Journal of Animal Ecology

British Ecological Society

Journal of Animal Ecology 2011 doi: 10.1111/j.1365-2656.2011.01813.x

REVIEW

Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts

Gregory E. Demas[1]*, Devin A. Zysling[2], Brianna R. Beechler[3], Michael P. Muehlenbein[4] and Susannah S. French[5]

[1]*Department of Biology, Center for the Integrative Study of Animal Behavior, Indiana University, 1001 E. 3rd St., Bloomington, IN 47405, USA;* [2]*Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, NY 14853, USA;* [3]*Department of Environmental Science, Oregon State University, Corvallis, OR 97331, USA;* [4]*Department of Anthropology, Indiana University, 701 E. Kirkwood Ave., Bloomington, IN 47405, USA; and* [5]*Department of Biology, Utah State University, 5305 Old Main Hill, Logan UT, 84322, USA*

Summary

1. Over the past decade, there has been a substantial increase in interest in the immune system and the role it plays in the regulation of disease susceptibility, giving rise to the field of eco-immunology.
2. Eco-immunology aims to understand changes in host immune responses in the broader framework of an organism's evolutionary, ecological and life-history contexts.
3. The immune system, however, is complex and multifaceted and can be intimidating for the nonimmunologist interested in incorporating immunological questions into their research. Which immune responses should one measure and what is the biological significance of these measures?
4. The focus of this review is to describe a wide range of eco-immunology techniques, from the simple to the sophisticated, with the goal of providing researchers with a range of options to consider incorporating in their own research programs.
5. These techniques were chosen because they provide relatively straightforward, biologically meaningful assessments of immune function, many of which can be performed across a range of ecological contexts (i.e. field vs. laboratory) and in a wide range of vertebrate animals without relying on species-specific reagents.
6. By incorporating assessments of immune function into their specific research questions, animal ecologists will gain a more comprehensive understanding of organism–environment interactions.

Key-words: disease ecology, eco-immunology, environment, immunity

Introduction

It is becoming increasingly apparent that immune responses play an important role in an organism's physiological, biochemical and behavioural responses to its environment and thus have the potential to shape the evolution of life-history strategies. 'Immunocompetence', an individual's capacity to mount an appropriate immune response following exposure to a pathogen, is a critical aspect of disease resistance and thus survival. Unfortunately, however, immunocompetence is not an easy concept to operationally define, as specific aspects of an immune response may be modulated differentially to optimize disease resistance to specific pathogens (McDade 2003; Pedersen & Fenton 2007). Within the past decade alone, a greater appreciation of how changes in host immunity can contribute to disease has developed among ecologists and evolutionary biologists alike, giving rise to the field of ecological immunology (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Schmid-Hempel & Ebert 2003). The goal of eco-immunology is to understand immune function as an adaptive response within the context of important life-history traits across a wide range of organisms (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Demas 2004; Martin, Weil & Nelson 2008b). Immunology, a research area once studied almost exclusively by immunologists using primarily laboratory and molecular-based approaches, has become an important area of ultimate and proximate investigation among environmentally oriented biologists as a means for understanding changes in disease susceptibility and resistance (French, Moore & Demas 2009; Hawley & Altizer 2011). As the title of this review suggests, the emerging field of eco-immunology has been traditionally been dominated by studies utilizing measures of delayed-type hypersensitivity (e.g. phytohaemagglutination-induced

*Correspondence author. E-mail: gdemas@indiana.edu

swelling responses), often as a singular measure of immunity. While this assay has been informative and has an important place in eco-immunology (see later), it is imperative for investigators to examine the immunity using a range of immune techniques that assess diverse aspects of the immune response to gain an accurate representation of an individual's 'immunocompetence'.

An astoundingly large number of immunological assays have been developed to allow investigators to assess specific aspects of immunity (i.e. innate, humoral and cell-mediated), predominantly in model systems (e.g. mice) and often within biomedical contexts. The wealth of available immune techniques has generally been a boon to investigators and has resulted in great strides in our understanding of environmental influences on immunity. However, it has also raised a substantial number of questions for the physiological ecologist and evolutionary biologist interested in studying immune responses as a proxy for individual fitness. Where does one begin? For example, is there a single technique one can use to assess overall immunity? If not, which set of techniques should be employed to gain a reasonable understanding of changes in immunity? What, if anything, does a lone immune measure tell us about an animal's fitness? Which techniques will work in my species of interest? How difficult is the technique to develop for nontraditional species? Can I use these techniques in the field or will I have to bring the animals into the laboratory to use them? Will I need to recapture the same animal repeatedly? These and other issues, while ultimately addressable, can be sufficiently intimidating to prevent physiological ecologists from incorporating eco-immunological techniques in their research.

The goal of the present review is to provide a brief introduction to a wide range of eco-immunology techniques potentially available in the physiological ecologist's toolkit as well as a description of the strengths, limitations and basic requirements of each technique. Some of these techniques have been briefly reviewed previously, with particular emphasis on avian eco-immunology (e.g. Salvante & Dufty 2006; Ardia & Schat 2008). Our goal is not to provide an exhaustive list of all possible techniques, and there will assuredly be viable techniques that we were unable to cover or perhaps even unaware of at the timing of this writing. Nor do we attempt to provide complete, step-by-step protocols for each of the techniques discussed. Rather, we have attempted to provide the investigator with sufficient information on a subset of well-established, commonly employed comparative eco-immunology techniques with the goal of convincing researchers that assessing immunity is not only possible but can prove both useful and informative. For the novice eco-immunologist who is interested but unsure of where to start within the field, these techniques should help to point them in the right direction. For the more experienced investigator already engaged in eco-immunological research, it is hoped that this review will expand their existing toolkit by providing additional options for consideration. In both cases, our goal is to provide researchers with a range of possible techniques for their research programs to gain a more integrative, comprehensive understanding of environment–immune interactions.

Despite the diversity of techniques available, the specific techniques we have included were chosen to address several broad requirements: (i) they provide a range of options covering the major components of immunity (e.g. innate, humoral and cell-mediated), (ii) they can be employed in nontraditional systems without requiring expensive species-specific reagents and antibodies, (iii) they are generally amenable to both field and laboratory studies, considering such issues as the ability to recapture or euthanize individuals. Although not all the techniques described in this review meet all the aforementioned conditions, we strived to provide sufficient breadth so that researchers interested in eco-immunology would be able to integrate one or more of these techniques in their own research programs. Before specific eco-immunology techniques are discussed, however, it is important that the reader have at least of working knowledge of the basics of the vertebrate immune system, which is provided later.

Primer of the vertebrate immune system

Virtually all organisms are exposed to a wide range of potential pathogens within their environments. Because of this fact, animals have evolved complex physiological responses, central among them are innate and acquired (adaptive) immunity, to resist or eliminate pathogens (Fig. 1) (Muehlenbein 2010). In general, innate responses are relatively rapid, nonspecific, germ line-encoded responses which serve as the initial line of defence against invading pathogens. Such defences include anatomical barriers (mucus membranes, skin), resident flora (nonpathogenic bacteria), humoral factors (lysozyme, complement and other acute phase proteins) and cellular responses [phagocytic cells like neutrophils, monocytes and macrophages; inflammatory mediators produced by basophils, mast cells and eosinophils; and natural killer (NK) cells]. The complement system includes enzymes which function to eliminate micro-organisms by promoting inflammatory responses, such as changes in local vascular permeability and entry of immune cells into infection sites. Complement also functions in the lysis of foreign cells through the formation of membrane attack complexes, and mediation of phagocytosis through the coating (opsonization) of pathogens and infected cells (Carroll 1998). Complement is also important for stimulating acquired (adaptive) immune responses (Dempsey *et al*. 1996). Acquired immune responses are typically slower, pathogen-specific responses that require activation and serve as additional line of defence. In this case, effector mechanisms allow fast, secondary responses during subsequent exposures. Collectively, the immune system comprises a collection of specialized cells whose integrated function is the differentiation of 'self' from 'non-self' and the elimination of foreign 'non-self' substances (Janeway *et al*. 2005), specifically those capable of damaging the host (Matzinger 2002).

The tissues and structures of the immune system, referred to as lymphoid organs, are connected to one another via a

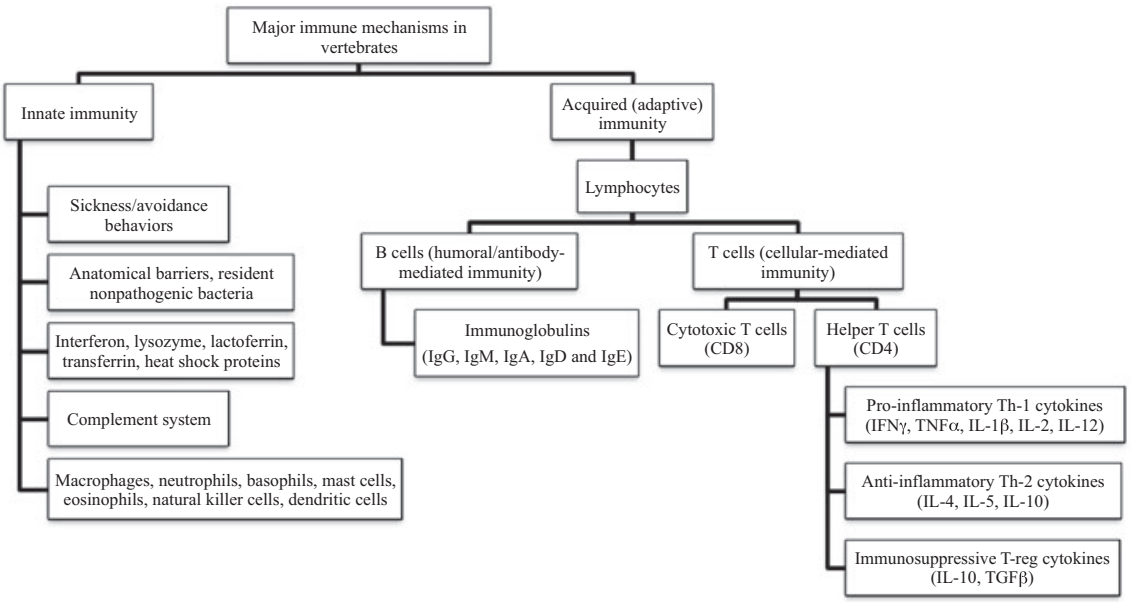

Fig. 1. Major immune mechanisms in vertebrates. Modified from Muehlenbein 2010.

type of circulatory system, the lymphatic system, which carries lymphocyte-containing lymph (fluid) throughout the body where these cells encounter a pathogen (Janeway *et al*. 2005). Among vertebrates, there are two main types of lymphoid organs – primary and secondary. Primary lymphoid organs include the bursa of Fabricius in birds and bone marrow in mammals and other vertebrates. These tissues are the source of stem cells, progenitor cells for all lymphoid tissues, and where a specific subtype of lymphocytes, B cells, mature (Janeway *et al*. 2005). The second primary lymphoid tissue is the thymus. It is within the thymus that lymphoid cells that have migrated from the bone marrow develop into a second type of lymphocyte, T cells. In addition to these primary lymphoid tissues, most vertebrates possess a host of secondary lymphoid tissues, including lymph nodes, spleen, appendix, tonsils, and Peyer's patches lining parts of the intestinal tract, which further serve to identify, sequester and eliminate pathogens via a host of immune responses (Janeway *et al*. 2005).

Cells of the immune system, called white blood cells (WBCs), all derive from common embryonic haematopoietic stem cells responsible for the production of most blood cell types. Among these pluripotent stem cells, some cells will undergo further development into lymphocytes, whereas others will differentiate into polymorphonuclear leucocytes (PMNs) or monocytes. Lymphocyte progenitor cells subsequently develop into B cells, T cells or NK cells. B cells produce antibodies that neutralize pathogens and their products, block binding of parasites to host cells, induce complement activation, promote cellular migration to sites of infection and enhance phagocytosis, among other actions. T cells do not produce antibodies; rather, they require direct contact with an infected cell to eliminate it. T cells can be further divided into different subtypes, including cytotoxic T cells (killer T cells; Tc), T helper cells (Th) and suppressor or regulatory T cells (Treg). Cytotoxic T cells destroy infected host cells by physical and chemical lysis, making T cells effective at destroying intracellular pathogens (e.g. viruses). In contrast, helper T cells serve a complementary function by secreting cytokines, glycoproteins that perform a variety of functions such as regulation of cell growth and development (Snapper 1996), as well as interacting with B cells to coordinate the production of antibodies. Th cells are further differentiated into several subtypes, most notably Th1 and Th2, depending on the specific patterns of cytokine production and their associated functions (Mosmann & Coffman 1989). Th-1 cytokines include, among others, interferon gamma (IFNγ), tumour necrosis factor alpha and beta (TNF α, β), and various interleukins (IL-1β, IL-2, IL-3, IL-12, etc.). These cytokines activate macrophages, neutrophils and NK cells, mediate inflammatory responses and cellular immunity (T cells), promote cytotoxicity towards tumour cells and enhance chemotaxis of leucocytes (Kobayashi *et al*., 1989). The Th-2, anti-inflammatory cytokines include many interleukins (IL-4, IL-5, IL-10, etc.) that induce humoral immunity and antibody production (B cells), eosinophil activation, mast cell degranulation, goblet cell hyperplasia, mucin production and intestinal mastocytosis (resulting in histamine release). This cytokine phenotype is important for protection against intestinal infections as well as the facilitation of allergic reactions (Barrett *et al*. 1988; Else & Finkelman 1998; Dinarello 2000). Despite the fact that Th-1 and Th-2 cytokines act antagonistically to one another, both are usually present within the host at any given time, although during infection one phenotype usually predominates.

Collectively, B and T cells coordinate an organism's acquired (adaptive) immune responses. NK cells, in contrast, are part of an innate immune response, and nonspecifically attack and lyse infected cells. Along with NK cells, PMNs serve as part of the innate immune response. PMNs consist

of three basic cell types: basophils, eosinophils and heterophils (called neutrophils in humans). Heterophils serve as phagocytes, targeting and killing cells that have been tagged with antibodies and complement proteins. Basophils and eosinophils also participate in innate immune responses, primarily by secreting proteins such as toxins, prostaglandins and histamine, as well as serving as antigen-presenting cells. Lastly, monocytes develop into macrophages, which are large leucocytes of the innate immune system that provide nonspecific defence against pathogens by phagocytosing pathogen-infected cells and activating B and T lymphocytes. Macrophages, along with lymphocytes, are also responsible for the production of cytokines (e.g. interleukins, tumour necrosis factors and interferons), soluble signalling molecules that coordinate a wide range of immunological and inflammatory responses to infection. Lastly, the body fluids of most vertebrates possess a wide range of soluble factors consisting of antimicrobial peptides (AMPs), lysozyme, complement proteins, transferrin and lectins that serve as part of the innate humoral immune response. As this section suggests, the immune system is a complex, multifaceted system that works in a coordinated fashion to destroy pathogens. Thus, it must be emphasized that single measures of immunity are rarely sufficient to capture the complexity of an immune response.

Techniques in eco-immunology

We begin with a description of a simple assessment of gross lymphoid organ mass. While measures of this sort are admittedly crude and not very informative on their own, they have historically provided investigators with an important 'point of entry' into the discipline of eco-immunology. Thus, a brief discussion of these techniques seems warranted. However, a word of caution is necessary for interpreting this and other descriptive measures (e.g. total cell counts). These assays, while potentially useful for determining the physiological state of an individual animal, do not provide information regarding the functional aspects of an immune response (i.e. how robust the response is). Thus, the remainder of the review focuses on more functional assays with which to assess specific aspects of immune responses (Table 1).

MORPHOLOGICAL AND HISTOLOGICAL MEASURES

Gross lymphoid tissue masses

Rationale. Historically, relative sizes of specific lymphoid tissues such as the spleen, thymus, or bursa of Fabricius (birds) have been assessed to provide initial information regarding the health of the immune system (Riddle & Krizenecky 1931; John 1994). As mentioned previously, while these measures are not strongly diagnostic of functional immune responses *per se*, they do provide an initial assessment of potential immune changes and are easy to perform, especially for those researchers who regularly assess morphological features of their animals. An initial assessment of immunity may be performed simply by removing and weighing specific gross lymphoid tissues including the spleen, thymus or bursa of Fabricius (birds) (Fänge & Silverin 1985). More detailed histological examinations may also be performed by preserving the tissues for future sectioning to assess structural components [e.g. amount of red (haemopoiesis) vs. white (immune) pulp in the spleen) of the tissue.

Requirements. Assessment of lymphoid tissue masses is easy to perform and only requires some basic surgical tools to remove tissue and a sensitive scale to weigh organs. This can be performed in the field or in the laboratory. Histological examination will require tissue processing via a microtome or cryostat and possibly staining with a common histological dye (e.g. haematoxylin and eosin) within a laboratory setting, but can be based on preserved samples taken in the field.

Benefits. This technique provides a relatively quick and simple assessment that does not require performing any assays and does not necessitate recapture of the animal. Results from these analyses, while not necessarily diagnostic of immune changes, can suggest whether further analyses with more sophisticated analyses might be warranted.

Limitations. Morphological changes do not always correlate with functional measures of immunity (e.g. a heavier spleen does not necessarily correlate with 'better' immunity). In fact, lymphoid tissues are often enlarged during infections (Ali & Behnke 1985). These measures should be performed only as an initial assessment of immunity and should always be performed in conjunction with more informative measures such as those described later. Obviously, this technique is a terminal measure requiring euthanasia of the study animal.

Haematological measures

Rationale. Analyses of the composition and concentration of the cellular components in blood have been frequently employed by functional ecologists, primarily because of the relative ease of use of these measures relative to other techniques. The primary measures include red blood cell (RBC) count, white blood cell (WBC) count, the classification of white blood cells (WBC differential), and haematocrit (packed cell volume). The most common haematological measures employed in past eco-immunology work has been basic WBC counts and various leucocyte ratios (e.g. heterophil : lymphocyte) (Birkhead, Fletcher & Pellatt 1998; Ots, Murumagi & Horak 1998; Moller & Petrie 2002; Nunn 2002; Semple, Cowlishaw & Bennett 2002). Although such measures are still employed, they are usually performed now in combination with other measures of innate and adaptive immunity (Millet *et al.* 2007; Lee *et al.* 2008).

Table 1. Overview of eco-immunology techniques

Technique	Type of immunity	Field/laboratory	Recapture required?	Type of sample	Amount needed	Key references
Gross lymphoid tissue masses	Morphological/ Histological	Collection: field or laboratory Analysis: field or laboratory	No (terminal measure)	Whole organs	Whole organs	Fänge & Silverin 1985; Ardia, 2005
Haematological measures	Morphological/ Histological	Collection: field or laboratory Analysis: field or laboratory	No	Cell smears	*c*. 1 drop whole blood	Weiss & Wardrop, 2010; Millet *et al*. 2007
Flow cytometry	Morphological/ Histological	Collection: field or laboratory Analysis: laboratory	No	Cells from whole blood or homogenized tissues	Dependent on cell type ($\geq 1 \times 10^5$ cells desirable)	Dhabhar *et al*. 1995; Baumgarth & Roederer 2000; Tella *et al*. 2008
Antimicrobial peptides	Innate	Collection: field or laboratory Analysis: laboratory	No	Tissue/epithelial samples, skin swab, or secretion collection by emersion, albumin (eggs)	Variable	Rollins-Smith *et al*. 2002; Ganz 2003;Gibble, Rollins-Smith & Baer 2008; Conlon & Sonnevend 2010
Complement proteins	Innate	Collection: field or laboratory Analysis: laboratory	No	Serum/plasma samples	25–100 μL	Klos *et al*. 1988; Seelen *et al*., 2005
Haemolytic complement	Innate	Collection: field or laboratory Analysis: laboratory	No	Serum/plasma samples	10–100 μL	Mayer 1948; Sinclair & Lochmiller 2000; Greives *et al*. 2006
NK cell cytotoxicity	Innate	Collection: field or laboratory Analysis: laboratory	No	Isolated mononuclear cells or whole blood	*c*. 250 000 isolate mononuclear cells or 100 uL of whole blood	Claus, Greil & Watzl 2009; Ferrández & De la Fuente 1996
Bacterial killing	Innate	Collection: field or laboratory Analysis: Field or laboratory	No	Serum/plasma or whole blood	1·5–200 μL	Tieleman *et al*. 2005; Millet *et al*. 2007; Liebl & Martin 2009
Macrophage phagocytosis	Innate	Collection: field or laboratory Analysis: laboratory	No	Isolated macrophages from body fluids or whole blood	*c*. 200 000 isolated macrophages	Grasman 2002; Kwiatkowska & Sobota 1999
Delayed-type hypersensitivity	Cell-mediated	Collection: field or laboratory Analysis: Field or laboratory	Yes	Consistently measurable surface	n/a	Martin *et al*. 2006a; Lee *et al*. 2006; Dhabhar 1998
Lymphocyte Proliferation	Cell-mediated	Collection: field or laboratory Analysis: laboratory	No	Cell suspensions for lymphoid tissues or whole blood	*c*. 100 000 lymphocytes or *c*. 100 μL whole blood	Cory *et al*. 1991; Bounous, Campagnoli & Brown 1992; Palacios *et al*. 2007

Table 1. (Continued)

Technique	Type of immunity	Field/laboratory	Recapture required?	Type of sample	Amount needed	Key references
Total immunoglobulins	Humoral	Collection: field or laboratory Analysis: laboratory	No	Serum/plasma samples	5–50 μL	Demas & Nelson 1996; Greives *et al*. 2006
Antigenic challenge	Humoral	Collection: field or laboratory Analysis: laboratory	Yes	Serum/plasma samples	25–100 μL	Hanssen *et al*. 2004; Demas & Nelson, 2003
Cytokines	Integrated	Collection: field or laboratory Analysis: laboratory	No	Serum/plasma samples or whole organs	50–100 μL	Graham *et al*., 2007; Sachdeva & Asthana 2007
Wound healing	Integrated	Collection: field or laboratory Analysis: field or laboratory	Yes	Images of wound site	n/a	Marucha & Sheridan, 1998; French & Moore 2008
Fever/sickness	Integrated	Collection: field or laboratory Analysis: field or laboratory	Maybe	Behaviour/body temperature/food intake measures	n/a	Dantzer 2006; Owen-Ashley *et al*., 2006a
Infectious disease models	Integrated	Collection: field or laboratory Analysis: laboratory	Yes	Serum/plasma samples, cells, whole organs, behaviours	Depends on measures	Staszewski & Boulinier 2004; Lemus, Vergara & Fargallo 2010

NK, natural killer.

Requirements. Manual cell counting usually involves either a thin smear or use of a haemocytometer. The stains of choice are usually Giemsa, Wright, Wright-Giemsa, azure-eosin and May-Grünwald. Several supplies are required, as is a microscope, slides, coplin jar, drying rack and a haematological atlas. Capillary blood samples can be used, as can venous blood with an anticoagulant (e.g. EDTA). Multiple slides should be observed from every subject organism, increasing accuracy despite requiring more time. Thin smears should be dried and fixed in anhydrous methanol. One simple method involves counting one hundred adult WBCs and classifying them by shape, cytoplasmic contents and colour as heterophils, basophils, eosinophils, monocytes or lymphocytes. When a blood sample is added via capillary action, cells are counted within a specific volume of fluid. Live cells can be differentiated from dead cells using trypan blue, a diazo stain that selectively colours dead cells blue.

For mammalian studies, it is also possible to automate this entire process by using either a haematology analyser or a flow cytometer (see later), although both can be very expensive. With the former, a lysing agent is added to the samples, which haemolyses the RBCs. WBCs then pass single-file through an electrified aperture. The cells are classified by type as they impede electrical flow proportional to their size and nuclear density. Other automated techniques include the use of radio frequency conductance and fluorescent staining. Nonmammalian vertebrates, however, possess nucleated RBC, which makes the use of automated techniques difficult. Although methods for measuring nucleated RBC are available and could be validated for these species (Tsuji *et al.* 1999), manual counts are typically necessary.

Benefits. One of the major benefits of haematological analyses is that several types of information can be gathered from a single sample, including WBC concentration and composition, and blood parasite infection and anaemia status, all from only a few microlitres of blood. Haematological analyses are relatively simple to conduct, although care and experience are required for them to be performed accurately (techniques and species values/ranges reviewed in Weiss & Wardrop, 2010). These methods also do not rely on specie-specific reagents and thus can performed on most species, assuming a blood sample can be obtained.

Limitations. A major shortcoming with haematological analyses is that they represent only a simple, gross measure of innate or constitutive immunity. WBCs can be elevated under a number of conditions, including infection, injury and allergy. This can make interpretation of WBC comparisons between individuals, populations and species difficult. A proper comparison would have to ensure that all animals are subject to the same external and internal stressors, including infection status. What if some animals have early-stage, subclinical infections that results in elevated WBCs compared with other animals in the same or different populations? Clearly, interpretation based on only a simple haematological measure is difficult and serious (and possibly inappropriate) assumptions may have to be made about the animals being surveyed. A more appropriate use of haematological measures would be to investigate changes in WBC profiles before, during and after some challenge, such as an inoculum [e.g. sheep red blood cells (SRBC)], vaccination or artificially controlled infection in otherwise immunologically naïve individuals (discussed later).

Flow cytometry

Rationale. By using the principles of light scattering, excitation and the emission of fluorochrome molecules, flow cytometers produce multiparameter data that can be used in the simultaneous analysis of numerous properties of single cells. Specifically, a suspension of cells is injected into a sheath flow that passes through the apparatus as a sequence of single cells across a light source (e.g. laser or mercury arc lamp). At this intersection, the cellular scattering of light and the emission of fluorescent probes are detected, which allows for the analysis of molecular and physical characteristics (e.g. size and granularity) (Robinson 2004; Sack, Tárnok & Rothe 2009). For example, lymphocytes, monocytes and granulocytes can be differentiated by their respective forward and sideward light scatter characteristics. For immunophenotyping purposes, it is common to use antibodies that recognize specific cell surface receptors (Baumgarth & Roederer 2000). In addition to immunophenotyping, specific staining with fluorescent antibodies allows for the assessment of immunological, biochemical or functional properties of cells in a heterogeneous population (Robinson 2004). Finally, it is possible to separate cells into specific populations based on a host of specified variables. Thus, cell populations of interest can be collected and used for further analysis (Herzenberg *et al.* 2002).

Requirements. To perform any of the methods described previously, a flow cytometer and associated data analysis software are required. In addition, all tissue and blood samples must be in a single cell suspension and the appropriate labelled antibodies must be obtained. Finally, because flow cytometry is a quantitative technology, calibration standards are necessary.

Benefits. There are a few known technologies that can evaluate such a diversity of parameters and cell characteristics on such small samples over a short period of time. Flow cytometers provide information on not only cell type, but also cell functioning. Further, the ability to separate specific cell populations through the process of cell sorting is unique and can allow for further detailed analyses (Herzenberg *et al.* 2002). Many different types of tissues can be used (e.g. lymph nodes, spleen, liver and lung) (Alitheen, McClure & McCullagh

2007; Martin *et al*. 2008a), and these methods have been validated in a variety of species including rodents, birds, reptiles and invertebrates (Dhabhar *et al*. 1995; Cárdenas, Jenkins & Dankert 2000; Tella *et al*. 2008; Zimmerman *et al*. 2010).

Limitations. Flow cytometers are expensive instruments to purchase and maintain, and require highly trained operators (although many universities maintain core facilities). The required reagents may also be costly, and species-specific antibodies are often necessary. Additionally, automated cell counting can be difficult and inaccurate in nonmammalian species with nucleated RBC (see Hematological Measures section). The methods require specifically processed samples such that solid tissues have to be disaggregated and live cells are necessary for many of the methods described earlier. This makes use in the field difficult, unless the study site is in close proximity to the laboratory and the necessary equipment is readily available.

INNATE IMMUNITY

Antimicrobial peptides

Rationale. Antimicrobial peptides are a highly conserved component of the innate immune response and are found within nearly all types of organisms. These small molecular weight proteins act as potent, broad-spectrum antibiotics against Gram-negative and Gram-positive bacteria, mycobacteria, enveloped viruses and fungi (Zasloff 2002). AMPs are usually positively charged and have both a hydrophilic and hydrophobic side that facilitates solubility in aqueous environments, as well as entry into lipid membranes. Once the AMP enters the microbial membrane, it can kill the target cell through a variety of mechanisms (Matsuzaki 1999; Shai 1999). In addition to the aforementioned actions, AMPs may also function as immunomodulators by altering gene expression, inhibiting cytokine production and promoting wound healing through receptor-dependent interactions (Bowdish *et al*. 2005; Oudhoff *et al*. 2010). Although AMPs are found in a variety of species, they are most often examined in anurans (e.g. frogs and toads) as these organisms possess a highly efficient defence system based on the secretion of bioactive compounds through the skin (Rollins-Smith *et al*. 2002; Gibble, Rollins-Smith & Baer 2008; Rollins-Smith 2009; Conlon & Sonnevend 2010). Specifically, AMPs are produced in specialized granular glands in the skin and each anuran species appears to produce its own unique set of peptides against a diversity of organisms (Nicolas & Mor 1995; Rollins-Smith *et al*. 2002). As mentioned previously, production of AMPs is an important means of host defence in all eukaryotes; AMPs such as defensins have been examined in epithelial tissues and leucocytes from a variety of different mammalian and avian species (see Ganz 2003). Thus, by measuring AMPs, one could obtain an approximation of innate 'first line of defence' immune mechanisms in additional species.

Requirements. To measure the efficacy in anurans, peptide secretions can by induced by subcutaneous injections of norepinephrine and quantified using a growth inhibition assay (Gibble, Rollins-Smith & Baer 2008; Conlon & Sonnevend 2010). Alternatively, AMPs can be also obtained from skin tissue samples (Rollins-Smith *et al*. 2002). Briefly, the pathogen of interest in the appropriate growth media is plated in a 96-well flat bottom microtitre plate with or without addition each peptide. Positive and negative controls are required and growth is often determined at days 1–7 by measuring increased optical density with an ELISA plate reader. The minimal inhibitory concentration is defined as the lowest concentration at which no growth is detectable. Individual peptide components in the secretions can also be separated using reversed-phase HPLC on octadecylsilyl-silica (C_{18}) columns after partial purification on Sep-Pak C_{18} cartridges and characterized structurally by automated Edman degradation and mass spectrometry (Conlon & Sonnevend 2010).

Benefits. This functional growth inhibition assay is relatively easy to perform and is useful in evaluating host resistance to potentially lethal skin infections. No species-specific reagents are necessary and although AMPs are generally evaluated in anurans, the aforementioned methods could be adapted and validated for additional species. Assessment of AMPs provides a powerful measure of the first lines of defence for many organisms.

Limitations. More detailed analyses of individual AMP components within skin secretions require specialized equipment (e.g. mass spectrophotometer and HPLC) that is both expensive and requires highly trained personnel to operate. Further, little is known regarding concentrations of AMPs on the skin of healthy, resting amphibians, and the mechanism of action for only a limited number of peptides has been studies in detail. Therefore, interpretation of results leading to generalized conclusions regarding immune responses may be difficult.

Quantification of complement proteins

Rationale. The complement pathway is part of the innate immune response and consists of a series of proteins present in the plasma. Activation of the complement system initiates an enzymatic cascade, which leads to bacterial cells lysis, the formation of chemotactic peptides that attract immune cells, and an increase in phagocytotic clearance of infected cells (Kuby 1997). Complement can also increase the permeability of vascular walls and cause inflammation (e.g. anaphylaxis). Most complement proteins exist in the plasma as inactive precursors that cleave and activate each other via a proteolytic cascade in response to three different mechanisms: the classical pathway, the alternative pathway and the lectin-induced pathway (Dunkelberger & Song 2009). These three systems

have divergent mechanisms by which they initiate the proteolytic cascade; however, they share most of their components and all three converge in the creation of a C3 convertase. This C3 convertase cleaves the C3 complement protein, leading ultimately to the formation of the membrane attack complex (MAC; C5b-9) and subsequent cell lysis. The classical pathway is activated by the recognition of foreign antigens by antibodies bound to the surface of cells. In contrast, the alternative and lectin-induced pathways are both antibody-independent (Dunkelberger & Song 2009). To quantitatively determine the levels of a complement component, commercially available kits are often used (e.g. ELISA and EIA) (Klos *et al*. 1988). To examine general complement activation via any pathway, the complement protein C3 and split products of C3 (C3a and C3b) and C5 (C5a and C5b) are recommended for measurement. Split products can be used to determine if activation has occurred because their increase only occurs when complement enzymes are formed and active. To evaluate the overall activity of the complement system, the terminal complex SC5b-9 can be measured.

Requirements. Measurement of the complement components cited earlier is usually accomplished via a commercially available ELISA kit; however, similar results could be obtained in the laboratory using appropriately validated ELISA methods and species-specific labelled antibodies. Appropriately processed serum samples, ELISA reagents, positive and negative controls for all components measured, and a plate reader are necessary.

Benefits. The assays described earlier are a quantitative way to measure complement production and the activation of the complement pathway. Additionally, multiple levels of the pathway may be analysed based on the chosen components. Complement components are involved in a variety of immune responses and act as the major effectors of the humoral immune response. Measurement of specific proteins may also provide a more comprehensive picture of immunity.

Limitations. A number of limitations are inherent in assessing complement activation via the methods described earlier. Like many plasma proteins, complement components exhibit a wide normal concentration range *in vivo*, thus making interpretations of results difficult. In addition, the accuracy of the detection methods can be variable. For example, some polyclonal antibodies used for ELISAs and other quantitative tests may react with both native molecules and their respective split products. Such cross-reactivity may result in overestimation of the concentrations of the tested-for components and hence should be taken into consideration when interpreting the results. In addition, as these assays are usually obtained from commercial suppliers, their use has been validated in a few species. Finally, as the complement system is very labile, the manner in which specimens are collected and processed can influence the results of testing. The impact of time, temperature and anticoagulants on the spontaneous generation of complement activation has been previously demonstrated (Mollnes, Garred & Bergseth 1988).

Haemolytic complement

Rationale. A haemolytic (CH_{50}) complement assay can be used to qualitatively measure overall pathway integrity, cell lysis and functional activity. The CH_{50} assay is based on a haemolytic assay in which an immune complex is formed on the surface of RBCs by antibodies that react with a cell surface antigen (see De Waal *et al*. 1988; Sinclair & Lochmiller 2000; Greives *et al*. 2006). When complement is activated by antigen-fixed antibodies on the cell surface, the cell is lysed by the MAC and haemoglobin is released. The formation of the MAC on the cell surface requires the sequential actions of all components of the classical and terminal pathways (i.e. final stages of complement activation common to both classical and alternate pathways) (Dunkelberger & Song 2009). Any break or interruption in the cascade will result in inactivation and decreased lysis. By titration of a complement source (e.g. serum), such that only a portion of the cells are lysed, the amount of active complement can be calculated. In general, the greater the amount of activated complement in serum, the greater the amount of cell lysis will occur. For precise titrations of haemolytic complement, the dilution of serum that will lyse 50% of the indicator red cells is determined as the CH_{50} (Mayer 1948). An alternative agglutination assay to measure haemolytic complement has also been described by Matson, Ricklefs & Klasing (2005). This alternative approach allows the measurement of natural antibodies without the need of an ELISA and also allows the measurement of complement-mediated lysis of cells. This method has been utilized successfully in both avian and reptilian systems (Matson, Ricklefs & Klasing 2005; Sparkman & Palacios 2009).

Requirements. Functional assays for complement should be performed with serum that was frozen at −70 °C within an hour after it was separated from the clot. In addition, refrigerated washed SRBC must be used within 30 days of preparation and cannot be frozen. Finally, rabbit anti-sheep red blood cell antibodies are required along with a plate reader that can measure absorbance at 405 nm.

Benefits. This functional assay is a relatively quick, inexpensive and easy way to evaluate complement activity. No species-specific reagents are necessary and the only piece of specialized equipment used is the plate reader to measure absorbance.

Limitations. Functional tests are often more sensitive than measuring protein concentrations and have the advantage of

measuring the native (active) components or pathways. However, unlike the quantitative tests mentioned previously, functional tests for complement are qualitative in nature. In the CH_{50} assay, although overall complement activity is examined, the activity of individual components cannot be assessed. In addition, substances that weakly activate the complement system may generate measurable local inflammatory responses (i.e. C3a) but are not 'strong' enough to initiate/reflect a significant change in whole complement activity and cell lysis (i.e. entire classical pathway ending at the MAC). As mentioned earlier, sample acquisition and handling can influence the results (Mollnes, Garred & Bergseth 1988). Finally, the arbitrary CH_{50} unit depends on the conditions of the assay and is therefore laboratory-specific. It is important to note that some anticoagulants (e.g. heparin) can interfere with complement and should be avoided (Mollnes, Garred & Bergseth 1988).

Natural Killer (NK) cell cytotoxicity

Rationale. Natural killer cells are an essential component of the innate immune response and are the first line of defence against many infectious agents in many vertebrates (Biron *et al*. 1999). Human patients with impaired NK cell activity often suffer from recurrent illness despite possessing a functional adaptive immune response (Biron, Byron & Sullivan 1989; Orange Jordan 2002). NK cells can be derived from either T- or non-T-cell lineages, sharing many functions with cytotoxic T cells. (Long *et al*. 2001). However, unlike cytotoxic T cells, NK cells are important in the initial innate immune response, as they do not require specific antigen presentation to initiate killing of certain cell lines (Janeway *et al*. 2005). NK cells respond to the presence of non-self-antigens and specific cytokines with the release of cytotoxic granules inducing apoptosis in the target cell (Claus, Greil & Watzl 2009) and the release of additional cytokines such as IFNλ or TNFα (Bryceson *et al*. 2006). The measurement of NK cell cytotoxicity is useful for understanding a component of the innate immune response to viral and neoplastic antigens (Ferrández & De la Fuente 1996; Biron *et al*. 1999). NK cell cytotoxicity assesses only the cytotoxic function of NK cells and does not address their cytokine releasing function.

Requirements. Natural killer cell cytotoxicity can be performed on isolated mononuclear cells (for example see Ferrández & De la Fuente 1996; Muñoz *et al*. 2000) or on whole blood (reviewed in Claus, Greil & Watzl 2009). Using whole blood, rather than isolated mononuclear cells, has the advantage of allowing crosstalk between NK cells and other components of the immune system, such as dendritic cells and cytokines (Bromelow *et al*. 1998), thus providing a more holistic view of NK cell cytotoxicity (Claus, Greil & Watzl 2009). In the 'gold standard' assay, whole blood or isolated mononuclear cells are incubated with radionuclide (typically ^{51}Cr) labelled target cells for 4 h. As target cells are destroyed by NK cells, they release chromium. Chromium release is then quantified by measuring the gamma radiation emitted by the radionuclide using a gamma counter (for an excellent description of the process see Ferrández & De la Fuente 1996).

This assay requires specialized equipment and an approved radionuclide facility, including a gamma counter, but none of the requirements are species specific. This method has been used on a variety of wildlife species including ectothermic vertebrates (Muñoz *et al*. 2000), marine mammals (De Guise *et al*. 1997) and birds (Fowles, Fairbrother & Kerkvliet 1997). This assay can be performed on any sample collected from an animal that contains NK cells. Historically, it has been reported that the assay must be performed promptly after sample collection. However, Claus, Greil & Watzl (2009) found that there was no decrease in NK cell cytotoxicity in whole blood incubated with cell growth media for 24 h prior to assessment when compared to NK cell cytotoxicity from whole blood assessed immediately after collection.

Benefits. Assessing NK cell cytotoxicity quantifies an important component of the initial immune response to many parasites, especially viruses (Biron *et al*. 1999). The described NK cell assay is a simple, widely used assay that does not require species-specific reagents.

Limitations. The major drawback of this technique is the requirement for a gamma counter and an appropriate facility in close proximity to a field site. This assay is also criticized for measuring only lysed target cells, and not accounting for NK cell concentration or the cytotoxic capacity of individual NK cells (Claus, Greil & Watzl 2009).

Bactericidal (Bacterial Killing) assay

Rationale. The bactericidal or bacterial killing assay (BKA) measures the capacity of fresh whole blood to kill bacteria *ex vivo* (Tieleman *et al*. 2005; Millet *et al*. 2007). Generally, whole blood is diluted in media, and a known number of live bacteria (*Escherichia coli* ATCC#8739 is one of the most commonly utilized strains) are added to this sample. After a brief incubation period, samples are plated and incubated on agar; bacterial colonies are counted following incubation and compared to reference plates (bacteria grown without blood). The degree of bacterial killing is then extrapolated from the number of colonies present on each plate. Both thawed samples of frozen serum and plasma can be used instead of whole blood, but it is best if these samples are used relatively soon (e.g. hour or days) after collection. Recent work has shown that repeated freeze-thaws and increased storage duration (over 20 days) of plasma samples significantly reduce killing ability (Liebl & Martin 2009). Also, bacterial killing ability

varies substantially across species and contexts, so some pilot work is almost always required to optimize dilutions of serum/plasma and bacterial strain (Matson *et al*. 2006; Buehler *et al*. 2008b; Rubenstein *et al*. 2008; Ruiz *et al*. 2010). More recently, a modified version of this assay has been proposed that utilizes spectrophotometry for the quantification of bacterial colonies that is reported to be more reliable and requires smaller sample volumes (Liebl & Martin 2009).

Requirements. The BKA is one of the simplest eco-immunology assays and requires the least amount of specialized equipment. While it is best to conduct the assay in sterile conditions under a laminar flow hood, biologists have been able to adapt this technique to the field by utilizing a relatively aseptic enclosure to prevent contamination of the agar culture plates. In addition to the blood samples, agar-coated plates (which can either be prepared in the laboratory or purchased commercially), a bacterial strain and an incubator are needed to conduct this assay. This assay is currently being adapted for use with human saliva (Muehlenbein *et al*., unpublished data), providing a much-needed noninvasive method to assess variation in immune responses within and between humans.

Benefits. This assay allows characterization of a functionally relevant immune response that involves that action of phagocytes (e.g. macrophages, heterophils and thrombocytes), opsonizing proteins (complement and acute phase proteins) and natural antibodies (predominantly IgM and IgA), depending on whether whole blood or serum is used. While the *E. coli* ATCC#8739 strain is complement-dependent, other strains can also be used that are complement-independent or that vary in their reliance on phagocytic activity for bacterial killing. Assessing responses to several strains allows investigators to determine specific immune mechanisms (e.g. complement and phagocytes) underlying experimental effects. Because the BKA assesses the ability to eliminate an actual pathogen, it provides a more functionally relevant assessment of host immune function compared with assays that assess isolated immune components or immune responses to relatively artificial antigens or mitogens.

Limitations. As with other assays performed in culture, the results of the BKA may not extrapolate to immune response *in vivo*. Further, this assay requires maintaining bacteria-coated culture plates under ascetic conditions in an incubator, which makes the assay itself difficult to perform in the field, although some studies have been able to perform bactericidal experiments in field laboratories using this or similar type assays (Tieleman *et al*. 2005; Buehler, Piersma & Irene Tieleman 2008a). This difficulty, however, can be eliminated if a laboratory facility is relatively close by to the field site.

Macrophage phagocytic ability

Rationale. Macrophages are important cell signalling agents that activate an appropriate immune response and are also are potent effector cells of the innate immune response (Kwiatkowska & Sobota 1999). Macrophages can attack pathogens by releasing toxic compounds or by actively ingesting and digesting a variety of pathogens using the process of phagocytosis. After the antigen contacts and binds to the macrophage cell membrane, the macrophage engulfs the antigen in a phagosome (Janeway *et al*. 2005). This membrane-bound phagosome then fuses with a lysozome and becomes a mature phagolysosome, capable of destroying the antigen (Vieira, Botelho & Grinstein 2002; Damiani & Colombo 2003). Determining the phagocytic ability of macrophages is an excellent measure of a specific host innate immune function that is important for the immediate killing of numerous pathogens, from whole bacteria to virally infected and injured host cells.

Requirements. The phagocytic assay requires that macrophages be isolated before adding a labelled antigen. Macrophages can be isolated from peritoneal fluids or whole blood using standard peripheral blood mononuclear cell isolation methods (Trust, Hooper & Fairbrother 1994; Fowles, Fairbrother & Kerkvliet 1997), followed by allowing macrophages to adhere to a surface and washing away the nonadherent lymphocytes and immature monocytes (Patton *et al*. 2005; Orsi *et al*. 2010). These isolated macrophages can then be combined with a labelled antigen, such as stained yeast or SRBC, and a microscope is used to count the number of macrophages engulfing particles and/or the number of particles being engulfed (Fairbrother *et al*. 1994; Lahvis *et al*. 1995). This protocol requires the use of live macrophages and must be performed with viable cells. This technique has been successful with macrophages that were previously cryopreserved (Grasman 2002).

Benefits. This technique provides a simple assessment of macrophage phagocytic activity – an important mechanism of the innate immune response. It requires very basic equipment (e.g. centrifuge and microscope) and does not require species-specific reagents or complex sampling, but rather can be performed using whole blood or peritoneal fluid collected from a variety of species (Grasman 2002).

Limitations. This technique must be performed promptly after sample collection or cells must be promptly cryopreserved to maintain cell viability. Unlike techniques that measure multiple aspects of immune function (for example bactericidal ability of whole blood or serum), macrophage phagocytic ability is a very specific measure of a particular immune response and cannot be used on its own as a descriptor of generalized immune function. In addition, this

technique only measures one component of macrophage function, phagocytic ability. The assay does not measure other important functions of macrophages in the immune system, including the release of cytokines and toxic compounds or the activation of an appropriate acquired immune response.

ACQUIRED IMMUNITY

Delayed-type hypersensitivity

Rationale. Delayed-type hypersensitivity (DTH) responses are localized antigen-specific responses eliciting swelling and redness at the site of antigen injection in immunized animals (Dhabhar 1998). A classic example is the tuberculosis skin screening test currently used in humans. DTH responses are predominantly T-cell-mediated responses resulting in the recruitment and activation of cytokines, macrophages, NK cells and cytotoxic T cells. This initial response is followed by the proliferation of T cells, which upon secondary exposure will elicit a more robust response than the primary response. It is important to clarify that many researchers in the field do not sensitize their study animals prior to antigen challenge and are therefore not measuring a DTH response (unless the animal has some form of prior exposure to the antigen). This initial priming is followed by a second injection at the same site *c*. 5–10 days later to stimulate the DTH response. The exact timing of the second injection is dependent on the antigen and study subject used both of which will affect the speed of T-cell memory formation necessary to mount a DTH response.

The most common antigens used to elicit DTH responses are 2,4-dinitro-1-fluorobenzene (DNFB) (Dhabhar 1998; Bilbo & Nelson 2003) and phytohaemagglutinin (PHA), which act as a T-cell mitogen and are derived from the red kidney bean (*Phaseolus vulgaris*) (Lee *et al*. 2006; Martin *et al*. 2006a). Less commonly used are keyhole limpet haemocyanin (KLH) and heat-killed *E. coli* bacteria (Smith *et al*. 2005; Martin, Hasselquist & Wikelski 2006b). Because these antigens cause proliferation of T cells to a localized area, the amount of swelling can be measured and roughly equated to the strength of the immune response.

Requirements. The most important requirement to measure the DTH swelling response is that the animal has a consistently measureable surface, such as, a pinna, wing web, fin or foot pad. Assessment of DTH is very straightforward and only requires a shaver for hair/feather removal if necessary, an antigen of your choice (e.g. DNFB and PHA), a syringe (wing-webs and foot pads) or acetone (pinnae) for antigen delivery, and calipers or a constant-loading dial micrometre to measure swelling. Because constant-loading dial micrometres exert constant pressure at the swelling site, they help to eliminate much of the user error common in hand-held calipers. Swelling at the injection site is measured *c*. 12, 24 and 48 h postsecondary injection. The amount of swelling is roughly equal to the strength of the cell-mediated immune response, and therefore more swelling is equivalent to a greater infiltration and proliferation of macrophages and lymphocytes. Data are presented as the change in thickness or swelling at the injection site; therefore a baseline or control thickness must be measured. There are two different methods of control utilized in DTH studies: first is assessing baseline, pre-antigen size at the challenge site and the second is to have a secondary location to serve as a control throughout the study (i.e. alternate wing and pinnae). Studies have demonstrated that either way is valid (Martin *et al*. 2006a).

Benefits. This technique provides a relatively quick and simple assessment that does not require performing any assays. It can be performed readily in the laboratory or the field, but does require recapture at specific points. Further, there are no specific antibodies required, and so DTH can be readily performed in nontraditional model systems.

Limitations. The largest limitation of this procedure is that not all animals have a surface location conducive to measuring swelling. The second major limitation is that DTH requires repeated sampling of the animal at specific time points which makes it difficult to perform on certain species in the field. Therefore, the study animals must either be held in the laboratory for a prolonged time or must be recaptured at specific time points if researcher is truly measuring DTH, and not simply primary responses. Individual hydration state and body condition can greatly affect the swelling response while not necessarily affecting immunity. Because of the relative ease of conducting DTH responses (e.g. PHA-induced wing web swelling), this technique has historically dominated the field of eco-immunology, (although additional measures are beginning to be incorporated). However, most researchers only measure swelling and not specific cellular recruitment at the site of swelling and therefore results have the potential to be misleading or unclear. Therefore, referring to DTH as 'T-cell-mediated immunity' or 'cell-mediated immunity', as has been repeatedly performed in the field, is an oversimplification.

In vitro lymphocyte proliferation

Rationale. When lymphocytes (either circulating or those harvested from lymphoid tissues) are challenged with a pathogen, they undergo marked proliferation. An important way to test the functional ability of lymphocytes to multiply in response to a pathogen is to test their proliferative ability under controlled conditions within the laboratory (Froebel *et al*. 1999). Because all proliferating cells actively incorporate thymidine into their DNA, this can be used to determine the degree of proliferation. By using a radioactively labelled

form of thymidine, tritiated thymidine (^{3}[H]thymidine), it is possible to quantify the amount of ^{3}[H]thymidine as a measure of cell proliferation. Typically, a specific lymphoid tissue (e.g. spleen) is removed from an animal, ground between glass slides to liberate lymphocytes and then added to culture media to maintain viable cells. ^{3}[H]thymidine is then added. To stimulate proliferation, a variety of mitogens can be employed which target specific lymphocyte subtypes. The most commonly employed mitogens include conconavalin A (Con A) and PHA which serve as T-cell mitogens, lipolysaccharide (LPS) which stimulates B cells predominately, and pokeweed mitogen which is a mixed B- and T-cell mitogen. More recently, a rapid colorimetric assay has been developed to determine proliferation based on the ability of the mitochondrial enzyme succinate-dehydrogenase to transform the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetra-zolium bromide) into a blue-coloured MTT formazan (Cory *et al*. 1991). Because the conversion of MTT conversion only occurs in living cells, the amount of formazan produced is proportional to the number of cells present and thus serves as a measure of proliferation (Demas, Klein & Nelson 1996). This method correlates well with ^{3}[H]thymidine (Bounous, Campagnoli & Brown 1992) and has the benefits of being rapid, cost-effective and does not require the use of radioactive materials (Bounous, Campagnoli & Brown 1992). Several kits employing this technology are now commercially available.

Requirements. *In vitro* cell proliferation can be performed on whole blood (Palacios *et al*. 2007, 2009) or with liberated cells from whole lymphoid tissues (e.g. splenocytes) (Demas, Klein & Nelson 1996). Because this is an *in vitro* technique, the assay requires maintaining cells in culture in a sterile environment and typically requires the use of a laminar flow hood while treating cells and a CO_2 incubator to maintain cells during the proliferative process. While samples can be taken under field or laboratory conditions, the assay itself is typically performed within a laboratory setting.

Benefits. Proliferation assays, like most *in vitro* assays, have the important benefit of examining immune responses under controlled conditions removed from other physiological influences. By using different mitogens, or even presorting cell types prior to the assay (e.g. via flow cytometry), one can determine the ability of specific immune cell subtypes to proliferate under very specific experimental conditions. Further, these assays do not typically rely on species-specific reagents. While some initial pilot work will be needed to optimize doses to a specific species, this technique can be used with a wide range of species and there can be no terminal measure with the use of whole blood.

Limitations. While lymphocyte proliferation is an important immune assay in the eco-immunologist's toolkit, the need to perform the assay under sterile (typically laboratory) conditions can make this assay less attractive to the field biologist. Furthermore, this technique is a less functional response in that while immune cells may proliferate more or less under certain experimental conditions, greater proliferation does not necessarily mean greater ability to destroy pathogens. As with any *in vitro* technique, one must be careful when extrapolating responses seen in a dish to those that might be demonstrated when using *in vivo* models, as these two responses may differ because of differences in experimental conditions. Also, this assay must be performed within *c*. 24 h of harvesting cells or taking blood samples and preservation of viable cells in media is typically required. Unlike other assays that rely on frozen samples if this assay is performed on lymphocytes harvested fresh from lymphoid tissues at necropsy, there is no opportunity to repeat the assay if it fails the first time. This is less of an issue, however, if whole blood is used.

HUMORAL IMMUNITY

Total Immunoglobulin (Ig) levels and antigenic challenge

Rationale. Antibodies are produced by B lymphocytes following exposure to an antigen and are only useful for eliminating pathogens that are in the blood or the extra-cellular matrices and spaces (Kuby 1997). In addition to antibodies produced in response to an immune challenge, constitutive (natural) antibodies provide a first line of defence against pathogenic attack and may reflect a state of immunological readiness (Greives *et al*. 2006; Lee *et al*. 2008). IgG is the most abundant antibody isotope in the blood and is responsible for coating micro-organisms so that macrophages and neutrophils can recognize and destroy them. IgM on the other hand has multiple functions including complement-mediated lysis and the formation of antibody-antigen complexes (Kuby 1997). The most common antigens used to elicit antibody responses include KLH, diphtheria–tetanus toxin and SRBC (Demas & Nelson 1996; Hanssen *et al*. 2004). Additionally, animals can be challenged with an ecologically relevant pathogen (i.e. pneumococcus and malaria) and disease-specific antibodies can be quantified. Following the antigenic challenge, blood samples are drawn at multiple time points postinjection to measure specific immunoglobulins (IgM and IgG titres are the most commonly measured because of their high blood concentrations). For quantification of both baseline constitutive antibody titres and antibody production in response to an antigenic challenge, an ELISA can be used (Demas, DeVries & Nelson 1997b; Drazen *et al*. 2000; Zysling & Demas 2007). Because SRBC do not readily bind to ELISA plates, challenges using this antigen will require an agglutination assay (Grasman 2010).

Requirements. Antigenic challenges require injection of the chosen antigen and then (potentially) multiple sample points

thereafter. Further, to measure immunological memory (secondary antibody responses) additional challenge points are necessary (Martin *et al*. 2008a). As with other ELISA methods, species-specific labelled antibodies, appropriate ELISA reagents, and a plate reader are necessary. Data are presented as the per cent change when compared with a positive control sample, yielding relative abundance of antibodies in samples rather than absolute concentrations (Demas, Drazen & Nelson 2003).

Benefits. Constitutive antibody measurements require only one blood sample; thus, this method is compatible with field studies and use on organisms with a low recapture rate. Antigenic challenge can be used to evaluate immunological memory and responses to a novel pathogen. Measurements of antibodies against a relevant pathogen can result in ecologically relevant conclusions regarding disease resistance and exposure. For both types of measurements, samples can be frozen and stored until further analysis.

Limitations. For measurements of constitutive antibodies, it is difficult to determine whether antibody titres reflect an actual baseline measurement or whether levels are elevated because of recent sickness or pathogen exposure. Antigenic challenges require one or more injections and multiple blood samples; thus, high recapture rates are required for use in the field. As a result, antigenic challenges are best suited for laboratory or captive studies. For both methods, species-specific antibodies are required and the antibody response is presented as the per cent change based on a positive control, which can vary between assays and laboratories.

INTEGRATED MEASURES

Cytokine profiles

Rationale. Cytokines are low molecular weight, biologically active proteins that function as intercellular messengers at very low concentrations (Janeway *et al*. 2005). Their actions are typically mediated by cell surface receptors located on the same (autocrine) or neighbouring cells (paracrine). The effects of their presence may be stimulatory or inhibitory and can include cell proliferation, activation or differentiation of the target cell, often a cell involved in host immune defence (Banks 2000). Cytokines are important in regulating the immune response in many disease processes (Bienvenu *et al*. 1998), as well as regulating immune responses of healthy animals. A single cytokine can affect a wide variety of cells and a single cell can produce a wide variety of cytokines that may be redundant in their activities (Banks 2000). However, specific cytokines can often be linked to specific immunological responses, making them useful to measure when trying to understand an immunological mechanism. For example, measuring cytokines associated with a Th1 response and cytokines with a Th2 response has helped clarify the immunological interactions seen with tuberculosis and macroparasites (Rook & Brunet 2005; Graham 2008).

Requirements. There are two types of cytokine assays: bioassays and immunoassays. Immunoassays have been used to quantify the amount of cytokine in a bodily fluid or tissue. This simple quantification can be performed by many techniques (reviewed in Sachdeva & Asthana 2007), but is most often performed by ELISA (Wadhwa & Thorpe 1998). Newer technologies (e.g. Multiplex) allow a large number of cytokines (20 or more) to be assessed simultaneously in a single assay requiring small (50 μL) samples, but these assays require specialized equipment and can be expensive. Both assays require an antibody to the cytokine, which is species-specific. However, as biologically active entities, cytokines cannot be fully characterized by physiochemical methods alone, so bioassays may also be performed (Meager 2006). Bioassays quantify the potency of a cytokine using a cultured cell line to assess the response of a target cell line to a given quantity of cytokine (Meager 2006). Bioassays take a longer time to perform, are less precise and are more technically demanding then immunoassays (Banks 2000). Bioassays measure only the amount of biologically active cytokine and international standards exist for the assays (Sachdeva & Asthana 2007). These assays require tissue culture facilities and results are often laboratory dependent (Thorpe *et al*. 1999).

Benefits. Immunoassays are relatively simple to standardize and perform and can provide an excellent overview of immune function when multiple cytokines are measured. With the recent advent of microarray technology, it is becoming possible to measure the cytokine milieu with one assay (Sachdeva & Asthana 2007). Bioassays help to confront some of the limitations of immunoassays (reviewed in Banks 2000) and quantify the biological function(s) of the cytokine(s) of interest.

Limitations. Immunoassays require species-specific and cytokine-specific reagents, so the assay can be limited by commercial availability. However, numerous antibodies exist and many cross-react between species so this limitation can be overcome. Bioassays require cell culture facilities and can be challenging to standardize.

Wound healing

Rationale. Wound healing is a multifaceted measure of immune function, used for many years in studies involving stress, susceptibility to bacterial infection and interleukin kinetics in laboratory animals (Padgett, Marucha & Sheridan 1998; Rojas *et al*. 2000, 2002; Mercado *et al*. 2002), and in studies of stress seasonality in small mammals (Kinsey, Pren-

dergast & Nelson 2003). Wound healing is a coordinated immune response, including an initial inflammatory response (Padgett, Marucha & Sheridan 1998; Rojas *et al*. 2002), mobilization and proliferation of platelets, granulocytes, cytokines, chemokines, fibroblasts and keratinocytes (Martin 1997; Padgett, Marucha & Sheridan 1998), all of which are stress sensitive. Wound healing is also a biologically relevant measure of immune function in most animals, which frequently incur wounds in their natural habitat.

Requirements. Assessment of wound healing is easy to perform and only requires some basic surgical tools to create and remove a skin biopsy, an anaesthetic, and a digital camera to photograph the resulting wound site including a scale reference. The animal will need to be either anaesthetized (smaller animals) or given a local anaesthetic such as lidocaine at the wound site. Animals with either fur or feathers may need to be shaved at the wound site prior to the biopsy procedure. Once anaesthetized, the animal receives a sterile cutaneous biopsy punch (e.g. Millitex). The biopsy size can be variable and should be appropriate for the study animal. This procedure can be performed in the field or in the laboratory.

The length of healing time should be based on initial pilot studies that document the comprehensive healing profile for the study animal (Padgett, Marucha & Sheridan 1998; Rojas *et al*. 2000, 2002; French, Matt & Moore 2006). To photograph the wounds, animals need to be secured in a uniform fashion with a scale reference (e.g. metric ruler) for size measurements. Digital images can be taken with either a digital camera (in the field) or a camera attached to a light microscope (in the laboratory). Images then can be analysed using image analysis software such as Image Pro (http://www.mediacy.com) or Image J (http://rsbweb.nih.gov/ij). Image J analysis software is easily attainable via download online and is free to all users. Measurements could potentially be collected by hand; however, the measurement accuracy would not be as high as with image analysis because of the small size of the wounds and wounds not healing in a uniform manner.

Benefits. Wound healing is a coordinated measure of immunity and thus a comprehensive immune response (Kiecolt-Glaser *et al*. 1995; Epstein, Singer & Clark 1999). It is both biologically and ecologically relevant to most animals. It can be performed without specific reagents or antibodies, and so can be performed in more nontraditional systems. Lastly, it can be performed readily in the field (French & Moore 2008), but is easier with territorial animals, because recapture at specific time points is necessary to asses healing rate over time.

Limitations. Wound healing requires an initial pilot study to determine the appropriate measurement time points in the healing profile to assess healing rate for a given animal model. This technique is not conducive for field use in some model systems because animals must be recaptured and wounds remeasured at specific time points. Like many other immune measures, wound healing is highly stress sensitive (French, Matt & Moore 2006), so results can be affected by experimental context. Lastly, the procedure also requires a minor surgery and anaesthetic, which usually require practice and specific IACUC approval for use.

Fever/sickness responses

Rationale. The assessment of fever and/or sickness responses is both a biologically relevant measure of immune function and an integrated immune response. Sickness responses generally include, but are not limited to fever, sickness behaviour (lethargy and mild anorexia), cytokine production and elevated glucocorticoid levels. The most common agent used to induce immune activation and sickness is LPS, a molecule present on the cells walls of Gram-negative bacteria, which acts on toll-like receptors (TLR) present on immune cells (specifically TLR4) and elicits an inflammatory and pyrogenic (fever-inducing) response without actually infecting the animal with an infectious agent. Therefore, immunizing an animal with LPS mimics a bacterial infection without actually getting the animal sick. When animals are treated with LPS in controlled doses, immune cells including macrophages, monocytes and Kupffer cells, which all express TLR4 receptors, bind LPS and stimulate an intracellular signalling cascade resulting in activation of transcription factors such as nuclear factor κB and related kinases (Mouihate *et al*. 2008). In addition, LPS activates the complement cascade, resulting in the production of anaphylatoxin (C5a), which in turn activates cyclo-oxygenases (COX-1, COX-2) and prostaglandins (PGs) (Mouihate *et al*. 2008). Approximately 30 min following LPS exposure, cells of the innate immune systems synthesize and secrete a range or pro-inflammatory cytokines, predominantly interleukin (IL) 1β, IL-6, and tumour necrosis factor α (TNFα). The experimental sickness is relatively short-term, as fever and inflammation has usually abated within 24–48 h (Dantzer & Kelley 2007); however, this response does require significant energetic investment. There are also readily available chemical agents (e.g. polyinosinic:polycytidylic acid) that induce viral-like responses via acting on TLR3.

Requirements. There are different ways to assess the fever and sickness response. First, sickness responses can be measured using a thermometer to track body temperature (fever), behaviour recording equipment to measure behavioural changes over time, a thermal gradient if studying an ectothermic animal to allow for behavioural thermoregulation, and a scale to monitor food intake over the course of the experiment. Sickness behaviour includes lethargy, reduced food intake, and elevated body temperature, all of which are monitored regularly over the typical 48-h LPS sickness response. All measures require a baseline, pre-sickness (i.e.,

pre-injection) measure for comparison. Researchers can also quantify cytokine profiles in response to sickness (see Cytokine section), as well as other indirect responses that may also change because of sickness response (e.g. leucocyte counts and antibody titres) (Dantzer *et al*. 1999; Dantzer 2006; Dantzer & Kelley 2007; Buehler *et al*. 2009). There is also a usual increase in circulating corticosterone in response to LPS injection (Owen-Ashley *et al*. 2006), which can be measured using either radioimmunoassay or an ELISA.

Benefits. This immune measure is an integrated immune response, including behaviour, without actually infecting the animal. The response is also rapid and certain endpoints such as behaviour and fever responses are relatively simple to measure. Some studies, involving avian species, have been conducted on free-living animals in the field (Owen-Ashley & Wingfield 2006; Owen-Ashley *et al*. 2006).

Limitations. Behaviour and sickness responses usually require repeated observations and handling of the study animals at specific time points, including baseline, which is usually only conducive to a laboratory setting. However, a few studies have successfully utilized radiotelemetry to reduce or eliminate the need for recapture (Hetem *et al*. 2008; Adelman *et al*. 2010). There is large variability in LPS potency, even within the same lot, making it difficult to calculate the appropriate dose. Additionally, there is also high individual variation in response to LPS, and variation according to season, pregnancy state and prior exposure (Prendergast *et al*. 2004; Owen-Ashley & Wingfield 2006; Owen-Ashley *et al*. 2006; Tsang, Fewell & Moore 2006; Hodyl *et al*. 2010). For example, studies have repeatedly shown that pregnant animals show hypothermic rather than pyrogenic responses to LPS in both laboratory rats and Siberian hamsters (Fofie & Fewell 2003; Wei, Li & Zhou 2007; French, SS, Chester, EM, & Demas, GE, unpublished data).

Infectious disease models

Rationale. Arguably, one of the more powerful study designs in eco-immunology is to artificially expose immunologically naïve animals to a well-characterized infectious organism (of known inoculum) and measure correlated changes in immune function and life-history traits during infection and after convalescence. Of course, such a design may be best suited for controlled laboratory conditions, and is not feasible in some species like humans. That said, various model systems have been successfully employed, including the coccidian *Caryospora* in Eurasian kestrels (*Falco tinnunculus*) (Lemus, Vergara & Fargallo 2010), the nematode *Trichostrongylus* in lambs (Wagland *et al*. 1984), the bacterium *Borrelia* in black-legged kittiwake (*Rissa tridactyla*) (Staszewski *et al*. 2007) and *Plasmodium* in several species (Benten *et al*. 1997; Eisen & DeNardo 2000), to name a few.

All of the above-mentioned immunological tests can be employed under these circumstances, including multiple WBC and immunoglobulin analyses throughout infection. Importantly, disease-specific antibody titres may be determined. Furthermore, measurements can be made after initial exposure (in naïve animals) and after subsequent exposures to assess adaptive immune functions.

When it is not feasible to infect an animal, an alternative study design may be to utilize a vaccine so as to induce an activated immune response (Staszewski & Boulinier 2004). Such a design has been employed using a variety of species, including the pied flycatcher (*Ficedula hypoleuca*), pheasant (*Phasianus colchicus*) and the blue tit (*Parus caeruleus*), all exposed to the diphtheria–tetanus vaccine (Svensson *et al*. 1998; Ilmonen, Taarna & Hasselquist 2000; Ohlsson *et al*. 2002). Similarly, the Newcastle disease virus has been used in the collared flycatcher (*Ficedula albicollis*) and the barn swallow (*Hirundo rustica*) (Nordling *et al*. 1998; Saino *et al*. 2002). Porcine respiratory and reproductive syndrome has been used in pigs (Spurlock *et al*. 1997), and yellow fever and typhoid vaccines in humans (Gandra & Scrimshaw 1961; Cooper *et al*. 1992).

Requirements. The basic requirements for this type of study are intensive, simply because one's animal population must be of a known infection/immune status, and because animals must be re-sampled (before, during, and after infection). In addition to all of the above-mentioned requirements for the immune assays of choice, one must often maintain one's own source of infectious agent, whether that be source animals or mosquitoes infected with malaria, or other forms of inocula. Animals should be free of infection and immunologically naïve prior to inoculation, which may require special pathogen-free colonies of laboratory animals or antimicrobial treatment of wild species. Furthermore, one's choice of host-pathogen system should be species-specific and ecologically relevant. That is, one should consider using a pathogenic organism or vaccine that induces a significant immune response in the model organism of choice.

Benefits. A study design which utilizes these methodologies is certainly in a better position to assess actual causality rather than simple correlations between immune activation and variation in different life-history traits or other endpoint criteria. Furthermore, trade-offs involving immunity may only be evident during immune activation in model organisms (Muehlenbein & Bribiescas 2005).

Limitations. The limitations of this study design are obvious: intensive repeated monitoring of well-characterized model species, using choice infectious organisms or vaccine paradigms. Such experimental designs may not be logistically or ethically feasible with certain host organisms, particularly humans.

Conclusions and additional ecological considerations

Regardless of which eco-immunology techniques are employed (Table 1), experimental context is paramount to the interpretation of immunological data. Immune responses are not fixed in nature; they are instead highly variable depending on context. The predominant factors to consider when measuring immunity are season, reproductive state, sex, energy/nutrient balance, individual stress and current/prior infection status. Perhaps most significant, immune responses require substantial energy to occur. As a result, an organism's energy balance can greatly alter their immunocompetence. For example, mounting an antibody response in house mice significantly elevated metabolic rate (Demas *et al*. 1997a). Limiting food or even specific nutrients can also inhibit innate and cell-mediated immune responses and lymphatic tissue mass (Glick *et al*. 1983; Lochmiller, Vestey & Jon 1993; Alonso-Alvarez & Tella 2001; French, DeNardo & Moore 2007). Many potential factors can influence individual energy balance, including but not limited to seasonal energy availability (Nelson & Demas 1996, 2004; Deerenberg *et al*. 1997; Nelson 2004; Owen-Ashley & Wingfield 2006), and reproductive state (Saad 1989; Deerenberg *et al*. 1997; Nordling *et al*. 1998; Moreno, Sanz & Arriero 1999; Olsson *et al*. 2000; Drazen, Trasy & Nelson 2003; Hanssen, Folstad & Erikstad 2003; French, DeNardo & Moore 2007).

Current or prior exposure to pathogens and parasites is also integral to the ability of an organism to mount an immune response. For example, an animal currently battling an infection would likely show elevated antibody titres and circulating leucocytes and this response could be mistaken for an elevated response to a concurrent experimental challenge being assessed. Prior exposure can also produce immunological memory, allowing previously exposed animals to produce more robust responses than previously unexposed animals. Conversely, recent or current exposure to infection may limit energy available to additional experimental immune challenges, rendering the animal immunocompromised. Although not all factors can be controlled within a study, they should be considered when interpreting immunological data. For example, testing for current infection status via baseline antibody titres and screening for parasite infections could greatly improve the interpretation of an animal's immunological responses (see earlier sections for specific techniques). Additionally, choosing the appropriate immunological approach is critical to a successful study.

The most basic, but perhaps most overlooked question in eco-immunology studies is 'which technique is right for my research'? Feasibility for a particular experimental design or study species dictates which techniques are viable. However, the question of relevancy to the experimental question and species being studied are often overlooked in eco-immunology studies. A practical first step is to investigate your organism's natural history. Is there a high incidence of a particular pathogen in the system? Is there high parasite prevalence in the organism's habitat? Is the organism frequently wounded defending mates or a territory? These factors help establish whether an innate, cell-mediated, or humoral response for example is more important and ecologically relevant to the organism. The question of biological/ecological relevancy is particularly important now that current evidence has identified physiological trade-offs occurring within the immune system (Zysling, Garst & Demas 2009). This evidence suggests that organisms are likely energetically 'favouring' particular immune responses that are most relevant to that organism and its environment. Therefore, examining isolated immune components such as PHA, although still informative, do not provide a comprehensive measure of the immune system and should not be interpreted as such.

The goal of this review was to provide a basic overview of some of the techniques available to eco-immunologists; we hope it has served its purpose. Before employing any new technique, however, it is highly recommended that investigators obtain published protocols specific to the technique of interest, as well as consult with relevant experts (e.g. immunologists, microbiologists and parasitologists). A modest amount of initial pilot work will allow researchers to maximize the utility of specific methodologies, tailoring them to one's specific experimental needs. More importantly, doing so will likely minimize the potential for serious problems with the techniques after the data are collected. In conclusion, we hope researchers will consider incorporating some of these techniques described here in their own research programs; by doing so, eco-immunologists will gain a more integrative, comprehensive understanding of environment–immune interactions.

References

Adelman, J.S., Córdoba-Córdoba, S., Spoelstra, K., Wikelski, M. & Hau, M. (2010) Radiotelemetry reveals variation in fever and sickness behaviours with latitude in a free-living passerine. *Functional Ecology*, **24**, 813–823.

Ali, N.M.H. & Behnke, J.M. (1985) Observations on the gross changes in the secondary lymphoid organs of mice infected with *Nematospiroides-dubius*. *Journal of Helminthology*, **59**, 167–174.

Alitheen, N., McClure, S. & McCullagh, P. (2007) Detection and quantification of IgM+ lymphocytes in fetal lamb spleen, liver and lymph nodes by flow cytometry. *Immunology and Cell Biology*, **85**, 391–393.

Alonso-Alvarez, C. & Tella, J.L. (2001) Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. *Canadian Journal of Zoology*, **79**, 101–105.

Ardia, D.R. (2005) Tree swallows trade off immune function and reproductive effort differently across their range. *Ecology*, **86**, 2040–2046.

Ardia, D.R. & Schat, K.A. (2008) Ecoimmunology. *Avian Immunology* (eds B. Kaspers & K.A. Schat), pp. 421–441. Elsevier Ltd, London.

Banks, R.E. (2000) Measurement of cytokines in clinical samples using immunoassays: problems and pitfalls. *Critical Reviews in Clinical Laboratory Sciences*, **37**, 131–182.

Barrett, K.E., Neva, F.A., Gam, A.A., Cicmanec, J., London, W.T., Phillips, J.M. & Metcalfe, D.D. (1988) The immune response to nematode parasites: modulation of mast cell numbers and function during *Strongyloides stercoralis* infections in nonhuman primates. *American Journal of Tropical Medicine and Hygiene*, **38**, 574–581.

Baumgarth, N. & Roederer, M. (2000) A practical approach to multicolor flow cytometry for immunophenotyping. *Journal of Immunological Methods*, **243**, 77–97.

Benten, W.P.M., Ulrich, P., Kuhn-Velten, W.N., Vohr, H.-W. & Wunderlich, F. (1997) Testosterone-induced susceptibility to *Plasmodium chabaudi* malaria: persistence after withdrawal of testosterone. *Journal of Endocrinology*, **153**, 275–281.

Bienvenu, J.A.D., Monneret, G., Gutowski, M.C. & Fabien, N. (1998) Cytokine assays in human sera and tissues. *Toxicology*, **129**, 55–61.

Bilbo, S.D. & Nelson, R.J. (2003) Sex differences in photoperiodic and stress-induced enhancement of immune function in Siberian hamsters. *Brain, Behavior, and Immunity*, **17**, 462–472.

Birkhead, T.R., Fletcher, F. & Pellatt, E.J. (1998) Sexual selection in the zebra finch *Taeniopygia guttata* : condition, sex traits and immune capacity. *Behavioral Ecology and Sociobiology*, **44**, 179–191.

Biron, C.A., Byron, K.S. & Sullivan, J.L. (1989) Severe herpesvirus infections in an adolescent without natural killer cells. *New England Journal of Medicine*, **320**, 1731–1735.

Biron, C.A., Nguyen, K.B., Pien, G.C., Cousens, L.P. & Salazar-Mather, T.P. (1999) Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annual Review of Immunology*, **17**, 189–220.

Bounous, D.I., Campagnoli, R.P. & Brown, J. (1992) Comparison of MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation assays using chicken splenocytes. *Avian Diseases*, **36**, 1022–1027.

Bowdish, D.M.E., Davidson, D.J., Scott, M.G. & Hancock, R.E.W. (2005) Immunomodulatory activities of small host defense peptides. *Antimicrobial Agents and Chemotherapy*, **49**, 1727–1732.

Bromelow, K.V., Galea-Lauri, J., O'Brien, M.E. & Souberbielle, B.E. (1998) A highly sensitive whole blood natural killer cell assay. *Journal of Immunological Methods*, **217**, 177–184.

Bryceson, Y.T., March, M.E., Ljunggren, H.-G. & Long, E.O. (2006) Activation, coactivation, and costimulation of resting human natural killer cells. *Immunological Reviews*, **214**, 73–91.

Buehler, D.M., Piersma, T. & Irene Tieleman, B. (2008a) Captive and free-living red knots *Calidris canutus* exhibit differences in non-induced immunity that suggest different immune strategies in different environments. *Journal of Avian Biology*, **39**, 560–566.

Buehler, D.M., Piersma, T., Matson, K. & Tieleman, B.I. (2008b) Seasonal redistribution of immune function in a migrant shorebird: annual-cycle effects override adjustments to thermal regime. *The American Naturalist*, **172**, 783–796.

Buehler, D.M., Encinas-Viso, F., Petit, M., Vezina, F., Tieleman, B.I. & Piersma, T. (2009) Limited access to food and physiological trade-offs in a long-distance migrant shorebird. II. Constitutive immune function and the acute-phase response. *Physiological and Biochemical Zoology*, **82**, 561–571.

Cárdenas, W., Jenkins, J.A. & Dankert, J.R. (2000) A flow cytometric approach to the study of crustacean cellular immunity. *Journal of Invertebrate Pathology*, **76**, 112–119.

Carroll, M.C. (1998) The role of complement and complement receptors in induction and regulation of immunity. *Annual Review of Immunology*, **16**, 545–568.

Claus, M., Greil, J. & Watzl, C. (2009) Comprehensive analysis of NK cell function in whole blood samples. *Journal of Immunological Methods*, **341**, 154–164.

Conlon, J.M. & Sonnevend, A. (2010) Antimicrobial peptides in frog skin secretions. In: *Antimicrobial Peptides* (eds A. Giuliani & A.C. Rinaldi). *Methods in Molecular Biology*, **618**, 3–14. Humana Press, Totowa, NJ, USA.

Cooper, A.L., Horan, M.A., Little, R.A. & Rothwell, N.J. (1992) Metabolic and febrile responses to typhoid vaccine in humans: effect of beta-adrenergic blockade. *Journal of Applied Physiology*, **72**, 2322–2328.

Cory, A.H., Owen, T.C., Barltrop, J.A. & Cory, J.G. (1991) Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Communications*, **3**, 207–212.

Damiani, M.T. & Colombo, M.I. (2003) Microfilaments and microtubules regulate recycling from phagosomes. *Experimental Cell Research*, **289**, 152–161.

Dantzer, R. (2006) Cytokine, sickness behavior, and depression. *Neurologic Clinics*, **24**, 441–460.

Dantzer, R. & Kelley, K.W. (2007) Twenty years of research on cytokine-induced sickness behavior. *Brain, Behavior, and Immunity*, **21**, 153–160.

Dantzer, R., Aubert, A., Bluthé, R.M., Gheusi, G., Cremona, S., Layé, S., Konsman, J.P., Parnet, P. & Kelley, K.W. (1999) Mechanisms of the behavioural effects of cytokines. *Cytokines, Stress, and Depression*, **461**, 83–105.

De Guise, S., Ross, P.S., Osterhaus, A.D.M.E., Martineau, D., Béland, P. & Fournier, M. (1997) Immune functions in beluga whales (*Delphinapterus leucas*): evaluation of natural killer cell activity. *Veterinary Immunology and Immunopathology*, **58**, 345–354.

De Waal, R.M.W., Schrijver, G., Bogman, M.J.T., Assmann, K.J.M. & Koene, R.A.P. (1988) An improved sensitive and simple microassay of mouse complement. *Journal of Immunological Methods*, **108**, 213–221.

Deerenberg, C., Arpanius, V., Daan, S. & Bos, N. (1997) Reproductive effort decreases antibody responsiveness. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **264**, 1021–1029.

Demas, G.E., Drazen, D.L. & Nelson, R.J. (2003) Reductions in total body fat decrease humoral immunity. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **270**, 905–911.

Demas, G.E. (2004) The energetics of immunity: a neuroendocrine link between energy balance and immune function. *Hormones and Behavior*, **45**, 173–180.

Demas, G.E., DeVries, A.C. & Nelson, R.J. (1997b) Effects of photoperiod and 2-deoxy-D-glucose-induced metabolic stress on immune function in female deer mice. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, **272**, R1762–R1767.

Demas, G.E., Drazen, D.L. & Nelson, R.J. (2003) Reductions in total body fat decrease humoral immunity. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **270**, 905–911.

Demas, G.E., Klein, S.L. & Nelson, R.J. (1996) Reproductive and immune responses to photoperiod and melatonin are linked in *Peromyscus* subspecies. *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral Physiology*, **179**, 819–825.

Demas, G.E. & Nelson, R.J. (1996) Photoperiod and temperature interact to affect immune parameters in adult male deer mice. *Journal of Biological Rhythms*, **11**, 94–102.

Demas, G.E., Chefer, V., Talan, M.I. & Nelson, R.J. (1997a) Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, **42**, R1631–R1637.

Dempsey, P.W., Allison, M.E., Akkaraju, S., Goodnow, C.C. & Fearon, D.T. (1996) C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science*, **271**, 348–350.

Dhabhar, F.S. (1998) Stress-induced enhancement of cell-mediated immunity. In: *Neuroimmunomodulation. Annals of the New York Academy of Sciences*, **840**, 359–372.

Dhabhar, F.S., Miller, A.H., McEwen, B.S. & Spencer, R.L. (1995) Effects of stress on immune cell distribution – dynamics and hormonal mechanisms. *Journal of Immunology*, **154**, 5511–5527.

Dinarello, C.A. (2000) Proinflammatory cytokines. *Chest*, **118**, 503–508.

Drazen, D.L., Trasy, A. & Nelson, R.J. (2003) Photoperiod differentially affects energetics of immunity in pregnant and lactating Siberian hamsters (*Phodopus sungorus*). *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, **81**, 1406–1413.

Drazen, D., Nelson, R., Bartness, T. & Demas, G. (2000) Sympathoadrenal regulation of photoperiodic changes in immune function in Siberian hamsters. Society for Neuroscience, New Orleans.

Dunkelberger, J.R. & Song, W.-C. (2009) Complement and its role in innate and adaptive immune responses. *Cell Research*, **20**, 34–50.

Eisen, R.J. & DeNardo, D.F. (2000) Life history of a malaria parasite (*Plasmodium mexicanum*) in its host, the western fence lizard (*Sceloporus occidentalis*): host testosterone as a source of seasonal and among-host variation? *Journal of Parasitology*, **86**, 1041–1045.

Else, K.J. & Finkelman, F.D. (1998) Invited review intestinal nematode parasites, cytokines and effector mechanisms. *International Journal for Parasitology*, **28**, 1145–1158.

Epstein, F.H., Singer, A.J. & Clark, R.A.F. (1999) Cutaneous wound healing. *New England Journal of Medicine*, **341**, 738–746.

Fairbrother, A., Fix, M., O'Hara, T. & Ribic, C. (1994) Impairment of growth and immune function of avocet chicks from sites with elevated selenium, arsenic, and boron. *Journal of Wildlife Diseases*, **30**, 222–233.

Fänge, R. & Silverin, B. (1985) Variation of lymphoid activity in the spleen of a migratory bird, the pied flycatcher (*Ficedula hypoleuca*; Aves, passeriformes). *Journal of Morphology*, **184**, 33–40.

Ferrández, M.D. & De la Fuente, M. (1996) Changes with aging, sex and physical exercise in murine natural killer activity and antibody-dependent cellular cytotoxicity. *Mechanisms of Ageing and Development*, **86**, 83–94.

Fofie, A.E. & Fewell, J.E. (2003) Influence of pregnancy on plasma cytokines and the febrile response to intraperitoneal administration of bacterial endotoxin in rats. *Experimental Physiology*, **88**, 747–754.

Fowles, J.R., Fairbrother, A. & Kerkvliet, N.I. (1997) Effects of induced hypo- and hyperthyroidism on immune function and plasma biochemistry in mallards (*Anas platyrhynchos*). *Comparative Biochemistry and Physiology. Part C: Pharmacology, Toxicology and Endocrinology*, **118**, 213–220.

French, S.S., DeNardo, D.F. & Moore, M.C. (2007) Trade-offs between the reproductive and immune systems: facultative responses to resources or obligate responses to reproduction? *The American Naturalist*, **170**, 79–89.

French, S.S., Matt, K.S. & Moore, M.C. (2006) The effects of stress on wound healing in male tree lizards (*Urosaurus ornatus*). *General and Comparative Endocrinology*, **145**, 128–132.

French, S.S. & Moore, M.C. (2008) Immune function varies with reproductive stage and context in female and male tree lizards, *Urosaurus ornatus*. *General and Comparative Endocrinology*, **155**, 148–156.

French, S.S., Moore, M.C. & Demas, G.E. (2009) Ecological immunology: the organism in context. *Integrative and Comparative Biology*, **49**, 246–253.

Froebel, K.S., Pakker, N.G., Aiuti, F., Bofill, M., Choremi-Papadopoulou, H., Economidou, J., Rabian, C., Roos, M.T.L., Ryder, L.P., Miedema, F. & Raab, G.M. (1999) Standardisation and quality assurance of lymphocyte proliferation assays for use in the assessment of immune function. *Journal of Immunological Methods*, **227**, 85–97.

Gandra, Y.R. & Scrimshaw, N.S. (1961) Infection and nutritional status: II. Effect of mild virus infection induced by 17-D yellow fever vaccine on nitrogen metabolism in children. *American Journal of Clinical Nutrition*, **9**, 159–163.

Ganz, T. (2003) Defensins: antimicrobial peptides of innate immunity. *Nature Reviews Immunology*, **3**, 710–720.

Gibble, R.E., Rollins-Smith, L. & Baer, K.N. (2008) Development of an assay for testing the antimicrobial activity of skin peptides against the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) using *Xenopus laevis*. *Ecotoxicology and Environmental Safety*, **71**, 506–513.

Glick, B., Taylor Jr, R.L., Martin, D.E., Watabe, M., Day, E.J. & Thompson, D. (1983) Calorie-protein deficiencies and the immune response of the chicken. II. Cell-mediated immunity. *Poultry Science*, **62**, 1889–1893.

Graham, A.L., Cattadori, I.M., Lloyd-Smith, J.O., Ferrari, M.J. & Bjørnstad, O.N. (2007) Transmission consequences of coinfection: cytokines writ large? *Trends in Parasitology*, **23**, 284–291.

Graham, A.L. (2008) Ecological rules governing helminth–microparasite coinfection. *Proceedings of the National Academy of Sciences*, **105**, 566–570.

Grasman, K.A. (2002) Assessing immunological function in toxicological studies of avian wildlife. *Integrative and Comparative Biology*, **42**, 34–42.

Grasman, K.A. (2010) In vivo functional tests for assessing immunotoxicity in birds. *Immunotoxicity Testing* (ed R.R. Dietert), pp. 387–398. Humana Press, Clifton, NJ, USA.

Greives, T.J., McGlothlin, J.W., Jawor, J.M., Demas, G.E. & Ketterson, E.D. (2006) Testosterone and innate immune function inversely covary in a wild population of breeding dark-eyed juncos (*Junco hyemalis*). *Functional Ecology*, **20**, 812–818.

Hanssen, S.A., Folstad, I. & Erikstad, K.E. (2003) Reduced immunocompetence and cost of reproduction in common eiders. *Oecologia*, **136**, 457–464.

Hanssen, S.A., Hasselquist, D., Folstad, I. & Erikstad, K.E. (2004) Costs of immunity: immune responsiveness reduces survival in a vertebrate. *Proceedings. Biological sciences/The Royal Society*, **271**, 925–930.

Hawley, D.M. & Altizer, S.M. (2011) Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. *Functional Ecology*, **25**, 48–60.

Herzenberg, L.A., Parks, D., Sahaf, B., Perez, O., Roederer, M. & Herzenberg, L.A. (2002) The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. *Clinical Chemistry*, **48**, 1819–1827.

Hetem, R.S., Mitchell, D., Maloney, S.K., Meyer, L.C.R., Fick, L.G., Kerley, G.I.H. & Fuller, A. (2008) Fever and sickness behavior during an opportunistic infection in a free-living antelope, the greater kudu (*Tragelaphus strepsiceros*). *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, **294**, R246–R254.

Hodyl, N.A., Walker, F.R., Krivanek, K.M., Clifton, V.L. & Hodgson, D.M. (2010) Prenatal endotoxin exposure alters behavioural pain responses to lipopolysaccharide in adult offspring. *Physiology & Behavior*, **100**, 143–147.

Ilmonen, P., Taarna, T. & Hasselquist, D. (2000) Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **267**, 665–670.

Janeway, C., Travers, P., Walport, M. & Shlomchik, M. (2005) *Immunobiology: The Immune System in Health and Disease*, 6th edn. Garland Science, New York, NY, USA.

John, J.L. (1994) The avian spleen: a neglected organ. *Quarterly Review of Biology*, **69**, 327–351.

Kiecolt-Glaser, J.K., Marucha, P.T., Mercado, A.M., Malarkey, W.B. & Glaser, R. (1995) Slowing of wound healing by psychological stress. *The Lancet*, **346**, 1194–1196.

Kinsey, S.G., Prendergast, B.J. & Nelson, R.J. (2003) Photoperiod and stress affect wound healing in Siberian hamsters. *Physiology and Behavior*, **78**, 205–211.

Klos, A., Ihrig, V., Messner, M., Grabbe, J. & Bitter-Suermann, D. (1988) Detection of native human complement components C3 and C5 and their primary activation peptides C3a and C5a (anaphylatoxic peptides) by ELISAs with monoclonal antibodies. *Journal of Immunological Methods*, **111**, 241–252.

Kobayashi, M., Fitz, L., Ryan, M., Hewick, R.M., Clark, S.C., Chan, S., Loudon, R., Sherman, F., Perussia, B. & Trinchieri, G. (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *The Journal of Experimental Medicine*, **170**, 827–845.

Kuby, J. (1997) *Immunology*. W. H. Freeman and Company, New York, NY, USA.

Kwiatkowska, K. & Sobota, A. (1999) Signaling pathways in phagocytosis. *Bioessays*, **21**, 422–431.

Lahvis, G.P., Wells, R.S., Kuehl, D.W., Stewart, J.L., Rhinehart, H.L. & Via, C.S. (1995) Decreased lymphocyte responses in free-ranging bottlenose dolphins (*Tursiops truncatus*) are associated with increased concentrations of PCBs and DDT in peripheral blood. *Environmental Health Perspectives*, **103**(Suppl 4), 67–72.

Lee, K., Martin, L., Hasselquist, D., Ricklefs, R. & Wikelski, M. (2006) Contrasting adaptive immune defenses and blood parasite prevalence in closely related Passer sparrows. *Oecologia*, **150**, 383–392.

Lee, K.A., Wikelski, M., Robinson, W.D., Robinson, T.R. & Klasing, K.C. (2008) Constitutive immune defences correlate with life-history variables in tropical birds. *Journal of Animal Ecology*, **77**, 356–363.

Lemus, J.A., Vergara, P. & Fargallo, J.A. (2010) Response of circulating T-lymphocytes to a coccidian infection: insights from a parasitization-vaccination experiment. *Functional Ecology*, **24**, 638–645.

Liebl, A.L. & Martin, L.B. (2009) Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry. *Functional Ecology*, **23**, 1091–1096.

Lochmiller, R.L. & Deerenberg, C. (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos*, **88**, 87–98.

Lochmiller, R.L., Vestey, M.R. & Jon, C.B. (1993) Relationship between protein nutritional status and immunocompetence in Northern Bobwhite Chicks. *The Auk*, **110**, 503–510.

Long, E.O., Barber, D.F., Burshtyn, D.N., Faure, M., Peterson, M., Rajagopalan, S., Renard, V., Sandusky, M., Stebbins, C.C., Wagtmann, N. & Watzl, C. (2001) Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158). *Immunological Reviews*, **181**, 223–233.

Martin, P. (1997) Wound healing – Aiming for perfect skin regeneration. *Science*, **276**, 75–81.

Martin, L.B., Hasselquist, D. & Wikelski, M. (2006b) Investment in immune defense is linked to pace of life in house sparrows. *Oecologia*, **147**, 565–575.

Martin, L.B., Weil, Z.M. & Nelson, R.J. (2008b) Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **363**, 321–339.

Martin, L.B., Han, P., Lewittes, J., Kuhlman, J.R., Klasing, K.C. & Wikelski, M. (2006a) Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. *Functional Ecology*, **20**, 290–299.

Martin, L.B., Navara, K.J., Bailey, M.T., Hutch, C.R., Powell, N.D., Sheridan, J.F. & Nelson, R.J. (2008a) Food restriction compromises immunological memory in deer mice (*Peromyscus maniculatus*) by reducing spleen-derived antibody producing B cell numbers. *Physiological and Biochemical Zoology*, **81**, 366–372.

Marucha, P.T., Kiecolt-Glaser, J.K. & Favagehi, M. (1998) Mucosal wound healing is impaired by examination stress. *Psychosomatic Medicine*, **60**, 362–365.

Matson, K.D., Ricklefs, R.E. & Klasing, K.C. (2005) A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Developmental & Comparative Immunology*, **29**, 275–286.

Matson, K.D., Cohen, A.A., Klasing, K.C., Ricklefs, R.E. & Scheuerlein, A. (2006) No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl. *Proceedings of the Royal Society B: Biological Sciences*, **273**, 815–822.

Matsuzaki, K. (1999) Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochimica et Biophysica Acta (BBA). Biomembranes*, **1462**, 1–10.

Matzinger, P. (2002) The danger model: a renewed sense of self. *Science*, **296**, 301–305.

Mayer, M.M. (1948) Complement and complement fixation. *Experimental Immunochemistry* (eds E.A. Kabat & M.M. Mayer). 100–112. Charles C. Thomas, Springfield, IL, USA.

McDade, T.W. (2003) Life history theory and the immune system: steps toward a human ecological immunology. *Yearbook of Physical Anthropology*, **46**, 100–125.

Meager, A. (2006) Measurement of cytokines by bioassays: theory and application. *Methods*, **38**, 237–252.

Mercado, A.M., Padgett, D.A., Sheridan, J.F. & Marucha, P.T. (2002) Altered kinetics of IL-1 alpha, IL-1 beta, and KGF-1 gene expression in early wounds of restrained mice. *Brain Behavior and Immunity*, **16**, 150–162.

Millet, S., Bennett, J., Lee, K.A., Hau, M. & Klasing, K.C. (2007) Quantifying and comparing constitutive immunity across avian species. *Developmental & Comparative Immunology*, **31**, 188–201.

Moller, A.P. & Petrie, M. (2002) Condition dependence, multiple sexual signals, and immunocompetence in peacocks. *Behavioral Ecology*, **13**, 248–253.

Mollnes, T.E., Garred, P. & Bergseth, G. (1988) Effect of time, temperature and anticoagulants on in vitro complement activation: consequences for collection and preservation of samples to be examined for complement activation. *Clinical and Experimental Immunology*, **73**, 484–488.

Moreno, J., Sanz, J.J. & Arriero, E. (1999) Reproductive effort and T-lymphocyte cell-mediated immunocompetence in female pied flycatchers *Ficedula hypoleuca*. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **266**, 1105–1109.

Mosmann, T.R. & Coffman, R.L. (1989) Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Advances in Immunology*, **46**, 111–147.

Mouihate, A., Harré, E.M., Martin, S. & Pittman, Q.J. (2008) Suppression of the febrile response in late gestation: evidence, mechanisms and outcomes. *Journal of Neuroendocrinology*, **20**, 508–514.

Muehlenbein, M.P. (2010) Evolutionary medicine, immunity and infectious diseases. *Human Evolutionary Biology* (ed. M.P. Muehlenbein), pp. 351–377. Cambridge University Press, New York, NY.

Muehlenbein, M.P. & Bribiescas, R.G. (2005) Testosterone-mediated immune functions and male life histories. *American Journal of Human Biology*, **17**, 527–558.

Muñoz, F.J., Galván, A., Lerma, M. & De la Fuente, M. (2000) Seasonal changes in peripheral blood leukocyte functions of the turtle *Mauremys caspica* and their relationship with corticosterone, 17-[beta]-estradiol and testosterone serum levels. *Veterinary Immunology and Immunopathology*, **77**, 27–42.

Nelson, R.J. (2004) Seasonal immune function and sickness responses. *Trends in Immunology*, **25**, 187–192.

Nelson, R.J. & Demas, G.E. (1996) Seasonal changes in immune function. *Quarterly Review of Biology*, **71**, 511–548.

Nelson, R.J. & Demas, G.E. (2004) Seasonal patterns of stress, disease, and sickness responses. *Current Directions in Psychological Science*, **13**, 198–201.

Nicolas, P. & Mor, A. (1995) Peptides as weapons against microorganisms in the chemical defense system of vertebrates. *Annual Review of Microbiology*, **49**, 277–304.

Nordling, D., Andersson, M., Zohari, S. & Gustafsson, L. (1998) Reproductive effort reduces specific immune response and parasite resistance. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **265**, 1291–1298.

Nunn, C.L. (2002) A comparative study of leukocyte counts and disease risk in primates. *Evolution*, **56**, 177–190.

Ohlsson, T., Smith, H.G., Råberg, L. & Hasselquist, D. (2002) Pheasant sexual ornaments reflect nutritional conditions during early growth. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **269**, 21–27.

Olsson, M., Wapstra, E., Madsen, T. & Silverin, B. (2000) Testosterone, ticks and travels: a test of the immunocompetence-handicap hypothesis in free-ranging male sand lizards. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **267**, 2339–2343.

Orange Jordan, S. (2002) Human natural killer cell deficiencies and susceptibility to infection. *Microbes and Infection*, **4**, 1545–1558.

Orsi, C.F., Colombari, B., Callegari, F., Todaro, A.M., Ardizzoni, A., Rossini, G.P., Blasi, E. & Peppoloni, S. (2010) Yessotoxin inhibits phagocytic activity of macrophages. *Toxicon*, **55**, 265–273.

Ots, I., Murumagi, A. & Horak, P. (1998) Haematological health state indices of reproducing great tits: methodology and sources of natural variation. *Functional Ecology*, **12**, 700–707.

Oudhoff, M.J., Blaauboer, M.E., Nazmi, K., Scheres, N., Bolscher, J.G. & Veerman, E.C. (2010) The role of salivary histatin and human cathelicidin LL-37 in wound healing and innate immunity. *Biological Chemistry*, **391**, 541–548.

Owen-Ashley, N.T., Turner, M., Hahn, T.P. & Wingfield, J.C. (2006) Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (*Zonotrichia leucophrys gambelii*). *Hormones and Behavior*, **49**, 15–29.

Owen-Ashley, N.T. & Wingfield, J.C. (2006) Seasonal modulation of sickness behavior in free-living northwestern song sparrows (*Melospiza melodia morphna*). *Journal of Experimental Biology*, **209**, 3062–3070.

Owen-Ashley, N.T., Turner, M., Hahn, T.P. & Wingfield, J.C. (2006) Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (*Zonotrichia leucophrys gambelii*). *Hormones and Behavior*, **49**, 15–29.

Padgett, D.A., Marucha, P.T. & Sheridan, J.F. (1998) Restraint stress slows cutaneous wound healing in mice. *Brain Behavior and Immunity*, **12**, 64–73.

Palacios, M.G., Cunnick, J.E., Winkler, D.W. & Vleck, C.M. (2007) Immunosenescence in some but not all immune components in a free-living vertebrate, the tree swallow. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 951–957.

Palacios, M.G., Cunnick, J.E., Vleck, D. & Vleck, C.M. (2009) Ontogeny of innate and adaptive immune defense components in free-living tree swallows, *Tachycineta bicolor*. *Developmental & Comparative Immunology*, **33**, 456–463.

Patton, K.M., McGuire, T.C., Hines, M.T., Mealey, R.H. & Hines, S.A. (2005) Rhodococcus equi-specific cytotoxic T lymphocytes in immune horses and development in asymptomatic foals. *Infection and Immunity*, **73**, 2083–2093.

Pedersen, A.B. & Fenton, A. (2007) Emphasizing the ecology in parasite community ecology. *Trends in Ecology & Evolution*, **22**, 133–139.

Prendergast, B.J., Hotchkiss, A.K., Bilbo, S.D. & Nelson, R.J. (2004) Peripubertal immune challenges attenuate reproductive development in male siberian hamsters (*Phodopus sungorus*). *Biology of Reproduction*, **70**, 813–820.

Riddle, O. & Krizenecky, N. (1931) Studies on the physiology of reproduction in birds XXVIII. Extirpation of thymus and bursa in pigeons with a consideration of the failure of thymectomy to reveal thymus function. *American Journal of Physiology*, **97**, 343–352.

Robinson, J. (2004) Flow cytometry. *Encyclopedia of Biomaterials and Biomedical Engineering*, 630–640.

Rojas, I.G., Padgett, D.A., Sheridan, J.F. & Marucha, P.T. (2000) Effects of restraint stress on susceptibility of bacterial infection during cutaneous wound healing. *Journal of Dental Research*, **79**, 393.

Rojas, I.G., Padgett, D.A., Sheridan, J.F. & Marucha, P.T. (2002) Stress-induced susceptibility to bacterial infection during cutaneous wound healing. *Brain Behavior and Immunity*, **16**, 74–84.

Rollins-Smith, L.A. (2009) The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochimica et Biophysica Acta (BBA). Biomembranes*, **1788**, 1593–1599.

Rollins-Smith, L.A., Doersam, J.K., Longcore, J.E., Taylor, S.K., Shamblin, J.C., Carey, C. & Zasloff, M.A. (2002) Antimicrobial peptide defenses against pathogens associated with global amphibian declines. *Developmental & Comparative Immunology*, **26**, 63–72.

Rook, G.A.W. & Brunet, L.R. (2005) Microbes, immunoregulation, and the gut. *Gut*, **54**, 317–320.

Rubenstein, D.R., Parlow, A.F., Hutch, C.R. & Martin Ii, L.B. (2008) Environmental and hormonal correlates of immune activity in a cooperatively breeding tropical bird. *General and Comparative Endocrinology*, **159**, 10–15.

Ruiz, M., French, S.S., Demas, G.E. & Martins, E.P. (2010) Food supplementation and testosterone interact to influence reproductive behavior and immune function in *Sceloporus graciosus*. *Hormones and Behavior*, **57**, 134–139.

Saad, A.H. (1989) Pregnancy-related involution of the thymus in the viviparous lizard, *Chalcides ocellatus*. *Thymus*, **14**, 223–232.

Sachdeva, N. & Asthana, D. (2007) Cytokine quantitation: technologies and applications. *Frontiers in Bioscience: A Journal and Virtual Library*, **12**, 4682–4695.

Sack, U., Tárnok, A. & Rothe, G. (2009) *Cellular Diagnostics. Basics, Methods and Clinical Applications of Flow Cytometry*. Karger, Basel.

Saino, N., Ferrari, R.P., Martinelli, S., Romano, M., Rubolini, D. & Møller, S. (2002) Early maternal effects mediated by immunity depend on sexual ornamentation of the male partner. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **269**, 1005–1009.

Salvante, K.G. & Dufty Jr, A.M. (2006) Techniques for studying integrated immune function in birds. *The Auk*, **123**, 575–586.

Schmid-Hempel, P. & Ebert, D. (2003) On the evolutionary ecology of specific immune defence. *Trends in Ecology & Evolution*, **18**, 27–32.

Seelen, M.A., Roos, A., Wieslander, J., Mollnes, T.E., Sjöholm, A.G., Wurzner, R., Loos, M., Tedesco, F., Sim, R.B., Garred, P., Alexopoulos, E., Turner, M.W. & Daha, M.R. (2005) Functional analysis of the classical, alternative, and MBL pathways of the complement system: standardization and validation of a simple ELISA. *Journal of Immunological Methods*, **296**, 187–198.

Semple, S., Cowlishaw, G. & Bennett, P.M. (2002) Immune system evolution among anthropoid primates: parasites, injuries and predators. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **269**, 1031–1037.

Shai, Y. (1999) Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by [alpha]-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochimica et Biophysica Acta (BBA). Biomembranes*, **1462**, 55–70.

Sheldon, B.C. & Verhulst, S. (1996) Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution*, **11**, 317–321.

Sinclair, J.A. & Lochmiller, R.L. (2000) The winter immunoenhancement hypothesis: associations among immunity, density, and survival in prairie vole (*Microtus ochrogaster*) populations. *Canadian Journal of Zoology*, **78**, 254–264.

Smith, A., Vollmer-Conna, U., Geczy, A., Dunckley, H., Bennett, B., Hickie, I. & Lloyd, A. (2005) Does genotype mask the relationship between psychological factors and immune function? *Brain, Behavior, and Immunity*, **19**, 147–152.

Snapper, C.M. (1996) *Cytokine Regulation of Humoral Immunity: Basic and Clinical Aspects*. Wiley, New York, NY, USA.

Sparkman, A.M. & Palacios, M.G. (2009) A test of life-history theories of immune defence in two ecotypes of the garter snake, *Thamnophis elegans*. *Journal of Animal Ecology*, **78**, 1242–1248.

Spurlock, M.E., Frank, G.R., Willis, G.M., Kuske, J.L. & Cornelius, S.G. (1997) Effect of dietary energy source and immunological challenge on growth performance and immunological variables in growing pigs. *Journal of Animal Science*, **75**, 720–726.

Staszewski, V. & Boulinier, T. (2004) Vaccination: a way to address questions in behavioral and population ecology? *Trends in Parasitology*, **20**, 17–22.

Staszewski, V., McCoy, K.D., Tveraa, T. & Boulinier, T. (2007) Interannual dynamics of antibody levels in naturally infected long-lived colonial birds. *Ecology*, **88**, 3183–3191.

Svensson, E., Raberg, L., Koch, C. & Hasselquist, D. (1998) Energetic stress, immunosuppression and the costs of an antibody response. *Functional Ecology*, **12**, 912–919.

Tella, J.L., Lemus, J.A., Carrete, M. & Blanco, G. (2008) The PHA test reflects acquired T-cell mediated immunocompetence in birds. *PLoS ONE*, **3**, e3295.

Thorpe, R., Wadhwa, M., Page, C. & Mire-Sluis, A. (1999) Bioassays for the characterization and control of therapeutic cytokines: determination of potency. *Developments in Biological Standardization*, **97**, 61–71.

Tieleman, I.B., Williams, J.B., Ricklefs, R.E. & Klasing, K.C. (2005) Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. *Proceedings of the Royal Society B: Biological Sciences*, **272**, 1715–1720.

Trust, K.A., Hooper, M.J. & Fairbrother, A. (1994) Effects of 7,12-dimethylbenz[*A*]anthracene on immune function and mixed-function oxygenase activity in the european starling. *Environmental Toxicology and Chemistry*, **13**, 821–830.

Tsang, M., Fewell, J.E. & Moore, S.L. (2006) LPS induced hypothermia in pregnant rats: a regulated thermoregulatory response. *Physiology & Behavior*, **89**, 235–240.

Tsuji, T., Sakata, T., Hamaguchi, Y., Wang, F.-s. & Houwen, B. (1999) New rapid flow cytometric method for the enumeration of nucleated red blood cells. *Cytometry*, **37**, 291–301.

Vieira, O.V., Botelho, R.J. & Grinstein, S. (2002) Phagosome maturation: aging gracefully. *Biochemical Journal*, **366**, 689–704.

Wadhwa, M. & Thorpe, R. (1998) Cytokine immunoassays: recommendations for standardisation, calibration and validation. *Journal of Immunological Methods*, **219**, 1–5.

Wagland, B.M., Steel, J.W., Windon, R.G. & Dineen, J.K. (1984) The response of lambs to vaccination and challenge with *Trichostrongylus colubriformis*: effect of plane of nutrition on, and the inter-relationship between, immunological responsiveness and resistance. *International Journal for Parasitology*, **14**, 39–44.

Wei, Y.L., Li, X.H. & Zhou, J.Z. (2007) Prenatal exposure to lipopolysaccharide results in increases in blood pressure and body weight in rats. *Acta Pharmacologica Sinica*, **28**, 651–656.

Weiss, D.J. & Wardrop, K.J. (2010) *Schalm's Veterinary Hematology*, Wiley-Blackwell, New York.

Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature*, **415**, 389–395.

Zimmerman, L.M., Vogel, L.A., Edwards, K.A. & Bowden, R.M. (2010) Phagocytic B cells in a reptile. *Biology Letters*, **6**, 270–273.

Zysling, D. & Demas, G. (2007) Metabolic stress suppresses humoral immune function in long-day, but not short-day, Siberian hamsters (*Phodopus sungorus*). *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, **177**, 339–347.

Zysling, D.A., Garst, A.D. & Demas, G.E. (2009) Photoperiod and food restriction differentially affect reproductive and immune responses in Siberian hamsters *Phodopus sungorus*. *Functional Ecology*, **23**, 979–988.

Received 16 July 2010; accepted 24 January 2011
Handling Editor: Mike Boots