship.

Neither changes in PHA response nor changes in total leukocyte concentration were related to changes in any measure of condition in the human-impacted colony. This may be because the PHA response and total leukocyte concentration under most conditions represent snapshots of immune activity at the time of challenge or measurement (Vinkler et al. 2010), while IgG acts as a more cumulative measure of past immune activity. However, it is also possible that the pattern emerges with IgG concentration only because the immune challenge presented by the human-impacted colony elicits a dominantly humoural immune response, which is what we would expect of primary antigen exposure during early immune system ontogeny (Freitas et al. 1991; Hall et al. 2002).

Although less likely given the young age of the pups sampled in this study and therefore the presumed immaturity of their acquired immune response capacity (Day 2007), it is also possible that infection drove the negative relationships observed in the human-impacted colony. If IgG was produced in response to established infection rather than antigen exposure, pups that experienced the greatest increases in IgG concentration may have decreased

most in condition due to the direct costs of infection (Schmid-Hempel 2011c), rather than the costs of IgG production (Eraud et al. 2005). However, such an effect of infection would be more likely to be evident in total leukocyte and PHA data, as well as IgG data, than an effect of primary antigen exposure, as we would expect most infections to affect all three parameters to some degree, even though the effect on IgG concentration would be more cumulative. In addition, even in hyper-immunised individuals, IgG that is specific to a single antigen makes up less that 5% of the total IgG in circulation (King et al. 2001). Chapter 2 showed that there was a 29.3 % colony difference in juvenile IgG concentration between the ages of 6 and 18 months (control colony intercept = 1.069, difference to human-impacted colony intercept = 0.313; units = relative optical density; Table 2.3), which suggests that the difference was driven by response to multiple antigens. None of the animals included in this study showed any outward signs of sickness, but, as discussed in Chapter 2, it was not possible to quantify pathogen burden or clinical indicators of disease. I was therefore unable to test whether there were higher levels of infection in the human-impacted colony compared with the control colony, or whether pathogen burden was positively correlated with IgG concentration.

However, as discussed in Chapter 2 in the context of colony differences in immune activity, whether loss of condition in the human-impacted colony reported in this chapter was due entirely to the effects of immunity, entirely to the effects of infection, or to a combination, these findings have implications for life history and disease risk in the Galapagos sea lion. If changes in IgG concentration in the human-impacted colony were protective against infection, then any down-regulation of the antibody response could increase disease risk to individuals and the population. Such down-regulation is plausible because food shortages are known to have a negative impact on humoural immune responses (Martin et al. 2007a), and the Galapagos sea lion is exposed to rapid decreases in food availability (Trillmich & Limberger 1985; Trillmich & Dellinger 1991; Mueller et al. 2011). On the other hand, changes in IgG concentration in the human-impacted colony may have been due to antigen exposure that was not associated with disease risk (e.g. bacteria in

human effluent; Rodriguez & Valencia 2000; Cordoba et al. 2008; Alava 2011). If the sensitivity of response to this exposure were not modulated in response to energy availability, then sea lions in the human-impacted colony could be at greater risk from climate-driven decreases in food supply, as antigenic pressure could drain energy and resources through their immune systems.

Given the evidence presented in Chapter 2 and in this chapter, the second of these two scenarios seems more realistic, as, in the apparent absence of disease, pups from the human-impacted colony that produced high concentrations of IgG lost condition. This may be because some individuals do not regulate their response to post-natal antigen exposure in response to food availability, as – if the negative correlation between antibody production and condition is indicative of a cost of immunity – they appear to mount detrimentally high antibody responses, even when disease risk appears to be absent. Therefore, in the long-term, pups in the human-impacted colony may be at greater risk of death from starvation and, if the effects were to extend to mothers through compensation provisioning behaviour, mothers would have less energy available for future reproduction (Williams et al. 2007). If such effects were sustained, they could undermine colony stability and contribute to a population decline.

It is noteworthy that the positive relationship between change in IgG concentration and change in mass per unit length in the control colony was only evident in females. This may be because males and females grow in different ways: the sexes may regulate their subcutaneous fat stores in a similarly condition-dependent manner, but perhaps only females modulate their relative investments in skeletal and tissue growth in this way. The fact that the negative relationship between change in IgG concentration and change in albumin concentration in juveniles was only observed in males is curious. Given that there were neither sex differences in change in albumin concentration nor in change in IgG concentration in juveniles (Chapter 2), this result suggests that the physiological correlates of changes in IgG

concentration in juvenile males are fundamentally different from those in females.

The detection of life history trade-offs in the wild is complicated by variation that is often difficult to account for (van Noordwijk & de Jong 1986; Wilson & Nussey 2010), especially when immune responses and disease processes are involved (Sandland 2003). By taking advantage of the unique ecology of the Galapagos sea lion and using a longitudinal sampling design, the analysis presented in this chapter has shown that ecological circumstances may modulate the relationship between immunity and condition in the wild. These results have important implications for the conservation of this wild mammal that is currently endangered by disease (Alava & Salazar 2006; Aurioles & Trillmich 2008; Levy et al. 2008), and may suggest that subtle anthropogenic impacts that are difficult to study in the wild may be more common than we currently appreciate. Globally, as human pressure on wild systems increases, it becomes ever more important to understand these effects and their potential contribution to population declines through interactions with resource availability and the phenotypic plasticity of traits that have evolved in environments without ubiquitous human impacts.

Chapter 4

Relationships between inbreeding estimates and immune measures vary between ecological contexts

Abstract

Inbreeding depression is an important consideration for evolutionary and conservation biologists, and has been shown to have effects on immunity and disease processes in the wild. This chapter explores the hypothesis that inbreeding has a negative impact on immunity by assessing correlations between a marker-based estimate of inbreeding and growth and immune measures in the Galapagos sea lion, a species in which inbreeding is likely to occur. This analysis explored the heterogeneity of these relationships across ecological contexts and between age classes. Although inbreeding is not a variable commonly analysed by ecological immunology studies, it can provide valuable insight into the association between observed phenotypic variation in immune activity and underlying patterns of genetic variation. The results showed that inbreeding estimates were negatively correlated with growth and IgG production in Galapagos sea lions pups. In addition, inbreeding estimates were positively correlated with total leukocyte concentration in juveniles, but only in those juveniles that were resident in the human-impacted colony, and which, therefore, were likely to be exposed to anthropogenic environmental impacts. These results suggest that inbreeding depression may be present in the Galapagos sea lion, and that the effects of inbreeding may interact with human influence to affect immune development and activity. These findings highlight the importance of inbreeding as a consideration for the conservation of small populations of threatened species, and demonstrate how testing for associations across ecological contexts can suggest insight into the mechanisms that influence the expression of inbreeding depression in natural populations.

Introduction

Inbreeding has long been known to have deleterious effects on fitness (Darwin 1876; Wright 1922), and these effects have been well documented in captive, laboratory and domesticated animals (Frankham 1995). Historically, the occurrence of inbreeding depression in natural populations was controversial, as evidence was scarcer from the wild than from other contexts. However, this is likely to have been due to detection difficulties rather than lack of occurrence (Frankham et al. 2002), and it is now generally accepted that inbreeding has effects on fitness in natural settings (Crnokrak & Roff 1999; Keller & Waller 2002; Brekke et al. 2010; Walling et al. 2011). The consequences of inbreeding in the wild are of particular interest to biologists working to conserve small populations of threatened species (Keller & Waller 2002; Brekke et al. 2010), as inbreeding depression may interact with other extinction threats, such as disease risk, through both direct and indirect effects on immunity (Acevedo-Whitehouse et al. 2003a; Reid et al. 2003; Acevedo-Whitehouse & Duffus 2009). Moreover, the extent to which the expression of inbreeding effects varies across ecological contexts is poorly explored. In this chapter, I tested the prediction that growth and immune variation would be associated with estimates of inbreeding in the Galapagos sea lion, and whether the expression of any of associations varied between ecological contexts defined by anthropogenic influence.

The estimation of inbreeding coefficients from pedigrees is complicated by the assumption that founding individuals are outbred and unrelated (Szulkin et al. 2010), by the difficulty of accounting for the chance events of Mendelian segregation (Forstmeier et al. 2012), and by the rarity of multi-generation pedigrees for natural populations (Frentiu et al. 2008). An alternative to pedigree-based calculation is to use data from neutral genetic markers to either directly estimate inbreeding coefficients using relatedness algorithms, or to summarise genetic diversity as multi-locus heterozygosity (MLH), as a proxy for inbreeding value. These methods have the advantage of being calculable for individuals sampled from a single cohort, and are therefore feasible in many study systems, especially given recent advances in

sequencing technology and the availability of resources for the study of genetics.

Associations between indices of MLH and many fitness-related traits, from birth weight (Coltman et al. 1998) to song complexity (Marshall et al. 2003), have been reported from wild animal populations (reviewed in Chapman et al. 2009). However, there is debate about whether indices of MLH calculated using a number of loci that is small relative to the size of the genome can be closely enough related to true inbreeding values for inbreeding depression to be invoked as the cause of these heterozygosity-fitness correlations (HFCs; Balloux et al. 2004; Pemberton 2008). Empirical comparisons in populations for which both pedigrees and marker data are available have shown that pedigree-based estimates of inbreeding coefficients and indices of MLH are not well correlated, especially when the mean and variance of inbreeding value are low (Slate et al. 2004; Overall et al. 2005; Grueber et al. 2011; Wetzel et al. 2012). However, whether this suggests that MLH indices are less accurate than pedigree-based estimates, or that both are inaccurate in different ways has been a topic of recent debate (Forstmeier et al. 2012).

An explanation offered to explain HFCs as an alternative to inbreeding depression is that of linkage between one or a few neutral markers and functional genes under balancing selection, which could give rise to the frequently observed patterns of heterosis (Balloux et al. 2004; Acevedo-Whitehouse et al. 2006, 2009; Hoffman et al. 2010a, 2010b). A recent reappraisal points out, however, that the uneven contribution of loci to HFCs is implicit in inbreeding theory (Szulkin et al. 2010), as weak inbreeding is not expected to lead to detectable identity disequilibrium (David et al. 2007; Szulkin et al. 2010). Therefore, the finding that HFCs are driven by variation at one or a few loci does not preclude inbreeding as their underlying cause. In addition, the statistical tests that have been employed to identify single-locus contributions to HFCs suffer from problems of power (Hoffman et al. 2010b) and non-independence (Szulkin et al. 2010).

To date, variation in the detection and strength of HFCs has largely been attributed to methodology, and there is consensus that increases in numbers of samples and markers will help elucidate their significance (Balloux et al. 2004; Chapman et al. 2009). Another way in which HFC studies are being developed as tools for investigating the impact of inbreeding in wild populations is through the inclusion of ecological heterogeneity in their study designs (Chapman et al. 2009). This approach takes advantage of natural variation in the expression of the consequences of inbreeding, for example, through episodic heterozygote advantage (Samollow & Soulé 1983) or the effects of environmental stress (Hoffman & Hercus 2000; Enders & Nunney 2012) to unravel the underlying mechanisms that drive them and shed light on their significance in the wild. For this to be possible, a study species must be prone to inbreeding and display variation in traits that may be impacted by inbreeding, both of which are the case in the Galapagos sea lion.

As described in the Chapter 1, the Galapagos sea lion (*Zalophus wollebaeki*) is endemic to the Galapagos archipelago and has a small population (20,000-40,000 animals; Aurioles & Trillmich 2008) that is spatially and genetically structured amongst small colonies (20-500 animals; Alava & Salazar 2006; Wolf et al. 2008). In addition, the Galapagos sea lion has a polygynous mating system (Wolf et al. 2005; Pörschmann et al. 2011) and is philopatric (Wolf & Trillmich 2007). These conditions are those under which we expect the mean and variance of inbreeding value to be high, and therefore the inevitable imprecision of marker-based inbreeding estimates to be minimised (Balloux et al. 2004; Szulkin et al. 2010; Grueber et al. 2011).

Growth and condition measures are known to covary with survival probability and reproductive success in many taxa (Schulte-Hostedde et al. 2005), including marine mammals (Hall et al. 2001, 2002), and are likely to be important components of fitness in this system, as the Galapagos sea lion is sensitive to fluctuations in marine productivity driven by unpredictable environmental variation (Trillmich & Limberger 1985; Trillmich & Dellinger 1991; Mueller et al. 2011). IgG concentration has been linked to survival probability in the Grey Seal (Hall et al. 2002), and immune variation may be

associated with Galapagos sea lion fitness given the patterns reported in Chapter 3 and the threat of pathogen pollution from domesticated animals (Cunningham et al. 2003; Alava & Salazar 2006; Aurioles & Trillmich 2008). In addition, inbreeding has been associated with infection in other study systems, which may be due in part to effects on immunity (Coltman et al. 1999; Acevedo-Whitehouse et al. 2003a; Townsend et al. 2010).

Given the many influences on immune variation in natural populations, it is unlikely that measures of immune variation are related to fitness in a straightforward way (e.g. Råberg et al. 2003). For this reason, the analysis presented here makes use of the immune variation between age classes and colonies to test predictions about when associations between inbreeding and immunity would most likely be expressed. This variation is described in Chapters 2 and 3 and is derived from data collected in two Galapagos sea lion colonies: one on an uninhabited island (control colony), and one located in a town (human-impacted colony); and two ecologically distinct age-classes: pups (0-3 months old), which are restricted to land and shallow tidal pools, and are dependent on their mothers for nutrition; and juveniles (6-24 months old), which swim out to sea, dive to depth and are capable of foraging independently.

I predicted: 1) that growth measures would be negatively correlated with inbreeding estimates across colonies and age classes; 2) that immune measures would be negatively correlated with inbreeding estimates in the control colony, where infection risk is likely to be relatively low (Chapter 1) and where immune variation appears to be condition-dependent (Chapter 3); and 3) that immune measures would be positively correlated with inbreeding estimates in the human-impacted colony, where infection risk is likely to be relatively high (Chapter 1). This third prediction rests on the assumptions that immune systems are capable of mounting detectable responses to infection, which is more likely to be the case in juveniles, and that more inbred individuals suffer higher infection rates (e.g. Coltman et al. 1999; Acevedo-Whitehouse et al. 2003a; Townsend et al. 2010).

Methods

Genotyping

I extracted genomic DNA from 166 Galapagos sea lion skin samples and amplified 23 polymorphic microsatellite loci previously developed for the Galapagos sea lion and other pinniped species (Wolf et al. 2006; Pörschmann et al. 2011). All genetic analyses were carried out at the University of Bielefeld, Germany, as described in Wolf et al. (2006) and Pörschmann et al. (2011). Sequencing was performed on an Applied Biosystems 3130 Sequencer (Life Technologies) and genotyping was performed in GENEMARKER (Soft Genetics, USA). I tested for deviations from Hardy-Weinberg equilibrium (HWE) using the Markov chain algorithm to estimate exact p-values without bias in GENEPOP'007 (Rousset 2008); and for null alleles and large allele dropout using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004).

Simulations

In order to make an evidence-based choice on the most appropriate neutral marker-based estimate of inbreeding value for this dataset, I performed simulations in COANCESTRY (Wang 2011). I used the empirical allele frequencies to simulate four populations of known relatedness structure, and compared the values of the inbreeding coefficient *f* with neutral marker-based inbreeding estimates and MLH indices calculated from the simulated genotypes. I calculated two moment estimators (Ritland 1996; Lynch & Ritland 1999) and two likelihood estimators of inbreeding (Milligan 2003; Wang 2007) in COANCESTRY (Wang 2011). In addition, I calculated three indices of MLH: standardised heterozygosity (SH, Coltman et al. 1999); internal relatedness (IR, Amos et al. 2001); and homozygosity by loci (HL, Aparicio et al. 2006) in the Rhh package (Alho et al. 2010) in R 2.14.1 (R Development Core Team, 2011). An advantage of the COANCESTRY (Wang 2011) likelihood framework is that it takes into account genotyping error rates, which are known accurately for this system because of the large number of individuals genotyped as part of the University of Bielefeld's Galapagos Sea Lion Project (mean error rate across loci = 0.003; Pörschmann et al. 2011).

Following Brekke et al. (2010) I simulated the first population with a uniform distribution of *f*, in which *f* increased in increments of 0.05 from 0 to 1, and each category included 100 individuals (n = 2,100). I simulated the second population with the same uniform distribution but limiting the range of *f* from 0 to 0.5 and increasing the number of individuals in each category to 200 (n = 2,200). I limited the range in this way after estimating inbreeding values from the data, and observing that estimators on the same scale as *f* were always below 0.5. Next, I used the triadic maximum likelihood estimator of inbreeding value (TML; Wang 2007) to estimate the empirical distribution of inbreeding values from the data. Then I simulated the third population using this distribution, adjusting the number of individuals in each inbreeding value category to match the total number of individuals in the first two simulated populations as closely as possible (n = 2,088). I simulated the fourth population using the empirical distribution of TML inbreeding value estimates and the empirical sample size (n = 166). Finally, I correlated the four inbreeding estimators and three MLH indices with the value of *f* in each simulated population.

Growth, immunity and parasite data

I sampled Galapagos sea lion pups shortly after birth and at 3-months-old, between which two ages they undergo a growth spurt (Mueller et al. 2011). To analyse pup growth I calculated absolute changes in body mass and length over time (see Chapter 3 for morphology data collection methods). I used the same measures to quantify immune variation in this chapter as in Chapter 3: immunoglobulin G (IgG) concentration, the *in vivo* inflammation response to phytohemagglutinin (PHA), and total leukocyte concentration; and for the same reasons: as they were possible in the greatest number of individuals at the greatest number of time points and therefore allowed for the greatest statistical power, and because they displayed variation with both colony and age (Chapter 2).

I set out to estimate the infection status and burden of the sampled Galapagos sea lions in three ways. First, I conducted external examinations during each capture to quantify infection with ectoparasites and the trematode *Philophthalmus zalophi* (Dailey et al. 2005; Chapter 2). Second, while carrying out differential white blood cell counts (Brock et al. 2012), I screened blood smears for blood-borne parasites, particularly microfilariae (Dailey et al 2001). Third, I screened faecal samples, which were collected by rectal swabbing during captures and opportunistically when known individuals were observed defecating on the beach, for the presence of hookworm eggs. I examined swab samples using direct smears and beach samples by flotation, using a McMaster chamber to count eggs after centrifugation and saturation of the sample with sugar solution (E. T. Lyons, personal communication).

Statistical analysis

In pups, I calculated changes in these three immune measures relative to changes in body mass, since they changed with age during this period of pup growth (Mueller et al. 2010; Chapter 2). Next, I tested for relationships between TML, sex, colony and estimated birthdate using ANOVAs and a linear model, respectively. Then I fitted the five growth and immune variables as responses in separate linear models that included TML, colony and their interaction as explanatory variables. I simplified these models by removing non-significant terms following Crawley (2007) until either all terms had been removed, or a minimum adequate model was achieved.

Relatively little growth occurs in juvenile Galapagos sea lions between the sampled ages of 6, 12, 18 and 24 months (Mueller et al. 2011). Therefore, in juveniles, I tested for an association between TML and raw values of body mass, mean body length, and the three measures of immune variation. First, I tested for differences in TML between colonies and sexes with linear mixed effect (LME) models that included individual identity and age as random effects. Then I fitted these five growth and immunity variables as responses in LME models that included TML, colony and their interaction as explanatory variables, as well as individual identity and age as random effects. I simplified

these models by the serial comparison of nested models using likelihood ratio tests following Zuur et al. (2009) until either all terms had been removed, or a minimum adequate model was achieved.

I checked all models for signs of heteroscedasticity, heterogeneity of variance, non-normality of error and the disproportionate influence of outliers. The results of these checks showed that the skewed distribution of TML led to a disproportionate influence of individuals with high TML values on model estimates, and worrisome heteroscedasticity. In order to solve these problems of model instability without losing information through the removal of outliers, I log-transformed TML values prior to analysis.

Locus contribution tests

For significant relationships between TML and immune variation, I explored the contribution of individual loci in a five-step selection process. Loci were considered to have standout effects if they fulfilled all of the following criteria: 1) if, for the overall relationship, the model in which TML was replaced with a set of two-level factors, each coding heterozygosity at a single locus, explained significantly more variation than the original model (Szulkin et al. 2010); 2) if the removal of the two-level factor coding heterozygosity at a locus from the full model including two-level heterozygosity factors for all loci significantly reduced explanatory power; 3) if the addition of the two-level factor coding heterozygosity at a locus to the null model significantly increased explanatory power; 4) if removing TML calculated without a locus from a model including TML calculated without that locus and a two-level factor coding heterozygosity at that locus did not significantly reduce explanatory power; and 5) if removing the two-level factor coding heterozygosity at a locus from a model including TML calculated without that locus and a two-level factor coding heterozygosity at that locus did significantly reduce explanatory power. I also tested for identity disequilibrium in RMES (David et al. 2007; Szulkin et al. 2010) to test for the correlation of heterozygosity across loci.

Next, I investigated the influence of locus variability on locus contribution as assessed by the above selection process. Although locus variability is controlled for by the calculation of indices of MLH and inbreeding estimators, it is rarely taken into account by analyses of single locus effects. First, for each significant relationship between TML and immune variation, I ranked the loci according to the chi-squared values of each of the four locus-specific contribution tests. I ranked chi-squared values in ascending order for tests 2, 3 and 5 and in descending order for test 4, so that the ranks reflected contributions in a consistent direction. Then, I fitted linear models of mean chi-squared rank with four aspects of locus variability: number of alleles, number of genotypes, maximum allele frequency and maximum genotype frequency.

Power analysis

In order to gain insight into how likely the combination of analysis approach and dataset used in this analysis was to detect a real effect of inbreeding, I conducted a tailored power analysis, using change in IgG concentration per kilogram of mass change (IgG mg mL^{-1} kg^{-1}) in pups as an example. First, I randomly sampled 40 of the 166 individuals from the population simulated to have true values of *f* distributed according to the empirical distribution of TML, as this was the number of data points used in the empirical analysis of the relationship between change in IgG mg mL^{-1} kg^{-1} and TML in pups. Then I randomly assigned the sampled individuals values of change in IgG mg mL^{-1} kg^{-1} from a normal distribution with the mean and standard deviation of the empirical change in IgG mg mL^{-1} kg^{-1} distribution. I adjusted this value according to each sampled individual's simulated value of *f* and a range of effect sizes of *f* on change in IgG mg mL^{-1} kg^{-1} that included the empirical effect size. Next, I calculated TML from the simulated genotypes of the sampled individuals and fit a linear model of assigned change in IgG mg mL^{-1} kg^{-1} value with TML as the only explanatory variable. I repeated this process 100 times for 20 effect size values, and recorded the percentage of models that reported a significant relationship for each effect size.

Additive genetic variance

Finally, to test whether any additive genetic variance for growth and immune variation was detectable, and whether this detectability differed between colonies, I calculated the triadic maximum likelihood estimator of relatedness between all possible pairs of individuals within colony and age-class subsets in COANCESTRY (Wang 2011). I classified individuals as either high or low for each growth and immune variation variable, according to whether their value was greater or smaller than the median of their subset. Then I used 1000 bootstrap replicates to test whether relatedness between pairs of individuals from the same half of the growth and immune variation distributions was significantly different from relatedness between those from different halves.

Results

Two of the 23 amplified loci showed significant deviations from HWE (Table 4.1): ZcwB07 (Hoffman et al. 2007) and ZcwG06 (Wolf et al. 2007a), but there was no evidence for the presence of null alleles or of large allele dropout. In all four simulated populations the correlation with *f* was higher for likelihood inbreeding estimators than it was for either moment estimators or MLH indices (Table 4.2). While MLH indices performed better in simulated populations with uniform distributions of *f*, moment estimators performed better when populations were simulated with more realistic, right-tailed *f* distributions (Table 4.2).

The mean value of TML across the sample was 0.05, and 11.5 % of individuals (19 out of 166) had values greater than 0.125. The empirical distribution of TML in the Galapagos sea lion was, therefore, similar to that described in a bird population (*Notiomystis cincta*) in which inbreeding depression has been demonstrated: mean = 0.08, 17.8 % of individuals (41 out of 230) with values greater than 0.125 (Brekke et al. 2010).

I encountered ectoparasites in 15 out of 487 external examinations carried out during sea lion captures. All 15 of these observations were of lice (*Antarctophthirus microchir*), nine findings were of a single louse and the remaining six were of two lice. Lice were most often to be found on the ventral surface of the abdomen just anterior to the hind flippers. Given the small number of sea lions positive for ectoparasite infection, I did not include this data in the analysis.

Eye examinations for the determination of *P. zalophi* infection status were possible during 207 captures; 126 of these individuals were infected and 81 were not. However, the burden of infection varied between infected individuals in a way that could not be reliably quantified. This was partly due to differences in individual behaviour: during many captures examination of the ocular cavity was difficult and incomplete, and in others it was not feasible at all. Therefore, despite the prevalence of this parasite, it was not possible to

quantify infection and burden across individuals consistently. Consequently, I also excluded this data from the analysis.

I screened 280 blood sears for the presence of blood-borne parasites such as microfilariae, but none showed any signs of such infections. I examined 79 faecal swabs collected during captures and 20 faecal samples collected from the beach. Only one of these beach samples was from a known individual, the others were collected in an attempt to validate the methods. However, no hookworm eggs were detectable in any of the samples.

TML neither varied significantly with sex (p = 0.23), colony (p = 0.74) or estimated birthdate (p = 0.78) in pups shortly after birth; nor with sex (N_{total} = 169, $N_{individuals}$ = 73, t = 0.21, p = 0.829) or colony (N_{total} = 169, $N_{individuals}$ = 73, t = -1.15, p = 0.250) in juveniles. The lack of a difference between the colonies is particularly relevant, as it suggests that despite the difference in colony size (Brock et al. 2012; Chapter 2), there is no colony difference in mean level of inbreeding. TML values did not depend on the structure of the dataset used for calculation; for example, TML values estimated for pups from the control colony were always highly correlated (r > 0.99) whether they were calculated in a dataset including control colony pups only, control colony animals only, pups from both colonies, or all animals. There was no evidence of identity disequilibrium, as g_2 was not significantly different from zero (g_2 = 0.0033, sd = 0.0027, p = 0.888, 1000 iterations).

Table 4.1 Hardy-Weinberg probability tests performed in GENEPOP'007 (Rousset 2008); estimation of exact *p*-values by Markov chain (20 batches, 5000 iterations per batch); null allele frequency estimated by maximum likelihood. HWE = Hardy-Weinberg equilibrium.

Locus	HWE probability test *p*-value	Null allele frequency
ZcwA05	0.975	0.000
Pv9	0.626	0.000
ZcwD01	0.696	0.043
Hg4.2	0.334	0.168
ZcwA12	0.434	0.000
ZcwE05	0.605	0.000
ZcwH09	0.326	0.087
ZcwD02	0.115	0.015
ZcwA07	0.363	0.000
ZcwB09	0.800	0.000
ZcwC03	0.928	0.000
ZcCgDh5.8	0.139	0.028
ZcwC11	0.972	0.001
Hg8.10	0.531	0.000
ZcCgDh7tg	0.851	0.000
Hg6.1	0.700	0.032
ZcwF07	0.204	0.000
ZcwE03	0.533	0.101
ZcwE12	0.793	0.118
ZcwE04	0.354	0.038
Pv11	0.088	0.269
ZcwG06	0.000 *	0.217
ZcwB07	0.000 *	0.086

Table 4.2 Mean correlations between simulated values of the inbreeding coefficient *f* and indices of multi-locus heterozygosity (MLH), moment estimators of inbreeding and likelihood estimators of inbreeding in four simulated populations. MLH indices were standardized heterozygosity (SH, Coltman et al. 1999), internal relatedness (IR, Amos et al. 2001) and homozygosity by loci (HL, Aparicio et al. 2006); moment estimators were Ritland (Ritland 1996) and Lynch and Ritland (Lynch & Ritland 1999); likelihood estimators were dyadic (Milligan 2003) and triadic methods (Wang 2007). "Uniform" distributions had an equal number of individuals in each inbreeding value category, while in "GSL" distributions the number of individuals in each inbreeding value category was proportional to empirical estimates from Galapagos sea lion data. See Table 4.3 for more detail.

			Mean correlation with *f*		
Distribution	Range of *f*	Number of individuals	Heterozygosity indices	Moment estimators	Likelihood estimators
Uniform	0 – 1.0	2,100	0.917	0.794	0.943
Uniform	0 – 0.5	2,200	0.726	0.618	0.767
GSL	0 – 0.5	2,088	0.416	0.437	0.512
GSL	0 – 0.5	166	0.326	0.388	0.404

Table 4.3 Correlations between simulated values of *f*, 4 inbreeding estimators and 3 indices of multi-locus heterozygosity (MLH), for different simulated distributions and ranges of *f*, and different sample sizes: (a) uniform distribution of *f* from 0 to 1 in steps of 0.05 with 100 individuals in each category (n = 2,100); (b) uniform distribution of *f* from 0 to 0.5 in steps of 0.05 with 200 individuals in each category (n=2,200); (c) simulated distribution of *f* modelled on empirical distribution estimated from Galapagos sea lion data, from 0 to 0.5 in steps of 0.05, (n = 2,088); (d) simulated distribution of *f* modelled on the empirical distribution estimated from Galapagos sea lion data from 0 to 0.5 in steps of 0.05, empirical sample size (n=166). MLH indices were standardized heterozygosity (SH, Coltman et al. 1999), internal relatedness (IR, Amos et al. 2001) and homozygosity by loci (HL, Aparicio et al. 2006); moment estimators were RIT (Ritland 1996) and LYRT (Lynch & Ritland 1999); likelihood estimators were dyadic (DML; Milligan 2003) and triadic methods (TML; Wang 2007).

(a)	True Value	RIT	LYRT	DML	TML	SH	IR
RIT	0.675						
LYRT	0.913	0.784					
DML	0.944	0.702	0.955				
TML	0.942	0.704	0.957	0.998			
SH	0.914	0.677	0.929	0.968	0.972		
IR	0.922	0.688	0.938	0.976	0.979	0.994	
HL	0.914	0.677	0.929	0.968	0.972	1.000	0.994

(b)	True Value	RIT	LYRT	DML	TML	SH	IR
RIT	0.519						
LYRT	0.718	0.811					
DML	0.764	0.594	0.857				
TML	0.769	0.599	0.864	0.992			
SH	0.720	0.521	0.784	0.907	0.919		
IR	0.738	0.555	0.811	0.929	0.940	0.982	
HL	0.720	0.521	0.784	0.907	0.919	1.000	0.982

(c)	True Value	RIT	LYRT	DML	TML	SH	IR
RIT	0.367						
LYRT	0.409	0.941					
DML	0.408	0.719	0.776				
TML	0.400	0.733	0.789	0.979			
SH	0.326	0.631	0.684	0.796	0.811		
IR	0.327	0.657	0.709	0.826	0.838	0.969	
HL	0.326	0.631	0.684	0.796	0.811	1.000	0.969

(d)	True Value	RIT	LYRT	DML	TML	SH	IR
RIT	0.400						
LYRT	0.473	0.850					
DML	0.518	0.586	0.786				
TML	0.505	0.602	0.805	0.981			
SH	0.407	0.476	0.665	0.784	0.805		
IR	0.434	0.524	0.713	0.822	0.841	0.966	
HL	0.407	0.476	0.665	0.784	0.805	1.000	0.966

The mean change in pup body mass from shortly after birth until 3 months of age was 6.01 kg (standard deviation = 1.81 kg). Over the same period, mean change in pup body length was 14.59 cm (standard deviation = 5.19 cm), and mean change in pup skinfold thickness was 0.075 cm (standard deviation = 0.153 cm). In pups change in body length was greater in the human-impacted colony than in the control colony ($t_{1,38}$ = -2.56, p = 0.014), and there was no difference in change in body length between the sexes ($t_{1,38}$ = 1.97, p = 0.055). Change in body mass was greater in male pups than female pups ($t_{1,38}$ = 2.59, p = 0.013), and there was no difference between colonies ($t_{1,38}$ = -1.77, p = 0.083). There was neither a sex difference ($t_{1,38}$ = 0.79, p = 0.43) nor a colony difference in change in pup skinfold thickness ($t_{1,38}$ = 0.29, p = 0.77).

In pups, changes in body length, body mass and IgG concentration per kilogram of mass change (IgG mg mL^{-1} kg^{-1}) were negatively correlated with TML, while changes in total leukocyte concentration and PHA response per kilogram of mass change (WBC 10^9 L^{-1} kg^{-1} and PHA mm kg^{-1}) were not related to TML (Fig. 4.1a-c; Table 4.4). There were no significant interactions between colony and TML in pups. The consistency of outcome across the two growth measures was to be expected as they were correlated (r = 0.72). Given the small sample size in pups, I was only able to apply single locus contribution tests 4 and 5 to pup data, and they did not identify any loci with consistent standout effects on the relationship between TML and change in IgG mg mL^{-1} kg^{-1}. In addition, the rank of locus contribution to this relationship was unrelated to the four measures of locus variability (p > 0.05).

In juveniles, mean change in mass between 6 months and 24 months was 13.61 kg (standard deviation = 3.41 kg), and mean change in length from 6 to 24 months was 19.36 cm (standard deviation = 5.59 cm). TML was not correlated with juvenile body length, IgG concentration or PHA response (Table 4.5). However, TML was negatively correlated with body mass in both colonies, and positively correlated with total leukocyte concentration in the human-impacted colony (Fig. 4.1d-e; Table 4.5). A model of total leukocyte concentration in the human-impacted colony in which TML was replaced by

factors coding heterozygosity at each locus did not explain the data better than the original model (χ^2 = 24.151, p = 0.33). No single locus was selected by the remaining four locus contribution tests, and there was no significant association between locus contribution rank and the four measures of locus variability ($p > 0.05$).

The power analysis showed that the statistical approach presented here had an 86 % chance of detecting an effect of true inbreeding value of the same size as the observed statistical effect of TML on change in IgG mg mL^{-1} kg^{-1} (slope = -0.595, Table 4.4; Fig. 4.2). Consistent with the detection of additive genetic variation for immune traits, pairs of pups from the same half of the change in IgG mg mL^{-1} kg^{-1} and WBC 10^9 L^{-1} kg^{-1} distributions in the control colony were more closely related than those from different halves of the distributions (Fig. 4.3a). Contrary to expectation, however, pairs of pups from the human-impacted colony from the same half of the growth rate distributions were less related than pairs from different halves (Fig. 4.3a). In control colony juveniles, pairs of individuals from the same half of the PHA response distribution were more closely related than those from different halves (Fig. 4.3b).

Table 4.4 Minimum adequate linear models of the effect of the triadic maximum likelihood estimator of inbreeding (TML; Wang 2007) on two growth and three immune variables in pups: 'Δ Length (cm)' = change in mean body length; 'Δ Mass (kg)' = change in body mass; 'Δ IgG (mg mL^{-1} kg^{-1})' = change in total immunoglobulin G concentration per kilogram of mass change; 'Δ WBC (10^9 L^{-1} kg^{-1})' change in total leukocyte concentration per kilogram of mass change; 'Δ PHA (mm kg^{-1})' = change in PHA response per kilogram of mass change. 'Ln' denotes natural logarithm, 'CC' control colony, and 'NS' denotes non-significance for cases in which all variables were removed during model simplification.

Response	Explanatory	N	Estimate	SE	*t*	*p*	R^2
Δ Length (cm)	Ln (TML)	40	-1.138	0.550	- 2.069	0.045 *	0.194
	Colony (CC)	-	-3.532	1.487	-2.375	0.022 *	-
Δ Mass (kg)	Ln (TML)	40	-0.558	0.194	-2.874	0.006 **	0.157
Δ IgG (mg mL^{-1} kg^{-1})	Ln (TML)	37	-0.595	0.189	-3.149	0.003 **	0.198
Δ WBC (10^9 L^{-1} kg^{-1})	Ln (TML)	38	NS	NS	NS	NS	NS
Δ PHA (mm kg^{-1})	Colony (CC)	40	-0.115	0.044	-2.604	0.013 *	0.129

Table 4.5 Linear mixed effect models of the effect of the triadic maximum likelihood estimator of inbreeding (TML; Wang 2007) and colony on two morphological and three immune variables in juveniles: 'Length (cm)' = mean body length; 'Mass (kg)' = body mass; 'IgG (mg mL^{-1})' = immunoglobulin G concentration; 'PHA (mm)' = PHA response; 'WBC (10^9 L^{-1})' total leukocyte concentration. The colony effect is reported as a contrast between the estimated colony intercepts. 'Ln' = natural logarithm; 'LRT' = likelihood ratio test, 'HIC' = human-impacted colony, 'CC' = control colony. The likelihood ratio tests reported are for the comparison between models including Ln (TML) and colony as main effects and those including only colony; except in the case of WBC (10^9 L^{-1}), in which the comparison is between a model including the interaction between Ln (TML) and colony and a model including only their main effects.

			Fixed effects				Random effects (variance)		
Response	N_{total}	$N_{individuals}$	Explanatory	Estimate	SE	*t*	Age	Identity	Residual
Mass (cm)	169	73	Ln (TML)	-0.548	0.260	-2.103	22.361	7.473	9.938
Length (cm)	169	73	Ln (TML)	NS	NS	NS	NS	NS	NS
IgG (mg mL^{-1})	149	69	Colony (CC)	-0.222	0.028	-7.830	<0.001	0.007	0.012
PHA (mm)	128	62	Ln (TML)	NS	NS	NS	NS	NS	NS
WBC (10^9 L^{-1})	165	72	Ln (TML) (HIC)	0.369	0.136	2.701	0.654	<0.001	1.646
			Colony (HIC)	-2.352	0.706	-3.329			
			Ln (TML) * Colony (CC)	-0.474	0.170	-2.788			

Figure 4.1

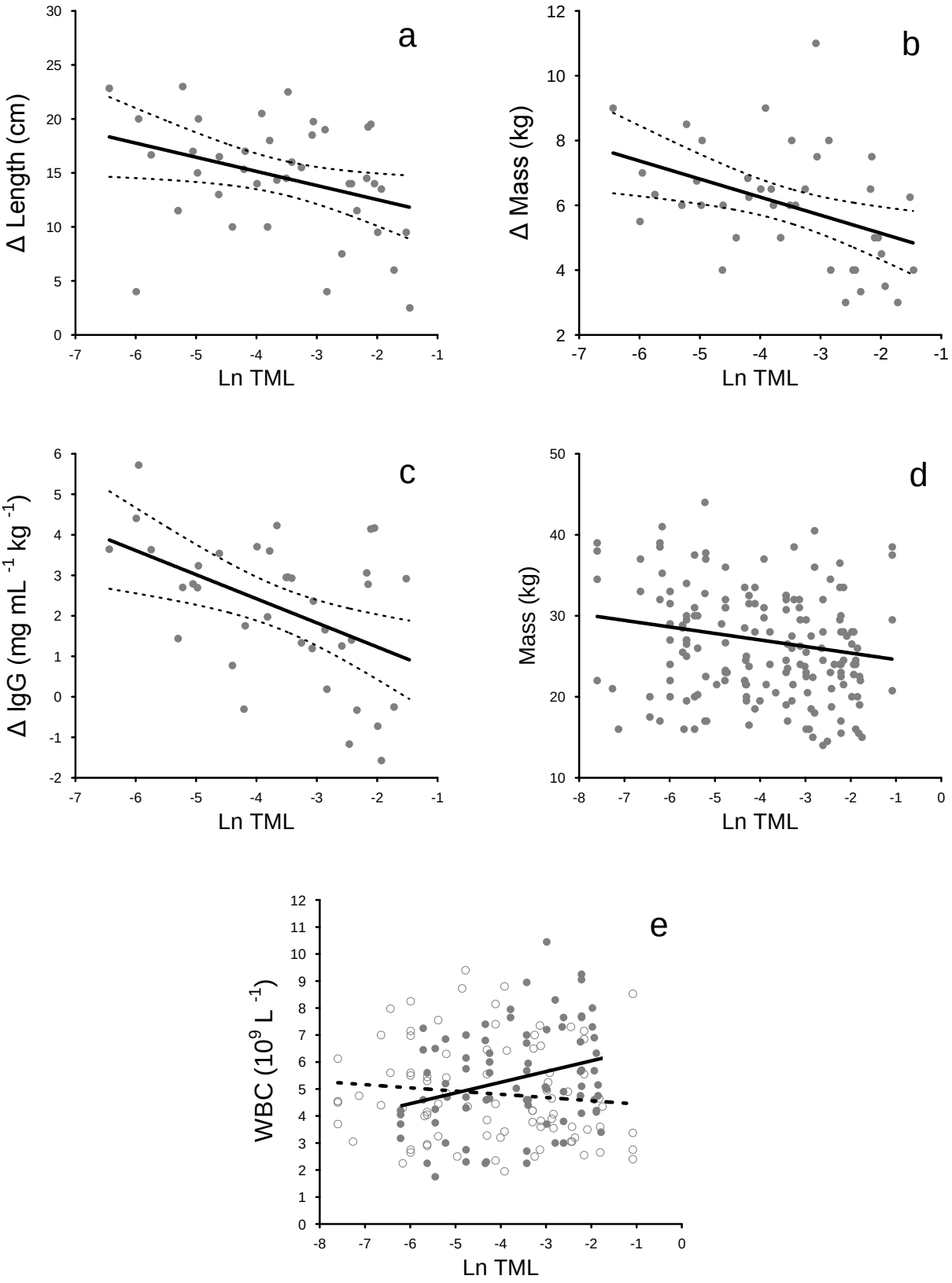
a
Δ Length (cm)
Ln TML
b
Δ Mass (kg)
Ln TML
c
Δ IgG (mg mL -1 kg -1)
Ln TML
d
Mass (kg)
Ln TML
e
WBC (10^9 L -1)
Ln TML

Figure 4.1 (legend) The predicted relationship between the triadic likelihood estimator of inbreeding (TML; Wang 2007) and (a) change in mean body length (cm) in pups from both colonies, (b) change in body mass (kg) in pups from both colonies, (c) change in total immunoglobulin G concentration per kilogram of change in mass (IgG mg mL^{-1} kg^{-1}) in pups from both colonies, (d) body mass (kg) in juveniles from both colonies, and (e) total leukocyte concentration (10^9 L^{-1}) in juveniles from the human-impacted colony (filled circles and solid line) and the control colony (open circles and broken line). 'Ln' = natural logarithm, 'Δ' = change in. See Tables 4.4 and 4.5 for more detail.

Figure 4.2

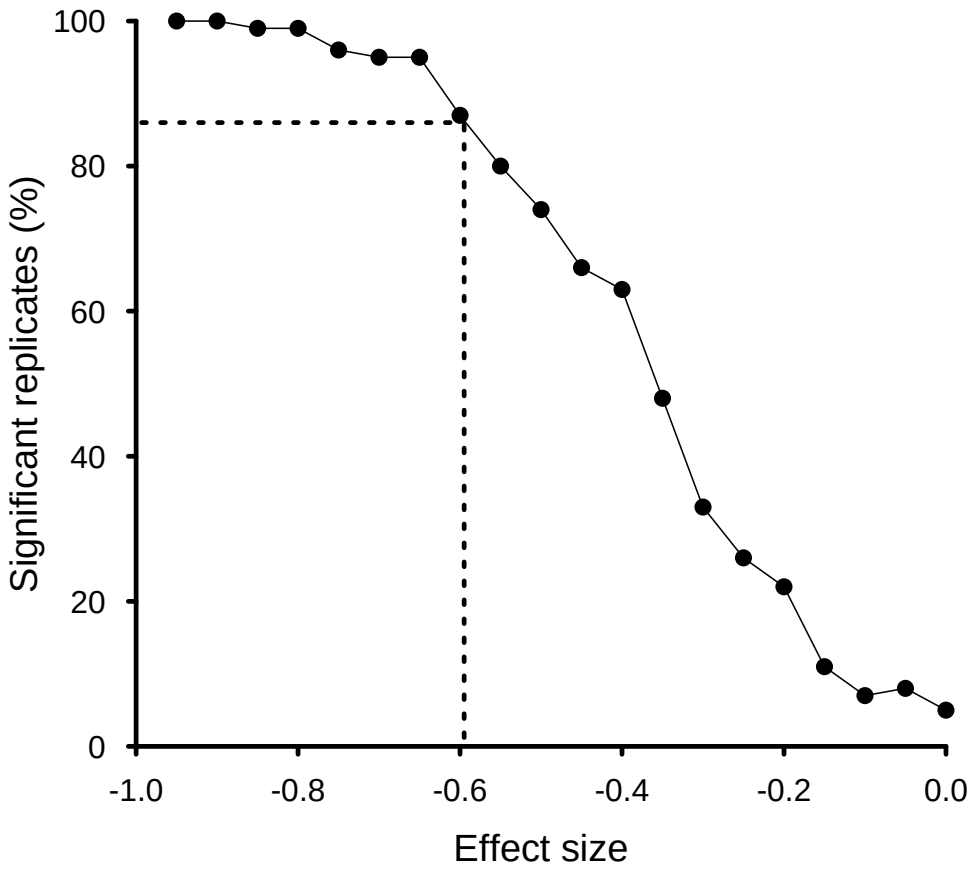

Figure 4.2 Influence of effect size on the probability of detecting an effect of inbreeding value (*f*) on change in IgG mg mL^{-1} kg^{-1} using the triadic likelihood estimator of inbreeding (TML; Wang 2007). The dotted line shows the empirical estimate for the effect of TML on change in IgG mg mL^{-1} kg^{-1} and the probability of detecting an equivalent effect of *f* (86 %) given the dataset.

Figure 4.3

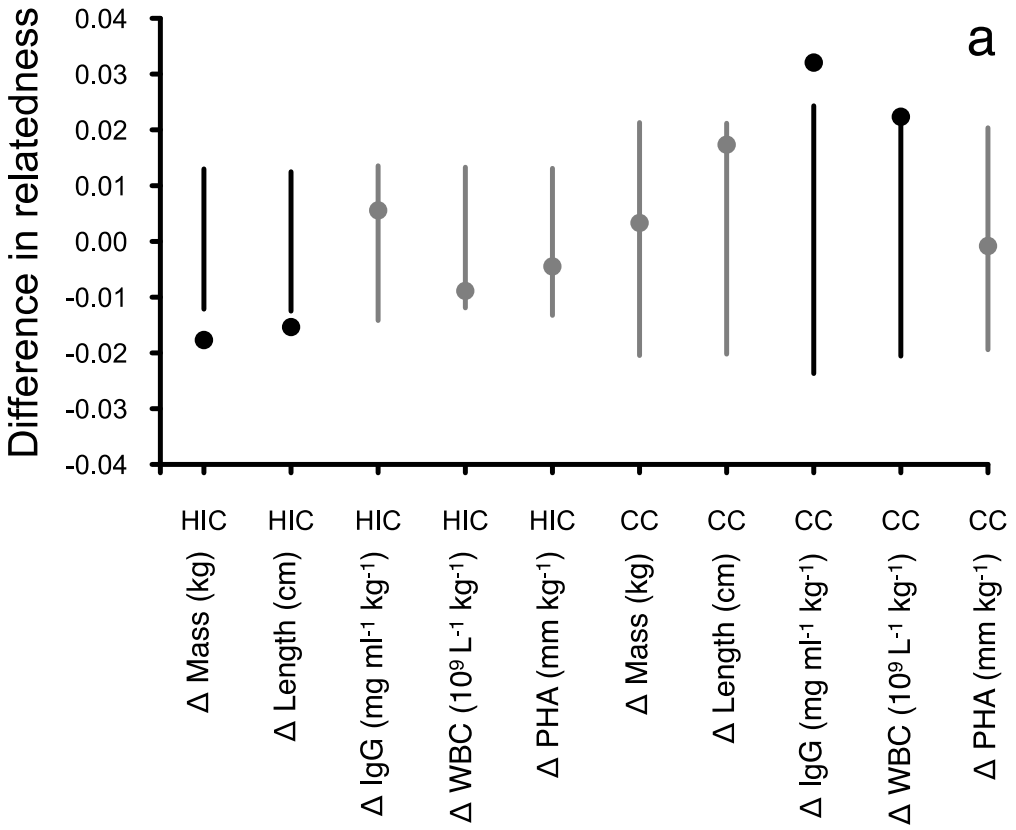

Figure 4.3a Tests for differences in relatedness between groups with trait values from either the same or different halves of the distributions for colony and age class subsets, in pups. Bars show 95 % bootstrapped confidence intervals (1000 replicates) and points are the observed mean differences between pairs of individuals from the same half and different halves of trait distributions. 'HIC' = human-impacted colony, 'CC' = control colony, 'Δ Length (cm)' = change in mean body length; 'Δ Mass (kg)' = change in body mass; 'Δ IgG (mg mL^{-1} kg^{-1})' = change in immunoglobulin G concentration per kilogram of mass change; 'Δ WBC (10^9 L^{-1} kg^{-1})' change in total leukocyte concentration per kilogram of mass change; 'Δ PHA (mm kg^{-1})' = change in PHA response per kilogram of mass change. Grey bars and circles represent tests where the observed difference in relatedness lay inside the 95 % bootstrapped confidence intervals, while black bars and circles represent those where the observed difference lay outside the 95 % bootstrapped confidence interval.

Figure 4.3 (cont.)

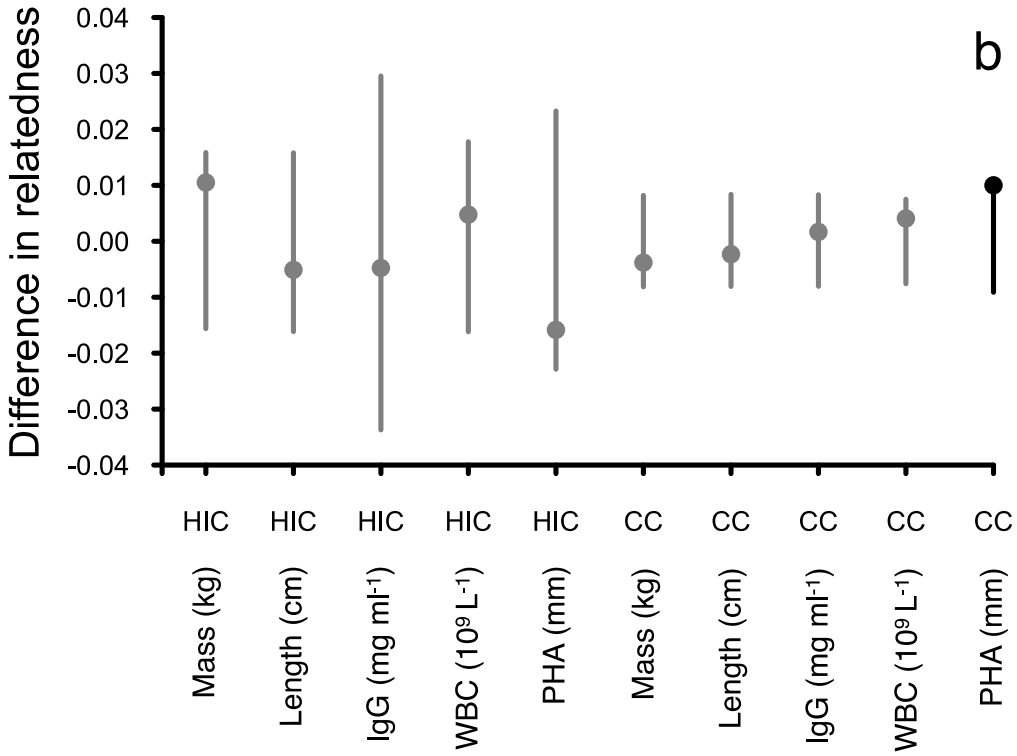

Figure 4.3b Tests for differences in relatedness between groups with trait values from either the same or different halves of the distributions for colony and age class subsets, in juveniles. Bars show 95 % bootstrapped confidence intervals (1000 replicates) and points are the observed mean differences between pairs of individuals from the same half and different halves of trait distributions. 'HIC' = human-impacted colony, 'CC' = control colony, 'Length (cm)' = mean body length; 'Mass (kg)' = body mass; 'IgG (mg mL^{-1})' = immunoglobulin G concentration; 'PHA (mm)' = PHA response; 'WBC (10^9 L^{-1})' total leukocyte concentration. Grey bars and circles represent tests where the observed difference in relatedness lay inside the 95 % bootstrapped confidence intervals, while black bars and circles represent those where the observed difference lay outside the 95 % bootstrapped confidence interval.

Discussion

This chapter has two main findings. First, as predicted, growth and immune variation was associated with inbreeding estimates in the endangered Galapagos sea lion. Second, the occurrence and nature of these associations varied between ecological contexts, suggesting heterogeneity in the expression of the underlying effects that may have been influenced by anthropogenic environmental impacts. The description of such patterns answers a call to incorporate more ecologically relevant variation into the design of investigations into the impacts of inbreeding in natural populations (Chapman et al. 2009). The results are consistent with the hypothesis that inbreeding has a negative effect on immune function in the wild, and that the expression of these effects is heterogeneous across ecological contexts. However, it should be noted that these results are correlative, and consistency of the results with the hypothesis of a negative effect of inbreeding on immune function is not the same as evidence for a causative link.

As predicted, the growth of pups from both colonies was consistently associated with TML, and there was a similar negative association between juvenile body mass and TML in both colonies. This suggests that inbreeding may have a negative impact on growth that is independent of the anthropogenic environmental impacts that define the majority of the differences between the two study colonies (Chapter 1). Body length may not have been related to TML in juveniles because body mass is a better indicator of overall growth, and therefore produces a clearer statistical signal. While changes in sea lion body length chart skeletal growth, changes in body mass may be more informative because they encode information about both skeletal and soft tissue growth. This possibility is supported by the stronger effect of TML on change in body mass than on change in body length in pups. It is also possible that there is greater error associated with the measurement of body length compared with body mass in sea lions.

When controlled for changes in body mass, change in IgG concentration was negatively correlated with TML in pups from both colonies. The majority of

Galapagos sea lion pups grow (Mueller et al. 2011) and produce IgG (Chapter 2) during this period of their ontogeny. IgG production during early immune system ontogeny is likely to be condition-dependent to some degree, which is why I controlled changes in IgG concentration for changes in mass: so that any relationship between IgG production and TML was not confounded by growth. Chapter 2 showed that pups from the human-impacted colony produced more IgG than those from the control colony.

As discussed in Chapter 2, this colony difference in IgG production is likely to have been caused by differences in post-natal antigen exposure (Chapter 2), which drives the build up of protective baseline populations of lymphocytes and antibodies (Freitas et al. 1991; Hall et al. 2002). As there was no interactive effect of TML with colony on change in IgG mg mL^{-1} kg^{-1} the results of this Chapter suggest that inbreeding influences this aspect of early immune system development, regardless of the degree of antigen exposure. This is congruent with findings from other systems that have shown inbreeding estimates to be negatively associated with measures of humoural immunity (Whiteman et al. 2006; Reid et al. 2007), and suggests that IgG production is positively correlated with fitness during this period of development in the Galapagos sea lion.

The fact that the statistical effect of TML on change in IgG mg mL^{-1} kg^{-1} was negative has interesting implications. The correlations reported in Chapter 3 suggested that pups from the human-impacted colony may have paid a physiological cost for IgG production: those that produced high concentrations of IgG lost condition, while those that produced lower concentrations of IgG gained condition. In the control colony, where lower concentrations of IgG were produced overall, the opposite was the case. This suggests that the cost of IgG production was particular to the human-impacted colony, where the relatively antigen-rich environment may have stimulated some pups to produce disadvantageously high concentrations of IgG. Therefore, the negative effect of TML on changes in IgG mg mL^{-1} kg^{-1} raises the intriguing, though speculative, possibility that inbred individuals are at an advantage

relative to outbred individuals in the human-impacted colony, at least in terms of their primary antibody response during early immune system ontogeny.

The positive correlation observed between TML and total leukocyte concentration in juveniles from the human-impacted colony suggests that inbreeding may be associated with an increase the number of circulating leukocytes under certain ecological circumstances. The involution of the thymus is likely to have taken place in juvenile sea lions (Day 2007) so total leukocyte concentration is more likely to represent mature immune system activity than ontogenetic leukocyte production (Bossart et al. 2001). Inbreeding and low heterozygosity have been shown to increase susceptibility to parasitism (Coltman et al. 1999; Acevedo-Whitehouse et al. 2003a; Townsend et al. 2010). In juveniles, inbreeding may, therefore, have compromised aspects of innate immunity (e.g. Whiteman et al. 2006; Reid et al. 2007), which could have led to relatively high infection rates (e.g. Coltman et al. 1999) in the human-impacted colony where infection risk is likely to be relatively high. Such infections could stimulate sustained cell-mediated immune responses that could increase total leukocyte concentrations (Bossart et al. 2001), which could explain the observed positive relationship with TML. However, evidence for this possibility remains scant without parasite data to complement the above findings.

The above proposition is plausible given that pups with high TML values from both colonies produced lower concentrations of IgG per kilogram of growth during early immune system ontogeny than those with low TML values. Given that even in hyper-immunised individuals IgG to a single antigen makes up less than 5 % of total IgG (King et al. 2001), it seems likely that these differences in concentration are associated with differences in repertoire diversity. In which case, juveniles with high TML values may have had less diverse B-lymphocyte and IgG repertoires, which may have decreased their chance of eliminating invading pathogens before they established infection, and could have led to sustained increases in total leukocyte concentrations. The positive relationship between TML and total leukocyte concentration may not have arisen in the control colony because juveniles there were exposed to

relatively few pathogens, which is what we would expect given the colony differences in human impact discussed in Chapter 1.

Investigations into the consequences of inbreeding should carefully consider the kind of selection that is likely to act on the traits under scrutiny. A general assumption of HFC studies is that phenotypic variation is linearly related to fitness and therefore under directional selection (Chapman et al. 2009). However, immune variables such as IgG concentration are unlikely to be so, due to the damage caused by immunopathology (van Boven & Weissing 2004), and different kinds of antibody responses have been empirically shown to be under different kinds of selection (Råberg et al. 2003). However, this does not mean that correlations between these kinds of traits and genetic diversity are uninteresting, as when and under what ecological circumstances such relationships are detectable could provide valuable insight into their underlying mechanisms (Chapman et al. 2009). As noted above, the results of this chapter suggest that the production of IgG may be positively related to fitness during early immune system ontogeny in the Galapagos sea lion, and that total leukocyte production may be negatively related to juvenile fitness under human influence, which may be linked to environmental stress and pathogen pollution (Cunningham et al. 2003). Immune traits are unlikely to be influenced by as many genes as traits such as survival or lifetime reproductive success, which is advantageous from the point of view of candidate gene studies aiming to elucidate the mechanisms that underlie patterns like those reported in this chapter (e.g. McCarthy et al. 2011). However, this also means that immune traits are likely to be less powerfully influenced by the genome-wide effects of inbreeding than other traits (Szulkin et al. 2010).

As g_2, the identity disequilibrium measure, was not estimated to be significantly different from zero, identity disequilibrium of our marker set was low (David et al. 2007). This suggests an absence of strong inbreeding but does not preclude the presence of weak inbreeding (Szulkin et al. 2010). Nevertheless, low identity disequilibrium is what would be expected if the observed correlations between phenotypic traits and TML were driven by variation at one or a few loci. The contribution of individual loci to HFCs

through linkage to functional genes under balancing selection is controversial, and a variety of statistical approaches have been proposed to identify these effects (Balloux et al. 2004; Acevedo-Whitehouse et al. 2006, 2009; Amos & Acevedo-Whitehouse et al. 2009). However, problems of power and non-independence mean it is difficult to assess the significance of these tests (Szulkin et al. 2010; Hoffman et al. 2010b). The approach of the locus contribution analysis presented here was an attempt to sidestep these statistical issues, and use a conservative selection procedure in which multiple redundant tests were applied serially to each locus. This reduced the probability of type I error at the expense of increasing the probability of type II error, which may be appropriate given that single-locus effects are expected to be rare (Szulkin et al. 2010). However, the results did not support a role for the effects of any individual loci, as no significant relationship between TML and immune variation passed the first selection test, and no locus was consistently selected by the remaining tests. Given the lack of evidence for single locus effects, it was not surprising that there was no observable effect of locus diversity on locus contribution. However, the exploration of locus variability remains an important consideration for studies that report statistical support for single locus effects.

Pairwise estimates of relatedness calculated from neutral marker data, like equivalent estimates of inbreeding value, are likely to be imprecise, especially when the mean and variance of relatedness in a population are low (Pemberton 2008). In addition, the use of relatedness coefficients to estimate additive genetic variance for traits in un-manipulated wild populations has been shown to have low power (Frentiu et al. 2008). Nevertheless, such methods can recover meaningful information about additive genetic variance for phenotypic traits, and alternative methods require more data than is generally available or more manipulation than is generally feasible (Frentiu et al. 2008). Given ecological context, neutral marker data can be used to test hypotheses about the relative strength of associations between relatedness and phenotypic traits. In the Galapagos sea lion control colony, immune traits thought to be heritable to some degree but also to be affected by immune challenge (e.g. the PHA response: Gleeson et al. 2005; Saks et al. 2006;

Bonneaud et al. 2009; Drobniak et al. 2010) are likely to be influenced to a proportionally greater extent by genetic than environmental variation, as it appears that control colony sea lions are subject to fewer immune challenges than those in the human-impacted colony (Chapter 2). Therefore, any detectable patterns of additive genetic variance for immune traits may have been more pronounced in the control colony. This prediction was supported by the PHA response in juveniles and by IgG and total leukocyte production in pups, as relatedness between pairs of individuals from different halves of these trait distributions was greater than that between pairs from the same half. The absence of the same pattern in the human-impacted colony suggests that the signal of additive genetic variance for the PHA response in juveniles and IgG and total leukocyte production in pups from the human-impacted colony may have been lost due to environmental influence on the expression of these immune traits. Curiously, the results showed the reverse of the predicted pattern for changes in body mass and length in pups from the human-impacted colony: pairs of individuals from the same halves of the growth rate distributions were less closely related than pairs from different halves. These results may be the effect of sampling artefacts, but it is also possible that they were influenced by the idiosyncratic breeding behaviour of the Galapagos sea lion (Pörschmann et al. 2011) or the dynamics of early growth in pinnipeds (Bolund et al. 2009; Mueller et al. 2011). However, without the appropriate data available to justify them, such *post hoc* explanations would be speculative.

This chapter has shown, that although the use of inbreeding estimates calculated using data from neutral genetic markers has its drawbacks (Pemberton 2008), these methods can yield useful and interesting information on the context-dependent expression of inbreeding consequences in natural populations. The findings have broad relevance to research on inbreeding in the wild, and implications for the conservation of small, endangered populations, such as that of the Galapagos sea lion. Since the results suggest that inbreeding may be linked to fitness in the Galapagos sea lion, it should be considered by population viability analyses (e.g. Salazar & Denkinger 2010), as it may interact with the influences fluctuating population size (Aurioles &

Trillmich 2008), climate driven changes in food availability (Mueller et al. 2011) and disease threat (Alava & Salazar 2006) to compound extinction risk.

Chapter 5

The threat of canine distemper virus to the Galapagos sea lion

Abstract

Predicting when and with what consequences pathogens jump from one species to another is important for maintaining and promoting human quality of life, animal welfare, economic prosperity and the persistence of biodiversity. The Galapagos sea lion is endangered by the threat of infectious disease from domesticated animals, in particular canine distemper virus (CDV) from dogs. This chapter used the tools of epidemiology to describe a CDV outbreak that occurred in the dogs on the Galapagos Islands in 2001, investigate the consequences of a repeat outbreak for the Galapagos sea lion, and explore the risk of disease spread from one Galapagos sea lion colony to another under different climate scenarios. It tested the hypotheses that straightforward domestic dog management could reduce the risk of Galapagos sea lion exposure to CDV, and that environmental variation could affect the risk of disease spread in the Galapagos sea lion through an effect on immunity. The results showed that if there were an outbreak of CDV in dogs on the island of San Cristobal, sea lions would be highly likely to come into contact with the virus; that dog vaccination and the control of the dog population size, the contact rate amongst dogs and the contact rate between dogs and sea lions would all significantly reduce CDV risk to the Galapagos sea lion; and that the risk of disease spread between sea lion colonies may be highest during mild El Niño conditions. In addition to providing evidence of practical value to the conservation management of an endangered species, this chapter serves as an example of how conservation biologists can address issues of infectious disease risk in their study systems using adaptable epidemiological models.

Introduction

As human populations grow and spread, so too do populations of livestock, pets and pests. This extends the interface between domesticated and wild animals, which increases the chance of pathogens passing from one group to the other. Infectious disease can threaten the persistence of endangered species (Daszak et al. 2000; de Castro & Bolker 2005; Smith et al. 2009), especially when acting in synergy with other anthropogenic pressures (Lafferty & Gerber 2002; Acevedo-Whitehouse & Duffus 2009), and can have negative impacts on the productivity and welfare of domesticated animals (e.g. Kitching et al. 2005). These are good reasons to develop epidemiological frameworks to assess infectious disease threats to wildlife from domesticated animals, and *vice versa*. Even in situations where risks and solutions appear simple, the non-linear dynamics of infectious disease can lead to counter-intuitive outcomes that epidemiological models can help us understand (Woolhouse 2011). This chapter aimed to use epidemiological tools to assess disease risk in the Galapagos sea lion and make evidence-based conservation recommendations.

As discussed in Chapter 1, the Galapagos Islands are not exempt from the dangers of introduced diseases, despite their geographical isolation. As the number of tourists, residents and domesticated animals arriving on the archipelago each year increases, so does the threat of disease to resident wildlife (Kilpatrick et al. 2006). The endemism and small population sizes that are typical for Galapagos species mean that infectious disease could significantly increase extinction risk on the Galapagos (Wikelski et al. 2004; Whiteman et al. 2006). The Galapagos sea lion (*Zalophus wollebaeki*) is endemic to the archipelago (Wolf et al. 2007a) and is classified as endangered for two reasons (Aurioles & Trillmich 2008). First, it is sensitive to the unpredictable variation in oceanic productivity (El Niño events) that characterises the Galapagos marine ecosystem (Trillmich & Limberger 1985; Dellinger & Trillmich 1991; Mueller et al. 2011), so its small population undergoes stochastic decreases in size (20,000–40,000 animals; Aurioles & Trillmich 2008). Second, due to the unique location of their colony, sea lions resident in Puerto Bazquerizo Moreno, San Cristobal, come into close contact

with the growing number of dogs, cats and rats in the surrounding town (Alava & Salazar 2006). This is thought to be increasing the risk that infectious diseases pass from domesticated animals to the Galapagos sea lion (Aurioles & Trillmich 2008), particularly in the case of canine distemper virus (CDV) from dogs (Alava & Salazar 2006).

CDV is an acute and strongly antigenic pathogen that is directly transmitted through the exchange of oral and nasal exudates and aerosols (Appel et al. 1987). CDV is a Paramyxovirus of the genus *Morbillivirus*, which contains the viruses that cause measles and rinderpest, and which infects a wide range of carnivores (Deem et al. 2000). Morbilliviruses are known to cause disease in variety of marine mammals (Di Guardo et al. 2005), and CDV caused a mass mortality event in Caspian seals in 2000 (Kuiken et al. 2006). Phocine distemper virus (PDV), which caused mass mortality events in European harbour seals in 1988 and 2002, is closely related to CDV, and is thought to have originated in dogs (Barrett et al. 2002).

There are an estimated 1,500 dogs currently on the Galapagos Islands, distributed between the four population centres on the islands of Santa Cruz, San Cristobal, Isabela and Floreana (Comité Interinstitucional para Manejo y Control de Especies Introducidas, CIMEI, Ecuador, unpublished data). In 2001 there was an outbreak of CDV in the dog population on Santa Cruz, which was followed a month later by an outbreak on Isabela and a single isolated case on San Cristobal (Cruz et al. 2003; Levy et al. 2008). Only in the town of Puerto Bazquerizo Moreno on San Cristobal is there an established Galapagos sea lion breeding colony. There is no serological evidence that Galapagos sea lions have been exposed to CDV and there are no recorded clinical cases of CDV or PDV in Galapagos sea lions (Levy et al. 2008). Therefore, the perceived threat of CDV to the Galapagos sea lion is based on the potential for the virus to be present in Galapagos dogs, and precedent cases of CDV transmission to novel carnivore hosts.

CDV is unlikely to be endemic in Galapagos dogs, as the population is much smaller than the minimum size of population in which CDV is thought to be

able to persist (Swinton et al. 1998; Almberg et al. 2010). For the purposes of this chapter, and to use the definitions of Haydon et al. (2002), Galapagos sea lions are the target population, Galapagos dogs are the source population and mainland dogs and Galapagos dogs together comprise the reservoir. Given the CDV outbreak in Galapagos dogs in 2001, there is likely to be a loose epidemiological connection between mainland and Galapagos dogs. A risk analysis approach, similar to that used to identify routes of mosquito introduction, could be used to identify how dogs are most likely to arrive on the Galapagos (Kilpatrick et al. 2006), as breaking this epidemiological connection would be the most effective way to reduce the risk of the Galapagos sea lions being exposed to CDV. However, given the continuing influx of dogs to the Galapagos Islands, the protocols already in place to prevent dogs arriving on the archipelago do not seem to be effective.

It is not known whether strains of CDV present on the Ecuadorian mainland would cause disease in Galapagos sea lions. That CDV caused disease in Caspian Seals (Kuiken et al. 2006) suggests a degree of compatibility between CDV and pinniped cell surface receptors. However, except for a single report from the New Zealand sea lion (Duignan et al. 2000), all cases of morbillivirus infection in pinnipeds have been reported in phocids (Kennedy-Stoskopf 2001). Therefore, it is possible that an aspect of otariid biology protects them from morbillivirus infection. However, it also possible, given that CDV has been reported from a range of terrestrial carnivores (Deem et al. 2000), that the predominance of morbillivirus reports in phocids relative to otariids is not due to CDV incompatibility with otariids, but rather to chance effects acting in combination with distribution and behaviour, both of which could affect the probability of exposure. A comparative study showed that the emergence of CDV in new hosts is usually associated with base changes in the virus (McCarthy et al. 2007). Whether or not a base change would be required, the more contacts there are between infectious dogs and sea lions, the more likely virus is to pass between them, given that high dose is thought to be required for a pathogen to jump the species barrier even in the case of compatibility (Woolhouse et al. 2005).

The goal of this chapter was to use epidemiological models to generate data that could be used for evidence-based decision making by the Galapagos National Park on how to most efficiently manage Galapagos dog populations in order to reduce the risk of disease transfer to Galapagos sea lions in the case of a CDV outbreak. Throughout, I use the phrase ‘disease transfer’ to mean the passing of a CDV-like virus particle from an infectious dog to a susceptible sea lion in which it subsequently establishes infection and causes disease. This differs from the epidemiological definition of spillover, which implies that the infection does not spread in the newly infected host (Fenton & Pedersen 2005), an assumption I do not make when modelling disease transfer, but one I address explicitly when modelling disease spread. The analyses that follow aim to: 1) describe the CDV outbreak that occurred in the dogs on the Galapagos island of Santa Cruz in 2001; 2) explore the consequences of a possible CDV outbreak in the dogs of San Cristobal on the risk of disease transfer to Galapagos sea lions and test the hypothesis that domestic dog management could reduce the risk of Galapagos sea lion exposure to CDV; and 3) explore the risk of a CDV-like infection spreading from one Galapagos sea lion colony to another under different climate scenarios and test the hypothesis that environmental variation could affect the risk of disease spread in the Galapagos sea lion through an effect on immunity.

Methods

CDV outbreaks in Galapagos dog populations

I constructed a stochastic discrete-time compartmental simulation model for CDV in a Galapagos dog population, following the generalised structure presented in Vynnycky & White (2010). The time-course of CDV outbreaks in naïve populations is generally short (Williams 2001), and that which occurred on Santa Cruz in 2001 lasted less than 3 months (Cruz et al. 2003; Levy et al. 2008). In addition, the commercial breeding of dogs on the Galapagos Islands and the transport of dogs to the archipelago are forbidden (Ecuadorian Law no. 67. RO/278). Therefore, I did not include births or deaths that were not caused by disease in any model.

I began simulations with a single infectious individual in an otherwise susceptible population of 509 dogs, the estimated number on Santa Cruz before the 2001 CDV outbreak (Cruz et al. 2003). Exposed individuals did not become infectious until the end of a latency period. Once they had been infectious, they either recovered or died; and once recovered, they were immune. I assumed that the latency period (mean = 7 days, standard deviation = 1 day) and an individual's mortality probability once infected were normally distributed (mean = 0.5, standard deviation = 0.05), and that the infectiousness period was negatively binomially distributed (r = 2, p = 0.2). Daily mortality rates were calculated using sampled values of infectiousness period and mortality probability. I chose these distributions in order to incorporate as much of the described variation in CDV pathogenesis in domesticated dogs as possible (Appel 1969, 1970, 1987; Williams 2001; Willoughby & Dawson 2001; Greene & Appel 2006; Almberg et al. 2010). In addition, according to the collated results of all the veterinary reports submitted to the Galapagos National Park at the time (Cruz et al. 2003) CDV caused 54 % mortality in Galapagos dogs during the 2001 outbreak on Santa Cruz (274 out of 509), which suggests that a mean mortality probability of 50 % is appropriate for the description of CDV in Galapagos dogs.

I calculated the daily number of contacts between infectious and susceptible dogs using a modification of the Reed-Frost equation:

$$\lambda_{d\text{-}d} = S_d * 1 - (1 - T_{d\text{-}d})^{I_d}$$

where S_d is the number of susceptible dogs, I_d the number of infectious dogs and $T_{d\text{-}d}$ a dog contact parameter. In stochastic models the Reed-Frost equation is normally used to calculate the risk of infection for susceptible individuals between time-points, and combines information on contact to infectious individuals and infection probability given contact (Vynnycky & White 2010). However, I was interested in these parameters in their own right, so split the calculation into two parts, first calculating the number of contacts as above, and then assigning the number of these contacts resulting in successful transmission of infection through random number generation and selection using an infection probability threshold, which I designated β for the purposes of this chapter. The numbers used in these calculations were counts not densities, which is appropriate for dogs given the small size and the isolation of the town, and for sea lions given the limited terrestrial habitat available to them, their spatial aggregation into colonies and philopatric behaviour (McCallum et al. 2001).

As $T_{d\text{-}d}$ and β are unknown for CDV in dogs on the Galapagos, I ran optimisation simulations to find the parameter values that best described the empirical data from the 2001 CDV outbreak on Santa Cruz, so they could be used to simulate CDV outbreaks on San Cristobal. I ran simulations with combinations of values of β (0 – 1) with values of $T_{d\text{-}d}$ (0.005 – 0.1), determining these ranges as approximately realistic using preliminary simulation analyses. I calculated the weekly incidence of CDV from 10 replicate simulations for each unique parameter value combination, and the mean sum of squares difference between these values and the empirical values that were available from the 2001 outbreak, which comprised the collated results of all the veterinary reports submitted to the Galapagos National Park (n = 7; Cruz et al. 2003). Since there is likely to be a time lag

between the beginning of an epidemic and the first reported case, I repeated the optimisation simulations four times, with offsets of one, two, three and four weeks between the beginning of the simulations and the empirical data. Then I used the parameters that best described the data to simulate CDV infection in the current population of dogs on San Cristobal, of which there are an estimated to 453, according to the organisation CIMEI, which is responsible for monitoring domesticated animals on San Cristobal.

Risk of disease transfer from dogs to sea lions

I used behavioural data collected over 68 hours of observation between May and November 2011 to estimate the daily contact rate between dogs and sea lions on San Cristobal (J. Denkinger, unpublished data); these behavioural surveys were carried out as part of a long-term sea lion monitoring program, co-ordinated by the University of San Francisco de Quito (J. Denkinger, personal communication). Behavioural interactions between sea lions and dogs occurred over periods of minutes and usually involved dogs running onto beaches and then off again after a period of exploration. I defined contacts between dogs and sea lions as behavioural interactions that could give rise to the transmission of CDV: when animals were physically close to one another (< 2 m) and barking, snorting or making open-mouth threats. The average number of these contacts per hour that could result in pathogen transmission was 0.9 (Denkinger, unpublished data). As behavioural observations were only performed during the coolest 6 hours of the day, I used 5.4 as a conservative estimate of the mean number of daily contacts, which amounts to assuming that there are no contacts during the hottest hours of the day.

To estimate the number of contacts between infectious dogs and sea lions during simulated outbreaks in dogs, I used a similar modification of the Reed-Frost equation as above:

$$\lambda_{d\text{-}s} = S_s * 1 - (1 - T_{d\text{-}s})^{I_d}$$

where S_s is the number of susceptible sea lions, I_d the number of infectious dogs and $T_{d\text{-}s}$ a dog to sea lion contact parameter. I solved for $T_{d\text{-}s}$ using the empirical estimate of the mean number of daily contacts between dogs and sea lions (5.4), the mean number of susceptible sea lions on land (mean = 518, n = 8; Actis 2012), and the current San Cristobal dog population size (453; CIMEI, unpublished data), which gave a $T_{d\text{-}s}$ value of 2.313313×10^{-5}.

I ran simulations in the San Cristobal dog population under a variety of conditions, mimicking dog population control measures that could be implemented by the Galapagos National Park, and summed the number of contacts between infectious dogs and sea lions over the duration of each simulation to estimate the relative risk of disease transfer. First, I tested the effect of changing the dog population size. While all other parameters were held constant including $T_{d\text{-}d}$ and β at the values selected by the optimisation analysis, I ran 100 replicate simulations for each of 10 values of dog population size, and calculated 95 % bootstrap confidence intervals (1000 replicates) around the total number of infectious dog to sea lion contacts. Second, I tested the potential of a vaccination programme to reduce the risk of disease transfer, by repeating the above procedure for 10 simulated dog populations, each containing a different proportion vaccinated individuals. As with recovered individuals, I assumed that vaccinated individuals were immune for life. Third, I tested the effect of changing the contact rate within the dog population by running simulations for 10 values of $T_{d\text{-}d}$. Fourth, I tested the effect of changing the contact rate between dogs and sea lions, by repeating the above for 10 values of $T_{d\text{-}s}$. As before, I began simulations with one infectious dog in an otherwise susceptible population. The structure of this model of CDV in Galapagos dogs is summarised in the schematic diagram in Figure 5.1, and the parameters are listed together in Table 5.1. The simulation ranges of the parameters were chosen to represent realistically possible management interventions that included the current best estimate of the parameter.

Figure 5.1

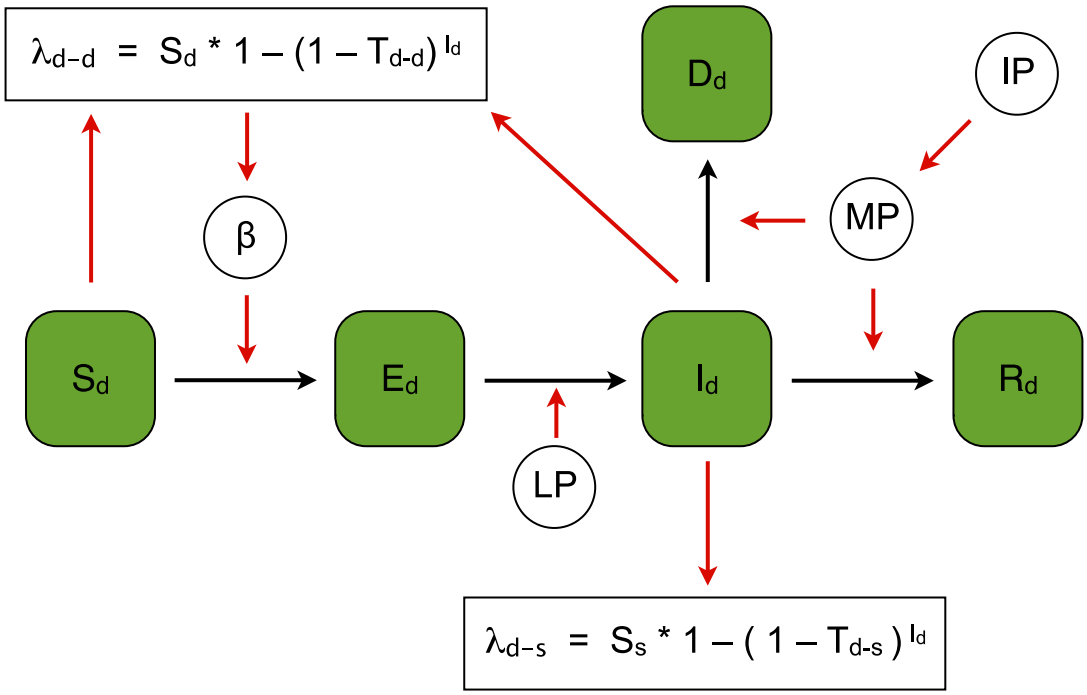

Figure 5.1 Schematic diagram of the stochastic discrete-time compartmental simulation model of CDV in Galapagos dogs. Clinical states are shown in green boxes and parameters are shown in black circles. Black arrows indicate the flow of individuals, and red arrows indicate the influence of parameters and numbers of individuals in certain clinical states. S_d represents susceptible dogs, E_d exposed dogs, I_d infectious dogs, R_d recovered dogs and D_d dead dogs. λ_{d-d} is the number of contacts between susceptible and infectious dogs per unit time; λ_{d-s} is the number of contacts between infectious dogs and sea lions per unit time; β is the probability of infection passing from an infectious to a susceptible dog on contact; T_{d-d} determines the contact rate amongst dogs; LP is the latency period; T_{d-s} determines the contact rate between dogs and sea lions; MP is the mortality probability; and IP is the infectiousness period.

Table 5.1 Parameter values used in the stochastic discrete-time compartmental simulation model of CDV in Galapagos dogs; β is the probability of infection passing from an infectious dog to a susceptible dog on contact, $T_{d\text{-}d}$ determines the contact rate amongst dogs and $T_{d\text{-}s}$ determines the contact rate between dogs and sea lions.

Parameter	Base model values and distributions	Other values
Latency period (days)	Normally distributed, mean = 7 days, SD = 1	-
Infectious period (days)	Negative binomially distributed, $r = 2$, $p = 0.2$	-
Mortality probability	Normally distributed, mean = 0.5, SD = 0.05	-
Santa Cruz population	509	-
β	0.1	Optimisation: 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0
$T_{d\text{-}d}$	0.02	Optimisation: 0.00, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10 Simulation: 0.001, 0.005, 0.010, 0.015, 0.020, 0.025, 0.030, 0.035, 0.040, 0.045
$T_{d\text{-}s}$	2.313313×10^{-5}	Simulation: 0.05×10^{-4}, 0.10×10^{-4}, 0.15×10^{-4}, 0.20×10^{-4}, 0.25×10^{-4}, 0.30×10^{-4}, 0.35×10^{-4}, 0.40×10^{-4}, 0.45×10^{-4}, 0.50×10^{-4}
San Cristobal population	453	Simulation: 50, 100, 150, 250, 500, 750, 1000, 1250, 1500, 1750, 2000
Vaccinated proportion	0	Simulation: 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9

Spread between sea lion colonies

In this section I assumed that a single sea lion had acquired a CDV-like infection, and modelled the spread of this infection in the San Cristobal sea lions to assess the risk of spread to a neighbouring colony. As sea lions haul out on land but forage at sea, it is necessary to take into account their movement in and out of the colony, particularly as it is assumed that the transmission of morbillivirus in pinnipeds only occurs on land (Swinton et al. 1998; Harris et al. 2008). GPS-tracking data from Galapagos sea lions resident in a colony in the central part of the archipelago has shown that adult females spend 64% of their time on land in their home colony (Jeglinski, unpublished data). There were an average of 518 sea lions present during the land censuses of the San Cristobal colony (n = 8; Actis 2012), so I estimated that there were 291 San Cristobal residents hauled out elsewhere on a typical day. For the purpose of this chapter, I assumed that these sea lions were all hauled out in a single hypothetical neighbouring colony. The average proportion of time that Galapagos sea lions spend at sea is 51.5% (Villegas-Amtmann et al. 2011; Jeglinski et al. 2012), so I estimated that there were an additional 859 San Cristobal residents that were in the sea on a typical day. The sum of these estimates gave a total estimate of 1668 sea lions resident in the San Cristobal colony.

To quantify the risk of infection being passed to sea lions resident in the hypothetical neighbouring colony, I extended the basic model structure developed for dogs so that each clinical state (susceptible, exposed, infectious and recovered) of the San Cristobal sea lions was possible on land in the San Cristobal colony, in the sea and on land in the neighbouring colony. For sea lions on land, I defined the daily probability of going to sea as the mean empirical proportion of time spent at sea (0.515; Villegas-Amtmann et al. 2011; Jeglinski et al. 2012). For animals in the sea, I defined the daily probability of hauling out as the mean empirical proportion of time spent on land (0.485; Villegas-Amtmann et al. 2011; Jeglinski et al. 2012). For sea lions hauling out, I defined the probability that they did so in their home colony, San Cristobal, as the empirical proportion of time spent hauled out in the home

colony (0.64; Jeglinski, unpublished data). This model structure broadly follows that of Harris et al. (2008), except that it includes only a single alternative haul out to the home colony, and mean daily probabilities of movement into and out of the sea in place of cumulative individual probabilities, as it is a compartmental rather than individual-based model.

I began simulations with a single infectious sea lion on land in the San Cristobal colony. I estimated the contact rate between sea lions present in their home colony using published data on the fine-scale spatial association of Galapagos sea lions into 'cliques' (Wolf et al. 2007b). This study described the number of conspecifics a sea lion associates with consistently, and I used this data to assign probabilities of 10 %, 27.5 %, 22.5 %, 17.5 %, 12.5 % and 10 % to 3, 4, 5, 6, 7 and 8 contacts per day respectively. Sea lions that were infected did not become infectious until the end of a latency period, once they had been infectious they either recovered or died, and once they had recovered they were immune. Since the spread of a CDV-like infection in the Galapagos sea lion is hypothetical and estimates of epidemiological parameters for morbillivirus infection in other pinniped species are similar to values observed in dogs (Swinton et al. 1998; Harris et al. 2008), I used the same distributions and values to describe the latency period, infectiousness period, mortality probability and β in sea lions as in dogs.

In order to gauge the probability of an infection spreading to the neighbouring colony, I added a contact term for San Cristobal residents hauled out in the neighbouring colony to neighbouring colony residents. Fine-scale spatial aggregation in the Galapagos sea lion is associated with relatedness (Wolf et al. 2007b; Wolf & Trillmich 2008) and association behaviour is therefore likely to depend on residency. As there were no data available on the contact rate between resident and non-resident sea lions ($T_{R\text{-}NR}$), I calculated a conservative estimate that would require 10 infectious San Cristobal residents to be hauled out in the neighbouring colony for a single contact between an infectious San Cristobal resident and a susceptible neighbouring colony resident to occur per day ($T_{R\text{-}NR} = 1.226257 \times 10^{-3}$). I did this by using the mean census Galapagos sea lion colony size of 82 individuals (n = 68; Alava

& Salazar 2006) as the number of sea lions present in the neighbouring colony on a typical day. Then I used the same modification of the Reed-Frost equation as above to estimate the number of contacts between infectious San Cristobal residents hauled out in the neighbouring colony and susceptible neighbouring colony residents:

$$\lambda_{R\text{-}NR} = 82 * 1 - (1 - T_{R\text{-}NR})^{I_R}$$

where I_R is the number of infectious San Cristobal residents hauled out in the neighbouring colony and $T_{R\text{-}NR}$ is a contact parameter that describes mixing between resident and non-resident sea lions. I summed the number of these contacts over the duration of simulations to gauge the relative probability of disease spread from San Cristobal to the neighbouring colony.

Initially I ran 100 simulations, and then investigated the potential effects of climatic variation on the risk of disease spread. First, as oceanic conditions are likely to affect the number of Galapagos sea lions (Mueller et al. 2011) and El Niño events are known to cause high mortality (Trillmich & Limberger 1985; Dellinger & Trillmich 1991), I tested the effect of changing the number of sea lions resident in the San Cristobal colony. I ran 100 replicate simulations for 10 values of the San Cristobal colony size, adjusting the numbers of sea lions on land in the San Cristobal colony, in the sea and on land in the neighbouring colony proportionally. Next, I investigated the effects of prolonging the infectiousness period. The high variation in mortality reported for CDV infections is thought to be linked to nutritional status and immune response: animals in poor condition are thought to be less able to mount timely and protective immune responses, so are likely to be infectious for longer (Appel 1969, 1970 & 1987; Williams 2001; Willoughby & Dawson 2001). Chapter 3 showed a negative correlation between condition and IgG production in the Galapagos sea lion, which suggests a trade-off between investment in resistance to starvation and immunity may exist in this species. Therefore, I prolonged the mean infectiousness period to simulate the possible negative effects of reduced food availability on the effectiveness of

infection control by the immune system. I ran 100 replicate simulations for 10 distributions of infectiousness period, decreasing the value of *p*, which has the effect of increasing the mean of a negative binomial distribution. Finally, I changed the San Cristobal sea lion colony size and infectiousness period simultaneously; using the same ranges as in the univariate runs. I ran 100 replicate simulations for 10 parameter combinations to simulate El Niño conditions of increasing severity. The structure of this model is summarised in the schematic diagram in Figure 5.2, and the parameters are listed together in Table 5.2. All analyses were performed in R 2.14.1 (R Development Core Team 2010) and run for 300 simulated days.

Figure 5.2

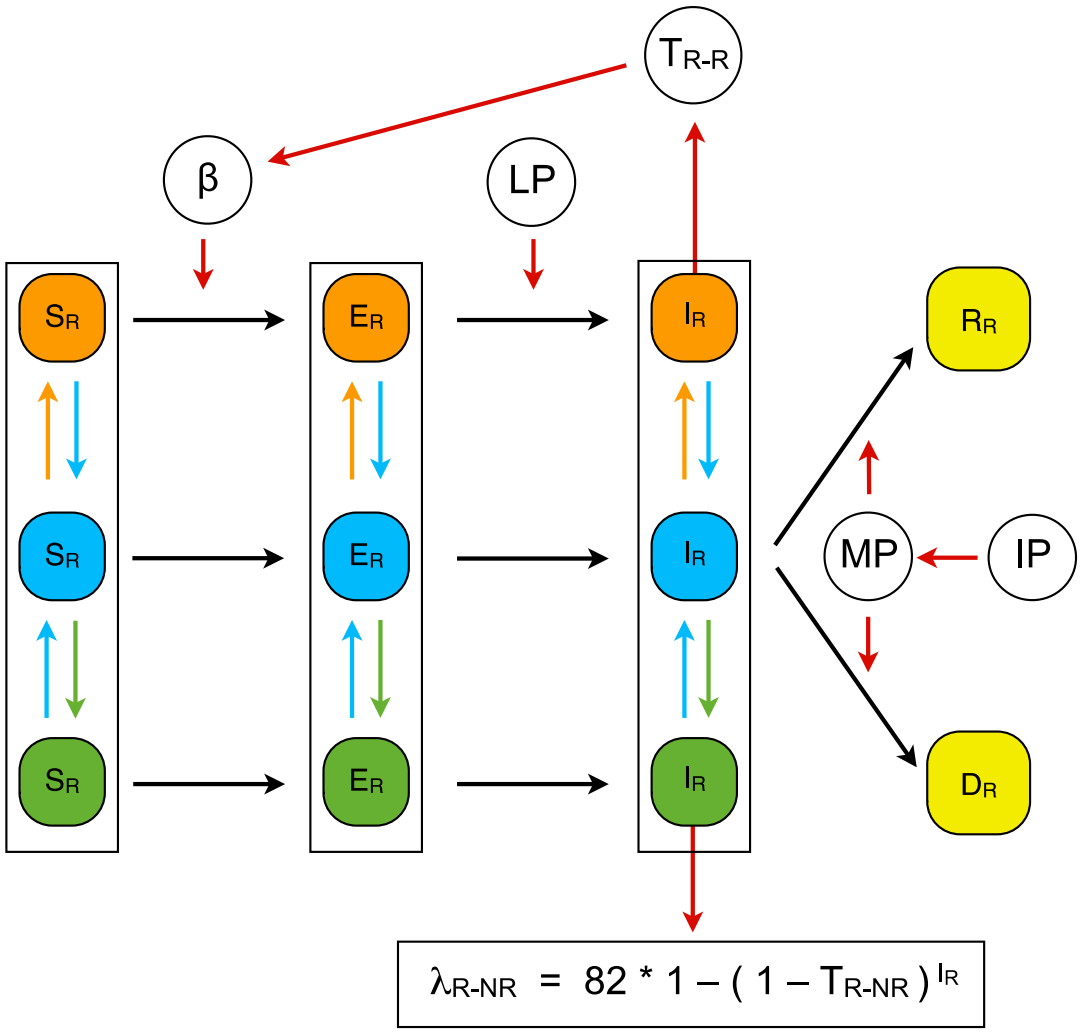

Figure 5.2 Schematic diagram of the stochastic discrete-time compartmental simulation model of CDV in Galapagos sea lions. Clinical states are shown in coloured boxes, parameters are shown in circles. Black arrows indicate the flow of individuals, and red arrows indicate the influence of parameters and numbers of individuals in certain clinical states. Coloured boxes represent sea lions that are resident in the San Cristobal colony, which are present either in the San Cristobal colony (orange), in the sea (blue) or in the neighbouring colony (green). Orange arrows indicate sea lions hauling out in the San Cristobal colony, green arrows indicate sea lions hauling out in the neighbouring colony and blue arrows indicate sea lions going to sea from both colonies. S_R are susceptible, E_R exposed, I_R infectious, R_R recovered and D_R dead sea lions that are resident in the San Cristobal colony. β is the probability of infection passing from an infectious sea lion to a susceptible sea lion upon contact; $T_{R\text{-}R}$ determines the contact rate amongst resident sea lions; LP is the latency period; $T_{NR\text{-}R}$ determines the contact rate between non-resident and resident sea lions; $\lambda_{R\text{-}NR}$ is the number of contacts between infectious resident sea lions and non-resident sea lions per unit time; MP is the mortality probability; and IP is the infectiousness period.

Table 5.2 Parameter values used in the stochastic discrete-time compartmental simulation model of CDV in Galapagos sea lions; β is the probability of infection passing from an infectious sea lion to a susceptible sea lion on contact, T_{R-R} determines the contact rate between resident sea lions, and T_{R-NR} determines the contact rate between resident and non-resident sea lions.

Parameter	Base model values and distributions	Simulation values
Latency period (days)	Normally distributed, mean = 7 days, SD = 1	-
Infectious period (days)	Negative binomially distributed, $r = 2$, $p = 0.2$	p = 0.2, 0.19, 0.18, 0.17, 0.16, 0.15, 0.14, 0.13, 0.12, 0.11
Mortality probability	Normally distributed, mean = 0.5, SD = 0.05	-
β	0.1	-
T_{R-R}	3, 4, 5, 6, 7, 8	-
T_{R-NR}	1.226257×10^{-3}	-
San Cristobal total colony size	1669	1669, 1508, 1347, 1186, 1024, 863, 702, 541, 380, 219
On land in San Cristobal colony	518	518, 468, 418, 368, 318, 268, 218, 168, 118, 68
On land in the neighbouring colony	291	291, 263, 235, 207, 179, 151, 123, 95, 66, 38
In the sea	859	859, 776, 694, 611, 528, 445, 362, 279, 196, 113
Daily probability of going to sea	0.515	-
Daily probability of hauling out	0.485	-
Probability of hauling out in home colony	0.64	-

Results

Simulations of CDV infection in the dogs of Santa Cruz best fit the empirical data with an offset of 3 weeks, and the optimisation analysis revealed a curve of β and $T_{d\text{-}d}$ parameter value combinations with low mean sum of squares difference (SSD) values (Fig. 5.3). I visually inspected the fit of the models with the ten lowest SSD values and confirmed that the model that minimised SSD best fit the empirical data (Fig. 5.4). Five of the 100 simulations run with these optimal parameter values did not result in outbreaks in the San Cristobal dog population.

As the size of the San Cristobal dog population increased, the number of contacts between infectious dogs and sea lions increased steadily from a dog population size of 50 to one of 2000 (Fig. 5.5). However, the proportion of simulations that resulted in at least one contact rose quickly between dog population sizes of 50 and 500. Increases in the proportion of vaccinated dogs would decrease the chance of disease transfer from dogs to sea lions, even if a relatively low proportion of the dog population were vaccinated (Fig. 5.6). Above a $T_{d\text{-}d}$ value of 0.001, the number of contacts between infectious dogs and sea lions increased rapidly with contact rate amongst dogs ($T_{d\text{-}d}$; Fig. 5.7). However, there was no further increase in the number of contacts between infectious dogs and sea lions beyond a $T_{d\text{-}d}$ value of 0.02, which was the value selected by the optimisation analysis. The number of contacts between infectious dogs and sea lions increased linearly across the tested range of contact rate between dogs and sea lions ($T_{d\text{-}s}$), but the proportion of simulations that resulted in at least one contact between an infectious dog and a sea lion did not (Fig. 5.8), as the likelihood of an infection spreading in the dog population is independent of contact between dogs and sea lions in this model framework.

Figure 5.3

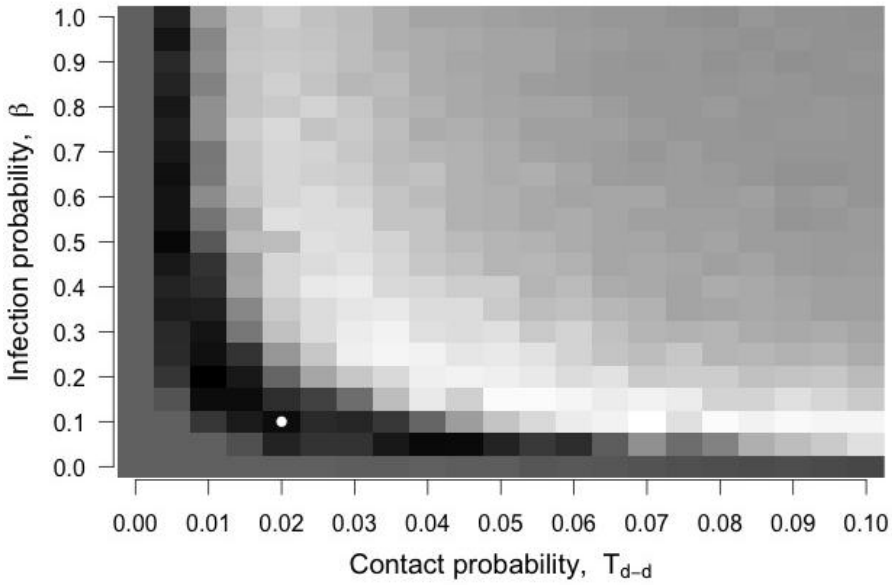

Figure 5.3 Results of the optimization analysis showing the mean sum of squares difference (SSD) between modelled and empirical weekly incidence of CDV in dogs on the Galapagos island of Santa Cruz. I ran each parameter combination 10 times and the darker a square, the lower the mean SSD. The filled white circle shows the parameter value combination that resulted in the lowest mean SSD value. Other parameter values were kept constant: latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; infectiousness period (IP): negatively binomially distributed, r = 2, p = 0.2; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; vaccinated proportion = 0; dog population size = 509; dog sea lion contact rate ($T_{d\text{-}s}$) = 2.313312×10^{-5}.

Figure 5.4

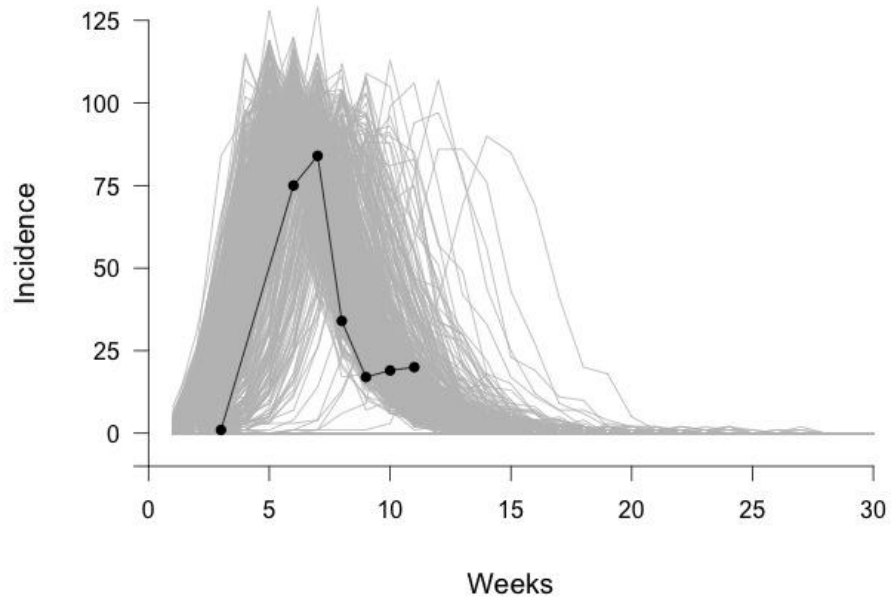

Figure 5.4 Modelled weekly incidence of CDV from 500 replicate simulations in the dogs on the Galapagos island of Santa Cruz, using parameters found by the optimization analysis ($T_{d\text{-}d}$ = 0.02, β = 0.1; grey lines). The black points show the empirical weekly incidence of CDV from the 2001 outbreak, plotted with an offset of three weeks. Other parameter values: latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; infectiousness period (IP): negatively binomially distributed, r = 2, p = 0.2; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; vaccinated proportion = 0; dog sea lion contact rate ($T_{d\text{-}s}$) = 2.313312×10^{-5}; dog population size = 509.

Figure 5.5

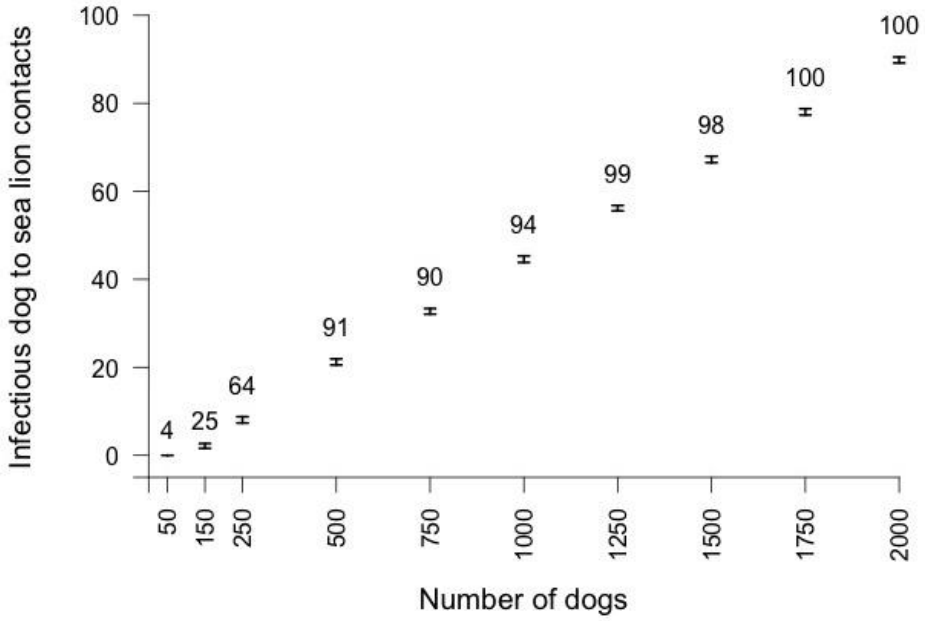

Figure 5.5 Effect of the San Cristobal dog population size on the number of contacts between infectious dogs and sea lions during CDV outbreaks in dogs. The error bars show the 95 % bootstrapped confidence intervals (1000 replicates) for 100 replicates for each value of dog population size. The numbers above the bars denote how many of the 100 replicates resulted in at least one contact between an infectious dog and a sea lion. Other parameter values: infection probability given contact (β) = 0.1; contact rate amongst dogs ($T_{d\text{-}d}$) = 0.02; latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; infectiousness period (IP): negatively binomially distributed, r = 2, p = 0.2; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; vaccinated proportion = 0; dog sea lion contact rate ($T_{d\text{-}s}$) = 2.313312×10^{-5}.

Figure 5.6

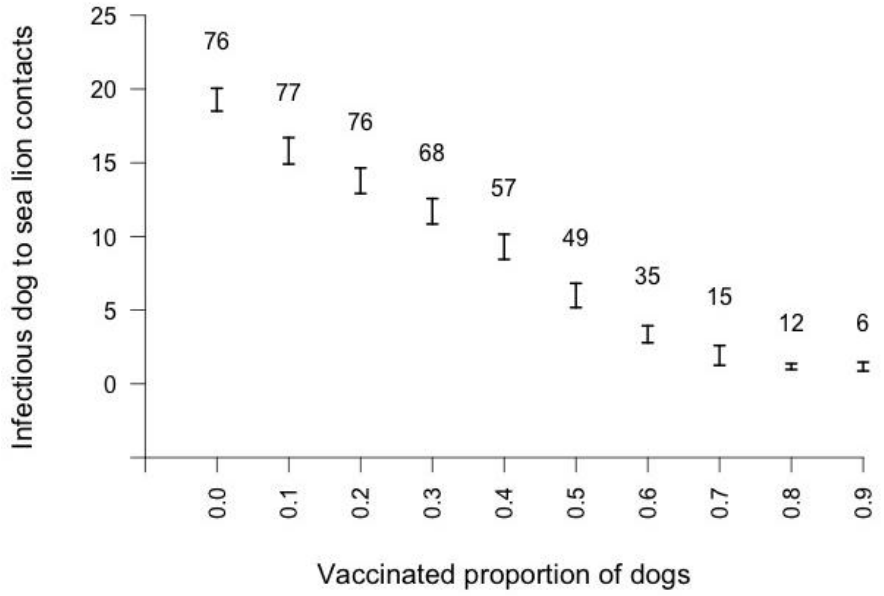

Figure 5.6 Effect of the proportion of vaccinated dogs on the number of contacts between infectious dogs and sea lions during CDV outbreaks in dogs on San Cristobal. The error bars show the 95 % bootstrapped confidence intervals (1000 replicates) for 100 replicates for each value of dog population size. The numbers above the bars denote how many of the 100 replicates resulted in at least one contact between an infectious dog and a sea lion. Other parameter values: infection probability given contact (β) = 0.1; contact rate amongst dogs ($T_{d\text{-}d}$) = 0.02; latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; infectiousness period (IP): negatively binomially distributed, r = 2, p = 0.2; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; dog population size = 509; dog sea lion contact rate ($T_{d\text{-}s}$) = 2.313312 x 10^{-5}.

Figure 5.7

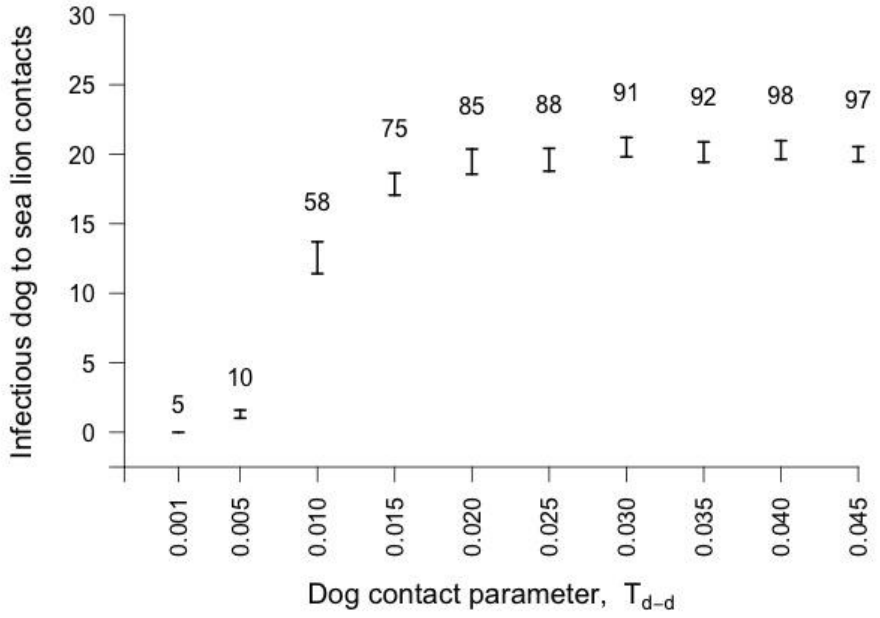

Figure 5.7 Effect of the contact rate amongst dogs ($T_{d\text{-}d}$) on the number of contacts between infectious dogs and sea lions during CDV outbreaks in the dogs on San Cristobal. The error bars show 95 % bootstrapped confidence intervals (1000 replicates) for 100 replicates for each value of $T_{d\text{-}d}$. The numbers above the bars denote how many of the 100 replicates resulted in at least one contact between an infectious dog and a sea lion. Other parameter values: infection probability given contact (β) = 0.1; latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; infectiousness period (IP): negatively binomially distributed, r = 2, p = 0.2; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; dog population size = 509; vaccinated proportion = 0; dog sea lion contact rate ($T_{d\text{-}s}$) = 2.313312×10^{-5}.

Figure 5.8

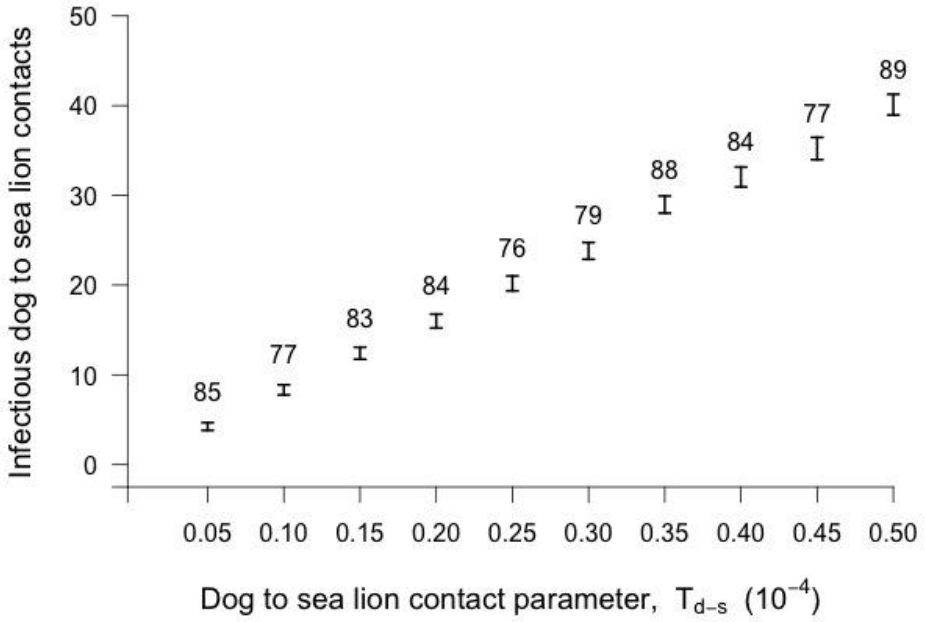

Figure 5.8 Effect of the contact rate between dogs and sea lions ($T_{d\text{-}s}$) on the number of contacts between infectious dogs and sea lions during CDV outbreaks in the dogs on San Cristobal. The error bars show 95 % bootstrapped confidence intervals (1000 replicates) for 100 replicates for each value of $T_{d\text{-}s}$. The numbers above the bars denote how many of the 100 replicates resulted in at least one contact between an infectious dog and a sea lion. Other parameter values: infection probability given contact (β) = 0.1; contact rate amongst dogs ($T_{d\text{-}d}$) = 0.02; latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; infectiousness period (IP): negatively binomially distributed, r = 2, p = 0.2; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; vaccinated proportion = 0; dog population size = 509.

Twenty-six of the 100 replicate simulations of CDV-like infection in sea lions resulted in outbreaks (Fig. 5.9). When CDV-like outbreaks occurred, they resulted in a mean number of contacts between infectious sea lions from San Cristobal and sea lions from the neighbouring colony of 75.12 (95 % bootstrapped confidence intervals, 1000 replicates: 72.07 – 78.34). As the number of sea lions in the San Cristobal colony decreased, the chance of spread to the neighbouring colony also decreased (Fig. 5.10). As the *p* value describing the negative binomial distribution of infectiousness period decreased, which has the effect of increasing the mean infectiousness period, the chance of spread to the neighbouring colony increased (Fig. 5.11). The El Niño simulations suggested that the chance of disease spread between colonies was lowest during severe El Niño conditions and highest during intermediate El Niño conditions (Fig. 5.12). Although the most severe scenario resulted in the lowest number of contacts, it generated the highest proportion of replicate simulations that resulted in at least one contact.

Figure 5.9

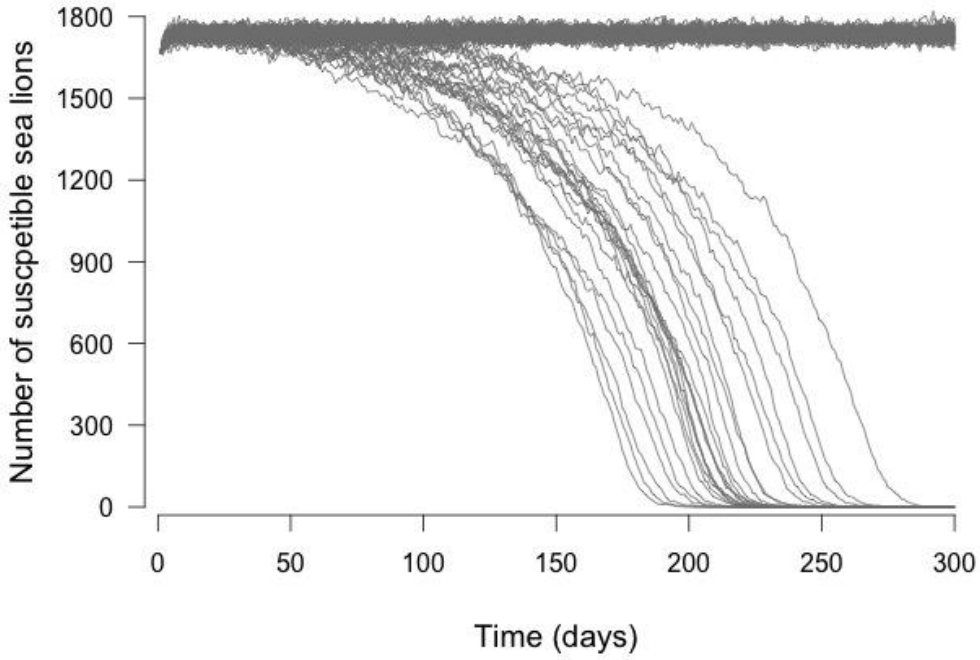

Figure 5.9 Changes in the total number of susceptible sea lions over the time course of 100 replicate CDV infection simulations in the sea lions of the Puerto Bazquerizo sea lion colony on San Cristobal. Twenty-six out of the 100 replicates resulted in outbreaks. Parameter values were: infection probability given contact (β) = 0.1; latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; infectiousness period (IP): negatively binomially distributed, r = 2, p = 0.2; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; contact rate amongst residents (T_R) = 3-8; contact rate between residents and non-residents ($T_{R\text{-}NR}$) = 1.226257×10^{-3}; sea lion colony size = 1669; initial number of sea lions present in home colony = 518; initial number of sea lions in the sea = 859; initial number of sea lions present in the neighbouring colony = 291; daily probability of leaving to sea = 0.515; daily probability of hauling out = 0.485; probability that a haul out is in home colony = 0.64.

Figure 5.10

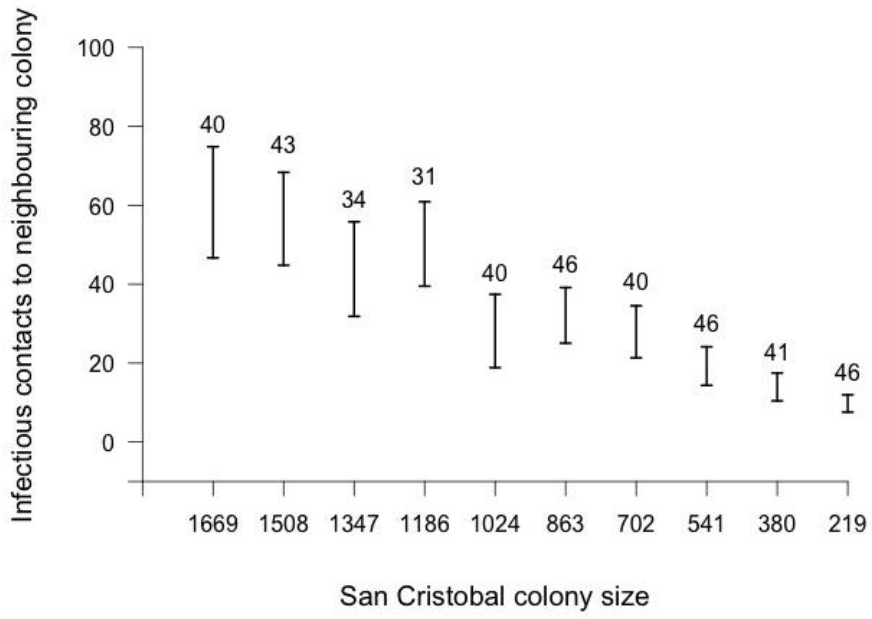

Figure 5.10 Effect of San Cristobal sea lion colony size on the number of contacts between infectious sea lions resident in the San Cristobal colony and susceptible sea lions resident in a hypothetical neighbouring colony, during CDV-like outbreaks in sea lions resident in the San Cristobal colony. The error bars show 95 % bootstrapped confidence intervals (1000 replicates) for 100 replicates for each value of colony size. The numbers above the bars denote how many of the 100 replicates resulted in at least one contact between an infectious San Cristobal resident and a neighbouring colony resident. Parameter values were: infection probability given contact (β) = 0.1; latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; infectiousness period (IP): negatively binomially distributed, r = 2, p = 0.2; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; contact rate amongst residents (T_R) = 3-8; contact rate between residents and non-residents ($T_{R\text{-}NR}$) = 1.226257 x 10^{-3}; daily probability of leaving to sea = 0.515; daily probability of hauling out = 0.485; probability that a haul out is in home colony = 0.64. See Table 5.2 for the initial numbers of sea lions in each location for each simulation.

Figure 5.11

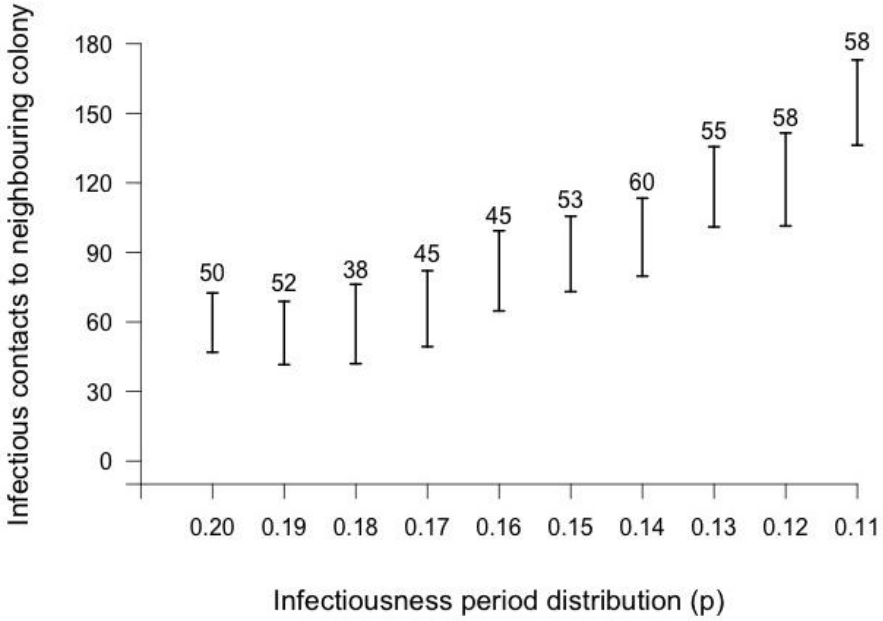

Figure 5.11 Effect of the infectiousness period on the number of contacts between infectious sea lions resident in the San Cristobal colony and susceptible sea lions resident in a hypothetical neighbouring colony, during CDV-like outbreaks in sea lions resident on San Cristobal. The error bars show 95 % bootstrapped confidence intervals (1000 replicates) for 100 replicates for each value of *p*, which increases the mean of the negative binomial distribution of infectiousness period as it decreases. The numbers above the bars denote how many of the 100 replicates resulted in at least one contact between an infectious San Cristobal resident and a neighbouring colony resident. Parameter values were: infection probability given contact (β) = 0.1; latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; contact rate amongst residents (T_R) = 3-8; contact rate between residents and non-residents ($T_{R\text{-}NR}$) = 1.226257×10^{-3}; sea lion colony size = 1669; initial number of sea lions present in home colony = 518; initial number of sea lions in the sea = 859; initial number of sea lions present in the neighbouring colony = 291; daily probability of leaving to sea = 0.515; daily probability of hauling out = 0.485; probability that a haul out is in home colony = 0.64.

Figure 5.12

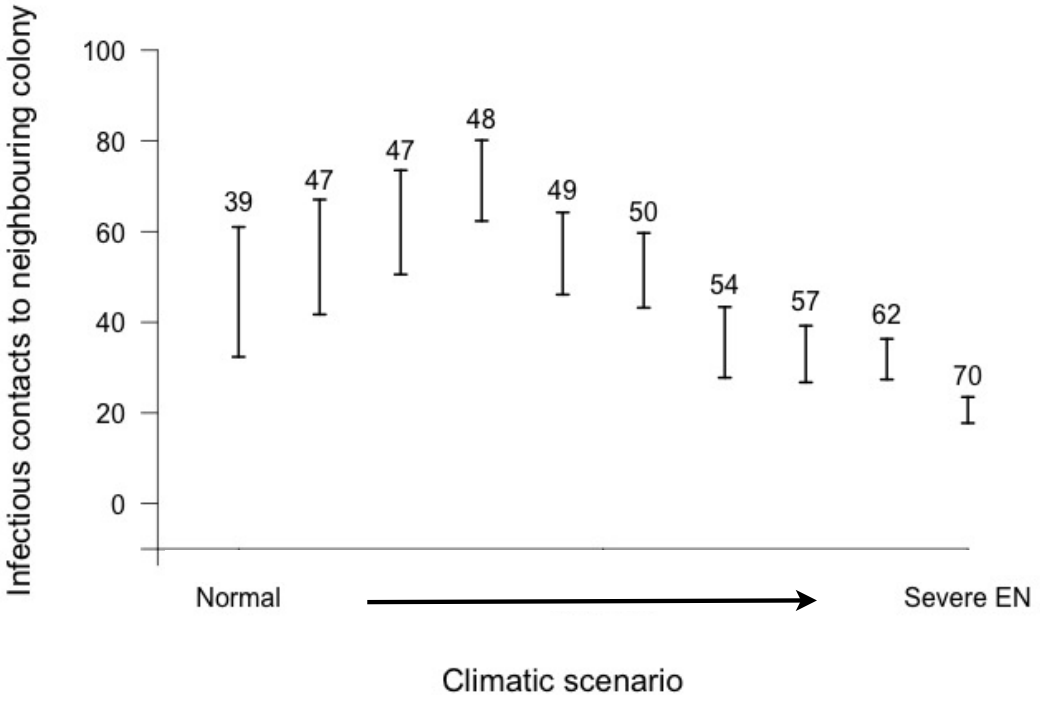

Figure 5.12 Combined effects of San Cristobal colony size and infectiousness period on the number of contacts between infectious sea lions resident in the San Cristobal colony and susceptible sea lions resident in a hypothetical neighbouring colony, during CDV-like outbreaks in sea lions resident in the San Cristobal colony. The 10 combinations of these two parameters simulated El Niño conditions of increasingly severity (Table 5.3). The error bars show 95 % bootstrapped confidence intervals (1000 replicates) for 100 replicates for each scenario. The numbers above the bars denote how many of the 100 replicates resulted in at least one contact between an infectious San Cristobal resident and a neighbouring colony resident. 'EN' denotes El Niño. Parameter values were: infection probability given contact (β) = 0.1; latency period (LP): normally distributed, mean = 7 days, standard deviation = 1 day; mortality probability (MP): normally distributed, mean = 0.5, standard deviation = 0.05; contact rate amongst residents (T_R) = 3-8; contact rate between residents and non-residents ($T_{R\text{-}NR}$) = 1.226257 x 10^{-3}; daily probability of leaving to sea = 0.515; daily probability of hauling out = 0.485; probability that a haul out is in home colony = 0.64.

Discussion

This chapter has three main findings: 1) given a CDV outbreak in dogs on the Galapagos island of San Cristobal, it is highly likely that Galapagos sea lions will come into contact with the virus; 2) as predicted, control of the dog population size, dog vaccination, management of the contact rate amongst dogs and the contact rate between dogs and sea lions would all significantly reduce the risk of disease transfer from dogs to sea lions; and 3) the risk of disease spread between sea lion colonies may be highest during mild El Niño conditions. This analysis serves as an example of how conservation biologists can customise the tools of epidemiology to address infectious disease threats in particular study systems, and generate results that policy makers and conservation practitioners can use for evidence-based decision-making (Lafferty & Gerber 2002).

CDV outbreaks in Galapagos dog populations

The models parameterised using laboratory data on CDV pathogenesis and following previous studies of morbillivirus epidemiology in carnivores (Swinton et al. 1998; Haydon et al. 2006; Guiserix et al. 2007; Almberg et al. 2010) fitted well to the small amount of data that was available from the 2001 CDV outbreak in Galapagos dogs (Cruz et al. 2003; Levy et al. 2008). Fitting models to incidence data by minimising sum of squares difference (SSD) values has the advantage of simplicity. However, it does not allow for the statistical distinction between alternative models. In other words, although the parameter value combination chosen by the optimisation analysis minimised the SSD, there were other combinations that appeared to produce similarly low SSD values that may have described the data equally well. Given the small number of empirical data points available for this analysis (n = 7), though, more complex model fitting procedures involving likelihood functions or particle filtering would be unlikely to be to provide deeper insight into model fit than the combination of SSD minimisation and visual inspection that I used here.

The comparison of the dog model with empirical data highlights an advantage of using stochastic discrete-time compartmental models (Vynnycky & White 2010) over deterministic alternatives when little empirical data is available. The incidence data that were available from the 2001 CDV outbreak in Galapagos dogs are bound to include noise due to variation in reporting and surveillance through time. Therefore, it is more appropriate to fit a model with parameters that cover all of the empirical data points through stochastic variation, than a deterministic model that would inevitably trade-off the goodness of fit to some data points against that to others. Stochastic models have the additional advantages of allowing for the calculation of confidence intervals and producing results that are intuitively understandable to policy makers and conservation practitioners (Woolhouse 2011).

The optimisation analysis, and the models simulating different management strategies, addressed the issue of the sensitivity of dog model outcomes to variation in parameter estimates. The optimisation analysis showed that weekly incidence, and how well it fit to the empirical data available from the 2001 epidemic, was sensitive to changes in both the infection probability given contact (β) and the contact probability ($T_{d\text{-}d}$). However, model fit was not sensitive to simultaneous changes in both parameters along the curve of parameter values shown in Fig. 5.3.

Risk of disease transfer from dogs to sea lions

Given current conditions, if there were an outbreak of CDV in the dogs of San Cristobal similar to that which occurred on Santa Cruz in 2001, Galapagos sea lions would be highly likely to come into contact with the virus. This exposure risk changes proportionally with dog population size, but is only minimised at very low dog population sizes. Dog population size was changed in this simulation analysis in order to gauge the effect of dog population control as a sea lion conservation management tool. However, it is unlikely that the Galapagos authorities would be able to reduce the number of dogs on San Cristobal to below 500 given the political resistance to dog extermination following the 2001 outbreak on Santa Cruz (Cruz et al. 2003). Nonetheless,

these results can also be used to predict the consequences of allowing the dog population to grow, in other words: the consequences of the Galapagos National Park not imposing a limit on the growth of the dog population. For example, allowing the dog population to increase from its current size (453; CIMEI, unpublished data) to 1,000 dogs would increase the chance of disease transfer given an outbreak by a factor or 2.1. The importation of dogs to the Galapagos Islands and their commercial breeding on the archipelago are already forbidden (Ecuadorian Law no. 67. RO/278), but domestic animal regulations are loosely enforced, especially in the municipal areas in which the Galapagos National Park has a relatively weak influence. Our results emphasise the importance of meaningful collaboration between the Galapagos municipal and National Park authorities, to effectively prevent the arrival of dogs from the mainland and to monitor and enforce dog-breeding regulations to prevent the growth of the dog population.

The efficacy of vaccination programmes is usually assessed on their ability to eliminate the chance of disease outbreak in the species receiving vaccination, which requires a minimum proportion of individuals in the population to be vaccinated (Haydon et al. 2006). However, this herd immunity threshold does not apply to the risk of disease transfer from dogs to sea lions. Therefore, the vaccination of even a low proportion of the dogs would significantly decrease the chance of disease transfer from dogs to sea lions, even if it would not eliminate or even decrease the chance of an outbreak in the dogs. Currently, no vaccination of Galapagos dogs is permitted, a regulation originally introduced to prevent vaccines causing disease (Articulo No. 56, Ley Especial de Galápagos, Registro Oficial N° 278, 1998; Cruz et al. 2003). However, given advances in vaccine development (van de Bildt et al. 2002) and the potential of even a low coverage dog vaccination programme to reduce disease risk to the Galapagos sea lion as demonstrated by the vaccination coverage simulation results presented here, the Galapagos authorities should consider the amendment of this legislation and the institution of a dog vaccination programme against CDV, using recently developed vaccines that are effective and safe.

The simulations run at different dog contact rates demonstrate how modelling can usefully describe the non-linearity of infectious disease dynamics (Woolhouse 2011). As we would expect, as the contact rate between dogs increased, so did the proportion of simulations that resulted in at least one contact and the chance of disease transfer from dogs to sea lions. However, both effects were non-linear: allowing the dog contact rate to increase above the value selected by the optimization analysis of 0.02, which is equivalent to a single dog coming into contact with an average of 9.06 other dogs per day, would not further increase the chance of disease transfer. However, decreasing $T_{d\text{-}d}$ from 0.02 to 0.015, 0.1 or 0.005, which are equivalent to a single dog contacting an average of 6.79, 4.53 and 2.27 other dogs per day, would decrease the risk of disease transfer given an outbreak by 8 %, 13 % and 93 % respectively. The majority of Galapagos dogs are companion animals, so human behaviour is likely to have a significant influence on their infectious disease dynamics (Alexander and McNutt 2010). These simulation results were generated to assess the potential benefits of education and awareness programmes aimed at reducing the number of contacts between dogs that the Galapagos authorities could implement. This could involve, for example, discouragement of dog walking during the busy evening promenade along the sea front, which would have the added advantage of reducing the number of contacts between dogs and sea lions, as the sea front walk is the interface between the dog and sea lion populations. The results also show that any decrease in the contact rate between dogs and sea lions would lead to a proportional decrease in disease transfer risk, which could be achieved through the introduction and enforcement of zoning laws to prevent dogs from accessing beaches, the control of stray dogs and the introduction and enforcement of laws surrounding dog registration and ownership; all of which are disease control strategies that the Galapagos authorities could plausibly implement.

As the relationship between dog-sea-lion contact and disease transfer risk is linear but that between dog-dog contact and disease transfer risk is curvilinear, together these results provide an example of how epidemiological models could be used to efficiently prioritise the investment of limited

conservation resources. If the Galapagos authorities had only enough resources to invest in either a programme to reduce dog-dog contact or one to reduce dog-sea-lion contact, these results could be used to assess which would result in the largest reduction of disease risk to sea lions in the case of an outbreak in the dogs. For example, if it could be calculated that it were feasible to lower $T_{d\text{-}d}$ to 0.005 for the same investment required to lower $T_{d\text{-}s}$ by 0.00001, the non-linearity of the relationship between $T_{d\text{-}d}$ and disease transfer risk shows that investing in lowering $T_{d\text{-}d}$ would be a more effective conservation strategy than investing in lowering $T_{d\text{-}s}$.

The simulation tests of different dog management strategies showed how sensitive the model outcome of 'total number of infectious dog to sea lion contacts' was to changes in the values of the parameters for: dog population size, the proportion of vaccinated dogs, contact rate amongst dogs and contact rate between dogs and sea lions. The ranges of these values were chosen to represent realistically possible management interventions. However, whether the sensitivity of the model outcomes to changes in these parameters is sufficient for them to be effective conservation measures, will depend on what practical changes conservation managers are able to achieve. This study does not aim to answer this last question, but rather to present model sensitivity in a form that is as useful as possible to conservation managers, for their further analysis based on specific knowledge of their capabilities and resources.

Spread between sea lion colonies

In dogs, the majority of simulations (95 %) resulted in disease outbreaks. This was not the case in sea lions, in which a minority of simulations resulted in outbreaks (26 %). However, the results suggested that if an outbreak were to occur in sea lions resident in the San Cristobal colony, it would be highly likely to spread to a neighbouring colony. The San Cristobal sea lion colony at Puerto Bazquerizo Moreno is unique in its location in the centre of a town. The results, in agreement with genetic data (Wolf et al. 2008), emphasise that it is epidemiologically connected to the rest of the Galapagos sea lion

population. Therefore, even though the San Cristobal sea lion colony at Puerto Bazquerizo Moreno lies outside of the protected zone of the Galapagos National Park, it should be a central consideration of conservation management plans for the Galapagos sea lion. However, it should be noted that the model of disease spread between sea lion colonies was less reliably parameterised than the model of spread in a Galapagos dog population, which was informed by clinical data on domestic dogs and empirical data collected during a real epidemic. Therefore the conservation recommendations made based on the sea lion model are more speculative than those based on the results of the dog model.

As San Cristobal colony size decreased, so did the chance of disease spread between colonies; and as the infectiousness period increased, the risk of spread increased. When San Cristobal colony size and infectiousness period and were changed together to simulate El Niño conditions of increasing severity, their antagonistic univariate effects gave rise to a curve of risk of disease spread, with the highest value resulting from intermediate levels of the two parameters. Since we can only speculate on how these parameters change with environmental conditions and one another, these simulations are less reliable tools for conservation planning than those conducted in dogs. However, they suggest a way in which unpredictable changes in food availability driven by changes in environmental conditions and disease threat, which are the two major threats to the Galapagos sea lion (Aurioles & Trillmich 2008), may interact. When the sea lion population declines to very low numbers, although the risk of disease spread is not eliminated, it is significantly reduced, which suggests that the mortality caused by extreme food limitation (e.g. Trillmich & Limberger 1985) may act as a buffer against the threat of disease spread in these extreme circumstances. Under intermediate conditions, though, it appears that the positive effect of infectiousness period on the risk of disease spread outweighs the negative influence of population size. Therefore, it is possible that if the Galapagos National Park could identify the environmental conditions under which Galapagos sea lion immunity is significantly compromised, but under which there is not a relatively high level of mortality due to starvation, they could

deploy their resources for the surveillance of CDV in dogs when these conditions arise. Further research into the combined effects of climate variability (e.g. Weng et al. 2007) and physiology could forge interesting links between ecological immunology (Martin et al. 2011), epidemiology and disease ecology (Hawley & Altizer 2011). The unusual variation in food availability in the Galapagos sea lion study system may have exaggerated effects on host immunity that could facilitate investigation of the influence of host immune plasticity (Graham et al. 2011) on infection dynamics in the wild.

Future model development

Although some work has been done on the influence of climatic variation on Galapagos sea lion life history (Trillmich & Limberger 1985; Mueller et al. 2011; Jeglinski et al. 2012), the results of the long-term study that begun in 2002 and is currently in progress (Wolf & Trillmich 2007) will allow the models presented here to be developed to be more explicit. The models developed in this chapter provide a platform on which more detailed versions can be built through the integration of data on behaviour, physiology and life history. For example, the assumption that sea lions can only transmit disease to members of their social clique (Wolf et al. 2007b) is likely to be an underestimate of the transmission potential within a sea lion colony. If the models presented here were developed into individual-based models (Grimm et al. 2006) and were parameterised using the social network data that is available for the Galapagos sea lion (Wolf & Trillmich 2008) they could more accurately predict pathogen transmission within colonies. It would also be beneficial to more accurately estimate between-colony mixing, which is an important consideration for analysis of disease spread in pinnipeds (Swinton et al. 1998; Hall et al. 2006). In terrestrial carnivores, such between group mixing can be estimated from observational and radio-tracking data (e.g. Craft et al. 2008, 2011). This is more of a challenge in pinnipeds, but could be addressed in the future with increasingly available GPS-tracking data and the emerging framework for integrating spatial with epidemiological analysis in marine mammals (Harris et al. 2008; Norman 2008). This would remove the constraint imposed by the simplifying assumption of a single hypothetical

colony and allow a more realistic, spatially explicit network of alternative haul out sites to be incorporated into the model.

Such spatially explicit, individual-based models would also allow for tests of more specific questions about disease risk in the Galapagos sea lion. For example, as the number of tourist operations in the Galapagos increases (Epler 2007) and boat traffic becomes heavier, Galapagos sea lions may change their movement and aggregation behaviour. If these changes led to more between-colony associations caused by reduced access to feeding areas, such anthropogenic impacts could increase the risk of disease spread. Other issues that could be addressed are the effects of sex differences in behaviour on disease risk (Skorping & Jensen 2004), which could be particularly relevant in a sexually dimorphic pinniped (Robertson et al. 2006); changes in animal and human behaviour caused by disease (e.g. Adelman et al. 2010); contact behaviour of San Cristobal sea lions when present in the neighbouring colony, as GPS-tracking may show that non-residents spatially aggregate with one another in preference to with residents; and the timing of reproduction relative to disease outbreaks. However, such explicit models require more detailed data than is currently available, and it is also possible that they would produce results that would be less easily communicable than those presented in this study, due to high complexity and low generality (Woolhouse 2011).

A further consideration that could be addressed with individual-based models and that would be of particular relevance to the dogs in this study system is that of body size. Body size has been shown to have an influence on infectious disease dynamics, an effect that is thought to be due to correlation with aspects of metabolism, physiology and life history (Bolzoni et al. 2008). The number of dog breeds on the Galapagos is increasing, and there are breeds of all sizes from dalmatians to cocker spaniels. In addition to affecting epidemiological dynamics, this increase in dog diversity may also be correlated with an increase in the diversity of the locations on mainland Ecuador whence they originate. This may increase the diversity of CDV strains that could arrive on the Galapagos, and therefore the chance that one

causes disease in sea lions, given that the emergence of CDV in novel hosts is usually associated with base changes in the virus (McCarthy et al. 2007). This risk could perhaps be modelled using data from comparative studies on CDV strains and host species (e.g. McCarthy et al. 2007; Nikolin et al. 2012) in model frameworks that incorporate pathogen genetic variation (McCormack et al. 2005).

Conclusion

Absolute risks are difficult to quantify, especially when they involve disease in wild animals (e.g. Harding et al. 2003). In this study I aimed to quantify risk in a relative way, and one that could be used by policy makers and conservation practitioners for assessing the most effective ways to reduce disease risk and prioritising resource investment. Although the models presented here make simplifying assumptions, they show how various measures of dog population control could reduce the risk of disease to the endangered Galapagos sea lion. It is up the Galapagos authorities to decide whether this evidence constitutes a “reasonable basis for action” (Woolhouse 2011). These models could also be used as a foundation for other integrated epidemiological analyses of disease risk that incorporate physiological and immunological variation, and that have potential for constructive development beyond the Galapagos sea lion.

Chapter 6

General discussion

The dual aim of this thesis has been to contribute to general understanding of vertebrate immune dynamics in the wild, and to the evidence-based conservation management of the endangered Galapagos sea lion. Here I summarise the findings of the data chapters in relation to one another, and highlight emergent themes and limitations. I also consider to what extent the limitations of the study system affected the significance of the ecological immunology findings, and whether the costs of any limitations were offset by the utility of the results to Galapagos sea lion conservation.

Results summary

This thesis set out to address three issues of ecological immunology using the Galapagos sea lion study system: the potential influence of human activity on immune system development, the physiological cost of investment in immunity, and the effects of inbreeding on immunity in the wild. In addition, it addressed the issue of disease threat to wildlife from domestic animals.

Chapter 2 tested the hypothesis that immune activity and investment would be higher in a human-impacted Galapagos sea lion colony than in a relatively undisturbed control colony. In the context of immune system ontogeny during the first two years of life, the results generally upheld this prediction, though the pattern was heterogeneous across immune measures and ages. This suggested that human activity and its associated environmental impacts may have indirectly influenced immune system ontogeny in the Galapagos sea lion.

Chapter 3 tested the hypothesis that changes in immune measures would be negatively correlated with changes in condition, due to an underlying trade-off between investment in immunity and energy storage and growth. The results

supported this prediction during early immune system ontogeny and across all three measures of condition, but only for a single immune measure and only in the human-impacted colony. In addition, and contrary to expectation, positive correlations were observed in the control colony between changes in immune measures and condition. Together these results suggested that IgG production during early immune system ontogeny may have incurred the physiological cost of reduced condition, but only under human influence.

Chapter 4 tested the hypothesis that inbreeding estimates would be negatively correlated with measures of growth and immunity. The results supported this prediction in the case of IgG production during early immune system ontogeny in both colonies, but the opposite pattern was observed for total leukocyte concentrations in juveniles from the human-impacted colony. This suggested that inbreeding may have had a negative impact on the development of humoural immune protection during early immune system ontogeny, and also that inbreeding may have led to higher levels of infection in juvenile sea lions exposed to human influence.

Chapter 5 tested the hypothesis that domestic dog management could reduce the risk of Galapagos sea lion exposure to CDV in the case of a CDV outbreak in Galapagos dogs. The results showed that dog vaccination, control of the dog population size, control of the contact rate amongst dogs and control of the contact rate between dogs and sea lions could all significantly reduce the risk of Galapagos sea lion exposure to CDV. Chapter 5 also tested whether environmental variation could affect the risk of disease spread between Galapagos sea lion colonies through an effect on food availability and immunity. Given the assumptions of the model, the results suggested that such an effect of environmental variation is possible, and that the risk of disease spread between Galapagos sea lion colonies may be greatest during El Niño conditions of intermediate severity.

Relevance to ecological immunology

Chapter 2 revealed considerable differences in several aspects of immunity between the two sampled Galapagos sea lion colonies. These differences were heterogeneously expressed across ages and immune measures. Nevertheless, they suggested an effect of habitat on immune development and activity, a phenomenon that has been described in other species (Lindström et al. 2004; Palacios et al. 2010), and that may have been driven by indirect human influence in the case of the Galapagos sea lion. Chapter 3 contextualised these colony differences through association with individual-level variation: the colony difference in the relationship between changes in immune measures and condition (Chapter 3) suggested that the colony differences in immune development and activity (Chapter 2) were physiologically significant, and could therefore play a biologically meaningful role in Galapagos sea lion life history.

The suggestion of a physiological cost of immunity in the human-impacted colony (Chapter 3) is highly relevant from the perspective of ecological immunology. As is the case in many wild studies – and as was unavoidably the case in this study – the evidence presented in the data chapters is largely correlative, and therefore does not allow for the inference of cause and effect. Even so, the results of Chapters 2 and 3 suggest that relatively high post-natal IgG production was associated with physiological costs in sea lions resident in the colony exposed to anthropogenic environmental impacts. The cost of immunity is integral to the theoretical foundation of ecological immunology (van Boven & Weissing 2004; Schmid-Hempel 2011b), yet evidence for this cost in wild mammals is rare (but see Hall et al. 2002; Graham et al. 2010). This is partly because its detection in the wild is complicated by variation that is often difficult to control for (van Noordwijk & de Jong 1983; Wilson & Nussey 2010), especially when disease and immune process are involved (Sandland 2003). This study has added to the body of evidence on how immune costs are expressed in the wild by making use of the idiosyncratic ecology of the Galapagos sea lion. Therefore, it contributes indirectly to wider research on how immunoheterogeneity is maintained in wild

populations (Graham et al. 2010) and how selection on immunity interacts with selection on other aspects of life history (Lazzaro & Little 2009).

The fact that relevant correlations were consistent across all three measures of condition in the human-impacted colony (Chapter 3) adds weight to the evidence in support of a cost of immunity in the Galapagos sea lion. This consistency also suggests that the cost of immunity was general, and likely to have involved a broadly relevant physiological trait such as net energy availability, rather than the availability of a specific resource, such as a particular micronutrient (Long & Nanthakumar 2004) or molecule involved in lipid transport or metabolism (Adamo et al. 2008; Trotter et al. 2011). The same was not true of immune variation, as despite colony differences in other immune measures, only changes in IgG concentration were negatively associated with changes in condition in the human-impacted colony. This suggests that the hypothesised immune cost was particular to components or processes that are related to IgG concentration. This proposition is plausible given that IgG production has previously been shown to incur biologically meaningful energetic costs and to cause degradation of condition (Eraud et al. 2005). Chapter 4 further highlighted the importance of IgG during early immune system ontogeny, and given its prominence emergent from the data chapters, I discuss IgG variation in more detail below.

Immunoglobulin G

As concluded in Chapter 2, colony variation in IgG concentration is most likely to have been driven by environmental factors. Therefore, the genetic perspective on IgG variation supplied by Chapter 4 may provide insight into the interaction between genetic and environmental influences on this aspect of immune variation, which emerged from the data chapters as important to Galapagos sea lion life history. Unlike in Chapters 2 and 3, the pattern of IgG variation described in Chapter 4 was consistent between colonies: sea lions from both colonies with relatively high inbreeding estimates produced less IgG during early immune system ontogeny. As discussed in Chapter 4, this is congruent with findings from other systems that have shown inbreeding

estimates to be associated with relatively low measures of humoural immunity (Whiteman et al. 2006; Reid et al. 2007), and suggests that IgG production is positively correlated with fitness during this period of development in the Galapagos sea lion.

If IgG production during this period of development were positively correlated with fitness, we would also expect it to be positively correlated with condition (Iverson 2002; Schulte-Hostedde et al. 2005). This was the case in the control colony but the reverse was true in the human-impacted colony. This may have been because the environmental effect of human influence superseded the genetic effect of inbreeding on IgG production in the human-impacted colony. In other words, in the control colony, IgG may have been produced in a broadly condition-dependent way, which would involve the influence of any genetic effects, including those of inbreeding. However, in the human-impacted colony the environmental stimulus to produce IgG may have outweighed such condition-dependent regulation. This possibility is supported by the fact that additive genetic variance for IgG production was detectable in the control colony but not in the human-impacted colony, which could have been due to environmental variation interrupting the statistical signal in the human-impacted colony.

Although speculative, this suggests the intriguing possibility that in the human-impacted colony relatively inbred sea lions may be at an advantage, at least in terms of their IgG production during early immune system ontogeny. However, inbreeding would be likely to have detrimental effects that would outweigh the possible advantage of relatively low IgG production during early development in the human-impacted colony, such as increased disease risk later in life (Coltman et al. 1999; Acevedo-Whitehouse et al. 2003a; Townsend et al. 2010.), which could explain why juveniles in the human-impacted colony with relatively high inbreeding value estimates had relatively high total leukocyte concentrations. In addition, without data on variation in exposure to PAMPs, antigens, allergens and pathogens (including parasites) between individuals within colonies, it is not possible to speculate on how much of the variation in IgG production was due to extrinsic environmental variation

compared with intrinsic differences in regulatory and investment strategy amongst individuals. The issue of individual variation in exposure is discussed further in the context of project limitations in the section that follows.

Bridging the gap between ecological immunology and the Galapagos sea lion

Chapter 2 showed that despite a lack of immunological reagents specifically adapted for use in the Galapagos sea lion, the application of the generalised techniques of ecological immunology was possible and allowed for a broad overview of the immune system. The combination of cellular with humoural immune components and cumulative with snapshot measures allowed for a more nuanced interpretation of immune activity and development than would have been possible with fewer types of measure. Chapter 2 also demonstrated that longitudinal sampling can bring clarity to the evaluation of immune variation in an ecological context, as colony differences in immune activity at single time points would have been less meaningful than the ontogenetic trajectories described in Chapter 2. Moreover, the longitudinal sampling design made possible the assessment of immune measure variability through time, and showed, for example, that serum protein concentrations were less variable than those of leukocytes. Chapters 2, 3 and 4 together showed that not only was it possible to describe immune variation in an informative way in the Galapagos sea lion, but that it was also possible to do so in the context of relevant ecological variation. Given the associations between immune activity and habitat (Chapter 2), condition (Chapter 3) and inbreeding estimates (Chapter 4), it is likely that the measures used to quantify immunity in this study were able to do so at a resolution relevant to life history in the Galapagos sea lion.

The idiosyncratic ecology of the Galapagos sea lion provided the advantage of a natural experiment-like set-up that facilitated the investigation of immunity in a wild vertebrate in an ecological context. However, it presented one major disadvantage, which was the low detectability of infection given the tools available to the project. Given the paucity of data on parasitism in the Galapagos sea lion, I originally aimed to quantify infection status and parasite

load based on expectations from other sea lion species. However, despite carrying out external examinations, screening faecal samples, screening blood smears, and conducting necropsies there were very few signs of infection, except for a small number of ectoparasites (*Orthohalarachne diminuata* and *Antarctophthirus microchir*; Dailey et al. 2005) and a single dead pup heavily infected with hookworm (*Uncinaria* sp.; Paras et al. 2003).

In principal, the inability to quantify infection and parasite density can be sidestepped by the use of specific antibody assays, as concentrations of antibodies to parasite-specific antigens are correlated with parasite exposure (Bradley & Jackson 2008). However, although they have been applied to other sea lion species (Acevedo-Whitehouse et al. 2003b; Burek et al. 2005; Lloyd-Smith et al. 2007; Castinel et al. 2008), only one such assay has been optimised for use in the Galapagos sea lion (Coria-Galindo et al. 2009), and it was not available for use as part of this project. Therefore, IgG concentration was the most precise measure of humoural immunity that was feasible, and although consideration of gamma globulin concentration and electrophoresis curve shape added context to the interpretation of IgG variation, it could not provide detailed insight into parasite exposure.

Without infection and parasite density data or appropriate specific antibody proxies it was not possible to determine to what extent the variation in IgG concentration between individuals within colonies was driven by exposure compared with responsiveness. In other words, to what extent individuals varied in their investment in immunity compared with other life history traits when exposed to the same environment. The distinction of variable investment under fixed exposure from fixed response to variable exposure is central to the questions about the drivers of immune variation and the maintenance of immunoheterogeneity in natural populations that ecological immunology aims to answer. In order to make this distinction possible and therefore enable investigation of the phenotypic plasticity of immune responses in the integrated context of life history, Graham et al. (2011) recommend measuring “host fitness, parasite density and relevant immune responses” simultaneously.

In the case of this study, and as discussed above, the lack of exposure data limited the depth of possible interpretation. This limitation applied equally to PAMP, antigen and allergen exposure, which may not be associated with disease risk but that nevertheless may stimulate changes in immune measures, as it did to pathogen and parasite exposure, which may be associated with disease risk. However, as discussed in Chapter 2, it is likely that the IgG variation described in this study was largely driven by the overall diversity of the post-natal antigen environment, rather than the presence of a particular molecule or organism. In this case, the expensive and time consuming process of adapting laboratory assays for the detection of common sea lion pathogens in the Galapagos sea lion may be unlikely to yield data that could explain ecologically relevant immune variation successfully. This observation emphasises that, despite its limitations, the application of the generalised tools of ecological immunology to little-known species in the wild can helpfully guide future work and can allow for the prioritisation of investment in the many possibilities of targeted veterinary and physiological investigation.

As discussed in Chapter 1, ecological immunology in the wild is best applied to species that have well described ecologies and that can be manipulated (Pedersen & Babayan 2011). As the Galapagos sea lion study system cannot be manipulated in the way that other wild study systems can be (e.g. Pedersen & Greives 2008; Palacios et al. 2010), the results of this study are correlative, which, as noted above, prohibits inference of cause and effect. Another limitation imposed by the nature of the study system, is that the Galapagos sea lion has a long generation time, which complicates the estimation of host fitness, one of the three key ingredients of observational ecological immunology datasets (Graham et al. 2011). Without long term data on known individuals that enable the calculation of robust indices of survival and reproductive success (e.g. Graham et al. 2010) host fitness can only be estimated through proxies such as body condition (Iverson 2002; Schulte-Hostedde et al. 2005). However, although they are imprecise as estimates of fitness, the fact that these measures can be performed over short time scales

may be a consolatory advantage when ecological conditions are changing fast and conservation management decisions are being made quickly, as is the case in the Galapagos sea lion.

Relevance to conservation

From the point of view of Galapagos sea lion conservation, the lack of data on exposure to, and infection by, particular parasites and pathogens means that the findings of this study do not translate as readily into conservation recommendations as they could have done. Nonetheless, the suggestion that IgG production was associated with costs in the human-impacted colony implies that an environmental aspect of this colony had an adverse effect on the resident sea lions. Subtle negative impacts of human activity on wildlife, such as the energetic drain through the immune system suggested by the results of this study, are ever more important to understand, as their cumulative effects may influence population dynamics, and therefore extinction risk, especially in small populations such as that of the Galapagos sea lion. Therefore, the evidence suggestive of a physiological cost imposed on the Galapagos sea lion by the environment of the human-impacted colony should be considered by conservation managers, and could be addressed through targeted further research on specific environmental components, such as the mircoorganismal content of sewage (Cordoba & Valencia 2000; Rodriguez et al. 2008; Alava 2011).

Chapter 5 demonstrated how the tools of epidemiology can be tailored to provide species-specific conservation recommendations in cases where disease is thought to contribute to extinction risk, as it is in the Galapagos sea lion (Alava & Salazar 2006; Aurioles & Trillmich 2008). These models are flexible enough to allow direct tests of the relative effectiveness of alternative conservation management practices, and therefore produce results that are amenable to translation into action. In addition, Chapter 5 demonstrated how the integration of the largely physiological approach of ecological immunology and the model-based approach of epidemiology could benefit conservation science. Such an integrated approach has diverse potential for future

development and could generate holistic models of disease risk that could be adapted for application to a variety of complex systems and drive coordinated conservation action plans.

Future work

There are two broad ways in which this project could be built upon and in which its contribution to ecological immunology could be expanded, both of which have been touched on above. The first is through long-term monitoring and the second is through more targeted, more specific data collection. The principal advantage of long-term monitoring is that it would enable robust estimation of fitness in the long-lived Galapagos sea lion. A long-term study on the Galapagos sea lion is underway (Mueller 2011) but the dataset has not yet matured to a point that permits the calculation of traits such as lifetime reproductive success. The maturation of this dataset will be useful not only because it will allow for the calculation of these traits, but also because it will enable the evaluation of fitness proxies such as body condition as predictors of lifetime reproductive success at different stages of development (e.g. Hamel et al. 2009).

Long-term study would also allow immune variation to be investigated in the context of life history variation that is not present during the first two years of life in the Galapagos sea lion. Sex differences in adult physiology have been a major driving force behind the theoretical and empirical development of ecological immunology (Hamilton & Zuk 1982; Folstad & Karter 1992; Westneat & Birkhead 1998; Roberts et al. 2004; Demas et al. 2011a). Given the high degree of sexual dimorphism found in otariids (Lindenfors et al. 2002), sexual maturity in the Galapagos sea lion is likely to be accompanied by significant divergence in life history, behaviour and physiology between the sexes, which could have important implications for immune variation. The study of adult female sea lions would also allow for the investigation of the role that maternal effects play in immune development and disease processes, a subject in which there is growing research interest (Stjernman & Little 2011; Garnier et al. 2012; Hasselquist et al. 2012). A further advantage

of long-term study in this system would be the opportunity to study the effects of stochastic environmental variation on immune dynamics (e.g. Graham et al. 2010), as sensitivity to the unpredictable variation of the oceanic environment of the Galapagos archipelago is an important aspect of Galapagos sea lion ecology (Trillmich & Limberger 1985; Trillmich & Dellinger 1991; Mueller 2011).

The specificity of the data collected by this project could be increased through the development of detection assays for parasites or antibodies to antigens expressed by common sea lion pathogens. In the case of hookworm infection, for example, a DNA detection assay could be developed. This could be optimised using hookworm samples collected from the single infected pup that was found during the course of this study (Chapter 2) and then applied to living pups (e.g. Traub et al. 2004). However, given that the screening of faecal samples in this study found no hookworm eggs, even if a DNA detection assay could be successfully developed, it may detect a naturally low level of infection in the Galapagos sea lion and one that is not linked to detectable variation in immunity. In addition, this approach may have a low probability of success, as the important variation in IgG concentration during early immune system ontogeny seems unlikely to be driven by one or few infective agents, but rather by the antigenic diversity of the post-natal environment.

Instead, there are other, less specific methods that could supply insight into what drives this variation in IgG concentration in young Galapagos sea lions. For example, natural antibody production during early development could be measured (Ochsenbein & Zinkernagel 2000; Whiteman et al. 2006; Ujvari et al. 2011), and its contribution to changes in IgG concentration assessed. In addition, bactericidal assays (Millet et al. 2007) could be used to test whether relatively high IgG concentrations and other aspects of Galapagos sea lion immune phenotype confer a generalised and functional immunological advantage. Humoural immune challenge tests (Demas et al. 2011b) could also be carried out to investigate whether Galapagos sea lion pups are capable of mounting acquired immune responses during early immune

system ontogeny, which would allow for more definitive interpretation of the significance of IgG concentrations during this period of development.

There are also a number of ways in which non-immunological aspects of Galapagos sea lion physiology could be more explicitly measured. The influence of sex differences on immune variation in adult sea lions, for example, could be investigated through the quantification of sex hormone concentrations (e.g. Browne et al. 2006). The measurement of hormone concentrations would also facilitate investigation of the potential effects of acute and chronic stress on immune variation (e.g. Myers et al. 2010). However, any such study on hormones would have to design a rigorous sampling regime and system of controls in order for the results to be reliably informative in an ecological context (Goymann 2012). In addition, further work could focus on better defining the difference in antigen environment between the colonies, not only by testing for the presence of pathogens in sea lions, domestic animals and sewage but also in the environment, such as tide pools, beaches and even on sea lion skin (e.g. Apprill et al. 2011).

An alternative way in which the results of this project could be used as a foundation for future work is by acting as motivation for the design of controlled laboratory experiments in model organisms. In other words, they could contribute to the feedback cycle discussed in Chapter 1 between ecological immunology carried out in the wild and laboratory-based immunology. This could involve experiments on the effects of the post-natal antigenic environment on mice, for example, during which test subjects could even be exposed to environmental samples from the sea lion colonies. Such laboratory studies would allow for the experimental dissection of the mechanisms underlying the immune variation described by this study.

Feedback into the design of laboratory experiments could also probe the interesting possibility of an interaction between environmental unpredictability and disease risk that was discussed in Chapters 2, 3 and 5. Given the time period between El Niño events (Salazar & Denkinger 2010) and the long generation time of the Galapagos sea lion (Mueller 2011), even within the

context of a long-term study it would be difficult to show how unpredictable environmental variation contributed to disease risk through trade-offs between investment in immunity and energy stores within individuals. However, such questions could be investigated in microcosmic model systems, manipulated in a way to mimic variation of the kinds experienced by the Galapagos sea lion. In moths, for example, the interactive influences of food quality and environmental variation on immunity have already been investigated in the laboratory (Triggs and Knell 2011).

Conclusion

In the introduction to Chapter 2 I claimed that recent developments in ecological immunology research 'pave the way' for studies on little-known species such as the Galapagos sea lion. In light of the emergent limitations discussed above, 'point the way' might have been a more appropriate metaphor, as the power of the results to provide insight into fundamental biological processes was limited by unpredictable mismatches between idiosyncrasies of the study system and the methodological tools available for its investigation. Nevertheless, despite these limitations, this project demonstrated that the application of ecological immunology to a species such as the Galapagos sea lion can provide insight into the dynamics of immunity in the wild, generate data that has practical value to conservation, and suggest specific avenues for constructive further research. Therefore, the risks implicit in the application of ecological immunology to a little-known species, and their expression in the Galapagos sea lion as the limitations discussed above, were, in the case of this project, outweighed by the utility of the results to ecological immunology and conservation.

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Appendix 1.1

Photographs and satellite images of the town and sea lion colony of Puerto Bazquerizo Moreno, San Cristobal (a-h; human-impacted colony; 0°54’07” S, 89°36’44” W) and the sea lion colony at Bahia Paraiso, Santa Fe (i-j; control colony; 0°48’15” S, 90°02’28” W); satellite images downloaded from Google Earth.

a

b

c

Jana Jeglinski

d

Jana Jeglinski

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h


Image © 2012 DigitalGlobe
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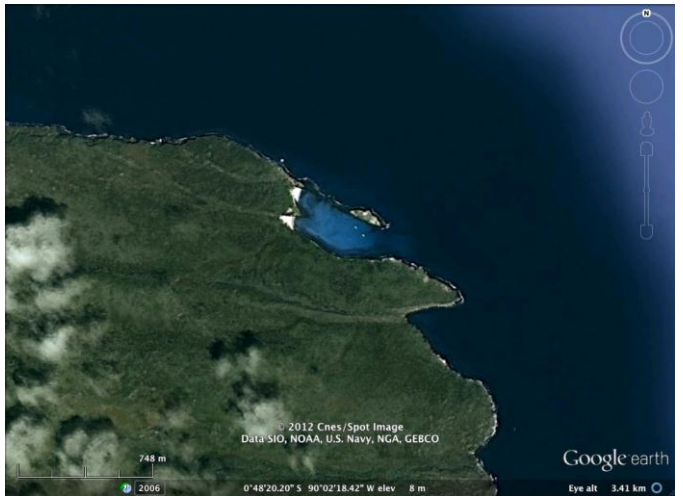
Google earth

i

j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Google earth