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A primer in ecoimmunology and immunology for wildlife research and management

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Two predictions of changing climate are the emergence of new diseases and the expansion of the ranges of existing parasites. Variation among individuals, especially in response to parasites, directly affects population dynamics and how populations respond to management. Immune function, therefore is a key individual-level trait that influences demographic characteristics and life-history traits because it directly affects the survival outcome of a parasitic challenge. Mounting an immune response is expensive in energy and resources and, thus, the principle of allocation predicts that trade-offs will occur with other energetically demanding tasks, such as survival or reproduction. Therefore, understanding immune function in wild animals is important for predicting how animal populations will respond to management, and we recommend that managers integrate data on immune function into larger studies of population dynamics and management of populations. In this review, we introduce how types of immune function are classified within traditional immunology and the emerging field of ecological immunology (ecoimmunology). We also review the resources available to wildlife managers for learning about techniques in ecoimmunology, and provide guidance for developing studies of immune function within larger projects on demography among populations.

Key words: adaptive immunity, constitutive immunity, ecoimmunology, immune function, innate immunity, individual variation, induced immunity, life history, parasite, population regulation

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Models of population dynamics and management plans are based on estimates of life-history parameters, such as survival and reproduction. Those life-history parameters directly affect how species or populations respond to management. Because individuals within a population vary greatly in survival and reproductive success (Clutton-Brock 1988), population managers and ecologists have become increasingly interested in how variation among individuals affects demographics at the level of the population (Sheldon

and Verhulst 1996, Hayes and Jenkins 1997). Although studies at the population level are important for conservation and management, these studies are substantially limited because of difficulty distinguishing among proximate causes of changes in population size, such as breeding success, survival, immigration, and emigration (Clutton-Brock and Sheldon 2010). Recently, individual-based studies have advanced our understanding of population ecology considerably (Clutton-Brock and Sheldon 2010). Data collection and analyses at the individual level are complementary to those at the population level, but individual-based studies provide further insight into the mechanisms of population change and selection for life-history traits (Stearns 1992, Lindstrom 1999, Testa 2004, Benton et al. 2006, Monteith et al. 2014b). An improved understanding of how individual heterogeneity affects population dynamics will continue to improve models of population dynamics and inform management decisions. To accomplish this task, one needs to answer the question: what mechanisms underlie heterogeneity among individuals in their life histories (Flatt et al. 2011)?

Why Measure Immune Function and Parasite Load?

A major component of the expanding field of ecological immunology (ecoimmunology) is understanding how ecology and evolution have shaped immune responses, and how immune responses, in turn, have shaped the ecology and evolution of wild organisms (Downs et al. 2014). Hypotheses developed to explain variation in life histories emphasize the role of physiology as an integrator between genetics and environmental effects on expressed phenotypes (Sibly and Calow 1986, Ricklefs and Wikelski 2002, Martin et al. 2011b, Cohen et al. 2012). Immune responses have received particular emphasis because of their integral role regulating physiology of animals (Ricklefs and Wikelski 2002, Martin et al. 2008, Demas et al. 2011a, Martin et al. 2011b, Cohen et al. 2012, Demas and Nelson 2012). For example, the same signaling molecules (e.g., growth hormones, sex hormones) are involved in the regulation of both immune function and reproduction (Downs et al. 2014 and references therein).

Immune responses are also integrated with other systems because they are costly in energy and nutrients (Demas et al. 1997, Lochmiller and Deerenberg 2000, Iseri and Klasing 2013). When resources are limited, the principle of allocation predicts that a trade-off will occur between competing processes, such as investment in immune function and investment in reproduction. Indeed, the immune system has been proposed as a mediator of long-term trade-offs between reproduction and survival (Sheldon and Verhulst 1996), and experimental work has shown that energetic and nutritional limitations result in trade-offs between immune responses with reproduction or growth rates (Lochmiller et al. 1993a, French et al. 2007a, French et al. 2007b).

Finally, immune responses are an important component of survival because of their role in regulating parasites (Alizon and van Baalen 2008, Day et al. 2011, Hawley and Altizer 2011, Downs et al. 2014, Klein et al. 2014). For the purposes of this discussion, we define parasite to include micro-parasites that cause disease (i.e., pathogens, including viruses, bacteria, and fungi) as well as macro-parasites (i.e., intestinal worms, ticks, and fleas) (Anderson and May 1979). Parasites are ubiquitous in the environment, and their role in regulating population size was recognized by Leopold (1933). The optimal intensity of immune response to a parasite infection, however, depends on a complex balance of costs and benefits. If responses are too weak, animals succumb to disease or parasites but, if too

vigorous, responses might damage the individual's own tissues or use resources that could otherwise be invested elsewhere (Raberg et al. 1998, Schmid-Hempel 2003, Viney et al. 2005, Zimmerman et al. 2014). Thus, natural selection should select for individuals that mount an appropriate intensity of immune response to parasites, making the immune system a focus for natural selection (Ardia et al. 2011). Immune responses, therefore, are important for understanding individual heterogeneity in survival and reproduction and the regulation of animal populations because they provide a mechanistic link between disease dynamics and consequences in host populations (Krebs 1995, Lochmiller 1996).

Implications of Studies of Immune Function for Management

Two predicted effects of climate change are the emergence of new parasites and the expansion of ranges of known parasites (Harvell et al. 2002). Diseases such as chronic wasting disease are already having adverse effects on populations of wildlife (Monello et al. 2014), and emerging fungi are contributing to the decline of amphibian populations worldwide (Blehert et al. 2009, Frick et al. 2010) and bat populations in North America (Daszak et al. 1999, Daszak et al. 2003, Stuart et al. 2004). In addition, macroparasites including winter ticks and lice are expanding their ranges and having decimating effects on populations of moose (*Alces alces*) (Drew and Samuel 1985, Samuel 2007) and black-tailed deer (*Odocoileus hemionus* ssp.) (Bildfell et al. 2004), respectively. In general, effects of parasites often are difficult to assess in free-ranging wildlife because of predation or scavenging of dead individuals (Wobeser 2007). Effects of parasites in populations of wildlife also may result in decreased productivity or recruitment, which are much more difficult to quantify than adult mortality but may have a stronger effect on population dynamics.

Models of disease spread in populations traditionally assume random variation in organismal responses to infections (Anderson and May 1979); however members of the population are not homogenous and variability exists among portions of the population as well as among individuals. Differences among individuals in immunocompetence also determine whether an individual resists or tolerates an infection. Resistance involves reducing parasite numbers, and thus reflects the ability of the host to kill an invading parasite, while tolerance involves minimizing fitness losses in responses to a particular parasite load (Caldwell et al. 1958, Simms 2000, Raberg et al. 2009, Downs et al. 2014). An individual with a high tolerance can maintain higher fitness during an infection despite having a high parasite loads. As a consequence, whether an individual is tolerant or resistant to a parasite will affect transmission rates within a population because a tolerant individual has the potential to infect many other individuals (Boots et al. 2009, Arsnoe et al. 2011). Understanding variation among individuals in immune function will provide a mechanistic understanding of whether an individual will adopt a strategy of tolerance or resistance, which will in turn inform disease dynamics (Hawley and Altizer 2011).

Effects of parasites may be more difficult to detect if a small portion of the population is affected or if individuals in poor nutritional condition are disproportionately affected (Caron et al. 2013). Given those difficulties in identification and detection, parasites may become established in wildlife populations with relatively few obvious indicators of the presence of that parasite (see, for example, Bleich et al. 2014). From a management perspective, a stronger understanding of how physiology correlates with, and potentially mediates, life-history traits will lead to the development of biomarkers that indicate animal

health and condition. These biomarkers may replace traditionally measured traits that often are difficult to quantify without repeated capture and sampling of individuals, which is often prohibited by cost, time, and the risk to the animals. Thus, biomarkers that are relatively easy to quantify and more cost effective may be more useful indices for large numbers of free-ranging animals, particularly if they provide insights into disease dynamics and factors affecting population regulation. Investigations into immune responses have the potential to yield biomarkers of this type (Sild and Hörak 2009).

In this review, we start by discussing briefly the importance of understanding nutritional condition when interpreting immunological assays (Arsnoe et al. 2011). We then review how the immune system is classified in traditional immunological and ecoimmunology studies. Finally, we introduce general techniques that help address questions about the immunological state of vertebrate animals, and we provide guidance for implementing those techniques. We do not attempt a complete review of all possible techniques. Rather, we provide references of key review papers and other resources that provide further information about immunological techniques currently being used in the field so that the reader may locate additional resources.

The Importance of Understanding Nutritional Condition

Nutritional condition (generally described as percent body fat) functions as the mechanism through which intraspecific competition for resources is mediated, and provides the most direct and sensitive measure of resource limitation for the organism (Parker et al. 2009, Monteith et al. 2013, Monteith et al. 2014b). Body fat is strongly related to survival and reproduction in animals because energy from fat is used during winter fasts, thermoregulation, migration, incubation, and lactation (Barboza et al. 2009). Moreover, fat reserves also are tied directly to productivity in adult females (Testa and Adams 1998, Keech et al. 2000, Cook et al. 2001, Stephenson et al. 2002, Stewart et al. 2005). Indeed, vital rates of large herbivores, in particular, generally respond to resource limitation in a predictable sequence beginning with decreased survival of young (corresponding to decreased recruitment), increased age at first reproduction, decreased reproduction by adults, and decreased adult survival (Gaillard et al. 1998, Gaillard et al. 2000, Eberhardt 2002, Monteith et al. 2014b). Processes such as reproduction or mounting an immune response demand resources above those necessary for maintenance (Wobeser 2007, Monteith et al. 2014a), and large herbivores have been shown to favor their own survival over reproduction (Stearns 1992, Morano et al. 2013, Monteith et al. 2014b). Changes in life-history characteristics operate through changes in nutritional condition, which is an integrator of both intake of forage and physiological demands of that organism (Monteith et al. 2014b). Because of our clear understanding that nutritional condition of animals directly influences survival and reproduction, indices of nutritional condition are useful for managers especially when fecundity and survival cannot be measured directly (Bishop et al. 2009). Body fat and protein reserves are a direct indication of nutritional status of animals and are often used to make predictions about survival and reproduction (Hobbs 1989, Moen et al. 1997, Stephenson et al. 2002). How fat reserves are tied to immune function and thus affect tradeoffs with reproduction and survival are still in need of further studies, especially with free-ranging species of wildlife.

Limitations in body protein and other macronutrients also are documented to lead to immunosuppression (Klasing 1998, Klasing and Leshchinsky 1999, Lochmiller

and Deerenberg 2000, Brunner et al. 2014). For example, protein reserves were critical for maintaining immunocompetence in juvenile cotton rats and bobwhite quail chicks (Lochmiller et al. 1993b, Vestey et al. 1993, Lochmiller et al. 1994). We recommend acquiring measures of nutritional condition, specifically protein and fat reserves, of individuals in studies investigating immune function in wild animals to better understand the relationship between nutritional condition and immunocompetence.

Finally, micronutrients, including trace minerals, also play an important rule in regulating immune responses (Chandra and Dayton 1982, Suttle and Jones 1989, Bhaskaram 2002). A detailed review these relationships is beyond the scope of this paper, but blood samples can also be used to quantify micronutrient levels in wildlife (Duffy et al. 2009) and we recommend investigating these levels in populations that are exhibiting signs of disease.

Classification of Immune Responses

General classification of immune function.—The front line of defense against infection is behavioral (Nelson et al. 1975, Murphy et al. 2007; Figure 1). The second line of defense against infection, and the first layer of the architecture of the immune system, is the epidermal layer that provides a physical barrier to invasion (Hofmeyr 2001, Murphy et al. 2007; Figure 1). The third line of defense is the physical barrier created by physiological conditions within the body, such as pH, which determine whether the internal environment is appropriate for the parasite (Hofmeyr 2001; Figure 1). The fourth line of defense is physiological. Traditionally the immune system is partitioned into two types of responses: innate and adaptive (Figure 1). Both systems consist of a multitude of cells and molecules

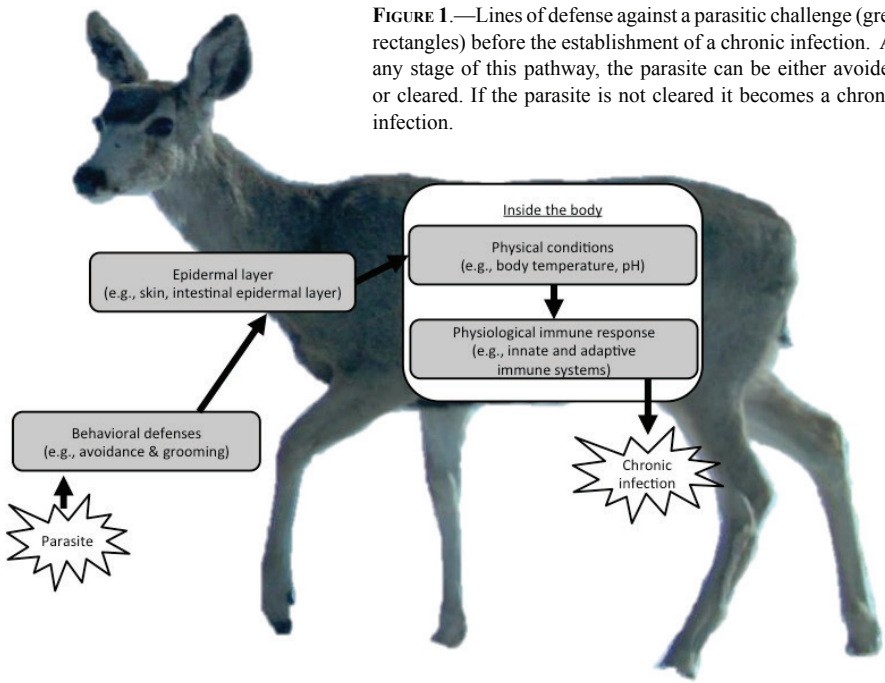

Figure 1.—Lines of defense against a parasitic challenge (grey rectangles) before the establishment of a chronic infection. At any stage of this pathway, the parasite can be either avoided or cleared. If the parasite is not cleared it becomes a chronic infection.

that interact in a complex manner to detect and eliminate parasites (Hofmeyr 2001). We focus on the physiological defense against infection and Appendix I includes a glossary of terms for aspects of the immune system.

Traditional immunological classification.—Innate immunity includes immediate responses following detection of a parasite and responses during the early stage of infection. Innate responses include the complement activity, cytokine cascades, the acute phase response, and the phagocytic system, which involves scavenger cells (e.g., macrophages) that detect and engulf extracellular molecules and material (Murphy et al. 2007). Some portions of the innate immune system are constitutive (always present), such as complement proteins, whereas some are induced, such as acute phase responses (Figure 2).

In contrast, adaptive immunity is the response of antigen-specific lymphocytes to an antigen, and includes the development of immunological memory (Murphy et al. 2007). Adaptive immunity includes responses of B cells involved in humoral immunity and responses of T cells involved in cellular immunity (Figure 2). Note that processes that are part of innate immunity induce adaptive responses. For example, macrophages recognize parasites and initiate innate immune responses, but macrophages also initiate an adaptive immune response by recruiting T cells to the site of infection and presenting antigens to initiate T-cell mediated killing of infected cells (Murphy et al. 2007).

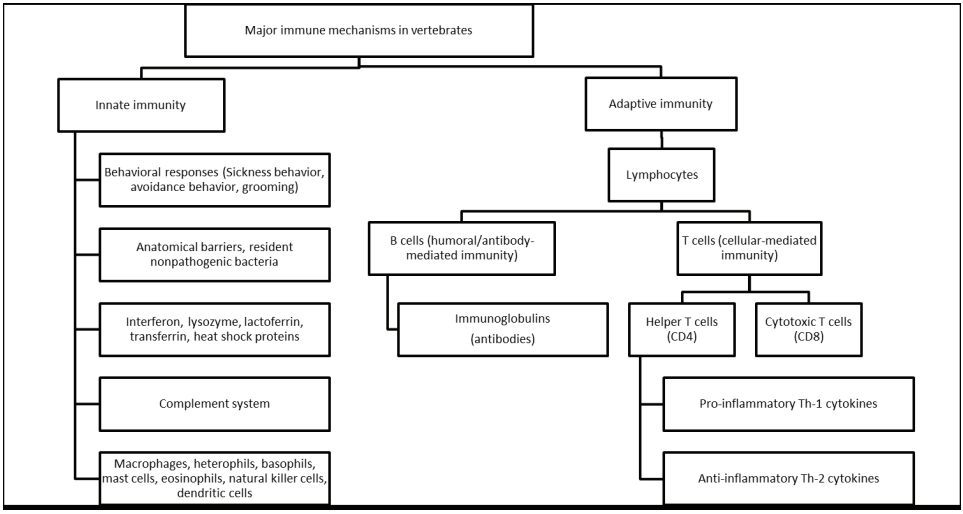

Figure 2.—Traditional classification of the immune system. Terms used in the figure are defined in Appendix I. This figure is modified from Demas et al. (2011) and Muehlenbein (2010).

Functional classification from ecoimmunology.—A helpful classification of immune function used in ecoimmunological studies involves classifying immune responses along two axes, which are not mutually exclusive: (1) a readiness axis ranging from constitutive to induced responses, and (2) a specificity axis ranging from non-specific to specific responses (Schmid-Hempel and Ebert 2003; Figure 3). Constitutive immune responses are always present, are capable of immediate physiological defense, and include defenses such as complement activity and phagocytosis (Schmid-Hempel and Ebert 2003). Induced immune responses are activated when a parasite is recognized, and include responses such

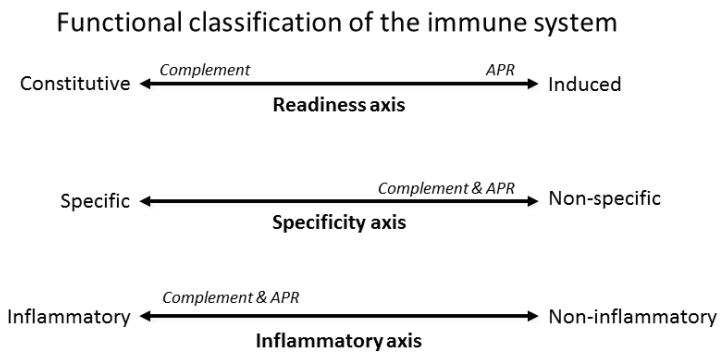

Figure 3.—Three functional axes are often used to describe immune responses in ecoimmunology studies: readiness, specificity, and inflammatory. Traditionally, immune responses are partitioned into innate and adaptive responses. No combination of functional axes describes all responses classified as innate or adaptive under the traditional classification; rather, each innate or adaptive response can be described as a combination of these three functional axes. This figure depicts the placement of two innate immune responses, the acute phase response (APR) and complement activity (complement), on the three functional axes.

as antibody production and the acute phase response (Schmid-Hempel and Ebert 2003). Non-specific immunity is characterized by responses that are not specific to an antigen and are used against numerous types of parasites and includes the complement pathway, local inflammation, and the acute phase response (Schmid-Hempel and Ebert 2003, Lee 2006). In contrast, specific immunity is characterized by responses that target specific antigens, and include toll-like receptors, antibody responses, and T-cell mediated killing (Schmid-Hempel and Ebert 2003, Lee 2006). Specific responses tend to cause less damage to the host than non-specific responses because they are specific to a parasite. An inflammatory axis, ranging non-inflammatory to inflammatory, is a commonly used third axis (Lee 2006; Figure 3).

Ecoimmunological classifications are helpful for hypothesis development because they divide the immune system into functional groups that have different relative costs of energy, resources, and immunopathology. Immunopathology is damage to the host caused by the actual immune responses rather than the parasite. For example, the release of reactive oxygen species during an inflammatory response can damage host tissues (Mates and Sanchez-Jimenez 1999). Note that these axes of ecoimmunological classification include both innate and adaptive immune responses, and no single ecoimmunological axis describes innate versus adaptive immune responses. For example, the acute phase response is part of the innate immune system, but is classified as inflammatory on the inflammation axis, induced on the readiness axis, and non-specific on the specificity axis (Figure 3). Likewise, complement activity is also traditionally classified as part of the innate immune system, but it is classified as constitutive, non-specific, and inflammatory under the ecoimmunological classification scheme (Figure 3). The type and strength of the immune response will determine the energetic, resource, and immunopathology costs associated with mounting an immune response. The type and strength of the immune response will therefore determine the selective pressures on that immune response and will inform predictions about how an individual will respond to endogenous and environmental forces.

Review Papers and Resources Available for Finding an Immune Assay

Demas et al. (2011b) and Boughton et al. (2011) provide broad and comprehensive reviews of techniques that are commonly used by ecoimmunologists. These reviews include tables describing the type of immune function measured by each assay, the pros and cons of each assay, and references of key papers that describe each assay. In addition to these broad reviews, numerous reviews of more specific topics have been published recently, including a review of developing serological assays to quantify antibody responses to specific parasites (Garnier and Graham 2014), molecular techniques to quantify gene expression and circulating protein levels in non-model species (Fassbinder-Orth 2014), and remote biomonitoring techniques that can be used to quantify heterogeneity in immune responses and disease dynamics in small, free-living animals (Adelman et al. 2014). In addition, Zimmerman et al. (2014) reviewed the role of specific cytokines, important signaling molecules that regulate the strength and type of immune response. Cytokines also integrate immune responses with responses of other physiological systems (e.g., endocrine) making them important in integrated, whole-animal responses to infection (Zimmerman et al. 2014). Cytokine expression can be measured using numerous molecular techniques reviewed by Frassbinder-Orth (2014). Finally, the National Science Foundation sponsored the Research Coordination Network in Ecoimmunology, which compiled laboratory protocols for many immune response assays on their website (www.ecoimmunology.org) in an effort to standardize techniques across laboratory groups.

Caveats and Considerations When Picking an Assay

The immune system is clearly complex and cannot be described with a single assay (Adamo 2004, Boughton et al. 2011, Demas et al. 2011b). This complexity leads to numerous methodological considerations and caveats when choosing an assay. First, choose an assay that measures the immune pathway of interest. This advice may seem obvious, but it is easy to pick an inappropriate assay because the immune system is complex. Second, we recommend functional assays, such as a bactericidal assay, over morphometric assays, such as counts of white blood cells. While morphometric assays provide information about the immune system (e.g., Nunn 2002, Nunn et al. 2003, Matson et al. 2006a), extrapolating from morphology to functionality, such as clearance of a parasite, is difficult (Demas et al. 2011b). Third, if interested in the immune system in general (e.g., immunocompetence), then measure many different parts of the immune system. Results for different parts likely will differ and it is difficult to extrapolate from single assays to overall immunocompetence. Fourth, a general limitation to the application of immunological techniques to new species is the lack of species-specific reagents (Demas et al. 2011b, Downs et al. 2014). Three general solutions exist: (1) develop species-specific reagents (e.g., Hibma and Griffin 1990, Hunter et al. 2008); (2) adapt reagents from a closely related species and calibrate the assay for the new species (e.g., Svensson et al. 1998, Graham et al. 2010, Brock et al. 2013); or (3) use assays that do not require species-specific reagents (e.g., Matson et al. 2006a, Sparkman and Palacios 2009). Finally, different types of samples yield different information. For example, samples of whole blood include white blood cells, but plasma and serum samples do not. As a result, only bactericidal assays using samples of whole blood include measurements of phagocytic activity, because white blood cells are required.

Choosing between induced and constitutive immune responses.—If the goal of the study is to understand overall immunocompetence, then a researcher should measure aspects of both induced and constitutive immunity because both contribute to the outcome of an immune challenge. If animals are sampled only once, constitutive immune responses can be quantified, but induced immune responses can also be measured only if individuals can be held for at least a couple of hours or if using remote biomonitoring techniques (Boughton et al. 2011, Adelman et al. 2014). Similarly if animals cannot be handled, techniques are restricted to behavioral observations. Regardless of the approach, single samples of immune function conflate within-individual and between-individual variation, and animals should be sampled multiple times if possible to disentangle these types of variation (Downs and Dochtermann 2014).

Considerations for constitutive immune responses.—Almost by definition, studies are restricted to constitutive immune responses if a single sample is taken from an individual upon capture. As described previously, constitutive responses are present without being induced by a parasite. A complication of measuring constitutive immunity is that parasite status of the individual is often unknown. Concentrations of constitutive components of immune function change during an induced response making it difficult to determine if concentrations are due to a parasitic challenge or differences in investment (Gabay and Kushner 1999, Tieleman et al. 2005). For example, the bactericidal assay is a functional assay that measures levels of complement, natural antibodies, and phagocytosis, and it is predictive of the ability of an individual to clear a microbial parasite when challenged (Tieleman et al. 2005, French et al. 2010). Aspects of the immune system involved in bactericidal capacity increase in blood when an individual is challenged by a parasite (Gabay and Kushner 1999, Tieleman et al. 2005). In a study of 12 species of wild passerine birds, Tieleman et al. (2005) found a negative correlation between bactericidal capacity and metabolic rates, but it was unclear whether this correlation was caused by differences in life histories or by differences in parasite load that elevated both metabolic rates and bactericidal capacity. This particular example is also an example of the broader caveat that correlation does not equal causation, something that should be considered carefully when interpreting correlative studies. This example also highlights the need to obtain measurements of parasite loads or disease environment to provide context for interpreting measures of immune function (Horrocks et al. 2011).

Considerations for induced immune responses.—Induced immune responses are activated by constitutive components of the immune system; therefore, measurements of induced responses measure a part of the integrated network that encompasses a full pathway (Boughton et al. 2011). An advantage of measuring induced responses is that they can provide a direct measure of the intensity of an immune response and the related fitness consequences. Induced responses can be experimentally stimulated with a living parasite (e.g., avian influenza virus (Arsnoe et al. 2011) or *Trichinella spiralis* (Dlugosz et al. 2013) or a non-parasitic stimulant. Examples of non-parasitic stimulants include heat-killed *Escherichia coli* (Tieleman et al. 2005, Lee et al. 2008), lipopolysaccharide (Downs et al. 2012), sheep red blood cells (Ardia et al. 2003), or a vaccine (Ilmonen et al. 2000). The information that can be gleaned from a study will depend on type of stimulant used and response measured. If a living parasite is used, then the physiological, behavioral, and fitness consequences of tolerating, resisting, or clearing an infection can be measured. For example, Allenby's gerbils (*Gerbillus andersoni allenhbyi*) experimentally challenged with fleas (*Synosternus cleopatrae*

pyramidis) experienced increased predation risk because of changes in foraging behavior (Raveh et al. 2011), but whether effects on physiology, behavior, and fitness were attributable to immune responses or to parasites could not be separated because a live parasite was used. Similarly, experimentally removing warble flies (*Hypoderma tarandi*) from female reindeer (*Rangifer tarandus tarandus*) had a positive effect on body mass (Ballesteros et al. 2012), but it is unclear whether this effect was caused directly by a reduction in the parasite load or caused indirectly by decreased immune responses. In contrast, non-parasitic stimulants do not have the ability to replicate or establish an infection, and are advantageous because it is clear that changes in physiology and behavior associated with the immune response rather than the parasite (Elin and Wolff 1976). Ideally, connections between immune responses to parasitic and non-parasitic challenges should be tested directly to confirm the degree of correlation between those two measurements.

Measuring induced immune responses requires holding the animal until a measureable response occurs or recapturing the individual numerous times. One way to quantify induced responses is to measure cytokines that are involved with initiating that response (Zimmerman et al. 2014). Depending on the species under investigation and which cytokine is chosen as the biomarker, it can take hours to days for cytokines to peak in blood (Dantzer 2001, Demas et al. 2011b). Animals have to be held for multiple days or recaptured to quantify these responses, which is possible for some species but may be logistically infeasible for others (e.g., Tieleman et al. 2005, Owen-Ashley and Wingfield 2007). Alternatively, scientists are making use of remote biomonitoring techniques, such as radio transmitters, to measure some immune responses, which eliminate the need for holding individuals of some species (Adelman et al 2014).

If interested in quantifying adaptive immunity, a way around the constraint of multiple capture events is to measure responses for a known disease using serological techniques (Garnier and Graham 2014). An advantage of this approach is the ability to measure a response to a natural infection and an ecologically relevant disease; a disadvantage, however, is that the initial infection load and date of infection are unknown. Nevertheless, information about the intensity of infection can be paired with information about the level of immune response to create a more complete picture about the dynamics of both the parasite and immune responses (Nussey et al. 2014).

Handling times and stress.—The interaction between stress responses and immune function must be considered when collecting samples from wild animals (Boughton et al. 2011). Stress hormones, such as glucocorticoids, are involved in the regulation of immune function and increase in the blood during capture, handling, and captivity (Sapolsky et al. 2000, Jacobson 2005). Acute stress increases glucocorticoids and often stimulates immune responses, whereas chronic stress increases glucocorticoids and suppresses immune responses (Martin et al 2009), but how stress affects immunity is specific to the type of response (Sapolsky et al. 2000). Indeed, zebra finches (*Taeniopygia guttata*) held in captivity had lower white blood cell counts and decreased skin swelling in responses to phytohaemagglutinin challenge (Ewenson et al. 2001), but house sparrows (*Passer domesticus*) held in captivity had increased inflammatory responses (Martin et al. 2011a). Thus, caution must be used when interpreting results from animals held for a long duration.

Even when animals are not held in captivity, the time between capture and processing can be sufficient enough to alter immune responses (Matson et al. 2006b, Buehler et al. 2008). Glucocorticoids begin to increase within 1–3 minutes in many species (Romero

and Romero 2002). Immune markers generally change at a slower pace but responses vary by species (Matson et al. 2006b, Buehler et al. 2008). For example, handling times of 1 hour reduced bactericidal capacity in three bird species, but not in two other bird species (Matson et al. 2006b). Buehler et al. (2008) recommended taking samples within 30 minutes of capture to avoid changes in immune measures caused by stress of capture. If recorded, handling times can be controlled for statistically and used to test for trends. Sometimes, especially when working with animals that are passively trapped, it is not possible to obtain blood samples quickly or to record handling times. Immune function can still be quantified, but the result will be an integrated measure of the individual's response that includes the intensity of the baseline immune response before capture and stress of capture and handling.

Notes on logistics.—When collecting samples in the field, a number of precautions must be considered. Owens (2011) reviewed blood collection techniques for birds and Boughton et al. (2011) reviewed some logistical considerations more thoroughly. Briefly, sterilize skin at the collection site, and ensure sterility of collection materials (e.g., cryovials, pipette tips). Also, take precautions to ensure viability of samples: e.g., dilute in appropriate media, reduce exposure to oxygen, cool or freeze rapidly (Boughton et al. 2011). If assays require living white blood cells, the assay must be run shortly after blood is collected and the sample cannot be frozen. Regardless of the assay, minimize time between collection of sample and performing the assay, and once in the laboratory, ensure sterility of media and buffers (Boughton et al. 2011). In addition, assays must be calibrated for each new species, and calibrating assays makes it difficult to compare results among species.

Soay Sheep: A Case study

Despite the cautionary notes above, the mechanistic insights gained by investigating immune responses in wildlife are worth the effort. A study of the unmanaged population of Soay sheep (*Ovis aries*) in Village Bay on Hirta, St. Kilda off the northwest coast of Scotland provides an excellent case study. The population has been monitored since 1985, and experiences periodic crashes caused by high mortality (Clutton-Brock and Pemberton 2003). Gut parasites were one factor contributing to these crashes; parasite loads increased during crash years (Clutton-Brock and Pemberton 2003). Individuals that invested in high levels of antibodies that were specific to the predominate gut parasite had lower parasite loads, and higher over-winter survival (Nussey et al. 2014). Nevertheless, Graham et al. (2010) found that although an indicator of investment in immunocompetence was positively associated with survival probability in crash years, it was always negatively associated with female fecundity and probability of a male siring an offspring. This research provides direct evidence of trade-offs between immune function and survival, which suggests a reason why more individuals do not mount a more intense immune response to gut parasites despite the positive association with survival (Graham et al. 2010, Nussey et al. 2014). This case study reveals the complexity of the interactions among types of immune responses, as well as between immune responses and life history traits, but it also highlights the importance of understanding immune function when investigating population dynamics.

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Appendix I: Glossary of Terms Related to the Immune System

The immune system is multifaceted and complex. Herein we present a glossary of terms related to functions of the immune system (also see Figure 1).

Acute phase response: An evolutionary conserved innate defense that is activated at the beginning of an infection. It is triggered by pro-inflammatory cytokines which orchestrate a cascade of immunological recruitment and inflammation, behavioral responses (e.g., sickness behavior), hormonal responses, and metabolic responses (e.g., febrile response) (Hart 1988, Kent et al. 1992, Owen-Ashley and Wingfield 2007). During the acute phase response, acute phase proteins are released from the liver, and these proteins are involved in antimicrobial activities including opsonization of bacteria, activation of complement, enhancement of phagocytosis, and scavenging minerals from the blood stream that are limiting for bacterial growth and replication (Weinberg 1974, Baumann and Gauldie 1994, Owen-Ashley and Wingfield 2007).

Adaptive immunity: The response of antigen-specific lymphocytes to antigen that includes development of immunological memory (Murphy et al. 2007). Adaptive immunity is distinct from the innate or non-adaptive phases of immunity, which are not mediated by antigen-specific lymphocytes (Murphy et al. 2007). Adaptive immunity includes responses of B cells that are involved in humoral immunity and T cells that are involved in cellular immunity.

Antibody: A protein that binds specifically to a particular substance—that is its antigen (Murphy et al. 2007). Antibodies are produced in plasma cells (terminally differentiated B cells) in response to infection and they bind to and neutralize parasites or prepare parasites for uptake and destruction by phagocytes (Murphy et al. 2007). Natural antibodies are present in small concentrations and bind to parasites that the individual has not encountered previously. Antibodies are known collectively as immunoglobulins, but there are six major classes of antibodies; IgA, IgD, IgE, IgG, IgM and IgY. **IgA** is the main antibody class secreted by mucosal lymphoid tissues; **IgD** appears on the surface of mature naïve B cells and are involved in mucosal immunology and stimulating basophiles to release proinflammatory and antimicrobial mediators; **IgE** is involved in the defense against macro-parasite infections (e.g., tape worms) and in allergic reactions; **IgM** is the first immunoglobulin to appear on the surface of B cells and the first to be secreted; **IgY** is found in birds and transferred to hatchlings from mothers (Murphy et al. 2007, Chen and Cerutti 2011, Edholm et al. 2011). In mammals, IgG, IgA, and IgM are part of the maternal immunity passed to offspring through milk (Carlier and Truyens 1995, Boulinier and Staszewski 2008)

Antigen: Any molecule that can bind specifically to an antibody; some antigens do not elicit antibody responses, rather they produce cellular immune responses (Murphy et al. 2007). Thus, an antigen is any molecule that can produce an adaptive immune response that is specific to that antigen. Antibodies bind directly to antigens in responses by B cells. In contrast, in responses involving T cells antigens are presented as peptide fragments bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells where they are recognized by T cells (Murphy et al. 2007).

Anti-inflammatory cytokines: Cytokines including interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-10 (IL-10) that inhibit inflammatory responses and cellular immunity (Muehlenbein 2010, Demas et al. 2011b). These cytokines are secreted by Type 2 helper T cells (Th-2) and are involved in humoral immunity (i.e., the production of antibodies by B cells); they suppress Type 1 helper T-cells (Th-1) (Delves and Roitt 2000, Zimmerman et al. 2014). Zimmerman et al. (2014) review the function of anti-inflammatory cytokines.

B cells: A lymphocyte that produces antibodies upon activation by an antigen (Murphy et al. 2007). Antibodies neutralize parasites and their products, block binding of parasites to host cells, induce complement activation, promote cellular migration to sites of infections, and enhance phagocytosis, among other actions (Demas et al. 2011b). B cells can recognize native or free antigens, in contrast to T cells that recognize antigens that have been processed by antigen-presenting cells (Muehlenbein 2010).

Basophils: White-blood cells that are part of the innate immune system and contain large granules that store a variety of proteins such as toxins, prostaglandins, and histamine that are secreted upon activation (Demas et al. 2011b). When activated by IgG, basophils cause a local or systemic immediate hypersensitivity reaction. This response is part of the immune response to parasites that are too large to be engulfed by macrophages and phagocytic cells including ectoparasites such as ticks and fleas (Wakelin 1996). Basophils play a role in allergy reactions (Delves and Roitt 2000). In contrast to mast cells that are found in connective tissues throughout the body, basophils circulate through the body in the blood (Delves and Roitt 2000).

Cellular-mediated immunity: Any adaptive immune response in which antigen-specific T cells have a main role.

Complement system: Part of the innate immune system activated during the early stages of infection. It is made up of a large number of different plasma proteins that interact with one another both to opsonize parasites for engulfment by phagocytes and to induce a series of inflammatory responses to help fight infection. The final components of the complement pathway damage certain bacteria by creating pores in the bacterial membrane resulting in lysing. The complement system can be activated by three pathways: the classical pathway is initiated when antibodies bind to the surface of a parasite; the lectin pathway is initiated when mannose-binding lectin or ficolin binds carbohydrate on surfaces of parasites; and the alternative pathway is initiated when component C3 of complement in plasma is spontaneously activated and binds to the surface of a parasite (Murphy et al. 2007).

Cytokine: A large family of proteins with a small molecular weight involved in regulating cellular activity, particularly within the immune system (Delves and Roitt 2000). Interactions between cytokines are complex and results in different endpoints depending on other cytokines present (Zimmerman et al. 2014). Cytokines also act as integrator molecules linking the immune system to other physiological systems and processes including milk production in mammals and stress responses (Watson 2009, Zimmerman et al. 2014).

Cytotoxic T cells (CD8, killer T cells): A T lymphocyte that typically carries the coreceptor CD8 and that kills its target cell through perforin and lysis (Murphy et al. 2007, Muehlenbein 2010). Cytotoxic T cells recognize complexes of peptides and major-histocompatibility-complex class I molecules displayed on the target cell membrane

(Murphy et al. 2007). They are important for eliminating intercellular parasites such as viruses (Muehlenbein 2010, Murphy et al. 2000).

Dendritic cells: White blood cells that are part of the innate immune system and involved in antigen presentation (Murphy et al. 2007). They are found in most tissues, including lymphoid tissues, and are classified into two functional groups (Murphy et al. 2007). Conventional dendritic cells take up antigen in the peripheral tissues, and then travel to the peripheral organs where they stimulate a T-cell responses (Murphy et al. 2007). Plasmacytoid dendritic cells take up and present antigens, but their main function is to produce large amounts of antiviral interferons. There are also follicular dendritic cells that present antigens to B cells in lymphoid follicles (Murphy et al. 2007).

Ectoparasite: see "Parasite"

Eosinophils: White blood cells that are part of the innate immune system; they attack extracellular parasites by the release of various chemical mediators (Muehlenbein 2010). They also help during recovery from an inflammatory response by releasing histaminase to degrade histamine (Venge 1990).

Extracellular parasite: see "parasite"

Heterophils (neutrophils in mammals): Phagocytic white-blood cells that are part of the innate immune system; they target and kill cell that have been tagged with antibodies and complement proteins (Demas et al. 2011b).

Humoral immunity: Immunity due to antibody response. Humoral immunity can be transferred to a naïve recipient by serum antibody, in contrast to cellular immunity that cannot be transferred to by serum antibody (Murphy et al. 2007).

Immunological memory: The ability of the immune system to recall an encounter with a specific antigen and to mount a qualitatively and quantitatively superior secondary immune response on reencountering the antigen. This process involves the generation of memory T and B cells during the primary immune response and is part of adaptive immunity (Delves and Roitt 2000).

Immunoglobulin: A family of proteins that includes antibodies and B-cell receptors (Murphy et al. 2007).

Innate immunity: The early, physiological immune response (Murphy et al. 2007). This branch of the immune system includes a variety of innate resistance mechanisms that recognize and respond to the presence of a parasite. The innate immune system is always present and does not increase with repeated exposure to a given parasite. It does discriminate between groups of similar parasites, for example gram-positive and gram-negative bacteria. Processes in the innate immune response are precursors to adaptive immune responses; without innate immune responses the adaptive immune responses are not activated (Murphy et al. 2007).

Intercellular parasites: see "parasite"

Leukocyte: White blood cell

Lymphocyte: A white-blood cell that is derived from a common lymph progenitor; includes natural killer cells, B cells and T cells (Murphy et al. 2007).

Macroparasite: see "parasite"

Macrophages: Mononuclear white blood cells that are involved in phagocytosis, cytokine secretion, chemotaxis, antigen processing and presentation.

Major histocompatibility complex (MHC): is a highly polymorphic cluster of genes that encodes a set of membrane glycoproteins called the MHC molecules that present antigenic peptides to T cells (Murphy et al. 2007).

Mast cells: White-blood cells that are part of the innate immune system and contain large granules that store a variety of proteins, such as histamine, that are secreted upon activation (Murphy et al. 2008). Mast cells are activated by IgG causing the production of a local or systemic immediate hypersensitivity reaction that is part of the immune response to parasites that are too large to be engulfed by macrophages and phagocytic cells (Wakelin 1996, Murphy et al. 2007). Mast cells also play a critical role in allergic reactions (Murphy et al. 2007). In contrast to basophils that circulate through the body in the blood, mast cells are found in connective tissues throughout the body (Delves and Roitt 2000).

Microparasites: see "parasite"

Natural killer cells: White-blood cells that are part of the innate immune system, and non-specifically attack and lyse infected cells (Demas et al. 2011b).

Neutrophils: see "Heterophils"

Parasite: Within the ecoimmunology literature, parasite is often defined broadly using the ecological definition of parasite (Anderson and May 1979), that is any species that makes a living by uses the resources of another species and causing the host species harm. Under this definition, parasites are often partitioned into two categories: microparasites and macroparasites. **Microparasites** include bacteria, viruses, fungi, protozoans, and other microscopic parasites. **Macroparasites** include helminthes, arthropods, and other macroscopic parasites. In traditional immunology, parasites are often partitioned into intercellular, extracellular, and ectoparasites. **Intercellular parasites** reside within cells; for example *Rickettsia* spp. and viruses. **Extracellular parasites** reside within the body, but are not within a cell; for example helminthes, *Escherichia coli*, *Mycoplasma* spp.*, Streptococcus pyrogenes*. **Ectoparasites** reside on the outside of a host's body; for example fleas and mites. Sometimes the word parasite is used as shorthand for macroparasite or extracellular parasite. Because different parts of the immune system target different types of parasites (in the broad sense), it is important to understand what type of parasite is being discussed in a particular study.

Pro-inflammatory cytokines: Cytokines that promote inflammatory responses including interferon-γ (IFNγ), interlukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF-α) (Muehlenbein 2010, Demas et al. 2011b). IL-2 and IFNγ are secreted by Type 1 helper T cells (Th-1) and promote cell-mediated immunity (i.e., the activation of macrophages and cytotoxic T cells) and are involved in defense against intracellular parasites (Delves and Roitt 2000, Zimmerman et al. 2014). IL-1β increases proliferation, phagocytosis, migration, and antibacterial activity of leukocytes, and induces fever and anorexia (Zimmerman et al. 2014). IL-12 is produced mainly by monocytes, macrophages, and dendritic cells; it is important in the defense against intracellular parasites and is the main driver of Th1 responses (Zimmerman et al. 2014). TNF-α is produced mainly by macrophages in responses to stimuli such as endotoxins, viruses, parasites, and other cytokines; it is a key cytokine involved in the activation of inflammation in responses to injury and infection, and the acute phase response including fever and sickness, in addition to inducing anorexia (Zimmerman et al. 2014). Zimmerman et al. (2014) review the function of proinflammatory cytokines.

Regulatory T cells: T cells that inhibit T-cell responses (Murphy et al. 2007).

Sickness behavior: A behavioral response that is part of the acute phase response and innate immunity. It includes reduced activity, anorexia (reduced food intake), adipsia (reduced water intake), and lethargy (Hart 1988).

Suppressor T cell: See "Regulatory T cell"

T cells: A type of lymphocyte. T cells can be further divided into different subtypes, notably cytotoxic T cells (killer T cells), T helper cells (Th), and suppressor or regulatory T cells (Treg). See definitions of each subtype for further details about each subtype.

Helper T cells (CD4): CD4 T cells that assist B cells in making antibodies in response to antigenic challenge; both Type 1 and Type 2 helper cells can carry out this function (Murphy et al. 2007).

Type 1 helper T cell (Th1): Cells that secrete cytokines interleukin-2 (IL-2) and interferon-γ (IFNγ) which promote cell-mediate immunity (i.e., the activation of macrophages and cytotoxic T cells). Th1 cytokines also inhibit Type 2 helper cells (Delves and Roitt 2000).

Type 2 helper T cell (Th2): Cells that secrete cytokines interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-6 IL-6) which promote humoral immunity (i.e., the production of antibodies by B cells). Th2 cytokines also inhibit Type 1 helper cells (Delves and Roitt 2000).